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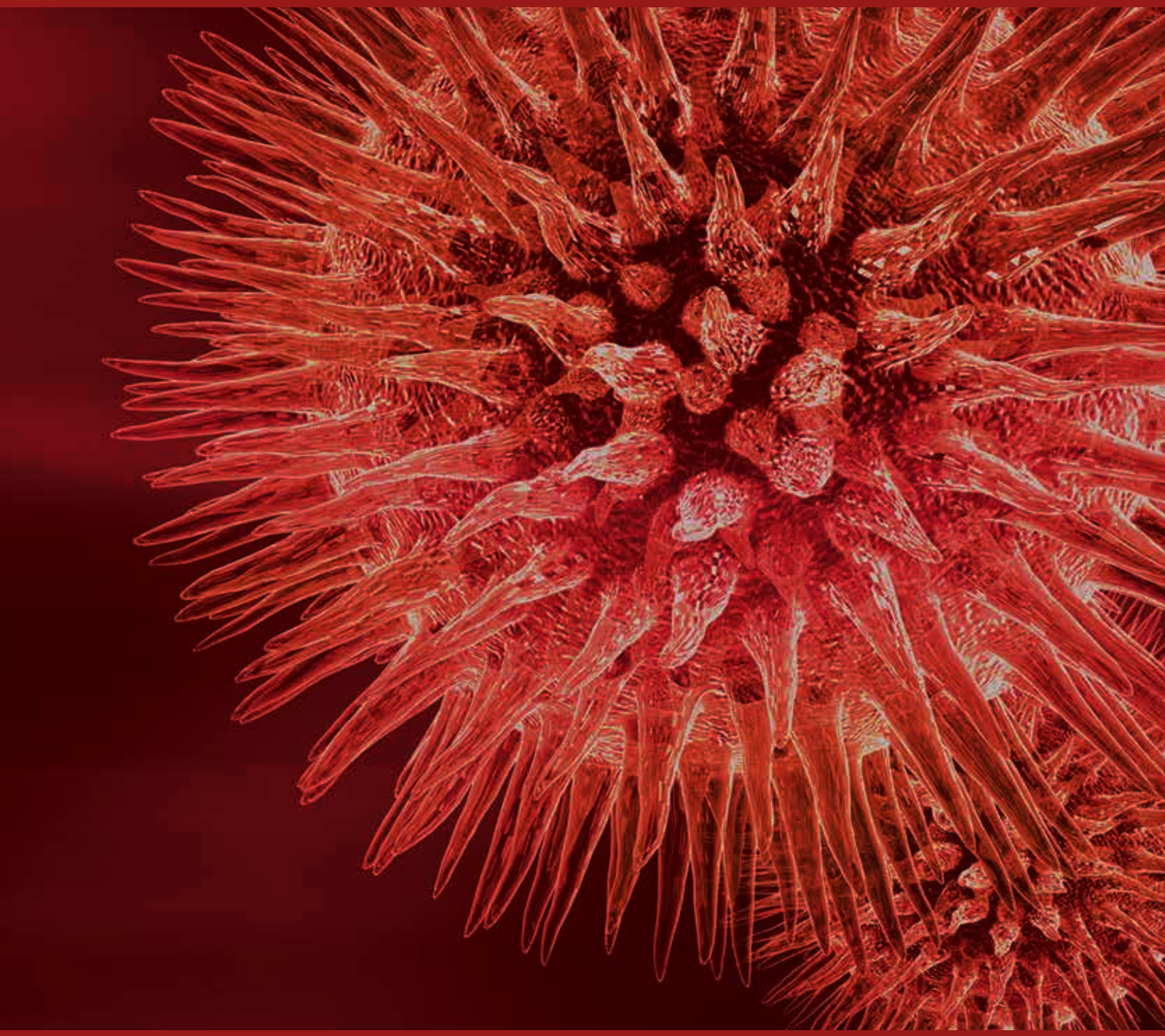
MicroRNAs in Cancer Management: Big Challenges for Small Molecules

Guest Editors: Paolo Gandellini, Elisa Giovannetti, and Francesco Nicassio



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Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 982156, 2 pages
http://dx.doi.org/10.1155/2015/982156

Editorial

MicroRNAs in Cancer Management: Big Challenges for Small Molecules

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Received 24 November 2014; Accepted 24 November 2014

In the last decade, the scientific community has been shaken by what we call the "noncoding revolution." We have witnessed the discovery of an increasing amount of RNA molecules, which play a critical role in normal physiology as well as disease, without providing any protein product. The smallest regulatory RNA species, known as the ~22 nucleotide-long microRNAs (miRNAs), were at the forefront of such a revolution.

According to the last release of miRNA database (*miRBase* http://www.mirbase.org—release 21) more than 35,000 miRNA species have been identified, of which a total of more than 2,500 mature miRNAs exist only in humans [1]. Since their first discovery in *C. elegans* in 1993, it has become clear that these tiny molecules have an enormous regulatory potential, being able to exert negative posttranscriptional regulation on hundreds of protein coding genes, even simultaneously, and ultimately act as master regulators of entire biological processes.

Besides their role in development, the involvement of miRNAs in human disease, and cancer in particular, has attracted major attention. In fact, deregulated miRNA expression could lead to aberrant expression of targeted oncogenes or tumor suppressors, thus resulting in tumor development and progression. Accordingly, altered expression of miRNAs has been observed in almost every tumor type, including haematological malignancies, carcinomas, sarcomas, and central nervous system neoplasms. Relevant examples of the role of miRNAs on specific cancer types are reported in this issue, with a focus on childhood acute lymphoblastic leukemia (original contribution by M. Duyu et al.), ovarian and cervical cancer (reviewed by Y. Kinose et al. and S. M. Díaz-González et al.), soft tissue sarcomas (reviewed by T. Fujiwara et al.), or medulloblastoma (reviewed by S. López-Ochoa et al.).

The unique pattern of altered miRNA expression provides a fingerprint that may serve for cancer diagnosis and prediction of patient's prognosis or response to treatment. In addition, a number of miRNAs have been shown to directly participate in tumorigenesis by acting as "oncoMirs" or "tumor suppressive miRNAs," thus becoming potential key targets or tools for anticancer therapy. The review by A. Saumet and colleagues suitably introduces the current approaches and applications of miRNAs in cancer and human diseases, from expression analyses aimed at identifying miRNAs potentially useful for tumor diagnosis or prognosis to the potential use of them as novel therapeutic agents.

The opportunities and challenges of miRNAs as cancer biomarkers have been addressed in the issue by H. Lan and colleagues. Specific focuses have been also provided on the clinicopathological significance of miR-155 in breast cancer (H. Zeng et al.), diagnostic and prognostic miRNAs in sarcomas (T. Fujiwara et al.), and miRNAs exploitable for prostate cancer risk assessment (A. Cannistraci et al.). Based on their relative stability in body fluids, a great effort has been devoted in the last years to the study of circulating miRNAs as noninvasive biomarkers for tumor

diagnosis or disease monitoring. Though attracting, miRNA quantification in liquid samples is far from being trivial. Challenges encountered in the field have been extensively addressed in the issue by P. Tiberio and colleagues.

Due to their nature as physiological molecules able to control multiple genes at the same time, miRNAs are also considered as very promising therapeutic targets or tools. As tumors typically evade cancer treatment by acquiring secondary mutations on targeted proteins, it appears more difficult for a tumor to escape from miRNA effects, which are directed on multiple proteins at the same time. Controlling the delivery and activity of miRNA-modulating agents into specific tissues or organs still appears as the "the big challenge," albeit a number of promising approaches are under validation. This contention is extensively explored in this issue in different contexts. The review by O. Fortunato et al. focuses on lung cancer, one of the big cancer killers, and discusses the most likely miRNA targets as well as the methodological approaches for *in vivo* delivery of miRNAs. The use of miRNA modulators for a direct antitumor effect, when administered alone or in combination with chemo- or radiotherapy, has been described in the prostate cancer context by A. Cannistraci and colleagues. Furthermore, the use of miRNAs for indirect anticancer effects has been reviewed by S. Gallach et al., who discussed on the possibility to modulate miR-126 or miR-92a to target tumor angiogenesis, thus limiting tumor growth and metastatic spread, or act on hypoxia. Finally the role of miRNAs in determining and regulating chemoresistance of pancreatic cancer has been discussed by I. Garajová and colleagues.

Overall the issue is intended to provide evidence of the potential usefulness of miRNAs in cancer management as well as focus on the still unsolved technical and conceptual challenges in miRNA research.

Paolo Gandellini
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Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 374924, 13 pages
http://dx.doi.org/10.1155/2015/374924

Review Article

Utility of MicroRNAs and siRNAs in Cervical Carcinogenesis

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Received 12 June 2014; Revised 13 October 2014; Accepted 14 October 2014

Academic Editor: Paolo Gandellini

MicroRNAs and siRNAs belong to a family of small noncoding RNAs which bind through partial sequence complementarity to 3′-UTR regions of mRNA from target genes, resulting in the regulation of gene expression. MicroRNAs have become an attractive target for genetic and pharmacological modulation due to the critical function of their target proteins in several signaling pathways, and their expression profiles have been found to be altered in various cancers. A promising technology platform for selective silencing of cell and/or viral gene expression using siRNAs is currently in development. Cervical cancer is the most common cancer in women in the developing world and sexually transmitted infection with HPV is the cause of this malignancy. Therefore, a cascade of abnormal events is induced during cervical carcinogenesis, including the induction of genomic instability, reprogramming of cellular metabolic pathways, deregulation of cell proliferation, inhibition of apoptotic mechanisms, disruption of cell cycle control mechanisms, and alteration of gene expression. Thus, in the present review article, we highlight new research on microRNA expression profiles which may be utilized as biomarkers for cervical cancer. Furthermore, we discuss selective silencing of HPV E6 and E7 with siRNAs which represents a potential gene therapy strategy against cervical cancer.

1. Introduction

Rapid advances in the study of microRNA expression profiles and siRNAs for silencing gene expression have led to many ongoing efforts to exploit these molecules as biomarkers and therapeutic agents, respectively, in the treatment of several cancers. A key feature of microRNAs and siRNAs is that they are not translated into proteins but rather function in the regulation of gene expression. The study of microRNAs which negatively regulate gene expression by either translational repression or target mRNA degradation, as well as siRNAs which are involved in the biological process of RNA interference, will have greater impact once these molecules are characterized in context of their function and their impact in human health and disease. Considerable progress has occurred into the area of microRNAs and siRNAs in recent years. The new knowledge has contributed to an improved understanding of the mechanism of microRNAs biogenesis and an emerging consensus about the function of microRNAs and their targets in several species including humans. One of the most successful approaches utilizes microRNAs as biomarkers in several diseases. The siRNAs, on the other

hand, represent a fast, cost-effective, and relatively simple tool for inducing downregulation of virtually any gene sequence in many species. Therefore, siRNA-based drugs may be the next generation of biochemical compounds because they are highly gene-specific due to nucleotide complementarity and have less challenging pharmacodynamics because siRNAs are biologic molecules.

Cervical cancer is the second most common type of cancer in women in worldwide, with about 500,000 new cases diagnosed and 270,000 women dying each year from this neoplasia [1]. The main causative agent is persistent infection with high-risk human papillomavirus (HPV) and the process of tumorigenesis is associated with well-defined clinical stages, each with unique features [1]. HPV E6 and E7 oncoproteins deregulate cell proliferation and inhibit the apoptotic mechanism mainly by targeting p53 and pRb tumor suppressor proteins, respectively. Additional genetic and epigenetic alterations disrupting cell cycle control are required to immortalize and transform the epithelial host cells [2]. Analysis of global mRNA expression, known as the transcriptome, has demonstrated that aberrant expression of cellular microRNAs has an important role in cervical carcinogenesis [3]. Prior studies have reported deregulation of microRNA expression profiles in various cervical cancer cell lines and tissues compared to normal tissue [3]. In addition, several groups are developing an attractive technology platform to selectively silence gene expression and target HPV E6 and E7 oncogenes, which have significant biological roles in the survival of cervical tumor cells *in vitro* as well as *in vivo*, which represents a potential gene therapy against cervical cancer.

2. MicroRNA Biogenesis

In microRNA biogenesis, events begin within the cell nucleus. Primary microRNAs (pri-microRNAs) are processed to generate an intermediate RNA called precursor microRNA (pre-microRNA). Pre-microRNAs are 60 to 110 nucleotides long and form secondary stem-loop-type structures. The processing of pri-microRNAs to generate pre-microRNAs is mediated by an RNase endonuclease type III called Drosha, which hydrolyzes the RNA strands at sites near the base of the stem-loop secondary structure [7–11]. The pre-microRNAs are then exported to the cytoplasm by the Ran guanine nucleotide exchanger (Ran-GTP) and the Exportin-5 receptor [12]. In the next step, the pre-microRNAs are processed by a second cytoplasmic RNase type III called Dicer to produce mature dsRNAs which are 19–25 nucleotides long. These dsRNAs are separated into single strands to generate mature microRNAs [13, 14]. In the next step of microRNA biogenesis, a single microRNA is incorporated into a ribonucleoprotein effector complex known as the RNAi-induced silencing complex (RISC) [15, 16]. The RISC with the mature microRNA guide incorporated identifies the target mRNA via nucleotide base complementarity and either produces endonucleolytic cleavage of the mRNA or induces translation arrest. Several studies have identified the Ago2, Dicer, and protein cofactors as components of RISC and have also determined the assembly mechanism [17–21]. The identification of R2D2 protein in *Drosophila*, RDE4 protein in *C. elegans*, and TRBP protein in humans [21–23] provided additional information about the molecular composition of RISC, as well as about the initiation and effector phases of the RNA interference mechanism. This evidence indicates that RISC activity is important during the initiation phase to generate microRNAs and suggests that RISC may be involved in later events of the RNA interference mechanism, that is, in the effector phase of posttranscriptional regulation of gene silencing. In the next stage of biogenesis, pre-microRNA processing and RISC assembly are functionally connected to catalyze multiple cycles of hydrolysis of specific target mRNA [15]. MicroRNAs 19–25 nucleotides in length are processed to produce mature microRNAs, which associate with RISC in order to recognize complementary mRNA sequences. MicroRNAs mediate their effects at the mRNA level by inhibiting translation or inducing cleavage of target mRNA, recognized by nucleotide complementarity between the microRNAs and mRNA. Perfect complementarity induces cleavage of mRNA, whereas partial complementarity with several mismatches leads to translation arrest [24]. Taken together, the evidence supports the idea that the RNA interference mechanism is a natural process of sequence-specific posttranscriptional gene silencing mediated by microRNAs in eukaryotic cells.

3. Disruption of MicroRNA Expression Profiles in Cervical Cancer

By comparing microRNA expression profiles between normal tissue and tumor tissue, studies have identified deregulated microRNAs and mRNAs, demonstrating an aberrant microRNA expression pattern in various malignancies [25]. Malignant changes such as tumorigenesis or tumor progression are associated with changes in the expression of multiple microRNAs, rather than a single microRNA regulating an oncogene or tumor suppressor target gene [26]. However, it is unclear whether microRNA expression is altered at the onset of cell transformation or as a consequence. Since microRNAs regulate the expression of their mRNA targets, it is expected that the over- or underexpression of microRNAs would have an effect on cellular phenotype [27]. For cancer research purposes, microRNAs can be divided in those with increased expression which target tumor suppressor protein and are known as oncomirs and those with decreased expression which generally target oncogenes and are referred to as tumor suppressor microRNAs. However, sometimes microRNAs overexpressed in cancer cells may act as tumor suppressors if they target oncogenes, and similarly microRNAs underexpressed in tumor cells sometime act as oncomirs if they target tumor suppressor genes. These classes of microRNAs have become an attractive target for gene therapy in recent years.

Several microRNAs with altered expression in cervical cancer have been identified and put forth as oncomirs or tumor suppressor genes. For instance, miR-10a, miR-106b, miR-21, miR-135b, miR-141, miR146, miR-148a, miR-214, and miR-886-5p have been proposed to act as oncomirs in cervical cancer, contributing to the development of cancer through

dysregulation of gene products involved in cell proliferation, apoptosis, or cell-cell adhesion [3, 5, 28–33]. The miR-10a is an oncomir found to be overexpressed in colon and pancreatic cancer [34] which functions in tumor invasion and metastasis. A study by Long et al. [28] found miR-10a to be overexpressed in 92.8% of cervical cancer tissues, with similar results reported by Volinia et al. [34]. In the Long et al. study, researchers found an inverse correlation in the expression of miR-10a and of Close Homolog of L1 (CHL1) transmembrane protein type 1, a protein involved in cell adhesion, in HeLa (HPV18+) and C33 A (HPV−) cells transfected with pri-miR-10a. In these cells a decrease in CHL1 mRNA levels was observed, suggesting that miR-10a targets CHL1. Long et al. propose that a decrease in CHL1 caused by miR-10a interference results in deregulation of the MAPK and PAK pathways which affect downstream molecules, contributing to cell growth, migration, and invasion of cells into other tissues. These findings suggest that, in cervical cancer, miR-10a may play an important role in tumor metastasis by regulating the cellular gene CHL1 [28].

The expression of miR-21 has been found to be altered in almost all types of cancer and this microRNA has been classified as an oncomir. miR-21 has been reported to be overexpressed in glioblastoma, uterine leiomyosarcoma, diffuse large B-cell lymphoma, breast, lung, stomach, prostate, colon, esophagus, head and neck cancer, and cervical cancer [35]. miR-21 is located on chromosome 17 at the 17q23.2 locus and the gene coding for pri-miR-21 is located within the intronic region of the protein-coding gene TMEM49, a human homolog of rat vacuole membrane protein [35]. Inhibition of miR-21 can induce cell cycle arrest and increase chemosensitivity to anticancer agents, offering evidence that miR-21 may function as an oncogene in human cancer [36–38]. miR-21 has been shown to negatively regulate the expression of cellular genes p53 and Cdc25, which are involved in regulation of cell proliferation, RECK and TPM1, which suppress metastasis, and PDCD4 and PTEN which induce apoptosis of malignant cells. Furthermore, the repression of PDCD4 by miR-21 has been proposed to generate feedback signaling or autoregulation by activating mitogenic signals throughout the RAS pathway, which leads to the induction of the AP-1 transcription factor. AP-1 may bind to specific recognition sites into the miR-21 promoter region to activate its transcription [35]. This evidence implicates miR-21 in multiple malignancy-related processes in cervical cancer including cell proliferation, apoptosis, invasion, and metastasis.

Another microRNA overexpressed in cervical cancer is miR-886-5p. Li et al. showed that miR-886-5p negatively regulates the expression of Bax. [33]. Bcl-2 and related proteins such as Bcl-XL increase cell survival, and their functions are countered by related proapoptotic proteins such as Bax and Bak. Specifically, the Bax gene codes for a proapoptotic protein that is inserted into the outer mitochondrial membrane in response to a cell death signal, causing release of cytochrome C and subsequent activation of the initiator caspase-9, resulting in apoptosis. Bax expression levels are reduced in cervical cancer cells. In HPV16+ H8 cells, low levels of Bax have been associated with decreased apoptosis and increased cell proliferation, and silencing miR-886-5p in HPV16+ SiHa cells increases levels of Bax and apoptosis. These data suggest that miR-886-5p regulates Bax expression via translational inhibition and this regulatory pathway may play an important role in cancer cervical development.

Some microRNAs act as tumor suppressor microRNAs which regulate oncogenes and are underexpressed in cervical cancer; examples of these include let-7c, miR-124, miR-126, miR-143, and miR-145 [3]. One of the first microRNAs studied was let-7. The 3′-UTR region of the human RAS gene contains multiple let-7 complementary sites (LCSs), enabling let-7 to regulate Ras expression [39]. RAS proteins are membrane proteins that regulate normal cell growth and differentiation throughout NF-kB transcription factor, PKB/Akt and MAPK kinases. In lung cancer, let-7 is underexpressed relative to normal tissue, while levels of RAS are significantly higher than in normal tissue, which is consistent with a potential let-7-mediated mechanism in cancer development. With regard to cervical cancer, let-7 is expressed at a frequency of 15% to 18% in individual normal cervical samples while its expression was not detected in three cancer cell lines (SW756, C4I, and SiHa cells) and detected at 0.2% in CasKi cells (HPV16+), 0.4% in ME-180 cells, and 7.2% in C33 A cells [40]. In HeLa cells expressing endogenous let-7 that were transfected with antisense oligonucleotides designed to inhibit let-7 activity, a ~70% increase in RAS protein levels was observed, strongly suggesting that let-7 negatively regulates the expression of RAS in human cervical cells [39].

Another important tumor suppressor microRNA in cervical cancer is miR-143. The genes targeted by miR-143 include k-Ras, Macc1, and Bcl-2, which are implicated in the ERK5 and MAPK signaling pathways [41, 42]. One of the target genes, Bcl-2, is an oncogene which acts to suppress cellular apoptosis; therefore, overexpression of Bcl-2 inhibits apoptosis in damaged cells, leading to uncontrolled cellular proliferation that drives the development of cancer. In addition, overexpression of the BCL-2 protein may contribute to metastasis in certain cancers. Liu et al. demonstrated that overexpression of miR-143 in HeLa cells resulted in suppression of Bcl-2, while knockdown of miR-143 increased Bcl-2 expression. Overexpression of miR-143 induced with anti-miR-Bcl-2 partially reversed the inhibition of cell proliferation and promoted apoptosis in the HeLa cells expressing miR-143 [43]. Another experimentally verified target of tumor suppressor microRNAs miR-143 and miR-145 is the cellular gene ERK5 (also known as MAPK7) a mitogen-activated protein kinase (MAPK) regulated by a wide range of mitogens and by cell stress, which promotes cell growth and proliferation in response to tyrosine kinase signaling [44]. In the bladder cancer cell line T24, prostate cancer cell lines LNCaP and C4-2, and the Burkitt lymphoma cell line Raji, ERK5 expression levels were found to be reduced and cell proliferation was inhibited in response to increased levels of miR-143 and miR-145 [40, 45–47]. Study of the role of ERK5 in cervical cancer and its regulation by miR-143 y miR-145 is certainly worthy of further investigation. Recently, Zhang et al. demonstrated that IFN-β is induced by miR-129-5p in HeLa cells (HPV18+) [48]. They identified that miR-129-5p overexpression inhibits the growth of HeLa cells and

that transfection of pre-miR-129-5p increased the arrest of HeLa cells and decreased the HPV18 E6 and E7 expression. The same group observed that miR-129-5p expression was induced by INF-β. In addition, they demonstrated that the SP1 transcription factor can be downregulated by overexpression of miR-129-5p throughout a binding site for miR-129-5p at SP1 3′-UTR. These data support the notion that induced expression of miR-129-5p by IFN-β suppresses the progression of cervical cancer cells by downregulating HPV18 E5 and E7 expression, and SP1 transcription factor is direct downstream, target of miR-129-5p.

4. Cell Checkpoints and Regulation of MicroRNAs in Cervical Cancer

A significant event in HPV-associated carcinogenesis is the induction of genetic instability and global disruption of cell gene expression principally by the HPV E6 and E7 oncoproteins, whose cell protein targets have been identified via molecular analysis. One of the main, well-defined targets of HPV E6 is the tumor suppressor protein p53 which acts as a checkpoint to maintain cell homeostasis. The p53 transcription factor is the tumor suppressor gene most frequently inactivated in human cancers and is involved in the control of cell proliferation and the response to genotoxic stress and DNA damage [49]. The inactivation of p53 by E6 affects multiple cell processes including apoptosis, cell cycle arrest, cellular differentiation, and senescence [50]. Thus, loss of p53 results in an increase in the genomic instability of the cell. On the other hand, the HPV E7 oncoprotein may interact with the pRb tumor suppressor family of proteins, another important checkpoint, to liberate transcription factors in the E2F family, thus stimulating the expression of multiple genes involved in cell cycle progression [50, 51]. In addition to their oncogenic and antiapoptotic effects, the E6 and E7 oncoproteins may modulate viral transcription and other cellular genes. Thus, HPV E6 and E7 have effects on several levels of cellular functions, such as cell cycle control and regulation of gene expression, and in combination they efficiently immortalize and transform human keratinocytes to promote carcinogenesis. Due to these properties, HPV E6 and E7 oncogenes are an important focus of research to improve understanding of the molecular mechanism of viral oncogenesis in humans and are considered good targets for gene therapy for cervical cancer.

Several mechanisms likely contribute to the global deregulation of microRNAs which has been reported in cervical cancer cells. For instance, there is growing evidence that microRNAs are critical components of several canonical and noncanonical signaling pathways that frequently undergo gain or loss of function in tumor cells, including those regulated by key molecules to maintain cell homeostasis. One such pathway involves the c-Myc gene, which encodes a helix-loop-helix transcription factor with several functions including inducing cell cycle progression and which represents a checkpoint in cell homeostasis. As such, c-Myc is one of the most frequently activated oncogenes in human cancers. In cervical cancer, independent of HPV E6 p53-degradative function, E6 interacts with c-Myc to enhance c-Myc binding to the hTERT promoter and induce hTERT expression [52]. Veldman et al. demonstrated that E6/c-Myc/hTERT regulation is mediated by the proximal Myc/Max-binding element (E-box) in the hTERT promoter, which is the main determinant of E6 and c-Myc responsiveness. Furthermore, c-Myc represses transcription when it binds to promoters by transcriptional activators such as Miz-1 (Myc-interacting zinc-finger protein 1), a transcription factor involved in transcriptional activation of p15 and p21 genes. In cervical cancer cells E7 has been shown to form a complex with Miz-1 which has effects on p21 regulation as well as on cell cycle progression in response to UV-induced DNA damage [53]. In addition to c-Myc directly controlling the expression of many protein-coding genes, there is an increased appreciation that its ability to reprogram microRNAs expression also contributes to its oncogenic process. O'Donnell et al. reported that the c-Myc oncogene directly regulates microRNA expression [54]. Moreover, Ota et al. demonstrated that miR-17-92 cluster is transactivated by c-Myc [55]. They performed ChIP assay which demonstrated that c-Myc interacts directly with a conserved binding site in the first intron of miR-17-92 primary transcript to activate its transcription. Other studies of microRNA regulation by c-Myc have revealed a wide role for this transcription factor in reprogramming microRNA expression. For example, Chang et al. demonstrated that c-Myc activation leads not only to induction of the miR-17-92 cluster but also to widespread repression of microRNAs including let-7 family, miR-29 family, miR-15a/16-1, and miR-34a which are known to have antitumorigenic activity [56]. Through ChIP assays they demonstrated that c-Myc associates with the core promoter of the microRNAs it represses, suggesting that this microRNA downregulation is a consequence of reduced transcription of pri-microRNAs. This evidence indicates that c-Myc overexpression inhibits the expression of several microRNAs such as let-7a-1/f-1/d, miR-15a/16-1, miR-22, miR-26a-2, miR-26b, miR29a/b-1, miR-29b-2/c, miR-50e/30c-1, miR-34a, and miR-146a, which may be the result of c-Myc binding to microRNA promoters.

Numerous downstream targets of the microRNA-17-92 cluster have been identified, providing evidence about the oncogenic mechanisms mediated by this microRNA. Downregulation of the proapoptotic genes (CL2L11/BIM) by multiple members of the microRNA-17-92 cluster likely explains these microRNAs' ability to block apoptosis and promote carcinogenesis processes [57]. MiR-20a is a member of the miR-17-92 cluster, and its function in cervical cancer cells is not clear. Kang et al. confirmed that miR-20a is upregulated in cervical cancer tissue [58]. Overexpression of miR-20a in the cervical cancer cell lines HeLa and C33A increased cell proliferation, migration, and invasion, whereas inhibition of miR-20a suppressed those functions. This same group found that the oncogene TNKS2 is directly upregulated by miR-20a, with effects on cervical cancer cell colony formation, migration, and invasion.

The main reported function of HPV E6 is to target p53 for degradation. Thus, it is certainly conceivable that HPV E6 is

able to regulate the expression of many cellular microRNAs through p53, which acts as a checkpoint to maintain cell homeostasis. The function of p53 is critical and more complex than originally thought, because it represses and/or activates coding and noncoding genes during their biogenesis and at the level of transcription. p53 binds to several microRNA promoters to induce repression. p53-mediated repression of miR-17-5p, miR-18a, miR-18b, miR-19a, miR-19b-1, miR-19-2, miR-20a, miR-20b, miR-25, miR-92-1, miR-92-2 miR-93, miR-106a, and miR-106b has been reported [59]. HPV E6-expressing cells show a p53 null phenotype; consequently, all microRNAs regulated by p53 are likely affected by E6. Wang et al. had reported that miR-34a is regulated by E6-dependent expression in cervical cancer [60]. They observed that cervical cancer tissues and cervical cancer-derived cell lines infected with high-risk HPVs display reduced expression of tumor suppressive miR-34a. The decrease in miR-34a expression correlates with the early productive phase and is attributed to the expression of HPV E6, which destabilizes p53, a known miR-34a transactivator. This same group demonstrated that knockdown of E6 in cervical cancer cell line (HPV16+ and HPV18+) by siRNAs leads to an increased expression of p53 and miR-34a and accumulation of miR-34a in G0/G1 phase cells. Furthermore, they demonstrated that miR-34a gene is a direct transcriptional target of p53 and its expression can be transactivated by the binding of p53 to a consensus p53 recognition site in the miR-34a promoter region. Finally, they found that p53 degradation by E6 leads to the decreased miR-34a in raft cultures, CIN, and cervical cancer tissues [60]. Given that miR-34a has effects on the expression of many cell cycle regulators, including cyclin E2, cyclin D1, CDK4, CDK6, E2F1, E2F3, E2F5, Bcl-2, SIRT1, and p18, the repression of p53 and miR-34a disrupts the multistep control of cell cycle progression, senescence, and apoptosis, resulting in disruption of cell differentiation and proliferation and leading to cell transformation [61].

The tumor suppressor microRNAs miR-15a and miR-16-1 are expressed as a microRNA cluster from an intron region of the DLEU2 (Deleted in Lymphocytic Leukemia 2) transcript and influence cell proliferation, survival, and invasion. Wang et al. demonstrated higher levels of miR-15a and miR16-1 expression in cervical cancer tissues compared to normal cervical tissues; however, the overexpression of this microRNA cluster does not appear to affect growth of cervical cancer cells [3]. To analyze whether miR-15a/miR-16-1 cluster overexpression is associated with high-risk HPV infection, Zheng and Wang compared miR16-1 expression levels in raft tissues derived from human foreskin keratinocytes with and without HPV18 infection and observed a twofold increase in miR-16-1 expression in the HPV18+ raft tissues [62]. When cells were infected with a retrovirus expressing HPV18 E6 and E7, expression of miR-16-1 was increased only in the raft tissues expressing HPV E7, but not in those expressing HPV E6, suggesting that while HPV oncoproteins regulate expression of the miR-15/16 cluster, E7 is specifically responsible for the increased expression of miR-16-1. This evidence indicates that these cell checkpoints can be studied to improve our understanding of the gene expression network in the context of biological systems. Figure 1 summarizes the main checkpoints and the pathways in which microRNAs are involved in cervical cancer cells.

5. Regulatory Genetic Networks Modulated by MicroRNAs in Cervical Cancer

Recently, Wang et al. described regulatory genetic networks in cervical cancer, which were constructed from databases of differentially expressed microRNA and related genes [59]. Three regulatory genetic networks were generated; the first was the differentially expressed network which includes differentially expressed genes, differentially expressed microRNAs, and host genes of differentially expressed microRNAs. The second network was constructed from related genes, related microRNAs, and host genes of cervical cancer-related microRNAs. The third network was the global network, which consists of all the elements extracted from the basic source data. In the first network, seven genes and ten microRNAs were experimentally validated as differentially expressed in cervical cancer. The subnetwork centered around PTEN was the principal component. TWIST1, miR-214, PTEN, and miR-21 function together as an ordered chain of control. The miR-21 targets its regulator PTEN, which formed a self-adapting feedback loop, creating a balance mechanism with STAT3 and miR-21 in the system as components. Thus, two host genes, two transcription factors, and one microRNA influence the expression of PTEN and miR-21. The circling between miR-21 and PTEN makes them dominant and dominating factor simultaneously and turns the pathway into a bidirectional net crux. In another partial network centered on miR-143, TP53 and TGFβ1 jointly have been shown to regulate miR-143. The second network expands on the differentially expressed network by including more genes and microRNAs whose association with cervical cancer is not as close as the differentially expressed ones. The related network is of a much larger scale and of higher complexity than the differentially expressed network. Certain factors are predominant, including microRNAs such as miR-21, miR-23b, miR-34, miR-143, and let-7c and transcription factors of PTEN, TP53, TP63, c-Myc, and k-Ras. Two more self-adapting feedback mechanisms were identified involving let-7c, miR-34, and c-Myc. c-Myc acts as transcription factor for let-7b and let-7c, which target k-Ras. This is accorded to inhibition of the transactivation function of c-Myc. In addition, c-Myc's overexpression could alter the suppressive function of let-7c. Meanwhile, another tumor suppressor, miR-34, has a local balance adjustment system with c-Myc as well. In the third network, interactions involving the previously identified transcription factors and their base sequences of 1000-nt were evaluated; these transcription factors were integrated with host genes and differentially expressed microRNAs to include 1000-nt transcription factors in the network system of cervical cancer. Self-adapting feedback was assessed and was found to exist between NF-kB and miR-21. NF-kB regulates miR-21, miR-214, and let-7b. MiR-21 and miR-214 are core

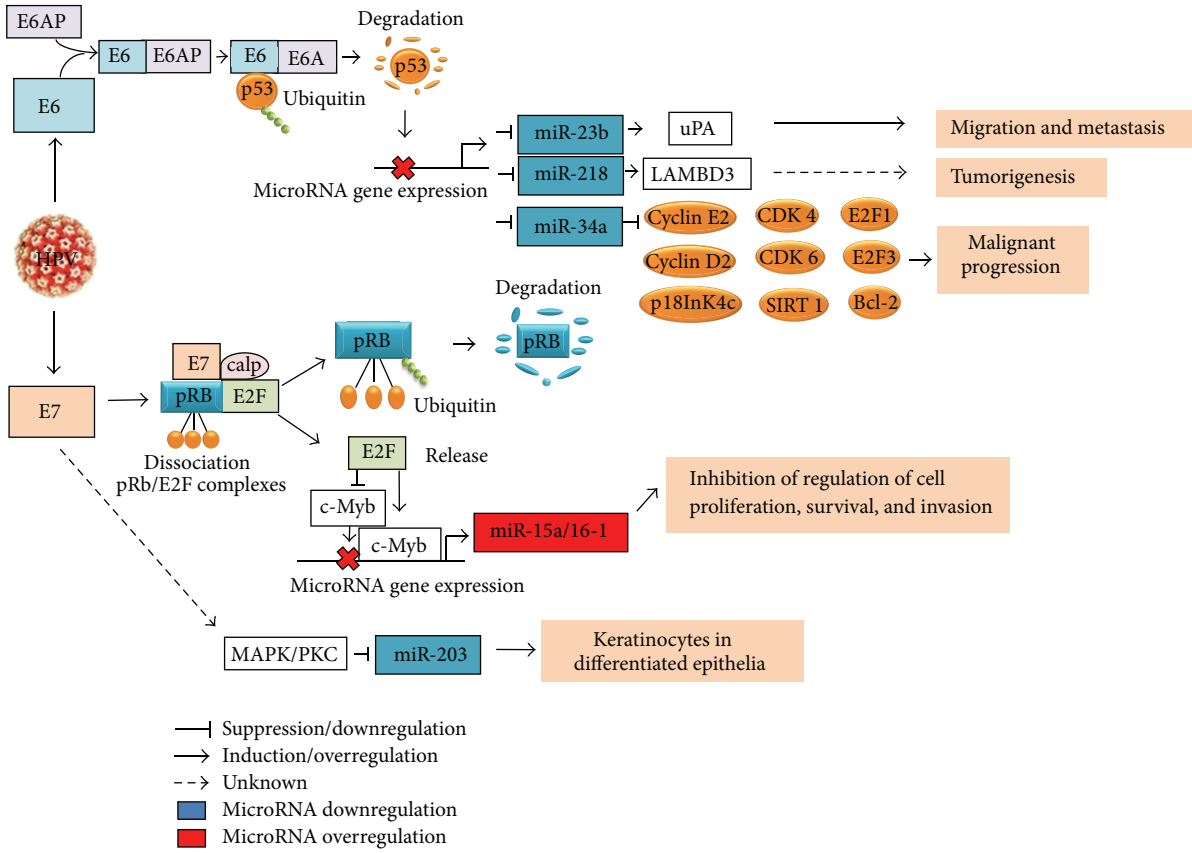

Figure 1: Schematic model of the interaction between microRNAs and factors involved in malignant transformation caused by HPV E6 and E7 expression in cervical cancer cell. Cervical cancer is the second most common cause of cancer mortality in women worldwide and persistent infection with HPV is the main etiologic agent. HPV E6 and E7 deregulate cellular proliferation and inhibit the apoptotic mechanism by targeting p53 and pRb, respectively. In addition, E6 disrupts the expression of miR-23b, miR-218, and miR-34a via p53 degradation and their expression is transactivated by the binding of p53 to consensus sites in the promoter regions, affecting the expression of cell cycle regulators, such as E2, cyclin D1, CDK4, CDK6, E2F1, E2F3, E2F5, Bcl-2, SIRT1, p18, uPA, and LAMBD3. In the overexpression of miR-15/16 cluster by E7, E2F1 transactivates the c-Myb expression and represses the c-Myc expression, and then the microRNA cluster regulation is controlled by binding of c-Myc or c-Myb to promoter region of microRNA cluster. The increased expression of miR-15a/miR-16-1 induces the inhibition of cell proliferation, survival, and invasion. The downregulation of miR-203 by E7 is mediated by MAPK/PKC pathway.

biological factors in the cervical cancer network and their appearance here supports the importance of these particular microRNAs. With the help of miR-21 and mir-214, NF-kB participates in the subsystem centered around PTEN and miR-21. This method of analysis could be useful to identify more core factors, other parallel networks and relevant motifs in cervical cancer carcinogenesis, and/or other tumorigenesis processes.

6. The MicroRNA Profile Expression in the Natural History of Cervical Cancer

Cervical cancer is histologically classified into squamous cell carcinomas (SCCs), which comprise about 80% of cervical cancers, adenocarcinomas (AdCAs), which comprise about 15%, and adenosquamous carcinomas, which comprise about 3% to 5%. According to the Bethesda classification, preneoplastic cell abnormalities that precede cervical cancer are divided into low-grade intraepithelial lesions (cervical intraepithelial neoplasia (CIN-1)) and high-grade lesions (CIN2-3). A number of studies have demonstrated deregulated patterns of microRNA expression in cell lines derived from cervical cancer tissue as well as in tissue samples; however, only a few have described the alteration of microRNA expression that occurs during the progression from normal cervical epithelium to high-grade CIN lesions to SCC or AdCAs. Table 1 summarizes the existing evidence in this area.

Wilting et al. determined that the expression profiles of microRNA in CIN2-3 are similar to normal epithelium infected with high-risk HPV, consistent with the concept that cervical lesions take approximately a decade to progress to invasive cervical carcinoma [6]. The authors identified 106 microRNAs that are differentially expressed ($P < 0.01$) in CIN2-3 and/or SCCs compared to normal epithelium. One set of 27 of these microRNAs showed differential

Table 1: Differentially expressed profile microRNAs in normal squamous epithelium, CIN1-3, and CC.

MicroRNAs expression	Normal tissue	CIN1–CIN3	CC	Reference
miR-26a, miR-143, miR-145, miR-99a, miR-203, miR-513, miR-29a, miR-199a	Upregulated	Downregulated	Downregulated	Pereira et al. [4]
miR-106a, miR-205, miR-197, miR-16, miR-27a, miR-142-5p	Upregulated	Downregulated	Downregulated	
miR-522, miR-512-3p	Upregulated	Upregulated	Downregulated	
miR-148a, miR-302b, miR-10a, miR-196a, miR-132	Downregulated	Upregulated	Upregulated	
miR-155, miR-92a, miR-92b, miR-224, miR-221, miR-222, miR-31, miR-182, miR-106a, miR-17, miR-20a, miR-20b, miR-15b, miR-16, miR-25, miR-185, miR-93	Downregulated	Downregulated	Upregulated	Li et al. [5]
let-7b, miR-145, miR-126, miR-199a-3p, miR-195, miR-29a, miR-375, miR-10b, miR29c, miR-218, miR-424, miR-100, miR-125b, miR99a	Upregulated	Upregulated	Downregulated	
miR-192, miR-135b, miR-101, miR-191, miR-34c-5p, miR-150, miR-125a-5p, miR-30a, miR-143, miR-146b-5p, miR-181b, miR-7g, miR-26a, miR-29a, miR-29c, miR-29b, miR-10a, miR-29a, miR-145	Downregulated	Upregulated "early transient"	Upregulated "early transient"	Wilting et al. [6]
miR-205, miR-27a, miR-27b, miR-221, miR-193a-3p, miR-212, miR-770-5p, miR-484, miR-636	Upregulated	Downregulated "early transient"	Downregulated "early transient"	
miR-28-5p, miR-338-5p, miR-206, miR-200a, miR-92b, let-7i, miR-181d, miR-92a, miR-30e, miR-34b, miR-592, miR-19b, miR-106b, miR-595, miR-34a, miR-25, miR-146a, miR-21	Downregulated	Upregulated "early continuous"	Upregulated "early continuous"	
miR-203, miR-638, miR-370, miR-575, miR-193b, miR-149, miR-210, miR-622, miR-23b, miR-493, miR-296-5p, miR-671, miR-134, miR-365	Downregulated	Downregulated	Upregulated "early continuous"	
miR-30c, miR-425, miR-24, miR-331-3p, miR-151-3p, miR-107, miR-652, miR-17, miR-9, miR-185, miR-339-5p, miR-18a, let-7-d, miR-17, miR-30d, miR-130b, miR-15a, miR-106a, miR-19a, miR-200c, miR-20b, miR-363, miR-155, miR-141, miR-93, miR-15b, miR-16	Downregulated	Upregulated "late"	Upregulated "late"	
miR-125b, miR-375, miR-99a, miR-188-5p, miR-148a, miR-671-5p, miR-199b-3p, miR-513b, miR-378, miR-195, miR-486-5p, miR-26b, miR-376a, miR-199a-5p, miR-497, miR-100, miR-660, miR-218	Upregulated	Upregulated	Downregulated "late"	

expression (false discovery rate FDR < 0.05) only in CIN2-3 compared to normal tissue and did not have differential expression in SCC compared to normal tissues (FDR > 0.1). These microRNAs were therefore designated microRNAs with transient early expression. A different set of 33 microRNAs showed concordant differential expression (one FDR < 0.05 and the other FDR < 0.1) in both CIN2-3 and SCC and were thus designated microRNAs with continuous early expression. A third set of 46 microRNAs showed differential expression (FDR < 0.05) restricted to SCC and were thus classified as microRNAs with late expression. These 46 microRNAs are potentially important in the progression of high-grade CIN lesions to invasive carcinomas. Finally, one set of 18 microRNAs showed significantly different expression between AdCA and SCC and could be used to accurately diagnose the different histological types of cervical cancer.

To characterize changes in microRNA expression associated with CIN progression, Pereira et al. analyzed the microRNA expression profile in CIN-1, CIN-3, and cervical cancer and identified 21 microRNAs with statistically significant differential expression between CIN-1 and/or CIN-3 and the pool of normal samples ($P < 0.05$) [4]. One set of eight microRNAs exhibited relative decreased expression in the transition from normal cervix to CIN1-3 and from CIN1-3 to cancer. Another set of six microRNAs displayed relative decreased expression in the transition

from normal cervix to CIN1/3 but increased expression from CIN1-3 to cervical cancer. Two microRNAs exhibited relative increased expression in the transition from normal cervix to CIN1-3 and decreased expression from CIN1-3 to cervical carcinoma. Interestingly, one set of five microRNAs displayed relative increased expression in the transition from normal cervix to CIN1/3 and from CIN1/3 to cancer. In another study, Li et al. compared HPV16+ SCC, HPV16+ CIN2-3, and normal cervical tissue and identified a set of 31 unique microRNAs with significant and continuous expression trends in the progression from normal tissue to cancer, with 14 microRNAs decreasing in expression and 17 microRNAs increasing in expression [5]. Among these, miR-218 was the most significantly downregulated, with $\log_2$ values of −1.15-fold and −4.83-fold changes in CIN2-3 and SCC, respectively. These data were validated with increased sample number for each HPV16+ histological type, which confirmed an identical microRNA expression profile to the primary screening. Lui et al. analyzed microRNA expression levels in human cell lines transformed with the HPV16/18 as well as in normal cervical cells and determined that miR-21 is overexpressed in HPV16/18+ cervical cancer cell lines derived at a rate of 45% compared to 13% expression in normal cells, miR-143 is not expressed in HPV+ cells, and let-7 and miR-196 are underexpressed in HPV-transformed cells compared to normal cervical cells [40]. In summary, this evidence supports the theory that microRNAs have specific expression profiles at different stages in the natural history of cervical cancer associated with high-risk HPV infection. The variation in the microRNA expression pattern is associated primarily with HPV E6 and E7 oncoprotein expression. By comparing the microRNA expression profile in CIN1-2-3 and cancer to normal tissue, it is possible to establish a specific genomic and/or transcriptomic signature of cervical tissue infected with HPV. Thus, microRNA expression profiles may be employed as biomarkers in the staging and prognosis of cervical cancer associated with high-risk HPV infection.

7. siRNAs for HPV Oncogenes as Potential Gene Therapy for Cervical Cancer

The silencing of genes by siRNAs is a potential mechanism to inactivate foreign DNA sequences and may be employed to silence the expression of HPV oncogenes in cervical cancer. The first studies carried out with synthetic siRNAs to silence HPV16 E6 and E7 oncogene expression were described by Jiang and Milner in 2002 [63]. In this study, the administration of siRNAs led to mRNA cleavage and the specific silencing of HPV16 E6 and E7 oncogene expression. Furthermore, E6 silencing induced expression of the p53 gene and transactivation of the p21 gene and decreased cell proliferation, while silencing of E7 induced cell death by apoptosis. These findings demonstrated for the first time that the expression of HPV E6 and E7 oncogenes may be specifically silenced by siRNAs in human tumor cervical cells HPV+.

Recent investigation has focused on silencing the HPV E6-E7 bicistron with siRNAs. These oncogenes are transcribed jointly, as a bicistron, which is the result of alternative splicing. The effect of silencing of both E6 and E7 with synthetic siRNAs against the HPV16 E6 oncogene in SiHa cells (HPV16+) has been described [64]. The data demonstrate inhibition of cell proliferation, p53 and pRb protein expression, and p21 induction. The effect of siRNAs against the HPV16 E6 oncogene on the E6-E7 bicistron has been studied *in vitro* as well as *in vivo*. Administration of siRNAs for E7 induces silencing of both oncogenes, while siRNAs for E6 inhibit E6 expression but do not affect E7 expression [65, 66]. These same studies analyzed the functionality of siRNAs for E6 and E7 and demonstrated induction of expression of p53, p16, p21, p27, and pRb, silencing of cyclin A gene, and induction of apoptosis in cervical cancer cells. Another group has reported the use of synthetic siRNAs to silence the HPV18 E6 oncogene [67]. This study demonstrated induction of apoptosis of CasKi cells (HPV16+), increased p53 and p21 expression, and expression of pRb. However, the siRNAs for HPV18 E6 did not affect HPV18 E7 expression. Sima et al. generated siRNA for HPV16 E7 in the pSIRE-DNR plasmid for the generation of small transcripts [68]. Their group demonstrated silencing of the HPV16 E6-E7 bicistron in SiHa and CaSki cells (HPV16+), with a resultant increase in expression of p53, p21, and pRb and induction of cervical tumor cell death by apoptosis. This evidence supports the idea that the silencing of HPV E6-E7 bicistron expression is dependent on the design of the siRNA sequences and suggests that the alternative splicing of HPV E6 and E7 oncogenes precedes the silencing by siRNAs.

8. Chemotherapeutic Drugs and siRNAs for HPV E6 and E7

Although the effect of chemotherapeutic drugs on p53 expression in cervical cancer cells is known, new research has focused on the association between the activation of p53 gene, the cytotoxic effect of drugs, and the silencing of HPV oncogenes with siRNAs. Different groups have analyzed the expression of p53 in HeLa cells (HPV18+) treated with siRNAs for HPV18 E6, combined with carboplatin, cisplatin, doxorubicin, etoposide, gemcitabine, mitomicine, mitoxantrone, oxaliplatin, paclitaxel, and/or topotecan treatment [69]. The researchers observed silencing of HPV18 E6 and E7 oncogenes, as well as an increase in p53 protein expression and changes in cytotoxicity dependent on the nature of each chemotherapeutical compound. A separate group found that the administration of siRNAs for HPV18 E6 generated in lentivirus, combined with cisplatin, in HeLa cells produced silencing of HPV18 E6 and E7 oncogenes, an increase in p53 expression, and death of cancer cells by cellular senescence [70]. This evidence suggests that the silencing of HPV E6 and E7 oncogenes with siRNAs can increase cellular sensitivity to the cytotoxic effects of drugs and that combined treatment may have a synergistic effect and reduce resistance to chemotherapeutical drugs, representing an advantage for treatment.

9. Transport Vehicles for siRNAs

When siRNAs are administered via lipofection to mammalian cells, one potential problem is cleavage of siRNAs by endogenous cellular endonucleases. An alternative technique to protect siRNAs from this cleavage is the synthesis of siRNAs with chemical modifications; however, this may induce undesirable collateral effects. Another problem that arises in the systemic administration of siRNAs is that there is no dose-dependent effect on target organs. In order to overcome these methodological complications, siRNAs for HPV oncogenes may be administered via a liposome-based system contained in biogels, which has been shown to cause specific silencing of E7 and induction of apoptosis of cancerous cells *in vitro* [71]. It has been demonstrated that when siRNAs are administered in CasKi cells (HPV16+), silencing of both oncogenes occurs and the cells die by apoptosis. The effects of siRNAs for E6 were evaluated in a murine tumor model, with resultant silencing of the viral oncogene as well as induction of tumor cell apoptosis and significant inhibition of the growth of the tumor mass *in vivo* [72]. In addition, atelocollagen has also been used as vehicle to administer siRNAs for HPV18 E6 and E7, with demonstration of the silencing of E6 and E7 oncogene expression, inhibition of cell proliferation, induction of the expression of pRb, and death of cancer cells by cellular senescence [73].

Although the silencing effects of siRNAs are evident, the half-life of these molecules after administration is relatively short, even when they are attached to transport molecules, which limits their application in preclinical or clinical trials. Furthermore, application of siRNA for HPV oncogenes in clinical studies requires development of highly specific siRNAs and more efficient systems for *in vivo* release. To this end, protocols have been developed to use lentiviruses as molecular vectors for siRNAs, as well as for the stable transfection and transduction of siRNAs in human cervical cancer cells [74]. The use of plasmids to clone siRNAs affords a longer half-life and greater stability to these molecules, and the most efficient molecular vehicles to release these plasmids are the lentiviruses. The lentiviruses have advantages such as the ability to infect dividing cells and resting cells with great efficiency, being nontoxic and only mildly immunogenic, and have been modified not to integrate themselves into the cellular genome. Their application has been studied in preclinical and clinical trials with encouraging results. In summary, siRNAs for HPV oncogenes that are generated in lentivirus have direct application in tumor sites and have been proven to be comparatively more useful than synthetic siRNAs.

10. Design of siRNA Sequences for HPV E6 and E7

A large volume of software has been developed and is available at diverse websites which allows for the design of more efficient and biologically functional siRNA sequences. For example, siDirect software has been used for the design of siRNA sequences that are highly efficient, with high specificity for the target gene sequence [75]. The siDirect software minimizes nonspecific complementarity of siRNA sequences to reduce binding to unrelated sequences. To do this, siDirect software uses a rigorous specificity measure called mismatch tolerance which involves identifying the minimal number of nonspecific sequences among the siRNA sequences and any sequence that does not correspond to the target sequence. Highly efficient siRNA sequences are selected from the algorithm based on the rules developed by Ui-Tei et al. [75, 76]. The siDirect software has been utilized to generate a set of several siRNAs for HPV16 E6 and E7 oncogenes [76, 77]. To evaluate the functionality of these siRNAs, the DNA inserts were cloned in the silencing plasmid psiCheck2. Highly specific silencing of E6 and E7 was demonstrated, along with suppression of the proliferation of HPV 16+ tumor cervical cells, an increase in the expression of p53 and p21, and morphologic and histochemical changes characteristic of cancer cell death by cell senescence. The functionality of siRNAs was also demonstrated *in vivo*, producing inhibition of tumor growth in an HPV16+ animal tumor model. Taken together, this evidence demonstrates that software-generated siRNAs are functional *in vitro* as well as *in vivo* and confirms that siRNAs can silence expression of HPV E6 and E7 oncogenes, with biological effects on cervical cancer cells.

Our group has also developed a protocol to silence the expression of HPV16 E6 and E7 oncogenes using siRNAs [78]. In this study, we designed siRNAs for HPV16 E6 and E7 with siRNA Target Finder software. The siRNAs for HPV16 E6 and E7 were designed for cloning into the pSilencer 1.0-U6 plasmid. The silencing of HPV16 E6 and E7 oncogenes and the biological effect of siRNAs on SiHa cells (HPV16+) were evaluated. SiHa cells showed a selective decreased expression of mRNA HPV16 E6 and E7 oncogenes and oncoproteins, as well as functional effects in cell proliferation, an increase in p53 and pRb protein expression, and features of autophagy and apoptosis, attributable to silencing of E6 and E7 oncogenes. In a murine tumor model, the administration of siRNAs reduced tumor growth rate. These findings suggest that selective silencing of HPV16 E6 and E7 oncogenes by siRNAs has significant biological effects on survival of human cancer cells *in vitro* and *in vivo* and represents a potential gene therapy against cervical cancer.

11. Transcriptome Regulation by siRNAs for HPV E6 and E7

Studies have examined the effect of silencing HPV E6 and E7 oncogenes using siRNAs on transcriptome regulation in human cervical cancer cells. Kuner et al. analyzed the transcriptome of HeLa cells and patient biopsies after silencing HPV 18 E6 and E7 with siRNAs generated in the silencing plasmid pSUPER [79]. The study identified 360 cellular genes which were negatively regulated and 288 genes which were positively regulated due to silencing of E6 and E7. Most of these genes are involved in biological processes that occur during development of the tumor cell, such as apoptosis

control, cell cycle regulation, formation of the mitotic spindle, processing of mRNA by splicing, metabolism, DNA replication and repair, nuclear transport, cell proliferation, and gene regulation by c-Myc. These findings complement previous studies which analyzed expression of the HPV E2 protein. E2 inhibits HPV E6 and E7 expression and alters the transcriptome expression in human tumor cervical cells. The potential of this type of studies lies in the fact that the basic cell pathways for viral transformation may be identified, which may be targets for the development of therapeutically strategies. With this strategy, new molecular biomarkers may be identified for diagnosis and prognosis of cervical cancer.

12. Conclusions and Perspectives

Review of the evidence regarding microRNA expression in cervical cancer reveals that the expression pattern of microRNAs in cancer cell lines and cervical (pre)malignant lesions provides valuable information about the role of microRNAs in the different stages of cervical carcinogenesis. The microRNAs may represent a promising disease biomarker in cervical cancer, as well as potential therapeutic targets in gene therapy. The microRNAs regulate a large number of target genes involved in cell proliferation, differentiation, and apoptosis, and there is evidence *in vitro* and *in vivo* of their function as oncogenes or tumor suppressors in cervical cancer. The transcriptional regulation of microRNAs via epigenetic mechanisms or transcription factors must be further studied to understand their role in the process of carcinogenesis. Other important aspects to consider are chromosomal damage that may lead to overexpression or downregulation of microRNAs and the genomic localization of microRNAs in fragile sites that may undergo amplification or deletions near HPV integration sites in cervical cancer. Additionally, microRNAs may enhance the current diagnostic technologies in cervical cancer. Identification of key microRNAs and cellular target genes involved in HPV-related pathways based on expression patterns in HPV-infected cervical tissues provides useful information about prognosis. The identification and subsequent functional evaluation of host microRNA expression profiles associated with HPV oncoproteins is the major challenge in utilizing microRNAs as molecular biomarkers, and better understanding of their role in cervical carcinogenesis may allow for the development of specific targeted strategies against cervical cancer. In regard to the function of siRNAs, the evidence supports the idea that administration of siRNAs for HPV E6 and E7 oncogenes induces silencing of viral oncogene expression. The administration of siRNAs has biological effects on human tumor cervical cells transformed by HPV, including activation of cell death by apoptosis, effects on cell senescence, synergistic cytotoxicity with drugs used in chemotherapy for cervical cancer, and inhibition of cancer cells' tumor growth potential *in vivo*. The relevance of the silencing properties of the E6 and E7 oncogenes will be better appreciated once they are applied in clinical protocols. This will require adequate analysis during design of siRNA sequences to induce silencing of the E6-E7 bicistron, selection of optimal cloning vectors for siRNAs, and selection of siRNA transport vehicles such as biogels or atelocollagen to protect them from the action of endonucleases and to allow for administration in a site-specific and dose-dependent manner, as well as the development of treatment schemes which combine siRNAs, chemotherapeutic drugs, and/or radiation therapy. In summary, the use of siRNAs as a gene therapy strategy against cervical cancer has great potential for success in the treatment of this malignancy.

In regard to circulating microRNAs, Wang et al. demonstrated that serum microRNAs can be used as noninvasive biomarker for cervical SCC patients [80]. They show that miR-646, miR-141*, and miR-542-3p expression levels were significantly different between cervical SCC serum samples and the control samples of a total of 765 analyzed circulating microRNAs. They identified that miR-21, miR-200a, miR-143, miR-15a, miR-181c, miR-646, and miR-370 expression levels in serum can be used as biomarkers to monitor therapeutic efficiency. This evidence suggests that select microRNAs target genes were predicted to affect main biological processes such as hormone-mediated signal pathways and chemotherapy responses.

Conflict of Interests

None of the authors have any financial conflict of interests related to the submitted paper.

Acknowledgments

This paper was carried out at the National Institute of Public Health in México and received federal financial support from the institute, as well as from the National Council of Science and Technology (CONACYT) with File nos. SALUD-2008-01-87130, SALUD-2009-01-111892, and CB-2011-01-169209. Sacnite del Mar Díaz-González was recipient of a Doctoral fellowship with no. 167887 from CONACYT, México.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 125094, 17 pages
http://dx.doi.org/10.1155/2015/125094

Review Article

MicroRNAs as Potential Biomarkers in Cancer: Opportunities and Challenges

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Received 6 June 2014; Accepted 5 September 2014

Academic Editor: Francesco Nicassio

MicroRNAs (miRNAs) are a group of small noncoding RNAs (ncRNAs) that posttranscriptionally regulate gene expression by targeting their corresponding messenger RNAs (mRNAs). Dysregulated miRNAs have been considered as a new type of "oncomiRs" or "tumor suppressors," playing essential roles in cancer initiation and progression. Using genome-wide detection methods, ubiquitously aberrant expression profiles of miRNAs have been identified in a broad array of human cancers, showing great potential as novel diagnostic and prognostic biomarkers of cancer with high specificity and sensitivity. The detectable miRNAs in tissue, blood, and other body fluids with high stability provide an abundant source for miRNA-based biomarkers in human cancers. Despite the fact that an increasing number of potential miRNA biomarkers have been reported, the transition of miRNAs-based biomarkers from bench to bedside still necessitates addressing several challenges. In this review, we will summarize our current understanding of miRNAs as potential biomarkers in human cancers.

1. Introduction

According to NIH (National Institutes of Health), a biomarker is described with a characteristic that it is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention [1]. Useful biomarkers can provide great insights into tumorigenesis and facilitate the development of improved therapies. Cancer is not only one of the biggest global killers but also one of the fastest growing causes of death [2]. Hunting for cancer biomarkers capable of providing diagnostic, prognostic, or therapeutic information has become a necessary but challenging work in cancer research. Currently, some available biomarkers such as PSA (prostate-specific antigen) for prostate cancer, CEA (carcinoembryonic antigen) for colorectal cancer, AFP (a-fetoprotein) for liver cancer, CA 125 for ovarian cancer, and CA 19–9 for pancreatic cancer invariably display an inferior sensitivity and specificity, despite their generally clinical applications. Due to high rates of "overdiagnosis" and "pseudodisease" by PSA test, the USPSTF (US Preventive Services Task Force) issued a grade D recommendation to recommend against routine PSA-based screening for prostate cancer in July 2012 [3]. Therefore, it is an urgent request for more reliable biomarkers to serve as precise indicators of developments and changes of cancer at the cellular levels and the patient's response of cancer therapy.

The discovery of miRNAs has captured increased attention of researchers recently. Following the Lin-4 in *C. elegans*, known as the first member of miRNAs, discovered in 1993 [4], more and more miRNAs have been found and investigated; herein, a number of miRNAs have been identified to essentially revolve in human carcinogenesis by acting as "oncoMirs" or "tumor suppressive miRNAs" [5]. Strikingly, based on the pervasive cancer-associated aberrant expression profiles of dysregulated miRNAs, together with the characteristics of miRNAs such as temporal and spatial specificity, sensitivity, and stability in both paraffin sections and body fluids, miRNAs have shown great potential as a new class of biomarkers in cancer. Furthermore, with the extensive use

of next generation of technologies such as miRNA microarrays and high-throughput deep sequencing techniques [6], translating biomarker into practice with increased diagnostic and therapeutic sensitivity and specificity would be less of a problem [7]. Among these contexts, the potential of miRNAs being involved in carcinogenesis as cancer biomarkers has been extensively investigated and developed.

In this review, we will overview recent advances in the dysregulation of tumor-associated miRNAs in both biopsies and circulation and highlight some critical challenges for the transition of miRNAs as cancer biomarkers from a research setting into clinical application.

2. Overview of miRNAs: Definition, Biogenesis, and Functions

miRNAs belong to the heterogeneous class of ncRNAs, of 22–24 nt RNAs that play important regulatory roles by targeting mRNAs for cleavage or translational repression [8]. By definition, miRNAs are small RNA molecules incapable of encoding proteins, but possessing important structural, catalytic, and regulatory functions. According to the latest miRBase release (v21, June 2014, http://www.mirbase.org/), there are 28645 entries representing hairpin precursor miRNAs from 223 species, expressing 35828 mature miRNA products. MiRNA genes are located either in independent noncoding DNA loci or in the introns of protein-coding genes [9]. Furthermore, up to 50% miRNAs are clustered on chromosomes with a common promoter [10] and cotranscribed to form different miRNAs families which share common mRNAs targets and biological processes because of their identical seed regions [11].

It is well established that miRNA biogenesis is a complex process which generally includes three main steps as follows. (i) In the nucleus, miRNA genes are transcripted by RNA polymerase II to form primary miRNA transcripts (pri-miRNAs) [12]. (ii) Subsequently, the Drosha RNase III endonuclease trims the pri-miRNA to liberate a pre-miRNA hairpin which is actively transported to the cytoplasm by Ran-GTP and exportin-5 [8, 13]. (iii) Its final maturation is processed in the cytoplasm where Dicer RNase III endonuclease cleaves the pre-miRNA into a single-stranded mature miRNA [13] and then the mature miRNA binds to proteins of the argonaute (Ago) family and assembles the RNA induced silencing complex (RISC) together to exert its further physiological functions.

After being incorporated into the RISC, the mature miRNA induces posttranscriptional gene silencing by tethering RISC to be partially complementary to the target mRNA predominantly found within the 3′-untranslated region (UTR) [14]. Inspiringly, a recent study showed that a single miRNA may repress more than 100 mRNAs on average and over 60% of human protein-coding genes are conserved targets of miRNAs [15]. Given this vast majority of mRNA targets regulated by miRNAs, aberrant miRNA expression profoundly influences a wide variety of cell regulation pathways important to cell proliferation [16], apoptosis [17], and stress responses [18]. In cancer disease phenotype, miRNAs serve exclusively as a novel class of oncogenes or tumor suppressor genes named "oncomiRs" or "tumor suppressive miRs" [19]. Following the first identified tumor suppressive miRNA gene miR-15a/miR-16-1 revolving in the development of B-cell lymphocytic leukemia [20], a growing number of oncomiRs have been validated as well as tumor suppressive miRs (more details are in the review [21]). Remarkably, it has been continually confirmed by numbers of studies that "oncomiRs" or "tumor suppressive miRs" revolve in a multitude of cancer-related signaling pathways. For example, miR-17 activates PI3K/AKT pathway and regulates tumor growth [22], the axis of p53-miRNA-34 network regulates Wnt pathway [23] and promotes EMT program [24], and let-7 leads to growth inhibition by negatively regulating RAS pathway [25]. Taken together, these observations give a miRNAs-mediated complex network model that connects oncogenic and tumor suppressive signaling pathways in human cancers, indicating that miRNAs may play a more indispensable role in the pathogenesis of human cancers than previously thought.

3. miRNAs as Potential Biomarkers in Cancer

3.1. Aberrant miRNA Expression Profiles in Cancer

3.1.1. Linkage of Aberrant miRNA Expression Profiles with Cancer Development. Aberrant expression of miRNAs in cancer is characterized by abnormal expression levels of mature or precursor miRNA transcripts in comparison with those in the corresponding normal tissues. A steadily growing number of reports have proven that human cancers frequently show an aberrant expression profile of miRNAs as well as pre-miRNAs. With various kinds of high-throughput sequencing platforms applied to analyze the genome-wide expression of miRNA genes these years, aberrant expression profiles of miRNAs either downregulated or upregulated have been discovered in a broad variety of human malignant cancers, including lymphoma [26], breast cancer [27], colorectal cancer [28], prostate cancer [29], and glioma [30].

Although the causes of aberrant miRNAs expression in cancer have not yet been fully elucidated, several defined mechanisms can be possible, including chromosomal abnormalities, genomic mutations, epigenetic changes, and alterations in miRNA biogenesis. Notably, some miRNA genes are frequently located at fragile sites and genomic regions related to cancer [31]. In other words, alterations of miRNAs expression can directly reflect the chromosomal or genomic changes of cancer-associated genes. Together with important roles of cancer-associated miRNAs identified in various types of cancer cell lines [32] and clinical tumor specimens [33–35], the aberrant expressions of miRNAs appear to show significant values of clinical applications.

3.1.2. Diagnostic, Prognostic, and Predictive Values of miRNA Signatures for Cancer. Given those circumstances above, alterations of miRNAs expression in different types of neoplasia constitute specific patterns of miRNA signature for certain types of cancer. Logically, these specific miRNA signatures

would be attractively conducive to early diagnosis, signifying the prognosis and predicting the response of cancer therapy.

(i) Diagnosis and Classification. It was observed that, with cancers derived from different epithelial and hematopoietic lineage having distinct miRNA profiles, miRNA signatures were able to discriminate them according to their developmental origins. For example, in a research trying to identify cancers with unknown primary tissue of origin by using microarray platform to evaluate the expressions of 47 miRNAs in 101 FFPE (formalin fixed and paraffin embedded) samples from primary or metastatic cancers, overall, the accuracy reached 100% for primary cancers and 78% for metastatic cancers. When the signature was applied to an independent published dataset of 170 samples, correct prediction was further verified in 86% of the metastasis cases [36].

For patients with malignant cancers, precise determination of tumor subtype significantly influences the decision-making of treatments. For example, In renal cell carcinoma (RCC), a research team developed a classification system applying method of decision trees that can distinguish different RCC subtypes in 94 different subtype cases using unique miRNA signatures analysis [37]. This system obtained a sensitivity of 97% for differentiating normal cases from RCC patients. And among the four types of RCC (clear cell RCC (ccRCC), papillary RCC (pRCC), oncocytoma, and chromophobe RCC (chRCC)), 100% for ccRCC subtype from the other fours, 97% for pRCC subtype from oncocytoma and chRCC, and 100% accuracy for distinguishing oncocytoma from chRCC subtype [37]. Moreover, another study by Gilad et al. validated a miRNA-based assay capable of differentiating between four main types of lung cancer in an independent set of 451 samples. They got a result for more than 90% of the samples with overall accuracy of 94%, with a similar performance observed in pathologic and cytologic samples [38]. Collectively, these findings above suggested that miRNAs signatures were able to serve as an accurate classification tool for different cancers in pathologic and cytologic samples.

Moreover, the need of biomarkers for cancer early diagnosis is extremely important because of the fact that survival and prognosis of patients extensively depend on the stage of tumor at the time of detection, with an early diagnosis generally associated with a better prognosis. Promisingly, miRNA signatures have been identified to possess a robust potential as early diagnosis biomarkers. For example, overexpression of miR-205 and miR-21 in ductal adenocarcinoma has been reported to precede phenotypic changes in the ducts [39], suggesting that aberrant miRNA production was an early event in the development of cancer and thus supporting the possibility to use them for early detection of cancers.

(ii) Prognostic Values. In addition to cancer diagnosis including distinguishing tissue of origin, subtypes, and early detection of cancer, miRNA signatures can be also valuable for cancer prognosis prediction, which has been vilified by a number of studies recently. The first evidence was proposed by Calin et al. in chronic lymphocytic leukemia (CLL), where a unique miRNA signature composed of 13 genes can differentiate CLL patients with abnormal expression of prognostic factors (ZAP-70 and IgV(H)) from normal cases. Besides, the same miRNA signature was associated with the presence or absence of disease progression [40]. Moreover, in lung cancer, a signature of five miRNAs has been used in the prediction of treatment outcome for non-small-cell lung carcinoma (NSCLC), among which high expression of miR-221 and let-7a is associated with good prognosis, as opposed to elevated miR-137, miR-372, and miR-182 with poor prognosis [41]. Likewise, high expression of the miR-183 family (miR-183, miR-182, and miR-96) was associated with overall poor survival in patients with lung cancer [42]. In addition, a recent study interrogated plasma miR-10b and miR-373 levels, which are known mediators of metastasis in breast cancer, and they uncovered association of these miRNAs for detecting the lymph node metastasis, thereby showing potential as prognostic biomarkers [43]. It is worth noting that one miRNA alone can also possess accurate predictive power which was clarified by a study in patients with breast cancer, they identified the fact that overexpression of miR-210 is associated with an increased risk of recurrence and a reduced chance of relapse-free survival, and miR-210 alone allowed prediction of prognosis to the same level as a 76-gene mRNA signature test (GENE76) [44]. In prostate cancer, miRNAs have also been connected to prognosis of PC, such as miR-141 and miR-375, which turned out to correlate with high Gleason score or lymph-node positive status in a second independent validation study [45]. Besides, signature of miR-410 and miR-645 was negatively associated with overall survival in advanced serous ovarian cancer [46]. Remarkably, a pattern of seven-miRNA signature recently identified in gastric cancer can predict overall survival and relapse-free survival [47], and, in patients with stage II colon cancer, a six-miRNA-based classifier established was an independent prognostic factor for disease recurrence [48].

(iii) Predictive Values for Response to Therapy. Apart from their use as prognostic biomarkers, miRNA signatures could also predict the responses to various cancer managements. The correlation between miRNA signatures and the responses of specific therapies is of great clinical value because of the involvement of miRNAs in chemoresistance identified in many studies [49, 50]. For instance, Meng et al. firstly found that inhibition of miR-21 and miR-200b increased sensitivity to gemcitabine in cholangiocarcinoma cell lines [51]. Subsequently, it was proven that high miR-21 expression involved in gemcitabine chemoresistance was able to predict significantly shorter overall survival (OS) in pancreatic cancer treated with gemcitabine [52]. Likewise, by conferring taxol resistance through the suppression of Bak1 expression, high expression of miR-125b in breast cancer predicted the poor response to taxol-based treatments [53]. In colorectal cancer, the presence of the KRAS mutation was associated with upregulation of miR-127-3p, miR-92a, and miR-486-3p and downregulation of miR-378, which constituted a miRNA signature capable of predicting colorectal cancers resistant to EGFR antagonists [54]. Additionally, it was demonstrated that let-7g and miR-181b may be indicators of chemotherapy response to S-1 based chemotherapy in CRC [55]. In glioma cancer, miR-181d upregulation may correspond to a better response to temozolomide, while upregulation of miR-21 may

predict poor response caused by temozolomide resistance [30]. Remarkably, in relapsed patients with NSCLC, the loss of heterozygosity of miR-128b, an EGFR regulator, was correlated with response to the EGFR inhibitor gefitinib [56]. In belief, given these specific miRNA signatures serving as predictors of response to various cancer management, it may be possible to improve patients' prognosis by selecting the right treatment course as soon after diagnosis as possible and permit rapid adaption of treatment to the acquisition of chemotherapeutic and radioresistance. What is more is that, by predicting response to molecular-targeted agents, the use of predictive biomarkers could be promisingly conducive to accelerate the development of new anticancer therapies in clinical trials [57].

In summary, based on the aberrant miRNA expression profiles identified in human cancers, miRNAs give us a totally new horizon in understanding the development management of cancer. Despite of some certain miRNAs commonly exhibiting altered expression across various kinds of tumors, particular types of cancers often express their exclusive patterns of miRNAs referring to their tissues of origin [33]. Such specific miRNA signatures exhibited in types of tumors mentioned above are also termed as "miRNome," which characterizes the malignant state and defines some of their clinicopathological features such as grade, stage, aggressiveness, vascular invasion, and proliferation index [31]. Given the great value of miRNA signatures for cancer diagnosis, prognosis and prediction of therapeutic responses, a comprehensive list of miRNAs identified as various biomarkers in different cancer types are summarized in Table 1.

3.2. Potential Source of miRNA-Based Biomarkers

3.2.1. Stability of miRNAs. In addition to the high specificity and sensitivity of miRNA biomarkers in cancer diagnosis as previously described, miRNAs are characterized by robust stability as well in stored RNA samples, including fixed tissue, blood, and other body fluids, which further supports their potential as biomarkers. Recent studies have systematically investigated the stability of miRNAs even after being subjected to severe conditions that would normally degrade mRNA or other types of larger RNAs. For example, in two similar studies, miRNA expression profiles in FFPE from colorectal cancer (CRC) samples dating back up to 10 year were assessed and compared with fresh frozen CRC samples; a high similarity of miRNAs profiles was observed. Moreover, differing formalin fixation times did not significantly influence miRNAs profiles in FFPE specimens in light of quantitative real-time polymerase chain reaction (qRT-PCR) [58, 59]. For another instance of extracellular miRNAs stability reported by Chen at al., they treated some miRNAs with or without RNase A digestion; then they surprisingly found that half of miRNAs remain intact after 3 hours of exposure to RNase A, but all high molecular weight RNAs set as controls were rapidly degraded. More importantly, after being treated with several harsh conditions including boiling, low/high pH (pH = 1/13), extended storage, or freeze-thaw cycles, miRNAs in sera still maintained potent intrinsic stability as expected [60]. Taken together, these data consistently revealed that miRNAs, particularly serum miRNAs, possess considerable resistance to the enzymatic cleavage by RNase A and various severe conditions, so as to maintain their intrinsic stability for long.

Generally, there are mainly three explanations for miRNAs' stability. Firstly, as a class of small molecules with a short length of only 20–24 nt, miRNAs tend to be less subjected to degradation during FFPE sample processing. Secondly, these small RNAs are taken into encapsulation by cell-derived lipid vesicles such as exosomes [61], microparticles [62], or apoptotic bodies [63]. However, the precise mechanisms of miRNAs' selective packaging remain elusive yet. Thirdly, the nuclease-resistant extracellular miRNAs were predominantly associated with RNA binding proteins such as argonaute proteins (Ago1 or Ago2), nucleophosmin (NPM1), or ribosomal proteins [64], which exert a robust protective effect on miRNAs from abundant nucleases in extracellular space. Overwhelmingly, Turchinovich et al. argued that most of the extracellular miRNAs are not exclusively associated with cell-exported exosomes; instead, a majority of them are floating outside exosomes to bind to Ago2 protein [64]. Nevertheless, no final conclusion has yet been reached on the main mechanism of how to maintain miRNAs' stability. But what we can confirm is that inherent stability of miRNAs in both FFPE and circulation perfectly presents great advantages for them as ideal biomarkers of cancer.

3.2.2. miRNAs in FFPET. Tissue biopsy specimen in the form of FFPET (FFPE tissue) is commonly used for tissue storage, which preserves good tissue integrity so as to provide excellent resources for discovery of disease-detecting biomarkers, not excepting the miRNAs-based biomarkers of cancer certainly. Better than mRNAs which are too unstable to be suitable for qRT-PCR, miRNAs are stable enough to be well preserved even over prolonged FFPE block storage and quantified from FFPET by qRT-PCR. In a word, miRNAs from substantial FFPET can provide tremendous source for cancer-related biomarkers.

Numerous studies have discovered and evaluated a broad variety of potential miRNAs biomarker candidates in FFPET which provide valuable information for cancer diagnosis and prognosis. A recent study used bioinformatics to screen miRNAs classifiers in 69 FFPE pancreatic specimens including benign, premalignant, and malignant. Different miRNA panels mapped to these lesions above, separate anyone from the two others with sensitivity and specificity up to 85%–100% [65]. Furthermore, in another study by Casanova-Salas et al. [66], an independent cohort of 273 FFPET samples was used for validation of new potential biomarkers by qRT-PCR. They assessed the relationship of two miRNAs (miR-182 and miR-187) most differentially expressed in prostate cancer with the clinicopathological characteristics and outcome of patients, and it was observed that miR-187 expression was decreased in advanced prostate cancer cases, which was consistent with microarray data. Moreover, the higher expression of miR-182 independently conferred a worse prognosis for BPFS and PFS using Cox proportional hazard multivariable analysis. Therefore, such results showed the promising potential of miRNAs for serving as biomarkers in cancer diagnosis or prognosis.

Table 1: Types of specific miRNAs biomarkers in different cancers.

Cancer	Type of biomarker	miRNAs biomarkers	Reference
Breast cancer	Diagnostic	miR-10b, -21, -30a, -92a, -125b, -141, -145, -200, -801, -155, -191, -203, -210	[112, 113]
	Prognostic	miR-10b, -373, miR-210	[43, 44]
	Predictive	miR-125b	[53]
Lung cancer	Diagnostic	miR-21, -155, miR-16, miR-17, -19b, -25, -29c, -30c, -106a, -126, -451, -660, -28-3p	[114–116]
	Prognostic	miR-221, let-7a, -137, miR-372, -182	[41, 42]
		miR-1, -15b, -16, -21, -126, -142-3p, -148a, -197	[116]
	Predictive	miR-128b	[56]
Liver cancer	Diagnostic	miR-222, -223, -181a, -181b, -181c, -200c, -203, -21, -224, -10b, -222, 126, -96	[117, 118]
	Prognostic	miR-21, -22, -26, -29, -31, -122, -124, -135a, -139, -145, -146a, -155, -200c, -221, -222, -222, -223	[119]
	Predictive	miR-21, -200b	[52]
Colon cancer	Diagnostic	miR-15b, -18a, -29a, -335, miR-17-3p, -20a, -21, -92, -601, -760, -29a	[120, 121]
	Prognostic	miR-141	[122]
	Predictive	miR-127-3p, -92a, -486-3p, -378, let-7g, miR-181b	[54, 55]
Pancreatic cancer	Diagnostic	miR-205, -21, miR-642b, -885-5p, -22	[39, 123]
		miR-145, -150, -223, -636, -26b, -34a, -122, -126*, -145, -150, -223, -505, -636, -885.5p	[124]
	Prognostic	miR-130b, miR-21, -105, -196a-2, -203, -210, -222, -452, -105, -127, -187, - 518a-2, - 30a-3p	[125, 126]
	Predictive	miR-21	[52]
Prostate cancer	Diagnostic	miR-30c, -622, -1285, miR-10b, -373, let-7c, -7e	[43, 127]
		miR-141, -375, miR-26a, -195	[128]
	Prognostic	miR-141, -375, miR-20a, -21, -141, -145, -221	[45, 129]
	Predictive	miR-21	[130]
Ovarian cancer	Diagnostic	miR-200 family, let-7 family, miR-21, -29a, -92, -93, miR-126, -127, -132, -155, -214, -182, -205, -144, -222, -302	[131–133]
	Prognostic	miR-410, -645, miR-200 family, miR-141, -429	[46, 134]
	Predictive	miR-23a, -27a, -30c, let-7g, -199a-3p, -181a, -181b, -213	[132]

Table 2: miRNAs as potential biomarkers in FFPE samples.

Cancer	miRNAs as biomarkers	Expression	Reference
Glioblastoma	miR-323, -329, -155, -210	Up	[135]
	miR-326, -130a	Down	[135]
	miR-21, -221	Up	[136]
	miR-128, -181b	Down	[136]
Head and neck cancer	miR-21, -31, -107, -138, -504, -10b	Up	[137]
	miR-16, -20a, -106b, -142-3p	Up	[138]
	miR-155, -423, -451, let-7i	Up	[138]
	miR-10a, -125b, -375	Down	[138]
Breast cancer	miR-21, -29b-2, -125b	Up	[139]
	miR-16, -155, -191, -196a	Up	[139]
	miR-34b, -145, -205, let-7a	Down	[140, 141]
Lung cancer	miR-21, -205	Up	[142]
	miR-16	Up	[115]
Liver cancer	miR-519d	Up	[143]
	miR-146a	Down	[144]
	miR-185	Down	[145]
Gastric cancer	miR-21, -221, -222	Up	[146]
	miR-10b, -21, -106a, -223, -338	Up	[47]
	miR-30a-5p, -31, -126, let-7a	Down	[47]
Pancreatic cancer	miR-192-5p, -202-3p, -337-5p, -130-3p	Up	[65]
	miR-187, -30a-3p	Up	[147]
	miR-21, -155, -203, -210, -222, let-7a	Up	[148]
	miR-200c	Down	[149]
Cervical cancer	miR-21, -27a, -34a, -146a	Up	[150]
	miR-155, -196a, -203, -221	Up	[150]
Ovarian cancer	miR-223	Up	[151]
	miR-9, -200a, -200b, -429	Down	[151]
Prostate cancer	miR-31, -143, -221	Up	[152]
	miR-126, -146a, -150	Down	[152]
	miR-182	Up	[66]
	miR-187	Down	[66]
	miR-203	Down	[153]
Colorectal cancer	miR-31, -135b	Up	[154]
	miR-21, -23a, -193a-3p, -338-5p	Down	[155]
Bladder	miR-10a-5p, -31-5p, -130a, -3p	Up	[156]
	miR-145, -30a-3p, -125b, -133a	Down	[157]
	miR-133b, -195, -199a	Down	[157]

In addition, other miRNAs identified as potential biomarkers in FFPET for various types of malignancies are summarized in Table 2.

3.2.3. miRNAs in Blood (Total Blood, Serum, Plasma, and Exosome). Apart from the diagnostic miRNA signatures identified in specimens from tumor tissues, studies have also shown the diagnostic and prognostic usefulness of miRNAs in circulation. In fact, except for miRNAs in the biopsies and tumor samples, a substantial number of cancer-related miRNAs can be also detected in blood including total blood, serum, and plasma, as well as other body fluids such as saliva, urine, breast milk, seminal fluid, and tears [67].

In fact, the intriguing phenomenon of cancer-associated nucleic acids in circulation has been identified for decades. Generally, from their intracellular origin, miRNAs can be secreted extracellularly in cell-derived extracellular vesicles such as exosomes or bound to other lipoproteins as mentioned above, although the concrete mechanisms for the origin of such miRNAs released by tumor cells have not yet been clarified and little is known about the mechanisms of how to specifically direct miRNAs into multivesicular bodies or exosomes.

However, what we know at present is that miRNAs in blood released by tumor cells may not simply act as a set of bystander product of tumor cells. It has been widely identified

that tumor-released (TR) exosomes could act as intercellular mediators transporting diverse types of proteins, mRNA, and miRNAs that can promote angiogenesis, cell proliferation, and cell survival, resulting in establishing an oncogenic niche systemically [68]. Recently, a paradigm of autocrine and paracrine miRNA signaling pathway contributing to microenvironment of cancer through TR exosomes has been proposed. For example, Skog et al. firstly found that the messengers encoding for EGFRvIII protein and miR-21 are transported in glioblastoma-derived exosomes and these molecules could be taken up by normal host cells and transformed into functional signals, stimulating proliferation of cancer cells [69]. Furthermore, Fabbri recently demonstrated that miR-21 and -29a secreted by tumor cells via exosomes could bind to toll-like receptor (TLR) 7/8 and activate these receptors on immune cells, leading to TLR-mediated NF-κB activation and secretion of prometastatic inflammatory cytokines that may ultimately lead to tumor growth and metastasis [70]. These observations above revealed that the mobile miRNAs entrapped in exosomes in blood could function as hormones, entering the circulation and travelling to distant organs to exert their hormone-like effects, leading to widespread consequences within the recipient cells at a distance from the donor cells.

Additionally, Pigati and colleagues verified the fact that the cancer-related miRNAs are specific to the cancer cells, which is a further consideration supporting detection of the circulating miRNAs to serve as molecular tumor markers. They found that the bulk of miR-451 and miR-1246 produced by malignant cells was selectively released into blood particularly, but the majority of these miRNAs produced by normal cells were retained intracellularly [71]. Their findings indicated that release of miRNAs into blood and other biological fluids is selective presumably with an explanation that the assortment of them might be changed by malignant transformation [72].

Therefore, with a stable form and extensively hormone-like effects, the noninvasively detectable cancer-specific miRNAs in circulation have been widely investigated and demonstrated to efficiently discriminate cancer patients from healthy people, predict the prognosis, and monitor the therapeutic response. Comprehensively, recent studies for discovery of potential miRNAs biomarkers for types of cancers in blood and other body fluids have been listed in Table 3.

In serum, following the first paradigm showed that the diagnostic and prognostic potential of miR-21 that was associated with increased relapse-free survival of diffuse large B-cell lymphoma (DLBCL) patients by Lawrie et al. [73] and lots of new candidate miRNA biomarkers in serum of cancer patients have been reported. For example, Chen et al. demonstrated that a profiling of 10 differentially expressed serum miRNAs selected from a sample set including 400 NSCLC cases and 220 controls provided a high sensitivity and specificity for NSCLC diagnosis. They used risk score analysis to evaluate the diagnostic value of the serum miRNAs profile system and found that these 10 miRNAs were able to distinguish NSCLC cases from controls with sensitivity of 93% and specificity of 90%. What is more is that these 10 serum miRNAs were correlated with the stage of NSCLC patients [74]. Moreover, in a recent study, Wu et al. reported 35 miRNAs targeting 11 genes involved in the TGF-β signaling pathway for their associations with survival among 141 highly expressed serum miRNAs collected from 391 patients with advanced NSCLC. They concluded that 17 miRNAs were significantly associated with 2-year patient survival using Cox regression model. Of these 17 miRNAs, miR-16 exhibited the most statistical significance, whose high expression was associated with an obviously better survival [75]. Findings of similar crosstalk between TGF-beta signaling and miRNAs machinery also have been clarified in other cancers such as CRC [76] and glioblastoma [77].

In plasma, Mitchell et al. firstly proved that miRNAs are readily measurable by direct cloning and sequencing from a plasma small RNA library. To further determine whether tumor-derived miRNAs enter the circulation at levels sufficient to be measurable as biomarkers, they established a mouse prostate cancer xenograft model system, collected the plasma, and isolated miRNAs for analyzing the different expression of miRNAs compared to the control. They found that levels of miR-629 and miR-660 were able to independently differentiate xenografted mice from controls with 100% sensitivity and 100% specificity [78]. Such conclusion, thereby, provided a firm grounding of the fact that that tumor-derived miRNAs measured in plasma can serve as noninvasion biomarkers for cancer detection. Examples are numerous. Plasma miR-21, miR-145, and miR-155 used in combination helped in distinguishing lung cancer from healthy smokers with 69.4% sensitivity and 78.3% specificity [79]. Combination of miR-148b, miR-409-3p, and miR-801 discriminated powerfully between breast cancer cases and healthy controls [80]. Three plasma microRNAs (miR-106b, miR-20a, and miR-221) had a statistically significant elevation in gastric cancer patients so as to serve as novel biomarkers for the early detection of GC [81]. Interestingly, the combination of miR-16, miR-196a, and CA19-9 was more effective for pancreatic cancer diagnosis, especially in early tumor screening [82]. Ng et al. revealed that expression of miR-92 in plasma is significantly elevated and differentiated CRC from gastric cancer or normal subjects of CRC patients with a high sensitivity of 89% and specificity of 70%; thus miR-92 in plasma could have roles as a potential marker for CRC screening [68]. Moreover, high expression of miR-27b, miR-148a, and miR-326 was associated with decreased progression-free survival of CRC patients [68].

Exosomes are a class of small (50–90 nm) membrane vesicles formed in the plasma membrane by the fusion of endocytic vesicles and extracellularly released into surrounding body fluids [83]. As is mentioned above, miRNAs passively released from normal or tumor-lysed cells may be preferentially secreted and packaged into exosomes that provide a protective effect on the stability of extracellular miRNAs [61]. Excitingly, it was discovered that the exosomal miRNAs content was similar to that in the original tumors [84], thus peaking researchers' interests in the use of exosomal miRNA profiles for diagnostic markers of cancer. For example, Hessvik compared miRNAs in PC-3 cells with the noncancerous prostate cell line by microarray and qRT-PCR analysis. It was found that the exosomal miRNA

Table 3: Potential miRNAs biomarkers for types of cancers in blood and other body fluids.

Cancer	Samples	miRNAs biomarkers	Expression	Reference
Glioblastoma	Serum	miR-15b, -23a, -133a, -150, -197, -497	Up	[158]
	Plasma	miR-128, -342-3p	Down	[30]
	CSF	miR-21, -17-5p, -200	Up	[30]
Head and neck	Plasma	miR-31, -10b, -24, -181, -184	Up	[87]
	Saliva	miR-31	Up	[87]
		miR-200a, -125a	Down	[87]
Breast cancer	Serum	miR-373, -155	Up	[159]
		miR-34a, -17	Down	[159]
		miR-222, -103, -23a, -29a, -23b, -24, -25	Up	[160]
	Plasma	miR-148b, -376c, -409-3p, -801	Up	[80]
Lung cancer	Serum	miR-21-3p, -205-5p, -205-3p, -141, -200c	Up	[92]
		miR-21, -24, -205, -30d	Up	[93]
	Plasma	miR-21, -155	Up	[114]
		miR-145	Down	[114]
	Exosome	miR-17-3p, -21, -106a, -146, -155, -191,-192, -203, -205, -210, -212, -214	Up	[161]
	Sputum	miR-205, -210, -708	Up	[90]
Liver cancer	Serum	miR-122	Up	[162]
		miR-500	Up	[163]
		miR-21, -122, -223	Up	[164]
		miR-21, -1, -25, -92a, -206, -375, let-7f	Up	[165]
		miR-16	Down	[166]
Gastric cancer	Serum	miR-221, -376c, -744	Up	[167]
		miR-1, -20a, -27a, -34, -423-5p	Up	[168]
	Plasma	miR-106b, -20a, -221	Up	[81]
		miR-21, -223, -218	Up	[169]
		miR-451, -486	Down	[170]
Pancreatic cancer	Serum	miR-21, -100, -155	Up	[171]
		miR-203, -369-5p, -376a, -375	Up	[172]
	Plasma	miR-21, -210, -155, -196a	Up	[82, 173]
	Exosome	miR-21, -17-5p	Up	[174]
Colorectal cancer	Serum	miR-29a	Up	[175]
	Plasma	miR-17-3p, -92	Up	[68]
		miR-92a, -29a	Up	[176]
		miR-27b, -148a, -326	Up	[177]
		miR-221	Up	[178]
		miR-15b, -17	Up	[179]
Ovarian cancer	Serum	miR-21, -92, -93, -126, -29a	Up	[131]
		miR-155, -127, -99b	Down	[131]
	Plasma	miR-205	Up	[180]
		let-7f	Down	[180]
	Exosome	miR-21, -141, -200a/b/c, -203, -205, -214	Up	[84]
Prostate cancer	Serum	miR-26a-1, -141, -375	Up	[181]
		miR-16, -195, -26a, let7i	Up	[128]
		miR-20b, -874, -1274a, -1207-5p, -93, -106a	Up	[182]
		miR-223, -26b, -30c, -24	Down	[182]
	Exosome	miR-107, -574-3p	Up	[85]
Bladder	Blood	miR-129, -133b	Up	[183]
		miR-26b-5p, -144-5p, -374-5p	Up	[184]
		miR-378g, -942, -106a-5p, -142-3p, -374a	Up	[185]
	Urine	miR-18a, -25, -187	Up	[89]
		miR-142-3p, -140-5p, -204	Down	[89]
		miR-214	Up	[91]
		miR-200, -155, -192, -205	Up	[186]

Table 3: Continued.

Cancer	Samples	miRNAs biomarkers	Expression	Reference
Large B-cell lymphoma	Serum	miR-21, -155, -210	Up	[73]
		miR-15a, -16-1, -29c, -155	Up	[187]
		miR-34a	Down	[187]
Leukemia	Serum	miR-181b-5p, -10a-5p, -93-5p	Up	[188]
		miR-129-5p, -155-5p, -320d	Up	[188]
		miR-29c, -223	Down	[189]

profile released by PC-3 was highly similar to the profile of corresponding parent cells. In a study of ovarian cancer, researchers found that levels of 8 exosomal miRNAs (miR-21, miR-141, miR-200a, miR-200c, miR-200b, miR-203, miR-205, and miR-214) previously demonstrated as diagnostic markers were similar between cellular and exosomal miRNAs, which were significantly distinct from profiles observed in benign disease [84]. In another study, exosomal miRNAs were profiled from the plasma of prostate cancer patients with or without metastases and a distinct set of 11 miRNAs was present at significantly greater amounts in patients with metastases compared to those without metastases. And the association of miR-141 and miR-375 among these 11 microRNAs was confirmed in plasma exosomes from a separate patient cohort with recurrent or nonrecurrent disease, thus demonstrating that changes of miRNAs concentration present in exosomes could be of great value for early detection and tumor staging diagnosis [85]. Collectively, these results suggested that miRNA profiles of circulating tumor exosomes could potentially be used as surrogate diagnostic markers for biopsy profiles and could also serve as potential diagnostic and prognostic biomarkers of cancer.

3.2.4. miRNAs in Other Body Fluids. Although the majority of studies identified circulating miRNAs in serum and plasma, miRNAs have also been detected in other body fluids such as urine, tears, breast milk, and seminal, amniotic, bronchial, lavage as well as pleural, peritoneal, and cerebrospinal fluids [67, 86]. Recent studies have confirmed the potential use of tumor-specific miRNAs as diagnostic or prognostic markers for cancer in these body fluids. For instance, miR-125a and miR-200a were identified with significantly lower levels in the saliva for oral cancer detection [87]. The RNA ratio of miRNA-126 : miRNA-152 enabled the detection of bladder cancer from urine with high specificity of 82% and sensitivity of 72% [88]. A subset of six miRNAs (miR-187, miR-18a*, miR-25, miR-142-3p, miR-140-5p, and miR-204) was capable of correctly classifying bladder urothelial cell carcinoma (UCC) patients with an overall sensitivity of 84.8% and specificity of 86.5% [89]. In combination of miR-205, miR-210 and miR-708 in sputum distinguished lung squamous cell carcinoma patients from normal subjects with 73% sensitivity and 96% specificity thus helping in the early detection of lung squamous cell carcinomas [90]. What is more is that urinary level of cell-free miR-214 could be an independent prognostic parameter for non-muscle-invasive bladder cancer recurrence [91]. Presence of miR-21 and miR-15b in cerebrospinal fluid (CSF) of glioma patients also has been identified as a source of biomarkers [30].

However, as a matter of fact, miRNAs, no matter in serum, plasma, exosomes, or other body fluids, may not be absolutely specific to a certain cancer type. MiR-21 in serum is exactly a representative example for this. From a serial of investigations, it is found that high expression of serum miR-21 in lung cancer associated with poor prognosis [92] and the dynamic change of serum miR-21 between postoperative and preoperative lung carcinoma patients can potentially serve as biomarkers for disease recurrence after surgery operation [93]. In other types of cancer, also, the level of serum miR-21 significantly elevated in patients with CRC and advanced adenoma thus exhibiting its potential diagnostic value for early detection of CRC [94], as well as the prognostic value because of the postoperative reductions in serum miR-21 level which occurred [95]. Observations of analogous diagnostic and prognostic significance also have been identified in esophageal squamous cell carcinomas [96], pancreatic cancer [97], hepatocellular carcinoma [98], and ovarian cancer [99]. Therefore, a concept that has to be noted again is the "miRNome" defined as the full complement of miRNAs in a cell, which emphasizes that different tissues have distinctive patterns of miRNome expression with each tissue presenting a specific signature [100]. In order to map to every type of cancer with higher accuracy and specificity, further studies with larger sample numbers are needed to allow generalized detection of unique patterns of miRNome toward every type of cancer, even before or after therapy, and thus may really help in the identification of useful biomarkers in cancer efficiently.

4. Methods for MiRNA Detection

Though miRNAs show striking potential as new biomarkers of human cancers in clinic settings and maintain high stability in both FFPET and body fluids, the methods for miRNAs' detection are still challenging yet for the intrinsic characterisitic of miRNAs such as small size, low level, a high degree of sequence similarity among various members, and tissue-specific or stage-specific expressions. Currently, there are various strategies for the detection of miRNAs, including qRT-PCR, in situ hybridization (ISH), enzymatic luminescence miRNA assay and northern blotting, microarrays, deep sequencing or next-generation sequencing (NGS), and nanopore technology et al. [101]. Herein, qRT-PCR is the most commonly used manner, quantifying the miRNome from low levels with high sensitivity and specificity, which is

very important for the lack of large amounts of RNA from clinical samples could be obtained. More importantly, qRT-PCR is more convenient and reliable than methods based on hybridization for its potential as it can be performed in high-throughput assays to allow for large-scale miRNAs detection at the same time and still with high specificity or sensitivity [102]. Therefore, qRT-PCR technique has become the gold standard of nucleic acid quantification currently [103]. Nevertheless, several limitations of qRT-PCR also hinder its use in practice. For example, the selection of inner reference genes is essential and critical for data normalization, but the determination of suitable reference genes as the normalizer remains as a highly controversial issue. As previous studies displayed, the commonly used reference genes include miR-16, miR-142-3p, 18S rRNA, miR-638, let-7a, miR-1249, miR-295, 5SRNA, U6, U6B, RNU38B, RNU43, RNU62, and SNORD43 [104]. Unfortunately, there is no single gene that is constitutively expressed in all cell types and under all experimental conditions [105]. Rigorous experimental justification for these data reported is deficient, and these data are contradicted and inconsistent in their crossing comparison, so none of them has been widely accepted as a standard control. Therefore, further studies are needed to develop a set of stable internal control genes for accurate quantification of miRNAs profiles by qRT-PCR even for each type of human cancers.

Microarray is another technique for miRNAs profiling based on nucleic acid hybridization between target molecules and their corresponding complementary probes. The miRNAs oligonucleotide probes are immobilized to glass slides through covalent crosslinking between the amino groups and the SAM (self-assembling monolayer), forming a ready-to-use miRNA microarray. When hybridized with the miRNA microarray, the isolated miRNAs labeled with fluorescent dye can emit fluorescence after being specifically bound to the corresponding probes. Through detecting the fluorescence signal intensity at different positions on the slides, relative quantities of the corresponding miRNAs can be evaluated consequently [106]. Furthermore, microarray-based techniques are particularly attractive for their powerful high-throughput function allowing for detecting the presence of a wide range of defined miRNAs simultaneously within numerous samples processed in parallel in a single experiment. However, deficiencies of microarray also exist, for example, background noise and crosshybridization problems as well as measuring only the relative abundances of previously discovered miRNAs [107].

Deep sequencing relying on next-generation sequencing machines appears to be another promising quantitative technology for miRNAs profiling, because it provides high-throughput sequencing enabling processing up to millions of sequence reads in parallel simultaneous with high speed [108]. In this method, adapters are ligated to the prepared cDNA libraries from RNA sample of interest, followed by the massively parallel sequencing of millions of individual cDNA molecules from the library. Bioinformatic analysis translates the sequence reads into miRNAs abundance levels with relative quantification and then discovers novel miRNAs differentially expressed and their associated genes by using digital approaches may be publicly available or custom-made software tools [107]. Compared with the microarray, deep sequencing technologies are not subject to the problems of background noise and crosshybridization; they measure absolute abundance over a wider dynamic range than microarrays and are not limited by array content, allowing for reflecting the actual picture of the miRNA profiles and the discoveries of novel miRNAs having not been previously identified [109].

Nanopore single-molecule technology, a novel method recently reported by several studies to electrically detect miRNAs in tissue or biofluids, requires no labeling, enzyme reactions, or amplification [110, 111]. Wang et al. showed that a nanopore sensor, which generates a target-specific signature signal by using a programmable oligonucleotide probe, were capable of quantifying subpicomolar levels of cancer-associated miRNAs and distinguishing single-nucleotide differences between miRNA family members [111]. Coinciding with the work of Wang et al., another study by Wanunu et al. used a 3 nm synthetic nanopore to detect the complex of liver miR-122a hybridized with the probe: miRNA duplex enriched through binding to the viral protein p19, and then the abundance of the duplex was quantified using a nanopore, providing accurate quantification for cellular miRNAs in tissues finally [110]. Taken together, these works by them demonstrated that the nanopore-based miRNAs techniques may with promising potential for quantitative miRNAs detection to discover more novel biomarkers and thus it would be validated as noninvasive method for early diagnosis in cancer patients.

5. Perspectives and Challenges

Years of research work have indicated that miRNAs play important regulatory roles in translational repression, eventually contributing to the cancer development. Indeed, gene profiling studies have identified a number of significant dysregulated miRNAs as "oncomiRs" or "tumor suppressors" in a variety of human cancers, and overwhelming amounts of data have strongly validated that the ubiquitously aberrant expressions of miRNAs were closely associated with the pathogenesis and progression of human malignancies. These dysregulated miRNAs constitute unique patterns of miRNome in different categories of cancers, which provide accurate fingerprints to detect and classify various cancers. Particularly, as circulating miRNAs in blood and other body fluids are readily detected by various techniques in a relatively noninvasive manner, the clinical application of miRNAs as a new generation of cancer biomarker is considered to be prospecting.

Despite the encouraging results, we are still confronted with many difficulties on the long way of transiting miRNAs as biomarker from bench to bedside. First, exactly as the proposing of the misconceptions that the 98% in human genome is noncoding and thus junk DNA, miRNAs are probably only the tip of the iceberg of noncoding genes in our complex genome. More miRNA transcripts remain to be excavated extensively. Second, more fundamental investigations are needed to further elucidate the exact miRNAs' functional roles in cancer biology. Apart from the pathway by targeting mRNAs for cleavage or translational repression, if

there are any other unrevealed mechanisms led to the translational repression, or even any other undiscovered regulation functions more than posttranscriptional regulation. Third, it is needed to further clarify the potential biological functions of circulating miRNAs, particularly the mechanisms of MVBs-contained miRNAs for cell-cell communication, tumor immune evasion, and tumor microenvironment. After all, the underlying mechanism of miRNAs secretion or the mechanisms of the selectivity of miRNAs packaging are still elusive. Fourth, more consistent and reliable miRNA signatures or miRNome in both FFPET and circulation are urgent to be established by adequately large sample size of cohort studies on multiple, independent populations. Fifth, methods for the detection and analysis of miRNAs should be further optimized. To ensure the miRNAs' detection results are as consistent as possible, a set of technical methods both miRNA extraction procedures and technology platforms should be compared with each other or optimized for different types of cancer patients. In addition, it is worth noting that further studies are needed to develop a set of stable inner reference genes to accurately quantify miRNAs even for each type of human cancers.

Despite such challenges that are a lot, the potential of miRNAs as a new class of cancer biomarkers is attractive and cannot be underestimated. MiRNAs are being expected to bring an exciting new dimension to the field of clinical management for human cancers in the near future.

Conflict of Interests

No potential conflicts of interests were disclosed regarding the publication of this paper.

Authors' Contribution

Huiyin Lan and Haiqi Lu contributed equally to the work.

Acknowledgments

This work was supported by National Natural Science Foundation of China (nos. 81301706, 81372178), Department of Health in Zhejiang Province (WKJ2012-2-013; 2013RCA029), and Zhejiang Provincial Natural Science Foundation of China (nos. Y13H160010 and LR12H16001).

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Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 731479, 10 pages
http://dx.doi.org/10.1155/2015/731479

Review Article

Challenges in Using Circulating miRNAs as Cancer Biomarkers

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Received 4 June 2014; Accepted 6 August 2014

Academic Editor: Paolo Gandellini

In the last years, circulating miRNAs have emerged as a new class of promising cancer biomarkers. Independent studies have shown the feasibility of using these small RNAs as tools for the diagnosis and prognosis of different types of malignancies as well as for predicting and possibly monitoring treatment response. However, despite an initial enthusiasm for their possible clinical application, widespread inconsistencies have been observed among the studies, and miRNA-based tools still represent the object of research within clinical diagnostic or treatment protocols. The poor overlap of results could be explained, at least in part, by preanalytical and analytical variables and donor-related factors that could generate artefacts, impairing an accurate quantification of circulating miRNAs. In fact, critical issues are represented by nonuniform sample choice, handling, and processing, as well as by blood cell contamination in sample preparation and lack of consensus for data normalization. In this review, we address the potential technical biases and individual-related parameters that can influence circulating miRNA studies' outcome. The exciting potential of circulating miRNAs as cancer biomarkers could confer an important advance in the disease management, but their clinical significance might not be proven without a global consensus of procedures and standardized protocols for their accurate detection.

1. Introduction

MicroRNAs (miRNAs) are highly conserved single-stranded small RNA molecules (~19–22 nucleotides long) that play a key role in posttranscriptional gene regulation [1, 2]. To date, more than 2,500 human miRNAs have been identified (miRBase V20, [3]). These small RNA molecules bind the 3′UTR region of their messenger RNA (mRNA) targets, inducing posttranscriptional gene regulation by either inhibition of translation or mRNA degradation [2, 4, 5], and are being widely investigated with multiple approaches in oncology [6]. Indeed, in the last decade their implication in different types of cancer has become clear [7], generating a great deal of enthusiasm in clinical and scientific communities. miRNA signatures from normal cancer tissues and metastases have been used to classify different types of cancer and have been shown to represent potential biomarkers for diagnosis, prognosis, and therapy [8, 9]. In addition, recent studies have shown that miRNAs can be released from cells (encapsulated in exosomes and/or bound to proteins and lipoproteins) and enter the circulation as a consequence of apoptotic and necrotic cell death, as well as of an active release [10–15]. As a result of miRNA release from cells, these molecules have been also found in several human body fluids (including blood, serum, plasma, urine, saliva, seminal fluid, and pleural effusion) [16], in a stable form protected from endogenous RNAses, thus making circulating miRNA levels well suited for noninvasive analysis in patient samples [17–19]. Independent studies have reported the feasibility of using circulating miRNAs as promising disease biomarkers and, in the context of malignancies, they have shown a potential as molecular tools for detection, prognosis, and treatment decision making of various cancers [17–23]. In this review, we focus on studies and technical issues concerning the evaluation of circulating miRNAs in blood, plasma, and serum, the biological fluids most analyzed as a source of cell-free miRNAs.

2. Circulating miRNAs as Promising Cancer Biomarkers

In the last few years, there has been increasing interest in circulating miRNAs as cancer biomarkers, due to their high

stability, their putative capability to be more informative than mRNA, and the noninvasiveness of their detection. Since their discovery in body fluids, considerable effort has been directed to investigate the relevance of these small RNAs in different diseases, and now there is much evidence of their potential clinical relevance as cancer biomarkers in different types of malignancies. The first study that identified specific circulating miRNAs associated to cancer was by Lawrie et al. [24], who in 2008 found high levels of miR-155, miR-210, and miR-21 in patients with diffuse large B-cell lymphoma and demonstrated a significant correlation between high levels of miR-21 and relapse-free survival. A literature survey concerning the principal findings related to the usefulness of specific circulating miRNAs (or miRNA signatures) as diagnostic, prognostic, and/or predictive parameters in different cancer entities has recently been published (reviewed in [23]). Plasma or serum miRNAs appear to display a promising potential mainly in the diagnosis of different solid tumors at preoperative level, thus suggesting the possibility of their utility as early-diagnostic tools. Accordingly, a very recent study performed in a large cohort of smoker individuals provided evidence that specific ratio-based miRNA signatures (including 24 distinct miRNAs assayed in plasma samples) have significant diagnostic and prognostic power to anticipate the detection of malignant lung cancers and to predict tumor aggressiveness [25].

It should be pointed out that circulating miRNAs might not only represent promising noninvasive diagnostic and prognostic tools but they could also be used to predict and monitor the efficacy of anticancer treatments. In this context, recent correlative studies within neoadjuvant or adjuvant chemotherapy trials identified many circulating miRNAs as associated with response to treatment and drug resistance [23]. For example, in HER2-positive breast cancer patients undergoing neoadjuvant therapy, plasma miR-210 levels were found to be associated to trastuzumab sensitivity, thus suggesting that plasma miR-210 levels might be used to predict and monitor response to therapies containing the monoclonal antibody [26]. In the context of adjuvant chemotherapy regimens, it has been shown that serum miR-21 levels can predict the benefit of gemcitabine treatment in advanced pancreatic cancer patients, suggesting that the miRNA might be used as a predictor of the chemosensitivity to this nucleoside analogue [27]. However, despite the fact that several published papers demonstrated the feasibility of using circulating miRNAs as putative cancer biomarkers, many preanalytical and analytical aspects, as well as donor-related factors, can interfere with accurate circulating miRNA quantification, and future studies have to take them into consideration.

3. Preanalytical and Analytical Variables Affecting Circulating miRNA Studies

In a recent work by Leidner et al. [28], the authors highlighted for the first time the widespread inconsistency across circulating miRNA studies, cautioning the scientific community about the huge variety of methodological parameters impairing circulating miRNA evaluation. Figure 1 summarizes the main preanalytical and analytical variables interfering with circulating miRNA analysis that are examined in detail in the following sections.

3.1. Sample Choice/Starting Material. Quantification of miRNAs present in the circulation can be performed from different sources of materials (i.e., whole blood, plasma, and serum). In a recent study, Pritchard et al. [29] demonstrated the influence of blood cell miRNAs (contained in red and white blood cells and in platelets) in circulating miRNA analysis. They verified that blood cells are substantial contributors to the presence of miRNAs in the circulation, markedly altering specific miRNA levels. For this reason, whole blood may not be considered a preferential biological fluid for circulating miRNA detection. However, even for the studies performed in plasma (liquid part of the blood containing fibrinogen and collected in the presence of an anticoagulant) and serum (liquid part of the blood obtained after the blood has clotted), the complete removal of cellular components that could impair accurate miRNA quantification is mandatory. Many research groups have compared different plasma/serum preparation protocols, analyzing differences between the two types of biological fluid in terms of circulating miRNA amount, but univocal results have not been obtained. In fact, McDonald et al. [30] reported that plasma presents higher level of specific miRNAs than does serum, which is in contrast to results shown by Mitchell et al. [17] and by Wang et al. [31]. To explain this difference, the latter group has hypothesized that the higher miRNA concentration observed in serum compared to plasma may be due to miRNA release from blood cells (such as platelets) during the coagulation process [31]. In addition, the inconsistency between the two body fluids could also be explained by the different methods used for the separation of plasma and serum from whole blood, which could lead to different amounts of blood cell contamination in these fluids. In fact, it is known that different centrifugation protocols can produce platelet-rich or platelet-poor plasma [32, 33]. Specifically, considering plasma preparation protocols, many research groups recommend a double step of centrifugation (even though slight differences regarding centrifugation speed and time can be found in the different protocols) for plasma separation in order to limit platelet contamination [33–35]. In addition, the choice of the anticoagulant used to collect blood for plasma preparation could impair further analysis. For many years, the use of heparin in blood collection tubes dedicated to RNA analysis has been avoided since the anticoagulant inhibits PCR amplification [32, 36–38]. However, we have recently demonstrated that if adequately treated (i.e., performing digestion with heparinase on extracted RNA), plasma samples derived from blood collected with heparin tubes may be suitable for miRNA expression analysis, without affecting miRNA detection performance [39]. EDTA and citrate, two anticoagulants commonly added to blood collection tubes, are suitable for miRNA detection without any additional treatment [38]. However, EDTA collection tubes should be

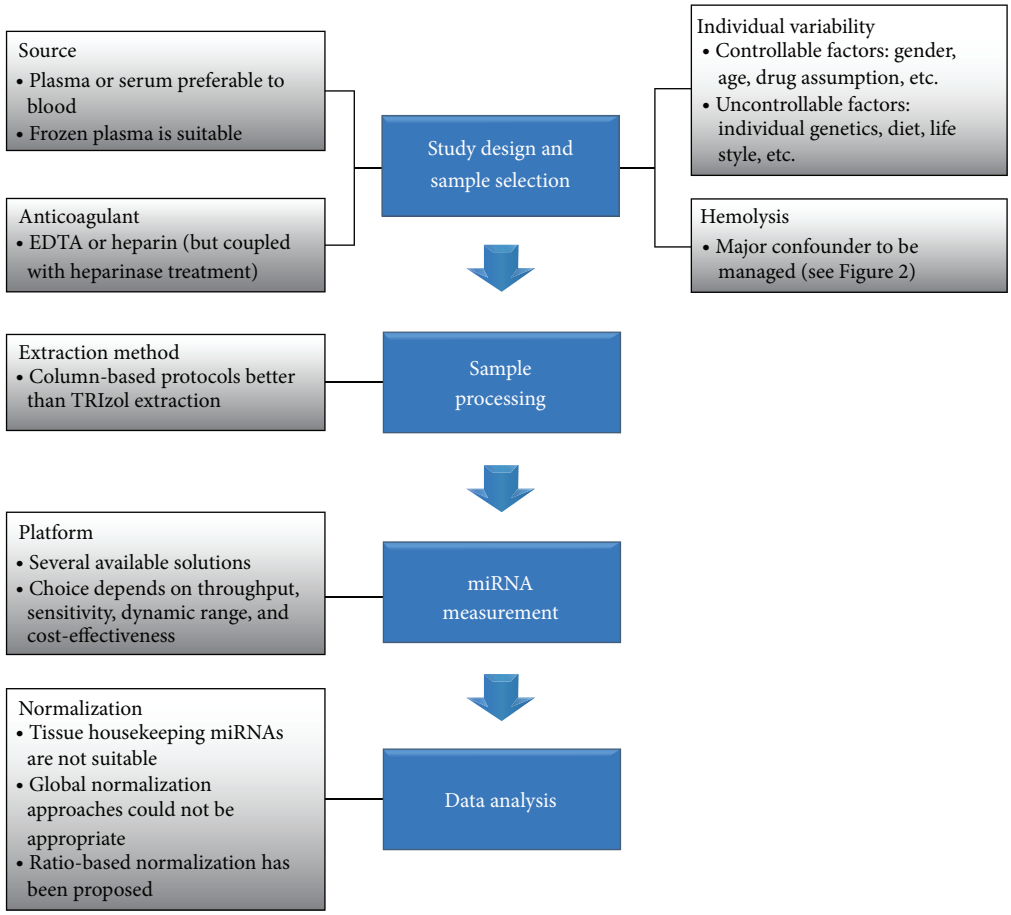

Figure 1: Flow chart summarizing the different steps and the main preanalytical and analytical confounders in circulating miRNA detection.

preferred to citrate because the latter may trigger hemolysis (see the following section about hemolysis interference on miRNA analysis) [40]. Finally, interesting findings have been obtained comparing fresh and frozen fluids. In accordance with the fact that miRNAs are highly stable in the circulation, minimal or no differences have been found between fresh and frozen specimens, even after repeated freeze-thaw cycles [17, 33, 41–43]. Nevertheless, it is advisable to avoid any unnecessary freeze-thawing, since, if miRNA degradation occurs (even to a limited extent), poorly represented miRNA species could be missed. Other critical concerns regarding the starting material have been raised. For example, since skin cells (containing tissue-specific miRNAs) could also contaminate the first blood draw [32], it would be more advisable to discard it. Moreover, the fact that plasma/serum samples from patients with an inflammatory process could be contaminated with a high number of white blood cells should be taken into account [32]. Exclusion of such patients from miRNA analysis could thus be advisable. In this section the technical issues known to be strictly associated with blood collection and serum/plasma preparation have been examined, but we cannot exclude a putative impact of other unknown factors on miRNA profiling. Thus, to minimize confounding effects, at least specific standard operating procedures (SOPs) for blood collection and plasma/serum preparation are needed.

3.2. Hemolysis Interference. It has been demonstrated that quantification of plasma/serum miRNAs can be impaired by the contamination of erythrocyte-specific miRNAs [30, 44, 45]. Such findings have important implications for the interpretation of circulating miRNA profile results. In fact, a recent study showed that, among tumor-associated circulating miRNAs reported in the literature, 58% were highly expressed in blood cells and that hemolysis alters circulating miRNA levels by up to 50-fold [29], starting to have an influence from an erythrocyte contamination as little as 0.008% [44]. To limit hemolysis impact, the main objective would be to prevent the phenomenon. *In vivo* hemolysis cannot be avoided, but by following a few guidelines together with the definition of SOPs, *in vitro* hemolysis could be dramatically reduced. Since it has been widely proven that the mere visual detection of samples (pink or red coloration) is not sufficiently sensitive in this context, the identification of hemolyzed samples remains a crucial issue for biomarker research [46]. In the field of circulating biomarker and miRNA research studies,

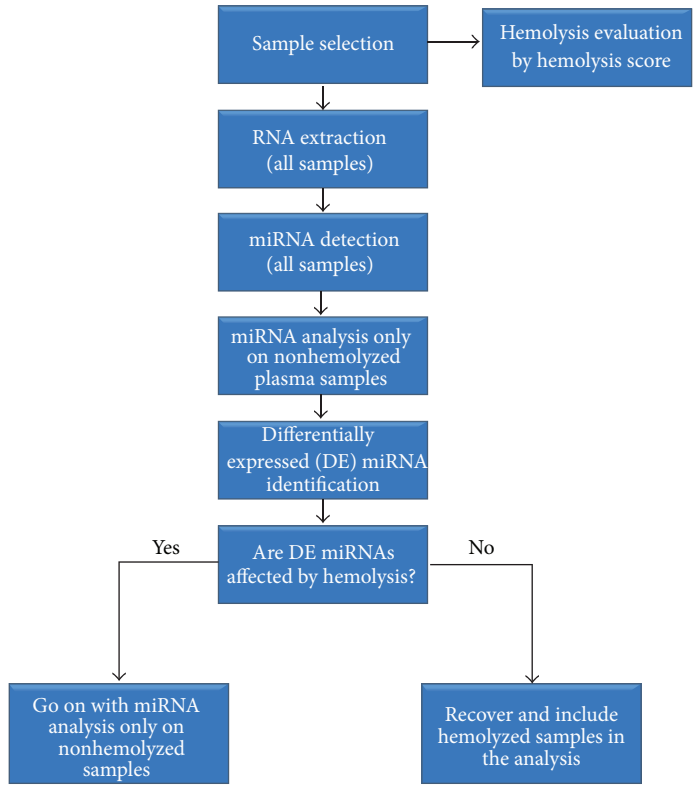

Figure 2: Proposed workflow for circulating miRNA studies.

many approaches have been applied to identify hemolyzed plasma/serum specimens. Spectrophotometric measurement of the main oxyhemoglobin peak absorbance at wavelength (λ) = 414 nm could in theory represent a simple way to identify hemolyzed samples [44]. However, measuring absorbance at λ = 414 nm cannot be used as a robust method to identify hemolyzed samples, since, for example, lipemia in plasma interferes with hemoglobin absorbance, causing an increase in absorbance values at λ = 414 even in the absence of any hemoglobin-related peak. We have recently proposed a simple, sensitive, and rapid to use, lipemia-independent, spectrophotometrically based procedure able to identify, as hemolyzed, those samples containing at least 6.1 mg/dL of free hemoglobin. The preanalytical method can be performed using a NanoDrop spectrophotometer (which requires a small volume for analysis) and is based on absorbance measurements at λ = 414 nm and at λ = 385 nm (as a lipemia indicator) [47]. Otherwise, alternative methods to identify hemolyzed specimens based on the detection of erythrocyte-specific miRNAs have been recently proposed [35, 48]. Such approaches are sustained by the recent finding that a very low percentage of hemolysis can elicit a considerable increase in erythrocyte-specific miRNA levels [44, 45]. Therefore, erythrocyte-specific-miRNA-based methods are in theory highly sensitive to identify hemolyzed samples. However, contrary to preanalytical methods, miRNA quantification requires sample processing (RNA extraction) and analysis (e.g., Real-Time PCR), which is sample and time consuming. Thus, miRNA-based procedures may not be suitable if a limited amount of plasma/serum specimens is available. In addition, circulating miRNA expression can be affected by several factors, including individual variability and medical conditions (see the following section). Therefore, miRNA-based methods could turn out to be not sufficiently accurate to discriminate hemolyzed samples from samples presenting altered erythrocyte-contained miRNA expression due to other conditions.

Once hemolyzed samples are identified (independently of the approach used), the choice of removing them from miRNA analysis could be questionable. In fact, in our opinion, circulating miRNA studies should be performed following the workflow proposed in Figure 2. Briefly, after hemolysis evaluation, all samples have to be subjected to miRNA detection, but only nonhemolyzed samples have to be initially used for marker discovery. Then, when interesting miRNAs have been identified, researchers should determine whether (and in what extent) the identified miRNAs are hemolysis affected. If identified miRNAs are not influenced by hemolysis, hemolyzed samples could be recovered and included in the analysis.

3.3. Extraction Methods. Several extraction methods have been used in circulating miRNA studies. They could be divided into two different main categories: guanidine/phenol/chloroform-based protocols and commercial kits using columns or beads. Several research groups have investigated the miRNA extraction issue by comparing different

protocols. First of all, McDonald et al. [30] measured four plasma/serum miRNAs to be able to compare four different extraction methods. They found that mirVana PARIS kit (Life Technologies) and miRNeasy Mini Kit (Qiagen) had the highest mean yield for miR-15b and miR-16 and for mi-24 and cel-miR-39, respectively. Similar findings have been obtained by Sourvinou et al. [49], who demonstrated that the mirVana PARIS kit and miRNeasy Mini Kit produced the highest yield of recovery for a spike-in miRNA compared to TRIzol extraction, with the first kit also obtaining a better performance than miRNeasy. However, Survinou's results were in contrast with those obtained by Kroh et al. [32] demonstrating that RNA extraction with miRNeasy led to a 2- to 3-fold increase in RNA yield compared to mirVana. More recently, Moret et al. [34] compared four different protocols for RNA extraction (using TRIzol-LS, mirVana PARIS kit, miRNeasy Serum/Plasma kit, and TRIzol-LS following mirVana kit) starting from both fresh and frozen plasma samples. The authors demonstrated that column-based methods performed better than TRIzol extraction, due to the presence of organic and phenolic contaminants in the TRIzol-extracted RNA. By comparing commercial column-based kits, they found that the miRNeasy kit obtained the highest RNA concentration and that the addition of a RNA carrier to the lysis buffer improved RNA recovery. According to such findings, Page et al. [42] compared three different commercial kits for RNA extraction and demonstrated that the miRNeasy Serum/Plasma kit produced the highest recovery of circulating RNA as regards quantity (in terms of amount of total RNA and evaluated as threshold cycle (CT) for individual miRNAs) and quality (addressed as RNA integrity). Of note, the authors also suggested that increasing starting material volume caused a less efficient recovery of miRNAs. More recently, another commercial kit has been developed for biofluid-specific RNA extraction (named miRCURY biofluids kit, Exiqon). In the only report comparing RNA extraction performed by this commercial kit with other RNA isolation methods, the authors found that the Exiqon biofluid-specific kit outperformed both Exiqon miRCURY cell and plant and Qiagen miRNeasy Serum/Plasma kits [50]. Notably, another benefit of this extraction method is the lack of a phase-separation step, thus leading to short processing time and ease of use. Despite the dissimilar results obtained in different studies, a common warning emerged about the use of TRIzol to extract miRNAs from body fluids. Conversely, no consistent results have been obtained regarding differences in column-based methods, suggesting that a great effort is still needed in comparing different extraction methods and working toward standardization.

Another warning that emerged from the studies regards plasma RNA quantification. Many research groups, independently of the specific miRNA detection methods used, have performed plasma RNA quantification by NanoDrop spectrophotometer before analyzing it for miRNA contents. However, RNA extracted from plasma/serum is undetectable by using NanoDrop spectrophotometer [30, 32, 34]. In addition, it should be considered that many diseases, including cancer, may cause a release of nucleic acids in the circulation, leading to a significant higher level of circulating RNA in cancer patients than in healthy subjects [51]. Thus, for circulating biomarker detection analysis, it could be more accurate to use an equal volume input rather than the same amount of RNA [52].

3.4. Detection Platforms. The accurate measurement of miRNAs, both in tissues and in circulation, poses several challenges related to their short lengths, their GC content, and the high sequence similarity within miRNA families [29]. In circulating miRNA quantification, the low abundance of these nucleic acids in body fluids has to be added to such concerns. Nonetheless, several technologies able to measure from one to thousands of miRNAs have been applied to the study of circulating miRNAs. Here we focus on three approaches that allow a medium/high throughput: amplification-based methods, hybridization-based miRNA microarrays, and massively parallel miRNA sequencing (miRNA-seq).

Amplification-Based Methods. Quantitative reverse transcription PCR (qRT-PCR) is a well-established method, considered as the "gold standard" for miRNA detection and is often intended as a single miRNA assay. However, assays enabling the measurement of a panel of miRNAs have been developed, such as microfluidic cards and arrays (e.g., TaqMan OpenArray and TaqMan TLDA microfluidic cards by Applied Biosystems, miRCURY LNA qPCR by Exiqon, miScript miRNA PCR Array by Qiagen). Despite the differences in the type of technology offered, all these panels allow the detection of about 700 miRNAs with high sensitivity, high specificity (single nucleotide discrimination), and high dynamic range, starting from less than 100 ng of RNA input. In addition, the data generated from this type of analysis are in terms of CT and, thus, do not necessitate further bioinformatics manipulation. However, such technology also presents some disadvantages, such as the possibility to detect only annotated miRNAs and the fact that only a medium throughput can be reached [23, 29, 52, 53]. Finally, it should be pointed out that a comparison between the two most commonly used qRT-PCR arrays (i.e., TLDA card and miRCURY array) demonstrated that both plates exhibit high reproducibility between technical replicates and that there was a significant correlation between results obtained with the two platforms [42].

Hybridization-Based miRNA Microarrays. Several commercial miRNA microarray platforms (e.g., GeneChip miRNA Array by Affimetrix and Human miRNA Microarray by Agilent) are available and can be used to measure circulating miRNAs. They are able to analyze up to thousands of miRNAs in one assay, but only among those already known and annotated in miRBase [3], starting from about 100 ng of total RNA. The methodology is high-throughput and is less expensive than amplification-based arrays, but it is typically considered to have lower dynamic range and specificity than qRT-PCR and miRNA-seq [23, 29, 52, 53]. Some cross-platform studies performed by our group and others [54, 55] have already highlighted limitations in correlation between results obtained with different systems for tissue miRNAs. The Agilent system emerged as one of those obtaining

the highest performances and is probably the most commonly used. In a pilot study that we recently published, the feasibility of such a platform in miRNA detection also from archival plasma samples was evaluated [56]. We found a very high correlation between technical replicates and a good correlation between different batches. However, we also noticed that since circulating miRNAs give lower signals than do tissue miRNAs, the increase in chip background, when approaching the expiration date, dramatically reduced the number of miRNAs that can be detected [56].

miRNA-Seq. miRNA quantification by miRNA-seq is an expanding approach useful especially for miRNA families differing for a single nucleotide as well as for isomiRs of varying length. It has the great advantage of allowing detection of both known and novel miRNAs, since only a portion of the miRNA population is presently *bona fide* annotated on miRBase. Standard protocols require a relatively large amount of starting material (~1 μg of RNA), which is hard to isolate from serum or plasma. However, adapted protocols have been recently proposed, giving the opportunity to obtain miRNA-seq data from as little as 5 ng of RNA extracted from plasma samples [57]. It is plausible to speculate that, in the near future, nucleic acid researches will be probably all performed using this methodology. However, for now, this technology is expensive and necessitates special equipment and expert bioinformaticians, so it cannot be considered as user and lab friendly.

3.5. Normalization and Data Analysis. Once we have obtained the data, the next challenge is normalization. As is known, technical variability among samples is expected due to issues such as variation in the starting material, RNA extraction, or reaction efficiency during labeling procedures or hybridization.

Housekeeping transcripts used for tissue miRNA analysis (e.g., RNU6 and RNU48) cannot be consistently detected in the circulation for their extensive RNAse-mediated degradation [31]. miR-16 is among the proposed miRNA housekeeping and probably is the most commonly reported in the literature. However, it is one of the most affected by hemolysis [29, 44] and thus may not be considered a preferential miRNA reference for data normalization. Independent studies have proposed other miRNAs as candidate housekeeping [58, 59], but a global consensus is still lacking.

In all assays measuring a relatively large number of miRNAs, global normalization methods can be applied. For amplification-based array data, a commonly used approach is to use a global measure of miRNA expression profiles, such as the mean or the median, as a calibrator. However, the number of detected miRNAs in the circulation with PCR-based methods is usually around 100, likely not large enough to apply a global normalization approach. Moreover, after the discovery phase, a limited number of candidate miRNA markers usually need further validations, both in technical and in independent case series. However, the same data normalization approach will not be applicable in such subsequent validation studies (usually performed with single assays or a custom card containing a limited number of miRNAs). To overcome this problem, Pizzamiglio et al. [60] recently proposed a comprehensive procedure that starting from a data-driven normalization method based on amplification-based array data identifies a small set of miRNAs to be used as reference for data normalization in view of subsequent validation studies. Alternatively, Kroh et al. [32] suggested a miRNA absolute quantification using a standard curve generated with a synthetic miRNA (synthetic oligonucleotides) to carry out by RT-PCR in parallel with biological samples. This approach appears feasible for individual miRNA marker quantification but cannot be used for multiplex miRNA evaluation. The same authors also proposed a normalization strategy based on spiked-in synthetic RNAs. They recommended the use of three *C. elegans* spiked-in control miRNAs during the denaturation of plasma/serum samples to normalize the biological variability that could affect the extraction efficiency. The synthetic exogenous miRNAs could be measured by RT-PCR together with target miRNA and their mean CT values could be used to express the miRNA data as median-normalized CT values [32].

Normalization is a key point also in microarray-based data [61]. Global normalization methods developed for gene expression analysis (e.g., lowess or quantile normalization) are regularly applied to miRNA expression profiling. However, the reduced number of features (compared with the number of genes measured within a gene expression microar ray) raises some doubts about the appropriateness of such methods in the context of miRNA profiling, and new strategies have been proposed, such as a modified least-variant set normalization for miRNA microarray [62].

Sequencing-based quantification of miRNAs is a relatively new technology compared to qRT-PCR and microarrays, and there is a minor standardization of the optimal workflow to be used. At present, the standard method to normalize miRNA-seq data is to divide the reads of each miRNA by the total number of reads mapping to the genome or to the known miRNAs. Methods developed for microarray normalization (e.g., lowess or quantile normalization) have also been applied to miRNA-seq data [63].

However, it is noteworthy that all global normalization methods (independently of the platform used) are based on the assumption that the same total amount of miRNAs is expected in all samples and that only a small percentage of miRNAs is differentially expressed, as both up- and downregulated. Such assumptions are often inappropriate for studies aimed to identify cancer-related circulating biomarkers, because in neoplastic patients, as already mentioned, mainly an increase in circulating miRNAs is expected compared with unaffected individuals. This implies that whereas a general upregulation is present in raw data, not only due to technical biases to be removed but also due to the biological phenomenon under investigation, such a trend could disappear after application of classical normalization strategies. Such a problem was clearly demonstrated in the case of an expected general miRNA downregulation as a consequence of inducible deletion of Dicer1 [64]. Consequently, although widely used in the literature, classical normalization methods

are not suitable in many situations when analyzing circulating miRNA profiling data. This implies that further research is needed to identify suitable methods, and, in this context, a ratio-based approach has been proposed [65], that is, like using, in turn, all miRNAs as housekeeping. Such an approach has the advantage of limiting *a priori* assumptions; however, high redundancy is generated in the data and it could become not trivial to establish which miRNA has a relevant role and which functions as a calibrator.

3.6. Individual Factors. Besides the potential technical biases discussed above, other critical variables that could have deep implications in the accurate interpretation of circulating miRNA biomarker studies are related to the intrinsic inter-individual variability and to the influence of disease-independent factors. Facing circulating miRNAs as cancer biomarker molecules, the first source of variability to be considered is related to the tumor itself. In fact, since miRNA expression patterns are extremely specific for individual cancers, miRNA circulating signatures are expected to be extremely specific for different cancer types or molecular subtypes and for distinct tumor features (e.g., hormone responsiveness, oncogene overexpression, etc.). Moreover, recent evidence suggests that individual variability, such as race [66] and gender [67, 68], as well as external factors and life-style, such as drug assumption [69], smoking habits [70], diet [71, 72], physical activity [73], and many other conditions, could contribute to affect miRNA levels in the circulation. Although some of the interindividual variables, such as race, gender, and age, can be easily analyzed and properly considered, there is also a large array of individual and environmental factors that are hardly verifiable and correctly taken into consideration in the evaluation of circulating miRNAs as disease biomarkers. For example, polymorphisms in miRNA chromosome loci have been described as responsible for differential miRNA expression. In particular, copy number variations (CNVs) occurring in coding regions of the genome also contain miRNA genes across all chromosomes [74]. CNVs that cause miRNA deregulation have been shown to play a role in the occurrence of different diseases [75, 76], and it could be plausible to hypothesize that this type of polymorphism contributes to differences in miRNA expression among individuals, probably also influencing the levels of specific circulating miRNAs.

Among the donor-related factors, diet can be considered as a critical potential confounder of miRNA profiling studies. In fact, miRNA expression profiles have been shown to be affected by a large number of dietary constituents such as resveratrol, curcumin, isoflavones, catechins, indoles, and many other compounds including vitamins A and D (reviewed in [77]). It has also been postulated that many of the miRNAs contained in food, once entered in the circulation, would be largely indistinguishable, at sequence and/or function levels, from endogenous miRNAs, and, in addition, they could cause changes in miRNA concentration through homeostatic mechanisms that regulate circulating miRNA-containing vehicles (including lipoprotein particles and exosomes) [72]. The influence of diet as well as that of other external factors such as the physical activity cannot be taken into account during case selection for circulating miRNA studies. Thus, in the evaluation of results, it has to be considered that miRNA levels could represent mainly a summary of individual behavior rather than of what one suffers from. Even though no extensive information is available, it is plausible that also intraindividual variability could affect circulating miRNAs evaluation because miRNA amount may vary in the same individual over time. Notably, pharmacological treatments could have a profound influence on circulating miRNA levels. A very common medication such as aspirin has, for example, been proven to reduce miR-126 levels in the plasma of patients with type 2 diabetes [69]. Importantly, the effects of chemotherapy and, possibly, of different type and/or treatment schedules of anticancer therapies on circulating miRNA levels could have a profound relevance when such molecules are under evaluation as prognostic cancer biomarkers or to monitor tumor progression. However, the effect of pharmacological treatments on circulating miRNA levels points out an important premise for their use as pharmacodynamic markers.

4. Conclusions

Circulating miRNAs hold great promise as cancer biomarkers. However, as we have discussed in this review, before translation into clinical practice, all circulating miRNA findings require further steps of validation and a proper standardization of all preanalytical and analytical procedures, in order to control for all potential technical biases. The development and the application of SOPs regulating procedures related to circulating miRNA analysis are in fact an imperative issue to successfully translate miRNA signatures in clinically meaningful tests. Standardized and consistent methods need to be applied at many levels, from whole blood collection to plasma/serum preparation, handling, and banking to RNA extraction and miRNA quantification, in order to keep at minimum the interlaboratory differences that could have high repercussion in miRNA results and also limiting incoherencies among different users. Once consensus procedures have been defined and included in circulating miRNA profiling, it will be possible to accurately interpret and compare different study results and, possibly, to identify those miRNAs acting as novel specific and sensitive cancer biomarkers. However, it is worth keeping in mind that if methodological parameters could be correctly considered in study design, some individual and environmental factors could not be properly evaluated and taken into account during circulating miRNA analysis, thus plausibly influencing circulating miRNA application in clinical practice.

Conflict of Interests

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

Acknowledgments

This work was supported by a fellowship and grant from the Associazione Italiana Ricerca sul Cancro (Special Program "Innovative Tools for Cancer Risk Assessment and Early Diagnosis," 5X1000/5 per mille, no. 12162) (fellowship for Paola Tiberio and grant for Maria Grazia Daidone), by grants from the Regione Lombardia-Call ricerca indipendente 2010 (no. 11902), from the EurocanPlatform and from the Italian Interministerial Committee for Economic Planning and the Ministry of Education, University and Research (Nanomax Flag and NEWTON projects), and by funds obtained through an Italian law that allows taxpayers to allocate a 0.5 percent share of their income tax contribution to a research institution of their choice. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the paper. None of the investigators have any potential conflicts of interest to disclose. The authors thank Mrs. Betty Johnston for proofreading the paper.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2015, Article ID 717509, 12 pages
http://dx.doi.org/10.1155/2015/717509

Review Article

Analysis of Chromosome 17 miRNAs and Their Importance in Medulloblastomas

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Received 6 June 2014; Revised 16 November 2014; Accepted 16 November 2014

Academic Editor: Elisa Giovannetti

MicroRNAs (miRNAs) are small sequences of nucleotides that regulate posttranscriptionally gene expression. In recent years they have been recognized as very important general regulators of proliferation, differentiation, adhesion, cell death, and others. In some cases, the characteristic presence of miRNAs reflects some of the cellular pathways that may be altered. Particularly medulloblastomas (MB) represent entities that undergo almost characteristic alterations of chromosome 17: from loss of discrete fragments and isochromosomes formation to complete loss of one of them. An analysis of the major loci on this chromosome revealed that it contains at least 19 genes encoding miRNAs which may regulate the development and differentiation of the brain and cerebellum. miRNAs are regulators of real complex networks; they can regulate from 100 to over 300 messengers of various proteins. In this review some miRNAs are considered to be important in MB studies. Some of them are miRNA-5047, miRNA-1253, miRNA-2909, and miRNA-634. Everyone can significantly affect the development, growth, and cell invasion of MB, and they have not been explored in this tumor. In this review, we propose some miRNAs that can affect some genes in MB, and hence the importance of its study.

1. Medulloblastoma

Medulloblastoma (MB) is the embryonal tumor of the cerebellum and the most common intracranial embryonal tumor. It develops in the posterior fossa, where there are subconscious motor nuclei of great importance, such as those of balance, posture, speech, swallowing, and other important functions.

It is the tumor with the second highest incidence in childhood and constitutes 20% of all childhood central nervous system (CNS) tumors, 70% of which occur in patients under 16 years. Its peak incidence is at age 7, but there are also reports of prenatal and neonatal cases [1, 2]. Seventy-five percent of these tumors are located in the vermis and show very characteristic features in neuroimaging, which allows identifying them [3]. Despite their intratumoral heterogeneity, the variety of histological subtypes, and the irregularity and variety of immunohistochemical results for different proteins, the differential diagnosis can be relatively simple with adequate clinical and radiological information, even in the case of an intraoperative frozen biopsy or a partial resection. High cell density, abundant mitosis, and apoptosis, as well as a great tendency to subarachnoid infiltration, are common features in all variants of MB [2, 3]. The identification of these subtypes has sometimes prognostic implications or can involve pathogenically separate groups. The World Health Organization [4] currently classifies MB in the following:

(1) classic MB;
(2) desmoplastic/nodular MB;

(3) MB with extensive nodularity;

(4) large-cell MB;

(5) anaplastic MB.

2. MB Subtypes

Before the 90s, MB was considered as a histologically uniform entity. In the year 1992, Giangaspero et al. identified a large-cell MB that also corresponded to a more aggressive MB group [5]. The cells in these tumors show large vesicular nuclei with prominent nucleoli and also frequently show amplification of the oncogene c-myc and an isochromosome 17q. This subtype of MB is associated with a poor prognosis; it spreads easily through the cerebrospinal canal. In 2000, Brown et al. analyzed a large group of 495 MB and found a large number of cases with large/anaplastic nuclei that also had different histologic and cytogenetic features [6]. Later, Lamont et al. (2004) demonstrated the usefulness of combining histopathologic features and molecular alterations to stratify patients with MB [7]. This group of patients with anaplastic MB and loss of chromosome 17 has lower survival rates than patients without loss of this chromosome. Afterwards, it was shown that chromosome 17 and its alterations are important markers to stratify patients with respect to their prognosis [8].

Finally, broader and deeper studies of MB using gene expression profiles, gene-microarray analysis, and gene polymorphism analysis, among other methods, have led specialists to establish a consensus that MB can be classified into the following 4 subgroups [9]:

subgroup 1, wingless-type (WNT);

subgroup 2, sonic hedgehog (SHH);

subgroup 3;

subgroup 4.

Each of these MB subgroups has characteristic molecular profiles and genetic alterations.

2.1. WNT Subgroup. To this subgroup belong between 10 and 15% of all MB cases [10]. This subgroup is characterized because 90% of the cases belonging to it present the typical histology of an MB; the patients are older than 3 years old (it can also occur in adults but never in children under 3 years); it has a good prognosis and rarely shows metastasis [11, 12].

2.2. SHH Subgroup. This subgroup comprises 25–30% of all MB cases. It is characterized by the presence of a desmoplastic reaction in histopathological analysis (40%). It occurs in patients under 3 years old or in very young adults over 16 years [13]. Half the adult cases of MB also belong to this subgroup. The prognosis of MB in this subgroup is good in patients under 3 years old and very young adults [14].

2.3. Subgroups 3 and 4. These subgroups were originally designated as non-WNT/non-SHH. The cases have some similarities in clinical presentation and molecular profile. Most of them have a typical histological pattern; a desmoplastic reaction may occur but is rare. Most cases are in children in both age groups. Both subgroups show a similar frequency of metastasis, but the cases in subgroup 3 have a poor prognosis while the cases in subgroup 4 have an intermediate prognosis [10, 14, 15].

Several cytogenetic and molecular biological studies have confirmed that the etiology of MB is related to the deletion of the short arm of chromosome 17 (17p) and that this deletion occurs in 25–50% of cases. Thus, it is important to study the genetic and molecular mechanisms regulating noncoding RNAs that form complex networks, such as in microRNAs (miRNAs), with at least 100 different genes. Thus, using a different approach to study and analyze MB cases has interesting and important consequences, as will be described later [16].

3. Importance of Chromosome 17

Chromosome 17 is one of the 23 pairs of human chromosomes; the anomalies and functions that have been studied with respect to the expression of the genes of this chromosome affect, among other organs, the nervous system, particularly the differentiation and cell and tissue maturation process.

In addition, several articles and databases that align the sequence of miRNAs and estimate mRNA targets suggest that much of the miRNAs encoded on chromosome 17 have regulatory activity at different stages of neuronal differentiation.

Mutations in the tumor protein 53 gene (TP53) and deletions in the 17p region are among the most common disorders encountered in primary solid tumors of different histological origin [17]; both types of disorders induce genomic instability in transformed cells. It has also been observed that the inactivation or loss of the TP53 gene is associated with a poor prognosis and advanced stages of cancer and with the progression of various neoplasias [18], suggesting that the inactivation of this gene or the deletion of the 17p region is of great importance in carcinogenesis, as observed in other examples such as breast, stomach, liver, and colon cancer and in head tumors [18, 19].

4. Chromosome 17 in MB

The first reports on the importance of the alterations of chromosome 17 in MB come from the work of Emadian et al. [20] and Steichen-Gersdorf et al. [21]. These studies showed that the allelic loss of regions of chromosome 17 is associated with poor prognosis when compared with the prognosis of patients with MB without these alterations.

The analysis of chromosome 17 alterations in four MB tumor lines and in an induction model of tumor implant showed that there are different types of alterations including the presence of a dicentric chromosome i(17q), two normal copies of chromosome 17, loss of telomere in 17p, and deletion in 17p11.2 [22].

Other studies have shown that loss of 17p and the gain of 1q correlated with poor survival. The gain of 17q without loss of 17p showed a tendency to better prognosis. The careful analysis of all data suggested that, in general, the loss of 17p is

a marker of poor prognosis, while the gain of 17q might be a new marker of good prognosis in patients with MB [8].

Based on these results, it can be noted that good and poor prognosis groups cannot be accurately differentiated according to the alterations of chromosome 17, but what is clear is the presence of genes that are important for the development of CNS, and that despite the great clinical utility of classifying the different subgroups of MB, these markers do not seem to discern in more detail the patients within these subgroups from the point of view of prognosis, strongly justifying the proposal of slightly finer markers able to discriminate between subgroups of response to treatment and that can reflect or participate in important or critical pathways of MB, especially in pediatric cases. Thus, some miRNAs are proposed as new markers, in this case molecular markers in MB that can be used to subclassify and distinguish between groups of good and poor prognosis.

5. Molecular Markers

A marker is a character or a gene that, due to linkage, may be used to indicate the presence of another gene; molecular markers are thus a necessary tool in many fields of biology such as the study of evolution, ecology, biomedicine, forensics, and diversity studies. In addition, they are used to locate and isolate genes of interest. At present, there are several molecular techniques that allow us to know indirectly what the proportions of genes in natural populations are as with the analysis of proteins or, in a direct manner, with DNA studies [23].

Advances in the study of molecular pathways, the identification of biomarkers, and new therapies have been important for the development of new methods of characterization and clinical management; they have also expanded the understanding of the molecular pathogenesis of some types of cancer [24].

In the case of MB, important advances have also been made in the study of the major gene-regulated pathways that are altered in this tumor. In fact, some of these pathways are related to signaling pathways during cerebellar development in the embryonic stage. Some of these pathways are the SHH, WNT, and that of the gene associated with notches in the edges of the wings in *Drosophila* (NOTCH). The deregulation of these pathways strongly affects cerebellar development and may participate in the formation of MB [16].

5.1. SHH Pathway. The sustained expression of the SHH pathway causes serious cerebellar disorders such as development of MB [25].

The association between the overactivation of the SHH pathway and the development of MB arose from the finding that patients with Gorlin syndrome are predisposed to the development of multiple tumors, including MB [26].

Later the genetic analysis studies came that showed that the patched gene (PTCH) gene of the SHH pathway is mutated in these patients [27, 28].

Mutations of other genes in this pathway have been subsequently described, such as smoothened (SMO) in 5% [29] and suppressor of fused (SUFU) in 9% of patients [30].

5.2. WNT Pathway. The first evidence demonstrating the involvement of the WNT pathway in the development of MB came from the genetic study of patients with Turcot syndrome, who are 92 times more likely to develop MB than the general population. These patients carry a germline mutation of the adenomatous polyposis coli gene (APC) gene involved in the WNT pathway [31]. Subsequently, it was shown that other MB cases also had mutations in other genes of the WNT pathway such as β-catenin and axin 1 [32, 33].

5.3. NOTCH Pathway. The Notch protein is a transmembrane protein and exists as a heterodimeric receptor. The extracellular domain contains repeated domains similar to the epidermal growth factor and is involved in the binding of ligands, preventing signaling in the absence of these. The participation of NOTCH-1 and NOTCH-2 in MB has been described. NOTCH-1 inhibits the proliferation of MB while NOTCH-2 promotes cell growth [34]. Furthermore, the expression of NOTCH-1 is so low that it is undetectable in some cases, while NOTCH-2 is overexpressed in MB [35].

6. MicroRNAs

In the past 10 years, great importance has been given to the various functions of RNA, as not only it participates in the process of gene expression, but there are also different types of noncoding RNAs such as the so-called microRNAs (miRNAs) that play an important role in the regulation of gene expression in animals, plants, and viruses and have a crucial role in processes of cell differentiation, development, and proliferation, in cell death and in the acquisition and maintenance of a particular phenotype (e.g., tumor), among many other examples [36]. The first miRNAs were discovered through genetic research of the nematode *Caenorhabditis elegans*. This is why we intend to explain the role played by miRNAs, from their biogenesis to the control of the expression of some genes. These small RNAs associated with multienzyme complexes are used for the recognition of complementary sequences in target messenger RNA (mRNA). The functional interaction between both of them induces the degradation of mRNA and consequently translational repression, a mechanism considered as another form of epigenetic regulation [37].

7. Biogenesis of miRNAs

The biosynthetic pathway of miRNAs includes several stages: initially, miRNAs are transcribed by RNA polymerase II to generate precursor molecules called pri-miRNA, with a modification 5′ (7-methyl guanosine) and a tail of poly(A) at the 3′ end. These transcripts may be up to several kilobases in length. A single pri-miRNA may contain one or several miRNAs. These primary transcripts self-align to their sequence, forming stem-loop structures. Subsequently, these pri-miRNA are processed in the nucleus by a protein complex called "microprocessor," formed by an RNase III called Drosha accompanied by the protein DiGeorge syndrome critical region gene 8 (DGCR8) (Figure 1), which recognizes the pri-miRNA and generates a smaller precursor

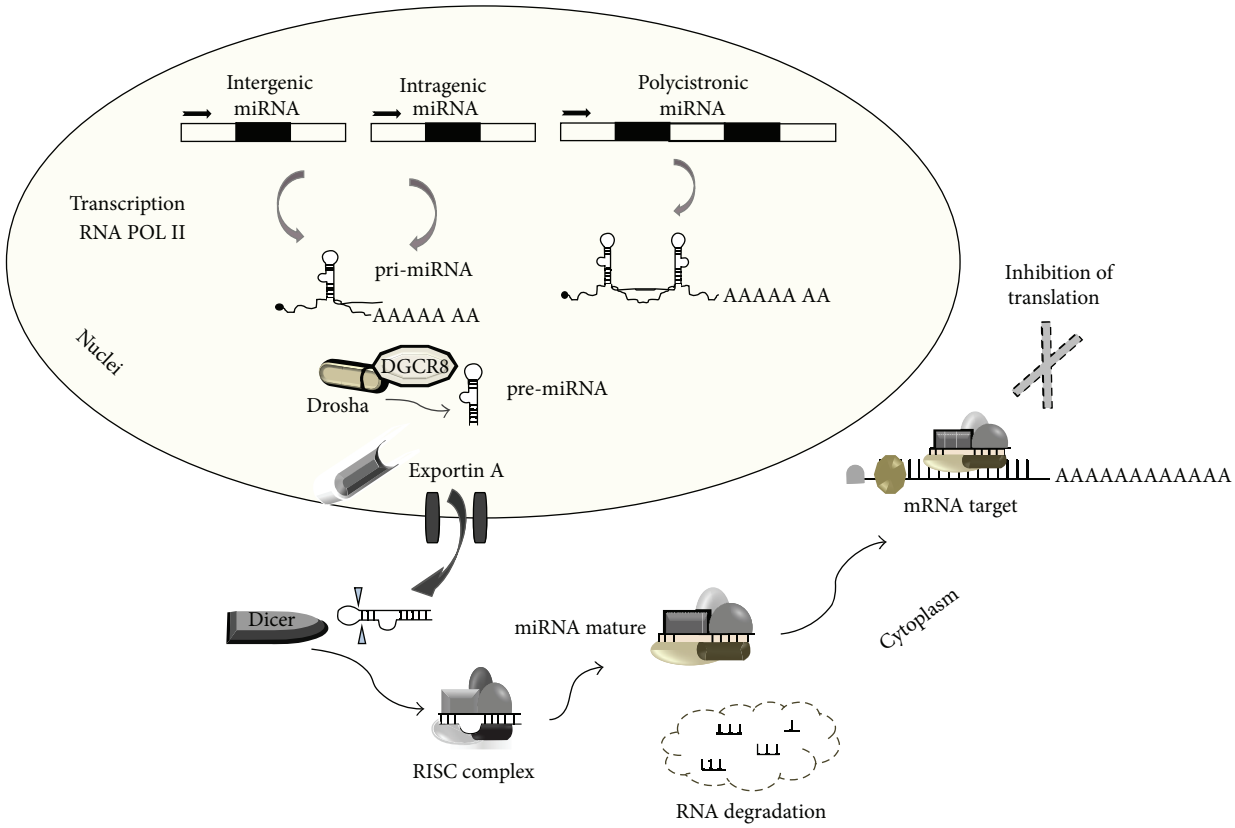

Figure 1: Biogenesis of miRNAs.

known as pre-miRNA, of between 60 and 100 nucleotides in length, forming a stem-loop structure. Exportin-5 is a nuclear export protein that recognizes and transports pre-miRNAs to the cytoplasm. In the cytoplasm, the Dicer enzyme is involved; this is a second RNase III enzyme that separates the pre-miRNAs to generate the mature miRNA with a length of 18–24 nucleotides. RNA induces the activation of the RNA-induced silencing complex (RISC), in which the main component is the Argonaute protein, which includes a guide strand of miRNAs (Figure 1). The posttranscriptional silencing mediated by miRNAs occurs either due to the specificity of homologous mRNAs or when the guide miRNA joins the RISC complex and it in turn recognizes the target mRNA and represses gene expression through the imperfect (in animals and viruses) or perfect (in plants) coupling of the 3′ untranslated region UTR region (most of cases) of the target mRNA, preventing the production of the protein [36, 38].

It has been demonstrated that miRNAs have many biological functions. Their targets range from molecules involved in the signaling pathway of proteins, such as enzymes and transcription factors, to RNA-binding proteins. The diversity and abundance of target genes offer a number of possibilities and combinations and suggest that miRNAs and their targets form, as mentioned above, a complex regulatory network intertwined with other cellular networks such as the signal transduction, metabolic pathways, gene regulation, and protein interaction networks. Therefore, it is essential to understand the general principles of the regulation exerted by miRNAs to understand how they participate in the regulation of different cellular processes and, consequently, to understand their function at system level.

8. miRNAs as Molecular Markers

Since the discovery in the 90s of miRNAs as potent epigenetic regulators that have a general inhibitory effect on gene expression, they have opened a new era in the study of the regulation and development of cancer. This also started an exploration of the possible therapeutic applications of miRNAs. Although most miRNAs have not yet been characterized in terms of function and the signaling pathways regulated by them, certain mammalian miRNAs have emerged as critical regulators of stem cell function, self-renewal, epithelial-mesenchymal transition (EMT), initiation of cancer, resistance to therapy, and the promotion of metastasis [39].

9. Studies of miRNAs in Medulloblastomas

Studies of miRNAs and MB show that none of the miRNAs that have been studied are encoded on chromosome 17. Few of the target proteins of these miRNAs are from epidermal growth factor receptor (EGFR), B-cell lymphoma 2 (Bcl-2), and cyclin-dependent kinase 6 (CDK6) to solute carrier family 16, member A11 (SLC16A11); and others have not been validated. Several miRNAs have been associated with both

poor and better prognoses when their levels are increased or decreased. Anyone can induce gain or loss of function. Some miRNAs are shown in Table 1 and summarize the information of four review articles published about miRNAs in MB [40–43].

10. Location of the Chromosome 17 miRNAs

Table 2 shows a review of the loci on chromosome 17 that, when mutated, may cause a disease. This illustrates a wide and diverse range of loci containing genes that may play an important role in the development of various diseases. This picture really changes when the loci of miRNAs encoded on chromosome 17 are located; these miRNAs can, through their multiple gene regulatory network, impact heavily on many diseases and disorders of the central nervous system development.

With this approach in mind, it would be possible to understand why the study of miRNAs can have a greater impact than the study of only the genes encoded on chromosome 17.

Figure 2 shows the loci of the main miRNAs as unique genes encoded along chromosome 17, whose targets are, according to http://targetscan.org/, involved in the development and differentiation of the central nervous system (Table 3).

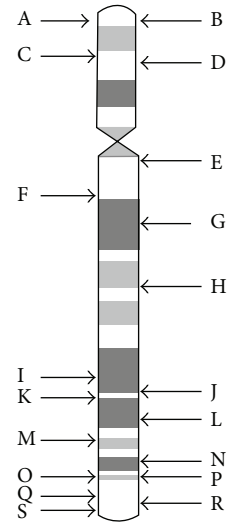

Figure 2: Location of the loci of the miRNAs described in Table 3, located on human chromosome 17.

11. Clinical Significance of Some miRNAs

Our working group, based on the importance of chromosome 17 described above and on the proven fact that alterations or losses in genes of that chromosome in MB mark tumors with a poor prognosis, took on the task of choosing for study four miRNAs that we consider important for their potential involvement in MB. The expression alterations of these miRNAs have not been explored yet or their role in the different subtypes of MB and in the prognosis and survival of patients. We have a tissue bank of MB of Mexican pediatric patients, for whom there is no information regarding the expression of miRNAs in Mexico and Latin America; therefore, we consider it very important to conduct this study in next time and later integrate the information obtained into another article.

The potential clinical applications of these miRNAs should be evaluated based on their expression profile. If the interest is their utility in tumor tissues, it will be important to check their expression, comparing normal and tumor tissue. In the case of MB, it will be necessary to evaluate and check the expression between tumor and normal cerebellum samples. Hence, two possibilities arise: one is that the phenotype is overexpressed in the tumor compared to normal tissue. In this case, it means that this overexpression is causing a loss of function of some important proteins that is allowing/promoting tumor development. In this case, it would be appropriate to reduce the levels of the mature form of the miRNA to restore the function lost by the group of proteins whose mRNAs are targets for these miRNAs. In the contrary case, when the expression of the mature miRNA is reduced in the tumor tissue and is highly expressed in normal tissue, this strongly suggests that a function is gained by the expression of a group of proteins that allow the manifestation and maintenance of the tumor phenotype. In this case, it would appropriate to introduce the active form of the miRNA to again control the function and allow regulating the function of the tumor cells. Following this pattern, it is possible to compare pairs of deregulated microRNAs (overexpressed or underexpressed) between 2 different tumor phenotypes: primary tumor and metastasis; sensitive and drug-resistant phenotype, equal to radiotherapy; angiogenic and nonangiogenic; low and high tumor grade; adenocarcinoma and squamous cell carcinoma, and so forth.

11.1. miRNA-1253. The mature sequence of this miRNA is **agagaagaagaucagccugca**. An analysis of the target sequences of miRNA-1253 in http://targetscan.org/ reveals the presence of 330 transcripts and http://mirdb.org/ shows 466 conserved binding sequences of this microRNA. This analysis inevitably reveals a complex network of signals that are difficult to group. There is no report in the literature that describes an important role of this microRNA in any cellular process.

However, it is noteworthy that the group of mRNA targets contains the protein called neuroblastoma suppression of tumorigenicity 1 (NBL1). This protein is an antagonist of the differentiation factors bone morphogenetic factor 2 (BMP2) and BMP4 [44]. Factors BMP2 and BMP4 play an important role in MB; Iantosca et al. (1999), when evaluating the biological effects of these factors on DAOY cells (a medulloblastoma cell line), reported that exposure to BMP2 and BMP4 can reduce apoptosis and increase cell number. These responses are specific to these factors, as neither BMP3 nor transforming growth factor-beta 1 (TGF-β1) or glial cell derived neurotrophic factor (GDNF) is able to produce this effect. These results have an important potential clinical implication, as the increase of miR-1253 levels can induce a

Table 1: Summary of expression pattern of some miRNAs studied in MB and their potential clinical [40–43].

miRNA	Expression pattern	Potential clinical	Cellular targets	Cellular function
miRNA-let7g	UP	POOR	ND	ND
miRNA-9	DOWN	ND	Trkc	Increase apoptosis low proliferation
miRNA-10b	UP	ND	ND	ERBB2 overexpression
miRNA-17/92	UP	POOR	ND	SHH Pathway
miRNA-19a	UP	POOR	ND	Associated with high risk
miRNA-21	UP	ND	Pdcd4	Metastatic process
miRNA-23b	UP	BETTER	ND	WNT pathway
miRNA-25	UP	ND	P57	Tumor suppressive function
miRNA-30b/miRNA-30d	UP	ND	ND	8q24.22-q24.23 amplification
miRNA-31	DOWN	ND	ND	Associated with high risk
miRNA-34a	DOWN	ND	MAGE-A DII1 Notch1 Notch2	Increase apoptosis Increase cell cycle Decrease cell proliferation Increasing senescence
miRNA-96	DOWN/UP	ND/ND	AKT/ND	Cell cycle G0/G1, G2/ND
miRNA-100	UP	POOR	ND	Targets predicted in carcinogenesis
miRNA-106b	UP	POOR	ND	Anaplastic histology
miRNA-124a	DOWN	POOR	CDK6 SLC16A1	Cell cycle Glycolysis
miRNA-125a	DOWN	ND	Trkc	Increase apoptosis low proliferation
miRNA-125b	DOWN	POOR	Smo	SHH Pathway
miRNA-125p	DOWN	ND	Smo Gli1	SHH Pathway
miRNA-128a	DOWN/UP	BETTER/ND	Bmi-1/ND	Targeting oncogene Bmi-1/associated MYC
miRNA-128b	UP	ND	ND	Associated with MYC
miRNA-129	DOWN	ND	ND	ND
miRNA-135a/miRNA-135b	UP	ND	ND	ERBB2 overexpression
miRNA-148a	UP	BETTER	ND	WNT pathway
miRNA-153	DOWN	ND	ND	ERBB2 overexpression
miRNA-181b	UP	ND	ND	Associated MYC overexpression
miRNA-182	DOWN/UP	ND/ND	AKT/ND	Increase cell cycle G0 and G2, Decrease cell proliferation and migration/WNT pathway
miRNA-183~96~	DOWN/UP	ND/POOR	AKT/ND	Increase cell cycle G0 and G2, Decrease cell/ND proliferation and migration
miRNA-186	DOWN	ND	MYC	Decrease cell proliferation
miRNA-191	UP	POOR	ND	ND
miRNA-193a	UP	BETTER	ND	WNT pathway
miRNA-199b-5p	UP	BETTER	HES1	Notch signaling
miRNA-214	UP	POOR	SUFU	SHH pathway

Table 1: Continued.

miRNA	Expression pattern	Potential clinical	Cellular targets	Cellular function
miRNA-218	DOWN	POOR	EGFR Bcl-2	ND
miRNA-224/452	UP	POOR	ND	WNT pathway
miRNA-324-5p	DOWN	POOR	Gli1	SHH pathway
miRNA-326	DOWN	POOR	ND	ND
miRNA-365	UP	BETTER	ND	WNT pathway
miRNA-383	DOWN	ND	PRDX3	Increase apoptosis Increase cell cycle G1 Decrease cell proliferation
miRNA-512-2/miRNA-512-5p	DOWN	ND	MYC	Decrease cell proliferation
miRNA-548d-1/miRNA-548d-2	DOWN	ND	MYC	Decrease cell proliferation
miRNA-935	DOWN	ND	KIAA0232 SLC5A3 TBC1D9 ZFAND6	ND

ND, not determined.

Table 2: Loci of several genes on chromosome 17 and the main diseases induced when some of these genes suffer mutations.

LOCUS	Diseases
17p13.3	Retinitis pigmentosa
17p13.3	Platelet ADP receptor defect bleedings
Chr.17	Lambert-Eaton myasthenic syndrome
17p13	Type 2 diabetes mellitus
17p13.1	Congenital ichthyosiform erythroderma, Nonbullous
17p13.2	Miller-Dieker syndrome
17p13-p12	Liver failure
17p11.2	Van Buchem disease
17p11.2	Birt-Hogg-Dubé syndrome
17q11-q12	T cell immunodeficiency, alopecia, nail dystrophy
Chr.17	Endometrial stromal tumors
17q11.2	Alzheimer's disease
17q11.2	Acute promyelocytic leukemia
17q21-q22	White sponge nevus
17q21	Naxos syndrome
17q21	Narcolepsy
17q21	Sanfilippo syndrome type 2
17q21.32	Glanzmann's thrombasthenia
17q22	Early breast cancer
17q22-q23	Mulibrey nanism
17q22-q23	Meckel-Gruber syndrome
17q24-q25	Usher syndrome
17q25	Acute myeloid leukemia
17q25	Retinitis pigmentosa
17q25	Alveolar soft part sarcoma

reduction in the concentrations of the NBL1 protein, which is an antagonist of the factors BMP2 and BMP4, thereby reducing apoptosis and increasing cell proliferation [45].

However, there is a contradictory report revealing that in retinoid-induced apoptosis in a model of tumor implant induced by a medulloblastoma cell line retinoids are able to induce the secretion of BMP2 in tumors of the cells sensitive to this agent, and that this signal is sufficient to produce apoptosis [46].

These results clearly reveal what the responses *in vitro* do not appear to correspond to the *in vivo* responses, so it is always necessary to validate and compare the results of the two assays. What is clear is that miRNA-1253 may have an important role in MB cells, negatively regulating an antagonist of the positive effects of the differentiation factors BMP2 and BMP4, thus causing a gain of function such as MB cell proliferation (Figure 3).

11.2. miRNA-2909. The mature sequence is **guuagggccaacaucucuugg**. This miRNA has around 97 miRNAs as targets according to http://targetscan.org/ and 151 according to http://mirdb.org/. A review of the literature reveals that the microRNA-2909 has been studied more extensively in cardiovascular disease, immune response, and the toxic response caused by arsenic, but no study has suggested a relation with central nervous system cells. However, it is important to mention that this microRNA is highly expressed in peripheral blood mononuclear cells isolated from patients with coronary artery disease and might be a diagnostic and prognosis marker for these patients with cardiovascular disease [47]. Moreover, Sharma et al. (2013) demonstrated that arsenic induces overexpression of miRNA-2909 and that this regulates the expression of important genes such as cyclin D1, reducing its levels and controlling the cell cycle [48].

Another effect of this microRNA is on the induction and maturation of T lymphocytes *in vitro* from mononuclear cells. MiRNA-2909 is capable of increasing the cell populations positive for cluster of differentiation 4 (CD4), CD25, and forkhead box P3 (Foxp3), as well as the populations of

Table 3: List of miRNAs encoded on chromosome 17 as single genes and their position, a target gene of each miRNA, its relevance for the nervous system, and its location on chromosome 17 illustrated in Figure 2.

mIRNA	Neurological importance	Location
miRNA-3183	Neurogenic differentiation 2 neuregulin 3	**A**
miRNA-1253	Neuroblastoma, suppression of tumorigenicity 1	**B**
miRNA-4314	Several neural targets	**C**
miRNA-4521	Neuropilin (NRP) and tolloid-(TLL-) like	**D**
miRNA-4522	Neurogenic differentiation 2	**E**
miRNA-632	Neurochondrin	**F**
miRNA-2909	Neuroblastoma RAS viral (v-ras) oncogene homolog	**G**
miRNA-2117	Neurofascin brain protein 44-like	**H**
miRNA-3185	Neural precursor cell expressed, developmentally down-regulated 4	**I**
miRNA-4736	Several neural targets	**J**
miRNA-4729	Several neural targets	**K**
miRNA-4737	Neurooncological ventral antigen 1	**T**
miRNA-633	Neurooncological ventral antigen 1	**M**
miRNA-5047	Neuroblastoma, suppression of tumorigenicity 1	**N**
miRNA-634	Neuroblastoma RAS viral (v-ras) oncogene homolog	**O**
miRNA-636	Neurooncological ventral antigen 1	**P**
miRNA-4739	Neurooncological ventral antigen 2	**Q**
miRNA-657	Neuroepithelial cell transforming 1	**R**
miRNA-4525	Neurochondrin	**S**

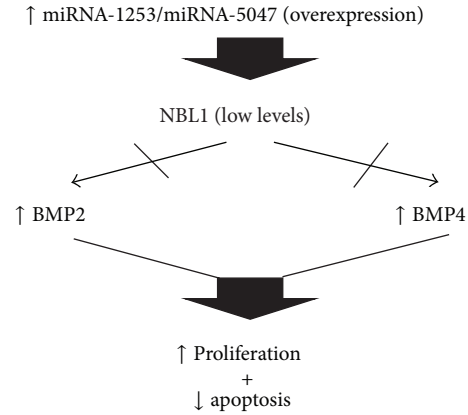

Figure 3: Pathway proposed in which miRNA-1253 and miRNA-5047 can deregulate some functions due to their overexpression and some cellular effects on proliferation and apoptosis in MB.

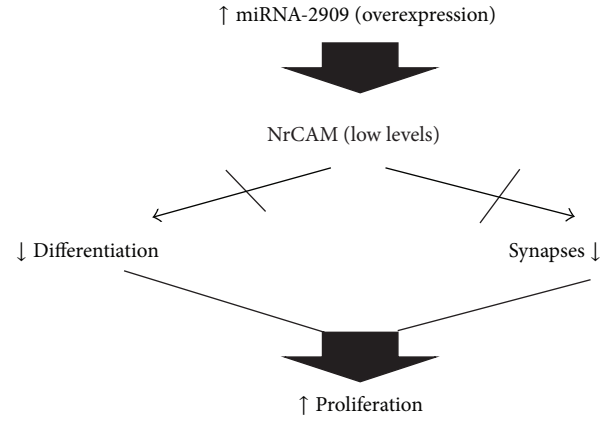

Figure 4: Pathway proposed in which overexpression of miRNA-1253 can affect NrCAM and results on proliferation in MB.

lymphocytes T helper 1 (Th1). Therefore, this miRNA can help in increasing the number of immune cells capable of protecting against viral infections and other pathogens [49].

The neuronal cell adhesion molecule (NrCAM) is among the target mRNAs of important proteins that have not yet been validated for this miRNA. It is a membrane protein widely expressed in the cerebellum, mainly during embryonic development. NrCAM is mainly expressed by Purkinje and Golgi cells in postnatal cerebellum, suggesting an important role in the maturation and stabilization of the synaptic connections in the cerebellum [50].

Thus, the overexpression of miRNA-2909 in MB can induce the degradation of this adhesion molecule and affect/activate some residual embryonic cells, which are the ones forming the MBs (Figure 4).

11.3. miRNA-5047. The mature sequence is **uugcagcugcgguu guaaggu**. There are no reports in the literature about this miRNA. A review of the mRNA sequences targeted by this microRNA revealed 356 transcripts according to http://targetscan.org/ and 333 according to http://mirdb.org/. It is noteworthy that this miRNA also targets the mRNA of the NBL1 protein, as in the case of miR-1253, so that an overexpression of these two miRNAs, miRNA-5047 and miRNA-1253, would ensure the degradation and significant reduction of the levels of this antagonist of the differentiation factors BMP2 and BMP4 and would have an effect on the apoptosis of MB cells (Figure 3). But one thing that stands out is that miR-5047 also targets Drosha ribonuclease III, which is involved in the processing of pri-microRNA into pre-miRNA in the nucleus. Thus, it is important to recognize that the expression of this microRNA can potentially affect the formation of pre-miRNAs and therefore the production of mature forms. The overall system for the processing and formation of miRNAs would be significantly affected.

11.4. miRNA-634. The mature sequence of this miRNA is **aaccagcaccccaacuuuggac**. An analysis of possible targets reveals 320 transcripts according to http://targetscan.org/ and 266 according to http://mirdb.org/. A review of the literature shows that this miRNA, and 39 others, is capable of controlling the human epidermal growth factor receptor 2 (HER2) signaling pathway and the cell replication pathway in breast cancer lines with amplification of this pathway [51].

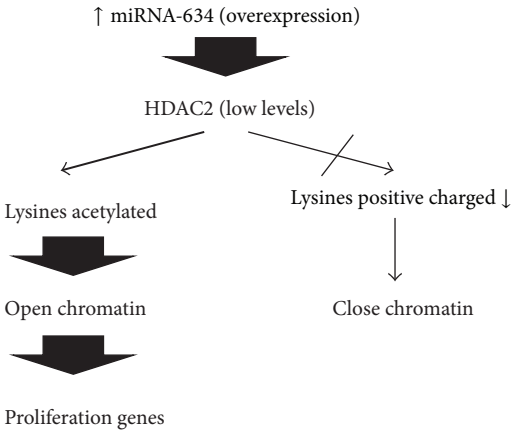

Figure 5: Pathway proposed in which overexpression of miRNA-634 can affect HDAC2 and open chromatin in MB.

Studies of the LN229 glioblastoma cell line are another example. Following the regulatory pathway of the cysteine-rich, angiogenic inducer 61 (CYR61) gene that activates cell proliferation and migration in these cells, with the aim of identifying a miRNA that controls it, it was found that miRNA-634 is among 3 miRNAs regulating this gene. This miRNA controlled cell proliferation but not migration. In addition, this miRNA can downregulate the mechanistic target of rapamycin (mTOR) pathway. These results demonstrate that miRNA-634 is an important regulator of the proliferation of glioblastoma cells [52]. It was also shown that this miRNA is overexpressed in chondrocytes of people without rheumatoid arthritis, so that a reduced or null expression of this microRNA may be involved in rheumatoid arthritis [53].

Among the important target sequences of this miRNA that are implicated in MB, one of the most interesting gene is the mRNA of histone deacetylase 2 HDAC2. This gene encodes an enzyme that deacetylates histone lysines. This increased expression of HDAC2 might cause an increase of positively charged deacetylated lysines that may be related to transcriptionally closed chromatin, repressing some of the genes under the control of HDAC2 in MB (Figure 5). If levels of miRNA-634 increase, it reduces HDAC2 and it turns on open chromatin and transcription of genes of proliferation among others [54].

11.5. miRNA-636. The mature sequence of this miRNA is **ugu gcuugcucgucccgcccgca**. The platform http://targetscan.org/ shows 656 sequences as potential targets for this miRNA, but http://www.mirdb.org/ shows only 173. There is a large discrepancy between the two platforms. When thinking about important targets in MB, it is worth noting that MB is thought to develop from cerebellar granule precursors. The SHH pathway is activated in these precursors and cell proliferation occurs with sustained activity of histone deacetylases HDACs. Several members of the HDAC family are expressed in the medulloblastoma, compared to what is observed in normal cerebellum. Thus, this miRNA may have important targets of upregulation through chromatin modification such as histone deacetylation [55].

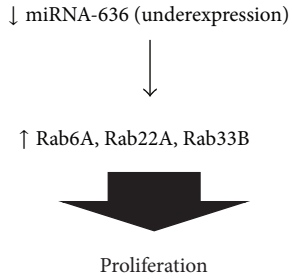

Figure 6: Pathway proposed in which underexpression of miRNA-636 can affect several Rab proteins and results on proliferation in MB.

A review of the main targets of miRNA-636 reveals important proteins of the rat sarcoma virus (Ras) pathway such as ras-related GTP binding proteins Rab6A, Rab22A, and Rab33B (Figure 6). This activation pathway may be involved in hepatic carcinoma. A low expression of miRNA-636 was observed in hepatocellular carcinoma cell samples, compared to normal liver samples. To verify this finding, *in vitro* experiments were performed with a liver carcinoma cell line, Hep3B; transfection with the mature form of miRNA-636 resulted in a significant reduction in cell proliferation and colony formation. This restoration of the levels of miRNA-636 significantly decreased the levels of Ras, supporting the results of the bioinformatic analysis of databases that predicts components of the Ras pathway as important targets of this miRNA [56].

12. Conclusions

After reviewing the historical progress of the description of molecular alterations in MB, based on the alterations of genetic pathways that have also been used to classify these tumors, it is important to note that, at present, the fact that Molecular Genetics is considered as a way of studying cellular functions from a different perspective from that of the pioneers of Classical Genetics and Cytogenetics, shows a clear picture of how Genetics itself has developed historically: from the guiding paradigm of genetic flow, one gene, and one protein, to the current paradigm of "one miRNA gene, multiple regulated genes." Since the beginning of the sequencing of the human genome, only recently it was possible to clarify and understand the important functions of these noncoding RNAs that were known as "junk RNA." If we make a clear and objective analysis, we must acknowledge that nature never produces unnecessary or "junk" actions or molecules because it has integrated millions of years of evolution that allowed it to know "intelligently" what it has and what it is good for; in addition, it will never work without profit, expending energy for something futile. The study of MB is only a pretext that helps understand more clearly how the new diagnostic, prognostic, and therapeutic approaches, based on Molecular Genetics, have started a revolutionary paradigm shift. What the study of Molecular Genetics makes clear is that the analysis based on miRNAs is teaching us how to integrate each element into a complex network such as that of biological systems, and that it is less and less possible to

manipulate and explain things using the reductionist point of view with which we have learned to observe and study diseases such as cancer. miRNAs have shown increasingly solid therapeutic potential because they are not able to act on and modify the nucleus, as was thought in gene therapy. Thus, miRNAs fully respect the "Pandora box" of the nucleus, where the DNA resides and which must not be moved or altered in order to perform cleaner manipulations and obtain the desired effects. This is now possible thanks to miRNAs, which act in the cytoplasm and exert their regulatory action from there reducing the undesirable effects caused by inducing mutations by insertion or silencing by a reintroduced gene.

The potential clinical applications of miRNAs are focused in the following: if some miRNA encoded in chromosome 17 in MB is deregulated as overexpressed relative to cerebellum, the strategy will be to induce its degradation and control the loss of function as differentiation, apoptosis, and cell adhesion; it will be by transfecting with the antagonistic or antagomir sequence of the miRNA. If a miRNA is underexpressed and there is a gain of function in processes such as proliferation, migration, and metastasis, then an important strategy is to reintroduce the mature sequence to regain control of the function by negatively controlling it.

Abbreviations

APC: Adenomatous polyposis coli gene
Bcl-2: B-cell lymphoma 2
BMP2: Bone morphogenetic factor 2
BMP4: Bone morphogenetic factor 4
CD4: Cluster of differentiation 4
CDK6: Cyclin-dependent kinase 6
CNS: Central nervous system
CYR6: Cysteine-rich, angiogenic inducer 61
DGCR8: DiGeorge syndrome critical region gene 8
EGFR: Epidermal growth factor receptor
EMT: Epithelial-mesenchymal transition
Foxp3: Forkhead box P3
GDNF: Glial cell derived neurotrophic factor
HDAC2: Histone deacetylase 2
HDACs: Histone deacetylases
HER2: Human epidermal growth factor receptor 2
MB: Medulloblastoma
mTOR: Mechanistic target of rapamycin
NBL1: Neuroblastoma suppression of tumorigenicity 1
NOTCH: Gene associated with notches
NRCAM: Neuronal cell adhesion molecule
PTCH: Patched gene
Ras: Rat sarcoma virus
RISC: RNA-induced silencing complex
SHH: Sonic hedgehog
SLC16A11: Solute carrier family 16, member A11
SMO: Smoothened
SUFU: Suppressor of fused
TGF-β1: Transforming growth factor-beta 1
Th1: Lymphocytes T helper 1
TP53: Tumor protein 53 gene
WNT: Wingless-type
UTR: Untranslated region.

Conflict of Interests

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

Acknowledgments

The authors thank Dirección de Investigación of the Hospital Infantil de México Federico Gómez for their support and CONACYT for the financial support received through the Grant SALUD-2012-C01-181456 of Fondos Sectoriales de Investigación en Salud y Seguridad Social, 2012.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 756975, 8 pages
http://dx.doi.org/10.1155/2014/756975

Review Article

Therapeutic Use of MicroRNAs in Lung Cancer

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Received 5 June 2014; Accepted 8 August 2014; Published 16 September 2014

Academic Editor: Paolo Gandellini

Lung cancer is a leading cause of cancer deaths worldwide. Although the molecular pathways of lung cancer have been partly known, the high mortality rate is not markedly changed. MicroRNAs (miRNAs) are small noncoding RNAs that actively modulate cell physiological processes as apoptosis, cell-cycle control, cell proliferation, DNA repair, and metabolism. Several studies demonstrated that miRNAs are involved in the pathogenesis of lung diseases including lung cancer and they negatively regulate gene and protein expression by acting as oncogenes or tumor suppressors. In this review we summarize the current knowledge on the role of miRNAs and their target genes in lung tumorigenesis and evaluate their potential use as therapeutic agents in lung cancer. In particular, we describe methodological approaches such as inhibition of oncogenic miRNAs or replacement of tumor suppressor miRNAs, both in *in vitro* and *in vivo* assays. Furthermore we discuss new strategies to achieve *in vivo* tissue specific delivery, potential off-target effects, and safety of miRNAs systemic delivery.

1. Introduction

Lung cancer is the most frequent cause of cancer mortality worldwide, accounting for more than 1.4 million deaths per year [1]. The two major histotypes of lung cancer are nonsmall cell lung cancer (NSCLC) (about 80% of all lung cancers) and small cell lung cancer (about 20%) [2].

Despite improvements in early diagnosis and new therapeutic strategies, the overall 5-year survival remains only of 10–20% [1]. The poor prognosis is due to diagnosis at advanced stage, tumor heterogeneity, and relatively limited understanding of lung cancer biology. Surgical resection is so far the most common treatment for early stages tumors, in combination with chemotherapeutic agents for advanced lung cancer patients or chemotherapy treatment alone for metastatic disease. The platinum-based treatment is commonly used in the clinical practice, with small benefit in survival of lung cancer patients. The identification of driver mutations and genetic rearrangements in approximately 50–60% of NSCLC has led to a change in the treatment of lung cancer patients [3–5], by identifying subgroups of patients characterized by different molecular profile. K-RAS mutations have been found in approximately 17% of all NSCLC, especially in adenocarcinomas (27%–34%), whereas the discovery of activating mutations in the EGFR gene (23%) and rearrangements of anaplastic lymphoma (ALK) (5%) were found and had also relevant impact in the treatment of lung cancer patient, through their responsiveness to tyrosine kinase inhibitor (TKI) agents, such as erlotinib, crizotinib, and gefitinib [6, 7].

Although new targeted therapies entering clinical trials have shown positive results, a large number of patients still remain without any potential known target for therapy. Therefore, the identification of novel treatment strategies is critical and essential for lung cancer management.

MicroRNAs (miRNAs) are small noncoding, 22 nt-long, RNAs that are able to bind complementary sequences of target mRNAs and to induce either their degradation or translational repression [8]. In mammals, miRNAs control the activity of more than 50% of all protein-coding genes [9]. MiRNAs are expressed in a tissue or developmental specific manner, thereby greatly contributing to cell-type-specific profiles of protein expression. MiRNAs potentially target hundreds of different mRNAs and thus coordinate or fine-tune the expression levels of many proteins, thus regulating a wide variety of cellular processes [10]. To date, more than 1000

Table 1: Principal miRNAs involved in lung carcinogenesis.

miRNAs	Expression in lung cancer	Cellular processes affected and targets	Reference
Let-7 family	Decreased	(i) Cell proliferation (KRAS, MYC, and HMGA2)	[28–30]
		(ii) miRNA maturation Dicer mediated	[31]
		(iii) Cell-cycle regulation (CDC25A, CDK6, and cyclin D2)	[32]
mir-34 family	Decreased	(i) Transcriptionally activated by p53	[40]
		(ii) TRAIL-induced cell death and cell proliferation (BCL-2, MET, and PDGFR-α/β)	[41–43]
mir-21	Increased	(i) Apoptosis, cellular proliferation, and migration (TPM1, PDCD4, and PTEN)	[50–53]
		(ii) TKI-treatment resistance	[54]
mir-17/92a cluster	Increased	(i) Transcriptionally regulated by c-MYC	[60]
		(ii) Cellular proliferation and cancer development (PTEN, HIF-1a CL2L11, CDKNA, and TSP-1)	[57, 61–63]
mir-15a/16 cluster	Decreased	Cell cycle regulation (cyclin D1, D2 and E1)	[66, 67]
mir-200 family	Decreased	Promotion of EMT and metastasization (ZEB transcription factors,CDH-1, and vimentin)	[68–73]
miRNA-29 family	Decreased	Epigenetic regulation of gene expression (DNMT-3A and DNMT-3B)	[74]
mir-221/mir-222	Increased	(i) TRAIL resistance and cellular migration (PTEN and TIMP3)	[75, 76]
		(ii) Transcriptionally regulated by EGFR and MET and gefitinib resistance (BIM and APAF1)	[77]
mir-548	Decreased	Tumor cell growth (CCND, ERBB2, DMNT3A, and DNMT3B)	[78]

human miRNAs were found in the genome and a considerable number of them were found deregulated in cancer cells compared to normal cells [11–14].

The rationale of miRNA therapy in lung cancer is based on two criteria: one is that miRNAs play an essential role in lung development [15] and their expression levels are deregulated in lung cancer compared to normal tissues [16] and also in the blood of patients [17] and the second one is that several studies demonstrated that modulation of miRNA expression, both *in vitro* and *in vivo*, can modify the cancer phenotype [18–22].

Different strategies of miRNAs therapeutics can be envisaged according to the expression status of miRNAs in the tumor: (i) miRNAs inhibition when it is overexpressed using antagomir, which are synthetic miRNAs complementary to the miRNAs of interest or miRNAs sponges, that compete with the targets of the miRNA and (ii) miRNAs replacement using oligonucleotide mimics or viral vectors when the miRNAs is downmodulated [23].

In this review, the role of those miRNAs, which regulate fundamental proteins and pathways involved in lung tumorigenesis, will be discussed. Furthermore, we will describe general therapeutic strategies involving inhibition of oncogenic miRNAs and replacement of tumor suppressor miRNAs including recent experimental methods developed to bypass blood clearance and poor intracellular uptake.

2. miRNA Role in Lung Carcinogenesis

Human cancers exhibit an altered expression of miRNAs with oncogenic or tumor-suppressive activity providing an explanation of their role in tumor development and progression. Recent studies reported an aberrant expression of miRNAs in lung tumor tissues when compared to the corresponding normal lung tissues, supporting miRNAs involvement in lung carcinogenesis (Table 1). Lethal-7 (let-7) is one of the earliest identified tumor suppressor miRNA in lung cancer and its downmodulation is associated with poor prognosis [16, 24–27]. Furthermore, let-7 can regulate several oncogenic pathways; for example, this miRNA negatively modulates multiple cell-cycle oncogenes as KRAS, mutation of which are commonly implicated in lung adenocarcinomas [28] MYC and HMGA2 [29, 30]. Furthermore, an involment of let-7 in the regulation of miRNA maturation process-Dicer mediated has also been described [31]. Let-7 overexpression in lung adenocarcinoma A549 cell line inhibits *in vitro* assays cell growth and reduces cell-cycle progression by targeting CDC25A, CDK6, and cyclin D2 [32]. Accordingly, *in vivo* experiments show that let-7b inhibits lung cancer cell xenografts in immunodeficient mice [33]. In addition, the therapeutic potential of let-7 in lung cancer treatment is supported also by experimental evidence that altered expression of let-7 family members associated with radiation resistance [34].

Mir-34 is a tumor suppressor miRNA largely investigated in cancers and its deregulation has been reported in various tumor types, including lung cancer [35–39]. Mir-34 members (a, b, and c) are regulated by p53 and mir-34 expression is frequently reduced in p53 mutant tumors [40]. mir-34 family members are involved in cell-cycle control, apoptosis, and cellular senescence through specific targeting of BCL-2, MYC, and MET genes [41]. In addition, Garofalo et al. showed

that mir-34a and mir-34c are downregulated in NSCLC cell lines and described an inverse correlation between PDGFR-α/β and miR-34a/c expression in lung tumor samples [42]. The inhibition of PDGFR-α and PDGFR-β by miR-34a/c replacement antagonizes tumorigenicity and increases sensitivity to TRAIL-induced cell death, suggesting an important therapeutic application for lung cancer. The potential therapeutic use of mir-34 is further supported by Kasinski and Slack, who investigated the effects of miR-34 replacement on tumor formation and progression in a mouse model [43].

Unlike let-7 and mir-34, mir-21 has been described as a oncogenic miRNA, and it was found overexpressed in lung cancer [44]. The expression levels of mir-21 were significantly higher in tissues from NSCLC patients with lymph node metastasis than in patients without lymph node invasion [45] and the diagnostic and prognostic values as biomarkers were confirmed in several works [45–49]. These data suggest that the overexpression of mir-21 plays a critical role in lung tumorigenesis. *In vitro* inhibition of mir-21 expression in NSCLC cell lines showed a deregulation of cellular mechanisms as programmed cell death, cellular proliferation, and migration. Validated target for mir-21 include TPM1, PDCD4, and PTEN [50–53] and recently mir-21 overexpression was indeed associated with the acquired resistance of EGFR-TKI in NSCLC, due to the activation of PI3K/AKT pathway through PTEN and PDCD4 inhibition by mir-21 [54]. The role of mir-21 as an oncogenic miRNA and its therapeutic value were also demonstrated in an *in vivo* assay using K-ras^{LA2} murine model of multifocal lung cancer, where the downmodulation of mir-21 expression resulted in a reduction of tumor number, incidence, and size [55].

Oncogenic activity was also reported for mir-17/92a cluster (mir-17, mir-18a, mir-19a, mir-20a, mir-19b, and mir-92), that is, upregulated in several cancer types, both hematopoietic and solid tumors [56–59]. miRNAs from this cluster are regulated directly by c-MYC and have a central role in the control of cellular proliferation [60]. Reporter assays revealed target sequences for mir-19a and mir-19b-1 in the 3′UTR of PTEN [61] and the introduction of mir-19a and mir-19b-1, or the full cluster, was sufficient to induce c-MYC-driven B-cell lymphoma development in mouse model by restoring PTEN expression levels [57, 62]. In lung cancer, this cluster is frequently amplified and in particular mir-17-5p and mir-20a are overexpressed. These miRNAs are fundamental for lung cancer development by targeting HIF-1a, PTEN, BCL2L11, CDKNA, and TSP-1 [63].

Several papers showed that mir-15a/16 are implicated in cell-cycle control and likely contribute to NSCLC tumorigenesis [64–67]. mir-15a/16 are frequently deleted or downregulated in squamous cell carcinomas and adenocarcinomas of the lung, and their expression is inversely correlated with the expression of cyclin D1. In NSCLC cell lines, physiologic concentrations of mir-15a/16 regulated cyclin D2 and cyclin E1 expression. Using luciferase reporter constructs of the different cyclin genes, miRNAs were able to downregulate luciferase activity and this effect was reverted by cotransfection with anti-mir-15a/16. In addition, mir-15a/16-induced cell-cycle arrest can be partially restored by overexpression of CCND1 or CCNE1.

Recent works investigated mir-200 family (mir-200b, mir-200a, mir-429, and mir-200c) and its role in the promotion of EMT in NSCLC through regulation of ZEB1 transcription factors and regulation of CDH-1 and vimentin expression [68–70]. In addition, in a mouse model that develops metastatic lung adenocarcinoma, overexpression of mir-200b locus in highly metastatic cells eliminated their ability to undergo EMT and metastasize and also this miRNA could be used to distinguish lung tumors cell lines based on their site of origin, with higher levels in cells from a primary tumor than in cell lines derived from metastatic sites. Recently Tejero and colleagues demonstrated that high levels of miR-141 and miR-200c are associated with shorter overall survival in a cohort of NSCLC patients with adenocarcinoma [71–73].

Among the reported miRNAs that are downregulated in lung cancer, the miRNA-29 family (29a, 29b, and 29c) expression in lung cancer tissue is associated with DNA methyltransferase DNMT-3A and DNMT-3B levels, two important enzymes for DNA methylation that are frequently upregulated and associated with poor prognosis [74]. The expression of mir-29 family members in lung cancer cell lines restored normal patterns of DNA methylation, inducing reexpression of tumor suppressor genes such as FHIT and WWOX that were previously silenced by methylation, thereby inhibiting tumorigenesis *in vitro* and *in vivo*. These findings support the role of mir-29s in epigenetic normalization of nonsmall cell lung cancer (NSCLC), providing a rationale for the development of miRNA-based therapeutic interventions for the treatment of lung cancer.

Finally, mir-221 and mir-222 are reported to be involved in lung cancer by targeting PTEN and TIMP3 tumor suppressors, inducing TRAIL resistance and enhancing cellular migration [75, 76]. Mir-221/222 are modulated by both epidermal growth factor (EGF) and MET receptors and were found to be involved in gefitinib resistance in cooperation with mir-30b and mir-30c, by inhibiting APAF1 and BIM cell death genes. Since MET amplification plays an important role in the resistance to anti-EGFR agents, a modulation of these miRNAs could have therapeutic applications to sensibilize lung tumors to TKI therapy [77].

Recently, the work of Hu et al. identified a new member of the miR-548 family in the intron of human FHIT gene, which is a tumor suppressor gene altered in human cancer as lung, head and neck, esophageal, stomach, pancreatic, and cervical cancer. This miRNA inhibited human tumor cell growth *in vitro* and *in vivo* xenograft mouse model targeting CCND1, ERBB2, DNMT3A, and DNMT3B [78].

3. miRNA as Therapeutic Agents

Delivery of miRNAs as synthetic miRNA mimics or antagomirs has emerged as a promising approach to treat cancer (Table 2). To date, MRX34 is the only phase I clinical trial for miRNA replacement in patients with primary liver cancer or with liver metastasis from other cancers (Mirna Therapeutics). MRX34 is based on the formulation of miR-34 mimic and the liposomal delivery technology SMARTICLES (Marina Biotech). Interestingly, mir-34, as mentioned above,

Table 2: Strategies for *in vivo* miRNA delivery.

	Type of particles	Advantages	Disadvantages	Reference
Synthetic	Mimic inhibitor	Safe and simple Easy to produce Low immunogenicity	Poor cellular uptake Rapid degradation and clearance	[94]
Neutral lipoplexes/liposomes	MaxSuppressor SLNs SMARTICLES siPORT	Low immunogenicity Lung accumulation Easy to produce Available commercially	Nonspecific uptake Low transfection efficiency Cytotoxicity	[79, 80, 84, 86]
Cationic lipoplexes/liposomes	PU-PEI DOTMA	Low immunogenicity Easy to produce High cellular uptake	Cytotoxicity Tend to form aggregates Moderate transfection efficiency Nonbiodegradability	[81, 83]
Viral vectors	Adenovirus *Lentivirus*	High transfection efficiency Stable expression	Difficult to produce High immunogenicity High toxicity	[33, 43]

is one of the most deregulated tumor suppressor miRNA in lung cancer suggesting also a potential use of this methodology for the treatments of lung cancer patients.

The first evidence of miRNAs replacement in lung cancer was reported in 2008. The authors showed that restoring let-7 expression affected tumor growth in xenograft models derived from lung cancer cells H460 or A549 (NSCLC cell lines carrying mutations in K-RAS gene) injected subcutaneously in immunodeficient mice. In this work, cells were transiently transfected with 30 nM of let-7 miRNA mimic prior to subcutaneous injection into NOD/SCID mice and a delay in tumor growth was observed. They also used a conditionally inducible K-RAS mutated mouse model ($Kras^{LSL\text{-}G12D/+}$) developing orthotopic lung adenocarcinoma to demonstrate that let-7 expression interferes with lung tumorigenesis. They treated mice intranasally with adenovirus expressing cre recombinase (Ad-cre) (5×10^8 pfu) to induce lung cancer growth and treatment with a second adenovirus expressing let-7 resulted in significant tumor growth inhibition [33].

Trang and coworkers investigated the therapeutic role of let-7 in established lung tumors [79]. They injected intratumorally 6.25 μg of let-7 mimics mixed with siPORT-amine (siPORT), a lipid-based reagent, in xenograft mouse models of H460 lung cancer cell line. Mice were treated on days 11, 14, 17, and 20 after xenograft implantation and tumor size measure on day 21 resulted in a robust tumor growth inhibition. This group also obtained a reduction in tumor growth with intranasal injections of lentiviral (10^6 pfu) or adenoviral (5×10^8 pfu) vectors expressing let-7 in $Kras^{LSL\text{-}G12D/+}$ mice. Local delivery by intratumorally or intranasal injection of miRNA demonstrated a significant tumor reduction but is probably inadequate for the clinical setting of miRNA therapy.

In 2010, Wiggins et al. reported a tumor growth reduction by miR-34 treatment in H460 and A549 NSCLC xenograft models in immunodeficient mice [80]. NOD/SCID mice were treated with different concentration of mir-34 mimic (5 and 1 mg/kg) formulated with MaxSuppressor *in vivo* RNALancerII, a neutral lipid emulsion (NLE) delivery reagent (BIOO Scientific, Inc.), injected intratumorally or intravenously on days 12, 15, and 18 after xenograft implantation. Interestingly, they performed a blood chemistry analysis, to test possible toxicity in liver, kidney, and heart, and observed that miRNA treatment was well tolerated. Using a different mouse model they proved that miRNA treatment did not induce nonspecific immune responses in mice by measuring serum cytokines. In summary, this study provided evidence for the safe and effective therapeutic delivery of a miRNA mimics. However, further studies have to demonstrate that miRNAs replacement has no effect on normal cells.

One year later, the same authors also showed that using NLE, rather than the standard cationic liposomes, it was possible to transfect miRNAs systemically by intravenous injections in mice and these particles with miRNA were taken up especially by lung tissue, thus showing that NLE could vehicle therapeutic miRNAs to lung tumors. Indeed, using NLE, $Kras^{LSL\text{-}G12D/+}$ mice were treated with 1 mg/kg of let-7 or mir-34 for 8 consecutive days and showed a significant reduction of tumor burden [81]. The authors suggest that NLE may bypass some of the shortcomings that can be attributed to charge of cationic lipids. These particles form less aggregates in biofluids, are not filtered by the liver, and do not adhere to the endothelium or are taken up by macrophages [82].

More recently, it was created a double transgenic mouse model, $Kras^{LSL\text{-}G12D/+}$; $Trp53^{LSL\text{-}R172H/+}$, where KRAS mutation was induced by Ad-cre in the cells with a p53 null background to recapitulate human lung carcinogenesis [43]. In this study, mir-34 was able to reduce progression of preformed tumors and also totally prevented tumor formation. For these experiments mice were intubated and 3×10^6 TUs of lentiviral particles expressing mir-34 were administrated directly in the thoracic region.

New methodologies for miRNA delivery were settled up in order to reduce cytotoxicity, to avoid liver capture, and to better target lung cells. Among these, the biodegradable cationic polyurethane short branch polyethylenimine (PU-PEI) system was conjugated with mir-145 and showed very high transfection efficiency when injected intratumoral in lung adenocarcinoma cancer stem cells (LAC-CSCs) xenograft models, suppressing the stem-like properties and

sensitizing them to chemo- or radiotherapy [83]. These non-viral gene vectors could represent a genetically and immunogenically safe delivery method for miRNAs-based therapies, but since intratumoral administration is not a feasible clinical route, PU-PEI should be subjected to further characterization for systemic delivery.

In an effort to find an appropriate delivery method of miRNAs for lung cancer treatment, Shi et al. used solid lipid nanoparticles (SLNs) containing dimethyldioctadecylammonium bromide (DDAB) that condense miRNAs and enhance cellular uptake. They demonstrated that intravenously injection of 0.5 mg of SLNs loaded with Cy3-miR-34 was taken up by lung tissue in just 1-2 hours, resulting in a cytosolic delivery of the miRNA [84]. Their results indicate that miSLNs enhance miR-34a uptake into the lung, due to the ability of the delivery vehicle to avoid liver capture and to be physically entrapped in the capillary of the lung compared to lipofectamine particles, which were captured by liver. Recently, Wu et al. developed a cationic lipoplexes-based carrier system containing miR-29b (LP-miR-29b) to treat mice by intravenous injection and reducing tumorigenicity of the A549 xenograft models [85]. LPs formulation contained DOTMA, a cationic lipid that confers a positive surface charge for cellular uptake, cholesterol to improve transfection efficiency and protect from degradation, and a short polyethyleneglycol molecule linked to vitamin E to increase the stability and circulation time of LPs *in vivo*. Furthermore, Cortez et al. developed a systemic delivery method using miR-200c-loaded liposomes (NOV340/miR-200c; SMARTICLES) to enhance radiosensitivity in a mouse model of lung cancer. They described mir-200c accumulation in tumor, liver, brain, and lung after subcutaneous injection in mice. Interestingly, NOV340/miR-200c is a liposomal nanoparticle that contains a synthetic, double-stranded mimic of the tumor suppressor miRNA miR-200c which is composed of amphoteric lipids that change their net charge depending on the pH of the environment, such that it becomes cationic at low pH and anionic at higher pH, to prevent unwanted interactions and to adhere only at tumor site [86].

4. Challenges for miRNA Therapy

The principal advantage of using miRNA as therapeutics agents is that they could target several genes of redundant pathways involved in cancer development [87]. For this reason, a small number of miRNAs could be used to achieve a broad silencing of protumoral pathways and they become more attractive than mixture of small interfering RNAs already used for therapy. In addition, miRNA mutations are extremely rare due to the small size of the sequence and probably a potential resistance to miRNA therapeutics would require multiple mutations in several genes. Furthermore, several studies demonstrated that relatively small changes in miRNAs and their corresponding targets expression could induce phenotypic alterations, reinforcing the idea that correction of a limited number of miRNA could revert the malignant phenotype [88]. The challenges for the use of microRNA in therapy, that have to be addressed, are tissue specific delivery, potential off-target effects and safety.

In this review, we described several strategies of miRNAs replacement/inhibition such as intratumoral injections and regulation of miRNAs expression by viral vectors which represent delivery routes unlikely to be applied in a clinical context. Intratumoral injections could be used only for a small number of easily accessible tumors. Similarly, modulation of miRNAs expression with viral vectors could potentially show the same weaknesses encountered in gene therapy, such as limited infectivity and problems in the transcription of the gene product. In addition, cancer cells frequently show deficiencies in the maturation of miRNA precursors so expression with a viral vector is a less preferable approach.

Thus, the most promising approach for miRNAs therapeutics is the systemic delivery of miRNA mimics or inhibitors. The main obstacle for the systemic delivery of miRNAs in lung cancer therapy is to determine their uptake in lung cancer cells; this delivery has to overcome miRNAs degradation by nucleases, renal clearance, failure to cross the capillary endothelium, ineffective endocytosis by target cells, and activation of the host immune responses [89–92]. A major aspect to be considered is how to deliver the therapeutic molecules into the target cells without inducing unwanted responses in cells other than the intended ones. Furthermore, other potential side effect of miRNAs therapy is that the same miRNA can affect hundreds of targets genes in different cell types and regulates different mechanisms. Therefore, miRNA modulation might have beneficial effects in one cell type but harmful effects in another. Further complication is the finding that miRNAs generated from each strand of the same hairpin RNA structure, termed 5p and 3p, may bind to different mRNAs and display different behaviour. For example, it has been reported that miR-125a-5p and miR-125a-3p, which are downregulated in NSCLC, exhibit distinct effects *in vitro* on the migration and invasion of lung cancer cells [93]. This information has direct implications for the design of miRNA gene therapy; in fact, when using a precursor miRNA inserted into a viral vector and both strands are produced, the function of each strand in the cells must be identified.

5. Conclusion

The challenges for the future development of miRNA therapy as the improvement of stability, delivery, and control of off-target effects of miRNAs need to be addressed.

We hope that some of the miRNAs strategies described above will be further developed to improve specificity and efficacy and finally will be used, alone or in combination with chemotherapy, for the treatment of lung cancer patients.

Conflict of Interests

Gabriella Sozzi and Mattia Boeri are coinventors for two patent applications regarding miRNA signature for lung cancer detection.

Authors' Contribution

Orazio Fortunato, Mattia Boeri, and Carla Verri contributed equally to this work.

Acknowledgments

This work was supported by grants from the Italian Association for Cancer Research (AIRC), IG research Grant nos. 10096, 1227, 11991, and 10068, and Special Program "Innovative Tools for Cancer Risk Assessment and early Diagnosis," 5x1000, no. 12162 and Italian Ministry of Health (RF-2010). Research funding was received from Gensignia Inc.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 146170, 17 pages
http://dx.doi.org/10.1155/2014/146170

Review Article

MicroRNA as New Tools for Prostate Cancer Risk Assessment and Therapeutic Intervention: Results from Clinical Data Set and Patients' Samples

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Received 6 June 2014; Revised 18 August 2014; Accepted 21 August 2014; Published 16 September 2014

Academic Editor: Paolo Gandellini

Prostate cancer (PCa) is one of the leading causes of cancer-related death in men. Despite considerable advances in prostate cancer early detection and clinical management, validation of new biomarkers able to predict the natural history of tumor progression is still necessary in order to reduce overtreatment and to guide therapeutic decisions. MicroRNAs are endogenous noncoding RNAs which offer a fast fine-tuning and energy-saving mechanism for posttranscriptional control of protein expression. Growing evidence indicate that these RNAs are able to regulate basic cell functions and their aberrant expression has been significantly correlated with cancer development. Therefore, detection of microRNAs in tumor tissues and body fluids represents a new tool for early diagnosis and patient prognosis prediction. In this review, we summarize current knowledge about microRNA deregulation in prostate cancer mainly focusing on the different clinical aspects of the disease. We also highlight the potential roles of microRNAs in PCa management, while also discussing several current challenges and needed future research.

1. Introduction

In developed Western countries, prostate cancer is the most common solid tumor diagnosed in men and one of the highest causes of cancer-related deaths after lung cancer [1]. For many years, digital rectal examination (DRE) represented the primary diagnostic test for prostate cancer detection. In the late 1980s, prostate specific antigen- (PSA-) screening was rapidly and widely adopted for PCa diagnosis [2]. However, in spite of the significant improvement in early detection and relapse assessment after radical prostatectomy, there is no evidence that the PSA-test reduces the risk of death for the disease. In fact, serum PSA level may be a consequence of different variable events, such as benign prostatic hyperplasia (BPH), inflammation of the gland, or pharmacological therapy, and it is not correlated with either predicting tumor aggressiveness or therapy responsiveness. Thus, PSA level evaluation inevitably affects the false-positive rate of prostate cancer detection, leading to overdiagnosis of patients who present nonneoplastic alteration of the prostate gland or clinically insignificant cancer [3, 4]. As a consequence of its low predictive value, PSA screening has caused extra diagnosis and overtreatment in cancer patients who are subject to invasive or radical procedures with significant side-effects and without effective benefits in quality of life. In the last few years, several active surveillance protocols have been approved for monitoring patients with low risk cancers [5]. This approach may strongly reduce patients' overtreatment and morbidity associated with surgery. However, a considerable group of low-risk patients may experience tumor progression. In this case, radical prostatectomy and radiotherapy represent the standard treatment for localized high-grade tumors [6, 7]. Nevertheless, a significant percentage of radical-treated patients (30–35%) may develop biochemical recurrence, with rising levels of PSA as a consequence of the presence of cancer cells [8]. Since PCa depends on hormone signaling for its growth and survival, androgen-deprivation therapy represents the first-line therapy for this stage of the

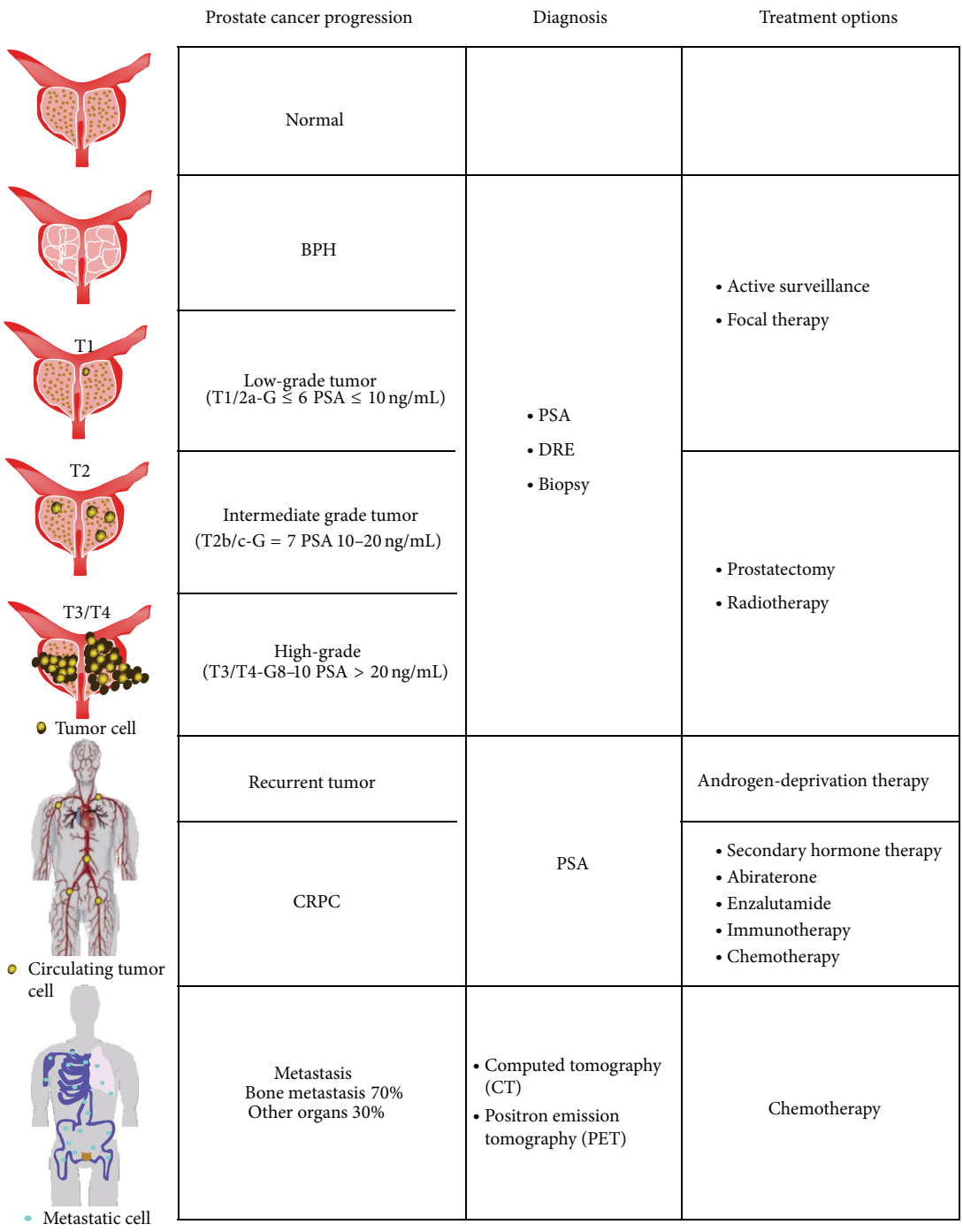

Figure 1: Representative scheme of prostate cancer tumor progression.

disease, with significant improvement in patient prognosis. However, within 2 years of treatment a significant percentage of these patients develop a castration-resistant form (CRPC) of the disease, which is ultimately responsible for PCa mortality [9]. Bone metastases occur in 70% of castration-resistant patients and are associated with impairment in quality of life due to the onset of skeletal-related events (SREs) such as pathologic fractures, spinal cord compression, need of surgery or radiotherapy on bone, hypercalcaemia, and bone pain (Figure 1).

Therefore, the identification of predictive biomarkers able to discriminate indolent tumors from aggressive ones would be helpful in reducing the risk of overdiagnosis, avoiding patients undergoing surgical/radiation therapies without any clear clinical benefits but complex side effects. Moreover, the consequent possibility to stratify patients on the basis of

their responsiveness to treatment would be helpful in guiding therapeutic decisions and in paving the way to personalized medicine.

MicroRNAs (or miRs) are a family of small noncoding RNA which are able to regulate gene expression at different levels [10]. miRs are interspersed in the genome as independent transcriptional units or within the open reading frame of a specific gene. They are transcribed by the RNA polymerase II and are processed through a series of endonucleolytic cleavages, from nucleus to cytoplasm, in a mature form of 22–25 nucleotide fragments which are able to regulate mRNA spatial and temporal translation/degradation through association with the RNA-induced silencing complex (RISC complex). Generally, sequences recognized by miRs are located in the 3′-untranslated region (3′-UTR) of coding RNA but several studies demonstrated that microRNAs can also bind to the 5′-UTR [11] and to the coding sequence [12] maintaining their regulatory properties. Furthermore, it has been shown that these small RNAs play a dual role in cellular regulation not only in inhibiting but also in activating gene expression through direct binding to target RNA [13]. Based on these observations, which highlight the complexity of this fine-tune control of protein expression, it is not surprising that microRNAs are involved in all basic cell processes, such as proliferation, stemness maintenance, differentiation, and apoptosis. Consequently, deregulation of their expression is significantly correlated with the etiology of many diseases, including cancer. The first evidence of microRNA's role in cancer came from the study of Calin and colleagues in 2002 [14]. They demonstrated that miR-15a and miR-16a were either absent or downregulated in approximately 70% of patients with B-cell chronic lymphocytic leukemia (B-CLL), due to the deletion of chromosome region 13q14.

Circulating microRNAs represent the new expanding field in the world of biomarker research. Indeed, they potentially epitomize the perfect candidates for diagnostic and prognostic purpose [15–17], since their expression reflects the molecular profile of tumor origin [18], and they are highly stable in body fluids and resistant to storage handling. In fact, it has been demonstrated that serum miRs remain stable after being subjected to severe conditions (boiling, high/low pH levels, and freeze-thaw cycles) [19] and preserved in long-term banked human serum samples. Although the mechanisms by which circulating microRNAs are protected from RNase digestion have not yet been extensively described, an increasing amount of evidence indicates that miRs can form complexes with RNA-binding proteins [20] or can be incorporated in cell-derived secreted exosomes [21]. In the tumor environment, tumor-derived exosomes act as carriers of genetic information and proteins destined to recipient cells, where their production is finely regulated by cancer cells. Thus, the incorporation of specific microRNAs in these microvescicles would not be exclusively considered a stochastic process due to the saturated levels of intracellular RNA, but also a functional molecular program which permits cancer cells to influence homeostasis of the surrounding microenvironment. A recent study gave further evidence of this mechanism, demonstrating that exosomes-mediated transfer of miR-105 efficiently destroys tight junctions of the vascular endothelial monolayer, prompting tumor metastasis [22]. Currently, a variety of circulating RNA detection methods is available but the most commonly used are Real-Time PCR, microarray, and deep sequencing [23]. Although, several technical biases are associated with each of these detection protocols including contamination of microRNA samples from other cellular sources (blood cells and stroma cells), there is a lack of unequivocal endogenous control due to the cell-free conditions, specificity of probes and enzyme efficiency rate. Consequently, published studies report conflicting data indicating the necessity of a standardized and robust method with universal parameters for circulating microRNA analysis. From this point on, our knowledge on microRNA-cancer connection has increased exponentially but it is far from complete. However, it is evident that microRNA analysis represents an innovative and specific tool for the improvement of diagnostic, prognostic, and therapeutic protocols. As a consequence of this assumption, an increasing number of studies analyzing genomic alteration of large cohorts of patients are evaluating also the microRNA expression profiles, producing a high quantity of data which could be useful to improve cancer management [24, 25]. Our review focuses on miR clinical relevance and how much our knowledge is expanding in this field. We selected all articles reporting applications and results obtained analyzing patient-derived samples, with a minor analysis of papers clearly oriented towards basic research.

In order to provide a better understanding of recent advances in miR-based biomarker validation for PCa, we divided articles into three main topics: (1) diagnosis, (2) prognosis, and (3) therapy.

2. MicroRNAs and PCa Diagnosis

In 2014, an estimate of 233,000 new cases of PCa will be diagnosed with a 12% mortality rate (National Cancer Institute, http://www.cancer.gov/). A significant percentage of patients who experience tumor development before the mean age represent the most difficult group to manage from a clinical and psychological perspective. Several approaches have been proposed to improve cancer diagnostic accuracy of the PSA test, including the measurement of PSA velocity (change over time), PSA density (ratio between protein blood-level and prostate volume), and PSA-free and protein-bound PSA levels. However, the clinical usefulness of these strategies remains solely experimental. Sensitive biomarkers are needed in order to reduce overdiagnosis, overtreatment, biopsy side effects, and psychological stress [26]. Since microRNA expression reflects the tumor of origin [27] and that it has been correlated with prostate cancer development and progression [28–30], miRs represent an intriguing and promising approach for improving specificity of diagnosis. To date, a distinctive microRNA signature able to distinguish between healthy and diseased patients has not been found but encouraging results have been obtained for PCa (Table 1). In 2006, Volinia and colleagues analyzed the expression profile of 228 miRs in 56 prostate tumor tissues and 7 normal controls [31]. Upregulation of miR-32, -26a, -181a, -93, -196a, -25, -92, and let-7i was confirmed also by Ambs [32] in a cohort

Table 1: MicroRNAs associated with PCa diagnosis.

References	Sample type	miRs deregulated	miRs selected as candidate biomarkers	
Taylor et al. (2010) [24]	113 PCa tissues 28 normal tissues	Large screening study	Taylor et al. 10.1016/j.ccr.2010.05.026	
Volinia et al. (2006) [31]	56 PCa tissues 7 normal tissues	39 upregulated 6 downregulated	Volinia et al. 10.1073/pnas.0510565103;	
Ambs et al. (2008) [32]	60 microdissected tumor tissues 16 normal tissues	21 upregulated 21 downregulated	miR-32, miR-26a, miR-181a, miR-93, miR-196a, miR25, miR-92 and let-7i	↑
Porkka et al. (2007) [38]	5 hormone-naïve PCa tissues 4 HRPC tissues 4 BPH tissues	14 upregulated 37 downregulated	PCa: miR-16, miR-99 and let-7 family	↓
			HRPC: miR-205, miR-100 and miR-30	↓
Ozen et al. (2008) [43]	16 PCa tissues and 10 normal tissues	9 upregulated 76 downregulated	Let-7, miR-30, miR-16	↓
Martens-Uzunova et al. (2012) [44]	102 PCa tissues and normal adjacent tissues	54 deregulated	miR-205	↓
Larne et al. (2013) [45]	49 PCa tissues 25 normal tissues	7 deregulated	miR-96-5p, miR-183-5p	↑
			miR-145-5, miR-221-5p	↓
			(combined in miQ score)	
Moltzahn et al. (2011) [46]	Serum samples from PCa ($n = 36$) and HD ($n = 12$)	6 upregulated 4 downregulated	miR-20b, miR-874, miR-1274a, miR-1207-5p, miR-93, miR-106a	↑
			miR-223, miR-26b, miR-30c, miR-24	↓
Bryant et al. (2012) [48]	Plasma samples from PCa ($n = 78$) and HD ($n = 28$) Urine samples from PCa ($n = 118$) and HD ($n = 17$)	12 deregulated	miR-107, miR-574-3p	↑
Srivastava et al. (2013) [49]	40 PCa tissues and 40 normal adjacent tissues. Urine samples from PCa ($n = 36$) and HD ($n = 12$)	2 downregulated	miR-205, miR-214	↓
Haj-Ahmad et al. (2014) [50]	Urine samples from PCa ($n = 8$) BPH ($n = 12$) patients and HD ($n = 10$)	17 deregulated (only 7 selected for further analysis)	miR-1825 (only in PCa)	↑
			miR-484 (in PCa and BPH)	↓
Schaefer et al. (2010) [54]	76 PCa and adjacent normal tissues	5 upregulated 10 downregulated	miR-96, miR-182, miR-182* miR-183 and miR-375	↑
			miR-16, miR-31, miR-125b, miR-145, miR-149, miR-181b, miR-184, miR-205, miR-221, miR-222	↓

PCa: prostate cancer; HRPC: hormone refractory prostate cancer; BPH: benign prostatic hyperplasia; HD: healthy donors, FFPE: formalin-fixed, paraffin-embedded.

of 76 microdissected tissues including 60 tumor specimens and 16 controls. In this study, the authors identified miR-101, -30c, and -195 significantly upregulated in patients with extraprostatic extension of cancer cells, suggesting a possible role in predicting the evolution of the disease. Interestingly, subsequent studies demonstrated the tumorigenic role of miR-181a and miR-196 in several types of cancers, regulating fundamental processes of malignant progression, such as epithelial to mesenchymal transition (EMT) [33–35] and invasive properties of the cells [36, 37].

In contrast to the previously described studies, Porkka and colleagues demonstrated a significant downregulation of microRNA expression levels correlating with PCa progression [38]. They evaluated a panel of 13 clinical prostate tissues, including 4 BPH, 5 hormone-naïve, and 4 hormone-refractory PCa tumors. Their analysis revealed 37 downregulated and 14 upregulated miRs in PCa specimens. Among downregulated microRNAs miR-16, miR-99, and let-7 family are well known tumor-suppressor genes [39–42]. Interestingly, reduced levels of miR-205, miR-100, and miR-30 family were observed only in hormone-refractory specimens suggesting a hypothetical prognostic role for CRPC prediction. Porkka's profile discretely overlapped the one generated by Ozen et al. [43]. In the latter study, the authors observed

a significant reduction in miR levels in 16 prostate cancer tissues compared with 10 normal prostate tissues. Among the 85 detectable miRs, 76 were downregulated, with a tendency toward a more global downregulation of miRs in case of early PSA recurrence. Stroma contamination could be one possible explanation for widespread downregulation of miRs in prostate cancer tissues. In fact, based on Ozen hypothesis, normal stromal tissues express higher levels of miR and thus, since cancers have less stroma than normal tissues, the relative expression of miRs would appear to be decreased. In line with this hypothesis, a more recent study confirmed the general downregulation of microRNA expression correlating with tumor progression [44]. The authors analyzed a group of 102 patient-derived tissues through microarray. Deregulation of 54 microRNAs was found to clearly segregate PCa specimens from normal adjacent tissues. Moreover, a panel of 25 miRs (13 downregulated and 12 upregulated) significantly correlated with poor clinical parameters, such as Gleason score, incidence of metastases, and E-Twenty-Six variant 1 (ETV1) alterations. Among the microRNAs deregulated in the aforementioned study, 13 of them were specifically analyzed by Larne and colleagues in a cohort of FFPE prostatic tissues derived from 49 prostate cancer patients and 25 men without PCa [45]. Single-assay qRT-PCR analysis revealed a signature of 7 deregulated microRNAs (miR-96-5p, -183-5p, -183-3p, -145-5p, -205-5p, -221-5p, and -409-5p) differentially expressed in PCa samples compared with healthy control. The most significant upregulated (miR-96-5p and miR-183-5p) and downregulated (miR-145-5p and miR-221-5p) microRNAs were combined together in order to obtain an miR index quote (miQ) which was able to discriminate with high accuracy prostate cancer from non-prostate cancer samples and to significantly predict tumor aggressiveness and patients' metastatic status. The predictive value of miQ was further validated in four different cohorts and, despite the differences in size, methodology, and experimental design, the results obtained indicate that miQ could represent a useful clinical biomarker reducing the intervariability between samples.

In recent years, the discovery of circulating microRNAs has resulted in a welcome and exciting change in biomarker research. Particularly for prostate cancer, the possibility to substitute invasive procedures, such as DRE and biopsy, will certainly improve patient care. In 2011, Moltzahn et al. compared microRNA serum levels of 12 healthy men and 36 PCa patients, divided in low risk, intermediate risk and high risk based on the CAPRA score [46, 47]. Ten miRs were substantially different between the healthy and all malignant samples. Four were downregulated in the cancer group (miR-223, -26b, -30c, and -24), and 6 were upregulated in the cancer group (miR-20b, -874, -1274a, -1207-5p, -93, and -106a). Two miRs shown a linear correlation between miR levels and cancer risk: miR-24 steadily decreased with risk, whereas miR-106a steadily increased with risk. A similar analysis was conducted by Bryant and colleagues in 2012 [48]. Using a qRT-PCR microarray panel, they analyzed changing in microRNA expression profiles comparing 78 plasma samples derived from prostate cancer patients and 28 healthy controls. A signature of 12 microRNAs was found to be significantly deregulated in PCa specimens and the upregulation of miR-107 and miR-574-3p in localized prostate cancer patients was also validated through single-assay analysis. Notably, these two microRNAs were present at significantly higher concentrations in the urine of men with cancer compared with the control, indicating their potential as noninvasive detectable biomarkers. Because of the ease of collection, and the fact that prostate cells are directly released into the urethra through prostatic ducts after DRE, urine has become the future for noninvasive biomarker testing. From the first study of Bryant, much effort has been devoted to microRNA detection in patients' urine seeing a rapid expansion in this branch of research. In 2013, Srivastava and colleagues analyzed the expressions of miR-205, miR-214, miR-221, and miR-99b in 36 PCa patients and 12 age and ethnicity matched healthy men [49]. miR-205 and miR-214 were found to be significantly downregulated in cancer samples compared with normal controls. More recently, a cohort of 30 patient-derived urine samples (8 PCa patients, 12 BPH patients, and 10 healthy men) were analyzed for their microRNA expression profiles [50]. From the deregulated group, a panel of 7 miRs (miR-1234, -1238, -1913, -486-5p, -1825, -484, and -483-5p) was selected for further analysis. Single-assay evaluation showed a significant modulation of miR-1825 and miR-484 which were, respectively, upregulated and downregulated in PCa samples compared with healthy controls. In the BPH group, the same trend was observed only for miR-484 whereas upregulation of miR-1825 was found to be variable among the samples. The expression pattern observed for these two miRs was not confirmed when patients were reevaluated two years later but combining data from microRNAs deregulation and abnormal PSA levels, the presence of prostate cancer was detected with 40% of sensitivity and 81% of specificity. These promising results testify that biomarkers detectable in body fluids, which can be obtained in a noninvasive manner, seem a good alternative as possible screening tool.

3. MicroRNAs and PCa Prognosis

Patient prognosis prediction and follow-up monitoring still represent the major challenges for clinical management of prostate cancer. Prostate specific antigen-screening has significantly improved tumor early detection and relapse assessment after radical prostatectomy. However, serum PSA level is not directly correlated with tumor aggressiveness or therapy sensitiveness and its usefulness in reducing cancer mortality rate is still under debate. MicroRNAs are gaining considerable attention in the clinical setting because their expression seems to accurately reflect the malignant evolution of cancer cells [27]. Moreover, the high stability in frozen and formalin-fixed and paraffin-embedded (FFPE) tissues [51] combined with the possibility to detect these small RNAs in body fluids, including serum, plasma, urine, and saliva [21], make them highly attractive as potential biomarkers. Based on this evidence, the number of studies focusing on microRNA expression profiles in prostate cancer has notably increased; however, results deriving from high-throughput approaches produced partially contradictory

reports. Lack of uniformity in proposed datasets for patient stratification is in part due to the different study design, underestimated treatments of the patients, methods of sample collection, presence of contaminating cells, and sensitivity and specificity of the platforms used. A summary of the most prognostic significant miR has been reported in Table 2. As a consequence of an abundant data in literature and extreme complexity of the discussion in prognostic values of the data, we divided this paragraph into two sections, one dedicated to tissue and the other to fluid samples.

3.1. Prognostic MicroRNAs in PCa Tissues. The first attempt to segregate patients through a microRNA profile screening came from primary tumor analyses. Although it may be questionable whether tumor tissue evaluation effectively predicts changes observed in cancer cells, which are not only spatial but also temporally regulated, the availability of a limitless quantity of banked tumor samples makes them a unique resource of information. The possibility of analyzing preserved specimens for retrospective studies is particularly advantageous for PCa, which is a slow-growing disease requiring a long-scheduled follow-up monitoring program to obtain significant correlation between biomarker expression and tumor progression.

3.1.1. Biochemical Recurrence (BCR) and MicroRNAs. Biochemical recurrence is defined as *de novo* rising of PSA blood levels after radical prostatectomy [52]. BCR is widely used as an early end point to assess treatment success and frequently prompts the initiation of secondary therapy in order to reduce the risk of metastasis formation. Several studies were attempted in order to establish a distinctive signature of microRNAs able to stratify patients on the basis of their risk in developing BCR. However, much controversy is still present in the literature possibly due to several biases (study design, sample collections, sensitivity, and specificity of platforms used) which are common in translational research. In 2009, Tong and colleagues analyzed the microRNA expression profiles of 40 FFPE tumor tissues divided in early biochemical relapse ($n = 20$) and nonrelapsed ($n = 20$) patients [53]. A signature of 16 microRNAs was able to segregate 75% of analyzed relapsed patients, excluding 85% of patients with no evidence of recurrence. Interestingly, single-assay qRT-PCR validates the upregulation of miR-16, miR-135b, miR-194, and miR-218 and downregulation of miR-140. In addition, the authors showed a significant reduction of miR-23b, -100, -145, -221, and -222 in prostate cancer tissues compared with normal adjacent tissues, giving a diagnostic relevance to their analysis. Another study performed by Schaefer and colleagues [54], identified a microRNA signature of 10 miRs downregulated (miR-16, -31, -125b, -145, -149, -181b, -184, -205, -221, and -222) and 5 upregulated (miR-96, -182, -182*, -183, and -375), differently expressed in tumor tissues compared with normal adjacent tissues in a cohort of 76 prostate cancer patients. Further validation experiments significantly correlate miR-31, -125b, -205, -222, and -96 with Gleason score and tumor stage. miR-96 expression was also associated with BCR, indicating its potential role as a prognostic biomarker. Notably, negative modulation of miR-205 in recurrent samples was confirmed also by Hulf et al. [55] who also demonstrated that this microRNA can impair cell viability of cancer cells through modulation of MED1 which has been correlated with castration-resistance acquisition [56]. Due to the high number of studies demonstrating the downregulation of miR-205 in patient-derived samples, the role of miR-205 in PCa biology has been further investigated in basic research, and recent reports demonstrated that this microRNA exerts its tumor-suppressive functions directly inhibiting the expression of the AR and its downstream signaling cascade, c-SRC oncogene and the antiapoptotic Bcl-2 protein [57–60]. Interestingly, Mittal and colleagues observed that the delivery of gemcitabine-conjugated and miR-205—complexed copolymers effectively reverses chemoresistance, invasion, and migration of pancreatic cancer cells and inhibits tumor growth using *in vivo* model [61]. All these results demonstrate the clinical relevance of microRNAs as therapeutic tools for treating cancer diseases and the significant advances in nanotechnology applied to medicine with a consequent increase in the number of curative options for diseased patients. Among downregulated microRNAs correlating with PCa cancer progression, let-7b and let-7c were demonstrated to be lost in patients with shorter disease-free survival time [62]. This result is in agreement with the demonstrated biological role of let-7c in suppressing the expression of androgen receptor (AR) in prostate cancer cells [63]. Interestingly, the let-7c/AR interaction was not mediated by the canonical mechanism of miR-mediated control of gene expression, but rather through the targeting of v-myc avian myelocytomatosis viral oncogene homolog (MYC), a recognized transcription factor for the AR. The microRNA—mediated regulation of the androgen receptor represents a new attractive way trying to unravel the different pathways, in which this molecule plays a pivotal role, and which are responsible for PCa progression. However, since we focused mainly in the clinical aspects of microRNAs biology, we decided not to discuss papers lacking in analyzing clinical samples. Nevertheless, in order to facilitate the comprehension of the importance of AR targeting for future perspectives, we listed in Table 3 several microRNAs involved in receptor regulation that have been validated in basic research studies.

Several microRNAs play a dual role in prostate cancer development and sometimes show opposite behavior in basic research compared to translational studies. This is the case of miR-100 and miR-221/-222. Basic research studies have demonstrated the tumor-suppressor function for miR-100, regulating several oncogenes, such as insulin-like growth factor 2 (IGF2) and mechanistic target of rapamycin (mTOR) [64, 65]. However, Leite et al. in 2011 [66] found a significant overexpression of these microRNAs in BCR prostate cancer samples. The same scenario is applicable to miR-221/-222 which are well known oncomiRs acting in various tumor types [67] and regulating cyclin-dependent kinase (CDK) inhibitors $p27^{Kip1}$ and $p57^{Kip2}$ [68, 69]. Moreover, the work of Sun et al. [70] indicated that miR-221/-222 are able to reduce the sensitivity of androgen-dependent cell lines to dihydrotestosterone (DHT) resulting in androgen

Table 2: MicroRNAs associated with PCa prognosis.

References	Sample type	Clinical parameters	miRs deregulated	miRs selected as candidate biomarkers	
Taylor et al. (2010) [24]	113 PCa tissues 28 normal tissues	Large screening study		Taylor et al. 10.1016/j.ccr.2010.05.026	
Martens-Uzunova et al. (2012) [44]	102 PCa tissues and normal adjacent tissues	High risk biochemical recurrence	12 upregulated 13 downregulated	miR-19a, miR-130a, miR-20a/106/93 miR-27, miR-143, miR-221/222	↑ ↓
Tong et al. (2009) [53]	40 FFPE prostatectomy Specimens (20 without early BCR 20 with early BCR)	Biochemical recurrence	2 upregulated 4 downregulated	miR-135, miR-194 (40% of case) miR-145, miR-221, miR-222	↑ ↓
Schaefer et al. (2010) [54]	76 PCa and adjacent normal tissues	Biochemical recurrence	5 upregulated 10 downregulated	miR-96	↑
Hulf et al. (2013) [55]	149 PCa and 30 matched normal tissues	Biochemical recurrence	1 downregulated	miR-205	↓
Schubert et al. (2013) [62]	BCR tissues and disease-free tissues	Biochemical recurrence	2 downregulated	let-7b and let-7c	↓
Leite et al. (2011) [66]	21 frozen BCR tissues 28 frozen disease-free tissues	Biochemical recurrence	4 upregulated	miR-100	↑
Karatas et al. (2014) [72]	82 PCa tissues (41 BCR and 41 disease-free)	Biochemical recurrence	3 downregulated	miR-1, miR-133b	↓
Selth et al. (2013) [102]	Serum samples from PCa patients (BCR = 8) disease-free = 8)	Biochemical recurrence	3 upregulated	miR-194 miR-146-3p	↑
Shen et al. (2012) [87]	Plasma samples from PCa (n = 82)	Castration resistance	2 upregulated	miR-21, miR-145	↑
Jalava et al. (2012) [90]	28 primary PCa tissues 14 CRPC tissues 12 BPH tissues	Castration resistance	4 upregulated 3 downregulated	miR-32, miR-148a, miR-590-5p, miR-21 miR-99a, miR-99b, miR-221	↑ ↓
Peng et al. (2011) [94]	6 primary PCa tissues 7 bone metastatic tissues	Metastasis	5 downregulated	miR-508-5p, miR-143, miR-145, miR-33a, miR-100	↓
Saini et al. (2011) [95]	36 PCa tissues 8 metastatic tissues 8 normal tissues	Metastasis	1 downregulated	miR-203	↓
Mitchell et al. (2008) [19]	Serum samples from metastatic PCa (n = 25) and age-matched HD (n = 25)	Metastasis	6 deregulated	miR-141	↑
Brase et al. (2011) [100]	Serum samples from localized PCa (n = 14) and metastatic PCa (n = 7)	Metastasis	5 upregulated	miR-141, miR-375	↑
Bryant et al. (2012) [48]	Serum samples from PCa (n = 72) and metastatic PCa (n = 47) Plasma samples from PCa (n = 55) and metastatic PCa (n = 24)	Metastasis	2 upregulated	miR-141 and miR-375	↑
Nguyen et al. (2013) [103]	Serum samples from localized PCa (n = 58) and metastatic CRPC (n = 26)	Castration resistance	3 upregulated 1 downregulated	miR-141, miR-375, miR-378* miR-409-3p	↑ ↓
Zhang et al. (2011) [88]	Serum samples from localized PCa (n = 20), ADPC (n = 20), CRPC DTX treated (n = 10) and BPH (n = 6)	Castration resistance	1 upregulated	miR-21	↑

FFPE: formalin-fixed, paraffin-embedded, BCR: biochemical recurrence, Pca: prostate cancer, CRPC: castration resistant prostate cancer, BPH: benign prostatic hyperplasia, HD: healthy donors.

Table 3: MicroRNAs regulating AR expression through direct targeting.

References	miRs involved in AR regulation
Hagman et al. (2013) [57]	miR-205
Qu et al. (2013) [132]	miR-185
Lin et al. (2013) [133]	miR-31
Sikand et al. (2011) [134]	miR-488*
Östling et al. (2011) [135]	miR-135b, miR-105b, miR-297, miR-299-3p, miR-34a, miR-34c, miR-371-3p, miR-421, miR-499a, miR-499b, miR-634, miR-654-5p, miR-9

independence acquisition. All these results contradict other various studies reporting a significant downregulation of miR-221/-222 expression in clinical samples representative of PCa progression, including castration-resistant and biochemical recurrence stages [38, 54, 71]. More recently, Karatas and colleagues described a significant downregulation of miR-1 and miR-133b in recurrent compared with disease-free patients [72]. As negative modulation of miR-1 is in agreement with its tumor-suppressive role in prostate cancer cells, modulating at least indirectly the expression of AR and consequently their proliferative capabilities [73], downregulation of miR-133b does not reflect recent reports in which its dependence on androgen receptor and its role in maintaining cell viability is described [74, 75].

Altogether, these observations demonstrated the fundamental role of the biological context in which microRNAs are located but, at the same time, they stress the need to bridge the gap between basic and translational research in order to optimize the efforts toward improving cancer management.

3.1.2. Castration Resistant Prostate Cancer (CRPC) and MicroRNAs. The concept of androgen deprivation for the treatment of advanced prostate cancer was developed by Huggins and Hodges in 1941 [76], and up until today, AR—target therapy remains the first line treatment for this disease. Almost all patients with advanced prostate cancer initially respond to androgen deprivation therapy (ADT), showing reduced PSA levels indicating a partial regression of residual tumor. However, this type of condition is transitory and invariably develops into a castration-resistant form which leads to the formation of bone metastases in a significant percentage of treated patients.

miR-21 is one of the most commonly deregulated oncomiR in cancer [77]. Validated targets of miR-21 enclose several genes mainly implicated in suppressing cell migration and invasion, including programmed cell death 4 (PDCD4), phosphatase and tensin homolog (PTEN), tropomyosin 1 (TPM1), sprouty homolog 2 (SPRY2) and the metalloprotease inhibitors TIMP3 and reversion-inducing-cysteine-rich protein with kazal motifs (RECK) [78–83]. In PCa biology, miR-21 expression increases together with clinical parameters (pathological stage, lymph node metastasis, capsular invasion, organ confined disease and Gleason score) and it is correlated with biochemical relapse, castration resistance and metastasis formation [66, 84, 85]. Significantly, an increase of miR-21 and miR-145 in D'Amico et al. score [86] high-risk compared with low-risk patients, were described by Shen et al. [87]. miR-21 was also described as predictive biomarker of PCa progression since its blood-levels are directly correlated with castration-resistant and metastatic states [88]. One of the molecular mechanisms through which miR-21 is able to regulate castration resistance process was described recently. It involves the activation of the epithelial to mesenchymal transition (EMT), through negative modulation of tumor-suppressor BTG2 [89]. A further microarray analysis identified a panel of miRs (miR-21, -32, -99a, -99b, -148a, -221, and -590-5p) differentially expressed in castration-resistant tumors compared to benign prostate hyperplasia [90], and the functional study revealed that also miR-32 can inhibit BTG2 expression, suggesting a prognostic and therapeutic role of this miR.

EMT seems to be a relevant process in escaping blockade of androgen signals. miR-205 and miR-30 are two tumor-suppressor microRNAs which can block the transition of cancer cells towards an undifferentiated and more aggressive state and both miRs have been negatively correlated with prostate cancer malignant evolution. miR-30 reduces expression levels of v-ets avian erythroblastosis virus E26 oncogene homolog (ERG) gene, which is one of the EMT-associated effectors and, more importantly, is the most frequently overexpressed oncogene in PCa activated by genomic fusion of TMPRSS2 and ERG genomic loci [91]. In the same way, miR-205 exerts its functions inhibiting the translation of EMT-related genes zinc-finger-E-box-binding homeobox 1 (ZEB1) and 2 (ZEB2/SIP1) [92]. Moreover, its ectopic expression in prostate cancer cell can impair dedifferentiation and invasive properties acquisition blocking cancer associate fibroblast (CAF) stimulation [93].

3.1.3. Bone Metastasis and MicroRNAs. Bone metastasis is a common and severe complication of late-stage prostate cancer. Complex interactions between tumor cells, bone cells, and a milieu of components in their microenvironment contribute to the osteolytic, osteoblastic, or mixed lesions present in patients with advanced forms of PCa. Despite the enormous efforts in unraveling the molecular mechanisms regulating bone metastasis, this remains the most clinically relevant but poorly understood aspect of the disease.

Analyzing a cohort of 13 patient-derived specimens (6 primary tumors and 7 bone metastatic PCa samples), Peng and colleagues identified 5 microRNAs (miR-508-5p, -143, -145, -33a, and -100) significantly decreased in bone metastasis [94]. miR-143 and miR-145 were also found to be inversely correlated with serum PSA levels and Gleason score. Further analysis in metastatic PC3 cell line demonstrated that overexpression of these two miRs can reduce invasive capabilities in increasing expression of E-Cadherin and consequently impairing EMT activation.

Similar results were obtained for miR-203 whose expression was significantly attenuated in bone metastatic tissues compared with normal tissues [95]. As a possible explanation

for this modulation, the authors demonstrated that miR-203 controls the expression of the EMT factor ZEB2 and the bone metastasis-related factor RUNX2. Moreover, this miR has been associated with "stemness" maintenance owing to its ability to inhibit the self-renewal-associated BMI1 polycomb ring finger oncogene (BMI1) [96].

Although the significant association between miR-15/-16 cluster and bone metastasis formation has not yet been confirmed, the role of these two microRNAs in PCa, and other cancer types, has been extensively evaluated [39]. Alteration of the miR-15a and miR-16-1 translates into multiple tumor-promoting processes through the derepression of key cell cycle-and apoptosis-related genes such as B-cell CLL/lymphoma 2 (BCL2), wingless-type MMTV integration site family-member 3A (WNT3A) and cyclin D1 (CCND1) [97]. miR-15 and miR-16 have been found to be downregulated also in cancer-associated fibroblasts (CAFs), promoting malignant transformation and progression [98]. Reduced miR-15a and -16-1 levels in CAFs result in FGF-2/FGFR1 axis activation, which ultimately increases the tumor-supportive capabilities of CAFs. Interestingly, delivery of synthetic miR-16 was shown to be able to inhibit the growth of metastatic prostate cancer cell lines in mouse bone [99]. This study not only suggests that loss of miR-16 is a predictor factor of metastatic colonization of bones but also that systemic delivery of miR-16 could represent a novel type of personalized-therapy for treating patients with advanced prostate cancer.

3.2. Prognostic MicroRNAs in Patient Body Fluids Circulatory System. The first report regarding the potential role of circulating microRNAs as predictive biomarkers in PCa, was published by Mitchell and colleagues in 2008 [19]. They analyzed a panel of six candidates miRs (miR-100, -125b, -141, -143, -296, and -205) in serum samples collected from 25 metastatic prostate cancer patients and 25 age-matched healthy donors. Among all the candidates, only miR-141 showed a significant overexpression in PCa patients and a further analysis revealed a moderate correlation with PSA levels. Similar results were obtained by Brase et al., who demonstrated that miR-141 is upregulated, together with miR-375, in sera of metastatic patients compared with nonmetastatic samples [100]. Interestingly, a significant deregulation of these two miRs was found within the nonmetastatic group (low-risk versus high-risk), indicating their potential role not only in prognosis but also in early diagnosis. The upregulation of miR-141 and miR-375 in plasma and serum samples of metastatic patients was also demonstrated in the aforementioned study of Bryant et al. [48]. The biological role of miR-141 in metastatic progression is still not fully understood, however its expression has been demonstrated to be positively correlated with expression of alkaline phosphatase (ALP), a marker of skeletal lesions and with increased level of bone metastatic lesions [101].

Circulating microRNAs can also be associated with biochemical recurrence and castration-resistant state acquisition. In 2013, Selth and colleagues revealed that miR-194 and miR-146-3p are overexpressed in sera of patients experiencing biochemical relapse [102]. Interestingly, they found also an increased level of miR-141, miR-375, and miR-200 in these patients but further validation analyses did not confirm a significant statistical relevance. On the contrary, in the study conducted by Nguyen et al., miR-141 and miR-375 were found strongly upregulated in a cohort of patients experiencing CRPC compared with localized PCa patients [103]. The same authors observed a significant correlation with castration resistant state also for upregulated miR-378* and for downregulated miR-409-3p. All these results, especially those associated with miR-141 and miR-375, confirmed that microRNA expression is extremely sensitive to molecular changes in cancer cells.

Similarly, miR-21 has been reported to be upregulated in CRPC patients. Zhang and colleagues analyzed a cohort of 6 BPH patients, 20 patients with localized PCa, 20 androgen-deprivation therapy responsive patients and 10 CRPC patients under docetaxel treatment, observing a positive correlation between miR-21 levels and tumor progression [88]. Intriguingly, further observations in the CRPC cohort revealed a direct correlation between high expression of miR-21 and unresponsiveness to chemotherapy, indicating that this microRNA can distinguish between patients that will benefit from the chemotherapeutic regimen and patients that will not.

4. MicroRNAs and PCa Therapy

In addition to the absence of reliable diagnostic and prognostic indicators, prostate cancer management is also impaired by the lack of tools in guiding treatment assignment and evaluating therapy response. Testosterone suppression represents the gold-standard first line treatment for men with recurrent PCa, but the majority of tumors evolve towards a castration-resistant state (CRPC) within 2 years. Despite such tangible advances in local and systemic therapy, the global management of PCa patients is still far from ideal, and many questions concerning the optimal treatment of both early and advanced forms still need to be addressed. The administration of targeted or conventional therapies requires accuracy of staging procedures and predictive biomarkers of prostate cancer patients response. Hormone therapy for prostate cancer is typically initiated using drugs that lower serum testosterone, often in combination with competitive androgen receptor antagonists, such as bicalutamide or Casodex (CDX). The initial response to ADT is significantly high in almost all treated patients but, within two years after the initial treatment, a significant group of these patients fails to be sensitive to this kind of treatment. Once this occurs, secondary hormone therapy is usually considered and it includes different types of antiandrogens. Recently, different drugs able to block androgen synthesis have been approved by the FDA, such as (i) Abiraterone Acetate (AA) which is a potent and selective inhibitor of CYP17, a protein required for androgen biosynthesis in the testes, adrenal glands and prostate tissue, and (ii) enzalutamide (Xtandi) which blocks the effects of androgens in stimulating the growth of the prostate cancer cells. Although initially effective at blocking tumor growth, these therapies eventually fail, leading to a lethal

Table 4: MicroRNAs associated with PCa therapy.

References	Sample type	Therapy	miRs deregulated	miRs selected as candidates	
Josson et al. (2008) [107]	LnCaP and LnCaP C4-2B	Radiation therapy	6 downregulated	miR-521	↓
Huang et al. (2013) [110]	PC3 radiation resistant cells	Radiation therapy	1 upregulated	miR-95	↑
Ribas et al. (2009) [85]	LNCaP and LAPC-4	Hormone therapy	Overexpressed	miR-21	↑
Ottman et al. (2014) [113]	LnCap CDX sensitive cells LnCap CDX non-sensitive cells	Androgen deprivation-therapy and casodex	21 upregulated 22 downregulated	http://www.molecularcancer.com /content/13/1/1	
Lehmusvaara et al. (2013) [114]	28 tumor tissues (n = 8 goserelin-treated patients n = 9 bicalutamide-treated patients n = 11 no treated-patients)	Endocrine treatment	10 deregulated	miR-9 and miR-17	↑
				miR-218	↓
Zhang et al. (2011) [88]	Serum samples from localized PCa (n = 20), ADPC (n = 20), CRPC DTX treated (n = 10) and BPH (n = 6)	Docetaxel	1 upregulated	miR-21 (in CRPC docetaxel resistant)	↑
Puhr et al. (2012) [116]	PC3 docetaxel-resistant cells	Docetaxel	2 downregulated	miR-200c, miR-205	↓
Lin et al. (2014) [118]	Serum and plasma samples from CRPC PCa (n = 97) before and after therapy	Docetaxel	46 deregulated	miR-200c, miR-200b, miR-146a, miR-222, miR-301b, miR-20a	
Kojima et al. (2010) [119]	PC3 paclitaxel-resistant cells	Paclitaxel	1 downregulated	miR-34a	↓
Fujita et al. (2010) [121]	PC3 and DU145	Paclitaxel	1 downregulated	miR-148	↓

PCa: prostate cancer; ADPC: androgen dependent prostate cancer; CRPC: castration resistant prostate cancer; BPH: benign prostatic hyperplasia; DTX: docetaxel; CDX: casodex.

drug-resistant condition, castration-resistant state. For men unresponsive to all forms of hormone treatment, the clinical protocol actually provides different therapeutic approaches, such as bisphosphonates administration, targeted therapies, immunotherapy (Sipuleucel-T), and chemotherapy. Recently, Radium-223 (Xofigo) has been approved as a new agent for bone metastasis treatment. It is a radioactive element that localizes in bone and its delivery may be effective at relieving bone pain, preventing complications and prolonging life expectancy [104]. The availability of biomarkers that discriminate patients with indolent or aggressive tumors would allow appropriate treatment early tumors doomed to become aggressive and metastatic. New molecular markers of therapy response will be essential in driving therapy decision-making of advanced tumors. Therefore, an overall improvement of prostate cancer management will need a comprehensive effort to devise new tools for patient stratification and prediction of therapy response. A future clinical use of miRs in the era of individualized oncology may satisfy the requirement for a patient-tailored therapeutic approach, based on personalized therapeutic choices guided by patient' molecular profiles. Biomarker signature for patient stratification and therapy decision is also expected to influence modern therapeutic approaches to prostate cancer treatment such as neo- or adjuvant systemic therapy, early chemotherapy, bisphosphonates, and targeted therapies. In fact, new targeted treatments such as Denosumab, Abiraterone, Sipuleucel-T, androgen receptor-, MET receptor-, and angiokinase-inhibitors will highly benefit from molecular biomarkers that support the decision-making process.

To date, an increasing number of published studies are examining the role of microRNAs as direct targets for prostate cancer therapy (Table 4). However, the majority of studies are based on prostate cancer cell line analysis which have no valuable clinical relevance.

4.1. Radiation Therapy. Radiation therapy (RT) is one of the treatment options for localized, high risk prostate cancer tumors which cannot be treated with radical prostatectomy. However, the risk of tumor regrowth following RT remains high for a number of cancer patients, despite modern radiation oncology techniques allowing specific delivery of high radiation doses directly to the tumor volume [105, 106]. Thus, radiation resistance remains an open issue to be solved. The role of microRNAs in RT is not yet fully understood and the few studies analyzing the effects of X-rays administration in changing miR expression profile come from basic research. In 2008, Josson and colleagues [107] observed a considerable downregulation of 6 miRs after irradiation of androgen-dependent LnCaP and androgen-independent LnCaP C4-2B cell lines. miR-521 was found to be downregulated to a greater extent in both cell lines and its forced expression increased LnCaP sensitivity to radiation-induced damages. The observed phenotype was demonstrated to be a consequence of Cockayne syndrome A (CSA), a DNA repair

protein, and manganese superoxide dismutase (MnSOD), an anti-apoptotic and antioxidant enzyme, regulation. All these results suggest that miR-521 could be a new potential tool in enhancing the efficacy of radiation treatment in PCa.

LnCaP cells were used as *in vitro* model for measuring radiation effects also by Li et al. [108] who demonstrated that overexpression of miR-106b is sufficient to override the cell-cycle arrest induced by irradiation, through downregulation of its validated target p21 [109]. Interestingly, this microRNA was previously found upregulated in PCa specimens [32] compared to normal control. Altogether, these results suggest a potential therapeutic role of miR-106b suppression. Another microRNA involved in radiation therapy resistance is miR-95, which was found to be upregulated in radiation resistant PC3 cell line compared to parental control [110]. A further analysis correlated resistance acquisition with suppression of the sphingosine-1-phosphate phosphatase 1 (SGPP1) which was demonstrated to be directly regulated by miR-95.

Despite this recent advance, little is known about the regulatory effects of miRs on radiation resistance acquisition and the molecular mechanisms involved. Consequently, a comprehensive analysis on the role of microRNAs in treatment responsiveness, especially in the clinical setting, is still required to improve patient prognosis.

4.2. Hormone Therapy. Androgen deprivation therapy is the standard treatment for patients who experience recurrence after surgical resection of the prostate but, within two years after the initial treatment, a significant group of these patients develop incurable forms. Several microRNAs have been associated with castration-resistant properties of the cells through AR regulation. Overexpression of miR-21, which is positively modulated by androgen receptor, is able to increase proliferation abilities of androgen-dependent LnCaP and LAPC-4 cell lines, overcoming cell cycle arrest induced by testosterone deprivation and anti-AR treatment. Moreover, the effect of miR-21 in evasion of castration-mediated growth arrest was also confirmed *in vivo*, giving further evidence of its role in PCa [85]. These results are in agreement with translation studies which indicate miR-21 as one of the microRNAs upregulated to major extent in prostate cancer progression and suggest future implications for personalized target therapies.

The opposite scenario has been described for the tumor-suppressor miR-331-3p [111]. This microRNA is able to reduce v-erb-b2 avian erythroblastic leukemia viral oncogene homolog 2 (ERBB2) expression, a known oncogene whose expression increases in advanced prostate cancer [112], and to impair AR signaling pathway. Furthermore, miR-331-3p increases the bicalutamide-induced inhibition of PSA expression.

Although the significant results obtained for miR-21 and miR-331-3p, changing in their expression was not confirmed by Ottman and colleagues [113]. They analyzed different LnCaP-derived cell lines with different sensitiveness to androgen withdrawal and CDX treatment. Comparing microRNA expression profiles, they found 21 upregulated and 22 downregulated miRs in both androgen-deprivation and CDX administration conditions. Interestingly, deregulation of several microRNAs was also confirmed in a study conducted on patient samples derived from a neoadjuvant trial consisting of 8 men treated with goserelin, 9 men treated with CDX ($n = 9$), and 10 men untreated, prior to prostatectomy [114]. Analysis of resected tumor tissues revealed an upregulation of miR-9 and miR-17 and a downregulation of miR-218 in agreement with Ottman's study. Furthermore, the authors identified a panel of miRs upregulated (miR-141, -375, -210, etc.) and downregulated (miR-204, -100, -125b, etc.) after hormone treatment, whose expression trend correlates with poor prognosis.

Although we are moving toward translational medicine, most studies still rely on prostate cancer cell lines as surrogates for therapy response, which is not favorable. As the attempts to unravel the molecular mechanisms responsible for castration-resistant state are producing intriguingly results, it is evident that shifting this knowledge into the clinical setting will provide a great benefit for patient outcome.

4.3. Chemotherapy. Chemotherapy is offered to suitable patients with CRPC who have failed other treatment options. However, 40–50% of patients with CRPC do not respond substantially to chemotherapy, with the median duration of response being 6–9 months [115].

EMT is an event frequently involved in chemotherapy resistance of cancer cells. Puhr et al. [116] reported that docetaxel-resistant prostate cancer cells underwent an epithelial-to-mesenchymal transition during the selection process, leading to diminished E-cadherin levels and upregulation of mesenchymal markers. This phenotype was accompanied by a significant downregulation of miR-200c and miR-205 which, once reexpressed, were able to rescue E-cadherin and increase apoptotic rate of resistant cells. Different studies demonstrated the correlation of miR-205 with enhanced cisplatin cytotoxicity, through negative modulation of autophagic pathway, and with docetaxel-resistance acquisition [116, 117]. Docetaxel-resistant cell lines were also analyzed by Lin et al. in order to identify candidate circulating microRNA biomarkers able to predict chemotherapy responsiveness [118]. After a first screening in naïve and resistant PC3 and DU145 cell lines, the authors selected a panel of 46 deregulated microRNAs which were further analyzed for their expression in plasma and serum samples derived from 97 CRPC patients who were stratified in responder and nonresponder groups. Six microRNAs (miR-200c, miR-200b, miR-146a, miR-222, miR-301b, and miR-20a) were significantly associated with therapy responsiveness on the basis of their pretreatment levels and posttreatment expression changing. Furthermore, 12 microRNAs (miR-200b, -200c, -200a, -429, -21, -590-5p, -375, -132, -20a, -20b, -25, and -222) were correlated with patient overall survival.

In PCa, miR-34a and miR-148 were associated with paclitaxel resistance. Restored expression of miR-34a is able to reduce proliferative capabilities of PC3 taxol-resistant cells through modulation of sirtuin 1 (SIRT1) and antiapoptotic BCL2 [119]. Interestingly, a similar effect was observed

Table 5: MicroRNAs associated with stemness properties acquisition.

References	miRs associated with stemness properties
Liu et al. (2012) [125]	miR-34a
Saini et al. (2012) [126]	miR-708
Huang et al. (2012) [127]	miR-143 and miR-145
Hsieh et al. (2013) [129]	miR-320

by Rokhlin et al. [120], whereby simultaneous overexpression of miR-34a and miR-34c resulted in increased p53-mediated apoptosis in response to doxorubicin treatment of LNCaP cells. The role of miR-148a in the response of hormone-refractory prostate cancer cells to chemotherapy was investigated by Fujita and colleagues [121]. Expression levels of miR-148a were found to be lower in both PC3 and DU145 hormone-refractory prostate cancer cells, compared to normal human prostate epithelial cells and LNCaP hormone-sensitive prostate cancer cells. Furthermore, forced expression of miR-148 in PC3 cells inhibited cell growth, cell migration, and cell invasion and increased sensitivity to paclitaxel through modulation of MSK1.

The theoretical concept of EMT is strictly correlated with the new evidence demonstrating the presence within the tumor mass of a stem-like subset of cells which are able to self-renew and drive cancer development and progression [122]. Moreover, increasing evidence indicates that these cells, which are called cancer stem cells (CSCs), are responsible for drug resistance, tumor recurrence, and metastasis formation [123, 124]. CSCs hypothesis suggests several possible explanations for the mainly unsolved questions of treating patients with cancer, such as local recurrence after treatment of solid tumor by radiation or chemotherapy and development of metastases that can appear many years after curative surgical treatment of primary tumor. The first who analyzed the role of microRNAs in stem-like prostate cancer cells were Liu and colleagues in 2011 (Table 5) [125]. They demonstrated that forced expression of miR-34a reduces purified $CD44^+$ stem-like prostate cancer cells and that it is sufficient to inhibit clonogenic expansion, tumor regeneration, and metastasis. In contrast, downregulation of miR-34a in $CD44^-$ cell lines promoted tumor development and invasive properties. Furthermore, the authors identified and validated CD44 itself as a direct and functional target of this microRNA. CD44 protein is also a direct target of the tumor-suppressor miR-708. As a consequence of this regulation, ectopic expression of miR-708 is able to inhibit the tumor-initiating capacity of prostate cancer cells *in vitro* and to reduce tumor progression in prostate cancer xenografts [126]. A further analysis revealed that low miR-708 expression was associated significantly with poor survival outcome, tumor progression, and recurrence in patients with prostate cancer. Finally, miR-320, -143, -145, and let-7 were also associated with suppression of stem-like properties of the cells [125, 127–129].

Taken together, these results establish a strong rationale for developing microRNA-based therapy for targeting prostate CSCs in order to eradicate the basal core of tumors and restore patient responsiveness to current pharmacological treatment.

5. Conclusion

In the last decade, the advent of PSA screening improved PCa detection but its low predictivity caused overdiagnosis and overtreatment with consequent increase in patient morbidity [5]. In addition, analysis of PSA blood levels does not represent *per se* an unequivocal method to assess the presence of prostate adenocarcinoma since it can be influenced by nonneoplastic alterations. Thus, patients necessarily undergo multiple local biopsies which up to now represent the standard approach for diagnosis. However, it is possible that also after anatomical and pathological evaluation of tissue biopsies, diagnosis still remains uncertain with consequent psychological stress for the patients. In this context, a lot of effort has been dedicated trying to improve our capabilities to manage cancer diseases and the exponential development of innovative technologies creating the bases for translating results from basic research to a real patients' clinical benefit. Thus, the role of microRNAs in prostate cancer provides a solid rationale for their further evaluation in clinical practice. In fact, microRNAs' ability to regulate almost all cellular processes makes them an attractive tool for disease management, including cancer. Furthermore, their high stability in patient-derived tissues and body fluids provides the possibility to perform noninvasive screening which are able to evaluate patients' natural history of the disease with high accuracy, due to the fact that microRNAs are representative of all tumor molecular changing during the time. The increasing sensitiveness and reliability of new technologies assure the analysis starting from low amount of material giving the opportunity to create new accurate and noninvasive tests. In particular for prostate cancer, the identification of microRNA signatures, correlating specifically with tumor properties, represents a new source of specific biomarkers and the future advantage for both diagnosis and prognosis is clearly evident. The increasing amount of studies analyzing miR expression profiles is a consequence of this assumption with the specific aim to identify innovative biomarkers able to distinguish nonneoplastic alteration of the prostate from localized tumors and indolent from aggressive tumors, in order to decrease the incidence of overdiagnosis/overtreatment and to guide therapeutic decision, respectively. In the era of personalized therapy, it is imperative to find new biomarkers able to mirror cancer aberrant molecular setting and to guarantee monitoring cancer progression during early and late stages. Thus, the possibility to create a personal molecular profiling of the tumors may determine the creation of an individual therapeutic protocol optimizing therapy benefit and reducing secondary effects of nonnecessary treatment. Finally, advancement in delivery systems (liposomes and nanoparticles among others) and molecule stability (LNA-modified anti-miR and microRNA-mimics) have paved the way for the use of microRNAs as effective drugs for integrated therapy [130, 131] and several clinical trials testing the efficacy of microRNAs are ongoing. This field may offer a great opportunity in terms of application in tumor disease, including PCa. Overall, improving prostate cancer management will provide both individual and social benefits due to the massive social impact of this malignancy, allowing at the same time a

significant rationalization of financial resources dedicated by the national health system.

Conflict of Interests

The authors declare that there is no conflict of interests that could be perceived as prejudicing the impartiality of the research reported.

Acknowledgments

This work was supported by AIRC, Bando Giovani Ricercatori 2012 and Fondazione Roma. The authors would like to thank Dr. Tania Merlino and Dr. Giuseppe Loreto for technical support. The authors regret not being able to cite many important primary studies owing to space limitations.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 249393, 11 pages
http://dx.doi.org/10.1155/2014/249393

Review Article

The Role of MicroRNAs in Ovarian Cancer

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Received 6 June 2014; Revised 22 August 2014; Accepted 27 August 2014; Published 10 September 2014

Academic Editor: Paolo Gandellini

Ovarian cancer is the most lethal of malignant gynecological tumors. Its lethality may be due to difficulties in detecting it at an early stage and lack of effective treatments for patients with an advanced or recurrent status. Therefore, there is a strong need for prognostic and predictive markers to diagnose it early and to help optimize and personalize treatment. MicroRNAs are noncoding RNAs that regulate target genes posttranscriptionally. They are involved in carcinogenesis, cell cycle, apoptosis, proliferation, invasion, metastasis, and chemoresistance. The dysregulation of microRNAs is involved in the initiation and progression of human cancers including ovarian cancer, and strong evidence that microRNAs can act as oncogenes or tumor suppressor genes has emerged. Several microRNA signatures that are unique to ovarian cancer have been proposed, and serum-circulating microRNAs have the potential to be useful diagnostic and prognostic biomarkers. Various microRNAs such as those in the miR-200 family, the miR-199/214 cluster, or the let-7 paralogs have potential as therapeutic targets for disseminated or chemoresistant ovarian tumors. Although many obstacles need to be overcome, microRNA therapy could be a powerful tool for ovarian cancer prevention and treatment. In this review, we discuss the emerging roles of microRNAs in various aspects of ovarian cancer.

1. Introduction

Ovarian cancer is the most lethal gynecological malignancy in developed countries. In the United States, ovarian cancer is the fifth leading cause of cancer death in women, with estimated 14270 deaths in 2014 [1]. It is often diagnosed at a late stage with peritoneal dissemination and massive ascites, and despite combined treatments of aggressive cytoreductive surgery with platinum- and taxane-based chemotherapy the 5-year survival rate remains only 30% [1]. One reason for this high mortality rate is the lack of an early detection method for ovarian cancer. Indeed, the 5-year survival rate at stages I-II is estimated to be approximately 90%. While pelvic examination, transvaginal ultrasonography, and serum CA125 are performed during routine diagnostic procedures, they have failed to detect the disease at an early stage and thus reduce the mortality [2]. Therefore, new approaches for detecting early stage ovarian cancer are urgently needed. Another cause of the high mortality rate is the difficulty of treating disseminated or recurrent ovarian cancer. Although the clinical response rate after platinum- and taxane-based chemotherapy is usually high initially, subsequent relapses and repetitive treatments using cytotoxic chemotherapies lead to an acquired resistance to those treatments. Therefore, most patients that suffer relapses finally succumb to their disease [3]. At the molecular level, a number of interesting genes and pathways that may play essential roles in the pathogenesis of ovarian cancer have already been identified. Many could serve as molecular targets for therapies, although effective treatments that substantially extend overall patient survival have not been established so far. Among these, the recently discovered microRNAs (miRNAs or miRs) constitute a novel layer of gene expression regulation and have been implicated in the etiology of ovarian cancer. This review summarizes the ways in which microRNAs are involved in the pathogenesis of ovarian cancer and discusses cumulative efforts to apply them to the creation of novel diagnostic tools or promising future therapies.

2. MicroRNAs and Cancer

MicroRNAs (miRs) are approximately 22-nucleotide noncoding RNAs, are highly conserved among a wide range of species, and are generally involved in posttranscriptional

gene regulation. MiRs negatively regulate genes expression by binding to the 3′-untranslated region (UTR) of target mRNAs. Since miRs do not require perfectly complementary target sites and recognize short sites complementary to their 5′-seed region (nucleotides 2–8 of the miRs), one miR can regulate hundreds of mRNAs and multiple miRs can regulate an individual mRNA [4]. MiRs are predicted to regulate approximately 60% of all human genes and are involved in processes such as development, differentiation, metabolism, proliferation, cell cycle, and inflammation and the immune system [5–8]. Currently, it is well known that miRs can be upregulated or downregulated in various human cancers. Overexpressed miRs may function as oncogenes by downregulating tumor suppressor genes, whereas the downregulated miRs may act as tumor suppressor genes by negatively regulating oncogenes [9]. Important insights into the functions of miRs in cancer have been provided through the demonstration that they are involved in known oncogenic pathways. Three human *RAS* oncogenes (*H*-, *K*-, and *N-RAS*) contain binding sites for the let-7 family of miRs in their 3′UTR [10]. Interestingly, the miRs of the let-7 family, which are typically downregulated in various tumors, have been shown to negatively regulate the *RAS* oncogenes, thereby acting as tumor suppressors [10, 11]. MiR-15 and miR-16 have been shown to target the *BCL2* oncogene, leading to its downregulation and, consequently, resulting in apoptosis in leukemic cells [12]. MiR-221 and miR-222 are examples of miRs that act as oncogenes. They do so by targeting and inhibiting the expression of the tumor suppressor gene, *p27Kip* [13]. High levels of these miRs were shown to result in low p27 protein expression and increased proliferation of cancer cells. There is also evidence of a role of miR in p53-induced cell death. It has been shown that p53 transcriptionally induces miR-34 expression, and this induction is important in p53-mediated apoptosis of cancer cells [7, 14, 15]. These studies only represent a fraction of the explosion of publications emphasizing the role of miRs in cancer biology and showing miR dysregulation in various malignancies, including ovarian cancer.

3. Drosha/Dicer and Ovarian Cancer

Drosha and Dicer are essential for the biogenesis of miRNA. Drosha, an RNase III enzyme, cleaves the pri-miRNA and releases a hairpin-structured pre-miRNA in the nucleus. After the pre-miRNA is exported to the cytoplasm, Dicer, another RNase III enzyme, cleaves the pre-miRNA and releases the miRNA duplexes. When mature miRNA duplexes are produced, they associate with Argonaute (Ago) proteins and form the RNA-induced silencing complex (RISC), resulting in the degradation or translational repression of specific target mRNAs. In 2008, Merritt et al. measured the mRNA levels of Drosha and Dicer in 111 clinical samples of epithelial ovarian cancer and analyzed the prognostic values [16]. Low Dicer expression is significantly associated with advanced stage ovarian cancer and low Drosha expression with suboptimal surgery. Low Dicer expression is an independent predictor of disease-specific survival in multivariate analysis, as well as high-grade histological finding and chemotherapy resistance. These results suggested that impaired processing of miRs by Dicer and Drosha is involved in the tumorigenesis of ovarian cancer and leads to poor clinical outcomes. Vaksman et al. showed that the metastatic sites of differential expression of Drosha, Dicer, Ago1, and Ago2 in ovarian cancers are different from those in primary carcinomas [17]. In their study, higher Ago2 protein expression in ovarian cancer before chemotherapy correlated with shorter progression free survival. The study group saw similar trends for both Ago1 and Ago2 with respect to overall survival, suggesting a pivotal role of these molecules in ovarian cancer progression. Kim et al. proved that high-grade serous carcinomas could arise from the Fallopian tube in mice by conditionally knocking out Dicer and phosphatase and tensin homolog (*Pten*), which is a key negative regulator of the PI3K pathway [18]. Collectively, these reports demonstrate that enzymes and proteins involved in miR biogenesis and processing are closely related to development and progression in ovarian cancer.

4. miR Expression Profiles in Ovarian Cancer

In 2008, studies using miR microarrays, cDNA microarrays, and tissue arrays demonstrated genome-wide transcriptional changes in ovarian cancer [19]. Numerous miRs are markedly downregulated in advanced stages or high-grade ovarian cancer, suggesting that miRs are involved in malignant transformation and tumor progression. Both genomic copy number loss and epigenetic alteration may account for this downregulation and contribute to genome-wide transcriptional dysregulation. The authors compared the miR expression profiles of 18 epithelial ovarian cancer (EOC) cell lines and 4 immortalized ovarian surface epithelium (IOSE) primary cultures. They showed that the expression levels of 35 miRs were significantly different between the EOC lines and IOSE lines. Of these, 31 miRs (88.6%, 31/35) were downregulated in the EOC lines compared with the IOSE lines, including the tumor suppressor miRs, let-7d [10], and miR-127 [20].

Iorio et al. reported different miR expression profiles between ovarian cancer tissues/cell lines and normal tissues. Of 29 miRs, they showed that only 4 (miR-141, miR-200a, miR-200b, and miR-200c) were upregulated and 25 were downregulated, including miR-199a, miR-140, miR-145, and miR-125b-1 in the cancer samples [21]. They also found that miR signatures were different between ovarian carcinoma histotypes (serous, endometrioid, clear cell, and mucinous). Calura et al. analyzed miR profiles characteristic of each EOC histotype at stage 1 and found robust miR markers for clear cell and mucinous histotypes. The clear cell histotype is characterized by higher expression of miR-30a-5p and miR-30a-3p, whereas mucinous histotype displays higher levels of miR-192 and miR-194 [22]. Nam et al. reported the miR expression profiles of 20 serous ovarian carcinomas using a miRNA microarray and compared them with normal samples [23]. In ovarian cancer, 11 miRs were upregulated

Table 1: Potential prognostic miRs for ovarian cancer, which are significant in multivariate analysis (modified from [27]).

Reference	microRNA	Patients	Prognosis	Endpoint
[29], 2013	miR-21 (Serum)	EOC	Poor	OS
[30], 2014	miR-25	EOC	Poor	OS
[31], 2009	miR-29b	SAC	Poor	DFS
[32], 2012	miR-100	EOC	Good	OS
[33], 2014	miR-150	EOC	Good	PFS, OS
[34], 2012	miR-187	EOC	Good	RFS, OS
[35], 2009	miR-200a	EOC	Good	RFS, OS
[28], 2011	miR-200c	EOC (Stage 1)	Good	PFS, OS
[36], 2013	miR-203	EOC	Poor	PFS, OS
[37], 2013	miR-221	EOC	Poor	OS
[38], 2010	miR-221/222 ratio	EOC	Good	OS
[39], 2014	miR-335	EOC	Good	RFS, OS
[40], 2011	miR-410 and miR-645	EOC	Good	OS

EOC: epithelial ovarian cancer; SAC: serous adenocarcinoma; OS: overall survival; DFS: disease-free survival; PFS: progression-free survival; RFS: recurrent-free survival.

(miR-16, miR-20a, miR-21, miR-23a, miR-23b, miR-27a, miR-93, miR-141, miR-200a, miR-200b, and miR-200c) and 12 were downregulated (miR-10b, miR-26a, miR-29a, miR-99a, miR-100, miR-125a, miR-125b, miR-143, miR-145, miR-199a, miR-214, and let-7b). Thus, these reports identified similar sets of dysregulated miRs. Vang et al. analyzed the miR expression profiles of primary serous ovarian cancers and their respective omental metastases using miRNA qPCR arrays [24]. Seventeen miRs showed differential expression in omental lesions compared to primary tumors. Among these, miR-146a and miR-150 were significantly increased in omental metastases, regulating enhancement of spheroid formation and cisplatin resistance.

The Cancer Genome Atlas project has analyzed mRNA expression, miRNA expression, promoter methylation, and DNA copy number in a total of 489 high-grade serous ovarian adenocarcinomas [25]. They reported that high-grade serous ovarian cancer was characterized by *TP53* mutations in almost every tumor (96%). In addition, there was a low but statistically significant prevalence of recurrent somatic mutations in 8 other genes including *NF1*, *BRCA1*, *BRCA2*, *RB1*, and *CDK12*. They also showed that ovarian cancers could be separated into 4 transcriptional subtypes, 3 miR subtypes, and 4 promoter methylation subtypes. Integrated genomic analysis revealed a miRNA-regulatory network that defined a robust integrated mesenchymal subtype associated with poor survival in 459 cases of serous ovarian cancer and 560 cases independent of cohort data [26]. Eight key miRs (miR-25, miR-29c, miR-101, miR-128, miR-141, miR-182, miR-200a, and miR-506) were identified and predicted to regulate 89% of the targets in this network. Recently, Davidson et al. summarized the clinical and diagnostic roles of miRs in ovarian carcinoma in their review of approximately 100 publications [27]. In addition, various miRs have also been identified as potential prognostic indicators and promise utility in future practice. These are summarized in Table 1 [28–40].

5. Plasma/Serum miRs as Early Diagnostic Biomarkers for Ovarian Cancer

Ovarian cancer is a disease for which noninvasive serum screening tests are highly desirable for early stage cancer detection. Emerging evidence shows that miRs exist not only in cells but also in circulating blood, reflecting tissue or organ conditions. miRs generated in the cytoplasm can not only affect the function of the cell in which they are produced, but they can also be released into the blood stream and are taken up to regulate the gene expression of distant target cells [41]. Circulatory miRs in blood are resistant to the degradation of RNase enzyme and remain stable [42, 43]. Lawrie et al. first described serum miRs in cancer patients and suggested that miRs have potential as minimally invasive diagnostic markers for diffuse large B cell lymphoma (DLBCL) and possibly other cancers [44]. They found that the levels of miR-21, miR-155, and miR-210 in serum from DLBCL patients were higher than in healthy controls. In addition, high miR-21 expression was associated with relapse-free survival.

The potential of circulating miRs as cancer biomarkers depends on their high stability and their capacity to reflect tumor status and predict therapy response [43]. Many studies have determined that circulating miRs remain stable after being subjected to harsh conditions that would normally degrade RNAs, such as boiling, extreme pH levels, extended storage time, and repetitive freeze-thaw cycles. This incredible stability is partly explained by the association of the miRs with protein complexes such as Ago2 and the presence of these small RNAs in circulating microvesicles such as exosomes. Arroyo et al. found that most circulating miRs in plasma are cofractionated with Ago2, suggesting that circulating Ago2 complexes are responsible for the stability of plasma miR [45]. The authors reported that approximately 90% of miRs in circulation are present in a non-membrane-bound form consistent with a RISC complex. Other proteins

Table 2: Potential diagnostic miRs for ovarian cancer.

Reference	Sample	Elevated miR	Decreased miR	Tumor histology (*n*)	Control (*n*)
[47], 2008	Exosome (serum)	miR-21, miR-141, miR-200a, miR-200c, miR-200b, miR-203, miR-205, miR-214		SAC (50)	BOA (10), HC (10)
[48], 2009	Serum	miR-21, miR-92, miR-93, miR-126, miR-29a	miR-155, miR-127, miR-99b	SAC (17), CAC (6), EAC or MAC (5)	HC (15)
[49], 2010	Whole blood	miR-30c-1-3p	miR-181a-3p, miR-342-3p, miR-450-5p	SAC (21), EAC (2), other (1) (relapsed)	HC (15)
[50], 2012	Serum	miR-200a, miR-200b, miR-200c		SAC (28)	HC (28)
[51], 2013	Serum		miR-132, miR-26a, let-7b, miR-145	SAC (18)	HC (12)
[52], 2013	Plasma	miR-16, miR-21, miR-191 (CAC, EAC) miR-16, miR-191, miR-4284 (SAC)		SAC (21), CAC (7), EAC (6), other (1)	EM (33), HC (20)
[53], 2013	Plasma	miR-205	let-7f	SAC (179), CAC (15), EAC (86), MAC (33), other (47)	HC (200)

SAC: serous adenocarcinoma; CAC: clear cell adenocarcinoma; EAC: endometrioid adenocarcinoma; MAC: mucinous adenocarcinoma; BOA: benign ovarian adenoma; HC: healthy control; EM: endometriosis.

may also be associated with circulating miRs. Vickers et al. presented evidence that high-density lipoprotein (HDL) transports endogenous miRs and delivers them to recipient cells with functional targeting capabilities and that the cellular export of miRs to HDL is regulated by neutral sphingomyelinase [46]. While only 10% of circulating miRs are packaged in microparticles such as exosomes, recent research has revealed that exosomal miRs can affect many aspects of physiological and pathological conditions. Taylor and Gercel-Taylor reported that the miR signatures of tumor-derived exosomes have the potential to be used as diagnostic biomarkers of ovarian cancer [47]. Exosomes are small (30–100 nm) lipoprotein vesicles that exist in body fluids. They contain proteins, mRNAs, and miRs and are thought to play important roles in intercellular communication. The researchers compared the expression profiles of 8 miRs (miR-21, miR-141, miR-200a, miR-200b, miR-200c, miR-203, miR-205, and miR-214) between cancer tissues and exosomes collected from the peripheral sera of the corresponding patients, since these had been previously demonstrated to be overexpressed in ovarian cancer. They showed that exosomal miR profiles from ovarian cancer patients were elevated, whereas the exosomal miRs could not be detected in normal healthy controls.

Resnick et al. compared 21 serum miRs between epithelial ovarian cancer patients and healthy controls [48]. MiR-21, miR-29a, miR-92, miR-93, and miR-126 were significantly overexpressed in the serum of ovarian cancer patients compared to controls, while miR-99b, miR-127, and miR-155 were significantly underexpressed. Häusler et al. investigated the whole blood-derived miR profiles of ovarian cancer patients [49]. A comparison between ovarian cancer patients and healthy controls detected 147 significantly dysregulated miRs. In particular, miR-30c-1-3p was significantly upregulated and miR-181a-3p, miR-342-3p, and miR-450b-5p were significantly downregulated in ovarian cancer patients. Kan et al. found that miR-200a, miR-200b, and miR-200c were significantly elevated in the serum of patients and suggested that their presence could be used as a predictor of ovarian cancer [50]. Chung et al. reported that serum miR-26a, miR-132, miR-145, and let-7b could be considered potential candidates as novel biomarkers of serous ovarian cancer [51]. Suryawanshi et al. identified 3 distinct miR signatures between healthy controls, patients with endometriosis, and patients with endometriosis-associated ovarian cancer [52]. They suggested that these signatures might serve as useful diagnostic markers for the discrimination of these diseases, which is often clinically difficult. Zheng et al. showed that plasma miR-205 and let-7f could be used as biomarkers for ovarian cancer detection, especially in patients with stage 1 disease [53]. These efforts strongly support the idea that the detection of ovarian cancer-associated miRs from the peripheral blood could become a valuable method for early diagnosis of this disease in future clinical practice. Improving the sensitivity and lowering the cost of such detection methods are both key goals for advancing the application of detecting serum miR in cancer patients. Plasma/serum miRs that are potentially useful for the diagnosis or detection of ovarian cancer are summarized in Table 2 [47–53].

6. Therapeutic Potential of miRs That Inhibit Ovarian Cancer Progression

With the progress in cancer profiling, treatments will soon be customized for each individual. Because each miR regulates the expression of hundreds of different genes, miRs can function as master coordinators, efficiently regulating and coordinating multiple cellular pathways and processes [5]. Thus, miRs have been suggested as possible therapeutic armaments against cancer. The therapeutic application of miRs involves two strategies, inhibiting oncogenic miRs by using

miRNA antagonists and replacement of tumor suppressor miRs to restore a loss-of-function [54].

6.1. miR-200 Family. Members of the miR-200 family (miR-141, miR-200a, miR-200b, miR-200c, and miR-429) are downregulated in the majority of ovarian cancers, as previously described [21, 23]. Marchini et al. reported that low levels of miR-200c can predict poor survival and are a biomarker of relapse in stage I epithelial ovarian cancer [28]. The miR-200 family plays a critical role in the suppression of epithelial-to-mesenchymal transition (EMT) and tumor cell migration, invasion, and metastasis by directly targeting *ZEB1* (zinc finger E-box-binding homeobox 1) and *ZEB2* [55, 56]. Both miR-141 and miR-200a target p38 and modulate the oxidative stress response, affecting tumorigenesis and chemosensitivity [55]. miR-200a or miR-200c inhibits cancer stem-like cell populations [56, 57]. Pecot et al. demonstrated that miR-200 members inhibit angiogenesis through direct and indirect mechanisms by targeting interleukin-8 and *CXCL1* secreted from the tumor epithelial and cancer cells. They showed the therapeutic potential of miR-200 delivery in treating ovarian cancer or other malignancies [58].

Furthermore, it has been reported that miR-200 family members are associated with chemosensitivity in ovarian cancer. Cochrane et al. found that class III tubulin (*TUBB3*), which encodes a tubulin isotype normally found only in neuronal cells, is a direct target of miR-200c [59, 60]. The restoration of miR-200c increased sensitivity to microtubule-binding chemotherapeutic drugs, paclitaxel, epothilone B, and vincristine and suppressed the expression of *TUBB3*. Van Jaarsveld et al. compared the miR expression profiles of cisplatin-sensitive and -resistant ovarian cancer cells, revealing that high expression of miR-141, miR-200c, miR-215, and miR-421 and low expression of miR-492-5p correlated with increased cisplatin resistance [61]. They also demonstrated that miR-141 directly targets *KEAP1*, and downregulation of *KEAP1* by miR-141 overexpression induced cisplatin resistance.

6.2. miR-199/214 Cluster. Chen et al. discovered that miR-199a regulates IKK expression, which modulates the inflammatory microenvironment in ovarian cancer [62]. Yin et al. showed that TWIST1 regulated the IKK/NF-κB and PTEN/AKT pathways through the miR-199a-2/miR-214 cluster [63]. miR-199a also targets *CD44* to suppress the tumorigenicity and multidrug resistance of ovarian cancer-initiating cells [64]. Epigenetic silencing of miR-199b-5p is associated with chemoresistance in ovarian cancer through the activation of JAG1/Notch1 signaling [65]. Yang et al. showed that miR-214 induced cell survival and cisplatin resistance through direct targeting of *PTEN* and inactivation of the AKT pathway [66]. Joshi et al. found that the expression of miR-199a is reduced in cancer cells by hypoxic stimuli, and exogenous expression of miR-199a decreased cell migration and metastasis of ovarian cancer cells by targeting the 3′-UTRs of HIF-1α and HIF-2α [67].

6.3. let-7 Paralogs. The let-7 family includes 12 human homologs that are considered tumor suppressors. These miRs are located in cancer-associated regions or in fragile sites [68]. Johnson et al. reported that the let-7 family negatively regulates let-60/*RAS*, whose 3′-UTR contains multiple let-7 complementary sites [10]. In ovarian cancer, let-7 is downregulated. It also targets the embryonic gene high mobility group A2 (*HMGA2*) more efficiently than *RAS* during early cancer progression [69, 70]. Shell et al. demonstrated that let-7 and *HMGA2* can be predictors of prognosis and that loss of let-7 expression indicates less differentiated cancer [70]. High-grade serous ovarian carcinoma (HG-SOC) is a heterogeneous, poorly classified, and lethal disease. Recently, meta-analysis of its transcriptome revealed let-7b as an unfavorable prognostic biomarker that can predict molecular and clinical subclasses of HG-SOC [71]. A let-7b-defined 36-gene prognostic survival signature outperformed many clinicopathological parameters. As for let-7e, Cai et al. suggested that it might act as a promising therapeutic target for improving sensitivity to cisplatin in ovarian cancer [72].

6.4. miR-506. From integrated genomic analysis, 8 key miRs (miR-25, miR-29c, miR-101, miR-128, miR-141, miR-182, miR-200a, and miR-506) were predicted to regulate 89% of the miR targets in the network [26]. In follow-up functional experiments, overexpression of miR-506 in ovarian cancer cells augmented E-cadherin expression, inhibited cell migration and invasion, and prevented TGF-β-induced EMT by targeting *SNAI2*, a transcriptional repressor of E-cadherin. In an orthotopic ovarian cancer mouse model, nanoparticle delivery of miR-506 significantly reduced tumor growth. Liu et al. reported that miR-506 also suppressed ovarian cancer cell proliferation and induced senescence by directly targeting the CDK4/6-FOXM1 axis [73].

6.5. miR-92a. miR-92a is in the miR-17/92 family cluster, which includes miR-17, miR-18, miR-19a, mir-19b, miR-20, and miR-92. Ohyagi-Hara et al. described the involvement of miR-92a in the expression of integrin α5, a known key player in ovarian cancer adhesion and dissemination [74, 75]. The levels of integrin α5 and miR-92a expression were significantly and inversely correlated in ovarian cancer cells. The forced expression of miR-92a in cancer cells markedly suppressed peritoneal dissemination *in vivo*, suggesting that targeting miR-92a may prove to be a novel and effective gene therapy for patients with ovarian cancer.

6.6. miR-31. Mitamura et al. analyzed miR-associated paclitaxel (PTX) chemoresistance in ovarian cancer cells [76]. Lower expression of miR-31 and higher expression of MET (also known as c-Met or hepatocyte growth factor receptor) were significantly correlated with PTX resistance and poor prognosis in ovarian cancer patients. miR-31 directly targets the 3′-UTR of MET and increases the PTX sensitivity of ovarian cancer cells in an animal model. Creighton et al. comprehensively profiled the expression of miRs and mRNAs in serous ovarian cancers, cell lines, and normal ovarian epithelium [77]. They discovered that miR-31, the least

regulated miR in serous ovarian cancer, repressed the cell cycle regulator *E2F2*, inhibited proliferation, and induced apoptosis. They revealed that loss of miR-31 is associated with defects in the TP53 (also called p53) pathway and functions in serous ovarian cancer, suggesting that patients with cancers that are deficient in TP53 activity might benefit from therapeutic delivery of miR-31.

6.7. miR-484. Vecchione et al. analyzed miR signatures associated with chemoresistance in 198 serous ovarian cancer samples along with clinical data and concluded that the presence miR-217, miR-484, and miR-617 was able to predict the chemoresistance of these tumors [78]. The response to chemotherapy is associated with tumor angiogenesis, and miR-484 has a potential to improve chemosensitivity through the modulation of tumor angiogenesis, by directly targeting *VEGFB* and *KDR* (formerly called *VEGFR2*).

6.8. Therapeutic Synergy between miR-520d-3p and EPHA2 siRNA. Nishimura et al. identified miR-520d-3p as a tumor suppressor upstream of *EPHA2*, whose expression correlated with favorable outcomes in clinical cohorts [79]. Dual inhibition of *EPHA2*, using *EPHA2* siRNA and nanoliposomes loaded with miR-520d-3p, showed better antitumor efficacy than either monotherapy *in vivo*. These data emphasize the feasibility of combined miRNA-siRNA therapy for cancer or other diseases.

7. miR and Tumor Microenvironment

The interaction of cancer cells with their microenvironment is essential for tumor development, tumor progression, and metastasis [80]. Tumor microenvironment is a collective term that includes the tumor's surrounding supportive stroma, the host immune system, and other humoral factors. Various miRs have the therapeutic potential by targeting not only tumor cells directly, but also cells surrounding tumor microenvironment.

7.1. Angiogenesis and miRs. Pecot et al. demonstrated that miR-200 inhibits angiogenesis through direct and indirect mechanisms by targeting interleukin-8 and CXCL1 that is secreted by tumor endothelial cells [58]. Using several experimental models, including models of ovarian cancer, they showed that the delivery of the members of the miR-200 family into the tumor endothelium led to marked reduction in metastasis and angiogenesis and induced vascular normalization, resulting in ovarian cancer regression. Xu et al. found that miR-145 acts as a tumor suppressor by indirectly downregulating the expression of hypoxia-inducible factor 1 (HIF-1) and vascular endothelial growth factor (VEGF) by targeting p70S6K1, in turn resulting in the inhibition of tumor growth and angiogenesis [81]. Similarly, miR-125b and miR-199a were also shown to act as tumor suppressors by targeting HIF-1α and VEGF in ovarian cancer cells, consequently reducing angiogenesis [82]. Lai et al. reported that miR-27a may play a central role in follicle-stimulating hormone- (FSH-) mediated angiogenesis in ovarian cancer. They showed that the ablation of miR-27a repressed FSH-induced expression of VEGF, Cox2, and survivin [83]. Because antiangiogenic therapy in ovarian cancer has been shown to be effective in several large phase III trials, these miRs could be used in the development of ovarian cancer therapies in the future [84, 85].

7.2. Cancer Associated Fibroblasts (CAFs) and miRs. Cancer cells change the surrounding normal stroma into tumor supportive environments during the processes of invasion and metastasis [86]. Cancer associated fibroblasts (CAFs) are a major component of the tumor stroma. They promote cancer cell invasion and enhance the viability of tumor cells. Mitra et al. found that, in ovarian CAFs, miR-31 and miR-214 are downregulated while miR-155 is upregulated compared to normal or tumor-adjacent fibroblasts [87]. Their results indicate that ovarian cancer cells reprogram fibroblasts to become CAFs through miRs and that targeting miRs in stromal cells has therapeutic potential.

7.3. Cancer-Associated Dendritic Cells (DCs) and miRs. Cancer-associated dendritic cells (DCs) represent the most frequent leukocyte subset to infiltrate solid tumors. These cancer-associated DCs are located around perivascular areas, where they deliver multiple proangiogenic and immunosuppressive mediators [88]. Huarte et al. demonstrated that the elimination of cancer-associated DCs delays ovarian cancer progression by boosting antitumor immunity [89]. Cubillos-Ruiz et al. showed that the activity of mature miR-155 can be augmented in tumor-associated DCs by delivering novel Dicer substrate RNA duplexes that mimic the structure of the endogenous precursor miR-155 hairpin [90]. The replenishing of miR-155 levels in DCs reprogrammed their immunosuppressive phenotype and boosted potent antitumor immune responses that abrogated the progression of established ovarian cancers.

8. Current Challenges in miRNA Delivery

The data presented in this review support a clinical role of miRs in ovarian cancer and suggest that miR-regulated pathways may be relevant targets in novel therapeutics. However, there remains a major challenge of miR-based cancer therapy with respect to systemic delivery *in vivo*. In particular, the problems related to specificity, efficiency, and safety pose major limitations. The keys for miR drug development are that the chemical structure must be stable *in vivo* and cell-permeable and should hybridize to the miR of interest with high specificity and affinity. Techniques for chemical modifications have been applied to enhance oligonucleotide stability and to acquire increased resistance to nucleases. Examples include 2-O-methyl-group- (OMe-) modified oligonucleotides and locked nucleic acid- (LNA-) modified oligonucleotides [91]. LNA-antimir-122 (miravirsen) is the first drug to successfully enter phase II trials for the treatment of hepatitis C virus (HCV) infection [92]. Thirty-six patients were randomly assigned to receive five weekly subcutaneous injections of miravirsen at doses of 3, 5, or 7 mg/kg or

Table 3: Potential therapeutic miRs for ovarian cancer.

miRNA		Target gene	Cellular function	Ovarian cancer cell line	Reference
miR-200 family	miR-141	KEEP1	Cisplatin resistance	A2780 (EOC), TOV112D (EAC), TOV21G (CAC)	[61]
	miR-141, miR-200a	MAPK14	Oxidative stress response, pactitaxel sensitivity	SKOV3 (EOC)	[55]
	miR-200a	ZEB2	EMT, stemness, migration, invasion	OVCAR3 (EOC)	[56]
	miR-200c	ZEB1, TUBB3	EMT, stemness, adhesion, migration, invasion, paclitaxel sensitivity	HEY (SAC), SKOV3 (EOC)	[57, 59, 60]
	miR-200a, miR-200b	IL8, CXCL1	Angiogenesis	HeyA8 (SAC), ES2 (CAC)	[58]
miR-199/214 cluster	miR-199a-5p	IKKB, HIF-1A, HIF-2A	Inflammation, chemosensitivity, migration, metastasis	A2780 (EOC), R454 (EOC), 01-28 (EOC), R182 (EOC), 01-19B (EOC), R1185 (EOC), primary culture	[62, 63, 67]
	miR-199a-3p	CD44	Stemness, chemosensitivity	primary culture	[64]
	miR-199b-5p	JAG1	Cisplatin sensitivity	A2780 (EOC), OV119 (EOC)	[65]
	miR-214	PTEN, CCL5	Proliferation, cell survival, cisplatin resistance, CAFs activity	A2780 (EOC), HeyA8 (SAC), SKOV3ip1 (EOC), OV119 (EOC), primary culture	[63, 66, 87]
let-7		HMGA2	Carcinogenesis	A2780 (EOC), HeyA8 (SAC), IGROV-1 (EOC)	[69, 70]
miR-506		SNAI2	EMT	HeyA8 (SAC), SKOV3 (EOC), OVCA420 (EOC), OVCA433 (EOC)	[26]
		CDK4, CDK6	Proliferation, senescence	HeyA8 (SAC), SKOV3 (EOC), OVCA432 (EOC), OVCA433 (EOC)	[73]
miR-92a		ITGA5	Adhesion, invasion, proliferation	A2780 (EOC), SKOV3ip1 (EOC), OVISE (CAC)	[74]
miR-31		MET	Paclitaxel sensitivity	KFr13 (EOC)	[76]
		CEBPA, STK40, E2F2	Proliferation	SKOV3 (EOC), OVCAR8 (EOC), OVCA433 (EOC)	[77]
miR-484		VEGFB, VEGFR2	Angiogenesis	SKOV3 (EOC), MDAH-2274 (EOC)	[78]
miR-502d-3p		EPHA2, EPHB2	Proliferation, migration, invasion	HeyA8 (SAC), SKOV3ip1 (EOC), ES2 (CAC)	[79]
miR-155		SATB1, CD200	DCs activity	ID8 (EOC, mouse)	[90]

CAFs: cancer associated fibroblasts; EMT: epithelial-mesenchymal transition; DCs: dendritic cells; EOC: epithelial ovarian cancer; EAC: endometrioid adenocarcinoma; CAC: clear cell adenocarcinoma; SAC: serous adenocarcinoma.

placebo over a 29-day period. Miravirsen resulted in a dose-dependent reduction in HCV RNA levels, and there were no observed dose-limiting adverse effects or escape mutations in the miR-122 binding sites of the HCV genome. Systemic delivery of miRNA, like that of other types of nucleic acids, activates the innate immune system leading to unexpected toxicities and significant undesirable side effects. When anti-inflammatory miRs are concurrently delivered as therapeutic agents, they may suppress the systemic immune response instead of causing immune toxicity [41]. Moreover, one of the biggest issues regarding miR therapy is the off-target effect of miRNAs. Since miRs are designed to target multiple pathways via imperfect matching with 3′-UTRs, they may cause unwanted silencing of tumor suppressor genes.

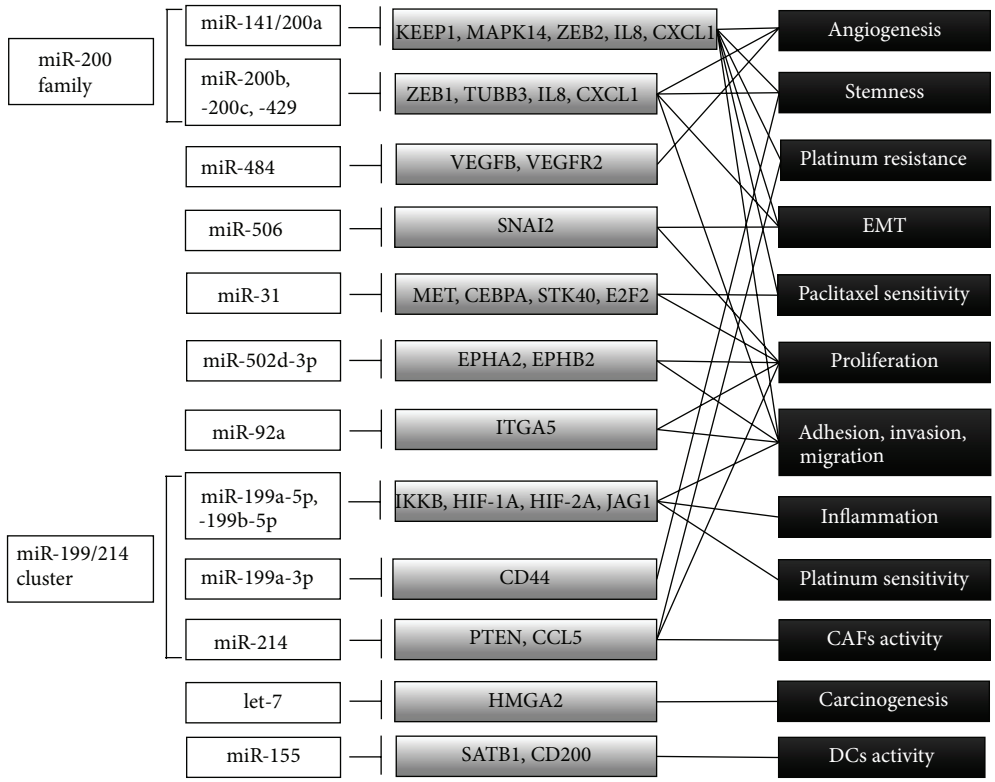

Figure 1: Schema of mechanism and target genes of potential therapeutic miRs for ovarian cancer.

Such off-target gene silencing may cause potential toxicities and reduced therapeutic effects [41]. A multifunctional nanoparticle delivering miRs, siRNAs, or miRNA cocktails to silence several oncogenic pathways and activate tumor suppressive ones may minimize unintended off-target effects and maximize the therapeutic effect. Although these problems remain unsolved, conquering them may render miR-based therapy an important armament for cancer therapy in the future. Further studies are required in order to successfully apply therapeutic miRs to ovarian cancer, and this could realize a potential use of miRs to drastically improve the prognosis of this disease.

9. Conclusion

Since 2005 and the discovery of miR-15a and miR-16-1 deletions in B-CLL [12], there have been an enormous number of reports regarding miR dysregulation in various cancer types. In addition to transcriptional regulation, posttranscriptional repression by miRs contributes to every cell/tissue function by fine-tuning large networks of genes. In the ovarian cancer field, many miR signatures from tumor cells/tissues or serum/plasma have been described so far. Since early detection tools are lacking, ovarian cancer is often diagnosed at a late stage. This substantially contributes to the high mortality rate of ovarian cancer. This review summarized that miR expression profiles are quite different in ovarian cancer compared to normal control tissue. Thus, in the near future, screening serum/plasma miRs might contribute to improved prognosis of ovarian cancer by enabling diagnosis at an early stage noninvasively.

Emerging evidence strongly supports the rationale that inhibition of overexpressed oncogenic miRs or substitution of tumor suppressive miRs might be novel treatment strategies for ovarian cancer therapy, as summarized in Table 3 and Figure 1. Optimization of the stabilizing method for miRs, improvement in delivery methods, and the control of off-target effects induced by miRs delivery appear to be the keys to future clinical applications.

Conflict of Interests

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

Acknowledgments

The authors thank Yuko Nishimura for her secretarial assistance. This work was supported by a Grant-in-Aid for scientific research from the Ministry of Education, Science, Sports and Culture of Japan (26293360 and 26670725 to Kenjiro Sawada, and 24249080 to Tadashi Kimura).

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Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 678401, 17 pages
http://dx.doi.org/10.1155/2014/678401

Review Article

Molecular Mechanisms Underlying the Role of MicroRNAs in the Chemoresistance of Pancreatic Cancer

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Received 2 July 2014; Accepted 28 July 2014; Published 28 August 2014

Academic Editor: Paolo Gandellini

Pancreatic ductal adenocarcinoma (PDAC) is an extremely severe disease where the mortality and incidence rates are almost identical. This is mainly due to late diagnosis and limited response to current treatments. The tumor macroenvironment/microenvironment have been frequently reported as the major contributors to chemoresistance in PDAC, preventing the drugs from reaching their intended site of action (i.e., the malignant duct cells). However, the recent discovery of microRNAs (miRNAs) has provided new directions for research on mechanisms underlying response to chemotherapy. Due to their tissue-/disease-specific expression and high stability in tissues and biofluids, miRNAs represent new promising diagnostic and prognostic/predictive biomarkers and therapeutic targets. Furthermore, several studies have documented that selected miRNAs, such as miR-21 and miR-34a, may influence response to chemotherapy in several tumor types, including PDAC. In this review, we summarize the current knowledge on the role of miRNAs in PDAC and recent advances in understanding their role in chemoresistance through multiple molecular mechanisms.

1. Introduction

A surprising revelation from the human genome project was that 75% of the genome is transcribed into RNA [1–3], but less than 2% is composed of protein-coding genes [4]. Noncoding RNAs (ncRNAs) represent an extremely interesting class of RNAs that can be divided into three types, according to the size. Short ncRNAs are <50 nucleotides (nt); those between 50 nt and 200 nt are referred to as midsize ncRNAs, while long ncRNAs (lncRNAs) are >200 nt [5–8]. miRNAs are a class of short ncRNAs containing approximately 19–24 nt. They have a key regulatory role in development, differentiation, and apoptosis of normal cells, as well as in the determination of the final phenotype of cancer cells, affecting carcinogenesis and metastatic potential [9]. Remarkably, miRNAs exhibit tissue-specific and disease-specific expression that could provide the basis for their development as novel diagnostic, prognostic, and/or predictive biomarkers, as well as therapeutic targets [8]. Furthermore, several studies have documented that selected miRNAs may influence response to chemotherapy [10].

Cancer chemoresistance can occur by multiple mechanisms. It can arise from physiological barriers to drug absorption or penetration into target tissues or from biological

mechanisms within individual tumor cells which reduce the effectiveness at their intended site of action, such as increased expression of enzymes involved in drug catabolism or anti-apoptotic proteins [10].

The dense stromal reaction which characterizes most PDACs has been frequently reported as the main cause of chemoresistance, preventing the drugs from reaching their intended site of action [11]. However, detailed genetic analyses have unraveled the pivotal mechanisms controlling pancreatic carcinogenesis, and cluster analysis of recurrently mutated genes has defined twelve different core pathways that lead to aberrant signaling in PDAC cells [12]. Such studies suggest that the best hope for the development of agents targeting critical points in the altered pathways lies in the study of mechanisms involved in gene expression regulation. Therefore, in the present review we summarize the role of miRNAs in PDAC and focus on the miRNA-based mechanisms of PDAC chemoresistance.

2. Discovery of miRNAs and Their Role in Cancer

The first miRNA molecule, lin-4, was identified in 1993 by Lee and collaborators [13]. In 2000, Reinhart et al. identified lethal-7 (let-7), another miRNA, and discovered its role in the posttranscriptional regulation of gene expression [14]. Currently, it has been reported that there are around 2,600 unique mature human miRNAs (miRBase version 20) [15]. miRNAs regulate more than one-third of all human genes, which suggest their remarkable influence on human biology [16]. It is known that more than 50% of miRNA genes are localized within genomic regions that are either frequently amplified or deleted in different tumor types, resulting in miRNAs deregulation and aberrant expression [17, 18]. The altered miRNAs may have different effects on the tumors [19]. Some of these miRNAs have been characterized as potent oncogenes (oncomiRs), while others have been identified as tumor suppressors (tsmiRs), based on the consequences of their expression on the phenotype of several experimental models [4]. OncomiRs, such as miR-21, are commonly upregulated in cancer [20], while tsmiRs, such as let-7, are downregulated [21], resulting in unique combinations of miRNAs (i.e., overexpressed oncomiRs and underexpressed tsmiRs) characterizing different tumors [22].

The multiple roles of these miRNAs can be explained by starting from the analysis of their biological synthesis and functions. Biosynthesis of miRNAs is a multistep process, involving both nuclear and cytoplasmic components [23]. Initially, they are transcribed in the nucleus by RNA polymerase II into large RNA precursors, called pri-miRNAs [24–26], which can be several hundreds to several thousands of nt in length. The first slicing step performed by the ribonuclease (RNase) III Drosha-DGCR8 (DiGeorge syndrome critical region 8) enzyme leads to the formation of 70-base long pre-miRNAs [27–29]. Pre-miRNAs are actively transported from the nucleus to the cytoplasm by Exportin-5 [30] where they are subjected to further cleavage by the RNase Dicer to achieve the final size, and each molecule is combined with proteins of the Argonaute (AGO) family to obtain its functional form [31–33], thus forming the miRNA-induced silencing complexes (miRISCs). Typically, these complexes bind to the 3′-untranslated region (3′ UTR) of target mRNA with perfect or near-perfect complementarity. When miRNAs form perfect base pairs with their target mRNA, they result in its degradation. Nevertheless, most of the human miRNAs bind to their target 3′-UTRs with imperfect complementarity and therefore induce translational repression [34]. A result of all these interactions is that the target sequence is not translated or there is a variation of translation and subsequently the encoded protein is modified or not produced at all. According to the role of this protein, this leads to structural or functional alterations to the involved cells, thus having a direct effect on their phenotype [9]. Remarkably, each miRNA can regulate the expression of numerous target genes and also the same target gene can be regulated by several types of miRNAs which create a complex network of interactions [35–37]. However, the regulatory role of miRNA in mRNA stability and translation into protein is a complex biological process, which is not restricted through the binding of miRNA only in the 3′-UTR of the mRNA [19]. miRNAs can also interact with the 5′ UTR of protein-coding genes and cause translational repression [38] or activation of the targeted proteins [39]. Similarly, miRNAs can also target the coding sequence and repress the translation of targeted genes [40]. Moreover, some miRNAs can interact with regulatory protein complexes, such as AGO2 and fragile X mental retardation-related protein 1 (FXR1), and indirectly upregulate the translation of a target gene [41].

The involvement of miRNAs in cancer was first discovered in a quest to identify tumor suppressors in the frequently deleted 13q14 region in chronic lymphocytic leukaemia (CLL), and the miRNA cluster miR-15a-miR-16-1 was found to be deleted or downregulated via epigenetic silencing in 69% of the patients [42]. One of the most striking themes in the study of miRNAs and cancer is indeed the large alteration of miRNA expression in malignant cells compared to their normal counterparts. Most cancers have a specific miRNA signature or "miRNome" that characterizes the malignant state and defines some of the clinicopathological features of the tumors (e.g., grade, stage, aggressiveness, vascular invasion, and/or proliferation index) [43]. miRNAs have a variety of roles in cancer development and progression [8], acting not only as tumor suppressors or oncogenes [44], but also as key activators or suppressors of tumor metastasis [45]. Variations in miRNA genes and their precursors, as well as the target sites and genes encoding components of the miRNA processing machinery, can affect the cell phenotype and disease susceptibility [46, 47]. Finally, a subclass of miRNAs, known as epi-miRNAs, can directly control the epigenetic variations [48], and miRNA expression can also be downregulated via promotor hypermethylation [49], adding another piece to the puzzle of regulatory gene expression networks.

Research in various cancers has found that miRNAs also have great potential as biomarkers for early diagnosis and prognosis [17, 50, 51]. In particular, circulating miRNAs have high translational potential as noninvasive biomarkers [52].

Indeed, miRNA expression levels can be detected in a variety of human specimens including both fresh and formalin-fixed paraffin-embedded tissues, fine-needle aspirates, and in almost all human body fluids, including serum, plasma, saliva, urine, and amniotic fluid [53, 54]. The impressive stability of miRNAs in tissues and biofluids is a key advantage over proteins and mRNAs [55]. Circulating miRNAs may have cellular or extracellular origin and are presumably not naked miRNA, which would be degradated within seconds due to the high levels of nucleases in blood. Several reports have demonstrated that stability results from the formation of complexes between circulating miRNAs and specific proteins [56]. Other studies have found miRNAs contained within circulating exosomes or other microvesicles, and it is also possible that cell lysis or an increase in the number of exosomes shed from the diseased cells can contribute to increased levels of certain circulating miRNAs [56]. miRNAs have been found within circulating exosomes or other microvescicles which can be taken up by acceptor cells, playing a role in cell-to-cell communication. Although the mechanism of secretion and incorporation of miRNAs has not been elucidated, secretory miRNAs may play a pivotal role as signaling molecules in physiological and pathological events. In general, there are three mechanisms of shedding which lead to release of vesicles into the extracellular space, that is, via exocytosis, budding of microvesicles directly from a plasma membrane or through the membranous microvesicles shed from cells during apoptosis [56]. However, before applying large-scale efforts to miRNA biomarker discovery, baseline parameters such as intraindividual and interindividual variability of miRNAs must be explored very carefully. Currently there are no validated guidelines for the collection and extraction of samples for miRNA analysis. Differences in specimen types (tissue type or plasma/serum) can have a profound effect on miRNA levels. For example, miRNA content in both plasma and serum can be influenced by cell remnant contamination from erythrocytes, leukocytes, or platelets. Standardization of many analysitical parameters is essential for the evaluation of miRNA as ideal biomarkers.

Further research is also necessary to understand whether miRNAs have clinical potential as prognostic factors and as predictive biomarkers for chemotherapy resistance in specific tumor types. The present review summarizes the current knowledge on the role of miRNAs in PDAC, reporting the most recent studies on miRNA-based mechanisms of chemoresistance.

3. miRNA and PDAC

PDAC is a highly aggressive malignancy and fourth leading cause of cancer-related death in developed countries [57]. The median survival after diagnosis is 2–8 months, and approximately only 3–6% of all patients with PDAC survive 5 years after diagnosis [58]. For resectable or borderline resectable patients (i.e., patients with stages T1, T2 or T3 tumors) surgical resection remains the cornerstone of management of PDAC. However, the average survival of resected patients is between 12 and 20 months, with a high probability of relapse [9]. Owing to vague symptoms in early stages, 80% of PDACs are diagnosed when already advanced, and no curative therapy is currently available [59–61].

Tumors of the pancreas are divided into those arising from the exocrine pancreas and those arising from the endocrine cells. PDACs represent 75% of exocrine malignancies [61]. It has been established that PDAC does not arise *de novo* but is preceded by histologically distinct noninvasive precursor lesions within the pancreatic ducts. The most common precursors are pancreatic intraepithelial neoplasia (PanIN), which show a defined histological progression from the low-grade PanIN-1, through to the intermediate-grade PanIN-2, and culminating in the high-grade PanIN-3 (carcinoma *in situ*) [62]. Key shared genetic alterations associated with PDAC progression include earliest genetic events such as mutation of K-RAS and overexpression of HER-2/neu. At later stages, inactivation of the p16 tumor suppressor gene occurs, followed by the loss of TP53, SMAD4, and BRCA2 signaling pathways and the genomic-transcriptomic alterations that facilitate cell cycle deregulation, cell survival, invasion, and metastases [4]. Importantly, several miRNAs functionally interact with these genetic lesions, as described in the following paragraphs (see also Figure 1).

3.1. K-RAS Mutations. Over 90% of PDACs harbor an activating K-RAS gene mutation. The vast majority of these mutations are at codon 12 and occur very early in pancreatic carcinogenesis [63]. K-RAS is a 21 kDa intracellular membrane bound protein that belongs to the GTPase superfamily [64, 65]. In physiological conditions, the GAP proteins and, specifically, the RAS GTPases do promote GTP hydrolysis and reversal of the RAS activation step [66]. During oncogenic transformation, the mutated RAS is constitutively activated and cannot be deactivated by the GAP proteins [65]. RAS signaling involves multiple branches (B-RAF, PI3K, and PLC pathways). Together, these branches cover most aspects of cellular life, including regulation of the cell cycle, differentiation, proliferation, and apoptosis [62]. Several recent studies have identified specific miRNAs that regulate the K-RAS signaling pathway in pancreatic oncogenesis and vice versa. Preclinical studies have shown that K-RAS regulates miR-21 expression levels in precancerous lesions and the peak of miR-21 expression correlates with the degree of progression to more aggressive forms [67]. K-RAS is also a direct target of miR-217; thus upregulation of miR-217 decreases K-RAS protein levels and reduces the constitutive phosphorylation of downstream AKT [68]. Another study identified K-RAS as a direct target of miR-96 [69]. Indeed, overexpression of miR-96 decreased cancer cell invasion, migration and slowed tumor growth and was associated with K-RAS downregulation [69]. Recent studies have shown that miR-126 and let-7d can also regulate K-RAS levels in PDAC. In particular miR-126 can directly target K-RAS; thus miR-126 downregulation can allow overexpression of K-RAS [70].

3.2. HER2/neu Overexpression. Up to 29% of PDACs have HER2 overexpression [71–73]. There is direct correlation between the expression levels of the Her2/neu and the shorter survival in patients with PDAC, suggesting that

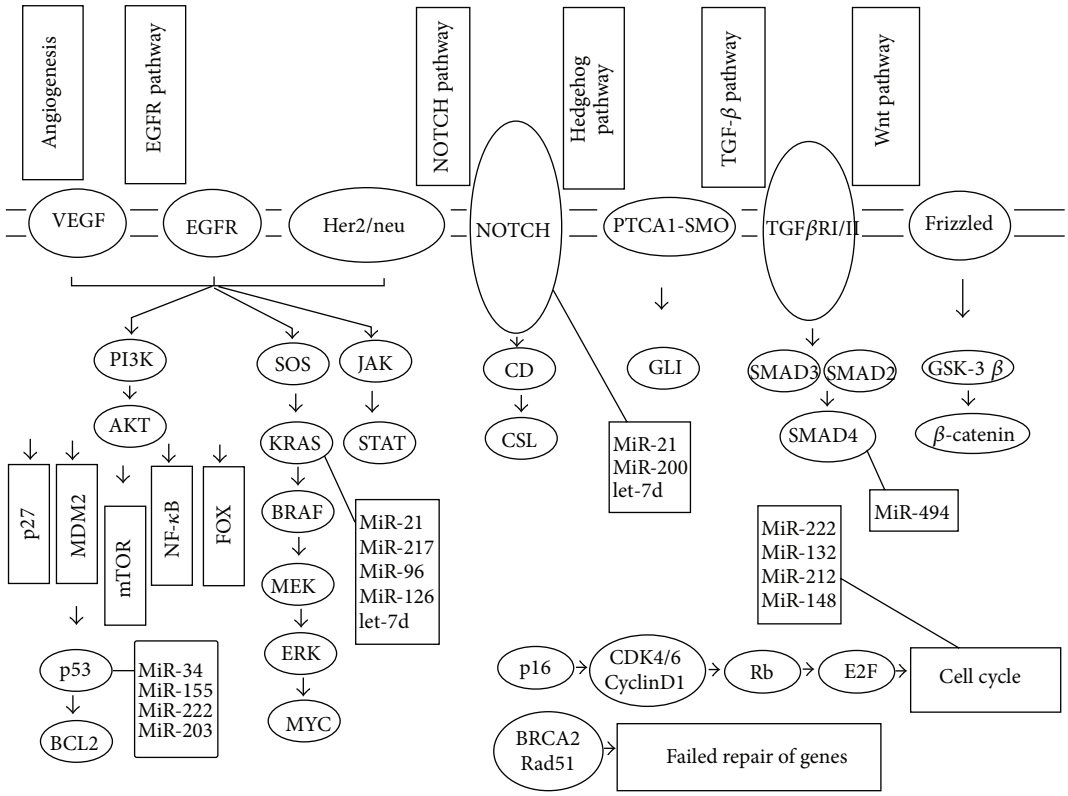

Figure 1: *MicroRNA and their involvement in oncologenic signaling cascades in pancreatic cancer. EGFR pathway.* Activation of the EGF receptor results in autophosphorylation of key tyrosine residues which subsequent activation of downstream signalling cascades including the RAS/extracellular signal regulated kinase (ERK) pathway, the phosphatidylinositol 3-kinase (PI3) pathway and the Janus kinase/Signal transducer and activator of transcription (JAK/STAT) pathway. All of them result in cell survival promotion. *Notch pathway*. A ligand on one cell induces a series of proteolytic cleavage events in a Notch receptor on a contacting cell. These cleavage events release the Notch intracellular domain (NICD), which translocates to the nucleus to activate the transcription of Notch target genes together with CSL (CBF1/Suppressor of Hairless/LAG-1). The notch signaling pathway is important for cell-cell communication, which involves gene regulation mechanisms that control multiple cell differentiation processes. *Hedgehog pathway*. Hedgehog is a secreted ligand that binds to its receptor, Patched (PTCA1). When PTCA1 is activated, it leads to inhibition of the Smoothened (Smo) receptor. Smo is then able to inhibit the phosphorylation and cleavage of Gli, which prevents the formation of repressive Gli (GliR) and promotes the formation of activated Gli (GliA). GliA then translocates into the nucleus and initiates transcription of target genes, which play a role in stem cell regulation. *TGF-β pathway*. TGF receptors are activated after binding with their ligand, which leads to further phosphorylation of receptor-regulated SMADs (mainly SMAD2 and SMAD3). Phosphorylated SMAD2/3 form heteromeric complexes with SMAD4, which accumulate in the nucleus and activate transcription of different genes, including those responsible for cell cycle arrest. *Wnt pathway*. In the absence of signal, action of the destruction complex (CKIα, GSK3β, APC, and Axin) creates a hyperphosphorylated β-catenin, which is a target for ubiqitination and degradation by the proteosome. Binding of Wnt ligand to a Frizzled/LRP-5/6 receptor complex leads to stabilization of hypophosphorylated β-catenin, which interacts with TCF/LEF proteins in the nucleus to activate transcription.

the HER2/neu signaling pathway is a central regulator of pancreatic oncogenesis [74].

The HER2/neu pathway has been primarily studied in breast cancer cell lines, where miR-21 expression levels correlate with the HER2/neu upregulation [65]. More recently, dysregulation of miR-125a-5p/125b and HER2 emerged as an early event in the gastric (intestinal-type) and esophageal (Barretts) oncogenesis [75]. In these oncogenic lesions, miR-125 expression correlates inversely with HER2 status. Therefore, miR-125a-5p/125b can be considered among the therapeutic targets in HER2-positive esophageal and gastric adenocarcinoma. Similarly, the role of newer anti-HER2 agents agents interacting with regulating miRNA in HER2-positive PDAC remains to be explored [74, 76].

3.3. p16/CDKN2A Inactivation. CDKN2A is a tumor suppressor gene which is somatically inactivated in approximately 95% of PDACs [77]. Most of these inactivating mutations lead to loss of function of the protein p16, the product of the CDKN2A gene. The p16 protein binds cyclin-dependent kinases 4 and 6 (CDK4 and CDK6) and specifically inhibits their pRb phosphorylating activity, which is required for G1/S transition [62]. Inherited mutations in the p16/CDKN2A gene cause the familial atypical multiple mole melanoma syndrome, with increased risk for developing PDAC and melanoma [78]. Several miRNAs that participate in the deregulation of the cell cycle genes are essential during PDAC development and progression. For example, miR-222 targets p27 and p57, which are both pivotal cell cycle inhibitors [79].

Other studies have shown that downregulation of miR-132 and miR-212 causes G2/M cell cycle arrest and results in reduced cell proliferation [80], while miR-148 directly targets AMP activated protein kinase (AMPK), which plays a key role as a master regulator of cellular energy homeostasis, and can induce cell cycle arrest and apoptosis [81].

3.4. TP53 Mutations. The TP53 gene is inactivated in 75 to 85% of PDACs [63]. Genetic inactivation of TP53 abrogates important cell functions, such as regulation of cellular proliferation and apoptosis in response to DNA damage. When cellular stress and DNA damage are detected, degradation of TP53 is inhibited by different mechanisms, leading to accumulation of its active form [82]. Preclinical studies have shown that TP53 directly regulates miR-34, which further downstream targets Notch, and therefore plays a role in the maintenance and survival of PDAC initiating cells [82]. Moreover, TP53-induced nuclear protein 1 gene has been described to be downregulated by miR-155, accelerating pancreatic tumor development [83]. MiR-222 and miR-203 are also able to target p53 and affect its function as a crucial regulator of the cell cycle [84].

3.5. SMAD4 Inactivation. The SMAD4 gene is inactivated in approximately 60% of PDACs [63]. The protein product of the SMAD4 gene is involved in the transmission of intracellular signals from transforming growth factor beta (TGFb) receptors within the cell membrane to the nucleus [85]. In normal cells, TGF-A receptors are activated after binding with their ligand, which leads to further phosphorylation of receptor-regulated SMADs (mainly SMAD2 and SMAD3). Phosphorylated SMAD2 and SMAD3 form heteromeric complexes with SMAD4, which accumulate in the nucleus and activate transcription of different genes, including those responsible for cell cycle arrest. This pathway is of key importance for pancreatic cells [62]. PDACs with loss of SMAD4 expression have higher rates of distant metastases and a poorer prognosis [86, 87]. A recent study showed that loss of SMAD4 in PDAC cells leads to increased levels of FOXM1, nuclear localization of β-catenin, and reduced levels of miR-494 [88]. Transgenic expression of miR-494 in PDAC cells produced the same effects as reducing expression of FOXM1 or blocking nuclear translocation of β-catenin, reducing cell proliferation, migration, and invasion, and increasing their sensitivity to gemcitabine. Reduced expression of miR-494 correlated with PDAC metastasis and reduced survival times of patients. This study suggested that miR-494 might be developed as a prognostic marker or a therapeutic target for patients with PDAC. Other studies have shown that in human PDAC specimens, the expression levels of both miR-421 and miR-483-3p are inversely correlated to SMAD4 expression and ectopic expression of these miRNAs significantly represses SMAD4 protein levels in PDAC cell lines, suggesting that they are potent regulators of SMAD4 in PDAC [89, 90].

3.6. BRCA2 and PALB2 Mutations. The BRCA2 gene is inactivated in fewer than 10% of PDACs [91]. Importantly, germline mutations in BRCA2 are associated with an increased risk of PDAC [92]. Similarly, germline truncating mutations in the PALB2 gene, which encodes for a BRCA2 binding protein [93], have been identified in ~3% of individuals with familial pancreatic cancer [94, 95]. Of note, a recent study for the prediction of BRCA1/2 mutation-associated hereditary breast cancer identified a 35-miRNA classifier for the prediction of BRCA1/2 mutation status with a reported 95% and 92% accuracy in the training and the test set, respectively [96]. These miRNA signatures might be of interest also in PDAC, in order to complement current patient selection criteria for gene testing by identifying individuals with high likelihood of being BRCA1/2 mutation carriers.

4. MicroRNA-Based Mechanisms of Anticancer Drug Resistance in PDAC

Chemotherapy remains the primary treatment for metastatic, nonresecable PDAC. However, the best currently available treatments prolong life by only a few months [97, 98], and PDAC chemoresistance renders most drugs ineffective.

Drug resistance can be divided into two groups: intrinsic or acquired. Intrinsic resistance is caused by a preexisting phenotype, whereas acquired resistance develops due to repeated use of the same drug. The most common reason for the acquisition of resistance to a broad range of anticancer drugs is the overexpression of one or more energy-dependent transporters that detect and eject anticancer drugs from cells, resulting in multidrug resistance (MDR) [10, 99]. However, drug resistance can occur for many causes, including increased drug efflux, alterations in drug target, DNA repair, cell cycle regulation, and evasion of apoptosis [100].

Up- and/or downregulation of miRNAs can influence the expression of multiple target mRNAs, and therefore multiple proteins, leading to variations in the chemosensitivity of cancer cells via various cellular processes. In particular, several miRNAs have been demonstrated to alter cellular response to anticancer agents via modulation of drug efflux and targets, cell cycle, survival pathways, and/or apoptotic response, as reported in the following paragraphs and in Figure 2.

4.1. Upregulation of Drug Efflux Transporters. Resistance to various anticancer agents has been associated with increased expression of drug efflux pumps [99], keeping the intracellular drug concentration below a cell-killing threshold [100]. miRNAs have also been shown to be involved in chemotherapy resistance through the regulation of ATP-binding cassette (ABC) membrane transporters [100]. They transport drugs from the cytosol to the extracellular space. Activation of the MDR1 gene results in overexpression of P-glycoprotein (P-gp) which is a multidrug efflux pump and confers cancer cell resistance to a broad spectrum of drugs [10, 101]. P-glycoprotein is localized at the apical level in cells membranes of different cellular compartments such as liver, intestine, kidney, and placenta. This strategic localization gives P-gp a crucial role as responsible for drugs absorption and accumulation [101]. It has been shown that miR-27a and miR-451 are activators of drug resistant process by modulation of MDR1/P-gp expression in human ovarian and cervical

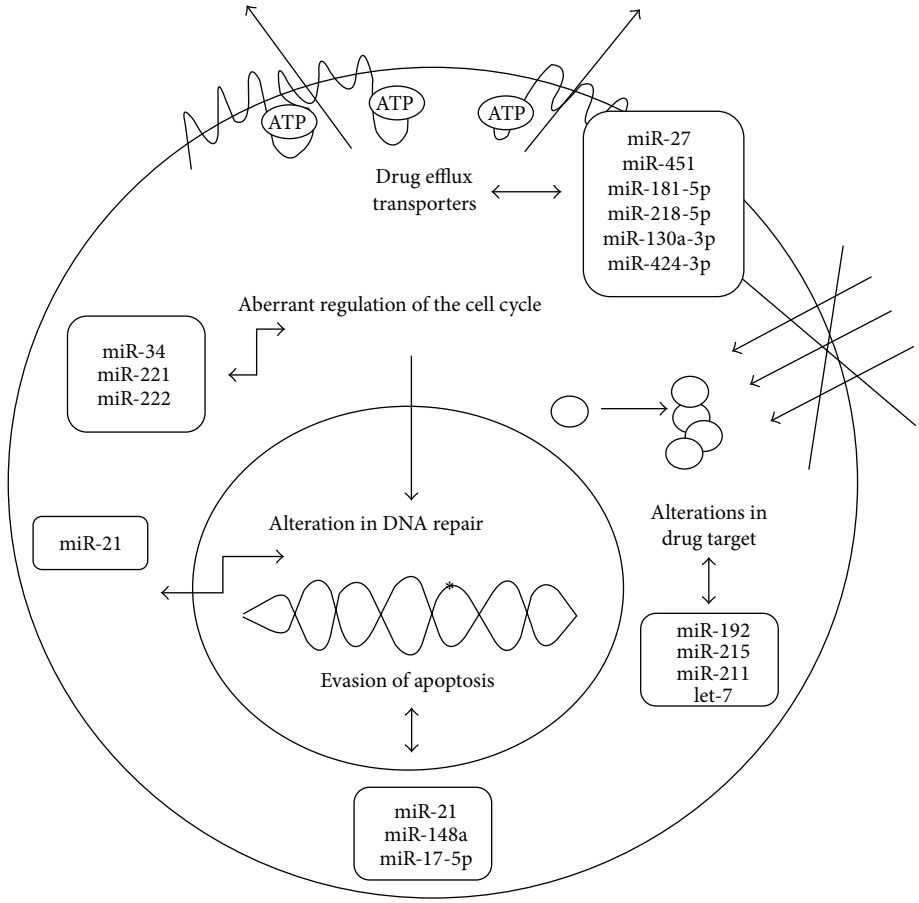

Figure 2: *MicroRNA and their involvement in anticancer drug resistance.* Drug resistance can occur at many levels, including drug efflux, alterations in drug target, DNA repair, cell cycle regulation, and evasion of apoptosis. Some selected miRNAs which have been demonstrated to alter these mechanisms are shown in this figure.

cancer cells [10, 102]. A recent study evaluated the role of miRNAs in MDR in PDAC, monitoring the modulation of some specific miRNAs by the treatment of a wild type cell line and in the corresponding cell line with P-gp overexpression and unsensitive to several antineoplastic treatments [103]. This study showed the different modulation of 4 miRNAs (miR-181a-5p, miR-218-5p, miR-130a-3p, and miR-424-3p), using a specific P-gp substrate, and suggested new molecular mechanisms potentially involved in chemoresistance, such as the modulation by miR-424 of the protein cullin 2, a scaffolding protein displaying a pivotal role in the assembly of the ubiquitin ligase system, thereby stabilizing HIF-1α.

4.2. Alterations in Drug Targets and DNA Repair. Chemoresistance can be caused by either quantitative (i.e., modulation of expression levels) or qualitative (i.e., mutation) alterations of the drug targets [100]. Examples of quantitative alterations have been reported for several antimetabolites, which influence various steps the metabolism of nucleic acids, through inhibition of key enzymes, such as thymidylate synthase and ribonucleotide reductase. MiR-192 and miR-215 target thymidylate synthase (TS), which is the main drug target of the fluoropyrimidine-based therapy in colorectal cancer, which is also used in PDAC patients [104]. However, down-regulation of TS by miR-192/215 did not lead to an increase in 5-FU sensitivity, suggesting that the activity of miR-192/215 was not mediated by TS. In contrast, overexpression of both miRNAs resulted in a reduction of cell proliferation and therefore diminished the effectiveness of S-phase specific drugs like 5-FU, suggesting that miR-192 and miR-215 can still play a role in 5-FU resistance.

Two recent studies suggested the key role of miR-211 in the modulation of ribonucleotide reductase subunit 2 (RRM2), which is an important cellular target of gemcitabine. This miRNA had significantly higher expression in long- versus short-OS PDAC patients, evaluating high-resolution miRNA profiles with Toray's 3D-Gene-miRNA-chip, detecting more than 1200 human miRNAs [105]. The preclinical analyses demonstrated that induction of the miR-211 expression in PDAC cells increased the sensitivity to gemcitabine through reduced expression of its target RRM2 [106]. Similarly, it has been demonstrated that let-7 negatively regulates RRM2 and let-7 expression is inversely correlated with RRM2 expression in gemcitabine-resistant PDAC cells. Additionally, silencing RRM2 or overexpression of let-7 was shown to sensitize PDAC cells to gemcitabine [107].

miRNAs can also alter cellular response to several anticancer drugs via interfering with DNA repair. In particular, the inhibition of ribonucleotide reductase by gemcitabine results in deoxyadenosine triphosphate depletion, causing DNA replication errors. Moreover, gemcitabine is incorporated into DNA and arrests DNA replication. Both the mispaired bases and the gemcitabine-modified DNA bases can be the substrates for postreplicative DNA mismatch repair (MMR) machinery [108], which influences cancer cell sensitivity.

Similarly, defects in MMR proteins have been associated with reduced or absent benefit from 5-FU adjuvant chemotherapy [109]. MMR alterations reduce the incorporation into DNA of the 5-FU metabolites that cause G2/M arrest and induce apoptosis after 5-FU treatment. Colorectal cancer cells with miR-21 overexpression exhibited significantly reduced 5-FU-induced G2/M damage arrest and apoptosis, suggesting that miR-21-dependent downregulation of core MMR component (hMSH2–hMSH6) might be responsible for both primary and acquired resistance to 5-FU [110, 111]. Of note, miR-21 is included in the miRNA metasignature for recognising PDAC [112, 113]. Furthermore, high miR-21, high miR-31, and low miR-375 tumoral expressions have been validated as independent prognostic biomarkers for poor overall survival in PDAC.

4.3. Aberrant Regulation of the Cell Cycle. The cell cycle is an ordered set of events, culminating in cell growth and division into two daughter cells. Uncontrolled cellular proliferation is one of the hallmarks of cancer, and these alterations are commonly caused by genetic damages to regulator genes such p16 and cyclin D1 that govern phosphorylation of the retinoblastoma protein (RB) and control exit from the G1 phase of the cell cycle or the tumor suppressor TP53, which can arrest growth by holding the cell cycle at the G1/S regulation point on DNA damage recognition [114]. Recent studies showed that the members of the miR-34 family are direct TP53 targets, and their upregulation induced apoptosis and cell cycle arrest [115]. The miR-34 family comprises three miRNAs, encoded by two different genes: miR-34a is encoded by its own transcript, whereas miR-34b miR-34c share a common primary transcript. Moreover, the promoter region of miR-34a, miR -34b, and miR -34c contains CpG islands. An aberrant CpG methylation reduces miR-34 family expression in multiple types of cancer, including PDAC [116]. Therefore a recent study investigated the functional significance of miR-34a in PDAC progression through its epigenetic restoration with chromatin modulators, demethylating agent 5-Aza-2′-deoxycytidine, and HDAC inhibitor Vorinostat [117]. The restoration of miR-34a in human PDAC and pancreatic cancer stem cells (CSCs) strongly inhibited cell proliferation, cell cycle progression, self-renewal, epithelial to mesenchymal transition, and invasion, while inducing apoptosis. These results provided not only mechanistic insight but also promising therapeutic approaches, which might also improve esponse to existing chemotherapies in PDAC.

Another example of protein of interaction between proteins regulating the cell cycle and miRNA is represented by Cyclin-dependent kinase inhibitor 1B (CDKN1B, p27, or p27Kip1), which is a cell cycle inhibitor and tumor suppressor. This enzyme has been identified as a direct target of miR-221 and miR-222 [53]. The expression of miR-221 is significantly upregulated in PDAC cell lines and tumor tissues compared to normal pancreatic duct epithelial cells and normal pancreas tissues and has been proposed as candidate plasma biomarkers in PDAC [118]. However, transfection of miR-221 inhibitor suppressed the proliferative capacity of PDAC cells with concomitant upregulation of CDKN1B, as well as of PTEN and PUMA, which are other tumor suppressors among the predicted targets of miR-221 [119]. The same study showed that the expression of miR-221 was modulated by the treatment with isoflavone mixture (G2535), formulated 3,3′-diindolylmethane (BR-DIM), or synthetic curcumin analogue (CDF), leading to the inhibition of cell proliferation and migration and supporting further studies on these potential nontoxic agents in novel targeted therapeutic strategy that are capable of downregulation of miR-221.

4.4. Evasion of Apoptosis. Apoptotic evasion is considered to be one of the main causes of chemotherapeutic and radiotherapeutic resistance that characterizes the most aggressive tumor [120]. Cancer cells can resist apoptosis if they have an overexpression of antiapoptotic proteins, involved in the two main apoptosis pathways, extrinsic and intrinsic. The extrinsic pathway is regulated mainly by "death receptors" of the TNF-receptor family, while the intrinsic pathway is regulated by Bcl-2 proteins. Various anticancer drugs such as antimetabolites, DNA cross-linking and intercalating agents, alkylating agents, topoisomerase I/II inhibitors, and TKIs have been reported to induce intrinsic or extrinsic apoptotic response in tumor cells, resulting in caspases activation [121]. Although the extrinsic and the intrinsic apoptosis pathways are activated by different stimuli, both these pathways can be regulated by specific miRNAs. For example, upregulation of Bcl-2, directly induced by miR-21, is associated with apoptosis, chemoresistance to gemcitabine, and proliferation of MIA PaCa-2 cells [110]. Using western blot and luciferase activity assay, Bcl-2 was identified also as a target of miR-148a, and the expression of Bcl-2 lacking in 3′UTR could abrogate the proapoptotic function of miR-148a in PANC-1 and AsPC-1 cells [122]. Similarly, exogenous expression of miR-204 and miR-320 reduced the protein level of their targets, Bcl-2 and Mcl-1, respectively. Mcl-1 is an antiapoptotic member of Bcl-2 family, and induction of miR-320 activity leads to apoptosis through Mcl-1 suppression, sensitizing cholangiocarcinoma cells to 5-FU [123]. However, miR-204 was also reported to be significantly downregulated in gemcitabine-resistant PDAC [124], and Li et al. identified the role of the entire miR-200 family of miRNAs in gemcitabine-resistant PDAC cells [125].

Conversely, miR-17-5p downregulates the proapoptotic member of the Bcl-2 protein family Bim, and PDAC cells transfected with miR-17-5p inhibitor showed growth inhibition, spontaneous apoptosis, higher caspase-3 activation, and increased chemosensitivity to gemcitabine [126]. Pathways delivering an antiapoptotic signal, such as PI3K/Akt, play also a pivotal role in the balance between proapoptotic and survival signals, which determines the fate of cancer cells. An increased miR-21 expression has been associated

with the activation of PI3K/AKT/mTOR pathway, while combination of anti-miR-21 strategies with drugs targeting PI3K/AKT/mTOR pathway reduced pAKT levels and enhanced apoptosis when used in combination with gemcitabine [127]. Importantly, the antiapoptotic role of miR-21 is possibly tumor specific, with inhibition of miR-21 increasing sensitivity and apoptosis induction by gemcitabine in PDAC and cholangiocarcinoma, but not in colon cancer cells [127]. This suggests that its oncogenic properties could be cell and tissue dependent and that its potential role in chemoresistance should be contextualized with respect to the tumor type and the treatment [128].

5. miRNA in PDAC Resistance to Conventional Therapy and Target Therapy

Pancreatic cancer is a genetically heterogenous disease with a very limited response to most treatments [129], including both conventional (also known as standard-dose chemotherapy, which includes chemotherapeutic agents and regimens that have been in use from the past 15 to 40 years) and targeted therapies (a newer type of cancer treatment that uses drugs or other substances to more precisely identify specific molecules involved in cell growth and survival and attack cancer cells) as described in the following paragraphs.

5.1. Conventional Chemotherapy. Conventional chemotherapy, also known as standard-dose chemotherapy, includes chemotherapeutic agents and regimens that have been in use from the past 15 to 40 years. The three different therapeutic options for PDAC in the metastatic setting include gemcitabine, as monotherapy or in combinations: the combination of 5-FU, leucovorin, irinotecan, and oxaliplatin (FOLFIRINOX), and the most recent combination of gemcitabine with nab-paclitaxel. Although only 20% of patients present with localized disease amenable to potentially curative resection, on the basis of a few randomized trials [130–132], the current accepted standard of care is adjuvant gemcitabine or 5-FU chemotherapy, while there have been no conclusions regarding the role or timing of adjuvant chemoradiation [133].

5.2. Gemcitabine Monotherapy and Gemcitabine-Based Combinations. Since 1997, gemcitabine is being used in metastatic PDAC. Patients receiving gemcitabine have a median survival of 6.2 months and a 1-year survival rate of 20% [134]. Meta-analysis of randomized trials with a combination of gemcitabine and platinum analogues or of gemcitabine and capecitabine suggested a survival benefit for these combinations for patients with a good performance status [135–137]. In contrast, an Italian phase III trial examining gemcitabine and cisplatin did not confirm a survival benefit for this combination [138]. In a retrospective study on laser-microdissected PDAC specimens patients with high miR-21 expression had a significantly shorter overall survival both in the metastatic and in the adjuvant setting. Multivariate analysis confirmed the prognostic significance of miR-21 [127]. The reduced expression of miR-21 was associated with benefit from gemcitabine treatment in two independent cohorts of PDAC patients [139, 140], as well as in a cohort of intraductal papillary mucinous neoplasms (IPMNs) of the pancreas [141]. These results might be explained by the effects of miR-21 expression on certain phenotypic characteristics in PDAC cell lines [139, 142]. Overexpression of miR-21 promotes cell proliferation, increases the metastatic ability through expression of matrix metalloproteinase-2 and metalloproteinase-9 as well as VEGF, and decreases gemcitabine sensitivity, whereas miR-21 repression delivers the opposite results [143]. Furthermore, as reported in the previous chapters, Hwang et al. [139] and Dong et al. [144] provided experimental evidence for a role of miR-21 in chemoresistance thorough modulation of apoptosis by directly regulating Bcl-2 and PTEN expression. More recently, Frampton et al. identified three miRNAs (miR-21, miR-23a, and miR-27a) that acted as cooperative repressors of a network of tumor suppressor genes that included PDCD4, BTG2, and NEDD4L [145]. In 91 PDAC samples from PDAC radically resected patients, high levels of a combination of these miRNAs were associated with shorter survival times. Thus, high expressors of this triple miRNA combination (miR-21/23a/27a) may be identified as having a much worse prognosis and may benefit from anti-miRNA therapy, although the best way to deliver such a treatment and potential off-target effects are unknown. Another recent study demonstrated that miR-10b might be a novel diagnostic and predictive biomarker for PDAC [146]. MiR-10b is indeed overexpressed in PDAC patients and reduced expression of miR-10b was associated with improved response to multimodality neoadjuvant therapy, likelihood of surgical resection, delayed time to metastasis, and increased survival [146]. Finally, several studies reported miR-155, among the miRNA which are commonly overexpressed in PDACs and their precursor lesions [147], and although only one study reported that its elevated expression correlated with shorter survival [84], Xia et al. [148] demonstrated that gemcitabine treatment induced the expression of miR-155 in PDAC cells suggesting its role in acquired chemoresistance. Other miRNAs that have been linked to gemcitabine chemoresistance in PDAC are reported in Table 1.

Gemcitabine plus nanoparticle albumin-bound nab-paclitaxel represents a novel, acceptable alternative to FOLFIRINOX. This combined therapy was associated with significantly higher objective response rate (23%) and significantly longer median overall (8.5 months) and progression-free survival (5.5 months), in comparison to gemcitabine alone [149]. Combination treatment with gemcitabine and nab-paclitaxel increases intratumoral gemcitabine levels attributable to a marked decrease in the primary gemcitabine metabolizing enzyme, cytidine deaminase. Correspondingly, paclitaxel reduced the levels of cytidine deaminase protein in cultured cells through reactive oxygen species-mediated degradation, resulting in the increased stabilization of gemcitabine [150]. Nab-paclitaxel alone or in combination with gemcitabine has been demostrated to reduce the desmoplastic stroma [151]. Moreover, it is hypothesized that the albumin-bound nab-paclitaxel may selectively accumulate in the pancreatic stroma via its binding to secreted protein acidic and rich in cysteine (SPARC) matricellular glycoprotein which binds albumin and is overexpressed in tumor stroma

Table 1: Selected miRNA candidates which are correlated to gemcitabine chemoresistance in pancreatic cancer.

miRNA	Expression	Targets	Reference
miR-21	Upregulated	EGFR, HER2/neu, PDCD4, BCL2, PTEN, TIMP2, and TIMP3	[139, 142]
miR-222 and miR-221	Upregulated	p27, PUMA, PTEN, and Bim	[84, 185]
miR-10a and miR-10b	Upregulated	HOXB8, HOXA1	[186, 187]
miR-214	Upregulated	PTEN, ING4	[188, 189]
mir-320c	Upregulated	SMARCC1	[190]
miR-155	Upregulated	PI3K SMG-1	[148]
miR-34°	Downregulated	BCL-2	[43]
Let-7	Downregulated	E2F2, c-Myc, KRAS, and MAPK	[125]
miR-142-5p	Downregulated	Unknown	[124]
miR-204	Downregulated	MIC-1	[124]
miR-200a, miR-200b, and miR-200c	Downregulated	EP300	[125, 191]

[57]. High SPARC expression has been correlated to poor survival outcome and has been suggested as a possible predictive biomarker for nab-paclitaxel in the phase-II trial [151]. However, no data on SPARC are available from the phase III trial and Neesse et al. showed that the effects of nab-paclitaxel were largely dose-dependent and that SPARC expression in the tumor stroma did not influence drug accumulation in a PDAC mouse model. Further studies are therefore warranted to evaluate tissue and plasma SPARC expression as a potential predictive biomarker for nab-paclitaxel [11].

No data are available on miRNA affecting nab-paclitaxel, but several miRNAs have been associated to resistance to paclitaxel. Regarding miRNA potentially affecting the drug target, TUBB3 has been unraveled as a target for miR-200c in ovarian and endometrial cancer cells, and the ectopic expression of this miRNA downregulated TUBB3 and enhanced sensitivity to microtubule-targeting agents, including paclitaxel [152].

As example of miRNA affecting survival pathway, miR-17-5p has been identified as one of most significantly downregulated miRNAs in paclitaxel-resistant lung cancer cells, which might cause upregulation of beclin 1 gene, one of the most important autophagy modulators [153]. Moreover, miRNA miR-17-5p, which is a member of the miR-17-92 cluster, is upregulated in pancreatic cancer and some present findings suggest that miR-17-5p plays important roles in pancreatic carcinogenesis and cancer progression and is associated with a poor prognosis in pancreatic cancer [154].

5.3. FOLFIRINOX (5-FU, Leucovorin, Irinotecan, and Oxaliplatin). A phase III trial using FOLFIRINOX regimen in PDAC patients has shown a response rate of 31.6%, a median survival of 11.1 months [155]. Therefore, FOLFIRINOX protocol confers a significant improvement in the overall survival in stage IV PDAC and can be considered as a novel therapeutic option for patients with a good performance status [136]. No predictive biomarkers are actually used in clinical practice, but a few studies suggested the role of candidate miRNAs to predict the sensitivity/resistance to 5-FU, and the other drugs in this regimen. 5-FU activity might indeed depend on the expression of its target TS, or by the modulation of cell cycle, and apoptosis induction by several miRNAs, as reported above.

Interestingly, a pharmacogenetic study evaluated 18 polymorphisms both in miRNA-containing genomic regions (primary and precursor miRNA) and in genes related to miRNA biogenesis with outcome in metastatic colorectal cancer patients treated with 5-FU and irinotecan [156]. A significant association with tumor response and time to progression was observed for the SNP rs7372209 in pri-miR26a-1. The genotypes CC and CT were favorable when compared with the TT variant genotype. Similarly, the SNP rs1834306, located in the pri-miR-100 gene, significantly correlated with a longer time to progression.

5.4. Targeted Therapy. From its introduction, cancer chemotherapy has been encumbered by its poor selectivity because most antineoplastic drugs are toxic also to fast-replicating cells of the blood compartment, skin cells, and gastrointestinal tract lining cells. This unsatisfactory situation and the development of technology leading to the sequencing of the genome have driven intensive researches and development over the last few decades towards more specific and less toxic anticancer drugs that block the growth and spread of cancer by interfering with specific molecules involved in tumor growth and progression and are therefore called "targeted therapies." Some of these therapeutic regimens especially designed to intercept deregulated dominant oncogenes have proven to be effective treatment in "oncogene addicted" tumors [157]. In particular, the epidermal growth factor receptor (EGFR) has been successfully targeted either by mAbs or small molecules inhibiting the tyrosine kinase domain. The mAb cetuximab blocks the extracellular domain of EGFR, thereby competing with the ligands and resulting in the inhibition of the receptor. This mAb is approved for the treatment of advanced colorectal cancer, while the EGFR-TKIs gefitinib and erlotinib have been approved as upfront therapy replacing chemotherapy in late-stage NSCLC patients harboring activating-EGFR mutations.

5.5. Anti-EGFR Therapy in PDAC. The SWOG group conducted a randomized Phase III clinical trial randomizing

patients with stages III-IV PDAC to receive either gemcitabine alone or in combination with cetuximab, which did not improve the clinical outcome. Negative results for this combination were also observed in the adjuvant setting [158]. Similarly, other EGFR and HER2 targeted therapies, including trastuzumab and lapatinib, have not shown a survival benefit in PDAC patients [136]. In contrast, a combination of gemcitabine and erlotinib has been approved for use by the United States Food and Drug Administration (FDA) and European Medicines Agency (EMEA) as a treatment for PDAC patients on the basis of a randomized trial, showing a overall gain in median survival of 2 weeks [159]. Examination of K-RAS mutational status and EGFR gene copy number in 26% of patients from this trial failed to identify either change as molecular predictors of response [160]. However, accumulating evidence suggests that dysregulation of specific miRNAs may be involved in the acquisition of cancer cell resistance to EGFR-targeted agents. In particular, miR-7 emerged as a critical modulator of a regulatory network for EGFR signaling in lung cancer cells, with the ability of coordinately downregulating the expression of several members of the EGFR signaling cascade [161]. The binding of c-Myc to the miR-7 promoter enhanced its activity, while ectopic miR-7 promoted cell growth and orthotopic tumor formation in nude mice. In these models, quantitative proteomic analysis revealed that miR-7 decreased levels of the Ets2 transcriptional repression factor ERF, which is a direct target of miR-7. Accordingly, the inhibition of miR-7 expression suppressed EGFR mRNA and protein expression in different lung cancer cell lines as well as the growth of the A549 lung adenocarcinoma cells [162]. Of note, miR-7 is preferentially expressed in endocrine cells of the developing and adult human pancreas [163]. However, its role in the regulation of the insulin growth factor-1 receptor expression might affect the development of diabetes-associated PDAC [164].

Other studies in lung cancer cell lines showed that decreased miR-424 levels were indicative of increased resistance to erlotinib, while the gefitinib resistant cell line-HCC827/GR had a significant upregulation of miR-214 [165]. The inhibition of miR-214 has been also correlated with decreased apoptosis and miR-214 and PTEN were indeed inversely expressed, while knockdown of miR-214 altered the expression of PTEN and p-AKT, resensitizing HCC827/GR to gefitinib. MiR-214 has been identified as aberrantly expressed in PDAC and *in vitro* experiments showed that overexpression of miR-214 decreased the sensitivity of the BxCP-3 cells to gemcitabine [166].

The sensitivity to erlotinib was also predicted by a 13-gene miRNA signature, identified in sensitive towards resistant lung cancer cell lines. Ontological annotation of these miRNA (miR-140-3p, miR-628-5p, miR-518f, miR-636, miR-301a, miR-34c, miR-224, miR-197, miR-205, miR135b, miR-200b, miR-200c, and miR-141) and their potential targets revealed enrichment in the components of EMT, including Wnt pathway, which may explain the ability of this signature to separate primary from metastatic tumor samples as well as why the treatment with TGFβ1 modulated both the expression of these miRNA and cell migration [167]. Interestingly, EMT has been inversely correlated with the response of cancers to EGFR-targeted therapy and the TGFβ-mitogen-inducible gene 6-miR200 network orchestrates the EMT-associated kinase switch hat induces resistance to EGFR inhibitors in primary tumor xenografts of patient-derived lung and pancreatic cancers carrying wild type EGFR [168]. These data support the low ratio of Mig6 to miR200 as a promising predictive biomarker of the response of PDAC to EGFR-TKIs.

6. miRNA Affecting PDAC Chemoresistance through Modulation of Its Microenvironment

PDAC is characterized by a dense fibrotic stromal matrix [11], composed of activated fibroblasts/stellate cells, inflammatory cells, and other cell types such as endothelial cells. PDAC is one of the most stroma-rich malignancies [169]. Such desmoplasia facilitates a mechanopathology known as growth-induced solid stress, resulting in collapsed or compressed intratumoral blood vessels or lymphatics, which respectively lead to increased hypoxia and interstitial fluid pressure, both attenuating chemosensitivity [170].

Hypoxia is an essential component of the PDAC microenvironment, as demonstrated by the characteristic avascular appearance on computed tomography and low oxygen tension measurements of these tumors [171, 172]. Several studies showed that hypoxia plays a pivotal role in cancer progression through induction of the hypoxia-inducible factor (HIF), which leads to increased expression of VEGF [173]. However, hypoxic conditions in solid malignancies may also confer resistance to conventional radiation and chemotherapy [174]. A functional link between hypoxia and miRNA expression was shown in colon and breast cancer cell lines [175] and in several other cancers, including PDAC [176]. MiR-210, in particular, is induced by hypoxia and the levels of this miRNA are significantly higher elevated in PDAC patients and may potentially serve as a useful biomarker for PDAC diagnosis [177]. Furthermore, miR-210 regulates the interaction between PDAC cells and stellate cells, promoting the progression and chemoresistance of tumor cells [178]. However, the same study showed that stellate cells-induced miR-210 upregulation was inhibited by inhibitors of ERK and PI3K/Akt pathways, suggesting novel therapeutic combinations to counteract the interaction between stellate cells and PDAC, which is at least in part responsible for the innate resistance to chemotherapy in pancreatic tumors by creating barriers against circulating therapeutic compounds.

Hypoxia induces also the overexpression of miR-21 [179], while the treatment with the novel curcumin-derived analogue CDF downregulated the expression of miR-21 and miR-210, as well as Nanog, Oct4, and EZH2 mRNAs, and the production of VEGF and IL-6. CDF also led to decreased cell migration/invasion, angiogenesis, and formation of pancreatospheres under hypoxia, supporting further studies on its role to overcome microenvironment-mediated chemoresistance of PDAC [180].

Other important factors playing a key role in PDAC microenvironment and chemoresistance include cells of

the immune response and CSCs. Recent data indicated that tumor-associated macrophages (TAMs), which are abundant in the microenvironment of PDAC, secrete protumorigenic factors that contribute not only to cancer progression and dissemination but also to chemoresistance by reducing gemcitabine-induced apoptosis. In particular, TAMs induce upregulation of cytidine deaminase, the enzyme that metabolizes gemcitabine following its transport into the cell [181]. Moreover, immune cells within the tumor microenvironment can also activate pancreatic stellate cells which orchestrate the strong desmoplasia that characterizes PDAC and the resulting hypoxia [182]. Importantly, several miRNAs, including miR-155, which is commonly overexpressed in PDAC, are involved in the control of macrophage production and activation, suggesting that reprogramming miRNA activity in TAMs and/or their precursors might be effective for controlling tumor progression/chemosensitivity [183].

The existence of CSCs has been widely accepted to be responsible for tumor aggressiveness in PDAC, because CSCs have the capacity for increased cell growth, cell migration/invasion, metastasis, and also treatment resistance. However, a recent study detected deregulated expression of over 400 miRNAs, including let-7, miR-30, miR-125b, and miR-335, in PDAC CD44+/CD133+/EpCAM+ (triple-marker-positive) CSCs [184]. In the same study, as a proof of concept, knockdown of miR-125b resulted in the inhibition of tumor aggressiveness, consistent with the downregulation of CD44, EpCAM, EZH2, and snail. These results clearly suggest the importance of miRNAs in the regulation of CSCs characteristics, and their potential role as novel targets to improve therapeutic efficacy.

7. Conclusions and Future Perspectives

PDAC is a common cause of cancer-death and has the worst prognosis of any major malignancy, with less than 5% of patients alive 5 years after diagnosis. miRNAs have been documented to be involved in PDAC tumorigenesis; progression and recent evidence support their utility as promising biomarkers in cancer diagnosis and prognosis. In the present review we evaluated studies on the association between candidate miRNAs and drug response/resistance. Importantly, miRNAs remain intact in routinely collected, formalin-fixed, paraffin-embedded tumor tissues, and biofluids, and hopefully, in the near future, the expression profiles of specific miRNAs could provide new information about resistance of individual tumors to different treatments before starting therapy, while modulation of the expression of other miRNAs during treatment might offer a new tool for the prediction of acquired resistance.

However, as with previous studies on gene profiling, most emerging miRNA signatures of chemoresistance are not overlapping and no conclusive evidence has been obtained on their clinical utility. The controversial results might be explained by different specimens (frozen versus paraffin-embedded, micro- versus nonmicrodissected), experimental platforms used (quantitative PCR versus different miRNA array or *in situ* hybridization systems), stage, and regimens as well as small sample size, ethnic differences, and lack of appropriate statistical analyses.

Additional studies in larger homogeneous populations with validated methodology are needed to clarify these issues. Furthermore, new analytical techniques, such as next-generation sequencing, may provide useful tools to understand the role of miRNA as effective biomarkers also starting from very small amount of tissues. The next step will then be to use the emerging miRNAs as markers within prospective trials, to see if they can aid clinical decision-making.

Conflict of Interests

The authors confirm that this paper content has no conflict of interests.

Acknowledgments

This work was partially supported by grants from NWO Stimuleringfonds Open Access, AIRC Start-Up grant, and Italian Minister of Research, PRIN-2009 (to Elisa Giovannetti). The authors would like to thank Dennis Poel (Vumc, Amsterdam) and Alessandro Augusti (University of Pisa) for their support and suggestions.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 642916, 10 pages
http://dx.doi.org/10.1155/2014/642916

Review Article

The Potential of MicroRNAs in Personalized Medicine against Cancers

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Received 7 June 2014; Accepted 6 August 2014; Published 28 August 2014

Academic Editor: Paolo Gandellini

MicroRNAs orchestrate the expression of the genome and impact many, if not all, cellular processes. Their deregulation is thus often causative of human malignancies, including cancers. Numerous studies have implicated microRNAs in the different steps of tumorigenesis including initiation, progression, metastasis, and resistance to chemo/radiotherapies. Thus, microRNAs constitute appealing targets for novel anticancer therapeutic strategies aimed at restoring their expression or function. As microRNAs are present in a variety of human cancer types, microRNA profiles can be used as tumor-specific signatures to detect various cancers (diagnosis), to predict their outcome (prognosis), and to monitor their treatment (theranosis). In this review, we present the different aspects of microRNA biology that make them remarkable molecules in the emerging field of personalized medicine against cancers and provide several examples of their industrial exploitation.

1. Introduction

Recent technological advances in the field of molecular biology have revolutionized not only basic biological concepts but also clinical practice, in particular in the field of anticancer treatment. Management of patients with cancer is often based on the identification of tumor morphology, which decides the treatment program a patient should be enrolled in. However, pan-genomic analyses of genetic and epigenetic alterations and gene expression profiles are providing important new insights into the pathogenesis and molecular classification of cancers [1]. These rapidly diversifying and improving technologies to analyze tumors have revealed distinctive genomic (DNA mutations and chromosomal alterations), epigenomic (e.g., DNA methylation profiles), and transcriptomic (RNA expression profiles) differences between tumors that improve their classification in distinct molecular subtypes [2]. It is rapidly becoming apparent that each tumor has a unique combination of coding and noncoding mutations that distinguish between patients' tumors and therefore have the potential to serve as "signatures" in personalized anticancer therapies. It is possible to tailor patient medical care through the combination of individual genomic studies, phenotypic histomorphological features, and patient clinical specificities [3]. This approach, referred to as "personalized" or "individualized" medicine, is distinct from the classical "generalized" medicine as the medical decisions and selection of optimal therapies are not indiscriminately applied to each patient but rather take into account several parameters that identify the specific status of a patient. Personalized medicine will improve prediction of susceptibility to diseases and will restrict the development of cancers by anticipating disease progression. The use of personalized medicine will also reduce emergence of chemoresistance through the selection of drugs deemed most effective for each patient [2, 3]. This medical strategy

will save time and improve cost effectiveness, not to mention significantly improving patients' quality of life by limiting the adverse effects of inappropriate treatments [2, 3].

One prerequisite for the development of personalized medicine is the identification of combinations of biomarkers to guide a physician's clinical decision. It is in this context that the potential of microRNAs (miRNAs), a particular class of small noncoding RNAs, has rapidly become apparent [4–6]. To date, more than two thousand human miRNAs have been identified [7]. These small RNAs orchestrate the expression of the genome at the posttranscriptional level and adapt the protein output to various intracellular or extracellular stimuli. As such, they impact many, if not all, cellular processes and their deregulation is causative of many human malignancies, including cancers [8–11]. A plethora of researchers have now implicated miRNAs in the initiation and progression of primary tumors, as well as in metastasis formation [12–14]. More than 12,600 publications related to miRNA and cancer are listed in the NCBI PubMed database and their number exponentially grows. Advantageously, cancer cell types tend to have a highly specific cellular repertoire of miRNAs [15–18]. The expression levels of miRNA can be monitored in a variety of human specimens, including fresh or formalin-fixed paraffin embedded (FFPE) tissues [19, 20], as well as in almost all human body fluids [21–25]. Moreover, recent studies revealed that specific miRNA expression levels in biological fluids are associated with chemotherapy responses [23, 26, 27]. Hence, in addition to their potential as targets of novel anticancer therapies, several aspects of miRNA biology make them excellent candidates as biomarkers to be used in innovative and noninvasive tests aimed at identifying various cancers (diagnosis), predicting their outcome (prognosis), and monitoring their treatments (theranosis) [21, 25, 28–31]. Here we review the different aspects of miRNA biology that establish their potential in the emerging field of personalized medicine against cancers. We also present several known limitations of their exploitation, as well as future challenges and ongoing industrial developments.

2. miRNA Biogenesis and Mechanism of Action

miRNAs are noncoding RNAs, typically ~18–22 nucleotides long, which are generated through a complex multistep process. Several excellent reviews have already thoroughly described this process (see [32–35]). We focus here on the events required for the understanding of this review. MiRNA genes are first transcribed by RNA polymerase II into long, capped, and polyadenylated primary miRNA precursors (pri-miRNAs). The pri-miRNAs are subsequently processed by the nuclear RNase III enzyme Drosha into precursor miRNAs (pre-miRNAs) [36–38]. The pre-miRNAs are exported from the nucleus to the cytoplasm where they are cleaved by the cytoplasmic RNase III enzyme Dicer into a double-stranded RNA duplex (miR-5p/miR-3p). Alternative pathways exist that bypass either the Drosha or the Dicer steps [39], but these pathways invariably produce a miR-5p/miR-3p duplex. The two strands of the duplex are then incorporated into the effector complex called the miRNP complex, which contains several proteins including the key Argonaute proteins. One strand of the miRNA duplex then redirects the miRNP onto RNAs that harbor partial sequence complementarity. The study of the mechanisms responsible for the recognition of RNAs by miRNAs is an intense field of research with rapidly evolving concepts (e.g., location of miRNA binding sites [40, 41]). Canonical models are based on imperfect base-pairing between the mature miRNA and the targeted RNA. The extent to which the 5′ end of the mature miRNA (referred to as the "seed") pairs with the targeted RNA is of particular importance for the efficacy of miRNA-target interactions [42]. The miRNP complex eventually induces mRNA degradation and/or repression of translation [43, 44]. While the former's mechanism relies on deadenylation and further exonucleolytic cleavage of the mRNA, the latter's remains unclear and is debated as translation repression could occur at different steps: inhibition of initiation, inhibition of elongation, cotranslational protein degradation, or premature termination of translation [43].

The expression of miRNAs is a tightly regulated process that is extremely sensitive to intra- and extracellular stimuli (e.g., hormones, vitamins, pharmacological molecules, or hypoxia) [13, 45–49]. As a consequence, each cell type, at a particular time and a particular location, harbors a particular miRNA repertoire. This important concept constitutes the basis of the remarkable interest in miRNAs within the field of oncology. The potential importance of miRNAs to medicine was first highlighted by the seminal findings of Chen et al. [50] who demonstrated that some miRNAs are expressed in hematopoietic cells and showed that their expression was dynamically regulated during early hematopoiesis and lineage commitment. Importantly, they showed that miR-181 was preferentially expressed in the B-lymphoid cells and that its ectopic expression in hematopoietic stem/progenitor cells led to an increased fraction of B-lineage cells. Thus, it was illustrated that it is possible to distinguish different cell types or different cellular conditions (i.e., treatment) based on miRNA profiling. Moreover, miRNA expression levels in somatic cells of male and female patients can differ, likely due to exposure to specific hormones (e.g., testosterone, estrogen, and androgen), an observation that can explain gender-related differences noted in disease outcome and pathogenesis [51, 52]. Similarly, the expression of some miRNAs can be linked to aging [53–55]. The specificity of the cellular miRNA repertoire and its sensitivity to a large panel of intra/extracellular stimuli and characteristics (including gender and age) have stimulated interest not only in basic research focused on deciphering the contribution of miRNAs to cancer development, but also in more applied research aimed at evaluating miRNAs' potential in cancer personalized medicine.

3. miRNAs and Cancer

Extensive research has shown that miRNAs play essential roles in cancer initiation, progression, and metastasis formation [56–59]. The miRNA expression levels in tumors can be up- or downregulated compared to normal tissue, and

several miRNAs have been directly implicated in tumorigenesis by acting either as "oncomirs" or tumor suppressor miRNAs [15, 60, 61]. Among them, we can cite the miR-17–92 cluster (several miRNAs transcribed in a single transcription unit/pri-miRNA), which was the first oncogenic miRNA locus described [62]. Conversely, the miR-34a is an important miRNA with tumor suppressor activity, which can be directly transactivated by p53 [60]. Its upregulation results in increased apoptosis and altered expression of genes related to cell cycle progression, apoptosis, and angiogenesis [63]. As observed for protein coding genes [64–68], individual miRNAs can behave as oncogenes in one cell type and as tumor suppressors in others [69, 70]. For example, miR-221 acts as an oncogene in liver cancer by downregulating the expression of the tumor suppress or phosphatase and tensin homolog (PTEN), but it acts as a tumor suppressor in erythroblastic leukaemia by reducing the expression of the KIT oncogene [69, 70]. This dual action can be attributed to specific cellular contexts which expose a miRNA to distinct transcriptional regulation and/or to different RNA targets [42, 71].

The changes in the miRNA repertoire observed in cancer can result from (1) various disruptive mechanisms occurring at genes (deletions, amplifications, or mutations of miRNA genes), (2) regulation of transcription (epigenetic silencing, deregulation of transcription factors), or (3) posttranscriptional regulation (deregulation of the miRNA biogenesis pathway) [13, 72, 73]. One of the first implications of miRNAs in cancer was the discovery that the gene encoding miR-15a and miR-16 is frequently deleted in chronic lymphocytic leukemia [12]. This observation was further supported by other miRNA genes in other types of cancers [74–76]. The transcriptional deregulation of miRNA genes is mechanistically similar to what is observed in the case of coding genes and relies on similar processes (DNA methylation, histone acetylation, defect in specific transcription factor binding) [77, 78]. We have, for instance, demonstrated that the PML-RARA oncogenic protein associated with acute promyelocytic leukemia represses retinoic acid-responsive miRNA genes similar to coding genes [79]. Likewise, in breast cancer cells, the antagonism between RARA and ESR1 initially observed in the case of coding genes [80] also occurs on miRNA genes [45]. The deregulation observed at the posttranscriptional level (i.e., biogenesis of miRNA) is manifestly more specific to miRNAs. For instance, the LIN28 protein, a developmentally regulated RNA binding protein, whose expression is reactivated in many human tumors, can specifically block the Drosha cleavage of the pri-miRNAs belonging to the let-7 family [81]. The expression of several proteins (e.g., Dicer, Drosha, and Argonaute 2) involved in the biogenesis, processing, or the action of the miRNAs can be perturbed in certain cancers with presumably even more broad impact on cell physiology [82, 83]. The combinatorics of varied sources of deregulation generates miRNA profiles are specific to cancer types/subtypes and are often associated with staging, progression, and response to chemotherapies [15–18, 26, 60, 84, 85], thereby providing a means for the development of miRNA-based diagnostic, prognostic, and/or theranostic tests.

4. miRNA and miRNA Target Site Alterations in Cancer

Alteration of miRNA-mediated posttranscriptional regulation can be the consequence of genomic variations specific to cancer. Studies have shown that genomic mutations observed in cancer cells can drastically perturb miRNA-mediated regulation by modifying either the sequence of the miRNAs or the sequence of their targets. Intensive efforts are developed to collect the relevant data and to develop tools for their analysis. The first studies assessing the impact of mutations on miRNA-mediated regulation focused on polymorphic mutations (single nucleotide polymorphism (SNP)) (see [86] for a review). Bioinformatics studies highlighted SNPs in cancer samples located in pri-miRNAs, pre-miRNA, mature miRNAs, and miRNA targets with a potential impact on miRNA biogenesis, or the process of miRNA-mediated posttranscriptional regulation [87–90]. Operating under the knowledge that mRNAs are predominantly targeted by miRNAs in their 3′UTRs [91], Bruno et al. used SNP data to create the miRdSNP database, which stores disease-associated SNPs located in the 3′UTRs of genes and are supported by the literature after manual curation of publications stored in PubMed [92]. The most recent version of miRdSNP (v.11.03) stores 175,351 SNPs in 3′UTRs with 630 disease-associated SNPs for 204 diseases (including ~30 cancers). While previous studies have mainly been focused on SNPs, an increasing number of studies provide access to patient-specific somatic SNVs. Last year, Bhattacharya et al. created SomamiR [93], the first comprehensive database of mutations from whole-genome sequencing of cancer samples obtained by extracting mutations specific to cancer samples when compared to matched normal samples. The database provides the community with germline and somatic mutations in miRNAs and target sites that have the potential to functionally altering miRNA regulation. Importantly, the database stores experimental information about the impact of the mutations on miRNA function and their association with cancer.

Bioinformatics analyses of SNVs in miRNA target sites are critical for predicting functionally impactful mutations, but the development of bioinformatics approaches has only recently started to be the focus of concerted genomewide efforts. By combining whole-genome sequencing data from The Cancer Genome Atlas pan-cancer data set with Argonaute crosslink immunoprecipitation (AGO-CLIP) data, Hamilton et al. [94] defined a set of miRNA target sites derived from AGO-CLIP that were mutated in cancer. The algorithm developed by Hamilton et al. was then used to identify thousands of SNVs in miRNA binding sites. By combining these datasets with mRNA expression, they highlighted expression changes correlating with mutations. Four out of six tested mutations successfully exhibited experimentally strong evidence of miRNA binding and regulation. An alternative approach for highlighting mutations that impact miRNA regulation is through analyses of the transcriptome. In [95], the authors identified 73,717 SNVs in UTRs from transcriptome data of non-small-cell lung cancer samples. This set of SNVs was processed to predict mutations

affecting miRNA secondary structure and target sites. The computational analysis highlighted 490 SNVs with potential effects on miRNA target sites; the SNVs in turn are associated with genes enriched in molecular mechanisms of cancer. In the recent years, it has become apparent that mutations in miRNAs and miRNA target sites that play a critical role in cancer development. As an increasing number of cancer whole-genome sequence datasets will become publicly available to the community in the near future, it is critical to develop dedicated computational tools for the identification of mutations altering miRNA-mediated regulation. This will enable the community to better understand the underlying causes of carcinogenesis at the level of miRNA regulation and promises to significantly contribute to the vision of personalized diagnosis and therapeutic treatment.

5. miRNA-Based Cancer Diagnosis

While cancer-specific mutations in miRNA genes and/or their targets can be detected by classical DNA sequencing technologies, miRNA expression profiling requires more specific approaches. Three types of miRNA profiling technologies are currently used: RT-qPCR, microarrays, and RNA sequencing. The RT-qPCR approach requires a particular reverse transcription step which is primed by a stem-loop oligonucleotide [96]. This primer can pair with the 3′ region of the mature miRNA or with an adapter than have been ligated to its 3′ end. This latter solution allows the use of one single RT primer while adding a ligation step. The PCR step can rely on either the Taqman or SYBRGreen technology. The RT-qPCR does not necessitate large amounts of RNA and is traditionally recognized as highly sensitive and specific. Several assays are commercially available either in a specific single miRNA format or as arrays that can correspond to hundreds of miRNAs (this number is limited by the plates used for qPCR). Conversely, microarrays can detect more miRNAs in one single experiment, but this approach is considered to be less specific. Both RT-qPCR and microarrays are targeted technologies that do not allow the detection of novel miRNAs that are constantly identified [97, 98]. As an alternative, RNA sequencing is obviously the most powerful profiling technology in terms of both specificity and sensibility, but its cost is still high (compared to RT-qPCR or microarray) and the data generated require substantial computational processing.

In addition to intracellular expression, miRNAs can be detected in extracellular compartments. The presence of specific extracellular and circulating miRNAs in several body fluids of cancer patients is now largely described [21, 22, 25]. These circulating miRNAs are particularly interesting in the context of personalized medicine because correlations between high levels of specific circulating miRNAs and the response to a given anticancer treatment have been observed [26, 88, 99]. For instance, levels of miR-21 were found elevated in the serum of patients suffering from metastatic hormone-refractory prostate cancer especially in those patients resistant to docetaxel-based chemotherapy [27]. Studies in gastric and bladder cancers also identified specific miRNAs involved in cisplatin resistance [100–102]. Although the molecular basis behind the secretion of miRNAs remains largely unknown, it appears to be specific. The secretion of miRNAs represents a potent mode of intercellular communication that can, for instance, create a favorable context for the implantation of metastasis and the formation of secondary tumors [99, 103]. The miRNAs circulating in body fluids also present the remarkable characteristic of being extremely stable, though the mechanistic basis of this resistance to degradation remains largely unclear [104]. One reason could lie in the fact that circulating miRNAs are packaged in exosomes or other microvesicles present in body fluids as well as associated with (lipo)proteins (HDL and Argonaute 2) [105–107].

Similar to mutations, several miRNA profiles in various human specimens and cancers have been collected and made publicly available in dedicated databases: PhenomiR [108], oncomiRDB [61], PROGmiR [109], miRò [110], and miRandola [111]. We acknowledge that revisiting these data can dampen enthusiasm for the diagnostic/prognostic potential of miRNAs [112, 113]. In fact, no matter the technology used or the tissue studied, several issues associated with standardization of samples manipulation, miRNAs extraction protocols, measurements, and statistical analyses still require improvements [114–116]. Several papers have previously tackled the importance of samples processing [117, 118]. For example, hemolysis occurring during blood collection can have significant impact on miRNA profiling in plasma/serum [119–122]. The evaluation of the quantity and quality of miRNAs isolated from biological samples is indeed a key step in miRNA profiling. Although methods for miRNA extraction are usually similar to that used in the case of total RNAs (with possibly only slight modifications required to retain the small RNA fraction), the sizes and relative abundance of ribosomal RNAs cannot give information about the integrity of the miRNA preparation. In addition, the quantification of miRNA preparations can only be accurate in samples where larger RNAs are not degraded as the degradation products can compromise this quantification. Moreover, the low concentration of RNAs present in certain body fluids makes the estimation of miRNAs abundance particularly difficult [123]. The measurement of miRNA expression can also be affected by certain compounds coextracted with RNAs [124]. Strikingly, it has been reported that short RNAs with low GC content may be selectively lost during extraction depending on the extraction methods [125]. In addition to these experimental steps, data standardization and normalization as well as the evaluation of their statistical significance must also be carefully defined.

Overall, it is likely that inconsistencies in any of the steps described above will impede the definition of robust cancer-specific miRNA signatures [112, 113], but a better definition and standardization of the protocols used will undoubtedly overcome these obstacles [104, 126]. Several companies have indeed decided to meet the challenge (e.g., Santaris Pharma, Rosetta Genomics, Cepheid, Prestizia-Theradiag, and IntegraGen [127]). These efforts have, for instance, revealed that the expression level of the miR-31-3p allows the identification of patients with wild-type KRAS metastatic colorectal cancer responding to anti-EGFR therapy [128]. With approximately

two-thirds of metastatic colorectal cancer patients being wild-type KRAS, this marker could help better use EGFR therapy and spare patients from inappropriate treatment.

6. miRNA-Based Anticancer Therapy

It is important to note that miRNA deregulations observed in cancers are not necessarily involved in carcinogenesis, but that such deregulation could constitute potent biomarkers nonetheless. In contrast, some miRNAs have been truly functionally implicated in the development/progression of cancer or in the integration of chemotherapies. In that specific case, miRNAs can represent appealing candidate targets for novel anticancer therapies [129–131]. In fact, some pharmaceutical companies are already finalizing preclinical research phases and proceeding to clinical trials (see below). In addition to pharmacological agents classically used in oncology and able to control transcription and miRNA expression [132, 133], two miRNA-specific technological approaches can be envisaged: (i) to downregulate or block the function of oncogenic miRNAs (miRNA antagonists) and (ii) to upregulate the expression of miRNAs that have a tumor-suppressive function (miRNA mimics). The ultimate goal of these manipulations would be to restore a nonpathogenic miRNA profile [129–131] but, even more interesting in the context of personalized medicine, they can also sensitize cancerous cells to a particular chemo/radiotherapy. In fact, some miRNAs are implicated in the integration of drug effect [100–102] and modulating these miRNAs would restore the sensitivity of drug-resistant cells to chemotherapy and would prevent tumor recurrence [134, 135], as exemplified in the case of microRNA-200c [136].

Current strategies to inhibit miRNAs are mainly based on antisense oligonucleotides (also known as anti-miRs including locked nucleic acids (LNA anti-miRs), tiny LNA anti-miRs, and antagomirs) which titer the targeted miRNA [137–140]. They usually involve the introduction of a chemically modified single stranded RNA that binds with high affinity to a miRNA of interest. Since pairing with the inhibitor is very stable, the targeted miRNA is unable to repress translation. LNA-mediated miRNA silencing was shown to be efficient *in vivo* even in non-human primates [141]. In fact, an LNA-based inhibitor of miR-122, miravirsen, is currently being tested in phase 2 clinical trials for the treatment of hepatitis C virus infection [142]. Another strategy used to inhibit miRNA is to introduce within the cells an artificial RNA decoy, also called miRNA sponge, which harbors several binding sites complementary to a miRNA of interest [143–145]. This miRNA sponge can be produced from a transgene allowing stable expression even *in vivo* [143]. It is interesting to note that this artificial strategy is in fact an endogenous regulatory process, which involves long non-coding RNAs, referred to as competing endogenous RNAs (ceRNAs), acting as miRNA sponges [146]. Whether this situation is widely encountered and could occur with different long noncoding RNAs is still debated [147], but similar strategies have also been described in the case herpesvirus saimiri, which produces an RNA decoy able to titer the miR-27 [148]. In addition to nucleic-acid based strategies (i.e., anti-miRs and miRNA sponges), small chemical molecules able to block the processing of the pre-miRNAs by Dicer are also envisaged [149].

On the other hand, artificial restoration of the expression or function of one or a limited number of miRNAs, also called "miRNA replacement therapy," can be achieved either with miRNA mimics (typically introduced in the cell as pre-miRNAs) or with miRNAs directly encoded by expression vectors. In many cases, the reintroduction of these miRNAs leads to a reactivation of pathways that are required for normal cellular function [150, 151]. It is worth mentioning that a clinical trial using a miR-34 mimic is already in progress [152, 153]. In preclinical studies, it was reported that the injection of miR-34a mimic extended the survival of tumor-bearing mice [58]. Another study demonstrated that systemic administration of a miR-34 in a pancreatic xenograft cancer model significantly inhibited tumor growth and induced cancer cell apoptosis [154]. In May 2013, the Mirna Therapeutics Company initiated a phase I study to evaluate the safety of MRX34, a liposome-formulated mimic of miR-34, in patients with unresectable primary liver cancer and advanced or metastatic cancer (ClinicalTrials.gov Identifier: NCT01829971). Likewise let-7 mimics are in preclinical development stages at Mirna Therapeutics.

In addition to these chemical and synthetic procedures, miRNA expression levels can also be adjusted through dietary manipulations. Several nutrients such as amino acids, carbohydrates, fatty acids, vitamins, and phytochemicals (curcumin, resveratrol) are indeed known to modulate miRNA expression levels [155, 156]. For instance, intake of dietary fiber is inversely associated with colorectal cancer risk [157]. The microbial anaerobic fermentation of dietary fiber produces short chain fatty acids (such as acetate, propionate, and butyrate) and butyrate, whose bioavailability is reduced in case of low fiber intake, was shown to decrease the expression of several oncogenic miRNAs in HCT-116 (miRs-17, -20a, -20b, -93, -106a, and -106b) [158]. Hence, although further studies are required to fully unveil the mechanisms underlying diet-mediated miRNA regulations, modulating food intake may contribute to novel miRNA-based anticancer strategies that could be easily adapted to patient's requirements.

7. Conclusion

The discovery of miRNAs, and their implication in cancer, has not only intensified the "noncoding RNA revolution" [159] but also opened up new prospects in biomarker and therapeutic target studies [26, 27]. These molecules harbor specific features (stability, easy manipulation, reasonably simple detection, and tissue specificity) that can guide individualized treatments and monitoring of cancers. Some limits still exist that may prevent their immediate large-scale exploitation, but collective efforts currently made by both academic and industrial researchers will certainly circumvent these constraints and rapidly transfer miRNAs from bench to bedside. We also anticipate that this particular field of research, and the field of personalized medicine as a whole, will encourage (not to say demand) the acquisition of novel expertise and competences by physicians in order to understand and

combine computational/experimental biology together with medical practices.

Conflict of Interests

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

Acknowledgments

The authors thank Rebecca Worsley Hunt for critical reading of the paper. They are grateful to Wyeth W. Wasserman for continuous support and acknowledge the Genome Canada/Genome BC for funding (ABC4DE Project).

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Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 878450, 14 pages
http://dx.doi.org/10.1155/2014/878450

Review Article

MicroRNAs: Promising New Antiangiogenic Targets in Cancer

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Received 6 June 2014; Accepted 18 July 2014; Published 14 August 2014

Academic Editor: Elisa Giovannetti

MicroRNAs are one class of small, endogenous, non-coding RNAs that are approximately 22 nucleotides in length; they are very numerous, have been phylogenetically conserved, and involved in biological processes such as development, differentiation, cell proliferation, and apoptosis. MicroRNAs contribute to modulating the expression levels of specific proteins based on sequence complementarity with their target mRNA molecules and so they play a key role in both health and disease. Angiogenesis is the process of new blood vessel formation from preexisting ones, which is particularly relevant to cancer and its progression. Over the last few years, microRNAs have emerged as critical regulators of signalling pathways in multiple cell types including endothelial and perivascular cells. This review summarises the role of miRNAs in tumour angiogenesis and their potential implications as therapeutic targets in cancer.

1. Introduction

MicroRNAs (miRNAs) were initially discovered in 1993 by Lee et al. while they were studying the *lin-4* gene. They showed that *lin-4* encodes a small RNA with antisense complementarity to the *lin-14* gene which resulted in reduced lin-14 protein expression and thus disrupted the regulation of developmental timing of the nematode *Caenorhabditis elegans* [1]. miRNAs were subsequently shown to inhibit their target genes through specific sequences which are complementary to the target messenger RNA (mRNA). This discovery resulted in a paradigm shift in our understanding of gene regulation because miRNAs are now known to repress thousands of target genes and to coordinate many physiological processes including, but not limited to, development, differentiation, cell proliferation, and apoptosis [2–4]. The aberrant expression or alteration of miRNAs also contributes to a range of human pathologies, including cancer [5–7].

2. MicroRNAs: Definition, Biogenesis, and Function

miRNAs are one class of small noncoding RNAs that are approximately 18–25 nucleotides in length; they are evolutionary conserved single-stranded RNA molecules which are involved in the specific regulation of gene expression in eukaryotes [8–10]; thousands have been identified in a wide variety of species. They can increase or decrease protein expression by binding to the 3′-untranslated region (UTR) or to other regions (e.g., the 5′-UTR, coding sequences) of target mRNA transcripts [11] and thus play a central role in gene regulation in both health and disease. miRNA genes are located in inter- or intragenic regions of protein-coding gene introns and/or exons and are transcribed from DNA but not translated into proteins; they can exist individually or form clusters (reviewed in [12]).

miRNA biogenesis starts with transcription from a miRNA gene by RNA polymerase II (pol II), generating a

primary transcript RNA (pri-miRNA) which is up to several kilobases in length [13] and which can be distinguished by the presence of an imperfect double stranded (ds) RNA region known as the stem-loop structure. This structure is recognised by the nuclear RNase III endonuclease Drosha and its cofactor DGCR8 (DiGeorge syndrome critical region 8) which together with other proteins form a complex known as the microprocessor complex that cleaves the pri-miRNA and releases an approximately 60–70 nt long precursor miRNA (pre-miRNA) [14–17]. The pre-miRNA is exported from the nucleus to the cytoplasm via the exportin-5 protein (RAN-GTP-dependent transporter) [18–20] and once the complex is in the cytoplasm, Dicer (RNase III endonuclease), with the help of TRBP (the human immunodeficiency virus transactivating) and AGO2 (argonaute 2), generates the final mature 18–25 nt ds miRNA, miRNA:miRNA* (the complementary miRNA strand, referred to as miRNA*) [21, 22]. The mature miRNA loses one of its strands and the remaining one is loaded onto an argonaute-containing RNA-induced silencing complex (RISC) to form a miRISC which mediates protein inhibition [23, 24].

Once a miRNA binds to its target gene, two mechanisms of action are known: (i) mRNA degradation and (ii) translational mRNA inhibition without degradation, the latter of which occurs in animals, including mammals [25]. In the first of these mechanisms the binding is completely complementary between the miRNA and mRNA sequences whereas in the second one, where the bound mRNA remains untranslated, the binding is not completely complementary, resulting in reduced target gene expression (Figure 1). Another important characteristic of miRNAs is that one single miRNA has the potential to regulate many target genes while any one gene can be targeted by multiple miRNAs, meaning that miRNAome-mRNAome interaction can be a complicated network.

Some data in humans have shown that about 30–50% of genes coding for proteins are controlled by miRNAs [26]; therefore, any signalling pathway or cellular mechanism could potentially be governed by them. The causes of miRNA dysregulation in cancer can result from various mechanisms including (reviewed in [27]) the deletion or amplification of miRNA-coding chromosomal regions [6, 28–30], mutations in the miRNA or the target site sequence of its respective gene(s) [31–34], epigenetic silencing of miRNA promoters [35–38], or the dysregulation of proteins upstream of the miRNA pathway such as cellular signalling and transcription factors [39–45]. Hence, the ability of miRNAs to simultaneously regulate several genes makes them a very attractive study target, especially, given that many tumour cell types have altered miRNA expression patterns. In particular, recent work has provided support for the idea that noncoding RNAs, and in particular miRNAs, may play important roles in physiological and pathological angiogenesis.

3. Tumour Angiogenesis

Tumour angiogenesis is the process by which new blood vessels form in neoplasms; it starts in the early stages of disease and is a crucial step in the growth and spread of tumours. Without forming new blood vessels tumours cannot grow beyond a certain size due to the lack of oxygen and other essential nutrients [46]. Neovascularization has a dual effect on the tumour: firstly it supplies nutrients, oxygen, and growth factors that stimulate tumour cell growth [47]. Secondly, in combination with lymphangiogenesis, it is a prerequisite for metastasis as it provides a site of entry into the circulation allowing shed tumour cells to travel through the bloodstream to reach remote organs [48]. This pathological angiogenesis is characterized by uncontrolled growth and disordered vasculature and appears when there is an imbalance between pro- and antiangiogenic factors [49].

In order to initiate the neovascularization, tumour cells may overexpress one or more angiogenic inducers, mobilise proangiogenic proteins from the extracellular matrix, or attract host cells such as macrophages which produce their own angiogenic proteins [50].

The activation of angiogenesis starts when preexisting vessels become permeabilised in response to stimulating factors such as VEGF (vascular endothelial growth factor), PLGF (placental growth factor), or ANG-1 (angiopoietin-1). The basement membrane and extracellular matrix (ECM) are locally degraded by extracellular matrix metalloproteinases (MMPs) allowing the underlying endothelial cells (ECs), which are attracted by the angiogenic stimulus produced by the tumour cells and the microenvironment, to migrate into the perivascular space [51]. In tumour vasculature, the pericyte coating is decreased or is inadequate, leading to the formation of fenestrations and/or transcellular holes; these incomplete basal membranes and the fact that tumour blood vessel walls can also be formed by both endothelial and tumour cells lead to the formation of vessels with irregular diameters and structural abnormalities [51, 52].

In summary, angiogenesis is regarded as an essential step in cancer development which promotes tumour progression and metastasis by providing an entry site into the circulation [53]. Angiogenesis has become the focus of intense study in recent years, for example, in the development of antiangiogenesis pharmacological agents as attractive antitumor targets [54, 55]. In addition, the response of the vascular endothelium to angiogenic stimuli is modulated by certain miRNAs which can be either proangiogenic or antiangiogenic. For this reason, the study of miRNAs and angiogenesis is likely to improve our understanding of the process of carcinogenesis and may lead to the identification of new therapeutic targets for cancer treatment.

4. Role of MicroRNAs in the Regulation of Angiogenesis

4.1. Enzymes Involved in miRNA Biogenesis. One approach to studying the biological relevance of miRNAs is by silencing their functions by mutating or disrupting Dicer, a critical enzyme involved in miRNA maturation [22]. Functional loss of Dicer results in profound vascular developmental abnormalities in both zebrafish and mice [56, 57], but the first evidence that miRNAs were involved in the regulation

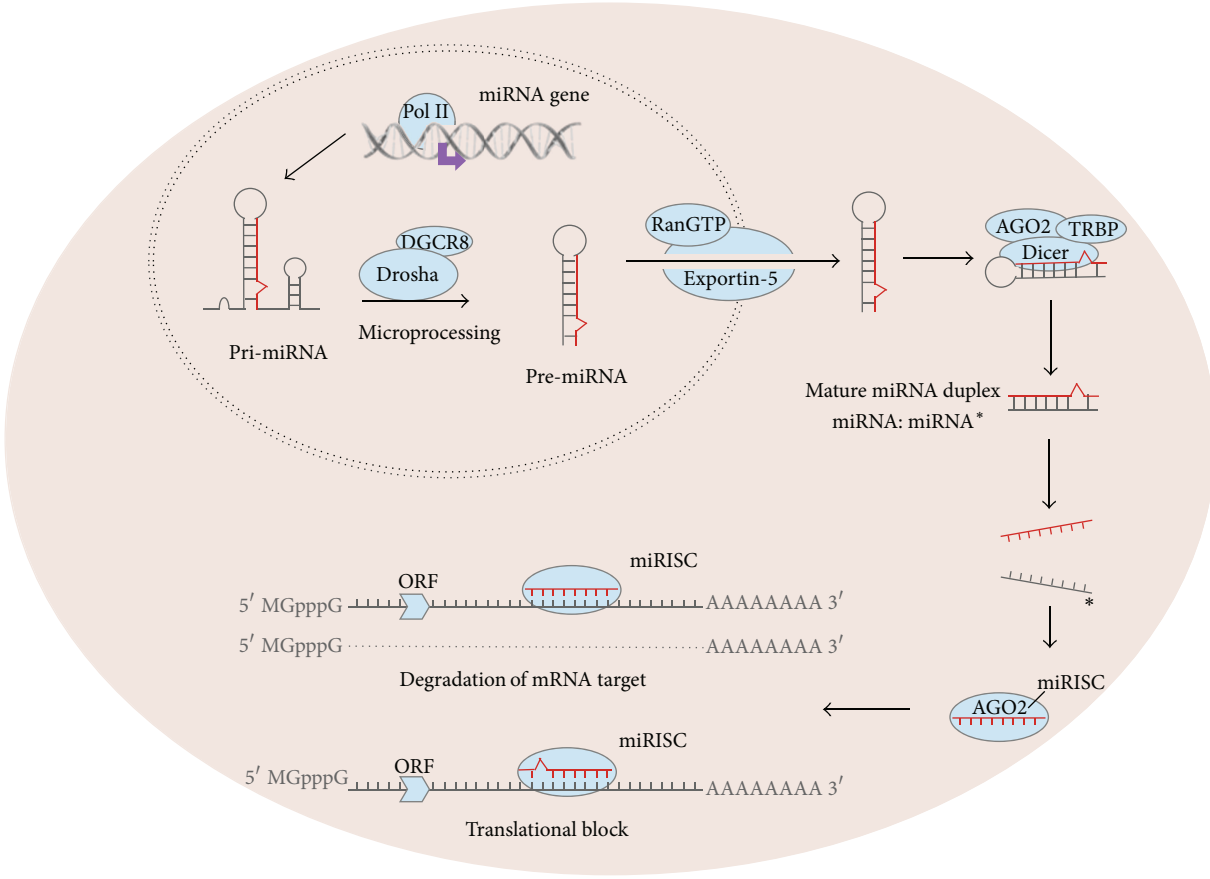

Figure 1: miRNA biogenesis: miRNA gene transcription generates primary miRNA (pri-miRNA) in the nucleus which is then cleaved by the microprocessor complex (Drosha and DGCR8), liberating pre-miRNA which is exported from the nucleus to the cytoplasm by exportin-5. Pre-miRNA is finally processed by Dicer and TRBP to obtain a mature miRNA with the capacity to bind to target mRNAs. AGO2: argonaute 2, DGCR8: DiGeorge syndrome critical region 8, miRISC: miRNA bound to RNA-induced silencing complex, ORF: origin replication frame, Pol II: polymerase II, and TRBP: the human immunodeficiency virus transactivating.

of angiogenesis during vascular development came from investigating mice with hypomorphic Dicer expression; these mice had a retarded phenotype and died as embryos between days 12.5 and 14.5 because Dicer is specifically required for the formation/maintenance of blood vessels in embryos and yolk sacs [58]. Furthermore, these mutant embryos also had altered *Vegf*, *Flt1*, *Kdr* (kinase insert domain receptor), and *Tie2* expression indicating that Dicer probably exerts its function because it is involved in the biogenesis of miRNAs that regulate the expression levels of these critical proangiogenic factors in mice [58]. Similarly, generation of mutant embryos disrupts Dicer in zebrafish and results in pericardial oedema and vascular defects [59]. Moreover, genetic silencing of Dicer and/or Drosha in HUVECs reduces EC proliferation, migration, capillary sprouting, and tube forming activity *in vitro* and, in the case of Dicer (but not Drosha), reduces angiogenesis *in vivo* [60, 61]. This difference in the effects of Dicer and Drosha might be due to a recently described alternative Drosha processing pathway which is miRNA-independent [62]. Another new study in bone marrow mice endothelial progenitor cells (EPCs) also showed that conditional ablation of Dicer led to the inhibition of angiogenesis and impaired tumour growth, demonstrating that functional Dicer is also necessary for bone marrow-mediated tumour angiogenesis [63]. Together, these studies reveal that Dicer and Drosha are prerequisite enzymes in miRNA processing and demonstrate the essential role of miRNAs in angiogenesis.

4.2. MicroRNAs and Endothelial Cells. Different cell types contribute to tumour neovascularization; among them, the endothelial and perivascular cells are generally acknowledged to play a central role in the angiogenesis process. miR-126 was suggested to be an endothelium specific miRNA, which modulates the endothelial phenotype *in vitro* and blood vessel integrity *in vivo,* respectively [64]. It is encoded by intron 7 of the *EGFL7* (EGF-like domain 7) gene, which encodes an EC-specific secreted peptide that acts as a chemoattractant and smooth muscle cell migration inhibitor [65–67]; both miR-126 and *Egfl7* have a similar EC expression pattern [68]. In concordance, it has been demonstrated that this miRNA is enriched in tissues with a high vascular component such as the lung and heart [69, 70]. miR-126 promotes angiogenesis in

response to VEGF and bFGF (basic fibroblast growth factor) through negative suppression regulators in signal transduction pathways [64, 68, 71]. Furthermore, miR-126 has been shown to be enriched in Flk-1 (kinase insert domain receptor; a type III VEGF receptor tyrosine kinase) positive cells derived from mouse embryonic bodies. miR-126 also directly regulates the vascular process by targeting *SPRED-1* (sprouty-related, *EVH1* domain containing 1), *VCAM1* (vascular cell adhesion molecule 1), and *PIK3R2* (phosphoinositide-3-kinase, regulatory subunit 2, also known as p85-β) resulting in posttranscriptional repression in HeLa cells [64]. miR-126 loss-of-function studies in both mice and zebrafish highlighted its importance in developmental and pathological angiogenesis affecting the EC function *in vivo* [64]. Targeted deletion of miR-126 in mice causes leaky vessels, hemorrhaging, and partial embryonic lethality, due to a loss of vascular integrity and defects in EC proliferation, migration, and angiogenesis; these vascular abnormalities are similar to those caused by diminished angiogenic growth factor signalling (e.g., by VEGF or FGF). miR-126 enhances MAP kinase signalling in response to VEGF and FGF and, in its absence, angiogenic growth factor signalling is reduced. This process may be regulated by *Vegf* suppression mediated by *Spred-1*, considering that it is a negative regulator of the RAS/MAP kinase pathway; therefore, miR-126 promotes blood vessel formation by repressing SPRED-1 expression [68]. Another finding was that miR-126 deletion inhibits VEGF-dependent AKT and ERK signalling derepressing the p85β subunit of *Pi3-kinase* and of *Spred-1*, respectively [71]. Finally, Png and colleagues reported that miR-126 regulates EC recruitment to metastatic breast cancer cells *in vitro* and *in vivo* [72]. According to these data, it seems that miR-126 contributes to the EC recruitment in physiological as well as in pathological conditions and might be a promising antiangiogenic target.

Other miRNAs have been found to regulate the angiogenic process by exerting an antiangiogenic function. Among these miR-221 and miR-222 are highly conserved miRNAs which are transcribed from a pri-miRNA located on the human X chromosome. These miRNAs are negative regulators of angiogenesis [73], have a proliferative effect on cancer cells [74], and are also expressed by growth factor-stimulated or quiescent ECs [11]; indeed, microarray data indicate that these are the most abundantly expressed miRNAs in HUVECs [73]. This latter study showed that these two miRNAs inhibit stem cell factor (SCF) by decreasing the abundance of c-KIT (tyrosine kinase receptor for SCF), thus blocking EC migration, proliferation, and angiogenesis *in vitro*. Their antiangiogenic activity was further demonstrated by their interaction with the c-KIT 3′-UTR in ECs [75] and this group also showed that these two miRNAs regulate endothelial nitric oxide synthase (eNOS) in ECs by silencing Dicer [61]. NO is synthesized by eNOS and is necessary for EC survival, migration, and angiogenesis [76]. However, binding sites for these miRNAs were not found in the eNOS 3′-UTR, suggesting that miR-221/222 are likely to indirectly regulate eNOS protein production. These miRNAs can also specifically promote cancer cell proliferation by regulating the *p27* (Kip1) tumour suppressor gene [74], indicating that the regulation of proliferation by miR-221/222 is cell-type specific. More recent studies have shown that these miRNAs control different target genes: miR-222 is involved in inflammation mediated by vascular growth factors [77], whereas miR-221 is required for vascular remodelling [78]. Similarly, a study performed in a murine model of liver tumorigenesis showed that miR-221 but not miR-222 accelerated tumour growth [79].

Similar to miR-221/222, the polycistronic miR-17-92 gene cluster (Cl3orf25), located on human chromosome 13q31.3, which encodes six mature miRNAs, miR-17, miR-18a, miR-19a, miR-20a, miR-19b, and miR-92a [80], is also highly expressed in ECs [81]. This cluster is amplified in several types of lymphoma and solid tumours [45, 82] and regulates vascular integrity and angiogenesis, promoting tumour neovascularization *in vivo* by downregulating antiangiogenic *THBS1* (thrombospondin 1) [83]. A recent study showed that while miR-17, miR-18a, and miR-19a expression were enhanced and miR-92a expression was reduced during EC differentiation, inhibiting these miRNAs did not affect EC differentiation [84]. Although the cluster is highly upregulated in several human tumour types, only miR-18a and miR-19a have a proangiogenic function during tumour angiogenesis [71, 85, 86]. In contrast, an antiangiogenic role for miR-17-92 cluster members has also been reported in two different studies in cultured ECs [86, 87]: the first reported an antiangiogenic role for miR-92a in ECs, where injection of miR-92a antagomirs (small synthetic RNAs that are perfectly complementary to the specific miRNA to inhibit its function) into mice promoted neovascularization in ischemic limbs. This antiangiogenic function was mediated by ITGA5 (integrin α5 subunit) repression and also indirectly suppressed eNOS production [87]. The second showed that overexpression of miR-17, miR-18a, miR-19a, miR-20a, and miR-19b inhibited EC sprouting, network formation, and cell migration, which was reversed when they were silenced [86]. However, the combined antagomir inhibition of miR-17 and miR-20a *in vivo* enhanced vessel invasion into subcutaneous tissue, although it did not enhance tumour angiogenesis; relevant targets for this miR-17 antiangiogenic activity include the cell cycle inhibitor *p21*, the S1P receptor EDG1, and the protein kinase JAK-1 [86]. A new study has shown that miR-17-3p controls the angiogenic process in HUVECs *in vitro* in a cell-autonomous manner by modulating the FLK-1 (VEGFR-2) expression implicated in the pleiotropic effects of angiogenesis. miR-17-3p negatively regulates FLK-1-mediated angiogenesis in ECs by rapidly downregulating expression via a 21 bp fragment from the *FLK-1* 3′-UTR [88]. Thus both the pro- and antiangiogenic properties of the miR-17-92 cluster seem to be related to the cellular microenvironment.

Inhibition of Dicer and Drosha by siRNAs reduces let-7f and miR-27b expression in ECs *in vitro*, and inhibitors for both miRNAs contribute to the reduction of *in vitro* angiogenesis and sprout formation, suggesting that let-7f and miR-27b promote angiogenesis by targeting antiangiogenic genes such as *THBS1* (using *in silico* analysis of predicted targets), although these targets have not yet been characterised [60, 75]. Furthermore, miR-214 overexpression in ECs significantly inhibited tubular sprouting, and, similarly, knockdown

of the quaking protein (a direct miR-214 target which is critical for vascular development) reduced proangiogenic growth factor expression and EC sprouting; moreover, miR-214 upregulation decreased the secretion of proangiogenic growth factors, including VEGF, which was reversed by inhibiting it [89].

Finally, Fang et al. reported that miR-93, a miRNA from the miR-106B-25 cluster and a paralog of the miR-17-92 cluster, has both pro- and antiangiogenic properties. It enhanced EC activities, including cell spreading and tube formation in a human breast carcinoma cell line by targeting the *LATS2* gene (large tumour suppressor kinase 2), whereas it was found to be upregulated in human breast carcinoma tissues [90].

The most important mechanisms and functions involved in EC regulation by miRNAs described above are summarized in Figure 2 and Table 1.

4.3. miRNAs and Hypoxia. Hypoxia, a key driver of angiogenesis, works primarily by inducing angiogenic factors via the HIF-1α (hypoxia-inducible factor-1 alpha) pathway. Hypoxia occurs during tumour development, and several hypoxia-regulated miRNAs have been identified in cancer cells, as detailed below.

miR-210 is the only miRNA so far identified which strongly responds to hypoxic stress in virtually all experimental systems *in vivo* and *in vitro* and in both normal and tumour cells under physiological hypoxic conditions [91]. Hypoxia in tumours is closely related with angiogenesis [92] and several proangiogenic factors are overexpressed in tumours as a response to a hypoxic microenvironment [93], VEGF being the best example [94, 95]. miR-210 and *VEGF* expression were closely correlated in breast cancer patients [96], showing a possible role for miR-210 in tumour angiogenesis. In support of this, two independent studies demonstrated that upregulation of miR-210 in normoxic HUVECs induced the formation of capillary-like structures and VEGF-dependent EC migration, while inhibiting it antagonised these processes [97, 98]. Furthermore, miR-210 induces angiogenesis in part repressing endothelial ligand ephrin-A3, which is an antiangiogenic factor [97]. In another study HUVECs cultured with exosomes derived from mouse breast cancer 4T1 cells which were transfected with miR-210 had significantly increased migration and capillary formation [99]. Taken together, this data suggests that miR-210 may be one of the angiogenesis-promoting factors released by tumour cells, therefore explaining the increased quantities of miR-210 found in the circulation of cancer patients [100, 101].

Two studies performed in four different murine tumour cell lines as well as the MCE-7 breast cancer cell line showed that miR-20b regulates angiogenesis by targeting VEGF and HIF-1α [102, 103].While repression of miR-20b enhanced HIF-1α and VEGF protein levels in normoxic conditions, hypoxic conditions increased miR-20b levels and decreased HIF-1α and VEGF levels. Overexpression of HIF-1α also downregulated miR-20b expression in normoxic tumour cells, whereas HIF-1α repression in hypoxic tumour cells caused miR-20b to increase. It is thought that this might be a novel molecular regulation mechanism through which miR-20b regulates HIF-1α and VEGF but which is also self-regulated by HIF-1α so that tumour cells continuously adapt to different oxygen concentrations [103]. In support of this idea Cascio et al. used hypoxia-mimicking conditions ($CoCl_2$ exposure) to demonstrate that VEGF expression in breast cancer cells is mediated by HIF-1α and STAT3 in a miR-20b-dependent manner. miR-20b decreased VEGF protein levels after $CoCl_2$ treatment, and *VEGF* mRNA downregulation by miR-20b was associated with reduced levels of nuclear HIF-1α and STAT3; STAT3 was also necessary for $CoCl_2$-mediated HIF1α nuclear accumulation and its recruitment to the *VEGF* promoter [102].

Additionally, miR-21 has been identified as one of the most important miRNAs associated with tumour growth and metastasis. Lei et al. confirmed that its overexpression in DU145 cells increases both HIF-1α and VEGF expression to promote tumour angiogenesis and that, similar to previous findings [103], HIF-1α (itself a key downstream miR-21 target) downregulation negated miR-21-induced tumour angiogenesis. miR-21 activates AKT and ERK 1/2 (extracellular signal-regulated kinases) by targeting PTEN (phosphatase and tensin homolog) which elevates HIF-1α and VEGF expression [104]. Interestingly, miR-21 is only upregulated by hypoxia in AKT2-expressing cells, and AKT2 confers greater resistance to hypoxia than AKT1 or AKT3. When miR-21 is upregulated in hypoxic conditions AKT2 downregulates PTEN which then activates the other two Akt isoforms. In addition, miR-21 also downregulates PDCD4 (programmed cell death 4) and sprouty 1 (Spry1) which, together with PTEN downregulation, confers resistance to hypoxia [105]. This group also confirmed the involvement of the AKT2/miR-21 pathway in angiogenesis *in vivo* in hypoxic human ovarian carcinoma cells and in the MMTV-PyMT (mouse mammary tumour virus-polyoma-middle T) breast cancer metastasis model. Taken together, these data indicate that a novel AKT2-dependent pathway is activated by hypoxia and that this promotes tumour resistance by inducing miR-21.

The miR-200 family plays a crucial role in epithelial-to-mesenchymal transition by controlling cell migration and polarity [106–108]. Delivery of miR-200b mimic into HMECs (human microvascular endothelial cells) suppressed the angiogenic response, whereas miR-200b-depleted HMECs exhibited elevated angiogenesis *in vitro*, as evidenced by Matrigel tube formation and cell migration assays [109]. Using different technologies, this group showed that (i) ETS-1 (avian erythroblastosis virus E26 (v-ets) oncogene homolog-1), an essential angiogenesis-related transcription factor, is a direct miR-200b target, (ii) some *ETS-1*-associated genes such as *MMP-11* and *VEGFR-2* were downregulated by miR-200b, and (iii) hypoxia and HIF-1α stabilisation inhibited miR-200b expression, increasing *ETS-1* expression. As miR-200b becomes downregulated in a hypoxic environment its expression is derepressed and angiogenesis is promoted [109]. A recent study on the A549 and HUVEC cell lines demonstrated that miR-200c regulates *VEGFR-2* expression, increasing cancer cell radiosensitivity by targeting the VEGF-VEGFR-2 pathway. Ectopic miR-200c expression in HUVECs significantly impaired angiogenesis, tubulogenesis,

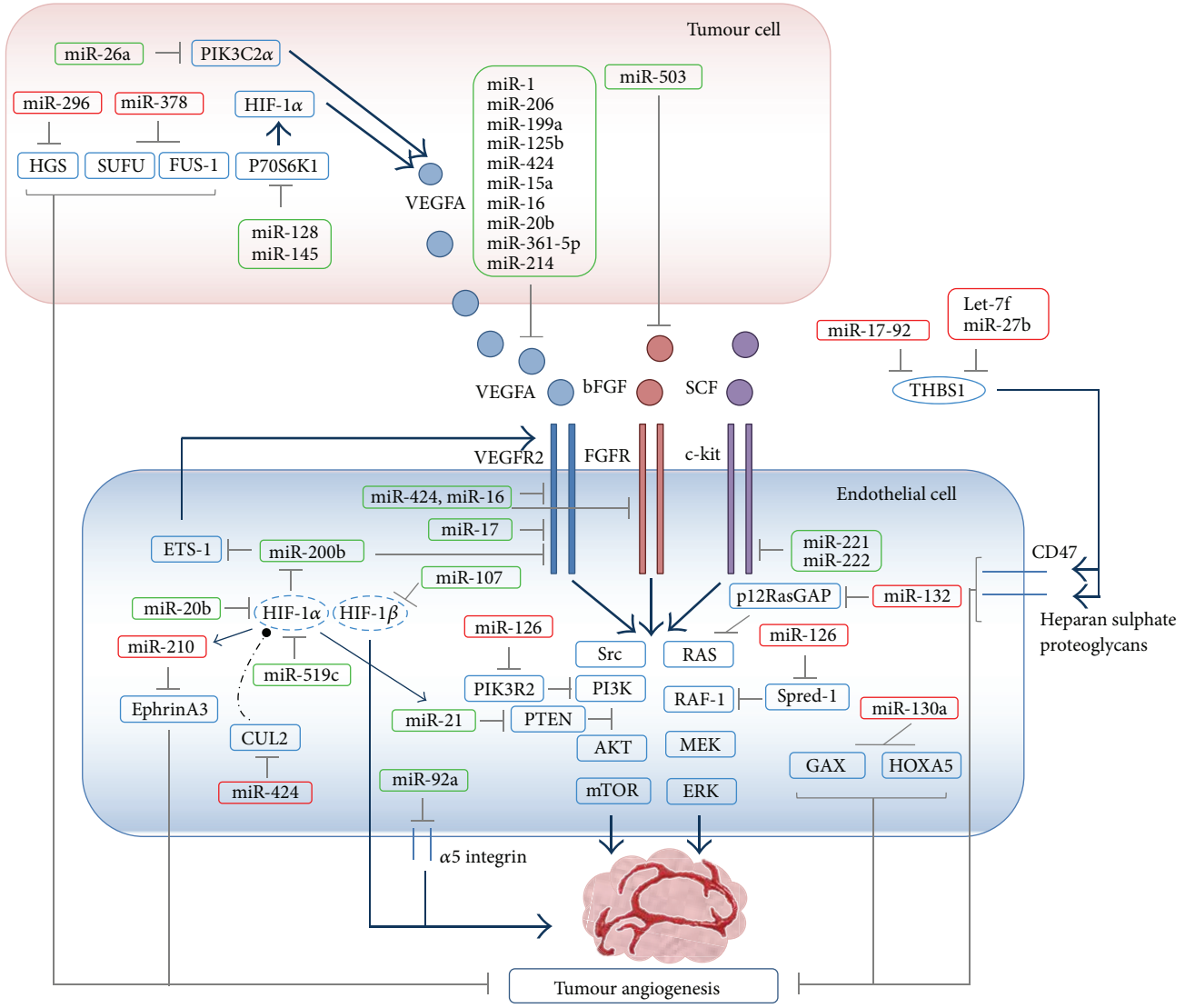

FIGURE 2: miRNAs involved in angiogenic process. Angiogenesis regulation conducted by different miRNAs is based on a complex network and is summarized in this figure. Red boxes indicate proangiogenic miRNA, green boxes indicate antiangiogenic miRNAs, and dashed circles indicate genes involved in molecular pathway taking place in both tumour and endothelial cells. Grey lines represent inhibitory processes while the blue lines with arrows represent activation processes and the dashed black line represents the ubiquitin-mediated degradation HGS (hepatocyte growth factor-regulated tyrosine kinase substrate), SUFU (suppressor of fused), FUS-1 (FUS RNA binding protein), PIK3C2α (phosphoinositide-3-kinase class 2α), THBS1 (thrombospondin-1), HIF-1α (hypoxia-inducible factor 1 alpha), HIF-1β (hypoxia-inducible factor-1 beta), VEGF (vascular endothelial growth factor), bFGF (basic fibroblast growth factor), Spred-1 (sprouty-related, EVH1 domain containing 1), PIK3R2 (phosphoinositide-3-kinase, regulatory subunit 2), SCF (stem cell factor), c-KIT (tyrosine-protein kinase kit), VEGFR-2 (vascular endothelial growth factor receptor 2), ERK (extracellular signal-regulated kinase), AKT (v-akt murine thymoma viral oncogene homolog 1), PTEN (phosphatase and tensin homolog), Ets-1 (avian erythroblastosis virus E26 (v-ets) oncogene homolog-1), fibroblast growth factor receptor-1 (FGFR-1), GAX (growth arrest homeobox) and HOXA5 (homeobox A5), RAS (v-ki-ras2 kirsten rat sarcoma viral oncogene homolog), RAF-1 (v-raf-1 murine leukemia viral oncogene homolog 1), Cul2 (Cullin 2), mTOR (mechanistic target of rapamycin serine/threonine kinase), and Src (v-src avian sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog).

and migration, whereas miR-200c suppression increased tube formation and migration by about 30% [110].

In addition to the key miRNAs discussed above, miR-107, miR-519c, and miR-424 have also been implicated in hypoxia-induced angiogenesis. Yamakuchi et al. showed that miR-107 decreases hypoxic signalling in human colon cancer cells by inhibiting *HIF-1β* and that it is transcriptionally regulated by p53; in addition, miR-107 overexpression in mouse tumour cells repressed tumour angiogenesis, growth, and VEGF expression [111]. Cha et al. identified miR-519c as another important hypoxia-independent regulator which directly binds the *HIF-1α* 3′UTR and thus causes a reduction in tumour angiogenesis. Overexpression of this miRNA significantly decreased HIF-1α protein levels and

Table 1: Angiogenic miRNAs related to cancer and their targets.

miRNA	Role	Function	Targets	Reference
Dicer	Function loss	Maturation of microRNAs	miRNAs	[56, 57] [58, 60, 61]
miR-126	Proangiogenic	Regulates the response of endothelial cells to VEGF	SPRED-1, PIK3R2, VECAM-1,	[64, 68, 71]
miR-221/222	Antiangiogenic and proangiogenic	Inhibitor of SCF	C-KIT, eNOS, p27	[73] [61] [74]
miR-17-92 cluster	Proangiogenic and antiangiogenic	Regulation of vascular integrity	THBS1, p21, S1P, JAK1 Flk-1 (VEGFR-2)	[83] [86] [88]
let-7f; miR-27b	Proangiogenic	↑EC-mediated angiogenesis	*ND	[75]
miR-214	Antiangiogenic	Tubular sprouting	Quaking	[89]
miR-93	Proangiogenic	Enhanced endothelial cell activity	LATS2	[90]
miR-210	Proangiogenic	Endothelial cell migration and formation of capillaries	Ephrin-A3	[97]
miR-20b	Antiangiogenic	Decreases levels of HIF1A and VEGF	VEGF, HIF-1α	[102, 103]
miR-21	Antiangiogenic	Induction of tumour angiogenesis, confers resistance to hypoxia	PTEN, PDCD4, Sprouty1	[104] [105]
miR-200 family	Antiangiogenic	Epithelial-mesenchymal transition	ETS-1	[109]
miR-200c	Antiangiogenic	Epithelial-mesenchymal transition	VEGFR-2	[110]
miR-107	Antiangiogenic	Hypoxia signalling	HIF-1β	[111]
miR-519c	Proangiogenic	Depletion of tumour angiogenesis	HIF-1α	[112]
miR-424	Proangiogenic and antiangiogenic	Destabilization of the E3-ligase assembly, increasing HIF-1α levels	CUL-2 VEGF VEGFR-2 FGFR-1	[113] [117]
miR-15a	Antiangiogenic	Control of the cell cycle, apoptosis, proliferation, and angiogenesis	BCL-2 VEGF-A	[116]
miR-16	Antiangiogenic	Controls VEGF expression and induces cell apoptosis	VEGF VEGFR-2 FGFR-1 BCL-2 VEGF-A	[117] [116]
miR-378	Proangiogenic	Cell survival and tumour growth	SUFU and FUS-1	[114, 119]
miR-296	Proangiogenic	Promotes angiogenesis by increasing levels of proangiogenic growth factor receptors	HGS	[120]
miR-199a	Antiangiogenic	Suppresses tumour angiogenesis via the HIF-1α/VEGF pathway	HER3	[121]
miR-125b	Antiangiogenic	Suppresses tumour angiogenesis via the HIF-1α/VEGF pathway	HER2 HER3	[121]
miR-361-5p	Antiangiogenic	Cancer development and progression	VEGF A	[122]
miR-1/206	Antiangiogenic	Regulation of VegfA expression	VEGF A	[124]
miR-10b	Proangiogenic	Regulation of endothelial cell division and migration	HOXD10, FLT1	[123]
miR-196b	Proangiogenic	*ND	*ND	[63]
miR-503	Antiangiogenic	Overexpression reduces tumour angiogenesis	FGF2, VEGFA	[130]
miR-128	Antiangiogenic	Decreases cell proliferation, tumour growth, and angiogenesis	P70S6K1	[131]

Table 1: Continued.

miRNA	Role	Function	Targets	Reference
mir-145	Antiangiogenic	Inhibition of tumour growth and angiogenesis	P70S6K1	[132]
miR-130a	Proangiogenic	Increases angiogenesis by targeting GAX and HOXA5 (antiangiogenic genes)	GAX, HOXA5	[133]
miR-132	Proangiogenic	Increases Ras activity	p120RasGAP	[134]
miR-26a	Antiangiogenic	Suppresses tumour growth and metastasis	PIK3C2α HGF	[135] [136]

*ND: not described.

reduced HUVEC tubulogenesis, whereas its inhibition by antagomirs had the opposite effect [112]. miR-424, which is increased in hypoxic ECs and during vascular remodelling *in vivo*, is thought to play an important role in postischemic vascular remodelling and angiogenesis. It inhibits CUL2 (Cullin 2) expression by targeting its 3′-UTR, stabilising HIF-1α, which then transcriptionally activates VEGF; similarly, EC transfection with miR-424 increases both HIF-1α and HIF-2α expression and increases proliferation and migration, presumably through the same VEGF-mediated mechanism [113].

In summary, many different studies have identified the functional targets and pathways involved in miRNA-mediated regulation of hypoxia (Figure 2 and Table 1), providing a rationale for a new therapeutic approach to suppressing hypoxia-induced tumour angiogenesis.

4.4. miRNAs and the VEGF Pathway. The first miRNAs which were directly associated with tumour biology by their downregulation or deletion were miR-15a and miR-16; expression of these miRNAs is reduced in response to hypoxia which increases VEGF expression [114]. These miRNAs also induce apoptosis in leukaemia cells by inhibiting *BCL-2* (an antiapoptotic protein) and blocking cell cycle progression, making them attractive antitumour targets which could be used to block tumour cell survival, proliferation, and VEGF-mediated angiogenesis [115]. miR-15a and miR-16 are significantly underexpressed in primary multiple myeloma (MM) cells as well as MM cell lines and their expression inversely correlates with VEGF in both human MM cell lines and normal plasma cells [116]. Moreover, miR-16 and another miRNA, miR-424, decrease VEGF, VEGFR-2, and FGFR-1 (fibroblast growth factor receptor-1) expression (all of which have been validated as miR-16 and miR-424 targets in ECs using mimetic microRNAs) and hence play an important role in regulating the cell-intrinsic angiogenic activity of ECs by increasing VEGF and bFGF signalling [117]. Dejean et al. showed that miR-16 directly interacts with *VEGF* mRNA at the 3′-UTR and that ALK expression leads to miR-16 downregulation, thus increasing VEGF levels. This was further supported by experiments in TPM3-ALK (conditional onco-ALK) MEF cells which showed that both ALK and HIF1α expression are a prerequisite for miR-16 downregulation; in agreement with these findings, increased miR-16 expression *in vivo* reduced angiogenesis and tumour growth [118].

miR-378 is another important angiogenic regulator. When this miRNAs is overexpressed in cancer cell lines, *SUFU* (suppressor of fused) and *FUS-1* (FUS RNA binding protein), two tumour suppressor genes, are downregulated, and as a consequence there is an increase in the levels of VEGF, thus increasing cell survival and reducing cell death [119].

Similarly, increased miR-296 expression activates angiogenesis in cultured ECs due to the suppression of HGS (hepatocyte growth factor-regulated tyrosine kinase substrate) which mediates VEGFR-2 and PDGFR-β (platelet derived growth factor receptor beta) degradation, whereas miR-296 inhibition reduces angiogenesis in tumour xenografts [120]. In contrast, He et al. identified another two miRNAs, miR-199a and miR-125b, which were downregulated in ovarian cancer tissues and cell lines, and overexpression of these miRNAs inhibits tumour-induced angiogenesis and is associated with a decrease in *VEGF* mRNA and protein expression [121]. A different miRNA, miR-361-5p, represses a miRNA recognition element located in a conserved downstream region of the *VEGFA* 3′-UTR and is inversely correlated with VEGFA expression in human squamous cell carcinoma (SCC) cells and in healthy skin, indicating that it may play a role in cancers [122].

Two studies in zebrafish have demonstrated that miR-1, miR-206, and miR-10 govern angiogenesis by targeting *vegf* [123, 124]. They negatively regulate developmental angiogenesis by controlling VegfA in muscle and thus angiogenic signalling to the endothelium. Interestingly, reducing the levels of VegfAa but not VegfAb rescued the increase in angiogenesis previously observed when miR-1/206 were knocked down [124]. miR-10 repression led to premature truncation of intersegmental vessel growth in the trunk of zebrafish larvae, and its overexpression promoted angiogenesis in both zebrafish and cultured HUVECs. miR-10 acts by directly regulating FLT1 (a cell-surface receptor that binds VEGF) and its soluble splice variant SFLT1. Its downregulation in zebrafish and HUVECs increases FLT1/SFLT1 protein levels, which binds VEGF with higher affinity than VEGFR-2 and therefore negatively regulates VEGFR-2 signalling pathway [123]. Moreover, miR-10b and miR-196b have been related to angiogenesis and cancer metastasis [125–129] and are both upregulated in murine ECs treated with tumour-conditioned medium, although only miR-10 responded to increased VEGF levels. These miRNAs are preferentially expressed in the vasculature of more invasive human breast tumours

and are upregulated by tumour-produced growth factors in human ECs [63]. Taken together, these studies establish miR-10, and perhaps miR-196b, as potential new targets for the selective modulation of angiogenesis [123].

Zhou et al. reported that miR-503 simultaneously downregulates *FGF2* and *VEGFA*. miR-503 expression is inhibited in hepatocarcinoma cells and primary tumours which may be due to an epigenetic mechanism; its overexpression reduces tumour angiogenesis *in vitro* and *in vivo*, and furthermore, its expression is downregulated by hypoxia via HIF1α, thus indicating an antiangiogenesis role in tumorigenesis [130]. Finally, other studies have indicated that miRNAs may function as tumour suppressors by targeting p70S6K1. Two independent studies, the first with miR-128 in glioma [131] and the second with miR-145 in colon cancer tissues [132], demonstrated that decreased p70S6K1 expression, mediated by these miRNAs, inhibits cell proliferation, tumour growth, and angiogenesis which is thought to be because HIF-1α and VEGF are both downstream to this molecule.

In conclusion, the key angiogenic factor VEGF appears to be regulated by several miRNAs including miR-191, miR-126, miR-155, miR-31, the miR-17-92 cluster, miR-10, miR196, and miR-1/206 (summarized in Figure 2 and Table 1); however, exhaustive studies on the implication of these miRNAs in therapeutic treatments are needed before these findings can be added to existing therapeutic anti-VEGF drugs.

4.5. miRNAs That Affect Other Pathways Implicated in Angiogenesis. Other angiogenesis-modulating miRNAs that do not affect any of the previously described targets include miR-132, miR-26a, and miR-130a (Figure 2 and Table 1), the latter of which inhibits the expression of two antiangiogenic genes: *GAX* (growth arrest homeobox) and *HOXA5* (homeobox A5) [133] and is produced in increased amounts by hECs (human embryonic carcinoma cells) in culture. Similarly, miR-132 is also highly upregulated in a human vasculogenesis model as well as in human tumour endothelium [134]. Its ectopic expression *in vitro* enhances EC proliferation and tubulogenesis. MiR-132 expression in hECs repressed p120RasGAP (its predicted target) increasing RAS activity and thus promoting angiogenesis, which could explain why p120RasGAP is expressed in normal but not in tumour endothelium [134]. Further, supporting this, the same group showed that the addition of anti-miR-132 inhibited angiogenesis in wild-type mice but not in mice with an inducible *Rasa1* (encoding p120RasGAP) deletion; in another experiment, targeted delivery of anti-miR-132 nanoparticles to the vessels restored p120RasGAP expression in the tumour endothelium, suppressing angiogenesis and decreasing the tumour burden in an orthotopic xenograft mouse model of human breast carcinoma. It is therefore thought that miR-132 acts as an angiogenic switch by suppressing endothelial p120RasGAP expression, resulting in Ras activation and induction of neovascularization which is counteracted by anti-miR-132 [134]. Taken together, these observations indicate that miR-132 may play an important role in pathological neovascularization downstream of multiple triggers, including tumour-derived growth factors, viral infections, and inflammation.

Recent studies in human HCCs (hepatocellular carcinoma cells) have demonstrated that miR-26a is implicated in tumour angiogenesis [135, 136]. Ectopic expression of miR-26a reduces VEGFA levels in HepG2 (human hepatocellular liver carcinoma cell line) cells. Furthermore, *in silico* analysis indicates that *PIK3C2α* is a downstream miR-26a target gene, and inhibition studies suggest that miR-26a decreases VEGFA expression in HCCs via the PI3K/AKT/HIF-1α/VEGFA pathway. Finally, VEGFA levels inversely correlate with miR-26a levels in HCC tumours [135], and there is also a correlation between miR-26a downregulation and increased angiogenic potential in HCCs [136]. In addition, HGF (hepatocyte growth factor) has been identified as a miR-26a target, demonstrating its antiangiogenic function which is at least partially mediated by inhibiting cMet (HGF-hepatocyte growth factor receptor) and its downstream signalling pathway, thus reducing VEGFA expression in HCCs and decreasing VEGFR-2 signaling in ECs [136].

5. "Angiogenic" miRNAs in the Era of Personalised Medicine

There are different therapeutic strategies for inhibiting miRNAs *in vivo* that are currently being evaluated in preclinical models (reviewed in [137]). These strategies include the following.

Antagomirs. They are a class of chemically engineered oligonucleotides which are able to silence endogenous miRNAs. They are specifically designed, chemically modified, cholesterol-conjugated single-stranded RNA analogues which are complementary to miRNA targets [138–140].

Locked Nucleic Acid- (LNA-) AntimiRs. They are antisense RNA oligonucleotides in which the ribose moiety of an LNA nucleotide is modified to increase stability and specificity. LNA nucleotides can be mixed with DNA or RNA residues in the oligonucleotide depending on the user's requirements [141].

MiR Sponge. This is miRNA-inhibiting transgene which expresses an mRNA which contains multiple tandem binding sites for an endogenous miRNA which is thus able to stably interact with the corresponding miRNA and prevent its association with its endogenous targets [142].

miR-Mask. This is a single-stranded 2′-O-methyl-modified antisense oligonucleotide which is fully complementary to the predicted miRNA binding sites in the 3′-UTRs of target mRNAs. The miR-mask is therefore able to obscure the access of the miRNA to its binding sites on the target mRNA and so impairs its inhibitory function [143].

Alternatively, there are strategies intended to restore miRNA levels, such as miRNA mimics. It is based on the use of double-stranded synthetic oligonucleotides that mimic endogenous miRNAs and are processed into the cell when they are transfected [144]. The expression vectors of these miRNAs are constructed with promoters that can enable the expression of certain miRNAs in a tissue-/tumour-specific

manner [145]. For instance, a liposome-formulated mimic of the tumour suppressor miR-34 named MRX34 (developed by Mirna Therapeutics) produced a complete tumour regression in orthotopic mouse models of hepatocellular carcinoma [146]. These results prompted the development of an ongoing phase I multicentre clinical trial (ClinicalTrials.gov identifier:NCT01829971) to evaluate the safety of MRX34 in patients with primary liver cancer or those with liver metastasis from other cancers.

In summary, although the pharmacological manipulation of miRNAs is still in its initial steps, there are scientific evidences demonstrating the potent modulatory impact of certain miRNAs on the angiogenic response.

6. Conclusions

miRNAs are implicated in most, if not all, signalling pathways and in many human diseases, including cancer. This review summarizes the role of miRNAs in the control of different pathways related to angiogenesis and, in particular, with the tumour neovascularization. To date there are groups of well characterized miRNAs implicated in regulating EC function and angiogenesis, making them attractive objectives in tumour angiogenesis. Hopefully, in a few years targeted use of specific miRNAs against angiogenic pathways in cancer will become a reality, allowing combining these with other types of antitumour strategies.

Conflict of Interests

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

Acknowledgments

This study was partially supported by a Grant from Ministerio de Ciencia e Inovación de España (TRA09-0132), Beca Roche en Onco-Hematología 2009, and Red Temática de Investigación Cooperativa en Cáncer (RD12/0036/0025).

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Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 592868, 15 pages
http://dx.doi.org/10.1155/2014/592868

Review Article

MicroRNAs in Soft Tissue Sarcomas: Overview of the Accumulating Evidence and Importance as Novel Biomarkers

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Received 6 June 2014; Accepted 9 July 2014; Published 4 August 2014

Academic Editor: Paolo Gandellini

Sarcomas are distinctly heterogeneous tumors and a variety of subtypes have been described. Although several diagnostic explorations in the past three decades, such as identification of chromosomal translocation, have greatly improved the diagnosis of soft tissue sarcomas, the unsolved issues, including the limited useful biomarkers, remain. Emerging reports on miRNAs in soft tissue sarcomas have provided clues to solving these problems. Evidence of circulating miRNAs in patients with soft tissue sarcomas and healthy individuals has been accumulated and is accelerating their potential to develop into clinical applications. Moreover, miRNAs that function as novel prognostic factors have been identified, thereby facilitating their use in miRNA-targeted therapy. In this review, we provide an overview of the current knowledge on miRNA deregulation in soft tissue sarcomas, and discuss their potential as novel biomarkers and therapeutics.

1. Introduction

Sarcomas are malignant tumors of mesenchymal origin. Mesenchymal tissue is defined as a complex of nonepithelial structures of the body, which exclusively comprise the reproductive, glia, hematopoietic, and lymphoid tissues. The word "sarcoma" is derived from the Greek word sarkoma, meaning "fleshy outgrowth," and can present as either a bone or soft tissue sarcomas [1]. Since the origin of soft tissue sarcomas has not been clarified, the classification system commonly used is based on histopathology. The world health organization (WHO) system is generally accepted as the basis for soft tissue tumor classification. According to the study based on the Surveillance, Epidemiology, and End Results (SEER), which included 26,758 cases from 1978 to 2001, leiomyosarcoma (LMS) was the most common form of sarcoma, accounting for 23% of all cases. Additional major histological types included in this study were malignant fibrous histiocytoma (MFH; 17%), liposarcoma (11%), dermatofibrosarcoma (10%), and rhabdomyosarcoma (RMS; 4%) [2]. Another report showed that MFH and LS are the most common types of soft tissue sarcomas in adults, accounting for 35%–45% of all sarcomas [3]. Notably, it is accepted that MHF does not show true histiocytic differentiation and its morphological pattern is shared by a variety of poorly differentiated malignancies. Accordingly, the diagnostic term MFH has been removed

from WHO classification, and such lesions, without using the outdated terminology, are now included in the new category of undifferentiated/unclassified sarcomas.

Treatment options for most patients with sarcomas include surgical resection and adjuvant chemo- and radiotherapy. Despite the development of combined modality treatments in recent years, a significant proportion of patients with sarcomas respond poorly to chemotherapy, leading to local recurrence or distant metastasis. Lung metastasis is the main cause of death among patients with soft tissue sarcomas [4, 5]. Thus, early detection of recurrent or metastatic disease or early decision making according to tumor response to chemotherapy could improve patient prognosis. However, there are no useful biomarkers for these purposes. Indeed, only imaging methods are mostly used to detect or monitor tumor development. Thus, the discovery of novel biomarkers to detect tumors, predict their drug sensitivity, and monitor them is one of the most important challenges that must be overcome.

There is a growing amount of evidence in favor of utilizing miRNA profiling in the diagnosis of soft tissue sarcomas. Despite their small size (~22 nucleotides), these endogenous noncoding RNAs have an enormous effect on gene expression and regulate a variety of physiological and pathological processes [6–8]. Over the past several years, it has become evident that dysregulation of many types of miRNAs has been associated with the initiation and progression of human cancers [9]. A number of many studies have indicated that miRNAs can act as either oncogenes or tumor suppressors. The recent discovery of miRNAs as novel biomarkers in human serum or plasma has represented a new approach for the diagnostic screening for malignant diseases [8]. In addition, some successful *in vivo* studies support the concept that they may be used as innovative therapeutics to address unmet needs, although they are not presently used as cancer therapeutics [7].

In this review, we overview the accumulating evidence of miRNAs in soft tissue sarcomas, highlighting their function in each histological type of soft tissue sarcoma and their clinical relevance. Further, we update the clinical trials on the basis of miRNA profiling using patient blood samples as well as addressing the potential of miRNAs as novel biomarkers and therapeutics for soft tissue sarcomas.

2. Aberrant miRNA Expression in Soft Tissue Sarcomas (Table 1)

2.1. Liposarcoma. Liposarcoma is one of the most common soft tissue sarcomas in adults and can be subdivided into the following four major types: atypical lipomatous tumor/well-differentiated liposarcoma (WDLS), myxoid liposarcoma (MLS), pleomorphic liposarcoma (PLS), and dedifferentiated liposarcoma (DDLS). DDLS is defined as a WDLS that shows an abrupt transition to a nonlipogenic sarcoma. In addition to distinctive morphologies, each of the subgroups has a different prognosis and treatment strategy. MLS is relatively chemosensitive in comparison to the other types [10]. Although the prognosis of WDLS is good, that of DDLS is much worse, with a survival rate of approximately 28%–30% at the 5-year follow-up [11].

Most reports on miRNA profiling of liposarcoma have been specific to DDLS. Based on deep sequencing of small RNA libraries and hybridization-based microarrays, Ugras et al. identified more than 40 miRNAs that were dysregulated in DDLS and not in normal adipose tissue and WDLS. The upregulated miRNAs included miR-21 and -26, while the downregulated miRNAs included miR-143 and -145 [12]. Furthermore, reexpression of miR-143 in DDLS cell lines inhibited cell proliferation and induced apoptosis through downregulation of BCL2, topoisomerase 2A, protein regulator of cytokinesis 1 (PRC1), and polo-like kinase 1 (PLK1) [12]. A similar approach was adopted by Zhang et al., who performed miRNA profiling to compare WDLS/DDLS and normal adipose tissue. They determined that miR-155 was upregulated in DDLS, and silencing of miR-155 in DDLS cells inhibited cell growth and colony formation, induced G1-S cell-cycle arrest *in vitro*, and blocked tumor growth *in vivo* [13]. Further, they determined that miR-155 directly targeted casein kinase 1α, which enhanced β-catenin signaling [13]. Renner et al. identified miR-218-1* and HS_303_a as being upregulated miRNAs and miR-144 and -1238 as being downregulated miRNAs relative to that in normal adipose tissues [14]. Using unbiased genome-wide methylation sequencing, Taylor et al. identified that miR-193b was downregulated in DDLS relative to normal adipose tissue and WDLS, whose putative miR-193b promoters were differentially methylated [15]. A DDLS study by Hisaoka et al. focused on calreticulin (*CALR*), an inhibitor of adipocyte differentiation, and identified decreased expression of miR-1257, which targets *CALR* [16].

MLS has a unique genomic abnormality characterized by t(12; 16)(q13; p11) translocation, which creates the TLS-CHOP chimeric oncoprotein. Borjigin et al. investigated the molecular functions of TLS-CHOP and revealed that miR-486 was downregulated in both TLS-CHOP-expressing fibroblasts and MLS [17]. Since plasminogen activator inhibitor-1 (*PAI-1*) was identified as a target of miR-486, TLS-CHOP-miR-486-PAI-1 might be critical for MLS tumorigenesis and development [17]. In the miRNA profiling of MLS relative to normal adipose tissue, Renner et al. determined that miR-9, -891a, and -888 were upregulated and miR-486-3p and -1290 were downregulated. Interestingly, this was consistent with the report by Borjigin et al., who also reported on dysregulated miRNAs in PLS relative to normal adipose tissue and demonstrated that miR-1249, -296-5p, and -455-5p were upregulated and miR-200b*, -200, and -139-3p were downregulated [14].

Recently published papers have demonstrated a clinical correlation with miRNA dysregulation and liposarcoma. In a single SNP array of 75 liposarcoma samples, Lee et al. identified frequent amplification of miR-26a-2c [18]. This miRNA was upregulated in not only WDLS/DDLS but also MLS. Importantly, high miR-26a-2 expression significantly correlated with poor patient survival in both types of liposarcoma, regardless of histological subtypes. An additional study revealed that the regulator of chromosome condensation and

Table 1: Deregulated miRNAs in soft tissue sarcomas.

Histology	miRNAs	Expression level	Function	miRNA target	Reference
Liposarcoma	miR-21, -26a (DDLS)	Increased	N/D	N/D	[12]
	miR-143, -145 (DDLS)	Decreased	Cell proliferation, apoptosis	*BCL2, Topoisomerase 2A, PRC1, and PLK1*	[12]
	miR-155 (DDLS)	Increased	Cell proliferation, colony formation, and tumor growth	*CK1α*	[13]
	miR-218-1* (DDLS)	Increased	N/D	N/D	[14]
	miR-144, -1238 (DDLS)	Decreased	N/D	N/D	[14]
	miR-193b (DDLS)	Decreased	N/D (methylated)	N/D	[15]
	miR-1257 (DDLS)	Decreased	N/D	*CALR*	[16]
	miR-486 (MLS)	Decreased	Cell proliferation	*PAI-1*	[17]
	miR-486-3p, -1290 (MLS)	Decreased	N/D	N/D	[14]
	miR-9, -891a, and -888 (MLS)	Increased	N/D	N/D	[14]
	miR-1249, -296-5p, and -455-5p (PLS)	Increased	N/D	N/D	[14]
	miR-200b*, -200, and -139-3p (PLS)	Decreased	N/D	N/D	[14]
	miR-26a-2 (DDLS, MLS)	Increased	Clonogenicity, adipocyte differentiation, and cell apoptosis	*RCBTB1*	[18]
Rhabdomyosarcoma	miR-1, -133a/b	Decreased	Myogenic differentiation, cell proliferation	*SRF, Cyclin D2*	[23, 25]
	miR-206	Decreased	Myogenic differentiation, cell growth, cell migration, tumor growth, and correlation with prognosis	*c-Met, PAX3, PAX7, CCDN2, HDAC4, and BAF53a*	[23, 25–30]
	miR-26a	Decreased	N/D	*Ezh2*	[31]
	miR-203	Decreased	Myogenic differentiation, cell proliferation, cell migration, and tumor growth	*p63, LIFR*	[32]
	miR-335 (ARMS)	Increased	N/D	*CHFR, HAND1, SP1*	[24]
	miR-29	Decreased	Cell cycle arrest, muscle differentiation, tumor growth	*YY1*	[28, 32]
	miR-183	Increased	Cell migration, and cell invasion	*EGR1, PTEN*	[37]
	miR-9*	Increased	Cell migration	*E-cadherin*	[38]
	miR-200c	Decreased	Cell migration	N/D	[38]
	miR-17-92 cluster (ARMS)	Increased	Correlation with prognosis in 13q31 amplified ARMS	N/D	[39]
	miR-485-3p	N/D	Drug resistance	*NF-YB*	[40]
Leiomyosarcoma	miR-1, -133a, and -133b	Increased	N/D	N/D	[24]
	miR-17-92 cluster (uterine LMS)	Increased	Smooth muscle differentiation	N/D	[42]
	let-7 (uterine LMS)	N/D	Cell proliferation	*HMGA2*	[43]
	miR-221 (uterine LMS)	Increased	N/D	N/D	[44]
	miR-320a	Increased	N/D	N/D	[45]
	miR-133a, -1, and -449a	Increased	N/D	N/D	[14]
	miR-483-5p, -656, and -323-3p	Decreased	N/D	N/D	[14]
Synovial sarcoma	miR-143	Decreased	N/D	*SSX1*	[24]
	miR-183	Increased	Cell migration, cell invasion	*EGR1*	[37]
	let-7e, miR-99b, miR-125a-3p	Increased	Cell proliferation	*HMGA2, SMARCA5*	[48]
	miR-200b*, -183, and -375	Increased	N/D	N/D	[14]
	miR-34b*, -142-5p, and -34c-3p	Decreased	N/D	N/D	[14]

Table 1: Continued.

Histology	miRNAs	Expression level	Function	miRNA target	Reference
MPNST	miR-34a	Decreased	Apoptosis	*MYCN, E2F2, and CDK4*	[49]
	miR-10b	Increased	Cell proliferation, migration, and invasion	*NF1*	[53]
	miR-21	Increased	Apoptosis	*PDCD4*	[50]
	miR-204	Increased	Cell proliferation, migration, and invasion	*HMGA2*	[52]
	miR-29c	Decreased	Cell invasion	*MMP2*	[51]
	miR-210, -339-5p	Increased	N/D	N/D	[51]
	miR-30d	Decreased	Apoptosis	*KPNB1*	[54]
Angiosarcoma	miR-520c-3p, -519a, and -520h	Increased	N/D	N/D	[55]
	miR-17-92 cluster (*myc*-amplified AS)	Increased	N/D	*THBS1*	[56]
Fibrosarcoma	miR-520c, -373	N/D	Cell growth, cell migration	*mTOR, SIRT1*	[58]
	miR-409-3p	N/D	Cell proliferation, tumor growth, vascularization, and metastasis	*ANG*	[59]
UPS	miR-126, -223, -451, and -1274b	Increased	N/D	N/D	[45]
	miR-100, -886-3p, -1260, -1274a, and -1274b	Decreased	N/D	*IMP3*	[45]
Epithelioid sarcoma	miR-206, -381, and -671-5p	Increased	N/D	*SMARCB1 (INI1)*	[69]
Kaposi's sarcoma	miR-155, -K12-11	N/D	N/D	*BACH-1, FOS, and LDOC-1*	[71, 76, 77]
	miR-155, -220/221, let-7	Decreased	Transition to tumorigenic endothelial cells	N/D	[79]
	miR-221/-222	Decreased	Cell migration	*ETS1, ETS2*	[80]
	miR-31	Increased	Cell migration	*FAT4*	[80]
	miR-15, 140	Increased	Transition to tumorigenic endothelial cells	N/D	[81]
	miR-24-2	Increased	N/D	N/D	[81]
Soft tissue sarcomas	miR-210	N/D	Correlates with age of tumor onset (male) and prognosis (female)	N/D	[82]

DDLS: dedifferentiated liposarcoma; MLS: myxoid liposarcoma; PLS: pleomorphic liposarcoma; LMS: leiomyosarcoma; ARMS: alveolar rhabdomyosarcoma; AS: angiosarcoma; MPNST: malignant peripheral nerve sheath tumor; UPS: undifferentiated pleomorphic sarcoma; N/D: no data.

BTB domain-containing protein 1 (*RCBTB1*) was one of the targets of miR-26a-2, which regulates cellular apoptosis [18].

2.2. Rhabdomyosarcoma. RMS is not only the most common soft tissue sarcoma in children under 15 years of age (representing 5%–8% of all pediatric malignancies) but also one of the most common soft tissue sarcomas in adolescents and young adults [19]. Histopathologically, RMS is classified into the following four subtypes: embryonal RMS (ERMS), alveolar RMS (ARMS), pleomorphic RMS (PRMS), and spindle cell/sclerosing RMS. Most patients with RMS are treated with chemotherapy, and depending on the size and location of the primary tumor, most will also undergo either radiotherapy or surgery. Adult patients who showed complete response to chemotherapy had a 5-year survival rate of 57% compared to only 7% for poor responders [20].

Since RMS has been predicted to originate from mesenchymal progenitor cells located in muscle tissue, most studies have focused on miRNAs that are involved in skeletal muscle development ("muscle-specific miRNAs") [21–23]. Global miRNA expression analysis was performed by Subramanian et al., which revealed that muscle-specific miRNAs (miR-1 and -133) were relatively downregulated in PRMS relative to normal skeletal muscle, and miR-335 was upregulated in ARMS relative to normal skeletal muscle [24]. miR-335 resides in intron 2 of *MEST*, which has been indicated to play a role in muscle differentiation. Furthermore, it shows high mRNA expression in ARMS. Notably, *MEST* is a downstream target of *PAX3*, the gene involved in the *PAX3-FKHR* fusion that is typical for ARMS. Rao et al. determined that miR-1 and -133a were drastically reduced in ERMS and ARMS cell lines [25]. Although these miRNAs affected cytostasis and differentiation in ERMS cells, this was not true for ARMS cells. Taulli et al. and Yan et al. examined the role of the muscle-specific miR-1 and -206 in RMS [26, 27]. They showed that their reexpression in RMS cells targeted *c-Met* mRNA to promote myogenic differentiation, decreased cell growth and migration, and inhibited tumor growth in xenografted mice. Furthermore, Li et al. reported on additional important targets. They showed that miR-1, -206, and -29 could regulate *PAX3* and *CCND2* expression [28]. Recently, Taulli et al. further pursued miR-206 targets. They focused on the BAF53a subunit of the SWI/SNF chromatin remodeling complex, which is an important molecule during myogenic differentiation. Indeed, the BAF53a transcript was present at significantly higher levels in primary RMS tumors compared with normal muscle. Silencing of BAF53a in RMS cells inhibited cell proliferation and anchorage-independent growth *in vitro*, inhibited ERMS and ARMS tumor growth, and induced myogenic differentiation *in vivo*, therefore, leading to the conclusion that failure to downregulate the BAF53a subunit may contribute to RMS pathogenesis [29].

Importantly, Missiaglia et al. demonstrated the clinical relevance of these muscle-specific miRNAs by using RT-PCR to investigate miR-1, -206, -133a, and -133b expression in 163 primary RMS samples [30]. The Kaplan-Meier curves showed a correlation between overall survival and miR-206 expression, whereas no correlation was observed with miR-1 or -133a/b. In particular, low miR-206 expression correlated with poor overall survival and was an independent predictor of shorter survival times in metastatic ERMS and ARMS cases without *PAX3/7-FOXO1* fusion genes [30]. Among the muscle-specific miRNAs, Ciarapica et al. found that miR-26a was also downregulated in RMS cells [31]. They further revealed that it may have a role in RMS pathogenesis via regulation of the expression of *Ezh2*, which regulates embryonic development through inhibition of homeobox gene expression [31]. miR-203 was also found to be downregulated in RMS by Diao et al. This occurred due to promoter hypermethylation and could be reexpressed by DNA-demethylating agents [32]. Reexpression of miR-203 suppressed tumor growth by directly targeting *p63* and *LIFR*, which lead to the inhibition of both the Notch and JAK1/STAT1/STAT3 pathways and promotion of myogenic differentiation [32].

Nonmuscle-specific miRNAs also have been reported as key molecules that function in RMS. Subramanian et al. showed that miR-29 was downregulated in RMS and acted as a tumor suppressor [24, 28, 33]. In the reports from Wang et al., NF-κB and YY1 downregulation caused derepression of miR-29 during myogenesis, whereas, in RMS, miR-29 was epigenetically silenced by an activated NF-κB-YY1 pathway. Reexpression of miR-29 in RMS inhibited tumor growth *in vivo* [33]. It has also been proposed that miR-29 can silence HDAC4 [34] or affect the Rybp epigenetic modifier [35], further promoting myogenic differentiation [21]. To date, HDAC inhibitors are promising agents for targeted therapy for metastatic RMS [36]. Sarver et al. reported that *EGR1* is regulated by miR-183 in multiple tumor types in addition to RMS, including synovial sarcoma and colon cancer [37]. Silencing of miR-183 in RMS cells revealed deregulation of a miRNA network composed of miR-183-EGR1-PTEN [37]. Armeanu-Ebinger et al. analyzed miRNA expression in ARMS and malignant rhabdoid tumor (MRT) in tissue samples and cell lines to identify their specific miRNA expression patterns. As a result, miR-9* was shown to be overexpressed in ARMS, whereas miR-200c was expressed at lower levels in ARMS than MRT [38]. Another important study on ARMS was reported by Reichek et al. They investigated the 13q31 amplicon that contains the miR-17-92 cluster gene and observed its significant overexpression in tumors with the 13q31 amplicon [39]. This was present in 23% of ARMS cases, especially in *PAX7-FKHR*-positive cases compared to *PAX3-FKHR*-positive and fusion-negative cases. Notably, high expression of the miR-17-91 cluster significantly correlated with poor prognosis in the 13q31-amplified group of patients, most of whom represented *PAX7-FKHR*-positive cases [39].

miRNA that is associated with drug resistant RMS has been reported. Chen et al. demonstrated that miR-485-3p was expressed at lower levels in drug-resistant lymphoblastic leukemia cells than in parental cells [40]. Facilitated by its promoter, miR-485-3p targets NF-YB, which may be a mediator of topoisomerase 2α [40]. They replicated these results in drug-sensitive and -resistant RMS cells and found that the miR-485-3p-Top2α-NF-YB pathway represented a general phenomenon associated with drug sensitivity.

2.3. Leiomyosarcoma. LMS is a malignant tumor showing smooth muscle differentiation. Soft tissue LMS usually occurs in middle-aged or older individuals, although it may develop in young adults and even in children [11]. It originates in retroperitoneal lesions (40%–45%), extremities (30%–35%), skin (15%–20%), and larger blood vessels (5%). Surgical resection is the most reliable treatment. Although the effectiveness of chemo- and radiotherapy is uncertain, a clear survival benefit of chemo- or radiotherapy is evident if surgical margins are not clear of tumor cells. For patients with LMS in the extremities, the reported local recurrence rate is 10%–25%, whereas the 5-year survival rate is 64% [41].

Accumulated studies on miRNA profiling of LMS have focused on those originating from the extremities and uterus. All studies have demonstrated upregulation of miRNAs in LMS relative to its benign counterparts such as leiomyoma or other soft tissue sarcomas. Subramanian et al. demonstrated that miR-1, -133a, and -133b, which play major roles in myogenesis and myoblast proliferation, are significantly overexpressed in LMS relative to normal smooth muscle [24]. Interestingly, miR-206, a miRNA that is highly expressed in normal skeletal muscle, was underexpressed in both LMS and normal smooth muscle [24]. Danielson et al. investigated miRNA profiling of uterine LMS and reported that the miR-17-92 cluster was overexpressed compared with myometrium [42]. Shi et al. focused on the overexpression of HMGA2 in uterine LMS and found that it is caused by let-7 repression [43]. Similarly, Nuovo et al. performed *in situ* hybridization and found that miR-221 was upregulated in uterine LMS but was not detected in leiomyomas or benign metastasizing leiomyomas [44]. Two recent reports have demonstrated miRNA dysregulation compared to the other sarcomas. Guled et al. profiled 10 high-grade LMS and 10 high-grade UPS samples with miRNA microarray and identified that miR-320a was upregulated in LMS relative to UPS [45]. In the examination of differentially expressed miRNAs in LMS compared to the other sarcoma subtypes, Renner et al. reported that miR-133a, -1, and -449a were upregulated, while miR-483-5p, -656, and -323-3p were downregulated [14]. These results were partly consistent with those of Subramanian et al. [24].

2.4. Synovial Sarcoma. Synovial sarcoma accounts for up to 10% of soft tissue sarcomas and includes two major histological subtypes, biphasic and monophasic [46]. They can occur anywhere in the body and feature local invasiveness and a propensity to metastasize [47]. Synovial sarcoma has a specific chromosomal translocation t(X; 18)(p11; q11) that leads to formation of an *SS18-SSX* fusion gene. Although treatment is based on surgery, adjuvant radio- or chemotherapy may be beneficial, particularly in high-risk patients. The 5-year overall survival is 55% for axial synovial sarcoma and 84% for extremity synovial sarcoma [47].

In the first report on miRNA profiling performed by Subramanian et al. in 2008, they utilized microarray, cloning, and northern blot analysis to demonstrate that miR-143 was downregulated in synovial sarcoma relative to GIST and LMS [24]. Since *SSX1* is predicted to be a target for miR-143 in *in silico* databases such as miRBase or TargetScan, it is speculated that its decreased expression in synovial sarcoma enables the production of the SS18-SSX1 oncoprotein. Sarver et al. focused on the molecular feature of synovial sarcoma that the SS18-SSX fusion protein represses *EGR1* expression through a direct association with the *EGR1* promoter. They investigated the correlation between *EGR1* and miR-183, which is significantly overexpressed in synovial sarcoma [37]. These studies found that miR-183 could target *EGR1* mRNA, which contributed to cell migration and invasion in synovial sarcoma cells. Through the functional analysis of many tumor cell lines, miR-183 was found to have an oncogenic role through the miR-183-EGR1-PTEN pathway in synovial sarcoma, RMS, and colon cancer [37]. Interestingly, Renner et al. also indicated that miR-183 is upregulated in synovial sarcoma relative to other sarcomas. Additional upregulated miRNAs demonstrating a >10-fold change were miR-200b* and -375, while the downregulated miRNAs showing >5.5-fold change included miR-34b*, -142-5p, and -34c-3p [14]. Hisaoka et al. examined the global miRNA expression in synovial sarcoma and compared the results to Ewing sarcoma and normal skeletal muscle. Unsupervised hierarchical clustering revealed 21 significantly upregulated miRNAs, including let-7e, miR-99b, and -125-3p [48]. Functional analysis based on the silencing of let-7e and miR-99b resulted in the suppression of cell proliferation and the expression of *HMGA2* and *SMARCA5*, the putative targets of these miRNAs [48].

2.5. Malignant Peripheral Nerve Sheath Tumor. Malignant peripheral nerve sheath tumor (MPNST) typically originates from cells constituting the nerve sheath, such as Schwann and perineural cells. Approximately 50% of MPNSTs occur sporadically, with the remaining originating in patients with neurofibromatosis type 1 (NF1) [11]. Patients with NF1 have high risk of developing MPNSTs, and most are aggressive tumors with a poor prognosis.

Many reports have investigated the global miRNA profiling of MPNSTs in comparison with benign counterparts such as neurofibromas. Subramanian et al. determined the gene expression signature for benign and malignant peripheral nerve sheath tumors, which indicated that *p53* inactivation occurs in majority of MPNSTs [49]. They also performed miRNA profiling of these tumor sets and found a relative downregulation of miR-34a expression in most MPNSTs, concluding that *p53* inactivation and the subsequent loss of miR-34a expression may significantly contribute to MPNST development [49]. Itani et al. utilized a similar approach and identified the overexpression of miR-21 in MPNSTs compared to neurofibromas. *In silico* research predicted programmed cell death protein 4 (*PDCD4*) as a putative target of miR-21 [50]. Functional analysis using an MPNST cell line indicated that silencing of miR-21 could induce apoptosis of MPNST cells [50]. Presneau et al. also compared miRNA profiling between MPNSTs and NFs and identified 14 downregulated and 2 upregulated miRNAs. The former included miR-29c, -30c, -139-5p, 195, -151-5p, 342-5p, 146a, -150, and -223, and the

latter included miR-210 and -339-5p [51]. Among them, miR-29c mimics reduced cell invasion of MPNST cells, regulating the expression of its target, *MMP2* [51]. Gong et al. identified the downregulated expression of miR-204 in MPNSTs in the same approach and reported *Ras* and *HMGA2* as the target molecules in MPNSTs [52]. Chai et al. utilized a different approach and found that miR-10b was upregulated in primary Schwann cells isolated from NF1 neurofibromas, and in cell lines and tumor tissues from MPNSTs [53]. Importantly, they showed that NF1 mRNA was the target for miR-10b. Zhang et al. focused on the expression of polycomb group protein enhancer of zeste homologue 2 (*Ezh2*), an important regulator for various human malignancies, and identified that it was significantly upregulated in MPNSTs [54]. Ezh2 inhibited miR-30d expression by binding to its promoter and an *in silico* database identified *KPNB1* as a miR-30d target. They concluded that EZH2-miR-30d-KPNB1 signaling was critical for MPNST survival and tumorigenicity [54].

2.6. Angiosarcoma. Angiosarcoma is a malignant tumor that recapitulates the morphological and functional characteristics of normal endothelium [11]. It accounts for less than 1% of all sarcomas and originates most commonly in the deep muscles of the lower extremities [3]. They are aggressive malignancies with a high rate of tumor-related death and more than half of all patients die within the first year [11].

In the web-accessible Sarcoma miRNA Expression Database (S-MED) generated by Sarver et al. [55], miRNAs that are significantly unregulated (>80-fold change) in angiosarcoma compared to other sarcomas included miR-520c-3p, -519a, and -520h (http://www.oncomir.umn.edu/). However, they have not been analyzed for their function in any cell lines. On the other hand, Italiano et al. investigated miRNA profiling based on *MYC* abnormalities in angiosarcoma. MYC amplification was identified in 3 out of 6 primary angiosarcomas and in 8 out of 12 secondary angiosarcomas by array-comparative genomic hybridization (aCGH) and FISH analysis. By comparing the miRNA profile of *MYC*-amplified and *MYC*-unamplified angiosarcomas using deep sequencing of small RNA libraries, they identified that the miR-17-92 cluster is preferentially overexpressed in *MYC*-amplified angiosarcoma. Since *MYC*-amplified angiosarcoma is associated with lower expression of thrombospondin-1 (*THBS1*), *MYC* amplification may be important in the angiogenic phenotype of angiosarcoma through upregulation of the miR-17-92 cluster, which downregulates *THBS1* expression [56].

2.7. Fibrosarcoma. Soft tissue fibrosarcoma is classified into infantile fibrosarcoma and adult fibrosarcoma. The infantile fibrosarcoma is histologically similar to classic adult fibrosarcoma but has a distinctive *ETV6-NTRK3* gene fusion and a favorable outcome. In contrast, >80% of adult fibrosarcoma cases were reported to be high-grade in the recent series of strictly defined cases [57].

To date, miRNA profiling has been limited to the fibrosarcoma cell line, HT1080. The first report came from Liu and Wilson, who investigated the correlation between matrix metalloproteinases (MMPs) and miR-520c and -373, which had been reported to play important roles in cancer cell metastasis as oncogenes [58]. Their data demonstrated that miR-520c and -373 suppressed the translation of *mTOR* and *SIRT1* by directly targeting the 3′-untranslated region (UTR). Since *mTOR* and *SIRT1* are negative regulators of *MMP9* via inactivation of the Ras/Raf/MEK/Erk signaling pathway and NF-κB activity, these miRNAs were found to increase MMP9 expression by directly targeting *mTOR* and *SIRT1* and stimulating cell growth and migration [58]. Another investigation using HT1080 cells was reported by Weng et al., who focused on the regulatory mechanism of angiogenin (ANG) expression. In their *in silico* analysis, they found that *ANG* mRNA was targeted by miR-409-3p via its 3′UTR and overexpression of miR-409-3p in HT1080 cells silenced *ANG* expression [59]. Furthermore, their *in vitro* and *in vivo* analyses demonstrated that miR-409-3p inhibited tumor growth, vascularization, and metastasis via silencing *ANG* expression [59].

2.8. Undifferentiated Pleomorphic Sarcoma. In 2002, WHO declassified MFH as a formal diagnostic entity and renamed it as an undifferentiated pleomorphic sarcoma (UPS) not otherwise specified (NOS) [60]. In 2013, UPS/MFH was categorized in the undifferentiated/unclassified sarcomas [61]. Undifferentiated/unclassified sarcomas account for up to 20% of all sarcomas and have no clinical or morphological characteristics that would otherwise place them under specific types of sarcomas. Genetic subgroups are emerging within this entity.

Guled et al. conducted miRNA profiling on a series of LMS and UPS samples to identify specific signatures useful for differential diagnosis. They profiled 10 LMS and 10 UPS samples, using two cultured human mesenchymal stem cell samples as controls. As a result, 38 human miRNAs were determined to be significantly differentially expressed in UPS compared to control samples [45]. In UPS samples, miR-126, -223, -451, and -1274b were significantly upregulated (>2-fold change) and miR-100, -886-3p, -1260, -1274a, and -1274b were significantly downregulated (>3-fold change) compared to control samples [45]. When comparing the profiles of LMS and UPS, miR-199-5p was highly expressed in UPS, while miR-320a was highly expressed in LMS [45]. They also revealed that several genes, including IMP3, ROR2, MDM2, CDK4, and UPA, were targets of differentially expressed miRNAs and validated their expression in both sarcomas by immunohistochemistry.

2.9. Epithelioid Sarcoma. Epithelioid sarcoma represents between 0.6% and 1.0% of sarcomas and is most prevalent in adolescents and young adults between 10 and 35 years of age [62, 63]. This tumor is the most common soft tissue sarcoma in the hand and wrist, followed by ARMS and synovial sarcoma [3]. Two clinicopathological subtypes are recognized: (1) the conventional or classic ("distal") form, characterized by its proclivity for acral sites and pseudogranulomatous growth pattern, and (2) the proximal-type ("large-cell") variant that originates mainly in proximal/truncal

regions and consists of nests and sheets of large epithelioid cells. The reported 5-year overall survival rates are 60%–80% [64–66] and the prognosis for patients with the proximal type is significantly worse than that for patients with the classic form [66–68].

Proximal-type epithelioid sarcoma has similarities with MRT, including the lack of nuclear immunoreactivity of *SMARCB1* (also known as *INI1*, *BAF47*, and *hSNF5*). Papp et al. hypothesized that miRNAs regulate *SMARCB1* expression and analyzed eight candidate miRNAs selected from *in silico* analysis. RT-PCR using tumor samples identified the overexpression of miR-206, -381, -671-5p, and -765 in epithelioid sarcomas [69]. Examination of the effect of miRNA transfections revealed that three of the overexpressed miRNAs (miR-206, miR-381, and miR- 671-5p) could silence *SMARCB1* mRNA expression in cell cultures. They concluded that the epigenetic mechanism of gene silencing by miRNAs caused the loss of *SMARCB1* expression in epithelioid sarcoma [69].

2.10. Kaposi's Sarcoma. Kaposi's sarcoma (KS) is the most common malignancy in untreated HIV-infected individuals. KS-associated herpesvirus (KSHV; also known as human herpesvirus 8) is the infectious cause of this neoplasm [70]. KSHV is a large DNA virus that encodes over 80 different proteins and is the causative agent of several diseases including not only KS but also the hyperproliferative B cell disorders, primary effusion lymphoma (PEL) and multicentric Castleman's disease [71]. Notably, recent discovery that KSHV encodes 12 miRNAs raises the possibility that these non-protein-coding gene products may contribute to viral-induced tumorigenesis [71–75].

Two groups have provided interesting evidence that KSHV-encoded miR-K-11 and miR-155 share a common set of mRNA targets (BACH-1, FOS, and LDOC-1) and binding sites; this finding implies a possible link between viral- and nonviral-mediated tumorigenesis [71, 76–78]. These are particularly interesting findings because miR-155 overexpression is associated with certain B cell lymphomas, raising the possibility that miR-K-11 expression may be one factor linking KSHV to B cell lymphoproliferative disease [78]. Other tumor-specific miRNAs have been reported by O'Hara et al. and Wu et al. O'Hara et al. profiled KS biopsies, PELs, normal tonsil tissue, and KSHV-infected and uninfected endothelial cells (ECs) because KS is a malignancy of ECs and is believed to be at the border between infection-induced hyperplasia and clonal neoplasia. As a result, multiple tumor suppressor miRNAs (miR-155, miR-220/221, and the let-7 family) are downregulated in KSHV-associated cancers, including PEL and KS [79]. Furthermore, they identified miR-143/145 as novel KS tumor-regulated miRNAs. Wu et al. also investigated a series of differentially expressed miRNAs and protein-coding genes associated with Kaposi's sarcomagenesis or KSHV infection. They found that the miR-221/222 cluster was downregulated, while miR-31 was upregulated in KS. Analysis of the putative miRNA targets revealed that ETS1 and ETS2 were downstream targets of miR-221/222, while FAT4 was one of the direct targets of miR-31 [80]. These molecules were involved in manipulating cell migration and motility. O'Hara et al. further analyzed pre-miRNA profiling of KS biopsies with well-established culture and mouse tumor models. As a result, increased miR-15 expression and decreased miR-221 demarked the malignant transition of endothelial cells, whereas increased miR-140 determined the degree of the transformation [81]. Interestingly, miR-24-2 pre-miRNA levels were strikingly elevated only in KS biopsies, thus, serving as a KS-specific biomarker [81].

2.11. Others. Greither et al. demonstrated a correlation of expression of a single miRNA with the age of tumor onset and the prognosis in a gender-specific manner in patients with soft tissue sarcomas. They focused on the expression levels of miR-210, a known hypoxia-regulated miRNA, since it is correlated with poor prognosis. In qRT-PCR analysis using the 78 tumor samples of soft tissue sarcomas, an intermediate expression of miR-210 was significantly correlated with poor prognosis of female patients with soft tissue sarcomas. They also found that miR-210 expression was significantly correlated with a 9.6-year later age of tumor onset in male patients with soft tissue sarcomas [82].

3. Comparison of Deregulated miRNAs in Bone Sarcomas and Soft Tissue Sarcomas

Extensive miRNA studies have been conducted on bone sarcomas such as osteosarcoma (OS), Ewing sarcoma, and chordoma [83–87]. Several deregulated miRNAs are commonly identified in soft tissue sarcomas and bone sarcomas, while several miRNAs are unique to their own histopathological classification of soft tissue sarcomas. Commonly upregulated miRNAs include miR-21 and the 17–92 cluster, whereas commonly downregulated miRNAs include miR-143, -1/206, -34a, and -100. miR-21 is upregulated in both DDLS and MPNST (Table 1) and also in OS [88]. miR-17-92 cluster is upregulated in ARMS, uterine LMS, angiosarcoma (Table 1), and in OS [89]. Indeed, these miRNAs are well-known oncomiRs that have also been identified in other cancers of the lung, stomach, esophagus, prostate, colon, ovaries, blood, pancreas, liver, and breasts [90–92]. Therefore, miR-21 and the miR-17-92 cluster have been considered to be representative oncomiRs for a wide variety of malignant neoplasms. On the other hand, miR-143 is commonly downregulated in DDLS, SS (Table 1), and OS [93], while miR-34a is downregulated in MPNST, OS, and Ewing sarcoma [86, 94]. These miRNAs are also widely reported as tumor-suppressor miRNAs in a variety of cancers such as breast, lung, colon, kidney, bladder, and skin cancer. Indeed, miR-34a is a direct transcriptional target of p53 [95], a central tumor suppressor, and p53 enhances the posttranscriptional maturation of several miRNAs with growth-suppressive function, including miR-16-1, miR-143, and miR-145, in response to DNA damage [96]. Therefore, miR-34a and -143 are classified as representative tumor suppressor miRNAs for a variety of malignancies including bone and soft tissue sarcomas. It is interesting that muscle-specific miR-1/206 is downregulated in RMS and

chordoma [97], but the molecular mechanisms of miR-1/206 downregulation in chordoma have not been elucidated.

miRNAs that are unique in their histology include miR-26a in DDLS and miR-203 in RMS (Table 1). To date, their deregulation have not been identified in other soft tissue sarcomas or bone sarcomas. Indeed, miR-26a has been reported as a key miRNA in adipocyte differentiation. Indeed, miR-26a has been reported as a key miRNA in adipocyte differentiation [18, 98], whereas miR-203 suppresses p63 and LIFR, which in turn leads to the downregulation of the Notch pathway and the LIFR-dependent JAK1/STAT1/STAT3 pathway [99]. These pathways are indispensable for the maintenance and proliferation of muscle satellite cells during normal muscle development and muscle regeneration, and also inhibits myogenic differentiation by repressing MEF2 and MyoD [100, 101]. Thus, these results indicate that the deregulation of miRNAs that correlate with the differentiation of normal cells and tissues may play an important role in tumorigenesis of mesenchymal origin.

4. Challenge for the Clinical Application of miRNA as a Novel Biomarker

Emerging reports have demonstrated that circulating miRNAs are useful for tumor detection. To date, studies on breast, colon, prostate, and ovarian cancers have shown the possibilities of circulating miRNAs as diagnostic and prognostic markers for each cancer [102–105]. The first report of circulating miRNAs as potential diagnostic markers in sarcomas was presented in 2010 [106]. To date, the studies on soft tissue sarcoma have been reported in two histological types [107]: RMS and MPNST (Table 2).

4.1. Rhabdomyosarcoma. The first trial of circulating miRNAs as novel biomarkers in sarcomas was performed using serum samples derived from patients with RMS. Miyachi et al. focused on muscle-specific miRNAs (miR-1, -133a, -133b, and -206) that were shown to be more abundantly expressed in myogenic tumors [106]. Expression levels of these muscle-specific miRNAs in RMS cell lines were analyzed and, compared to those in neuroblastoma, Ewing sarcoma, and MRT cell lines, miR-206 was most abundantly expressed in RMS cells. Notably, these results were reflected in culture supernatants of RMS cell lines. They also confirmed that muscle-specific miRNAs were significantly upregulated in RMS tumor specimens. In their analysis of muscle-specific miRNA serum levels in patients with RMS and without RMS, serum levels of these miRNAs were significantly higher in the former. Among these miRNAs, normalized serum miR-206 showed the highest sensitivity and specificity among muscle-specific miRNAs [106]. Importantly, miR-206 expression levels decreased after RMS treatment compared to the pretreatment condition. This result was consistent with the evidence based on the previous studies using RMS tissues [26, 27, 30], indicating that miRNA deregulation in patient tissue specimens could reflect those in patient serum.

4.2. Malignant Peripheral Nerve Sheath Tumor. A recent report from Weng et al. has shown the possibility of miRNAs representing novel, noninvasive biomarkers for the diagnosis of MPNST. They performed genome-wide serum miRNA expression analysis in order to distinguish MPNST patients with and without NF1. Solexa sequencing was applied to screen for differentially expressed miRNAs in pooled serum from 10 patients with NF1, 10 patients with sporadic MPNST, and 10 patients with NF1 and MPNST. On the basis of validation studies on more patient sets, miR-801 and -214 showed higher expression in patients with sporadic MPNST and patients with NF1 and MPNST than patients with NF1 [108]. In addition, miR-24 was significantly upregulated only in patients with NF1 and MPNST. Therefore, they concluded that the combination of the three miRNAs (miR-801, -214, and -24) could distinguish patients with sporadic MPNST from those with NF1 and MPNST [108].

5. Conclusions and Future Directions

Sarcomas are distinctly heterogeneous tumors of mesenchymal origin [4, 84, 109, 110]. More than 100 sarcoma subtypes have been described [11]; however, this variety can present a diagnostic challenge because their clinical and histopathological characteristics are not always distinct [111]. In these past three decades, genetic exploration has greatly improved the diagnosis for soft tissue sarcomas, including the identification of fusion genes in soft tissue sarcomas such as synovial sarcoma, MLS, or clear cell sarcoma. The identification of miRNAs specific to histological subtypes may be a novel breakthrough for sarcoma research. As shown in Tables 1 and 2, a variety of miRNAs have been detected by various approaches. These miRNAs include those related to chromosomal translocation of each subtype or those associated with the cell differentiation of the normal counterpart. An important step forward has been achieved on the basis of miRNA research for further understanding of sarcomagenesis and sarcoma development.

To date, there are few useful biomarkers to monitor tumor development, which is one of the important problems in soft tissue sarcomas. However, several researchers have shown the possibility of miRNAs as novel biomarkers for monitoring sarcomas or for their differential diagnosis using patient-derived serum or plasma. Since these trials of "liquid biopsy" have been limited to a few histological subtypes, further exploration to include a variety of subtypes is expected. In addition, there is no evidence for miRNAs serving as biomarkers that reflect drug resistance. These miRNAs would help clinicians to determine the optimal individual treatment options, thus leading to the improvement of the patients' prognosis. Another problem is that there are not a few cases that cannot be classified into the current histological classification. In such cases, miRNA profiling may help in obtaining a differential diagnosis or creating a novel category of histopathological classification.

Emerging reports indicate the possibility of "miRNA therapeutics" in bone sarcomas. For example, supplementary administration of miR-143 mimic or miR-133a inhibitor into

Table 2: Studies on circulating miRNAs in the serum of patients with soft tissue sarcomas.

Histology	Promising circulating miRNAs	Study design	Samples	Sample size	Methods	Number of miRNAs examined	Normalization	References
Rhabdomyosarcoma	miR-206	RMS versus non-RMS versus healthy individual	Serum	8 RMS patients versus 23 non-RMS patients versus 17 healthy controls	qRT-PCR	4 miRNAs	miR-16	[106]
Malignant peripheral nerve sheath tumor	miR-24, 801, and 214	Sporadic MPNST versus NF1 MPNST versus NF1	Serum	(Screening) 10 sporadic MPNSTs versus 10 NF1 MPNSTs versus 10 NF1 (Validation) 83 sporadic MPNSTs versus 61 NF1 MPNSTs versus 90 NF1	Solexa sequencing, qRT-PCR	Genome-wide profiling	cel-miR-39	[108]

RMS: rhabdomyosarcoma; MPNST: malignant peripheral nerve sheath tumor; NF1: neurofibromatosis type 1.

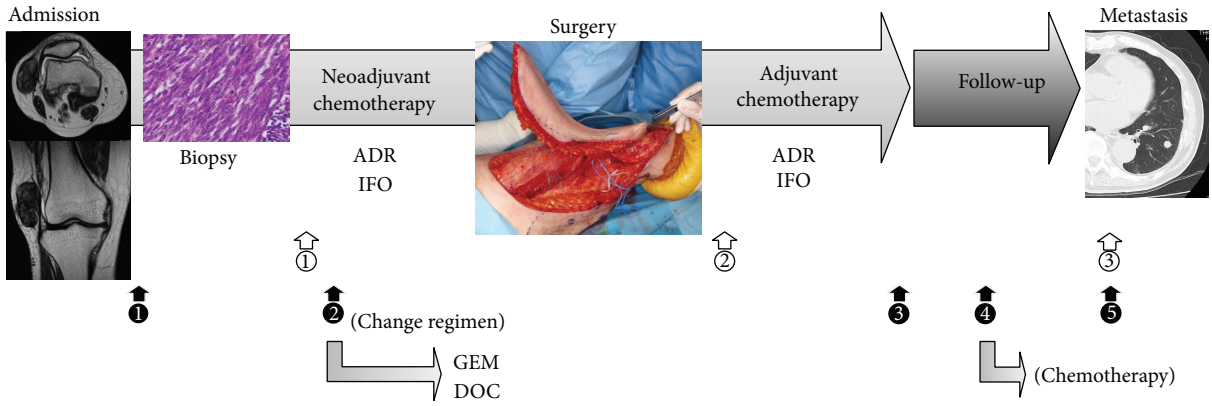

Figure 1: Examples of clinical applications of miRNAs as biomarkers and therapeutics for patients with soft tissue sarcoma. As therapeutics: ① combination with neoadjuvant chemotherapy, ② combination with adjuvant chemotherapy, and ③ combination with chemotherapy for metastasis. As biomarkers: ❶ diagnosis, ❷ determination of drug resistance, ❸ monitoring after treatment for primary lesions, ❹ detection for micrometastasis, and ❺ monitoring after treatment for metastasis. ADR: adriamycin; IFO: ifosfamide; GEM: gemcitabine; DOC: docetaxel.

osteosarcoma-bearing mice using conventional chemotherapy has been shown to inhibit osteosarcoma lung metastasis [84, 93]. We have now identified some *in vivo* trials for soft tissue sarcomas, most of which utilize viral transduction into cells prior to xenografting into mice, while few trials have utilized systemic administration of oligonucleotide. The high number of mRNAs targeted by a single miRNA may represent an advantage compared to specific gene silencing by siRNA. Notably, this method also means that each miRNA can modulate several molecular pathways with potentially unpredictable side effects. Identification of the miRNAs that are critical and specific to each sarcoma (among the reported miRNAs as shown in Table 1) would be an important step to the clinical application of "miRNA therapeutics."

While some issues remain unresolved regarding the monitoring of circulating miRNA as biomarkers or the efficacy of miRNA delivery, novel trials for noninvasive miRNA-based diagnosis and for highly efficacious "miRNA therapeutics" will be a worthwhile step for clinical applications in the near future (Figure 1).

Abbreviations

WDLS: Well-differentiated liposarcoma
MLS: Myxoid liposarcoma
DDLS: Dedifferentiated liposarcoma
RMS: Rhabdomyosarcoma
ARMS: Alveolar rhabdomyosarcoma
ERMS: Embryonal rhabdomyosarcoma
LMS: Leiomyosarcoma
MPNST: Malignant peripheral nerve sheath tumor
MFH: Malignant fibrous histiocytoma
MRT: Malignant rhabdoid tumor
UPS: Undifferentiated pleomorphic sarcoma.

Conflict of Interests

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

Acknowledgment

The authors acknowledge a grant-in-aid for Scientific Research on Applying Health Technology from the Ministry of Health, Labor and Welfare of Japan.

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Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 724209, 7 pages
http://dx.doi.org/10.1155/2014/724209

Research Article

The Clinicopathological Significance of MicroRNA-155 in Breast Cancer: A Meta-Analysis

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Received 15 May 2014; Accepted 3 July 2014; Published 3 August 2014

Academic Editor: Elisa Giovannetti

Objective. Previous studies demonstrated that the associations between expression level of microRNA-155 (miR-155) and clinicopathological significance of breast cancer remained inconsistent. Therefore, we performed a meta-analysis based on eligible studies to summarize the possible associations. *Methods*. We identified eligible studies published up to May 2014 by a comprehensive search of PubMed, EMBASE, CNKI, and VIP databases. The analysis was performed with RevMan. 5.0 software. *Results*. A total of 15 studies were included. The results of meta-analysis showed that miR-155 was positively correlated with breast cancer with standardized mean difference (SMD) = 1.22. Elevated miR-155 was found in Her-2 positive or lymph node metastasis positive, or p53 mutant type breast cancer. But the result showed to be insignificant in TNM comparison. With respect to estrogen receptor alpha (ER) and progesterone receptor (PR) status, both of them showed significant associations with SMD = −1.2 and −1.85, respectively. *Conclusion*. MiR-155 detection might have a diagnostic value in breast cancer patients. It might be used as an auxiliary biomarker for different clinicopathological breast cancer.

1. Introduction

Development of tumors is considered to be a complex multistep process, including the activation of oncogenes and the silencing of tumor suppressors [1]. MicroRNAs (miRNAs) are kinds of highly conserved, approximately 20~22 nucleotide noncoding RNAs. Endogenous miRNAs inhibit the expression of target genes in the translation level by assembling to RNA-induced silence complex (RISC) through parts of base pairing with the 3′ untranslated region (UTR) of target mRNAs [2–4].

Recent studies have shown that miRNAs were involved in various physiological and pathological processes, and an increasing number of evidences prove that the abnormal expression of miRNAs has a direct correlation with the occurrence and development of cancers. The abnormal expression and dysfunction of miRNAs may lead to cellular disorder and finally result in diseases or even cancers [5]. Thus miRNAs may be exploited as new kinds of molecular targets for diagnosing and treating cancers.

At present, in studies that focused on the association between miRNAs and tumors, abnormal expression of miR-155 in patients with breast cancer has gained substantial attention. As an oncogene, high expression of miR-155 was considered as a breast cancer risk factor. Suppressor cytokine signaling-1 (*socs1*) is a constant target gene of miR-155 in breast cancer cells in the human evolutionary process. The expression of miR-155 and socs1 is negative correlated [6]. The study by Kong et al. has shown that miR-155 participates in the process of epithelial to mesenchymal transition (EMT) and infiltration in NMuMG cells [7], suggesting that miR-155 could be used as a diagnostic marker for breast cancer metastasis. Our group has reported multiple miRNA network clusters involving in antiestrogen resistance in breast cancer; miR-155 was one of the most important dysregulated miRNAs, indicating that miR-155 might be utilized as a diagnostic marker for breast cancer endocrine therapy [8].

As the most common malignant tumor in the world among women, it had been anticipated that there would be 1.5 million women being newly diagnosed by 2010 in 2004

[9]. In Chinese population, breast cancer has an incidence rate of 16.39 per 100000 Chinese women and seriously affects people's lives and health. Among cancers that happened in Chinese women of economically developed provinces and cities, breast cancer has the highest incidence and is the fourth most common cause of cancer death [10]. Recently, there were many investigations referring to the relationship between miR-155 and breast cancer, but because of the small sample size, discrepancy exists among different studies more or less. Therefore, in order to provide a more considerable, clearer, and more systematic recognition of the expression of miR-155 in breast cancer, we collected the data related to miR-155 expression in breast cancer to carry out the meta-analysis.

2. Materials and Methods

2.1. Search Strategy. The literature retrieval was performed by two independent reviewers (Hui Zeng and Cheng Fang). All studies included in the meta-analysis were selected by searching the PubMed, EMBASE, CNKI and VIP databases up to May 2014 using the following keywords: "miR-155" or "microRNA-155" and "breast cancer." All references in these studies were examined to identify additional research that was not indexed by the databases. We selected published articles written in English or Chinese.

2.2. Including and Excluding Criteria. Including criteria include (1) being related to breast cancer and miR-155, (2) patients that are confirmed by pathology, (3) sufficient data, including mean value and standard deviation or other data that can result in mean value and standard deviation, and (4) measurement methods and experiment group that are the same or almost same.

Excluding criteria include (1) reviews, comments, or letters, (2) low-quality or incomplete data, (3) abstract only and lack of the full text, without author's reply, and (4) reduplicate publication. For duplicate articles, only the most recent or largest data set was selected.

2.3. Data Extraction. Data were extracted from eligible articles independently by two of the authors, with any disagreement resolved by consensus. The following information was collected in a predefined data collection form: first author's name, publication year, country, sample type, total number of cases and controls, quantitative methods, and publication language.

2.4. Literature Quality Evaluation. Based on the results of the system, we used grading method [11] recommended by the GRADE system to evaluate quality of evidence. Evidence quality classification is as follows: (I) high quality: further research would not change the credibility of evaluation results about the curative effect; (II) medium quality: further research is likely to affect the credibility of evaluation results about the curative effect and may change the assessment results; (III) low quality: further research is likely to affect the credibility of evaluation results about the curative effect, and the assessment results are very likely to change; (IV) very low quality: any curative effect evaluation results are uncertain.

2.5. Statistic Analysis. Rev-Man 5.0 software which was recommended by Cochrane collaboration was used in this meta-analysis. Rev-Man which is short for review manager is the software used for preparing and maintaining Cochrane reviews. Results can be presented graphically with the software.

Firstly, heterogeneity between studies was assessed by χ^2-based Q-tests and I^2 tests, where I^2 (%) > 50% or $P < 0.10$ was considered significantly heterogeneous [12]. The random effect model (DerSimonian-Laird) [13] was used to assess pooled odds ratios (ORs) when significant heterogeneity was observed. Otherwise, the fixed effect model (Mantel-Haenszel) [14] was used. Sensitivity analysis was used to analyze the stability of the text results by omitting one study at a time. For continuous data, if quantitative method is the same, we adopted the weighted mean difference (MD) as our analysis index. If they used different measuring instruments or units for the same variable or there was large difference among the mean value of numerical analysis, the standardized mean difference (SMD) was adopted for analysis. We calculated 95% confidence interval (CI) of all analysis. At the same time, the funnel chart is used to determine publication bias. Data input and monitor were done by two researchers.

3. Results

3.1. Study Characteristics. For the initial inspection, 65 related English articles and 53 Chinese articles were obtained by literature search from the PubMed, EMBASE, CNKI, and VIP databases. After titles and abstracts were screened, 86 articles were excluded because of irrelevant or duplicate records. The full texts of the remaining 32 records were carefully reviewed. Among these articles, seven articles were abandoned for overlapped data, and five articles were excluded because of review papers. Another five were excluded due to data that were incomplete or inappropriately calculated or original data that could not be obtained despite attempts to contact the authors. Therefore, 15 [15–29] articles were considered in the present meta-analysis. Among them, 13 discussed the different expression level of miR-155 between breast cancer samples and normal samples, and another 2 only discussed it between different subtype breast cancers. One [27] was included in ER and PR analysis, and another [28] was included in TNM and p53 analysis. The specific retrieval process was shown in Figure 1. The study characteristics included in the meta-analysis were listed in Table 1.

3.2. Results of Meta-Analysis. A total of 13 studies including 791 breast cancer samples and 509 normal samples were collected in this section. As shown in Figure 2, we observed an elevated miR-155 expression in breast cancer samples (SMD = 1.22, 95% CI = 0.65–1.78, $P < 0.00001$).

However, high heterogeneity was observed in the analysis, and sensitivity analyses indicated that the study from Lu et al. [22] was mainly responsible for the observed heterogeneity. When we excluded this study, the high heterogeneity was significantly decreased and the association was still significantly different (for SMD = 0.58, 95% CI = 0.46–0.71, $P < 0.00001$, P for heterogeneity = 0.15, $I^2 = 31\%$). Sensitivity analyses

Table 1: Characteristics of studies included in this meta-analysis.

Reference	Year	Origin	Sample size (case/control)	Quantitative method	Language
Chen et al. [15]	2012	China	Tissue 92/92	qRT-PCR	English
Fu and Zhang [16]	2011	China	Tissue 38/38	qRT-PCR	Chinese
Hafez et al. [17]	2012	Egypt	Tissue 40/40	qRT-PCR	English
Han et al. [18]	2013	China	Serum 45/22	qRT-PCR	Chinese
Heneghan et al. [19]	2010	Ireland	Blood 83/63	qRT-PCR	English
Huang et al. [20]	2013	China	Plasma 55/30	qRT-PCR	Chinese
Iorio et al. [21]	2005	Italy	Tissue 76/6	microarray	English
Lu et al. [22]	2012	China	Tissue 67/67	qRT-PCR	English
Ouyang et al. [23]	2014	China	Tissue 3/3	microarray	English
Mar-Aguilar et al. [24]	2013	Mexico	Serum 61/10	qRT-PCR	English
Shao et al. [25]	2013	China	Serum 165/120	qRT-PCR	Chinese
Sun et al. [26]	2012	China	Serum 103/55	qRT-PCR	English
Wang et al. [27]	2010	China	Tissue 58/58	qRT-PCR	English
Wang and Zhang [28]	2011	China	Serum 20/10	qRT-PCR	Chinese
Zheng et al. [29]	2012	China	Tissue 45/45	qRT-PCR	Chinese

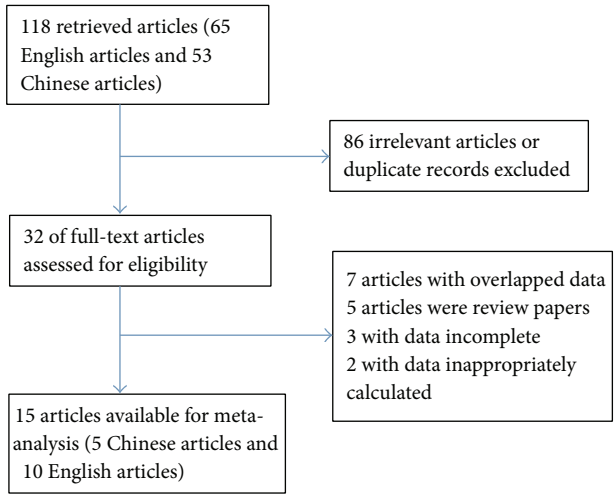

Figure 1: Flow diagram of study identification.

indicated that the pooled SMD was consistently significant by omitting one study at a time for the last studies. We then did a subgroup analyses to investigate the expression level of miR-155 in different sample types. As the study of Lu et al. [22] mainly contributed to the high heterogeneity, we excluded it in the subgroup analyses. In both tissue sample and blood sample, higher miR-155 expression level was detected in breast cancer samples than that in normal samples (with SMD = 0.63, 95% CI = 0.39–0.87, $P < 0.00001$, SMD = 0.56, 95% CI = 0.41–0.71, $P < 0.00001$, resp., Figure 3).

In subtype analysis, the expression level of miR-155 was significantly correlated with estrogen receptor alpha (ER), progesterone receptor (PR), Her-2, lymph node metastasis, tumor size, and p53 status. In subtype analysis of different ER and PR status breast cancer, miR-155 was significantly less expressed in ER+ or PR+ breast cancer. But it was highly expressed in Her-2+ or lymph node metastasis positive breast cancer compared with Her-2− or lymph node metastasis negative breast cancer. Comparing breast cancer with tumor size >2 cm with that <2 cm, the one with larger tumor may be along with higher miR-155 expression level. When breast cancer with wild p53 type and breast cancer with mutant p53 type were compared, higher miR-155 expression was detected in mutant p53 type breast cancer. However, the miR-155 expression level in different TNM grades breast cancer samples showed no significant difference (Table 2).

3.3. Evaluation of Publication Bias. We assessed the publication bias of the literature by the funnel plot. The funnel plot showed that most of the researches lay in the top of the funnel and rare in the base. The shape of the funnel plot did not reveal any evidence of obvious asymmetry (Figure 4). Egger's test and Begg's test showed $P > 0.05$.

4. Discussion

Although the pathogenesis of breast cancer has not been completely understood, the occurrence, development, and metastasis of cancer are sure to be genes participated. In recent years, miRNA studies started a new field for cancer research; there were studies showing [21, 30–32] that miR-155 might be closely related with breast cancer and played a crucial role in the development of it. Zhu et al. [33] found that miR-155 expression in breast cancer tissues was higher than that in normal tissue, lymph node metastasis and the level of estrogen receptor alpha (ER) and progesterone receptor (PR) were associated with the expression of miR-155 level in the study. Wang et al. [27] also showed significant associations between the expression of miR-155 level and the status of ER and PR. In this meta-analysis, we analyzed the correlation between expression level of miR-155 and characteristics of breast cancer. We did our best to do a comprehensive search to avoid publication bias and closely followed both including and excluding criteria. We evaluated the publication bias by

Study or subgroup	BC Mean	BC SD	BC Total	NBC Mean	NBC SD	NBC Total	Weight	Standard mean difference IV, random, 95% CI
Chen et al. 2012	0.9571	0.193	10	0.6757	0.1133	10	6.8%	1.70 [0.65, 2.76]
Fu and Zhang 2011	0.352	0.523	38	0.149	0.38	38	8.5%	0.44 [−0.02, 0.89]
Hafez et al. 2012	0.1109	0.298	40	0.0076	0.122	40	8.5%	0.45 [0.01, 0.89]
Han et al. 2013	−0.707	1.284	45	−1.966	1.201	22	8.3%	0.99 [0.45, 1.53]
Heneghan et al. 2010	3.1	0.6	83	2.9	0.7	63	8.7%	0.31 [−0.02, 0.64]
Huang et al. 2013	0.01908	0.00635	55	0.0162	0.00461	30	8.5%	0.49 [0.04, 0.94]
Iorio et al. 2005	3.97	2.62	76	1.43	0.23	6	7.5%	0.99 [0.15, 1.84]
Lu et al. 2012	0.006349	0.000875	67	0.001401	0.0001875	67	7.0%	7.78 [6.77, 8.78]
Ouyang et al. 2014	0.59	0.19	3	0.1	0.03	3	2.3%	2.88 [−0.33, 6.09]
Mar-Aguilar et al. 2013	96.18	201.125	61	2.125	1.04	10	7.9%	0.50 [−0.18, 1.17]
Shao et al. 2013	2.18	1.12	165	1.48	0.66	120	8.8%	0.73 [0.49, 0.97]
Sun et al. 2012	1.202	3.056	103	0.23	0.164	55	8.7%	0.39 [0.06, 0.72]
Zheng et al. 2012	−0.444	0.054	45	−0.87	0.911	45	8.5%	0.65 [0.23, 1.08]
Total (95% CI)			**791**			**509**	**100.0%**	**1.22 [0.65, 1.78]**

Heterogeneity: $\tau^2 = 0.93$; $\chi^2 = 210.20$, df = 12 ($P < 0.00001$); $I^2 = 94\%$
Test for overall effect: $Z = 4.23$ ($P < 0.0001$)

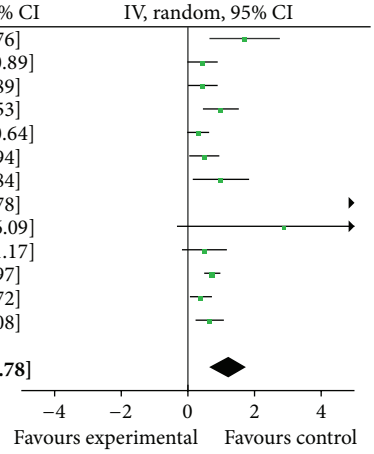

Figure 2: Random effects standardized mean difference (SMD) for the association of miR-155 expression level and breast cancer. BC: breast cancer and NBC: nonbreast cancer. The central of the square means the study-specific SMD and the horizontal lines correspond to the study-specific 95% CI.

Study or subgroup	BC Mean	BC SD	BC Total	NBC Mean	NBC SD	NBC Total	Weight	Standard mean difference IV, fixed, 95% CI
6.1.1 Tissue								
Chen et al. 2012	0.9571	0.193	10	0.6757	0.1133	10	1.4%	1.70 [0.65, 2.76]
Fu and Zhang 2011	0.352	0.523	38	0.149	0.38	38	7.6%	0.44 [−0.02, 0.89]
Hafez et al. 2012	0.1109	0.298	40	0.0076	0.122	40	8.0%	0.45 [0.01, 0.89]
Iorio et al. 2005	3.97	2.62	76	1.43	0.23	6	2.2%	0.99 [0.15, 1.84]
Ouyang et al. 2014	0.59	0.19	3	0.1	0.03	3	0.2%	2.88 [−0.33, 6.09]
Zheng et al. 2012	−0.444	0.054	45	−0.87	0.911	45	8.7%	0.65 [0.23, 1.08]
Subtotal (95% CI)			**212**			**142**	**28.0%**	**0.63 [0.39, 0.87]**
Heterogeneity: $\chi^2 = 7.88$, df = 5 ($P = 0.16$); $I^2 = 37\%$								
Test for overall effect: $Z = 5.21$ ($P < 0.00001$)								
6.1.2 Blood								
Han et al. 2013	−0.707	1.284	45	−1.966	1.201	22	5.4%	0.99 [0.45, 1.53]
Heneghan et al. 2010	3.1	0.6	83	2.9	0.7	63	14.5%	0.31 [−0.02, 0.64]
Huang et al. 2013	0.01908	0.00635	55	0.0162	0.00461	30	7.7%	0.49 [0.04, 0.94]
Mar-Aguilar et al. 2013	96.18	201.125	61	2.125	1.04	10	3.5%	0.50 [−0.18, 1.17]
Shao et al. 2013	2.18	1.12	165	1.48	0.66	120	26.6%	0.73 [0.49, 0.97]
Sun et al. 2012	1.202	3.056	103	0.23	0.164	55	14.4%	0.39 [0.06, 0.72]
Subtotal (95% CI)			**512**			**300**	**72.0%**	**0.56 [0.41, 0.71]**
Heterogeneity: $\chi^2 = 7.73$, df = 5 ($P = 0.17$); $I^2 = 35\%$								
Test for overall effect: $Z = 7.45$ ($P < 0.00001$)								
Total (95% CI)			**724**			**442**	**100.0%**	**0.58 [0.46, 0.71]**
Heterogeneity: $\chi^2 = 15.84$, df = 11 ($P = 0.15$); $I^2 = 31\%$								
Test for overall effect: $Z = 9.08$ ($P < 0.00001$)								
Test for subgroup differences: $\chi^2 = 0.23$, df = 1 ($P = 0.63$); $I^2 = 0\%$								

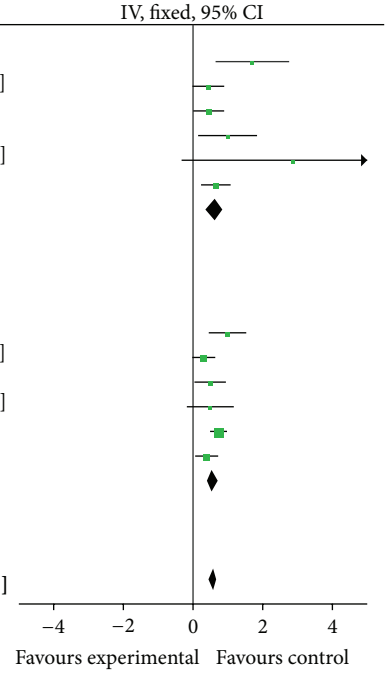

Figure 3: Fixed effects standardized mean difference (SMD) for the association of miR-155 expression level and subgroup breast cancer. BC: breast cancer and NBC: nonbreast cancer. The central of the square means the study-specific SMD and the horizontal lines correspond to the study-specific 95% CI.

using the Egger's test and Begger's test, resulting in $P > 0.05$. It indicated that no significant publication bias existed. In the analysis of the homogeneity of the literatures that had been incorporated, we found that most had heterogeneity. In this case, we preferred random effects model.

According to the results, we found that the expression level of miR-155 in breast cancer sample was greater than that in nonbreast cancer sample. Results of most articles reported are almost consistent. Liu et al. [34] showed in their article that the expression level in breast cancer group was

Table 2: Analysis of miR-155 expression level and different subtype breast cancer.

Comparisons	N Studies	Heterogeneity I^2 (%)	Heterogeneity $P(Q)$	Model[a]	Effect size SMD	Effect size $P(Z)$
ER+ Versus ER−	7 [16–18, 20, 22, 27, 29]	96	<0.00001	R	−1.2	0.03
PR+ Versus PR−	7 [16–18, 20, 22, 27, 29]	97	<0.00001	R	−1.85	0.01
Her2+ Versus Her2−	4 [20, 22, 25, 29]	50	0.11	F	0.32	0.007
LNM+ Versus LNM−	7 [15–18, 20, 22, 29]	97	<0.00001	R	2.62	0.0001
TNM I/II Versus III/IV	8 [15–18, 22, 26, 28, 29]	96	<0.00001	R	−0.94	0.08
TS 1 Versus 2/3	5 [16, 18, 20, 22, 29]	92	<0.00001	R	−0.58	<0.0001
P53 WT Versus MT	3 [16, 25, 28]	65	0.06	R	−0.79	0.02

N number of studies, $P(Q)$ P value of Q test for heterogeneity, $P(Z)$ P value of Z test for significant test, LNM lymph node metastasis, TS tumor size, WT wild type, MT mutant type.
[a]R: random-effect model; F: fixed-effect model.

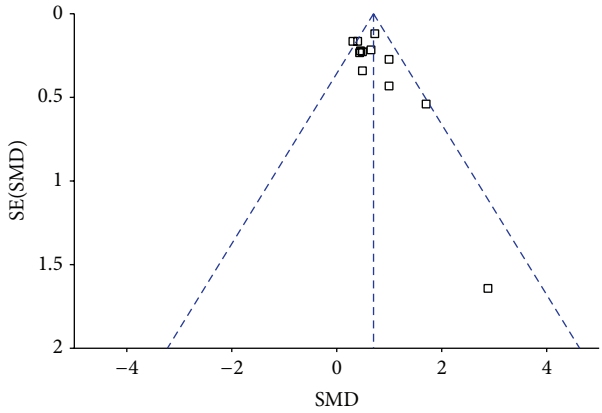

Figure 4: Funnel plots of studies included in the overall analysis.

3.2-fold higher than that in nonbreast cancer group. Other studies also have shown the miR-155 expression in breast cancer sample are at least 2-fold higher than that in nonbreast cancer sample; for example, Hui et al. [35] reported 2.4-fold overexpression in breast cancer; Sun et al. [26] reported 2.94-fold overexpression. When analyzing the different expression folds of miR-155 in breast cancer samples relative to normal samples of available data [6, 17, 26, 34–37], we discovered that the expression folds of miR-155 in breast cancer samples were higher than that in normal samples with the average expression about 6 times folds. In the subgroup analysis by sample type, the associations between miR-155 expression level and breast cancer showed to be significant in both tissue and blood sample. Therefore the detection of the expression level of miR-155 in blood can be used in diagnosing breast cancer as an auxiliary molecular marker.

According to our analysis, the higher expression level of miR-155 in breast cancer samples than that in normal samples was detected. However, the level in different clinical pathology breast cancer samples also showed to be inconsistent. So we merged into available literatures for the analysis. Pooled results for different subtypes showed that the expression level of miR-155 was not statistically significant associated with TNM-staging, but the expression level of miR-155 in lymph node metastasis positive group was significantly higher than that in lymph node metastasis negative group.

ER and PR were proteins which played important roles in the regulation of the growth and differentiation of breast cancer [38]. According to our analysis, significantly higher expression level of miR-155 was detected in both ER- and PR- group.

Gene p53, as a tumor suppressor, is located on the short arm of chromosome 17. It can inhibit cell transformation and activity of cancer gene. But mutant gene p53 can cause cell transformation, unlimited cell growth, and cancer. Gene p53 mutation may be the most important deterioration factor of breast cancer [39]. In the study, it showed that miR-155 was significantly overexpressed in breast cancer with p53 mutant type compared with breast cancer with p53 wild type.

Her-2 is a prooncogene which is also located on chromosome 17. It is recognized to be one of the most closely related gene to breast cancer. MiR-155 is overexpressed in Her-2+ breast cancer compared with Her-2− breast cancer.

Although our meta-analysis represented a quantified synthesis of all available studies, some limitations should be noticed. First, this meta-analysis was conducted based on case-control studies, which might encounter recall and selection bias. Second, in subgroup analyses by sample type and subtype analyses, the number of the studies was relatively small. A further analysis in the subtype analysis could not be performed. Third, lack of the original data of available studies limited our further evaluation of potential associations.

In conclusion, we can demonstrate that miR-155 is one of the most significant altered miRNAs in breast cancer. And the overexpression of miR-155 is not so related to TNM stage, but it is closely related to lymph node metastasis, p53 status, and hormone receptor status of breast cancer patients. More sizable sample based clinical investigations should be conducted before miR-155 can be applied as an auxiliary diagnostic biomarker.

Conflict of Interests

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

Authors' Contribution

Hui Zeng and Cheng Fang contribute equally to this work.

Acknowledgments

The project is sponsored by SRF for ROCS, SEM. This work is supported by National Natural Science Foundation of China (Grants nos. 30873044 and 81272372 to XL) and Korean National Cancer Center (NCC) Grant (NCC-1210460 to SN).

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Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 967585, 7 pages
http://dx.doi.org/10.1155/2014/967585

Clinical Study

Prospective Evaluation of Whole Genome MicroRNA Expression Profiling in Childhood Acute Lymphoblastic Leukemia

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Received 26 February 2014; Revised 7 April 2014; Accepted 22 April 2014; Published 14 May 2014

Academic Editor: Elisa Giovannetti

Dysregulation of microRNA (miRNA) expression contributes to the pathogenesis of several clinical conditions. The aim of this study is to evaluate the associations between miRNAs and childhood acute lymphoblastic leukemia (ALL) to discover their role in the course of the disease. Forty-three children with ALL and 14 age-matched healthy controls were included in the study. MicroRNA microarray expression profiling was used for peripheral blood and bone marrow samples. Aberrant miRNA expressions associated with the diagnosis and outcome were prospectively evaluated. Confirmation analysis was performed by real time RT-PCR. miR-128, miR-146a, miR-155, miR-181a, and miR-195 were significantly dysregulated in ALL patients at day 0. Following a six-month treatment period, the change in miRNA levels was determined by real time RT-PCR and expression of miR-146a, miR-155, miR-181a, and miR-195 significantly decreased. To conclude, these miRNAs not only may be used as biomarkers in diagnosis of ALL and monitoring the disease but also provide new insights into the potential roles of them in leukemogenesis.

1. Introduction

The prevalence of cancer is 11–15/100.000 among children and leukemia is the most common malignancy with an incidence of 30.2%, hence a major cause of mortality and morbidity [1, 2]. Acute lymphoblastic leukemia (ALL) accounts for 75% of childhood leukemia [2]. Event-free-survival (EFS) rates of childhood ALL exceeded 80% with current treatment protocols and relapse as the main reason for therapy failure [3]. Pediatric leukemia has distinct features when compared to adult leukemia, especially in prognosis and treatment options [4]. There are also individual differences among patients of the same age and risk group in response to treatment and in prognosis. Genetic factors are seen as one of the likely culprits for these discrepancies. Recently, microRNAs have been shown to be important molecules in developmental and oncogenic processes. They are 19–25 nt long posttranscriptional regulator ribonucleic acid (RNA) molecules [5]. They target mRNAs, inhibit the translation into proteins, and silence specific gene expression [6]. Aberrant expression of miRNAs has been observed in many types of cancer, showing either tumor suppressive or oncogenic activity [7, 8]. The number of studies regarding the relationship between adult cancers and miRNAs is gradually increasing; however, there are still a relatively low number of studies regarding childhood malignancies. Moreover, there have been even fewer studies focusing specifically on the association of miRNAs with childhood ALL [9–15]. The aim of the present study is to identify the relevant miRNAs in the diagnosis of

childhood ALL and evaluate their effect in the course of the disease.

2. Materials and Methods

2.1. Patients. A total of 45 children with newly diagnosed and untreated childhood ALL and 15 age-matched control subjects (normal peripheral blood (PB) and bone marrow (BM) smears) were enrolled in the study. Patients were consecutively included from the inpatient oncology and hematology departments of 3 hospitals. Previously diagnosed leukemia cases were excluded from the study as the miRNA expression levels might have been altered as a result of previous treatment. ALL diagnoses were confirmed via a BM aspirate showing at least 30% blast cells, in accordance with the FAB classification. All patients were diagnosed according to standard morphological, cytochemical, and immunophenotypic criteria. Patients were treated primarily with Berlin-Frankfurt-Munster- (BFM-) based national ALL protocol. This protocol was modified slightly with regard to methotrexate dosing and cranial irradiation. Following the BFM-ALL 1995 protocol, risk groups were categorized as standard (SRG), intermediate (IRG), and high (HRG) risk groups based on their age, leukocyte count, immunophenotyping, cytogenetic changes, early response to prednisone therapy, and BM remission. Patients received four drug induction regimens consisting of prednisone, asparaginase, vincristine, and daunorubicin. Complete remission (CR) was defined as a normocellular marrow with less than 5% blast cells. Patients' characteristics, such as age, sex, white blood cell (WBC) count, FAB classification, and treatment response, are available in Table 1.

Table 1: Demographics, laboratory results, and response to the treatment in ALL patients.

Sex	Female *n* (%)	21 (48.8)
	Male *n* (%)	22 (51.2)
Mean age (year old)		6.8 ± 4.5
White blood cells	Mean (mm^3)	47.930
	Range (mm^3)	487–474.000
	Subtype	T-lineage: 9
		B-lineage: 34
Blast BM	Mean (%)	85
	Range (%)	52–100
Blast PB	Mean (%)	51
	Range (%)	2–100
Characteristic	Status	*n* (%)
Risk group	Standard risk	13 (30)
	Intermediate risk	20 (47)
	High risk	10 (23)
Steroid response	Good	36
	Poor	7
Response at 33 day	Remission	43
	Not remission	0
Survival	Alive	39
	Dead	4
Relapse	Yes	0
	No	39
	Dead	4

ALL: acute lymphoblastic leukemia, BM: bone marrow, PB: peripheral blood.

2.2. Study Design. The cases were evaluated prospectively. The enrollment period for each patient in the study was 18 months, plus 2 years of followup. MicroRNA microarray profiling was performed on all patients at day 0 and in control cases. The significant miRNAs were confirmed by real time RT-PCR. Six months after treatment, significantly dysregulated and validated miRNAs at day 0 were again analyzed by real time RT-PCR. The change in miRNA levels over time was determined by comparing the ratio at day 0 with the ratio of those measured at 6 months. During this period, 4 patients died. Among our study population, 3 cases (2 from patient group and 1 from control group) were also excluded following RNA isolation process, due to insufficient signalization during microarray work. The significance of miRNAs that were obtained from PB samples was compared with BM samples. Aberrant miRNA expressions associated with the diagnosis, differential diagnosis, and outcome of ALL were evaluated.

2.3. RNA Extraction and Microarray Study. RNA isolation was performed on each BM and peripheral blood (PB) samples obtained from both patient and control groups. Total RNA was isolated by using Qiazol, which was then followed by miRNeasy Mini Kit (Qiagen, Valencia, USA) as per the manufacturer's instructions. Genome wide microRNA microarray profiling was performed by using a microRNA biochip platform (Febit, Heidelberg, Germany). The platform consisted of 1136 microRNA probes (Sanger, miRBase 12.0). In short, 0.7 micrograms of microRNA was labelled using miRVANA labeling kit (Ambion, USA) and then dried via SpeedVac (Thermo, Germany). Dried samples were then treated with 18 microL of hybridization buffer (Febit Biomed GmbH, Heidelberg, Germany) and placed into the biochip platform overnight. Following hybridization and washing, signals were measured. The signal enhancement procedure was processed with Geniom Real Time Analyzer (GRTA) and detection pictures were evaluated by using Geniom Wizard software. The signal intensities for all miRNAs were extracted from the raw data for each array. After background correction, the median signal intensity of the seven replicate intensity values of each miRNA was obtained. Normalization was conducted by using the freely available R software (http://www.R-project.org/).

The RNA quality control was determined by using the NanoDrop ND-1000 in which the ratios of 230/260 and 260/280 were 2. The RNA integrity number was determined by using Agilent 2100 Bioanalyzer (Agilent Technologies) and was ≥7.

The array data including raw data has been deposited in Gene Expression Omnibus (GEO) with the accession number GSE56489.

2.4. Quantitative Real Time Polymerase Chain Reaction and Validation. Significantly dysregulated miRNAs were validated by Quantitative PCR (LightCycler 480, Roche Applied Science, Mannheim, Germany) after comparing the BM microarray miRNA expressions of patients with the controls. RNA was reverse-transcribed to cDNA by using a cDNA synthesis kit (Exiqon). For miRNA quantification, the miRCURY LNA Universal RT microRNA PCR system (Exiqon) was used in combination with the predesigned primers (Exiqon). A master mix was designed for each primer set in accordance with the recommendations of the real time RT-PCR setup for "individual assays," suggested in the kit. The reaction conditions consisted of polymerase activation/denaturation at 95°C for 10 min. For miRNA quantification, 40 amplification cycles at 95°C for 10 sec and 60°C for 1 min were performed; this was then followed by signal detection. The Delta-Delta-Ct algorithm was used to determine relative gene expression, and SNORD48 and U6 were used for housekeeping genes.

2.5. Statistical Analysis. According to mean values, only those miRNAs whose "fold change" value demonstrated ±2-fold or more expression difference were included in the study. miRNAs whose "False Discovery Rate" (FDR) corrected P value <0.05 were considered significant. FDR was determined by CLC Main Workbench 5 (CLC Bio, Denmark). Heat map and cluster analysis were performed for grouping of the miRNA data. Student's t-test was used to make comparisons in different groups by CLC Main Workbench.

2.6. Consent. This study was approved by the Local Ethics Committee, and written consent was taken from all parents of children that participated in the study.

3. Results

The patient group consisted of 43 cases with ALL in which 34 cases (79%) were B-lineage, and 9 cases (21%) were T-lineage ALL. The mean age in the ALL group was 6.8 ± 4.5. Fourteen cases constituted the control group and the mean age was 6.6 ± 5.1. The ratio of female/male was calculated 0.9 in ALL and 0.8 in the control group (Table 1). The distribution of ALL cases, according to the risk groups, was as follows: 13 cases (30%) were in the SRG, 20 cases (47%) were in the IRG, and 10 cases (23%) were in the HRG. Upon followup, 4 ALL patients (9%) (2 cases with SRG B-lineage ALL, 2 cases with HRG B-lineage ALL) died due to infectious complications (Table 1). Risk groups and response to the treatment of ALL cases are summarized in Table 1.

3.1. miRNA Analysis. miRNAs from PB samples were not correlated with BM samples. Significant miRNAs in PB of patients when compared to control cases were totally different to miRNAs in BM. Therefore, all analysis and considerations in different categories were made by using BM samples. We propose using BM samples for the miRNA analysis in hematological malignancies because they reflect the leukemic process more efficiently, when compared to PB. With reference to the controls' ($n = 14$), significantly dysregulated miRNAs in BM samples according to the microarray study ($n = 43$) are shown in Table 2. A total of 13 miRNAs (miR-548i, miR-708, miR-181b, miR-449a, miR-146a, miR-155, miR-181a, miR-3121, miR-181a, miR-128, miR-1323, miR-195, and miR-587) showed upregulation and 2 miRNAs (miR-640, miR-145) showed downregulation. Heat map and cluster analysis in ALL patients and control cases (FDR P value <0.05) were given in Figure 1. Confirmation analysis of significantly dysregulated miRNAs in the patient group by real time RT-PCR revealed 5 upregulated miRNAs (miR-128, miR-146a, miR-155, miR-181a, and miR-195) in ALL (Table 3). Following 6 months of treatment, the level of miR-146a, miR-155, miR-181a, and miR-195 which were found to be upregulated at diagnosis and validated by real time RT-PCR decreased significantly (Table 3). The change in expression level of miR-128, though decreased by 50%, was not significant throughout this period. The expression change of these relevant miRNAs was also presented in Figure 2.

The most significantly upregulated miRNAs and their fold changes in T-lineage ALL and B-lineage ALL are summarized in supplemental Table 1 (see Supplementary Table 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2014/967585). miR-548i, miR-3140, and miR-181b in T-lineage ALL and miR-708, miR-181b, and miR-369-3p in B-lineage ALL were the most distinctive miRNAs among all.

4. Discussion

In this study a total of 1136 miRNAs were studied in children with ALL using microarray platform to reveal their contribution with regard to diagnosis, classification, and treatment period. Microarray study revealed 15 miRNAs dysregulated, when compared to the control cases. The results of real time RT-PCR for 5 miRNAs (miR-128, miR-146a, miR-155, miR-181a, and miR-195) were consistent with our microarray results; however, the other 10 miRNAs (miR-548i, miR-708, miR-181b, miR-369-3p, miR-449a, miR-3121, miR-181a, miR-1323, miR-587, and miR-181a-2) contradicted the results of our microarray study. This discrepancy may be due to the small sample size ($n = 43$) used for microarray assay [16]. Array platforms are specifically used for high throughput studies to define significantly dysregulated miRNAs and involve background correction, normalization, and summarization, from which it is recommended that the results be confirmed via other methods. Real time RT-PCR is accepted as a "gold standard" for the confirmation of array platforms [17]; therefore, our data is based on RT-PCR results. After 6 months of follow-up period following chemotherapy, miRNA expression profiles were reevaluated, and their potential involvement in cancer biology was assessed. As presented in Table 3 and shown in Figure 2, the level of all oncogenic miRNAs except miR-128 which were confirmed by real time RT-PCR significantly decreased. This data has not been reported in the literature.

miR-155 is an oncogenic miRNA which has been shown to be dysregulated in many studies and is suggested as a putative prognostic factor [18, 19]. After 6 months of followup

Table 2: Significant miRNA profile compared to control cases in the microarray study and validation results in real time RT-PCR. According to mean values, only those miRNAs whose "fold change" value demonstrated ± 2-fold or more expression difference were included in the study. miRNAs whose "False Discovery Rate" (FDR) corrected P value <0.05 were considered significant. FDR is calculated by using CLC Main Workbench 5 (CLC Bio, Denmark). A total of 13 miRNAs showed upregulation (shown in bold) and 2 miRNAs showed downregulation.

miRNA	Microarray					High/Low Expression (n)*	RT-PCR				High/Low Expression (n)*
	Control	ALL	Ratio	P value	FDR P		Control	ALL	$\log_2$RR	P	
hsa-miR-548i	1.84	22.12	12.50	$3.90E-03$	0.03	23/0	0.000112	0.007942	6.15	0.590	21/0
hsa-miR-708	46.55	488.77	10.00	$6.55E-04$	0.01	21/7	0.002949	19.01167	12.65	0.291	28/3
hsa-miR-181b	72.46	467.47	6.25	$7.09E-06$	$3.83E-04$	36/3	0.0468	48.42431	10.02	0.251	33/2
hsa-miR-449a	6.66	23.84	3.57	$2.69E-03$	0.03	14/0	0.003653	0.013143	1.85	0.163	7/0
hsa-miR-146a	326.65	1135.73	3.45	$8.31E-05$	$1.79E-03$	35/2	0.068189	24.37251	8.48	0.008	35/1
hsa-miR-155	170.68	515.25	3.03	$7.93E-05$	$1.79E-03$	32/2	0.063027	31.66987	8.97	0.009	34/0
hsa-miR-181a*	104.93	298.17	2.86	$6.54E-03$	0.04	25/4	0.012341	0.766604	5.96	0.123	35/2
hsa-miR-3121	12.04	33.64	2.78	$6.95E-03$	0.04	21/0	0.023803	0.001278	−4.22	0.209	0/0
hsa-miR-181a	661.35	1810.68	2.70	$3.66E-03$	0.03	29/7	0.770556	80.63069	6.71	0.002	34/2
hsa-miR-128	720.72	1713.49	2.38	$2.67E-06$	$2.88E-04$	30/0	0.245556	11.3962	5.54	0.009	33/4
hsa-miR-1323	34.21	81.96	2.38	$1.05E-03$	0.01	25/2	0.000686	0.000024	−4.84	0.199	0/0
hsa-miR-195	962.76	2251.43	2.33	$9.67E-04$	0.01	28/4	0.012433	1.250504	6.65	<0.001	32/3
hsa-miR-587	33.83	69.93	2.08	$5.67E-03$	0.04	17/4	0.000523	0.000146	−1.84	0.326	1/0
hsa-miR-640	91.53	45.07	−2.03	$7.18E-03$	0.04	3/30	0.050227	0.000498	−6.66	0.191	0/0
hsa-miR-145	498.27	197.73	−2.52	$8.09E-05$	$1.79E-03$	2/35	1.201444	14.55961	3.60	0.440	26/10

FDR: false discovery rate, ALL: acute lymphoblastic leukemia.

*"High/Low Expression (n)" means the number of cases in which particular miRNA expression is over the normal range. Normal range was accepted for the 95% CI of control cases.

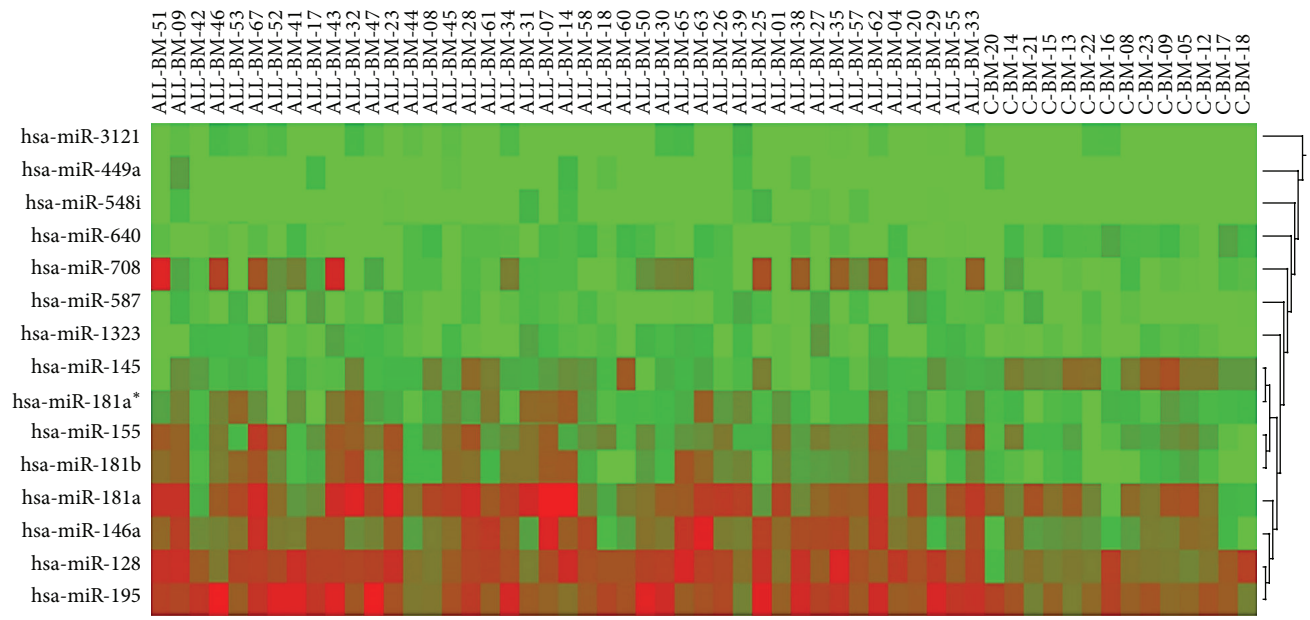

Figure 1: Heat map and cluster analysis in ALL patients and control cases (FDR P value <0.05). The figure shows the relative expression of bone marrow (ALL-BM) miRNAs in ALL patients and control cases (c-BM). miRNAs are in columns representing the 15 miRNAs, samples in rows representing 43 ALL patients and 14 control cases. The color scale shown on the top illustrates the expression level of the indicated miRNA across all samples: red means that a miRNA expression value is higher than its average expression across all samples (upregulated), and green means a lower expression value (downregulated).

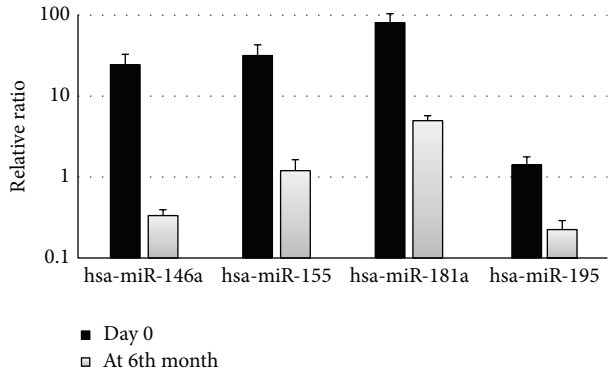

Figure 2: Diagram of significantly changed miRNAs in ALL patients after 6 months of treatment. The figure shows the change in expression levels of 4 significantly dysregulated miRNAs after 6 months of treatment. The change in miRNA levels over time was determined by comparing the ratio at day 0 with the ratio of those measured at 6 months by using quantitative real time RT-PCR.

Table 3: Significant miRNAs after validation by real time RT-PCR at day 0 were given. Those miRNAs were reevaluated after 6 months of treatment and the expression change of all miRNAs except miR-128 was significant.

	miRNA	RR
Validated by RT-PCR (at diagnosis)	hsa-miR-128	11.396200
	hsa-miR-146a	24.372519
	hsa-miR-155	31.669875
	hsa-miR-181a	80.630694
	hsa-miR-195	1.250504
Real time RT-PCR (after 6 months)	hsa-miR-128	6.073660
	hsa-miR-146a	0.334798
	hsa-miR-155	1.201844
	hsa-miR-181a	4.987600
	hsa-miR-195	0.206393

RR: relative ratio.

including chemotherapy, it was found to be downregulated in our study. It can be speculated that this result is the reflection of the fact that after 6 months, blasts which overexpress miR-155 have disappeared. Although miR-155 is a potent inhibitor of myeloid differentiation and was found to be upregulated particularly in AML patients [20, 21], it has been reported that this miRNA is also an important factor in lymphopoiesis and immune response [22–24]. The study by Wang et al. has shown that miR-155 was expressed at a significantly higher level in ALL than in AML [25]; therefore, it can be speculated that, in view of our data combined with the literature, miR-155 could be used as a valuable biomarker in the diagnosis and maintenance of ALL.

miR-146 has been reported to have a role in innate immunity by downregulating *TRAF6* and *IRAK1* genes, and miR-146a is regulated by *NF-kappa* [26, 27]. Combined with miR-155 and miR-181a, which were found to be overexpressed in our study, those 3 miRNAs are associated with genes involved in innate immunity and inflammation, including *TLR4*, *TLR8*, *IRF8*, and *IL6R* [28]. In the light of the information reported in the literature, altered expression

of miR-146a, miR-155, and miR-181a could be suggested as additional factors which may lead to leukemia development by causing deleterious effect on normal immune function [29, 30]. *miR-181a* has been reported to have a role in the development and in the differentiation of both B cells and cytotoxic T cells [31, 32]. It has also been shown that miR-181a repressed the expression of genes which play a role in thymocyte maturation, such as *Bcl-2*, *CD69*, and the T-cell receptor, and the members of the miR-181 family were found to be significantly overexpressed in T-cell leukemia in our study. These findings support the suggestion that future studies should focus on miR-181 family in the management of ALL.

Studies have shown that *miR-195* has a regulatory function in the cell cycle and cell proliferation [33, 34]. It is expressed in many cancers including chronic lymphocytic leukemia which was one of the 5 most expressed in our patients [35]. However, there is no study in the literature regarding the impact of this miRNA on acute leukemia patients.

miR-128 together with let-7b and miR-223 was found to be the most discriminatory miRNAs involving ALL and AML [36, 37]. miR-128 was found to be expressed at a significantly higher level in ALL than in AML; therefore, in the light of our results, miR-128 can be used to diagnose and discriminate ALL and AML cases accurately.

Some miRNAs are already known to be related to specific subtypes of pediatric ALL. As reported by Schotte et al., genetic subtypes such as MLL-rearranged, TEL-AML1 positive, hyperdiploid, and drug-resistant leukemic cells display characteristic miRNA signatures in pediatric ALL [38]. Fulci et al. have studied 470 miRNAs in adult ALL patients and identified miRNAs discriminative of the ALL subsets, namely, T-cell and B-cell ALL [39]. miRNAs which were highly discriminative of the different subgroups in this study were not consistent with the results of the present study. The inconsistency between these two studies may be caused by the different age group characteristics. Wang et al. studied 23 miRNAs, which were reported to be associated with hematopoiesis and/or leukemogenesis, to show differentially expressed miRNAs in ALL and AML [25]. Among those identified as differentially expressed miRNAs, miR-128 is remarkable because it also showed significant overexpression in our study.

The limitation of our study lies in the studying of expression levels in specimens containing both normal and blast cells where the results may be affected by the contribution of normal cells. The mean blast cell level in our study, however, was found to be 85% (range: 52–100) meaning this issue may be avoided.

When the age group and its characteristics of childhood leukemia are considered, our data could add important contributions to the literature. The first is the studying of the largest miRNA profile in ALL and the presentation of novel miRNAs associated with leukemogenesis; the second contribution is identifying miRNAs as discriminative of T-lineage versus B-lineage ALL. Moreover, our results confirmed the importance of certain miRNAs such as miR-128, miR-146a, and miR-181a in childhood ALL. The final and possibly the most important contribution is the prospective design of our study that we were able to evaluate miRNAs throughout a treatment period.

In conclusion, the discovery of miRNAs and their association with disease have provided valuable information on potential diagnostic and/or prognostic biomarkers, as well as monitoring the disease progression. In our study, miR-128, miR-146a, miR-155, miR-181a, and miR-195 were found to be significantly dysregulated which may help provide new insights into the diagnosis and prognosis of childhood ALL. Further studies, with larger subject numbers, are needed to clearly demonstrate the effect of miRNAs in leukemogenesis and its practical implications.

Conflict of Interests

The authors declare that they have no conflict of interests regarding the publication of this paper.

Authors' Contribution

Muhterem Duyu and Burak Durmaz contributed equally to this work.

Acknowledgment

The authors would like to thank the Scientific and Technological Research Council of Turkey (TUBITAK) for financial support under Grant 109S076.

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