International Journal of Genomics

Noncoding RNAs in Health and Disease

Lead Guest Editor: Michele Purrello
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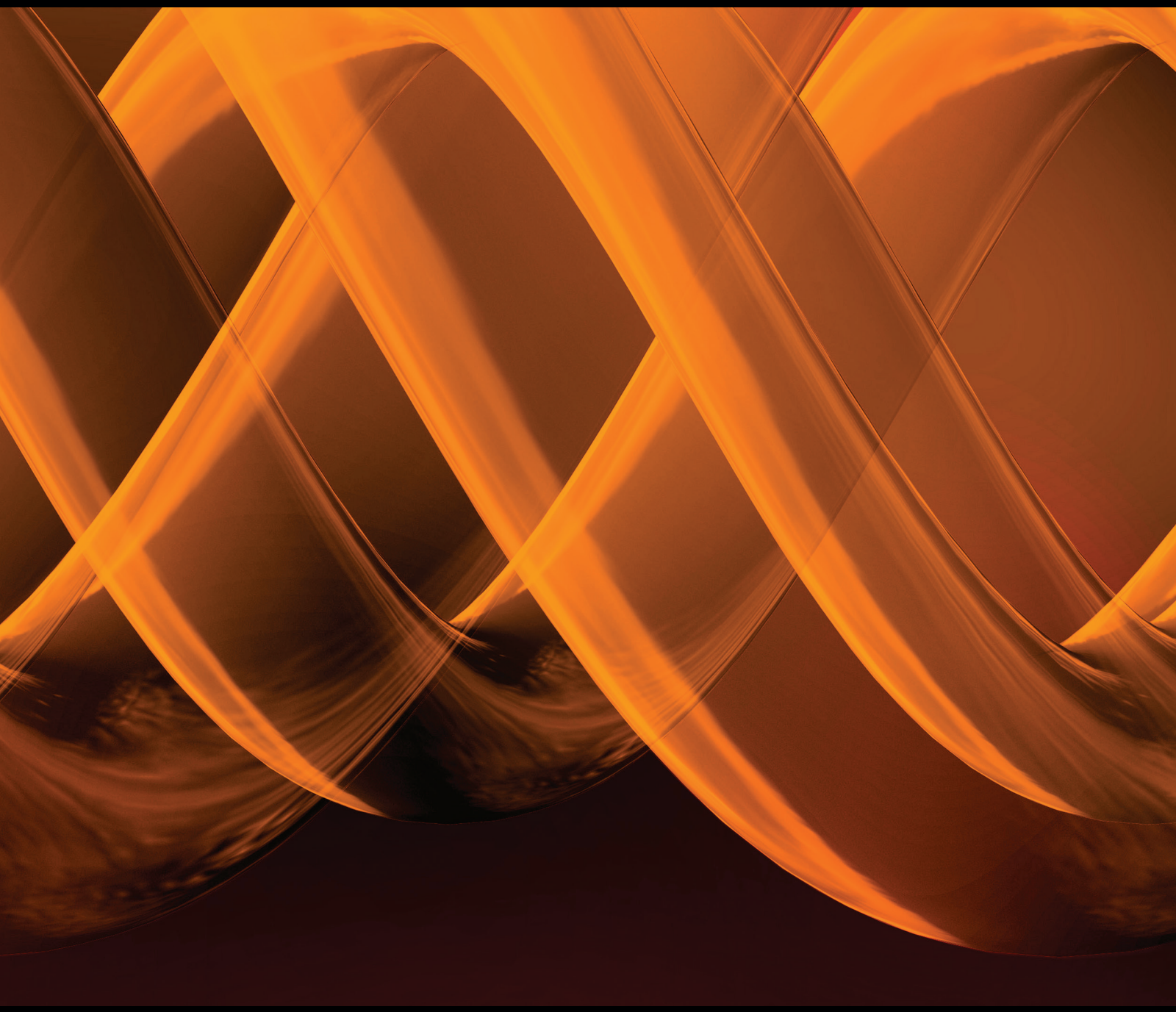

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Editorial Board

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Editorial
Noncoding RNAs in Health and Disease

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Genome and RNA sequencing have reliably demonstrated that up to 90% of the human genome may be transcribed. According to actual estimates, the *proteome genome* (i.e., all genomic sequences involved in mRNA synthesis) occupies only up to 4% of the human genome [1]. The remaining part, which may be provisionally named as *noncoding RNA genome* (*ncRNA genome*), comprises genes encoding a plethora of structurally and functionally different RNAs other than mRNAs: since most of these apparently do not code for proteins, they have been labelled noncoding RNAs (ncRNAs) [2]. These data have determined a radical paradigm shift in biomolecular medicine and modified our vision of genome structure and functions. They also changed objectives and perspectives of genomic research. As reminded above, the *ncRNA genome* very likely occupies a very large molecular space; accordingly, it seems logical to hypothesize that the molecular explanation of important biopathological phenomena (i.e., normal development and differentiation together with their pathological alterations) is to be found in these regions. It is interesting to remark that they were once deemed to be devoid of any biological function and inappropriately labeled as *garbage DNA*. Different parameters are actually applied to classify and analyze ncRNAs: (1) molecular length and structure [e.g., microRNAs (miRNAs), long ncRNAs (lncRNAs), and circular RNAs (circRNAs)]; (2) mode of expression (housekeeping ncRNAs versus cell type-specific ncRNAs); (3) molecular mechanism of action (miRNAs are mechanically the best characterized ncRNAs); (4) organism where they are expressed (eukaryotes, prokaryotes, and Archaea). Concerning this last point, ncRNAs have been detected in organisms at all evolutionary levels, including bacteria: this confirms their high functional biomolecular importance within living organisms. This special issue comprises 5 research and 6 review articles, which describe several aspects of the molecular structure and functions of ncRNAs (miRNAs, lncRNAs, and circRNAs), both in physiology and in pathology. Identification and characterization of structure and function of new RNA molecules are easier and faster to obtain than in the past thanks to the recent progress in high-throughput molecular biology techniques and bioinformatic analysis, especially applied to next-generation sequencing (NGS). Among ncRNAs, miRNAs are the most known and well characterized: they belong to the class of small ncRNAs and act as negative regulators of gene expression at posttranscriptional level [3–6]. Each of them is estimated to regulate the expression of up to 200 different target mRNAs; accordingly, miRNAs occupy a leading position within molecular cell networks and their function is critical in regulating both physiological and pathological processes (e.g., cancer and neurodegenerative and neuropsychiatric diseases). Research in the field of miRNAs, and ncRNAs in general, ranges from investigation of pathways that are involved in their use as therapeutic agents and as diagnostic and prognostic biomarkers. All these aspects are dealt with within this special issue. I. Grossi and colleagues review the characterized functions of miR-193a-3p, both as

physiological negative regulator of cell cycle progression in several cell types (i.e., endothelial colony forming cells, myofibers, and uterine epithelial cells) and as tumour suppressor downregulated in several cancers. The same authors suggest an in-depth analysis aimed to unravel the role of miR-193a-3p in Parkinson's disease or schizophrenia. Study of physiological processes as ageing can suggest how a specific pathway is perturbed in pathological condition: this is the case of the paper by P. Balaskas and colleagues who suggest a battery of miRNAs for future functional studies in osteoarthritis based on their analysis of differential expression of miRNAs in joints or cartilage during ageing. B. Banelli and colleagues review the involvement of miRNAs in the pathogenesis of glioblastoma multiforme (GBM), encompassing the interplay between miRNAs and epigenetic cell networks as well as the proposed role of miRNAs as candidates for innovative therapies in GBM. Biomolecular effects of miRNAs are strictly related to cell context; for instance, a specific miRNA can act as a tumour suppressor or oncogene in different cancers, depending on the targets it recognizes in a specific biomolecular context. A. Izzo and colleagues deal with this issue in a paper on the involvement of altered miRNAs expression in heart defects of Down syndrome fetuses. M. Rizzo et al. describe the *targetome* of miR-28-5p in the prostate cancer cell context. Identification of molecular mechanisms and pathways regulated by miRNAs is critical to expand knowledge on cell physiological processes or to determine onset and progression of a disease: a major aim of this work is to find new and effective therapeutic targets. Another expanding field of interest is the search for ncRNAs batteries as noninvasive diagnostic (i.e., liquid biopsies), prognostic, and predictive biomarkers. M. Barchitta and colleagues comprehensively review the role of miRNAs as potential biomarkers for adverse pregnancy outcomes (i.e., preeclampsia, spontaneous abortion, or preterm birth) and prenatal environmental exposure; M. Celano and colleagues focus on the possible use of miRNAs as sensitive and specific biomarkers for diagnosis and prognosis of thyroid cancers. If miRNAs likely are among the best functionally characterized ncRNAs, lncRNAs are the most represented RNA species in eukaryotic cells. Accordingly, the scientific community is making a great effort to functionally characterize these molecules, most of which are to date *orphan of function*. S. C. Credendino and colleagues have identified new isoforms of lncRNA Klhl14-AS, assaying their expression in a panel of mouse tissues and paving the way to their functional characterization. A. Matjašič and colleagues deal with the exploitation of lncRNAs as potential biomarkers for a detailed classification of gliomas by integrating their aberrant expression with that of miRNAs. A. Bolha and colleagues review the literature on the recently discovered circRNAs, by focusing on what is known about their involvement in cancer etiology and their use as effective biomarkers. It was becoming evident that cell networks cannot be appropriately investigated without considering the interplay between coding and noncoding RNAs. As reviewed by M. Ragusa and colleagues, the main challenge of ncRNA research in the next few years will be to unravel how ncRNAs can regulate each other in a cell type-specific biomolecular context and how this intricate interactions contribute to the final cell phenotype. A final and very important consideration is that we are firmly convinced that all present and future improvements (both theoretical and technological) of the scientific community should be considered in light of bioethics: the thirst for knowledge is one of the most sublime characteristics of man, but at its foundation there is always an ethical motivation. In today's scientific research, the experiment, in itself, can modify irreversibly what we are together with all that surrounds us (see CRIPSR/Cas9 technology). We must therefore be able to answer the following question: can genetic self-transformation be considered a legitimate means to free ourselves and increase individual autonomy? We also must ask ourselves if this aspect of liberal genetics may become something that could compromise individuals' self-understanding. As H. Jonas has revealed, "The value of all values is the possibility of value" [7].

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Davide Barbagallo
Gaetano Vittone
Massimo Romani
Michele Purrello

References

[1] M. Kellis, B. Wold, M. P. Snyder et al., "Defining functional DNA elements in the human genome," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 111, no. 17, pp. 6131–6138, 2014.

[2] K. R. Chi, "The dark side of the human genome," *Nature*, vol. 538, no. 7624, pp. 275–277, 2016.

[3] D. Barbagallo, A. Condorelli, M. Ragusa et al., "Dysregulated miR-671-5p / CDR1-AS / CDR1 / VSNL1 axis is involved in glioblastoma multiforme," *Oncotarget*, vol. 7, no. 4, pp. 4746–4759, 2016.

[4] D. Barbagallo, S. Piro, A. G. Condorelli et al., "miR-296-3p, miR-298-5p and their downstream networks are causally involved in the higher resistance of mammalian pancreatic α cells to cytokine-induced apoptosis as compared to β cells," *BMC Genomics*, vol. 14, no. 1, p. 62, 2013.

[5] M. Ragusa, C. Barbagallo, L. Statello et al., "Non-coding landscapes of colorectal cancer," *World Journal of Gastroenterology*, vol. 21, no. 41, pp. 11709–11739, 2015.

[6] V. Di Pietro, M. Ragusa, D. Davies et al., "MicroRNAs as Novel Biomarkers for the Diagnosis and Prognosis of Mild and Severe Traumatic Brain Injury," *Journal of Neurotrauma*, vol. 34, no. 11, pp. 1948–1956, 2017.

[7] H. Jonas, *Tecnica, Medicina Ed Etica*, Einaudi, Torino, Italy, 1997.

Hindawi
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Review Article

Circular RNAs: Biogenesis, Function, and a Role as Possible Cancer Biomarkers

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Circular RNAs (circRNAs) are a class of noncoding RNAs (ncRNAs) that form covalently closed continuous loop structures, lacking the terminal 5′ and 3′ ends. CircRNAs are generated in the process of back-splicing and can originate from different genomic regions. Their unique circular structure makes circRNAs more stable than linear RNAs. In addition, they also display insensitivity to ribonuclease activity. Generally, circRNAs function as microRNA (miRNA) sponges and have a regulatory role in transcription and translation. They may be also translated in a cap-independent manner *in vivo*, to generate specific proteins. In the last decade, next-generation sequencing techniques, especially RNA-seq, have revealed great abundance and also dysregulation of many circRNAs in various diseases, suggesting their involvement in disease development and progression. Regarding their high stability and relatively specific differential expression patterns in tissues and extracellular environment (e.g., body fluids), they are regarded as promising novel biomarkers in cancer. Therefore, we focus this review on describing circRNA biogenesis, function, and involvement in human cancer development and address the potential of circRNAs to be effectively used as novel cancer diagnostic and prognostic biomarkers.

1. Introduction

Noncoding RNAs (ncRNAs) represent a large, complex, and heterogeneous group of RNA molecules that can be classified into two major classes, according to the size of their transcripts: the small ncRNAs (<200 bp) and the long ncRNAs (lncRNAs) (>200 bp) [1, 2]. The small ncRNAs include microRNAs (miRNAs), small nuclear RNAs (snRNAs), PIWI-interacting RNAs (piRNAs), small interfering RNAs (siRNAs), small nucleolar RNAs (snoRNAs), and others [1], whereas lncRNAs comprise long intergenic ncRNAs (lincRNAs), intronic ncRNAs, macroRNAs, sense ncRNA, antisense RNAs, and others [2]. In addition, circular RNAs (circRNAs) have been recently identified as a relatively large class of ncRNAs, which are widespread and abundant in a variety of eukaryotic organisms and involved in multiple biological processes [3, 4]. CircRNAs may vary in length significantly, most of them being longer than 200 nt. However, some exonic and intronic circRNAs were shown to be shorter than 200 or even 100 nt [5].

CircRNAs form covalently closed continuous loop structures, without terminal 5′ caps and 3′ polyadenylated tails. They are generated by alternative splicing of pre-mRNA transcripts, where an upstream splice acceptor is joined to a downstream splice donor, in the process of back-splicing [6–8]. In the last decade, RNA-seq and other next-generation sequencing techniques have enabled a significant breakthrough in circRNA discovery, leading to the identification and characterization of a large number of circRNAs in humans and other eukaryotes [9, 10]. Several research groups have demonstrated a conservation of circRNA expression across mammals. In addition, circRNAs appear to be stably expressed in a cell/tissue-dependent and developmental stage-specific manner [3, 9, 11, 12]. Emerging evidence reveals the importance of circRNA involvement in regulating gene expression at transcriptional and posttranscriptional levels, and, furthermore, dysregulation in circRNA expression correlates with irregularities in developmental processes and various disease states, including cancer [13–16]. Regarding the observed correlation between altered circRNA

expression profiles and a cancer patient's clinical characteristics and circRNA's structural features that enable their abundance and stability in various biological samples, we focus this review on describing circRNA involvement in cancer development. In addition, we address the potential of circRNAs to be effectively used as novel biomarkers in cancer.

2. Biogenesis of CircRNAs

The majority of circRNAs originate from exons of protein-coding genes, frequently consisting of 1–5 exons [9]. However, they may be also formed from intronic, noncoding, antisense, 3′ UTR, 5′ UTR, or intergenic genomic regions [9, 17]. CircRNAs are generated by a spliceosome-mediated pre-mRNA back-splicing which connects a downstream splice donor site (5′ splice site) to an upstream acceptor splice site (3′ splice site) [18]. Similar to canonical (linear) splicing, back-splicing appears to be extensively regulated by canonical *cis*-acting splicing regulatory elements and *trans*-acting splicing factors. However, the regulation process of back-splicing in controlling circRNA production fundamentally differs from that of linear splicing, where the same combinations of splicing regulatory elements and factors have distinct or even opposite activity [18]. In addition, a single gene locus can produce various circRNAs through alternative back-splice site selection, when compared to canonical splicing of linear RNAs [19]. Generally, circRNAs can be generated by canonical and noncanonical splicing [20]. Regarding their biogenesis from different genomic regions, circRNAs can be categorized into four types, as determined by RNA-seq: exonic circRNAs (ecircRNAs) [9, 11, 12], circular intronic RNAs (ciRNAs) [17], retained-intron or exon-intron circRNAs (EIciRNAs) [11, 21], and intergenic circRNAs [9]. Schematic representation of ecircRNA, ciRNA, EIciRNA, and intergenic circRNA biogenesis is shown in Figure 1.

EcircRNAs are the most abundant circRNA type, accounting for over 80% of identified circRNAs, and are predominantly located in the cytoplasm [3, 9, 12], though the exact process of nuclear export remains to be elucidated. It has been suggested that ecircRNAs may escape from the nucleus during mitosis [22]. Although the exact mechanism of circRNAs biogenesis remains unclear, three models of circRNA formation have been proposed, including lariat-driven circularization (exon skipping) [12] (Figure 1(b)), intron pairing-driven circularization [12] (Figure 1(c)), and resplicing-driven circularization [23] (Figure 1(f)). It has been demonstrated that exon circularization depends on several genomic features, essential for promoting circularization. In general, exons comprising circRNAs are longer than average exons, which is especially notable for single-exon circRNAs, being approximately 3-fold longer, when compared to other expressed exons [3, 12]. In addition, several transcriptome analyses indicated a significant correlation between the presence of flanking intronic regions, containing the reverse complementary sequences (e.g., Alu elements) that may promote intron pairing, and exon circularization [12, 24, 25]. Normally, flanking introns containing inverted tandem repeats, involved in back-splicing and circRNA production, tend to be longer than introns generally, but some can be shorter than average [11]. It has also been demonstrated that relatively short (30–40 nt) inverted repeats are sufficient for intron base pairing and subsequent circRNA formation [26]. However, not all intronic tandem repeats can support exon circularization. In some cases, increased stability of intron base pairing prevented circRNA formation [26]. During the biogenesis process of circRNAs, introns may not be spliced out completely but are retained between the encircled exons in the newly generated circRNA. This phenomenon results in the formation of EIciRNAs [21]. The important role of RNA-binding proteins (RBPs) has been demonstrated in the regulation of circRNA production, which can act as *trans*-acting activators or inhibitors of the circRNA formation mechanism. Quaking (QKI) and muscleblind (MBL/MBNL1) proteins can bind to specific sequence motifs of flanking introns on linear pre-mRNA sequences, thus linking the two flanking introns together, promoting cycling and subsequent circRNA generation [27, 28] (Figure 1(d)). The process is similar to an intron pairing-driven circularization model, only that here, RBPs, after binding to specific putative binding sites, dimerize which leads to pre-mRNA looping. Conversely, the RNA-editing enzyme adenosine deaminase acting on RNA (ADAR) antagonizes circRNA production by direct binding and weakening RNA duplexes (e.g., inverted Alu repeats), through the action of adenosine-to-inosine (A-to-I) editing [25] (Figure 1(e)). High ADAR expression destabilizes intron base pairing interactions, impairing pre-mRNA looping and decreasing the likelihood of pre-mRNA circularization and circRNA formation, for a subset of circRNAs [25, 29].

CiRNA formation differs from that of ecircRNA and EIciRNA (Figure 1(g)). Stable ciRNAs can be formed, when intron lariats escape the usual intron debranching and subsequent degradation, following the canonical spliceosome-mediated pre-mRNA splicing [17]. CiRNA biogenesis depends mainly on a 7 nt GU-rich element near the 5′ splice site and an 11 nt C-rich element near the branch point site. During back-splicing, the two elements bind into a lariat-like intermediate, containing the excised exons and introns, and are cut out by the spliceosome [17, 30]. Then, generated stable lariats undergo 3′ tail degradation, which results in the formation of the final ciRNA molecule [17]. Generally, ciRNAs may be sensitive to RNA debranching enzymes and can be distinguished from ecircRNAs by the presence of a 2′–5′ junction, a residue of the lariat structure, which is evidently absent in ecircRNAs [17]. CiRNAs, along with EIciRNAs, are predominantly located in the nucleus and are believed to be involved in regulating expression of local genes in *cis* [17, 21]. In addition, sequence analyses have shown a weak but significant enrichment of conserved nucleotides between few ciRNAs and intergenic circRNAs [9]. However, there is currently very little information on the overall characteristics and biogenesis processes of intergenic circRNAs.

Despite a good deal of information on circRNA biogenesis, relatively little is known about the metabolic processing of these molecules within cells. Since circRNAs are abundant and highly stable and show resistance to

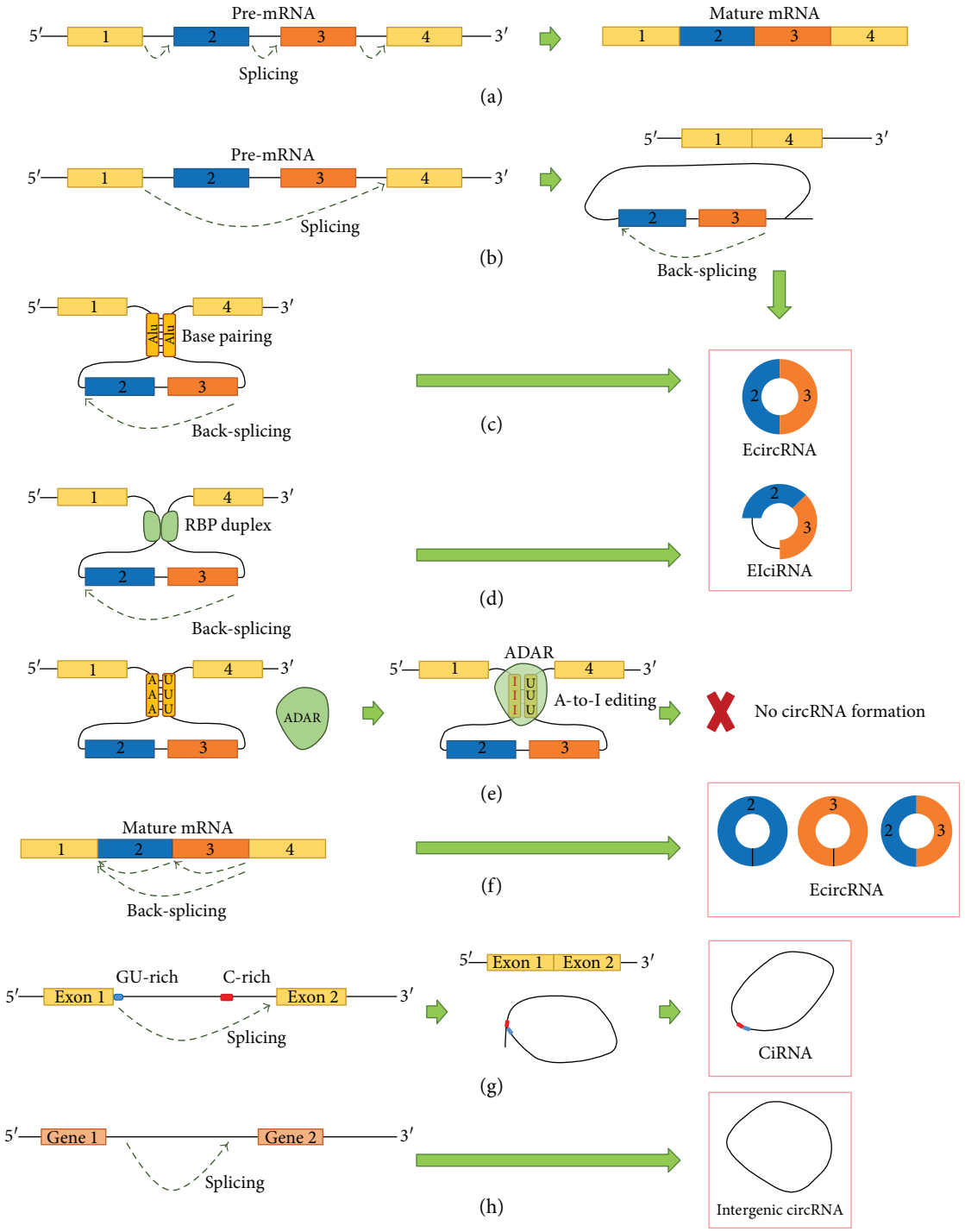

Figure 1: Schematic representation of circRNA biogenesis. (a) Canonical pre-mRNA splicing, yielding a mature mRNA molecule. (b) Lariat-driven circularization (exon skipping). Following canonical splicing, exons in exon-containing lariats undergo back-splicing and circularization, which results in the formation of ecircRNA or EIciRNA molecules. (c) Intron pairing-driven circularization, utilizing coupling of flanking introns by direct base pairing between *cis*-acting regulatory elements that contain reverse complementary sequences (e.g., Alu repeats). Intron pairing is followed by back-splicing and exon circularization. (d) CircRNA biogenesis, mediated by *trans*-acting factors, such as RNA-binding proteins (RBPs) (e.g., QKI, MBL/MBNL1) that bind to specific sequence motifs of flanking introns on linear pre-mRNA, dimerize, and facilitate back-splicing and exon circularization. (e) Regulation of circRNA biogenesis by the RNA-editing enzyme ADAR. ADAR destabilizes intron base pairing interactions through the action of adenosine-to-inosine (A-to-I) editing, which impairs pre-mRNA looping and diminishes exon circularization. (f) Resplicing-driven circularization. EcircRNAs may be formed from mature mRNA exons that undergo back-splicing and circularization. (g) Formation of ciRNAs from intron lariats that escape the usual intron debranching and degradation, following the canonical pre-mRNA splicing. (h) Formation of intergenic circRNAs. This figure is adapted from Wang et al. [151].

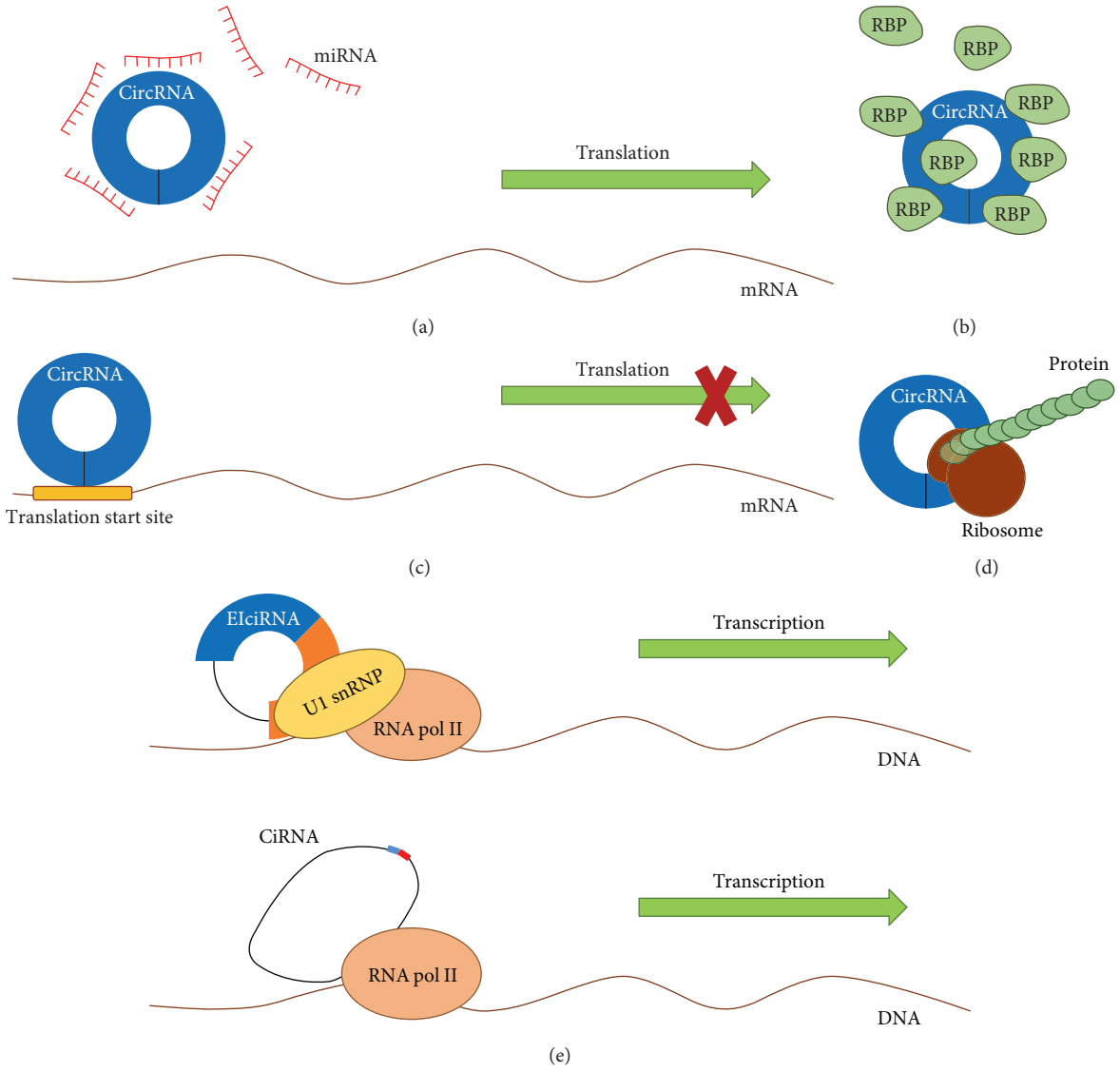

Figure 2: Schematic representation of circRNA functions. (a) CircRNAs may act as miRNA sponges by competing for miRNA binding sites, diminishing the effect of miRNA-mediated regulatory activities. (b) CircRNAs may act as protein sponges, by binding RNA-binding proteins (RBPs). (c) Some circRNAs may regulate protein expression by sequestering mRNA translation start sites. (d) CircRNAs may be translated to form functional proteins. (e) CircRNAs (e.g., EIciRNAs and ciRNAs) may interact with transcription complexes and enhance the expression of their parental genes.

exonucleases (e.g., RNase R) [9, 31], they may accumulate in the cell with a possible toxic effect. A study employing three cell lines has shown that the aggregated excessive circRNAs could be effectively eliminated from cells via released vesicles such as exosomes and microvesicles [32]. An additional study has also demonstrated similar results, where excessive circRNAs were enriched over their linear isoforms within extracellular vesicles, when compared to the producing cells [33].

3. Function of CircRNAs

CircRNAs have important functions in regulating gene expression and may act as miRNA sponges, RBP sponges, and regulators of transcription and translation [21, 34, 35]. Also, several circRNAs have shown the ability to be translated into peptides [18, 36–39]. Schematic representation of these circRNA functions is shown in Figure 2.

miRNAs are approximately 22 nt long ncRNAs, involved in posttranscriptional regulation of gene expression, which act by direct binding to specific target sites within UTRs of mRNAs [40, 41]. The result of such miRNA-mediated mRNA targeting is either blockage of the translation process or complete degradation of the bound mRNA molecule [41]. CircRNAs can act like miRNA sponges by competing for miRNA binding sites (Figure 2(a)), thus diminishing the effect of miRNA-mediated regulatory activities (e.g., posttranscriptional repression) [9, 34]. Indeed, it has been demonstrated that overexpression of miRNA sponge-acting circRNAs increases the expression of miRNA

targets, whereas knockdown of these circRNAs had the opposite effect [34]. The human cerebellar degeneration-related protein 1 antisense (CDR1as) circRNA, also known as a circular RNA sponge for miR-7 (ciRS-7), is the most well characterized circRNA with miRNA sponge function. It contains more than 70 selectively conserved miRNA target sites and associates with Argonaute (AGO) proteins in a miR-7-dependent manner [34]. Overexpression of ciRS-7 leads to a significant decrease in miR-7 activity and results in increasing the miR-7 target gene expression level [34]. CiRS-7 is highly expressed in mammalian brain during neuronal development [29]. Studies in mice have revealed an overlapping coexpression of ciRS-7 and miR-7 in brain tissues, which indicates that this circRNA may be crucial for normal neuronal development [34]. In addition to miR-7 binding sites, ciRS-7 contains an additional binding site for miR-671. The combination of ciRS-7 and miR-671 triggers the linearization and AGO2-mediated cleavage of ciRS-7, which enables the release of the absorbed miR-7 molecules [42]. Mouse testis-specific sex-determining region Y (*Sry*) linear isoform is expressed in the developing genital ridge and has a fundamental role as a transcription factor in sex determination [43]. However, in adult testes, the circular form of *Sry* is expressed, with its circularization being dictated by promotor usage and dependent on intron pairing-driven circularization [43, 44]. *Sry* circRNA serves as the miR-138 sponge and contains 16 miR-138 binding sites [34]. Furthermore, cir-ZNF609 [45], mm9_circ_012559 [46], and circRNAs from the human C_2H_2 zinc finger gene family [47] have also been assumed to function as miRNA sponges. Overall, when compared to linear miRNA sponges, circRNAs have proven themselves as more stable and therefore more effective [48, 49]. Also, their expression is not affected upon miRNA binding [34]. On the other hand, several studies implied that most circRNAs do not act like miRNA sponges, since the majority does not have more miRNA binding sites than colinear mRNAs [47, 50].

It has been demonstrated that circRNAs can bind to several RBPs (Figure 2(b)), including AGO [9, 34], RNA polymerase II [17], QKI [27], EIF4A3 [51], and MBL [28]. In addition, some ecircRNAs can store, sort, or localize RBPs and presumably regulate the RBP function by acting as competing elements, in a similar way as they affect miRNA activity [9, 35].

CircRNAs form a large group of transcriptional and posttranscriptional regulators, and their direct involvement in regulating gene expression has been demonstrated in several studies [9, 17, 21]. CircRNAs (e.g., EIciRNAs and ciRNAs) abundant in the nucleus displayed little target miRNA binding sites, and knockdown of these circRNAs commonly resulted in reduced expression of their parental genes [17, 21]. EIciRNAs, such as circEIF3J and circPAIP2, interact with U1 small nuclear ribonucleoprotein (snRNP) and RNA polymerase II and upregulate their parental genes in *cis* [21] (Figure 2(e)). Thus, these findings strongly imply that circRNAs could function as scaffolds for RBPs regulating transcription, as it had been determined for several lncRNAs [52, 53]. During splicing, circRNAs and their corresponding linear isoforms may compete with each other for biogenesis [28]. However, the generated circular RNA forms may promote both circRNA and mRNA expression [21]. In addition, some circRNAs may also regulate protein expression by sequestering mRNA translation start sites [22] (Figure 2(c)).

It was shown that peptides could be translated from ecircRNAs both *in vitro* and *in vivo*, when RNA molecules contained the internal ribosomal entry site (IRES) elements or prokaryotic ribosome binding sites [36, 54, 55] (Figure 2(d)). A recent study has strongly supported the fact that a subset of *Drosophila* endogenous circRNAs has the ability to be translated *in vivo* in a cap-independent manner. Many of these translating ribosome-associated circRNAs (ribo-circRNAs) shared the start codon with the hosting gene, had evolutionary conserved stop codons, and encoded at least one identifiable ribo-circRNA-specific protein domain, implying these proteins are functional [37]. Another endogenous protein-coding circRNA, circ-ZNF609, has been identified in murine and human myoblasts. Circ-ZNF609 is involved in regulating myoblast proliferation and is generated from the second exon of its host gene. Its open reading frame (ORF) contains a start codon, common with the linear transcript, and an in-frame stop codon, created upon circularization. Circ-ZNF609 is translated into a protein in a splicing-dependent and cap-independent manner. However, molecular activity of circ-ZNF609-derived proteins still needs to be determined [38]. It was also shown that the N^6-methyladenosine (m^6A) RNA modification promotes endogenous circRNA translation in human cells. The study revealed that m^6A motifs are enriched in many circRNAs and enable protein translation in a cap-independent manner, involving the m^6A reader YTHDF3 and translation initiation factors eIF4G2 and eIF3A [39]. In addition, association of many circRNAs with polysomes has been determined [37–39]. These emerging insights into circRNA protein-coding ability and their further characterization may eventually reveal a vast assortment of thus far uncharacterized proteins and throw light on the processes they are involved in. Thus, it is getting progressively clearer that beside their initially proposed regulatory role as ncRNAs, circRNAs may also represent a novel type of protein-coding RNA.

4. CircRNA Databases and Detection Tools

4.1. CircRNA Databases. There are several existing circRNA databases that summarize and integrate the data obtained from large-scale circRNA identification studies, utilizing next-generation sequencing technology, which were performed by different research groups. These databases enable a transparent and comprehensive view on the spatiotemporal presence and function of circRNAs in various biological processes in different organisms, tissues, and cell types. The 9 most acknowledged circRNA databases, containing information on human and animal circRNAs, are presented in Table 1, along with their main features.

The circ2Traits database is a compiled collection of data on circRNAs, categorized according to their potential association with specific human diseases. CircRNAs are grouped based on the number of disease-associated SNPs,

Table 1: Databases containing circRNA data.

Name	URL	Description	References
circ2Traits	http://gyanxet-beta.com/circdb/	A database containing information on disease-associated circRNAs and their complete putative interaction networks with miRNAs, mRNAs, and lncRNAs in specific diseases.	[56]
circBase	http://www.circbase.org/	A collection of merged and unified datasets of circRNAs, with evidence supporting their expression	[57]
CircInteractome	https://circinteractome.nia.nih.gov/	A web tool designed for predicting and mapping RBP and miRNA binding sites on reported circRNAs	[51]
CircNet	http://circnet.mbc.nctu.edu.tw/	A database providing information on known and novel circRNAs, circRNA-miRNA-gene regulatory networks, and tissue-specific circRNA expression profiles	[58]
CIRCpedia	http://www.picb.ac.cn/rnomics/circpedia/	A database holding information on identified and annotated back-splicing and alternative splicing in circRNAs from human, mouse, fly, and worm samples	[19]
circRNADb	http://reprod.njmu.edu.cn/circrnadb	A comprehensive database for human circRNAs with protein-coding annotations	[59]
starBase v2.0	http://starbase.sysu.edu.cn/	A database for decoding predicted interaction networks between lncRNAs, miRNAs, circRNAs, mRNAs, and RBPs from large-scale CLIP-seq data	[60]
deepBase v2.0	http://deepbase.sysu.edu.cn/	A platform for annotating, discovering, and characterizing small ncRNAs, lncRNAs, and circRNAs from next-generation sequencing data	[61]
TSCD	http://gb.whu.edu.cn/TSCD/	An integrated database designed for depositing features of human and mouse tissue-specific circRNAs	[62]

AGO interaction sites, and their potential interaction with disease-associated miRNAs, as determined by genome-wide association studies. circ2Traits also stores complete putative miRNA-circRNA-mRNA-lncRNA interaction networks for each of the described diseases [56].

The circBase database provides merged and unified datasets of circRNAs and the evidence supporting their expression. It is a database repository, containing circRNA data from various samples of several species, including human, mouse, fruit fly, and nematode. circBase enables exploring of public circRNA datasets and also provides custom python scripts, needed to discover novel circRNA from user's own (RiboMinus) RNA-seq data. All circRNA transcripts deposited in circBase have been annotated, their putative splice forms predicted and, where applicable, alignments of reads spanning head-to-tail junctions provided [57].

CircInteractome is a web tool designed for mapping RBP and miRNA binding sites on human circRNAs. CircInteractome also enables the identification of potential circRNAs that act as RBP sponges, designing junction-spanning primers for specific circRNA detection, designing siRNAs for circRNA silencing, and identifying potential IRES [51]. However, CircInteractome displays limited ability to predict RBP and miRNA interactions when circRNAs form secondary or tertiary structures. Thus, experimental validation is often needed to reliably verify RBP and miRNA functional sites [51].

The CircNet database provides information on novel circRNAs, integrated miRNA target networks, expression profile of circRNA isoforms, genomic annotation of circRNA isoforms, and sequence features of circRNA isoforms. CircNet also provides tissue-specific circRNA expression profiles, circRNA-miRNA-gene regulatory networks, and a thorough expression analysis of previously reported and novel circRNAs. Furthermore, CircNet generates an integrated regulatory network that illustrates the regulation between circRNAs, miRNAs, and genes [58].

CIRCpedia is an integrative database, which employs the CIRCexplorer2 characterization pipeline for identifying and annotating back-splicing and alternative splicing in circRNAs across different cell lines. Identified back-splicing and alternative splicing in circRNAs, together with novel exons, are formatted and classified for being easily searched, browsed, and downloaded. Currently, CIRCpedia contains information on circRNAs from human, mouse, fly, and worm samples [19].

The circRNADb database is a comprehensive database for human circRNAs with protein-coding annotations. circRNADb provides detailed information on circRNA genomic properties, exon splicing, genome sequences, annotated protein-coding potential, IRES, ORF, and corresponding references [59].

The starBase v2.0 database has been developed to systematically identify RNA-RNA and protein-RNA interaction networks from large-scale CLIP-seq datasets, generated by independent studies. starBase v2.0 distinctive features facilitate annotation, graphic visualization, analysis, and discovery of miRNA-mRNA, miRNA-circRNA, miRNA-pseudogene, miRNA-lncRNA, and protein-RNA interaction networks and RBP binding sites [60].

deepBase v2.0 is a platform for annotating and discovering small ncRNAs, lncRNAs, and circRNAs from next-generation sequencing data. deepBase v2.0 provides a set of tools to decode evolution, spatiotemporal expression patterns, and functions of diverse ncRNAs across 19 species from 5 clades, including human, mouse, fruit fly, and nematode. The platform also provides an integrative, interactive, and versatile web graphical interface to display multidimensional data and facilitates transcriptomic research and the discovery of novel ncRNAs [61].

TSCD (Tissue-Specific CircRNA Database) is an integrated database designed for depositing features of human and mouse tissue-specific circRNAs. TSCD provides a global view on tissue-specific circRNAs and holds information on their genomic location and conservation [62].

4.2. CircRNA Detection Tools. The detection and integration of newly discovered circRNAs into circRNA databases are predominantly dependent on complex bioinformatics analyses of large-scale RNA-seq data. In order to identify and annotate novel circRNAs, RNA-seq data undergoes thorough and rigorous analysis utilizing various state-of-the-art circRNA detection tools and software packages. A comprehensive overview and evaluation of 11 different circRNA detection computational pipelines have been summarized in a recent review by Zeng et al. [63]. Among the described circRNA detection tools, which were also compared with regard to their precision and sensitivity, were circRNA_finder [64], CIRCexplorer [24], DCC [65], find_circ [9], UROBORUS [66], PTESFinder [67], KNIFE [68], CIRI [69], MapSplice [70], segemehl [71], and NCLscan [72]. In general, these circRNA detection tools can be divided into two categories, based on the different strategies used for circRNA identification, according to the dependency on genome annotation [63]. In the pseudo-reference-based [73] or candidate-based approach [22], all possible combinations of candidate circRNAs are constructed. Each candidate comprises two well-annotated exons in which the exon order is topologically inconsistent with the reference genome. The candidate is regarded as a circRNA if at least one RNA-seq read has been identified, which maps to its noncolinear junction site. The strategy relies on candidate circRNAs that are constructed from preexisting gene models and does not detect circRNAs from unannotated transcripts [22, 73]. Conversely, the fragmented-based [73] or segmented read approach [22] does not rely on genome annotation. In this approach, RNA-seq reads are mapped to genomic locations de novo. Reads that cannot be mapped directly are split into two or more segments, and each segment is mapped separately to the reference genome. CircRNA back-splicing junctions are identified, when segmented reads are mapped in a noncolinear manner [22, 73]. As described by Zeng et al. [63], the tools that use the pseudo-reference-based approach to detect circRNAs include PTESFinder, KNIFE, and NCLscan, whereas circRNA_finder, CIRCexplorer, DCC, find_circ, UROBORUS, CIRI, MapSplice, and segemehl utilize the fragmented-based approach. In addition, several integrated tools aimed to identify circRNAs with a protein-coding potential have been developed, including CircPro [74], IRESite [75], CPAT [76], Pfam 31.0 [77], PhyloCSF [78], and ORF Finder from the NCBI database. Also, the TopHat-Fusion algorithm has been designed to detect circRNAs that are derived from gene fusion events [79].

Despite several useful state-of-the-art circRNA detection tools exist, their regular upgrades and the introduction of novel, improved methods, with even higher precision and sensitivity, are a prerequisite to overcome current and future challenges in circRNA identification and characterization studies.

5. CircRNAs in Cancer

Current knowledge about the involvement of circRNAs in cancer development and progression is limited, and the role of circRNAs as miRNA sponges has been proposed as the most frequent mechanism of circRNA activity in tumor cells [80]. Generally, miRNAs are included in various cell processes, including cellular differentiation, development, proliferation, and apoptosis, where they play an important role as regulators of gene expression [81]. These miRNA-mediated processes are frequently deregulated in cancer and can contribute to cancer initiation and progression [81]. Since many circRNAs regulate miRNA action through sponge-like binding (several are presented in Table 2), dysregulation in circRNA expression may affect their interaction with tumor-associated miRNAs, indicating an important role of circRNAs in regulating cancer.

There is emerging evidence that miR-7 can directly downregulate oncogenes in a variety of cancers [82, 83]. miR-7 has been shown to be involved in suppressing melanoma [84], breast cancer [85], glioma [86], gastric cancer [87], liver cancer [88], non-small-cell lung cancer (NSCLC) [89], colorectal cancer [90], and other cancer types. Since circRNA ciRS-7 acts as a miR-7 sponge, quenching the activity of miR-7 may increase the expression levels of miR-7 target oncogenes, resulting in a decreased tumor suppression [83]. Thereby, the ciRS-7/miR-7 axis is likely involved in cancer-related pathways and cancer development and progression [83]. However, despite its potential oncogenic properties, only few studies have revealed ciRS-7 involvement in cancer development through miR-7 binding. CiRS-7-mediated oncogenic activity, acting partly through targeting miR-7, was recently demonstrated in cancer tissues of hepatocellular carcinoma (HCC) patients. When compared to healthy adjacent tissues, ciRS-7 expression levels were significantly upregulated and ciRS-7 expression inversely correlated with miR-7 [91]. In addition, knockdown of ciRS-7 in HCC cell lines suppressed cell invasion and proliferation through miR-7 targeting [91]. Similarly, elevated expression of ciRS-7 was determined in colorectal cancer tissues, when compared to those of the adjacent normal mucosa, which was positively associated with tumor size, T stage, lymph node metastasis, and poor overall survival [92]. Downregulation of ciRS-7 increased miR-7 expression and significantly suppressed colorectal cancer cell proliferation and invasion. As

Table 2: CircRNAs associated with cancer.

CircRNA	Gene symbol	Cancer type	Expression	Fold change[a]	Function	References
CiRS-7/CDR1as*	*CDR1*	HCC	Up	NA	Sponge: miR-7	[91]
		HCC*	Down[b]	NA	Sponge: miR-7	[137]
		Colorectal	Up	NA	Sponge: miR-7	[92]
		Colorectal*	Up	2.4	Sponge: miR-7	[132]
		GBM	Down	3.5	Target of miR-671-5p Association with cell proliferation and migration	[94]
Circ-Foxo3	*FOXO3*	Breast	Down	NA	Sponge: miR-22, miR-136, miR-138, miR-149, miR-433, miR-762, miR-3614-5p, miR-3622b-5p Association with apoptosis-related proteins Foxo3, MDM2, p53, and Puma and cell cycle proteins CDK2 and p21	[95–97]
Circ-ABCB10	*ABCB10*	Breast	Up	5.0–10.0	Sponge: miR-1271	[98]
Hsa_circ_001569	*ABCC1*	Colorectal	Up	NA	Sponge: miR-145	[99]
		HCC	Up	NA	Promoting tumor growth	[100]
CircHIPK3	*HIPK3*	HCC	Up	NA	Sponge: miR-124	[101]
		Bladder	Down	4.6	Sponge: miR-558	[103]
Cir-ITCH	*ITCH*	ESCC	Down	NA	Sponge: miR-7, miR-17, miR-214 Inhibition of the Wnt/β-catenin pathway	[104]
		Colorectal	Down	NA	Sponge: miR-7, miR-20a Inhibition of the Wnt/β-catenin pathway	[105]
		Lung	Down	NA	Sponge: miR-7, miR-214 Inhibition of the Wnt/β-catenin pathway	[106]
Hsa_circ_0067934	*PRKCI*	ESCC	Up	8.8	Promoting cell proliferation and migration	[107]
Circ-ZEB1.5 Circ-ZEB1.19 Circ-ZEB1.17 Circ-ZEB1.33	*ZEB1*	Lung	Down	NA	Sponge: miR-200a-3p	[58]
CircMYLK	*MYLK*	Bladder	Up	NA	Sponge: miR-29a-3p	[109]
CircRNA_100290	*SLC30A7*	OSCC	Up	6.9	Sponge: miR-29 family	[110]
Hsa_circ_0016347	*KCNH1*	Osteosarcoma	Up	NA	Sponge: miR-214	[111]
Hsa_circ_0001564	*CANX*	Osteosarcoma	Up	NA	Sponge: miR-29c-3p	[114]
CircRNA_100269	*LPHN2*	Gastric	Down	NA	Sponge: miR-630	[116]
Hsa_circ_0020397	*DOCK1*	Colorectal	Up	NA	Sponge: miR-138	[117]
Hsa_circ_0000069	*STIL*	Colorectal	Up	≥1.0	Promoting cell proliferation, invasion, and migration	[118]
Circ-TTBK2	*TTBK2*	Glioma	Up	NA	Sponge: miR-217	[121]
cZNF292	*ZNF292*	Glioma	Up	NA	Promoting cell proliferation and tube formation	[122]
f-circRNA	*PML/RARα*[c]	APL	Up	NA	Promoting cell proliferation, transformation, and tumorigenesis	[123]
CircTCF25*	*TCF25*	Bladder	Up	21.4	Sponge: miR-103a-3p, miR-107	[129]
Hsa_circ_001988*	*FBXW7*	Colorectal	Down	NA	ND	[130]
Hsa_circRNA_103809*	*ZFR*	Colorectal	Down	3.6	Sponge: miR-511-5p, miR-130b-5p, miR-642a-5p, miR-532-3p, miR-329-5p	[131]
Hsa_circRNA_104700*	*PTK2*	Colorectal	Down	4.2	Sponge: miR-141-5p, miR-500a-5p, miR-509-3p, miR-619-3p, miR-578	[131]
CircRNA_100876*	*RNF121*	NSCLC	Up	1.2	Sponge: miR-136	[133]

Table 2: Continued.

CircRNA	Gene symbol	Cancer type	Expression	Fold change[a]	Function	References
Hsa_circ_0001649*	*SHPRH*	HCC*	Down	NA	Sponge: miR-1283, miR-4310, miR-182-3p, miR-888-3p, miR-4502, miR-6811, miR-6511b-5p, miR-1972 Promoting metastasis	[127]
		Gastric*	Down	NA	ND	[141]
Hsa_circ_0005075*	*EIF4G3*	HCC	Up	NA	Sponge: miR-23b-5p, miR-93-3p, miR-581, miR-23a-5p Promoting cell adhesion	[128]
CircZKSCAN1*	*ZKSCAN1*	HCC	Down	NA	Inhibition of cellular growth, migration, and invasion	[138]
Hsa_circ_0005986	*PRDM2*	HCC	Down	2.9	Sponge: miR-129-5p	[139]
Hsa_circ_0004018*	*SMYD4*	HCC	Down	NA	Sponge: miR-30e-5p, miR-647, miR-92a-5p, miR-660-3p, miR-626	[140]
Hsa_circ_002059*	*KIAA0907*	Gastric	Down	NA	ND	[16]
Hsa_circ_0000096*	*HIAT1*	Gastric	Down	NA	Sponge: miR-224, miR-200a Inhibition of cell growth and migration	[142]
Hsa_circ_0001895*	*PRRC2B*	Gastric	Down	NA	ND	[143]
Hsa_circ_0006633*	*FGGY*	Gastric	Down	NA	ND	[144]
Hsa_circ_0000190*	*CNIH4*	Gastric	Down	NA	ND	[145]
Hsa_circ_0003159*	*CACNA2D1*	Gastric	Down	NA	ND	[146]
CircPVT1*	*PVT1*	Gastric	Up	NA	Sponge: miR-125 family Promoting cell proliferation	[148]
Hsa_circ_100855*	*C11orf80*	LSCC	Up	10.5	ND	[150]
Hsa_circ_104912*	*DENND1A*	LSCC	Down	4.7	ND	[150]

HCC: hepatocellular carcinoma; GBM: glioblastoma multiforme; ESCC: esophageal squamous cell carcinoma; OSCC: oral squamous cell carcinoma; APL: acute promyelocytic leukemia; NSCLC: non-small-cell lung cancer; LSCC: laryngeal squamous cell cancer. Up: upregulated; down: downregulated. NA: not available (data is presented in a graphical format in the original report). ND: not determined. *Potential cancer biomarker. [a]Fold change values, relative to normal controls. [b]Expression levels were not statistically significant. [c]One or more f-circRNAs were generated from PML/RARα fusion gene, a product of the most recurrent cancer-associated aberrant chromosomal translocation in APL. In addition, other chromosomal translocations may also generate f-circRNAs.

demonstrated, ciRS-7 blocked miR-7 activity and positively regulated the expression of *EGFR* and *IGF-1R* oncogenes, indicating that the ciRS-7/miR-7 axis was associated with colorectal cancer progression. However, ciRS-7 may also regulate colorectal cancer progression through other mechanisms than as a miR-7 sponge [92]. Conversely, miR-7 overexpression has also been associated with upregulated oncogenes in several tumor cell lines and advanced colorectal cancer tissues, when compared to healthy controls [93]. This suggests that miR-671-mediated degradation of ciRS-7 may diminish the ciRS-7-mediated miR-7 inhibition and enhance miR-7 levels in tumor cells [83]. As a consequence, such miR-671 action may contribute to the increase in downstream target oncogenes (e.g., *EGFR* and *XIAP*) and promote vascularization, metastasis, and amplification of tumor cells [83]. It was also demonstrated that overexpression of miR-671-5p in glioblastoma multiforme (GBM) biopsies and cell lines increased the migration and proliferation rates of GBM cells [94]. Furthermore, overexpression of miR-671-5p negatively correlated with the expression of ciRS-7, *CDR1*, and *VSNL1*, which implied that the miR-671-5p/CDR1as/CDR1/VSNL1 axis was functionally altered in GBM [94].

The tumor suppressor gene *FOXO3* encodes two ncRNAs, the pseudogene Foxo3P and circ-Foxo3, both of which may act as miRNA sponges. Foxo3P and circ-Foxo3 were highly expressed in noncancerous cells and could function as miRNA sponges for several cancer-associated miRNAs, including miR-22, miR-136, miR-138, miR-149, miR-433, miR-762, miR-3614-5p, and miR-3622b-5p. However, among the two, circ-Foxo3 appeared to possess a stronger sponging effect on these miRNAs [95]. In human breast cancer cell lines, Foxo3P and circ-Foxo3 promoted the translation of Foxo3 mRNA by binding regulatory miRNAs and increased Foxo3-mediated apoptosis. Also, mouse xenograft models for breast cancer showed arrested tumor growth in the presence of circ-Foxo3 due to apoptosis, induced through combining circ-Foxo3, Foxo3P, and Foxo3 activity, when compared to controls [95]. Ectopic expression of circ-Foxo3 can result in the formation of the circ-Foxo3-p21-CDK2 ternary complex, arising from binding circ-Foxo3 to cell cycle proteins CDK2 and p21. The circ-Foxo3-p21-CDK2 ternary complex can suppress cell cycle progression and inhibit tumor growth [96]. In addition, expression of circ-Foxo3 was significantly increased during breast cancer cell apoptosis, where circ-Foxo3 effectively

bound to p53 and MDM2 proteins. As demonstrated, elevated expression of circ-Foxo3 increased Foxo3 protein levels but repressed p53 activity by promoting MDM2-induced p53 ubiquitination and subsequent degradation. Overexpression of circ-Foxo3 decreased the interaction between Foxo3 and MDM2, increased Foxo3 activity, and promoted cell apoptosis, through upregulating Puma expression [97]. Beside circ-Foxo3, circ-ABCB10 is another circRNA associated with breast cancer. Circ-ABCB10 was significantly upregulated in breast cancer tissues, and its function as a sponge for miR-1271 has been determined. Furthermore, *in vitro* circ-ABCB10 knockdown suppressed proliferation and increased apoptosis of breast cancer cells [98].

Hsa_circ_001569 was significantly overexpressed in colorectal cancer tissues and was positively correlated with the degree of clinical features (TNM stage) [99]. Hsa_circ_001569 may act as a miR-145 sponge and represses the transcriptional activities of miR-145, enabling upregulation of miR-145 target genes *E2F5*, *BAG4*, and *FMNL2*. Thus, it acts as a positive regulator in cell proliferation and invasion of colorectal cancer [99]. Elevated expression levels of hsa_circ_001569 were also determined in HCC tissues, when compared to adjacent normal tissues. As in colorectal cancer, expression of hsa_circ_001569 correlated with tumor differentiation and TNM stages in HCC. The inhibitory effect on HCC cell proliferation and tumor growth through hsa_circ_001569 silencing was also demonstrated [100].

Among many dysregulated circRNAs in several cancer types, a significant upregulation of circHIPK3 in HCC has been demonstrated, when compared with its expression in matched normal tissues [101]. CircHIPK3 could bind to 9 miRNAs with its 18 potential binding sites, including the tumor-suppressive miR-124, thus inhibiting its activity. Furthermore, circHIPK3 silencing significantly inhibited human cancer cell proliferation [101]. By inhibiting miR-124 activity, circHIPK3 might influence the proliferation of tumor cells in prostate cancer through several oncogenes, including *iASPP* [102]. CircHIPK3 was also significantly downregulated in bladder cancer tissues and cell lines, where it negatively correlated with cancer grade, invasion, and lymph node metastasis [103]. Mechanistic studies revealed that circHIPK3 abundantly sponged miR-558 and suppressed heparanase (HPSE) expression, which is involved in regulating tumor invasion and metastasis. In addition, overexpression of circHIPK3 effectively inhibited migration, invasion, and angiogenesis of bladder cancer cells *in vitro* and suppressed bladder cancer growth and metastasis *in vivo*, mainly through targeting the miR-558/heparanase axis [103].

CircRNA cir-ITCH may act as a miRNA sponge for cancer-associated miR-7, miR-17, and miR-214 in esophageal squamous cell carcinoma (ESCC) [104] and miR-7 and miR-20a in colorectal cancer [105] and as a sponge for miR-7 and miR-214 in lung cancer [106]. Cir-ITCH expression was downregulated in ESCC, colorectal, and lung cancer tissues, when compared to adjacent peritumoral tissues. In all three cancer types, cir-ITCH activity could increase the level of ITCH protein, a regulator of several tumor-associated proteins, which is involved in the inhibition of the Wnt/β-catenin signaling pathway. Therefore, cir-ITCH likely plays an inhibitory role in ESCC and colorectal and lung cancer, through promoting ITCH-mediated ubiquitination and subsequent proteasome-mediated degradation of phosphorylated Dvl2 scaffold protein, which impairs the canonical Wnt/β-catenin signaling [104–106].

Involvement of another circRNA in promoting ESCC has been recently demonstrated. Hsa_circ_0067934 was significantly overexpressed in ESCC tissues, when compared to adjacent healthy tissues, and its expression positively correlated with tumor differentiation, T stage, and TNM stage [107]. *In vitro* studies revealed that hsa_circ_0067934 promoted ESCC cell proliferation, and its presence in the cytoplasm suggested that hsa_circ_0067934 was involved in posttranscriptional regulation of the ESCC cell cycle [107]. However, the exact molecular function of hsa_circ_0067934 still needs to be determined. Nevertheless, it would be interesting to assess the possibility of correlation between hsa_circ_0067934 and cir-ITCH in the development of ESCC, due to their apparent contrary modes of action.

Identification of four circRNAs associated with lung cancer has been performed, based on computational predictions utilizing transcriptome sequencing datasets, by using the CircNet database. As demonstrated, circRNAs circ-ZEB1.5, circ-ZEB1.19, circ-ZEB1.17, and circ-ZEB1.33 were upregulated in normal lung tissues, when compared to lung cancer samples, and are presumably implicated in lung cancer suppression by binding to miR-200a-3p [58], which has been reported to target *ZEB1* and to promote cancer initiation [108]. Similarly, bioinformatics approaches utilizing correlated coexpression networks of bladder cancer revealed a probable interaction between lncRNA H19 and circRNA circMYLK, demonstrating their ability to competitively bind to miR-29a-3p. Such miR-29a-3p targeting might increase the expression of *DNMT3B*, *VEGFA*, and *ITGB1* oncogenes, which suggested a possible involvement of H19 and circMYLK in the development, growth, and metastasis of bladder cancer [109].

CircRNA_100290 was upregulated and coexpressed with CDK6, a member of the cyclin-dependent kinase family, in oral squamous cell carcinoma (OSCC) tissues, when matched with noncancerous tissue samples [110]. CircRNA_100290 could directly bind to miR-29 family members, including miR-29a, miR-29b, and miR-29c. Since CDK6 has been determined as the direct target of miR-29b, circRNA_100290 evidently regulates CDK6 expression through sponging miR-29. Furthermore, knockdown of circRNA_100290 decreased the expression of CDK6, induced G1/S arrest, inhibited proliferation of OSCC cell lines *in vitro*, and decreased the growth of tumors *in vivo*. Thus, circRNA_100290 likely functions as a regulator of cell cycle and cell proliferation [110].

Involvement of two circRNAs in regulating osteosarcoma has been demonstrated recently. However, no correlation between the two in promoting osteosarcoma has been determined yet. Hsa_circ_0016347 was significantly upregulated in osteosarcoma tissues and cell lines, when compared to adjacent nontumor tissues and normal osteoblasts, and has been shown to sponge miR-214 [111], which is a known tumor promoter in osteosarcoma [112, 113]. By inhibiting

miR-214 activity, hsa_circ_0016347 increased the expression level of caspase-1, a direct target of miR-214, thus enabling the formation of favorable tumor microenvironment and promoting proliferation, invasion, and metastasis of osteosarcoma cells [111]. Also, overexpression of hsa_circ_0016347 increased either the size or the number of pulmonary metastasis tumors [111]. In addition to hsa_circ_0016347, a significant upregulation of hsa_circ_0001564 has been determined in osteosarcoma tissues and cell lines, which acted as a miR-29c-3p sponge [114]. Through inhibiting miR-29c-3p activity, hsa_circ_0001564 promoted tumorigenesis of osteosarcoma by regulating cell cycle and proliferation of osteosarcoma cells [114].

As demonstrated before, circRNA_100269 has been included in a group of circRNAs constituting the four-circRNA-based classifier, which was used to predict the early recurrence of stage III gastric cancer after radical surgery [115]. Further analysis has revealed a significantly downregulated level of circRNA_100269 in gastric cancer tissues, than in the corresponding adjacent healthy tissues, which correlated with histological subtypes and the node invasion number [116]. The study suggested that circRNA_100269 inhibits gastric cancer cell proliferation via inhibiting miR-630 activity, whose expression was negatively correlated with that of circRNA_100269 [116]. However, no confirmation of such circRNA_100269 action has been performed *in vivo*.

A negative correlation between expression profiles of miR-138 and hsa_circ_0020397 was determined in colorectal cancer cells, where hsa_circ_0020397 was significantly upregulated [117]. As determined, hsa_circ_0020397 acted as a miR-138 sponge and promoted the expression of miR-138 targets TERT and PD-L1, which promoted viability and invasion of colorectal cancer cells and inhibited their apoptosis [117]. In addition, circRNA hsa_circ_0000069 was also associated with colorectal cancer and was significantly upregulated in colorectal cancer tissues and cell lines, when compared to healthy controls [118]. Elevated expression of hsa_circ_0000069 correlated with the tumor TNM stage and could promote colorectal cancer cell proliferation, invasion, and migration *in vitro* [118]. However, a more detailed mechanism of hsa_circ_0000069 function still needs to be determined.

miR-217 is a tumor-suppressive miRNA, associated with various cancer types, including epithelial ovarian cancer [119] and gastric cancer [120]. In glioma, miR-217 negatively correlated with the pathological grades of tumors and exerted tumor-suppressive activity in glioma cells [121]. CircRNA circ-TTBK2 was significantly upregulated in glioma tissues and cell lines and acted as a miR-217 sponge. By sequestering miR-217 activity, circ-TTBK2 enabled higher expression of oncogenic proteins HNF1β and Derlin-1, which promoted cell proliferation, migration, and invasion, while inhibiting apoptosis of glioma cells [121]. As demonstrated, miR-217 expression was negatively regulated by circ-TTBK2 expression in an AGO2-dependent manner and there was a reciprocal repression feedback loop between circ-TTBK2 and miR-217 [121]. In addition, circ-TTBK2 knockdown in combination with miR-217 overexpression led to tumor regression *in vivo* [121].

In addition to circRNAs that predominantly act like miRNA sponges, few circRNAs appear not to function in such a manner (Table 2). It has been demonstrated that downregulation of cZNF292 suppresses human glioma tube formation via the Wnt/β-catenin signaling pathway. Thus, cZNF292 downregulation also resulted in inhibition of glioma cell proliferation and cell cycle progression [122]. However, the exact mechanism of cZNF292 activity still needs to be determined. Furthermore, chromosomal translocations may give rise to oncogenic fusion proteins that are often involved in the onset and progression of various cancers. Such cancer-associated chromosomal translocations may also result in the formation of fusion circRNAs (f-circRNAs), which are produced from transcribed exons of genes affected by these oncogenic translocations [123]. Among several distinctive chromosomal translocations in leukemia, *PML*/*RAR*α is the most frequent translocation in acute promyelocytic leukemia (APL) [124], which can generate one or more f-circRNAs from this fusion gene [123]. In addition, *MLL*/*AF9* aberrant translocation also generated several f-circRNAs in APL [123]. As demonstrated, f-circRNAs in combination with other oncogenic stimuli, including oncogenic fusion proteins, played an important role in promoting APL cell proliferation, transformation, and tumorigenesis progression *in vivo* [123]. In addition to APL, f-circRNAs have also been identified in SK-NEP-1 sarcoma and H3122 lung cancer cell lines [123]. Thus, this study strongly implied that f-circRNAs may have a potential diagnostic and therapeutic value in cancer.

In addition to the above-listed circRNAs, several research groups have identified a vast assortment of circRNAs, by using RNA-seq and other next-generation sequencing techniques that are likely involved in mechanisms which promote various cancers. The majority of the generated data can be obtained from several circRNA databases, which are presented in Table 1. Despite candidate cancer-specific circRNAs are getting discovered on a regular basis, the data currently remains insufficient to definitely associate individual circRNAs with a specific mechanism promoting a certain cancer type. However, it has recently become clear that circRNAs may represent promising biomarkers for various cancer types.

6. CircRNAs as Cancer Biomarkers

CircRNAs are abundant and highly stable molecules, exhibiting high cell/tissue and developmental stage specificity [9, 11]. The unique circular structure makes circRNAs insensitive to ribonucleases and enables them to exist intact in various tissues and body fluids. It has been shown that circRNAs may be stably expressed and present in relatively high quantities in human blood [125], saliva [126], and exosomes [33]. These characteristics make circRNAs ideal candidates as noninvasive biomarkers for cancer diagnosis, prognosis, and treatment. In addition, some circRNAs may correlate with age, gender, TNM stage, metastasis, and tumor size as it was determined for gastric cancer [16], HCC [127, 128], and colorectal cancer [99], additionally implying their suitability as cancer biomarkers. A number

of circRNAs that have been associated with human cancer are presented in Table 2. From these circRNAs, several have been tested for their diagnostic performance and may eventually become novel biomarkers for cancer diagnosis in clinical practice.

6.1. CircRNAs as Biomarkers for Bladder Cancer. CircRNA circTCF25 was found to be highly expressed in bladder cancer tissues, when compared to healthy controls. The analysis was performed by using a total of 40 paired snap-frozen bladder carcinoma and matched paracarcinoma tissue samples. The study showed that circTCF25 promotes proliferation and metastasis of urinary bladder carcinoma by acting as a sponge for miR-103a-3p and miR-107, which resulted in upregulated CDK6 expression [129]. The data also suggested that circTCF25 may be a new promising biomarker for bladder cancer [129]. However, the diagnostic performance of this circRNA still needs to be determined.

6.2. CircRNAs as Biomarkers for Colorectal Cancer. CircRNA hsa_circ_001988 has been identified in colorectal cancer and has been significantly downregulated in colorectal cancer tissues, when compared to those of the matched normal mucosa ($n = 31$) [130]. Evaluation of the diagnostic performance of hsa_circ_001988 has shown its sensitivity of 68.0% and specificity of 73.0%. The receiver operating characteristic curve (ROC) analysis showed an area under the ROC curve (AUC) of 0.788, indicating that hsa_circ_001988 may become a novel potential biomarker in the diagnosis of colorectal cancer [130].

CircRNAs hsa_circRNA_103809 and hsa_circRNA_104700 were also significantly downregulated in colorectal cancer tissues, where hsa_circRNA_103809 correlated with lymph node metastasis and TNM stage and hsa_circRNA_104700 with distal metastasis [131]. Analysis of both circRNAs was performed on 170 paired colorectal cancer tissues and matched adjacent noncancerous tissue samples. The evaluated diagnostic performances for hsa_circRNA_103809 (AUC 0.699) and hsa_circRNA_104700 (AUC 0.616) indicated that both circRNAs may serve as reliable biomarkers for colorectal cancer [131]. However, beside their dysregulation and putative miRNA binding site determination [131], the exact mechanisms of function for both circRNAs in colorectal cancer development have not been elucidated yet.

Despite its assumed involvement in promoting various cancer types, mainly due to its ability to sponge miR-7, the clinical significance of ciRS-7 in colorectal cancer was only recently demonstrated. CiRS-7 was significantly upregulated in tumor tissues of colorectal cancer patients and correlated with advanced tumor stage, tumor depth, and metastasis [132]. The study included a training cohort comprised of 153 primary colorectal cancer tissues and 44 matched normal mucosa tissues and an additional independent validation cohort ($n = 165$). Correlation of upregulated ciRS-7 expression levels with poor patient survival strongly suggested that ciRS-7 might serve as a novel prognostic biomarker in colorectal cancer patients [132]. *In vitro* experiments revealed that ciRS-7 inhibited miR-7 activity and activated the EGFR/RAF1/MAPK pathway, which linked ciRS-7 activity with colorectal cancer progression and aggressiveness [132]. Regarding the data obtained from the study, ciRS-7 suppression could increase the expression levels of miR-7 and reduce EGFR-RAF1 activity. Thus, therapeutic targeting of ciRS-7 might represent a potential treatment option for patients with colorectal cancer [132].

In addition, elevated expression levels of circRNA circ-KLDHC10 in serum samples of colorectal cancer patients were determined, when compared to those in healthy controls ($n = 11$ for both sample groups). Since circ-KLDHC10 was abundant in exosomes, it has the potential to serve as a novel circulating biomarker for colorectal cancer [33]. However, its oncogenic activity and diagnostic performance in colorectal cancer still need to be determined.

6.3. CircRNAs as Biomarkers for Non-Small-Cell Lung Cancer (NSCLC). CircRNA_100876 was significantly upregulated in NSCLC tissues, when compared to their paired adjacent nontumorous tissues ($n = 101$), and its elevated levels closely correlated with lymph node metastasis and advanced tumor staging in NSCLC [133]. As determined in a previous study, circRNA_100876 could regulate MMP-13 expression through inhibiting miR-136 activity and thus participated in chondrocyte extracellular matrix degradation [134]. Since MMP-13 is often overexpressed in lung cancer and can increase the risk of metastasis [135, 136], circRNA_100876 might be involved in tumor cell growth, progression, and metastasis in NSCLC, by regulating MMP-13 expression as a miRNA sponge [133]. The Kaplan-Meier survival analysis showed significantly shorter overall survival times of NSCLC patients with elevated circRNA_100876 expression levels, when compared to patients with low expression levels of circRNA_100876. Therefore, circRNA_100876 could be gradually used as a novel prognostic biomarker for NSCLC [133].

6.4. CircRNAs as Biomarkers for Hepatocellular Carcinoma (HCC). Hsa_circ_0001649 was significantly downregulated in HCC tissues, when compared to paired adjacent healthy liver tissues ($n = 89$), and its expression levels correlated with tumor size and the occurrence of tumor embolus in HCC [127]. Hsa_circ_0001649 may play a role in tumorigenesis and metastasis of HCC through sponge-like activity toward several miRNAs, including miR-1283, miR-4310, miR-182-3p, miR-888-3p, miR-4502, miR-6811, miR-6511b-5p, and miR-1972 [127]. The evaluated diagnostic performance (sensitivity 81.0%; specificity 69.0%; and AUC 0.63) indicated that hsa_circ_0001649 might serve as a novel potential biomarker for HCC, with relatively high degrees of accuracy, specificity, and sensitivity [127].

Another circRNA associated with HCC is hsa_circ_0005075, which was significantly upregulated in HCC tissues, when compared to paired adjacent normal liver tissues ($n = 30$) [128]. Hsa_circ_0005075 correlated with tumor size and showed a great diagnostic potential with a sensitivity of 83.3%, specificity of 90.0%, and AUC of 0.94 [128]. In addition, the circRNA-miRNA-mRNA interaction network revealed that hsa_circ_0005075 could potentially interact

with miR-23b-5p, miR-93-3p, miR-581, and miR-23a-5p. The study assumed that through its miRNA sponge-like activity, hsa_circ_0005075 may participate in regulating cell adhesion during HCC development, which is involved in cancer cell proliferation, invasion, and metastasis [128].

The relationship between ciRS-7 and clinical features of HCC was also demonstrated. In the study, ciRS-7 expression was upregulated in 39.8% ($n = 43$) and downregulated in 60.2% ($n = 65$) tissues of HCC patients, when compared to matched nontumor tissues ($n = 108$) [137]. Even though ciRS-7 expression was slightly higher in HCC tissues, the overall ciRS-7 expression levels were downregulated and not significantly different from those in healthy controls [137], which was in contrast with a previous study describing ciRS-7 involvement in HCC [91]. However, upregulated ciRS-7 expression significantly correlated with patient age, serum AFP levels, and hepatic microvascular invasion (MVI), which suggested ciRS-7 expression may be associated with deterioration and metastasis of HCC [137]. Also, ciRS-7 could promote MVI by inhibiting miR-7 and disrupting the PIK3CD/p70S6K/mTOR pathway [137]. The ROC curve analysis showed that ciRS-7 was related to MVI in HCC tissues with an AUC of 0.68, implying ciRS-7 expression level could predict MVI. Considering these results, the study indicated ciRS-7 may not be a key factor in HCC tumorigenesis, but rather a risk factor for MVI in HCC [137].

The zinc finger family gene *ZKSCAN1* can generate linear *ZKSCAN1* mRNA and circular circZKSCAN1 isoforms, both of which were associated with different regulatory roles in the development of HCC, mostly through inhibiting growth, migration, and invasion of HCC cells [138]. CircZKSCAN1 was significantly downregulated in HCC tissues, when compared to paired adjacent healthy tissues ($n = 102$), and its expression levels correlated with tumor numbers, cirrhosis, vascular invasion, MVI, and tumor grade [138]. The ROC analysis showed the AUC of circZKSCAN1 was 0.834 with a sensitivity of 82.2% and specificity of 72.4%, indicating circZKSCAN1 could be used as a biomarker to effectively differentiate cancerous tissues from adjacent noncancerous tissues in HCC [138].

In addition, two relatively recently identified tumor-suppressive circRNAs were associated with clinical characteristic of patients with HCC. Low expression levels of hsa_circ_0005986 correlated with chronic hepatitis B family history, tumor diameters, MIV, and Barcelona Clinic Liver Cancer staging system (BCLC) stage [139]. The analysis was performed on 81 paired HCC and matched nontumorous tissue samples. As determined, hsa_circ_0005986 regulated the HCC cell cycle and proliferation, by acting as a miR-129-5p sponge and through promoting *Notch1* gene expression [139]. However, despite the study suggested hsa_circ_0005986 could be used as a novel HCC biomarker, no diagnostic performance of this circRNA has been performed. Similar to hsa_circ_0005986, the decreased expression levels of hsa_circ_0004018 in HCC tissues correlated with AFP level, tumor diameters, differentiation, BCLC stage, and TNM stage, when compared to those in paired paratumorous tissues ($n = 102$) [140]. miRNA target prediction analysis revealed that hsa_circ_0004018 could sponge miR-30e-5p, miR-647, miR-92a-5p, miR-660-3p, and miR-626, additionally implying its role in tumorigenesis of HCC [140]. The evaluated diagnostic performance (sensitivity 0.716; specificity 0.815; and AUC 0.848) along with its HCC stage-specific expression profile highlighted hsa_circ_0004018 as a suitable biomarker for HCC diagnosis, capable of distinguishing HCC tissues from healthy and chronic hepatitis tissues [140].

6.5. CircRNAs as Biomarkers for Gastric Cancer. Significantly downregulated expression profiles of hsa_circ_002059 have been determined in gastric cancer tissues, when compared to paired adjacent nontumor tissues ($n = 101$) [16]. In addition, hsa_circ_002059 levels in plasma were significantly different between 36 paired plasma samples from pre- and postoperative gastric cancer patients. Also, lower expression levels of hsa_circ_002059 were significantly correlated with a patient's distal metastasis, TNM stage, gender, and age. Evaluated diagnostic performance of hsa_circ_002059 has shown its sensitivity of 81.0%, specificity of 62.0%, and AUC of 0.73, indicating hsa_circ_002059 represents a potential stable biomarker for gastric cancer [16].

Beside its role as a potential biomarker in HCC, hsa_circ_0001649 has also been associated with diagnosis of gastric cancer. Hsa_circ_0001649 was significantly downregulated in gastric cancer tissues, when compared to their paired paracancerous histological normal tissues ($n = 76$), and its expression levels correlated with pathological differentiation [141]. Analysis of hsa_circ_0001649 serum expression levels between paired pre- and postoperative serum samples ($n = 20$) of gastric cancer patients showed that hsa_circ_0001649 was significantly upregulated in serum after surgery. Also, hsa_circ_0001649 expression levels were more significantly decreased in poor and undifferentiated tumors than in well-differentiated ones, indicating its potential negative correlation with gastric cancer pathological differentiation [141]. The estimated diagnostic value of hsa_circ_0001649 determined by the ROC analysis showed the AUC of 0.834, with a sensitivity and specificity of 0.711 and 0.816, respectively [141]. These results suggest hsa_circ_0001649 may become a novel noninvasive biomarker for early detection of primary gastric cancer.

Hsa_circ_0000096 is a tumor-suppressive circRNA that affects gastric cancer cell growth and migration through suppressing the expression levels of cell cycle-associated (cyclin D1, CDK6) and migration-associated (MMP-2, MMP-9) proteins. Furthermore, hsa_circ_0000096 may also interact with 17 types of miRNA, including miR-224 and miR-200a [142]. Hsa_circ_0000096 was significantly downregulated in gastric cancer tissues (compared to paired adjacent nontumorous tissues; $n = 101$), and cell lines and its aberrant expression correlated with invasion and TNM stage [142]. The ROC analysis showed the AUC of hsa_circ_0000096 was 0.82. Intriguingly, the AUC was increased to 0.91 when a combination of hsa_circ_0000096 and a previously described hsa_circ_002059 was used [142]. Thus, these results suggest that hsa_circ_0000096 could be used independently or in combination with hsa_circ_002059 for effective diagnosis of gastric cancer.

In addition, four circRNAs have been recently proposed as biomarkers for gastric cancer, all of which were statistically significantly downregulated in gastric cancer tissues, correlated with different clinical characteristics, and showed an excellent diagnostic potential with relatively high accuracy, specificity, and sensitivity [143–146]. Hsa_circ_0001895 expression levels were downregulated in 69.8% ($n = 67$) gastric cancer tissues, compared to paired adjacent normal tissues ($n = 96$), and significantly correlated with cell differentiation, Borrmann type, and tissue CEA expression. The evaluated diagnostic performance of hsa_circ_0001895 showed the AUC was up to 0.792 with a sensitivity and specificity of 67.8% and 85.7%, respectively. The optimal cutoff value was 9.53 [143]. Interestingly, better sensitivity and specificity were obtained with the use of hsa_circ_0001895, when compared to common gastric cancer biomarkers CEA, CA19-9, and CA72-4, which showed only 20.1–27.6% sensitivity individually or 48.2% when used in combination [147]. This indicates hsa_circ_0001895 may be effectively used for screening and predicting the prognosis of gastric cancer [143]. Downregulation of hsa_circ_0006633 was associated with cancer distal metastasis and tissue CEA levels. In the study, 96 paired gastric cancer tissues and their adjacent nontumorous tissues were used. The evaluated diagnostic performance (sensitivity 0.60; specificity 0.81; and AUC 0.741) and increased hsa_circ_0006633 levels in plasma samples suggested that this circRNA could be used as a novel noninvasive biomarker for screening gastric cancer [144]. Hsa_circ_0000190 levels in gastric cancer tissues were correlated with tumor diameter, lymphatic metastasis, distal metastasis, TNM stage, and CA19-9 levels. However, in plasma samples, hsa_circ_0000190 correlated only with CEA levels [145]. The analysis was performed by using 104 paired gastric cancer tissues and their adjacent nontumor tissues, 104 plasma samples from gastric cancer patients, and 104 plasma samples from healthy controls. The diagnostic potential of hsa_circ_0000190 was determined in tissue (sensitivity of 0.721, specificity of 0.683, and AUC of 0.75) and plasma (sensitivity of 0.414, specificity of 0.875, and AUC of 0.60) samples. When tissue and plasma hsa_circ_0000190 were combined, the AUC was increased to 0.775, with a sensitivity and specificity of 0.712 and 0.750, respectively [145]. Moreover, when compared to CEA and CA19-9, hsa_circ_0000190 had a much higher sensitivity and specificity in the screening of gastric cancer [145]. Low hsa_circ_0003159 tissue levels in gastric cancer patients correlated with gender, distal metastasis, and TNM stage. The analysis was performed on 108 paired gastric cancer tissues and adjacent nontumorous tissue samples. The determined sensitivity and specificity were 0.852 and 0.565, respectively. The AUC of hsa_circ_0003159 was 0.75, which indicated that hsa_circ_0003159 may be also used as a biomarker for the diagnosis of gastric cancer [146].

In contrast to the above-listed circRNAs, circPVT1 expression levels were significantly upregulated in gastric cancer cells and tissues, when compared to paired healthy controls ($n = 187$) [148]. As demonstrated, circPVT1 may promote gastric cancer proliferation through acting as a sponge toward the miR-125 family members [148]. The Kaplan-Meier analysis confirmed that circPVT1 could serve as an independent prognostic biomarker for the overall survival and disease-free survival of patients with gastric cancer. Furthermore, the combined detection of expressed circPVT1 and its linear isoform from the *PVT1* oncogene enhanced the prognosis of patients with gastric cancer [148]. In addition, statistically significant upregulation of hsa_circ_0058246 was detected in tumor specimens of gastric cancer patients with poor clinical outcomes ($n = 43$). Also, patients who suffered recurrence of gastric cancer ($n = 12$) had a significant increase in hsa_circ_0058246 expression levels [149]. However, further studies are needed to convincingly demonstrate the suitability of hsa_circ_0058246 in diagnosis and prognosis of gastric cancer.

6.6. CircRNAs as Biomarkers for Laryngeal Squamous Cell Cancer (LSCC). Microarray and subsequent qRT-PCR analyses of laryngeal squamous cell cancer (LSCC) tissues have shown hsa_circ_100855 as the most upregulated and hsa_circ_104912 as the most downregulated circRNA in LSCC [150]. Hsa_circ_100855 levels were significantly higher in LSCC tissues and in patients with T3-4 stage, neck nodal metastasis, or advanced clinical stage. Conversely, hsa_circ_104912 levels were significantly lower in LSCC tissues than in corresponding adjacent nonneoplastic tissues. The study included 4 matched samples of LSCC tissues and corresponding adjacent nonneoplastic tissues for microarray analysis and 52 matched cancerous and noncancerous tissues for qRT-PCR analysis. Despite no diagnostic performance of either circRNAs has been determined, hsa_circ_100855 and hsa_circ_104912 may both serve as potential biomarkers and therapeutic targets for LSCC [150].

7. Conclusions and Perspectives

CircRNAs appear to be stably expressed in a cell/tissue-dependent and developmental stage-specific manner and have been shown to be dysregulated in different cancers. They are generally more stable than miRNAs and lncRNAs, several of which are currently recognized as relatively well-established biomarkers in cancer diagnosis. However, before circRNAs could be routinely used as effective biomarkers for early cancer diagnosis and prognosis, several important issues should be addressed, including the determination of their diagnostic performances for specific cancer types. As demonstrated above, several circRNAs have shown satisfactory diagnostic performances in distinguishing tumor from healthy tissues and between specific cancer types. However, suitable circRNAs as independent cancer biomarkers have not been identified yet. CircRNAs could be used in combination with RNA-based and other conventional cancer biomarkers, such as CEA, CA125, CA153, PSA, and AFP, for more specific cancer diagnosis and accurate cancer prognosis. In addition, their stability and abundance in exosomes suggest that circRNAs may represent a new class of exosome-based noninvasive cancer biomarkers. CircRNAs may also have a potential in targeted cancer treatment, where they could be utilized as sponges to bind to aberrantly expressed regulatory RNAs and proteins (e.g., RBPs), thus diminishing

their oncogenic activity. However, to achieve this, further insights into circRNA's molecular mechanisms and functions in circRNA-mediated diseases are a prerequisite, before such treatments may become applicable. Also, identification of dysregulated circRNAs in other body fluids, such as urine and cerebrospinal fluid, may be beneficial for noninvasive cancer diagnosis. To conclude, circRNAs represent promising novel biomarkers for various cancer types and have a great potential to be effectively used in clinical practice in the near future.

Conflicts of Interest

The authors declare that they have no competing interests.

References

[1] J. Sana, P. Faltejskova, M. Svoboda, and O. Slaby, "Novel classes of non-coding RNAs and cancer," *Journal of Translational Medicine*, vol. 10, no. 1, p. 103, 2012.

[2] G. St Laurent, C. Wahlestedt, and P. Kapranov, "The landscape of long non-coding RNA classification," *Trends in Genetics*, vol. 31, no. 5, pp. 239–251, 2015.

[3] J. Salzman, C. Gawad, P. L. Wang, N. Lacayo, and P. O. Brown, "Circular RNAs are the predominant transcript isoform from hundreds of human genes in diverse cell types," *PLoS One*, vol. 7, no. 2, article e30733, 2012.

[4] S. P. Barrett and J. Salzman, "Circular RNAs: analysis, expression and potential functions," *Development*, vol. 143, no. 11, pp. 1838–1847, 2016.

[5] E. Lasda and R. Parker, "Circular RNAs: diversity of form and function," *RNA*, vol. 20, no. 12, pp. 1829–1842, 2014.

[6] S. P. Barrett, P. L. Wang, and J. Salzman, "Circular RNA biogenesis can proceed through an exon-containing lariat precursor," *eLife*, vol. 4, article e07540, 2015.

[7] C. Schindewolf, S. Braun, and H. Domdey, "*In vitro* generation of a circular exon from a linear pre-mRNA transcript," *Nucleic Acids Research*, vol. 24, no. 7, pp. 1260–1266, 1996.

[8] S. Starke, I. Jost, O. Rossbach et al., "Exon circularization requires canonical splice signals," *Cell Reports*, vol. 10, no. 1, pp. 103–111, 2015.

[9] S. Memczak, M. Jens, A. Elefsinioti et al., "Circular RNAs are a large class of animal RNAs with regulatory potency," *Nature*, vol. 495, no. 7441, pp. 333–338, 2013.

[10] P. L. Wang, Y. Bao, M. C. Yee et al., "Circular RNA is expressed across the eukaryotic tree of life," *PLoS One*, vol. 9, no. 3, article e90859, 2014.

[11] J. Salzman, R. E. Chen, M. N. Olsen, P. L. Wang, and P. O. Brown, "Cell-type specific features of circular RNA expression," *PLoS Genetics*, vol. 9, no. 9, article e1003777, 2013.

[12] W. R. Jeck, J. A. Sorrentino, K. Wang et al., "Circular RNAs are abundant, conserved, and associated with ALU repeats," *RNA*, vol. 19, no. 2, pp. 141–157, 2013.

[13] C. E. Burd, W. R. Jeck, Y. Liu, H. K. Sanoff, Z. Wang, and N. E. Sharpless, "Expression of linear and novel circular forms of an *INK4/ARF*-associated non-coding RNA correlates with atherosclerosis risk," *PLoS Genetics*, vol. 6, no. 12, article e1001233, 2010.

[14] A. Bachmayr-Heyda, A. T. Reiner, K. Auer et al., "Correlation of circular RNA abundance with proliferation – exemplified with colorectal and ovarian cancer, idiopathic lung fibrosis, and normal human tissues," *Scientific Reports*, vol. 5, no. 1, p. 8057, 2015.

[15] W. J. Lukiw, "Circular RNA (circRNA) in Alzheimer's disease (AD)," *Frontiers in Genetics*, vol. 4, p. 307, 2013.

[16] P. Li, S. Chen, H. Chen et al., "Using circular RNA as a novel type of biomarker in the screening of gastric cancer," *Clinica Chimica Acta*, vol. 444, pp. 132–136, 2015.

[17] Y. Zhang, X. O. Zhang, T. Chen et al., "Circular intronic long noncoding RNAs," *Molecular Cell*, vol. 51, no. 6, pp. 792–806, 2013.

[18] Y. Wang and Z. Wang, "Efficient backsplicing produces translatable circular mRNAs," *RNA*, vol. 21, no. 2, pp. 172–179, 2015.

[19] X. O. Zhang, R. Dong, Y. Zhang et al., "Diverse alternative back-splicing and alternative splicing landscape of circular RNAs," *Genome Research*, vol. 26, no. 9, pp. 1277–1287, 2016.

[20] Y. Dong, D. He, Z. Peng et al., "Circular RNAs in cancer: an emerging key player," *Journal of Hematology & Oncology*, vol. 10, no. 1, p. 2, 2017.

[21] Z. Li, C. Huang, C. Bao et al., "Exon-intron circular RNAs regulate transcription in the nucleus," *Nature Structural & Molecular Biology*, vol. 22, no. 3, pp. 256–264, 2015.

[22] W. R. Jeck and N. E. Sharpless, "Detecting and characterizing circular RNAs," *Nature Biotechnology*, vol. 32, no. 5, pp. 453–461, 2014.

[23] T. Kameyama, H. Suzuki, and A. Mayeda, "Re-splicing of mature mRNA in cancer cells promotes activation of distant weak alternative splice sites," *Nucleic Acids Research*, vol. 40, no. 16, pp. 7896–7906, 2012.

[24] X. O. Zhang, H. B. Wang, Y. Zhang, X. Lu, L. L. Chen, and L. Yang, "Complementary sequence-mediated exon circularization," *Cell*, vol. 159, no. 1, pp. 134–147, 2014.

[25] A. Ivanov, S. Memczak, E. Wyler et al., "Analysis of intron sequences reveals hallmarks of circular RNA biogenesis in animals," *Cell Reports*, vol. 10, no. 2, pp. 170–177, 2015.

[26] D. Liang and J. E. Wilusz, "Short intronic repeat sequences facilitate circular RNA production," *Genes & Development*, vol. 28, no. 20, pp. 2233–2247, 2014.

[27] S. J. Conn, K. A. Pillman, J. Toubia et al., "The RNA binding protein quaking regulates formation of circRNAs," *Cell*, vol. 160, no. 6, pp. 1125–1134, 2015.

[28] R. Ashwal-Fluss, M. Meyer, N. R. Pamudurti et al., "circRNA biogenesis competes with pre-mRNA splicing," *Molecular Cell*, vol. 56, no. 1, pp. 55–66, 2014.

[29] A. Rybak-Wolf, C. Stottmeister, P. Glazar et al., "Circular RNAs in the mammalian brain are highly abundant, conserved, and dynamically expressed," *Molecular Cell*, vol. 58, no. 5, pp. 870–885, 2015.

[30] G. J. Talhouarne and J. G. Gall, "Lariat intronic RNAs in the cytoplasm of *Xenopus tropicalis* oocytes," *RNA*, vol. 20, no. 9, pp. 1476–1487, 2014.

[31] H. Suzuki, Y. Zuo, J. Wang, M. Q. Zhang, A. Malhotra, and A. Mayeda, "Characterization of RNase R-digested cellular RNA source that consists of lariat and circular RNAs from pre-mRNA splicing," *Nucleic Acids Research*, vol. 34, no. 8, article e63, 2006.

[32] E. Lasda and R. Parker, "Circular RNAs co-precipitate with extracellular vesicles: a possible mechanism for circRNA clearance," *PLoS One*, vol. 11, no. 2, article e0148407, 2016.

[33] Y. Li, Q. Zheng, C. Bao et al., "Circular RNA is enriched and stable in exosomes: a promising biomarker for cancer diagnosis," *Cell Research*, vol. 25, no. 8, pp. 981–984, 2015.

[34] T. B. Hansen, T. I. Jensen, B. H. Clausen et al., "Natural RNA circles function as efficient microRNA sponges," *Nature*, vol. 495, no. 7441, pp. 384–388, 2013.

[35] M. W. Hentze and T. Preiss, "Circular RNAs: splicing's enigma variations," *The EMBO Journal*, vol. 32, no. 7, pp. 923–925, 2013.

[36] J. T. Granados-Riveron and G. Aquino-Jarquin, "The complexity of the translation ability of circRNAs," *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms*, vol. 1859, no. 10, pp. 1245–1251, 2016.

[37] N. R. Pamudurti, O. Bartok, M. Jens et al., "Translation of circRNAs," *Molecular Cell*, vol. 66, no. 1, pp. 9–21.e7, 2017.

[38] I. Legnini, G. Di Timoteo, F. Rossi et al., "Circ-ZNF609 is a circular RNA that can be translated and functions in myogenesis," *Molecular Cell*, vol. 66, no. 1, pp. 22–37.e9, 2017.

[39] Y. Yang, X. Fan, M. Mao et al., "Extensive translation of circular RNAs driven by N^6-methyladenosine," *Cell Research*, vol. 27, no. 5, pp. 626–641, 2017.

[40] D. P. Bartel, "MicroRNAs: target recognition and regulatory functions," *Cell*, vol. 136, no. 2, pp. 215–233, 2009.

[41] D. P. Bartel, "MicroRNAs: genomics, biogenesis, mechanism, and function," *Cell*, vol. 116, no. 2, pp. 281–297, 2004.

[42] T. B. Hansen, E. D. Wiklund, J. B. Bramsen et al., "miRNA-dependent gene silencing involving Ago2-mediated cleavage of a circular antisense RNA," *The EMBO Journal*, vol. 30, no. 21, pp. 4414–4422, 2011.

[43] B. Capel, A. Swain, S. Nicolis et al., "Circular transcripts of the testis-determining gene *Sry* in adult mouse testis," *Cell*, vol. 73, no. 5, pp. 1019–1030, 1993.

[44] R. A. Dubin, M. A. Kazmi, and H. Ostrer, "Inverted repeats are necessary for circularization of the mouse testis *Sry* transcript," *Gene*, vol. 167, no. 1-2, pp. 245–248, 1995.

[45] L. Peng, G. Chen, Z. Zhu et al., "Circular RNA ZNF609 functions as a competitive endogenous RNA to regulate AKT3 expression by sponging miR-150-5p in Hirschsprung's disease," *Oncotarget*, vol. 8, no. 1, pp. 808–818, 2017.

[46] K. Wang, B. Long, F. Liu et al., "A circular RNA protects the heart from pathological hypertrophy and heart failure by targeting miR-223," *European Heart Journal*, vol. 37, no. 33, pp. 2602–2611, 2016.

[47] J. U. Guo, V. Agarwal, H. Guo, and D. P. Bartel, "Expanded identification and characterization of mammalian circular RNAs," *Genome Biology*, vol. 15, no. 7, p. 409, 2014.

[48] M. S. Ebert, J. R. Neilson, and P. A. Sharp, "MicroRNA sponges: competitive inhibitors of small RNAs in mammalian cells," *Nature Methods*, vol. 4, no. 9, pp. 721–726, 2007.

[49] F. C. Tay, J. K. Lim, H. Zhu, L. C. Hin, and S. Wang, "Using artificial microRNA sponges to achieve microRNA loss-of-function in cancer cells," *Advanced Drug Delivery Reviews*, vol. 81, pp. 117–127, 2015.

[50] X. You, I. Vlatkovic, A. Babic et al., "Neural circular RNAs are derived from synaptic genes and regulated by development and plasticity," *Nature Neuroscience*, vol. 18, no. 4, pp. 603–610, 2015.

[51] D. B. Dudekula, A. C. Panda, I. Grammatikakis, S. De, K. Abdelmohsen, and M. Gorospe, "CircInteractome: a web tool for exploring circular RNAs and their interacting proteins and microRNAs," *RNA Biology*, vol. 13, no. 1, pp. 34–42, 2016.

[52] J. L. Rinn and H. Y. Chang, "Genome regulation by long noncoding RNAs," *Annual Review of Biochemistry*, vol. 81, no. 1, pp. 145–166, 2012.

[53] T. R. Mercer, M. E. Dinger, and J. S. Mattick, "Long noncoding RNAs: insights into functions," *Nature Reviews Genetics*, vol. 10, no. 3, pp. 155–159, 2009.

[54] R. Perriman and M. Ares Jr., "Circular mRNA can direct translation of extremely long repeating-sequence proteins in vivo," *RNA*, vol. 4, no. 9, pp. 1047–1054, 1998.

[55] C. Y. Chen and P. Sarnow, "Initiation of protein synthesis by the eukaryotic translational apparatus on circular RNAs," *Science*, vol. 268, no. 5209, pp. 415–417, 1995.

[56] S. Ghosal, S. Das, R. Sen, P. Basak, and J. Chakrabarti, "Circ2Traits: a comprehensive database for circular RNA potentially associated with disease and traits," *Frontiers in Genetics*, vol. 4, p. 283, 2013.

[57] P. Glazar, P. Papavasileiou, and N. Rajewsky, "circBase: a database for circular RNAs," *RNA*, vol. 20, no. 11, pp. 1666–1670, 2014.

[58] Y. C. Liu, J. R. Li, C. H. Sun et al., "CircNet: a database of circular RNAs derived from transcriptome sequencing data," *Nucleic Acids Research*, vol. 44, no. D1, pp. D209–D215, 2016.

[59] X. Chen, P. Han, T. Zhou, X. Guo, X. Song, and Y. Li, "circRNADb: a comprehensive database for human circular RNAs with protein-coding annotations," *Scientific Reports*, vol. 6, no. 1, article 34985, 2016.

[60] J. H. Li, S. Liu, H. Zhou, L. H. Qu, and J. H. Yang, "starBase v2.0: decoding miRNA-ceRNA, miRNA-ncRNA and protein-RNA interaction networks from large-scale CLIP-Seq data," *Nucleic Acids Research*, vol. 42, no. D1, pp. D92–D97, 2014.

[61] L. L. Zheng, J. H. Li, J. Wu et al., "deepBase v2.0: identification, expression, evolution and function of small RNAs, LncRNAs and circular RNAs from deep-sequencing data," *Nucleic Acids Research*, vol. 44, no. D1, pp. D196–D202, 2016.

[62] S. Xia, J. Feng, L. Lei et al., "Comprehensive characterization of tissue-specific circular RNAs in the human and mouse genomes," *Briefings in Bioinformatics*, article bbw081, 2016.

[63] X. Zeng, W. Lin, M. Guo, and Q. Zou, "A comprehensive overview and evaluation of circular RNA detection tools," *PLoS Computational Biology*, vol. 13, no. 6, article e1005420, 2017.

[64] J. O. Westholm, P. Miura, S. Olson et al., "Genome-wide analysis of *Drosophila* circular RNAs reveals their structural and sequence properties and age-dependent neural accumulation," *Cell Reports*, vol. 9, no. 5, pp. 1966–1980, 2014.

[65] J. Cheng, F. Metge, and C. Dieterich, "Specific identification and quantification of circular RNAs from sequencing data," *Bioinformatics*, vol. 32, no. 7, pp. 1094–1096, 2016.

[66] X. Song, N. Zhang, P. Han et al., "Circular RNA profile in gliomas revealed by identification tool UROBORUS," *Nucleic Acids Research*, vol. 44, no. 9, article e87, 2016.

[67] O. G. Izuogu, A. A. Alhasan, H. M. Alafghani, M. Santibanez-Koref, D. J. Elliott, and M. S. Jackson, "PTESFinder: a computational method to identify post-transcriptional exon shuffling (PTES) events," *BMC Bioinformatics*, vol. 17, no. 1, p. 31, 2016.

[68] L. Szabo, R. Morey, N. J. Palpant et al., "Statistically based splicing detection reveals neural enrichment and tissue-specific induction of circular RNA during human fetal development," *Genome Biology*, vol. 16, no. 1, p. 126, 2015.

[69] Y. Gao, J. Wang, and F. Zhao, "CIRI: an efficient and unbiased algorithm for *de novo* circular RNA identification," *Genome Biology*, vol. 16, no. 1, p. 4, 2015.

[70] K. Wang, D. Singh, Z. Zeng et al., "MapSplice: accurate mapping of RNA-seq reads for splice junction discovery," *Nucleic Acids Research*, vol. 38, no. 18, article e178, 2010.

[71] S. Hoffmann, C. Otto, G. Doose et al., "A multi-split mapping algorithm for circular RNA, splicing, trans-splicing and fusion detection," *Genome Biology*, vol. 15, no. 2, article R34, 2014.

[72] T. J. Chuang, C. S. Wu, C. Y. Chen, L. Y. Hung, T. W. Chiang, and M. Y. Yang, "NCLscan: accurate identification of non-co-linear transcripts (fusion, *trans*-splicing and circular RNA) with a good balance between sensitivity and precision," *Nucleic Acids Research*, vol. 44, no. 3, article e29, 2016.

[73] I. Chen, C. Y. Chen, and T. J. Chuang, "Biogenesis, identification, and function of exonic circular RNAs," *Wiley Interdisciplinary Reviews RNA*, vol. 6, no. 5, pp. 563–579, 2015.

[74] X. Meng, Q. Chen, P. Zhang, and M. Chen, "CircPro: an integrated tool for the identification of circRNAs with protein-coding potential," *Bioinformatics*, vol. 33, no. 20, pp. 3314–3316, 2017.

[75] M. Mokrejs, T. Masek, V. Vopalensky, P. Hlubucek, P. Delbos, and M. Pospísek, "IRESite—a tool for the examination of viral and cellular internal ribosome entry sites," *Nucleic Acids Research*, vol. 38, Supplement 1, pp. D131–D136, 2010.

[76] L. Wang, H. J. Park, S. Dasari, S. Wang, J. P. Kocher, and W. Li, "CPAT: coding-potential assessment tool using an alignment-free logistic regression model," *Nucleic Acids Research*, vol. 41, no. 6, article e74, 2013.

[77] R. D. Finn, P. Coggill, R. Y. Eberhardt et al., "The Pfam protein families database: towards a more sustainable future," *Nucleic Acids Research*, vol. 44, no. D1, pp. D279–D285, 2016.

[78] M. F. Lin, I. Jungreis, and M. Kellis, "PhyloCSF: a comparative genomics method to distinguish protein coding and non-coding regions," *Bioinformatics*, vol. 27, no. 13, pp. i275–i282, 2011.

[79] D. Kim and S. L. Salzberg, "TopHat-Fusion: an algorithm for discovery of novel fusion transcripts," *Genome Biology*, vol. 12, no. 8, article R72, 2011.

[80] Y. Wang, Y. Mo, Z. Gong et al., "Circular RNAs in human cancer," *Molecular Cancer*, vol. 16, no. 1, p. 25, 2017.

[81] R. J. Taft, K. C. Pang, T. R. Mercer, M. Dinger, and J. S. Mattick, "Non-coding RNAs: regulators of disease," *The Journal of Pathology*, vol. 220, no. 2, pp. 126–139, 2010.

[82] J. Zhao, Y. Tao, Y. Zhou et al., "MicroRNA-7: a promising new target in cancer therapy," *Cancer Cell International*, vol. 15, no. 1, p. 103, 2015.

[83] T. B. Hansen, J. Kjems, and C. K. Damgaard, "Circular RNA and miR-7 in cancer," *Cancer Research*, vol. 73, no. 18, pp. 5609–5612, 2013.

[84] K. M. Giles, R. A. M. Brown, M. R. Epis, F. C. Kalinowski, and P. J. Leedman, "miRNA-7-5p inhibits melanoma cell migration and invasion," *Biochemical and Biophysical Research Communications*, vol. 430, no. 2, pp. 706–710, 2013.

[85] X. Kong, G. Li, Y. Yuan et al., "MicroRNA-7 inhibits epithelial-to-mesenchymal transition and metastasis of breast cancer cells via targeting FAK expression," *PLoS One*, vol. 7, no. 8, article e41523, 2012.

[86] W. Wang, L. X. Dai, S. Zhang et al., "Regulation of epidermal growth factor receptor signaling by plasmid-based microRNA-7 inhibits human malignant gliomas growth and metastasis in vivo," *Neoplasma*, vol. 60, no. 03, pp. 274–283, 2013.

[87] X. Zhao, W. Dou, L. He et al., "MicroRNA-7 functions as an anti-metastatic microRNA in gastric cancer by targeting insulin-like growth factor-1 receptor," *Oncogene*, vol. 32, no. 11, pp. 1363–1372, 2013.

[88] Y. Fang, J. Xue, Q. Shen, J. Chen, and L. Tian, "MicroRNA-7 inhibits tumor growth and metastasis by targeting the phosphoinositide 3-kinase/Akt pathway in hepatocellular carcinoma," *Hepatology*, vol. 55, no. 6, pp. 1852–1862, 2012.

[89] S. Xiong, Y. Zheng, P. Jiang, R. Liu, X. Liu, and Y. Chu, "MicroRNA-7 inhibits the growth of human non-small cell lung cancer A549 cells through targeting BCL-2," *International Journal of Biological Sciences*, vol. 7, no. 6, pp. 805–814, 2011.

[90] N. Zhang, X. Li, C. W. Wu et al., "MicroRNA-7 is a novel inhibitor of YY1 contributing to colorectal tumorigenesis," *Oncogene*, vol. 32, no. 42, pp. 5078–5088, 2013.

[91] L. Yu, X. Gong, L. Sun, Q. Zhou, B. Lu, and L. Zhu, "The circular RNA Cdr1as act as an oncogene in hepatocellular carcinoma through targeting miR-7 expression," *PLoS One*, vol. 11, no. 7, article e0158347, 2016.

[92] W. Tang, M. Ji, G. He et al., "Silencing CDR1as inhibits colorectal cancer progression through regulating microRNA-7," *OncoTargets and Therapy*, vol. 10, pp. 2045–2056, 2017.

[93] Y. Nakagawa, Y. Akao, K. Taniguchi et al., "Relationship between expression of onco-related miRNAs and the endoscopic appearance of colorectal tumors," *International Journal of Molecular Sciences*, vol. 16, no. 1, pp. 1526–1543, 2015.

[94] D. Barbagallo, A. Condorelli, M. Ragusa et al., "Dysregulated miR-671-5p / CDR1-AS / CDR1 / VSNL1 axis is involved in glioblastoma multiforme," *Oncotarget*, vol. 7, no. 4, pp. 4746–4759, 2016.

[95] W. Yang, W. W. Du, X. Li, A. J. Yee, and B. B. Yang, "Foxo3 activity promoted by non-coding effects of circular RNA and Foxo3 pseudogene in the inhibition of tumor growth and angiogenesis," *Oncogene*, vol. 35, no. 30, pp. 3919–3931, 2016.

[96] W. W. Du, W. Yang, E. Liu, Z. Yang, P. Dhaliwal, and B. B. Yang, "Foxo3 circular RNA retards cell cycle progression via forming ternary complexes with p21 and CDK2," *Nucleic Acids Research*, vol. 44, no. 6, pp. 2846–2858, 2016.

[97] W. W. Du, L. Fang, W. Yang et al., "Induction of tumor apoptosis through a circular RNA enhancing Foxo3 activity," *Cell Death and Differentiation*, vol. 24, no. 2, pp. 357–370, 2017.

[98] H. F. Liang, "Circular RNA circ-ABCB10 promotes breast cancer proliferation and progression through sponging miR-1271," *American Journal of Cancer Research*, vol. 7, no. 7, pp. 1566–1576, 2017.

[99] H. Xie, X. Ren, S. Xin et al., "Emerging roles of circRNA_001569 targeting miR-145 in the proliferation and invasion of colorectal cancer," *Oncotarget*, vol. 7, no. 18, pp. 26680–26691, 2016.

[100] H. Jin, M. Fang, Z. Man, Y. Wang, and H. Liu, "Circular RNA 001569 acts as an oncogene and correlates with aggressive characteristics in hepatocellular carcinoma," *International Journal of Clinical and Experimental Pathology*, vol. 10, no. 3, pp. 2997–3005, 2017.

[101] Q. Zheng, C. Bao, W. Guo et al., "Circular RNA profiling reveals an abundant circHIPK3 that regulates cell growth by sponging multiple miRNAs," *Nature Communications*, vol. 7, 2016.

[102] J. Chen, H. Xiao, Z. Huang et al., "MicroRNA124 regulate cell growth of prostate cancer cells by targeting iASPP," *International Journal of Clinical and Experimental Pathology*, vol. 7, no. 5, pp. 2283–2290, 2014.

[103] Y. Li, F. Zheng, X. Xiao et al., "CircHIPK3 sponges miR-558 to suppress heparanase expression in bladder cancer cells," *EMBO Reports*, vol. 18, no. 9, pp. 1646–1659, 2017.

[104] F. Li, L. Zhang, W. Li et al., "Circular RNA ITCH has inhibitory effect on ESCC by suppressing the Wnt/β-catenin pathway," *Oncotarget*, vol. 6, no. 8, pp. 6001–6013, 2015.

[105] G. Huang, H. Zhu, Y. Shi, W. Wu, H. Cai, and X. Chen, "*cir-ITCH* plays an inhibitory role in colorectal cancer by regulating the Wnt/β-catenin pathway," *PLoS One*, vol. 10, no. 6, article e0131225, 2015.

[106] L. Wan, L. Zhang, K. Fan, Z. X. Cheng, Q. C. Sun, and J. J. Wang, "Circular RNA-ITCH suppresses lung cancer proliferation via inhibiting the Wnt/β-catenin pathway," *BioMed Research International*, vol. 2016, Article ID 1579490, 11 pages, 2016.

[107] W. Xia, M. Qiu, R. Chen et al., "Circular RNA has_circ_0067934 is upregulated in esophageal squamous cell carcinoma and promoted proliferation," *Scientific Reports*, vol. 6, no. 1, article 35576, 2016.

[108] S. D. Hsu, Y. T. Tseng, S. Shrestha et al., "miRTarBase update 2014: an information resource for experimentally validated miRNA-target interactions," *Nucleic Acids Research*, vol. 42, no. D1, pp. D78–D85, 2014.

[109] M. Huang, Z. Zhong, M. Lv, J. Shu, Q. Tian, and J. Chen, "Comprehensive analysis of differentially expressed profiles of lncRNAs and circRNAs with associated co-expression and ceRNA networks in bladder carcinoma," *Oncotarget*, vol. 7, no. 30, pp. 47186–47200, 2016.

[110] L. Chen, S. Zhang, J. Wu et al., "circRNA_100290 plays a role in oral cancer by functioning as a sponge of the miR-29 family," *Oncogene*, vol. 36, no. 32, pp. 4551–4561, 2017.

[111] H. Jin, X. Jin, H. Zhang, and W. Wang, "Circular RNA hsa-circ-0016347 promotes proliferation, invasion and metastasis of osteosarcoma cells," *Oncotarget*, vol. 8, no. 15, pp. 25571–25581, 2017.

[112] Z. Xu and T. Wang, "miR-214 promotes the proliferation and invasion of osteosarcoma cells through direct suppression of LZTS1," *Biochemical and Biophysical Research Communications*, vol. 449, no. 2, pp. 190–195, 2014.

[113] W. Allen-Rhoades, L. Kurenbekova, L. Satterfield et al., "Cross-species identification of a plasma microRNA signature for detection, therapeutic monitoring, and prognosis in osteosarcoma," *Cancer Medicine*, vol. 4, no. 7, pp. 977–988, 2015.

[114] J. F. Li and Y. Z. Song, "Circular RNA hsa_circ_0001564 facilitates tumorigenesis of osteosarcoma via sponging miR-29c-3p," *Tumour Biology*, vol. 39, no. 8, article 1010428317709989, 2017.

[115] Y. Zhang, J. Li, J. Yu et al., "Circular RNAs signature predicts the early recurrence of stage III gastric cancer after radical surgery," *Oncotarget*, vol. 8, no. 14, pp. 22936–22943, 2017.

[116] Y. Zhang, H. Liu, W. Li et al., "CircRNA_100269 is downregulated in gastric cancer and suppresses tumor cell growth by targeting miR-630," *Aging*, vol. 9, no. 6, pp. 1585–1594, 2017.

[117] X. L. Zhang, L. L. Xu, and F. Wang, "Hsa_circ_0020397 regulates colorectal cancer cell viability, apoptosis and invasion by promoting the expression of the miR-138 targets TERT and PD-L1," *Cell Biology International*, vol. 41, no. 9, pp. 1056–1064, 2017.

[118] J. Guo, J. Li, C. Zhu et al., "Comprehensive profile of differentially expressed circular RNAs reveals that hsa_circ_0000069 is upregulated and promotes cell proliferation, migration, and invasion in colorectal cancer," *OncoTargets and Therapy*, vol. 9, pp. 7451–7458, 2016.

[119] J. Li, D. Li, and W. Zhang, "Tumor suppressor role of miR-217 in human epithelial ovarian cancer by targeting IGF1R," *Oncology Reports*, vol. 35, no. 3, pp. 1671–1679, 2016.

[120] H. Wang, X. Dong, X. Gu, R. Qin, H. Jia, and J. Gao, "The microRNA-217 functions as a potential tumor suppressor in gastric cancer by targeting GPC5," *PLoS One*, vol. 10, no. 6, article e0125474, 2015.

[121] J. Zheng, X. Liu, Y. Xue et al., "TTBK2 circular RNA promotes glioma malignancy by regulating miR-217/HNF1β/Derlin-1 pathway," *Journal of Hematology & Oncology*, vol. 10, no. 1, p. 52, 2017.

[122] P. Yang, Z. Qiu, Y. Jiang et al., "Silencing of cZNF292 circular RNA suppresses human glioma tube formation via the Wnt/β-catenin signaling pathway," *Oncotarget*, vol. 7, no. 39, pp. 63449–63455, 2016.

[123] J. Guarnerio, M. Bezzi, J. C. Jeong et al., "Oncogenic role of fusion-circRNAs derived from cancer-associated chromosomal translocations," *Cell*, vol. 165, no. 2, pp. 289–302, 2016.

[124] G. A. Dos Santos, L. Kats, and P. P. Pandolfi, "Synergy against PML-RARa: targeting transcription, proteolysis, differentiation, and self-renewal in acute promyelocytic leukemia," *The Journal of Experimental Medicine*, vol. 210, no. 13, pp. 2793–2802, 2013.

[125] S. Memczak, P. Papavasileiou, O. Peters, and N. Rajewsky, "Identification and characterization of circular RNAs as a new class of putative biomarkers in human blood," *PLoS One*, vol. 10, no. 10, article e0141214, 2015.

[126] J. H. Bahn, Q. Zhang, F. Li et al., "The landscape of microRNA, Piwi-interacting RNA, and circular RNA in human saliva," *Clinical Chemistry*, vol. 61, no. 1, pp. 221–230, 2015.

[127] M. Qin, G. Liu, X. Huo et al., "Hsa_circ_0001649: a circular RNA and potential novel biomarker for hepatocellular carcinoma," *Cancer Biomarkers*, vol. 16, no. 1, pp. 161–169, 2016.

[128] X. Shang, G. Li, H. Liu et al., "Comprehensive circular RNA profiling reveals that hsa_circ_0005075, a new circular RNA biomarker, is involved in hepatocellular crcinoma development," *Medicine*, vol. 95, no. 22, article e3811, 2016.

[129] Z. Zhong, M. Lv, and J. Chen, "Screening differential circular RNA expression profiles reveals the regulatory role of circTCF25-miR-103a-3p/miR-107-CDK6 pathway in bladder carcinoma," *Scientific Reports*, vol. 6, no. 1, article 30919, 2016.

[130] X. Wang, Y. Zhang, L. Huang et al., "Decreased expression of hsa_circ_001988 in colorectal cancer and its clinical

significances," *International Journal of Clinical and Experimental Pathology*, vol. 8, no. 12, pp. 16020–16025, 2015.

[131] P. Zhang, Z. Zuo, W. Shang et al., "Identification of differentially expressed circular RNAs in human colorectal cancer," *Tumour Biology*, vol. 39, no. 3, article 1010428317694546, 2017.

[132] W. Weng, Q. Wei, S. Toden et al., "Circular RNA ciRS-7—a promising prognostic biomarker and a potential therapeutic target in colorectal cancer," *Clinical Cancer Research*, vol. 23, no. 14, pp. 3918–3928, 2017.

[133] J. T. Yao, S. H. Zhao, Q. P. Liu et al., "Over-expression of circRNA_100876 in non-small cell lung cancer and its prognostic value," *Pathology, Research and Practice*, vol. 213, no. 5, pp. 453–456, 2017.

[134] Q. Liu, X. Zhang, X. Hu et al., "Circular RNA related to the chondrocyte ECM regulates MMP13 expression by functioning as a miR-136 'sponge' in human cartilage degradation," *Scientific Reports*, vol. 6, 2016.

[135] X. Yu, F. Wei, J. Yu et al., "Matrix metalloproteinase 13: a potential intermediate between low expression of microRNA-125b and increasing metastatic potential of non-small cell lung cancer," *Cancer Genetics*, vol. 208, no. 3, pp. 76–84, 2015.

[136] C. P. Hsu, G. H. Shen, and J. L. Ko, "Matrix metalloproteinase-13 expression is associated with bone marrow microinvolvement and prognosis in non-small cell lung cancer," *Lung Cancer*, vol. 52, no. 3, pp. 349–357, 2006.

[137] L. Xu, M. Zhang, X. Zheng, P. Yi, C. Lan, and M. Xu, "The circular RNA ciRS-7 (Cdr1as) acts as a risk factor of hepatic microvascular invasion in hepatocellular carcinoma," *Journal of Cancer Research and Clinical Oncology*, vol. 143, no. 1, pp. 17–27, 2017.

[138] Z. Yao, J. Luo, K. Hu et al., "*ZKSCAN1* gene and its related circular RNA (circ*ZKSCAN1*) both inhibit hepatocellular carcinoma cell growth, migration, and invasion but through different signaling pathways," *Molecular Oncology*, vol. 11, no. 4, pp. 422–437, 2017.

[139] L. Fu, Q. Chen, T. Yao et al., "Hsa_circ_0005986 inhibits carcinogenesis by acting as a miR-129-5p sponge and is used as a novel biomarker for hepatocellular carcinoma," *Oncotarget*, vol. 8, no. 27, pp. 43878–43888, 2017.

[140] L. Fu, T. Yao, Q. Chen, X. Mo, Y. Hu, and J. Guo, "Screening differential circular RNA expression profiles reveals hsa_circ_0004018 is associated with hepatocellular carcinoma," *Oncotarget*, vol. 8, no. 35, pp. 58405–58416, 2017.

[141] W. H. Li, Y. C. Song, H. Zhang et al., "Decreased expression of hsa_circ_00001649 in gastric cancer and its clinical significance," *Disease Markers*, vol. 2017, Article ID 4587698, 6 pages, 2017.

[142] P. Li, H. Chen, S. Chen et al., "Circular RNA 0000096 affects cell growth and migration in gastric cancer," *British Journal of Cancer*, vol. 116, no. 5, pp. 626–633, 2017.

[143] Y. Shao, L. Chen, R. Lu et al., "Decreased expression of hsa_circ_0001895 in human gastric cancer and its clinical significances," *Tumour Biology*, vol. 39, no. 4, article 1010428317699125, 2017.

[144] R. Lu, Y. Shao, G. Ye, B. Xiao, and J. Guo, "Low expression of hsa_circ_0006633 in human gastric cancer and its clinical significances," *Tumour Biology*, vol. 39, no. 6, article 1010428317704175, 2017.

[145] S. Chen, T. Li, Q. Zhao, B. Xiao, and J. Guo, "Using circular RNA hsa_circ_0000190 as a new biomarker in the diagnosis of gastric cancer," *Clinica Chimica Acta*, vol. 466, pp. 167–171, 2017.

[146] M. Tian, R. Chen, T. Li, and B. Xiao, "Reduced expression of circRNA hsa_circ_0003159 in gastric cancer and its clinical significance," *Journal of Clinical Laboratory Analysis*, e22281, 2017.

[147] Y. Liang, W. Wang, C. Fang et al., "Clinical significance and diagnostic value of serum CEA, CA19-9 and CA72-4 in patients with gastric cancer," *Oncotarget*, vol. 7, no. 31, pp. 49565–49573, 2016.

[148] J. Chen, Y. Li, Q. Zheng et al., "Circular RNA profile identifies circPVT1 as a proliferative factor and prognostic marker in gastric cancer," *Cancer Letters*, vol. 388, pp. 208–219, 2017.

[149] Y. Fang, M. Ma, J. Wang, X. Liu, and Y. Wang, "Circular RNAs play an important role in late-stage gastric cancer: circular RNA expression profiles and bioinformatics analyses," *Tumour Biology*, vol. 39, no. 6, article 1010428317705850, 2017.

[150] L. Xuan, L. Qu, H. Zhou et al., "Circular RNA: a novel biomarker for progressive laryngeal cancer," *American Journal of Translational Research*, vol. 8, no. 2, pp. 932–939, 2016.

[151] F. Wang, A. J. Nazarali, and S. Ji, "Circular RNAs as potential biomarkers for cancer diagnosis and therapy," *American Journal of Cancer Research*, vol. 6, no. 6, pp. 1167–1176, 2016.

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Review Article

MicroRNA in Glioblastoma: An Overview

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Glioblastoma is the most aggressive brain tumor and, even with the current multimodal therapy, is an invariably lethal cancer with a life expectancy that depends on the tumor subtype but, even in the most favorable cases, rarely exceeds 2 years. Epigenetic factors play an important role in gliomagenesis, are strong predictors of outcome, and are important determinants for the resistance to radio- and chemotherapy. The latest addition to the epigenetic machinery is the noncoding RNA (ncRNA), that is, RNA molecules that are not translated into a protein and that exert their function by base pairing with other nucleic acids in a reversible and nonmutational mode. MicroRNAs (miRNA) are a class of ncRNA of about 22 bp that regulate gene expression by binding to complementary sequences in the mRNA and silence its translation into proteins. MicroRNAs reversibly regulate transcription through nonmutational mechanisms; accordingly, they can be considered as epigenetic effectors. In this review, we will discuss the role of miRNA in glioma focusing on their role in drug resistance and on their potential applications in the therapy of this tumor.

1. Introduction

Epidemiological studies estimate that more than 250,000 new cases of central nervous system (CNS) tumors worldwide are expected every year with variable incidence rates ([1] and http://www.cancer.gov/types/brain/hp/adult-brain-treatment-pdq). Although glioblastoma is considered a rare tumor (Orphanet 360), it accounts for 4% of all cancer deaths making it as one of the deadliest human tumors. According to the current classification [2], approximately 38% of these tumors are at high grade (WHO III, anaplastic astrocytoma or AA and WHO IV, glioblastoma or GB) and hence are virtually lethal.

Given the extremely limited success of the standard treatment in prolonging survival in GB patients, considerable efforts were undertaken to develop targeted therapies that could significantly improve the outcome of these patients [3]. In this respect, epigenetics and epigenetic modulators have become a preferred field of investigation because of their influence in many aspects of cancer [4, 5].

Epigenetics, at large, is the mechanism utilized by living cells to decode and utilize properly the information contained in the raw DNA sequence. In practice, epigenetics consists in a "code" that lays on top of the genetic code and translates the simple information into function [6]. By definition, epigenetics does not change the "content of the information" (i.e., the sequence) and acts through reversible modifications like cytosine methylation at CpG doublets, postsynthetic modifications of the histones (acetylation, methylation, phosphorylation, etc.), and changes in the chromatin conformation. In the last years, a new class of effectors has been added to the epigenetic machinery: the microRNAs or in general, the noncoding RNAs that are capable of reversibly interfering with the transcription and translation of the genes without altering DNA sequence as expected for an epigenetic mechanism.

In this review, we will discuss some general aspects of miRNA in glioma focusing on the circuitry between miRNA and other epigenetic determinants like DNA methylation in this tumor, their role in drug resistance, and their potential therapeutic implications.

Table 1: Molecular characteristics of glioblastoma subtypes according to methylation, expression, mutation, copy number variations patterns, and clinical outcome.

IDH1/*IDH2* status	Wild type			Mutated	
MGMT	Mostly unmethylated			Methylated	
Methylation status	CIMP−			CIMP+	
Mutations	*TERT*			*ATRX-TP53*	*CIC-FUBP1*
CNV	EGFR+	NF1−		PDGFRA+	PDGFRA+; 1p19q−
Molecular subtype	Classical	Mesenchymal	Neural	Proneural	
Outcome	Very poor outcome			Significantly improved outcome	Significantly improved outcome. Better response to TMZ than 1p19q+

2. Epigenetics and Epigenetic Networks in Glioblastoma

Transcriptional profiling has delineated four major molecular subtypes of glioblastoma that could be better characterized by mutational, copy number variation, and methylation analyses [7–9]. In particular, this classification defines two clinical groups of GBs with distinct treatment response and outcome (Table 1). Overall, GB could be classified as "primary," a group that includes three transcriptional subtypes (classical, mesenchymal, and neural) and "secondary" glioblastoma derived from the evolution of low-grade GB that include the transcriptional proneural subtype and that can be loosely subdivided in two subgroups according to the mutational and chromosomal status. The average survival is 31 months for secondary glioblastoma and only 15 months for the patients with primary GB. As can be seen in Table 1, the major features that distinguish primary from secondary GB can be considered, at large of epigenetic nature, namely, *MGMT* methylation status and the CpG island methylator phenotype (CIMP).

One of the first evidences of the primary role of epigenetic mechanisms in GB was the discovery of the effect of the inactivation by methylation of the *MGMT* gene on the sensitivity to the alkylating agent temozolomide (TMZ). In two seminal papers published in 2005 [10, 11], Stupp et al. and Hegi et al. established that the inactivation of the *MGMT* gene by DNA methylation in the tumor is associated with better survival in GB patients treated with TMZ and radiotherapy compared to the patients with unmethylated *MGMT*. Hypermethylation of *MGMT* occurs also in primary GB; however, it is a characteristic feature of secondary glioblastoma and is the "iceberg tip" of a more extensive alteration of the DNA methylation status known as "CpG island methylator phenotype" or CIMP.

The concept of CIMP was developed in 1999 by Toyota et al. that showed the concomitant presence of hypermethylation at many different CpG sites in a subset of colorectal cancer patients with distinct clinicopathological characteristics and favorable outcome [12]. Since then, CIMP was described in many other tumors (for a recent review, see [13]) although it is not clear if the CIMP phenotype is tissue-specific or if all CIMP+ tumors represent a class of tumors with similar characteristics. Moreover, the molecular parameters, including the methylation cut-off levels and the genes to be considered to positively assess the presence of CIMP in a given tumor are not well established. The clinical characteristics of CIMP+ tumors differ; indeed, it differs in GB [8], colon carcinoma [14], myeloid leukemia [15], and breast cancer [16]. The CIMP+ phenotype is a predictor of better outcome whereas in other tumors like neuroblastoma [17, 18] and melanoma [19], CIMP+ tumors are associated with poor prognosis. A possible explanation for the nonuniversal clinical significance of CIMP is the absence of accurate criteria that define CIMP so that the true phenotype of the tumor cannot be always assessed.

In GB, the CIMP phenotype clearly distinguishes the primary (CIMP−) from the secondary (CIMP+) tumors and is tightly associated with inactivating mutations of the *IDH1* and *IDH2* genes [8]. The mechanistic link between *IDH* mutations and CIMP was discovered in 2012 when it was demonstrated that IDH1 is an epigenetic controller that modulates the pattern of histone and DNA methylation. This occurs through the inhibition by D-2-hydroxyglutarate of the jumonji histone lysine demethylases (KDM) and of the TET-hydroxylases that convert 5-methylcytosine into 5-hydroxyl-methylcytosine thus leading to the accumulation of 5-methylcytosine.

Recently, histone modifications also have gained importance in GB and the possibility of pharmacological intervention on histone deacetylases (HDAC) has been exploited [20]. Moreover, the involvement of *KDM* genes in GB development and drug resistance has been demonstrated [21–23].

MicroRNAs, a class of noncoding RNAs, are considered epigenetic modifiers because they control the functionality of the genome by base pairing of nucleotides 2–8 of their sequence to the 3'UTR of mRNA forming the so-called "silencing complex" [24]. Since their inhibitory function is sequence-specific and does not involve the permanent alteration of the DNA sequence, miRNAs are considered an integral part of the epigenetic machinery.

In glioblastoma, as in many other tumors, the remodeling of the epigenome is an important aspect of the biology of the tumor [25, 26] and the interaction between epigenetic factors and the cell signaling cascade appears as a promising target for new therapeutic approaches [20, 23, 25, 27, 28].

3. The Interplay between Tumor Suppressing and Oncogenic miRNA in Glioblastoma

From the current release, 21 of the miRNA database lists 2588 mature and 1881 precursor human miRNA sequences

Table 2: miRNA involved in the regulation of EZH2.

miRNA	Action	Reference
Let-7a	Direct targeting of EZH2 in nasopharyngeal carcinoma, inhibition of glioma growth by targeting K-RAS	[206, 207]
miR-26a	Inhibits growth of nasopharyngeal carcinoma targeting EZH2	[208]
miR-101	miR-101 downregulation in GB results in EZH2-induced proliferation regulating the methylation status of CPBE1	[209, 210]
miR-124	Modulates the proliferation of epatocarcinoma cells by direct targeting of EZH2	[211]
miR-138	Blocks GB tumorigenicity by EZH2-CDK4/6-pRb-E2F1 signaling cascade	[212]
miR-214	Targeting of EZH2 in skeletal muscles	[213]
miR-708	Inhibits GB cell proliferation targeting EZH2, AKT1, MMP2, CCND1, Parp-1, and Bcl-2	[214]

(http://www.mirbase.org/cgi-bin/browse.pl?org=hsa). Each of these miRNA can modulate the expression of several mRNAs, and each mRNA can be modulated by several miRNA generating an extraordinary complex regulatory network. In a literature survey of miRNA deregulated in GB, it was found that the majority of them ($n = 253$) were overexpressed compared to normal brain tissue, 95 were downmodulated, and conflicting results were reported for 17 of them [29].

The genes targeted by deregulated microRNAs in GB are involved in many pathways including cell proliferation, resistance to apoptosis, autophagy, invasion and metastasis, angiogenesis, and drug resistance. Since microRNAs have multiple targets in different tissues, they may have oncogenic (oncomiR) or antioncogenic effects depending upon the biological context.

Several miRNAs acting as tumor suppressor genes have been identified; some of them are unique of glioblastoma whereas others are involved also in other tumors. In principle, all miRNA interfering with the histone methyltransferase *EZH2* (Table 2) can be considered as tumor suppressors, in particular let-7 which inhibits also oncogenes like *MYC* and *K-RAS* [30, 31] and is capable of inhibiting glioblastoma cell proliferation [32]. miR-128 and miR-34a are two examples of miRNA acting as tumor suppressor in glioblastoma. miR-128 is an antiproliferative miRNA that interferes with multiple pathways targeting genes involved in glioblastoma pathogenesis like *EGFR* and *PDGFRA* [33] and *WEE1* [34] and *E2F3a* [35]. miR-34a interferes with cell proliferation through multiple targets (*CDK6*, *CCND1*, *NOTCH*, and others). When the functionality of miR-34a is restored, this miRNA acts as a tumor suppressor gene reducing cell proliferation and invasion [36]. MiR-124 and miR-137 are two microRNA significantly downregulated in high-grade gliomas and *in vitro* can induce phenotypic changes, growth arrest, and differentiation in glioma stem cells and thus can be considered oncosuppressive miRNA [37]. Most deregulated miRNAs in GB interfere with cell proliferation pathways, particularly those of *EGFR* and *AKT*. A prototype of this group of miRNA is miR-7 whose transfection in GBM cells leads to decreased invasiveness and increased apoptosis fulfilling the basic requirements of a tumor suppressor [38, 39].

Many miRNAs are upregulated in glioblastoma and can be functionally classified as oncomiR. Historically, miR-21 was the first oncogenic miRNA to be identified [40] and can target a set of oncosuppressor genes including *PTEN* [41] and the metalloproteinase inhibitor *TIMP3* which is involved in extracellular matrix remodeling, tumor infiltration, and angiogenesis [42, 43]. Thus, miR-21 targets an entire network of tumor suppressor genes and its inhibition by complementary oligonucleotides blocks GB cell growth *in vitro* and *in vivo* [44]. It is reasonable to hypothesize that the delivery of an inhibitor of miR-21 at the tumor site might be a useful addition to the standard therapy.

The targeting of multiple oncosuppressor genes easily explains the oncogenic mechanism of miR-21. On the contrary, the oncogenic properties of miR-221and miR-222, overexpressed in a variety of tumors including GB, have several oncogenic functions including the inactivation of the cell cycle suppressors p27 and p57 [45, 46].

Apoptosis and autophagy are two mechanisms utilized to eliminate dysfunctional or otherwise stressed cells, and resistance to apoptosis is one of the hallmarks of cancer cells. Unsurprisingly miRNA can target several genes at the center of both mechanisms. Oncogenic antiapoptotic miRNAs like mir-21 [42], miR-221/mir-222 [47], and miR-335 [48] are overexpressed in glioblastoma and interfere with the p53/Bcl-2/PUMA and TGF-β signaling (miR-21/miR-221/miR-222) or with the potential tumor suppressor *DAAM1* (miR-335). Conversely, proapoptotic oncosuppressive miRNAs like miR-218 and miR-451 are downmodulated in GB [49, 50]. Interestingly, downmodulation of miR-221/miR-222 restores the p53 pathway, activates apoptosis, and sensitizes GB cells to TMZ [51]. In addition to its antiapoptotic effect, miR-21, along with miR-17, inhibits autophagy. Downregulation of these miRNA increases the sensitivity of GB cells to TMZ and radiation expanding the cell population undergoing apoptosis [52, 53].

Experimental models indicate that in GB exists a subpopulation of cells possessing the characteristics of neural stem cells that are responsible for continuous proliferation and drug resistance [54, 55]. miRNA profiling revealed that glioma cells have an expression profile remarkably similar to that of embryonic and neural precursor cells and distinct from that of a normal adult brain [56]. Interestingly, a set of 71 miRNA deregulated in human spontaneous GB is remarkably similar to that of chemically induced mouse glioma suggesting a common pattern of cancer development [56]. The miRNAs deregulated in GB and in neural precursor cells are clustered in seven genomic regions and have been associated with many other cancers like the mir-17 family cluster [57–59], miR-182-183 cluster [60], miR-302-367 and miR-372 [59, 61, 62], and the Dlk1 domain [63].

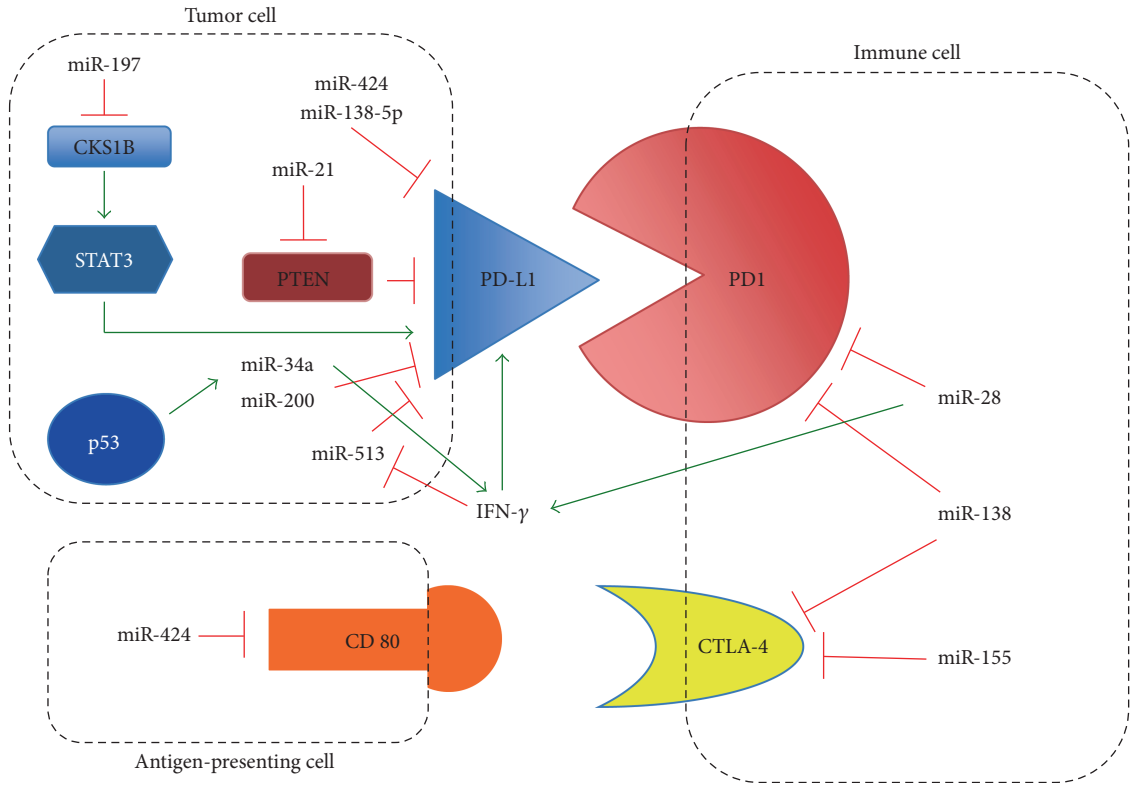

Figure 1: Interactions between miRNA and immune checkpoints. This nonexaustive scheme shows the major interactions between miRNA and immune checkpoint molecules. Red and green arrows indicate the suppressive or activating interactions, respectively.

In GB, recurrent chromosomal aberrations are lacking; nevertheless, chromosomal instability (CIN) is considered an important mechanism for the establishment and maintenance of tumor heterogeneity [64]. CIN has the dual function of responding to various stressing conditions while being, at the same time, the origin of further genome destabilization. The comparison by genome-wide analysis between long-survival GB outlier patients (OS > 33 months) and short-term survivors (OS < 7 months) showed significantly lower genetic alterations in the short-term survivors than in long-term survivors [65]. The increased genomic instability of long-surviving patients might be responsible for the increased vulnerability of the cells to the standard radio- and chemotherapy. Along with this line, it was shown that glioma stem cells have high CIN that accounts for maintaining tumor heterogeneity and that increasing CIN further abolishes tumorigenicity as if an upper limit of genetic instability exists to initiate tumor formation [66].

Unsurprisingly, miRNAs are implicated in the molecular mechanisms of CIN and the intervention of these molecules into chromosomal instability has been studied in several tumors but, to the best of our knowledge, not yet in glioblastoma. Several miRNAs, like miR-26a and miR-28-5p, interfere with genes involved in cell replication and cell cycle checkpoints [67, 68] while others like miR-1255b, miR-148b*, and miR-193b* reduce homologous recombination at G1 thus maintaining genomic stability [69]. Other miRNAs like miR-214 [70], miR-137 [71], miR-1255b, miR-148b*, and miR-193b* [69] regulate at a different level the DNA repair mechanisms.

4. miRNA Targeting Immune Checkpoints and Inflammatory Molecules in Glioblastoma

Targeting the immune checkpoint gene PD1, its ligand PDL1, and other genes like CTLA-4 has raised considerable attention in the recent years because of the dramatic antitumor effect exerted by antibodies against these molecules particularly in tumors with limited therapeutic options like lung cancer and melanoma (reviewed in [72, 73]). In this respect, glioblastoma is not an exception and GB escapes T-cell killing by activating immune checkpoints [74]. In an experimental model of glioma, the blockade of three immune checkpoints (IDO, CTLA-4, and PD1) significantly increased the survival of tumor-bearing mice [75]. However, these findings might not be generally applicable because of the extreme heterogeneity of this tumor and the absence of solid predictive biomarkers of sensitivity to immune checkpoints inhibitors [76]. Immune checkpoints can be modulated by microRNAs [77], and Figure 1 summarizes some of the interaction of the complex network between miRNA, genes, and immune checkpoints.

Intuitively, this complex network requires an extremely precise tuning since immune checkpoint molecules can be blocked by a given miRNA (e.g., miR-34a and miR-138) that, at the same time, can indirectly promote the

expression of cytokines that in turn induce the expression of the same checkpoint molecules that are targeted by the miRNA.

Microglia and astrocytes, along with macrophages, are part of the glioma microenvironment, astrocytes are part of the brain immune system as they express cytokines and chemokines, and glioma cells produce and are targets of inflammatory molecules [78, 79]. Glioma cells produce IL-1 which is a potent inducer of angiogenesis and invasion and in glial cells, strongly upregulates miR-155 implicated in inflammation-mediated cancer development [80]. Besides miR-155, other IL-1-induced miRNAs involved in inflammation, miR-21, and miR-146 are upregulated in gliomas [81]. Interestingly, miR-146 is a negative regulator of astrocyte-mediated inflammation [82], and upregulation of this miRNA decreases the expression of its target TRAF6 that is linked to seizure frequency in glioma patients suggesting that miR-146 could be involved in the epileptogenic focus surrounding the tumor [81].

5. Invasiveness and the Blood Brain Barrier as Escape Mechanisms from Therapy

An important mechanism contributing to the failure of treatment in GB is the invasiveness of the tumor. Brain is a particular environment that is made impermeable to external molecules by the blood brain barrier (BBB); this prevents the efficient targeting of glioma cells with antineoplastic drugs unless the BBB is severely damaged as in glioma above 2–4 mm [83]. Nevertheless, GB can escape treatment also because of its capacity to infiltrate the brain forming niches in regions where the BBB is intact. Invasiveness is part of the epithelial to mesenchymal transition (EMT), a mechanism through which cells lose the epithelial phenotype and acquire that of mesenchymal cells. Several miRNAs are involved in EMT or in general into the mechanisms of invasion; they include miR-21, miR-146, miR-10b, and miR-7 that target directly or indirectly metalloproteinase inhibitors [84, 85], adhesion molecules, and other genes involved in metastasis and cell invasion [86–88].

GB is a highly vascularized tumor, and this feature likely contributes to the invasive and proliferative capacity of the tumor and to the shielding of GB cells behind the BBB. A set of miRNA (miR-296, miR-125b, and others) can directly or indirectly fine-tune angiogenic factors and promote neoangiogenesis in GB [89, 90]. In GB, angiogenesis can be stimulated also by other mechanisms like hypoxia through the targeting of *HIF3A* by miR-210-3p that results in the overexpression of VEGF [91]. Interference with VEGF is not the only angiogenic mechanisms exerted by miRNA in glioma; indeed, neovascularization can be promoted by miR-93 that targets integrin-β8 involved in cell-cell and cell-matrix interactions [92]. The transport mechanisms of miRNA across the BBB is still debated and could involve extracellular vesicles (EV) like exosomes that could serve as a communication tool in nonpathologic situations [93] or between tumor cells and their environment and trigger cell proliferation [94].

MicroRNAs have also an important role in drug and radioresistance as will be described in another part of this review.

6. Circulating miRNA as Glioma Biomarkers

Circulating nucleic acids and circulating tumor cells [95, 96] are extensively exploited as tumor markers to predict outcome and to monitor the response to therapy. Importantly, in GB, response to therapy often results in enhancement of the captation of the contrast medium that can be disguised as progression ("pseudoprogression") challenging the imaging assessment of the disease [97]. The distinction between true progression and pseudoprogression is a diagnostic need required for optimizing patient's care.

Overall, both blood and cerebrospinal fluid are a source of circulating biomarkers and relevant glioma mutations like those of *IDH1*/*IDH2* can be detected in circulating DNA [98]. On the contrary, circulating tumor cells are so scarce in glioma that, with the available technologies, their clinical potentials remain an open issue. Similarly, serum proteomics has not yet provided clinically useful results [99].

Extracellular vesicles (EV) are an attractive source of circulating biomarkers because they act as a cargo for many types of molecules that are protected from degradation [100]. EV are released by all cells to enable the communication between nonadjacent cells, and EV secretion is an early response of cancer cells to a variety of stress conditions including treatment [101]. Interestingly, EV are not randomly loaded and their content seems to reflect the biology of the donor cells making EV an ideal source of circulating biomarkers [102]. Although the utilization of EV in the clinical context is still in its infancy, promising results were obtained in two small GB trials. One of these was aimed at comparing the level of the DNA repair enzymes *MGMT* and *APNG* in the parental tissue and in EV before and after TMZ treatment [103]. In the second study, exosome mRNA was examined to study the changes of expression of immune markers and cytokines after inoculation of a tumor vaccine in glioblastoma patients [104]. Both studies demonstrate that in principle, molecules carried by EV can be utilized to develop robust assays to monitor disease progression in glioblastoma.

Different studies reported the miRNA profiling in the plasma of glioma patients or evaluated the level of defined circulating miRNA known to be involved in this tumor. A restricted signature of eleven miRNAs was selected through a systematic review of the literature and utilized to screen a small cohort of GB patients, and the results showed that the level of expression of miR-497 and miR-125b could distinguish between low- and high-grade glioma [105]. In principle, these types of markers could be very useful to monitor the evolution of primary low-grade glioma into secondary GB for better timing the beginning of therapeutic procedures. The expression of miRNA deregulated in GB was included in several studies on circulating biomarkers. Indeed, miR-21 was found overexpressed in plasma of glioblastoma patients compared to normal controls while miR-128 and miR-342-3p were downmodulated in the same set of patients [106].

Expression of these miRNA returned to baseline levels after treatment suggesting that circulating miRNA can be utilized to monitor disease response to treatment and disease relapse [106]. Interestingly, a recent whole miRNA profiling from the plasma of a relatively large set of glioblastoma patients identified a signature predicting disease-free and overall survival independently from other clinicopathological factors [107].

In conclusion, there are strong indications that circulating biomarkers have the potential to recapitulate the molecular complexity of GB and that they could gain clinical relevance. Nevertheless, more informative biomarkers are needed to develop robust and reproducible assay before a liquid biopsy could become a standard clinical practice.

7. MicroRNA and DNA Methylation: Interplay between Epigenetic Factors

The effects of miRNA dysregulation have been extensively studied initially at the level of single interaction between miRNA and its target gene or genes in a countless number of pathologic and physiologic conditions and more recently as components of signatures or within functional pathways. Intriguingly, miRNA can also be subjected to epigenetic control through DNA methylation and histone modifications [108] thus establishing a complex interplay capable of interfering, directly or indirectly, with multiple pathways in extraordinary complex networks that have been partially explored in several tumors including glioblastoma [109–115].

The effect of miRNA on epigenetic modifier genes and the influence of DNA methylation on miRNA expression in glioblastoma have been studied in some detail. In particular, targeting of *DNMT3a* and *DNMT3b* by miRNA-29, miRNA-29a, and miRNA-148 was observed, and it is generally believed that this interaction may contribute to the general hypomethylation seen in cancer [116–118]. However, the direct link between the expression of the miRNA-29 family and of miRNA-148 and the methylation status of glioblastoma cells has not yet been studied.

EZH2 is a histone methyltransferase that catalyzes the trimethylation of H3 at lysine 27 (H3K27), a postsynthetic modification of H3 leading to transcriptional inactivation [119]. Furthermore, EZH2 promotes the de novo DNA methylation interacting with DNMT3A and DNMT3B [120]. In glioblastoma, and other tumors, EZH2 is overexpressed and acts as an oncogene with multiple mode of actions including cell invasion utilizing largely tumor-specific mechanisms [121, 122], cell cycle progression, maintenance of cell stemness [123], and, last but not least, the development of drug resistance [123, 124] and inhibition of apoptosis [119]. It thus appears that EZH2 is at the center of many cancer-related pathways and that it must be kept under stringent transcriptional control. Several miRNAs, reported in Table 2, and lncRNAs are integral components of mechanisms that regulate *EZH2* expression; however, the role of some of them in GB has not yet been investigated or experimentally proven.

Although manipulating EZH2 expression may seem a promising and logical strategy for the therapy of GB and other tumors, it must be reminded that knocking down a gene that masters DNA and histone methylation will epigenetically influence a vast number of genes with unpredictable effects. Indeed, it was shown that prolonged inhibition of EZH2 results in GB tumor progression whereas short-term inhibition improves survival in animal models [125]. However, it is likely that the major benefits from EZH2 inhibition will derive from appropriate scheduling of cytotoxic and epigenetic drugs as recently proposed [27].

Acetylation and deacetylation of histones H3 and H4 are postsynthetic modifications that contribute to the switching between permissive (acetylated) and repressed (deacetylated) conformation of the chromatin [126]. Acetylation and deacetylation are driven by two sets of enzymes: histone acetyltransferase (HAT) and histone deacetylases (HDAC) that include several variants. In glioblastoma, the expression of *HDAC1* and *HDAC3* is inversely correlated with survival of GB patients, whereas that of *HDAC4, HDAC5*, *HDAC6*, and *HDAC11* is positively correlated with survival of glioma patients [127]. *HDAC1* is a known target of miR-449 and miR-874 [128, 129] but the clinical relevance of the expression pattern of these miRNA in GB is not known. *HDAC4* is targeted by miR-1 and miR-155 [130, 131]; in contrast with the *HDAC* expression data, exogenously expressed miR-1 that putatively should interfere with *HDAC4* acts as a tumor suppressor gene prolonging survival in an animal model [132]. On the contrary, the expression of miR-155 in glioma is prevalent in high-grade tumors with a worse prognosis [133].

Several other genes belonging to the epigenetic machinery are targeted by miRNA; their involvement in glioblastoma is not yet well established, and they will not be discussed here.

Besides controlling epigenetic modifier genes, miRNA can be subjected to epigenetic control. This control can be exerted at three levels: DNA methylation, histone modification, and combined DNA methylation and histone modification. Approximately half of the known miRNAs are hosted in CpG -rich regions and are thus potential targets of DNA methylation; indeed, the effect of DNMT inhibitors has been tested on several cancer cells showing the activation of the miRNA-target gene axis [114, 115, 134]. To the best of our knowledge, the systematic analysis of miRNA silenced by DNA methylation in glioma has not yet been performed; nevertheless, several examples of miRNA silenced by DNA methylation in GB have been described along with the functional effects of their reexpression [135].

miR-211 targets *MMP9*, activates the caspase-9/caspase-3 apoptotic cascade, and was found to be hypermethylated in GB [136]. miR-204, methylated and downregulated in glioma, when activated suppresses the expression of stem transcription factor SOX4, reduces cell invasion, and prolongs survival in animal models [137]. miR-23 is hypermethylated in GB and is reactivated by 5-Azacytidine treatment leading to cell cycle arrest [138]. miR-145 is underexpressed in astrocytoma compared to normal brain, functionally acts as a tumor suppressor gene targeting *SOX2*, a stem-maintaining

gene, and reduces proliferation and migration of GB cells targeting CTGF and NEDD9 [135, 139, 140]. miR-137 is epigenetically inactivated in many cancers, and its expression is diminished in GB and in glioma stem cells. Reexpression of miR-137, hypermethylated in GB tumor samples, promotes neural differentiation and decreases the expression of stem cell markers (Oct4, SOX2, and Nanog) [141]. Furthermore, miR-137 is also an inhibitor of EZH2 [142]. In one of the most comprehensive methylation analysis of miRNA promoter regions to date [143], 29 miRNAs differentially methylated in high-grade glioma were identified. The hypermethylation (and low expression) of three of them, miR-155, miR-210, and miR-355, was a strong predictor of better outcome and longer PFS. However, upon validation in different patient series and in multivariate models, only miR-155 remained of prognostic value independently from other indicators like histology, *MGMT* methylation, and *IDH1*/*IDH2* mutation. Therefore, miR-155 can be considered both as an oncomiR in GBM with multiple biological roles including the activation of the NFkB pathway [143]. On the other hand, miR-155 could act also on the immune cell compartment by downmodulating the immune checkpoint molecule CTLA-4 exerting the function of a tumor suppressor miRNA (Figure 1 and [144]). miR-181c, another example of miRNA downregulated by epigenetic mechanisms in glioblastoma, targets the NOTCH2 pathway and is important in self-renewal, proliferation, and invasion of GB cells [145]. This miRNA was sorted out by chromatin immunoprecipitation/sequencing screening as a region containing H3K4me3 and H3K27ac marks partially overlapping with a CpG-rich region close to miR-181c that is hypermethylated in GB [146].

8. miRNA and Chemo- and Radioresistance in Glioblastoma

The response to treatment in GB patients is variable and probably depends from tumor heterogeneity that originates from genetic and epigenetic alterations which can influence the behavior of the disease. In this respect, the relation between miRNA and chemo- and radiotherapy has been extensively exploited to search for new possible therapeutic targets or to predict and improve the response to treatment.

Earlier preclinical studies showed that cisplatin could increase the efficacy of TMZ by decreasing the activity of MGMT [147] but several clinical trials have tested the activity of cisplatin in GB patients with limited success [148, 149]. Indeed, GB cells after an initial and positive response to cisplatin develop chemoresistance. Many biological pathways underlie the resistance to cisplatin and platinum derivatives [150], and several miRNAs contribute to the reduction of platinum sensitivity. Let-7b seems to be involved in cisplatin resistance affecting the cyclin D1 pathway [151], and miR-873, which targets Bcl-2, is downregulated in a time-dependent manner by cisplatin and, if overexpressed, increases apoptosis in cisplatin-resistant GB cells [152].

Temozolomide is, at the moment, the first-line drug for high-grade glioma treatment independently from the methylation status of *MGMT* (https://www.cancer.gov/types/brain/hp/adult-brain-treatment-pdq#link/_1089_toc). Several mechanisms of resistance to TMZ have been identified (reviewed in [153]), and epigenetic mechanisms, besides *MGMT* methylation, are explored as possible effectors of constitutive or acquired TMZ resistance in GB patients. In this respect, a substantial body of evidence gained mostly in preclinical models supports the idea that many miRNAs interfere with the response of the cells to TMZ.

As discussed above, miR-21 is consistently upregulated in astrocytic tumors (grade II–IV) [154] and downmodulates an entire set of oncosuppressor genes [41, 155, 156]. Indeed, miR-21 has antiapoptotic activity in glioblastoma cells [40] and treatment of GB cells with TMZ results in miR-21 overexpression while its inhibition with specific anti-miR-21 results in high apoptotic levels upon treatment with TMZ [157].

The *AEG-1* (astrocyte elevated gene-1), overexpressed in GB tumor samples, favors the infiltration capabilities of established GB cell lines [158], and its downmodulation by siRNA sensitizes the cells to TMZ. *AEG-1* is directly targeted by miR-136 that, when exogenously overexpressed, increases the cytotoxic activity of TMZ [159]. In principle, the expression of miR-136 could be utilized as an indicator of drug response in GB patients.

Direct targeting of genes controlling the apoptotic pathway is another mechanism capable to modulate TMZ resistance in GB cells. For example, miR-139 inhibits the expression of the antiapoptotic gene *Mcl-1*, a member of the *Bcl-2* family, and sensitizes GB cells to the effect of TMZ [160]. Similarly, miR-143 targets several genes involved in the pathogenesis of cancer like *K*- and *N-RAS*, *Bcl-2*, and *IGF-IR*. The overexpression of miR-143 sensitizes GB cells to apoptosis induced by TMZ and inhibits invasion and proliferation, and this effect has been attributed to the direct targeting of *N-RAS* and, indirectly, to the dephosphorylation of *AKT* and to the downmodulation of *HIF* and *VEGF* as a result of N-*RAS* inhibition [161].

A more direct link with TMZ resistance is attributed to miRNA targeting directly *MGMT*. The inhibition of *MGMT* through different mechanisms besides DNA methylation silencing may at least partly explain the positive response to treatment in patients without methylation of *MGMT*. In this respect, miR-603 and miR-181d directly target and independently coregulate *MGMT* inducing sensitivity to TMZ [162].

As mentioned in a previous section of this review, miR-29c is a direct inhibitor of the de novo DNA methyltransferases *DNMT3a*/*DNMT3b* and is an indirect suppressor of *MGMT* via silencing of Sp1, a *MGMT* transcription factor. Interestingly, forced expression of miR-29c, which is downmodulated in glioblastoma, sensitizes cells to TMZ [163].

Along with chemotherapy, radiotherapy is an integral part of the clinical management of GB and different miRNAs are involved in radiosensitization or radioresistance.

Low levels of ATM protein are a major determinant of radiosensitivity in glioblastoma, and *ATM* is the target of different miRNAs such as miR-100 and miR-26a. High level of miR-100 expression was found in the radiosensitive glioma cell line; on the other hand, its ectopic expression in

radioresistant cells downmodulates ATM and sensitizes the cells to ionizing radiation [164]. Ionizing radiation induces ATM expression (and radioresistance), and miR-26a restores radiosensitivity by targeting ATM [164]. It thus appears that drug and radioresistance in GB are controlled by an array of miRNA that directly or indirectly interferes with multiple pathways involved in drug and radiation response.

9. miRNA and Innovative Therapies in Glioblastoma

The development of multiomics strategies has led to impressive advancements of the knowledge on the mechanisms behind cell transformation and has opened the possibility of selectively targeting cancer cells in many types of tumors including GB [165–168].

In principle, a drug-based "biologic therapy" is aimed at changing the cell phenotype through the use of molecules capable of blocking well-defined pathways. This can be achieved either through the functional inhibition of the enzymatic activities of a given protein or through the ablation of the protein itself. The first strategy leaves the protein unmodified while the second acts on the expression of the target protein and, in theory, should be more effective.

Transcriptional inhibition of a given gene can be obtained by RNA interference, a mechanism originally described in worms [169] and later in higher organisms [170–172]. In practice, it was observed that double-stranded RNA delivered into the cells caused the degradation of the target mRNA and this system is now widely employed for the transient or stable gene inactivation. MicroRNA, because of their hairpin and partially complementary structure, can be considered as an endogenous form of interfering RNA that depending on the extent of complementarity with their targets can either stop the translation or promote the degradation of the mRNA.

A major question to be answered is if miRNA modulation of gene transcription is powerful enough to have a therapeutic consequence in glioma also in view of the necessity of obtaining an adequate delivery at the tumor site. While *in vitro* assays demonstrated the feasibility of this approach, the *in vivo* translation of these studies appears a much more complex task. The partial knowledge of the miRNA networks, pathways, target genes, and of their interplay in healthy and diseased cells adds further difficulties to the short-term therapeutic utilization of these strategies.

One of the questions that need to be answered is if miRNA has the potential to enter the routine clinical practice. Along with this review, we have seen that suppressing certain oncomiR (i.e., miR-21) or inducing the expression of tumor suppressor miRNA like let-7 has dramatic effects on cell behavior and suppress GBM viability. Nevertheless, many major issues still remain, first of all, the problem of delivery, and also the choice between monospecific synthetic siRNA and polyspecific miRNA mimics or miRNA antagonists. siRNAs have the obvious advantage to selectively target specific pathway components while miRNA can interfere with multiple pathways at once. However, the off-target effects of the miRNA have to be carefully evaluated. Furthermore, if a siRNA cocktail seems a reasonable tool, the utilization of a miRNA cocktail seems more complex also because of the conspicuous off-target effect of this cocktail and because of the interactions between different miRNAs [114].

9.1. Biological Therapies in GB: The Delivery Issue. In Glioblastoma, the presence of the BBB represents a major challenge to the utilization of miRNA in therapy because if the BBB is damaged and permeable at the tumor site, its integrity is maintained at the infiltrating tumor areas that are those responsible for tumor relapse after initial surgery and radiochemotherapy [83]. Nevertheless, some preliminary results support the use of antago-miR or miRNA mimics in the therapy of glioma although the issue of the active concentration that can be achieved at the tumor site needs to be taken into consideration.

The ideal goals of the delivery across the BBB are as follows:

(i) to increase the local drug concentration

(ii) to increase the possibility of using drugs that do not pass through the BBB

(iii) to increase the possibility of reaching the tumor niches surrounded by integral BBB that are responsible for tumor relapse

(iv) to increase the possibility of using antitumor drugs in low-grade glioma protected by a functional BBB

Delivery systems can be passive or active. The objective of the passive methods is the permeabilization of the BBB with hyperosmotic agents, surfactant, ultrasounds, and electromagnetic waves to transiently open the tight cell-cell junctions of the BBB [173]. In this respect, a randomized phase III clinical trial showed that the combined treatment of TMZ and pulsed electric fields is superior to the standard TMZ treatment [174]. The direct infusion of drugs or other bioactive molecules at the site of the lesion after craniotomy, even if highly selective, was found of limited utility because of the poor diffusion in the perilesional area where the tumor niches are [175].

Active transport toward the lesion is considered, in general, a more efficient mode to selectively deliver drugs or other molecules within the brain. The most promising active delivery systems are those based on nanoparticles of less than 200 nm [176] that carry on their surface molecules that can be recognized by specific receptors on the BBB, like transferrin, lactoferrin, transferrin receptor, and glutathione [177].

The most commonly utilized carriers for drug delivery in the CNS are liposomes at a single or double layer of approximately 100 nm of diameter that are engineered with molecules for tumor targeting [178, 179]. Some liposomal formulations have entered into the clinical practice, and others are being tested in clinical trials [179–183].

Other utilized delivery systems are the polymeric colloids (PDP) [184–186] or other colloidal formulations (LNC)

[187–189] that can be modified to pass the BBB and to target the tumor utilizing two ligands [185]. The delivery systems based on nanoparticles are highly promising but their toxicity, biocompatibility, and payload retention must be carefully evaluated [190, 191].

9.2. Targeting Glioblastoma Cells with miRNA. Convention-enhanced delivery, a drug delivery method based on catheters stereotactically implanted to infuse the treatments directly to the tumor site, was utilized to deliver let-7a into the brain of mice xenografted with an aggressive GB. This treatment was well tolerated and was effective in reducing the expression of *HMGA2*, one of the targets of let-7a [192].

Although direct delivery of miRNA into the brain seems to be effective, intuitively non- or minimally invasive drug delivery methods may be preferable. In this respect, nanoparticles seem a very promising strategy and were exploited to deliver at the tumor site not only a variety of drugs but also miRNA [193]. In principle, nanoparticles should overcome the poor systemic stability of oligonucleotides and improve their delivery; as said above, different nanoparticle formulations are available each with advantages and disadvantages but they can all be engineered to target the tumor site and, in the case of brain tumors, to transit the BBB. To date, the most common carriers are targeted liposomes of 100 nm [178, 194] that are being tested in animal models [180, 181].

Several types of nanoparticles have been utilized to carry a number of miRNA and to test their biological effects. For example, antago-miR-21 carried by RNP were utilized to successfully rescue the expression of antioncogenic *PTEN* and of *PDCD4* and to promote tumor regression in a model system [195]. Similarly, antago-miR-21, delivered by poly(lactic-*co*-glycolic acid) (PLGA), sensitizes the effect of TMZ *in vitro* [196].

Another interesting example of cooperative treatment in glioblastoma is provided by a multifunctional delivery system MSNPs (mesoporous silica nanoparticle) charged with TMZ molecules and decorated by an anti-miR-221 PNA-octaarginine conjugate (R8-PNA221) that increases the biological effect of TMZ in drug-resistant cells [197]. A similar effect was seen with miR-34a encapsulated in a polyglycerol scaffold [198].

Mesenchymal stem cells (MSC) are an interesting and potentially very effective method to target sites of injury or of inflammation and tumors for therapeutic purposes [199, 200], and it was demonstrated that functional miRNA can be conveyed to neural progenitor cells by cocultivation with appropriately engineered MSC [201].

As mentioned above, miR-10b is involved in tumor invasion and is an optimal therapeutic target because of its high and generalized expression in all GB subtypes [86–88]. A preclinical in vivo study focused on the inhibition of miR-10b in an orthotopic GB xenograft model compared the results of different delivery methods utilizing as endpoint the inhibition of the tumor growth [202]. Brain injections, systemic injections, and intracranial osmotic pumps were compared, and each one showed weak and strong points. The antagonist of miR-10b administered by the three routes resulted in the inhibition of miR-10b and in turn reactivated its target genes, attenuated tumor growth, and prolonged survival. Considering the possible translation from the bench to the bedside, the systemic injections of miR-10b inhibitor were less invasive compared to the other routes and had minimal or no side effects on extracranial tissues and with a good delivery through the BBB.

miRNA "sponges" are oligonucleotide sequences that contain many binding sites for a specific miRNA or miRNA family and act as competitive inhibitors of the binding of the miRNA to their targets [203]. The utility of these "sponges" in GB was recently demonstrated for miR-23b in an orthotopic *in vivo* model and showed the reduction of angiogenesis, migration and invasion, and in turn the malignancy of the tumor [204]. Circular RNAs (circRNA) are natural examples of sponges that are highly resistant to degradation and that are now subject of in-depth investigations because of their strong regulatory activity on miRNA [205].

10. Conclusions

MicroRNAs are epigenetic regulatory molecules that possessing multiple targets have a profound impact on cell physiology and pathology. MicroRNAs are players of the "epigenetic orchestra" that fine-tune the coordinate transcription of the genetic information. It is quite clear that control exerted by miRNA is extraordinary complex, that indeed a single miRNA can bind many genes, and that each gene can be recognized by many miRNAs in an extremely complex direct and indirect regulatory circuitry. Obviously, mastering this network could have dramatic effects on cell behavior.

Therefore, it is not surprising that although our knowledge of the complex effects and interactions between miRNA and genome is still incomplete, the potential implications of miRNA for the diagnosis and prognosis, for the patients' stratification and for their personalized therapy, were not overlooked. However, in order to translate the impressive basic knowledge so far gained on miRNA onto the clinical practice, several issues urgently need to be addressed. Besides the technicisms of delivery and targeting, the major problem remains that of understanding the miRNA effects not anymore at the level of single miRNA-target interaction, but utilizing a "holistic" approach to fully appreciate the balance between miRNA and target genes of opposite functions.

It is quite clear that this will be a highly demanding, but exciting, task for the scientists of the immediate future.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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References

[1] E. Crocetti, A. Trama, C. Stiller et al., "Epidemiology of glial and non-glial brain tumours in Europe," *European Journal of Cancer*, vol. 48, no. 10, pp. 1532–1542, 2012.

[2] D. N. Louis, A. Perry, G. Reifenberger et al., "The 2016 World Health Organization classification of tumors of the central nervous system: a summary," *Acta Neuropathologica*, vol. 131, no. 6, pp. 803–820, 2016.

[3] R. Chen, A. L. Cohen, and H. Colman, "Targeted therapeutics in patients with high-grade gliomas: past, present, and future," *Current Treatment Options in Oncology*, vol. 17, no. 8, p. 42, 2016.

[4] A. D. Kelly and J. J. Issa, "The promise of epigenetic therapy: reprogramming the cancer epigenome," *Current Opinion in Genetics & Development*, vol. 42, pp. 68–77, 2017.

[5] S. Biswas and C. M. Rao, "Epigenetics in cancer: fundamentals and beyond," *Pharmacology & Therapeutics*, vol. 173, pp. 118–134, 2017.

[6] R. G. Gosden and A. P. Feinberg, "Genetics and epigenetics — nature's pen-and-pencil set," *The New England Journal of Medicine*, vol. 356, no. 7, pp. 731–733, 2007.

[7] Cancer Genome Atlas Research Network, "Comprehensive genomic characterization defines human glioblastoma genes and core pathways," *Nature*, vol. 455, no. 7216, pp. 1061–1068, 2008.

[8] H. Noushmehr, D. J. Weisenberger, K. Diefes et al., "Identification of a CpG island methylator phenotype that defines a distinct subgroup of glioma," *Cancer Cell*, vol. 17, no. 5, pp. 510–522, 2010.

[9] D. Sturm, H. Witt, V. Hovestadt et al., "Hotspot mutations in H3F3A and IDH1 define distinct epigenetic and biological subgroups of glioblastoma," *Cancer Cell*, vol. 22, no. 4, pp. 425–437, 2012.

[10] M. E. Hegi, A. C. Diserens, T. Gorlia et al., "MGMT gene silencing and benefit from temozolomide in glioblastoma," *The New England Journal of Medicine*, vol. 352, no. 10, pp. 997–1003, 2005.

[11] R. Stupp, W. P. Mason, M. J. van den Bent et al., "Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma," *The New England Journal of Medicine*, vol. 352, no. 10, pp. 987–996, 2005.

[12] M. Toyota, N. Ahuja, M. Ohe-Toyota, J. G. Herman, S. B. Baylin, and J. P. Issa, "CpG island methylator phenotype in colorectal cancer," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 15, pp. 8681–8686, 1999.

[13] B. F. Miller, F. Sanchez-Vega, and L. Elnitski, "The emergence of pan-cancer CIMP and its elusive interpretation," *Biomolecules*, vol. 6, no. 4, 2016.

[14] M. Van Rijnsoever, H. Elsaleh, D. Joseph, K. McCaul, and B. Iacopetta, "CpG island methylator phenotype is an independent predictor of survival benefit from 5-fluorouracil in stage III colorectal cancer," *Clinical Cancer Research*, vol. 9, no. 8, pp. 2898–2903, 2003.

[15] A. D. Kelly, H. Kroeger, J. Yamazaki et al., "A CpG island methylator phenotype in acute myeloid leukemia independent of IDH mutations and associated with a favorable outcome," *Leukemia*, 2017, in press.

[16] F. Fang, S. Turcan, A. Rimner et al., "Breast cancer methylomes establish an epigenomic foundation for metastasis," *Science Translational Medicine*, vol. 3, no. 75, article 75ra25, 2011.

[17] M. Abe, M. Ohira, A. Kaneda et al., "CpG island methylator phenotype is a strong determinant of poor prognosis in neuroblastomas," *Cancer Research*, vol. 65, no. 3, pp. 828–834, 2005.

[18] B. Banelli, D. F. Merlo, G. Allemanni, A. Forlani, and M. Romani, "Clinical potentials of methylator phenotype in stage 4 high-risk neuroblastoma: an open challenge," *PLoS One*, vol. 8, no. 5, article e63253, 2013.

[19] A. Tanemura, A. M. Terando, M. S. Sim et al., "CpG island methylator phenotype predicts progression of malignant melanoma," *Clinical Cancer Research*, vol. 15, no. 5, pp. 1801–1807, 2009.

[20] I. Y. Eyupoglu and N. E. Savaskan, "Epigenetics in brain tumors: HDACs take center stage," *Current Neuropharmacology*, vol. 14, no. 1, pp. 48–54, 2016.

[21] B. Banelli, E. Carra, F. Barbieri et al., "The histone demethylase KDM5A is a key factor for the resistance to temozolomide in glioblastoma," *Cell Cycle*, vol. 14, no. 21, pp. 3418–3429, 2015.

[22] B. B. Liau, C. Sievers, L. K. Donohue et al., "Adaptive chromatin remodeling drives glioblastoma stem cell plasticity and drug tolerance," *Cell Stem Cell*, vol. 20, no. 2, pp. 233–246.e7, 2017.

[23] G. R. Sareddy, B. C. Nair, S. K. Krishnan et al., "KDM1 is a novel therapeutic target for the treatment of gliomas," *Oncotarget*, vol. 4, no. 1, pp. 18–28, 2013.

[24] D. P. Bartel, "MicroRNAs: genomics, biogenesis, mechanism, and function," *Cell*, vol. 116, no. 2, pp. 281–297, 2004.

[25] A. Kinnaird, S. Zhao, K. E. Wellen, and E. D. Michelakis, "Metabolic control of epigenetics in cancer," *Nature Reviews. Cancer*, vol. 16, no. 11, pp. 694–707, 2016.

[26] M. W. Coolen, C. Stirzaker, J. Z. Song et al., "Consolidation of the cancer genome into domains of repressive chromatin by long-range epigenetic silencing (LRES) reduces transcriptional plasticity," *Nature Cell Biology*, vol. 12, no. 3, pp. 235–246, 2010.

[27] B. Banelli, A. Daga, A. Forlani et al., "Small molecules targeting histone demethylase genes (KDMs) inhibit growth of temozolomide-resistant glioblastoma cells," *Oncotarget*, vol. 8, no. 21, pp. 34896–34910, 2017.

[28] K. Helin and D. Dhanak, "Chromatin proteins and modifications as drug targets," *Nature*, vol. 502, no. 7472, pp. 480–488, 2013.

[29] H. G. Moller, A. P. Rasmussen, H. H. Andersen, K. B. Johnsen, M. Henriksen, and M. Duroux, "A systematic review of microRNA in glioblastoma multiforme: micro-modulators in the mesenchymal mode of migration and invasion," *Molecular Neurobiology*, vol. 47, no. 1, pp. 131–144, 2013.

[30] S. M. Johnson, H. Grosshans, J. Shingara et al., "RAS is regulated by the let-7 microRNA family," *Cell*, vol. 120, no. 5, pp. 635–647, 2005.

[31] V. B. Sampson, N. H. Rong, J. Han et al., "MicroRNA let-7a down-regulates MYC and reverts MYC-induced growth in Burkitt lymphoma cells," *Cancer Research*, vol. 67, no. 20, pp. 9762–9770, 2007.

[32] S. T. Lee, K. Chu, H. J. Oh et al., "Let-7 microRNA inhibits the proliferation of human glioblastoma cells," *Journal of Neuro-Oncology*, vol. 102, no. 1, pp. 19–24, 2011.

[33] T. Papagiannakopoulos, D. Friedmann-Morvinski, P. Neveu et al., "Pro-neural miR-128 is a glioma tumor suppressor that targets mitogenic kinases," *Oncogene*, vol. 31, no. 15, pp. 1884–1895, 2012.

[34] S. Wuchty, D. Arjona, A. Li et al., "Prediction of associations between microRNAs and gene expression in glioma biology," *PLoS One*, vol. 6, no. 2, article e14681, 2011.

[35] Y. Zhang, T. Chao, R. Li et al., "MicroRNA-128 inhibits glioma cells proliferation by targeting transcription factor E2F3a," *Journal of Molecular Medicine*, vol. 87, no. 1, pp. 43–51, 2009.

[36] F. Sun, H. Fu, Q. Liu et al., "Downregulation of CCND1 and CDK6 by miR-34a induces cell cycle arrest," *FEBS Letters*, vol. 582, no. 10, pp. 1564–1568, 2008.

[37] J. Silber, D. A. Lim, C. Petritsch et al., "miR-124 and miR-137 inhibit proliferation of glioblastoma multiforme cells and induce differentiation of brain tumor stem cells," *BMC Medicine*, vol. 6, p. 14, 2008.

[38] W. Wang, L. X. Dai, S. Zhang et al., "Regulation of epidermal growth factor receptor signaling by plasmid-based microRNA-7 inhibits human malignant gliomas growth and metastasis in vivo," *Neoplasma*, vol. 60, no. 3, pp. 274–283, 2013.

[39] B. Kefas, J. Godlewski, L. Comeau et al., "microRNA-7 inhibits the epidermal growth factor receptor and the Akt pathway and is down-regulated in glioblastoma," *Cancer Research*, vol. 68, no. 10, pp. 3566–3572, 2008.

[40] J. A. Chan, A. M. Krichevsky, and K. S. Kosik, "MicroRNA-21 is an antiapoptotic factor in human glioblastoma cells," *Cancer Research*, vol. 65, no. 14, pp. 6029–6033, 2005.

[41] F. Meng, R. Henson, H. Wehbe-Janek, K. Ghoshal, S. T. Jacob, and T. Patel, "MicroRNA-21 regulates expression of the PTEN tumor suppressor gene in human hepatocellular cancer," *Gastroenterology*, vol. 133, no. 2, pp. 647–658, 2007.

[42] T. Papagiannakopoulos, A. Shapiro, and K. S. Kosik, "MicroRNA-21 targets a network of key tumor-suppressive pathways in glioblastoma cells," *Cancer Research*, vol. 68, no. 19, pp. 8164–8172, 2008.

[43] J. H. Qi, Q. Ebrahem, N. Moore et al., "A novel function for tissue inhibitor of metalloproteinases-3 (TIMP3): inhibition of angiogenesis by blockage of VEGF binding to VEGF receptor-2," *Nature Medicine*, vol. 9, no. 4, pp. 407–415, 2003.

[44] M. F. Corsten, R. Miranda, R. Kasmieh, A. M. Krichevsky, R. Weissleder, and K. Shah, "MicroRNA-21 knockdown disrupts glioma growth in vivo and displays synergistic cytotoxicity with neural precursor cell delivered S-TRAIL in human gliomas," *Cancer Research*, vol. 67, no. 19, pp. 8994–9000, 2007.

[45] F. Fornari, L. Gramantieri, M. Ferracin et al., "MiR-221 controls CDKN1C/p57 and CDKN1B/p27 expression in human hepatocellular carcinoma," *Oncogene*, vol. 27, no. 43, pp. 5651–5661, 2008.

[46] J. K. Gillies and I. A. Lorimer, "Regulation of p27Kip1 by miRNA 221/222 in glioblastoma," *Cell Cycle*, vol. 6, no. 16, pp. 2005–2009, 2007.

[47] C. Z. Zhang, J. X. Zhang, A. L. Zhang et al., "MiR-221 and miR-222 target PUMA to induce cell survival in glioblastoma," *Molecular Cancer*, vol. 9, p. 229, 2010.

[48] M. Shu, X. Zheng, S. Wu et al., "Targeting oncogenic miR-335 inhibits growth and invasion of malignant astrocytoma cells," *Molecular Cancer*, vol. 10, p. 59, 2011.

[49] H. Xia, Y. Yan, M. Hu et al., "MiR-218 sensitizes glioma cells to apoptosis and inhibits tumorigenicity by regulating ECOP-mediated suppression of NF-κB activity," *Neuro-Oncology*, vol. 15, no. 4, pp. 413–422, 2013.

[50] Y. Nan, L. Han, A. Zhang et al., "MiRNA-451 plays a role as tumor suppressor in human glioma cells," *Brain Research*, vol. 1359, pp. 14–21, 2010.

[51] L. Chen, J. Zhang, L. Han et al., "Downregulation of miR-221/222 sensitizes glioma cells to temozolomide by regulating apoptosis independently of p53 status," *Oncology Reports*, vol. 27, no. 3, pp. 854–860, 2012.

[52] H. S. Gwak, T. H. Kim, G. H. Jo et al., "Silencing of microRNA-21 confers radio-sensitivity through inhibition of the PI3K/AKT pathway and enhancing autophagy in malignant glioma cell lines," *PLoS One*, vol. 7, no. 10, article e47449, 2012.

[53] S. Comincini, G. Allavena, S. Palumbo et al., "microRNA-17 regulates the expression of ATG7 and modulates the autophagy process, improving the sensitivity to temozolomide and low-dose ionizing radiation treatments in human glioblastoma cells," *Cancer Biology & Therapy*, vol. 14, no. 7, pp. 574–586, 2013.

[54] P. B. Dirks, "Brain tumor stem cells: the cancer stem cell hypothesis writ large," *Molecular Oncology*, vol. 4, no. 5, pp. 420–430, 2010.

[55] T. J. Abou-Antoun, J. S. Hale, J. D. Lathia, and S. M. Dombrowski, "Brain cancer stem cells in adults and children: cell biology and therapeutic implications," *Neurotherapeutics*, vol. 14, no. 2, pp. 372–384, 2017.

[56] I. Lavon, D. Zrihan, A. Granit et al., "Gliomas display a microRNA expression profile reminiscent of neural precursor cells," *Neuro-Oncology*, vol. 12, no. 5, pp. 422–433, 2010.

[57] S. Ambs, R. L. Prueitt, M. Yi et al., "Genomic profiling of microRNA and messenger RNA reveals deregulated microRNA expression in prostate cancer," *Cancer Research*, vol. 68, no. 15, pp. 6162–6170, 2008.

[58] S. Volinia, G. A. Calin, C. G. Liu et al., "A microRNA expression signature of human solid tumors defines cancer gene targets," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 7, pp. 2257–2261, 2006.

[59] T. Uziel, F. V. Karginov, S. Xie et al., "The miR-17~92 cluster collaborates with the Sonic Hedgehog pathway in medulloblastoma," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 8, pp. 2812–2817, 2009.

[60] E. Bandres, E. Cubedo, X. Agirre et al., "Identification by real-time PCR of 13 mature microRNAs differentially expressed in colorectal cancer and non-tumoral tissues," *Molecular Cancer*, vol. 5, p. 29, 2006.

[61] Q. Huang, K. Gumireddy, M. Schrier et al., "The microRNAs miR-373 and miR-520c promote tumour invasion and metastasis," *Nature Cell Biology*, vol. 10, no. 2, pp. 202–210, 2008.

[62] J. Yu, F. Wang, G. H. Yang et al., "Human microRNA clusters: genomic organization and expression profile in leukemia cell lines," *Biochemical and Biophysical Research Communications*, vol. 349, no. 1, pp. 59–68, 2006.

[63] L. Zhang, S. Volinia, T. Bonome et al., "Genomic and epigenetic alterations deregulate microRNA expression in human epithelial ovarian cancer," *Proceedings of the National*

Academy of Sciences of the United States of America, vol. 105, no. 19, pp. 7004–7009, 2008.

[64] H. H. Heng, S. W. Bremer, J. B. Stevens et al., "Chromosomal instability (CIN): what it is and why it is crucial to cancer evolution," *Cancer Metastasis Reviews*, vol. 32, no. 3-4, pp. 325–340, 2013.

[65] S. Peng, H. Dhruv, B. Armstrong et al., "Integrated genomic analysis of survival outliers in glioblastoma," *Neuro-Oncology*, vol. 19, no. 6, pp. 833–844, 2017.

[66] K. M. Godek, M. Venere, Q. Wu et al., "Chromosomal instability affects the tumorigenicity of glioblastoma tumor-initiating cells," *Cancer Discovery*, vol. 6, no. 5, pp. 532–545, 2016.

[67] L. Castellano, A. Dabrowska, L. Pellegrino et al., "Sustained expression of miR-26a promotes chromosomal instability and tumorigenesis through regulation of CHFR," *Nucleic Acids Research*, vol. 45, no. 8, pp. 4401–4412, 2017.

[68] M. P. Hell, C. R. Thoma, N. Fankhauser, Y. Christinat, T. C. Weber, and W. Krek, "miR-28-5p promotes chromosomal instability in VHL-associated cancers by inhibiting Mad2 translation," *Cancer Research*, vol. 74, no. 9, pp. 2432–2443, 2014.

[69] Y. E. Choi, Y. Pan, E. Park et al., "MicroRNAs down-regulate homologous recombination in the G1 phase of cycling cells to maintain genomic stability," *eLife*, vol. 3, article e02445, 2014.

[70] Z. Wang, H. Yin, Y. Zhang et al., "miR-214-mediated downregulation of RNF8 induces chromosomal instability in ovarian cancer cells," *Cell Cycle*, vol. 13, no. 22, pp. 3519–3528, 2014.

[71] Y. Qin, S. Zhang, S. Deng et al., "Epigenetic silencing of miR-137 induces drug resistance and chromosomal instability by targeting AURKA in multiple myeloma," *Leukemia*, vol. 31, no. 5, pp. 1123–1135, 2017.

[72] M. G. Dal Bello, A. Alama, S. Coco, I. Vanni, and F. Grossi, "Understanding the checkpoint blockade in lung cancer immunotherapy," *Drug Discovery Today*, vol. 22, no. 8, pp. 1266–1273, 2017.

[73] D. Schadendorf, D. E. Fisher, C. Garbe et al., "Melanoma," *Nature Reviews Disease Primers*, vol. 1, article 15003, 2015.

[74] M. Preusser, M. Lim, D. A. Hafler, D. A. Reardon, and J. H. Sampson, "Prospects of immune checkpoint modulators in the treatment of glioblastoma," *Nature Reviews Neurology*, vol. 11, no. 9, pp. 504–514, 2015.

[75] D. A. Wainwright, A. L. Chang, M. Dey et al., "Durable therapeutic efficacy utilizing combinatorial blockade against IDO, CTLA-4, and PD-L1 in mice with brain tumors," *Clinical Cancer Research*, vol. 20, no. 20, pp. 5290–5301, 2014.

[76] A. D. Garg, L. Vandenberk, M. Van Woensel et al., "Preclinical efficacy of immune-checkpoint monotherapy does not recapitulate corresponding biomarkers-based clinical predictions in glioblastoma," *OncoImmunology*, vol. 6, no. 4, article e1295903, 2017.

[77] M. A. Smolle, H. N. Calin, M. Pichler, and G. A. Calin, "Noncoding RNAs and immune checkpoints-clinical implications as cancer therapeutics," *The FEBS Journal*, vol. 284, no. 13, pp. 1952–1966, 2017.

[78] N. A. Charles, E. C. Holland, R. Gilbertson, R. Glass, and H. Kettenmann, "The brain tumor microenvironment," *Glia*, vol. 59, no. 8, pp. 1169–1180, 2011.

[79] I. Yang, S. J. Han, G. Kaur, C. Crane, and A. T. Parsa, "The role of microglia in central nervous system immunity and glioma immunology," *Journal of Clinical Neuroscience*, vol. 17, no. 1, pp. 6–10, 2010.

[80] E. Tili, J. J. Michaille, D. Wernicke et al., "Mutator activity induced by microRNA-155 (miR-155) links inflammation and cancer," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 12, pp. 4908–4913, 2011.

[81] A. S. Prabowo, J. van Scheppingen, A. M. Iyer et al., "Differential expression and clinical significance of three inflammation-related microRNAs in gangliogliomas," *Journal of Neuroinflammation*, vol. 12, p. 97, 2015.

[82] A. Iyer, E. Zurolo, A. Prabowo et al., "MicroRNA-146a: a key regulator of astrocyte-mediated inflammatory response," *PLoS One*, vol. 7, no. 9, article e44789, 2012.

[83] K. E. Schlageter, P. Molnar, G. D. Lapin, and D. R. Groothuis, "Microvessel organization and structure in experimental brain tumors: microvessel populations with distinctive structural and functional properties," *Microvascular Research*, vol. 58, no. 3, pp. 312–328, 1999.

[84] Y. Li, Y. Wang, L. Yu et al., "miR-146b-5p inhibits glioma migration and invasion by targeting MMP16," *Cancer Letters*, vol. 339, no. 2, pp. 260–269, 2013.

[85] G. Gabriely, T. Wurdinger, S. Kesari et al., "MicroRNA 21 promotes glioma invasion by targeting matrix metalloproteinase regulators," *Molecular and Cellular Biology*, vol. 28, no. 17, pp. 5369–5380, 2008.

[86] L. Sun, W. Yan, Y. Wang et al., "MicroRNA-10b induces glioma cell invasion by modulating MMP-14 and uPAR expression via HOXD10," *Brain Research*, vol. 1389, pp. 9–18, 2011.

[87] J. Lin, S. Teo, D. H. Lam, K. Jeyaseelan, and S. Wang, "MicroRNA-10b pleiotropically regulates invasion, angiogenicity and apoptosis of tumor cells resembling mesenchymal subtype of glioblastoma multiforme," *Cell Death & Disease*, vol. 3, article e398, 2012.

[88] D. G. Wu, Y. Y. Wang, L. G. Fan et al., "MicroRNA-7 regulates glioblastoma cell invasion via targeting focal adhesion kinase expression," *Chinese Medical Journal*, vol. 124, no. 17, pp. 2616–2621, 2011.

[89] S. Wang and E. N. Olson, "AngiomiRs—key regulators of angiogenesis," *Current Opinion in Genetics & Development*, vol. 19, no. 3, pp. 205–211, 2009.

[90] T. Wurdinger, B. A. Tannous, O. Saydam et al., "miR-296 regulates growth factor receptor overexpression in angiogenic endothelial cells," *Cancer Cell*, vol. 14, no. 5, pp. 382–393, 2008.

[91] R. Agrawal, P. Pandey, P. Jha, V. Dwivedi, C. Sarkar, and R. Kulshreshtha, "Hypoxic signature of microRNAs in glioblastoma: insights from small RNA deep sequencing," *BMC Genomics*, vol. 15, p. 686, 2014.

[92] L. Fang, Z. Deng, T. Shatseva et al., "MicroRNA miR-93 promotes tumor growth and angiogenesis by targeting integrin-β8," *Oncogene*, vol. 30, no. 7, pp. 806–821, 2011.

[93] N. Kosaka, H. Iguchi, Y. Yoshioka, K. Hagiwara, F. Takeshita, and T. Ochiya, "Competitive interactions of cancer cells and normal cells via secretory microRNAs," *The Journal of Biological Chemistry*, vol. 287, no. 2, pp. 1397–1405, 2012.

[94] J. Skog, T. Wurdinger, S. van Rijn et al., "Glioblastoma microvesicles transport RNA and proteins that promote tumour

growth and provide diagnostic biomarkers," *Nature Cell Biology*, vol. 10, no. 12, pp. 1470–1476, 2008.

[95] X. Han, J. Wang, and Y. Sun, "Circulating tumor DNA as biomarkers for cancer detection," *Genomics, Proteomics & Bioinformatics*, vol. 15, no. 2, pp. 59–72, 2017.

[96] S. B. Liang and L. W. Fu, "Application of single-cell technology in cancer research," *Biotechnology Advances*, vol. 35, no. 4, pp. 443–449, 2017.

[97] B. M. Ellingson, C. Chung, W. B. Pope, J. L. Boxerman, and T. J. Kaufmann, "Pseudoprogression, radionecrosis, inflammation or true tumor progression? Challenges associated with glioblastoma response assessment in an evolving therapeutic landscape," *Journal of Neuro-Oncology*, 2017, in press.

[98] B. Boisselier, J. Gallego Perez-Larraya, M. Rossetto et al., "Detection of IDH1 mutation in the plasma of patients with glioma," *Neurology*, vol. 79, no. 16, pp. 1693–1698, 2012.

[99] D. Capper, M. Simon, C. D. Langhans et al., "2-hydroxyglutarate concentration in serum from patients with gliomas does not correlate with IDH1/2 mutation status or tumor size," *International Journal of Cancer*, vol. 131, no. 3, pp. 766–768, 2012.

[100] J. C. Contreras-Naranjo, H. J. Wu, and V. M. Ugaz, "Microfluidics for exosome isolation and analysis: enabling liquid biopsy for personalized medicine," *Lab on a Chip*, 2017, in press.

[101] P. Kucharzewska and M. Belting, "Emerging roles of extracellular vesicles in the adaptive response of tumour cells to microenvironmental stress," *Journal of Extracellular Vesicles*, vol. 2, 2013.

[102] M. Morishita, Y. Takahashi, M. Nishikawa et al., "Quantitative analysis of tissue distribution of the B16BL6-derived exosomes using a streptavidin-lactadherin fusion protein and iodine-125-labeled biotin derivative after intravenous injection in mice," *Journal of Pharmaceutical Sciences*, vol. 104, no. 2, pp. 705–713, 2015.

[103] H. Shao, J. Chung, K. Lee et al., "Chip-based analysis of exosomal mRNA mediating drug resistance in glioblastoma," *Nature Communications*, vol. 6, p. 6999, 2015.

[104] L. Muller, S. Muller-Haegele, M. Mitsuhashi, W. Gooding, H. Okada, and T. L. Whiteside, "Exosomes isolated from plasma of glioma patients enrolled in a vaccination trial reflect antitumor immune activity and might predict survival," *OncoImmunology*, vol. 4, no. 6, article e1008347, 2015.

[105] G. Regazzo, I. Terrenato, M. Spagnuolo et al., "A restricted signature of serum miRNAs distinguishes glioblastoma from lower grade gliomas," *Journal of Experimental & Clinical Cancer Research*, vol. 35, no. 1, p. 124, 2016.

[106] Q. Wang, P. Li, A. Li et al., "Plasma specific miRNAs as predictive biomarkers for diagnosis and prognosis of glioma," *Journal of Experimental & Clinical Cancer Research*, vol. 31, p. 97, 2012.

[107] H. Zhao, J. Shen, T. R. Hodges, R. Song, G. N. Fuller, and A. B. Heimberger, "Serum microRNA profiling in patients with glioblastoma: a survival analysis," *Molecular Cancer*, vol. 16, no. 1, p. 59, 2017.

[108] Z. Wang, H. Yao, S. Lin et al., "Transcriptional and epigenetic regulation of human microRNAs," *Cancer Letters*, vol. 331, no. 1, pp. 1–10, 2013.

[109] M. J. Ha, V. Baladandayuthapani, and K. A. Do, "DINGO: differential network analysis in genomics," *Bioinformatics*, vol. 31, no. 21, pp. 3413–3420, 2015.

[110] J. D. Doecke, Y. Wang, and K. Baggerly, "Co-localized genomic regulation of miRNA and mRNA via DNA methylation affects survival in multiple tumor types," *Cancer Genetics*, vol. 209, no. 10, pp. 463–473, 2016.

[111] H. Lopez-Bertoni, B. Lal, A. Li et al., "DNMT-dependent suppression of microRNA regulates the induction of GBM tumor-propagating phenotype by Oct4 and Sox2," *Oncogene*, vol. 34, no. 30, pp. 3994–4004, 2015.

[112] P. Jha, R. Agrawal, P. Pathak et al., "Genome-wide small noncoding RNA profiling of pediatric high-grade gliomas reveals deregulation of several miRNAs, identifies downregulation of snoRNA cluster HBII-52 and delineates H3F3A and TP53 mutant-specific miRNAs and snoRNAs," *International Journal of Cancer*, vol. 137, no. 10, pp. 2343–2353, 2015.

[113] M. Maugeri, D. Barbagallo, C. Barbagallo et al., "Altered expression of miRNAs and methylation of their promoters are correlated in neuroblastoma," *Oncotarget*, vol. 7, no. 50, pp. 83330–83341, 2016.

[114] F. Parodi, R. Carosio, M. Ragusa et al., "Epigenetic dysregulation in neuroblastoma: a tale of miRNAs and DNA methylation," *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms*, vol. 1859, no. 12, pp. 1502–1514, 2016.

[115] D. Barbagallo, A. Condorelli, M. Ragusa et al., "Dysregulated miR-671-5p/CDR1-AS/CDR1/VSNL1 axis is involved in glioblastoma multiforme," *Oncotarget*, vol. 7, no. 4, pp. 4746–4759, 2016.

[116] M. Fabbri, R. Garzon, A. Cimmino et al., "MicroRNA-29 family reverts aberrant methylation in lung cancer by targeting DNA methyltransferases 3A and 3B," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 40, pp. 15805–15810, 2007.

[117] R. Garzon, S. Liu, M. Fabbri et al., "MicroRNA-29b induces global DNA hypomethylation and tumor suppressor gene reexpression in acute myeloid leukemia by targeting directly DNMT3A and 3B and indirectly DNMT1," *Blood*, vol. 113, no. 25, pp. 6411–6418, 2009.

[118] A. M. Duursma, M. Kedde, M. Schrier, C. le Sage, and R. Agami, "miR-148 targets human DNMT3b protein coding region," *RNA*, vol. 14, no. 5, pp. 872–877, 2008.

[119] Y. Yin, S. Qiu, and Y. Peng, "Functional roles of enhancer of zeste homolog 2 in gliomas," *Gene*, vol. 576, 1, Part 2, pp. 189–194, 2016.

[120] E. Vire, C. Brenner, R. Deplus et al., "The polycomb group protein EZH2 directly controls DNA methylation," *Nature*, vol. 439, no. 7078, pp. 871–874, 2006.

[121] F. Crea, E. M. Hurt, and W. L. Farrar, "Clinical significance of polycomb gene expression in brain tumors," *Molecular Cancer*, vol. 9, p. 265, 2010.

[122] G. Ren, S. Baritaki, H. Marathe et al., "Polycomb protein EZH2 regulates tumor invasion via the transcriptional repression of the metastasis suppressor RKIP in breast and prostate cancer," *Cancer Research*, vol. 72, no. 12, pp. 3091–3104, 2012.

[123] S. H. Kim, K. Joshi, R. Ezhilarasan et al., "EZH2 protects glioma stem cells from radiation-induced cell death in a MELK/FOXM1-dependent manner," *Stem Cell Reports*, vol. 4, no. 2, pp. 226–238, 2015.

[124] T. Y. Fan, H. Wang, P. Xiang et al., "Inhibition of EZH2 reverses chemotherapeutic drug TMZ chemosensitivity in glioblastoma," *International Journal of Clinical and Experimental Pathology*, vol. 7, no. 10, pp. 6662–6670, 2014.

[125] N. A. de Vries, D. Hulsman, W. Akhtar et al., "Prolonged Ezh2 depletion in glioblastoma causes a robust switch in cell fate resulting in tumor progression," *Cell Reports*, vol. 10, no. 3, pp. 383–397, 2015.

[126] V. G. Allfrey and A. E. Mirsky, "Structural modifications of histones and their possible role in the regulation of RNA synthesis," *Science*, vol. 144, no. 3618, p. 559, 1964.

[127] N. Dali-Youcef, S. Froelich, F. M. Moussallieh et al., "Gene expression mapping of histone deacetylases and co-factors, and correlation with survival time and 1H-HRMAS metabolomic profile in human gliomas," *Scientific Reports*, vol. 5, article 9087, 2015.

[128] E. J. Noonan, R. F. Place, D. Pookot et al., "miR-449a targets HDAC-1 and induces growth arrest in prostate cancer," *Oncogene*, vol. 28, no. 14, pp. 1714–1724, 2009.

[129] N. Nohata, T. Hanazawa, T. Kinoshita et al., "Tumour-suppressive microRNA-874 contributes to cell proliferation through targeting of histone deacetylase 1 in head and neck squamous cell carcinoma," *British Journal of Cancer*, vol. 108, no. 8, pp. 1648–1658, 2013.

[130] J. F. Chen, E. M. Mandel, J. M. Thomson et al., "The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation," *Nature Genetics*, vol. 38, no. 2, pp. 228–233, 2006.

[131] S. K. Sandhu, S. Volinia, S. Costinean et al., "miR-155 targets histone deacetylase 4 (HDAC4) and impairs transcriptional activity of B-cell lymphoma 6 (BCL6) in the Emu-miR-155 transgenic mouse model," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 109, no. 49, pp. 20047–20052, 2012.

[132] A. Bronisz, Y. Wang, M. O. Nowicki et al., "Extracellular vesicles modulate the glioblastoma microenvironment via a tumor suppression signaling network directed by miR-1," *Cancer Research*, vol. 74, no. 3, pp. 738–750, 2014.

[133] X. Wu, Y. Wang, T. Yu et al., "Blocking MIR155HG/miR-155 axis inhibits mesenchymal transition in glioma," *Neuro-Oncology*, vol. 19, no. 9, pp. 1195–1205, 2017.

[134] J. Roman-Gomez, X. Agirre, A. Jimenez-Velasco et al., "Epigenetic regulation of microRNAs in acute lymphoblastic leukemia," *Journal of Clinical Oncology*, vol. 27, no. 8, pp. 1316–1322, 2009.

[135] H. K. Lee, S. Finniss, S. Cazacu et al., "Mesenchymal stem cells deliver synthetic microRNA mimics to glioma cells and glioma stem cells and inhibit their cell migration and self-renewal," *Oncotarget*, vol. 4, no. 2, pp. 346–361, 2013.

[136] S. Asuthkar, K. K. Velpula, C. Chetty, B. Gorantla, and J. S. Rao, "Epigenetic regulation of miRNA-211 by MMP-9 governs glioma cell apoptosis, chemosensitivity and radiosensitivity," *Oncotarget*, vol. 3, no. 11, pp. 1439–1454, 2012.

[137] Z. Ying, Y. Li, J. Wu et al., "Loss of miR-204 expression enhances glioma migration and stem cell-like phenotype," *Cancer Research*, vol. 73, no. 2, pp. 990–999, 2013.

[138] J. Geng, H. Luo, Y. Pu et al., "Methylation mediated silencing of miR-23b expression and its role in glioma stem cells," *Neuroscience Letters*, vol. 528, no. 2, pp. 185–189, 2012.

[139] H. K. Lee, A. Bier, S. Cazacu et al., "MicroRNA-145 is downregulated in glial tumors and regulates glioma cell migration by targeting connective tissue growth factor," *PLoS One*, vol. 8, no. 2, article e54652, 2013.

[140] M. C. Speranza, V. Frattini, F. Pisati et al., "NEDD9, a novel target of miR-145, increases the invasiveness of glioblastoma," *Oncotarget*, vol. 3, no. 7, pp. 723–734, 2012.

[141] A. Bier, N. Giladi, N. Kronfeld et al., "MicroRNA-137 is downregulated in glioblastoma and inhibits the stemness of glioma stem cells by targeting RTVP-1," *Oncotarget*, vol. 4, no. 5, pp. 665–676, 2013.

[142] K. E. Szulwach, X. Li, R. D. Smrt et al., "Cross talk between microRNA and epigenetic regulation in adult neurogenesis," *The Journal of Cell Biology*, vol. 189, no. 1, pp. 127–141, 2010.

[143] M. G. Schliesser, R. Claus, T. Hielscher et al., "Prognostic relevance of miRNA-155 methylation in anaplastic glioma," *Oncotarget*, vol. 7, no. 50, pp. 82028–82045, 2016.

[144] Y. Zhang, E. Sun, X. Li et al., "miR-155 contributes to Df1-induced asthma by increasing the proliferative response of Th cells via CTLA-4 downregulation," *Cellular Immunology*, vol. 314, pp. 1–9, 2017.

[145] J. Ruan, S. Lou, Q. Dai, D. Mao, J. Ji, and X. Sun, "Tumor suppressor miR-181c attenuates proliferation, invasion, and self-renewal abilities in glioblastoma," *Neuroreport*, vol. 26, no. 2, pp. 66–73, 2015.

[146] E. Ayala-Ortega, R. Arzate-Mejia, R. Perez-Molina et al., "Epigenetic silencing of miR-181c by DNA methylation in glioblastoma cell lines," *BMC Cancer*, vol. 16, p. 226, 2016.

[147] S. D'Atri, G. Graziani, P. M. Lacal et al., "Attenuation of O 6-methylguanine-DNA methyltransferase activity and mRNA levels by cisplatin and temozolomide in Jurkat cells," *The Journal of Pharmacology and Experimental Therapeutics*, vol. 294, no. 2, pp. 664–671, 2000.

[148] J. C. Buckner, K. V. Ballman, J. C. Michalak et al., "Phase III trial of carmustine and cisplatin compared with carmustine alone and standard radiation therapy or accelerated radiation therapy in patients with glioblastoma multiforme: North Central Cancer Treatment Group 93-72-52 and Southwest Oncology Group 9503 Trials," *Journal of Clinical Oncology*, vol. 24, no. 24, pp. 3871–3879, 2006.

[149] A. A. Brandes, U. Basso, M. Reni et al., "First-line chemotherapy with cisplatin plus fractionated temozolomide in recurrent glioblastoma multiforme: a phase II study of the Gruppo Italiano Cooperativo di Neuro-Oncologia," *Journal of Clinical Oncology*, vol. 22, no. 9, pp. 1598–1604, 2004.

[150] D. W. Shen, L. M. Pouliot, M. D. Hall, and M. M. Gottesman, "Cisplatin resistance: a cellular self-defense mechanism resulting from multiple epigenetic and genetic changes," *Pharmacological Reviews*, vol. 64, no. 3, pp. 706–721, 2012.

[151] Y. Guo, K. Yan, J. Fang, Q. Qu, M. Zhou, and F. Chen, "Let-7b expression determines response to chemotherapy through the regulation of cyclin D1 in glioblastoma," *Journal of Experimental & Clinical Cancer Research*, vol. 32, p. 41, 2013.

[152] X. Chen, Y. Zhang, Y. Shi et al., "MiR-873 acts as a novel sensitizer of glioma cells to cisplatin by targeting Bcl-2," *International Journal of Oncology*, vol. 47, no. 4, pp. 1603–1611, 2015.

[153] K. Messaoudi, A. Clavreul, and F. Lagarce, "Toward an effective strategy in glioblastoma treatment. Part I: resistance mechanisms and strategies to overcome resistance of glioblastoma to temozolomide," *Drug Discovery Today*, vol. 20, no. 7, pp. 899–905, 2015.

[154] A. Conti, M. Aguennouz, D. La Torre et al., "miR-21 and 221 upregulation and miR-181b downregulation in human grade

II-IV astrocytic tumors," *Journal of Neuro-Oncology*, vol. 93, no. 3, pp. 325–332, 2009.

[155] I. A. Asangani, S. A. Rasheed, D. A. Nikolova et al., "MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer," *Oncogene*, vol. 27, no. 15, pp. 2128–2136, 2008.

[156] S. Zhu, M. L. Si, H. Wu, and Y. Y. Mo, "MicroRNA-21 targets the tumor suppressor gene tropomyosin 1 (TPM1)," *The Journal of Biological Chemistry*, vol. 282, no. 19, pp. 14328–14336, 2007.

[157] S. T. Wong, X. Q. Zhang, J. T. Zhuang, H. L. Chan, C. H. Li, and G. K. Leung, "MicroRNA-21 inhibition enhances in vitro chemosensitivity of temozolomide-resistant glioblastoma cells," *Anticancer Research*, vol. 32, no. 7, pp. 2835–2841, 2012.

[158] L. Emdad, D. Sarkar, S. G. Lee et al., "Astrocyte elevated gene-1: a novel target for human glioma therapy," *Molecular Cancer Therapeutics*, vol. 9, no. 1, pp. 79–88, 2010.

[159] H. Wu, Q. Liu, T. Cai, Y. D. Chen, F. Liao, and Z. F. Wang, "MiR-136 modulates glioma cell sensitivity to temozolomide by targeting astrocyte elevated gene-1," *Diagnostic Pathology*, vol. 9, p. 173, 2014.

[160] R. Y. Li, L. C. Chen, H. Y. Zhang et al., "MiR-139 inhibits Mcl-1 expression and potentiates TMZ-induced apoptosis in glioma," *CNS Neuroscience & Therapeutics*, vol. 19, no. 7, pp. 477–483, 2013.

[161] L. Wang, Z. M. Shi, C. F. Jiang et al., "MiR-143 acts as a tumor suppressor by targeting N-RAS and enhances temozolomide-induced apoptosis in glioma," *Oncotarget*, vol. 5, no. 14, pp. 5416–5427, 2014.

[162] D. Kushwaha, V. Ramakrishnan, K. Ng et al., "A genome-wide miRNA screen revealed miR-603 as a MGMT-regulating miRNA in glioblastomas," *Oncotarget*, vol. 5, no. 12, pp. 4026–4039, 2014.

[163] S. Xiao, Z. Yang, X. Qiu et al., "miR-29c contribute to glioma cells temozolomide sensitivity by targeting O6-methylguanine-DNA methyltransferases indirectely," *Oncotarget*, vol. 7, no. 31, pp. 50229–50238, 2016.

[164] W. L. Ng, D. Yan, X. Zhang, Y. Y. Mo, and Y. Wang, "Over-expression of miR-100 is responsible for the low-expression of ATM in the human glioma cell line: M059J," *DNA Repair (Amst)*, vol. 9, no. 11, pp. 1170–1175, 2010.

[165] B. C. Yoo, K. H. Kim, S. M. Woo, and J. K. Myung, "Clinical multi-omics strategies for the effective cancer management," *Journal of Proteomics*, 2017, in press.

[166] J. H. Uhm and A. B. Porter, "Treatment of glioma in the 21st century: an exciting decade of postsurgical treatment advances in the molecular era," *Mayo Clinic Proceedings*, vol. 92, no. 6, pp. 995–1004, 2017.

[167] D. Mangani, M. Weller, and P. Roth, "The network of immunosuppressive pathways in glioblastoma," *Biochemical Pharmacology*, vol. 130, pp. 1–9, 2017.

[168] J. Polivka Jr., J. Polivka, L. Holubec et al., "Advances in experimental targeted therapy and immunotherapy for patients with glioblastoma multiforme," *Anticancer Research*, vol. 37, no. 1, pp. 21–33, 2017.

[169] A. Fire, S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver, and C. C. Mello, "Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans," *Nature*, vol. 391, no. 6669, pp. 806–811, 1998.

[170] S. M. Hammond, E. Bernstein, D. Beach, and G. J. Hannon, "An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells," *Nature*, vol. 404, no. 6775, pp. 293–296, 2000.

[171] N. Shrivastava and A. Srivastava, "RNA interference: an emerging generation of biologicals," *Biotechnology Journal*, vol. 3, no. 3, pp. 339–353, 2008.

[172] P. J. Paddison, "RNA interference in mammalian cell systems," *Current Topics in Microbiology and Immunology*, vol. 320, pp. 1–19, 2008.

[173] R. Karim, C. Palazzo, B. Evrard, and G. Piel, "Nanocarriers for the treatment of glioblastoma multiforme: current state-of-the-art," *Journal of Controlled Release*, vol. 227, pp. 23–37, 2016.

[174] R. Stupp, S. Taillibert, A. A. Kanner et al., "Maintenance therapy with tumor-treating fields plus temozolomide vs temozolomide alone for glioblastoma: a randomized clinical trial," *Journal of the American Medical Association*, vol. 314, no. 23, pp. 2535–2543, 2015.

[175] W. M. Pardridge, "Drug and gene delivery to the brain: the vascular route," *Neuron*, vol. 36, no. 4, pp. 555–558, 2002.

[176] W. Lu, Y. Zhang, Y. Z. Tan, K. L. Hu, X. G. Jiang, and S. K. Fu, "Cationic albumin-conjugated pegylated nanoparticles as novel drug carrier for brain delivery," *Journal of Controlled Release*, vol. 107, no. 3, pp. 428–448, 2005.

[177] Y. Chen and L. Liu, "Modern methods for delivery of drugs across the blood-brain barrier," *Advanced Drug Delivery Reviews*, vol. 64, no. 7, pp. 640–665, 2012.

[178] R. I. Pakunlu, Y. Wang, M. Saad, J. J. Khandare, V. Starovoytov, and T. Minko, "In vitro and in vivo intracellular liposomal delivery of antisense oligonucleotides and anticancer drug," *Journal of Controlled Release*, vol. 114, no. 2, pp. 153–162, 2006.

[179] H. I. Chang and M. K. Yeh, "Clinical development of liposome-based drugs: formulation, characterization, and therapeutic efficacy," *International Journal of Nanomedicine*, vol. 7, pp. 49–60, 2012.

[180] G. Charest, L. Sanche, D. Fortin, D. Mathieu, and B. Paquette, "Glioblastoma treatment: bypassing the toxicity of platinum compounds by using liposomal formulation and increasing treatment efficiency with concomitant radiotherapy," *International Journal of Radiation Oncology, Biology, Physics*, vol. 84, no. 1, pp. 244–249, 2012.

[181] M. I. Koukourakis, S. Koukouraki, I. Fezoulidis et al., "High intratumoural accumulation of stealth liposomal doxorubicin (Caelyx) in glioblastomas and in metastatic brain tumours," *British Journal of Cancer*, vol. 83, no. 10, pp. 1281–1286, 2000.

[182] S. Sengupta, B. Thaci, A. C. Crawford, and P. Sampath, "Interleukin-13 receptor alpha 2-targeted glioblastoma immunotherapy," *BioMed Research International*, vol. 2014, Article ID 952128, 8 pages, 2014.

[183] Y. Zhang, H. Jeong Lee, R. J. Boado, and W. M. Pardridge, "Receptor-mediated delivery of an antisense gene to human brain cancer cells," *The Journal of Gene Medicine*, vol. 4, no. 2, pp. 183–194, 2002.

[184] A. Ambruosi, S. Gelperina, A. Khalansky, S. Tanski, A. Theisen, and J. Kreuter, "Influence of surfactants, polymer and doxorubicin loading on the anti-tumour effect of poly(-butyl cyanoacrylate) nanoparticles in a rat glioma model," *Journal of Microencapsulation*, vol. 23, no. 5, pp. 582–592, 2006.

[185] Y. Liu and W. Lu, "Recent advances in brain tumor-targeted nano-drug delivery systems," *Expert Opinion on Drug Delivery*, vol. 9, no. 6, pp. 671–686, 2012.

[186] G. Tosi, L. Costantino, B. Ruozi, F. Forni, and M. A. Vandelli, "Polymeric nanoparticles for the drug delivery to the central nervous system," *Expert Opinion on Drug Delivery*, vol. 5, no. 2, pp. 155–174, 2008.

[187] E. Allard, N. T. Huynh, A. Vessieres et al., "Dose effect activity of ferrocifen-loaded lipid nanocapsules on a 9L-glioma model," *International Journal of Pharmaceutics*, vol. 379, no. 2, pp. 317–323, 2009.

[188] E. Allard, C. Passirani, E. Garcion et al., "Lipid nanocapsules loaded with an organometallic tamoxifen derivative as a novel drug-carrier system for experimental malignant gliomas," *Journal of Controlled Release*, vol. 130, no. 2, pp. 146–153, 2008.

[189] A. L. Laine, A. Clavreul, A. Rousseau et al., "Inhibition of ectopic glioma tumor growth by a potent ferrocenyl drug loaded into stealth lipid nanocapsules," *Nanomedicine*, vol. 10, no. 8, pp. 1667–1677, 2014.

[190] G. Bhabra, A. Sood, B. Fisher et al., "Nanoparticles can cause DNA damage across a cellular barrier," *Nature Nanotechnology*, vol. 4, no. 12, pp. 876–883, 2009.

[191] N. Gou, A. Onnis-Hayden, and A. Z. Gu, "Mechanistic toxicity assessment of nanomaterials by whole-cell-array stress genes expression analysis," *Environmental Science & Technology*, vol. 44, no. 15, pp. 5964–5970, 2010.

[192] B. Halle, E. G. Marcusson, C. Aaberg-Jessen et al., "Convection-enhanced delivery of an anti-miR is well-tolerated, preserves anti-miR stability and causes efficient target derepression: a proof of concept," *Journal of Neuro-Oncology*, vol. 126, no. 1, pp. 47–55, 2016.

[193] A. Ganju, S. Khan, B. B. Hafeez et al., "miRNA nanotherapeutics for cancer," *Drug Discovery Today*, vol. 22, no. 2, pp. 424–432, 2017.

[194] D. Goren, A. T. Horowitz, D. Tzemach, M. Tarshish, S. Zalipsky, and A. Gabizon, "Nuclear delivery of doxorubicin via folate-targeted liposomes with bypass of multidrug-resistance efflux pump," *Clinical Cancer Research*, vol. 6, no. 5, pp. 1949–1957, 2000.

[195] T. J. Lee, J. Y. Yoo, D. Shu et al., "RNA nanoparticle-based targeted therapy for glioblastoma through inhibition of oncogenic miR-21," *Molecular Therapy*, vol. 25, no. 7, pp. 1544–1555, 2017.

[196] J. S. Ananta, R. Paulmurugan, and T. F. Massoud, "Nanoparticle-delivered antisense microRNA-21 enhances the effects of temozolomide on glioblastoma cells," *Molecular Pharmaceutics*, vol. 12, no. 12, pp. 4509–4517, 2015.

[197] A. Bertucci, E. A. Prasetyanto, D. Septiadi et al., "Combined delivery of temozolomide and anti-miR221 PNA using mesoporous silica nanoparticles induces apoptosis in resistant glioma cells," *Small*, vol. 11, no. 42, pp. 5687–5695, 2015.

[198] Z. Shatsberg, X. Zhang, P. Ofek et al., "Functionalized nanogels carrying an anticancer microRNA for glioblastoma therapy," *Journal of Controlled Release*, vol. 239, pp. 159–168, 2016.

[199] S. Amano, S. Li, C. Gu et al., "Use of genetically engineered bone marrow-derived mesenchymal stem cells for glioma gene therapy," *International Journal of Oncology*, vol. 35, no. 6, pp. 1265–1270, 2009.

[200] A. U. Ahmed, N. G. Alexiades, and M. S. Lesniak, "The use of neural stem cells in cancer gene therapy: predicting the path to the clinic," *Current Opinion in Molecular Therapeutics*, vol. 12, no. 5, pp. 546–552, 2010.

[201] H. K. Lee, S. Finniss, S. Cazacu, C. Xiang, and C. Brodie, "Mesenchymal stem cells deliver exogenous miRNAs to neural cells and induce their differentiation and glutamate transporter expression," *Stem Cells and Development*, vol. 23, no. 23, pp. 2851–2861, 2014.

[202] N. M. Teplyuk, E. J. Uhlmann, G. Gabriely et al., "Therapeutic potential of targeting microRNA-10b in established intracranial glioblastoma: first steps toward the clinic," *EMBO Molecular Medicine*, vol. 8, no. 3, pp. 268–287, 2016.

[203] M. S. Ebert and P. A. Sharp, "MicroRNA sponges: progress and possibilities," *RNA*, vol. 16, no. 11, pp. 2043–2050, 2010.

[204] L. Chen, K. Zhang, Z. Shi et al., "A lentivirus-mediated miR-23b sponge diminishes the malignant phenotype of glioma cells in vitro and in vivo," *Oncology Reports*, vol. 31, no. 4, pp. 1573–1580, 2014.

[205] T. B. Hansen, T. I. Jensen, B. H. Clausen et al., "Natural RNA circles function as efficient microRNA sponges," *Nature*, vol. 495, no. 7441, pp. 384–388, 2013.

[206] K. Cai, Y. Wan, G. Sun, L. Shi, X. Bao, and Z. Wang, "Let-7a inhibits proliferation and induces apoptosis by targeting EZH2 in nasopharyngeal carcinoma cells," *Oncology Reports*, vol. 28, no. 6, pp. 2101–2106, 2012.

[207] X. R. Wang, H. Luo, H. L. Li et al., "Overexpressed let-7a inhibits glioma cell malignancy by directly targeting K-ras, independently of PTEN," *Neuro-Oncology*, vol. 15, no. 11, pp. 1491–1501, 2013.

[208] L. Yu, J. Lu, B. Zhang et al., "miR-26a inhibits invasion and metastasis of nasopharyngeal cancer by targeting EZH2," *Oncology Letters*, vol. 5, no. 4, pp. 1223–1228, 2013.

[209] M. Smits, J. Nilsson, S. E. Mir et al., "miR-101 is down-regulated in glioblastoma resulting in EZH2-induced proliferation, migration, and angiogenesis," *Oncotarget*, vol. 1, no. 8, pp. 710–720, 2010.

[210] L. Xiaoping, Y. Zhibin, L. Wenjuan et al., "CPEB1, a histone-modified hypomethylated gene, is regulated by miR-101 and involved in cell senescence in glioma," *Cell Death & Disease*, vol. 4, article e675, 2013.

[211] F. Zheng, Y. J. Liao, M. Y. Cai et al., "The putative tumour suppressor microRNA-124 modulates hepatocellular carcinoma cell aggressiveness by repressing ROCK2 and EZH2," *Gut*, vol. 61, no. 2, pp. 278–289, 2012.

[212] S. Qiu, D. Huang, D. Yin et al., "Suppression of tumorigenicity by microRNA-138 through inhibition of EZH2-CDK4/6-pRb-E2F1 signal loop in glioblastoma multiforme," *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, vol. 1832, no. 10, pp. 1697–1707, 2013.

[213] A. H. Juan, R. M. Kumar, J. G. Marx, R. A. Young, and V. Sartorelli, "Mir-214-dependent regulation of the polycomb protein Ezh2 in skeletal muscle and embryonic stem cells," *Molecular Cell*, vol. 36, no. 1, pp. 61–74, 2009.

[214] P. Guo, J. Lan, J. Ge, Q. Nie, Q. Mao, and Y. Qiu, "miR-708 acts as a tumor suppressor in human glioblastoma cells," *Oncology Reports*, vol. 30, no. 2, pp. 870–876, 2013.

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Review Article

Molecular Crosstalking among Noncoding RNAs: A New Network Layer of Genome Regulation in Cancer

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Over the past few years, noncoding RNAs (ncRNAs) have been extensively studied because of the significant biological roles that they play in regulation of cellular mechanisms. ncRNAs are associated to higher eukaryotes complexity; accordingly, their dysfunction results in pathological phenotypes, including cancer. To date, most research efforts have been mainly focused on how ncRNAs could modulate the expression of protein-coding genes in pathological phenotypes. However, recent evidence has shown the existence of an unexpected interplay among ncRNAs that strongly influences cancer development and progression. ncRNAs can interact with and regulate each other through various molecular mechanisms generating a complex network including different species of RNAs (e.g., mRNAs, miRNAs, lncRNAs, and circRNAs). Such a hidden network of RNA-RNA competitive interactions pervades and modulates the physiological functioning of canonical protein-coding pathways involved in proliferation, differentiation, and metastasis in cancer. Moreover, the pivotal role of ncRNAs as keystones of network structural integrity makes them very attractive and promising targets for innovative RNA-based therapeutics. In this review we will discuss: (1) the current knowledge on complex crosstalk among ncRNAs, with a special focus on cancer; and (2) the main issues and criticisms concerning ncRNAs targeting in therapeutics.

1. Introduction

When the Human Genome Project (HGP) began in the late 1990s, researchers hypothesized that our genome comprised about 100,000 protein-coding genes [1]. Over the years, this estimate has been continuously downsized. In 2001, the International Human Genome Sequencing Consortium (IHGSC) published the initial sequence of the human genome and proposed that the number of protein-coding genes was about 30,000 [2]. At the same time, Celera Genomics (a competitor group of IHGSC) estimated this number at 26,000 [3]. In 2004, when the final draft of the human genome was published, this number was further reduced to 24,500 [4], but in 2007 an additional analysis established that it was around 20,500 [5]. More recently, new studies updated the number of human protein-coding genes to 19,000 [6]. This estimate is particularly surprising, because it would suggest that less than 2% of the whole human genome encodes for proteins; accordingly, the keystone of *Homo sapiens* complexity could lie in the 98% of our DNA (the genome *dark matter*), which does not encode proteins but would be endowed with critical regulatory functions. In the last decade, two important scientific initiatives supported by the US National Institutes of Health (i.e., the projects ENCODE and Roadmap Epigenomics) reported seminal data on hundreds of thousands of functional regions in the human genome, whose function is to supervise gene expression [7, 8]. These data suggested that much more space in our genome is committed to regulatory than to structural functions. Moreover, these studies proposed that about 80% of the human genome

is dynamically and pervasively transcribed, mostly as non-protein-coding RNAs (ncRNAs). The biological relevance of the noncoding transcriptome has become increasingly undeniable over the last few years. Studies of comparative genomics showed that the relative proportion of genome space, occupied by the proteome-encoding genome as opposed to the regulatory (non-protein-encoding) genome is very variable among evolutionarily distant species; for instance, the protein-coding genome represents almost the entire genome of the unicellular yeast *Saccharomyces cerevisiae*, whereas it constitutes only 2% of mammalian genomes [9]. Moreover and intriguingly, the noncoding transcriptome is frequently altered in major diseases, including cancer [10–12]. These observations strongly suggest that ncRNAs are closely related to the complexity of higher eukaryotes and that their dysfunction may result in pathological phenotypes. RNA is a structurally versatile molecule, able to perform several molecular functions. By simple base pairing with other nucleic acids, RNA can recognize and bind both DNA and RNA targets in a very specific manner and regulate their transcription, processing, editing, translation, or degradation. An intriguing field for future explorations is the tridimensional folding of RNA molecules, which confers them allosteric properties: this increases the range of potential molecular interactors (including proteins); additionally, dynamic conformational changes can be triggered by ligand binding. Moreover and different from proteins, RNA can be rapidly transcribed and degraded making it a very dynamic molecule that can be quite rapidly synthesized without additional time and energetic costs of translation [13]. For all these reasons, over the past few years, ncRNAs have been extensively studied because of the significant biological roles that they play in regulation of cellular mechanisms. Noncoding RNA genes can generally be divided into two major categories by their transcript sizes: (1) long noncoding RNAs (lncRNAs) are longer than 200 nucleotides; and (2) small noncoding RNAs have a length equal to or lower than 200 nucleotides [i.e., microRNAs (miRNAs), small interfering RNAs (siRNAs), small nuclear RNAs (snRNAs or U-RNAs), small nucleolar RNAs (snoRNAs), PIWI-interacting RNAs (piRNAs), and tRNAs] [14]. To date, most research efforts have been focused on how ncRNAs (in particular, miRNAs) modulate the expression of protein-coding genes and their roles in human pathophysiology. However, recent evidence has shown the existence of unexpected interplay among ncRNAs, which influences cell physiology and diseases. In addition to the canonical multilayered control of expression of protein-coding genes (briefly described below), ncRNAs can interact with and regulate each other through various molecular mechanisms generating a complex network including different species of RNAs. In such a regulatory network, ncRNAs also compete among each other for binding to mRNAs, thus acting as competing endogenous RNAs (ceRNAs). In this review, we will summarize the current knowledge on the complex crosstalk among ncRNAs (including miRNAs, lncRNAs, and circRNAs) and how they could reciprocally interact to regulate cancer progression and dissemination.

1.1. miRNAs. miRNAs are 18–25 nucleotides long, evolutionarily conserved, single-stranded RNAs, which negatively modulate the expression of their target mRNAs (more than 60% of protein-coding genes) by binding to the $3'$-UTR of specific mRNA targets, leading either to their translational repression, cleavage, or decay [15–17]. This binding occurs through a specific miRNA region (named *seed region*), which is a contiguous string of at least 6 nucleotides beginning at position two of the $5'$ of the molecule [18]. The block of translation is due to the inhibition of mRNA $5'$-cap recognition and interference on the interaction between the mRNA and the 60S ribosomal subunit, while mRNA degradation is promoted by mechanisms of decapping and deadenylation [19]. These molecular mechanisms are mediated by an RNA-induced silencing complex (RISC) that includes proteins belonging to the Argonaute (AGO) family; specifically, RISC endonuclease activity depends exclusively on AGO2 protein [20]. A single miRNA can control the expression of several mRNAs, and a single mRNA may be targeted by more than one miRNA, thus creating a complex interplay of cooperative regulation [21]. To date, more than 2500 mature miRNAs have been included in the *miRbase* database [22].

Extensive studies have shown that miRNAs control pivotal cellular processes, (e.g., cell proliferation, differentiation, migration, cell death, and angiogenesis), thus contributing to the pathogenesis of diseases such as cancer. Indeed, several miRNAs have been identified as potential oncogenes or tumor suppressors in cancer development and progression [23]. In the last two decades, their mutations and altered expression were reported to be causally related to the neoplastic features of the cells, thus providing new perspectives for the understanding of the complex regulatory networks that rule tumor biology [24]. miRNA dysfunctions exert a pleiotropic effect on the expression of their mRNA targets impairing the functioning of biological networks. It has been convincingly demonstrated that different cancer histotypes display specific miRNA expression patterns: this phenomenon would be helpful to improve diagnosis of poorly differentiated tumors and predict prognosis in cancer [25, 26]. Moreover, multiple experimental evidence has shown that miRNAs can be also secreted by cancer cells into bodily fluids, sending oncogenic signals through circulation, which could advantageously mold the extracellular tumor environment [27]. These discoveries gave a new intriguing diagnostic and prognostic role to circulating miRNAs, paving the way for their potential use as noninvasive molecular RNA markers in clinical management of cancer patients [28–30].

1.2. lncRNAs. lncRNAs are the most heterogeneous class of non-protein-coding RNAs with lengths ranging from 200 nt to 100,000 nt. They include transcripts that may be classified as (a) intergenic lncRNAs, (b) intronic lncRNAs, (c) sense or antisense transcripts, (d) pseudogenes, and (e) retrotransposons [14]. Currently, LNCipedia 4.0 records more than 118,000 human lncRNAs, which are usually expressed in a developmental and tissue-specific manner [31]. lncRNAs regulate gene expression at different levels, including chromatin modification, alternative splicing, and protein localization and activity [32]. Such a wide range of mechanisms is due to their ability to bind to DNA, RNAs, and proteins. lncRNAs, thanks to their binding to promoter DNA, can

prevent the access of transcription factors to their own promoter binding sites and impede the transcription of specific genes (e.g., DHFR) [33]. Some lncRNAs (e.g., HOTAIR) are associated with chromatin-modifying complexes (e.g., polycomb repressive complex 2) to regulate epigenetic silencing of target genes [34]. Much evidence has also shown that lncRNAs may work as molecular scaffolds to connect two or more proteins in functional complexes or can serve to localize protein complexes to appropriate cellular compartments [35]. Antisense lncRNAs can target, by direct sequence complementarity, their antisense mRNAs and, accordingly, modulate alternative splicing processes or protect 3′-UTR from miRNA binding, increasing the stability of mRNAs (e.g., ZEB2-AS1, BACE1-AS) [36, 37]. Several recent studies have shown that lncRNAs are critically involved in a wide range of biological processes, such as cell cycle regulation, pluripotency, differentiation, and cell death [38–41]. Dysregulation of lncRNA activity has been frequently reported in association to diseases, including several types of cancer. Specifically, upregulated lncRNAs in cancer seem to possess tumor-promoting abilities, whilst downregulated lncRNAs exhibit tumor-suppressive roles [42–47]. Although several lncRNAs have been reported to be dysregulated in neoplastic phenotypes, their mechanistic role in cancer biology has not been satisfactorily explained for most of them. However, scientific evidence strongly suggests a promising role for lncRNAs as cancer-related biomarkers and potential targets for innovative therapeutic approaches.

1.3. circRNAs. Circular RNAs (circRNAs) represent a recently discovered class of noncoding RNAs, composed of single-stranded, covalently closed, exonuclease-resistant circular transcripts [48]. Although the existence of circular RNAs has been known since the 70s [49], for a long time such molecules were considered only by-products of pre-mRNA processing and therefore interpreted as artifacts of aberrant RNA splicing [50]. However, recent advances in RNA sequencing technologies have revealed a ubiquitous, and in some cases abundant, expression of endogenous circRNAs in mammalian genomes [51, 52]. circRNAs are a circularized isoform of linear protein-coding genes generated through backsplicing, a molecular process that is different from the canonical splicing of linear RNAs. Circular RNA biogenesis can occur both from exons (exonic circRNAs or ecircRNAs), through different mechanisms of backsplicing and introns (intronic circRNAs or ciRNAs), when lariat introns escape typical debranching processes [53]. Currently, about 35,000 circRNAs are reported in the *circBase* database [54], but molecular functions and biological processes, in which they are involved, remain elusive for most of them. Recent emerging evidence convincingly suggests that circRNAs may play an important role in RNA-RNA interactions. In some instances, circRNAs exhibit multiple binding sites for the same miRNA and represent a potential *molecular sponge* for sequestering the most abundant miRNAs [55]. In other words, circRNAs may negatively regulate the function of miRNAs, and, thus, protect miRNA targets, by acting as competing endogenous RNAs. As some papers would suggest that ceRNA role of circRNAs could not be their main function in cell biology, other molecular functions have been proposed for circRNAs (a) to bind and sequester RNA binding proteins (RBPs) [56–58] and (b) to be translated into proteins when recognized by ribosomes in the presence of internal ribosome entry sites (IRESs) [59, 60]. As circRNAs are potentially able to control different layers of gene expression, it is not surprising that their dysregulation is associated with human pathologies, including cancer [61–63]. Most reports that connect circRNAs and tumors mainly concern comparative gene expression profiling studies between tumor and normal samples. These investigations have shown that circRNAs are frequently downregulated in several types of cancer (e.g., colorectal cancer, ovarian cancer, and gastric cancer) [64–66]. Just few of these studies attempted to functionally explain how abnormal expression of circRNAs could impair physiological cell homeostasis and thus promote cancer phenotypes [67–69].

2. Noncoding RNAs: Different Ways to Interplay among Each Other

Interplay between ncRNAs obviously occurs because of sequence complementarity; for instance, ncRNAs may share miRNA response elements (MREs) with mRNAs and thus be targeted in the same manner [70]. The effects of miRNAs binding to other ncRNAs (i.e., lncRNAs and circRNAs) could be twofold: on the one hand, miRNAs could be sequestered and prevented from acting on the protein-coding mRNAs; on the other hand, miRNA binding to lncRNAs and circRNAs could promote their decay, similarly to mRNAs. In the next paragraphs, we will discuss the different mechanisms of ncRNA interaction and their influence on cancer biology.

2.1. miRNAs Induce Degradation of lncRNAs. Several papers have reported that miRNAs can bind lncRNAs and promote their degradation contributing to cancer processes (Table 1). lncRNAs are structurally similar to mRNAs; indeed, they have 5′-caps and 3′-poly(A) tails [71]; accordingly, the proteins involved in the regulation of decapping, deadenylation, and degradation of mRNAs may also control the turnover of lncRNAs by binding of specific miRNAs.

UCA1 (urothelial cancer associated 1), an lncRNA upregulated in several tumors (i.e., bladder cancer, tongue squamous cell carcinoma, breast cancer, and ovarian cancer) [72–75], possesses two predicted binding sites for miR-1, a well-known tumor suppressor miRNA. The binding of miR-1 to UCA1 has been confirmed by luciferase reporter assay in bladder cancer and, accordingly, *in vitro* upregulation of miR-1 induced UCA1 downregulation and caused a decreased cell growth and migration and also an augmented apoptosis. Such functional effects were reverted after UCA1 overexpression and silencing of AGO2, suggesting that miR-1 was able to downregulate UCA1 expression in an AGO2-mediated manner [76].

MALAT1 (metastasis-associated lung adenocarcinoma transcript 1) is one of the most studied and abundant lncRNAs: its expression was initially associated with metastasis in non-small-cell lung carcinoma (NSCLC) [77], but then

Table 1: miRNAs inducing degradation of lncRNAs.

miRNA	lncRNA/circRNA target	Tumor	miRNA role	PMID
let-7b	lincRNA-p21	Cervical carcinoma	Tumor suppressor	22841487
let-7b, let-7i	HOTAIR	Cervical carcinoma	Tumor suppressor	24326307
miR-1	UCA1	Bladder cancer	Tumor suppressor	25015192
miR-9	MALAT1	Hodgkin lymphoma, glioblastoma	Tumor suppressor	23985560
miR-21	CASC2	Renal cell carcinoma	Oncogene	27222255
miR-21	CASC2	Glioblastoma	Oncogene	25446261
miR-21	GAS5	Breast cancer	Oncogene	23933812
miR-34a	HOTAIR	Prostate cancer	Tumor suppressor	23936419
miR-101	MALAT1	Esophageal squamous cell carcinoma	Tumor suppressor	25538231
miR-125b	HOTTIP	Hepatocellular carcinoma	Tumor suppressor	25424744
miR-125b	MALAT1	Bladder cancer	Tumor suppressor	24396870
miR-141	H19	Gastric cancer	Tumor suppressor	26160158
miR-141	HOTAIR	Renal carcinoma	Tumor suppressor	24616104
miR-217	MALAT1	Esophageal squamous cell carcinoma	Tumor suppressor	25538231
miR-671	CDR1AS	Glioblastoma	Oncogene	26683098

This table reports for each miRNA: (1) its lncRNAs/circRNA target; (2) tumor where such interaction was reported; (3) its function in cancer (oncogene or tumor suppressor); and (4) bibliographic reference reported as Pubmed ID (PMID).

its deregulation has been reported in several other neoplastic diseases [78–80]. The 3′ end of MALAT1 is cleaved by RNase P and RNase Z, producing a tRNA-like ncRNA, called mascRNA (MALAT1-associated small cytoplasmic RNA), which will be exported into the cytoplasm [81], while most of the MALAT1 molecules are localized to nuclear speckles where they regulate alternative splicing of specific pre-mRNAs [82]. Moreover, MALAT1 may bind CBX4 (chromobox 4), a component of polycomb repressive complex 1 (PRC1), and modulate its localization in interchromatin granules, leading to activation or inhibition of gene expression [83]. Through these molecular mechanisms, MALAT1 controls the expression of several genes related to cell cycle and metastatic processes, thus influencing cell proliferation, migration, and invasion. Recent publications reported that MALAT1 is a target of a number of tumor suppressor miRNAs, which could induce its degradation and suppress its oncogenic effects. Leucci et al. reported miRNA-mediated regulation of MALAT1 in the nucleus of Hodgkin lymphoma and glioblastoma cell lines through direct binding of miR-9 to two different MREs in an AGO2-dependent manner [84]. There is evidence of a posttranscriptional regulation of MALAT1 by miR-101 and miR-217 in esophageal squamous cell carcinoma (ESCC) cells [85]. MiR-101 and miR-217 are functionally involved in several cancers as tumor suppressors and exhibited a significant negative correlation with MALAT1 in ESCC tissue samples and adjacent normal tissues. Enforced expression of miR-101 and miR-217 significantly repressed MALAT1 expression, leading to inhibition of cell growth, invasion, and metastasis in ESCC cells [85]. In bladder cancer, MALAT1 is inversely expressed with miR-125b. This miRNA was partially complementary with MALAT1 and bound it in *in vitro* models. MiR-125b was downregulated in bladder cancer, and its overexpression decreased the expression of MALAT1, causing an inhibition of bladder cancer cell proliferation, motility, and activation of apoptosis [86].

Additionally, miR-125b was also identified as a posttranscriptional regulator of HOTTIP (HOXA distal transcript antisense RNA) in hepatocellular carcinoma (HCC) [87]. HOTTIP is one of the most upregulated lncRNAs in HCC, also in early stages of HCC onset, and maps in antisense position to the distal end of the HOXA gene cluster. HOTTIP promotes tumor growth and metastasis *in vitro* and *in vivo* through regulation of the expression of its neighboring HOXA genes (e.g., HOXA10, HOXA11, and HOXA13). MiR-125b has been reported to be frequently downregulated in HCC, and a negative correlation of expression between miR-125b and HOTTIP existed in such cancer. The interaction between miR-125b and HOTTIP was validated by luciferase reporter assay; this was confirmed by ectopic expression of miR-125b that induced downmodulation of HOTTIP [87].

HOTAIR (HOX antisense intergenic RNA) is one of the most intensively studied lncRNAs, as it is frequently associated with different neoplasias. HOTAIR exerts its oncogenic functions by working as a scaffold to assemble polycomb repressive complex 2 (PRC2) on the HOXD gene cluster and inducing the transcriptional silencing of multiple metastasis suppressor genes (e.g., the protocadherin gene family) [34, 88]. HOTAIR is posttranscriptionally destabilized by several tumor suppressor miRNAs in different cancers. Chiyomaru et al. reported a functional binding between miR-34a and HOTAIR in prostate cancer cell lines treated with genistein, an isoflavone with antitumor activity: miR-34a directly bound to two MREs within HOTAIR RNA and lowered its levels [89]. Yoon et al. reported that human antigen R (HuR), let-7b, let-7i, and AGO2 cooperatively bind HOTAIR and promote HOTAIR decay, thus inhibiting the processes of ubiquitination and proteolysis of Ataxin-1 and

Snurportin-1, promoted by HOTAIR [90]. Interestingly, HuR and let-7b/AGO2 complex also decreased the stability of lincRNA-p21, an oncogenic lncRNA that reduced translation of beta-catenin and JUNB (JunB proto-oncogene, subunit of transcription factor AP-1) mRNAs in human cervical carcinoma HeLa cells [91]; even if in other experiments HuR was not able to transfer let-7b to AGO2 [92]. In another paper by Chiyomaru et al., it was reported that HOTAIR expression is negatively correlated to that of miR-141 in renal carcinoma cells (RCC) [93]. MiR-141 belongs to the miRNA-200 family, which has been reported to inhibit epithelial-mesenchymal transition (EMT) by ZEB1 (zinc finger E-box-binding homeobox 1) repression and E-cadherin upregulation [94]. MiR-141 was able to target and cleave HOTAIR in an AGO2-dependent manner, and such molecular action downregulated the expression of ZEB2 (zinc finger E-box-binding homeobox 2) induced by HOTAIR [93].

Expression of miR-141 was also found to be negatively correlated to that of lncRNA H19 (H19, imprinted maternally expressed transcript) in gastric cancer [95]. H19, an oncofetal lncRNA, is highly expressed during embryogenesis [96] and is upregulated in several cancers, including gastric cancer [97]. H19 acts as the primary miRNA precursor of miR-675, which in turn targets and represses RB1 (RB transcriptional corepressor 1) mRNA [98]. Overexpression of H19 enhances tumor cell growth and induces EMT; additionally, H19 modulates miRNA processing through its interaction with proteins involved in this molecular process (i.e., Drosha, Dicer). MiR-141 was shown to bind H19 in gastric cancer, and suppress H19 expression and its tumor-promoting functions [95].

MiR-21 is the most commonly upregulated miRNA in cancer: its genetic locus is often amplified in solid tumors, and its expression is promoted by a variety of cancer-related *stimuli* [99]. MiR-21 enhances cell proliferation, migration, and invasion by targeting several tumor suppressor genes, such as CCL20, CDC25A, PDCD4, and PTEN [100–103]. Recent findings showed that some lncRNAs could be added to the *repertoire* of miR-21 targets. Zhang et al. reported that expression of miR-21 and lncRNA GAS5 (growth arrest-specific 5) is negatively correlated in breast cancer and that miR-21 binds a miR-21-binding site in exon 4 of GAS5, thus inducing AGO2-mediated suppression of GAS5 [104]. GAS5 is an lncRNA with tumor-suppressive properties: its overexpression sensitizes cancer cells to UV or doxorubicin and decreases tumor proliferation and cell invasion. Interestingly, GAS5 also negatively regulated miR-21 at the posttranscriptional level through the RISC complex, suggesting the existence of a reciprocal negative feedback loop between GAS5 and miR-21 [104]. In two different studies on renal cell carcinoma and glioblastoma, it has been shown that miR-21 targeted and suppressed the expression of the tumor suppressor lncRNA CASC2 (cancer susceptibility candidate 2) in an AGO2-dependent manner [105, 106]. Indeed, the overexpression of miR-21 abrogated the inhibition of proliferation, migration, and the induction of apoptosis promoted by CASC2. Notably, when CASC2 was upregulated, miR-21 expression decreased: this suggests reciprocal repression between miR-21 and CASC2 [106].

The first experimental evidence that lncRNAs may be targeted by miRNAs was reported for the antisense transcript of the cerebellar degeneration-related protein 1 (CDR1, also known as CiRS-7 or CDR1AS), which is a circular RNA produced by a backsplice event [107]. MiR-671, a nuclear-enriched miRNA, induced cleavage of CDR1AS in an AGO2-dependent manner. Repression of miR-671 promoted the upregulation of both CDR1AS and CDR1, suggesting that CDR1AS was able to stabilize the sense transcript CDR1. Currently, this represents the only report on circRNA targeted and degraded by a miRNA. The interaction between miR-671 and CDR1AS could affect the biopathological molecular asset of glioblastoma multiforme (GBM), the most prevalent and aggressive cancer originating in the central nervous system, mainly in the brain. Indeed, Barbagallo et al. demonstrated that miR-671-5p is significantly upregulated in GBM. Enforced expression of miR-671-5p increased migration and decreased proliferation rates of GBM cell lines, suggesting its potential role as a novel oncomiRNA in GBM [108]. Expression of miR-671 was inversely correlated to that of CDR1AS and CDR1 in GBM biopsies and the expression of CDR1AS and CDR1 decreased when the miR-671 mimic was used, suggesting that the interaction of these molecules could be functionally altered in a GBM model [108].

2.2. lncRNAs as Decoys of miRNAs. The most explored mechanism of functional interactions between lncRNAs and miRNAs is based on sharing the same miRNA target sequence in both lncRNAs and mRNAs. In this way, lncRNAs are able to sequester miRNAs away from mRNAs, functioning as "miRNA sponges" or "miRNA decoys." Through such a competitive endogenous mechanism of interaction, lncRNAs decrease the quantity of available miRNAs and increase, accordingly, translations of their mRNA targets. lncRNAs, working as *competitive endogenous* RNAs, have been extensively described in molecular circuits involved in tumors (Table 2).

EWSAT1 (Ewing sarcoma-associated transcript 1) is an lncRNA with oncogenic functions in Ewing's sarcoma and nasopharyngeal carcinoma (NPC). EWSAT1 has two MREs for the miR-326/330-5p cluster and promoted the development and progression of tumors functioning as a ceRNA for these miRNAs, which in turn induced the expression of Cyclin D1, target of miRNAs from the miR-326/330-5p cluster [109].

Xia et al. showed that both lncRNA FER1L4 (FER-1-like family member 4, pseudogene) and PTEN (phosphatase and tensin homolog) mRNA had binding sites for oncomiR miR-106a-5p and were downregulated in gastric cancer [110]. As FER1L4 behaved as a ceRNA for miR-106a-5p, FER1L4 downregulation released miR-106a-5p that targeted PTEN mRNA, reducing its expression. Dysregulation of FER1L4-miR-106a-5p-PTEN axis increased cell proliferation by promoting the G0/G1 to S phase transition [110].

FTH1P3 (ferritin heavy chain 1 pseudogene 3) has been shown to function as a molecular sponge for miR-224-5p in oral squamous cell carcinoma (OSCC) [111]. Overexpression of FTH1P3 promoted proliferation and colony formation in

Table 2: lncRNAs acting as decoy of miRNAs.

lncRNA	miRNA target	Tumor	lncRNA role	PMID
CCAT1	let-7	Hepatocellular carcinoma	Oncogene	25884472
EWSAT1	miR-326/−330-5p cluster	Nasopharyngeal carcinoma	Oncogene	27816050
FER1L4	miR-106a-5p	Gastric cancer	Tumor suppressor	26306906
FTH1P3	miR-224-5p	Squamous cell carcinoma	Oncogene	28093311
FTX	miR-374a	Hepatocellular carcinoma	Tumor suppressor	27065331
GAS5	miR-135b	Non-small cell lung cancer	Tumor suppressor	28117028
H19	let-7a, let-7b	Breast cancer	Oncogene	28102845
HOST2	let-7b	Epithelial ovarian cancer	Oncogene	25292198
HOTAIR	miR-1	Hepatocellular carcinoma	Oncogene	27895772
HOTAIR	miR-152	Gastric cancer	Oncogene	26187665
HULC	miR-372	Liver cancer	Oncogene	20423907
lincRNA-RoR	miR-145	Breast cancer	Oncogene	25253741
lincRNA-RoR	miR-145	Endometrial cancer	Oncogene	24589415
LOC100129148	miR-539-5p	Nasopharyngeal carcinoma	Oncogene	28328537
MALAT1	miR-1	Breast cancer	Oncogene	26676637
MALAT1	miR-145	Cervical cancer	Oncogene	26311052
NEAT1	miR-449-5p	Glioma	Oncogene	26242266
PVT1	miR-152	Gastric cancer	Oncogene	28258379
PVT1	miR-186	Gastric cancer	Oncogene	28122299
RMRP	miR-206	Gastric cancer	Oncogene	27192121
SPRY4-IT1	miR-101-3p	Bladder cancer	Oncogene	27998761
TUG1	miR-145	Bladder cancer	Oncogene	26318860
TUG1	miR-299	Glioblastoma	Oncogene	27345398
TUG1	miR-300	Gallbladder carcinoma	Oncogene	28178615
TUG1	miR-9-5p	Osteosarcoma	Oncogene	27658774
TUSC7	miR-10a	Hepatocellular carcinoma	Tumor suppressor	27002617
TUSC7	miR-211	Colon cancer	Tumor suppressor	23558749
TUSC7	miR-23b, miR-320d	Gastric cancer	Tumor suppressor	25765901
UCA1	miR-143	Breast cancer	Oncogene	26439035
UCA1	miR-16	Bladder cancer	Oncogene	26373319
UCA1	miR-204-5p	Colorectal cancer	Oncogene	27046651
UCA1	miR-216b	Hepatocellular carcinoma	Oncogene	25760077
UCA1	miR-485-5p	Epithelial ovarian cancer	Oncogene	26867765
UCA1	miR-507	Melanoma	Oncogene	27389544
XIST	miR-139-5p	Hepatocellular carcinoma	Oncogene	28231734
XIST	miR-181a	Hepatocellular carcinoma	Tumor suppressor	28388883
XIST	miR-34a-5p	Nasopharyngeal carcinoma	Oncogene	27461945
XIST	miR-92b	Hepatocellular carcinoma	Tumor suppressor	27100897

This table reports for each lncRNA: (1) miRNA sponged; (2) tumor where such interaction was reported; (3) its function in cancer (oncogene or tumor suppressor); and (4) bibliographic reference reported as Pubmed ID (PMID).

OSCC cells and the upregulation of FZD5 (frizzled class receptor 5), target of miR-224-5p and an oncogene involved in activation of Wnt/β-catenin signaling.

It has been demonstrated that lncRNA GAS5 acts as a tumor suppressor in NSCLC by targeting and suppressing miR-135b [112]. GAS5 is downregulated in NSCLC and its expression is inversely correlated to that of miR-135b. After exposure to irradiation, expression of GAS5 and miR-135b was altered, as GAS5 was overexpressed whereas miR-135b was downregulated. Ectopic overexpression of GAS5 led to miR-135b downregulation, repression of cell proliferation, invasion, and improved radiosensitivity [112].

High expression of lncRNA H19 in breast cancer stem cells (BCSCs) is functionally critical for stemness maintenance [113]. In these cells, H19 functions as a molecular sponge for let-7a/b, leading to upregulation of pluripotency factor LIN28, a let-7 target that is highly abundant in BCSCs. Intriguingly, H19 is reciprocally repressed by its

targets let-7a/b, but this negative feedback loop can be interfered with by LIN28 because of its ability to inhibit let-7a/b expression [113]. Let-7b expression is also buffered by lncRNA HOST2 (human ovarian cancer-specific transcript 2) in ovarian cancer cells. By binding to let-7b, HOST2 negatively regulates its availability and induces the expression of its oncogenic targets that enhance cell growth and motility in ovarian cancer [114].

Let-7 decoy by lncRNAs was also reported by Deng et al. Upregulation of lncRNA CCAT1 (colon cancer associated transcript 1) in HCC tissues was associated with increased cell proliferation and migration [115]; these oncogenic activities were mediated by its molecular sponge function for let-7: inhibition of let-7 caused upregulated expression of let-7 targets: HMGA2 (high mobility group AT-hook 2) and MYC (MYC proto-oncogene, bHLH transcription factor). Interestingly, other studies reported that MYC, by binding to CCAT1 promoter, induces CCAT1 transcription in colon cancer and gastric carcinoma [116, 117], suggesting the existence of a positive feedback loop between CCAT1 and MYC mediated by let-7 decoy.

Recent works reported the inhibitory effect of HOTAIR on miRNAs functions in different neoplasias. Su et al. found that HOTAIR was highly expressed in HCC tissues and promoted HCC cell proliferation and progression of tumor xenografts [118]. These oncogenic effects were partially due to HOTAIR ability of repressing miR-1 expression. Moreover, also miR-1 was able to negatively regulate HOTAIR expression, thus generating a reciprocal repression feedback loop between these two ncRNAs [118]. Other experimental evidence showed that HOTAIR was capable of binding and downregulating miR-152 in gastric cancer [119]. HOTAIR overexpression in gastric cancer tissues led to decreased expression of miR-152 and to upregulation of its target, HLA-G (human leukocyte antigen G), which in turn facilitated tumor escape mechanisms [119]. Downregulation of miR-152 in gastric cancer could be also caused by PVT1 (plasmacytoma variant translocation 1), an oncogenic lncRNA that acts as a precursor of six miRNAs (i.e., miR-1204, miR-1205, miR-1206, miR-1207-5p, miR-1207-3p, and miR-1208) [120]. Indeed, PVT1 had three MREs for miR-152 and suppressed its expression inducing the upregulation of miR-152 targets (i.e., CD151, FGF2) [121]. Upregulation of PVT1 in gastric cancer was also associated with inhibition of miR-186 function. Indeed, PVT1 bound miR-186 and induced upregulation of HIF-1α (Hypoxia-inducible factor 1-alpha subunit), a target of miR-186 which was related to poor prognosis and invasiveness in gastric cancer [122].

Wang et al. studied in liver cancer the molecular sponge action of lncRNA HULC (highly upregulated in liver cancer). HULC was able to downregulate several miRNAs, including miR-372. Repression of miR-372 enhanced the translation of its target gene, PRKACB (protein kinase cAMP-activated catalytic subunit beta), which in turn promoted phosphorylation of protein CREB1 (cAMP responsive element-binding protein-1) and affected deacetylation and methylation of histones [123]. This process resulted in alterations of chromatin organization and increased expression of HULC, thus showing that HULC was involved in an autoregulatory loop that mantained its abundant expression in liver cancer [123].

Jin et al. reported an association between MALAT1 upregulation and tumor growth and metastasis in triple-negative breast cancer (TNBC) tissues [124]. These tumorigenic properties of MALAT1 were mediated by its ability to decoy miR-1 and, consequently, increase the expression of miR-1 target, SNAI2 (snail family transcriptional repressor 2), also named Slug, an oncogene involved in regulation of cancer cell invasion. Moreover, overexpression of miR-1 was able to reduce MALAT1 expression, demonstrating a reciprocal negative loop between lncRNA and miRNA [124]. The miRNA sponge function of MALAT1 was also reported for cervical cancer [125]. Indeed, MALAT1 levels were found to be more abundant in radio-resistant than in radio-sensitive cancers. Moreover, expression of MALAT1 and of its potential binding partner, miR-145, reverted in response to irradiation. The authors demonstrated that there was a reciprocal repression between MALAT1 and miR-145, which regulated the molecular mechanisms of radioresistance of cervical cancer [125].

Notably, tumor suppressor miR-145 was frequently reported to be buffered by lncRNAs in cancer models. MiR-145 negatively regulated cell invasion in TNBC, and its downregulation was related to overexpression of lincRNA-RoR (long intergenic ncRNA Regulator of Reprogramming), which acted as competitive endogenous RNA for miR-145 [126]. LincRNA-RoR-mediated downregulation of miR-145 led to upregulation of ARF6 (ADP-ribosylation factor 6), which is strongly involved in metastatic processes; indeed, ARF6 affected E-cadherin localization and impaired cell-cell adhesion, promoting cell invasion in TNBCs [126]. Zhou et al. reported a further effective interaction between lincRNA-RoR and miR-145 in endometrial cancer. LincRoR functioned as a miR-145 sponge by repressing the miRNA-mediated degradation of core stem cell transcription factors (i.e., Nanog, Oct4, and Sox2), thereby maintaining the pluripotency of endometrial cancer stem cells [127].

Decoying of miR-145 was performed also by TUG1 (taurine upregulated 1), which is a well-known oncogenic lncRNA, frequently upregulated in cancer and functionally related to several aggressive features of tumors. In bladder cancer, TUG1 decreased the expression of miR-145 and caused upregulation of ZEB2, miR-145 target, promoting EMT, and increasing the metastatic proneness of bladder cancer cells [128]. The ceRNA role of TUG1 was also proved in other tumors. Overexpression of TUG1 was involved in glioblastoma angiogenesis by modulation of endothelial cell proliferation, migration, and tube formation. These cellular processes were mediated by TUG1 interaction with miR-299, which was downregulated in glioblastoma. In fact, knockdown of TUG1-induced upregulation of miR-299 and concomitant decrease of VEGFA (vascular endothelial growth factor A), target of miR-299. These molecular events resulted in a reduced tumor microvessel density in xenograft glioblastoma models [129]. Ma et al. showed that upregulation of TUG1 in gallbladder carcinoma (GBC) was related to GBC cell proliferation and metastasis, and such oncogenic activities were, at least partly, due to the sponge activity of

TUG1 that bound miR-300 and negatively regulated its expression [130]. In osteosarcoma, TUG1 acted as a ceRNA by sponging miR-9-5p, inducing the upregulation of transcription factor POU2F1 (POU class 2 homeobox 1) [131]. POU2F1 is frequently upregulated in osteosarcoma and is involved in cell proliferation, differentiation and immune and inflammatory processes. Because POU2F1 is a target of miR-9-5p, silencing of TUG1-inhibited cell proliferation and colony formation, while inducing G0/G1 cell cycle arrest and apoptosis. These cellular processes were mediated by upregulation of miR-9-5p and repression of POU2F1 expression [131].

The tumor suppressor TUSC7 (tumor suppressor candidate 7; also named LOC285194) is an lncRNA transcriptionally induced by TP53 (tumor protein 53); it was initially discovered as depleted in osteosarcoma, inducing abnormal proliferation of osteoblasts, and associated with poor survival of osteosarcoma patients. Competitive endogenous binding between TUSC7 and onco-miRNAs has been frequently reported as associated with cancer-related processes. Wang et al. studied the biopathological meaning of strong downregulation of TUSC7 in HCC [132]. They found that ectopic expression of TUSC7 inhibited cell metastasis, invasion, and EMT, by functioning as a competitive sponge for miR-10a. Moreover, this miRNA was able to promote the EMT process in HCC through directly binding and repressing EPHA4 (EPH tyrosine kinase receptor A4) [132]. Moreover, exon 4 of TUSC7 harbors two binding sites for miR-211 [133]. In colon cancer, miR-211 enhanced cell growth, but this effect was reverted by enforced expression of TUSC7, which buffered the activity of miR-211 [133]. The tumor suppressor role of TUSC7 was also demonstrated in gastric cancer. TUSC7, downregulated in gastric cancer, was an independent prognostic marker of disease-free survival in patients, and its ectopic expression suppressed cancer cell growth both in *in vitro* and *in vivo* models, in part by negatively regulating the expression of miR-23 [134].

Unquestionably, one of the most iconic lncRNA acting as miRNA sponge is UCA1, which was reported to bind and repress several miRNAs in multiple tumors. UCA1 binding to miR-143 was proved in breast cancer, where UCA1 was able to modulate cell growth and apoptosis by downregulating miR-143: this in turn led to upregulation of BCL2 (BCL2, apoptosis regulator) and ERBB3 (erb-b2 receptor tyrosine kinase 3) [135]. The role of UCA1 in bladder cancer was associated with ROS (reactive oxygen species) metabolism [136]. Silencing of UCA1 decreased ROS production and promoted mitochondrial glutaminolysis in bladder cancer cells. In these cells, UCA1 acted as a ceRNA by sponging and downregulating miR-16. This induced the upregulation of GLS2 (Glutaminase 2), one of the miR-16 targets, which enhanced glutamine uptake and the rate of glutaminolysis, which is known to increase in cancer cells. UCA1-induced GLS2 maintained the redox balance and protected cancer cells by reducing excessive ROS production [136]. Oncogenic activity of UCA1 in CRC was the result of its decoy function for miR-204-5p, a critical tumor-suppressive miRNA [137]. UCA1, upregulated in CRC, inhibited miR-204-5p activity, thus promoting the upregulation of miRNA targets CREB1, BCL2, and RAB22A (RAB22A, member RAS oncogene) and regulating cell proliferation and apoptosis [137]. UCA1 upregulation in HCC was associated to cell growth and metastasis; these processes were induced by UCA1 binding to miR-216b and resulted in miR-216b downregulation [138]. Decreased levels of miR-216b led to the derepression of its target FGFR1 (fibroblast growth factor receptor 1) and the activation of ERK pathway [138]. Association between UCA1 and metastatic process was also reported for epithelial ovarian cancer [139]. In fact, UCA1 promoted the expression of MMP14 (matrix metallopeptidase14), a key protein involved in cell invasion, by working as a molecular sponge of miR-485-5p, a miRNA targeting MMP14 [139]. FOXM1 (forkhead box protein M1) is a transcription factor critical for G2/M-phase transition and DNA damage response, and it is also a target of miR-507. UCA1-mediated regulation of FOXM1 was discovered, in melanoma cells, to be based on the ceRNA function of UCA1 for miR-507, resulting in an increased malignant ability of these cells [140].

Finally, a ceRNA role in cancer was also reported for XIST (X-inactivate specific transcript). XIST was the first lncRNA to be functionally characterized, and it is considered the major effector of the X inactivation process during development in female mammals [141]. Its dysregulation was found in several tumors (e.g., breast cancer, glioblastoma, and hepatocellular carcinoma), suggesting that XIST could have a potential diagnostic power in cancer [142–144]. *In vitro* downregulation or upregulation of XIST was associated with altered cell proliferation, metastasis, and apoptosis in several cancer models. Song et al. discovered that XIST overexpression was related to metastasis and poor prognosis of NPC patients [145]. XIST induced the upregulation of E2F3 (E2F transcription factor 3), which is a critical protein for tumor cell proliferation. The authors demonstrated that XIST-promoted activation of E2F3 was caused by the competitive sponge role of XIST for miR-34a-5p (a well-known tumor suppressor miRNA), which targets E2F3 [145]. On the other hand, Chang et al. showed that XIST acts as tumor suppressor and inhibits metastatization and progression in HCC by binding miR-181a and reducing its availability; XIST induces PTEN upregulation, thus decreasing cell proliferation, invasion, and migration [146].

2.3. circRNAs as miRNA Sponges. circRNAs are considered new potential players among ceRNAs: they may harbor shared MREs and compete for miRNA binding with mRNAs [69]. Indeed, circRNAs competitively suppress the activity of miRNAs by adsorbing and sequestering them. As miRNAs are strongly involved in nearly all aspects of cellular physiology and perform pivotal roles in initiation and progression of cancer, circRNAs could reasonably be considered as a new class of RNA molecules closely associated with regulation of proliferation, differentiation, and metastatic processes (Table 3).

Zheng et al. reported that circ-TTBK2 (tau tubulin Kinase 2) is significantly upregulated in glioma tissues and cell lines, differently from its linear counterpart [147]. Overexpression of circ-TTBK2 is associated with increased cell proliferation rate, invasion, and decreased apoptosis.

Table 3: circRNAs acting as miRNA sponges.

circRNA	miRNA target	tumor	circRNA role	PMID
circRNA_0005075	miR-23b-5p, miR-93-3p, miR-581, miR-23a-5p	Hepatocellular carcinoma	Oncogene	27258521
circRNA_001569	miR-145	Colorectal cancer	Oncogene	27058418
circRNA_100290	miR-29 family	Oral cancer	Oncogene	28368401
Cdr1as	miR-7	Hepatocellular carcinoma	Oncogene	27391479
cir-ITCH	miR-7, miR-20a	Colorectal cancer	Tumor suppressor	26110611
cir-ITCH	miR-7, miR-17, miR-214	Esophageal squamous cell carcinoma	Tumor suppressor	25749389
ciR-SRY	miR-138	Cholangiocarcinoma	Oncogene	27671698, 23446431
cir-TTBK2	miR-217	Glioma	Oncogene	28219405

This table reports for each circRNA: (1) miRNAs sponged; (2) tumor where such interaction was reported; (3) its function in cancer (oncogene or tumor suppressor); and (4) bibliographic reference reported as Pubmed ID (PMID).

Circ-TTBK2 harbors MREs for miR-217, which has a tumor-suppressive role in glioma cells. In fact, circ-TTBK2 and miR-217 interact with each other in an AGO2-dependent manner and upregulation of circ-TTBK2 induced the malignant behavior of glioma cells via downregulation of miR-217. Thus, HNF1β (HNF1 homeobox B), a direct target of miR-217, was derepressed and bound to the promoter of Derlin-1 increasing its expression. Finally, Derlin-1 was able to promote cell proliferation, migration, and invasion and inhibit apoptosis of glioma cells by activating PI3K/AKT and ERK pathways. Moreover, restoration of miR-217 expression reversed the circ-TTBK2-induced promotion of cancer progression, suggesting a reciprocal negative feedback between circ-TTBK2 and miR-217 [147].

MiR-145 is a well-known tumor suppressor miRNA in CRC targeting the oncogenes ERK5 (mitogen-activated protein kinase 7) and IRS1 (insulin receptor substrate 1); furthermore, its ability to predict survival of CRC patients was also shown. In a study by Xie et al., it was demonstrated that downregulation of miR-145 in CRC was mechanistically explained by the role of circ_001569 acting as a miRNA sponge to directly inhibit miR-145 action [148]. Circ_001569 was found to be upregulated in CRC tissues and correlated with progression and aggressiveness of the disease. Notably, circ_001569 did not directly affect miR-145 expression, but through a *sponge mechanism* it inhibited its posttranscriptional activity; accordingly, it upregulated its targets E2F5 (E2F transcription factor 5), BAG4 (BCL2-associated athanogene 4), and FMNL2 (formin-like 2), which were responsible for cell proliferation and invasion promotion by circ_001569 [148].

Further work on CRC, investigating the role of cir-ITCH on the biopathology of this cancer, found a potential interaction between cir-ITCH and either miR-7 or miR-20a [149]. Cir-ITCH was downregulated in CRC tissues and its ectopic expression led to decreased cell proliferation. This cellular effect was due to cir-ITCH sponge activity for miR-7 and miR-20a; both can bind the 3′-UTR of ITCH (Itchy E3 Ubiquitin Protein Ligase), which is the linear isoform of cir-ITCH. Cir-ITCH-induced upregulation of ITCH promoted the ubiquitination and degradation of phosphorylated DVL2 (dishevelled segment polarity protein 2) and, accordingly, inhibited the Wnt/β-catenin pathway, by repressing the expression of MYC and CCND1 (cyclin D1) [149]. Interestingly, other authors found very similar findings in ESCC: cir-ITCH worked as a miRNA sponge for miR-7, miR-17, and miR-214, increased ITCH expression, and promoted ubiquitin-mediated DVL2 degradation, thus inhibiting canonical Wnt signaling [150].

Besides the cir-ITCH-induced decoy function for miR-7 described above, sponging of miR-7 by CDR1AS was one of the earliest and the most studied ceRNA mechanisms in ncRNA biology, which is also related to cancer. Expression of CDR1AS was found to be elevated in HCC tissues and inversely correlated to miR-7 expression, which was poorly expressed in the same samples [151]. Despite the oncogenic role of miR-7 (previously reported for CRC and ESCC), this miRNA exhibited tumor-suppressive properties in HCC. CDR1AS has sixty-three MREs for miR-7 and strongly suppresses its activity. Knockdown of CDR1AS promoted the expression of miR-7 and suppressed its targets, CCNE1 (cyclin E1) and PIK3CD (phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit delta): this molecular cascade resulted in a reduction of cell proliferation and invasion in HCC [151].

By expression profiling in OSCC, Chen et al. identified the upregulation of a circRNA named circRNA_100290, which was functionally related to abnormal control of cell cycle and cellular proliferation in OSCC cells [152]. circRNA_100290 worked as a miRNA sponge for several members of the miR-29 family, decreasing the quantity of available miR-29s and, accordingly, promoting translation of one of their targets, CDK6 (cyclin-dependent kinase 6), which in turn could induce transition from G1 to S phase in cancer [152].

The first circular transcript identified was Sry circRNA: its encoding gene maps to the sex-determining region of human Y chromosome and was discovered as highly expressed in adult mouse testis [153]. Initially, Sry circRNA was considered an artifact of aberrant RNA splicing and no specific function was attributed to it. The role of Sry circRNA has recently begun to be investigated. Sry circRNA harbors sixteen putative target sites for miR-138 and its function as a miR-138 sponge was demonstrated by Hansen et al. [55]. Currently, no experimental evidence of Sry circRNA-miR-138 axis dysregulation has been reported in cancer; however, as reviewed by Zhao and Shen, miR-138 could target different cancer-related transcripts [154]. For instance,

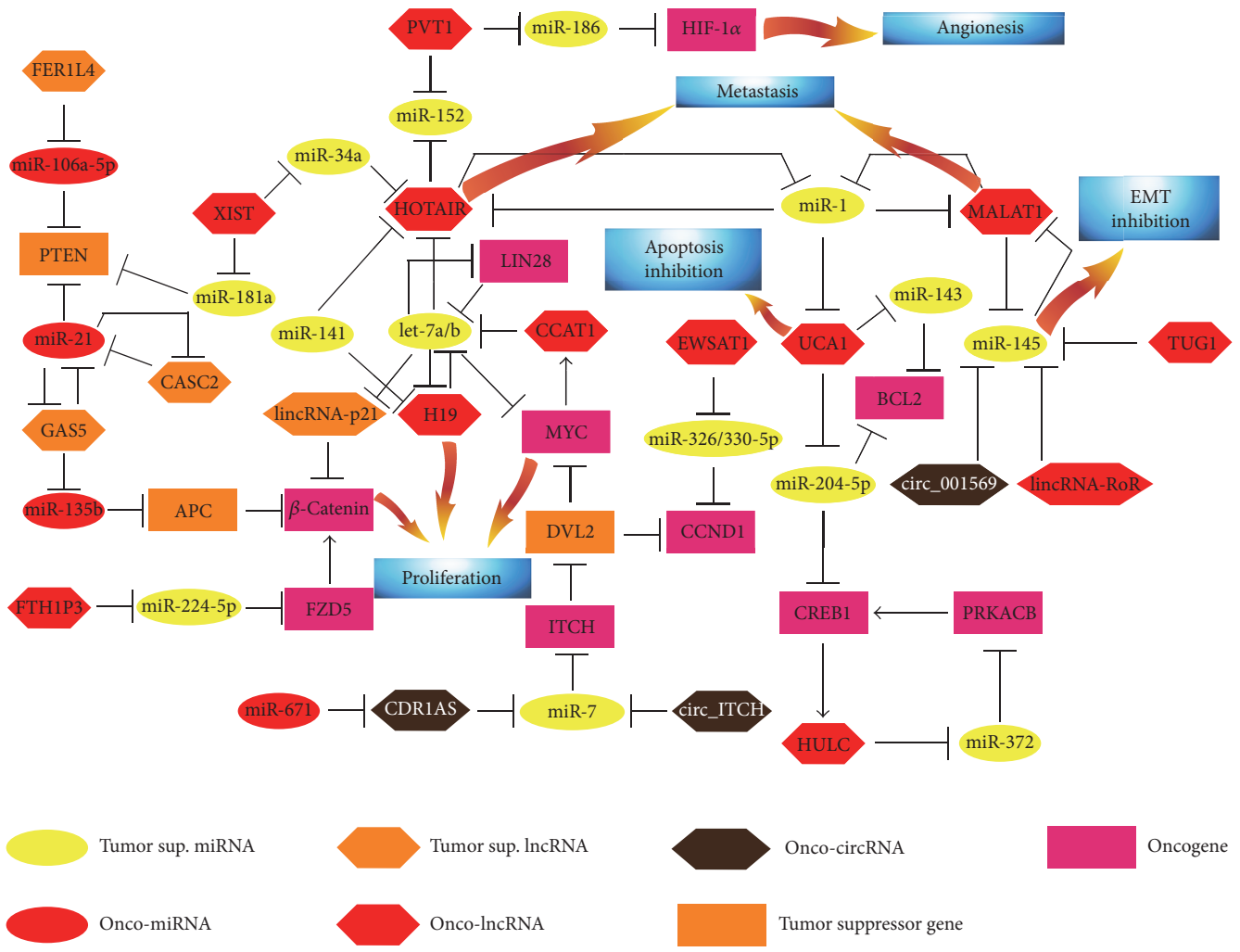

Figure 1: Network of noncoding crosstalking in cancer. Molecular interplay among ncRNAs (i.e., miRNAs, lncRNAs, and circRNAs) in cancer. RNA-RNA interactions were retrieved from papers cited in this review. Lines with arrowheads represent expression activation, those with bars represent expression inhibition.

downregulation of miR-138 promoted the malignant progression in cholangiocarcinoma by its target RhoC (ras homolog gene family, member C) [155]. These observations could suggest that the role of competitive endogenous binding between Sry circRNA and miR-138 would be worthy of in-depth analysis in cancer phenotypes.

3. Noncoding RNA Network: Future Perspectives for New Therapeutic Approaches

The existence of a complex RNA-based regulatory signaling, which controls cancer-related pathways, is evident from the experimental evidence collected to date. Such a partially hidden network of RNA-RNA interactions pervades and defines the correct functioning of canonical protein-coding pathways, classically involved in proliferation, differentiation, and invasion in cancer (Figure 1). The complexity of this *noncoding landscape* is dramatically expanded by the presence of several positive and negative regulatory loops: these make RNA signaling very robust and persistent, though complex and hard to functionally unveil. From a network biology point of view, it is possible to identify some *ncRNA hubs* that are a crossroad among different RNA-based circuits; accordingly, they represent a keystone of network structural integrity. For instance, the tumor suppressor miR-1 could repress and be sponged by the three most potent oncogenic lncRNAs, HOTAIR, UCA1, and MALAT1, which, in turn, could inhibit dozens of miRNAs with tumor-suppressive properties [76, 118, 124]. The signaling passing through let-7a/b appears extremely complex and pronged. Let-7a/b could be considered a crossroad of multiple interplays among cancer-related ncRNAs: let-7a/b and MYC are reciprocally negatively regulated through lncRNA CCAT1 [115], but MYC expression could be indirectly impaired by miR-7, which, in turn, is buffered by different circRNAs [156]. Moreover, let-7a/b could indirectly suppress the β-catenin pathway, which in a different way could be activated by lncRNA FTHIP3 [111], but also is regulated by molecular axis miR-21-GAS5-miR-135b [112]. This unexpected crosstalking between ncRNA signaling could shed a light on

expression relationships among ncRNAs and mRNAs, which have been frequently reported in cancer literature, but to date have not been satisfactorily explained [157–159]. This *scenario* is made more complex by the tissue-specific expression pattern of all ncRNAs, which could effectively influence the occurrence of specific interactions among ncRNAs. In other words, specific and effective functional interplays among ncRNAs in a particular biological system could occur only if RNA molecules, binding each other, are present at appropriate concentrations. Effectiveness of Ago binding to miRNAs and their targets is dependent on the relative concentration of the miRNA and its target pool [160, 161]. Effective Ago binding occurs when the miRNA : target ratio is close to one but rises dramatically with increasing miRNA : target ratios [162]. Only the most abundant miRNAs show detectable activity, while poorly expressed miRNAs (<100 copies per cell) possess exiguous regulatory properties [163]. However, functional binding between a miRNA and its target can be perturbed by overexpression of other RNAs with multiple shared MREs (e.g., other mRNAs, lncRNAs, and circRNAs) [164]. Such competition among different RNA molecules occurs in a threshold-like manner [165]. Mathematical models predict ceRNA functional effects when miRNA and target levels are near equimolar [166]. However, when the target pool exceeds the threshold set by the buffering miRNA concentration plus the equilibrium dissociation constant (KD) of the miRNA : target interaction, smaller changes in target (i.e., ceRNA) concentration could result in remarkable changes in the concentration of free unrepressed targets [165, 167]. In fact, poorly expressed miRNAs appear to be more susceptible to ceRNA control than more abundant miRNAs. This phenomenon could explain why in *in vitro* experiments a specific miRNA, when ectopically overexpressed, degraded its lncRNA target, but at the same time the enforced upregulation of lncRNA suppressed miRNA activity (e.g., miR-1/MALAT1, miR-21/GAS5) [104, 124]. Taken together, these considerations strongly suggest that miRNA functionality and the switch to ceRNA-promoted repression of miRNAs would be based on the stoichiometric equilibrium among miRNAs and ceRNAs. Based on these observations, physiological ceRNA expression changes could not affect highly expressed miRNAs; however, the relationship between cellular abundance of RNAs and effectiveness of competitive endogenous interactions remains to be fully unveiled in pathological models, in which strong dysregulation of specific ceRNAs could be present [162, 166, 168].

In spite of unclear stoichiometric relationships among ncRNAs in cancer, multiple experimental evidence shows that *in vitro* and *in vivo* modulation of ncRNAs strongly impair aggressive properties of cancer cells. The emerging role of ncRNAs as key regulators of cancer-related signaling makes them very attractive and promising targets for novel, potentially groundbreaking therapeutic approaches. RNA-based therapeutics has several advantages compared to other strategies. RNAs are molecules more *druggable* than proteins, because their targeting is mainly based on nucleic acid complementarity; therefore, an RNA-based drug would be quite easy to design and inexpensive to synthesize (i.e., ASOs, ribozymes, and aptamers) [14]. It is worth stressing that the development of RNA therapeutic strategies has to challenge the redundancy and complexity of the multiple regulatory loops, present in the ncRNA network. It would be quite naive to hypothesize to slow down *in vivo* tumor progression by targeting a single ncRNA molecule: this would be very hard also for protein-based drugs. This axiom should lead researchers to develop multitargeted RNA therapies to improve their impact on oncogenic signaling. In theory, the β-catenin pathway, frequently hyperactivated in cancers, could be effectively attenuated by simultaneous silencing of miR-21, miR-135b, and FTH1P3 together with restoring physiologic levels of GAS5, CASC2, and miR-224-5p. Furthermore, simultaneous repression of HOTAIR, MALAT1, and UCA1 with reactivation of miR-1 would result in a pleiotropic favorable effect on different cancer-related processes, such as cancer growth, metastatic behavior, and cell death. Such a synergic approach based on simultaneous administration of miRNA mimics and siRNAs against ncRNAs in *in vitro* and *in vivo* models has already provided encouraging results. Ideally, such therapeutic approaches would be greatly improved by innovative knockout technologies (such as CRISPR/CAS9), which would avoid potential saturation of RISC complexes, typically occurring by using siRNAs or miRNA mimics [169]. The main issue related to ncRNA therapeutics is to develop efficient delivery systems, which should be able to maintain RNA stability in the circulation and guarantee an effective tissue-specific uptake, as well as minimize off-target side effects. Rapid progress in drug delivery technologies has provided promising chemical and nanotechnological resources well adaptable to RNA therapeutics: chemical modifications of antisense molecules (e.g., steroids and cholesterol) [170], adenoviral vectors [171], cationic liposomes [172], and polymer-based nanoparticles [173]. Recently, an exosomal-based miRNA delivery system has been developed. Such a system appears to be very promising because exosomes are less toxic and better tolerated by the organism and naturally protect their molecular cargo in the blood [174].

4. Conclusions

A better knowledge on the complex interplay among ncRNAs, together with the development of selective methods for RNA delivery to cancer cells, will provide great benefits for cancer treatment. Needless to say, researchers will have to overcome many technical challenges to develop effective RNA-based anticancer strategies realistically applicable to patients. Before ncRNA targeting is pervasively applied in clinical settings, it will be indispensable to organize large collaborative efforts between research institutes and industry to fully realize the clinical potential of this very promising approach.

Conflicts of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] R. Nowak, "Mining treasures from 'junk DNA'," *Science*, vol. 263, no. 5147, pp. 608–610, 1994.

[2] E. S. Lander, L. M. Linton, B. Birren et al., "Initial sequencing and analysis of the human genome," *Nature*, vol. 409, no. 6822, pp. 860–921, 2001.

[3] J. C. Venter, M. D. Adams, E. W. Myers et al., "The sequence of the human genome," *Science*, vol. 291, no. 5507, pp. 1304–1351, 2001.

[4] International Human Genome Sequencing Consortium, "Finishing the euchromatic sequence of the human genome," *Nature*, vol. 431, no. 7011, pp. 931–945, 2004.

[5] M. Clamp, B. Fry, M. Kamal et al., "Distinguishing protein-coding and noncoding genes in the human genome," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 49, pp. 19428–19433, 2007.

[6] I. Ezkurdia, D. Juan, J. M. Rodriguez et al., "Multiple evidence strands suggest that there may be as few as 19,000 human protein-coding genes," *Human Molecular Genetics*, vol. 23, no. 22, pp. 5866–5878, 2014.

[7] ENCODE Project Consortium, E. Birney, J. A. Stamatoyannopoulos et al., "Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project," *Nature*, vol. 447, no. 7146, pp. 799–816, 2007.

[8] B. E. Bernstein, J. A. Stamatoyannopoulos, J. F. Costello et al., "The NIH roadmap epigenomics mapping consortium," *Nature Biotechnology*, vol. 28, no. 10, pp. 1045–1048, 2010.

[9] J. Sana, P. Faltejskova, M. Svoboda, and O. Slaby, "Novel classes of non-coding RNAs and cancer," *Journal of Translational Medicine*, vol. 10, p. 103, 2012.

[10] T. Huang, A. Alvarez, B. Hu, and S. Y. Cheng, "Noncoding RNAs in cancer and cancer stem cells," *Chinese Journal of Cancer*, vol. 32, no. 11, pp. 582–593, 2013.

[11] R. Fatima, V. S. Akhade, D. Pal, and S. M. Rao, "Long non-coding RNAs in development and cancer: potential biomarkers and therapeutic targets," *Molecular and Cellular Therapies*, vol. 3, no. 1, p. 5, 2015.

[12] J. R. Prensner and A. M. Chinnaiyan, "The emergence of lncRNAs in cancer biology," *Cancer Discovery*, vol. 1, no. 5, pp. 391–407, 2011.

[13] S. Geisler and J. Coller, "RNA in unexpected places: long non-coding RNA functions in diverse cellular contexts," *Nature Reviews Molecular Cell Biology*, vol. 14, no. 11, pp. 699–712, 2013.

[14] M. Ragusa, C. Barbagallo, L. Statello et al., "Non-coding landscapes of colorectal cancer," *World Journal of Gastroenterology*, vol. 21, no. 41, pp. 11709–11739, 2015.

[15] R. C. Friedman, K. K. Farh, C. B. Burge, and D. P. Bartel, "Most mammalian mRNAs are conserved targets of microRNAs," *Genome Research*, vol. 19, no. 1, pp. 92–105, 2009.

[16] A. Wilczynska and M. Bushell, "The complexity of miRNA-mediated repression," *Cell Death and Differentiation*, vol. 22, no. 1, pp. 22–33, 2015.

[17] R. C. Lee, R. L. Feinbaum, and V. Ambros, "The C. Elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14," *Cell*, vol. 75, no. 5, pp. 843–854, 1993.

[18] S. L. Ameres and P. D. Zamore, "Diversifying microRNA sequence and function," *Nature Reviews Molecular Cell Biology*, vol. 14, no. 8, pp. 475–488, 2013.

[19] A. Valinezhad Orang, R. Safaralizadeh, and M. Kazemzadeh-Bavili, "Mechanisms of miRNA-mediated gene regulation from common downregulation to mRNA-specific upregulation," *International Journal of Genomics*, vol. 2014, Article ID 970607, 15 pages, 2014.

[20] R. W. Carthew and E. J. Sontheimer, "Origins and mechanisms of miRNAs and siRNAs," *Cell*, vol. 136, no. 4, pp. 642–655, 2009.

[21] D. P. Bartel, "MicroRNAs: genomics, biogenesis, mechanism, and function," *Cell*, vol. 116, no. 2, pp. 281–297, 2004.

[22] A. Kozomara and S. Griffiths-Jones, "miRBase: annotating high confidence microRNAs using deep sequencing data," *Nucleic Acids Research*, vol. 42, Database issue, pp. D68–D73, 2014.

[23] L. A. Macfarlane and P. R. Murphy, "MicroRNA: biogenesis, function and role in cancer," *Current Genomics*, vol. 11, no. 7, pp. 537–561, 2010.

[24] C. M. Croce, "Causes and consequences of microRNA dysregulation in cancer," *Nature Reviews Genetics*, vol. 10, no. 10, pp. 704–714, 2009.

[25] S. Volinia, G. A. Calin, C. G. Liu et al., "A microRNA expression signature of human solid tumors defines cancer gene targets," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 7, pp. 2257–2261, 2006.

[26] C. Jay, J. Nemunaitis, P. Chen, P. Fulgham, and A. W. Tong, "miRNA profiling for diagnosis and prognosis of human cancer," *DNA and Cell Biology*, vol. 26, no. 5, pp. 293–300, 2007.

[27] M. Ragusa, L. Statello, M. Maugeri et al., "Highly skewed distribution of miRNAs and proteins between colorectal cancer cells and their exosomes following cetuximab treatment: biomolecular, genetic and translational implications," *Oncoscience*, vol. 1, no. 2, pp. 132–157, 2014.

[28] R. Ma, T. Jiang, and X. Kang, "Circulating microRNAs in cancer: origin, function and application," *Journal of Experimental & Clinical Cancer Research*, vol. 31, no. 1, p. 38, 2012.

[29] G. Cheng, "Circulating miRNAs: roles in cancer diagnosis, prognosis and therapy," *Advanced Drug Delivery Reviews*, vol. 81, pp. 75–93, 2015.

[30] M. Ragusa, C. Barbagallo, L. Statello et al., "miRNA profiling in vitreous humor, vitreal exosomes and serum from uveal melanoma patients: pathological and diagnostic implications," *Cancer Biology & Therapy*, vol. 16, no. 9, pp. 1387–1396, 2015.

[31] P. J. Volders, K. Helsens, X. Wang et al., "LNCipedia: a database for annotated human lncRNA transcript sequences and structures," *Nucleic Acids Research*, vol. 41, Database issue, pp. D246–D251, 2013.

[32] L. Nie, H. J. Wu, J. M. Hsu et al., "Long non-coding RNAs: versatile master regulators of gene expression and crucial players in cancer," *American Journal of Translational Research*, vol. 4, no. 2, pp. 127–150, 2012.

[33] I. Martianov, A. Ramadass, A. Serra Barros, N. Chow, and A. Akoulitchev, "Repression of the human dihydrofolate reductase gene by a non-coding interfering transcript," *Nature*, vol. 445, no. 7128, pp. 666–670, 2007.

[34] L. Li, B. Liu, O. L. Wapinski et al., "Targeted disruption of Hotair leads to homeotic transformation and gene derepression," *Cell Reports*, vol. 5, no. 1, pp. 3–12, 2013.

[35] J. M. Engreitz, N. Ollikainen, and M. Guttman, "Long non-coding RNAs: spatial amplifiers that control nuclear structure and gene expression," *Nature Reviews Molecular Cell Biology*, vol. 17, no. 12, pp. 756–770, 2016.

[36] M. Beltran, I. Puig, C. Pena et al., "A natural antisense transcript regulates Zeb2/Sip1 gene expression during Snail1-induced epithelial-mesenchymal transition," *Genes & Development*, vol. 22, no. 6, pp. 756–769, 2008.

[37] T. Liu, Y. Huang, J. Chen et al., "Attenuated ability of BACE1 to cleave the amyloid precursor protein via silencing long noncoding RNA BACE1AS expression," *Molecular Medicine Reports*, vol. 10, no. 3, pp. 1275–1281, 2014.

[38] A. Rosa and M. Ballarino, "Long noncoding RNA regulation of pluripotency," *Stem Cells International*, vol. 2016, Article ID 1797692, 9 pages, 2016.

[39] A. Fatica and I. Bozzoni, "Long non-coding RNAs: new players in cell differentiation and development," *Nature Reviews. Genetics*, vol. 15, no. 1, pp. 7–21, 2014.

[40] M. Kitagawa, K. Kitagawa, Y. Kotake, H. Niida, and T. Ohhata, "Cell cycle regulation by long non-coding RNAs," *Cellular and Molecular Life Sciences*, vol. 70, no. 24, pp. 4785–4794, 2013.

[41] Y. Su, H. Wu, A. Pavlosky et al., "Regulatory non-coding RNA: new instruments in the orchestration of cell death," *Cell Death & Disease*, vol. 7, no. 8, article e2333, 2016.

[42] P. Zhang, P. Cao, X. Zhu et al., "Upregulation of long non-coding RNA HOXA-AS2 promotes proliferation and induces epithelial-mesenchymal transition in gallbladder carcinoma," *Oncotarget*, vol. 8, no. 20, pp. 33137–33143, 2017.

[43] S. Deguchi, K. Katsushima, A. Hatanaka et al., "Oncogenic effects of evolutionarily conserved noncoding RNA ECONEXIN on gliomagenesis," *Oncogene*, vol. 36, no. 32, pp. 4629–4640, 2017.

[44] F. Yun-Bo, L. Xiao-Po, L. Xiao-Li, C. Guo-Long, Z. Pei, and T. Fa-Ming, "LncRNA TUG1 is upregulated and promotes cell proliferation in osteosarcoma," *Open Medicine*, vol. 11, no. 1, pp. 163–167, 2016.

[45] J. Tian, X. Hu, W. Gao et al., "Identification of the long noncoding RNA LET as a novel tumor suppressor in gastric cancer," *Molecular Medicine Reports*, vol. 15, no. 4, pp. 2229–2234, 2017.

[46] G. Luo, D. Liu, C. Huang et al., "LncRNA GAS5 inhibits cellular proliferation by targeting P27Kip1," *Molecular Cancer Research*, vol. 15, no. 7, pp. 789–799, 2017.

[47] Z. Li, C. Jin, S. Chen et al., "Long non-coding RNA MEG3 inhibits adipogenesis and promotes osteogenesis of human adipose-derived mesenchymal stem cells via miR-140-5p," *Molecular and Cellular Biochemistry*, vol. 433, no. 1-2, pp. 51–60, 2017.

[48] J. Liu, T. Liu, X. Wang, and A. He, "Circles reshaping the RNA world: from waste to treasure," *Molecular Cancer*, vol. 16, no. 1, p. 58, 2017.

[49] H. L. Sanger, G. Klotz, D. Riesner, H. J. Gross, and A. K. Kleinschmidt, "Viroids are single-stranded covalently closed circular RNA molecules existing as highly base-paired rod-like structures," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 73, no. 11, pp. 3852–3856, 1976.

[50] C. Cocquerelle, B. Mascrez, D. Hetuin, and B. Bailleul, "Mis-splicing yields circular RNA molecules," *The FASEB Journal*, vol. 7, no. 1, pp. 155–160, 1993.

[51] J. Salzman, C. Gawad, P. L. Wang, N. Lacayo, and P. O. Brown, "Circular RNAs are the predominant transcript isoform from hundreds of human genes in diverse cell types," *PLoS One*, vol. 7, no. 2, article e30733, 2012.

[52] W. R. Jeck, J. A. Sorrentino, K. Wang et al., "Circular RNAs are abundant, conserved, and associated with ALU repeats," *RNA*, vol. 19, no. 2, pp. 141–157, 2013.

[53] I. Chen, C. Y. Chen, and T. J. Chuang, "Biogenesis, identification, and function of exonic circular RNAs," *Wiley Interdisciplinary Reviews RNA*, vol. 6, no. 5, pp. 563–579, 2015.

[54] P. Glazar, P. Papavasileiou, and N. Rajewsky, "circBase: a database for circular RNAs," *RNA*, vol. 20, no. 11, pp. 1666–1670, 2014.

[55] T. B. Hansen, T. I. Jensen, B. H. Clausen et al., "Natural RNA circles function as efficient microRNA sponges," *Nature*, vol. 495, no. 7441, pp. 384–388, 2013.

[56] E. Lasda and R. Parker, "Circular RNAs: diversity of form and function," *RNA*, vol. 20, no. 12, pp. 1829–1842, 2014.

[57] J. U. Guo, V. Agarwal, H. Guo, and D. P. Bartel, "Expanded identification and characterization of mammalian circular RNAs," *Genome Biology*, vol. 15, no. 7, p. 409, 2014.

[58] Y. Enuka, M. Lauriola, M. E. Feldman, A. Sas-Chen, I. Ulitsky, and Y. Yarden, "Circular RNAs are long-lived and display only minimal early alterations in response to a growth factor," *Nucleic Acids Research*, vol. 44, no. 3, pp. 1370–1383, 2016.

[59] C. Y. Chen and P. Sarnow, "Initiation of protein synthesis by the eukaryotic translational apparatus on circular RNAs," *Science*, vol. 268, no. 5209, pp. 415–417, 1995.

[60] N. R. Pamudurti, O. Bartok, M. Jens et al., "Translation of CircRNAs," *Molecular Cell*, vol. 66, no. 1, pp. 9–21.e7, 2017.

[61] M. Huang, Z. Zhong, M. Lv, J. Shu, Q. Tian, and J. Chen, "Comprehensive analysis of differentially expressed profiles of lncRNAs and circRNAs with associated co-expression and ceRNA networks in bladder carcinoma," *Oncotarget*, vol. 7, no. 30, pp. 47186–47200, 2016.

[62] W. W. Du, W. Yang, E. Liu, Z. Yang, P. Dhaliwal, and B. B. Yang, "Foxo3 circular RNA retards cell cycle progression via forming ternary complexes with p21 and CDK2," *Nucleic Acids Research*, vol. 44, no. 6, pp. 2846–2858, 2016.

[63] H. Li, X. Hao, H. Wang et al., "Circular RNA expression profile of pancreatic ductal adenocarcinoma revealed by microarray," *Cellular Physiology and Biochemistry*, vol. 40, no. 6, pp. 1334–1344, 2016.

[64] A. Bachmayr-Heyda, A. T. Reiner, K. Auer et al., "Correlation of circular RNA abundance with proliferation – exemplified with colorectal and ovarian cancer, idiopathic lung fibrosis, and normal human tissues," *Scientific Reports*, vol. 5, no. 1, 8057 pages, 2015.

[65] Y. Dou, D. J. Cha, J. L. Franklin et al., "Circular RNAs are down-regulated in KRAS mutant colon cancer cells and can be transferred to exosomes," *Scientific Reports*, vol. 6, no. 1, article 37982, 2016.

[66] W. Sui, Z. Shi, W. Xue et al., "Circular RNA and gene expression profiles in gastric cancer based on microarray chip technology," *Oncology Reports*, vol. 37, no. 3, pp. 1804–1814, 2017.

[67] L. Wan, L. Zhang, K. Fan, Z. X. Cheng, Q. C. Sun, and J. J. Wang, "Circular RNA-ITCH suppresses lung cancer proliferation via inhibiting the Wnt/β-catenin pathway," *BioMed Research International*, vol. 2016, Article ID 1579490, 11 pages, 2016.

[68] Z. Zhong, M. Lv, and J. Chen, "Screening differential circular RNA expression profiles reveals the regulatory role of circTCF25-miR-103a-3p/miR-107-CDK6 pathway in bladder carcinoma," *Scientific Reports*, vol. 6, article 30919, 2016.

[69] S. Memczak, M. Jens, A. Elefsinioti et al., "Circular RNAs are a large class of animal RNAs with regulatory potency," *Nature*, vol. 495, no. 7441, pp. 333–338, 2013.

[70] X. Su, J. Xing, Z. Wang, L. Chen, M. Cui, and B. Jiang, "microRNAs and ceRNAs: RNA networks in pathogenesis of cancer," *Chinese Journal of Cancer Research*, vol. 25, no. 2, pp. 235–239, 2013.

[71] J. J. Quinn and H. Y. Chang, "Unique features of long non-coding RNA biogenesis and function," *Nature Reviews Genetics*, vol. 17, no. 1, pp. 47–62, 2016.

[72] M. Xue, X. Li, W. Wu et al., "Upregulation of long non-coding RNA urothelial carcinoma associated 1 by CCAAT/enhancer binding protein alpha contributes to bladder cancer cell growth and reduced apoptosis," *Oncology Reports*, vol. 31, no. 5, pp. 1993–2000, 2014.

[73] Z. Fang, L. Wu, L. Wang, Y. Yang, Y. Meng, and H. Yang, "Increased expression of the long non-coding RNA UCA1 in tongue squamous cell carcinomas: a possible correlation with cancer metastasis," *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology*, vol. 117, no. 1, pp. 89–95, 2014.

[74] J. Huang, N. Zhou, K. Watabe et al., "Long non-coding RNA UCA1 promotes breast tumor growth by suppression of p27 (Kip1)," *Cell Death & Disease*, vol. 5, article e1008, 2014.

[75] L. Zhang, X. Cao, L. Zhang, X. Zhang, H. Sheng, and K. Tao, "UCA1 Overexpression predicts clinical outcome of patients with ovarian cancer receiving adjuvant chemotherapy," *Cancer Chemotherapy and Pharmacology*, vol. 77, no. 3, pp. 629–634, 2016.

[76] T. Wang, J. Yuan, N. Feng et al., "Hsa-miR-1 downregulates long non-coding RNA urothelial cancer associated 1 in bladder cancer," *Tumour Biology*, vol. 35, no. 10, pp. 10075–10084, 2014.

[77] P. Ji, S. Diederichs, W. Wang et al., "MALAT-1, a novel noncoding RNA, and thymosin β4 predict metastasis and survival in early-stage non-small cell lung cancer," *Oncogene*, vol. 22, no. 39, pp. 8031–8041, 2003.

[78] Y. Huo, Q. Li, X. Wang et al., "MALAT1 predicts poor survival in osteosarcoma patients and promotes cell metastasis through associating with EZH2," *Oncotarget*, vol. 8, no. 29, pp. 46993–47006, 2017.

[79] Y. Li, Z. Wu, J. Yuan et al., "Long non-coding RNA MALAT1 promotes gastric cancer tumorigenicity and metastasis by regulating vasculogenic mimicry and angiogenesis," *Cancer Letters*, vol. 395, pp. 31–44, 2017.

[80] R. Lei, M. Xue, L. Zhang, and Z. Lin, "Long noncoding RNA MALAT1-regulated microRNA 506 modulates ovarian cancer growth by targeting iASPP," *OncoTargets and Therapy*, vol. 10, pp. 35–46, 2017.

[81] J. E. Wilusz, S. M. Freier, and D. L. Spector, "3' end processing of a long nuclear-retained noncoding RNA yields a tRNA-like cytoplasmic RNA," *Cell*, vol. 135, no. 5, pp. 919–932, 2008.

[82] V. Tripathi, J. D. Ellis, Z. Shen et al., "The nuclear-retained noncoding RNA MALAT1 regulates alternative splicing by modulating SR splicing factor phosphorylation," *Molecular Cell*, vol. 39, no. 6, pp. 925–938, 2010.

[83] J. A. West, C. P. Davis, H. Sunwoo et al., "The long noncoding RNAs NEAT1 and MALAT1 bind active chromatin sites," *Molecular Cell*, vol. 55, no. 5, pp. 791–802, 2014.

[84] E. Leucci, F. Patella, J. Waage et al., "microRNA-9 targets the long non-coding RNA MALAT1 for degradation in the nucleus," *Scientific Reports*, vol. 3, p. 2535, 2013.

[85] X. Wang, M. Li, Z. Wang et al., "Silencing of long noncoding RNA MALAT1 by miR-101 and miR-217 inhibits proliferation, migration, and invasion of esophageal squamous cell carcinoma cells," *The Journal of Biological Chemistry*, vol. 290, no. 7, pp. 3925–3935, 2015.

[86] Y. Han, Y. Liu, H. Zhang et al., "Hsa-miR-125b suppresses bladder cancer development by down-regulating oncogene SIRT7 and oncogenic long non-coding RNA MALAT1," *FEBS Letters*, vol. 587, no. 23, pp. 3875–3882, 2013.

[87] F. H. Tsang, S. L. Au, L. Wei et al., "Long non-coding RNA HOTTIP is frequently up-regulated in hepatocellular carcinoma and is targeted by tumour suppressive miR-125b," *Liver International*, vol. 35, no. 5, pp. 1597–1606, 2015.

[88] R. A. Gupta, N. Shah, K. C. Wang et al., "Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis," *Nature*, vol. 464, no. 7291, pp. 1071–1076, 2010.

[89] T. Chiyomaru, S. Yamamura, S. Fukuhara et al., "Genistein inhibits prostate cancer cell growth by targeting miR-34a and oncogenic HOTAIR," *PLoS One*, vol. 8, no. 8, article e70372, 2013.

[90] J. H. Yoon, K. Abdelmohsen, J. Kim et al., "Scaffold function of long non-coding RNA HOTAIR in protein ubiquitination," *Nature Communications*, vol. 4, p. 2939, 2013.

[91] J. H. Yoon, K. Abdelmohsen, S. Srikantan et al., "LincRNA-p21 suppresses target mRNA translation," *Molecular Cell*, vol. 47, no. 4, pp. 648–655, 2012.

[92] J. H. Yoon, M. H. Jo, E. J. White et al., "AUF1 promotes let-7b loading on Argonaute 2," *Genes & Development*, vol. 29, no. 15, pp. 1599–1604, 2015.

[93] T. Chiyomaru, S. Fukuhara, S. Saini et al., "Long non-coding RNA HOTAIR is targeted and regulated by miR-141 in human cancer cells," *The Journal of Biological Chemistry*, vol. 289, no. 18, pp. 12550–12565, 2014.

[94] S. M. Park, A. B. Gaur, E. Lengyel, and M. E. Peter, "The miR-200 family determines the epithelial phenotype of cancer cells by targeting the E-cadherin repressors ZEB1 and ZEB2," *Genes & Development*, vol. 22, no. 7, pp. 894–907, 2008.

[95] X. Zhou, F. Ye, C. Yin, Y. Zhuang, G. Yue, and G. Zhang, "The interaction between MiR-141 and lncRNA-H19 in regulating cell proliferation and migration in gastric cancer," *Cellular Physiology and Biochemistry*, vol. 36, no. 4, pp. 1440–1452, 2015.

[96] O. Lustig, I. Ariel, J. Ilan, E. Lev-Lehman, N. De-Groot, and A. Hochberg, "Expression of the imprinted gene H19 in the human fetus," *Molecular Reproduction and Development*, vol. 38, no. 3, pp. 239–246, 1994.

[97] H. Li, B. Yu, J. Li et al., "Overexpression of lncRNA H19 enhances carcinogenesis and metastasis of gastric cancer," *Oncotarget*, vol. 5, no. 8, pp. 2318–2329, 2014.

[98] W. P. Tsang, E. K. Ng, S. S. Ng et al., "Oncofetal H19-derived miR-675 regulates tumor suppressor RB in human colorectal cancer," *Carcinogenesis*, vol. 31, no. 3, pp. 350–358, 2010.

[99] J. Ribas, X. Ni, M. Castanares et al., "A novel source for miR-21 expression through the alternative polyadenylation of VMP1 gene transcripts," *Nucleic Acids Research*, vol. 40, no. 14, pp. 6821–6833, 2012.

[100] B. Vicinus, C. Rubie, S. K. Faust et al., "miR-21 functionally interacts with the 3'UTR of chemokine CCL20 and down-regulates CCL20 expression in miR-21 transfected colorectal cancer cells," *Cancer Letters*, vol. 316, no. 1, pp. 105–112, 2012.

[101] P. Wang, F. Zou, X. Zhang et al., "microRNA-21 negatively regulates Cdc25A and cell cycle progression in colon cancer cells," *Cancer Research*, vol. 69, no. 20, pp. 8157–8165, 2009.

[102] I. A. Asangani, S. A. Rasheed, D. A. Nikolova et al., "MicroRNA-21 (miR-21) post-transcriptionally downregulates tumor suppressor Pdcd4 and stimulates invasion, intravasation and metastasis in colorectal cancer," *Oncogene*, vol. 27, no. 15, pp. 2128–2136, 2008.

[103] S. Roy, Y. Yu, S. B. Padhye, F. H. Sarkar, and A. P. Majumdar, "Difluorinated-curcumin (CDF) restores PTEN expression in colon cancer cells by down-regulating miR-21," *PLoS One*, vol. 8, no. 7, article e68543, 2013.

[104] Z. Zhang, Z. Zhu, K. Watabe et al., "Negative regulation of lncRNA GAS5 by miR-21," *Cell Death and Differentiation*, vol. 20, no. 11, pp. 1558–1568, 2013.

[105] Y. Cao, R. Xu, X. Xu, Y. Zhou, L. Cui, and X. He, "Down-regulation of lncRNA CASC2 by microRNA-21 increases the proliferation and migration of renal cell carcinoma cells," *Molecular Medicine Reports*, vol. 14, no. 1, pp. 1019–1025, 2016.

[106] P. Wang, Y. H. Liu, Y. L. Yao et al., "Long non-coding RNA CASC2 suppresses malignancy in human gliomas by miR-21," *Cellular Signalling*, vol. 27, no. 2, pp. 275–282, 2015.

[107] T. B. Hansen, E. D. Wiklund, J. B. Bramsen et al., "miRNA-dependent gene silencing involving Ago2-mediated cleavage of a circular antisense RNA," *The EMBO Journal*, vol. 30, no. 21, pp. 4414–4422, 2011.

[108] D. Barbagallo, A. Condorelli, M. Ragusa et al., "Dysregulated miR-671-5p / CDR1-AS / CDR1 / VSNL1 axis is involved in glioblastoma multiforme," *Oncotarget*, vol. 7, no. 4, pp. 4746–4759, 2016.

[109] P. Song and S. C. Yin, "Long non-coding RNA EWSAT1 promotes human nasopharyngeal carcinoma cell growth in vitro by targeting miR-326/-330-5p," *Aging*, vol. 8, no. 11, pp. 2948–2960, 2016.

[110] T. Xia, S. Chen, Z. Jiang et al., "Long noncoding RNA FER1L4 suppresses cancer cell growth by acting as a competing endogenous RNA and regulating PTEN expression," *Scientific Reports*, vol. 5, article 13445, 2015.

[111] C. Z. Zhang, "Long non-coding RNA FTH1P3 facilitates oral squamous cell carcinoma progression by acting as a molecular sponge of miR-224-5p to modulate fizzled 5 expression," *Gene*, vol. 607, pp. 47–55, 2017.

[112] Y. Xue, T. Ni, Y. Jiang, and Y. Li, "LncRNA GAS5 inhibits tumorigenesis and enhances radiosensitivity by suppressing miR-135b expression in non-small cell lung cancer," *Oncology Research*, vol. 25, no. 6, pp. 1027–1037, 2017.

[113] F. Peng, T. T. Li, K. L. Wang et al., "H19/Let-7/LIN28 reciprocal negative regulatory circuit promotes breast cancer stem cell maintenance," *Cell Death & Disease*, vol. 8, no. 1, article e2569, 2017.

[114] Y. Gao, H. Meng, S. Liu et al., "LncRNA-HOST2 regulates cell biological behaviors in epithelial ovarian cancer through a mechanism involving microRNA let-7b," *Human Molecular Genetics*, vol. 24, no. 3, pp. 841–852, 2015.

[115] L. Deng, S. B. Yang, F. F. Xu, and J. H. Zhang, "Long non-coding RNA CCAT1 promotes hepatocellular carcinoma progression by functioning as let-7 sponge," *Journal of Experimental & Clinical Cancer Research*, vol. 34, p. 18, 2015.

[116] X. He, X. Tan, X. Wang et al., "C-Myc-activated long noncoding RNA CCAT1 promotes colon cancer cell proliferation and invasion," *Tumour Biology*, vol. 35, no. 12, pp. 12181–12188, 2014.

[117] F. Yang, X. Xue, J. Bi et al., "Long noncoding RNA CCAT1, which could be activated by c-Myc, promotes the progression of gastric carcinoma," *Journal of Cancer Research and Clinical Oncology*, vol. 139, no. 3, pp. 437–445, 2013.

[118] D. N. Su, S. P. Wu, H. T. Chen, and J. H. He, "HOTAIR, a long non-coding RNA driver of malignancy whose expression is activated by FOXC1, negatively regulates miRNA-1 in hepatocellular carcinoma," *Oncology Letters*, vol. 12, no. 5, pp. 4061–4067, 2016.

[119] B. Song, Z. Guan, F. Liu, D. Sun, K. Wang, and H. Qu, "Long non-coding RNA HOTAIR promotes HLA-G expression via inhibiting miR-152 in gastric cancer cells," *Biochemical and Biophysical Research Communications*, vol. 464, no. 3, pp. 807–813, 2015.

[120] G. B. Beck-Engeser, A. M. Lum, K. Huppi, N. J. Caplen, B. B. Wang, and M. Wabl, "Pvt1-encoded microRNAs in oncogenesis," *Retrovirology*, vol. 5, no. 1, 4 pages, 2008.

[121] T. Li, X. L. Meng, and W. Q. Yang, "Long noncoding RNA PVT1 acts as a "sponge" to inhibit microRNA-152 in gastric cancer cells," *Digestive Diseases and Sciences*, 2017.

[122] T. Huang, H. W. Liu, J. Q. Chen et al., "The long noncoding RNA PVT1 functions as a competing endogenous RNA by sponging miR-186 in gastric cancer," *Biomedicine & Pharmacotherapy*, vol. 88, pp. 302–308, 2017.

[123] J. Wang, X. Liu, H. Wu et al., "CREB up-regulates long non-coding RNA, HULC expression through interaction with microRNA-372 in liver cancer," *Nucleic Acids Research*, vol. 38, no. 16, pp. 5366–5383, 2010.

[124] C. Jin, B. Yan, Q. Lu, Y. Lin, and L. Ma, "Reciprocal regulation of Hsa-miR-1 and long noncoding RNA MALAT1 promotes triple-negative breast cancer development," *Tumour Biology*, vol. 37, no. 6, pp. 7383–7394, 2016.

[125] H. Lu, Y. He, L. Lin et al., "Long non-coding RNA MALAT1 modulates radiosensitivity of HR-HPV+ cervical cancer via sponging miR-145," *Tumour Biology*, vol. 37, no. 2, pp. 1683–1691, 2016.

[126] G. Eades, B. Wolfson, Y. Zhang, Q. Li, Y. Yao, and Q. Zhou, "lincRNA-RoR and miR-145 regulate invasion in triple-negative breast cancer via targeting ARF6," *Molecular Cancer Research*, vol. 13, no. 2, pp. 330–338, 2015.

[127] X. Zhou, Q. Gao, J. Wang, X. Zhang, K. Liu, and Z. Duan, "Linc-RNA-RoR acts as a "sponge" against mediation of the differentiation of endometrial cancer stem cells by microRNA-145," *Gynecologic Oncology*, vol. 133, no. 2, pp. 333–339, 2014.

[128] J. Tan, K. Qiu, M. Li, and Y. Liang, "Double-negative feedback loop between long non-coding RNA TUG1 and miR-145 promotes epithelial to mesenchymal transition and radioresistance in human bladder cancer cells," *FEBS Letters*, vol. 589, no. 20, Part B, pp. 3175–3181, 2015.

[129] H. Cai, X. Liu, J. Zheng et al., "Long non-coding RNA taurine upregulated 1 enhances tumor-induced angiogenesis through inhibiting microRNA-299 in human glioblastoma," *Oncogene*, vol. 36, no. 3, pp. 318–331, 2017.

[130] F. Ma, S. H. Wang, Q. Cai et al., "Long non-coding RNA TUG1 promotes cell proliferation and metastasis by negatively regulating miR-300 in gallbladder carcinoma," *Biomedicine & Pharmacotherapy*, vol. 88, pp. 863–869, 2017.

[131] C. H. Xie, Y. M. Cao, Y. Huang et al., "Long non-coding RNA TUG1 contributes to tumorigenesis of human osteosarcoma by sponging miR-9-5p and regulating POU2F1 expression," *Tumour Biology*, vol. 37, no. 11, pp. 15031–15041, 2016.

[132] Y. Wang, Z. Liu, B. Yao et al., "Long non-coding RNA TUSC7 acts a molecular sponge for miR-10a and suppresses EMT in hepatocellular carcinoma," *Tumour Biology*, vol. 37, no. 8, pp. 11429–11441, 2016.

[133] Q. Liu, J. Huang, N. Zhou et al., "LncRNA loc285194 is a p53-regulated tumor suppressor," *Nucleic Acids Research*, vol. 41, no. 9, pp. 4976–4987, 2013.

[134] P. Qi, M. D. Xu, X. H. Shen et al., "Reciprocal repression between TUSC7 and miR-23b in gastric cancer," *International Journal of Cancer*, vol. 137, no. 6, pp. 1269–1278, 2015.

[135] Y. L. Tuo, X. M. Li, and J. Luo, "Long noncoding RNA UCA1 modulates breast cancer cell growth and apoptosis through decreasing tumor suppressive miR-143," *European Review for Medical and Pharmacological Sciences*, vol. 19, no. 18, pp. 3403–3411, 2015.

[136] H. J. Li, X. Li, H. Pang, J. J. Pan, X. J. Xie, and W. Chen, "Long non-coding RNA UCA1 promotes glutamine metabolism by targeting miR-16 in human bladder cancer," *Japanese Journal of Clinical Oncology*, vol. 45, no. 11, pp. 1055–1063, 2015.

[137] Z. Bian, L. Jin, J. Zhang et al., "LncRNA-UCA1 enhances cell proliferation and 5-fluorouracil resistance in colorectal cancer by inhibiting miR-204-5p," *Scientific Reports*, vol. 6, no. 1, article 23892, 2016.

[138] F. Wang, H. Q. Ying, B. S. He et al., "Upregulated lncRNA-UCA1 contributes to progression of hepatocellular carcinoma through inhibition of miR-216b and activation of FGFR1/ERK signaling pathway," *Oncotarget*, vol. 6, no. 10, pp. 7899–7917, 2015.

[139] Y. Yang, Y. Jiang, Y. Wan et al., "UCA1 functions as a competing endogenous RNA to suppress epithelial ovarian cancer metastasis," *Tumour Biology*, vol. 37, no. 8, pp. 10633–10641, 2016.

[140] Y. Wei, Q. Sun, L. Zhao et al., "LncRNA UCA1-miR-507-FOXM1 axis is involved in cell proliferation, invasion and G0/G1 cell cycle arrest in melanoma," *Medical Oncology*, vol. 33, no. 8, p. 88, 2016.

[141] C. J. Brown, B. D. Hendrich, J. L. Rupert et al., "The human XIST gene: analysis of a 17 kb inactive X-specific RNA that contains conserved repeats and is highly localized within the nucleus," *Cell*, vol. 71, no. 3, pp. 527–542, 1992.

[142] P. C. Schouten, M. A. Vollebergh, M. Opdam et al., "High XIST and low 53BP1 expression predict poor outcome after high-dose alkylating chemotherapy in patients with a BRCA1-like breast cancer," *Molecular Cancer Therapeutics*, vol. 15, no. 1, pp. 190–198, 2016.

[143] Y. Yao, J. Ma, Y. Xue et al., "Knockdown of long non-coding RNA XIST exerts tumor-suppressive functions in human glioblastoma stem cells by up-regulating miR-152," *Cancer Letters*, vol. 359, no. 1, pp. 75–86, 2015.

[144] L. K. Zhuang, Y. T. Yang, X. Ma et al., "MicroRNA-92b promotes hepatocellular carcinoma progression by targeting Smad7 and is mediated by long non-coding RNA XIST," *Cell Death & Disease*, vol. 7, article e2203, 2016.

[145] P. Song, L. F. Ye, C. Zhang, T. Peng, and X. H. Zhou, "Long non-coding RNA XIST exerts oncogenic functions in human nasopharyngeal carcinoma by targeting miR-34a-5p," *Gene*, vol. 592, no. 1, pp. 8–14, 2016.

[146] S. Chang, B. Chen, X. Wang, K. Wu, and Y. Sun, "Long non-coding RNA XIST regulates PTEN expression by sponging miR-181a and promotes hepatocellular carcinoma progression," *BMC Cancer*, vol. 17, no. 1, p. 248, 2017.

[147] J. Zheng, X. Liu, Y. Xue et al., "TTBK2 circular RNA promotes glioma malignancy by regulating miR-217/HNF1beta/Derlin-1 pathway," *Journal of Hematology & Oncology*, vol. 10, no. 1, 52 pages, 2017.

[148] H. Xie, X. Ren, S. Xin et al., "Emerging roles of circRNA_001569 targeting miR-145 in the proliferation and invasion of colorectal cancer," *Oncotarget*, vol. 7, no. 18, pp. 26680–26691, 2016.

[149] G. Huang, H. Zhu, Y. Shi, W. Wu, H. Cai, and X. Chen, "Cir-ITCH plays an inhibitory role in colorectal cancer by regulating the Wnt/beta-catenin pathway," *PLoS One*, vol. 10, no. 6, article e0131225, 2015.

[150] F. Li, L. Zhang, W. Li et al., "Circular RNA ITCH has inhibitory effect on ESCC by suppressing the Wnt/beta-catenin pathway," *Oncotarget*, vol. 6, no. 8, pp. 6001–6013, 2015.

[151] L. Yu, X. Gong, L. Sun, Q. Zhou, B. Lu, and L. Zhu, "The circular RNA Cdr1as act as an oncogene in hepatocellular carcinoma through targeting miR-7 expression," *PLoS One*, vol. 11, no. 7, article e0158347, 2016.

[152] L. Chen, S. Zhang, J. Wu et al., "circRNA_100290 plays a role in oral cancer by functioning as a sponge of the miR-29 family," *Oncogene*, vol. 36, no. 32, pp. 4551–4561, 2017.

[153] B. Capel, A. Swain, S. Nicolis et al., "Circular transcripts of the testis-determining gene *Sry* in adult mouse testis," *Cell*, vol. 73, no. 5, pp. 1019–1030, 1993.

[154] Z. J. Zhao and J. Shen, "Circular RNA participates in the carcinogenesis and the malignant behavior of cancer," *RNA Biology*, vol. 14, no. 5, pp. 514–521, 2017.

[155] Q. Wang, H. Tang, S. Yin, and C. Dong, "Downregulation of microRNA-138 enhances the proliferation, migration and invasion of cholangiocarcinoma cells through the upregulation of RhoC/p-ERK/MMP-2/MMP-9," *Oncology Reports*, vol. 29, no. 5, pp. 2046–2052, 2013.

[156] T. B. Hansen, J. Kjems, and C. K. Damgaard, "Circular RNA and miR-7 in cancer," *Cancer Research*, vol. 73, no. 18, pp. 5609–5612, 2013.

[157] X. Zhou, X. Xu, J. Wang, J. Lin, and W. Chen, "Identifying miRNA/mRNA negative regulation pairs in colorectal cancer," *Scientific Reports*, vol. 5, article 12995, 2015.

[158] S. Zadran, F. Remacle, and R. D. Levine, "miRNA and mRNA cancer signatures determined by analysis of expression levels in large cohorts of patients," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 47, pp. 19160–19165, 2013.

[159] J. Seo, D. Jin, C. H. Choi, and H. Lee, "Integration of microRNA, mRNA, and protein expression data for the identification of cancer-related microRNAs," *PLoS One*, vol. 12, no. 1, article e0168412, 2017.

[160] G. Mullokandov, A. Baccarini, A. Ruzo et al., "High-throughput assessment of microRNA activity and function using microRNA sensor and decoy libraries," *Nature Methods*, vol. 9, no. 8, pp. 840–846, 2012.

[161] A. Arvey, E. Larsson, C. Sander, C. S. Leslie, and D. S. Marks, "Target mRNA abundance dilutes microRNA and siRNA activity," *Molecular Systems Biology*, vol. 6, p. 363, 2010.

[162] A. D. Bosson, J. R. Zamudio, and P. A. Sharp, "Endogenous miRNA and target concentrations determine susceptibility to potential ceRNA competition," *Molecular Cell*, vol. 56, no. 3, pp. 347–359, 2014.

[163] B. D. Brown, B. Gentner, A. Cantore et al., "Endogenous microRNA can be broadly exploited to regulate transgene expression according to tissue, lineage and differentiation state," *Nature Biotechnology*, vol. 25, no. 12, pp. 1457–1467, 2007.

[164] M. S. Ebert, J. R. Neilson, and P. A. Sharp, "MicroRNA sponges: competitive inhibitors of small RNAs in mammalian cells," *Nature Methods*, vol. 4, no. 9, pp. 721–726, 2007.

[165] S. Mukherji, M. S. Ebert, G. X. Zheng, J. S. Tsang, P. A. Sharp, and A. van Oudenaarden, "MicroRNAs can generate thresholds in target gene expression," *Nature Genetics*, vol. 43, no. 9, pp. 854–859, 2011.

[166] U. Ala, F. A. Karreth, C. Bosia et al., "Integrated transcriptional and competitive endogenous RNA networks are cross-regulated in permissive molecular environments," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 18, pp. 7154–7159, 2013.

[167] C. Bosia, A. Pagnani, and R. Zecchina, "Modelling competing endogenous RNA networks," *PLoS One*, vol. 8, no. 6, article e66609, 2013.

[168] M. Jens and N. Rajewsky, "Competition between target sites of regulators shapes post-transcriptional gene regulation," *Nature Reviews Genetics*, vol. 16, no. 2, pp. 113–126, 2015.

[169] A. L. Jackson and P. S. Linsley, "Recognizing and avoiding siRNA off-target effects for target identification and therapeutic application," *Nature Reviews Drug Discovery*, vol. 9, no. 1, pp. 57–67, 2010.

[170] C. Lorenz, P. Hadwiger, M. John, H. P. Vornlocher, and C. Unverzagt, "Steroid and lipid conjugates of siRNAs to enhance cellular uptake and gene silencing in liver cells," *Bioorganic & Medicinal Chemistry Letters*, vol. 14, no. 19, pp. 4975–4977, 2004.

[171] M. B. Mowa, C. Crowther, and P. Arbuthnot, "Therapeutic potential of adenoviral vectors for delivery of expressed RNAi activators," *Expert Opinion on Drug Delivery*, vol. 7, no. 12, pp. 1373–1385, 2010.

[172] S. Mallick and J. S. Choi, "Liposomes: versatile and biocompatible nanovesicles for efficient biomolecules delivery," *Journal of Nanoscience and Nanotechnology*, vol. 14, no. 1, pp. 755–765, 2014.

[173] E. Miele, G. P. Spinelli, E. Miele et al., "Nanoparticle-based delivery of small interfering RNA: challenges for cancer therapy," *International Journal of Nanomedicine*, vol. 7, pp. 3637–3657, 2012.

[174] D. Ha, N. Yang, and V. Nadithe, "Exosomes as therapeutic drug carriers and delivery vehicles across biological membranes: current perspectives and future challenges," *Acta Pharmaceutica Sinica B*, vol. 6, no. 4, pp. 287–296, 2016.

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Research Article

The miRNA Pull Out Assay as a Method to Validate the miR-28-5p Targets Identified in Other Tumor Contexts in Prostate Cancer

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miR-28-5p is an intragenic miRNA which is underexpressed in several tumor types showing a tumor suppressor (TS) activity. Routinely, the known miR-28-5p targets are validated in specific tumor contexts but it is unclear whether these targets are also being regulated in other tumor types. To this end, we adopted the miRNA pull out assay to capture the miR-28-5p targets in DU-145 prostate cancer (PCa) cells. Firstly, we demonstrated that miR-28-5p acts as a TS-miRNA in PCa, affecting cell proliferation, survival, and apoptosis. Secondly, we evaluated the enrichment of the 10 validated miR-28-5p targets in the pull out sample. We showed that E2F6, TEX-261, MAPK1, MPL, N4BP1, and RAP1B but not BAG1, OTUB1, MAD2L1, and p21 were significantly enriched, suggesting that not all the miR-28-5p targets are regulated by this miRNA in PCa. We then verified whether the miR-28-5p-interacting targets were regulated by this miRNA. We selected E2F6, the most enriched target in the pull out sample, and demonstrated that miR-28-5p downregulated E2F6 at the protein level suggesting that our approach was effective. In general terms, these findings support the miRNA pull out assay as a useful method to identify context-specific miRNA targets.

1. Introduction

It is well known that the deregulation of miRNA expression is one of the causes or contributory causes of cancer development. miRNAs may act as tumor suppressors (TS), oncogenes, or both depending on the tumor context [1]. miR-28-5p is an intragenic miRNA downregulated in several tumor types, such as hepatocellular carcinoma [2], renal cell carcinoma [3], natural killer/T-cell lymphoma [4], B-cell lymphoma [5], colorectal cancer (CRC) [6], and CRC liver metastasis [7, 8], although in some cases, an increased expression level of miR-28-5p has been observed (ovarian, esophageal, and cervical cancer) [9–11]. Most of the papers regarding the role of miR-28-5p in tumors suggested a prevalent tumor suppressor activity of this miRNA *in vitro* [2, 3, 5, 6, 12]. Very recently, it has been demonstrated that the miR-28-5p reexpression in xenograft models of Burkitt (BL) and diffuse large B-cell lymphoma (DLBCL) as well as in a BL murine model blocked tumor growth, opening the way to miR-28-5p-based replacement therapy as a novel therapeutic strategy for these diseases [13].

The molecular targets through which miR-28-5p exerts its anti- or proproliferative role are only partially known. For example, miR-28-5p reduced cell growth and migration in hepatocellular carcinoma [2] and in CRC [6] cells inhibiting the expression of IGF-1, CCND1, and HOXB3 genes. In

addition, miR-28-5p acted as a TS-miRNA in renal cell carcinoma by directly repressing the expression of RAP1B [3] and in B-cell lymphoma by directly inhibiting BAG1 expression, a gene involved in the MAP-kinase pathway regulation [5].

To date, there are no data either on the role of miR-28-5p in prostate cancer (PCa) or on the targets regulated by this miRNA in PCa cells. In this work, we evaluated whether the miR-28-5p targets validated in other types of tumors were regulated in PCa cells using the miRNA pull out assay, a technique that allows the isolation of all the targets of a given miRNA in specific biological contexts. We demonstrated that miR-28-5p exerted TS activity in PCa cells and that not all validated miR-28-5p targets are regulated by this miRNA in the PCa context.

2. Materials and Methods

2.1. Cell and Culture Conditions. DU-145 and A-549 cells were grown in RPMI Medium 1640 (EuroClone) whereas PC-3 cells were grown in HAM's medium (EuroClone) and MCF-7 cells in DMEM low glucose (EuroClone). 10% FBS (fetal bovine serum, EuroClone), 1% penicillin/streptomycin (2 mM, EuroClone), and 1% L-glutamine (2 mM, Sigma-Aldrich) were added to the medium. The cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2.

2.2. Transfection. Transient transfections of double-stranded miRNA mimics (miR-28a-5p) or negative control (miR-NC) (GenePharma) in DU-145 cells were carried out using Lipofectamine 2000 (Thermo Fisher): 1.5×10^5 cells were seeded in P30 dishes and after 48 hours, cells were transfected with miRNA mimic using 10 μl of Lipofectamine according to the protocol provided by the manufacturer. The suspension of the transfected cells was used for cellular and molecular assays.

2.3. Cell Proliferation. 1×10^5 cells were seeded in a series of 30 mm diameter dishes and grown for 96 hours. At 24-hour intervals, cells were collected and counted.

2.4. Cell Cycle Analysis. Cell cycle analysis was performed as follows: 5×10^5 cells were fixed with 95% cold ethanol and labelled with 300 μl of 50 μl/ml propidium iodide (Sigma-Aldrich) solution. After overnight incubation at 4°C, the cell cycle analysis was performed with Accuri™ C6 flow cytometer (BD Biosciences). Using specific software supplied with the instrument, the percentage of cells in each phase of the cell cycle was determined considering the parameters SSC-H/FL2-A.

2.5. Survival Assay. Survival was measured as follows: cells were collected and seeded at cell density of 200 cells/60 mm diameter culture dish to allow colony formation. After 10–12 days, dishes were stained with 0.1% CV and the ratio (number of colonies/number of seeded cells) was used to calculate the fraction of surviving cells.

2.6. Apoptosis Assay. Apoptosis was measured as follows: 1×10^6 cells were suspended in 300 μl Binding Buffer 1X and left at room temperature for 15 minutes. Thereafter, cell labelling was done according to the kit Annexin V-FITC. Cells were then passed through flow cytometer BD Accuri C6 (BD Biosciences) and analyzed using FL3-H/FL1-H parameters.

2.7. miRNA Pull Out Assay. The miRNA pull out assay was performed as described in Rizzo et al. [14]. Briefly, DU-145 cells were transfected using Lipofectamine 2000 (Thermo Fisher) with 60 nM of either miR-28a-5p duplex (ds-miR-28a_{CT}) or a mix of 3′ biotin-tagged miR-28a-5p 8tU (nucleotide 8 was a thiouridine) and miR-28a-5p 18tU duplexes (ds-miR-28a_{BIO}). The day after transfection, cells were irradiated with UV (365 nm, 2 J/cm^2) using Bio-Link crosslinking (BLX) (Ambrose Lourmat) and total RNA extracted adding TRIzol reagent (Thermo Fisher) directly to adherent cells and following the instructions provided by the manufacturer. 15 μg of RNA was incubated for 4 hours at 4°C with 100 μl of streptavidin-conjugated beads (Streptavidin Sepharose High Performance, GE Healthcare), and the RNA complexed with the beads recovered using the Trizol protocol. We performed three biological replicates obtaining three miR-28_{CT} (miR-28 control) and three miR-28_{BIO} (miR-28) pull out samples.

2.8. Quantification of miRNAs and mRNAs (qRT-PCR). Total RNA was extracted from 1×10^6 cells using the miRNeasy mini kit following the manufacturer's recommendations. 1 μg of total RNA was retrotranscribed using either the miScript II RT kit (Qiagen) or the QuantiTect Reverse Transcription Kit (Qiagen) for the miRNA or the mRNA quantification, respectively. The reverse transcription was performed following the manufacturer's instructions. miRNAs and the mRNAs were quantified with Rotor-Gene Q 2plex (Qiagen), using the miScript SYBR Green PCR Kit (Qiagen) and the SsoAdvanced™ SYBR® Green Supermix (Bio-Rad), respectively, according to the manufacturer protocols. The relative quantification was performed using the Rotor-Gene Q software, normalizing to the internal controls (U6, SNORD55, and SNORD110 for miRNA and GAPDH, ACTB, and HPRT for mRNA). The relative miR-28a-5p expression level in tumor cell lines was evaluated with respect to the normal cell RNA (FirstChoice Human Total RNA, Ambion). All reactions were performed in triplicate, and the results are the mean of three biological replicates.

2.9. Western Blot Analysis. Proteins were extracted from cell pellets using lysis buffer (1 M Tris HCl pH 8, Triton X-100 1%, and Na deoxycholate 0.25%) with the addition of PMSF 1 mM. The proteins were then quantified colorimetrically using the BioRad protein Assay Reagent (Bio-Rad). Absorbance was measured at 595 nm with ChroMate microplate reader (Awareness Technology). The proteins were separated on polyacrylamide gels SDS-PAGE (10%, gel precast Mini-PROTEAN® TGX Stain-Free™, Bio-Rad) and transferred to 0.2 μm nitrocellulose membranes by electro blotting using the Trans-Blot Turbo Blotting System (Bio-Rad). The resulting blots were blocked with 5% nonfat dry milk solution in TBST. Anti-GAPDH (Cell Signaling) (1 : 20000), anti-E2F6 (Santa Cruz Biotechnology) (1 : 500), and PARP-1 (Santa

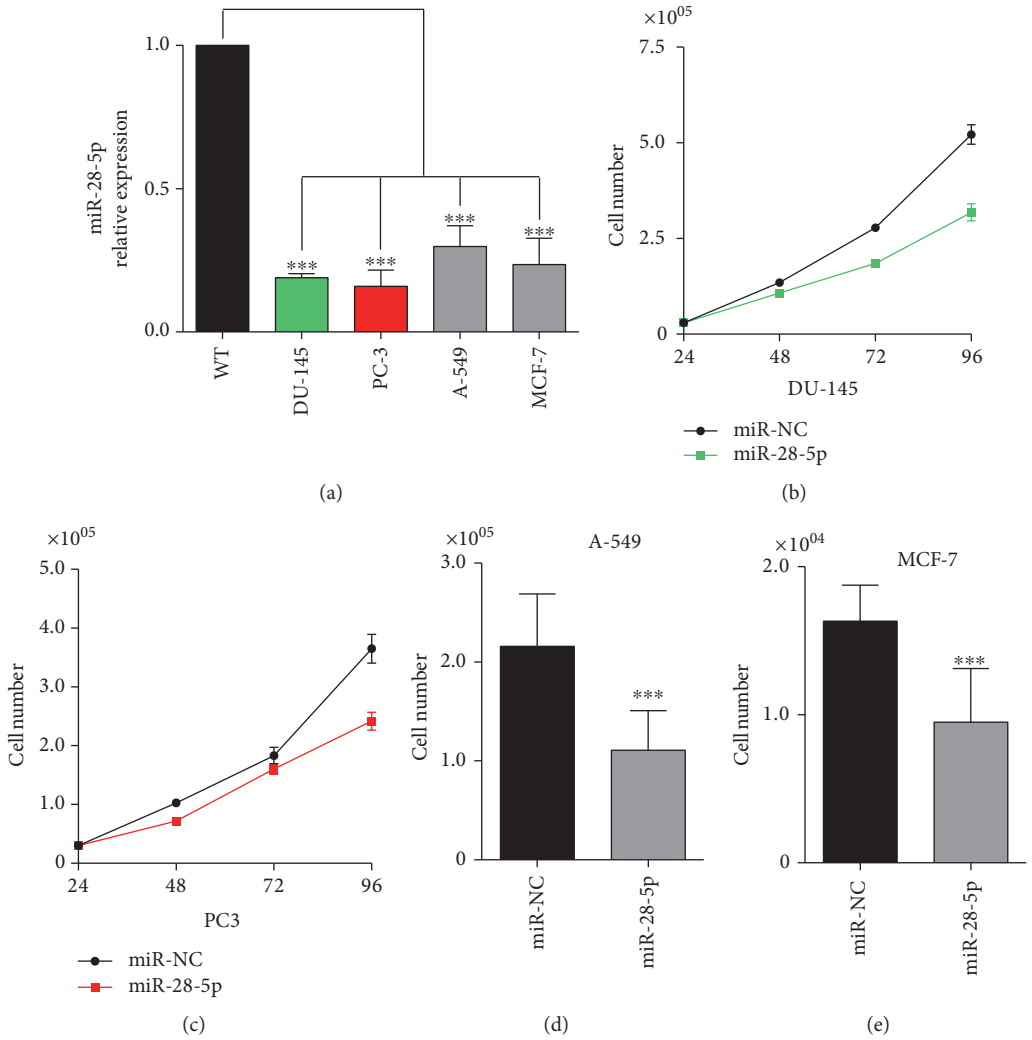

Figure 1: miR-28-5p expression and effect on tumor cells. (a) Analysis of the miR-28-5p expression level with qRT-PCR in prostate (PC-3 and DU-145), lung (A-549), and breast (MCF-7) cancer cell lines compared to the normal cell RNA. Cell proliferation of DU-145 (b), PC-3 (c), A-549 (d), and MCF-7 (e) cells at different time points or at 96 hours after the miR-28-5p reexpression. $^{***}P < 0.001$, unpaired t-test.

Cruz Biotechnology) (1 : 500) primary antibodies were used. Incubation was performed overnight at 4°C, and bands were revealed after incubation with the recommended secondary antibody coupled to peroxidase using ECL (GE Healthcare). Scanned images were quantified using ImageJ software and normalized to GAPDH.

2.10. Statistical Analyses. Results are expressed as mean SD of at least three independent experiments, and data are analyzed using Student's t-test ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$).

3. Results

3.1. miR-28-5p Exerted a TS Activity in PCa Cells. To investigate the role of miR-28-5p in PCa, we first evaluated its expression in two PCa cell lines (DU-145 and PC-3) compared to normal cells (Figure 1(a)). We also evaluated the expression of miR-28-5p in lung (A-549) and breast (MCF-7) cancer cells, demonstrating that miR-28-5p was markedly downregulated in all the analyzed cancer cell lines.

In order to test whether miR-28-5p behaves as a TS in PCa cells, we first measured cell proliferation of DU-145 and PC-3 cells after miR-28-5p reexpression. Data showed a significant inhibition of cell proliferation of both PCa cell lines (Figures 1(b) and 1(c)). Similar results were obtained when the miR-28-5p was transfected in breast and colon cancer cell lines (Figures 1(d) and 1(e)).

To further investigate the biological effects of the miR-28-5p in PCa, we checked the colony-forming ability (CFA) and the cell cycle after miRNA reexpression in DU-145 cells. Data showed that miR-28-5p reexpression resulted in both a significant reduction of CFA (Figure 2(a)) and a slight but significant increase of cells in G1 phase (Figure 2(b)), suggesting that the proliferation was negatively affected. Finally, we demonstrated that the miR-28-5p reexpression increased apoptosis in DU-145 cells (Figures 2(c) and 2(d)). Overall data suggested that the miR-28-5p acts as a TS-miRNA in

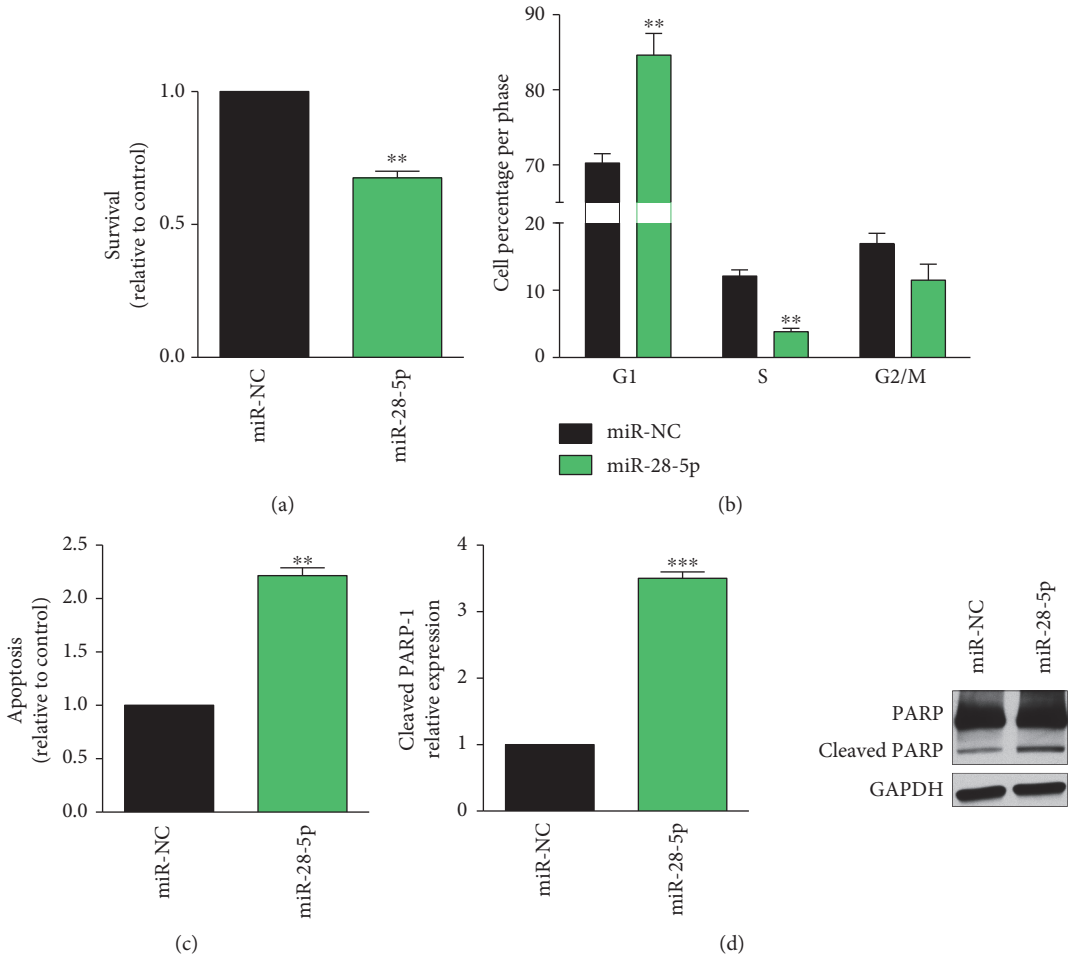

Figure 2: Effects of miR-28-5p reexpression on DU-145 cells. Cell survival (a) and cell cycle (b) in DU-145 cells after miR-28-5p reexpression. Apoptosis analysis measured with both annexin assay (c) and western blot of PARP-1 and cleaved PARP-1 (d) in miR-28-5p-transfected DU-145 cells. $^{**}P < 0.01$ and $^{***}P < 0.001$, unpaired t-test.

PCa, probably by regulating key pathways involved not only in tumor cell proliferation but also in tumor cell survival.

3.2. Some Validated miR-28-5p Targets Interacted with miR-28-5p in PCa. To investigate which targets were regulated by miR-28-5p in PCa, we transfected this miRNA in DU-145 cells and the miRNA pull out assay was performed [14]. This technique allowed the capture and the isolation of the miR-28-5p/target complexes using a biotinylated version of miR-28-5p. We considered the miR-28-5p targets deposited in miRTarBase (http://mirtarbase.mbc.nctu.edu.tw), in particular, the ones validated with the luciferase reporter assay (Table 1).

Using qRT-PCR, we checked the enrichment of these targets in the pool of miR-28-5p-captured targets (miR-28 pull out sample) and found that RAP1B, N4BP1, MPL, MAPK1, TEX-261, and E2F6 were enriched by more than 2-fold in the miR-28-5p pull out sample (Figure 3(a)). These results suggested that not all the miR-28-5p-validated targets interact and, as a consequence, may be regulated by miR-28-5p in PCa cells.

To verify whether the enrichment of the selected targets in the miR-28-5p pull out sample was indicative of the miRNA regulatory function, we selected the most enriched one, that is, E2F6, and determined its expression after the miR-28-5p reexpression in DU-145 cells. We demonstrated

Table 1: miR-28-5p targets validated with gene reporter assay according to miRTarBase.

miR-28-5p target	Tumor type	Reference
p21	Choriocarcinoma cells	[15]
MPL	Myeloproliferative neoplasms	[16]
N4BP1	Myeloproliferative neoplasms/ ovarian cancer	[9]
OTUB1	Myeloproliferative neoplasms	[16]
TEX-261	Myeloproliferative neoplasms	[16]
MAPK1	Myeloproliferative neoplasms	[16]
E2F6	Myeloproliferative neoplasms	[16]
MAD2L1	B-cell lymphomas	[5]
BAG1	B-cell lymphomas	[5]
RAP1B	B-cell lymphomas/renal cell carcinoma	[3, 5]

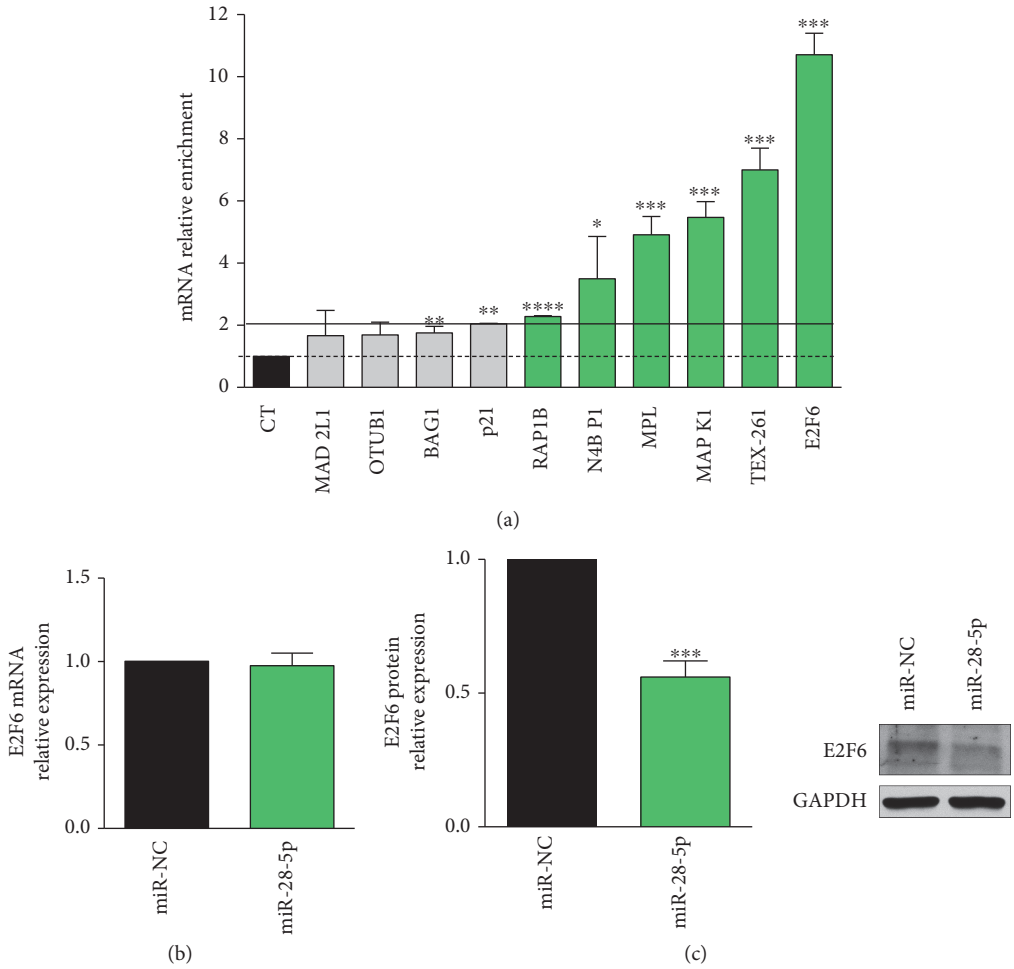

Figure 3: miR-28-5p target validation. (a) miR-28-5p-validated target enrichment quantified by qRT-PCR in miR-28-5p pull out sample compared to miR-28-5p control pull out sample (CT). E2F6 mRNA (b) and protein (c) quantification in DU-145 cells transfected with miR-28-5p or miR-NC. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ unpaired t-test.

that E2F6 was inhibited by miR-28-5p reexpression only at the protein level (Figures 3(b) and 3(c)), indicating that E2F6 was regulated by miR-28-5p in PCa.

4. Discussion

miRNAs are key inhibitors of gene expression that play a pivotal role in tumor development and progression affecting genes and pathways involved in all the hallmarks of cancer [17, 18]. By regulating oncogenes or TS genes, they can act, respectively, as a TS or oncogenes, although it is known that the role of a specific miRNA in cancer is not absolute but strongly related to the tumor context [1]. Therefore, it is not surprising that miR-28-5p may act as an oncogene (e.g., [9]) or as a TS (e.g., [13]), even though in most tumors, it showed an antiproliferative effect. It is possible that, depending on the expression level of the targets or on the effect of regulators which interfere with miRISC binding or function, not all the targets of a particular miRNA can be bound and regulated by the miRNA in a specific context [19, 20].

In this work, performing the miR-28-5p pull out assay, we have explored whether the known miR-28-5p targets, deposited in miRTarBase and validated with at least the luciferase reporter assay, interacted and were regulated by miR-28-5p in PCa. Under this strategy was that, among all miR-28-5p-validated targets, the ones that interact with miR-28-5p in PCa have higher chance to be regulated by the miRNA in this tumor. We first evaluated the miR-28-5p role in PCa, and we demonstrated for the first time that this miRNA is underexpressed in these cells and that its reexpression inhibited cell proliferation and survival. These data led us to conclude that miR-28-5p acted as a TS-miRNA in PCa. As it has been demonstrated that miR-28-5p negatively regulated genes involved in tumor cell growth in lung [12] as well as in colon rectal [6] cancer cells, we checked the expression level and the effects of miR-28-5p in lung and colon cancer cell lines as a positive control.

Using the miRNA pull out assay, we found that not all the validated miR-28-5p targets were enriched in the miR-28 pull out sample. Among the enriched targets that are more strongly associated with cancer (i.e., RAP1B, N4BP1, MAPK1, and E2F6), almost all are protumoral. Indeed, both MAPK1 and RAP1B, a Ras-related small GTP-binding protein that acts as GTPase in several signaling cascades, are

proproliferative proteins involved as oncogenes in the development and progression of several tumor types (e.g., [21, 22]). In particular, it has been demonstrated that miR-28-5p suppressed cell proliferation and migration by directly inhibiting RAP1B in renal cell carcinoma [22]. These observations are consistent with a possible regulation by miR-28-5p, suggesting the utility of our approach to identify context-specific miRNA targets. In the same way, it has been reported that when PCa evolves from a benign to more aggressive stage, it becomes resistant to apoptosis due to the increased expression of antiapoptotic proteins [23] such as E2F6 [24]. Indeed, we demonstrated that E2F6, the most enriched target in the miR-28-5p pull out sample, was regulated by miR-28-5p at the posttranscriptional level. We can speculate that the target enrichment level in the miRNA pull out sample might facilitate the identification of the targets affected by the regulation of the miRNA. In addition, we also showed that the miR-28-5p reexpression induced apoptosis in DU-145 cells. Given that DU-145 cells (androgen independent PCa cell line) are an advanced PCa *in vitro* model, miR-28-5p reexpression may be taken into consideration as a novel therapeutic approach for PCa at this stage. Furthermore, we demonstrated that E2F6 is regulated by miR-28-5p in DU-145 cells; thus, it is conceivable that E2F6 could be one of the mediators of the apoptosis induced by miR-28-5p reexpression.

In conclusion, in this work, we demonstrated that the capture of the targets that interact with a given miRNA in a specific tumor is a suitable approach to identify the subset of targets that have a higher probability of being regulated by that miRNA in the context under evaluation. In the future, the identification of all the miR-28-5p targets (miR-28-5p targetome) could help to decipher the genes and pathways affected by the regulation of this miRNA in PCa.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

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References

[1] H. Ling, L. Girnita, O. Buda, and G. A. Calin, "Non-coding RNAs: the cancer genome dark matter that matters!," *Clinical Chemistry and Laboratory Medicine*, vol. 55, no. 5, pp. 705–714, 2017.

[2] X. Shi and F. Teng, "Down-regulated miR-28-5p in human hepatocellular carcinoma correlated with tumor proliferation and migration by targeting insulin-like growth factor-1 (IGF-1)," *Molecular and Cellular Biochemistry*, vol. 408, no. 1-2, pp. 283–293, 2015.

[3] C. Wang, C. Wu, Q. Yang et al., "miR-28-5p acts as a tumor suppressor in renal cell carcinoma for multiple antitumor effects by targeting RAP1B," *Oncotarget*, vol. 7, no. 45, pp. 73888–73902, 2016.

[4] S. B. Ng, J. Yan, G. Huang et al., "Dysregulated microRNAs affect pathways and targets of biologic relevance in nasal-type natural killer/T-cell lymphoma," *Blood*, vol. 118, no. 18, pp. 4919–4929, 2011.

[5] C. Schneider, M. Setty, A. B. Holmes et al., "MicroRNA 28 controls cell proliferation and is down-regulated in B-cell lymphomas," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 111, no. 22, pp. 8185–8190, 2014.

[6] M. I. Almeida, M. S. Nicoloso, L. Zeng et al., "Strand-specific miR-28-5p and miR-28-3p have distinct effects in colorectal cancer cells," *Gastroenterology*, vol. 142, no. 4, pp. 886–896.e9, 2012.

[7] J. M. Sayagués, L. A. Corchete, M. L. Gutiérrez et al., "Genomic characterization of liver metastases from colorectal cancer patients," *Oncotarget*, vol. 7, no. 45, pp. 72908–72922, 2016.

[8] P. Vychytilova-Faltejskova, M. Pesta, L. Radova et al., "Genome-wide microRNA expression profiling in primary tumors and matched liver metastasis of patients with colorectal cancer," *Cancer Genomics Proteomics*, vol. 13, no. 4, pp. 311–316, 2016.

[9] J. Xu, N. Jiang, H. Shi, S. Zhao, S. Yao, and H. Shen, "miR-28-5p promotes the development and progression of ovarian cancer through inhibition of N4BP1," *International Journal of Oncology*, vol. 50, 2017.

[10] Z. Huang, L. Zhang, D. Zhu et al., "A novel serum microRNA signature to screen esophageal squamous cell carcinoma," *Cancer Medicine*, vol. 6, no. 1, pp. 109–119, 2017.

[11] S. M. Wilting, P. J. Snijders, W. Verlaat et al., "Altered microRNA expression associated with chromosomal changes contributes to cervical carcinogenesis," *Oncogene*, vol. 32, no. 1, pp. 106–116, 2013.

[12] Y. C. Choi, S. Yoon, Y. Byun et al., "MicroRNA library screening identifies growth-suppressive microRNAs that regulate genes involved in cell cycle progression and apoptosis," *Experimental Cell Research*, vol. 339, no. 2, pp. 320–332, 2015.

[13] N. Bartolomé-Izquierdo, V. G. de Yébenes, A. F. Álvarez-Prado et al., "miR-28 regulates the germinal center reaction and blocks tumor growth in preclinical models of non-Hodgkin lymphoma," *Blood*, vol. 129, no. 17, pp. 2408–2419, 2017.

[14] M. Rizzo, G. Berti, F. Russo, S. Fazio, M. Evangelista, and G. Rainaldi, "Discovering the miR-26a-5p targetome in prostate cancer cells," *Journal of Cancer*, vol. 8, no. 14, pp. 2729–2739, 2017.

[15] S. Wu, S. Huang, J. Ding et al., "Multiple microRNAs modulate p21Cip1/Waf1 expression by directly targeting its 3′ untranslated region," *Oncogene*, vol. 29, no. 15, pp. 2302–2308, 2010.

[16] M. Girardot, C. Pecquet, S. Boukour et al., "miR-28 is a thrombopoietin receptor targeting microRNA detected in a fraction of myeloproliferative neoplasm patient platelets," *Blood*, vol. 116, no. 3, pp. 437–445, 2010.

[17] V. G. Manasa and S. Kannan, "Impact of microRNA dynamics on cancer hallmarks: an oral cancer scenario," *Tumour Biology*, vol. 39, no. 3, 2017.

[18] R. Rupaimoole and F. J. Slack, "MicroRNA therapeutics: towards a new era for the management of cancer and other diseases," *Nature Reviews Drug Discovery*, vol. 16, no. 3, pp. 203–222, 2017.

[19] A. E. Pasquinelli, "MicroRNAs and their targets: recognition, regulation and an emerging reciprocal relationship," *Nature Reviews Genetics*, vol. 13, no. 4, pp. 271–282, 2012.

[20] N. Cloonan, "Re-thinking miRNA-mRNA interactions: intertwining issues confound target discovery," *BioEssays*, vol. 37, no. 4, pp. 379–388, 2015.

[21] W. Li, J. Liang, Z. Zhang et al., "MicroRNA-329-3p targets MAPK1 to suppress cell proliferation, migration and invasion in cervical cancer," *Oncology Reports*, vol. 37, no. 5, pp. 2743–2750, 2017.

[22] Y. Li, Y. Liu, F. Shi, L. Cheng, and J. She, "Knockdown of Rap1b enhances apoptosis and autophagy in gastric cancer cells via the PI3K/Akt/mTOR pathway," *Oncology Research*, vol. 24, no. 5, pp. 287–293, 2016.

[23] K. L. Mahon, S. M. Henshall, R. L. Sutherland, and L. G. Horvath, "Pathways of chemotherapy resistance in castration-resistant prostate cancer," *Endocrine-Related Cancer*, vol. 18, no. 4, pp. R103–R123, 2011.

[24] N. Bhatnagar, X. Li, S. K. Padi, Q. Zhang, M. S. Tang, and B. Guo, "Downregulation of miR-205 and miR-31 confers resistance to chemotherapy-induced apoptosis in prostate cancer cells," *Cell Death & Disease*, vol. 1, article e105, 2010.

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Research Article

Identifying Novel Glioma-Associated Noncoding RNAs by Their Expression Profiles

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Long noncoding RNAs (lncRNAs) and microRNAs (miRNAs) play a significant role in cancer development as regulators of protein-coding genes. Their dysregulation was in some extent already associated with glioma, the most aggressive primary brain tumours in adults. The correct diagnosis and treatment selection due to high tumour heterogeneity might be difficult and inadequate, resulting in poor prognosis. Studies of expression patterns of noncoding RNAs (ncRNAs) could provide useful insight in glioma molecular development. We used the qPCR approach to screen and investigate the expression of lncRNAs that were previously deregulated in other cancer types. The study showed altered expression levels for numerous lncRNAs across histologically different glioma samples. Validation of few lncRNAs showed association of expression levels with histological subtype and/or malignancy grade. We also observed deregulated and subtype-distinctive expression for four lncRNA-associated miRNAs. Expression of few lncRNAs and miRNA was also associated with patients' survival, showing potential prognostic value. Several ncRNAs, some already related to glioma and some, to the best of our knowledge, investigated for the first time, might be of greater importance in glioma molecular development and progression. Finding the subtype-specific lncRNA and/or miRNA expression patterns may contribute additional information for a more objective classification.

1. Introduction

Long noncoding RNAs (lncRNAs) are a group of noncoding RNAs (ncRNAs), classified by the length longer than 200 bp ranging up to 100 kb, and do not appear to have coding potential [1, 2]. Based on their loci of origin, lncRNAs can be classified as intergenic/intronic, sense/antisense, bidirectional, or overlapping with protein-coding genes or other ncRNAs [3, 4]. Their lack of coding potential does not mean lack of function; their role in cancer development and progression was already confirmed, although mechanisms of their function are still not fully understood [5] and have yet to be investigated in detail. With progression in expression profiling, tens of functional examples have arisen that associate lncRNAs with diverse range of cellular processes [2, 3]. Mechanistically, various lncRNAs act as the cotranscriptional regulators of protein-coding genes [6] and are involved in various biological and developmental pathways, including the acquisition and maintenance of cell characteristics [7]. Certain lncRNAs were found to be closely associated with initiation, proliferation, invasion, and recurrence of glioma and may therefore be exploited for the purposes of subclassification, diagnosis, and prognosis [8]. Genetic and epigenetic changes that permit the survival of abnormal cells and formation of tumour mass often include genes that control critical biological processes and pathways, such as cell proliferation, adhesion, migration, and differentiation [9]. In tumour, deregulated cell proliferation is a critical event required for tumour to grow and invade, that is, for tumour progression [10]. Regulatory lncRNAs can intercede both tumour suppressor and oncogenic effects [3, 5, 11], and their aberrant expression emphasizes their role in variety of diseases [5, 12]. Altered expression of certain cancer type-specific lncRNAs can reflect disease progression [13] and

might serve as potential biomarkers [11, 12]. Another interesting and also the most characterized group of ncRNAs are microRNAs (miRNAs), small, ~22-nucleotide long ncRNAs, which negatively regulate the expression of mRNA by binding on the untranslated mRNA and preventing their translation [14, 15]. In addition to lncRNAs, miRNAs can also function as tumour-suppressors or oncogenes. They can be expressed as single genes, gene clusters, or from introns of protein-coding as well as nonprotein-coding genes such as lncRNAs [16].

lncRNAs are widely expressed in mammalian nervous system [7], and several were identified to be specifically linked with neuro-oncological disorders [4, 17], including tumours of central nervous system (CNS) [7]. Glioma are the most common form of primary brain tumours in adults, and despite the progress in therapy, the prognosis of patients, especially those with glioblastoma (GBM), remains ruthless [18]. The lack of effective drugs and lack of inefficient treatment are probably consequences of glioma's heterogeneous and invasive nature. Regarding the latter, the exact pathways of glioma biogenesis and progression are thus still in question [19]. Numerous studies underline the association of changed expression profiles of specific lncRNAs with tumour histological grade [1, 3]. Aberrant lncRNA expression affects the expression of the target gene, which in turn leads to cell dysfunction and disease progression [2, 20], and may have a direct role in driving the disease state [3]. Indeed, altered expression of lncRNAs and miRNAs has been correlated with development of various cancer types and they could also help elucidate the mechanisms underlying glioma malignant transformation [12, 13]. In addition, as some glioma subtypes are known to have distinct molecular features [21], expression analyses of coding and noncoding genes can also provide additional information to further distinguish them (classification biomarkers) [13]. Only a few years ago, the golden standard for glioma classification has based mainly on their morphological features, including cells of origin, degree of differentiation, and grade of anaplasia [21]. However, histological diagnoses of glioma tend to be very difficult since many cases are morphologically alike, partly due to the lack of specific (particularly molecular) distinguishable markers [13]. The number of protein-coding genes only differs little between organisms, and as seen, they are not sufficient to properly explain the human gene expression complexity. It is suggested that the expression complexity could be explained right with these noncoding but transcriptionally active genes [22]. Certain lncRNAs, such as *H19*, *MALAT*, and *linc-POU3F3*, have been shown to be involved in cancer progression, and also glioma of different malignancy grades show different patterns of lncRNA expression [22–24]. Also, expression of miRNAs shows biomarker potential, used as diagnostic support or for prognostic and therapeutic application [25, 26].

The purpose of this study was to examine the lncRNA expression profile of 90 lncRNAs, previously reported to be involved in various types of cancer (Human LncProfiler™ qPCR Array Kit (System Biosciences, CA, USA)), in glioma samples and to characterize potential glioma-related lncRNAs. Expression of several selected, differentially expressed lncRNAs was validated on a bigger number of biological replicates. We searched which of validated lncRNAs might encode, be coexpressed, or coregulated with miRNAs. Those miRNAs were also analysed for potential correlation with their host or coregulated lncRNAs. At last, we evaluated the prognostic value of aberrant ncRNA's expression.

Table 1: Clinicopathological characteristics of patients in the study.

Patients' demographics		
Number of patients	64	
Sex (female/male)	36/28 (1.3 : 1)	
Median age at diagnosis (years) (min.–max.)	51.8 (3.9–83.1 years)	
Number < 50 years	29	
Number > 50 years	35	
Glioma classification	*Glioma subtype*	*WHO grade*
(i) Astrocytoma (AC)	4 diffuse AC	WHO II
	6 anaplastic AC	WHO III
	4 secondary GBM	WHO IV
	31 primary GBM	
(ii) Oligodendroglioma (ODG)	14 anaplastic ODG	WHO III
(iii) Oligoastrocytoma (OAC)	5 anaplastic OAC	WHO III
Tumour location		
(i) Frontal or temporal	32	
(ii) Other regions	16 (16 na)	

na: not applicable.

2. Materials and Methods

2.1. Tissue Samples and Clinicopathological Data. The study was approved by the National Medical Ethics Committee of the Republic of Slovenia (115/5/14). Sixty-four tumours that were surgically removed from the patients between 2008 and 2012 were chosen for the study. We randomly chose 17 glioma tissue samples for initial expression profiling from our previous glioma data set, with respect to appropriate RNA yield and integrity values. Expression validation set further included 60 glioma tissue samples, of which there were also 13 samples from the initial profiling set.

Tumours were stabilized in RNA*later* (Applied Biosystems, USA) immediately after surgical biopsy and incubated at 4°C for 7 days. After incubation, samples were stored at −20°C until RNA extraction, as recommended by the manufacturer. All tumours were evaluated by a neuropathologist in order to assess glioma subtype and tumour grade (see Table 1). The tumour biopsies used in the study belonged to 36 female and 28 male patients (median age at diagnosis: 51.8 years ± SD 16.2). As the expression study was conducted in the year 2012, sample classification is based primarily on a neuropathologist's evaluation of morphological features and previous diagnosis if existed and only in some cases based on the status of p53 and 1p/19q codeletion. Samples of diffuse and anaplastic astrocytoma were united in one subgroup, that is, grade II + III astrocytoma, for simpler

comparison with other subtypes. For reference RNA, we used the FirstChoice Human Brain Reference Total RNA (cat. number 6050, Ambion, Invitrogen, USA) (further referred to as reference RNA), obtained from the brain tissue of 23 individuals without any signs of neurodegeneration.

2.2. RNA Extraction from Fresh Tissue. RNA from all the samples used in primary profiling set and validation set was extracted following the same isolation protocol. Total RNA from up to 30 mg of each tissue was isolated using TRIzol reagent (Invitrogen, USA), followed by purification using miRNeasy Mini Kit (Qiagen, Germany), according to the manufacturer's instructions. The yield was measured spectrophotometrically using NanoDrop-1000 (Thermo Scientific, USA), and the quality was evaluated on Bioanalyzer 2100 (Agilent Technologies, USA). After the RNA extraction and quality assessment, the samples with appropriate RNA yield and integrity values (concentration higher than 100 ng/μL and RIN > 5.5) were considered for expression profiling and further validation analyses.

2.3. LncRNA Expression Profiling Using Human LncProfiler qPCR Array Kit. We performed a primary expression profiling of 90 lncRNAs that have already been associated with different types of cancer (Human LncProfiler qPCR Array Kit (System Biosciences, CA, USA)). lncRNA profiling of these 90 lncRNAs was performed using qPCR amplification of cDNA from an initial set of 17 glioma samples of different histopathological characteristics and normal brain reference RNA (control).

First strand of cDNA was synthesized using Human LncProfiler qPCR Array Kit with oligo-dT and random hexamers, according to the manufacturer's instructions. All qPCR reactions were carried out in triplicates and included a negative control. Quantification was performed using the ABI Prism 7900 sequence detection system (Applied Biosystems, USA). Cycling conditions were as follows: 2 minutes at 50°C, 10 minutes at 95°C, and 40 cycles of 15 seconds at 95°C, and one minute at 60°C. The signal was collected at the endpoint of each cycle. A cohort of five reference genes was simultaneously analysed for the normalization process (*18S rRNA*, *RNU43*, *GAPDH*, *LAMIN A/C*, and *U6 snRNA*), and the optimum reference genes were identified using NormFinder algorithm [27]. Expression values of lncRNAs in glioma were compared to values of normal brain reference RNA, and results were statistically analysed.

2.4. Validation of Selected lncRNAs. For more reliable results, results need to be validated on a larger number of biological replicates. Upon statistical analysis, we selected 7 lncRNAs (*EGO-A*, *7SL*, *RNCR3*, *MEG3*, *HOTAIR*, *ZFAS1*, and *JPX*) for validation and to further investigate their differential expression on a cohort of 60 glioma samples and normal brain reference RNA (control). For the subsequent analyses of lncRNA expression, cDNA was generated with Protoscript M-MuLV Taq RT-PCR kit using random primers (New England Biolabs, UK) according to the manufacturer's instructions, except were otherwise indicated. Reverse transcription reactions were prepared in 10 μl master mix with 500 ng of total RNA. Expression analyses were validated using the TaqMan or SybrGreen-based qPCR technology, dependent on probes availability. All qPCR reactions were carried out on Rotor-Gene Q (Qiagen, Germany), and each sample was analysed in duplicate. Thermal conditions were applied as follows: initial denaturation at 95°C for 15 minutes and 40 cycles of denaturation at 95°C for 15 seconds and annealing at 60°C for 1 minute, and following amplification melting curves were acquired. The signal was collected at the endpoint of each cycle.

2.4.1. TaqMan-Based Technology. qPCR reactions for *MEG3*, *RNCR3*, *JPX*, and *ZFAS1* were prepared with lncRNA-specific TaqMan assay (*MEG3* Assay ID Hs01098508_mi; *RNCR3* Assay ID Hs01039195_gi; *JPX* Assay ID Hs03681129_m1, *ZFAS1* Assay ID Hs03300756_m1) (all Applied Biosystems, USA) and FastStart Essential DNA Probes Master Mix (Roche, Germany) or TaqMan Gene Expression Master Mix (Applied Biosystems, USA) in 10 μl reaction volume. Reference genes *GAPDH* and *18S rRNA* were analysed in both conditions.

2.4.2. SybrGreen-Based Technology. qPCR reactions for *EGO-A*, *7SL*, and *HOTAIR* were prepared with designed, lncRNA-specific primers (*EGO-A:* F – CTTCTCCTCCAGG CCATACC, R – CCATTGTGTAGCCCCG; *7SL:* F – CTGT AGTCCCAGCTACTCG, R – CCCGGGAGGTCACCAT ATT; and *HOTAIR:* F – CAGTGGGGAACTCTGACTCG, R – GTGCCTGGTGCTCTCTTACC) and Power Sybr®Green PCR Master Mix (Applied Biosystems, USA) in 10 μl reaction volume. *GAPDH* and *U6 snRNA* were used as reference genes.

2.5. Quantitative Real-Time PCR of miRNAs. miRNAs were analysed using either the miScript system (SybrGreen, Qiagen, Germany) or TaqMan-based technology (Applied Biosystems, USA) according to their corresponding lncRNAs. All the reagents were from Qiagen or Applied Biosystems, respectively, except where otherwise indicated. qPCR was carried out using the Rotor-Gene Q (Qiagen, Germany).

2.5.1. miScript System (Qiagen, Germany). All of the steps were performed according to manufacturer's protocol, except where otherwise indicated. miScript reverse transcription kit was used for RT in a 10 μl reaction master mix with 100 ng of total RNA. All of the qPCR reactions were performed in duplicate, and following amplification melting curves were acquired. MicroRNAs *miR-196a* and *miR-125b* were tested relatively to *RNU6B* and *SCARNA17* (*RNU6B*, *SCARNA17*, *SNORA73A*, *and RNU1B* were tested as reference genes). The signal was collected at the endpoint of every cycle.

2.5.2. TaqMan-Based Technology. All of the steps were performed according to manufacturer's protocol, except where otherwise indicated. *RNU6B*, *RNU48*, *RNU58A*, and *HY3* were tested as references genes. MicroRNAs *miR-124a* and *miR-770* were tested relatively to *RNU6B* and *RNU58A*. Briefly, the 10 μl RT reaction master mix was performed with 10 ng of total RNA sample. The cDNA was diluted 30-fold, and 4.5 μl was used for each 10 μl qPCR reaction. The qPCR

reactions were performed in duplicates, and the signal was collected at the endpoint of every cycle.

2.6. Computational and Statistical Analysis. Relative quantification of lncRNA and miRNA levels of the target gene was calculated using the $\Delta\Delta C_T$ and represents the difference between ΔC_T tumour RNA and ΔC_T reference RNA, normalized to expression of endogenous controls [28]. If $\Delta\Delta C_T$ was significantly (2σ) higher or lower than zero, the expression was considered to be significantly different. The calculation method was used for computational analyses for both lncRNA profiling and validation of differentially expressed lncRNA and miRNA.

Calculated $\Delta\Delta C_T$ values and the R programming language were used for unsupervised hierarchical clustering analysis, with Pearson correlation for distance metric. All statistical tests were performed using the IBM SPSS Statistics 20. software (IBM Corporation, New York, USA). Not all cases had all data available. Factor analysis was performed using the principal component extraction method and the direct oblimin as the rotation method. We used the Mann–Whitney 2-independent samples test to determine which of the 90 lncRNAs are differentially expressed, and results are graphically presented as log values on the heat map. For determining significant differences in expression (using $\Delta\Delta C_T$) of lncRNAs and miRNAs between glioma subtypes, we used the Kruskal-Wallis k-independent test (multiple comparisons). Mann–Whitney 2-independent samples test was performed to cross test expression differences between two subtypes. The expression differences were considered statistically significant when the differences in tested groups reached or were below $p \leq 0.05$. The relative quantification of lncRNA and miRNA levels is graphically presented as average fold change ($2^{-\Delta\Delta CT}$). The Pearson's correlation coefficient was used to define expression relationships and Kaplan–Meier estimate for constructing survival curves (analysed by log-rank test). To assess the relative risk for each factor, we performed the univariate and multivariate Cox regression analyses using R language. We used the $\Delta\Delta C_T$ values, and two-sided p value < 0.05 was regarded as significant.

3. Results

3.1. Profiling Shows Numerous lncRNAs with Altered Expression. lncRNA profiling revealed changes in expression levels for numerous lncRNA (74/90 lncRNAs analysed) in the primary set of 17 glioma samples compared to normal brain reference RNA (pooled RNA samples from 23 donors, commercially available). Unsupervised hierarchical clustering (Figure 1) upon similarity of gene expression profiles between tumour samples showed roughly three molecular groups; however, gene expression-based groups did not coincide with samples' histological subtypes, suggesting the heterogeneous lncRNA expression background of morphologically similar tumours. Factor analysis, with the principal component extraction method based on 74 gene expression profiles, showed two principle components, which together account for 60.8% of variance in gene expression (Figure 2). The component 1, accountable for 46.8% of variance, separates samples in two main groups, which remain more or less the same with component 2 (accountable for 13.95% of variance). Sample grouping again showing differences in lncRNA expression profile vary between glioma subtype and to some extent coincided with unsupervised hierarchical clustering (comparing Figures 1 and 2).

3.2. Expression Validation of Seven lncRNAs. Among differentially expressed lncRNAs, we further validated seven lncRNAs (*7SL*, *EGO-A*, *HOTAIR*, *JPX*, *MEG3*, *RNCR3*, and *ZFAS1*) on a bigger cohort of glioma samples of different WHO malignancy grades and histopathological subtypes. Expression of lncRNAs *MEG3*, *JPX*, *RNCR3*, and *ZFAS1* significantly differed between low and high malignancy grade (Mann–Whitney test; $\Delta\Delta C_T$ values) (Figure 3). *MEG3*, *JPX*, *RNCR3*, and *ZFAS1* showed significant decrease with tumour malignant progression.

Next, we grouped the samples upon their histological subtype into five groups and compared lncRNA's expression between all five using the Kruskal-Wallis test ($\Delta\Delta C_T$ values). lncRNA expression patterns for corresponding glioma subtype are graphically presented in Figure 4. We observed significant differences across all samples for each of the lncRNAs. Expression of *HOTAIR* and *ZFAS1* was found overexpressed, and *MEG3*, *JPX*, and *RNCR3* were substantially underexpressed. *EGO-A* showed overexpression in all subtypes, except in secondary GBMs. Expression of *7SL* was found near normal level in oligodendroglioma and oligoastrocytoma, but underexpressed in astrocytoma, primary, and secondary GBMs.

3.3. There Are Distinctive lncRNA Expression Patterns between Glioma Subtypes. Kruskal-Wallis test does not tell between which subgroups the differences in expression significantly differ. Using the Mann–Whitney test, we compared the $\Delta\Delta C_T$ values of specific lncRNA of histologically determined subtypes among each other to establish subtype expression (Table 2) (for all graphic comparisons see graphs on Figure 4). *HOTAIR* exhibited much higher expression in oligodendroglioma and oligoastrocytoma compared to astrocytic tumours. *ZFAS1* was overexpressed in oligodendroglioma and oligoastrocytoma when cross tested with primary GBMs, which showed near normal expression levels. *MEG3* and *JPX* expression showed especially lower levels in primary GBMs compared to other astrocytic glioma. We also observed similar pattern for *RNCR3* with much lower expression levels in GBMs. And as observed before, expression of *MEG3*, *JPX*, and *RNCR3* decreases with malignancy grade (Figure 3). Significant differences were also found for *7SL* expression between oligodendroglioma and astrocytoma. Especially different pattern was observed for *EGO-A* in secondary GBMs.

3.4. Changed Expression of lncRNA-Related miRNA. In the subset of significantly changed lncRNAs we found that four are also encoding miRNAs. *7SL* was recently suggested to be regulated by *miR-125b* [29]. Wei et al. also suggest *miR-125b* to be a potential biomarker of glioma [30]. *HOTAIR* has been

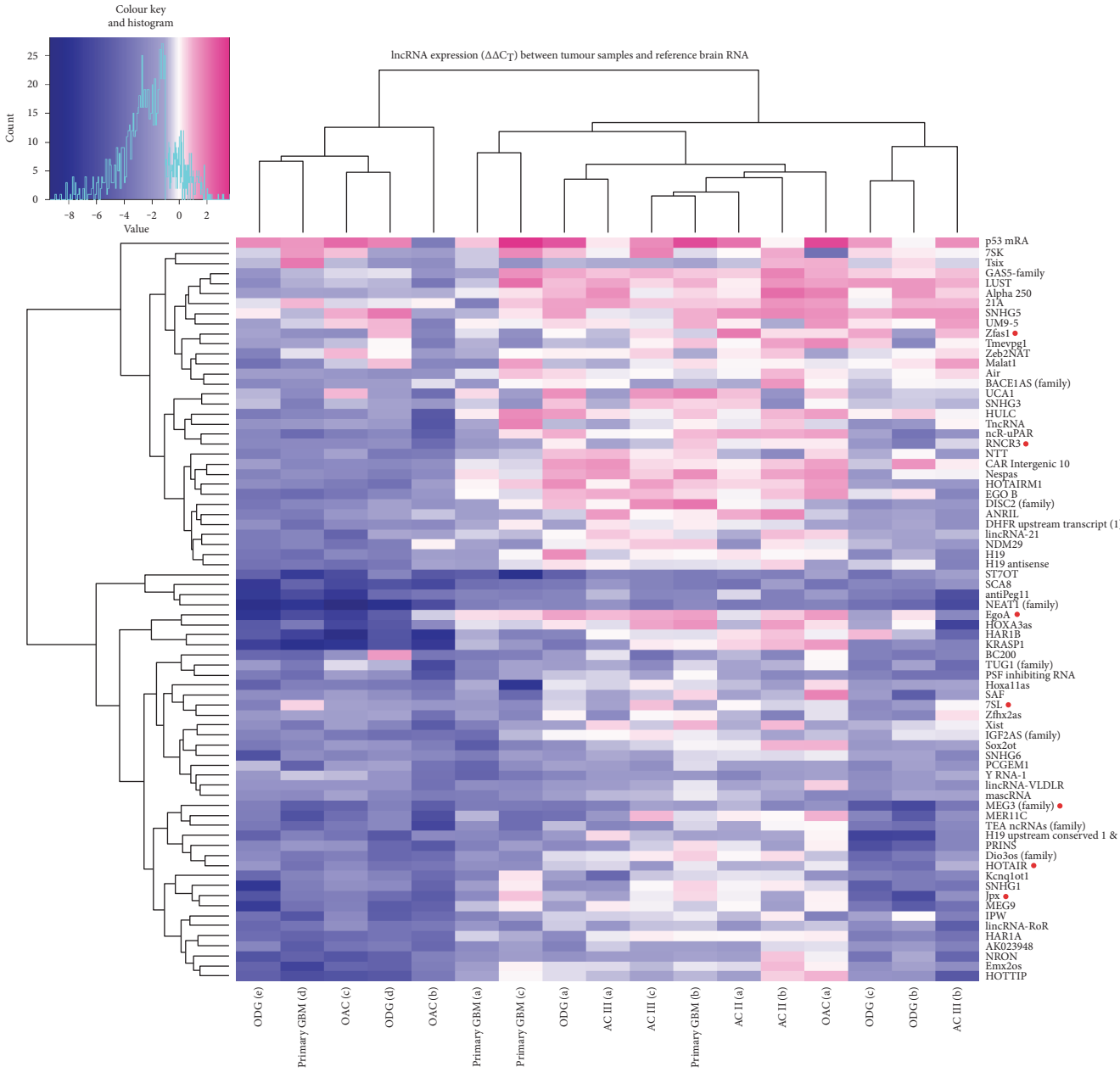

FIGURE 1: Heat map of lncRNA profiling analyses of glioma samples. The figure represents $\Delta\Delta C_T$ values of differentially expressed lncRNAs in glioma samples compared to human brain reference RNA. Data are presented on a colour scale where shades of blue represent decreased expression and pink as increased expression, with $\Delta\Delta C_T$ cut-off values set at −1 and 1. On the top of the figure is presented unsupervised Pearson's hierarchical clustering of samples. AC: astrocytoma of WHO grade II or III; OAC: oligoastrocytoma; ODG: oligodendroglioma. Genes denoted by red dot were selected for further qPCR validation and analysis.

found to be coregulated with *miR-196a* [31], and genes for *RNCR3* and *MEG3* are acting as host genes for *miR-124a* and *miR-770* [32], respectively. We determined the expression levels of these four miRNAs and performed a lncRNA-miRNA linear correlation analysis to determine their possible relationship.

Expression levels of *miR-124a* and *miR-196a* significantly differ between the WHO malignancy grades (Mann–Whitney test; $\Delta\Delta C_T$ values) (Figure 5). We observed significant differences in expression of *miR-770* among all glioma subtypes ($p < 0.001$). It has been decreased in all samples of astrocytoma, oligoastrocytoma, and oligodendroglioma and in majority of GBMs (expression varied). Expression significantly changed in oligodendroglioma and oligoastrocytoma compared to astrocytic subtypes (Figure 6(a)). *miR-196a* was also decreased in all groups ($p = 0.001$) and was significantly lower in oligodendroglioma, astrocytoma, and oligoastrocytoma compared to primary GBMs (2- to 4-fold) and secondary GBMs (up to 10-fold). In a proportion (~36%) of primary GBMs, *miR-196a* was slightly overexpressed (Figure 6(a)). For *miR-124a*, expression was decreased in all glioma subtypes when compared to reference RNA, but there was no significant

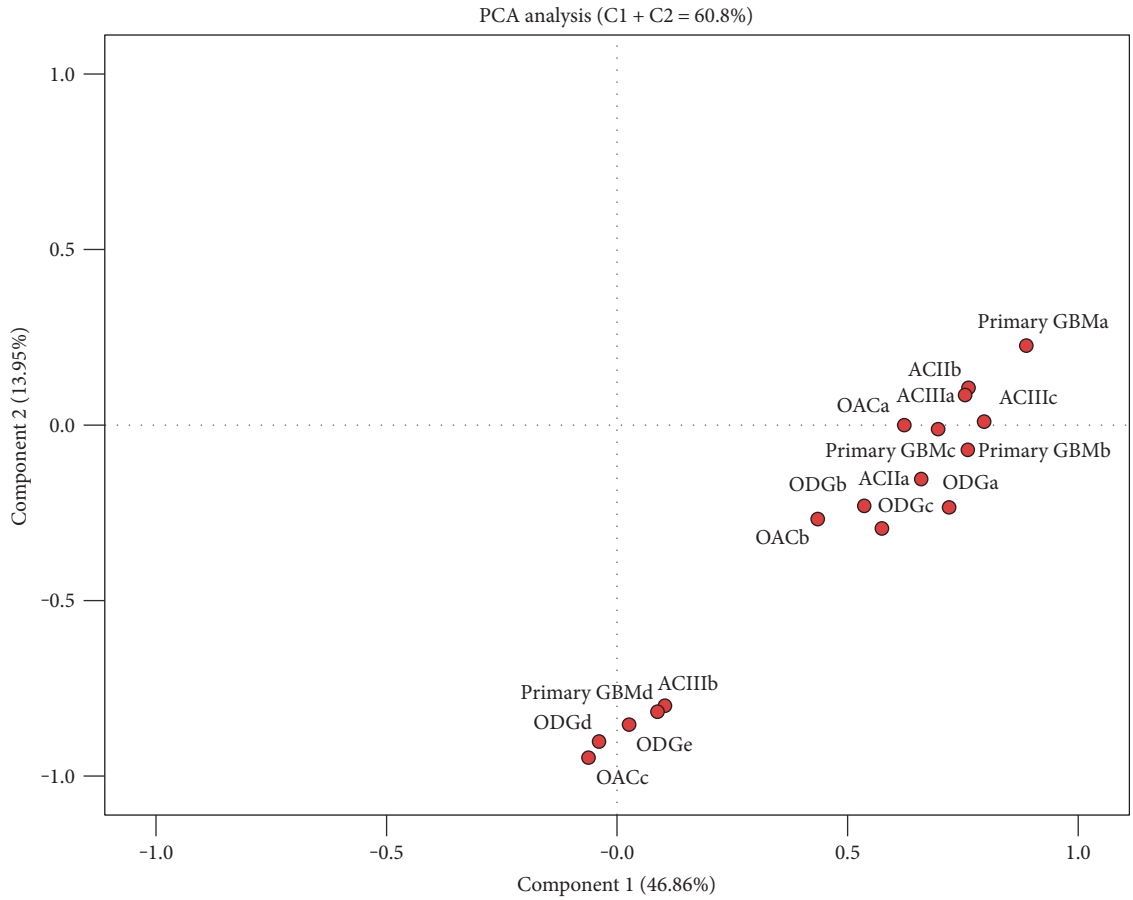

Figure 2: Principal component analysis of individual samples, generated with expression of 74 differentially expressed lncRNAs. AC: astrocytoma of grade II or III; GBMs: glioblastoma; ODG: oligodendroglioma; OAC: oligoastrocytoma. Letters at the end mean a consecutive sample.

change between different glioma subtypes. Also, *miR-125b* showed underexpression, except in secondary GBMs, for which we observed significant change comparing to other subtypes ($p = 0.017$) (Figure 6(a)). When comparing expression values of miRNAs between two different glioma subtypes, all miRNAs were shown to be differentially expressed between at least two glioma subtypes, except *miR-124a*, which did not reach statistically significant difference in expression between any two groups of samples (Figure 6(a)).

We observed moderate negative linear correlation ($r_s = -0.373$, $p = 0.005$) between expression of *miR-770* and *MEG3*, and positive correlation ($r_s = 0.291$, $p = 0.028$) between expression of *miR-124a* and *RNCR3* in all glioma samples. Both lncRNAs act as miRNA host genes. *miR-125b* and *7SL* and *miR-196a* and *HOTAIR* did not show significant correlation (Figure 6(b)). However, the lncRNA-miRNA relationship results provided are only suggestive as functional studies are required for stronger conclusion.

3.5. ncRNA's Expression Is Associated with Patients Age at Diagnosis and Survival Time. Expression of certain lncRNAs seems to be associated with patient's age at the time of diagnosis as correlation analysis showed moderate negative association for *MEG3* ($r_s = -0.336$, $p = 0.009$, $R^2 = 0.113$), *ZFAS1* ($r_s = -0.368$, $p = 0.004$, $R^2 = 0.164$), and *RNCR3* ($r_s = -0.431$, $p = 0.001$, $R^2 = 0.186$).

Also, *miR-196a* showed moderate association with patient's age at time of diagnosis ($r_s = 0.290$, $p = 0.039$).

The Kaplan-Meier estimate showed better survival for patients who were younger than 50 years at the time of diagnosis (Pearson's correlation $r_s = -0.482$, $p < 0.001$), but no difference regarding the sex or tumour location (Figure 7). Also, survival significantly differs between astrocytoma, primary GBMs, and oligodendroglioma. Because of a low sample number of oligoastrocytoma and secondary GBMs, we did not include them in the survival plot regarding the subtype ($p = 0.001$) (Figure 7). For determining possible association of gene's differential expression and survival, expression values of ncRNAs analysed were classified as low or high based upon individual ΔΔCt mean expression value. Kaplan-Meier plots and further analysis of survival differences (log-rank analysis) showed significant differences in survival for expression levels of *MEG3*, *ZFAS1*, *RNCR3*, *miR-770*, *miR-125b*, and *miR-196a* (Figure 8). These ncRNAs also showed significant association with survival when considering only the expression of astrocytoma, primary GBMs, and oligodendroglioma (data not shown). To identify the potential

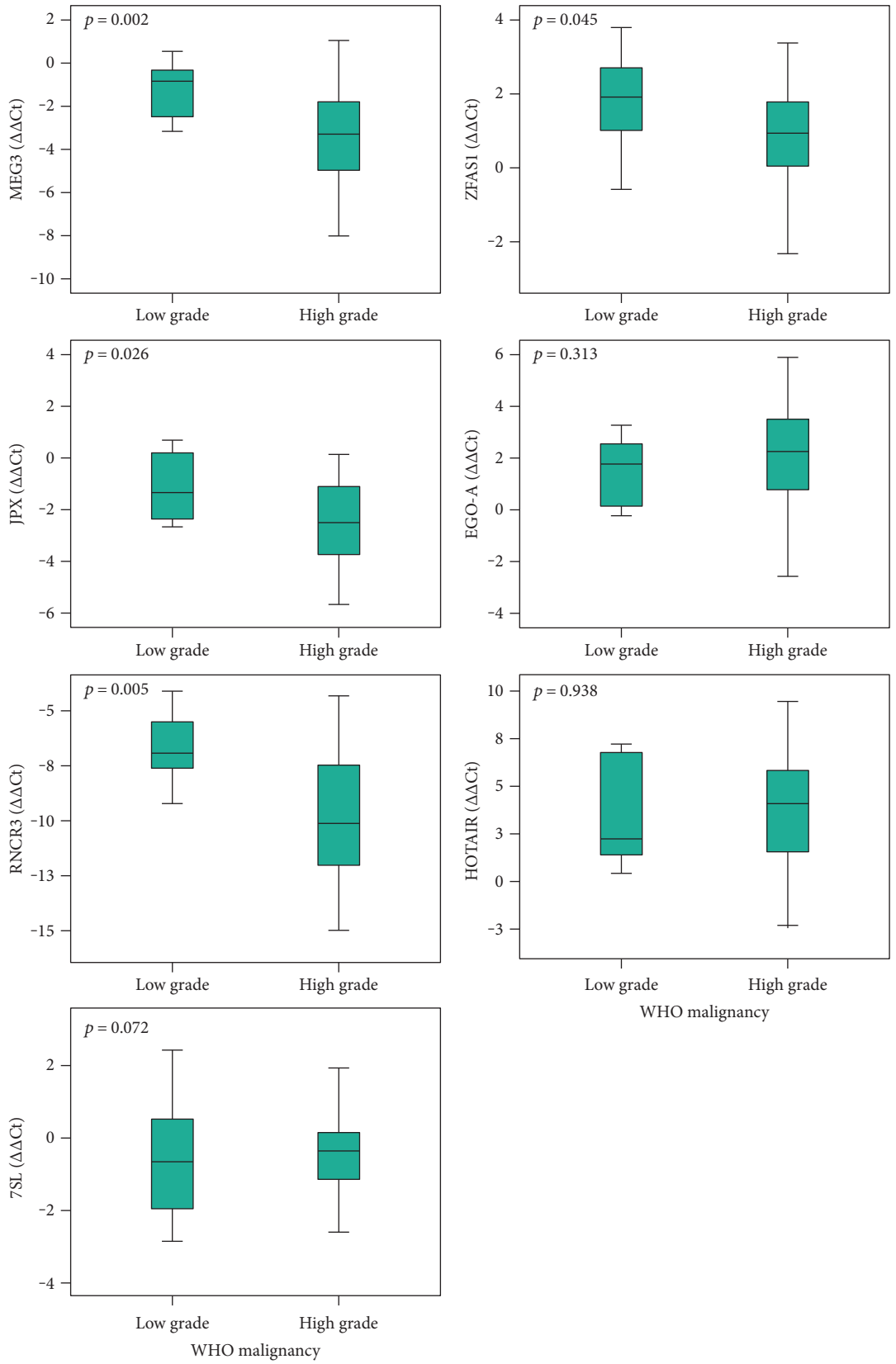

Figure 3: qPCR results of lncRNA gene expression regarding the WHO malignancy grade. Expression analyses of 7 lncRNAs, previously determined as differentially expressed, between low and high malignancy grade. Data are presented as $\Delta\Delta C_T$ box plots ± standard error.

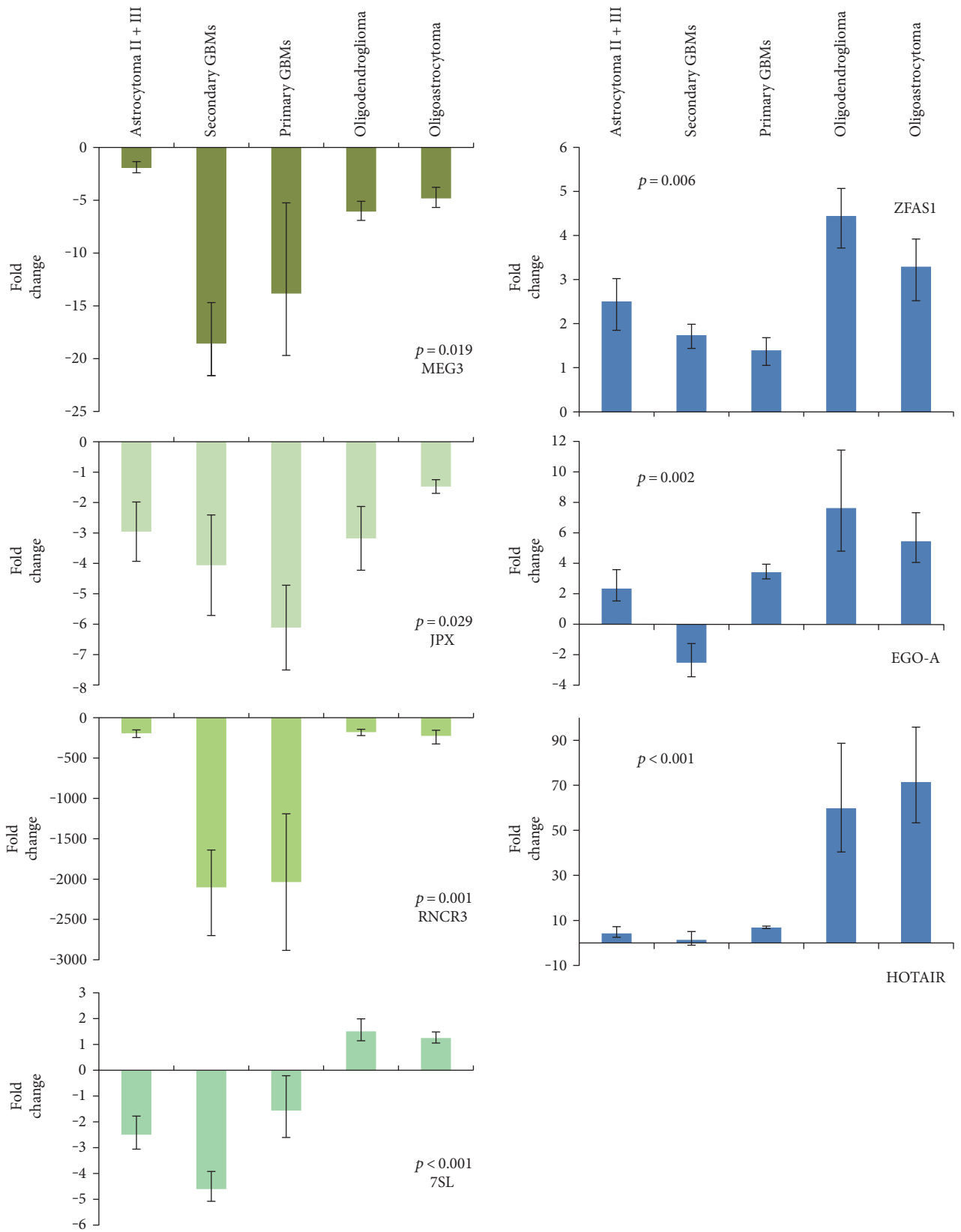

Figure 4: qPCR results of lncRNA gene expression in glioma subtypes. Expression analyses of glioma samples and human brain reference RNA for a subset of seven lncRNAs previously determined as differentially expressed. Data are presented as mean fold change (FC) ± standard FC error. p shows statistical significance for Kruskal-Wallis test of expression differences among all five subgroups (threshold set at $p < 0.05$).

Table 2: Significantly different expression of lncRNA between glioma subtypes (represented as p value).

Mann–Whitney 2-independent test	Secondary glioblastoma	Primary glioblastoma	Anaplastic oligodendroglioma	Anaplastic oligoastrocytoma
Grade II + III astrocytoma	*EGO-A* 0.016	*RNCR3* 0.001 *MEG3* 0.001	*HOTAIR* 0.004 *EGO-A* 0.010 *7SL* 0.005	*EGO-A* 0.024 *7SL* 0.038
	Secondary glioblastoma	*HOTAIR* 0.039 *EGO-A* 0.006 *7SL* 0.011	*HOTAIR* 0.003 *EGO-A* 0.004 *7SL* 0.003	*HOTAIR* 0.034 *EGO-A* 0.021 *7SL* 0.021
		Primary glioblastoma	*HOTAIR* 0.001 *RNCR3* 0.001 *ZFAS1* 0.001 *JPX* 0.019 *7SL* < 0.001	*HOTAIR* 0.041 *RNCR3* 0.018 *ZFAS1* 0.036 *JPX* 0.014
			Anaplastic oligodendroglioma	/

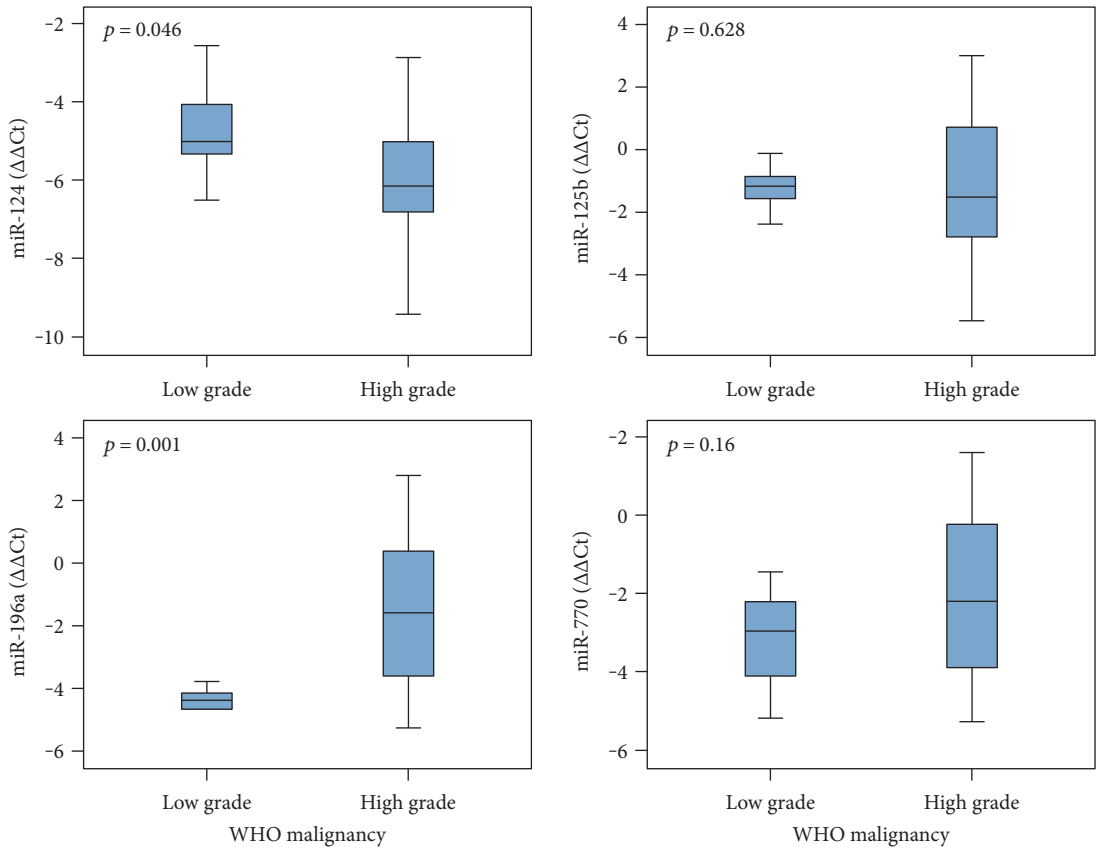

Figure 5: miRNA expression levels regarding the WHO malignancy grade. Expression analyses of four lncRNA-associated miRNAs between low and high malignancy grade glioma. Data are presented as $\Delta\Delta C_T$ box plots ± standard error.

prognostic value of ncRNA expression, we used the univariate Cox proportional hazards regression analysis. We found significant association of survival with patient's age at diagnosis and expression of *MEG3*, *ZFAS1*, *RNCR3*, *miR-125b*, *miR-196a*, and *miR-770*, respectively. For the multivariate analysis, we took into consideration only the parameters with $p < 0.05$ in the univariate analysis. Results showed patient's age at diagnosis and expression of *ZFAS1*, *RNCR3*, *miR-125b*, and *miR-196a* as potential independent prognostic variables.

4. Discussion

Although many of lncRNAs known are involved in a variety of cancers, only a few have been associated with a particular cancer type [12]. lncRNA expression profiling revealed

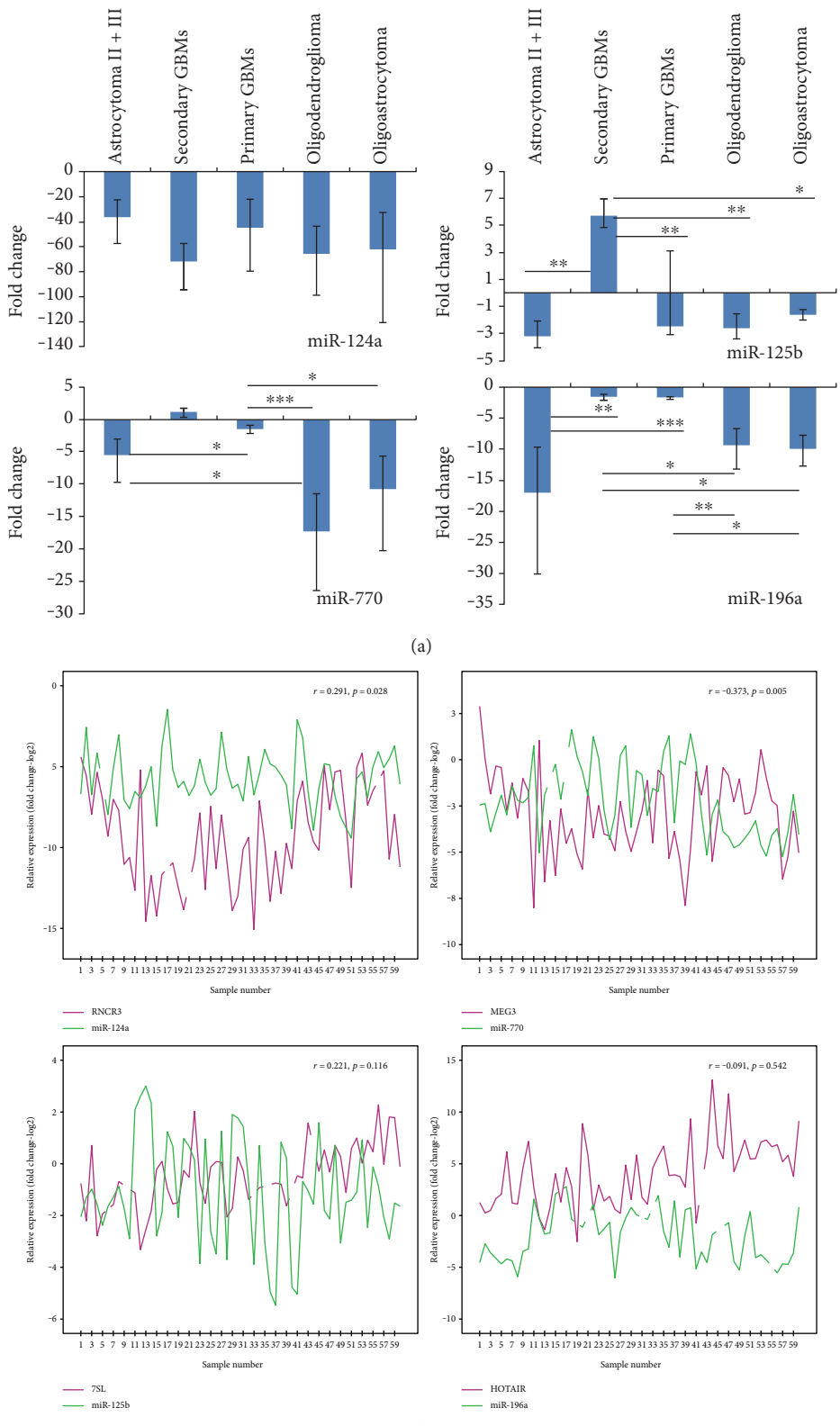

Figure 6: Expression results of miRNAs related to differentially expressed lncRNAs in glioma subtypes. (a) Expression analyses of miRNA, found to be associated with lncRNAs analysed, on the same cohort of sample as for lncRNAs as well as on human brain reference RNA. Data are presented as mean fold change (FC) ± standard FC error. Bars with asterisks represent significance for in-between-group comparison ($^{*}0.01 \leq p < 0.05$; $^{**}0.001 \leq p < 0.01$; $^{***}p < 0.001$). (b) Correlation relationship of expression levels in individual sample for miRNA and its related lncRNA. Letter r represents the magnitude of correlation and p the significance value.

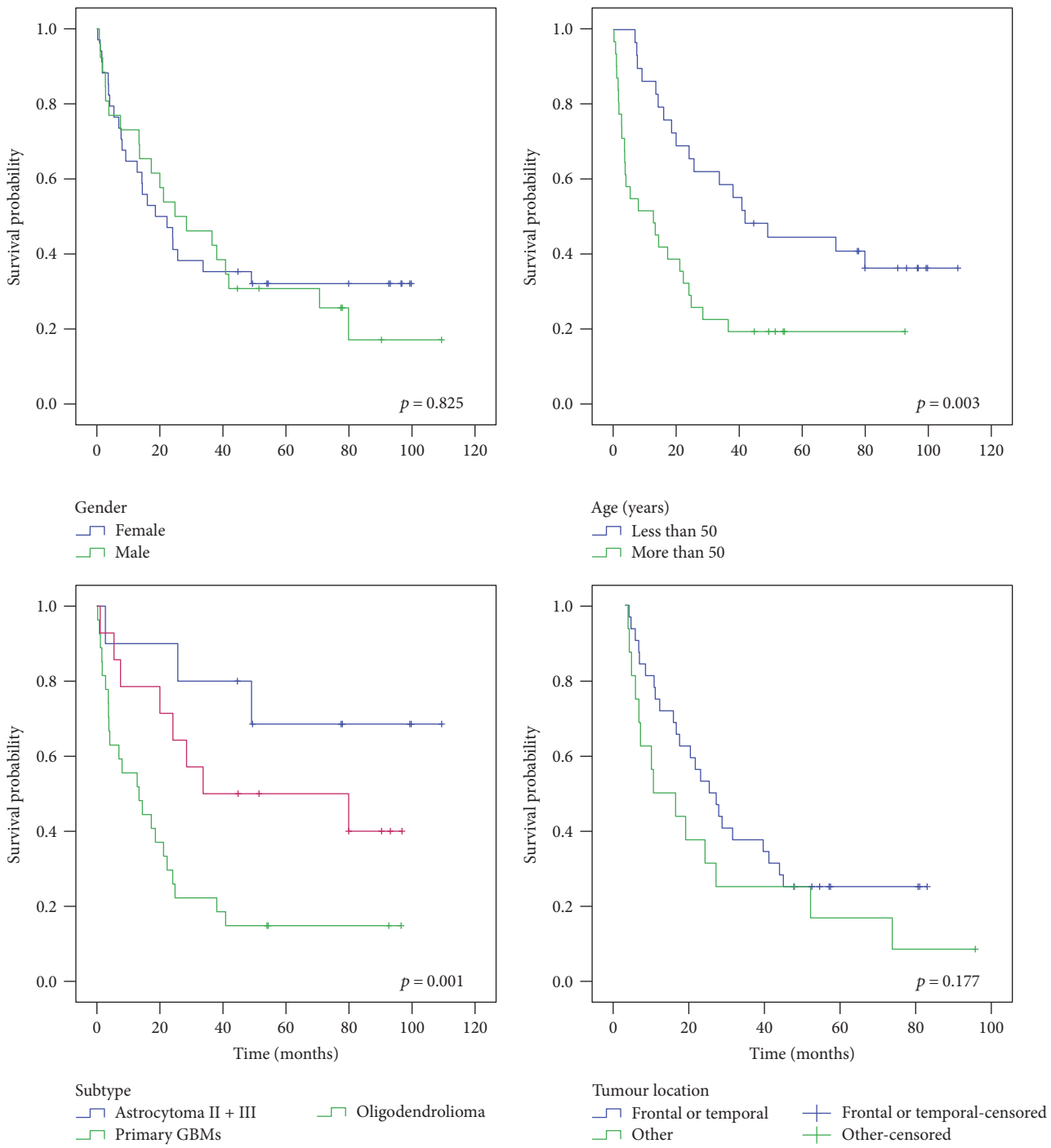

Figure 7: Kaplan-Meier survival curve analysis shows poor prognosis for patients older than 50 years at time of diagnosis and with diagnosed GBM.

numerous lncRNA to be widely expressed in glioma, thus once again acknowledging the complexity of the brain and brain tumours and interweaving of different molecular levels. Tumour sample clustering based on expressional profile of 74 lncRNAs did not coincide with histopathological subtype, which suggests heterogeneous global expression of lncRNAs. However, determining glioma lncRNA expression profiles may be an especially helpful step in studying glioma expression network regarding lncRNAs' crucial regulatory roles [33]. And furthermore, expression patterns of specific lncRNAs or smaller gene-sets could potentially help us refine glioma subclassification, especially since one of the biggest problems in diagnosis is the morphological similarity of tumours. Indeed, Zhang et al. found novel tumour-related lncRNAs to be associated with malignancy grade and histological differentiation [13], and also our results indicate the existence of measurable differences of individual lncRNA or miRNA expression in glioma, also between histopathological

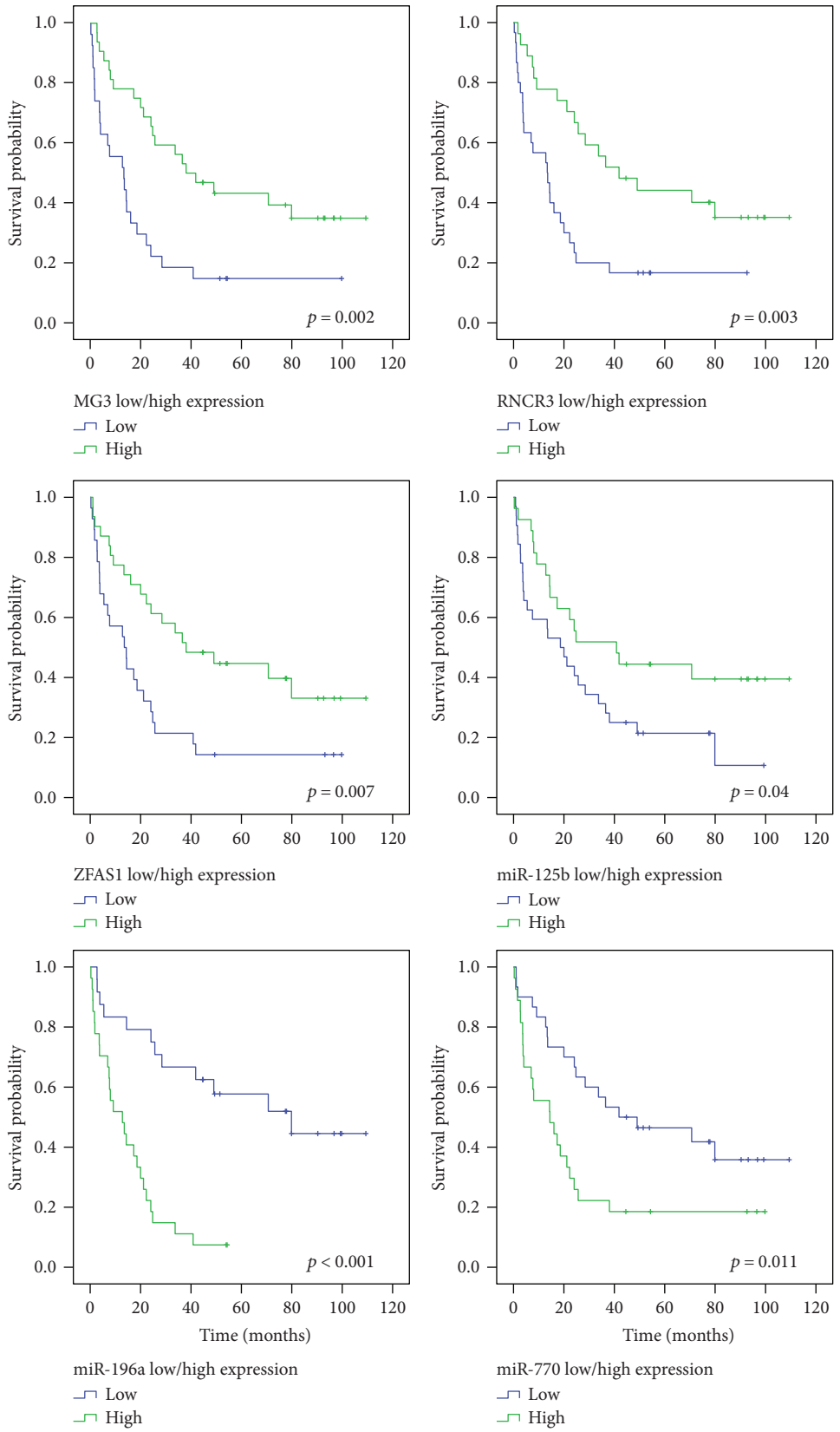

Figure 8: Expression of lncRNAs and miRNAs shows prognostic significance. Expression of individual sample was classified either low or high regarding the gene's ΔΔCt mean expression value.

subtypes and malignancy grades. Last but not the least, numerous lncRNAs show prognostic value in different human cancer types, including glioma [34–38]. Just recently, but in contrast to our findings, Gao et al. found elevated expression of *ZFAS1* in glioma as an independent, unfavourable prognostic factor [39]. Similarly, increased expression of *NEAT1* [40], *HOTAIR* [41], and *MALAT1* [23, 38] was also found associated with poor prognosis in glioma. Zhang et al. explored a six-lncRNA signature as a set of prognostic genes in glioma—*PART1*, *MGC21881*, *MIAT*, *GAS5*, and *PAR5* were correlated with prolonged survival and *KIAA0495* in association with the poorer [34].

Among seven validated lncRNAs, there is already a considerable research and data for roles of *HOTAIR* and *MEG3* in various cancer types [31, 42–52]. And just recently, Gao et al. reported *ZFAS1*'s oncogenic potential in glioma tumours [39]. lncRNAs can exhibit oncogenic or tumour suppressive functions [11]. Regarding processes of tumour growth, all three were found to be involved in cell proliferation in glioma [39, 53–55] and various other cancer types [56–61]. As an oncogenic lncRNA, *HOTAIR* is involved in epigenetic gene silencing, cell growth, and progression of various cancers [42–44]. A number of studies show that increased expression of *HOTAIR* enhances cell proliferation and invasion of cancer cells and has been associated with tumour progression and prognosis [45, 55, 56, 59, 62]. Overall high expression of *HOTAIR* in our glioma samples indicates its association also with glioma development and was already found essential for glioblastoma proliferation [55, 62, 63]. Expression of *HOTAIR* has been found to differentiate between astrocytoma, oligodendroglioma, and/or oligoastrocytoma, suggesting its use as a potential biomarker in distinguishing morphologically similar cases [41].

On the contrary, *MEG3* expression has been strongly decreased in glioma and its expression decreases with malignancy grade, thus acting as a tumour suppressor and contributing to glioma progression [18, 64]. As a putative tumour suppressor, it inhibits cell proliferation/DNA synthesis by stimulating the expression of tumour suppressor p53 [65] and modulating the binding of p53 on the promoter of its target genes [66]. On the other hand, *MEG3* is also able to inhibit cell proliferation and promote cell apoptosis in the p53-independent manner [64]. In line with the latter, its decreased levels are also associated with poorer survival. Overexpression of *MEG3* in human glioma cell lines inhibits cell proliferation and promotes cell apoptosis [18, 64].

Also found to be involved in p53-dependent cell cycle control is *ZFAS1* [54], an antisense lncRNA localized at the 5′ end of the protein-coding gene *ZNFX1* [67] that is widely expressed in numerous tissues, including the brain (http://www.proteinatlas.org). In their research of *ZFAS1* in glioma tissues and cell lines, Lv et al. [68] and Gao et al. [39] found its increased expression correlates with tumour stage and poor survival, matching with our results. Significantly increased *ZFAS1* expression indicates its oncogenic function [54]—its silencing decreases cell's proliferation through G1 cell cycle arrest [68]. Moreover, by regulating the epithelial-mesenchymal transition (EMT) and Notch signalling pathway, it could contribute to glioma progression [39]. In addition to lncRNA's oncogenic or tumour suppressive effect, one can also exhibit both effects [3, 5, 11]. *ZFAS1* was found downregulated in breast carcinoma, which suggests *ZFAS1* also as a possible tumour suppressor [67].

On the contrary to abovementioned lncRNAs, only little or none is known about the expression and function of lncRNAs *EGO-A*, *RNCR3*, *JPX*, and *7SL* in glioma. Abdelmohsen et al. reported *7SL* to be highly expressed in various cancer tissues and by repressing *p53* mRNA translation consequently promoting cancer cell growth [69]. *7SL* is a small RNA component of cytoplasmic SRP (signal recognition particle) complex, a guide that directs nascent secretory proteins towards the endoplasmic reticulum and is necessary for synthesis of normal, active proteins. It is essential for translocation across the membrane of endoplasmic reticulum [70], and its dysregulation may possibly result in impaired protein synthesis machinery. Thus, it is not surprising that *7SL* is ubiquitously expressed in numerous tissues, including the brain [71]. Our study showed decreased *7SL* levels in astrocytic tumours and increased levels in oligodendroglioma and oligoastrocytoma. These expression differences between oligodendroglioma and astrocytoma may be an additional genetic parameter for distinguishing these subtypes among each other, especially since expression is near normal levels in oligodendroglioma (and oligoastrocytoma).

Expression levels of *EGO-A* (eosinophil granule ontogeny isoform A) were significantly increased in all glioma subtypes, with the exception of secondary GBMs, which can be probably attributed to the small sample number. It is worth mentioning that secondary GBMs are relatively rare, partly because precursor low-grade or anaplastic astrocytoma develop at younger age than other GBMs, and some patients succumb before the disease progresses, and partly because they are mistakenly classified as primary GBMs [72]. *EGO-A* function is not yet known [73], but its potential role in glioma biogenesis may be implied by the chromosomal location, since its host gene *ITPR1* (inositol triphosphate receptor type 1) is encoded in close proximity to *EGR-1* (early growth response 1), a transcriptional regulator of genes required for induction of mitosis, cell differentiation, and growth [74]. Expression of *EGR-1* gene in glioma cells is induced by overexpression of *EGFR* and *PDGFR* genes, thus suggesting *EGR-1* as a connection of growth factor stimulation with gene expression changes [74]. Association of *EGR-1* gene with expression of growth factor might also explain the differences in expression level of *EGO-A* between the two GBM subtypes (small sample number aside), since *EGFR* overexpression is typical for primary GBMs, but is rare in secondary GBMs [72]. However, whether the correlation between expression levels of *EGR-1* and *EGO-A* loci exists is yet to be determined. Xu et al. found expression of *EGO* transcript to be downregulated in breast cancer and to play an important role in progression of breast cancer and prognosis, thus serving as potential prognostic target [75].

RNCR3 (retinal noncoding RNA 3) is highly expressed in the brain, yet we have not found any reported studies of its expression in glioma, and to date very little is known about its biological function. Especially, it was identified as

a precursor of the *miR-124a*, the most abundant miRNA in the vertebrate CNS and necessary for normal brain development [76]. Expression level of *miR-124a* was significantly decreased in anaplastic astrocytoma and GBMs, when compared to normal brain tissue [77], and also in oligodendroglioma [78]. We found substantially decreased expression of *miR-124a* and *RNCR3* in primary and secondary GBMs and a positive correlation between expression of these two ncRNAs. Since the *RNCR3* is a precursor for *miR-124a*, decreased expression of *miR-124a* could be a possible consequence of reduced *RNCR3* expression. Low expression of *RNCR3* also indicates lower survival probability. Regarding the findings, we can infer that *RNCR3* and its encoded *miR-124a* may be implicated in the development and progression of glioma. *miR-124a* is the most extensively investigated miRNAs in glioma cell proliferation. In the earliest study, it has been shown that upregulation of miR-124a induces glioblastoma cell cycle arrest. These results suggested that targeted delivery of *miR-124* to glioblastoma tumour cells may be therapeutically efficacious for the treatment of this disease [77]. Fowler et al. [79] found an ectopic expression of *miR-124a* significantly inhibiting GBM migration and invasion, which once more supports its role in glioma progression. Moreover, restoration of *miR-124a* could inhibit glioma cell proliferation and invasion through *miR-124a* blocking the expression of the *IQGAP1* gene and downstream β-catenin and cyclin D1 [80] and *PIM1* in astrocytoma cancer cells [81]. Additionally, transfection with *miR-124* inhibitor rescued the proliferative ability of human glioma cells. Results demonstrated that miR-124 is an important downstream target gene of Hedgehog signalling and that *glioma-associated oncogene-miR-124-AURKA* axis is essential for the proliferation and growth of human glioma cells [82].

Expression of miR-770 showed decreased levels in lower malignancy grades and a better survival probability. The expression was in an inverse correlation to its host gene, that is, MEG3, and regarding the findings of miR-770 being a downstream transcriptional target of Wnt/β-signalling pathway [83], it is reasonable to propose for MEG3 and miR-770 possibly having different mechanisms regulating their expression. There are only few investigations performed about the role of *miR-770* in carcinogenesis; moreover, only one study is investigating its role in proliferation. By acting as a sponge for *miR-770*, leading to its downregulation, lncRNA *PCGEM1* stimulates proliferation of osteoarthritic synoviocytes [84]. Whether or not this is also the case in glioma cells is yet to be investigated. Analyses of miRNA-lncRNA-mRNA interactions using their expression profiles alone are not enough to further understand the potential relationships between different RNA molecules [85]. Differences in lncRNA and its associated miRNAs' expression can also be the consequence of different stability of the transcript, splicing, promoter methylation, or miRNA's maturation [26].

miR-196a is concurrently upregulated with *HOTAIR* in gastrointestinal stromal tumours (GISTs) [31], but was downregulated in our glioma subtypes, with no association to *HOTAIR*. It has been shown that *miR-196a* is expressed in lower levels in oligodendrogliomas, astrocytomas, and oligoastrocytomas compared to glioblastoma, similar to our results [86]. *miR-196a* expression seems to be highly associated with patient's survival as its higher expression indicates poorer prognosis and shorter survival time, coinciding with HOTAIR overexpression survival prediction and their suggested coregulated expression [41]. Investigating the oncogenic effects of *miR-196a in vivo* and *in vitro* revealed that it induces and promotes proliferation and suppresses apoptosis through inhibition of the IκBα [87].

We were not able to detect any significant correlation between expression patterns of *miR-125b* and *7SL*, although there is an inverse overall expression in oligoastrocytoma, oligodendroglioma, and secondary GBMs. *miR-125b* has already been shown to be upregulated in primary GBMs (but downregulated in GBM cell lines) [88] and in oligodendroglioma [78]. Its overexpression promotes glioma cell proliferation and inhibits cell apoptosis, thus supporting the suggested oncogenic role [89, 90].

5. Conclusions

Studies of lncRNAs and miRNAs over the past decade increasingly reveal the importance of ncRNAs in cancer development and progression. All in all, our results corroborate the involvement of noncoding RNAs in the complex nature of primary brain tumours. We show that several lncRNAs, some already related to glioma and some investigated for the first time, could importantly contribute to glioma development. Expression level of several lncRNAs and certain lncRNA-associated miRNAs significantly changes between the analysed glioma subtypes and malignancy grades. Moreover, their possible correlation with miRNAs suggests a complex interplay of lncRNA-miRNA in regulating gene expression in glioma. Observing high expressional variability of the results opens numerous further questions and presents the foundation for further research of noncoding RNA implication in glioma formation and development, which will conceivably provide new glioma-specific biomarkers and targets for treatment of the disease. Although our research does not show/determine the exact mechanism of ncRNAs in tumour development, it does show that the analyses of expressional screening represent guidelines for further research and are efficient in the search of new cancer-related genes. It has been already accepted that lncRNAs have an immense role in glioma pathophysiology, but the question is which lncRNAs are implicated in malignant transformation and how they contribute to each level of tumorigenesis.

Ethical Approval

The study was approved by the Slovene National Ethics Committee and was therefore performed in accordance with the ethical standards, laid down in the 1964 Declaration of Helsinki and its later amendments. Informed consent of the patient for conducting the research was not necessary.

Disclosure

The funding organization had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflicts of Interest

All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers and bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements) or nonfinancial interest (such as personal or professional relationships, affiliations, knowledge, or beliefs) in the subject matter or materials discussed in this manuscript.

Authors' Contributions

Alenka Matjašič, Mojca Tajnik, Emanuela Boštjančič, and Damjan Glavač contributed in the conception and design of the study. Boštjan Matos and Mara Popović performed the acquisition and diagnosis of glioma specimens. Alenka Matjašič, Emanuela Boštjančič, and Mojca Tajnik contributed in the development of the methodology and performed the experiment. Alenka Matjašič and Emanuela Boštjančič performed the analysis and interpretation of data. Alenka Matjašič, Mojca Tajnik, Emanuela Boštjančič, Mara Popović, Boštjan Matos, and Damjan Glavač were responsible for the writing, review, and/or revision of the manuscript. All authors have read and approved the manuscript. Alenka Matjašič, Mojca Tajnik, and Emanuela Boštjančič contributed equally to this work.

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References

[1] M. Esteller, "Non-coding RNAs in human disease," *Nature Reviews Genetics*, vol. 12, pp. 861–874, 2011.

[2] J. L. Rinn and H. Y. Chang, "Genome regulation by long noncoding RNAs," *Annual Review of Biochemistry*, vol. 81, pp. 145–166, 2012.

[3] X. Li, Z. Wu, X. Fu, and W. Han, "Long noncoding RNAs: insights from biological features and functions to diseases," *Medicinal Research Reviews*, vol. 33, pp. 517–553, 2013.

[4] I. A. Qureshi and M. F. Mehler, "Emerging roles of non-coding RNAs in brain evolution, development, plasticity and disease," *Nature Reviews Neuroscience*, vol. 13, pp. 528–541, 2012.

[5] M. Huarte and J. L. Rinn, "Large non-coding RNAs: missing links in cancer?," *Human Molecular Genetics*, vol. 19, pp. R152–R161, 2010.

[6] J. S. Mattick, "The genetic signatures of noncoding RNAs," *PLoS Genetics*, vol. 5, article e1000459, 2009.

[7] I. A. Qureshi, J. S. Mattick, and M. F. Mehler, "Long non-coding RNAs in nervous system function and disease," *Brain Research*, vol. 1338, pp. 20–35, 2010.

[8] K. M. Kiang, X. Q. Zhang, and G. K. Leung, "Long non-coding RNAs: the key players in glioma pathogenesis," *Cancer*, vol. 7, pp. 1406–1424, 2015.

[9] R. Alelu-Paz, N. Ashour, A. Gonzalez-Corpas, and S. Ropero, "DNA methylation, histone modifications, and signal transduction pathways: a close relationship in malignant gliomas pathophysiology," *Journal of Signal Transduction*, vol. 2012, Article ID 956958, 8 pages, 2012.

[10] G. I. Evan and K. H. Vousden, "Proliferation, cell cycle and apoptosis in cancer," *Nature*, vol. 411, pp. 342–348, 2001.

[11] E. M. Reis and S. Verjovski-Almeida, "Perspectives of long non-coding RNAs in cancer diagnostics," *Frontiers in Genetics*, vol. 3, p. 32, 2012.

[12] E. A. Gibb, C. J. Brown, and W. L. Lam, "The functional role of long non-coding RNA in human carcinomas," *Molecular Cancer*, vol. 10, p. 38, 2011.

[13] X. Zhang, S. Sun, J. K. Pu et al., "Long non-coding RNA expression profiles predict clinical phenotypes in glioma," *Neurobiology of Disease*, vol. 48, pp. 1–8, 2012.

[14] S. D. Fouse and J. F. Costello, "Epigenetics of neurological cancers," *Future Oncology (London, England)*, vol. 5, pp. 1615–1629, 2009.

[15] D. E. Handy, R. Castro, and J. Loscalzo, "Epigenetic modifications: basic mechanisms and role in cardiovascular disease," *Circulation*, vol. 123, pp. 2145–2156, 2011.

[16] Y. K. Kim and V. N. Kim, "Processing of intronic micro RNAs," *The EMBO Journal*, vol. 26, pp. 775–783, 2007.

[17] E. A. Gibb, E. A. Vucic, K. S. Enfield et al., "Human cancer long non-coding RNA transcriptomes," *PLoS One*, vol. 6, article e25915, 2011.

[18] P. Wang, Z. Ren, and P. Sun, "Overexpression of the long non-coding RNA MEG3 impairs in vitro glioma cell proliferation," *Journal of Cellular Biochemistry*, vol. 113, pp. 1868–1874, 2012.

[19] E. Hulleman and K. Helin, "Molecular mechanisms in gliomagenesis," *Advances in Cancer Research*, vol. 94, pp. 1–27, 2005.

[20] J. Y. Tang, J. C. Lee, Y. T. Chang et al., "Long noncoding RNAs-related diseases, cancers, and drugs," *The Scientific World Journal*, vol. 2013, Article ID 943539, 7 pages, 2013.

[21] M. Pojo and C. B. Marques, "Molecular hallmarks of gliomas," in *Molecular Targets of CNS Tumors*, dM Garami, Ed., pp. 177–200, InTech, Croatia, 2011.

[22] X. Shi, M. Sun, H. Liu, Y. Yao, and Y. Song, "Long non-coding RNAs: a new frontier in the study of human diseases," *Cancer Letters*, vol. 339, pp. 159–166, 2013.

[23] K. X. Ma, H. J. Wang, X. R. Li et al., "Long noncoding RNA MALAT1 associates with the malignant status and poor prognosis in glioma," *Tumour Biology: the Journal of the International Society for Oncodevelopmental Biology and Medicine*, vol. 36, pp. 3355–3359, 2015.

[24] H. Guo, L. Wu, Q. Yang, M. Ye, and X. Zhu, "Functional linc-POU3F3 is overexpressed and contributes to tumorigenesis in glioma," *Gene*, vol. 554, pp. 114–119, 2015.

[25] H. Rothenmund, H. Singh, B. Candas et al., "Hereditary colorectal cancer registries in Canada: report from the Colorectal Cancer Association of Canada consensus meeting; Montreal, Quebec; October 28, 2011," *Current Oncology (Toronto, Ont)*, vol. 20, pp. 273–278, 2013.

[26] L. F. Gulyaeva and N. E. Kushlinskiy, "Regulatory mechanisms of microRNA expression," *Journal of Translational Medicine*, vol. 14, p. 143, 2016.

[27] C. L. Andersen, J. L. Jensen, and T. F. Orntoft, "Normalization of real-time quantitative reverse transcription-PCR data: a model-based variance estimation approach to identify genes suited for normalization, applied to bladder and colon cancer data sets," *Cancer Research*, vol. 64, pp. 5245–5250, 2004.

[28] K. J. Livak and T. D. Schmittgen, "Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) method," *Methods (San Diego, Calif)*, vol. 25, pp. 402–408, 2001.

[29] S. Jalali, D. Bhartiya, M. K. Lalwani, S. Sivasubbu, and V. Scaria, "Systematic transcriptome wide analysis of lncRNA-miRNA interactions," *PLoS One*, vol. 8, article e53823, 2013.

[30] X. Wei, D. Chen, T. Lv, G. Li, and S. Qu, "Serum microRNA-125b as a potential biomarker for glioma diagnosis," *Molecular Neurobiology*, vol. 53, pp. 163–170, 2014.

[31] T. Niinuma, H. Suzuki, M. Nojima et al., "Upregulation of miR-196a and HOTAIR drive malignant character in gastrointestinal stromal tumors," *Cancer Research*, vol. 72, pp. 1126–1136, 2012.

[32] P. P. Amaral, M. B. Clark, D. K. Gascoigne, M. E. Dinger, and J. S. Mattick, "lncRNAdb: a reference database for long noncoding RNAs," *Nucleic Acids Research*, vol. 39, pp. D146–D151, 2011.

[33] Q. Liao, C. Liu, X. Yuan et al., "Large-scale prediction of long non-coding RNA functions in a coding-non-coding gene co-expression network," *Nucleic Acids Research*, vol. 39, pp. 3864–3878, 2011.

[34] X. Q. Zhang, S. Sun, K. F. Lam et al., "A long non-coding RNA signature in glioblastoma multiforme predicts survival," *Neurobiology of Disease*, vol. 58, pp. 123–131, 2013.

[35] Z. Du, T. Fei, R. G. Verhaak et al., "Integrative genomic analyses reveal clinically relevant long noncoding RNAs in human cancer," *Nature Structural & Molecular Biology*, vol. 20, pp. 908–913, 2013.

[36] Y. Sun, Z. Wang, and D. Zhou, "Long non-coding RNAs as potential biomarkers and therapeutic targets for gliomas," *Medical Hypotheses*, vol. 81, pp. 319–321, 2013.

[37] M. Sun, F. Y. Jin, R. Xia et al., "Decreased expression of long noncoding RNA GAS5 indicates a poor prognosis and promotes cell proliferation in gastric cancer," *BMC Cancer*, vol. 14, p. 319, 2014.

[38] M. Sun, R. Xia, F. Jin et al., "Downregulated long noncoding RNA MEG3 is associated with poor prognosis and promotes cell proliferation in gastric cancer," *Tumour Biology: the Journal of the International Society for Oncodevelopmental Biology and Medicine*, vol. 35, pp. 1065–1073, 2014.

[39] K. Gao, Z. Ji, K. She, Q. Yang, and L. Shao, "Long non-coding RNA ZFAS1 is an unfavourable prognostic factor and promotes glioma cell progression by activation of the Notch signaling pathway," *Biomedicine & Pharmacotherapy = Biomedecine & Pharmacotherapie*, vol. 87, pp. 555–560, 2017.

[40] C. He, B. Jiang, J. Ma, and Q. Li, "Aberrant NEAT1 expression is associated with clinical outcome in high grade glioma patients," *APMIS: Acta Pathologica, Microbiologica, et Immunologica Scandinavica*, vol. 124, pp. 169–174, 2016.

[41] J. X. Zhang, L. Han, Z. S. Bao et al., "HOTAIR, a cell cycle-associated long noncoding RNA and a strong predictor of survival, is preferentially expressed in classical and mesenchymal glioma," *Neuro-Oncology England*, vol. 15, pp. 1595–1603, 2013.

[42] R. A. Gupta, N. Shah, K. C. Wang et al., "Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis," *Nature*, vol. 464, pp. 1071–1076, 2010.

[43] R. Kogo, T. Shimamura, K. Mimori et al., "Long noncoding RNA HOTAIR regulates polycomb-dependent chromatin modification and is associated with poor prognosis in colorectal cancers," *Cancer Research*, vol. 71, pp. 6320–6326, 2011.

[44] X. B. Lv, G. Y. Lian, H. R. Wang, E. Song, H. Yao, and M. H. Wang, "Long noncoding RNA HOTAIR is a prognostic marker for esophageal squamous cell carcinoma progression and survival," *PLoS One*, vol. 8, article e63516, 2013.

[45] B. Cai, X. Q. Song, J. P. Cai, and S. Zhang, "HOTAIR: a cancer-related long non-coding RNA," *Neoplasma*, vol. 61, pp. 379–391, 2014.

[46] X. Zhang, Y. Zhou, K. R. Mehta et al., "A pituitary-derived MEG3 isoform functions as a growth suppressor in tumor cells," *The Journal of Clinical Endocrinology and Metabolism*, vol. 88, pp. 5119–5126, 2003.

[47] C. Braconi, T. Kogure, N. Valeri et al., "microRNA-29 can regulate expression of the long non-coding RNA gene MEG3 in hepatocellular cancer," *Oncogene*, vol. 30, pp. 4750–4756, 2011.

[48] K. H. Lu, W. Li, X. H. Liu et al., "Long non-coding RNA MEG3 inhibits NSCLC cells proliferation and induces apoptosis by affecting p53 expression," *BMC Cancer*, vol. 13, p. 461, 2013.

[49] R. Qin, Z. Chen, Y. Ding, J. Hao, J. Hu, and F. Guo, "Long non-coding RNA MEG3 inhibits the proliferation of cervical carcinoma cells through the induction of cell cycle arrest and apoptosis," *Neoplasma*, vol. 60, pp. 486–492, 2013.

[50] L. Ying, Y. Huang, H. Chen et al., "Downregulated MEG3 activates autophagy and increases cell proliferation in bladder cancer," *Molecular BioSystems*, vol. 9, pp. 407–411, 2013.

[51] G. Luo, M. Wang, X. Wu et al., "Long non-coding RNA MEG3 inhibits cell proliferation and induces apoptosis in prostate cancer," *Cellular Physiology and Biochemistry: International Journal of Experimental Cellular Physiology, Biochemistry, and Pharmacology*, vol. 37, pp. 2209–2220, 2015.

[52] J. Li, E. B. Bian, X. J. He et al., "Epigenetic repression of long non-coding RNA MEG3 mediated by DNMT1 represses the p53 pathway in gliomas," *International Journal of Oncology*, vol. 48, pp. 723–733, 2016.

[53] Y. Chen, Y. Bian, S. Zhao, F. Kong, and X. Li, "Suppression of PDCD4 mediated by the long non-coding RNA HOTAIR inhibits the proliferation and invasion of glioma cells," *Oncology Letters*, vol. 12, pp. 5170–5176, 2016.

[54] N. Thorenoor, P. Faltejskova-Vychytilova, S. Hombach et al., "Long non-coding RNA ZFAS1 interacts with CDK1 and is involved in p53-dependent cell cycle control and apoptosis in colorectal cancer," *Oncotarget*, vol. 7, no. 1, pp. 622–637, 2016.

[55] K. Zhang, X. Sun, X. Zhou et al., "Long non-coding RNA HOTAIR promotes glioblastoma cell cycle progression in an EZH2 dependent manner," *Oncotarget United States*, vol. 6, pp. 537–546, 2015.

[56] S. N. Min, T. Wei, X. T. Wang, L. L. Wu, and G. Y. Yu, "Clinicopathological and prognostic significance of homeobox transcript antisense RNA expression in various cancers: a meta-analysis," *Medicine*, vol. 96, article e7084, 2017.

[57] W. Guo, Z. Dong, S. Liu et al., "Promoter hypermethylation-mediated downregulation of miR-770 and its host gene MEG3, a long non-coding RNA, in the development of gastric cardia adenocarcinoma," *Molecular Carcinogenesis*, vol. 56, 2017.

[58] W. P. Chak, R. W. Lung, J. H. Tong et al., "Downregulation of long non-coding RNA MEG3 in nasopharyngeal carcinoma," *Molecular Carcinogenesis*, vol. 56, pp. 1041–1054, 2017.

[59] Y. Yu, F. Lv, D. Liang et al., "HOTAIR may regulate proliferation, apoptosis, migration and invasion of MCF-7 cells through regulating the P53/Akt/JNK signaling pathway," *Biomedicine & Pharmacotherapy = Biomedecine & Pharmacotherapie*, vol. 90, pp. 555–561, 2017.

[60] L. Pan, W. Liang, M. Fu et al., "Exosomes-mediated transfer of long noncoding RNA ZFAS1 promotes gastric cancer progression," *Journal of Cancer Research and Clinical Oncology*, vol. 143, pp. 991–1004, 2017.

[61] F. Nie, X. Yu, M. Huang et al., "Long noncoding RNA ZFAS1 promotes gastric cancer cells proliferation by epigenetically repressing KLF2 and NKD2 expression," *Oncotarget*, vol. 8, pp. 38227–38238, 2017.

[62] X. Zhou, Y. Ren, J. Zhang et al., "HOTAIR is a therapeutic target in glioblastoma," *Oncotarget*, vol. 6, pp. 8353–8365, 2015.

[63] C. Pastori, P. Kapranov, C. Penas et al., "The bromodomain protein BRD4 controls HOTAIR, a long noncoding RNA essential for glioblastoma proliferation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 112, pp. 8326–8331, 2015.

[64] Y. Zhou, X. Zhang, and A. Klibanski, "MEG3 noncoding RNA: a tumor suppressor," *Journal of Molecular Endocrinology*, vol. 48, pp. R45–R53, 2012.

[65] X. Zhang, K. Rice, Y. Wang et al., "Maternally expressed gene 3 (MEG3) noncoding ribonucleic acid: isoform structure, expression, and functions," *Endocrinology*, vol. 151, pp. 939–947, 2010.

[66] M. U. Kaikkonen, M. T. Lam, and C. K. Glass, "Non-coding RNAs as regulators of gene expression and epigenetics," *Cardiovascular Research*, vol. 90, pp. 430–440, 2011.

[67] M. E. Askarian-Amiri, J. Crawford, J. D. French et al., "SNORD-host RNA Zfas1 is a regulator of mammary development and a potential marker for breast cancer," *RNA (New York, NY)*, vol. 17, pp. 878–891, 2011.

[68] Q. L. Lv, S. H. Chen, X. Zhang et al., "Upregulation of long noncoding RNA zinc finger antisense 1 enhances epithelial-mesenchymal transition in vitro and predicts poor prognosis in glioma," *Tumour Biology: the Journal of the International Society for Oncodevelopmental Biology and Medicine*, vol. 39, article 1010428317695022, 2017.

[69] K. Abdelmohsen, A. C. Panda, M. J. Kang et al., "7SL RNA represses p53 translation by competing with HuR," *Nucleic Acids Research*, vol. 42, pp. 10099–10111, 2014.

[70] C. Zwieb, R. W. van Nues, M. A. Rosenblad, J. D. Brown, and T. Samuelsson, "A nomenclature for all signal recognition particle RNAs," *RNA (New York, NY)*, vol. 11, pp. 7–13, 2005.

[71] J. C. Castle, C. D. Armour, M. Löwer et al., "Digital genome-wide ncRNA expression, including SnoRNAs, across 11 human tissues using polyA-neutral amplification," *PLoS One*, vol. 5, article e11779, 2010.

[72] H. Ohgaki and P. Kleihues, "The definition of primary and secondary glioblastoma," *Clinical Cancer Research: an Official Journal of the American Association for Cancer Research*, vol. 19, pp. 764–772, 2013.

[73] D. Rose and P. F. Stadler, "Molecular evolution of the non-coding eosinophil granule ontogeny transcript," *Frontiers in Genetics*, vol. 2, p. 69, 2011.

[74] K. Kaufmann and G. Thiel, "Epidermal growth factor and platelet-derived growth factor induce expression of Egr-1, a zinc finger transcription factor, in human malignant glioma cells," *Journal of the Neurological Sciences*, vol. 189, pp. 83–91, 2001.

[75] S. P. Xu, J. F. Zhang, S. Y. Sui et al., "Downregulation of the long noncoding RNA EGOT correlates with malignant status and poor prognosis in breast cancer," *Tumour Biology: the Journal of the International Society for Oncodevelopmental Biology and Medicine*, vol. 36, pp. 9807–9812, 2015.

[76] R. Sanuki, A. Onishi, C. Koike et al., "miR-124a is required for hippocampal axogenesis and retinal cone survival through Lhx2 suppression," *Nature Neuroscience*, vol. 14, pp. 1125–1134, 2011.

[77] J. Silber, D. A. Lim, C. Petritsch et al., "miR-124 and miR-137 inhibit proliferation of glioblastoma multiforme cells and induce differentiation of brain tumor stem cells," *BMC Medicine*, vol. 6, p. 14, 2008.

[78] P. T. Nelson, D. A. Baldwin, W. P. Kloosterman, S. Kauppinen, R. H. Plasterk, and Z. Mourelatos, "RAKE and LNA-ISH reveal microRNA expression and localization in archival human brain," *RNA (New York, NY)*, vol. 12, pp. 187–191, 2006.

[79] A. Fowler, D. Thomson, K. Giles et al., "miR-124a is frequently down-regulated in glioblastoma and is involved in migration and invasion," *European Journal of Cancer*, vol. 47, pp. 953–963, 2011.

[80] S. H. Lu, X. J. Jiang, G. L. Xiao, D. Y. Liu, and X. R. Yuan, "miR-124a restoration inhibits glioma cell proliferation and invasion by suppressing IQGAP1 and β-catenin," *Oncology Reports*, vol. 32, pp. 2104–2110, 2014.

[81] D. Deng, L. Wang, Y. Chen et al., "MicroRNA-124-3p regulates cell proliferation, invasion, apoptosis, and bioenergetics by targeting PIM1 in astrocytoma," *Cancer Science*, vol. 107, pp. 899–907, 2016.

[82] L. Xu, H. Liu, Z. Yan, Z. Sun, S. Luo, and Q. Lu, "Inhibition of the Hedgehog signaling pathway suppresses cell proliferation by regulating the Gli2/miR-124/AURKA axis in human glioma cells," *International Journal of Oncology*, vol. 50, pp. 1868–1878, 2017.

[83] W. J. Wu, J. Shi, G. Hu et al., "Wnt/β-catenin signaling inhibits FBXW7 expression by upregulation of microRNA-770 in hepatocellular carcinoma," *Tumour Biology: the Journal of the International Society for Oncodevelopmental Biology and Medicine*, vol. 37, pp. 6045–6051, 2016.

[84] Y. Kang, J. Song, D. Kim et al., "PCGEM1 stimulates proliferation of osteoarthritic synoviocytes by acting as a sponge for miR-770," *Journal of Orthopaedic Research: Official Publication of the Orthopaedic Research Society*, vol. 34, pp. 412–418, 2016.

[85] L. Guo, Y. Zhao, S. Yang, H. Zhang, and F. Chen, "An integrated analysis of miRNA, lncRNA, and mRNA expression profiles," *BioMed Research International*, vol. 2014, Article ID 345605, 12 pages, 2014.

[86] Y. Guan, M. Mizoguchi, K. Yoshimoto et al., "MiRNA-196 is upregulated in glioblastoma but not in anaplastic astrocytoma and has prognostic significance," *Clinical Cancer Research: an*

Official Journal of the American Association for Cancer Research, vol. 16, pp. 4289–4297, 2010.

[87] G. Yang, D. Han, X. Chen et al., "MiR-196a exerts its oncogenic effect in glioblastoma multiforme by inhibition of IkappaBalpha both in vitro and in vivo," *Neuro-Oncology*, vol. 16, pp. 652–661, 2014.

[88] S. A. Ciafrè, S. Galardi, A. Mangiola et al., "Extensive modulation of a set of microRNAs in primary glioblastoma," *Biochemical and Biophysical Research Communications*, vol. 334, pp. 1351–1358, 2005.

[89] X. Li, J. Zheng, L. Chen, H. Diao, and Y. Liu, "Predictive and prognostic roles of abnormal expression of tissue miR-125b, miR-221, and miR-222 in glioma," *Molecular Neurobiology*, vol. 53, no. 1, pp. 577–583, 2014.

[90] H. F. Xia, T. Z. He, C. M. Liu et al., "MiR-125b expression affects the proliferation and apoptosis of human glioma cells by targeting Bmf," *Cellular Physiology and Biochemistry: International Journal of Experimental Cellular Physiology, Biochemistry, and Pharmacology*, vol. 23, pp. 347–358, 2009.

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Research Article

Tissue- and Cell Type-Specific Expression of the Long Noncoding RNA Klhl14-AS in Mouse

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lncRNAs are acquiring increasing relevance as regulators in a wide spectrum of biological processes. The extreme heterogeneity in the mechanisms of action of these molecules, however, makes them very difficult to study, especially regarding their molecular function. A novel lncRNA has been recently identified as the most enriched transcript in mouse developing thyroid. Due to its genomic localization antisense to the protein-encoding Klhl14 gene, we named it Klhl14-AS. In this paper, we highlight that mouse Klhl14-AS produces at least five splicing variants, some of which have not been previously described. Klhl14-AS is expressed with a peculiar pattern, characterized by diverse relative abundance of its isoforms in different mouse tissues. We examine the whole expression level of Klhl14-AS in a panel of adult mouse tissues, showing that it is expressed in the thyroid, lung, kidney, testis, ovary, brain, and spleen, although at different levels. In situ hybridization analysis reveals that, in the context of each organ, Klhl14-AS shows a cell type-specific expression. Interestingly, databases report a similar expression profile for human Klhl14-AS. Our observations suggest that this lncRNA could play cell type-specific roles in several organs and pave the way for functional characterization of this gene in appropriate biological contexts.

1. Introduction

In the last few years, genome comparison across different species highlighted that, on the evolutionary scale, organismal complexity is directly correlated with the extent of the nonprotein coding (herein referred as noncoding) fraction of the total genome [1, 2]. Moreover, the discovery that the vast majority of the genome is transcribed revealed that the noncoding portion of eukaryote transcriptomes largely exceeds the protein-coding one, both in abundance and in complexity [3, 4]. These observations, together with accumulating evidences of a plethora of RNA-mediated gene regulatory networks, strongly suggested, hence, that noncoding RNAs are key players in the establishment of biological complexity [2, 5].

Noncoding RNAs are mainly classified according to their length into small and long noncoding RNAs (lncRNAs). LncRNAs are RNA molecules longer than 200 nucleotides and without any evident or conserved open reading frame. They are often 5′ capped, polyadenylated, and spliced and have been described to be expressed at levels lower than protein-coding genes but in a more tissue- and cell-specific

manner [6–10]. Many of these transcripts have been reported to have potential functional orthologs in different species, despite the low sequence conservation [8, 11–13]. Although only few lncRNAs have been functionally characterized [14, 15], the number of lncRNAs identified in different cell types and correlating with different biological processes has been rising [1, 16, 17]. Such increase strongly suggests that they have multiple considerable functions, mainly mediated by their ability to associate with different classes of biomolecules. Indeed, long noncoding RNAs can be able to bind to other nucleic acids (either DNA or RNA) by base pairing to complementary sequences and/or to bind to proteins by folding in secondary structures [1, 18–20]. Nevertheless, the intrinsic pleiotropic mechanism of action of these molecules represents the major challenge in the study of the functional role of each individual lncRNA.

Recently a poorly characterized gene, reported as long noncoding RNA, emerged as the most enriched in E10.5 mouse thyroid bud transcriptome, compared to that of the whole embryo [21]. To the best of our knowledge, only few data exist in the literature about this gene, which are essentially based on genome-wide expression profiling of lncRNAs in mouse [9]. We firstly named it Thybe1 (thyroid bud enriched 1); however, the analysis of its genomic localization revealed that it is in antisense and partially overlapping with the protein-encoding gene Klhl14 in a head to head arrangement; thus, a more standard name for this gene could be Klhl14-AS. In this paper, we analyze the Thybe1/Klhl14-AS expression pattern in adult mouse tissues, both by quantitative real-time reverse-transcriptase- (qRT-) PCR and by in situ hybridization (ISH). We also identify previously unknown splicing isoforms of this gene in mouse and explore available data on human Klhl14-AS expression, thus providing an important contribution to the characterization of this novel lncRNA.

2. Results and Discussion

2.1. Genomic Organization and Identification of Alternative Transcripts of Mouse Klhl14-AS Gene. To shed light on the Klhl14-AS expression profile, we looked at different databases such as NCBI [22], UCSC [23], and Ensembl [24] to obtain information about gene structure and expression. In mouse genome, the gene is named 4930426D05Rik and mapped to chromosome 18qA2, as reported in Ensembl. In human genome, its ortholog is named AC012123.1 and mapped to chromosome 18q12.1. The comparison of the genomic context of mouse and human locus reveals that the regions are clearly syntenic and show high grade of conservation of blocks of sequences. In both genomes, Klhl14-AS partially overlaps with the protein-coding gene Klhl14 in a head-to-head antisense arrangement (Figure 1(a)). Mouse Klhl14-AS produces several different transcripts (Figure 1(b)) differing between databases both in number and in structure (see Supplementary Figure S1 available online at https://doi.org/10.1155/2017/9769171), while in human, it is reported to produce a single isoform (Figure 1(c)). We thus referred to Ensembl for further analyses, because it reports the higher number of alternative transcripts for mouse. In Figure 1(b), it is shown that mouse Klhl14-AS produces four transcripts named 4930426D05Rik 002, 003, 004, and 005. These transcripts differ for the transcription start site and/or the exons, mainly at the 5′ moiety, while three out of four show a similar 3′ end, with only Rik04 showing a longer 3′ exon. All the isoforms, however, share three regions indicated in Figure 1(b) by blue, orange, and green rectangles.

As Klhl14-AS was identified in the developing thyroid [21], we decided to investigate which of the reported transcripts was expressed in the adult mouse gland. We thus designed appropriate primer sets and amplified total mouse thyroid cDNA. Surprisingly, only one out of the used oligo sets, including the most internal ones, gave rise to amplicons (see Figure S2 and Table S1). This prompted us to experimentally map 5′ and 3′ ends of thyroid isoforms by performing RACE experiments. To this aim, we started from the 5′ end of the common region in the blue box and the 3′ end of the common region in the green box (Figure 1(b)), by identifying a 5′ terminus and a polyadenylated 3′ end (data not shown). Moreover, we considered also a more upstream CAGE site reported in Encode, in a region shared by most 5′ exons of the Rik002 and Rik005 isoforms. Two different primer sets were thus designed, starting from the just described putative ends. The oligo starting from the 5′ RACE sequence was used in set 1, whereas the oligo starting from the most upstream sequence reported in databases was used in set 2 (Figure S3). For each oligo set, the reverse primer was specifically designed to optimize the amplification conditions, although reverse primers are largely overlapping and both mapped very closely to the 3′ end identified by RACE. RT-PCR performed with oligo set 1 produced two products, named A and B, while that performed with oligo set 2 produced three fragments, named C, D, and E (Figure 2, see the thyroid lane). The sequences of the isoforms amplified were mapped to the mouse genome (mm10) using BLAT (see Materials and Methods), revealing that only the two of them, A and D, correspond to the previously characterized splicing variants, Rik004 and Rik005, respectively. Importantly, we identified three novel isoforms, B, C, and E, which have not been previously annotated (Figure 1(b)). It is worth noting that we found a region that is amplified only using oligo set 1 (Figure 1(b), pink rectangle). The retention of this region only in transcripts A and B suggests that at least two different TSSs could exist for this gene. The finding that Klhl14-AS is transcribed in several different isoforms in the same organ suggests that they could play different roles through the binding of different interactors. Moreover, the presence of more than one TSS suggests that Klhl14-AS transcription could be differentially regulated in different conditions.

2.2. Expression Profile of Klhl14-AS in Adult Mouse Tissues. We asked if Klhl14-AS was expressed in other organs. To answer this question, we performed the above described amplifications in diverse adult mouse tissues. Such analysis indicated that Klhl14-AS transcripts are differentially expressed in different tissues and that the two used oligo sets amplify the same molecular species in all tested tissues (Figure 2). Indeed, isoforms A and B are expressed in the

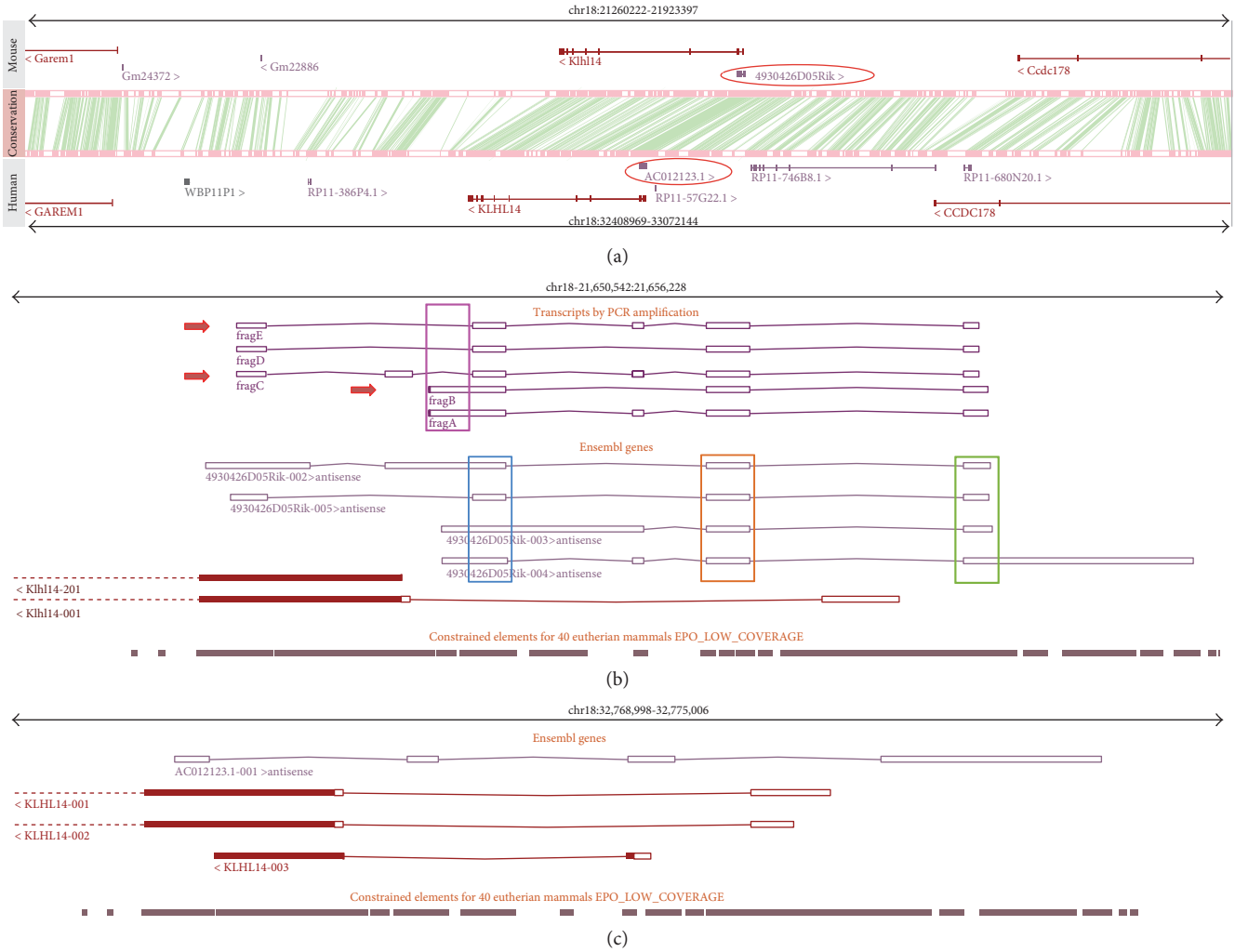

Figure 1: Genomic context of the Klhl14 S/AS pair in mouse and human. In (a), two genomic segments from mouse and human containing the Klhl14 S/AS pair and flanking genes are displayed. The regions are clearly syntenic, and the presence of an antisense noncoding gene (red circle) is conserved in both species. Pink boxes indicate conserved blocks, and green lines represent their correspondences. In (b) and (c), the slices are zoomed in to show the entire AS genes and the Klhl14 gene portion in overlap for the mouse (b) and human (c) genome, respectively. The constrained elements displayed as grey boxes represent regions of sequence conservation in mammals according to Ensembl. In (b), the regions shared by all the transcripts are indicated by the blue, orange, and green boxes. Fragment A to fragment E represent the isoforms identified in this study with the new ones indicated by the red arrows. The region in the pink rectangle is amplified exclusively with one set of oligos.

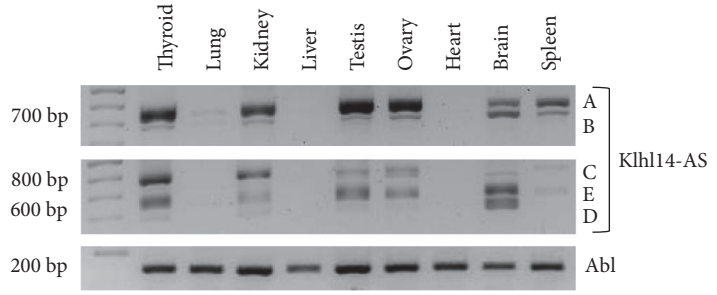

Figure 2: Klhl14-AS isoform expression in adult mouse tissues. Klhl14-AS transcripts were amplified by RT-PCR on total RNA from adult mouse organs. Three different PCR reactions were performed on the same template cDNA. Upper image: A and B isoforms were amplified by using the oligo set 1; middle image: C, D, and E were obtained by using oligo set 2; lower image: Abelson (Abl) amplification was performed as the control (for details see Materials and Methods). Data are representative of three independent experiments.

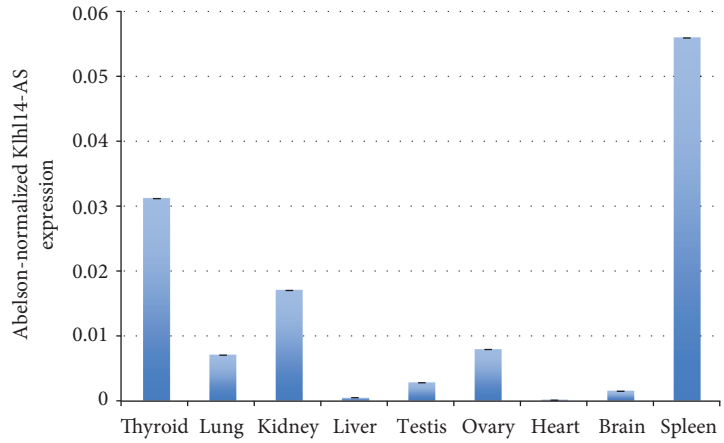

Figure 3: Klhl14-AS expression in adult mouse organs. Quantitative real-time RT-PCR was performed by amplifying a region shared by Klhl14-AS isoforms on total RNA from adult mouse organs. The data are reported as normalized by Abelson expression. Three replicates for each experimental point were performed. Error bars represent standard deviation of normalized ct values. Data are representative of three independent experiments.

thyroid, kidney, testis, ovary, brain, and spleen, although at different extents. A is mostly represented in the testis and ovary, being also strongly expressed in the thyroid and kidney. B is relatively more abundant in the brain than in other organs. The transcript C is mostly expressed in the thyroid and kidney. D is weakly expressed in the thyroid and kidney, while it is highly expressed only in the brain. E is mostly represented in the thyroid, testis, ovary, and brain. It is worth noting that all the identified transcripts are expressed in the thyroid gland, whereas none of the transcripts amplified by oligo set 2 are expressed in the spleen. Lastly, the liver and heart are negative for Klhl14-AS expression, while the lung shows a weak signal only with oligo set 1. Such data reveal a tissue-specific pattern of Klhl14-AS alternative transcript expression.

To overcome the complexity of the alternatively spliced isoform expression, we analyzed Klhl14-AS expression by using a primer set amplifying a region that is common between all known isoforms (overlapping the common splice junction between the regions included in the orange and green rectangles in Figure 1). Whole Klhl14-AS expression among adult tissues was evaluated through qRT-PCR, showing that it is expressed in several organs at different extents. This quantitative analysis confirms that the lncRNA is not expressed in the liver and heart; weakly expressed in the brain, lung, ovary, and testis; and more strongly expressed in the kidney, spleen, and thyroid gland (Figure 3). However, qRT-PCR, such as the standard PCR previously performed, measures the RNA in the whole organ, thus missing the possible differences between different cells types in the same organ. This technical limit could lead to underestimating Klhl14-AS presence in specific cell types. To deal with this problem, we performed ISH analysis by using a Klhl14-AS-specific probe that allowed to clearly define the lncRNA distribution in specific cellular types. A sense probe was used as a control (Figure S4). ISH performed on adult mouse organs, while confirming qRT-PCR results, revealed that Klhl14-AS is expressed in a cell type-restricted manner in the context of each organ. As expected, Klhl14-AS staining is absent in the liver and heart (Figure 4(a)). Unexpectedly, Klhl14-AS staining is absent also in the lungs (Figure 4(a)), where the qRT-PCR showed a detectable although weak positivity (Figure 3). One possible explanation is that ISH has a lower detection capability than qRT-PCR; thus, a weak level of expression could be undetectable by ISH. Conversely, all other organs positive for Klhl14-AS expression by qRT-PCR show a clear signal by ISH, with differences in the distribution between specific cell types (Figure 4(b)). In the thyroid, the lncRNA is highly expressed in follicular cells, representing the epithelial component of thyroid follicles. In the kidney, Klhl14-AS is mainly expressed in the renal corpuscles of the cortex (see 200x magnification). In the spleen, it is expressed in the rounded areas of white pulp consisting of lymphoid cells, while it is not detected in the red pulp (see 25x magnification). In the testis, the lncRNA is expressed in the seminiferous tubules, while resulting negative in the Leydig cells of the intertubular connective tissue (see 200x magnification). In the ovary, Klhl14-AS is expressed in the follicles, showing a strong signal in granulosa cells of the preovulatory follicles, while it was negative in the oocyte and theca cells (see 200x magnification). Figure 4(c) shows the ISH of the brain, where Klhl14-AS expression is strongly detected in the hippocampus and the in cortex (see 200x magnification).

We also did a survey of existing data about the human Klhl14-AS ortholog expression pattern. Gtex annotations in the UCSC database, obtained by RNA sequencing, report that the lncRNA is strongly expressed in the thyroid but weakly expressed in the spleen, testis, kidney cortex, and fallopian tube (Supplementary Figure S5) (http://bit.ly/klhl14-as). The expression pattern of human Klhl14-AS overlaps in part to that shown here in mouse, at least for the organs with the highest expression levels. The differences observed for some organs (i.e. brain, ovary) could be due either to the different experimental approaches or to real differences between the two species.

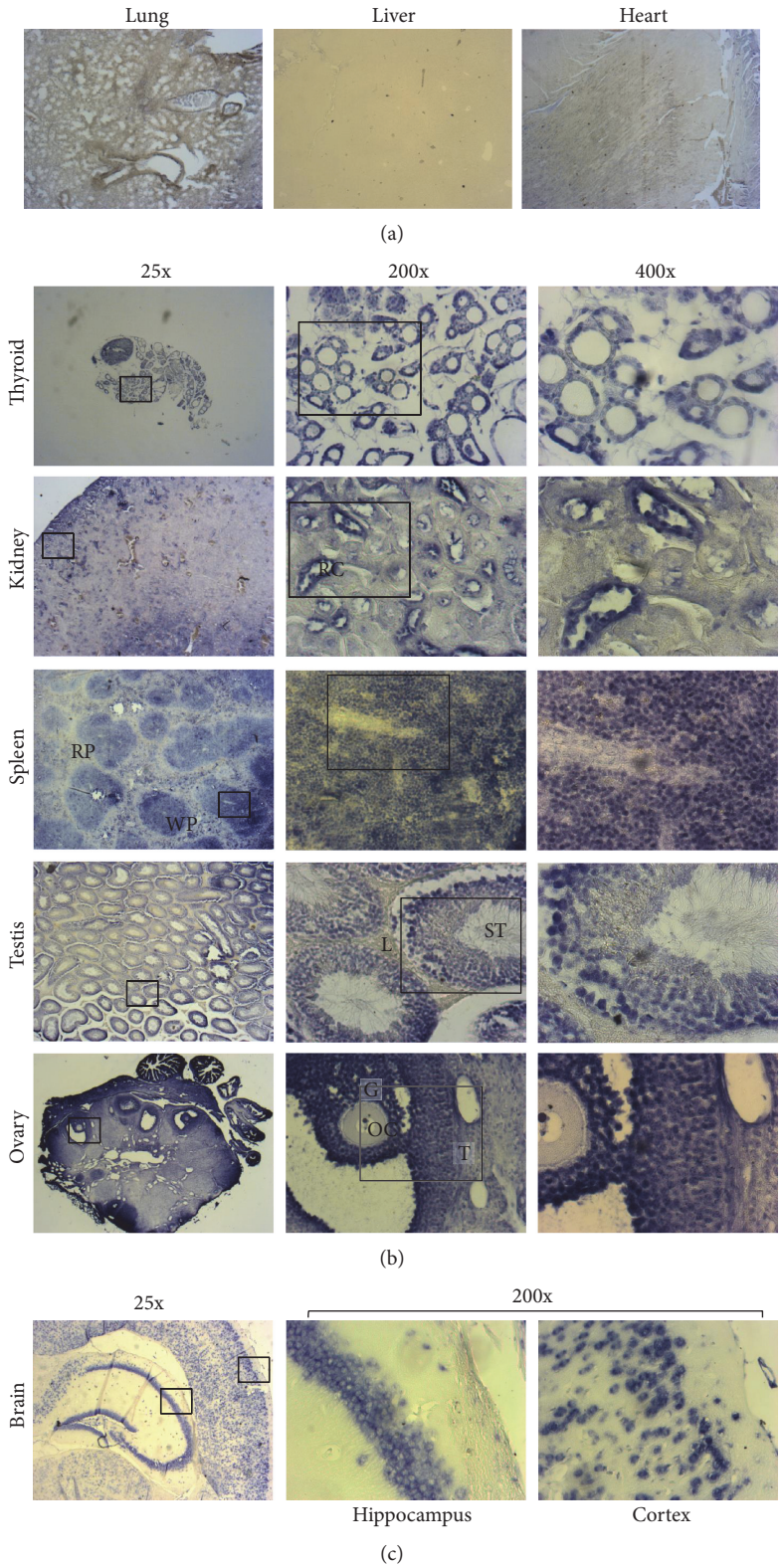

Figure 4: Cell type-specific expression of Klhl14-AS in adult mouse organs. In situ hybridization was performed on paraffin-embedded sections with a probe recognizing all Klhl14-AS isoforms (for details, see Materials and Methods). (a) The lung, liver, and heart are negative for Klhl14-AS staining. 25x magnifications are shown. (b) The thyroid, kidney, spleen, testis, and ovary are positive for Klhl14-AS staining. For each organ, three different magnifications are shown: 25x, 200x, and 400x. 200x magnifications correspond to the areas boxed in 25x pictures, while 400x magnifications correspond to areas boxed in 200x pictures. RC: renal corpuscle; RP: red pulp; WP: white pulp; L: Leydig cells; ST: seminiferous tubule; OC: oocyte; G: granulosa cells; T: thecal cells. All the images are reduced by 70% compared to the original ones. Data are representative of three independent experiments.

The conservation of Klhl14-AS sequence and genomic organization, together with a partially overlapping expression pattern, is suggestive of an important biological role of Klhl14-AS.

3. Conclusion

Identifying the biological role and the mechanism of action of long noncoding RNAs is among the most challenging issues in functional genetics studies, where the setting of appropriate experimental models is a critical issue, given their tissue specificity. Klhl14-AS was the first lncRNA identified in the thyroid gland, and here, we demonstrate that it is also expressed in very different tissues such as those of the kidney and brain. Moreover, we identify previously uncharacterized Klhl14-AS alternatively spliced transcripts and describe the differential expression pattern in several organs, suggesting that each isoform could play a specific role in a given physiological context. Indeed, the functional specificity of Klhl14-AS could arise by the combination of different variants with cell type-specific interactors that likely take place in different tissues. Taken together, our data represent the starting point for the characterization of this lncRNA, which is likely to be involved in relevant biological phenomena and possibly in different diseases.

4. Materials and Methods

4.1. Animal Experiments. All animal experiments were performed in accordance with the Italian and European guidelines and were approved by the local Ethical Committee and by the Italian Ministry of Health. Animals were maintained under specific pathogen-free conditions in the animal house facility of the Dipartimento di Medicina Molecolare e Biotecnologie Mediche. A total of eight wild-type C57BL/6 mice of both sexes were used for both molecular and histological analyses.

4.2. Transcript Mapping. 5′ and 3′ ends were investigated with the use of 3′ RACE System (Invitrogen 18373-027) and 5′ RACE System (Invitrogen 18374-041) according to the manufacturer's specifications.

cDNA from adult mouse tissues were amplified with the Pwo SuperYield DNA Polymerase (Roche 04 340 850 001) using oligos representing the ends obtained through RACE experiments and the 5′ oligo designed on the second upstream CAGE site reported in UCSC—Klhl14-AS CAGE II: CGCGTACTGCATGCGGGTCTCA, Klhl14-AS 5′RACE: GAGAGAGGAACAACAATCAAGGC, Klhl14-AS 3′RACE: GGGGATTAGAGTTTATTTTTGTCATCTC, and Klhl14-AS 3′ RACE inner: ATTCATCCAGATCACAGCTAAG.

The sequences from the identified isoforms in FASTA format were mapped to the mouse genome (mm10) using BLAT (https://genome.ucsc.edu/cgi-bin/hgBlat), and the results were exported in PSL format. The results in PSL format were uploaded as a custom track in the UCSC browser and converted into GTF using the UCSC table browser. The genome browser images were generated using the Ensembl genome browser (v88) for human (GRCh38.p10) and mouse (GRCm38.p5) where a custom track was created and uploaded to visualize the PCR fragment mappings in GTF format on the mouse genome. The genome browser screenshots were exported in PDF format, and processing of the vector graphics was done in Inkscape (v0.48.4).

4.3. Quantitative Real-Time PCR. Total RNA was isolated from mouse organs as previously described [25] using TRIzol® Reagent (Invitrogen 15596026) according to the manufacturer's specifications. Each sample corresponds to a single organ except the thyroid. Due to the small size of mouse thyroid, two glands were pooled for total RNA extraction. Total cDNA was generated with the SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen 18080051), according to the manufacturer's specifications. Real-time PCR on total cDNA was performed with iTaq™ Universal SyBR® Green Supermix (Bio-Rad 172-5124) using gene-specific oligos—Klhl14-AS F: GGCTCCTCTCCACTCACTTTC, Klhl14-AS R: TCAGCTCAGCAGCGAAGTC, Abelson F: TCGGACGTGTGGGCATT, and Abelson R: CGCATGA GCTCGTAGACCTTC.

4.4. In Situ Hybridization. Organs were fixed in 4% PFA (overnight, 4°C), washed in saline solution, dehydrated in solutions at increasing ethanol concentration from 70% to 100% (overnight, 4°C), and paraffin-embedded at 60°C after xylene soaking. The period of each step is determined according to the size of the processed sample.

Paraffin-embedded samples were sliced in 7 μm sections and analyzed. To perform the in situ hybridization, the sections were deparaffinized in xylene and rehydrated with EtOH 100% to EtOH 50%. After rehydration, the hybridization was performed as described in Fagman et al. [21], using a specific probe for Klhl14-AS amplified with Pwo SuperYield DNA Polymerase from adult mouse thyroid cDNA using the following oligos—Klhl14-AS sp6: GGCTGAACAGGA AGGGACCCT and Klhl14-AS T7: CAGATCACAGCTAA GAAAAAAGC.

PCR product was purified using the USB® PrepEase® Gel Extraction Kit (Affymetrix 78756). Digoxigenin-labelled riboprobes (sense and antisense) were obtained using the DIG-labeling RNA kit (Roche Diagnostics Basel, Switzerland) following the manufacturer's instructions. No signal was detected with the sense riboprobes (not shown). Images were obtained using an Axioskop microscope equipped with an Axiocam 105 color digital camera (Zeiss, Oberkochen, Germany). Images were processed using the Axion Vision software.

Disclosure

The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflicts of Interest

The authors declare that they have no conflict of interest with the contents of this article.

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References

[1] A. Fatica and I. Bozzoni, "Long non-coding RNAs: new players in cell differentiation and development," *Nature Reviews Genetics*, vol. 15, no. 1, pp. 7–21, 2014.

[2] A. Pasut, A. Matsumoto, J. G. Clohessy, and P. P. Pandolfi, "The pleiotropic role of non-coding genes in development and cancer," *Current Opinion in Cell Biology*, vol. 43, pp. 104–113, 2016.

[3] S. Djebali, C. A. Davis, A. Merkel et al., "Landscape of transcription in human cells," *Nature*, vol. 489, no. 7414, pp. 101–108, 2012.

[4] N. Maeda, T. Kasukawa, R. Oyama et al., "Transcript annotation in FANTOM3: mouse gene catalog based on physical cDNAs," *PLoS Genetics*, vol. 2, no. 4, article e62, 2006.

[5] F. F. Costa, "Non-coding RNAs: meet thy masters," *BioEssays*, vol. 32, no. 7, pp. 599–608, 2010.

[6] M. N. Cabili, C. Trapnell, L. Goff et al., "Integrative annotation of human large intergenic noncoding RNAs reveals global properties and specific subclasses," *Genes & Development*, vol. 25, no. 18, pp. 1915–1927, 2011.

[7] T. Derrien, R. Johnson, G. Bussotti et al., "The GENCODE v7 catalog of human long noncoding RNAs: analysis of their gene structure, evolution, and expression," *Genome Research*, vol. 22, no. 9, pp. 1775–1789, 2012.

[8] A. C. Marques and C. P. Ponting, "Catalogues of mammalian long noncoding RNAs: modest conservation and incompleteness," *Genome Biology*, vol. 10, no. 11, article R124, 2009.

[9] T. R. Mercer, M. E. Dinger, S. M. Sunkin, M. F. Mehler, and J. S. Mattick, "Specific expression of long noncoding RNAs in the mouse brain," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 2, pp. 716–721, 2008.

[10] D. Vucicevic, O. Corradin, E. Ntini, P. C. Scacheri, and U. A. Ørom, "Long ncRNA expression associates with tissue-specific enhancers," *Cell Cycle*, vol. 14, no. 2, pp. 253–260, 2015.

[11] M. Guttman, I. Amit, M. Garber et al., "Chromatin signature reveals over a thousand highly conserved large non-coding RNAs in mammals," *Nature*, vol. 458, no. 7235, pp. 223–227, 2009.

[12] I. Ulitsky, A. Shkumatava, C. H. Jan, H. Sive, and D. P. Bartel, "Conserved function of lincRNAs in vertebrate embryonic development despite rapid sequence evolution," *Cell*, vol. 147, no. 7, pp. 1537–1550, 2011.

[13] S. Basu, Y. Hadzhiev, G. Petrosino et al., "The Tetraodon nigroviridis reference transcriptome: developmental transition, length retention and microsynteny of long non-coding RNAs in a compact vertebrate genome," *Scientific Reports*, vol. 6, article 33210, 2016.

[14] A. R. Bassett, A. Akhtar, D. P. Barlow et al., "Considerations when investigating lncRNA function in vivo," *eLife*, vol. 3, article e03058, 2014.

[15] M. Morlando, M. Ballarino, A. Fatica, and I. Bozzoni, "The role of long noncoding RNAs in the epigenetic control of gene expression," *ChemMedChem*, vol. 9, no. 3, pp. 505–510, 2014.

[16] R. A. Flynn and H. Y. Chang, "Long noncoding RNAs in cell-fate programming and reprogramming," *Cell Stem Cell*, vol. 14, no. 6, pp. 752–761, 2014.

[17] M. Huarte, "The emerging role of lncRNAs in cancer," *Nature Medicine*, vol. 21, no. 11, pp. 1253–1261, 2015.

[18] M. Guttman and J. L. Rinn, "Modular regulatory principles of large non-coding RNAs," *Nature*, vol. 482, no. 7385, pp. 339–346, 2012.

[19] F. P. Marchese and M. Huarte, "Long non-coding RNAs and chromatin modifiers: their place in the epigenetic code," *Epigenetics*, vol. 9, no. 1, pp. 21–26, 2014.

[20] J. E. Wilusz, H. Sunwoo, and D. L. Spector, "Long noncoding RNAs: functional surprises from the RNA world," *Genes & Development*, vol. 23, no. 13, pp. 1494–1504, 2009.

[21] H. Fagman, E. Amendola, L. Parrillo et al., "Gene expression profiling at early organogenesis reveals both common and diverse mechanisms in foregut patterning," *Developmental Biology*, vol. 359, no. 2, pp. 163–175, 2011.

[22] NCBI Resource Coordinators, "Database resources of the national center for biotechnology information," *Nucleic Acids Research*, vol. 45, no. D1, pp. D12–D17, 2017.

[23] C. Tyner, G. P. Barber, J. Casper et al., "The UCSC genome browser database: 2017 update," *Nucleic Acids Research*, vol. 45, no. D1, pp. D626–D634, 2017.

[24] B. L. Aken, P. Achuthan, W. Akanni et al., "Ensembl 2017," *Nucleic Acids Research*, vol. 45, no. D1, pp. D635–D642, 2017.

[25] M. De Menna, V. D'Amato, A. Ferraro et al., "Wnt4 inhibits cell motility induced by oncogenic Ras," *Oncogene*, vol. 32, no. 35, pp. 4110–4119, 2013.

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Review Article

MicroRNAs as Biomarkers in Thyroid Carcinoma

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Optimal management of patients with thyroid cancer requires the use of sensitive and specific biomarkers. For early diagnosis and effective follow-up, the currently available cytological and serum biomarkers, thyroglobulin and calcitonin, present severe limitations. Research on microRNA expression in thyroid tumors is providing new insights for the development of novel biomarkers that can be used to diagnose thyroid cancer and optimize its management. In this review, we will examine some of the methods commonly used to detect and quantify microRNA in biospecimens from patients with thyroid tumor, as well as the potential applications of these techniques for developing microRNA-based biomarkers for the diagnosis and prognostic evaluation of thyroid cancers.

1. Introduction

Thyroid cancer is the most frequently diagnosed endocrine malignancy, and its prevalence has increased markedly over the last decade [1]. Neoplastic transformation can occur in either the follicular or parafollicular cells of the gland. In the former case, the results range from differentiated tumors—papillary thyroid carcinomas (PTCs), follicular thyroid carcinomas (FTCs), and Hürthle cell carcinomas—to the rarer poorly differentiated and anaplastic thyroid carcinomas (PDTCs and ATCs, resp.). Transformation of the parafollicular cells produces medullary thyroid carcinomas (MTCs). Approximately, a percentage of MTCs are familial, and this category includes those diagnosed as part of the multiple endocrine neoplasia type 2 syndrome [2]. Approximately, 80% of all differentiated thyroid carcinomas (DTCs) are PTCs. These tumors have a very good prognosis, thanks to the available tool (cytological examination of fine-needle aspiration biopsy (FNAB)) which allows an early diagnosis and the efficacy of the current treatment. It involves surgery and radioactive iodine to eliminate residual and/or locoregionally recurrent disease and, in some cases, also distant metastases. This approach is not an option for patients with MTCs or for those whose tumors (PDTCs and ATCs for the most part) are no longer able to concentrate iodine. This defect is the result of impaired expression/function of the sodium/iodine symporter (NIS) or thyroperoxidase (TPO) caused by oncogene-activated signaling that leads to thyrocyte dedifferentiation [3–5]. For these tumors, novel therapeutic strategies are being actively investigated [6, 7].

For many years, assays of serum thyroglobulin and calcitonin levels have played important roles in the diagnosis and follow-up of thyroid cancer [8, 9]. Thyroglobulin is produced exclusively by follicular thyroid cells. In patients with DTC who have undergone total thyroidectomy and radioiodine remnant ablation, its presence in the serum is thus considered a marker of persistent or recurrent disease (locoregional or at distant site metastases) [10]. Calcitonin, a product of the parafollicular C-cells, serves a similar purpose in the follow-up of patients operated on for MTC [11]. Both markers, however, have several well-documented limitations involving specificity and sensitivity [11, 12], and with the increasing prevalence of thyroid malignancy, the need for noninvasive thyroid cancer biomarkers with higher accuracy, sensitivity,

and specificity has become more pressing. Interest in this field has been sparked by our increasing understanding of the expression of microRNAs (miRNAs) in patients with thyroid tumors [13]. In this review, we will examine the growing body of evidence supporting the use of these small, noncoding RNA species to diagnose and predict the behavior of thyroid cancers, as well as the techniques currently used to detect and quantify their presence in tissues and other biological samples.

2. miRNAs in Thyroid Cancer

miRNAs are endogenous, noncoding RNAs with lengths ranging from 19 to 25 nucleotides. They play major roles in the posttranscriptional regulation of gene expression [14–16]. In general, miRNAs downmodulate the expression of a target gene by diminishing the stability of its transcript and/or inhibiting its translation [14–16]. By reducing the abundance of specific proteins in this manner, miRNAs exert fundamental modulatory effects on many physiological processes, including those involved in pre- and postnatal developments. Therefore, it is not surprising that their dysregulated expression is a feature of several pathological conditions, including neoplastic disease [17–20]. The first description of a link between aberrant miRNA expression and cancer was published in 2002 [21]. Since then, the number of miRNAs known to be encoded by the human genome has grown rapidly. A recent look at the miRBase database revealed over 2000 annotated human miRNAs [22], and their numbers are expected to increase [23].

The first published information on the role of miRNAs in thyroid tumorigenesis emerged in 2005 [24] and was followed by several other studies focusing on this issue. Thyroid tumors (and other cancers as well) display alterations involving various components of the machinery responsible for the complex process of miRNA biogenesis. Downregulated transcription of DICER has recently been observed in malignant thyroid tissues and cell lines, as compared with normal thyroid tissues and benign thyroid neoplasms, and this alteration was correlated with features indicative of tumor aggressiveness (extrathyroidal extension, lymph node and distant metastases, and recurrence) and with the presence of the *BRAFV600E* mutation [25]. A large cohort study of MTCs found that tumors harboring *RET* mutations exhibited upregulated expression of certain genes involved in miRNA biogenesis, as compared with their RET-wildtype counterparts, while no significant differences were observed between the expression levels of these genes in *RAS*-mutant and *RAS*-wildtype MTCs [26].

Specific patterns of miRNA expression have also been identified in a large number of studies performed on thyroid carcinomas [21, 27–30], and several miRNAs were found overexpressed or downregulated in major types of thyroid tumors [13, 31, 32]. Recent meta-analyses have attempted to provide a clearer overview of the miRNAs most commonly dysregulated in specific thyroid cancer histotypes. Several groups have reported overexpression of miRNA-146b, miRNA-221, miRNA-222, and miRNA-181b in PTCs, as compared with levels in normal thyroid tissues, and this upregulation is positively correlated with tumor aggressiveness [33–37]. Three of these four miRNAs, miRNA-146b, miRNA-221, and miRNA-222, are also upregulated in FTC, Hürthle cell thyroid carcinomas, and ATC [38–40]. In contrast, miRNA-197 and miRNA-346 are upregulated specifically in FTC [29, 41]. Members of the miRNA-17-92 cluster are highly expressed in ATC, as they are in other aggressive cancers [39], suggesting that dysregulation of this miRNA cluster influences the oncogenic process. Of note, increased expression of miRNAs-21, miRNA-183, and miRNA-375 has been associated with persistent and metastatic disease in MTC patients [42]. miRNA downregulations appear to be more variably associated with specific types of thyroid cancer. An exception is the downregulated expression of miRNAs belonging to the miRNA-200 and miRNA-30 families, which is associated exclusively with ATCs and is therefore suspected to play key roles in the acquisition of particularly aggressive tumor phenotypes [13, 39]. Table 1 shows the main miRNA dysregulations found in thyroid tumors, together with their documented associations with oncogenic mutations and their validated molecular targets. Among the latter, a functional role in oncogenic transformation of thyroid cancer cells is played by proto-oncogene receptor tyrosine kinase (KIT), C-X-C motif chemokine ligand 12 (CXCL12), connective tissue growth factor (CTGF), NF-kB, programmed cell death 4 (PDCD4), and yes-associated protein (YAP) (see Table 1), all involved in the regulation of cell proliferation, migration, invasion, and survival.

3. miRNA Detection in Biological Samples

miRNA expression patterns can be rich sources of biological information. Analysis of variations in these patterns can provide clues as to how different cellular processes are modulated under both physiological and pathological conditions [54]. Various diseases are associated with significant changes in the miRNA profile of involved tissues, and most of these changes have been reported in different kinds of cancer [55]. miRNAs display good stability in a variety of human biospecimens, including cell lines, fresh-frozen and formalin-fixed tissues, FNAB, blood plasma and serum, and urine [56]. Moreover, their levels provide more immediate and also more specific information on current physiological and pathological conditions than other molecules in these specimens [57]. Their importance in the modulation of gene expression and their remarkable stability in human biospecimens have led to develop a variety of approaches, and platforms have been developed to isolate and study miRNA expression, with the aim to identify profiles or single miRNAs associated with specific pathological condition.

miRNA profiling begins with the isolation of total RNA. The extraction protocols used for this purpose are often slightly modified to enrich the fraction containing miRNAs and other small RNA species. Widely used methods for miRNA extraction fall into two main categories: chemical methods and column-based methods. The principal advantages and disadvantages of each are summarized in Figure 1.

The isolated miRNA is then quantified and subjected to quality assessment. The quantity obtained is specimen

Table 1: Known targets for deregulated miRNAs in thyroid tumors and association with genetic alterations.

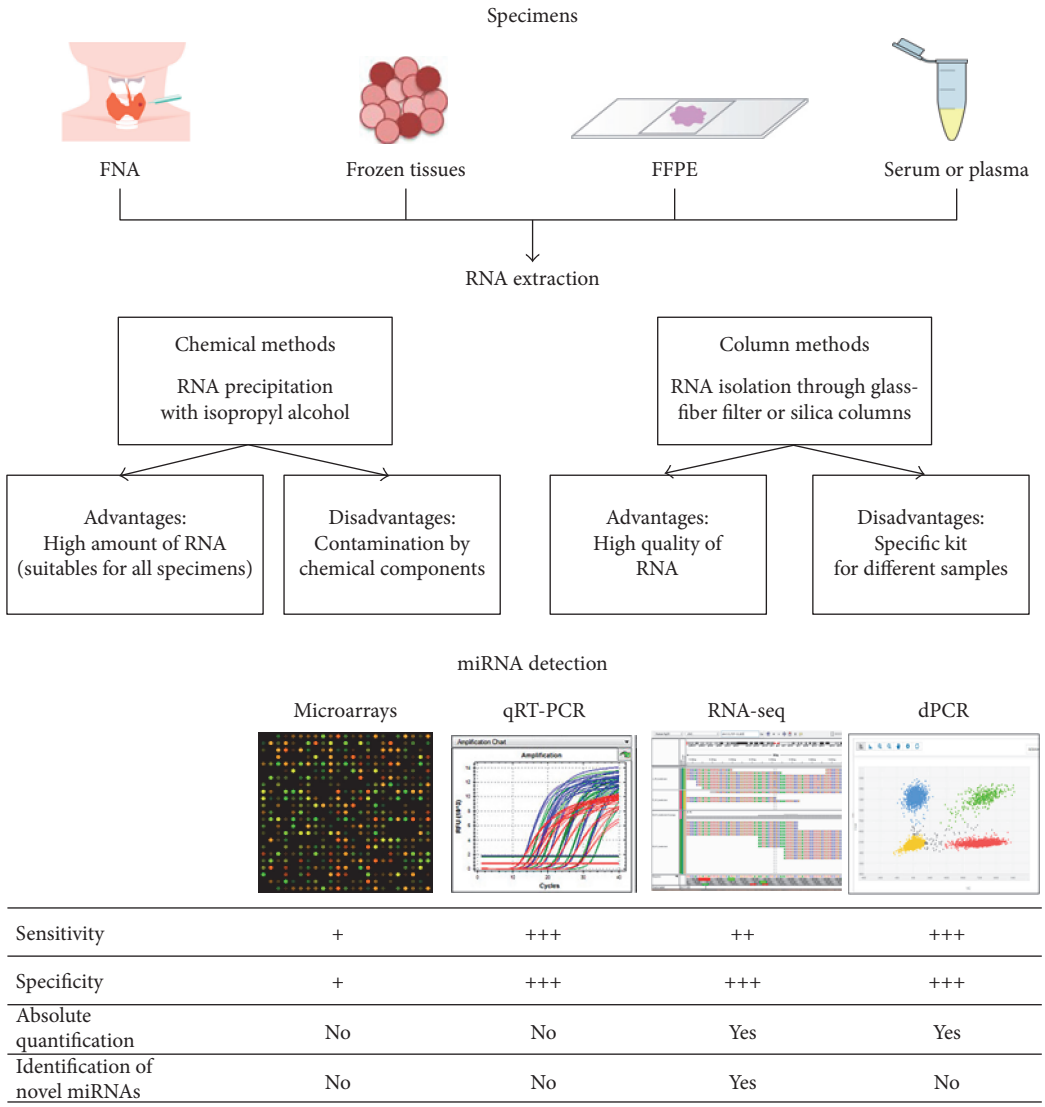
Histotype	miRNA expression (↑/↓)*	Oncogenic alteration	Molecular target	Reference
PTC	↑ 146b, 221, 222	n. d.	KIT	[24]
	↑ 181b, 221, 222	n. d.	n. d.	[43]
	↑ 187	RET/PTC, RAS	n. d.	[27]
	↑ 146b, 221, 222; ↓ 187	BRAF V600E		
	↑ 146b	BRAF V600E	n. d.	[33]
	↑ 221	BRAF V600E	n. d.	[34]
**	↑ 451	n.d.	n.d.	[44]
	↓ 137	n. d.	CXCL12	[45]
	↓ 451a	n.d.	n.d.	[46]
FTC	↑ 197, 346	n. d.	n. d.	[42]
	↑ 181b, 187	n. d.	n. d.	[27]
	↑ 221 ↓ 574-3p	n. d.	n. d.	[29]
	↑ 146b, 183, 221 ↓ 199b	n. d.	n. d.	[38]
	↓ 199a-5p	n. d.	CTGF	[47]
Hürtle	↑ 187, 197	n. d.	n. d.	[27]
	↑ 885-5p	n. d.	n. d.	[29]
	↑ 885-5p ↓ 138, 768-3p	n. d.	n. d.	[40]
ATC	↑ 137, 205, 302c	n. d.	n. d.	[27]
	↑ 221, 222	n. d.	n. d.	[48]
	↑ 146a	n. d.	NF-kB	[49]
	↓ 30, 200	n. d.	n. d.	[39]
MTC	↑ 130a, 138, 193a-3p, 373, 498 ↓7, 10a,29c, 200b-200c	n. d.	n. d.	[50]
	↑ 9, 21, 127, 154, 183, 224, 323, 370, 375	n. d.	PDCD4	[28, 51]
	↓ 129-5p	RET	n. d.	[30]
	↑ 183, 375	n. d.	n. d.	[52]
	↑ 10a, 375 ↓455	n.d.	YAP	[53]

(*) ↑/↓: upregulated/downregulated; (**): PTC with lymph node metastasis. ATC: anaplastic thyroid carcinoma; BRAF: b-type rapidly accelerated fibrosarcoma; CTGF: connective tissue growth factor; CXCL12: C-X-C motif chemokine ligand 12; FTC: follicular thyroid carcinoma; KIT: proto-oncogene receptor tyrosine kinase; MTC: medullary thyroid carcinoma; n. d.: not determined; PDCD4: programmed cell death 4; PTC: papillary thyroid carcinoma; RAS: rat sarcoma; RET/PTC: rearranged during transfection/papillary thyroid carcinoma; YAP: yes-associated protein.

specific, whereas the quality of the RNA depends on the extraction method used. The RNA is then ready for miRNA profiling. Four well-established methods are currently used to analyze miRNA expression: microarrays, quantitative reverse-transcription PCR (qRT-PCR), high-throughput sequencing (RNA-seq), and digital PCR (dPCR).

Microarray analysis was one of the first methods used for parallel analysis of large numbers of miRNAs. The miRNAs in a biological sample are labeled using fluorescent, chemical, or enzymatic techniques and then hybridized to DNA-based probes on the array. Microarray-based profiling allows rapid processing of the high number, and its cost is relatively low cost. However, it is the least sensitive and least specific of the miRNA profiling methods, and it does not allow the identification of novel targets [58].

QRT-PCR is probably the most popular method currently used for miRNA detection. It entails reverse transcription of miRNA to cDNA, followed by real-time monitoring of the accumulation of polymerase reaction products. Commercially available, customizable plates and microfluidic cards can be designed to examine a small set of miRNAs or to provide more comprehensive coverage. For qRT-PCR detection of hundreds of miRNAs, platforms are available with preplated PCR primers distributed across microfluidic cards containing nanoliter-scale wells. This approach is more specific and sensitive than microarray profiling. An internal control must be used for relative quantification of the expression; a standard curve can be used to obtain absolute quantification. Like microarray profiling, qRT-PCR cannot identify novel miRNAs [59].



Sensitivity	+	+++	++	+++
Specificity	+	+++	+++	+++
Absolute quantification	No	No	Yes	Yes
Identification of novel miRNAs	No	No	Yes	No

Figure 1: miRNA detection workflow. miRNAs can be isolated from different biospecimens. To isolate miRNAs, widely used methods are chemical and column-based techniques. After quantification step, samples are ready for miRNA profiling. Among widely used techniques, there are four established methods: microarray, quantitative PCR (qRT-PCR), massive parallel sequencing (RNA-seq), and digital PCR (dPCR). The sensitivity and specificity are classified as follows: + (low), ++ (moderate), +++ (high). FFPE: formalin-fixed paraffin embedded; FNA: fine-needle aspiration.

RNA-seq is currently the most expensive technique for miRNA profiling, but it is also the most informative. It provides quantification data as well as sequence data and can therefore be used to identify novel miRNAs or sequence variations. A cDNA library of small RNAs is prepared from the samples of interest. This is followed by an adaptor ligation step and immobilization of the cDNA on a support (solid phase for solid-phase PCR, bead-based for emulsion PCR). These steps are followed by massively parallel sequencing of millions of cDNA molecules from the library. This approach allows simultaneous analysis of the expression patterns of a huge number of targets [60].

Digital PCR allows quantitative analysis of miRNA expression without internal controls. It is the most sensitive technique and the only one that can directly quantify miRNA in terms of absolute copy numbers. It involves the partitioning of a cDNA sample into multiple parallel PCR reactions. The reaction is performed with nanofluidics partitioning or emulsion chemistry, based on the random distribution of the sample on a specific support. It is superior to previously described methods in terms of sensitivity and precision, and it is the technique most widely used to study miRNA expression in plasma or serum samples, where there are no stable endogenous controls [61].

4. miRNAs as Diagnostic Markers in Thyroid Cancer

After clinical and ultrasound assessment of the likelihood of malignancy, most thyroid nodules are subjected to FNAB for cytological examination [62, 63]. This approach has shown good accuracy in discriminating most DTCs from

Table 2: Studies of miRNAs in FNAB samples.

Samples	Histological diagnosis*	miRNA expression (↑/↓)**	Reference
8 (malignant)	PTC	↑ 181b, 221, 222	[43]
62 (8 malignant, 5 benign, 49 n.d.)	7 PTC, 1 Hürtle	↑ 146b, 155, 187, 197, 221; 222, 224	[27]
115 (37 malignant, 78 benign)	10 FTC or Hürtle (27 n.d.)	↑ 138	[65]
27 (20 malignant, 7 benign)	PTC	↑ 221	[66]
128 (88 malignant, 40 benign)	3 ATC, 13 FTC, 72 PTC	↑ 146b, 187, 221 ↓ 30d	[67]
141 (58 malignant, 83 benign)	58 PTC	↑ 146b, 155, 221	[68]
118 (70 malignant, 48 benign)	70 PTC	↑ 146b, 222	[69]
120 (45 malignant, 75 benign)	1 FTC, 2 ATC, 4 MTC, 8 Hürtle, 30 PTC	↑ 221, 222	[70]
44 (24 malignant, 20 benign)	24 FTC	↓ 148b-3p, 484	[64]

(*) related to malign samples; (**) ↑/↓: upregulated/downregulated. ATC: anaplastic thyroid carcinoma; FNAB: fine-needle aspiration biopsy; FTC: follicular thyroid carcinoma; MTC: medullary thyroid carcinoma; PTC: papillary thyroid carcinoma.

benign lesions. However, in a nonnegligible proportion of cases, the cytology is indeterminate [10]. In these cases, the evaluation of molecular markers in the aspirate can often allow more confident presurgical differentiation of benign and malignant lesions. miRNAs are one of the novel classes of molecular markers that are being used to improve the diagnosis of thyroid cancer [13, 64]. Several studies have shown that a miRNA-based signature in FNABs can be used to discriminate benign from malignant thyroid nodules (Table 2).

A recent meta-analysis [71] of 543 patients with benign ($n = 277$) or malignant ($n = 266$) thyroid nodules indicates that miRNA analysis of fine-needle aspirates (FNAs) allows significantly more accurate individuation of the malignant lesions than conventional cytology. More recently, Paskas et al. [70] assessed the performance of a panel of four markers, the BRAF V600E mutation, miRNA-221, miRNA-222, and galectin-3 protein, developing an algorithm for distinguishing benign and malignant thyroid nodules. In particular, among the 120 nodules of the study, the proposed algorithm classified 62 cases as benign (against the 75 cases observed with the conventional cytology classification), 9 false negative cases, and 6 false positive cases, with a sensitivity of 73.5%, a specificity of 89.8%, and an accuracy of 75.7%, thereby allowing over half the patient cohort to avoid unnecessary surgery. In a cohort of 118 samples of PTCs, Panebianco et al. [69] developed a statistical model that accurately differentiates malignant from benign indeterminate lesions on thyroid FNAs using a panel of two miRNAs and two genes (miRNA-146b, miRNA 222, KIT, and TC1). More recently, Stokowy et al. [64] have identified that two miRNA markers might improve the classification of mutation-negative thyroid nodules with indeterminate FNA. In this study, it was observed that miRNA-484 and miRNA-148b-3p identify thyroid malignancy with a sensitivity of 89% and a specificity of 87% in a subset of 44 FNA samples.

As for ATCs, most of the studies conducted thus far have failed to produce statistically significant data since the number of tumor samples examined is invariably low [67, 72]. At present, no data are available on the potential of miRNA assays for diagnosis of MTC.

Recently, improved diagnosis of cancer has been achieved by assaying cancer-derived materials isolated from peripheral blood samples [73]. These "liquid biopsies" provide a genetic snapshot of the whole-tumor landscape, including both primary and metastatic lesions [74]. Relatively few reports are available on the expression and clinical significance of circulating miRNAs in patients with thyroid cancer, particularly those with less common tumors, such as MTC, PDTC, and ATC. As shown in Table 3, the studies published to date have focused mainly on patients with PTC, but the results are nonetheless characterized by high variability. Several elements can contribute to these highly variable results, including the number of patients of each study and/or sample-related factors (i.e., gender, sample collection time), preanalytical factors (i.e., sample type, storage conditions, and/or sample processing), and experiment-related factors (i.e., RNA extraction protocol, quantification methods). In addition, only few studies reported the isoforms of the miRNAs identified. Circulating levels of miRNA-146b-5p, miRNA-221-3p, and miRNA-222-3p in PTC patients have been found to be higher than those in healthy controls [75, 76, 84], while miRNA-222 and miRNA-146b levels also reportedly discriminate between PTCs and benign nodules [75, 80, 83]. Plasma levels of miRNA-21 in FTC patients are reportedly higher than those found in patients with benign nodules or PTC, whereas miRNA-181a is more highly expressed in PTC patients than in those with FTC [81]. In PTC patients, circulating levels of miRNA-146a-5p, miRNA-146b-5p, miRNA-221-3p, and miRNA-222-3p have been shown to decline after tumor excision [75, 76, 83, 84].

5. miRNAs as Prognostic Markers in Thyroid Cancer

miRNA profiling of thyroid cancers can also provide prognostic information useful for defining optimal management strategies. Recent studies have demonstrated that expression levels of certain miRNAs in thyroid tumor tissues are associated with clinic-pathological characteristics, such as tumor size, multifocality, capsular invasion, extrathyroidal extension, and both lymph node and distant metastases. Tumor

Table 3: Circulating miRNAs as diagnostic biomarkers in thyroid carcinoma.

Histotype	Sample type	miRNA	Up/downregulated	Reference
PTC	Serum	let-7e, 151-5p, 222	Up	[75]
	Plasma	146b, 222	Up	[76]
	Serum	190	Up	[77]
		95	Down	
	Plasma	hsa-let7b-5p, hsa-miR-10a-5p, hsa-miR-93-5p, hsa-miR-191	Up	[78]
		hsa-miR-146a-5p, hsa-miR-150-5p, hsa-miR-199b-3p, has-miR-342-3p	Down	
	Plasma	let-7i, 25-3p, 140-3p, 451a	Up	[79]
	Plasma	146b, 155	Up	[80]
	Plasma-derived exosomes	31-5p, 126-3p, 145-5p, 181a	Up	[81]
	Plasma	9-3p, 124-3p	Up	[82]
	Serum	222	Up	[83]
		21	Down	
	Serum	24-3p, 28-3p, 103a-3p, 146a-5p, 146b-5p, 191-5p, 221-3p, 222-3p	Up	[84]
FTC	Plasma-derived exosomes	21	Up	[81]

FTC: follicular thyroid carcinoma; PTC: papillary thyroid carcinoma.

size displays associations with tissue levels of miRNA-221, miRNA-222, miRNA-135b, miRNA-181b, miRNA-146a, and miRNA-146b [35, 85–87]. The latter two are also associated with multifocality [86, 87]. The single study that analyzed the association between miRNA expression and capsular or vascular invasion identified three miRNAs (miRNA-146b, miRNA-221, and miRNA-222), whose levels were significantly elevated in tumor tissue samples of PTC that had invaded vascular structures and/or the capsule. Extrathyroidal extension has been associated with higher levels of miRNA-221, miRNA-222, miRNA-146a, miRNA-146b, miRNA-199b-5p, and miRNA-135b [35, 87–89]. Expression levels of miRNA-221, miRNA-222, miRNA-21-3p, miRNA-146a, miRNA-146b, and miRNA-199b-5p are reportedly higher in patients with lymph node metastases [85, 86, 89, 90], and miRNA-146b and miRNA-221 are also associated with the presence of distant metastases [86]. Chou and coworkers showed that overall survival rates among patients with higher miRNA-146b expression levels are significantly decreased relative to those associated with lower tumor levels of this miRNA. Overexpression of miRNA-146b significantly increases cell proliferation, migration, and invasiveness and causes resistance to chemotherapy-induced apoptosis [91]. Higher levels of miRNA-146a, miRNA-146b, miRNA-221, and miRNA-222 display positive associations with higher TNM stage (III/IV versus I/II) [35, 85–87]. Risk of recurrence, defined according to the American Thyroid Association (ATA) guidelines, has been positively associated with higher expression of miRNA-146b-5p, miRNA-146b-3p, miRNA-21-5p, miRNA-221, miRNA-222-3p, miRNA-31-5p, miRNA-199a-3p/miRNA-199b-3p, miRNA-125b, and miRNA-203 and lower expression levels of miRNA-1179, miRNA-7-2-3p, miRNA-204-5p, miRNA-138, miRNA-30a, and let-7c [37, 92].

Notably, the studies and findings discussed above are related exclusively to PTCs (Table 4).

Only two studies have investigated the role of miRNAs as prognostic markers in FTC and MTC [52, 93]. The study by Jikuzono and coworkers involved a comprehensive quantitative analysis of miRNA expression in tumor tissue from minimally invasive FTCs (MI-FTC) [93]. The subgroup of tumors that had metastasized ($n = 12$) exhibited significantly higher levels of miRNA-221-3p, miRNA-222-3p, miRNA-222-5p, miRNA-10b, and miRNA-92a than the nonmetastatic subgroup ($n = 22$). Expression of these miRNAs was also upregulated in widely invasive FTCs (WI-FTC; $n = 13$), which are characterized by distant metastasis and a worse prognosis. Logistic regression analysis identified one of these miRNAs, miRNA-10b, as a potential tool for predicting outcomes in cases of metastatic MI-FTC [93]. The second study, conducted by Abraham and coworkers, found that overexpression of miRNA-183 and miRNA-375 in MTCs ($n = 45$) was associated with lateral lymph node metastasis, residual disease, distant metastases, and mortality [52].

Recent studies have also looked at circulating miRNAs in patients with PTCs, which are showing undeniable promise as novel predictors of early disease relapse (Table 5).

In these patients, circulating levels of miRNA-146b-5p, miRNA-221-3p, miRNA-222-3p, and miRNA-146a-5p have been shown to decline after tumor excision [75, 76, 81, 83, 84]. Notably, serum levels of miRNA-221-3p and miRNA-146a-5p also appear to predict clinical responses to treatment, with significantly increased levels observed at the 2-year follow-up in PTC patients with structural evidence of disease, including some whose serum thyroglobulin assays remained persistently negative [84]. The association of thyroid cancer with circulating levels of miRNA-146b-5p, miRNA-221-3p, and miRNA-222-3p has been strengthened

Table 4: Tissue miRNAs as prognostic biomarkers in PTC.

miRNA	Tumor size	Multifocality	Capsular invasion	Vascular invasion	ETE	LN metastases	Distant metastases	Overall survival	TNM stage	ATA risk	References
1179										*	Rosignolo et al. [37]
125b										*	Geraldo and Kimura [92]
135b	*				*						Wang et al. [35]
138										*	Geraldo and Kimura [92]
146a		*			*	*			*		Sun et al. [87]
146b	*	*	*	*	*	*	*	*	*	*	Wang et al. [35], Acibucu et al. [86], Sun et al. [87], Chou et al. [91], Geraldo and Kimura [92]
146b-3p										*	Rosignolo et al. [37]
146b-5p										*	Rosignolo et al. [37]
181b	*										Sun et al. [85]
199a-3p/199b-3p										*	Rosignolo et al. [37]
											Rosignolo et al. [37]
199b-5p					*	*					Peng et al. [89]
203										*	Geraldo and Kimura [92]
204-5p										*	Rosignolo et al. [37]
21										*	Geraldo and Kimura [92]
21-3p						*					Huang et al. [90]
21-5p										*	Rosignolo et al. [37]
221	*		*	**	*	*	*		*	*	Wang et al. [35], Sun et al. [85], Acibucu et al. [86], Wang et al. [88], Geraldo and Kimura [92]
222	*		*	*	*	*			*	*	Wang et al. [35], Sun et al. [85], Acibucu et al. [86], Wang et al. [88], Geraldo and Kimura [92]
222-3p										*	Rosignolo et al. [37]
30a										*	Geraldo and Kimura [92]
31-5p										*	Rosignolo et al. [37]
7–2-3p										*	Rosignolo et al. [37]
let-7c										*	Geraldo and Kimura [92]

*: information included in indicated studies; ATA: American Thyroid Association; ETE: extrathyroidal extension; LN: lymph node.

Table 5: Circulating miRNAs as prognostic biomarkers for PTC follow-up.

Study	Number of cases	Samples	miRNA	Findings
Yu et al. [75]	9	Pre- and postoperative serum (5–15 days)	151-5p, 222	Decreased after tumor excision
Lee et al. [76]	32	Pre- and postoperative plasma (2–6 weeks)	221, 222, 146b	Decreased after tumor excision
Li et al. [79]	7	Pre- and postoperative plasma (4–7 days)	25-3p, 451a	Decreased after tumor excision
Samsonov et al. [81]	10	Pre- and postoperative plasma-derived exosomes (7–10 days)	126-3p, 145-5p, 146a-5p, 181a-5p, 206, 21-5p, 221-3p, 223-3p, 31-5p	Decreased after tumor excision
Yoruker et al. [83]	31	Pre- and postoperative serum (5 weeks)	221, 222, 151-5p, 31	Decreased after tumor excision
Rosignolo et al. [84]	44	Pre- and postoperative serum (30 days)	146a-5p, 221-3p, 222-3p, 146b-5p, 28-3p, 103a-3p, 191-5p, 24-3p	Decreased after tumor excision

PTC: papillary thyroid carcinoma.

by evidence of their upregulated expression in PTC [37, 94], FTC [27, 95], and ATC [27] tissues and by their association with tumor aggressiveness.

6. Conclusion

Analysis of miRNA expression levels and detection of circulating miRNAs can be used for the early diagnosis of thyroid cancer and for monitoring treatment responses. Compared with circulating messenger RNAs, circulating miRNAs are emerging as more promising biomarker candidates because they are more stable and tissue specific. miRNAs can also be assessed in other biological samples, such as FNABs, which can be obtained with minimally invasive procedures to easily identify a specific profile, which makes specific miRNAs optimal diagnostic and/or prognostic biomarkers. Improved standardization of methods used to assay circulating miRNAs will allow more extensive use of this approach in defining individualized treatment strategies for thyroid cancer patients.

Conflicts of Interest

The authors declare no conflict of interests.

Authors' Contributions

Marilena Celano and Francesca Rosignolo contributed equally to this work.

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References

[1] C. M. Kitahara and J. A. Sosa, "The changing incidence of thyroid cancer," *Nature Reviewes Endocrinology*, vol. 12, no. 11, pp. 646–653, 2016.

[2] M. E. Cabanillas, D. G. McFadden, and C. Durante, "Thyroid cancer," *Lancet*, vol. 388, no. 10061, pp. 2783–2795, 2016.

[3] M. Schlumberger, L. Lacroix, D. Russo, S. Filetti, and J. M. Bidart, "Defects in iodide metabolism in thyroid cancer and implications for the follow-up and treatment of patients," *Nature Clinical Practice Endocrinology & Metabolism*, vol. 3, no. 3, pp. 260–269, 2007.

[4] E. Puxeddu, C. Durante, N. Avenia, S. Filetti, and D. Russo, "Clinical implications of BRAF mutation in thyroid carcinoma," *Trends in Endocrinology and Metabolism*, vol. 19, no. 4, pp. 138–145, 2008.

[5] M. Xing, "Molecular pathogenesis and mechanisms of thyroid cancer," *Nature Reviews Cancer*, vol. 13, no. 3, pp. 184–199, 2013.

[6] S. Bulotta, M. Celano, G. Costante, and D. Russo, "Emerging strategies for managing differentiated thyroid cancers refractory to radioiodine," *Endocrine*, vol. 52, pp. 214–221, 2016.

[7] V. Ernani, M. Kumar, A. Y. Chen, and T. K. Owonikoko, "Systemic treatment and management approaches for medullary thyroid cancer," *Cancer Treatment Reviews*, vol. 50, pp. 89–98, 2016.

[8] G. Costante, C. Durante, Z. Francis, M. Schlumberger, and S. Filetti, "Determination of calcitonin levels in C-cell disease: clinical interest and potential pitfalls," *Nature Clinical Practice. Endocrinology & Metabolism*, vol. 5, no. 1, pp. 35–44, 2009.

[9] A. G. Gianoukakis, "Thyroglobulin antibody status and differentiated thyroid cancer: what does it mean for prognosis and surveillance?," *Current Opinion in Oncology*, vol. 27, no. 1, pp. 26–32, 2015.

[10] B. R. Haugen, E. K. Alexander, K. C. Bible et al., "2015 American Thyroid Association management guidelines for adult patients with thyroid nodules and differentiated thyroid cancer: the American Thyroid Association guidelines task force on thyroid nodules and differentiated thyroid cancer," *Thyroid*, vol. 26, no. 1, pp. 1–133, 2016.

[11] Y. J. Bae, M. Schaab, and J. Kratzsch, "Calcitonin as biomarker for the medullary thyroid carcinoma," *Recent Results in Cancer Research*, vol. 204, pp. 117–137, 2015.

[12] B. S. Indrasena, "Use of thyroglobulin as a tumour marker," *World Journal of Biological Chemistry*, vol. 8, no. 1, pp. 81–85, 2017.

[13] M. Boufraqech, J. Klubo-Gwiezdzinska, and E. Kebebew, "MicroRNAs in the thyroid," *Best Practice & Research. Clinical Endocrinology & Metabolism*, vol. 30, no. 5, pp. 603–619, 2016.

[14] D. P. Bartel, "MicroRNAs: target recognition and regulatory functions," *Cell*, vol. 136, no. 2, pp. 215–233, 2009.

[15] G. C. Shukla, J. Singh, and S. Barik, "MicroRNAs: processing, maturation, target recognition and regulatory functions," *Molecular and Cellular Pharmacology*, vol. 3, no. 3, pp. 83–92, 2011.

[16] S. Oliveto, M. Mancino, N. Manfrini, and S. Biffo, "Role of microRNAs in translation regulation and cancer," *World Journal of Biological Chemistry*, vol. 8, no. 1, pp. 45–56, 2017.

[17] M. Lagos-Quintana, R. Rauhut, W. Lendeckel, and T. Tuschl, "Identification of novel genes coding for small expressed RNAs," *Science*, vol. 294, no. 5542, pp. 853–858, 2001.

[18] J. Lu, G. Getz, E. A. Miska et al., "MicroRNA expression profiles classify human cancers," *Nature*, vol. 435, no. 7043, pp. 834–838, 2005.

[19] A. M. Ardekani and M. M. Naeini, "The role of MicroRNAs in human diseases," *Avicenna Journal of Medical Biotechnology*, vol. 2, no. 4, pp. 161–179, 2010.

[20] M. V. Iorio and C. M. Croce, "Causes and consequences of microRNA dysregulation," *Cancer Journal*, vol. 18, no. 3, pp. 215–222, 2012.

[21] G. A. Calin, C. D. Dumitru, M. Shimizu et al., "Frequent deletions and downregulation of micro- RNA genes miR15 and miR16 a 13q14 in chronic lymphocytic leukemia," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 24, pp. 15524–15529, 2002.

[22] A. Kozomara and S. Griffiths-Jones, "miRBase: annotating high confidence microRNAs using deep sequencing data," *Nucleic Acids Research*, vol. 42, pp. D68–D73, 2014.

[23] E. Londin, P. Loher, A. G. Telonis et al., "Analysis of 13 cell types reveals evidence for the expression of numerous novel primate- and tissue-specific microRNAs," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 112, no. 10, pp. E1106–E1115, 2015.

[24] H. He, K. Jazdzewski, W. Li et al., "The role of microRNA genes in papillary thyroid carcinoma," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 52, pp. 19075–19080, 2005.

[25] P. Erler, X. M. Keutgen, M. J. Crowley et al., "Dicer expression and microRNA dysregulation associate with aggressive features in thyroid cancer," *Surgery*, vol. 156, no. 6, pp. 1342–1350, 2014.

[26] C. Puppin, C. Durante, M. Sponziello et al., "Overexpression of genes involved in miRNA biogenesis in medullary thyroid carcinomas with RET mutation," *Endocrine*, vol. 47, no. 2, pp. 528–536, 2014.

[27] M. N. Nikiforova, G. C. Tseng, D. Steward, D. Diorio, and Y. E. Nikiforov, "MicroRNA expression profiling of thyroid tumors: biological significance and diagnostic utility," *The Journal of Clinical Endocrinology and Metabolism*, vol. 93, no. 5, pp. 1600–1608, 2008.

[28] C. Mian, G. Pennelli, M. Fassan et al., "MicroRNA profiles in familial and sporadic medullary thyroid carcinoma: preliminary relationships with RET status and outcome," *Thyroid*, vol. 22, no. 9, pp. 890–896, 2012.

[29] M. Dettmer, A. Vogetseder, M. B. Durso et al., "MicroRNA expression array identifies novel diagnostic markers for conventional and oncocytic follicular thyroid carcinomas," *The Journal of Clinical Endocrinology and Metabolism*, vol. 98, no. 1, pp. E1–E7, 2013.

[30] L. Duan, X. Hao, Z. Liu, Y. Zhang, and G. Zhang, "MiR-129-5p is down-regulated and involved in the growth, apoptosis and migration of medullary thyroid carcinoma cells through targeting RET," *FEBS Letters*, vol. 588, no. 9, pp. 1644–1651, 2014.

[31] M. N. Nikiforova, S. I. Chiosea, and Y. E. Nikiforov, "MicroRNA expression profiles in thyroid tumors," *Endocrine Pathology*, vol. 20, no. 2, pp. 85–91, 2009.

[32] C. S. Fuziwara and E. T. Kimura, "MicroRNAs in thyroid development, function and tumorigenesis," *Molecular and Cellular Endocrinology*, 2016, in press.

[33] C. K. Chou, R. F. Chen, F. F. Cho et al., "miR-146b is highly expressed in adult papillary thyroid carcinomas with high risk features including extrathyroidal invasion and the $BRAF^{V600E}$ mutation," *Thyroid*, vol. 20, no. 5, pp. 489–494, 2010.

[34] Y. L. Zhou, C. Liu, X. X. Dai, X. H. Zhang, and O. C. Wang, "Overexpression of miR-221 is associated with aggressive clinicopathologic characteristics and the BRAF mutation in papillary thyroid carcinomas," *Medical Oncology*, vol. 29, no. 5, pp. 3360–3366, 2012.

[35] Z. Wang, H. Zhang, L. He et al., "Association between the expression of four upregulated miRNAs and extrathyroidal invasion in papillary thyroid carcinoma," *OncoTargets and Therapy*, vol. 6, pp. 281–287, 2013.

[36] T. Stokowy, D. Gawel, and B. Wojtas, "Differences in miRNA and mRNA profile of papillary thyroid cancer variants," *International Journal of Endocrinology*, vol. 2016, Article ID 1427042, 10 pages, 2016.

[37] F. Rosignolo, L. Memeo, F. Monzani et al., "MicroRNA-based molecular classification of papillary thyroid carcinoma," *International Journal of Oncology*, vol. 50, no. 5, pp. 1767–1777, 2017.

[38] B. Wojtas, C. Ferraz, T. Stokowy et al., "Differential miRNA expression defines migration and reduced apoptosis in follicular thyroid carcinomas," *Molecular and Cellular Endocrinology*, vol. 388, no. 1-2, pp. 1–9, 2014.

[39] C. S. Fuziwara and E. T. Kimura, "MicroRNA deregulation in anaplastic thyroid cancer biology," *International Journal of Endocrinology*, vol. 2014, Article ID 743450, 8 pages, 2014.

[40] R. Petric, B. Gazic, K. Goricar, V. Dolzan, R. Dzodic, and N. Besic, "Expression of miRNA and occurrence of distant metastases in patients with Hürthle cell carcinoma," *International Journal of Endocrinology*, vol. 2016, Article ID 8945247, 6 pages, 2016.

[41] F. Weber, R. E. Teresi, C. E. Broelsch, A. Frilling, and C. Eng, "A limited set of human microRNA is deregulated in follicular thyroid carcinoma," *The Journal of Clinical Endocrinology & Metabolism*, vol. 91, no. 9, pp. 3584–3591, 2006.

[42] Y. H. Chu and R. V. Lloyd, "Medullary thyroid carcinoma: recent advances including microRNA expression," *Endocrine Pathology*, vol. 27, no. 4, pp. 312–324, 2016.

[43] P. Pallante, R. Visone, M. Ferracin et al., "MicroRNA deregulation in human thyroid papillary carcinomas," *Endocrine-Related Cancer*, vol. 13, no. 2, pp. 497–508, 2006.

[44] Z. Wang, H. Zhang, P. Zhang, J. Li, Z. Shan, and W. Teng, "Upregulation of miR-2861 and miR-451 expression in papillary thyroid carcinoma with lymph node metastasis," *Medical Oncology*, vol. 30, no. 2, p. 577, 2013.

[45] S. Dong, M. Jin, Y. Li, P. Ren, and J. Liu, "miR-137 acts as a tumor suppressor in papillary thyroid carcinoma by targeting CXCL12," *Oncology Reports*, vol. 35, no. 4, pp. 2151–2158, 2016.

[46] E. Minna, P. Romeo, M. Dugo et al., "miR-451a is underexpressed and targets AKT/mTOR pathway in papillary thyroid carcinoma," *Oncotarget*, vol. 7, no. 11, pp. 12731–12747, 2016.

[47] D. Sun, S. Han, C. Liu et al., "Microrna-199a-5p functions as a tumor suppressor via suppressing connective tissue growth factor (CTGF) in follicular thyroid carcinoma," *Medical Science Monitor*, vol. 22, pp. 1210–1217, 2016.

[48] S. Schwertheim, S. Sheu, K. Worm, F. Grabellus, and K. W. Schmid, "Analysis of deregulated miRNAs is helpful to distinguish poorly differentiated thyroid carcinoma from papillary thyroid carcinoma," *Hormone and Metabolic Research*, vol. 41, no. 6, pp. 475–481, 2009.

[49] F. Pacifico, E. Crescenzi, S. Mellone et al., "Nuclear factor-?b contributes to anaplastic thyroid carcinomas through up-regulation of miR-146a," *The Journal of Clinical Endocrinology and Metabolism*, vol. 95, no. 3, pp. 1421–1430, 2010.

[50] L. Santarpia, G. A. Calin, L. Adam et al., "A miRNA signature associated with human metastatic medullary thyroid carcinoma," *Endocrine-Related Cancer*, vol. 20, no. 6, pp. 809–823, 2013.

[51] G. Pennelli, F. Galuppini, S. Barollo et al., "The PDCD4/miR-21 pathway in medullary thyroid carcinoma," *Human Pathology*, vol. 46, no. 1, pp. 50–57, 2015.

[52] D. Abraham, N. Jackson, J. S. Gundara et al., "MicroRNA profiling of sporadic and hereditary medullary thyroid cancer identifies predictors of nodal metastasis, prognosis, and potential therapeutic targets," *Clinical Cancer Research*, vol. 17, no. 14, pp. 4772–4781, 2011.

[53] J. Hudson, E. Duncavage, A. Tamburrino et al., "Overexpression of miR-10a and miR-375 and downregulation of YAP1 in medullary thyroid carcinoma," *Experimental and Molecular Pathology*, vol. 95, no. 1, pp. 62–67, 2013.

[54] Y. Tay, J. Rinn, and P. P. Pandolfi, "The multilayered complexity of ceRNA crosstalk and competition," *Nature*, vol. 505, no. 7483, pp. 344–352, 2014.

[55] G. Di Leva, M. Garofalo, and C. Croce, "MicroRNAs in cancer," *Annual Review of Pathology*, vol. 9, pp. 287–314, 2014.

[56] J. A. Makarova, M. U. Shkurnikov, D. Wicklein et al., "Intracellular and extracellular microRNA: an update on localization and biological role," *Progress in Histochemistry and Cytochemistry*, vol. 51, no. 3-4, pp. 33–49, 2016.

[57] D. H. Buitrago, S. K. Patnaik, K. Kadota, E. Kannisto, D. R. Jone, and P. S. Adusumilli, "Small RNA sequencing for profiling microRNAs in long-term preserved formalin-fixed and paraffin-embedded non-small cell lung cancer tumor specimens," *PLoS One*, vol. 10, no. 3, pp. 1–12, 2015.

[58] E. A. Hunt, D. Broyles, T. Head, and S. K. Deo, "MicroRNA detection: current technology and research strategies," *Annual Review of Analytical Chemistry*, vol. 8, pp. 217–237, 2015.

[59] L. Moldovan, K. E. Batte, J. Trgovcich, J. Wisler, C. B. Marsh, and M. Piper, "Methodological challenges in utilizing miRNAs as circulating biomarkers," *Journal of Cellular and Molecular Medicine*, vol. 18, no. 3, pp. 371–390, 2014.

[60] C. C. Pritchard, H. H. Cheng, and M. Tewari, "MicroRNA profiling: approaches and considerations," *Nature Reviews Genetics*, vol. 13, no. 5, pp. 358–369, 2012.

[61] J. F. Huggett and A. Whale, "Digital PCR as a novel technology and its potential implications for molecular diagnostics," *Clinical Chemistry*, vol. 59, no. 12, pp. 1691–1693, 2013.

[62] H. Gharib, E. Papini, R. Paschke et al., "American Association of Clinical Endocrinologists, Associazione Medici Endocrinologi, and European Thyroid Association medical guidelines for clinical practice for the diagnosis and management of thyroid nodules," *Endocrine Practice*, vol. 16, no. 1, pp. 1–43, 2010.

[63] R. Paschke, L. Hegedus, E. Alexander, R. Valcavi, E. Papini, and H. Gharib, "Thyroid nodule guidelines: agreement, disagreement and need for future research," *Nature Reviews. Endocrinology*, vol. 7, no. 6, pp. 354–361, 2011.

[64] T. Stokowy, B. Wojtas, B. Jarzab et al., "Two-miRNA classifiers differentiate mutation-negative follicular thyroid carcinomas and follicular thyroid adenomas in fine needle aspirations with high specificity," *Endocrine*, vol. 54, no. 2, pp. 440–447, 2016.

[65] M. R. Vriens, J. Weng, I. Suh et al., "MicroRNA expression profiling is a potential diagnostic tool for thyroid cancer," *Cancer*, vol. 118, no. 13, pp. 3426–3432, 2012.

[66] H. Mazeh, I. Mizrahi, D. Halle et al., "Development of a microRNA-based molecular assay for the detection of papillary thyroid carcinoma in aspiration biopsy samples," *Thyroid*, vol. 21, no. 2, pp. 111–118, 2011.

[67] R. Shen, S. Liyanarachchi, W. Li et al., "MicroRNA signature in thyroid fine needle aspiration cytology applied to "atypia of undetermined significance" cases," *Thyroid*, vol. 22, no. 1, pp. 9–16, 2012.

[68] P. Agretti, E. Ferrarini, T. Rago et al., "MicroRNA expression profile helps to distinguish benign nodules from papillary thyroid carcinomas starting from cells of fine-needle aspiration," *European Journal of Endocrinology*, vol. 167, no. 3, pp. 393–400, 2012.

[69] F. Panebianco, C. Mazzanti, S. Tomei et al., "The combination of four molecular markers improves thyroid cancer cytologic diagnosis and patient management," *BMC Cancer*, vol. 15, p. 918, 2015.

[70] S. Paskas, J. Jankovic, V. Zivaljevic et al., "Malignant risk stratification of thyroid FNA specimens with indeterminate cytology based on molecular testing," *Cancer Cytopathology*, vol. 123, no. 8, pp. 471–479, 2015.

[71] Y. Zhang, Q. Zhong, X. Chen, J. Fang, and Z. Huang, "Diagnostic value of microRNAs in discriminating malignant thyroid nodules from benign ones on fine-needle aspiration samples," *Tumour Biology*, vol. 35, no. 9, pp. 9343–9353, 2014.

[72] S. Forte, C. La Rosa, V. Pecce, F. Rosignolo, and L. Memeo, "The role of microRNAs in thyroid carcinomas," *Anticancer Research*, vol. 35, no. 4, pp. 2037–2047, 2015.

[73] M. Ilié and P. Hofman, "Pros: can tissue biopsy be replaced by liquid biopsy?," *Translational Lung Cancer Research*, vol. 5, no. 4, pp. 420–423, 2016.

[74] E. Larrea, C. Sole, L. Manterola et al., "New concepts in cancer biomarkers: circulating miRNAs in liquid biopsies," *International Journal of Molecular Sciences*, vol. 17, no. 5, p. 627, 2016.

[75] S. Yu, Y. Liu, J. Wang et al., "Circulating microRNA profiles as potential biomarkers for diagnosis of papillary thyroid carcinoma," *The Journal of Clinical Endocrinology and Metabolism*, vol. 97, no. 6, pp. 2084–2092, 2012.

[76] J. C. Lee, J. T. Zhao, R. J. Clifton-Bligh et al., "MicroRNA-222 and MicroRNA-146b are tissue and circulating biomarkers of recurrent papillary thyroid cancer," *Cancer*, vol. 119, no. 24, pp. 4358–4365, 2013.

[77] S. Cantara, T. Pilli, G. Sebastiani et al., "Circulating miRNA95 and miRNA190 are sensitive markers for the differential diagnosis of thyroid nodules in a Caucasian population," *The Journal of Clinical Endocrinology and Metabolism*, vol. 99, no. 11, pp. 4190–4198, 2014.

[78] M. E. Graham, R. D. Hart, S. Douglas et al., "Serum microRNA profiling to distinguish papillary thyroid cancer from benign thyroid masses," *Journal of Otolaryngology - Head & Neck Surgery*, vol. 44, no. 1, p. 33, 2015.

[79] M. Li, Q. Song, H. Li, Y. Lou, and L. Wang, "Circulating miR-25-3p and miR-451a may be potential biomarkers for the diagnosis of papillary thyroid carcinoma," *PLoS One*, vol. 10, no. 8, article e0135549, 2015.

[80] Y. S. Lee, Y. S. Lim, J. C. Lee et al., "Differential expression levels of plasma-derived miR-146b and miR-155 in papillary thyroid cancer," *Oral Oncology*, vol. 51, no. 1, pp. 77–83, 2015.

[81] R. Samsonov, V. Burdakov, T. Shtam et al., "Plasma exosomal miR-21 and miR-181a differentiates follicular from papillary thyroid cancer," *Tumour Biology*, vol. 37, no. 9, pp. 12011–12021, 2016.

[82] S. Yu, X. Liu, Y. Zhang et al., "Circulating microRNA124-3p, microRNA9-3p and microRNA196b-5p may be potential signatures for differential diagnosis of thyroid nodules," *Oncotarget*, vol. 7, no. 51, pp. 84165–84177, 2016.

[83] E. E. Yoruker, D. Terzioglu, S. Teksoz, F. E. Uslu, U. Gezer, and N. Dalay, "MicroRNA expression profiles in papillary thyroid carcinoma, benign thyroid nodules and healthy controls," *Journal of Cancer*, vol. 7, no. 7, pp. 803–809, 2016.

[84] F. Rosignolo, M. Sponziello, L. Giacomelli et al., "Identification of thyroid-associated serum microRNA profiles and their potential use in thyroid cancer follow-up," *Journal of the Endocrine Society*, vol. 1, no. 1, pp. 3–13, 2017.

[85] Y. Sun, S. Yu, Y. Liu, F. Wang, Y. Liu, and H. Xiao, "Expression of miRNAs in papillary thyroid carcinomas is associated with BRAF mutation and clinicopathological features in Chinese patients," *International Journal of Endocrinology*, vol. 2013, Article ID 128735, 10 pages, 2013.

[86] F. Acibucu, H. S. Dökmetaś, Y. Tutar, Ş. Elagoz, and F. Kilicli, "Correlations between the expression levels of microRNA146b, 221, 222 and p27Kip1 protein mRNA and the clinicopathologic parameters in papillary thyroid cancers," *Experimental and Clinical Endocrinology & Diabetes*, vol. 122, pp. 137–143, 2014.

[87] M. Sun, S. Fang, W. Li et al., "Associations of miR-146a and miR-146b expression and clinical characteristics in papillary thyroid carcinoma," *Cancer Biomarkers*, vol. 15, pp. 33–40, 2015.

[88] P. Wang, W. Meng, M. Jin, L. Xu, E. Li, and G. Chen, "Increased expression of miR-221 and miR-222 in patients with thyroid carcinoma," *African Journal of Biotechnology*, vol. 11, pp. 2774–2781, 2012.

[89] Y. Peng, C. Li, D. C. Luo, J. W. Ding, W. Zhang, and G. Pan, "Expression profile and clinical significance of microRNAs in papillary thyroid carcinoma," *Molecules*, vol. 19, pp. 11586–11599, 2014.

[90] Y. Huang, D. Liao, L. Pan et al., "Expressions of miRNAs in papillary thyroid carcinoma and their associations with the $BRAF^{V600E}$ mutation," *European Journal of Endocrinology*, vol. 168, pp. 675–681, 2013.

[91] C. K. Chou, K. D. Yang, F. F. Chou et al., "Prognostic implications of miR-146b expression and its functional role in papillary thyroid carcinoma," *The Journal of Clinical Endocrinology & Metabolism*, vol. 98, pp. E196–E205, 2013.

[92] M. V. Geraldo and E. T. Kimura, "Integrated analysis of thyroid cancer public datasets reveals role of post-transcriptional regulation on tumor progression by targeting of immune system mediators," *PLoS One*, vol. 10, pp. 1–21, 2015.

[93] T. Jikuzono, M. Kawamoto, H. Yoshitake et al., "The miR-221/222 cluster, miR-10b and miR-92a are highly upregulated in metastatic minimally invasive follicular thyroid carcinoma," *International Journal of Oncology*, vol. 42, pp. 1858–1868, 2013.

[94] Cancer Genome Atlas Research Network, "Integrated genomic characterization of papillary thyroid carcinoma," *Cell*, vol. 159, pp. 676–690, 2014.

[95] M. Dettmer, A. Perren, H. Moch, P. Komminoth, Y. E. Nikiforov, and M. N. Nikiforova, "Comprehensive microRNA expression profiling identifies novel markers in follicular variant of papillary thyroid carcinoma," *Thyroid*, vol. 23, pp. 1383–1389, 2013.

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Research Article

Overexpression of Chromosome 21 miRNAs May Affect Mitochondrial Function in the Hearts of Down Syndrome Fetuses

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Dosage-dependent upregulation of most of chromosome 21 (Hsa21) genes has been demonstrated in heart tissues of fetuses with Down syndrome (DS). Also miRNAs might play important roles in the cardiac phenotype as they are highly expressed in the heart and regulate cardiac development. Five Hsa21 miRNAs have been well studied in the past: miR-99a-5p, miR-125b-2-5p, let-7c-5p, miR-155-5p, and miR-802-5p but few information is available about their expression in trisomic tissues. In this study, we evaluated the expression of these miRNAs in heart tissues from DS fetuses, showing that miR-99a-5p, miR-155-5p, and let-7c-5p were overexpressed in trisomic hearts. To investigate their role, predicted targets were obtained from different databases and cross-validated using the gene expression profiling dataset we previously generated for fetal hearts. Eighty-five targets of let-7c-5p, 33 of miR-155-5p, and 10 of miR-99a-5p were expressed in fetal heart and downregulated in trisomic hearts. As nuclear encoded mitochondrial genes were found downregulated in trisomic hearts and mitochondrial dysfunction is a hallmark of DS phenotypes, we put special attention to let-7c-5p and miR-155-5p targets downregulated in DS fetal hearts and involved in mitochondrial function. The let-7c-5p predicted target *SLC25A4/ANT1* was identified as a possible candidate for both mitochondrial and cardiac anomalies.

1. Introduction

Down Syndrome (DS) is a major cause of congenital heart defects (CHD), mainly represented by atrioventricular canal defect (AVCD), ventricular septal defect (VSD), and tetralogy of Fallot (TOF) [1]. Most of them derive from the abnormal development of the endocardial cushions [1, 2]. Defects of the outflow tract are also frequent.

Attempts to identify chromosome 21 (Hsa21) genes possibly contributing to the DS phenotype have focused in the past on the Down syndrome critical region (DSCR). The DSCR hypothesis assumed that one or more genes in this region may be sufficient to produce the specific DS features when present in three copies [3].

A chromosome segment spanning from *D21S3* to *PFKL* in band 21q22.3 was considered a critical region for cardiac anomalies in DS (DS-CHD) [4]. Later, a 5.4 Mb genomic region was identified in the DS mouse model Dp (16) associated to congenital heart defects similar to that observed in DS subjects [5]. This region, which spans from *Tiam1* and *Kcnj6* and includes 52 Hsa21 ortholog genes, was further narrowed to 3.7 Mb [6] from *Ifnar1* and *Kcnj6* (35 Hsa21 ortholog genes). The two CHD critical regions described by Barlow and by Liu, identified according to different criteria, were mapped to very different loci of Hsa21. Discrepancies like this one suggest today that the origins of trisomic phenotypes are more complicated than formerly assumed and that they possibly involve multiple gene interactions.

Another interesting detail is that only about 50% of DS individuals manifest CHD. This means that we cannot hypothesize a simple correlation between gene overexpression and cardiac alterations. Therefore, a more extensive analysis of both transcriptome data and pathway perturbations must be applied to identify the complex molecular defects underlying CHD in DS.

In a previous study, we applied the microarray technology to analyze genome-wide expression profiles of the heart of DS fetuses with and without CHD [7]. The rationale for analyzing fetal tissues was based on the concept that CHD are thought to arise from anomalies in cardiac morphogenesis. By this approach, it was found that Hsa21 gene expression was globally upregulated 1.5 fold in trisomic samples, in general agreement with the gene dosage hypothesis. More than 400 genes located on other chromosomes were also differentially expressed, either upregulated or downregulated, between trisomic and nontrisomic hearts. Functional class scoring and gene set enrichment analyses of these genes revealed a global downregulation of nuclear-encoded mitochondrial genes (NEMGs) and upregulation of genes encoding extracellular matrix proteins. These data indicate that dosage-dependent upregulation of Hsa21 genes causes dysregulation of the genes responsible for mitochondrial function and for the extracellular matrix organization in the fetal heart of trisomic subjects and suggest that these alterations might be a prelude to heart defects. However, no significant differences in gene expression in hearts from DS fetuses (18–22 gestational weeks) with CHD could be found [7].

Throughout that study only the expression of protein-coding RNAs was analyzed even though noncoding RNAs, such as microRNAs (miRNAs), expressed in the heart, might be implicated in determining the CHD as they control protein expression in development, differentiation, and metabolism [8].

Recent findings indicate the involvement of microRNAs in mouse cardiac development and diseases [9–12]. In the context of DS, this can occur by 2 mechanisms: (i) Hsa21 miRNAs could be overexpressed as a consequence of the trisomy and could affect target genes directly or indirectly involved in heart morphogenesis, and (ii) Non-Hsa21 dysregulated miRNAs might affect target genes involved in heart morphogenesis.

In this study, we have investigated the former mechanism in heart tissues from DS fetuses with and without CHD.

Hsa21 encodes several classes of noncoding RNAs, the most enriched being long noncoding RNAs, while miRNAs are the less represented [13]. The most recent annotation of miRNA database (miRBase, release 21) has indicated that the Hsa21 harbors at least 29 miRNAs.

Five of them, namely miR-99a-5p, let-7c-5p, miR-125b-2-5p, miR-155-5p, and miR-802-5p, were first identified (Figure 1). Their involvement in different DS associated phenotypes has been established, and several of their targets have been experimentally validated [14–20].

Few information is available about the expression of Hsa21-derived miRNAs in human heart tissues and their possible role in cardiomyogenesis.

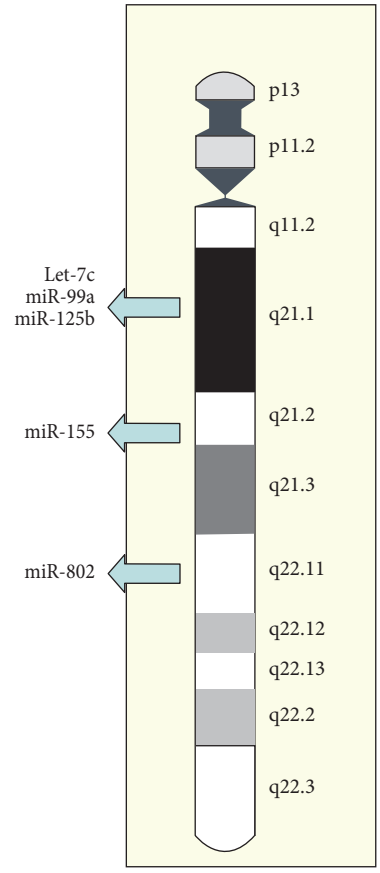

Figure 1: Hsa21 miRNAs. miR-99a-5p, let-7c-5p, and miR-125b-2-5p are located on Hsa21 in the sense orientation within an intron of the *C21orf34* gene, located at the beginning of q21.1 band. miR-99a-5p and let-7c-5p are only 659 bp apart, whereas miR-125b-2-5p lies just over 50.000 bp downstream of let-7c. miR-155-5p is located within the *BIC* gene, almost 9 Mb downstream from the *C21orf34* gene at Hsa21 genomic position q21.1. Finally, miR-802-5p is located just over 10 million bp downstream from the *BIC*/*miR-155* gene in the antisense orientation within intron 1 of the *RUNX1* gene at position q22.11.

We have analyzed the expression of 5 well-studied Hsa21 miRNAs to determine if they are dysregulated as consequence of trisomy and if their dysregulation might affect molecular mechanisms involved in mitochondrial function and heart development. To this aim, a bioinformatic analysis of Hsa21 miRNA target prediction by different databases was performed and cross-validated using the gene expression profiling dataset we previously generated for trisomic fetal hearts.

2. Materials and Methods

2.1. Samples. Cardiac tissues were obtained from fetuses at 18–22 weeks of gestation after therapeutic abortion according to protocols approved by our Institutional Ethical Committee. Tissues and RNAs were stored at the Telethon Bank of Fetal Biological Samples at the University of Naples. For this study, 3 hearts from euploid fetuses (NH), 3 from fetuses

with DS without CHD (DH), and 3 from fetuses with DS and CHD (CDH) were analyzed. For let-7c mimic and inhibitor transfection, 2 previously characterized [21, 22] primary lines of euploid (N-HFF) and trisomic (DS-HFF) fetal fibroblasts were, respectively, used. Fibroblasts from biopsies were cultured in T25 flasks (BD Falcon) with Chang Medium B + C (Irvine Scientific) supplemented with 1% penicillin/streptomycin (Gibco) at 37°C in 5% CO_2 atmosphere; all the analyses described throughout this study were carried out at cell culture passages 4-5.

2.2. RNA Extraction and Quantitative Real-Time PCR. Total RNA from each sample was extracted using TRIzol Reagent (Gibco/BRL Life Technologies Inc., Gaithersburg, MD) and was reverse-transcribed using iScript cDNA synthesis kit (Bio-Rad). qRT-PCR was performed using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) on a Bio-Rad iCycler CFX96 Touch Real-Time PCR Detection System according to the manufacturer's protocols. Primer pairs (MWG-Biotech, Ebersberg, Germany) were designed using the Primer 3 software (http://bioinfo.ut.ee/primer3-0.4.0/primer3; date last accessed 2015) to obtain amplicons ranging from 100 to 150 base pairs. In order to test primer efficiency, serial dilutions of cDNAs generated from selected samples, which expressed the target genes at a suitable level, were used to generate standard curves for each gene. qRT-PCR results are presented as relative mRNA levels normalized against reference control values. The GAPDH housekeeping gene was chosen as reference gene. Primer sequences of analyzed genes are the following *SLC25A4*/*ANT1*-F: GGGTTTC AACGTCTCTGTCC; *SLC25A4*/*ANT1*-R: TCCAGCTCAC AAAAATGTGC; *DICER*-F: CTGATGGAATTAGAAGA AGCACTTAAT; *DICER*-R: ACCAGGGTCCCAGAACT ACC; *GAPDH*-F: TGCACCACCAACTGCTTAGC; and *GAPDH*-**R:** GGCATGGACTGTGGTCATGAG.

For miRNA reverse transcription miScript II RT Kit (Qiagen) was used, and the mature miRNAs, mir-99a-5p, let-7c-5p, mir-125b-2-5p, mir-155-5p, and mir-802-5p, were quantified using miScript Primer Assay system and miScript SYBR Green PCR Kit (Qiagen). RNAUS and RNA5S were used as reference genes.

For end point PCR, assays were performed using the PCR Master Mix (2X) Kit (Thermo Fisher #K0171). Amplification products were visualized on 2% agarose gel and quantified using the Fiji software (http://www.fiji.sc) [23].

2.3. Bionformatic Analysis. Predicted miRNA-mRNA interactions were retrieved from 9 different algorithms (DIANA-microT [24], TargetScan [25], PITA [26], TargetMiner [27], miRDB [28], RNA22 [29], Pictar [30], and MiRanda [31]) using as input the sequences/IDs for the miRNA of interest. Before merging the date, divergent database annotations were unified by converting transcript-wise predictions to the gene level (Ensembl gene ID) using the BiomaRt package [32]. The lists of targets predicted by each database were subsequently marked with Affymetrix probe IDs using the related annotation package and finally merged with the list of probes ($n = 279$) found significantly downregulated in DS hearts compared to euploid controls [7]. For further analyses, we brought forward miR targets predicted by at least two databases, with a P value ≤ 0.05 and fold change ≤ -1.2 as defined in the original paper [7].

Gene ontology (GO) functional class scoring of the lists of significantly downregulated genes was performed using the Web-based GEne SeT Analysis Toolkit V2 (http://www.webgestalt.org) [33]. Special attention was given to mitochondria-related categories and pathways.

2.4. miRNA Mimic Transfection. For let-7c-5p upregulation, a let-7c miRNA mimic (miScript miRNA Mimics, Qiagen) was transfected in 2 N-HFF lines. Cells were plated in a concentration of 70,000/well on 24 well plates (BD Falcon) and after 24 hours were transfected with a miRNA mimic using the INTERFERin transfection reagent (Polyplus-transfection). A fluorescent siRNA (AllStars Neg. siRNA AF 488, Qiagen) has been used to monitor the efficiency of the chosen transfecting agent. Forty-eight hours after the transfection cells were harvested and *ANT1* and *DICER* expression were evaluated, cells treated with the INTERFERin transfection agent only were used as mock control for all experiments performed after transfection.

2.5. miRNA Inhibitor Transfection. For let-7c-5p downregulation, an Anti-hsa-let-7c miRNA (miScript miRNA Inhibitor, Qiagen) was transfected in 2 DS-HFF lines. Cells were plated in a concentration of 150,000/dish on 3,5 cm petri dishes (BD Falcon) and after 24 hours were transfected with the inhibitor using the INTERFERin transfection reagent (Polyplus-transfection). Forty-eight hours after transfection cells were harvested for RNA collection while 72 hours after transfection protein lysates were obtained.

2.6. Immunoblot Analysis. Cells were lysed in radioimmunoprecipitation assay buffer (1% Triton; 0,5% sodium deoxycholate; 0,1% sodium dodecyl sulfate; 0,15 M NaCl; 0,05 tris-HCl; and pH 7.2) supplemented with protease inhibitors. For western blot analysis, proteins were separated on SDS-PAGE (Mini-Protean TGX gels, Bio-Rad), gels were blotted onto Immobilon-P (Millipore, Bedford, MA, USA) for overnight, and the membranes were blocked in 5% nonfat dry milk in PBS plus 0,05% Tween 20 for 2 h or overnight before the addition of the primary antibody for 2 h. The primary antibodies used were anti-*SLC25A4*/*ANT1* mAb (ab 110322, Abcam) and anti-vinculin (N-19) (sc-7649, Santa Cruz).

The filters were washed three times in PBS plus 0.05% Tween 20 before the addition of horseradish peroxidase-conjugated secondary antibodies for 45 min. Horseradish peroxidase was detected with ECL (Pierce).

2.7. Statistics. The Student's t-test was applied to evaluate the statistical significance of data in this study. The threshold for statistical significance (P value) was set at 0.05.

3. Results

We evaluated, by qRT-PCR, the expression of Hsa21 miRNAs in fetal heart samples from 6 DS individuals, 3 with

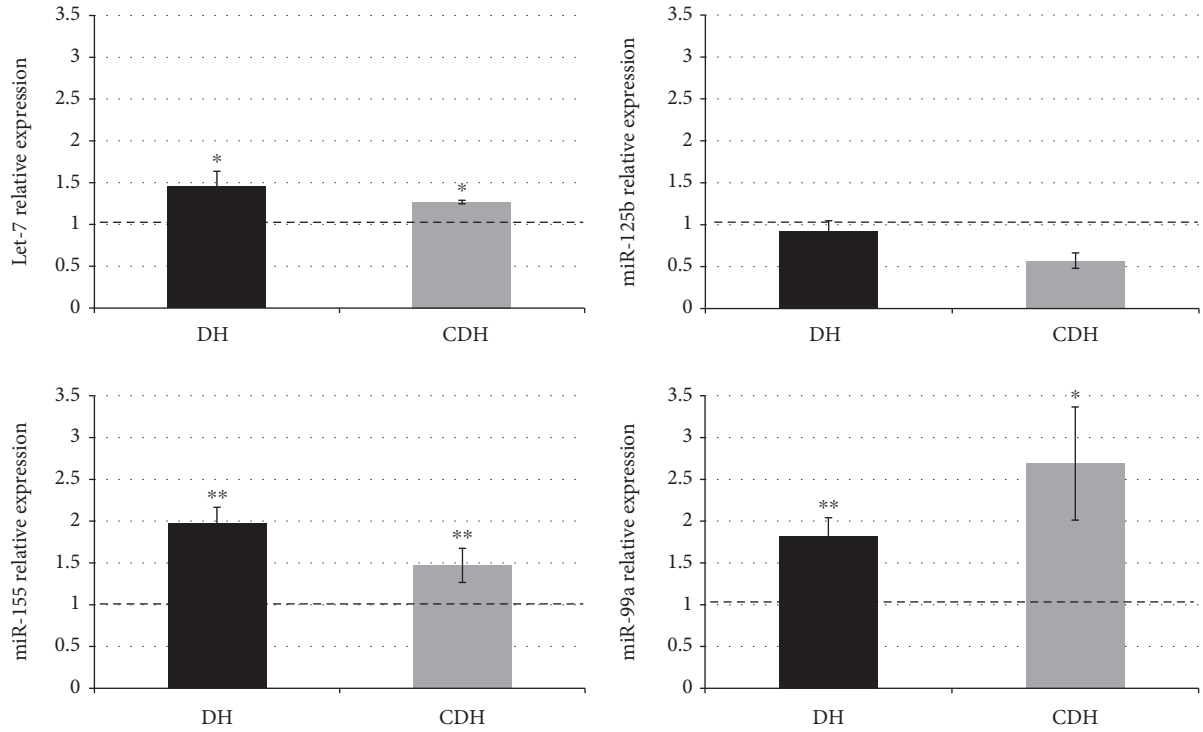

Figure 2: Hsa21 miRNA expression evaluated by qRT-PCR in fetal hearts. Let-7c-5p, miR-99a-5p, and miR-155-5p were upregulated in trisomic hearts without cardiopathy (DH) and with cardiopathy (CDH). miR-125b-2-5p was variably expressed and not upregulated. Results are expressed as relative mean values ± SEM of 3 DH and 3 CDH samples, compared with control hearts (NH) set equal to 1. $^{*}P < 0.05$, $^{**}P < 0.001$. P value expresses statistical significance for trisomic versus nontrisomic sample comparisons.

CHD (CHD samples) and 3 without CHD (DH samples), and from 3 nontrisomic controls (NH samples).

We found that miR-99a-5p, miR-125b-2-5p, let-7c-5p, and miR-155-5p were expressed in fetal hearts at 18–22 gestational weeks. miR-802-5p was not expressed. miR-99a-5p, miR-155-5p, and let-7c-5p were overexpressed in trisomic hearts when compared with euploid ones, whereas miR-125b-2-5p was not dysregulated and was quite variably expressed (Figure 2). The comparison between heart tissues from fetuses with and without CHD indicated that miR-155-5p and let-7c-5p did not show significant differences between DH and CDH samples while miR-99a-5p was differentially expressed even though the sample size is too small to obtain a statistical significance.

As miRNAs could affect protein expression by either interfering with RNA translation or promoting mRNA degradation [34], we looked at mRNA expression of target genes of overexpressed miRNAs by using the dataset of our previous study by which we investigated gene expression profiling in the same hearts [7].

We considered as target list the sum of all targets predicted by 9 different software from miRBase annotation, and we filtered them according to gene expression microarray results. The rationale of our approach is based on the notion that overexpression of Hsa21 miRNAs can result in downregulation of specific target genes possibly involved in DS phenotype. For each miRNA, we compared target genes predicted at least by 2 different software with the list of genes downregulated in trisomic hearts with a fold change (FC) > |1,2| and P value< 0.05. Using these criteria, we unraveled that 85 targets of let-7c-5p (Supplementary Table 1 available online at https://doi.org/10.1155/2017/8737649), 33 of miR-155-5p (Supplementary Table 2), and 10 of miR-99a-5p (Supplementary Table 3) were expressed in fetal heart and downregulated in trisomic samples.

Over-representation analysis (ORA) of these lists, performed using the Web-based GEne SeT Analysis Toolkit (http://www.webgestalt.org) [33], demonstrated a highly significant enrichment ($P < 0.0001$) of the cell component gene ontology category "mitochondrion" for let-7c-5p and miR-155-5p downregulated targets, with a cluster of 26 and 8 genes, respectively (Tables 1 and 2). No mitochondria-related genes were found among targets of miR-99a-5p.

We focused our attention on the *SLC25A4*/*ANT1* gene (indicated in bold in Table 1), which was predicted by 5 databases as a let-7c-5p target gene and already proposed as a target of both human and murine let-7b [35–37]. Its downregulation in heart tissue was confirmed by qRT-PCR (Supplementary Figure 1). According to the STarMirDB, a database of microRNA binding sites based on cross-linking immunoprecipitation (CLIP) data (http://sfold.wadsworth.org/starmirDB.php) [38], seven binding sites for let-7c-5p are located in the *SLC25A4*/*ANT1* sequence. One of them has been mapped to a conserved 3'UTR seed site (seed type 7mer-A1) [39] with a logistic probability of 0.68.

We were interested in this gene because of its central role in oxidative phosphorylation (OXPHOS). Our hypothesis

Table 1: Targets of let-7c-5p significantly downregulated in DS hearts and belonging to gene ontology cellular component category "mitochondrion." Twenty-six genes were observed instead of the expected 6.49 genes with $P < 0.00001$.

Gene symbol	Description	FC
AKAP8	A-kinase anchoring protein 8	−1,361
COX10	COX10, heme A: farnesyltransferase cytochrome c oxidase assembly factor	−1,307
COX5A	Cytochrome c oxidase subunit 5A	−1,263
DLAT	Dihydrolipoamide S-acetyltransferase	−1,678
DLST	Dihydrolipoamide S-succinyltransferase	−1,724
FKBP4	FK506 binding protein 4	−1,558
GADD45GIP1	GADD45G interacting protein 1	−1,664
GHITM	Growth hormone inducible transmembrane protein	−1,477
HSPB7	Heat shock protein family B (small) member 7	−1,422
MECP2	Methyl-CpG binding protein 2	−1,22
MPC1	Mitochondrial pyruvate carrier 1	−1,339
MRPL33	Mitochondrial ribosomal protein L33	−1,333
MRS2	MRS2, magnesium transporter	−1,416
NDUFS3	NADH: ubiquinone oxidoreductase core subunit S3	−1,314
NMT1	N-myristoyltransferase 1	−1,443
NOL7	Nucleolar protein 7	−1,299
PANK2	Pantothenate kinase 2	−1,361
PCCB	Popionyl-CoA carboxylase beta subunit	−1,335
PDHA1	Pyruvate dehydrogenase (lipoamide) alpha 1	−1,524
PGS1	Phosphatidylglycerophosphate synthase 1	−1,233
SDHC	Succinate dehydrogenase complex subunit C	−1,23
SLC25A12	Solute carrier family 25 member 12	−1,408
SLC25A4	Solute carrier family 25 member 4	**−1,55**
TIMM23	Translocase of inner mitochondrial membrane 23	−1,508
UQCC1	Ubiquinol-cytochrome c reductase complex assembly factor 1	−1,517
UQCRFS1	Ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1	−1,292

Table 2: Targets of miR-155-5p significantly downregulated in DS hearts and belonging to gene ontology cellular component category "mitochondrion." Eight genes were observed instead of the expected 2.34 genes with $P < 0.00001$.

Gene Symbol	Description	FC
DLAT	Dihydrolipoamide S-acetyltransferase	−1,678
HSPB7	Heat shock protein family B (small) member 7	−1,422
LPIN1	Lipin 1	−1,838
MECP2	Methyl-CpG binding protein 2	−1,22
NDUFS3	NADH: ubiquinone oxidoreductase core subunit S3	−1,314
NT5C	5′, 3′-nucleotidase, cytosolic	−1,524
SIRT5	Sirtuin 5	−1,255
YWHAQ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein theta	−1,364

was that the *SLC25A4*/*ANT1* downregulation could be an effect of the specific interaction between let-7c-5p and this gene at the seed sequence. To validate this hypothesis, we performed a transfection experiment of a let-7c-5p mimic miRNA in euploid fibroblasts in which *SLC25A4*/*ANT1* is expressed. As control, we evaluated also the expression of *DICER*, since it is a let-7c-5p validated target [40]. We transfected fibroblasts at different concentrations of miRNA mimic for 48 h, and we measured *SLC25A4*/*ANT1*, *DICER*, and *GAPDH* expression by end point PCR. As shown in Figure 3, at 25 nM miRNA dosage (lanes 13-14), a reduction of target amplification is already appreciable with respect to nontransfected cells (lanes 1-2) or cells transfected with transfection reagent only (lanes 4-5). As expected, *GAPDH* amplification did not show variations.

We further evaluated *SLC25A4*/*ANT1* and *DICER* expression by qRT-PCR showing that after let-7c mimic transfection (25 nM), their expression significantly decreased

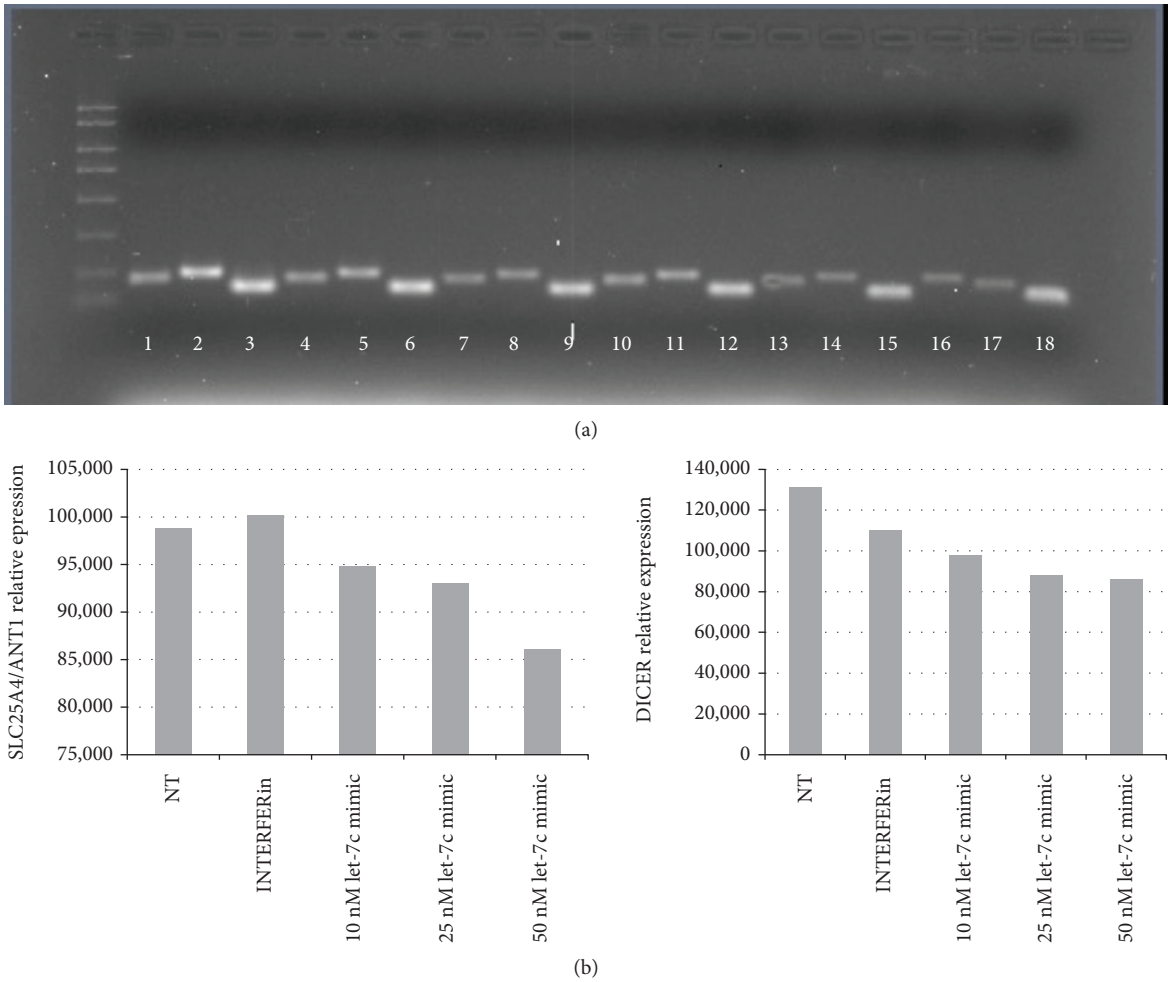

Figure 3: End point PCR of *SLC25A4*/*ANT1*, *DICER*, and *GAPDH* in euploid fibroblasts transfected with a let-7c-5p mimic. (a) Agarose gel of *SLC25A4*/*ANT1*, *DICER*, and *GAPDH* amplification in: Not transfected cells (lanes 1–3); cells + INTERFERin (lanes 4–6); cells + 5 nM miRNA mimic (lanes 7–9); cells + 10 nM miRNA mimic (lanes 10–12); cells + 25 nM miRNA mimic (lanes 13–15); cells + 50 nM miRNA mimic (lanes 16–18). Amplification products of *SLC25A4*/*ANT1*: lanes 1, 4, 7, 10, 13, and 16; amplification products of *DICER*: lanes 2, 5, 8, 11, 14, and 17; amplification products of *GAPDH*: lanes 3, 6, 9, 12, 15, and 18. (b) Densitometric analysis of *SLC25A4*/*ANT1* and *DICER* amplification products obtained by ImageJ software. At 25 nM miRNA mimic concentration, a reduction of target amplification is appreciable.

if compared with cells transfected with the transfecting agent only (Figure 4).

Finally, we performed the reciprocal experiment using a let-7c-5p inhibitor in trisomic fibroblasts collecting both RNA and proteins. After treatment with 25 nM inhibitor, which reduced let-7c-5p expression by 35%, the expression of *SLC25A4*/*ANT1* was significantly reduced both at mRNA (a) and protein (b-c) level (Figure 5).

4. Discussion

We have analyzed the expression of 5 Hsa21 miRNAs in trisomic fetal hearts at 18–22 gestational weeks to investigate whether they are affected by the gene dosage effect observed for most of the Hsa21 genes. Three out of five, namely let-7c-5p, miR-99a-5p, and miR-155-5p, were significantly overexpressed, if compared with euploid controls. The upregulation was not higher than 2.5 folds in agreement

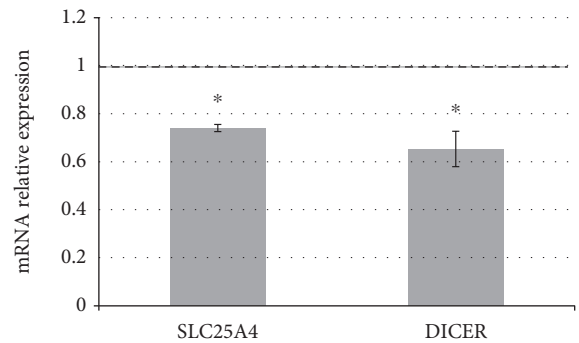

Figure 4: *SLC25A4*/*ANT1* and *DICER* expression by qRT-PCR. Transfection of let-7c-5p mimic induces a significant decrease of *SLC25A4*/*ANT1* and *DICER* expression in euploid fibroblasts. Results are expressed as relative mean values ± SEM of three different determination, compared with cells treated only with transfecting agent (set equal to 1). $^{*}P < 0.05$.

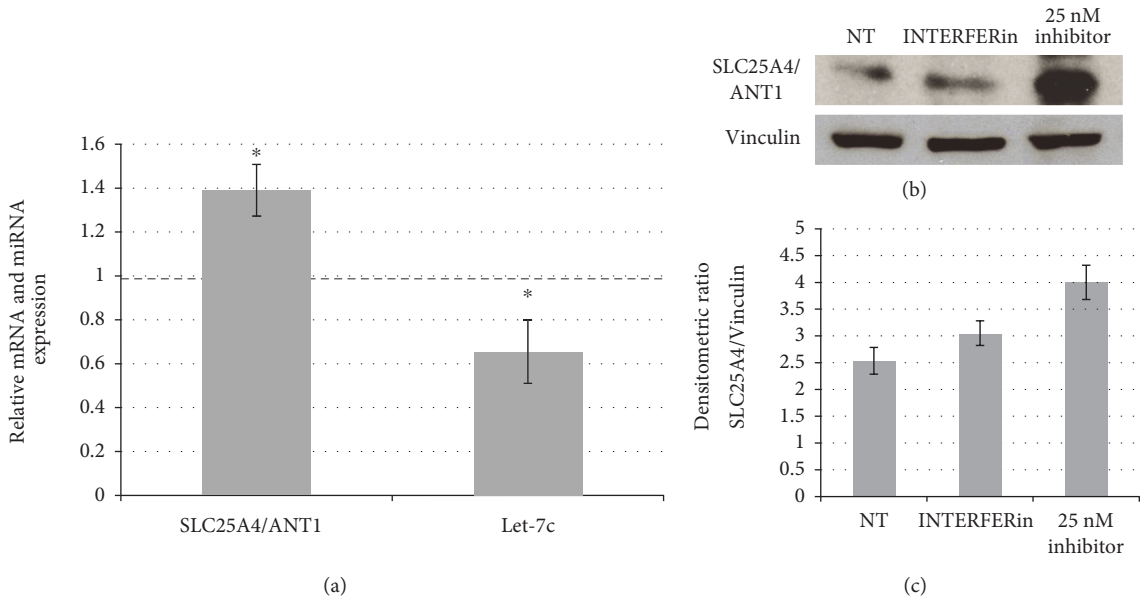

Figure 5: *SLC25A4*/*ANT1* after let-7c-5p inhibition in trisomic fibroblasts. (a) Transfection of let-7c-5p inhibitor in DS-HFFs induces a significant increase of *SLC25A4*/*ANT1* expression and a decrease of let-7c-5p, as detected by qRT-PCR. Results are expressed as relative mean values ± SEM of three different determination, compared with cells treated only with transfecting agent (set equal to 1). (b) Representative immunoblot of SLC25A4/ANT1 in nontransfected cells (NT), cells transfected only with transfecting agent and cells transfected with 25 nM let-7c-5p inhibitor. Vinculin was used as loading control. (c) Densitometric SLC25A4/ANT1 relative measurement after 72 h treatment. Protein expression was significantly increased after inhibiting treatment if compared with mock transfected cells. The bars show relative mean values ± SEM of two DS-HFFs in duplicate; $^{*}P < 0.05$.

with a gene dosage effect. In the same samples, miR-802-5p was not expressed and miR-125b-2-5p was normoregulated. This was not surprising as the miR-125b-1-5p, which maps to 11q24.1, encoding an identical miRNA, may mask the eventual overexpression.

Comparison between hearts from fetuses with cardiopathies versus hearts from fetuses without cardiopathies showed that miR-99a-5p was more expressed in the latter samples even though more samples are needed to reach a significant result due to a high variability.

In a recent paper, the role of mmu-let-7c and mmu-miR-99a in cardiomyogenesis was investigated using an overexpression strategy in murine embryonic stem cells. By overexpressing these miRNAs at early stage of differentiation, the authors demonstrated that they are involved in heart development as let-7c induces cardiogenesis while miR-99a appears to repress it by altering the *Smad2* signaling [18].

The overexpression of Hsa21 miRNAs can result in downregulation of specific target genes possibly involved in DS phenotype. To select relevant miRNA targets we have used the following criteria:

(i) Bioinformatics prediction

(ii) mRNA decreased expression in heart samples from DS fetuses

(iii) Inclusion in the category of mitochondria related genes

(iv) Involvement in normal and/or abnormal heart development

Among the selected genes, *SLC25A4*/*ANT1*, a predicted let-7c-5p target downregulated in trisomic hearts, appeared as a potential candidate for both mitochondrial dysfunction and CHD in DS. *SLC25A4* (Solute carrier family 25 member 4) or *ANT1* (Adenine nucleotide translocator 1) functions as a gated pore that translocates ADP and ATP between cytoplasm and mitochondria, regulating the intracellular energetic balance. Furthermore, its dysregulation has been associated to mitochondrial cardiomyopathies [41].

Humans have four *ANT* isoforms that are encoded by four different genes and are distributed in a tissue specific pattern. The human SLC25A4/*ANT1* gene is primarily expressed in the heart and in the skeletal muscle [42]. It is downregulated in DS fetal hearts and fibroblasts in which let-7c-5p is upregulated. qRT-PCR after let-7c-5p overexpression showed an inverse relationship with *SLC25A4*/*ANT1*, which was downregulated. The reverse experiment using miRNA inhibitor in trisomic cells demonstrated a significant increase of RNA and protein expression of the gene while let-7c-5p expression was decreased if compared with mock controls. These results may be considered an additional experimental support of our hypothesis even though further experiments will be necessary to definitely validate that *SLC25A4*/*ANT1* is a let-7c-5p target, which is beyond the purpose of this paper. To this aim, we plan to perform a luciferase assay to demonstrate a direct interaction.

OXPHOS deficiency and mitochondrial dysfunction have been associated with developmental mechanisms of DS [43]. *SLC25A4*/*ANT1* is one of the 37 NEMGs downregulated after *NRIP1* overexpression in the GEO GSE 19836

experiment [38] that we reanalyzed in Izzo et al. [22]. After *NRIP1* silencing in trisomic cells [23], *SLC25A4*/*ANT1* expression increased, thus allowing a more efficient exchange of ATP and an improvement of mitochondrial activity in DS samples. Accordingly, genetic inactivation of the heart isoform results in mtDNA damage and increased reactive oxygen species [44].

The above described strategy to select targets relevant for mitochondrial function and heart development was applied also to the miR-155-5p. Thirty-three predicted target genes of miR-155-5p have been found downregulated in trisomic fetal hearts if compared with euploid ones. Eight of them, including *MECP2* gene, which is a validated target [37, 45], are included in the cell component GO category "mitochondrion" suggesting an impact of this miRNA on mitochondrial function. *MECP2* is included also among the let-7c-5p predicted targets.

It was recently reported that the Hsa21 miR-155-5p regulates mitochondrial biogenesis by targeting mitochondrial transcription factor A (*TFAM*) [46]. Surprisingly, after studying both DS fibroblasts and heart samples, the authors conclude that the regulation of *TFAM* by the miRNA impacts mitochondrial biogenesis in the euploid setting but not in the DS setting. We suggest that a possible explanation may lie in the fact that mitochondrial biogenesis is already so decreased in DS cells to mask further defects. *TFAM* was not identified by our strategy because its heart expression at 18–22 gestational weeks is too low to be analyzed by microarray technology.

Finally miR-155-5p has been hypothesized to be an inducer of cardiac hypertrophy [47]. Its inhibition might have clinical potential to counteract this pathology.

5. Conclusions

In this study, we demonstrated the overexpression of three Hsa21 miRNAs, miR-99a-5p, miR-155-5p, and let-7c-5p, in the heart of trisomic fetuses as a likely consequence of gene dosage effect. Bioinformatic analysis demonstrated that two of these miRNAs, let-7c-5p and miR-155-5p, count several predicted targets among genes involved in mitochondrial function that were found to be downregulated in trisomic fetal hearts. Among the let-7c predicted targets, we identified and partially validated *SLC25A4*/*ANT1*, a gene encoding the main translocator of ADP/ATP across the mitochondrial membrane, which plays a major role in mitochondrial function.

Our results also support the hypothesis that the overexpression of miR-155-5p might have a potential impact on mitochondrial biogenesis.

Both miRNAs, let-7c-5p and miR-155-5p, should be taken into account while investigating the molecular mechanisms causing cardiac malformations in DS.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Antonella Izzo and Rosanna Manco are joint first authors.

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References

[1] S. C. Park, R. A. Mathews, J. R. Zuberbuhler, R. D. Rowe, W. H. Neches, and C. C. Lenox, "Down syndrome with congenital heart malformation," *American Journal of Diseases of Children*, vol. 131, no. 1, pp. 29–33, 1977.

[2] C. Ferencz, C. A. Neill, J. A. Boughman, J. D. Rubin, J. I. Brenner, and L. W. Perry, "Congenital cardiovascular malformations associated with chromosome abnormalities: an epidemiologic study," *The Journal of Pediatrics*, vol. 114, no. 1, pp. 79–86, 1989.

[3] J. R. Korenberg, X. N. Chen, R. Schipper et al., "Down syndrome phenotypes: the consequences of chromosomal imbalance," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 91, no. 11, pp. 4997–5001, 1994.

[4] G. M. Barlow, X. N. Chen, Z. Y. Shi et al., "Down syndrome congenital heart disease: a narrowed region and a candidate gene," *Genetics in Medicine*, vol. 3, no. 2, pp. 91–101, 2001.

[5] C. Liu, P. V. Belichenko, L. Zhang et al., "Mouse models for Down syndrome-associated developmental cognitive disabilities," *Developmental Neuroscience*, vol. 33, no. 5, pp. 404–413, 2011.

[6] C. Liu, M. Morishima, X. Jiang et al., "Engineered chromosome-based genetic mapping establishes a 3.7 Mb critical genomic region for Down syndrome-associated heart defects in mice," *Human Genetics*, vol. 133, no. 6, pp. 743–753, 2014.

[7] A. Conti, F. Fabbrini, P. D'Agostino et al., "Altered expression of mitochondrial and extracellular matrix genes in the heart of human fetuses with chromosome 21 trisomy," *BMC Genomics*, vol. 8, p. 268, 2007.

[8] V. Ambros and X. Chen, "The regulation of genes and genomes by small RNAs," *Development*, vol. 134, no. 9, pp. 1635–1641, 2007.

[9] Y. Zhao, J. F. Ransom, A. Li et al., "Dysregulation of cardiogenesis, cardiac conduction, and cell cycle in mice lacking miRNA-1-2," *Cell*, vol. 129, no. 2, pp. 303–317, 2007.

[10] B. Yang, H. Lin, J. Xiao et al., "The muscle-specific microRNA miR-1 regulates cardiac arrhythmogenic potential by targeting GJA1 and KCNJ2," *Nature Medicine*, vol. 13, no. 4, pp. 486–491, 2007.

[11] A. Care, D. Catalucci, F. Felicetti et al., "MicroRNA-133 controls cardiac hypertrophy," *Nature Medicine*, vol. 13, no. 5, pp. 613–618, 2007.

[12] E. van Rooij and E. N. Olson, "microRNAs put their signatures on the heart," *Physiological Genomics*, vol. 31, no. 3, pp. 365-366, 2007.

[13] A. Letourneau and S. E. Antonarakis, "Genomic determinants in the phenotypic variability of Down syndrome," *Progress in Brain Research*, vol. 197, pp. 15–28, 2012.

[14] T. S. Elton, S. E. Sansom, and M. M. Martin, "Trisomy-21 gene dosage over-expression of miRNAs results in the haploinsufficiency of specific target proteins," *RNA Biology*, vol. 7, no. 5, pp. 540–547, 2010.

[15] P. Sethupathy, C. Borel, M. Gagnebin et al., "Human microRNA-155 on chromosome 21 differentially interacts with its polymorphic target in the AGTR1 3′ untranslated region: a mechanism for functional single-nucleotide polymorphisms related to phenotypes," *American Journal of Human Genetics*, vol. 81, no. 2, pp. 405–413, 2007.

[16] R. M. O'Connell, D. S. Rao, A. A. Chaudhuri et al., "Sustained expression of microRNA-155 in hematopoietic stem cells causes a myeloproliferative disorder," *The Journal of Experimental Medicine*, vol. 205, no. 3, pp. 585–594, 2008.

[17] M. Ozen, C. J. Creighton, M. Ozdemir, and M. Ittmann, "Widespread deregulation of microRNA expression in human prostate cancer," *Oncogene*, vol. 27, no. 12, pp. 1788–1793, 2008.

[18] A. Coppola, A. Romito, C. Borel et al., "Cardiomyogenesis is controlled by the miR-99a/let-7c cluster and epigenetic modifications," *Stem Cell Research*, vol. 12, no. 2, pp. 323–337, 2014.

[19] X. Wu, Z. Gong, L. Sun, L. Ma, and Q. Wang, "MicroRNA-802 plays a tumour suppressive role in tongue squamous cell carcinoma through directly targeting MAP2K4," *Cell Proliferation*, vol. 50, no. 3, 2017.

[20] S. Malinge, S. Izraeli, and J. D. Crispino, "Insights into the manifestations, outcomes, and mechanisms of leukemogenesis in Down syndrome," *Blood*, vol. 113, no. 12, pp. 2619–2628, 2009.

[21] C. Piccoli, A. Izzo, R. Scrima et al., "Chronic pro-oxidative state and mitochondrial dysfunctions are more pronounced in fibroblasts from Down syndrome foeti with congenital heart defects," *Human Molecular Genetics*, vol. 22, no. 6, pp. 1218–1232, 2013.

[22] A. Izzo, R. Manco, F. Bonfiglio et al., "NRIP1/RIP140 siRNA-mediated attenuation counteracts mitochondrial dysfunction in Down syndrome," *Human Molecular Genetics*, vol. 23, no. 16, pp. 4406–4419, 2014.

[23] J. Schindelin, I. Arganda-Carreras, E. Frise et al., "Fiji: an open-source platform for biological-image analysis," *Nature Methods*, vol. 9, no. 7, pp. 676–682, 2012.

[24] M. Reczko, M. Maragkakis, P. Alexiou, I. Grosse, and A. G. Hatzigeorgiou, "Functional microRNA targets in protein coding sequences," *Bioinformatics*, vol. 28, no. 6, pp. 771–776, 2012.

[25] J. W. Nam, O. S. Rissland, D. Koppstein et al., "Global analyses of the effect of different cellular contexts on microRNA targeting," *Molecular Cell*, vol. 53, no. 6, pp. 1031–1043, 2014.

[26] M. Kertesz, N. Iovino, U. Unnerstall, U. Gaul, and E. Segal, "The role of site accessibility in microRNA target recognition," *Nature Genetics*, vol. 39, no. 10, pp. 1278–1284, 2007.

[27] S. Bandyopadhyay and R. Mitra, "TargetMiner: microRNA target prediction with systematic identification of tissue-specific negative examples," *Bioinformatics*, vol. 25, no. 20, pp. 2625–2631, 2009.

[28] N. Wong and X. Wang, "miRDB: an online resource for microRNA target prediction and functional annotations," *Nucleic Acids Research*, vol. 43, Database issue, pp. D146–D152, 2015.

[29] K. C. Miranda, T. Huynh, Y. Tay et al., "A pattern-based method for the identification of MicroRNA binding sites and their corresponding heteroduplexes," *Cell*, vol. 126, no. 6, pp. 1203–1217, 2006.

[30] A. Krek, D. Grun, M. N. Poy et al., "Combinatorial microRNA target predictions," *Nature Genetics*, vol. 37, no. 5, pp. 495–500, 2005.

[31] D. Betel, A. Koppal, P. Agius, C. Sander, and C. Leslie, "Comprehensive modeling of microRNA targets predicts functional non-conserved and non-canonical sites," *Genome Biology*, vol. 11, no. 8, article R90, 2010.

[32] S. Durinck, P. T. Spellman, E. Birney, and W. Huber, "Mapping identifiers for the integration of genomic datasets with the R/Bioconductor package biomaRt," *Nature Protocols*, vol. 4, no. 8, pp. 1184–1191, 2009.

[33] J. Wang, D. Duncan, Z. Shi, and B. Zhang, "WEB-based GEne SeT AnaLysis Toolkit (WebGestalt): update 2013," *Nucleic Acids Research*, vol. 41, Web Server issue, pp. W77–W83, 2013.

[34] J. Brennecke, A. Stark, R. B. Russell, and S. M. Cohen, "Principles of microRNA-target recognition," *PLoS Biology*, vol. 3, article e85, p. 3, 2005.

[35] A. Helwak, G. Kudla, T. Dudnakova, and D. Tollervey, "Mapping the human miRNA interactome by CLASH reveals frequent noncanonical binding," *Cell*, vol. 153, no. 3, pp. 654–665, 2013.

[36] S. W. Chi, J. B. Zang, A. Mele, and R. B. Darnell, "Argonaute HITS-CLIP decodes microRNA-mRNA interaction maps," *Nature*, vol. 460, no. 7254, pp. 479–486, 2009.

[37] C. H. Chou, N. W. Chang, S. Shrestha et al., "miRTarBase 2016: updates to the experimentally validated miRNA-target interactions database," *Nucleic Acids Research*, vol. 44, no. D1, pp. D239–D247, 2016.

[38] W. Rennie, S. Kanoria, C. Liu et al., "STarMirDB: a database of microRNA binding sites," *RNA Biology*, vol. 13, no. 6, pp. 554–560, 2016.

[39] D. P. Bartel, "MicroRNAs: target recognition and regulatory functions," *Cell*, vol. 136, no. 2, pp. 215–233, 2009.

[40] S. Tokumaru, M. Suzuki, H. Yamada, M. Nagino, and T. Takahashi, "let-7 regulates Dicer expression and constitutes a negative feedback loop," *Carcinogenesis*, vol. 29, no. 11, pp. 2073–2077, 2008.

[41] L. Palmieri, S. Alberio, I. Pisano et al., "Complete loss-of-function of the heart/muscle-specific adenine nucleotide translocator is associated with mitochondrial myopathy and cardiomyopathy," *Human Molecular Genetics*, vol. 14, no. 20, pp. 3079–3088, 2005.

[42] K. Li, C. K. Warner, J. A. Hodge et al., "A human muscle adenine nucleotide translocator gene has four exons, is located on chromosome 4, and is differentially expressed," *The Journal of Biological Chemistry*, vol. 264, no. 24, pp. 13998–14004, 1989.

[43] S. Arbuzova, T. Hutchin, and H. Cuckle, "Mitochondrial dysfunction and Down's syndrome," *BioEssays*, vol. 24, no. 8, pp. 681–684, 2002.

[44] V. Subramaniam, P. Golik, D. G. Murdock et al., "MITOCHIP assessment of differential gene expression in the skeletal muscle of Ant1 knockout mice: coordinate regulation of OXPHOS, antioxidant, and apoptotic genes," *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, vol. 1777, no. 7-8, pp. 666–675, 2008.

[45] X. Bofill-De Ros, M. Santos, M. Vila-Casadesus et al., "Genome-wide miR-155 and miR-802 target gene identification in the hippocampus of Ts65Dn Down syndrome mouse model by miRNA sponges," *BMC Genomics*, vol. 16, p. 907, 2015.

[46] A. Quinones-Lombrana and J. G. Blanco, "Chromosome 21-derived hsa-miR-155-5p regulates mitochondrial biogenesis by targeting mitochondrial transcription factor A (TFAM)," *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, vol. 1852, no. 7, pp. 1420–1427, 2015.

[47] H. Y. Seok, J. Chen, M. Kataoka et al., "Loss of MicroRNA-155 protects the heart from pathological cardiac hypertrophy," *Circulation Research*, vol. 114, no. 10, pp. 1585–1595, 2014.

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Review Article

Biological Function of MicroRNA193a-3p in Health and Disease

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MicroRNAs (miRNAs) are a class of small noncoding RNAs that act mainly as negative regulators of gene expression. Several studies demonstrated that miRNAs take part in numerous biological processes, such as proliferation, apoptosis, and migration. The dysregulation of miRNAs has been frequently observed in different types of disease, including cancer. Here, we provide a comprehensive review on the human miR-193a-3p by considering its role in both physiological and pathological contexts. Different mechanisms involved in regulating miR-193a-3p expression have been reported, including epigenetic modifications and transcription factors. In physiological contexts, miR-193a-3p seemed able to limit proliferation and cell cycle progression in normal cells. Remarkably, several publications demonstrated that miR-193a-3p acted as a tumor suppressor miRNA in cancer by targeting different genes involved in proliferation, apoptosis, migration, invasion, and metastasis. Furthermore, the downregulation of miR-193a-3p has been observed in many primary tumors and altered levels of circulating miR-193a-3p have been identified in serum or plasma of cancer patients and subjects affected by Parkinson's disease or by schizophrenia. In a clinical perspective, further studies are needed to explore the antitumor effects of the miR-193a-3p mimics delivery and the relevance of this miRNA detection as a possible diagnostic and prognostic biomarker.

1. Introduction

MicroRNAs (miRNAs) constitute a biologically very important class of small, noncoding RNAs, about 18–22 nucleotides (nt) long that mainly act as negative regulators of gene expression at posttranscriptional level by controlling the translation and stability of mRNA target. It is known that a miRNA may target several mRNAs as well as a mRNA can be under the control of several miRNAs. Most of the findings reported in the literature show clearly that miRNAs play an important role in several physiological and pathological processes exerting a highly precise regulation of most mRNA expression.

In this review, we focused on the human miR-193a-3p since the increasing number of evidences has described its importance in several biological functions. Moreover, the role as an important tumor suppressor miRNA has recently emerged in both liquid and solid tumors. According to UCSC Genome Browser (Human Dec. 2013 Assembly - GRCh38/hg38) [1], miR-193a coding gene, defined as *MIR193a*, is located on human chromosome 17q11.2 (chr17:31,558,803-31,560,358) (Figure 1(a)). By analyzing the region of 2000 bp spanning *MIR193a*, a typical CpG island is identified (chr17:31558803-31560358) in which miR-193a coding sequence is embedded. Interestingly, *MIR193a* is found internal to a sequence that displays a high level of enrichment of H3K27Ac, H3K4Me1, and H3K4Me3 histone marks. In detail, the acetylation of lysine 27 of the H3 histone protein, the monomethylation of lysine 4 of the H3 histone protein, and the trimethylation of lysine 4 of the H3 histone protein have been associated with enhanced transcription, enhancer, and active promoter, respectively. In addition, a regulatory region characterized by transcription factor binding sites is found upstream *MIR193a* (chr17:31558470-31559544) indicating that miR-193a coding sequence is localized in an active transcriptional region. The pre-miR-193a generates two mature miRNAs, miR-193a-3p and miR-193a-5p, depending on the arm that is processed during miRNA biogenesis (Figure 1(b)). Consequentially, the different sequence that characterizes miR-193a-3p and miR-193a-5p determines distinct target sets for each miRNA. In this review, we focused on the miR-193a-3p biological and molecular mechanisms both from the physiological and the pathological perspectives.

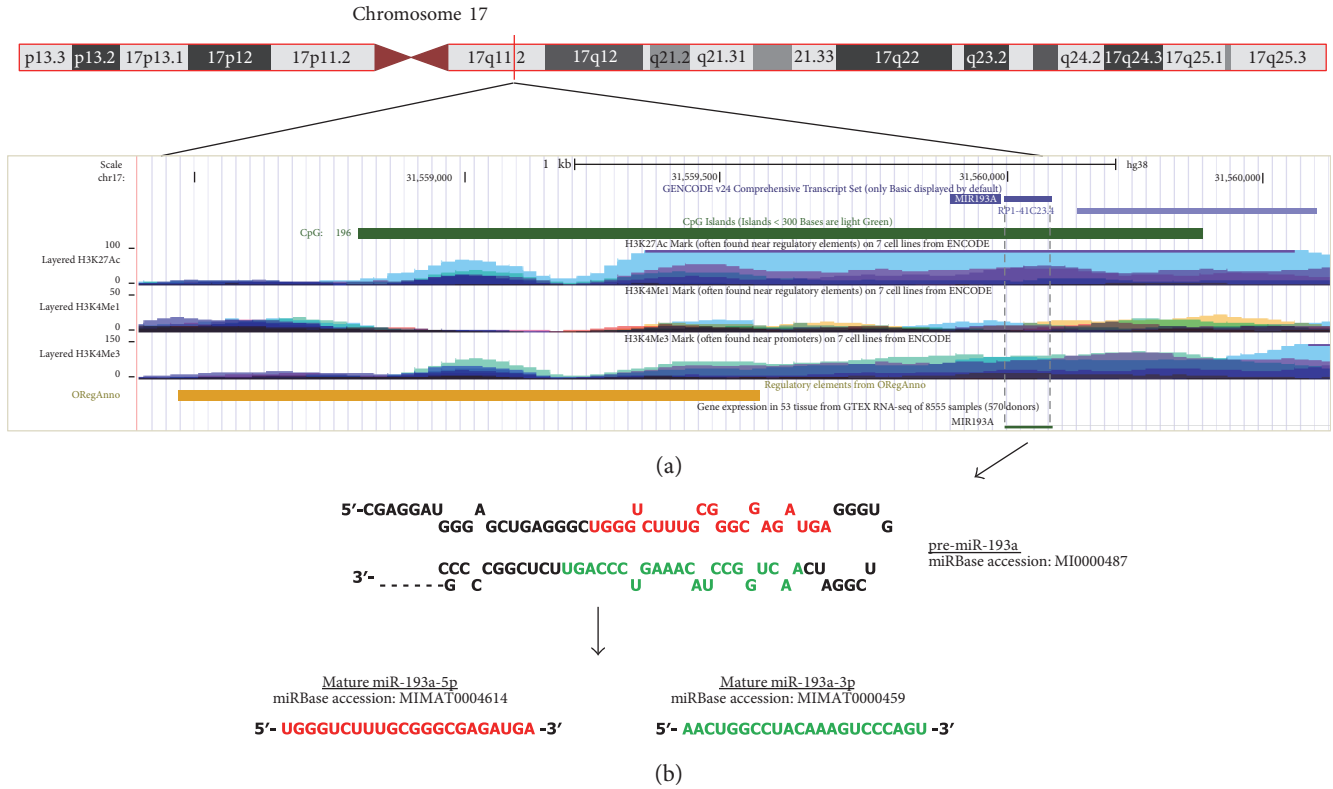

Figure 1: Genomic location of miR-193a coding sequence, stem-loop hairpin structure of pre-miR-193a and miR-193a-3p/miR-193a-5p sequences. (a) The analysis of the genomic region coding miR-193a referred to Genome Browser (https://genome-euro.ucsc.edu/). *MIR193a* coding sequence is located on human chromosome 17q11.2 characterized by a typical CpG island (in green). The layered H3K27Ac, H3K4Me1, and H3K4Me3 show the levels of histone marks across the genome in 7 cell lines (data obtain from ENCODE on the basis of ChiP-seq assay). By default, this track displays data from a number of cell lines in the same vertical space and each of the cell line is associated with a particular color. The regulatory element (in orange) is described as transcription factor binding sites by ORegAnno (open regulatory annotation). (b) The *MIR193a* gene is transcribed into a precursor (pre-miR-193a) with 88 nucleotides that is processed during miRNA biogenesis to yield mature miR-193a-5p (in red) and mature miR-193a-3p (in green) with 22 nucleotides in length.

Furthermore, the mechanisms involved in its expression regulation are also addressed. Finally, we highlighted the aberrant expression of miR-193a-3p both at tissue and at circulating levels in several pathological conditions, including cancer, in order to offer novel insights in the role of miR-193a-3p as a diagnostic and prognostic biomarker.

2. The Regulation of miR-193a-3p Expression

miR-193a-3p is highly conserved across several *Hominidae* (humans, chimpanzees, orangutans, and rhesus) and other mammals (*Mus musculus*, *Bos taurus*, and *Canis familiars*), as indicated in the microRNA viewer database (last update February 28, 2012) [2]. Several mechanisms, including transcription factors, DNA methylation, and competing endogenous RNAs (ceRNAs), have been reported to be involved in the dysregulation of miR-193a-3p in pathological contexts (Table 1). These evidences unmistakably suggest a multifactorial regulation of miR-193a-3p at transcriptional or posttranscriptional level with the possibility of a context-dependent activation of specific mechanisms.

2.1. Transcription Factors and Regulatory Proteins. Like protein-coding genes, the expression of miRNAs may be under the control of transcription factors (TFs) that bind to specific DNA sequences in the miR promoter and may act either as transcriptional activators or as repressors. The transcription silencing by specific TFs was reported to play a critical role in the inactivation of miR-193a-3p in different pathological contexts. Iliopoulos et al. showed that the downregulation of miR-193a-3p was driven by Max, the Myc-associated factor X, and RXRα, a nuclear receptor, both involved in the processes causing cellular transformation of breast epithelial cells. Using chromatin immunoprecipitation (ChIP) and siRNA-mediated inhibition, they demonstrated that Max and RXRα bound directly to the miR-193a regulatory region and repressed its transcription in ER-Src-transformed cells [3]. Similarly, Li et al. revealed that the downregulation of miR-193a-3p was strongly associated with fusion protein AML1/ETO expressed in hematopoietic cells isolated from patients affected by acute myeloid leukemia (AML) with t(8;21). In this pathological context, AML1/ETO acted as a transcriptional repressor by localizing the AML1 binding site on the *MIR193a* upstream region and

Table 1: Regulation of miR-193a-3p expression by different mechanisms.

Mechanisms of regulation	Effect on miR-193a-3p expression	Sample type	Experimental procedures	Ref.
Transcription factors:				
Max and RXRα	Downregulation	Transformed breast epithelial cells	ChIP; siRNA-mediated inhibition experiments	[3]
AML1/ETO	Downregulation	AML cell lines primary AML samples with t(8;12)	Luciferase reporter assay; ChIP	[4]
HNF4α	Upregulation	Liver from mice with liver-specific knockout of HNF4α	miRNA microarray and qPCR in Hnf4α-LivKO mice	[5]
DNA methylation:				
DNA hypermethylation	Downregulation	AML cell lines AML primary samples	MSP Bisulfite sequencing	[9]
DNA hypermethylation	Downregulation	OSC carcinoma cells and primary AML samples	COBRA Bisulfite sequencing	[8]
DNA hypermethylation	Downregulation	NSCL cancer cells NSCL specimens	MSP Bisulfite sequencing	[10, 11]
DNA hypermethylation	Downregulation	Highly metastatic osteosarcoma cells	Bisulfite sequencing	[12]
DNA hypomethylation	No effect	HCC cell lines HCC specimens	MSP	[14]
DNA hypomethylation	No effect	Mesothelioma	MSP	[13]
Regulatory protein:				
XB130	Downregulation	Thyroid carcinoma cells	shNA-mediated inhibition and ectopic expression experiments	[7]
Competing endogenous RNA (ceRNA) network:				
Linc00152	Downregulation	Colon cancer cells	RIP; luciferase reporter assay	[17]
LncRNA-UCA1	Downregulation	NSCL cells	RIP; luciferase reporter assay	[18]

recruiting HDAC and DNMTs. The chromatin remodeling complex formed by AML1/ETO and the DNA hypermethylation triggered the silencing of miR-193a-3p in t(8;21) AML [4].

Other two factors, hepatocyte nuclear factor α (HNF4α) and XB130, may also have a relevant role in the regulation of miR-193a-3p. HNF4α is a regulator of hepatic gene expression essential for liver development and function. The lacking of HNF4α expression in the liver of young adult mice (*Hnf4α*-LivKO) determined the downregulation of some miRs, including miR-193a that is in cluster with miR-365 on the chromosome 11 of *Mus musculus* [5]. XB130 is a member of the actin filament-associated protein (AFAP) family affecting the downstream signaling PI3k/Akt pathway by functioning as an adaptor protein and tyrosine kinase substrate [6]. In human thyroid carcinoma WRO cells and MRO cells, the gene silencing of XB130 by stable transfection of short hairpin increased both pri-miR-193a and its mature form (miR-193a-3p), while the ectopic expression of XB130 induced their downregulation [7]. These data indicated that the regulation of miR-193a-3p may be mediated by HNF4α and XB130 in a healthy liver and thyroid carcinoma, respectively. However, further studies based on ChIP and gene reporter assays are needed in order to examine the direct and specific mechanisms that link these factors to miR expression.

2.2. Epigenetic Regulation by DNA Methylation. The hypermethylation of CpG islands located around miR genes is a key mechanism of epigenetic downmodulation of miRs that acts as a tumor suppressor in specific tumors. The *MIR193A* gene is embedded in a 1556 bp CpG island that counts 196 CpG sites (Figure 1(a)). Several studies have found that altered DNA methylation occurring in the CpG sites of the miR-193a promoter in different types of tumors. The miR-193a-3p was silenced in oral squamous cell carcinoma (OSCC) cell lines and in primary tumors through aberrant DNA methylation of the CpG sites near the miR coding sequence as verified by COBRA (combined bisulfite restriction analysis) assay and bisulfite sequencing [8]. Gao et al. demonstrated that the promoter hypermethylation repressed miR-193a-3p expression in acute myeloid leukemia (AML). The authors studied the DNA methylation levels in several leukemia cell lines and bone marrow (BM) samples from AML patients and healthy donors by bisulfite sequencing and methylation-specific PCR (MSP). Treatment with the inhibitor of DNA methylation 5-azacytidine (5-aza-dC) restored miR-193a-3p expression and reduced its target, the oncogene c-kit. In this situation, the growth inhibition and the induction of apoptosis and differentiation of AML cells were observed [9]. miR-193a was also found tumor specifically methylated in patients with non-small-cell lung cancer (NSCLC) [10]. Treatment with 5-aza-dC upregulated

miR-193a-3p expression, impaired cell proliferation ability, and promoted apoptosis in NSCLC cells via downregulation of one of miR-193a-3p targets, the antiapoptotic myeloid leukemia cell sequence-1 (Mcl-1) [11]. Finally, Pu et al. found that both miR-193a-3p and miR-193a-5p were hypermethylated and downregulated in a metastatic osteosarcoma cell line [12].

Although altered DNA methylation levels have been associated with several types of tumor, this cannot be generalized. In malignant pleural mesothelioma (MPM), miR-193a-3p was inhibited when compared to normal pleura, but the DNA hypermethylation of miR-193a-associated CpG island was not responsible for the inhibition of miR-193a-3p in MPM cells as verified by MSP [13]. Interestingly, the results obtained in human hepatocellular carcinoma (HCC) by Grossi et al. were in line with those in MPM. The authors demonstrated that the downmodulation of miR-193a-3p in HCC was not mediated by DNA methylation in a cohort of 30 matched peritumoral and HCC tissues from bioptic samples. However, the miR-193a-3p CpG sites resulted methylated in the differentiated HepG2 cells and the treatment with 5-aza-dC led to miR-193a-3p increasing as observed also by Ma and colleagues [14, 15]. These results would point toward a variable miR-193a dependence on CpG DNA methylation in HCC. In conclusion, it has been proved that DNA hypermethylation of CpG island associated to *MIR193A* gene was responsible for miR-193a-3p downmodulation in certain types of cancer which in turn led to increased expression levels of miR-193a-3p targets involved in cell malignant behavior. In other types of cancer, DNA methylation may not contribute to the regulation of this miR.

2.3. The Competing Endogenous RNA (ceRNA). In the recent years, the important results obtained through the application of high-throughput RNA-seq have shed a light on the complex landscape of long noncoding RNAs (lncRNAs). These are RNA typically longer than 200 nucleotides without protein-coding potential. Several studies established the biological functions of lncRNAs comprising transcriptional and posttranscriptional regulation and chromatin modification. Furthermore, some lncRNAs have been characterized for their ability to regulate miRNA function by competing for miRNA binding and decreasing the negative effect of miRNAs on their targets. For this reason, these lncRNAs have been named as competing endogenous RNAs (ceRNAs) or miRNA sponges [16].

Consistently with the competing endogenous RNA role of lncRNAs, two different ceRNAs have been found to target miR-193a-3p where the physical association with mature miR-193a-3p has been demonstrated by RNA immunoprecipitation (RIP) and luciferase reporter assays. In particular, oncogenic Linc00152 (long intergenic noncoding RNA 152) and LncRNA-UCA1 (urothelial carcinoma-associated 1) competitively bind miR-193a-3p in colon cancer and NSCLC cell lines, respectively [17, 18]. Interestingly, Linc00152 and UCA1 functioned as miRNA sponges and suppressed the endogenous effect of miR-193a-3p by silencing the miR target ERBB4. In addition, the overexpression of Linc00152 or UCA1 increased cell growth through modulation of ERBB4 while this effect was attenuated by transfection of miR-193a-3p mimics in both cell lines.

These evidences strongly suggested that the ceRNA regulatory network should be considered as a mechanism involved in the dysregulation of miR-193a-3p.

3. Expression Profile of miR-193a in Human Normal Tissues

To provide complete information on the global expression profile of miR-193a-3p in normal tissues, we referred to data deposited in Genome Browser. The data have been reported as the median gene expression levels in 51 tissues and 2 cell lines, based on RNA-seq data obtained from the NIH Genotype-Tissue Expression (GTEx) project [19, 20]. This release is based on data from 8555 tissue samples obtained from the postmortem of 570 adult individuals with no evidence of disease. As indicated in Figure 2, the expression of miR-193a (without discriminating between miR-193a-3p and miR-193a-5p) has been detected in all tissues, with the exception of the bladder, some brain components (hippocampus, nucleus accumbens, and spinal cord), and cervix (endocervix) where miR-193a expression was not found. Adipose and breast tissues displayed the highest miR-193a expression level.

4. Biological Function of miR-193a-3p in Development and in Cell Physiology

Very little is known on the biological function of miR-193a-3p in cell physiology. To the best of our knowledge, there have not been reports on the role of this miR in development. Concerning its role in cell physiology, the main available data were obtained from studies on the following: (a) cord-blood and peripheral blood endothelial colony-forming cells (CB/PB-ECFC) derived from donations of healthy subjects; (b) from skeletal muscle specimens of control subjects (CTRL) with no sign of muscle pathology detectable by immunohistochemistry; and (c) from endometrial epithelial cells of healthy volunteer women aged 18 through 36 years old. In particular, it has been found that miR-193a-3p was one of the 25 miRNAs differentially regulated in CB-ECFC versus PB-ECFC [21]. It was highly expressed in the less-proliferative PB-ECFCs where its inhibition using anti-miR molecules improved the *in vitro* proliferation, migration, and vascular tubule formation of these cells. Conversely, miR-193a-3p was expressed at low amount in the proliferative CB-ECFCs with its *in vitro* ectopic overexpression limiting the proliferation and the cell cycle progression of these cells and consequent reduction of their vascular tubule formation and cell migration. Altogether, the data obtained by Khoo et al., by using the miRnome studies, in silico miRNA target database analyses combined with proteome arrays and luciferase reporter assays in miR mimic-treated ECFCs, allowed to identify the negative regulatory role of miR-193a-3p in the vascular function of these cells and in the proliferation and migration abilities of these cells via directly targeting the HMGB1 expression (Table 2). Thus, this miR has a regulatory role in cell physiology of ECFCs

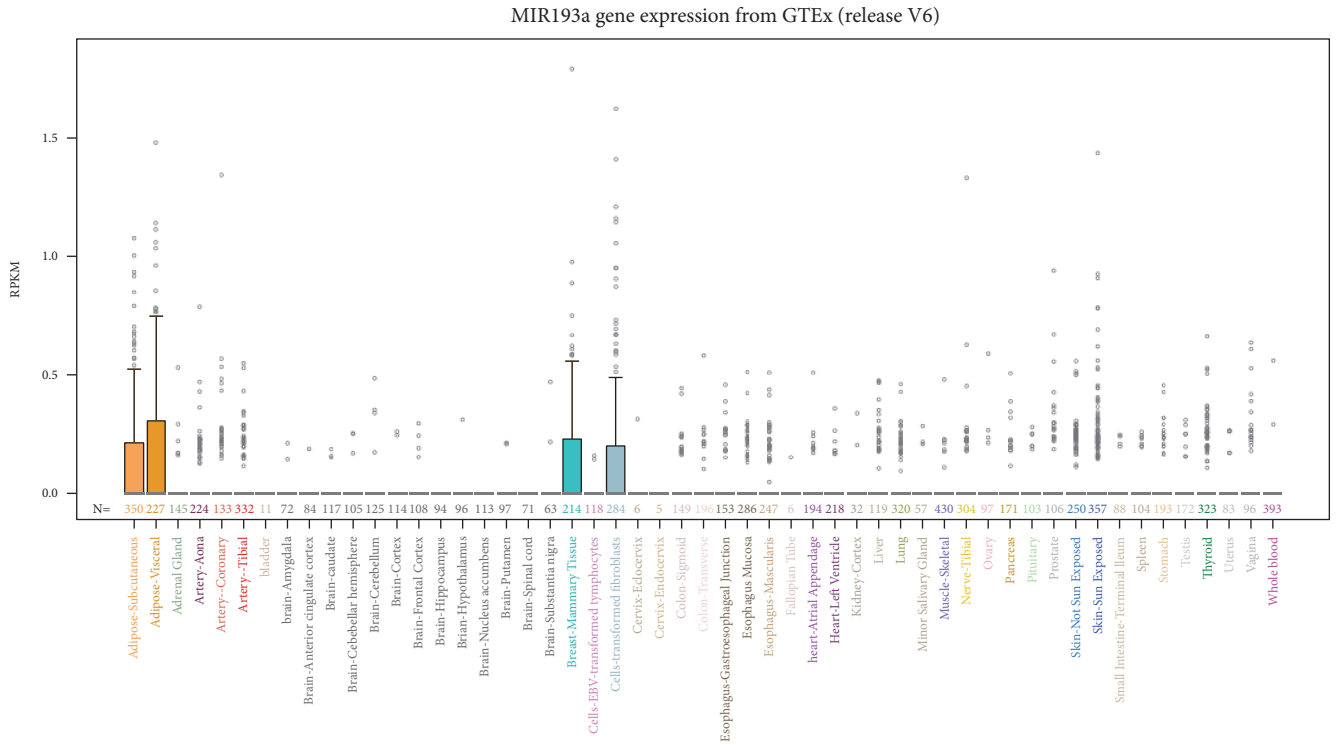

FIGURE 2: Expression profile of miR-193a in normal tissues. The expression level of miR-193a is reported for 51 tissues and 2 cell lines (EBV-transformed lymphocytes and transformed fibroblasts) and is referred to GTEx project collected in Genome Browser. Expression values are shown in RPKM (reads per kilobase of transcript per million mapped reads). The height of each bar represents the median expression level across all samples for a tissue, and points are outliers if they are above or below 1.5 times the interquartile range. Each color represents a specific tissue, conformed to GTEX consortium publication convention.

that are considered circulating endothelial lineage progenitors. Targeting the miR itself by anti-miR molecules may improve the abilities of PB-ECFC cells in proliferation and migration, in their angiogenic function thus contributing to a positive clinical outcome in ischemic diseases (stroke, myocardial infarction, and limb ischemia).

For skeletal muscle, a miRNA profiling approach combined with bioinformatics analyses and qPCR experimental validation has identified 11 miRNAs including miR-193a-3p involved in the homeostasis of normal myofibers. In particular, downregulation of miR-193a-3p has been associated with events contributing to the myofiber alterations of patients with myotonic dystrophy type 2 (DM2, OMIM 602688) [22]. DM2 is an autosomal dominant multisystemic disorder affecting the skeletal muscles, the heart, the eye, the central nervous system, and the endocrine system. The findings reported by Greco et al. clearly showed that the level of miR-193a-3p downmodulation contributed to the DM2 miRNA score allowing to distinguish the muscle specimens of DM2 patients from those of controls. However, these results do not permit to outline a hypothesis on the functional role in normal myofibers of miR-193a-3p as well as those of the other 10 miRNAs deregulated in DM2 muscle biopsies.

Finally regarding the human endometrium, several data suggested a regulatory function of miRNAs during the physiological cycle phases. In particular, Kuokkanen et al. provided strong evidence of the hormonal regulation of miR-193a-3p expression in isolated uterine epithelial cells derived from midreproductive aged women [23]. These authors examined miRNAs at two stages: (a) in uterine epithelial cells derived from late proliferative phase biopsies (cycle day, CD, 12 ± 1) to target the time of maximal endometrial response to female steroid hormone estradiol-17 beta (E2) and (b) from secretory biopsies specimens from midluteal phase on CDs 19 through 23 to target the endometrial window of receptivity and maximum P4 (progesterone) action. The findings obtained using a genomic profiling of miRNAs and mRNAs clearly showed that miR-193a-3p is one of the 12 miRs found to be upregulated in the midsecretory phase samples. This expression is suggestive of a role in downregulating some cell cycle genes in the secretory phase thereby suppressing proliferation of the endometrial epithelial cells in this specific physiological context. Further, these data demonstrate hormonal regulation in miRNA (i.e., miR-193a-3p) expression in a human endometrium.

In summary, the overexpression of miR-193a-3p in cultured normal cells derived from physiological contexts, in particular in PB-ECFC cells and in midsecretory uterine epithelial cells, seemed to limit cell proliferation and cell cycle progression. However, the lack of data (KO, KI, and conditional KD) in the development of an organism (i.e., *Mus musculus* and *Danio rerio*) carrying the ortholog *MIR193A* (Gene Card data) has prevented the proposal of hypothesis on the role of miR-193a-3p during development [24].

Table 2: miR-193a-3p gene targets validated experimentally.

Target genes	Functions	Cell line	References
K-Ras	Oncogene involved in many functions: antiapoptotic activity, angiogenesis, motility, cell growth	Breast cancer Lung cancer	[3, 29]
PLAU	Promotes cancer invasion and metastasis; the interaction of uPA with its receptor induced also cell proliferation, migration, and expression of specific genes	Transformed breast epithelial cells Breast cancer HCC BCa	[3, 32, 33, 40]
Mcl-1	Antiapoptotic gene, member of the Bcl-2 family	HeLa cells Ovarian cancer	[30, 31]
ERBB4	Induces a variety of cellular responses including mitogenesis and differentiation; triggers proliferation, invasion, and migration	Lung cancer NSCLC	[27, 37]
S6 K2	Promote cell survival	NSCLC	[37]
PepT1	Transporter involved in the low uptake of small bacterial peptides in a normal colon and of dietary proteins	Epithelial colorectal adenocarcinoma	[46]
c-kit	Oncogene. Activates many pathways involved in proliferation, differentiation, migration, and survival	AML	[9]
Aml1/Eto	Chimeric protein associated with the nuclear corepressor/histone deacetylase complex to block hematopoietic differentiation	AML	[4]
HDAC3	Regulates transcription and modulates cell growth an apoptosis	AML	[4]
DNMT3a	De novo DNA methylation	AML	[4]
Cyclin D1	Cell cycle progression	AML Breast cancer	[4] [28]
E2F6	Transcription factor with a main role in the control of the cell cycle	OSCC	[8]
Rab27B	Increased invasion and metastasis in cancer	Osteosarcoma	[12]
HMGB1	Tissue repair and regeneration, migration, angiogenesis, endothelial recruitment, and proliferation	Endothelial colony forming cell, ECFC	[21]
HYOU-1	Cytoprotective role in hypoxia-induced cellular perturbation	Endothelial colony-forming cell, ECFC	[21]
PSEN1	Promote cell proliferation	Esophageal squamous cell carcinoma Bladder cancer	[45]
E2F1	Transcription factor; control of the cell cycle and apoptosis	HCC	[15]
SRSF2	Regulates constitutive and alternative splicing; induces proapoptotic splice forms of apoptotic genes	HCC	[15]
HIC2	Putative transcriptional factor	BCa	[40]
HOXC9	Transcription factor; role in morphogenesis	BCa	[43]
ING5	Inhibit cell growth and induce apoptosis	BCa	[42]
LOXL4	Biogenesis of connective tissue: catalyzes the first step in the formation of crosslinks in collagens and elastin	BCa	[41]
SLC7A5	Large neutral amino acid transporter small subunit 1	Thyroid carcinoma	[7]
JNK1	MAP kinases involved in proliferation, differentiation, transcription regulation, and development	Breast cancer	[26]

5. miR-193a-3p Functions as Tumor Suppressor miRNA in Cancer

It is widely documented that the aberrant expression of miRNAs has a critical impact on cell biological processes and contributes to a number of pathological conditions, such as cancer. To the best of our knowledge, all published data pointed toward a role of miR-193a-3p as tumor suppressor miRNA (ts-miRNA) in both solid and liquid cancers since it impaired tumor cell aggressive properties by targeting oncogenes. In addition, miR-193a-3p is found downregulated in transformed cells and its downregulation seemed to be required for cellular transformation in two isogenic models (breast epithelial cells and fibroblasts) [3]. By considering a small cohort of cancer patients (only 36 cases), Yi et al. found that miR-193a-3p is upregulated in 24/36 esophageal squamous cell carcinoma (ESCC) tissues compared with adjacent normal tissues and the downregulation of miR-193a-3p by a synthesized inhibitor decreases migration and proliferation and promotes apoptosis in ESCC cells [25]. For these reasons, they described miR-193a-3p as an oncogenic miRNA in ESCC and suggested further studies to define the controversial role of miR-193a-3p in ESCC.

5.1. miR-193a-3p Limits Cancer Cell Proliferation and Impairs Cell Cycle Progression. Many studies have confirmed

that miR-193a-3p has a significant role in the regulation of cancer cell growth. In particular, miR-193a-3p directly targeted JNK-1, a tyrosine kinase, since the ectopic expression of miR-193a-3p determined the dysregulation of cell cycle components including the decrease of CDK4, PIK3CA, and cyclin D1 and the overexpression of p27. In association to miR-124 and miR-147, miR-193a-3p has been shown to coregulate and inhibit G1/S transition and proliferation in breast cancer and glioblastoma cell lines [26]. Moreover, miR-193a-3p repressed cell proliferation of AML cells through the inhibition expression of c-kit, an oncogene encoding a transmembrane glycoprotein belonging to the type III receptor tyrosine kinase family [9]. In the same clinical context, miR-193a-3p has been also found to directly regulate the expression of DNMT3a, HDAC3, and cyclin D1 consequently blocking the cell cycle progression during granulopoiesis and inducing the differentiation of myeloid precursors [4]. It has also been shown that miR-193a-3p decreased the abilities of proliferation by the expression inhibition of some TFs, including E2F6, and other genes involved in the growth of several cancer types, for example, K-Ras, ERBB4, and cyclin D1 [3, 8, 27, 28]. In particular, it has been demonstrated that miR-193a-3p negatively regulated K-Ras in lung cancer cells by binding two 3′UTR sites that have not been reported previously to be mutated in cancer. The overexpression of miR-193a-3p not only downregulated K-Ras but also reverted the whole protein signature associated with the signaling downstream of K-Ras identified by proteomic analysis of lung cancer samples. The authors clearly determined the effects of miR-193a-3p on the cell aggressive properties via the targeting of K-Ras. Interestingly, miR-193a-3p decreased cell cycle progression (G1-S) and cell proliferation *in vitro* and blocked colony formation in three-dimensional cultures. These findings have been translated into exciting ex vivo and *in vivo* experiments. For ex vivo experiments, the authors harvested lung-heart blocks from Sprague Dawley rats. To create a metastatic ex vivo 4D lung model, the lungs were decellularized and placed in a bioreactor with an oxygenator, pump with the right main stem ligated with silk suture, and A549 lung adenocarcinoma human epithelial cells were seeded in the left lung through the tracheal cannula. The use of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) nanoliposomes to deliver miR-193a-3p reduced the number of viable cells and impaired the presence of cancer cells in contralateral lobe that is indicative of metastasis formation in this model. In addition, this ex vivo model allowed the collection of circulating tumor cells (CTCs) from the perfused cell media present in a bioreactor bottle. Interestingly, cells derived from a 4D model treated with miR-193a-3p showed less proliferation ability than those from untreated model. In orthotopic xenograft K-Ras-mutated lung tumor models, miR-193a-3p encapsulated in DOPC nanoliposomes showed a reduction of tumor growth and metastasis at various sites [29].

5.2. miR-193a-3p Induces Cell Death Mainly by Promoting Apoptosis. When considering the miRNA target genes validated in different cancers, the role of miR-193a-3p in affecting genes with the consequence of promoting apoptosis stood out. In fact, among the putative targets, Mcl-1 is the most validated one involved in the programmed cell death. Mcl-1 is a multidomain protein belonging to the Bcl-2 family that binds and sequesters the BH3-only proapoptotic Bcl-2 family members (Bim, Bid, Bik, Noxa, and Puma) which in turn induce Bak and Bax homo-oligomerization and activation. Kwon et al. demonstrated that miR-193a-3p expression was induced by ionizing irradiation in U-251 glioma cells and HeLa cells. miR-193a-3p negatively regulated Mcl-1 and promoted apoptosis by inducing ROS accumulation and DNA damage [30]. The direct binding of miR-193a-3p and Mcl-1 has also been demonstrated in human ovarian cancer cell line where the overexpression of miR-193a-3p induced the activation of caspase 3/7 and resulted in apoptotic cell death [31]. Furthermore, the transfection with miR-193a-3p mimics in MPM cells reduced Mcl-1 protein level and increased the number of late apoptotic cells. In addition, the release of lactate dehydrogenase (LDH) from MPM cells transfected with miR-193a-3p could suggest that miR-193a-3p induced cell death at least in part by the induction of necrosis. The ability of miR-193a-3p to promote apoptosis was further demonstrated in MPM xenograft models when targeted by miR mimics delivered using EDV nanocells, bacterially-derived minicells that can be packaged with a variety of cargoes and be delivered to tumors via bispecific antibodies attached on surface [13]. Interestingly, Salvi et al. showed that the combination of miR-193a-3p mimics and sorafenib had additional effects on HCC inhibition of cell proliferation and induction of apoptosis suggesting that miR-193a-3p could also play an important role in promoting the sensitivity to sorafenib [32], the only innovative drug used for advanced HCC.

5.3. miR-193a-3p Impairs Cancer Migration, Invasion, and Metastasis. Tumor cell invasion and metastasis are events of primary importance in the prognosis of cancer patients. Metastatic cells are able to invade the basal membrane (BM) and extracellular matrix (ECM), to penetrate and move into the lymphatic or vascular circulation and to produce a secondary tumor by extravasation process and subsequent cell proliferation. Several evidences have highlighted the roles of miRNAs in these complex processes.

Recently, it has been demonstrated that miR-193a-3p acted as a negative regulator of urokinase-type plasminogen activator (uPA) in breast cancer and HCC cell lines and the high expression of miR-193a by mimics transfection strongly inhibited uPA expression and decreased cell aggressive properties [3, 32, 33]. uPA is a serine protease which converts the proenzyme plasminogen into the serine protease plasmin, thus making malignant cancer cells able to degrade BM and ECM. Furthermore, the interaction with its receptor, uPAR, leads to the activation of different intracellular signaling pathways, altering cell proliferation and migration abilities and expression of specific genes. The essential role played by uPA in migration has been well characterized in pathological context like cancer, and its overexpression was detected in various tumors, at both mRNA and protein level, representing an unfavorable prognostic factor [34–36].

Recently, Yu et al. validated miR-193a-3p as a negative regulator of ErbB4, belonging to the ErbB family of tyrosine kinase receptors and the ribosomal protein S6K2, both playing a critical role in cell movement, growth, and development [37]. The expression of miR-193a-3p and miR-193a-5p was positively associated with cellular invasion and migration by assessing human lung cancer cell with high metastatic potential (SPC-A-1sci) previously established from weakly metastatic cell (SPC-A-1) through *in vivo* selection in NOD/SCID mice [38]. Furthermore, the overexpression of miR-193a-3p inhibited migration, invasion, and epithelial mesenchymal transition *in vitro* and impaired the formation of metastasis *in vivo*. In addition, the protein profile of SPC-A-1sci cells stably transfected with miR-193a-3p has been determined by using a proteomic approach (iTRAQ and Nano LC-MS/MS) followed by DAVID (database for annotation, visualization, and integrated discovery; http://david.abcc.ncifcrf.gov) and STRING analysis. Interestingly, 112 proteins resulted differentially expressed (62 upregulated and 50 downregulated) compared with miR control-transfected cells, and some of them have been associated to lung cancer metastasis and proliferation [39].

Similarly, Pu et al. reported that miR-193a-3p and miR-193a-5p were downregulated in osteosarcoma cells defined as highly tumorigenic and metastatic (MG63.2) in respect to less metastatic parental MG63 cell line. The abilities of MG63.2 cells to invade and migrate resulted decreased by restoring the miR-193a-3p expression level using transient transfection of miR mimics. Correspondingly, the inhibition of miR-193a-3p by antagomiR transfection in MG63 cells induced invasive properties. Furthermore, the authors identified Rab27, a member of the RabGTPase family, as a direct target of miR-193a-3p. By Cignal reporter finder assay, they showed that the Rab27 knockdown repressed some pathways that were clearly implicated in metastasis, including TGFβ, Myc/Max, and ATF2/ATF3/ATF4. These results indicated the negative impact of miR-193a-3p on cancer invasion by repressing Rab27B and its downstream pathways in osteosarcoma cells [12].

Taken together, these evidences strongly suggest a potential role of miR-193a-3p as a metastasis-preventing miRNA. However, further work will be required to explore the exact molecular pathways by which this miRNA could exert its functions.

5.4. miR-193a-3p Modulates Drug Resistance in Cancer Cells. To date, chemotherapy still represents one of the most used therapeutic options for the treatment of solid tumors worldwide. However, the clinical efficacy of these treatments is limited by the onset of drug resistance and the side-effects of the drug both contributing to reduce cancer patients' positive outcome. Even if the specific regulatory mechanism involved in chemoresistance remains very often unclear, increasing evidences showed that miRNAs can have a crucial role in chemosensitivity by regulating cancer-related genes. As indicated in Figure 3(a), miR-193a-3p seems to be implicated in the activation of drug resistance pathways via repressing different targets. By a systematic analysis that compared the H-bc multidrug resistant bladder cancer (BCa) cells versus 5637 sensitive ones, Lv et al. reported that miR-193a-3p was silenced by DNA hypermethylation in the sensitive cells, the cell line presenting the lowest IC_{50} to different drugs. In chemoresistant BCa cells, miR-193a-3p decreased the expression of the following targets: SRSF2, PLAU, HIC2 [40], LOXL4 [41], ING-5 [42], HOXC9 [43], and PSEN-1 [44]. The last target has been considerate also relevant in chemo- and radioresistant esophageal cancer cell [45]. The results obtained through pathway reporter system assays revealed that the reduced level of these targets affected the activities of five signaling pathways in resistant cells. In particular, DNA damage, NF-κB, and Myc/Max pathways were found with lower activities, while Notch and oxidative stress pathways resulted activated in resistant cell compared with sensitive cells. In addition, the modulation of miR-193a-3p level by the injection of antagomir or agomir molecules in either resistant or sensitive BCa cells reversed the chemoresistance in tumor xenografts nude mice. Similarly, miR-193a-3p expression was found increased by DNA hypomethylation in HCC cells presenting resistance to 5-fluorouracil (5-Fu). miR-193a-3p seemed to induce antiapoptotic signals in 5-Fu resistant cells by suppressing SRSF2, a splicing factor that preferentially upregulates the proapoptotic form of caspase 2 (CASP2L) to the antiapoptotic form CASP2S [15]. These intriguing data will require further investigations since it is not clear how the overexpression of miR-193a-3p can dictate chemoresistance even if its tumor suppressor functions have been well established in many primary cancers. In support of this notion, Yue et al. reported that the reduced activity of miR-193a-3p caused by the sponge effect of Linc00152 was related to oxaliplatin (L-OHP) resistance in colon cancer both *in vitro* and *in vivo* (Figure 3(b)). Linc00152 is usually overexpressed in human colon cancer tissues and is associated with poor prognosis in patients undergoing L-OHP treatment after surgery. Interestingly, Linc00152 competitively bound miR-193a-3p inducing the upregulation of its ERBB4 target and the consequent activation of AKT signaling pathway which, in turn, conferred resistance [17].

6. Gene Annotation Analysis on Predicted and Experimentally Validated miR-193a-3p Targets

It is well known that a given miR can regulate the expression of hundreds of targets, and conversely, dozens of miRs can target a single mRNA. For hsa-miR-193a-3p, TargetScan 7.1 predicted 293 putative target genes with 168 displaying the highest score (cumulative weighted context++ score < -0.24). The functional annotation analysis conducted using DAVID 6.8 on the candidate target genes of miR-193a-3p highlighted 8 biological KEGG pathways overrepresented as statistically significant ($p < 0.05$). They were the following: microRNA in cancer, ErbB signaling pathway, GnRH signaling pathway, acute myeloid leukemia, PI3K-Akt signaling pathway, Ras signaling pathway, pathways in cancer, and focal adhesion. The importance of these pathways is indicated by the fact that they are highly relevant

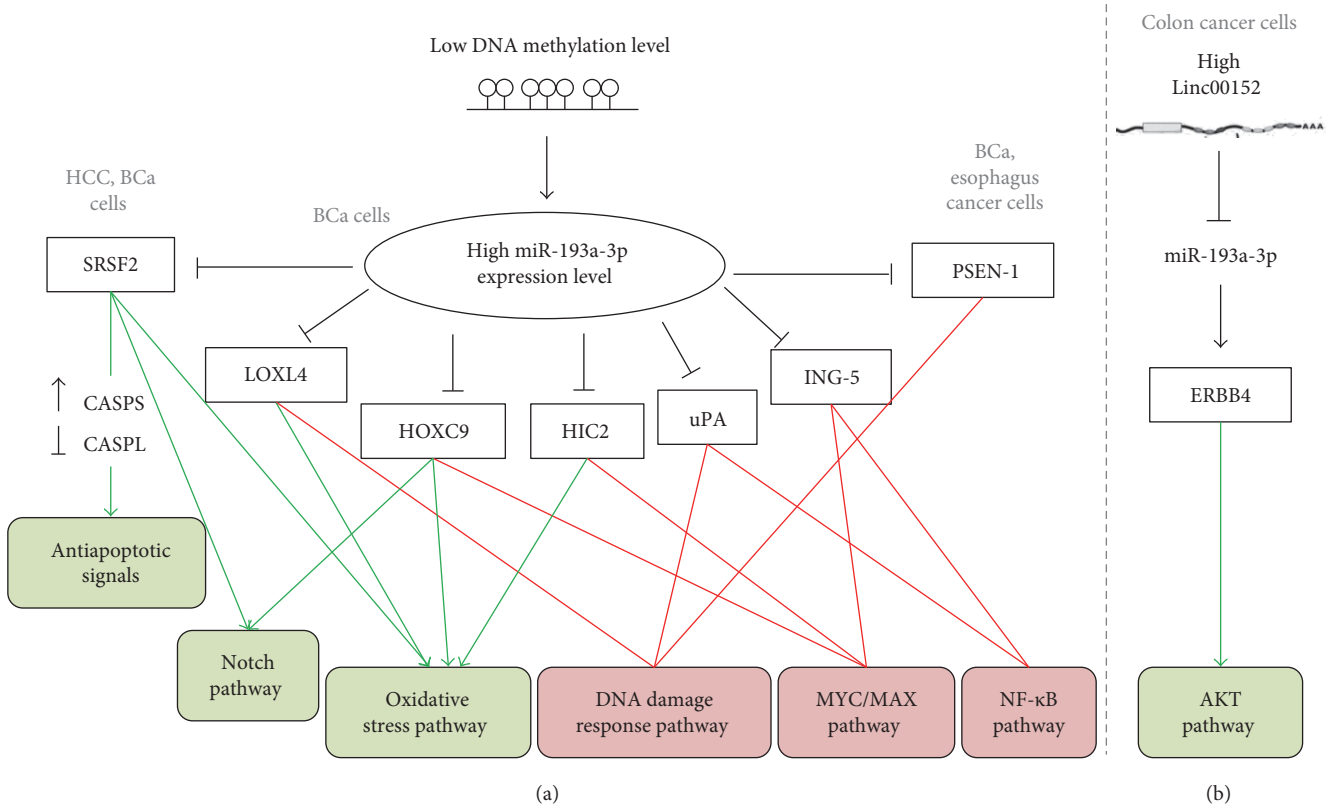

Figure 3: Proposed model for the role of miR-193a-3p in the regulation of the chemoresistance in different cancer cell lines. (a) Involvement of high miR-193a-3p expression level in chemoresistance in HCC cells, BCa cells, and esophageal cancer cells. (b) Low levels of miR-193a-3p are involved in resistance to oxaliplatin in colon cancer cells. Altered expression of miR-193a-3p will adversely affect immediate targets indicated inside black boxes. In turn, these targets will affect several downstream pathways with green arrows and red lines representing functional activation and repression, respectively.

in the processes of onset, progression, and metastasis of several types of cancer. In addition, among the 24 experimentally validated and published miR-193a-3p targets (Table 2), the KEGG pathway enrichment analysis outlines 6 terms ($p < 0.05$): acute myeloid leukemia, chronic myeloid leukemia, microRNA in cancer, proteoglycans in cancer, Erb signaling pathway, pancreatic cancer, and pathways in cancer. This bioinformatics analysis underscored once again the key role of miR-193a-3p in cancer contexts.

7. miR-193a-3p as Diagnostic and Prognostic Biomarker

It is well known that the aberrant miRNA expression has a critical impact on many cell biological processes and contributes to a number of pathological conditions. For this reason, the study of miRNA expression profile in pathological contexts is necessary to support the clinical significance of a specific miRNA and its possible role as a diagnostic and prognostic biomarker.

7.1. Dysregulation of miR-193a-3p in Cancer Tissues. Consistent with the role of ts-miRNA, miR-193a-3p was found downregulated in the majority of primary cancer tissues, such as HCC [14, 47], NSCLC [37, 48], MPM [13], and AML [4, 9]. Furthermore, the low expression of miR-193a-3p was significantly related to reduced overall survival (OS) and disease-free survival (DFS) of HCC patients indicating its possible prognostic role in this cancer type [14]. In NSCLC, the expression of miR-193a-3p was negatively correlated to tumor size, lymph node metastasis (LNM), and TNM stages. Interestingly, miR-193a-3p was reported as downregulated in BRAF mutation with respect to wild-type melanoma [49] suggesting that miR-193a-3p may have a role in BRAF-associated events.

7.2. Circulating miR-193a-3p Levels in Pathological Conditions. Several data have assessed that circulating microRNAs in human body fluids (i.e., serum and plasma) offer unique opportunities as biomarkers for early diagnosis of clinical conditions [50]. Indeed, some small ncRNAs have been found highly stable under extreme conditions (i.e., extreme pH and temperature and ribonuclease digestion) and numerous studies of circulating microRNA profiling have been conducted for several diseases. In regard to circulating miR-193a-3p, the use of next-generation sequencing and qPCR revealed different levels of this miR in patients with schizophrenia or with Parkinson's disease (PD) when compared with control subjects. Regarding PD, serum miRNA level obtained from a small number of patients

revealed that miR-193a-3p was among the panel of four miRNAs significantly decreased in the PD patients compared to controls. Furthermore, miR-193a-3p could also be used to distinguish the HY-stage1 in PD patients from healthy controls [51]. In schizophrenia, the higher level of plasma miR-193a-3p (and miR-130b) in patients compared to controls was determined by the global plasma miRNAs profiling in a test cohort of 164 schizophrenia patients and 187 control subjects and subsequently validated by qRT-PCR in an independent cohort of 400 schizophrenia patients [52]. We think that these findings are extremely interesting, but further studies are needed in order to support the detection of circulating miR-193a-3p as a noninvasive biomarker for these diseases.

Regarding cancer, circulating miR-193a-3p levels were found increased in many malignancies. By comparing two independent miRNA microarrays, one in tissue and one in blood of colorectal cancer patients, Yong et al. identified higher levels of miR-193a-3p (in combination to miR-23a and miR-338-5p) in cancer patients, and the positive correlation was demonstrated between tissue and blood samples [53]. The high-throughput TaqMan low-density array (TLDA) combined with qPCR validation allowed to establish the high level of miR-193a-3p included in two different five-serum miRNA panels, either in renal cell carcinoma (RCC) or in NSCLC patients. It was clearly demonstrated by ROC analysis that the 5-miRNA-based panels (miR-193a-3p, miR-362, miR-572, miR-425-5p, and miR-543) had a high sensitivity and specificity in the discrimination of patients with early-stage RCC from healthy controls [54]. In NSCLC, the effectiveness of the 5-miRNA panel (miR-193a-3p, miR-483-5p, miR-214, miR-25, and miR-7) in discriminating cancer patients from normal subjects was confirmed in a multiethnic and multicentric study in which 438 participants from both China and America were enrolled (221 NSCLC patients, 161 normal controls, and 56 benign nodules) [55]. By using the same experimental approaches (TLDA followed by qPCR), Wu et al. identified significantly elevated levels of miR-193a-3p in sera from patients with esophageal squamous cell carcinoma (ESCC). The authors indicated that miR-193a-3p may be used to discriminate between ESCC cases and healthy controls with high sensitivity and specificity in a cohort of 63 patients and 63 controls. The level of circulating miR-193a-3p was reduced after ESCC surgical removal indicating that this miR may have been originally secreted by the tumor cells [56]. Interestingly, in a retrospective longitudinal phase 3 biomarker study, a set of 5 serum miRNAs (miR-193a-3p, miR-369-5p, miR-672, miR-429, and let-7i*) was identified as a specific biomarker for the surveillance and preclinical screening of HCC in a high-risk population of patients infected by hepatitis B virus (HBV). In particular, the different expression levels of these miRNAs (including the downregulation of miR-193a-3p) were identified in HBV patients who developed HCC (preclinical HCC patients) compared to the HBV group that did not develop HCC [57].

Although the origin of circulating miRNAs remains unclear, it has been reported that they may originate through different pathways including passive leakage from broken cells, active secretion via microvesicles, and active secretion through an RNA-binding protein-dependent pathway that has been suggested as the major source of circulating miRNAs [58, 59].

Extracellular vesicles (EVs) are mediators of intercellular communications during several physiopathological processes such as differentiation, tissue repair, proliferation, and apoptosis, and they are released from both cancer cells and noncancer cells. Among the EVs, exosomes are small vesicles (50–150 nm) able to transport and deliver proteins, mRNAs, and ncRNAs including miRs from a donor to recipient cells [60]. Oh et al. demonstrated that the exosomes containing miR-193a-3p were able to induce differentiation of F11 cells (rat dorsal root ganglion and mouse neuroblastoma hybrid cells). By a microfluidic assay that collected real-time images of exosomes migration, they verified that miR-193a-3p was a neurogenic miR that promoted the differentiation of recipient undifferentiated cells [61]. Teng et al. demonstrated that miR-193a-3p was present into exosomes obtained from tissue and cell culture media and serum, derived from primary mouse colon tumors and human liver metastasis of colon cancer. In severe disease, the high level of miR-193a-3p into the exosomes led to the reduction of cytoplasmatic miR-193a-3p that in turn promoted the progression of premetastatic cells to metastatic ones. The authors found that overexpression of MVP (major vault protein) transported miR-193a-3p from the tumor cells to exosomes. On the contrary, MVP knockout determined miR-193a-3p accumulation in tumor cells triggering the inhibition of cell proliferation and cell cycle G1 arrest due to miR-193a-3p binding to its target, caprin-1 [62].

8. Conclusions and Perspectives

In the last decade, an increasing number of evidences have proved the biological importance of miRNAs in physiological contexts and a huge number of studies have pointed to the fact that dysregulation of miRNAs plays a fundamental role in several pathological conditions, including cancer. In the present report, we focused on miR-193a-3p because several findings support its role as tumor suppressor miR both in solid and in liquid tumors leading to believe that the detection of this miR at tissue and/or circulating level may be employed as a diagnostic and prognostic biomarker for certain types of tumors. It is worth to outline that the functional role of a given miR can be tissue- or tumor-type dependent and that few data indicate a possible oncogenic role of miR-193a-3p in ESCC; nevertheless, we think that only strong clinical evidence, as well as biological studies of the miR mimics on the proliferation of ESCC cell lines will elucidate this possible role of miR-193a-3p in this specific context of human cancer.

From a general point of view, miR-193a-3p had been studied in *Homo sapiens* and, to the best of our knowledge, no information is available in development. Regarding the biological function in physiological contexts, certain types of human normal cells seem to require high expression level of miR-193a-3p when they do not need to proliferate. As a consequence, the cancer cells that usually need high proliferative capacity show low expression levels of this miR.

Different factors can contribute to the regulation of *MIR193a* expression, including TFs, DNA methylation and, at posttranscriptional level, ceRNAs. The alteration of these factors is context dependent and determines the aberrant expression of miR-193a-3p in cancer. The elucidation of these mechanisms may allow extending our knowledge on the level of miR-193a-3p dysregulation.

As already mentioned, the dysregulation of a given microRNA may alter the expression of hundreds of genes in cancer affecting the entire network in which targets are involved. By considering all experimental validation studies in cancer, genes targeted by miR-193a-3p are involved in several biological processes, including proliferation, apoptosis, migration, and metastasis. To acquire major advancements in knowledge and comprehension of the canonical and non-canonical mRNA targets, more studies involving the use of proteomics profiling and RNA pull down with biotinylated microRNA mimics are needed.

To date, different clinical trials have demonstrated the use of miRNA-based therapy as a promising strategy for the treatment of different diseases, making miRNA highly relevant for a clinical use [63]. In this regard, the delivery of miR-193a-3p mimics by nanosized particles could represent a novel therapeutic tool for the treatment of cancer since it may hamper tumor aggressive properties in tumor xenograft models by restoring the miR original levels. On the other hand, the local delivery of anti-miR-193a-3p molecules could be an effective intervention for local ischemic diseases. These findings may pave the way to further studies aimed to elucidate the possible use of miR-193a-3p for experimental therapeutic procedures.

Finally, compelling evidences indicated that miR-193a-3p is detectable not only in primary cancer tissues but also at circulating level (in exosomes or not) in cancer patients indicating the possible diagnostic and prognostic value of miR-193a-3p. In addition, altered circulating levels of this miR have been identified in subjects affected by Parkinson's disease or schizophrenia. Further studies are really necessary to verify whether the detection of miR-193a-3p may be helpful for the characterization of these two diseases.

Conflicts of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] W. J. Kent, C. W. Sugnet, T. S. Furey et al., "The human genome browser at UCSC," *Genome Research*, vol. 12, no. 6, pp. 996–1006, 2002.

[2] A. Kiezun, S. Artzi, S. Modai, N. Volk, O. Isakov, and N. Shomron, "miRviewer: a multispecies microRNA homologous viewer," *BMC Research Notes*, vol. 5, p. 92, 2012.

[3] D. Iliopoulos, A. Rotem, and K. Struhl, "Inhibition of miR-193a expression by Max and RXRα activates K-Ras and PLAU to mediate distinct aspects of cellular transformation," *Cancer Research*, vol. 71, no. 15, pp. 5144–5153, 2011.

[4] Y. Li, L. Gao, X. Luo et al., "Epigenetic silencing of microRNA-193a contributes to leukemogenesis in t(8;21) acute myeloid leukemia by activating the PTEN/PI3K signal pathway," *Blood*, vol. 121, no. 3, pp. 499–509, 2013.

[5] H. Lu, X. Lei, J. Liu, and C. Klaassen, "Regulation of hepatic microRNA expression by hepatocyte nuclear factor 4 alpha," *World Journal of Hepatology*, vol. 9, no. 4, pp. 191–208, 2017.

[6] R. Zhang, J. Zhang, Q. Wu, F. Meng, and C. Liu, "XB130: a novel adaptor protein in cancer signal transduction," *Biomedical Reports*, vol. 4, no. 3, pp. 300–306, 2016.

[7] H. Takeshita, A. Shiozaki, X. H. Bai et al., "XB130, a new adaptor protein, regulates expression of tumor suppressive microRNAs in cancer cells," *PLoS One*, vol. 8, no. 3, article e59057, 2013.

[8] K. Kozaki, I. Imoto, S. Mogi, K. Omura, and J. Inazawa, "Exploration of tumor-suppressive microRNAs silenced by DNA hypermethylation in oral cancer," *Cancer Research*, vol. 68, no. 7, pp. 2094–2105, 2008.

[9] X. N. Gao, J. Lin, Y. H. Li et al., "MicroRNA-193a represses c-kit expression and functions as a methylation-silenced tumor suppressor in acute myeloid leukemia," *Oncogene*, vol. 30, no. 31, pp. 3416–3428, 2011.

[10] G. Heller, M. Weinzierl, C. Noll et al., "Genome-wide miRNA expression profiling identifies miR-9-3 and miR-193a as targets for DNA methylation in non-small cell lung cancers," *Clinical Cancer Research*, vol. 18, no. 6, pp. 1619–1629, 2012.

[11] J. Wang, B. Yang, L. Han et al., "Demethylation of miR-9-3 and miR-193a genes suppresses proliferation and promotes apoptosis in non-small cell lung cancer cell lines," *Cellular Physiology and Biochemistry*, vol. 32, no. 6, pp. 1707–1719, 2013.

[12] Y. Pu, F. Zhao, W. Cai, X. Meng, Y. Li, and S. Cai, "MiR-193a-3p and miR-193a-5p suppress the metastasis of human osteosarcoma cells by down-regulating Rab27B and SRR, respectively," *Clinical & Experimental Metastasis*, vol. 33, no. 4, pp. 359–372, 2016.

[13] M. Williams, M. B. Kirschner, Y. Y. Cheng et al., "miR-193a-3p is a potential tumor suppressor in malignant pleural mesothelioma," *Oncotarget*, vol. 6, no. 27, pp. 23480–23495, 2015.

[14] I. Grossi, B. Arici, N. Portolani, G. De Petro, and A. Salvi, "Clinical and biological significance of miR-23b and miR-193a in human hepatocellular carcinoma," *Oncotarget*, vol. 8, no. 4, pp. 6955–6969, 2017.

[15] K. Ma, Y. He, H. Zhang et al., "DNA methylation-regulated miR-193a-3p dictates resistance of hepatocellular carcinoma to 5-fluorouracil via repression of SRSF2 expression," *The Journal of Biological Chemistry*, vol. 287, no. 8, pp. 5639–5649, 2012.

[16] J. Liz and M. Esteller, "lncRNAs and microRNAs with a role in cancer development," *Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms*, vol. 1859, no. 1, pp. 169–176, 2016.

[17] B. Yue, D. Cai, C. Liu, C. Fang, and D. Yan, "Linc00152 functions as a competing endogenous RNA to confer oxaliplatin resistance and holds prognostic values in colon cancer," *Molecular Therapy*, vol. 24, no. 12, pp. 2064–2077, 2016.

[18] W. Nie, H. J. Ge, X. Q. Yang et al., "LncRNA-UCA1 exerts oncogenic functions in non-small cell lung cancer by targeting

miR-193a-3p," *Cancer Letters*, vol. 371, no. 1, pp. 99–106, 2016.

[19] GTEx Consortium, "The genotype-tissue expression (GTEx) project," *Nature Genetics*, vol. 45, no. 6, pp. 580–585, 2013.

[20] L. J. Carithers, K. Ardlie, M. Barcus et al., "A novel approach to high-quality postmortem tissue procurement: the GTEx project," *Biopreservation and Biobanking*, vol. 13, no. 5, pp. 311–319, 2015.

[21] C. P. Khoo, M. G. Roubelakis, J. B. Schrader et al., "miR-193a-3p interaction with HMGB1 downregulates human endothelial cell proliferation and migration," *Scientific Reports*, vol. 7, article 44137, 2017.

[22] S. Greco, A. Perfetti, P. Fasanaro et al., "Deregulated microRNAs in myotonic dystrophy type 2," *PLoS One*, vol. 7, no. 6, article e39732, 2012.

[23] S. Kuokkanen, B. Chen, L. Ojalvo, L. Benard, N. Santoro, and J. W. Pollard, "Genomic profiling of microRNAs and messenger RNAs reveals hormonal regulation in microRNA expression in human endometrium," *Biology of Reproduction*, vol. 82, no. 4, pp. 791–801, 2010.

[24] B. Mandriani, S. Castellana, C. Rinaldi et al., "Identification of p53-target genes in Danio rerio," *Scientific Reports*, vol. 6, article 32474, 2016.

[25] Y. Yi, J. Chen, C. Jiao et al., "Upregulated miR-193a-3p as an oncogene in esophageal squamous cell carcinoma regulating cellular proliferation, migration and apoptosis," *Oncology Letters*, vol. 12, no. 6, pp. 4779–4784, 2016.

[26] S. Uhlmann, H. Mannsperger, J. D. Zhang et al., "Global microRNA level regulation of EGFR-driven cell-cycle protein network in breast cancer," *Molecular Systems Biology*, vol. 8, p. 570, 2012.

[27] H. Liang, M. Liu, X. Yan et al., "miR-193a-3p functions as a tumor suppressor in lung cancer by down-regulating ERBB4," *The Journal of Biological Chemistry*, vol. 290, no. 2, pp. 926–940, 2015.

[28] K. W. Tsai, C. M. Leung, Y. H. Lo et al., "Arm selection preference of microRNA-193a varies in breast cancer," *Scientific Reports*, vol. 6, article 28176, 2016.

[29] E. G. Seviour, V. Sehgal, D. Mishra et al., "Targeting KRas-dependent tumour growth, circulating tumour cells and metastasis in vivo by clinically significant miR-193a-3p," *Oncogene*, vol. 36, no. 10, pp. 1339–1350, 2017.

[30] J. E. Kwon, B. Y. Kim, S. Y. Kwak, I. H. Bae, and Y. H. Han, "Ionizing radiation-inducible microRNA miR-193a-3p induces apoptosis by directly targeting Mcl-1," *Apoptosis*, vol. 18, no. 7, pp. 896–909, 2013.

[31] H. Nakano, Y. Yamada, T. Miyazawa, and T. Yoshida, "Gain-of-function microRNA screens identify miR-193a regulating proliferation and apoptosis in epithelial ovarian cancer cells," *International Journal of Oncology*, vol. 42, no. 6, pp. 1875–1882, 2013.

[32] A. Salvi, I. Conde, E. Abeni et al., "Effects of miR-193a and sorafenib on hepatocellular carcinoma cells," *Molecular Cancer*, vol. 12, p. 162, 2013.

[33] H. Noh, S. Hong, Z. Dong, Z. K. Pan, Q. Jing, and S. Huang, "Impaired MicroRNA processing facilitates breast cancer cell invasion by upregulating urokinase-type plasminogen activator expression," *Genes & Cancer*, vol. 2, no. 2, pp. 140–150, 2011.

[34] A. Salvi, B. Arici, A. Alghisi, S. Barlati, and G. De Petro, "RNA interference against urokinase in hepatocellular carcinoma xenografts in nude mice," *Tumour Biology*, vol. 28, no. 1, pp. 16–26, 2007.

[35] G. D. Petro, D. Tavian, A. Copeta, N. Portolani, S. M. Giulini, and S. Barlati, "Expression of urokinase-type plasminogen activator (u-PA), u-PA receptor, and tissue-type PA messenger RNAs in human hepatocellular carcinoma," *Cancer Research*, vol. 58, no. 10, pp. 2234–2239, 1998.

[36] J. A. Foekens, H. A. Peters, M. P. Look et al., "The urokinase system of plasminogen activation and prognosis in 2780 breast cancer patients," *Cancer Research*, vol. 60, no. 3, pp. 636–643, 2000.

[37] T. Yu, J. Li, M. Yan et al., "MicroRNA-193a-3p and -5p suppress the metastasis of human non-small-cell lung cancer by downregulating the ERBB4/PIK3R3/mTOR/S6K2 signaling pathway," *Oncogene*, vol. 34, no. 4, pp. 413–423, 2015.

[38] D. Jia, M. Yan, X. Wang et al., "Development of a highly metastatic model that reveals a crucial role of fibronectin in lung cancer cell migration and invasion," *BMC Cancer*, vol. 10, p. 364, 2010.

[39] W. Deng, M. Yan, T. Yu et al., "Quantitative proteomic analysis of the metastasis-inhibitory mechanism of miR-193a-3p in non-small cell lung cancer," *Cellular Physiology and Biochemistry*, vol. 35, no. 5, pp. 1677–1688, 2015.

[40] L. Lv, H. Deng, Y. Li et al., "The DNA methylation-regulated miR-193a-3p dictates the multi-chemoresistance of bladder cancer via repression of SRSF2/PLAU/HIC2 expression," *Cell Death & Disease*, vol. 5, article e1402, 2014.

[41] H. Deng, L. Lv, Y. Li et al., "miR-193a-3p regulates the multidrug resistance of bladder cancer by targeting the LOXL4 gene and the oxidative stress pathway," *Molecular Cancer*, vol. 13, p. 234, 2014.

[42] Y. Li, H. Deng, L. Lv et al., "The miR-193a-3p-regulated ING5 gene activates the DNA damage response pathway and inhibits multi-chemoresistance in bladder cancer," *Oncotarget*, vol. 6, no. 12, pp. 10195–10206, 2015.

[43] L. Lv, Y. Li, H. Deng et al., "MiR-193a-3p promotes the multi-chemoresistance of bladder cancer by targeting the HOXC9 gene," *Cancer Letters*, vol. 357, no. 1, pp. 105–113, 2015.

[44] H. Deng, L. Lv, Y. Li et al., "The miR-193a-3p regulated PSEN1 gene suppresses the multi-chemoresistance of bladder cancer," *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, vol. 1852, no. 3, pp. 520–528, 2015.

[45] F. Meng, L. Qian, L. Lv et al., "miR-193a-3p regulation of chemoradiation resistance in oesophageal cancer cells via the PSEN1 gene," *Gene*, vol. 579, no. 2, pp. 139–145, 2016.

[46] X. Dai, X. Chen, Q. Chen et al., "MicroRNA-193a-3p reduces intestinal inflammation in response to microbiota via down-regulation of colonic PepT1," *The Journal of Biological Chemistry*, vol. 290, no. 26, pp. 16099–16115, 2015.

[47] Y. Liu, F. Ren, Y. Luo, M. Rong, G. Chen, and Y. Dang, "Down-regulation of MiR-193a-3p dictates deterioration of HCC: a clinical real-time qRT-PCR study," *Medical Science Monitor*, vol. 21, pp. 2352–2360, 2015.

[48] F. Ren, H. Ding, S. Huang et al., "Expression and clinicopathological significance of miR-193a-3p and its potential target astrocyte elevated gene-1 in non-small lung cancer tissues," *Cancer Cell International*, vol. 15, p. 80, 2015.

[49] S. Caramuta, S. Egyhazi, M. Rodolfo et al., "MicroRNA expression profiles associated with mutational status and

survival in malignant melanoma," *The Journal of Investigative Dermatology*, vol. 130, no. 8, pp. 2062–2070, 2010.

[50] S. Gilad, E. Meiri, Y. Yogev et al., "Serum microRNAs are promising novel biomarkers," *PLoS One*, vol. 3, no. 9, article e3148, 2008.

[51] H. Dong, C. Wang, S. Lu et al., "A panel of four decreased serum microRNAs as a novel biomarker for early Parkinson's disease," *Biomarkers*, vol. 21, no. 2, pp. 129–137, 2016.

[52] H. Wei, Y. Yuan, S. Liu et al., "Detection of circulating miRNA levels in schizophrenia," *The American Journal of Psychiatry*, vol. 172, no. 11, pp. 1141–1147, 2015.

[53] F. L. Yong, C. W. Law, and C. W. Wang, "Potentiality of a triple microRNA classifier: miR-193a-3p, miR-23a and miR-338-5p for early detection of colorectal cancer," *BMC Cancer*, vol. 13, p. 280, 2013.

[54] C. Wang, J. Hu, M. Lu et al., "A panel of five serum miRNAs as a potential diagnostic tool for early-stage renal cell carcinoma," *Scientific Reports*, vol. 5, p. 7610, 2015.

[55] C. Wang, M. Ding, M. Xia et al., "A five-miRNA panel identified from a multicentric case-control study serves as a novel diagnostic tool for ethnically diverse non-small-cell lung cancer patients," *eBioMedicine*, vol. 2, no. 10, pp. 1377–1385, 2015.

[56] C. Wu, C. Wang, X. Guan et al., "Diagnostic and prognostic implications of a serum miRNA panel in oesophageal squamous cell carcinoma," *PLoS One*, vol. 9, no. 3, article e92292, 2014.

[57] L. Li, J. G. Chen, X. Chen et al., "Serum miRNAs as predictive and preventive biomarker for pre-clinical hepatocellular carcinoma," *Cancer Letters*, vol. 373, no. 2, pp. 234–240, 2016.

[58] K. Zen and C. Y. Zhang, "Circulating microRNAs: a novel class of biomarkers to diagnose and monitor human cancers," *Medicinal Research Reviews*, vol. 32, no. 2, pp. 326–348, 2012.

[59] H. W. Liang, F. Gong, S. Y. Zhang, C. Y. Zhang, K. Zen, and X. Chen, "The origin, function, and diagnostic potential of extracellular microRNAs in human body fluids," *WIREs RNA*, vol. 5, no. 2, pp. 285–300, 2014.

[60] H. Valadi, K. Ekstrom, A. Bossios, M. Sjöstrand, J. J. Lee, and J. O. Lötvall, "Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells," *Nature Cell Biology*, vol. 9, no. 6, pp. 654–U672, 2007.

[61] H. J. Oh, Y. Shin, S. Chung, D. W. Hwang, and D. S. Lee, "Convective exosome-tracing microfluidics for analysis of cell-non-autonomous neurogenesis," *Biomaterials*, vol. 112, pp. 82–94, 2017.

[62] Y. Teng, Y. Ren, X. Hu et al., "MVP-mediated exosomal sorting of miR-193a promotes colon cancer progression," *Nature Communications*, vol. 8, article 14448, 2017.

[63] R. Rupaimoole and F. J. Slack, "MicroRNA therapeutics: towards a new era for the management of cancer and other diseases," *Nature Reviews Drug Discovery*, vol. 16, no. 3, pp. 203–222, 2017.

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Research Article

MicroRNA Profiling in Cartilage Ageing

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Osteoarthritis (OA) is the most common age-related joint disorder in man. MicroRNAs (miRNA), a class of small noncoding RNAs, are potential therapeutic targets for regulating molecular mechanisms in both disease and ageing. Whilst there is an increasing amount of research on the roles of miRNAs in ageing, there has been scant research on age-related changes in miRNA in a cartilage. We undertook a microarray study on young and old human cartilages. Findings were validated in an independent cohort. Contrasts between these samples identified twenty differentially expressed miRNAs in a cartilage from old donors, derived from an OA environment which clustered based on OA severity. We identified a number of recognised and novel miRNAs changing in cartilage ageing and OA including miR-126: a potential new candidate with a role in OA pathogenesis. These analyses represent important candidates that have the potential as cartilage ageing and OA biomarkers and therapeutic targets.

1. Introduction

Osteoarthritis (OA) is the most common degenerative disease of joints affecting approximately 10% and 18% of men and women, respectively, over the age of 60 years [1]. It mainly affects the hands, knees, and hips with symptoms including pain, joint stiffness, and movement impairment leading to reduced quality of life [2]. The molecular mechanisms of OA though not fully understood are related to abnormal joint metabolism and an imbalance between anabolic and catabolic processes [3]. This imbalance leads to pathological changes in the joint. These present mainly thinning and progressive degradation of articular cartilage: the connective tissue that protects the joint from friction and mechanical load injury [4]. Other pathological changes include thickening of the subchondral bone, inflammation of the synovium, and formation of osteophytes [5]. Current treatments are aimed principally at relieving the symptoms rather than treating the disease. However, many patients ultimately undergo joint replacement surgery for end-stage OA. This is because the molecular mechanisms underlying this heterogeneous, age-related disease are poorly characterized.

OA is a multifactorial disease with known risk factors including genetics [6], sex, obesity, and joint injury [7]. However, the most common risk factor is age [5]. The progression and initiation of OA is facilitated by numerous stimuli and circumstances including changes in the homeostatic balance due to age. Age-related cell senescence can affect chondrocyte homeostasis and metabolism, by increasing the expression of enzymes such as matrix metalloproteinases and aggrecanases, which break down the extracellular matrix of cartilage, promoting OA development [8]. Additionally, age-related inflammation (termed inflamm-ageing) promotes the expression of cell signalling molecules, such as interleukins and other cytokines. These act as mediators of matrix degradation, contributing to OA progression [9].

MicroRNAs (miRNAs or miRs) are short (~22 nt) noncoding RNAs. They have emerged as critical cell homeostasis regulators which function through posttranscriptional modulation of gene expression by binding and repressing the expression of specific mRNA targets [10]. miRNA genes are found within intergenic or intragenic regions and are transcribed into double-stranded stem-loop structures called the primary transcript. The primary transcript is processed by the microprocessor complex, consisting of the ribonuclease DROSHA, and DiGeorge syndrome critical region 8 protein into precursor miRNAs which are exported in the cytoplasm through exportin-5 [11]. Precursor miRNAs are incorporated into the RNA-induced silencing complex. These are cleaved further by the endoribonuclease Dicer to form the single-stranded mature miRNA [12]. miRNA-mediated expression is accomplished through perfect or imperfect complementarity between the miRNA and the mRNA target. Ultimately, in animals, this leads to inhibition of translation, mRNA degradation, or both [13].

It is estimated that one-third of human genes are targeted by miRNAs [14]. This makes miRNAs potential therapeutic targets for regulating both disease and ageing molecular mechanisms. Indeed, several miRNAs have been found to play an important role in cartilage development and homeostasis, and dysregulation of specific miRNAs has been linked to OA [15–17]. This suggests miRNAs as feasible novel candidates for OA treatment targets and clinical biomarkers [18]. However, whilst there have been an increasing number of studies interrogating specific miRNAs as regulators of cartilage-specific processes in OA [19], few studies have assessed the contribution of cartilage ageing in the miRNA dysregulation evident in OA. One study found an age-related increase in miR-199a-3p and miR-193b contributing to a downregulation in collagen type II, aggrecan and SOX9, along with reduced proliferation and a reduction in miR-320c [17]. MiR-24, which regulates p16INK4a, was found to link age-related senescence and chondrocyte terminal differentiation-associated matrix remodelling in OA [20]. Furthermore, Miyaki et al. observed that miR-140 null mice developed an age-related OA-like pathology due to elevated ADAMTS5 [16].

Our previous studies have identified age-related changes in miRNAs in tendon [21], bone marrow-derived mesenchymal stem cells [22], and chondrocytes engineered from MSCs [23] and cartilage [24]. In this study, we investigated, for the first time, miRNA expression in ageing knee cartilage in order to understand further cartilage ageing and determine how this may contribute to OA. Establishing miRNAs differentially expressed in joints or cartilage during ageing and/or OA can provide basis for functional studies and potentially lead to development of novel, miRNA-based interventions against cartilage, and joint degeneration during ageing and OA.

2. Materials and Methods

All reagents were from Thermo Fisher Scientific, unless stated.

2.1. Samples. For microarray analysis, femoral intercondylar notch full-thickness cartilage from male human knees of young normal ($n = 6$; mean age ± SD 22.7 ± 4.1 years) was collected at the time of anterior cruciate ligament repair. OA cartilage was from old male ($n = 6$; 66.4 ± 15.9 years) human knees collected at the time of total knee arthroplasty. For qRT-PCR validation, an independent cohort was used which consisted of young knee cartilage from the intercondylar notch $n = 9$ (mean age ± SD 23.7 + −3.8), old "normal" cartilage from the lateral femoral condyle $n = 5$ (68.6 ± 3.8), and old OA cartilage $n = 8$ (63.1 ± 8.1) from the medial femoral condyle cartilage. All old specimens came from patients with a diagnosis of OA on preoperative knee radiographs using Kellgren and Lawrence scoring [25]. All cartilages taken were macroscopically normal. Medical ethics permission was received (Maastricht University Medical Centre approval IDs: MEC 08-4-028 and 14-4-038).

2.2. RNA Isolation. RNA was extracted from a cartilage once pulverised into a powder with a dismembranator (Mikro-S, Sartorius, Melsungen, Germany) under liquid nitrogen. Total RNA was extracted using the mirVana RNA isolation kit (Life Technologies, Paisley, UK) according to the manufacturer's instructions. The RNA samples were quantified using a Nanodrop spectrophotometer (NanoDrop Technologies, Wilmington, USA). The integrity of the RNA was assessed on the Agilent 2100 Bioanalyzer system using an RNA Pico chip.

2.3. Microarrays. 600–900 ng of total RNA was labelled using the Affymetrix FlashTag Biotin HSR RNA labelling kit according to the manufacturer's instructions. Following FlashTag labelling, the biotin-labelled samples were stored at −20°C prior to hybridisation onto Affymetrix GeneChip miRNA 4.0 for 17.5 hours at 48°C 60 rpm in an Affymetrix hybridisation oven 645.

Following hybridisation, the arrays were washed using Affymetrix hybridisation wash and stain kit on the GeneChip Fluidics station 450 using fluidics script FS450_0002 and scanned using the Affymetrix GeneChip scanner 3000 7G.

2.4. Data Analysis. CEL files were generated using the Affymetrix GeneChip Command Console Software, and Expression Console software was used to quality control array performance. The miRNA expression data measured using Affymetrix miRNA 4.0 arrays were preprocessed using Affymetrix Expression Console with optioned method RMA for data normalisation [26]. The further statistical analyses were carried out on the 2578 miRNA probe set for *Homo sapiens* extracted from all probes and were used to determine both the detected and differentially expressed (DE) miRNAs.

The presence of each probe in the young and old samples was tested. In each test, the p value of the six samples was combined using Fisher's combined p value methods. The expression was dereplicated to a transcript level by averaging replicated probes. The p value associated with the presence of dereplicated expression was assigned by combining the replicated probes using Fisher's combined p test.

The DE analyses on the contrasting two sample conditions were performed through linear models using limma package in R environment [27]. The significance of log fold change (logFC) values for miRNAs was evaluated using *t*-tests, and the *p* values associated with logFC values were adjusted for multiple testing using the false discovery rate (FDR) approach [28]. Significantly, DE were defined as those with an FDR-adjusted *p* value <5%. Sequence data have been submitted to National Centre for Biotechnology Information Gene Expression Omnibus (NCBI GEO); E-MTAB-5715.

2.5. Integrated miR-mRNA Analysis and Functional Enrichment Analysis. In order to identify putative miRNA targets, bioinformatics analysis was performed by uploading DE miRNA data into the microRNA target filter module within Ingenuity Pathway Analysis software (IPA, Qiagen Redwood City, CA, USA) to produce a network of potential miRNA gene targets. Targets were then filtered on a confidence of experimentally observed or highly predictive and on the cell chondrocyte. ToppGene was used for functional enrichment analysis of the miRNA targets using ToppGene [29] with a Bonferroni FDR of less than 0.05. Biological process gene ontology (GO) terms and associated FDR values generated through ToppGene were then summarised, and the network was visualised using REViGO [30] and Cytoscape [31].

2.6. Real-Time Polymerase Chain Reaction (qRT-PCR). Validation of the microarray analysis results in the dependent and independent cohorts of human knee cartilage samples was carried out using real-time quantitative PCR (qRT-PCR) analysis. Total RNA was extracted and quantified as above. cDNA was synthesized using 200 ng RNA and the miScript II RT Kit according to the manufacturer's protocol (Qiagen, Crawley, UK). qPCR mastermix was prepared using the miScript SYBR Green PCR Kit (Qiagen, Crawley, UK) and the appropriate miScript Primer Assay (Qiagen, Crawley, UK) (Supplementary file 1 available online at https://doi.org/10.1155/2017/2713725) using 1 ng/μl cDNA according to the manufacturer's guidelines. Real-time PCR was undertaken using an Applied Biosystems 7300 Real-Time PCR System (Applied Biosystems, Paisley, Scotland, UK). Relative expression levels were normalised to U6 snoRNA and calculated using the $2^{-\Delta Ct}$ method [32].

2.7. Statistical Analysis. For statistical evaluation of qRT-PCR results, a Mann–Whitney test was performed using GraphPad Prism version 7.03 for Windows, (GraphPad Software, La Jolla California USA, https://www.graphpad.com); *p* values are indicated.

3. Results

3.1. Microarray Analysis Overview. A data quality assessment report generated revealed that the quality of the data was good and consistent for all 12 arrays. The distribution for log expression signal was highly similar in signal distribution, and using a boxplot for relative log expression signal, no arrays were outliers (data not shown). The outcomes of variation assessment were visualised in Figures 1(a) and 1(b). The young samples were correlated closely together. However, the old samples were clustered into three distinct groupings as demonstrated by the correlation coefficient matrix heat map (Figure 1(a)). Principal component analysis (PCA) plot of the log expression signal for 12 arrays revealed that the samples from the young were clustered tightly together and could be separated from the old samples. However, the samples from the old group scattered in a very wide range as three subpopulations. Samples 7, 8, and 10 (cluster 1) were more similar to the samples from the young group and had the lowest KL scores: 1. Cluster 2 consisting of samples 11 and 12 had KL scores of 4 and sample 9 had KL score of 2 (Figure 1(b)). Based on the multidimensional scaling (MDS) plot, subsequently, four different selections of the old samples were made and compared to the young samples generating four result sets of DE analysis. Selection 1 includes all 6 old samples; selection 2 includes O_7, O_8, O_10, O_11, and O_12; selection 3 includes O_9, O_11, and O_12; and selection 4 includes O_7, O_8, and O_10.

3.2. miRNA Expression Profiling and Dysregulation. Of the 2578 human miRNAs represented on the Affymetrix GeneChip miRNA 4.0 microarray, 303 and 416 were detected above background in the young and old samples, respectively (Supplementary file 2). Using a cutoff of false discovery-adjusted *p* value <0.05, for selection 1 there were 20 DE miRNAs (Figure 2 and Table 1), for selection 2 there were 22 DE, for selection 3 there were 189 DE (Supplementary file 3), and for selection 4 there were 10 DE (Table 2).

3.3. Identification of Potential Target Genes of DE miRNAs. In order to investigate the position of the DE miRNAs in the chondrocyte expression network, we determined their putative target genes using IPA. This was undertaken for two datasets: (1) selection 1, the DE miRNAs were derived from all young samples compared to all old samples and (2) DE miRNAs derived from the young versus selection 4 (representing only the old samples with lowest K&L scores). This was because, we hypothesise, this latter set is most likely to be predominantly age-related changes. These presumed mRNAs were input into a gene ontology and visualised. (1) Putative target genes regulated by 11 of the 20 DE miRNAs were identified from the dysregulated genes in selection 1 (all young versus all old) in order to determine the functional significance. The microRNA target filter in IPA was used to integrate computational algorithms with multiple miRNA databases (Supplementary file 4). These presumed mRNAs were input into the gene ontology tool ToppGene, and then, biological processes were visualised in Revigo and Cytoscape (Figure 3(a)). The top biological processes were skeletal tissue development (FDR 9.29E11), regulation of cell proliferation (FDR 9.29E11), and ossification (FDR 1.18E9) (Supplementary file 3). The young samples compared to selection 4 gave putative target genes for six of the 10 DE miRNAs (Supplementary file 5). Biological processes are visualised in Figure 3(b), and the complete list is visualised in Supplementary file 5. The main biological processes were skeletal

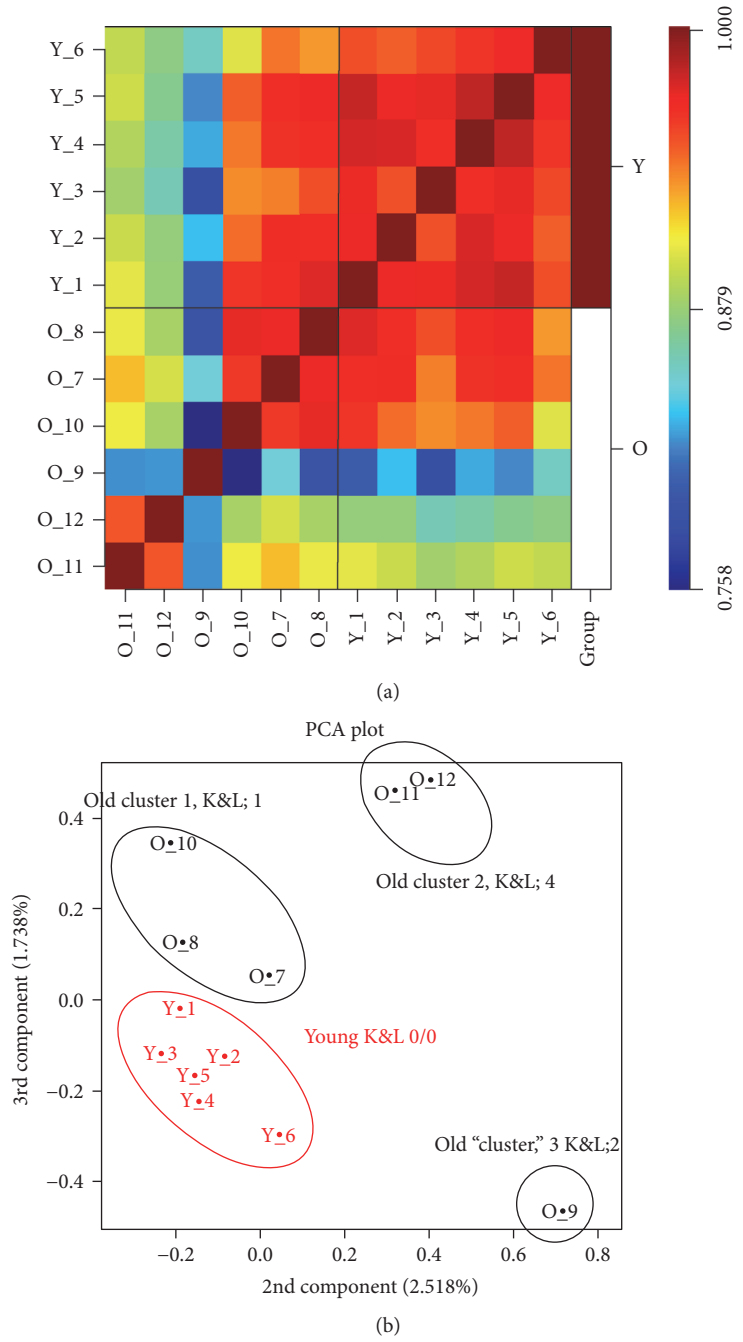

Figure 1: Variation data between the expressions for 12 microarray samples. (a) The heat map of hierarchical clusters of correlations among the samples. Pearson's correlation coefficients were computed using logarithm-transformed miRNA expression data from all miRNA probes that were detected. (b) A 2-D PCA plot of the second and third components from PCA of logarithm-transformed miRNA abundance data. The Kellgren and Lawrence scores (K&L) for the groups are shown on the PCA plot. H: young, O: old.

system development (FDR 3.15E07), homeostatic process (6.84E07), and positive regulation of signalling (6.22E06).

3.4. qRT-PCR Validation of miRNAs. To validate the changes in miRNA expression detected by microarray platform, qRT-PCR analyses using RNA from both dependent (original RNA extracted from the young normal and old OA donors used in microarray analysis) and independent cohorts were performed. An independent cohort was selected based on the samples with equivalent K&L scores to the samples used in the microarray. For the independent cohort, the K&L score from the young donors was 0, old donors mean ± SD, old "normal" 1.3 ± 0.9, and old OA 3.0 ± 0.8.

For the dependent cohort, 10 DE miRNAs from the contrast young normal versus selection 3 were selected as we decided to focus on miRNA changes due to age and OA (Table 3). The expression of miRNAs, miR-126-3p, -200c-3p, -424-3p, and -483-5p, was significantly lower in the

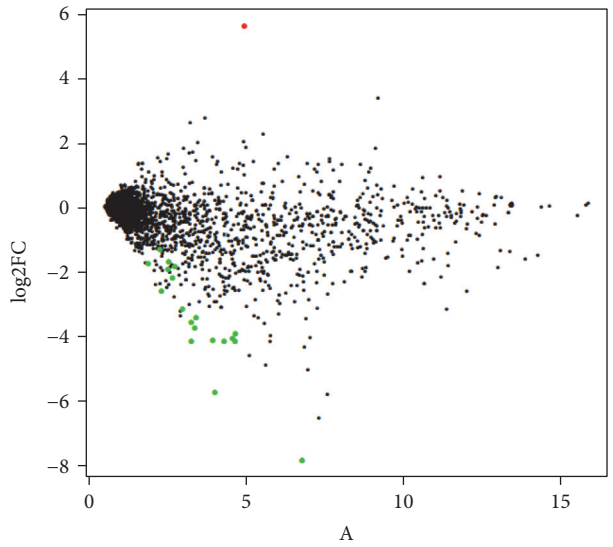

Figure 2: Cartilage expression profiling using an MA plot. The MA plot contrasts the log2 fold change (log2FC) against the mean intensity of all 12 arrays. The coloured spots represent DE small RNAs (FDR < 0.05), green dots reduced expression in the old OA samples, and red dots increased expression in the old OA samples. 20 miRs were significantly dysregulated; one increased in OA and 19 decreased in OA.

Table 1: Table demonstrating the 20 DE miRNAs in the young normal versus old OA cartilages.

miR	Log fold change	FDR adjusted
miR-126-3p	−7.81	0.01
miR-708-5p	−5.72	0.01
miR-489-3p	−4.14	0.01
miR-422a	−4.14	0.03
miR-378i	−4.14	0.03
miR-1273f	−4.10	0.01
miR-378f	−4.05	0.03
miR-150-5p	−3.91	0.02
miR-5585-3p	−3.73	0.01
miR-1273d	−3.55	0.01
miR-7111-5p	−3.41	0.01
miR-6875-5p	−3.13	0.00
miR-424-3p	−2.58	0.03
miR-6830-5p	−2.16	0.04
miR-6833-5p	−1.89	0.03
miR-6795-5p	−1.80	0.02
miR-4716-3p	−1.74	0.02
miR-4428	−1.68	0.02
miR-5010-5p	−1.29	0.02
miR-486-5p	5.64	0.00

FDR: false discovery rate.

old OA samples compared to the young normal samples (Supplementary file 6A) confirming microarray results. However, the expression of miRs, 146-5p, -424-3p, -181-5p, -let 7f-1-3p, -let 7b-5p, -150-5p, -21-5p, although following the same pattern of expression, changed between the two groups as in microarray analysis and did not reach significance (Supplementary file 6B).

Table 2: Table demonstrating the 10 DE miRNAs in the young normal versus old "selection 4" cartilages.

miR	Log fold change	FDR adjusted
hsa-miR-486-5p	5.98	0.00
hsa-mir-210	2.16	0.02
hsa-miR-4521	1.94	0.04
hsa-let-7a-1	0.93	0.04
hsa-miR-423-5p	0.82	0.02
hsa-miR-6795-5p	−1.33	0.02
hsa-miR-6774-5p	−1.42	0.04
hsa-miR-7111-5p	−2.51	0.04
hsa-miR-6824-5p	−2.76	0.03
hsa-miR-6875-5p	−2.93	0.02

FDR: false discovery rate.

To further validate the results of the microarray analysis, we performed qRT-PCR analysis of the expression of miRs, -21-5p, -146a-5p, -181a-5p, and -483a-5p, on an independent cohort of samples (young normal compared to old OA). These miRNAs were chosen based on the fold change of their expression (by microarray), predicted or validated target gene set, and/or known or predicted function in cartilage maintenance and degradation. The expression of all miRNAs tested was significantly lower in the old OA samples compared to the young normal samples (Figure 4(a)). This was in agreement with the results from the dependent cohort. Additionally, in the young normal compared to the old "normal" cartilage, the miRs, -126 and -424, were validated as reduced in expression in the old normal samples in agreement with the microarray results (Figure 4(b)).

4. Discussion

A strong correlation exists between the age of an organism and OA, whilst ageing has a clear effect on cartilage gene expression [24]. One potential mechanism capable of regulating global alterations to a particular tissue is modification to the miRNA system. miRNAs appear to control ageing at the level of organism lifespan, tissue, and cellular senescence. The expression of many miRNAs has been demonstrated to be significantly altered with ageing. Indeed, many of these miRNAs have been identified as regulators of ageing at each of these levels. To begin to elucidate the role that miRNAs play in the global changes observed in cartilage with ageing, we undertook a microarray analysis of the young and old human cartilages. Sex-related alterations were mitigated with the use of samples from males only. We identified unique signatures which were altered with ageing and/or OA as we characterised the expression of miRNAs in knee cartilage ageing as well as DE miRNAs dependent on the severity of OA (as determined by K&L score).

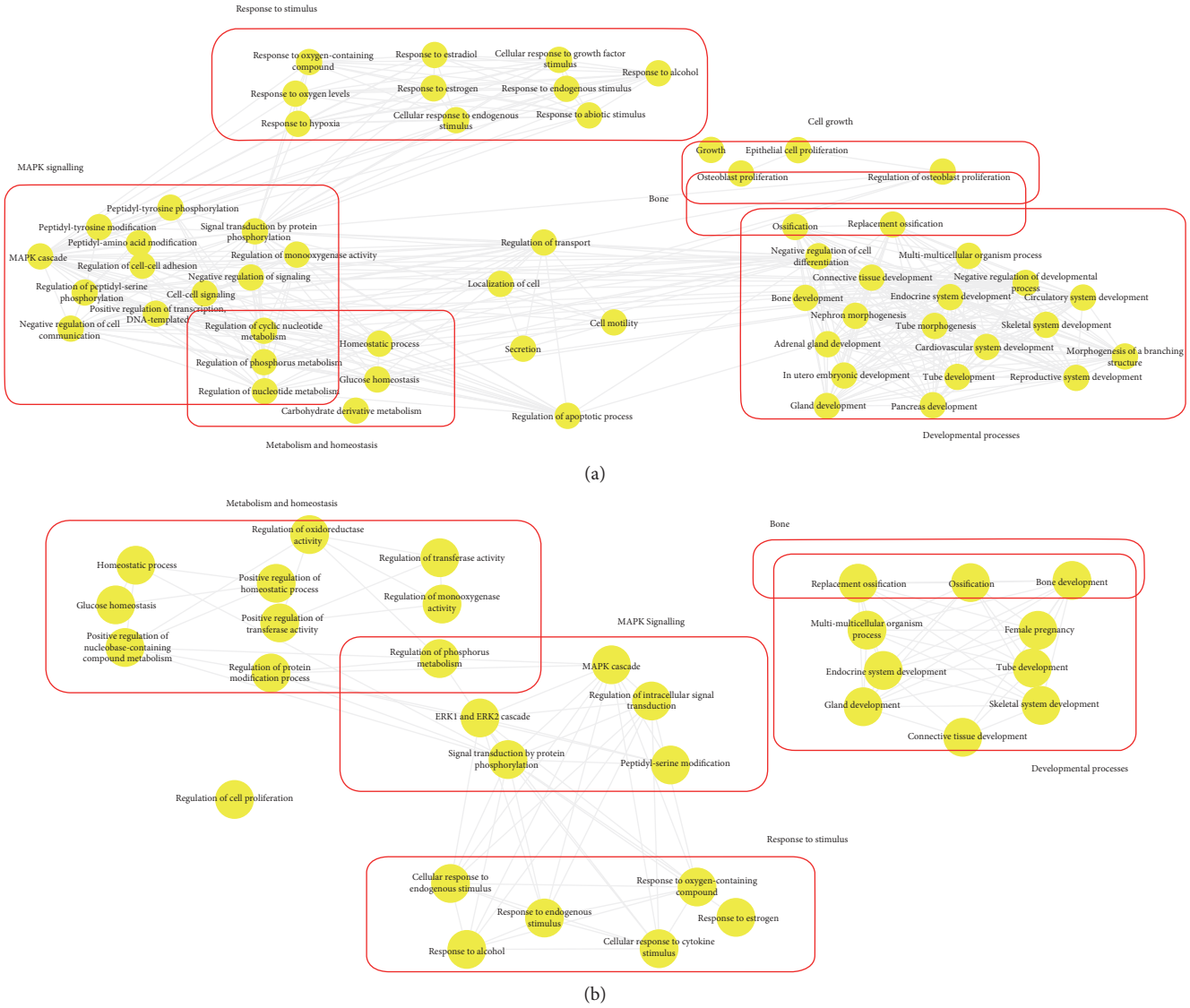

Figure 3: The position of the DE miRNAs in the chondrocyte expression network. Gene ontology biological processes associated with dysregulated miRNA targets were identified following TargetScan filter module in IPA. ToppGene was used to perform functional enrichment analysis on predicted miRNA targets to highlight biological processes most significantly affected by dysregulated miRNA-mRNA interactions. GO terms (FDR < 0.05) were summarized and visualised using REViGO and Cytoscape. Allowed similarity setting in Revigo was medium. The main clusters of biological processes significantly influenced by dysregulated miRs in (a) all young compared to all old samples and (b) all young samples compared to selection 4. The line width specifies the amount of similarity.

In the initial microarray study, the cartilage samples were removed from the femoral intercondylar notch in both the young and old donors. This site was selected as we had access to this tissue from both donor groups. We recognise that a limitation of the study is that, although the cartilage was taken from a macroscopically normal area in the old donors, this was from an OA joint environment. Furthermore, in order to validate our microarray results, we used both the dependent cohort (to validate the platform) and an independent cohort to further validate some of our DE miRNAs in additional biological donors. The samples collected from the old donors for the latter experiment were removed from the protected (lateral; described here as old normal) or unprotected (medial; described here as old OA) condyles following TKA.

Our initial microarray analysis determined 20 DE miRNAs. However, the old donors were clustered in three groups which correlated with the severity of K&L scores. We therefore repeated the microarray analysis with each of the clusters removed. In the analysis, "selection 4," the samples which were most different from the young (with the highest K&L scores) were removed identifying ten DE miRNAs. We hypothesize that these miRs represent the most likely dysregulated miRNAs principally due to age. These were miR-486-5p, -210, -4521, let-7a-1, -423-5p, -6795-5p, -6774-5p, -7111-5p, -6824-5p, and -6875-5p. Next, we used

Table 3: Summary of DE miRNAs detected by microarray analysis and selected for qRT-PCR validation.

miRNA	Expression in OOA samples compared to YN	
	Microarray analysis	qPCR analysis
let 7b-5p	↓	↓
let 7f-1-3p	↑	↑
21-5p	↓	↓
126-3p	↓	↓
146-5p	↓	↓
150-5p	↓	↓
181-5p	↓	↓
200c-3p	↓	↓
424-3p	↓	↓
483-5p	↓	↓

OOA: old osteoarthritic; YN: young normal.

TargetScan to find miRNA putative target genes. Gene ontology was then undertaken on these genes in an effort to explore the position of the DE miRNAs in the chondrocyte expression network and cartilage ageing. In both the contrast between all young and all old samples, and the young versus "selection 4" (most likely affected by age or low K&L score), we identified significant biological processes. These included changes in apoptosis and cell proliferation, metabolism and homeostasis, and response to stimulus (altered nutrient sensing). Processes such as altered nutrient sensing and changes to homeostasis are some of the hallmarks of cell ageing [7]. In the young samples, compared to "selection 4," we identified two miRNAs which are known to interact with ageing pathways. These were let-7 (cellular senescence and stem cell exhaustion) and miR-486 (altered nutrient sensing) [33]. Additionally, let-7 and miR-486 (which affect protein synthesis and mitochondrial function) have previously been identified as reduced in muscle ageing [34].

The clustering of old samples into subgroups was expected. This was as it is accepted that, although cartilage may appear grossly normal, its gene and protein expression can be affected when it is in an OA environment. Indeed, we have previously described that transcriptomes from chondrocytes in late-stage OA are similar whether cartilage is harvested from intact (protected, generally the lateral femoral condyle) or fibrillated (unprotected, generally the medial femoral condyle) areas within the knee [35]. However, others have described that in cartilage gene expression, changes are evident in different stages of OA [36, 37]. One problem with the identification of clusters apparently relating to the K&L scores was that this reduced the power of the study. Furthermore, when attempting to validate the results of the microarray with the dependent cohort using qRT-PCR, this led to large variations within the old donor groups. Higher variations in gene or miRNA expression in the old group are generally not unexpected. This is due to complexity of the ageing process, and comorbidities change occurring during ageing in the musculoskeletal system. The samples used in the old group were from two of the clusters. Thus, whilst most of the miRNAs tested with qRT-PCR showed changes in the same direction as the microarray, some did not reach statistical significance.

We believe that the analysis of the young compared to "selection 3" represents changes due to age and/or OA. These old samples represented those with the highest K&L scores compared to the young. Within this dataset, we identified 13 miRNAs known to affect the hallmarks of ageing; 11 of which were downregulated in ageing. These were for cellular senescence: let-7 and miR-146b-5p; stem cell exhaustion: let-7 and miR-29b; altered nutrient sensing: miR-120 and miR-320e; changes in gene regulation: miR-143, miR-193a, miR-200c, and miR-29b; mitochondrial dysfunction: miR-145 and miR- 349; DNA damage: miR-192, miR-24, and miR-21; inflammageing: miR-21; and loss of telomeres: miR-34a [33]. Additionally, a number of the DE miRNAs in this contrast had previously been identified in the pathogenesis of OA including miR-27b [38], miR-483 [39], miR-146 [40], miR-145 [41], and miR-675 [42]. In this study, the expression of each of these miRNAs was reduced compared to the young normal cartilage. Finally, a number of miRNAs, which have roles in cartilage homeostasis, including miR-337 [43], miR-302 [44], miR-181 [45], mir-193 [17], miR-135 [46], and miR-24 [20] were in this group. Additional work is required to decipher fully the role of this set of miRNAs in cartilage homeostasis, ageing, and OA.

Among microRNAs, DE expressed in microarray and in the dependent cohort were miRNAs, miR-126, -200c, and -424 (Supplementary file 6), whereas among miRNAs, DE expressed in microarray and independent cohort included miRNAs: -21, -146, -181 (Figure 4). MiR-483 was validated as DE expressed between the young and old OA samples in both dependent and independent cohorts. Indeed, this miRNA has been previously shown to be involved in the pathogenesis of OA [38]. It was downregulated during ageing and OA in our studies, and others have shown its positive role in cartilage maintenance [38].

Among interesting DE miRNAs in our study were miR-21, previously shown by us to be dysregulated in equine tissue during ageing [24], and also classified as "inflamma-miR" due to its major role in regulating inflammation [47]. MiR-181 demonstrated to regulate chondrocyte apoptosis in OA [48], and miR-424 previously suggested to play a role in OA [49] was also DE. Interestingly, miR-424 was also DE in the young normal compared to the old normal cohort and may also represent an age-related miR.

MiR-200c has been linked to osteogenic differentiation and proinflammatory responses by targeting interleukins 6 and 8 and chemokine (C-C motif) ligand. These are important mediators involved in OA inflammation [50]. In addition, miR-146a has been reported to play a role in cartilage homeostasis and preservation [51]. Yamasaki et al. [52] reported that expression of miR-146a was lower in late OA cartilage compared to early stages. This is in agreement with our results, where expression of miR-146a was significantly lower in the old OA donors from the dependent and independent cohorts.

Interestingly, our study has provided a new miRNA candidate, potentially regulating OA pathogenesis: miR-126. So far, little evidence exists on the role of miR-126 in joint

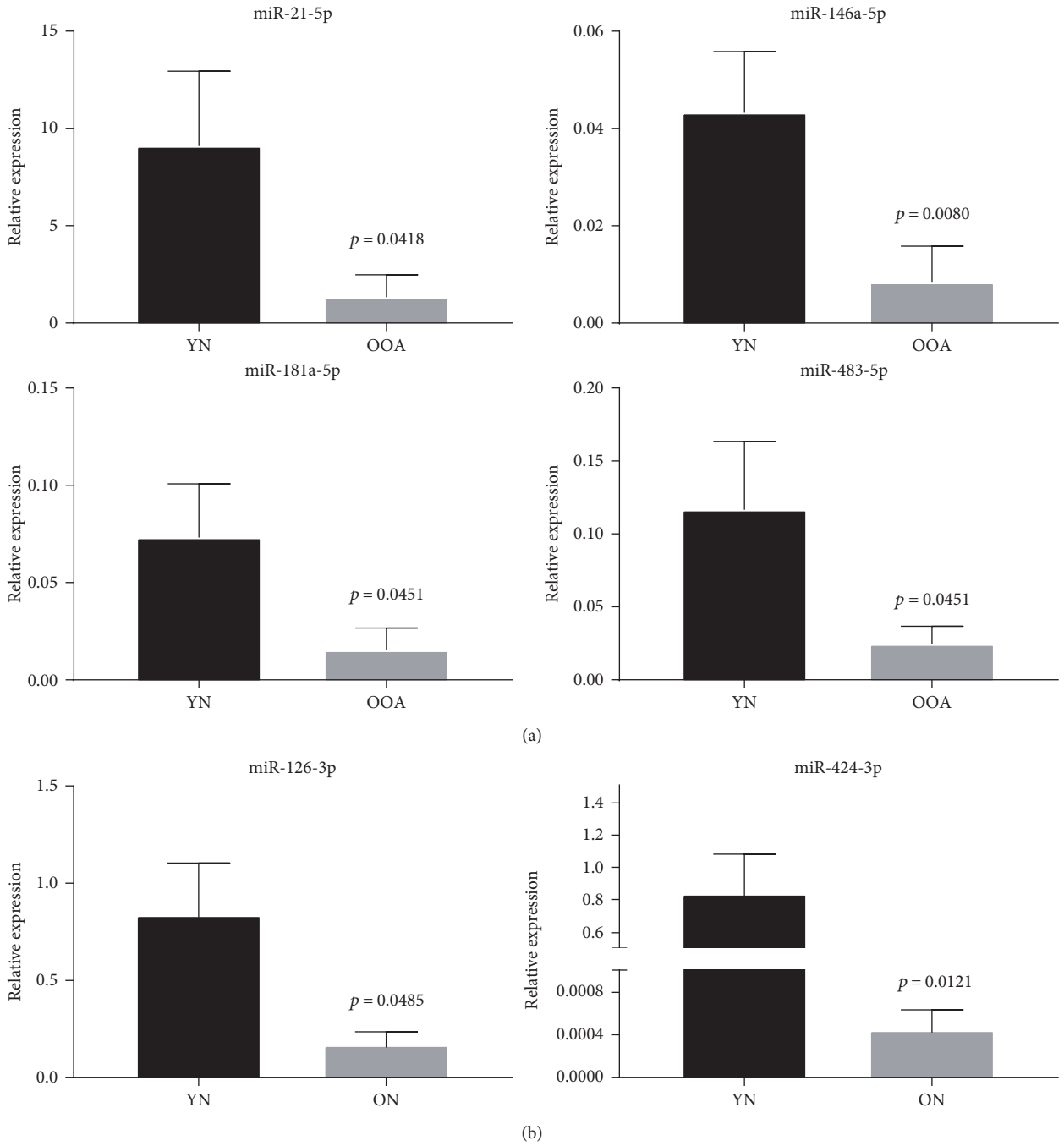

Figure 4: Validation of microarray results using qRT-PCR in an independent cohort. (a) Relative expression of miRNAs between the young normal and old OA cartilages. qRT-PCR results show relative expression normalised to Rnu-6 gene, young samples $n = 7$-8, old OA samples $n = 5$–7. (b) Relative expression of miRNAs between the young normal and old normal samples in an independent cohort. Results show the young normal samples ($n = 8$) and the old normal samples ($n = 3$-4). Mann–Whitney test was performed using GraphPad prism version 7.03; p values are indicated. YN: young normal; OOA: old OA; ON: old normal.

pathology and OA. MiR-126 has been demonstrated to regulate angiogenesis and de novo vascularisation [53], as well as inflammation [54]. As cartilage is an avascular tissue, this may suggest a potential role of vascularisation, or lack of thereof in OA development. Previously, increased miR-126 expression has been described as promoting matrix-dependent cell attachment and increased cell to cell interactions between perivascular and endothelial cells during angiogenesis. Here, reduced miR-126 expression led to a less stable cell to matrix attachment network [55], in concordance with the tissue changes observed in OA. Moreover, Borgonio Cuadra et al. has reported elevated levels of miR-126 in the plasma of OA patients [56]. However, as they mention, expression levels of intra and extracellular miRNAs may differ significantly. Therefore, it is not surprising that we found reduced miR-126 expression in knee cartilage from the

OA patients. Moreover, a few studies have linked miR-126 to ageing [57, 58]. Although, these studies were not relevant to cartilage homeostasis and OA, they provide indications of a possible role of miR-126 in cell ageing and senescence. Future functional studies will provide evidence on the extent to which miRNAs regulate OA development and the potential of miRNA-based interventions to ameliorate OA.

5. Conclusions

For the first time, we demonstrated changes in miRNAs in human knee cartilage ageing and OA. These represent miRNAs with known roles in ageing and/or OA as well as novel candidates for further functional studies. Importantly, our work provides critical evidence on the potential function of biological processes of miRNAs in cartilage ageing and OA. Further work is ongoing to determine the functional significance of specific miRNA candidates identified in this study with the aim of providing candidates as diagnostic biomarkers and therapeutic targets for OA treatment.

Disclosure

Tim Welting and Mandy Peffers are joint last authors.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

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References

[1] R. Wittenauer, L. Smith, and K. Aden, *Update on 2004 Background Paper, BP 6.12 Osteoarthritis*, World Health Organization, Geneva, 2013.

[2] J. Martel-Pelletier, A. J. Barr, F. M. Cicuttini et al., "Osteoarthritis," *Natural Reviews. Disease Primers*, vol. 2, article 16072, 2016.

[3] M. B. Mueller and R. S. Tuan, "Anabolic/catabolic balance in pathogenesis of osteoarthritis: identifying molecular targets," *PM & R: the journal of injury, function, and rehabilitation*, vol. 3, no. 6, Supplement 1, pp. S3–11, 2011.

[4] C. Vinatier, C. Merceron, and J. Guicheux, "Osteoarthritis: from pathogenic mechanisms and recent clinical developments to novel prospective therapeutic options," *Drug Discovery Today*, vol. 21, no. 12, pp. 1932–1937, 2016.

[5] D. Chen, J. Shen, W. Zhao et al., "Osteoarthritis: toward a comprehensive understanding of pathological mechanism," *Bone Research*, vol. 5, article 16044, 2017.

[6] J. B. van Meurs, "Osteoarthritis year in review 2016: genetics, genomics and epigenetics," *Osteoarthritis and Cartilage*, vol. 25, no. 2, pp. 181–189, 2017.

[7] R. F. Loeser, J. A. Collins, and B. O. Diekman, "Ageing and the pathogenesis of osteoarthritis," *Nature Reviews Rheumatology*, vol. 12, no. 7, pp. 412–420, 2016.

[8] Y. S. Li, W. F. Xiao, and W. Luo, "Cellular aging towards osteoarthritis," *Mechanisms of Ageing and Development*, vol. 162, pp. 80–84, 2017.

[9] P. K. Sacitharan and T. L. Vincent, "Cellular ageing mechanisms in osteoarthritis," *Mammalian Genome*, vol. 27, no. 7-8, pp. 421–429, 2016.

[10] Y. P. Li, X. C. Wei, P. C. Li et al., "The role of miRNAs in cartilage homeostasis," *Current Genomics*, vol. 16, no. 6, pp. 393–404, 2015.

[11] D. O'Carroll and A. Schaefer, "General principals of miRNA biogenesis and regulation in the brain," *Neuropsychopharmacology*, vol. 38, no. 1, pp. 39–54, 2013.

[12] S. Miyaki and H. Asahara, "Macro view of microRNA function in osteoarthritis," *Nature Reviews Rheumatology*, vol. 8, no. 9, pp. 543–552, 2012.

[13] J. Krol, I. Loedige, and W. Filipowicz, "The widespread regulation of microRNA biogenesis, function and decay," *Nature Reviews Genetics*, vol. 11, no. 9, pp. 597–610, 2010.

[14] B. P. Lewis, C. B. Burge, and D. P. Bartel, "Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets," *Cell*, vol. 120, no. 1, pp. 15–20, 2005.

[15] W. Chen, L. Chen, Z. Zhang et al., "MicroRNA-455-3p modulates cartilage development and degeneration through modification of histone H3 acetylation," *Biochimica et Biophysica Acta*, vol. 1863, no. 12, pp. 2881–2891, 2016.

[16] S. Miyaki, T. Sato, A. Inoue et al., "MicroRNA-140 plays dual roles in both cartilage development and homeostasis," *Genes & Development*, vol. 24, no. 11, pp. 1173–1185, 2010.

[17] T. Ukai, M. Sato, H. Akutsu, A. Umezawa, and J. Mochida, "MicroRNA-199a-3p, microRNA-193b, and microRNA-320c are correlated to aging and regulate human cartilage metabolism," *Journal of Orthopaedic Research*, vol. 30, no. 12, pp. 1915–1922, 2012.

[18] X. M. Yu, H. Y. Meng, X. L. Yuan et al., "MicroRNAs' involvement in osteoarthritis and the prospects for treatments," *Evidence-Based Complementary and Alternative Medicine*, vol. 2015, Article ID 236179, 13 pages, 2015.

[19] R. Vicente, D. Noël, Y. M. Pers, F. Apparailly, and C. Jorgensen, "Deregulation and therapeutic potential of microRNAs in arthritic diseases," *Nature Reviews Rheumatology*, vol. 12, no. 4, pp. 211–220, 2016.

[20] D. Philipot, D. Guérit, D. Platano et al., "p16INK4a and its regulator miR-24 link senescence and chondrocyte terminal differentiation-associated matrix remodeling in osteoarthritis," *Arthritis Research & Therapy*, vol. 16, no. 1, article R58, 2014.

[21] M. J. Peffers, Y. Fang, K. Cheung, T. K. Wei, P. D. Clegg, and H. L. Birch, "Transcriptome analysis of ageing in uninjured

human Achilles tendon," *Arthritis Research & Therapy*, vol. 17, p. 33, 2015.

[22] M. J. Peffers, J. Collins, Y. Fang et al., "Age-related changes in mesenchymal stem cells identified using a multi-omics approach," *European Cells & Materials*, vol. 31, pp. 136–159, 2016.

[23] M. J. Peffers, K. Goljanek-Whysall, J. Collins et al., "Decoding the regulatory landscape of ageing in musculoskeletal engineered tissues using genome-wide DNA methylation and RNASeq," *PLoS One*, vol. 11, no. 8, article e0160517, 2016.

[24] M. J. Peffers, X. Liu, and P. D. Clegg, "Transcriptomic profiling of cartilage ageing," *Genomics Data*, vol. 2, pp. 27-28, 2014.

[25] J. H. Kellgren and J. S. Lawrence, "Radiological assessment of osteo-arthrosis," *Annals of the Rheumatic Diseases*, vol. 16, no. 4, pp. 494–502, 1957.

[26] R. A. Irizarry, B. Hobbs, F. Collin et al., "Exploration, normalization, and summaries of high density oligonucleotide array probe level data," *Biostatistics*, vol. 4, no. 2, pp. 249–264, 2003.

[27] G. K. Smyth, *Bioinformatics and Computational Biology Solutions Using R and Bioconductor*, Springer, New York, 2005.

[28] Y. Benjamini and Y. Hochberg, "Controlling the false discovery rate: a practical and powerful approach to multiple testing," *Journal of the Royal Statistical Society Series B*, vol. 57, pp. 289–300, 1995.

[29] J. Chen, E. E. Bardes, B. J. Aronow, and A. G. Jegga, "ToppGene suite for gene list enrichment analysis and candidate gene prioritization," *Nucleic Acids Research*, vol. 37, no. Web Server issue, pp. W305–W311, 2009.

[30] F. Supek, M. Bošnjak, N. Škunca, and T. Šmuc, "REVIGO summarizes and visualizes long lists of gene ontology terms," *PLoS One*, vol. 6, no. 7, article e21800, 2011.

[31] P. Shannon, A. Markiel, O. Ozier et al., "Cytoscape: a software environment for integrated models of biomolecular interaction networks," *Genome Research*, vol. 13, no. 11, pp. 2498–2504, 2003.

[32] K. J. Livak and T. D. Schmittgen, "Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) method," *Methods*, vol. 25, no. 4, pp. 402–408, 2001.

[33] L. W. Harries, "MicroRNAs as mediators of the ageing process," *Genes (Basel)*, vol. 5, no. 3, pp. 656–670, 2014.

[34] J. M. Valentine, S. Ghosh, Y. Suh, and N. Musi, "Physical activity reverts miRNA signatures in aged human muscle," in *Proceeding from the 2016 Barshop Symposium on Aging*, Texas, USA, 2016.

[35] S. R. Tew, B. T. McDermott, R. B. Fentem, M. J. Peffers, and P. D. Clegg, "Transcriptome-wide analysis of messenger RNA decay in normal and osteoarthritic human articular chondrocytes," *Arthritis & Rhematology*, vol. 66, no. 11, pp. 3052–3061, 2014.

[36] T. Sato, K. Konomi, S. Yamasaki et al., "Comparative analysis of gene expression profiles in intact and damaged regions of human osteoarthritic cartilage," *Arthritis and Rheumatism*, vol. 54, no. 3, pp. 808–817, 2006.

[37] M. Geyer, S. Grässel, R. H. Straub et al., "Differential transcriptome analysis of intraarticular lesional vs intact cartilage reveals new candidate genes in osteoarthritis pathophysiology," *Osteoarthritis and Cartilage*, vol. 17, no. 3, pp. 328–335, 2009.

[38] N. Akhtar, Z. Rasheed, S. Ramamurthy, A. N. Anbazhagan, F. R. Voss, and T. M. Haqqi, "MicroRNA-27b regulates the expression of matrix metalloproteinase 13 in human osteoarthritis chondrocytes," *Arthritis and Rheumatism*, vol. 62, no. 5, pp. 1361–1371, 2010.

[39] S. Diaz-Prado, C. Cicione, E. Muiños-López et al., "Characterization of microRNA expression profiles in normal and osteoarthritic human chondrocytes," *BMC Musculoskeletal Disorders*, vol. 13, p. 144, 2012.

[40] L. Jin, J. Zhao, W. Jing et al., "Role of miR-146a in human chondrocyte apoptosis in response to mechanical pressure injury in vitro," *International Journal of Molecular Medicine*, vol. 34, no. 2, pp. 451–463, 2014.

[41] A. Martinez-Sanchez, K. A. Dudek, and C. L. Murphy, "Regulation of human chondrocyte function through direct inhibition of cartilage master regulator SOX9 by microRNA-145 (miRNA-145)," *The Journal of Biological Chemistry*, vol. 287, no. 2, pp. 916–924, 2012.

[42] K. A. Dudek, J. E. Lafont, A. Martinez-Sanchez, and C. L. Murphy, "Type II collagen expression is regulated by tissue-specific miR-675 in human articular chondrocytes," *The Journal of Biological Chemistry*, vol. 285, no. 32, pp. 24381–24387, 2010.

[43] N. Zhong, J. Sun, Z. Min et al., "MicroRNA-337 is associated with chondrogenesis through regulating TGFBR2 expression," *Osteoarthritis and Cartilage*, vol. 20, no. 6, pp. 593–602, 2012.

[44] D. Subramanyam, S. Lamouille, R. L. Judson et al., "Multiple targets of miR-302 and miR-372 promote reprogramming of human fibroblasts to induced pluripotent stem cells," *Nature Biotechnology*, vol. 29, no. 5, pp. 443–448, 2011.

[45] J. Song, M. Lee, D. Kim, J. Han, C. H. Chun, and E. J. Jin, "MicroRNA-181b regulates articular chondrocytes differentiation and cartilage integrity," *Biochemical and Biophysical Research Communications*, vol. 431, no. 2, pp. 210–214, 2013.

[46] Z. Li, M. Q. Hassan, S. Volinia et al., "A microRNA signature for a BMP2-induced osteoblast lineage commitment program," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 105, no. 37, pp. 13906–13911, 2008.

[47] F. Olivieri, M. R. Rippo, A. D. Procopio, and F. Fazioli, "Circulating inflamma-miRs in aging and age-related diseases," *Frontiers in Genetics*, vol. 4, p. 121, 2013.

[48] X. F. Wu, Z. H. Zhou, and J. Zou, "MicroRNA-181 inhibits proliferation and promotes apoptosis of chondrocytes in osteoarthritis by targeting PTEN," *Biochemistry and Cell Biology*, vol. 95, no. 3, pp. 4374–4444, 2017.

[49] Z. Xia, P. Ma, N. Wu et al., "Altered function in cartilage derived mesenchymal stem cell leads to OA-related cartilage erosion," *American Journal of Translational Research*, vol. 8, no. 2, pp. 433–446, 2016.

[50] L. Hong, T. Sharp, B. Khorsand et al., "MicroRNA-200c represses IL-6, IL-8, and CCL-5 expression and enhances osteogenic differentiation," *PLoS One*, vol. 11, no. 8, article e0160915, 2016.

[51] J. Li, J. Huang, L. Dai et al., "miR-146a, an IL-1beta responsive miRNA, induces vascular endothelial growth factor and chondrocyte apoptosis by targeting Smad4," *Arthritis Research & Therapy*, vol. 14, no. 2, article R75, 2012.

[52] K. Yamasaki, T. Nakasa, S. Miyaki et al., "Expression of MicroRNA-146a in osteoarthritis cartilage," *Arthritis and Rheumatism*, vol. 60, no. 4, pp. 1035–1041, 2009.

[53] S. Wang, A. B. Aurora, B. A. Johnson et al., "The endothelial-specific microRNA miR-126 governs vascular integrity and

angiogenesis," *Developmental Cell*, vol. 15, no. 2, pp. 261–271, 2008.

[54] A. Zernecke, K. Bidzhekov, H. Noels et al., "Delivery of microRNA-126 by apoptotic bodies induces CXCL12-dependent vascular protection," *Science Signaling*, vol. 2, no. 100, article ra81, 2009.

[55] L. Pitzler, M. Auler, K. Probst et al., "miR-126-3p promotes matrix-dependent perivascular cell attachment, migration and intercellular interaction," *Stem Cells*, vol. 34, no. 5, pp. 1297–1309, 2016.

[56] V. M. Borgonio Cuadra, N. C. González-Huerta, S. Romero-Córdoba, A. Hidalgo-Miranda, and A. Miranda-Duarte, "Altered expression of circulating microRNA in plasma of patients with primary osteoarthritis and in silico analysis of their pathways," *PLoS One*, vol. 9, no. 6, article e97690, 2014.

[57] J. Fiedler, E. Grönniger, A. Pfanne et al., "Identification of miR-126 as a new regulator of skin ageing," *Experimental Dermatology*, vol. 26, no. 3, pp. 284–286, 2017.

[58] F. Olivieri, M. Bonafè, L. Spazzafumo et al., "Age- and glycemia-related miR-126-3p levels in plasma and endothelial cells," *Aging (Albany NY)*, vol. 6, no. 9, pp. 771–787, 2014.

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Review Article

The Role of miRNAs as Biomarkers for Pregnancy Outcomes: A Comprehensive Review

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Several studies showed that altered expression of the miRNA-ome in maternal circulation or in placental tissue may reflect not only gestational disorders, such as preeclampsia, spontaneous abortion, preterm birth, low birth weight, or macrosomia, but also prenatal exposure to environmental pollutants. Generally, the relationships between environmental exposure, changes in miRNA expression, and gestational disorders are explored separately, producing conflicting findings. However, validation of tissue-accessible biomarkers for the monitoring of adverse pregnancy outcomes needs a systematic methodological approach that takes also into account early-life environmental exposure. To achieve this goal, exposure to xenochemicals, endogenous agents, and diet should be assessed. This study has the aim to provide a comprehensive review on the role of miRNAs as potential biomarkers for adverse pregnancy outcomes and prenatal environmental exposure.

1. Introduction

MicroRNAs (miRNAs) are endogenous, short, noncoding molecules, which play a role in the mechanism of posttranscriptional gene expression by suppressing translation of protein-coding genes or cleaving target mRNAs [1–3]. A peculiar characteristic of miRNAs is represented by the fact that one miRNA can regulate the expression of several genes, while one gene can be targeted by different miRNAs [4], which means that miRNAs can regulate up to 30% of the human genome [3]. In fact, miRNAs represent important epigenetic mechanisms of regulation that can control complex processes such as cell growth, differentiation, stress response, and tissue remodeling, that, under particular conditions, can play a key role in many disease states [5, 6], including gestational disorders. In particular, miRNAs may reflect pathological gestational conditions [7], such as preeclampsia [8–10], spontaneous abortion [11–13], preterm birth [6, 14, 15], macrosomia [16, 17], or low birth weight [18]. Thus, their detection in maternal circulation makes miRNAs good candidate biomarkers to monitor the progression of normal pregnancy and the presence of gestational diseases [19], for the prevention and treatment of adverse pregnancy outcomes.

The aim of the present study was to provide a comprehensive review on the role of miRNA characterization as potential biomarkers for monitoring the most common adverse pregnancy outcomes with a focus on the influence of environmental exposure during pregnancy.

2. miRNAs and Preeclampsia

Preeclampsia (PE) is a leading global cause of maternal and perinatal mortality, affecting up to 8% of pregnancies [20]. PE is the result of impaired placental development and maladaptation to the gestational conditions which leads to clinical disturbs [21]. According to the clinical management guidelines for obstetrician-gynecologists, a pregnant woman can be diagnosed with PE when she suffers from blood pressure of 140 mm Hg systolic or higher or 90 mm Hg diastolic or higher that occurs after 20 weeks of gestation in a woman with previously normal blood pressure and proteinuria. PE

Table 1: Studies reporting an association between alteration of miRNA expression and PE.

Authors and year	Study design	Enrolled population	Samples	Gestational age at sampling (weeks)	Techniques	↑ miRNAs	↓ miRNAs
Wu et al., 2012 [23]	Retrospective	10 cases + 10 controls	Maternal serum	37–40	miRNA microarray + qRT-PCR	miR-24, miR-26a, miR-103, miR-130b, miR-181a, miR-342-3p, and miR-574-5p (cases)	
Choi et al., 2013 [8]	Retrospective	21 cases + 20 controls	Placental tissue	35–40	miRNA microarray + qRT-PCR	miR-24, miR-26a, miR-103, miR-130b, miR-181a, miR-342-3p, and miR-574-5p (cases)	miR-21 and miR-223 (cases)
Ura et al., 2014 [24]	Retrospective	24 cases + 24 controls	Maternal serum	12–14	TLDA + qRT-PCR	miR-1233, miR-520, miR-210 (cases)	miR-144 (cases)
Li et al., 2013 [9]	Retrospective	*First step*:4 mild PE + 4 severe PE + 4 controls	Maternal serum	32–38	SOLiD sequencing	miR-519d, miR-517b, miR-517c, miR-26b, miR-221, miR-521, miR-378, miR-519a, miR-520h, miR-125b, miR-29a, miR-125a-5p, miR-114, miR-30a, miR-518c, miR-27a, miR-519e, miR-130a, miR-515-3p, miR-299a-5p, miR-518b, miR-23a, miR-23b, miR-34a, miR-424, miR-525-3p, miR-199a-5p, miR-29b, miR-99a, miR-21, miR-145, miR-512-5p, and miR-30b (mild and severe PE)	miR-15b, miR-223, miR-320c, miR-185, miR-107, miR-451, and let-7f (mild and severe PE)
						miR-19a, miR-10a, miR-144, miR-151b, miR-144, miR-182, and miR-19b (severe PE)	miR-19a, miR-144, miR-19b, and miR-25 (mild PE)
		Validation step: 16 mild PE + 22 severe PE + 32 controls		28–38	qRT-PCR	miR-141, miR-29a, (mild PE)	
						miR-141, miR-221, and miR-29a (severe PE)	miR-144 (mild PE)
Munaut et al., 2016 [10]	Retrospective	23 cases + 44 controls	Maternal serum	31-32	qRT-PCR	miR-210-3p, miR-210-5p, miR-1233-3p, and miR-574-5p (PE women)	—
Sandrim et al., 2016 [25]	Retrospective	*Screening*: 5 cases + 4 controls	Maternal plasma	35	PCR array + qRT-PCR	miR-885-5p (PE women)	miR-376c-3p, miR-19a-3p, and miR-19b-3p (PE women)
		Case control study: 19 cases + 14 controls		35		miR-885-5p (PE women)	—

Table 1: Continued.

Authors and year	Study design	Enrolled population	Samples	Gestational age at sampling (weeks)	Techniques	↑ miRNAs	↓ miRNAs
		Replication study: 8 cases + 8 controls		35		miR-885-5p (PE women)	—
Hromadnikova et al., 2017 [26]	Retrospective	21 PE + 58 controls	Maternal plasma	10-11	qRT-PCR	miR-517-5p, miR-518b, and miR-520h (PE women)	—

qRT-PCR: quantitative real-time polymerase chain reaction; TLDA: TaqMan low-density array; PE: preeclampsia; IUGR: intrauterine growth restriction.

can also be diagnosed in a severe form if the pregnant woman suffers from one of more of the following symptoms: blood pressure of 160 mm Hg systolic or higher or 110 mm Hg diastolic or higher on two occasions at least 6 hours apart while the patient is on bed rest; proteinuria of 5 g or higher in a 24-hour urine specimen or 3+ or greater on two random urine samples collected at least 4 hours apart; oliguria of less than 500 mL in 24 hours; cerebral or visual disturbances; pulmonary edema or cyanosis; epigastric or right upper-quadrant pain; impaired liver function; thrombocytopenia; and fetal growth restriction [22]. In order to prevent severe consequences for both the mother and the fetus, it is essential to identify women at risk of PE at an early stage [20]. In this scenario, much progress has been made to characterize miRNAs as potential biomarkers able to identify women at risk of PE. Up to date, several studies described the differential regulation of miRNAs in pregnant women suffering from PE and in healthy controls (Table 1). In 2012, Wu and collaborators detected 15 differentially expressed miRNAs in serum of severe preeclamptic women and healthy controls: miR-574-5p, miR-26a, miR-151-3p, miR-130a, miR-181a, miR-130b, miR-30d, miR-145, miR-103, miR-425, miR-221, miR-342-3p, and miR-24 were upregulated, while miR-144 and miR-16 were downregulated in PE women. Among these fifteen miRNAs, seven were further confirmed as upregulated in PE women by quantitative RT-PCR (miR-24, miR-26a, miR-103, miR-130b, miR-181a, miR-342-3p, and miR-574-5p; fold change, FC = 1.89–3.77) [23]. miR-26a and miR-342-3p were also detected as upregulated in PE women by Choi and colleagues, who isolated from formalin-fixed and paraffin-embedded samples of the placenta thirteen miRNAs (miR-92b, miR-197, miR-342-3p, miR-296-5p, miR-26b, miR-25, miR-296-3p, miR-26a, miR-198, miR-202, miR-191, miR-95, and miR-204) significantly overexpressed (FC = 2.03–12.28). Conversely, they detected miR-21 and miR-223 as underexpressed in women suffering from severe PE (FCs = 0.33 and 0.40, resp.) [8]. A study by Ura et al. detected 19 differentially expressed miRNAs in severe PE women: 12 were upregulated (miR-1233, miR-650, miR-520a, miR-215, miR-210, miR-25, miR-518b, miR-193a-3p, miR-32, miR-204, miR-296-5p, and miR-152), while 7 were downregulated (miR-126, miR-335, miR-144, miR-204, miR-668, miR-376a, and miR-15b). The results obtained by TaqMan array analysis were validated through quantitative real-time PCR, that confirmed that severe PE is associated with the upregulation of miR-1233, miR-520, and miR-210 (FC = 3.1–5.4) and the downregulation of miR-144 (FC = 0.39) during the early stages of pregnancy [24].

In 2013, a two-step study identified 51 miRNAs differentially expressed in women suffering from severe or mild PE compared to healthy pregnant women. The first step used a sequencing method to identify 22 upregulated miRNAs and 5 downregulated miRNAs in plasma of women suffering from severe PE. Furthermore, the researchers showed that 33 miRNAs were upregulated and 6 miRNAs were downregulated in plasma of mild preeclamptic women. The second step of the study focused on four different miRNAs (miR-141, miR-144, miR-221, and miR-29a) selected among the 51 previously detected, in order to validate the results on a larger number of samples through a RT-PCR method. The results from this second step confirmed the upregulation of miR-141, miR-221, and miR-29a in women suffering from severe PE and the upregulation of miR-141 and miR-29a and the downregulation of miR-144 in women suffering with mild PE [9]. In a retrospective study by Munaut and colleagues, four different miRNAs (miR-210-3p, miR-210-5p, miR-1233-3p, and miR-574-5p) were identified as upregulated in serum of PE women [10], while Sandrim and collaborators found miR-885-5p significantly overexpressed in plasma of PE women (FC = 4.5) [25].

In a recent study by Hromadnikova and colleagues, the differential expression pattern of C19MC miRNAs was detected in plasma of preeclamptic women compared to healthy controls. Particularly, levels of miR-517-5p, miR-518b, and miR-520h increased during the first trimester of gestation in women who developed PE (FC = 3.1–8.9) [26].

3. miRNAs and Spontaneous Abortion

Abortion, defined by the World Health Organization (WHO) as "any interruption of pregnancy before 28 weeks of gestation with a dead fetus" [27], is the most common complication in human reproduction, with an incidence ranging from 50 to 70% of all conceptions [28]. The spontaneous interruption of two or more pregnancies consists of a different disorder, which is the recurrent pregnancy loss (RPL), that affects 5% of pregnant women suffering from this disease [29]. Although the known causes of RPL are cytogenetic abnormalities, antiphospholipid syndrome, uterine anomalies, hereditary thrombophilia, autoimmunity, sperm quality, and environmental factors, it is not possible to understand its etiology in most of the cases [30].

Table 2: Studies reporting an association between alteration of miRNA expression and spontaneous abortion.

Authors and year	Study design	Enrolled population	Samples	Gestational age at sampling (weeks)	Techniques	↑ miRNAs	↓ miRNAs
Wang et al., 2012 [31]	Retrospective	12 cases + 10 controls	Villi	7	Microarray + qRT-PCR	miR-133a	
Dong et al., 2014 [34]	Retrospective	20 cases + 15 controls	Villi and decidua	7	Microarray + qRT-PCR	miR-184, miR-187, and miR-125b-2 (villi RPL)—miR-517c, miR-519a-1, miR-522, miR-520h, miR-184 (decidua RPL)	miR-520f, miR-3175, and miR-4672 (villi RPL)
Li and Li., 2016 [11]	Retrospective	20 cases + 20 controls	Decidua	7–10	qRT-PCR	miR-34a, miR-155, miR-141, miR-125a, and miR-125b (RPL)	miR-24 (RPL)
Qin et al., 2016 [13]	Retrospective	27 cases + 28 controls	Maternal plasma	7	miRNA array + qRT-PCR	miR-320b, miR-146b-5p, miR-221-3p, and miR-559 (cases)	miR-101-3p (cases)

qRT-PCR: quantitative real-time polymerase chain reaction; RPL: recurrent pregnancy loss.

Unexplained recurrent pregnancy loss (URPL) is a major challenge in the obstetric field as it lacks of both safe and effective therapies and reliable methods of early diagnosis [13].

In the uterus, miRNAs can regulate the expression of genes associated with the anti-inflammatory response at the time of peri-implantation and can also have a role in the maternal-fetal immune tolerance [30]. Table 2 shows the results of studies that have investigated the role of miRNA regulation in RPL; among these, a study conducted on 12 childless Chinese women with three or more spontaneous miscarriages at the 7th week of gestation and on women with induced abortion, showed that miR-133a was highly overexpressed in the villi of the RPL cases (FC = 32.4) [31]. Furthermore, JEG-3 cell lines were cultured and transfected with pre-miR-133a. Luciferase reporter assays and subsequent Western blot analysis showed that cell lines transfected with pre-miR-133a had a decreased HLA-G expression when compared with control cell lines [31]. HLA-G is a nonclassical major histocompatibility complex which is expressed in the placenta during the full length of gestation and almost solely in the extravillous trophoblasts at the fetal-maternal interface [32]. The peculiar localization of HLA-G suggests that it plays a crucial role in the maternal immune tolerance to the fetus [31]; however, it is known that its expression is associated with spontaneous abortion [33]. Thus, the study by Wang and colleagues provides evidences that miR-133a is involved in the pathogenesis of RPL by reducing the translation of HLA-G by binding its 3′-UTR [31].

Further analysis on the identification of miRNA profiles in villi of RPL cases has been conducted by Dong and colleagues in 2014. In their study, differences in miRNA expression were observed in villus tissues and decidua tissues obtained from 20 Chinese women suffering from RPL, compared to those in 15 clinically normal controls. In the villus of RPL women, 41 miRNAs were found as downregulated, whereas four were upregulated. In the decidua of RPL women, seven miRNAs were overexpressed. However, to further filter the key miRNAs involved in the RPL processes, new fold change criteria were selected (≥2 or ≤0.2): miR184, miR187, and miR125b-2 were significantly overexpressed in the villus of RPL women, whereas miR520f, miR3175, and miR4672 were downregulated. As far as the decidua is concerned, miR517c, miR591a-1, miR522, miR520h, and miR184 were found upregulated in RPL women, compared to those in normal controls. High levels of miR184 were found in both samples, suggesting that it is involved in RPL [34].

In 2016, Li and Li isolated NK (Natural Killer) cells from decidua tissue of both women suffering from URPL and healthy controls and identified six differentially expressed miRNAs: miR-34a, miR-155, miR-141, miR-125a, and miR-125b were upregulated (FC = 1.85–3.96), while miR-24 was downregulated, in the URPL group, compared with the those in healthy controls (FC = 0.64) [11]. Interestingly, members of the miR-24 family are regulators of p53 and thus involved in the mechanisms of apoptosis and cell proliferation [35]; however, this was the first study showing a possible association with RPL [11].

Further studies on URPL have been conducted by Qin and colleagues, who screened circulating miRNAs isolated from plasma of women suffering from URPL and healthy controls. Their findings showed that 6 miRNAs had differential expression between cases and controls and thus could serve as a potential candidate biomarker for URPL. Particularly, miR320b, miR146b-5p, miR221-3p, and miR559 were upregulated (FC = 3.06–4.79) whereas miR101-3p was downregulated (FC = 0.21). However, the use of miRNAs as noninvasive diagnostic biomarkers has not been established yet and the sample size of the study was small. Thus, studies on larger populations are needed in order to validate the potential role of these miRNAs as biomarkers for URPL [13].

4. miRNAs and Preterm Birth

Preterm birth (PTB) is defined as birth occurring earlier than the 37th week of gestation or before 259 days since the first day of a woman's last menstrual period [36]. Premature

Table 3: Studies reporting an association between alteration of miRNA expression and PTB.

Authors and year	Study design	Enrolled population	Samples	Gestational age at sampling (weeks)	Techniques	↑ miRNAs	↓ miRNAs
Elovitz et al., 2014 [14]	Retrospective	10 cases + 10 controls	Cervical cells	<37	Affymetrix GeneChip miRNA array + qRT-PCR	miR-143, miR-145 (cases)	—
Elovitz et al., 2015 [6]	Retrospective	40 cases + 40 controls	Maternal serum	<30	miRNA array	miR-4695-5P, miR-665 (stem-loop), and miR-887 (stem-loop) (cases)	miR-200a (cases)
Sanders et al., 2016 [15]	Prospective	60 pregnant women, 4 lost at follow-up	Cervical cells	<37	NanoString	miR-21, miR-30e, miR-142, miR-148b, miR-29b, and miR-223 (cases)	

qRT-PCR: quantitative real-time polymerase chain reaction.

birth, especially very premature birth, is a major cause of neonatal mortality, morbidity, disability [37], and cognitive impairment, in both childhood and adolescence [38]. In fact, complications of preterm birth are the single largest direct cause of neonatal deaths, responsible alone for 35% of the world's 3.1 million deaths a year, and the second most common cause of under 5-year-old deaths after pneumonia [39]. Although risk factors for PTB such as inflammation [40] and cervical length [41] are known, the major obstacle to the assessment of impactful interventional strategies against PTB is represented by the lack of understanding of the critical molecular mechanisms involved in its pathogenesis [14].

Epigenetic dysregulation may contribute to a substantial part of the risk of PTB [42], and miRNA modulation may represent an epigenetic mechanism that may be both a biomarker of risk and a target potentially amenable to future interventions [15]. Hassan and colleagues provided the first evaluation of a differential miRNA expression in cervical tissue of women undergoing term labor and delivery: in fact, among the 226 miRNA expressed in the cervical tissue, three (miR-223, miR-34b, and miR-34c) were differentially expressed and upregulated, compared to woman not in labor. However, the study wasn't able to investigate on the changes of the cervix during the progression of the pregnancy [43]. The process of connective tissue remodeling in the cervix, during pregnancy, occurs in four stages (softening, ripening, dilation, and repair), which are overlapping in time but uniquely regulated [44]. Elovitz and collaborators gave a substantial contribution on this topic, setting up a "RNA PAP" method that demonstrates that a noninvasive molecular assessment of human cervix during pregnancy is feasible. The "RNA PAP" technique is like a Pap smear and involves the collection of ectocervical cells through a cytobrush that can be performed in each trimester of pregnancy. The molecular analysis on the miRNA-ome, conducted on samples collected with this method, showed that miRNA profiles in cervical cells may distinguish women who are at risk for PTB. Among the 99 differentially expressed miRNA between women undergoing PTB and controls, 24 had a >2-fold change in expression. Among these, only two (miR-143 and miR-145) were increased in women who eventually had a PTB (FC = 11.5 and 12.34, resp.) and both were negatively correlated to cervical length. [14]. Further studies, conducted by Elovitz and colleagues on miRNA isolated from serum of pregnant women, showed an alteration in the structure of four different miRNAs (miR-200a, miR-4695-5P, miR-665, and miR887) between PTB women and controls: miR-200a and miR-4695-5P were in their mature form, whereas miR-665 and miR887 were in their nonactive stem-loop form. However, miRNA profiles in the maternal blood were not significantly different in women who were destined to have a preterm, compared with a term birth. The results are in contrast with previous findings but may suggest that PTB is a "local" disturb, with molecular and cellular changes at the level of the reproductive tissues [6]. The role of local miRNAs in reproductive tissues might help to significantly advance understanding of preterm birth. Sanders et al. showed that six miRNAs (miR-21, miR-30e, miR-142, miR-148b, miR-29b, and miR-223), isolated from cervical cells, were significantly overexpressed in women who had a shorter gestation. Interestingly, three of these miRNAs (miR-21, miR-142, and miR-223) have not been isolated in any previous studies concerning PTB or gestational age in general [15]. Table 3 summarizes the miRNAs involved in the etiology of PTB.

5. miRNAs and Birth Weight

The WHO has defined "low birth weight" (LBW) as a weight at birth of less than 2500 grams [45]. Infants' LBW can be caused both by PTB or restricted fetal intrauterine growth [46], and it is a risk factor for fetal and neonatal mortality, growth, and cognitive development inhibition and chronic diseases in adult life [47]. On the other hand, macrosomia has been defined by the American College of Obstetricians and Gynecologists as a weight at birth over 4000 grams, regardless of the gestational age or greater than the 90th percentile for gestational age, adjusting for neonatal sex and ethnicity [48]. Maternal overweight and metabolic disorders such as diabetes mellitus type 2 or gestational diabetes mellitus play a key role in macrosomia [49] which is known since the '80s for leading to complications such as prenatal asphyxia, trauma, and fetal death [50]. Several studies have been conducted in order to find an association between miRNAs and these birth outcomes with the aim to establish biomarkers useful for the diagnosis of these disorders (Table 4). As far as LBW is concerned, Song and colleagues studied the

Table 4: Studies reporting an association between alteration of miRNA expression and altered BW.

Authors and year	Gestational disorder	Study design	Enrolled population	Samples	Gestational age at sampling (weeks)	Techniques	↑ miRNAs	↓ miRNAs
Song et al., 2013 [18]	LBW	Retrospective	10 cases + 20 controls	Placental tissue + maternal serum	Delivery	qRT-PCR	miR-517a (maternal serum and placental tissue of cases)	
Wang et al., 2014 [51]	LGA, IUGR	Retrospective	30 LGA + 30 IUGR + 30 controls	Placental tissue	Delivery	qRT-PCR	mirR-519a (IUGR)	miR-518b (IUGR)
Wang et al., 2014 [51]	LGA	Retrospective	30 LGA + 30 IUGR + 30 controls	Placental tissue	Delivery	qRT-PCR	—	—
Hromadnikova et al., 2017 [26]	IUGR	Retrospective	18 IUGR + 58 controls	Maternal plasma	10	qRT-PCR	—	—
Hu et al., 2014 [16]	Macrosomia	Retrospective	60 cases + 60 controls	Maternal serum	16–20	TLDA − qRT-PCR	—	miR-376a
Li et al., 2015 [52]	Macrosomia	Retrospective	57 cases + 100 controls	Placental tissue + maternal serum	Placenta (delivery) serum (NA)	qRT-PCR	miR-18a, miR-19a, miR-20a, miR-19b, and miR-92a (placental tissue of cases)	miR17, miR18a, miR19a, and miR92a (maternal serum of cases)
Ge et al., 2015 [53]	Macrosomia	Retrospective	35 cases + 20 controls	Maternal serum	18–28	TLDA + qRT-CR	miR-523-3p, miR-3a-3p, and miR-16-5p (cases)	miR-221-3p, miR-143-3p, miR-18a-5p miR-141-3p, and miR200c-3p (cases)
Zhang et al., 2016 [54]	Macrosomia	Retrospective	67 cases + 64 controls	Placental tissue	Delivery	qRT-PCR	miR-21	miR-143
Jiang et al., 2015 [17]	Macrosomia	Retrospective	60 cases + 60 controls	Maternal plasma	16–20 weeks and 1 week from delivery	TLDA + qRT-PCR	—	miR-21
Miura et al., 2015 [55]	Birth weight	Cross-sectional	82 pregnant women	Maternal serum	37-38	qRT-PCR		

LBW: low birth weight; qRT-PCR: quantitative real-time polymerase chain reaction; IUGR: intrauterine growth restriction; LGA: large for gestational age; TLDA: TaqMan low-density array.

expression of placenta-specific miR-517a in placental tissue and maternal serum. The expression of mir-517a was higher both in the placenta of LBW infants (FC = 12.33) and in the serum of women who delivered a LBW baby (FC = 8.03). Further analysis conducted on cultured cells suggested that mir-517a inhibits trophoblast invasion, leading to abnormal placentation and, thus, to LBW [18]. Further studies, conducted on placenta-specific miRNAs, showed that the expression of miR-518b and miR-519a was altered in infants with IUGR, when compared to large for gestational age (LGA) infants and healthy babies: specifically, miR-518b was down regulated (FC = 0.46), whereas miR-519a was up regulated (FC = 1.91) [51]. miR-518b and the other C19MC miRNAs were recently studied also by Hromadnikova and colleagues, who screened first the trimester blood of IUGR subjects and healthy controls. However, no significant difference in plasma levels of C19MC miRNAs was found between the two groups [26]. Researches on macrosomia are more numerous. In 2014, a two-phase study conducted on 120 participants showed aberrant expression of miR37a, which was downregulated in serum of pregnant women who delivered infants with macrosomia [16]. In 2015, Li and colleagues

Table 5: Studies reporting an association between alteration of miRNA expression and environmental exposure.

Authors and year	Exposure	Study design	Enrolled population	Samples	Techniques	↑ miRNAs	↓ miRNAs
Li et al., 2015 [56]	Organic and inorganic environmental pollutants	Prospective	110 clinical normal pregnant women	Placental tissue	NanoString	miR-651 (Pb exposure) miR-1537 (Cd exposure) miR-188-5p (PBDE high brominated congener 209 exposure) miR-1537 (PCB congener 52 and 10 and total PCB exposure)	miR-151p, miR-10a, miR-193b, miR-1975, miR-423-5p, miR-520d-3p, miR-96, miR-252a, miR-518d-5p, miR-520a-5p, miR-190, let-7a, let-7b, let-7c, let-7d, let-7g, and let-7i (Hg exposure) let-7f, miR-146a, miR-10a, and miR-431 (Pb exposure) let-7c (PBDE low brominated congener 99 exposure)
Tsamou et al., 2016 [57]	$PM_{2.5}$, NO_2	Prospective	210 mother-newborn pairs	Placental tissue	qRT-PCR	*1st trimester* miR-20a, miR-21 ($PM_{2.5}$ exposure) miR-21 (NO_2 exposure) *2nd trimester* — *3rd trimester* —	*1st trimester* — *2nd trimester* miR-16, miR-21, miR-146a, and miR-222 ($PM_{2.5}$ exposure) miR-20a, miR-21, and miR-146a (NO_2 exposure) *3rd trimester* miR-146a, ($PM_{2.5}$ exposure)
LaRocca et al., 2016 [58]	Endocrine Disrupting Chemicals	Prospective	179 women	Maternal urine + placental tissue	PCR Array + qRT-PCR	mir-15a-5p (Σ* *phenols*, Σ *parabens* male placentas) mir-185 (Σ *DEHPm*, Σ *LMW*)	mir-15a-5p (Σ *phenols*, Σ *parabens* female placentas and *nonparabens*, overall) miR-142-3p (Σ *phenols*, Σ *parabens*, Σ *nonparabens* overall) mir-185 (Σ *phthalates*, Σ *HMW*, overall)

qRT-PCR: quantitative real-time polymerase chain reaction; ∗ indicates the sum of all the chemicals.

investigated on the role of miRNA17-92 cluster in macrosomia, with a study on both the placenta and serum from 57 mothers who delivered infants with macrosomia and 100 healthy controls. The results on placenta showed the upregulation of miR17, miR18a, miR19a, miR19b, miR20a, and miR92a in samples coming from mothers with macrosomic babies. As far as the maternal serum is concerned, miR17, miR18a, miR19a, and miR92a were downregulated in plasma of mothers with macrosomic infants. This result has high diagnostic sensitivity and specificity for macrosomia, as suggested by the ROC curve analysis [52]. Further studies on miRNA profile in serum of pregnant women were conducted by Ge and collaborators, investigating 45 pregnant women who eventually delivered infants with macrosomia and 30 women who eventually delivered healthy infants. Firstly, samples of women with fetal macrosomia and women with a healthy pregnancy were analysed through TaqMan low-density array (TLDA). Particularly, 143 miRNAs were found differentially expressed: 43 were up regulated and 100 were down regulated in women with fetal macrosomia. Among these, 12 miRNAs were chosen for the validation through qRT-PCR. Four out of the 12 selected miRNAs were upregulated (miR-661, miR-523-3p, miR-125a-5p, and miR-30a-3p) while 8 were downregulated (miR-181a-5p, miR-200c-3p, miR-143-3p, miR-221-3p, miR-16-5p, miR-141-3p, miR-18a-5p, and miR-451). All the results by TLDA were confirmed by qRT-PCR with the exception of miR-16-5p that had opposite results. ROC curve analysis was performed in order to test the characteristic of the differentially expressed miRNAs. Downregulated miR-523-3p, miR200c-3p, and miR141-3p showed a higher efficiency in distinguishing between woman with fetal macrosomia and women with normal pregnancy; thus, further analysis through qRT-PCR was conducted in order to verify their specificity for macrosomia. In fact, further analysis on serum of 16 pregnant women suffering from PE showed that miR141-3p and miR200c-3p were downregulated in women with fetal macrosomia, also when compared to those in preeclamptic woman, whereas miR-523-3p did not show a significant difference between the two groups [53]. In 2016, a study on placental expressed miRNAs on 67 samples of macrosomic placental tissues and 64 normal placental tissues showed that high levels of miR-21 and low levels of miR-143 are associated with macrosomia [54]. MiR-21 was also found associated to macrosomia by Jiang and colleagues; however, findings of this study on maternal serum showed that levels

of miR-21 were significantly lower in serum of women delivering macrosomic infants when compared to healthy controls [17]. Miura and collaborators found an association between circulating miR-21 levels in maternal serum and not only infants' birth weight but also maternal body mass index. However, no significant association was found between circulating miR-21 levels and placental weight [55].

6. miRNAs and Environmental Exposure during Pregnancy

Studies on birth cohorts using miRNAs as biomarkers in pregnancy have been mostly conducted to assess the association between the exposure to environmental pollutants and changes in the miRNA-ome in placental samples (Table 5). A study on placental samples from the National Children's Study (NCS) Vanguard birth cohort investigated on the association between miRNA profile in placentas and prenatal exposure to dichlorodiphenyldichloroethylene (DDE), bisphenol A (BPA), polybrominated diphenyl ethers (PBDEs), polychlorinated biphenyls (PCBs), arsenic (As), mercury (Hg), lead (Pb), and cadmium (Cd). Results showed that exposure to high levels of Hg was associated to decreased levels of miR-151p, miR-10a, miR-193b, miR-1975, miR-423-5p, miR-520d-3p, miR-96, miR-252a, miR-518d-5p, miR-520a-5p, miR-190, and numerous miRNAs belonging to the let-7 family. Exposure to high levels of Pb was associated not only to lower levels of the let-7 family miRNAs and miR-190 but also to lower levels of miR-146a, miR-10a, and miR-431, while the levels of miR-651 were increased. Exposure to high levels of Cd and PCBs were associated with high levels of miR-1537. However, the study did not take into account birth outcomes and analysed only 110 placentas collected from the larger NCS Vanguard birth cohort [56].

In 2016, a study analysed sample tissues of placenta from the ENVIR*ON*AGE birth cohort to assess a possible association between exposure to air pollutants ($PM_{2.5}$ and NO_2) and altered expression of placental miRNAs. As far as $PM_{2.5}$ is concerned, exposure to the pollutants in the first trimester of pregnancy was positively associated with increased expression of miR-20a and miR-21, while exposure during the second trimester was associated with lower expression of miR-16, miR-20a, miR-21a, miR-222, and miR146a, which levels were even lower during the third trimester. Exposure to NO_2 during the first trimester was positively associated with an increased expression of miR-21, whereas the exposure to the pollutant during the second trimester of pregnancy showed an association with lower levels of miR-20a, miR-21, and miR-146a. Although this study has a larger sample size (210 mother-newborns pairs) than the other previously described, it does not take into account pregnancy outcomes [57].

A study conducted on 179 samples of urines and placentas of first-trimester mothers and their infants, coenrolled in the Harvard Epigenetic Birth Cohort and in the Predictors of Preeclampsia Study, investigated on the alteration of placental miRNA expression and exposure to phthalate metabolites and phenol. After a pilot study on 48 samples, LaRocca and colleagues selected 29 differentially expressed miRNAs to be further investigated in the full study. The most relevant results showed that increased levels of total phenols in the urine were associated with lower expression of miR-142. Particularly, increased levels of parabens were associated with lower expression of miR-142 both in urine and in the placenta. Increasing levels of nonparabens in urine was also associated to lower levels of miR-15a-5p. However, only in female newborn placentas, the expression levels of miR-15a-5p were found significantly associated to an increasing exposure to total phenols. As far as the phenols were concerned, the expression of the majority of tested miRNAs was positively associated to levels of BPA and BP-3 in urines. Results about phthalates showed that an increased exposure to low molecular weight phthalates was associated to lower levels of miR-185 expression in placenta. The result was probably due to MEP (monoethyl phthalate), which was the compound presenting the stronger association with miR-185. Moreover, the expression of the majority of the tested miRNAs was associated to the exposure to monocarboxyisooctyl phthalate (MOCP). However, no association between the expression of the miRNAs altered by the maternal chemical exposure and gestational age, birth weight, or birth length was found [58].

7. Discussion

Several studies have investigated on the association between circulating levels of miRNAs and birth outcomes, to establish potential biomarkers for the prevention of the most common gestational disorders. In fact, the aforementioned scientific literature well describes aberrant miRNA expression in mother-child pairs with adverse pregnancy outcomes, suggesting that miRNAs may serve as useful prevention and clinical tools.

However, results from several works are inconsistent and the spectrums of miRNAs observed by different studies are rather controversial. Such inconsistency or discrepancies can be attributed to differences in sample type, sample handling, gestational age at sampling, techniques used for miRNA profiling, and population characteristics, such as age, ethnicity, and lifestyles.

Particularly, the results could be influenced by the different sources of miRNAs, such as the placenta, umbilical cord blood, or maternal sera. In biomarker-related studies, both these noninvasive and easily obtainable samples are suitable sources of miRNAs, reflecting early-life experience during pregnancy. However, changes in miRNA expression, using the cord blood and placenta as starting material, could be analysed to understand the etiology of adverse pregnancy outcomes, because the obtained expression profiles reflect the environmental exposure toward the end of pregnancy.

To investigate the relationship between placental-specific miRNAs and pregnancy outcomes, researchers compared the expression of miRNAs isolated from the placentas between patients affected by the disorders and healthy controls [8, 51, 54, 56–58]. Since placental tissues are obtained at the time of delivery, it is unclear whether the aberrant miRNA expression is the cause or the consequence of the disorder. Accordingly, only two studies analysed the

expression of specific miRNAs both in the placenta and maternal serum [18, 52].

In fact, maternal plasma and serum could be useful to monitor changes in miRNA expression throughout pregnancy and in specific gestational periods, allowing the development of screening biomarkers. Other sample sources were maternal urine [58], decidua [11, 34], villi [31, 34], and cervical cells [14, 15].

Blood-derived biomarkers could be routinely monitored, representing the preferred specimens for noninvasive diagnosis. However, gestational age at sampling could influence the levels of circulating miRNAs. Among the reviewed studies, gestational age at sampling depended on the study design and sample type, with a range from 7 to 40 weeks. As a result, it is discouraged to compare findings from studies which evaluate miRNA expression levels at different gestational age. To overcome this issue, studies have to include results for each trimester of the pregnancy, as provided by Tsamou et al. and LaRocca et al. [57, 58].

Endogenous degrading properties, elapsed time from collection, temperature during transport, anticoagulant, and stabilising agents are key factors that affect the quality of biological samples and expression analyses. However, among the reviewed studies, it is unclear whether sample processing and storage were appropriate.

To date, there are several methods to examine miRNA profiling, such as quantitative real-time PCR (qRT-PCR), microarrays, and direct sequencing. Although each method has advantages and limitations, qRT-PCR seems to have better sensitivity than array technologies for miRNA profiling [59]. On the other hand, qRT-PCR approach is limited in the number of detectable miRNAs compared with microarrays. Next-generation sequencing (NGS) offers important advantages over other technologies, representing the best platform for miRNA discovery. However, although NGS is extremely sensitive, qRT-PCR is still the only platform capable of generating absolute quantification [59]. Differences in techniques used for miRNA profiling are also a contributing factor controversial in the specific miRNA expression. The suitable approach, mostly used, was to identify disease-related differences in miRNA expression by miRNA microarray or second-generation sequencing in small subgroups, followed by the validation by qRT-PCR on a larger sample size [8, 9, 13, 14, 16, 23–25, 31, 34, 53, 58]. However, other studies just used qRT-PCR techniques to validate candidate biomarkers identified through literature search or based on previous findings [10, 11, 18, 26, 51, 52, 54, 55, 57]. In addition, variability between studies was also observed in data reporting. In fact, the majority of studies used 2-fold change and 0.5-fold change thresholds to define marked differential miRNA expression [8, 13, 14, 18, 24, 25, 31]. However, others reported as differentially expressed also miRNA with an expression of only 1.5-fold changes [11, 23, 51]. Thus, to improve accuracy and avoid discrepancies, it is important to develop standardized methodologies both in preanalytical and analytical activities.

An additional limitation of studies mentioned in this paper was the size of the enrolled population. Although several evidences demonstrate the potential application of miRNAs in the monitoring of adverse pregnancy outcomes, data interpretation must be prudent because of the small sample size.

In this context, a major involvement of large birth cohorts and biobanks is warranted.

Moreover, the validation of tissue-accessible biomarkers for the monitoring of adverse pregnancy outcomes needs a systematic approach that takes also into account early-life environmental exposure [60]. To achieve this goal, exposure to xenochemicals, endogenous agents, and diet should be assessed through the collection of questionnaire data, as well as biological samples.

At the best of our knowledge, few studies have investigated miRNAs involved in causal pathways between early-life exposure and adverse pregnancy outcomes [58]. Generally, the relationships between environmental exposure, changes in miRNA expression, and gestational disorders are explored separately, producing conflicting findings due to methodological and biospecimen heterogeneity.

8. Conclusion

The overall aim of this review was to summarize the effects of miRNAs and environmental exposure on pregnancy outcomes. Although several studies reported differential miRNA expression in gestational disorders, we arose the need of more standardized methodologies in both preanalytical and analytical levels, as well as a major involvement of large birth cohorts and biobanks. Particularly, the potential to transform biobanks into well-characterized longitudinal epidemiological studies has become crucial to the conduct of large-scale genomic and epigenomic researches. The set of exposure information, clinical data, and biological samples, collected in human biobanks, constitutes a resource to conduct further analyses for the characterization and validation of miRNAs as biomarkers in both preventive and diagnostic strategies for gestational disorders.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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References

[1] M. Montagnana, E. Danese, G. Lippi, and C. Fava, "A narrative review about blood laboratory testing for early prediction of preeclampsia: chasing the finish line or at the starting blocks?" *Annals of Medicine*, vol. 49, no. 3, pp. 240–253, 2017.

[2] H. Guo, N. T. Ingolia, J. S. Weissman, and D. P. Bartel, "Mammalian microRNAs predominantly act to decrease target mRNA levels," *Nature*, vol. 466, pp. 835–840, 2010.

[3] D. Baek, J. Villen, C. Shin, F. D. Camargo, S. P. Gygi, and D. P. Bartel, "The impact of microRNAs on protein output," *Nature*, vol. 455, pp. 64–71, 2008.

[4] D. Sayed and M. Abdellatif, "MicroRNAs in development and disease," *Physiological Reviews*, vol. 91, no. 3, pp. 827–887, 2011.

[5] D. P. Bartel, "MicroRNAs: genomics, biogenesis, mechanism, and function," *Cell*, vol. 116, pp. 281–297, 2004.

[6] M. A. Elovitz, L. Anton, J. Bastek, and A. G. Brown, "Can microRNA profiling in maternal blood identify women at risk for preterm birth?" *American Journal of Obstetrics and Gynecology*, vol. 212, article 782.e1-5, 2015.

[7] D. M. M. Prieto and U. R. Markert, "MicroRNAs in pregnancy," *Journal of Reproductive Immunology*, vol. 88, no. 2, pp. 106–111, 2011.

[8] S. Y. Choi, J. Yun, O. J. Lee et al., "MicroRNA expression profiles in placenta with severe preeclampsia using a PNA-based microarray," *Placenta*, vol. 34, no. 9, pp. 799–804, 2013.

[9] H. Li, Q. Ge, L. Guo, and Z. Lu, "Maternal plasma miRNAs expression in preeclamptic pregnancies," *BioMed Research International*, vol. 2013, Article ID 970265, 9 pages, 2013.

[10] C. Munaut, L. Tebache, S. Blacher, A. Noël, M. Nisolle, and F. Chantraine, "Dysregulated circulating miRNAs in preeclampsia," *Biomedical Reports*, vol. 5, no. 6, pp. 686–692, 2016.

[11] D. Li and J. Li, "Association of miR-34a-3p/5p, miR-141-3p/5p, and miR-24 in decidual natural killer cells with unexplained recurrent spontaneous abortion," *Medical Science Monitor*, vol. 22, pp. 922–929, 2016.

[12] Y. Zhu, H. Lu, Z. Huo et al., "MicroRNA-16 inhibits feto-maternal angiogenesis and causes recurrent spontaneous abortion by targeting vascular endothelial growth factor," *Scientific Reports*, vol. 6, article 35536, 2016.

[13] W. Qin, Y. Tang, N. Yang, X. Wei, and J. Wu, "Potential role of circulating microRNAs as a biomarker for unexplained recurrent spontaneous abortion," *Fertility and Sterility*, vol. 105, no. 5, pp. 1247–1254.e3, 2016.

[14] M. A. Elovitz, A. G. Brown, L. Anton, M. Gilstrop, L. Heiser, and J. Bastek, "Distinct cervical microRNA profiles are present in women destined to have a preterm birth," *American Journal of Obstetrics and Gynecology*, vol. 210, no. 3, article 221.e1-11, 2014.

[15] A. P. Sanders, H. H. Burris, A. C. Just et al., "microRNA expression in the cervix during pregnancy is associated with length of gestation," *Epigenetics*, vol. 10, no. 3, pp. 221–228, 2015.

[16] L. Hu, J. Han, F. Zheng et al., "Early second-trimester serum microRNAs as potential biomarker for nondiabetic microsomia," *BioMed Research International*, vol. 2014, Article ID 394125, 6 pages, 2014.

[17] H. Jiang, Y. Wen, L. Hu, T. Miao, M. Zhang, and J. Dong, "Serum MicroRNAs as diagnostic biomarkers for macrosomia," *Reproductive Sciences*, vol. 22, no. 6, pp. 664–671, 2015.

[18] G. Y. Song, W. W. Song, Y. Han, D. Wang, and Q. Na, "Characterization of the role of microRNA-517a expression in low birth weight infants," *Journal of Developmental Origins of Health and Disease*, vol. 4, no. 6, pp. 522–526, 2013.

[19] G. Fu, J. Brkic, H. Hayder, and C. Peng, "MicroRNAs in human placental development and pregnancy complications," *International Journal of Molecular Sciences*, vol. 14, no. 3, pp. 5519–5544, 2013.

[20] E. Steegers, P. Von Dadelszen, J. Duvekot, and R. Pijnenborg, "Pre-eclampsia," *Lancet*, vol. 376, pp. 631–644, 2010.

[21] J. M. Roberts and C. A. Hubel, "The two stage model of preeclampsia: variations on the theme," *Placenta*, vol. 30, Supplement A, pp. S32–S37, 2009.

[22] American College of Obstetricians and Gynecologists (ACOG) practice bulletin, "Diagnosis and management of preeclampsia and eclampsia," *Obstetrics and Gynecology*, vol. 99, pp. 159–167, 2002.

[23] L. Wu, H. Zhou, H. Lin et al., "Circulating microRNAs are elevated in plasma from severe preeclamptic pregnancies," *Reproduction*, vol. 143, no. 3, pp. 389–397, 2012.

[24] B. Ura, G. Feriotto, L. Monasta, S. Bilel, M. Zweyer, and C. Celeghini, "Potential role of circulating microRNAs as early markers of preeclampsia," *Taiwanese Journal of Obstetrics & Gynecology*, vol. 53, no. 2, pp. 232–234, 2014.

[25] V. C. Sandrim, M. R. Luizon, A. C. Palei, J. E. Tanus-Santos, and R. C. Cavalli, "Circulating microRNA expression profiles in pre-eclampsia: evidence of increased miR-885-5p levels," *BJOG : An International Journal of Obstetrics and Gynaecology*, vol. 123, no. 13, pp. 2120–2128, 2016.

[26] I. Hromadnikova, K. Kotlabova, K. Ivankova, and L. Krofta, "First trimester screening of circulating C19MC microRNAs and the evaluation of their potential to predict the onset of preeclampsia and IUGR," *PLoS One*, vol. 12, no. 2, article e0171756, 2017.

[27] World Health Organization, *Manual of the International Statistical Classification of Diseases, Injuries and Cause of Death, 1965 Revision*, World Health Organization, Geneva, 1967.

[28] R. Rai and L. Regan, "Recurrent miscarriage," *Lancet*, vol. 368, pp. 601–611, 2006.

[29] Practice Committee of the American Society for Reproductive Medicine, "Evaluation and treatment of recurrent pregnancy loss: a committee opinion," *Fertility and Sterility*, vol. 98, pp. 1103–1111, 2012.

[30] Y. W. Jung, Y. J. Jeon, H. Rah et al., "Genetic variants in microRNA machinery genes are associate with idiopathic recurrent pregnancy loss risk," *PLoS One*, vol. 9, no. 4, article e95803, 2014.

[31] X. Wang, H. Zhao, B. Li et al., "Evidence that miR-133a causes recurrent spontaneous abortion by reducing HLA-G expression," *Reproductive Biomedicine Online*, vol. 25, no. 4, pp. 415–424, 2012.

[32] D. S. Goldman-Wohl, I. Ariel, C. Greenfield, J. Hanoch, and S. Yagel, "HLA-G expression in extravillous trophoblasts is an intrinsic property of cell differentiation: a lesson learned from ectopic pregnancies," *Molecular Human Reproduction*, vol. 6, pp. 535–540, 2000.

[33] M. Cecati, S. R. Giannubilo, M. Emanuelli, A. L. Tranquilli, and F. Saccucci, "HLA-G and pregnancy adverse outcomes," *Medical Hypotheses*, vol. 76, pp. 782–784, 2011.

[34] F. Dong, Y. Zhang, F. Xia et al., "Genome wide miRNA profiling of villus and decidua of recurrent spontaneous abortion patients," *Reproduction*, vol. 148, no. 1, pp. 33–41, 2014.

[35] X. He, L. He, and G. J. Hannon, "The guardian's little helper: microRNAs in the p53 tumor suppressor network," *Cancer Research*, vol. 67, pp. 11099–11101, 2007.

[36] WHO, "WHO: recommended definitions, terminology and format for statistical tables related to the perinatal period and use of a new certificate for cause of perinatal deaths. Modifications recommended by FIGO as amended October 14, 1976," *Acta Obstetricia et Gynecologica Scandinavica*, vol. 56, pp. 247–253, 1977.

[37] P. Y. Ancel, F. Goffinet, and EPIPAGE 2 Writing Group, "EPIPAGE 2: a preterm birth cohort in France in 2011," *BMC Pediatrics*, vol. 14, p. 97, 2014.

[38] J. P. Boardman, S. J. Counsell, D. Rueckert et al., "Abnormal deep grey matter development following preterm birth detected using deformation-based morphometry," *NeuroImage*, vol. 32, pp. 70–78, 2006.

[39] H. Blencowe, S. Cousens, D. Chou et al., "Born too soon: the global epidemiology of 15 million preterm births," *Reproductive Health*, vol. 10, no. article S2, 2013Supplement 1, 2013.

[40] S. Trivedi, M. Joachim, T. McElrath et al., "Fetal-placental inflammation, but not adrenal activation, is associated with extreme preterm delivery," *American Journal of Obstetrics and Gynecology*, vol. 206, article 236.e1-8, 2012.

[41] S. S. Hassan, R. Romero, D. Vidyadhari et al., "Vaginal progesterone reduces the rate of preterm birth in women with a sonographic short cervix: a multicenter, randomized, double-blind, placebo-controlled trial," *Ultrasound in Obstetrics & Gynecology*, vol. 38, pp. 18–31, 2011.

[42] A. K. Knight and A. K. Smith, "Epigenetic biomarkers of preterm birth and its risk factors," *Genes (Basel)*, vol. 7, no. 4, 2016.

[43] S. S. Hassan, R. Romero, B. Pineles et al., "MicroRNA expression profiling of the human uterine cervix after term labor and delivery," *American Journal of Obstetrics and Gynecology*, vol. 202, article 80.e1-8, 2010.

[44] R. A. Word, X. H. Li, M. Hnat, and K. Carrick, "Dynamics of cervical remodeling during pregnancy and parturition: mechanisms and current concepts," *Seminars in Reproductive Medicine*, vol. 25, no. 1, pp. 69–79, 2007.

[45] World Health Organization, *International Statistical Classification of Diseases and Related Health Problems, Tenth Revision*, World Health Organization, Geneva, 1992.

[46] M. S. Kramer, "Determinants of low birth weight: methodological assessment and meta-analysis," *Bulletin of the World Health Organization*, vol. 65, no. 5, pp. 663–737, 1987.

[47] United Nations Children's Fund and World Health Organization, *Low Birthweight: Country, Regional and Global Estimates*, UNICEF, New York, 2004.

[48] S. K. Ng, A. Olog, A. B. Spinks, C. M. Cameron, J. Searle, and R. J. McClure, "Risk factors and obstetric complications of large for gestational age births with adjustments for community effects: results from a new cohort study," *BMC Public Health*, vol. 10, p. 460, 2010.

[49] A. Levy, A. Wiznitzer, G. Holcberg, M. Mazor, and E. Sheiner, "Family history of diabetes mellitus as an independent risk factor for macrosomia and cesarean delivery," *Journal of Maternal- Fetal and Neonatal Medicine*, vol. 23, no. 2, pp. 148–152, 2010.

[50] M. E. Boyd, R. H. Usher, and F. H. McClean, "Fetal macrosomia: prediction, risks, and proposed management," *Obstetrics and Gynecology*, vol. 61, pp. 715–722, 1983.

[51] D. Wang, Q. Na, W. W. Song, and G. Y. Song, "Altered expression of miR-518b and miR-519a in the placenta is associated with low fetal birth weight," *American Journal of Perinatology*, vol. 31, no. 9, pp. 729–734, 2014.

[52] J. Li, L. Chen, Q. Tang et al., "The role, mechanism and potentially novel biomarker of microRNA-17-92 cluster in macrosomia," *Scientific Reports*, vol. 5, article 17212, 2015.

[53] Q. Ge, Y. Zhu, H. Li, F. Tian, X. Xie, and Y. Bai, "Differential expression of circulating miRNAs in maternal plasma in pregnancies with fetal macrosomia," *International Journal of Molecular Medicine*, vol. 35, no. 1, pp. 81–91, 2015.

[54] J. T. Zhang, Q. Y. Cai, S. S. Ji et al., "Decreased miR-143 and increased miR-21 placental expression levels are associated with macrosomia," *Molecular Medicine Reports*, vol. 13, no. 4, pp. 3273–3280, 2016.

[55] K. Miura, A. Higashijima, Y. Hasegawa et al., "Circulating levels of maternal plasma cell-free miR-21 are associated with maternal body mass index and neonatal birth weight," *Prenatal Diagnosis*, vol. 35, no. 5, pp. 509–511, 2015.

[56] Q. Li, M. A. Kappil, A. Li et al., "Exploring the associations between microRNA expression profiles and environmental pollutants in human placenta from the National Children's Study (NCS)," *Epigenetics*, vol. 10, no. 9, pp. 793–802, 2015.

[57] M. Tsamou, K. Vrijens, N. Madhloum, W. Lefebvre, C. Vanpoucke, and T. S. Nawrot, "Air pollution-induced placental epigenetic alterations in early life: a candidate miRNA approach," *Epigenetics*, vol. 22, 2016.

[58] J. LaRocca, A. M. Binder, T. F. McElrath, and K. B. Michels, "First-trimester urine concentrations of phthalate metabolites and phenols and placenta miRNA expression in a cohort of U.S. women," *Environmental Health Perspectives*, vol. 124, no. 3, pp. 380–387, 2016.

[59] L. Moldovan, K. E. Batte, J. Trgovcich, J. Wisler, C. B. Marsh, and M. Piper, "Methodological challenges in utilizing miRNAs as circulating biomarkers," *Journal of Cellular and Molecular Medicine*, vol. 18, no. 3, pp. 371–390, 2014.

[60] A. Chango and I. P. Pogribny, "Considering maternal dietary modulators for epigenetic regulation and programming of the fetal epigenome," *Nutrients*, vol. 7, no. 4, pp. 2748–2770, 2015.