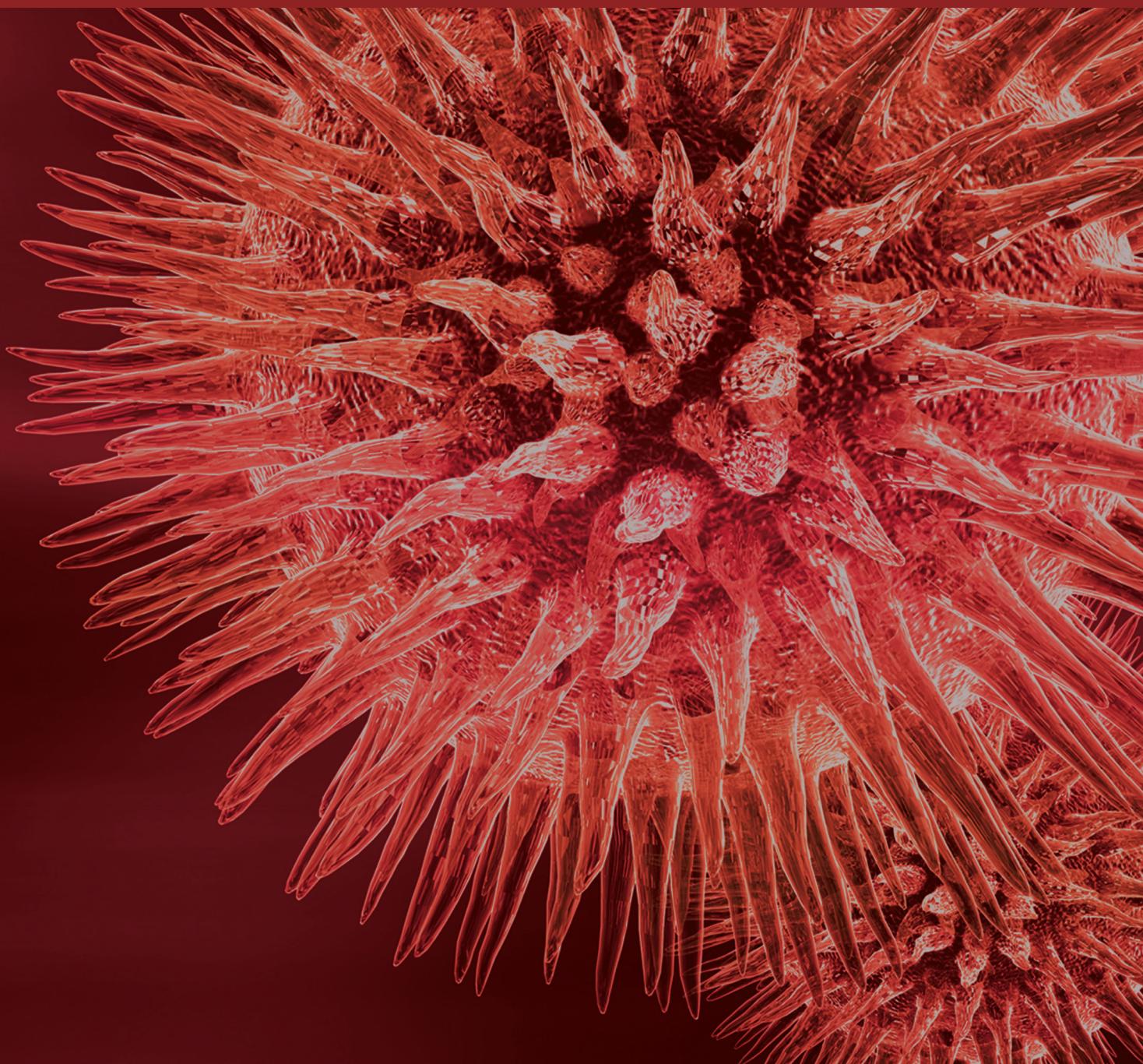


MicroRNAs: Emerging Novel Targets of Cancer Therapies

Guest Editors: Chengfeng Yang, Yiguo Jiang, Ajay P. Singh, and Fumitaka Takeshita



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Editorial

MicroRNAs: Emerging Novel Targets of Cancer Therapies

Chengfeng Yang,¹ Yiguo Jiang,² Ajay P. Singh,³ and Fumitaka Takeshita⁴

¹*Department of Physiology, Center for Integrative Toxicology, 4171 Biomedical Physical Sciences, Michigan State University, East Lansing, MI 48824, USA*

²*Institute for Chemical Carcinogenesis, State Key Laboratory of Respiratory Disease, Guangzhou Medical University 195, Dongfeng Road West, Guangzhou, Guangdong 510182, China*

³*Department of Oncologic Sciences, Mitchell Cancer Institute, University of South Alabama, 1660 Springhill Avenue, Mobile, AL 36604-1405, USA*

⁴*Division of Molecular and Cellular Medicine, National Cancer Center Research Institute, 1-1 Tsukiji 5-chome, Chuo-ku, Tokyo 104-0045, Japan*

Correspondence should be addressed to Chengfeng Yang; yangcf@msu.edu

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MicroRNAs (miRNAs) are a large family of small noncoding RNAs (~22 nucleotide long) that negatively regulate protein-coding gene expression posttranscriptionally by interacting with messenger RNAs (mRNAs), causing either their degradation or translation inhibition. While miRNAs were first discovered as important regulators of developmental timing, subsequent studies have shown that their deregulations are critically involved in various diseases, including cancer. This is evident from the crucial roles they play in regulating a wide range of cellular processes such as cell survival, apoptosis, cell cycle progression and proliferation, cell-cell interaction, differentiation, and motility.

It has been found that miRNA expression levels are altered in all types of cancer and they play important roles in almost all aspects of cancer pathogenesis such as initiation, promotion, metastasis, and responses to drug treatment. These recognitions and other accumulating evidences clearly suggest that miRNAs can serve as novel targets for cancer therapies. Indeed, targeting abnormally-expressed miRNAs has been shown to have great potential in suppressing primary tumor growth, reducing tumor metastasis, and overcoming anticancer drug resistance. It is anticipated that miRNAs can be valuable cancer-specific targets and novel miRNA-based targeted therapies can be formulated to provide effective treatments for cancer. The purpose of this special issue is to provide readers with an overview

of research findings on the roles of miRNAs in cancer development, progression, diagnosis and treatment, and how miRNA expression may be regulated. This issue will also update the readers about the challenges and possibilities of miRNAs to emerge as future cancer therapeutics.

A. L. Oom et al. first briefly review the potential value of miRNAs as cancer diagnostic markers, which is followed by the detailed discussion on the potential role of miRNAs in cancer therapy. The authors review literatures reporting the role of miRNAs in sensitizing of or mediating resistance to traditional and targeted cancer therapies. A nice summary about studies on miRNAs in combination with traditional cancer therapies is provided in a table. The authors also discuss the current studies on miRNA delivery systems. This review ends with discussion on challenges and perspectives of developing miRNAs as effective cancer therapies.

The review paper, authored by K. Otsuka and T. Ochiya, discusses the role of miRNAs in tumor development by uniquely focusing on the interplay between the classic tumor suppressor p53 and the emerging tumor regulatory miRNAs. The authors review recent findings showing both the regulation of expression of various miRNAs by p53 and the direct targeting of p53 by miRNAs. Particularly, this review summarizes up to date research findings about the critical roles of various miRNAs in regulation of cell cycle and apoptosis in the p53 pathways.

Y. Fujita, K. Kuwano, T. Ochiya, and F. Takeshita provide an in-depth review of current findings showing the potential of circulating miRNAs as diagnostic biomarkers for lung cancer. This review discusses the general mechanism of miRNA release into extracellular spaces and the impact of extracellular vesicle-encapsulated circulating miRNAs on cancer progression. Moreover, this review also summarizes current studies in detail showing the potential of miRNAs as circulating biomarkers in cancer diagnosis, particularly for lung cancer.

N. H. Phuah and N. H. Nagoor review the regulation of miRNAs by natural compounds. Recent studies have shown that natural agents such as curcumin, resveratrol, genistein, epigallocatechin-3-gallate, indole-3-carbinol, and 3,3'-diindolylmethane exert their antiproliferative and/or proapoptotic effects through the regulation of one or more miRNAs. This review summarizes current research findings about the regulation of miRNAs by above natural compounds. The review also discusses the potential of integrating natural agents with conventional chemotherapeutic drugs, thus, enhancing their efficacy.

The review article by S. K. Srivastava, S. Arora, C. Averett, S. Singh, and A. P. Singh discusses the effect of naturally occurring phytochemicals on miRNA expression. Particularly, the authors focus on the underlying molecular mechanisms of miRNA regulation by phytochemicals and their functional significance in cancer development and progression. Moreover, the translational potential of the relevant research findings is also discussed.

In closing, the guest editors of this special issue would like to thank all the reviewers for their timely and excellent review and journal management team for efficient processing of papers from submission through publication.

*Chengfeng Yang
Yiguo Jiang
Ajay P. Singh
Fumitaka Takeshita*

Review Article

Modulation of MicroRNAs by Phytochemicals in Cancer: Underlying Mechanisms and Translational Significance

Sanjeev K. Srivastava,¹ Sumit Arora,¹ Courey Averett,¹ Seema Singh,¹ and Ajay P. Singh^{1,2}

¹ Mitchell Cancer Institute, University of South Alabama, Mobile, AL 36604, USA

² Department of Biochemistry and Molecular Biology, University of South Alabama, Mobile, AL 36688, USA

Correspondence should be addressed to Ajay P. Singh; asingh@health.southalabama.edu

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MicroRNAs (miRNAs) are small, endogenous noncoding RNAs that regulate a variety of biological processes such as differentiation, development, and survival. Recent studies suggest that miRNAs are dysregulated in cancer and play critical roles in cancer initiation, progression, and chemoresistance. Therefore, exploitation of miRNAs as targets for cancer prevention and therapy could be a promising approach. Extensive evidence suggests that many naturally occurring phytochemicals regulate the expression of numerous miRNAs involved in the pathobiology of cancer. Therefore, an understanding of the regulation of miRNAs by phytochemicals in cancer, their underlying molecular mechanisms, and functional consequences on tumor pathophysiology may be useful in formulating novel strategies to combat this devastating disease. These aspects are discussed in this review paper with an objective of highlighting the significance of these observations from the translational standpoint.

1. Introduction

MicroRNAs (miRNAs) are endogenous, small noncoding RNA molecules that posttranscriptionally regulate gene expression. miRNAs bind to the 3'-UTRs of target mRNA with partial or complete complementarity, thus causing translational repression or target messenger RNA (mRNA) degradation [1]. An individual miRNA can regulate the expression of multiple genes; conversely, a single mRNA can be targeted by many miRNAs. To date, about 2,469 miRNAs have been identified in humans [2], and more than one-third of all human genes are potentially regulated by miRNAs [3]. Extensive studies have shown that miRNAs not only are involved in the process of cell development and differentiation but also play a critical role in carcinogenesis [4]. Emerging data suggest that several classes of naturally occurring, plant-derived compounds (phytochemicals) could potentially regulate the expression of several miRNAs involved in cancer.

Phytochemicals are nonnutritive plant chemicals that have various applications including anti-inflammatory and

anticancer. These phytochemicals are widely distributed in various fruits, vegetables, herbs, beverages, and many other dietary supplements. Numerous studies have demonstrated that the intake of fruit- and vegetable-rich foods decreases the occurrence of cancer [5–7]. So far, more than 10,000 phytochemicals have been identified [8], and a significant number of phytochemicals show anticancer potential with no or minimal toxicity to normal cells [9]. Interestingly, around 47% of FDA approved anticancer drugs are derived from plants [10, 11]. Moreover, these phytochemicals could be used as a single chemotherapeutic agent or in association with standard anticancer drugs. Phytochemicals can increase the efficacy of anticancer drugs synergistically, while reducing the toxic side effects of the standard chemotherapeutic drugs [12, 13]. These phytochemicals exert their anticancer effects through modulation of multiple molecular targets affecting various signaling pathways [8, 14, 15]. In the present review paper, we focus our attention on the regulation of miRNAs by some of the phytochemicals such as resveratrol, epigallocatechin-3-gallate (EGCG), curcumin, camptothecin

(CPT), and diindolylmethane (DIM) for cancer prevention and therapy.

2. Biogenesis of miRNA and Mechanism of Gene Silencing

The biosynthesis of miRNAs begins with gene transcription by RNA polymerase II or RNA polymerase III into primary miRNA (pri-miRNA) transcripts inside the nucleus. These pri-miRNAs are comprised of either a cluster or single miRNAs folded into a hairpin stem-like structure [1]. This long pri-miRNA is processed by the sequential, endonucleolytic cleavage of the transcript by the microprocessor complex, containing drosha and DGCR8, into a 65–70-nucleotide precursor miRNA (pre-miRNA). This pre-miRNA is then exported from the nucleus to the cytoplasm by the nuclear export factor Exportin-5/Ran-GTP and cleaved by RNase III endonuclease, Dicer/TRBP, and argonaute 2 (Ago2) miRNA duplex [16] into an ~22-nucleotide product. This duplex miRNA then unwinds to generate a single-stranded miRNA. One of the strands enters in the RNA-induced silencing complex (RISC) along with Ago proteins and directs the complex to target mRNA. This binding causes either target mRNA degradation or inhibition of translation [1].

3. Biological Significance of miRNAs in Cancer: Tumor Suppressors and Oncogenes

Based on *in vitro* and *in vivo* studies, miRNAs have been characterized as tumor suppressors or oncogenes. In this section we will discuss some of the reports from a long list of tumor suppressor/oncogenic miRNAs that have been experimentally validated.

Numerous studies have shown that the expression of the tumor suppressor Let-7 is significantly downregulated or lost in various cancers [17–19]. It has been shown that restoration of Let-7 inhibited tumor growth in a K-RAS lung cancer model [20]. Furthermore, decreased expression of let-7 correlated with shorter survival in non-small-cell lung cancer [21]. Other miRNAs such as miR-15a and miR-16 are either deleted or downregulated in most cases of the chronic lymphocytic leukemia, and their overexpression induced apoptosis [22]. Moreover, it has been reported that miR-16 and miR-15 were downregulated in multidrug-resistant human gastric cancer cells, and restoration of these miRNAs sensitized the cancer cells to chemotherapeutic drugs [23]. Takeshita and coworkers demonstrated the growth inhibitory role of miR-16 in prostate cancer cells [24]. The levels of miR-34 are significantly decreased in various cancers, and its restoration was reported to inhibit angiogenesis and malignant behavior, yet at the same time it induced apoptosis and cell cycle arrest [25, 26]. Additionally, the role of miR-34 in the inhibition of tumor-initiating cells has also been suggested [27]. In mixed lineage leukemia, the overexpression of miR-495 inhibited cell viability and reduced leukemogenesis *in vivo* [28]. In one of our recent studies, we investigated the significance of miR-150 downregulation in pancreatic cancer [29]. We demonstrated that the restoration of miR-150 inhibited the

MUC4 oncoprotein. Consequently, the growth and malignant potential of pancreatic cancer cells were suppressed [29, 30]. The role of miR-451 as a tumor suppressor miRNA by inhibiting cancer cell migration, invasion, and growth is well known [31–33]. Further, Kovalchuk and coworkers demonstrated that miR-451 sensitizes MCF-7/DOX-resistant cells to doxorubicin cytotoxicity [34]. Xu and coworkers demonstrated that miR-203 overexpression effectively inhibited cell proliferation and induced apoptosis and cell cycle arrest. Moreover, its overexpression also inhibited tumor growth in a mouse model [35].

To date, several miRNAs have been identified that act as oncogenes [36]. miR-17-92 is an oncogenic gene cluster [37]; restoration of this cluster in MYC-driven B-cell lymphomas suppressed apoptosis and enhanced tumorigenicity [37]. Ma et al., 2010, demonstrated that miR-27a, which is overexpressed in pancreatic cancer, plays an oncogenic role by targeting tumor suppressor Spry2 [38]. Inhibition of miR-27a decreased growth, clonogenicity, and migration of pancreatic cancer cells. miR-21 is a widely studied oncogenic miRNA which is frequently overexpressed in various malignancies [39, 40]. Inactivation of miR-21 results in apoptosis induction, inhibition of growth, and malignant progression [39–41]. In a transgenic mice model, miR-155 induced polyclonal pre-B-cell proliferation resulting in B-cell leukemia [42]. miR-373 and miR-520c are known to promote migration, invasion, and metastasis of breast cancer cells by targeting CD44 expression [43]. Moreover, the roles of miR-373 and miR-520c in promoting migration and invasion of prostate cancer cells have also been reported [44]. In pancreatic cancer, miR-424-5p is overexpressed, and its high expression has been reported to be associated with enhanced proliferation and apoptosis resistance through downregulating SOCS6 [45]. miR-10b is overexpressed in various malignancies and promotes cell migration, invasion, and metastasis [46–48]. Moreover, a high expression of miR-10b correlated with disease progression [47, 49]. miR-221 and miR-222 are known to facilitate tumor cell growth, malignant potential, and EMT in multiple malignancies such as prostate, breast, and thyroid cancer [50–53].

Moreover, overexpression of miR-221 and miR-222 was shown to impart tamoxifen-resistance in breast cancer cells [54]. Altogether, these findings indicate that miRNAs play important roles as oncogenes in cancer cells.

4. Modulation of miRNAs by Phytochemicals

miRNAs are being considered as attractive targets for cancer prevention and therapy due to their oncogenic or tumor suppressor activities. Various studies have suggested that the modulation of miRNAs serves as one of the key mechanisms in the anticancer activities of a variety of phytochemicals (Table 1). Below, we describe some of the phytochemicals which are known to regulate miRNA expression in cancer.

4.1. Resveratrol. Resveratrol is a stilbenoid that has been shown to have anticancer activities against various cancers

TABLE 1: Phytochemical-mediated regulation of miRNAs in cancer. Modulation of miRNA expression by certain phytochemical agents and effect on cancer pathobiology.

Phytochemical	miRNA	Function	Reference
Resveratrol	↑ miR-141, miR-663, and miR-200c ↓ miR-17, miR-21, miR-25, miR-92a-2, miR-103-1, and miR-103-2	Invasiveness, EMT, and metastasis	[57–61, 114]
EGCG	↑ miR-16, miR-210, miR-7-1, miR-34a, and miR-99a ↓ miR-21, miR-98-5p, miR-92, miR-93, and miR-106b	Apoptosis Proliferation, anchorage-independent growth, and drug resistance	[62–66]
Genistein	↑ miR-16 ↓ miR-221 and miR-222	Apoptosis Growth	[67, 68]
Curcumin	↑ miR-15a, miR-16, and miR-186* ↓ miR-21	Apoptosis Metastasis	[80, 82, 83]
Quercetin	↑ let-7 ↓ miR-27a	Growth Apoptosis	[88, 89]
Camptothecin	↓ miR-125b	Apoptosis	[92]
DIM	↑ miR-21, miR-200b, miR-200c, let-7b, let-7c, let-7d, let-7e, and miR-146a	Growth, EMT, drug resistance, invasion, and metastasis	[95–97]

including breast cancer, lung cancer, glioma, prostate cancer, colon cancer, and neuroblastoma [55, 56]. Resveratrol reduced the expression of numerous oncogenic miRNAs, namely, miR-17, miR-21, miR-25, miR-92a-2, miR-103-1, and miR-103-2, in human colon cancer cells [57]. Moreover, in the same study, tumor suppressor miR-663 levels were shown to be restored in human colon cancer cells after the treatment of resveratrol. In another study, Hagiwara et al. reported that resveratrol treatment upregulated miR-141 and resulted in a significant reduction of invasiveness, whereas resveratrol-induced miR-200c expression caused reversal of EMT through downregulation of Zeb1 and upregulation of E-cadherin [58]. It has been demonstrated that the anti-cancer effect of resveratrol on pancreatic cancer cells was due to inhibition of oncogenic miR-21 [59]. Moreover, the synergistic antitumor activity of resveratrol and miR-200c has been demonstrated in human lung cancer cells [60]. In colon cancer cells, resveratrol inhibited the cell growth and induced apoptosis through upregulating miR-34a expression [61].

4.2. Epigallocatechin-3-gallate (EGCG). Epigallocatechin-3-gallate is a polyphenol flavonoid that possesses significant antioxidant and anticancer properties. It has been shown that EGCG induces apoptosis in hepatocellular carcinoma through enhanced expression of miR-16 [62]. Increased expression of miR-16 resulted in inhibition of its target antiapoptotic Bcl-2, followed by mitochondrial dysfunction, cytochrome c release, and subsequent apoptosis. EGCG also inhibited the expression of miR-21 followed by repression of androgen receptor (AR) signaling and, consequently, a reduction of prostate cancer cell growth [63]. In lung cancer, EGCG upregulated the expression of miR-210, which led to the inhibition of proliferation and anchorage-independent growth [64]. EGCG enhanced the efficacy of cisplatin through downregulation of miR-98-5p in A549 non-small lung cancer cells [65]. A combination of N-(4-hydroxyphenyl) retinamide and

EGCG decreased the expression of oncogenic miRs (miR-92, miR-93, and miR-106b) and enhanced the expression of tumor suppressor miRs (miR-7-1, miR-34a, and miR-99a) which resulted in growth inhibitory effects in human malignant neuroblastoma cells [66].

4.3. Genistein. Genistein is another important polyphenol that showed significant anticancer effects through the regulation of miRNAs. Genistein treatment was shown to enhance apoptosis synergistically with miR-16 in human chronic lymphocytic leukemia cells [67]. In a study on prostate cancer, genistein both downregulated miR-221 and miR-222 and restored tumor suppressor gene aplasia Ras homolog member I (ARHI) expression, which ultimately resulted in anticancer effects [68]. In another study on prostate cancer, genistein inhibited the migration and invasion of PC3 and DU145 cells through downregulating oncogenic miR-151 [69]. Xu et al. have shown that treatment of ovarian cancer cells with genistein caused an inhibition of cell growth and migration through suppression of miR-27a [70]. Further, genistein has been shown to upregulate the tumor suppressor miR-574-3p in prostate cancer cells [71]. Moreover, it has been observed that genistein exerted its antitumor effect in prostate cancer via downregulation of miR-1260b [72]. Genistein treatment downregulated oncogenic miR-1260b and resulted in inhibition of Wnt-signalling in renal cancer cells [72]. miR-223 expression was found to be downregulated in pancreatic cancer cells after genistein treatment that correlated with cell growth inhibition and induction of apoptosis [73]. Genistein also plays a tumor suppressor role through inhibition of miR-27a in pancreatic cancer cells [74].

4.4. Curcumin. Curcumin is a constituent of turmeric (*Curcuma longa*) and has been used as an important component of spice in Indian food and as a traditional medicine in Asian

countries for many decades [75]. It possesses chemopreventive and chemotherapeutic activities against many tumors [75–78]. Curcumin exerts its therapeutic effects by regulating miRNAs known to play an important role in cancer [79]. Yang et al. have shown that curcumin upregulated miR-15a and miR-16 in MCF-7 breast cancer cells which caused an induction of apoptosis [80]. In another study, curcumin treatment resulted in the upregulation of tumor suppressor miR-203 in bladder cancer that led to apoptosis induction and diminished proliferation, migration, and invasion [81]. Curcumin has also been shown to induce tumor suppressor miR-186* expression to promote apoptosis in lung cancer [82]. Moreover, curcumin inhibited the transcriptional regulation of oncogenic miR-21 in colon cancer, causing inhibition of growth, invasion, and metastasis [83]. Zhao et al. provided evidence that curcumin exerts its cytotoxic effects against SKOV3 ovarian cancer cells largely through upregulation of miR-9 [84]. Another tumor suppressor, miR181b, has been demonstrated to be induced by curcumin, and it inhibited breast cancer metastasis via downregulation of the inflammatory cytokines CXCL1 and CXCL2 [85]. High levels of miR-221 expression have been correlated with shorter survival in pancreatic cancer patients, suggesting that miR-221 could be an oncogenic miRNA [86]. In the same study, the synthetic curcumin analogue (CDF) has been found to suppress the expression of miR-221 and upregulate the expression of PTEN, p27 (kip1), p57 (kip2), and PUMA, followed by inhibition of cell proliferation and migration of pancreatic cancer cells. Thus, altogether, these studies provide evidence that curcumin modulates the expression of miRNA signatures in cancer cells to confer its anticancer activity.

4.5. Quercetin. Intake of a quercetin-rich diet has been demonstrated to modulate the expression of 48 unique miRNAs. These miRNAs have been reported to decrease tumor metastasis and invasion (miR-146a/b, 503, and 194), inhibit cell proliferation (miR-125a, 155, let-7 family, 302c, 195, 26a, 503, and 215), induce apoptosis (miR-125a, 605, 26b, let-7g, 34a, 491, and 16), and upregulate tumor suppressor miRNAs (let-7 family, miR-125a, 183, 146a, 98, 19b, 106a, and 381) [87]. Del Follo-Martinez et al. reported that quercetin treatment induced apoptosis in colorectal cancer cells when used along with resveratrol. The underlying mechanism of apoptosis induction is the downregulation of oncogenic miR-27a [88]. In another study, quercetin, when used with catechins, was shown to enhance the expression of let-7 in pancreatic cancer cells followed by K-ras inhibition and reduction of the advancement of pancreatic cancer [89].

4.6. Camptothecin (CPT). Camptothecin, an alkaloid isolated from bark of *Camptotheca acuminata*, is a potent chemotherapeutic agent against a variety of tumors [90–92]. CPT was demonstrated to reduce the expression of miR-125b significantly, which led to the upregulation of Bak1 and p53 and resulted in apoptosis of human cervical cancer and myelogenous leukemia cells [92]. In a recent study, camptothecin was shown to inhibit HIF-1 α by enhancing the levels of miR-155, miR-17-5p, and miR-18a in HeLa cells [93].

4.7. Diindolylmethane (DIM). Diindolylmethane is an active compound that is generated in the stomach through the metabolic conversion of indole-3-carbinol (I3C), present in cruciferous vegetables [94]. DIM regulates the expression of numerous miRNAs involved in cancer development and progression. DIM has been demonstrated to induce the expression of certain miRNAs, such as miR-200 and let-7 families, that led to the reversal of EMT and enhanced chemosensitivity in gemcitabine-resistant pancreatic cancer cells [95]. DIM also induced the expression of miR-146a, which resulted in reduced pancreatic cancer cell invasion via inhibition of metastasis-associated protein 2 (MTA-2), interleukin-1 receptor-associated kinase 1 (IRAK-1), and NF κ B [96]. Moreover, Jin observed that DIM inhibited breast cancer cell growth by enhancing the expression of miR-21 which led to the degradation of its target Cdc25A [97]. Formulated 3,3'-diindolylmethane (BR-DIM) has been shown to be capable of downregulating miR-221, resulting in growth inhibition of pancreatic cancer cells [86].

5. Mechanism of miRNA Regulation by Phytochemicals

There have been several aberrantly expressed miRNAs identified in various cancer types. Unfortunately, the precise mechanisms that regulate the normal expression of miRNAs or their deregulation in cancer remain unclear. Growing evidence suggests that aberrant transcriptional regulation, epigenetic changes, alterations in miRNA biosynthesis machinery, mutations, or DNA copy number could contribute to miRNA dysregulation in human cancer [98]. Experimental studies on phytochemicals revealed that the expression of various miRNAs can be regulated by phytochemicals. In the following section, we provide an overview for the regulation of miRNAs by phytochemicals through various mechanisms such as epigenetic, transcriptional, and miRNA processing (Figure 1).

5.1. Transcriptional Regulation of miRNAs. Various studies indicate the involvement of certain transcription factors in the regulation of miRNA expression and the subsequent modulation of pathological conditions in cancer. Thus, transcription factor-mediated miRNA regulation is one critical aspect of study. Several groups have observed that phytochemicals modulate miRNA expression through transcriptional regulation (Figure 1). Wang and coworkers demonstrated that treatment with EGCG results in the transcriptional activation of miR-210 in lung cancer by promoting the binding of hypoxia inducible factor-1 α (HIF-1 α) to the hypoxia response element present in the promoter region of miR-210 [64]. Transcription factor activator protein (AP-1), an important regulator of genes involved in cell proliferation and extracellular matrix production [99], is an upstream regulator of miR-21 [100]. Mudduluru and coworkers demonstrated that curcumin inhibits the transcriptional regulation of miR-21 by downregulating AP-1 to suppress tumor growth, invasion, and metastasis of colorectal cancer [83]. Another transcription factor, CCAAT/enhancer binding protein beta

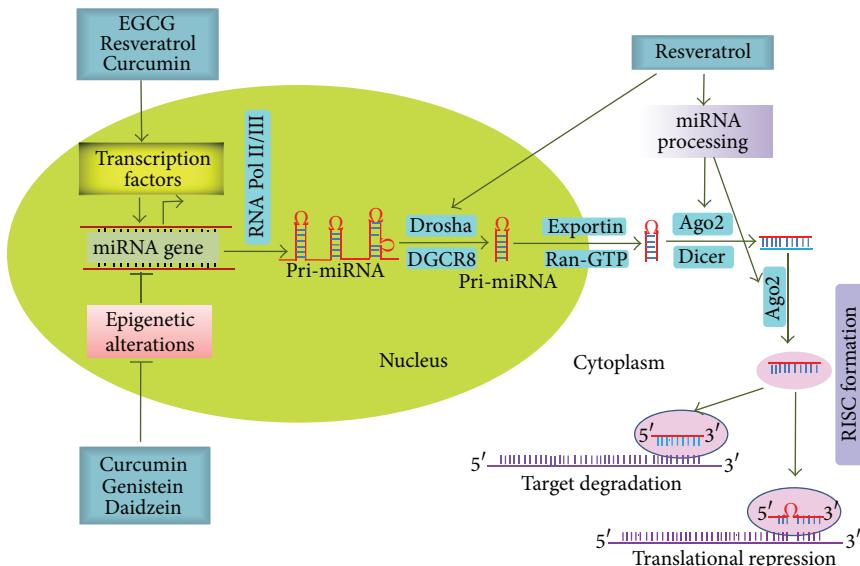


FIGURE 1: Mechanistic overview of miRNA regulation by phytochemicals. Phytochemicals regulate the expression of miRNAs through modulation of transcription factors and inducing epigenetic modifications and by interfering with processes associated with miRNA maturation.

(C/EBP- β), negatively regulates tumor suppressor miR-145 by directly interacting through the putative C/EBP- β binding site present in the miR-145 promoter. It was also shown that resveratrol treatment resulted in the decreased activation of C/EBP- β which subsequently induced the expression of miR-145 in breast cancer cells [101]. Androgen receptor (AR) directly regulates miR-21 by binding to the miR-21 promoter in prostate cancer [102]; Siddiqui and coworkers (2011) demonstrated that EGCG inhibited prostate cancer cell growth by decreasing the level of AR and miR-21 [63]. A study conducted by Hagiwara and coworkers has shown that resveratrol transcriptionally upregulated the expression of several tumor suppressor miRNAs such as miR-141, miR-26a, miR-195, miR-126, miR-340, miR-34a, miR-193b, miR-335, miR-200c, miR-497, miR-196a, and miR-125a-3p in MDA-MB-231 breast cancer cells [58]. It is known that p53 is a tumor suppressor gene and a transcription factor which functions by either causing growth arrest or inducing apoptosis [103]. Emerging data suggest that p53 transcriptionally regulates several miRNAs [104]. A number of studies have shown that curcumin regulates the expression of several miRNAs that are transcriptional targets of p53 such as miR-22, miR-15/16a, miR-34, and miR-21 [80, 83, 105–108]. Considering all these observations, it is speculated that curcumin regulates the expression of p53, which in turn modulates the expression of several miRNAs.

5.2. Epigenetic Regulation of miRNAs. Epigenetic regulation is defined as modifications of the genome without any change to the nucleotide sequence. Supporting evidence suggests that epigenetic modification, like aberrant CpG methylation or histone modifications, contributes to the dysregulation of gene expression in tumor cells. Pharmacologic inhibition of

DNA methyltransferase (DNMT) causes DNA demethylation and upregulates the expression of miRNAs. Saini and coworkers provided evidence that curcumin treatment resulted in the hypomethylation of miR-203 promoter and subsequent upregulation of miR-203, which is epigenetically silenced in various malignancies [81]. Treatment with a curcumin analogue, difluorinated curcumin, restored the expression of miR-34a and miR-34c by working as a demethylating agent in colon cancer cells [109]. Rabiau and coworkers performed miRNA expression profiling following the treatment with the flavonoids genistein and daidzein in prostate cancer cells [110]. Their investigation revealed a significant upregulation of miR-548b-5p in PC3 and miR-15a in LNCaP cells by genistein and daidzein through regulation at the epigenetic level [110]. Zaman et al. demonstrated that miR-145 is inactivated as a result of its promoter methylation in prostate cancer; treatment with genistein demethylated the promoter of miR-145 which resulted in an increased level of miR-145 [111]. In a separate study on prostate cancer, isoflavone efficiently demethylated the promoter region of miR-29a and miR-1256 and subsequently upregulated their expression [112]. All these observations suggest the significant role of phytochemicals in the regulation of miRNAs at the epigenetic level (Figure 1).

5.3. Regulation of miRNA Processing by Phytochemicals. miRNA processing can be regulated at various steps, and any alteration in processing either increases or decreases the level of miRNAs [113]. The role of phytochemical-modulated expression of miRNAs through the regulation of proteins involved in miRNA processing has been recently investigated, as shown in Figure 1. Hagiwara and coworkers investigated and reported that resveratrol treatment significantly increased the expression of Ago2 and results in enhanced

levels of tumor suppressor miRNAs such as miR-16, miR-141, miR-143, and miR-200c in MDA-MB-231 cells [58]. Moreover, resveratrol treatment resulted in the enhanced level of miR-663 and pre-miR-663 by interfering with the drosha mediated processing of pri-miR-663 that resulted in the inhibition of miR-155, which is overexpressed in many cancers [114]. These studies provide evidence that phytochemicals regulate the expression of miRNAs epigenetically and transcriptionally and by controlling miRNA processing.

6. Conclusion and Future Perspective

miRNAs are a novel class of gene regulators. Thus far, we have only been able to see just a small part of the complexity of cellular regulation by miRNAs with regard to all of the different functions that miRNAs can perform. miRNAs are aberrantly expressed in most cancers and this has been correlated throughout cancer initiation and progression; hence, miRNAs represent very attractive and novel targets for cancer therapy. Phytochemicals display an inimitable ability to alter the level of miRNAs involved in regulation of cancer pathobiology by modulating the expression of miRNAs through a variety of mechanisms, namely, epigenetic, transcriptional, and miRNA processing. In addition, phytochemicals increase the chemosensitivity of conventional therapeutic drugs through modulating miRNAs. As a result, phytochemicals can be exploited for designing therapeutic approaches in combination with conventional therapies to improve cancer treatment and prevention strategies. Despite the potent anticancer activities of phytochemicals, there are some concerns such as specific targeting and bioavailability. To overcome these hurdles in the use of phytochemicals, different approaches are being explored, namely, chemical modification, synthetic formulation, delivery by nanoparticles, and so forth. Hence, there is a wide scope for the development of phytochemicals into commercial drugs to efficaciously prevent and treat cancer.

Conflict of Interests

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

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Review Article

Genetic Networks Lead and Follow Tumor Development: MicroRNA Regulation of Cell Cycle and Apoptosis in the p53 Pathways

Kurataka Otsuka^{1,2} and Takahiro Ochiya¹

¹ Division of Molecular and Cellular Medicine, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan

² Division of Research and Development, Kewpie Corporation, Sengawa-cho, Chofu-shi, Tokyo 182-0002, Japan

Correspondence should be addressed to Kurataka Otsuka; kurataka.otsuka@kewpie.co.jp and Takahiro Ochiya; tochiya@ncc.go.jp

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During the past ten years, microRNAs (miRNAs) have been shown to play a more significant role in the formation and progression of cancer diseases than previously thought. With an increase in reports about the dysregulation of miRNAs in diverse tumor types, it becomes more obvious that classic tumor-suppressive molecules enter deep into the world of miRNAs. Recently, it has been demonstrated that a typical tumor suppressor p53, known as the guardian of the genome, regulates some kinds of miRNAs to contribute to tumor suppression by the induction of cell-cycle arrest and apoptosis. Meanwhile, miRNAs directly/indirectly control the expression level and activity of p53 to fine-tune its functions or to render p53 inactive, indicating that the interplay between p53 and miRNA is overly complicated. The findings, along with current studies, will underline the continuing importance of understanding this interlocking control system for future therapeutic strategies in cancer treatment and prevention.

1. Introduction

Cancer is commonly an age-related disease triggered by the accumulation of genomic mutations that lead to the dysregulation of tumor-suppressive genes and/or protooncogenes. For example, the functions of *TP53* (tumor-suppressive gene) and *c-MYC* (oncogene) have been extensively investigated, and their critical roles in complexly regulating tumorigenesis, including cell-cycle progression/arrest, apoptosis, senescence, and energy metabolism, have been uncovered [1–4]. Specifically, the significance of tumor suppressor p53 has been suggested by the fact that DNA mutation or loss of *TP53* is observed in many types (over 50%) of human tumors and by the possibility that the dysfunctions affect the p53 signaling network in over 80% of tumors [5, 6]. As a transcriptional activator, the p53 protein induces various kinds of tumor-suppressive genes, such as *p21* (G_1 /S-arrest), *14-3-3σ* (G_2 /M-arrest), and *PUMA* (apoptosis) [7–10]. p53 has also been reported to negatively regulate specific proteins: for instance, the p53-mediated repression of the cell-cycle regulators, such

as cyclin-dependent kinase 4 (CDK4) and cyclin E2, may lead to cell-cycle arrest [10, 11]. These prove the pivotal roles of p53 as a cellular gatekeeper.

Recently, it has been realized that small noncoding RNAs known as microRNAs (miRNAs) contribute to many human diseases, including cancers; that a general downregulation of miRNAs is observed in cancers as compared with normal tissues; and that miRNA expression profiles can be used to classify poorly differentiated tumors [12]. In addition, some kinds of miRNAs are shown to be connected to a well-studied tumor-suppressive or oncogenic network [13]. It remains to be investigated how miRNAs are regulated by transcription factors, but it is suggested that p53 enters the miRNA world to control the expression patterns of some miRNAs and promote cell-cycle arrest and apoptosis through the miRNA effector pathway. *miR-34a* is one of the representative miRNAs under the direct control of p53, and this upregulation induces cell-cycle arrest and apoptosis [14–18]. Moreover, there are many studies about miRNA effects on cell proliferation and survival in cancers, with attention

given to the interplay between p53 and the miRNA network. In this review, we will focus on the regulation of the cancer cell cycle and apoptosis by miRNA linked with the p53 axis. We will also summarize the key miRNAs concerned with the cell cycle and apoptosis in cancers.

2. miRNA Discovery, Biogenesis, and Mechanism

The first miRNAs discovered were *lin-4* and *let-7*, both of which are the key regulators in the pathway controlling the timing of postembryonic development in *Caenorhabditis elegans* [19–21]. After this discovery, miRNAs have been identified in diverse organisms, such as worms, flies, mice, humans, and plants. Several miRNAs are conserved among different species, indicating that these miRNAs might have important functions and modulate gene expression. Currently, in humans, over 2,000 microRNAs have been identified or predicted based on the miRBase database (<http://www.mirbase.org/>). Computational analyses suggest that about 5,300 genes contain miRNA target sites: ~30% of human genes might be subject to the translational regulation of miRNAs [22, 23].

miRNAs are initially transcribed by RNA polymerase II/III into primary transcripts (pri-miRNAs) [24, 25], which are processed by the complex of RNase III enzyme, Drosha, and its partner DGCR8 [26]. The pri-miRNAs are converted into ~65 nucleotides (nt) of a stem-loop precursor (pre-miRNA) [27]. These pre-miRNAs are transported to cytoplasm by Exportin-5/Ran-GTP and processed by another RNase III, Dicer, to generate a double-strand RNA of about 19–25 nt in length [28–30]. One strand of miRNA gives rise to the mature miRNA, which is incorporated into the RNA-induced silencing complex (RISC). The miRNAs guide the RISC complex to the 3'-untranslated region (3'-UTR) of the target mRNAs, leading to the translational repression or destabilization of the mRNA [31, 32]. In animal systems, the recognition of target mRNA usually requires the “seed” sequence, which is 2–8 nt from the 5'-end of the miRNA [22, 33]. Unlike with plant systems, because of this imperfect complementarity, there are extensive base-pairings to the sequence of mRNAs, and this makes it more complicated to predict miRNA targets and study miRNA biology. Recently, it has been shown that animal miRNAs can induce the degradation of target mRNAs (mRNA degradation and decay) besides translational repression: inhibition of translation elongation; cotranslational protein degradation; competition for the cap structure; and inhibition of ribosomal subunit joining [34–37]. However, the exact order and impact of these events still need to be investigated further.

3. p53 Transactivation Function in a Relationship with Tumorigenesis

Based on numerous studies at both structural and functional levels, p53 is known as a key player in genome stability and tumor suppression. In an unstressed condition, the expression level of p53 is kept low by the activity of an E3

ubiquitin ligase, mouse double minute 2 (MDM2) [38–40]. Under stressed conditions, p53 is activated in response to diverse intrinsic and extrinsic signals, such as DNA damage, oncogene activation, and hypoxia. As a sequence-specific transcription factor, the activated p53 acts directly on cancer-associated pathways to suppress tumor progression by modulating cell-cycle arrest, senescence, apoptosis, angiogenesis, or invasion and metastasis [41–43]. There are also demonstrations showing that p53 is involved in the regulation of DNA repair, oxidative stress, energy metabolism, and differentiation [44–48]. The approach of genome-wide analyses has identified many p53-binding sites and p53-regulated genes which are related to tumorigenesis and various stress signals [49, 50]. Recent works have highlighted that p53 directly induces some specific miRNAs which function as tumor suppressors through a novel transcriptional mechanism. Now, although unknown aspects of the mechanism still need to be investigated, the cooperative contribution of p53 and miRNAs has been shown to be more important for tumor formation and development.

4. miRNA Network with p53: Cell Cycle and Apoptosis

4.1. miR-34 Family. In 2007, several groups reported that the *miR-34* family members are direct p53 targets and that their expression level is strongly upregulated by genotoxic stress in a p53-dependent manner, inducing cell-cycle arrest and apoptosis [14–16, 51, 52]. In mammals, the *miR-34* family is composed of *miR-34a*, *miR-34b*, and *miR-34c*, which are encoded by two different genes in the *miR-34a* and *miR-34-b/c* loci. With the overexpression of the *miR-34* family in certain kinds of cell lines, microarray analyses unveiled hundreds of putative candidate target genes of *miR-34s* [15, 16, 18]. Actually, ectopic expression of *miR-34s* promotes cell-cycle arrest in the G₁ phase, senescence, and apoptosis by directly repressing CDK4, CDK6, cyclin E2, E2F3, MYC, and B-cell CLL/lymphoma 2 (BCL-2) [53]. Note that the triggering event of cell-cycle arrest or apoptosis by *miR-34s* depends on the cell type and context, and the expression level of *miR-34s* would affect the decision to proceed [15, 17, 54]. As seen in the decreased expression of *miR-34s* in several types of malignant cancers, the *miR-34* family powerfully prevents tumorigenesis in general.

In addition to the *miR-34* family, p53 is also engaged in the direct regulation of the transcriptional expression of additional miRNAs, such as *miR-107*, *miR-143/145*, *miR-192/194/215*, *miR-200c/141*, the *let-7* family, and the *miR-17-92* cluster (Figure 1 and Table 1).

4.2. miR-107. *miR-107* is encoded within an intron of pantothenate kinase 1 (*PANK1*), and *miR-107* and its host gene are directly activated by p53 under hypoxia condition or with the treatment of DNA damage agents [55, 56]. Hypoxia induces angiogenesis, which is essential for solid tumors to grow in severe environments. *miR-107* inhibits hypoxia signaling and antiangiogenesis by repressing the expression of hypoxia inducible factor-1 β (HIF-1 β), which interacts with HIF-1 α to form the HIF-1 transcription factor complex [55].

TABLE 1: Key microRNAs regulated by p53.

miRNA	Genomic location	Cancer type	Target	Phenotype	References
<i>miR-34s</i>	1p36 and 11q23	Colon cancer, neuroblastoma, pancreatic cancer, CLL, NSCLC, OSCC, breast cancer, bladder cancer, kidney cancer, melanoma	CDK4, CDK6, cyclin E2, E2F3, MYC BCL-2	Cell-cycle arrest apoptosis	[14– 16, 18, 51, 53, 54]
<i>miR-107</i>	10q23	Colon cancer, breast cancer	CDK6, P130	Cell-cycle arrest	[55–57]
<i>miR-145</i>	5q23	Colon cancer, breast cancer, MDS, prostate cancer	MYC, E2F3, cyclin D2, CDK4, CDK6	Cell-cycle arrest	[58–61]
<i>miR-192/215</i>	1q41 and 11q13	Colon cancer, lung cancer, multiple myeloma, renal cancer	CDC7, MAD2L1	Cell-cycle arrest	[66–69]
<i>miR-200c</i>	12p13	Breast cancer, ovarian cancer	FAP-1	Apoptosis	[71, 81]
<i>let-7</i>	Multiple locations (11 copies)	Lung cancer, colon cancer, ovarian cancer, breast cancer, lymphoma	CDK6, CDC25A, cyclin D, CDC34, MYC, E2F1, E2F3	Cell-cycle arrest	[61, 83–90]
<i>miR-15a/16-1</i>	13q14	B-CLL, pituitary adenomas, gastric cancer, NSCLC, prostate cancer, ovarian cancer, pancreatic cancer	CDK1, CDK2, CDK6, cyclin D1, D3, E1 BCL-2	Cell-cycle arrest apoptosis	[98–112]

CLL: chronic lymphocytic leukemia; NSCLC: non-small cell lung cancer; OSCC: oral squamous cell carcinoma; MDS: myelodysplastic syndromes; B-CLL: B-cell chronic lymphocytic leukemia.

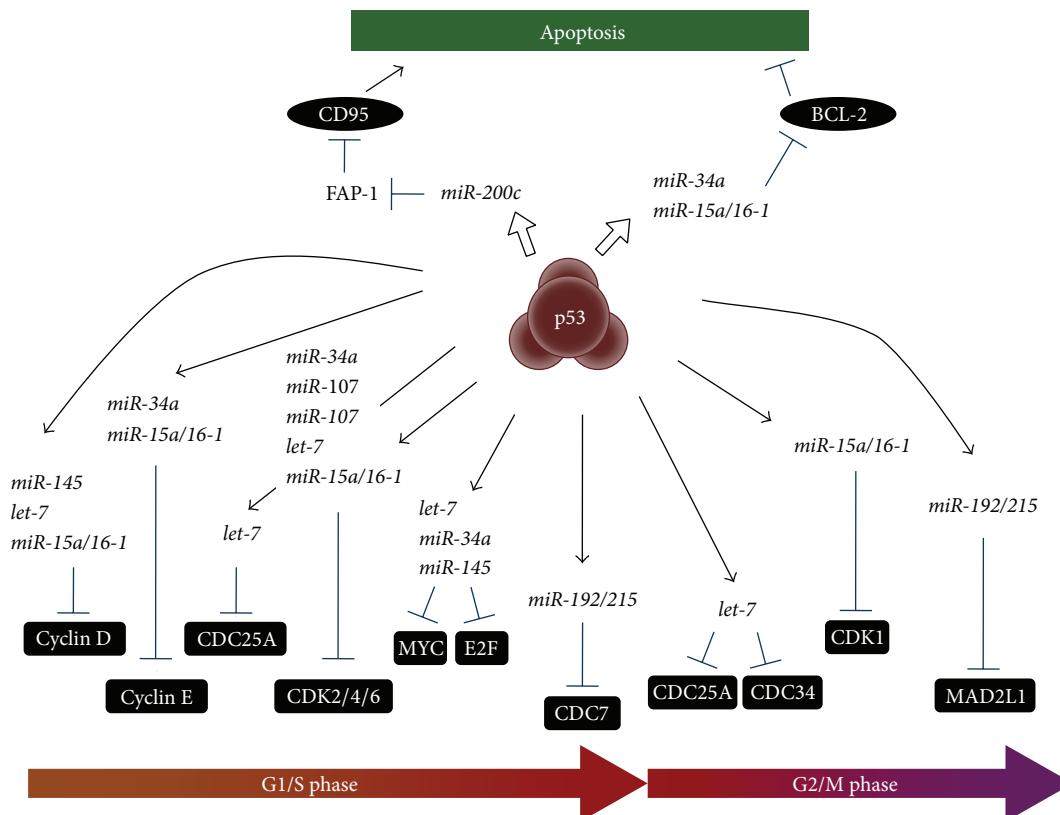


FIGURE 1: p53-induced miRNAs control cell cycle and cell survival. p53 directly induces many kinds of miRNAs, which repress cell-cycle regulators and/or antiapoptotic proteins and contribute to cell-cycle arrest and apoptosis. The miRNAs regulating apoptosis are shown in the top part of this figure, and the miRNAs regulating the cell cycle are at the bottom.

Furthermore, *miR-107* promotes cell-cycle arrest in the G₁/S phase via targeting the cell-cycle activator CDK6 and the antimitogenic p130 [56]. Nevertheless, *miR-107* has another aspect for directly targeting *DICER1* mRNA and the high level of *miR-107* might affect the production and function of p53-induced miRNAs [57].

4.3. *miR-145*. It has been reported that the expression of *miR-145* is frequently decreased in colon tumors, breast and prostate cancers and that the chromosomal region (chromosome 5 [5q32-33] within a 4.09 kb region) is deleted in myelodysplastic syndrome, suggesting *miR-145* acts as a tumor suppressor [58–61]. The expression of *miR-145* is transcriptionally induced by p53, and *miR-145* downregulates c-MYC, E2F3, cyclin D2, CDK4, and CDK6 and leads to G₁ cell-cycle arrest [62, 63].

Recently, it has been found that *miR-145* contains several CpG sites in its promoter region and that the expression of *miR-145* is affected by epigenetic events such as DNA methylation [60]. The CpG regions are located adjacent to p53 response element upstream of *miR-145*, and DNA hypermethylation inhibits p53 from binding to *miR-145*. In addition to this miRNA, it has been reported that *miR-34a*, *miR-124a*, and *miR-127* are downregulated by DNA methylation [64].

4.4. *miR-192/215*. *miR-192* and *miR-215* share a similar seed sequence and are composed of two clusters: the *miR-215/miR-194-1* cluster on chromosome 1 (1q41) and the *miR-192/miR-194-2* cluster on chromosome 11 (11ql3.1) [65]. *miR-192* and *miR-215* are downregulated in colon cancers, lung cancers, multiple myeloma, and renal cancers [66–69]. Some studies have suggested that these miRNAs are also under the control of p53 and can induce p21 expression and cell-cycle arrest in a partially p53-dependent manner [66, 70]. Gene expression analyses indicated that *miR-192* and *miR-215* target a number of transcripts that regulate DNA synthesis and the G₁ and G₂ cell-cycle checkpoints, such as CDC7 and MAD2L1 [70]. Therefore, *miR-192/215* functions as a tumor suppressor contributing to the G₁ and G₂/M cell-cycle arrest.

4.5. *miR-200c*. It is well known that p53 acts as an important regulator in modulating epithelial-mesenchymal transition (EMT) that is implicated in tumor progression, metastasis, and the correlation of poor patient prognosis [71, 72]. The p53-induced *miR-200c* represses EMT by targeting the E-cadherin transcriptional repressors ZEB1 and ZEB2, Krüppel-like factor 4 (KLF4), and the polycomb repressor BMI1, all of which are involved in the maintenance of stemness [73–80]. Moreover, *miR-200c* contributes to the induction of apoptosis in cancer cells via the apoptosis-inducing receptor CD95 by targeting the apoptosis-inhibitor FAS-associated phosphatase 1 (FAP-1) [81].

4.6. *let-7a* and *let-7b*. *let-7* is known to be important for the regulation of development and is evolutionarily conserved across bilaterian phylogeny [82]. In humans, some *let-7* gene clusters are located in fragile regions involved in cancers [61]. In lung cancers, it has been reported that the downregulated

expression of *let-7* members is correlated with poor prognosis [83, 84]. Recent works suggested that *let-7a* and *let-7b* expression is dependent on p53 in response to genotoxic stress and *let-7* miRNAs target CDK6, CDC25A, cyclin D, CDC34, and MYC [85–89]. On the other hand, *let-7a-d* and *let-7i* are direct targets of E2F1 and E2F3 during the G₁/S transition and are repressed in E2F1/3-null cells [90]. The *let-7* family plays multiple roles in the regulation of the cell cycle and goes a long way toward suppressing tumor progression.

4.7. *miR-17-92 Cluster*. The *miR-17-92* cluster consists of *miR-17-5p*, *miR-17-3p*, *miR-18a*, *miR-19a*, *miR-20a*, *miR-19b*, and *miR-92-1*. Some of these are known to be oncogenic, as suggested in the research showing that the cluster is upregulated in human B-cell lymphoma and amplified in malignant lymphoma [91, 92].

Different from the miRNAs mentioned above, *miR-17-92* miRNAs are more or less repressed transcriptionally by p53 under hypoxia, which leads to the p53-mediated apoptosis [93]. The p53-binding site overlaps with the TATA box of the *miR-17-92* promoter region, and p53 prevents the TATA-binding protein (TBP) transcription factor from binding to the site during hypoxic conditions. Moreover, *miR-17-92* is transcriptionally regulated by c-Myc [94]. Although c-Myc is repressed by p53 activation under some stress conditions, the repression of *miR-17-92* is not dependent on c-MYC but on p53 under hypoxia [93, 95].

Note that some members of *miR-17-92* are likely to function as tumor suppressors in different cancers. For example, in breast cancer, *miR-17-5p* represses the expression of the nuclear receptor coactivator amplified in breast cancer 1 (*AIB1*) that enhances the transcription activity of E2F1 to promote the cell proliferation of breast cancer cells [96]. A recent study showed that *miR-17-3p* reduces tumor growth by targeting MDM2 in glioblastoma cells [97].

4.8. *miR-15a/miR-16-1*. *miR-15a* and *miR-16-1* were identified to be deleted and/or downregulated in approximately 68% of B-cell chronic lymphocytic leukemia (B-CLL) [98], as is the case in pituitary adenomas [99], gastric cancer cells [100], prostate cancer [101–104], non-small cell lung cancer [105, 106], ovarian cancer [107], and pancreatic cancer [108], which indicates their important functions for tumor formation. The miRNAs are encoded by an intron of a long noncoding RNA gene, deleted in lymphocytic leukemia 2 (*DLEU2*), and *DLEU2* (*miR-15a/miR-16-1*) was shown to be transactivated by p53 [109]. In addition, p53 regulates the expression level of precursor and mature *miR-15a* and *miR-16-1* as well as *miR-143* and *miR-145* [110]. It has been reported that *miR-15a/miR-16-1* negatively regulates the antiapoptotic protein BCL-2 and the cell-cycle regulators, such as CDK1, CDK2, and CDK6, and cyclins D1, D3, and E1 [102, 110–112].

5. miRNAs Regulating Negative Regulators of p53

It has been shown that MDM2 negatively controls the stability and transcription activity of p53, which attenuates the tumor-suppressive functions of p53 [40]. Actually, overexpression

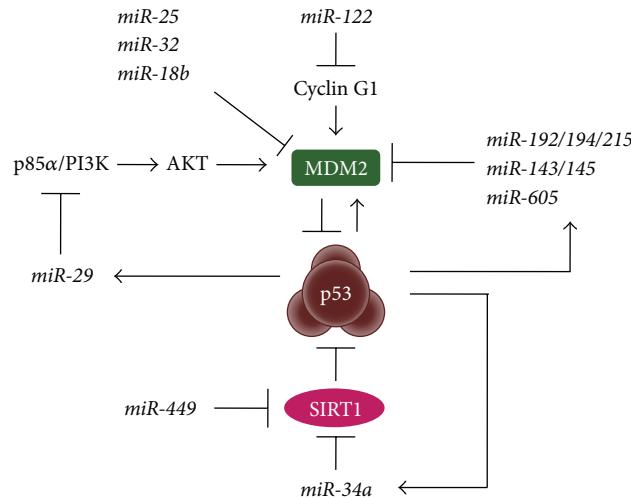


FIGURE 2: Indirect p53 regulation with miRNAs. p53 controls its stability and activity with the p53-inducible miRNAs that directly or indirectly target the negative regulators (MDM2 and SIRT1). *miR-25*, *miR-32*, *miR-18b*, and *miR-449* are not direct targets of p53 but repress the negative regulators and lead to p53 activation.

of MDM2 is often found in many types of human cancers, such as soft tissue sarcomas, brain tumors, and head and neck squamous cell carcinomas [113, 114]. On the flip side, p53 inhibits MDM2 expression using several miRNAs and establishes the regulatory circuit between p53 and MDM2 (Figure 2). For instance, *miR-192/194/215*, *miR-143/145*, and *miR-605*, which are the transcriptional targets of p53, directly inhibit MDM2 expression [68, 114, 115]. *miR-29* family members are also p53-inducible miRNAs and indirectly control the MDM2 level by targeting p85 α , a regulatory subunit of PI3 kinase (PI3K), in the PI3K/AKT/MDM2 axis [116, 117]. Furthermore, the *miR-29* family directly suppresses cell division cycle 42 (CDC42) and PPMID phosphatase, both of which negatively regulate p53 [116, 117]. While a liver-specific *miR-122* is not a transcriptional target of p53, the miRNA increases p53 activity through the downregulation of cyclin G1, which inhibits the recruitment of phosphatase 2A (PP2A) to dephosphorylate MDM2 and causes the decrease of MDM2 activity [118, 119]. Recent studies indicated that tumor-suppressive miRNAs, *miR-25*, *miR-32*, and *miR-18b* are also not transcriptionally regulated by p53 but affect the p53 pathway by targeting *MDM2* mRNA directly [120, 121].

Besides MDM2, a NAD-dependent deacetylase, silent information regulator 1 (SIRT1), increases the level of deacetylated p53 and negatively regulates the p53 activity [122, 123]. SIRT1 is targeted by the p53-inducible *miR-34a* and joins the positive feedback loop connecting the miRNA, SIRT1, and p53 (Figure 2) [124]. Additionally, *miR-449* participates in this regulatory circuit as the miRNA possesses a very similar seed sequence of *miR-34* members [125–127]. *miR-449* is upregulated by E2F1, not by p53, and *miR-34* and *miR-449* bring in an asymmetric network to balance the functions between p53 and E2F1.

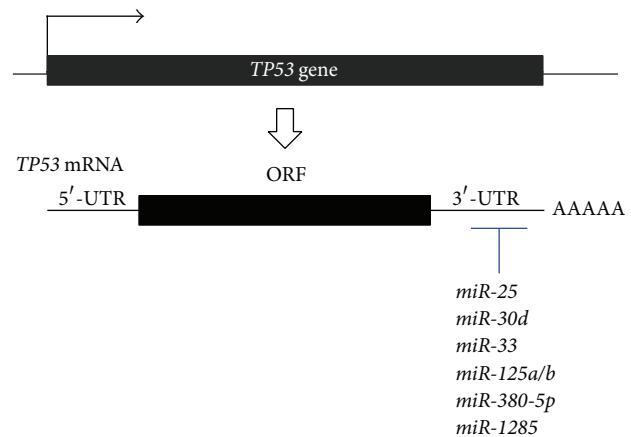


FIGURE 3: Direct p53 regulation by miRNAs. miRNAs directly interact with *TP53* mRNA by binding to sites in the 3'-UTR. This interaction inhibits the translation of mRNA, resulting in the repression of p53 activity. ORF: open reading frame.

6. miRNAs Directly Targeting *TP53* mRNA

As is the case in the control of negative regulators of p53 via miRNAs, p53 itself is repressed by several miRNAs through direct interaction with the 3'-UTR of *TP53* mRNA (Figure 3).

miR-125b is a first-identified p53-repressive miRNA and blocks the p53 expression level to suppress apoptosis in human neuroblastoma and lung fibroblast cells; in contrast, the knockdown of *miR-125b* leads to the opposite results [128]. Plus, *miR-125a*, an isoform of *miR-125*, was suggested to inhibit the translation of *TP53* by binding to a region of the 3'-UTR [129]. The high expression of *miR-125b* is associated with poor prognosis in patients with colorectal cancer [130].

Some studies have shown that *miR-125b* represses factors in the p53 network, including apoptosis regulators like *PUMA*, insulin-like growth factor-binding protein 3 (*IGFBP3*), and *BCL2*-antagonist/killer 1 (*BAK1*) and cell-cycle regulators like *cyclin C*, *CDC25C*, and cyclin-dependent kinase inhibitor 2C (*CDKN2C*) [131]. These suggest that *miR-125b* modulates and buffers the p53 pathway.

Subsequently, *miR-504* was reported to directly repress the p53 protein level and reduce the p53-mediated apoptosis and cell-cycle arrest in response to stress, and its overexpression promotes the tumorigenicity of colon cancer cells *in vivo* [132]. Additionally, *miR-380-5p*, *miR-33*, and *miR-1285* can downregulate the p53 protein expression by directly binding to the two sites in the 3'-UTR of *TP53*, resulting in the reduction of apoptosis and cell-cycle arrest [133–135]. Indeed, *miR-380-5p* is highly expressed in neuroblastomas with neuroblastoma-derived v-myc myelocytomatosis viral-related oncogene (*MYCN*) amplification, and the high expression level correlates with poor diagnosis [133]. More recently, *miR-30d* and *miR-25* also directly interacted with the 3'-UTR of *TP53* to decrease the p53 level. So then, these miRNAs affect apoptotic cell death, cell-cycle arrest, and cellular senescence in some cell lines, such as multiple myelomas, colon cancer, and lung cancer cells [136–138]. When taken together, the miRNAs targeting *TP53* would hinder p53 from exerting its tumor-suppressive functions (senescence, apoptosis, cell-cycle arrest, etc.) under stressed conditions.

7. Concluding Remarks

For more than a decade, small noncoding RNAs have become increasingly central to the study of tumor biology. The accumulating evidence of cancer-associated miRNAs reveals the missing link between classic tumor-suppressive networks and complex oncogenic pathways. In a stress situation, p53 directly induces various protein-coding genes such as *p21* and *PUMA* to contribute to cell-cycle arrest and apoptosis and, furthermore, utilizes tumor-suppressive miRNAs, such as *miR-34s*, *miR-107*, and *miR-145* (Figure 1 and Table 1). Some of the p53-inducible miRNAs target p53-negative regulators (*MDM2* and *SIRT1*), which creates a positive feedback loop to reinforce p53 stability and activity (Figure 2). However, as expected, miRNAs are not always on p53's side: p53-repressive miRNAs (*miR-125s*, *miR-504*, *miR-380-5p*, etc.) reduce the p53 expression level by binding to a region of the 3'-UTR of *TP53* mRNA and result in the inhibition of cell-cycle arrest and apoptosis (Figure 3). There will be more than one way to arrest the cell-cycle and/or induce apoptosis, and the balance between miRNAs and tumor suppressors might be crucial in deciding which strategy to adapt.

For future diagnostic and therapeutic advances, more extensive studies will be needed to find hidden messages in the tumor-suppressive networks of miRNA. The regulatory mechanism of the p53-miRNA circuit has been excellently shown, but the upstream regulators of almost all miRNAs are unknown at this time. What is more, regardless of computational prediction, the downstream targets of miRNA are hard to identify exactly because of the imperfect complementarity

and the possibility that miRNAs can bind to not only the 3'-UTR but also the 5'-UTR and coding regions.

In recent years, the competitive endogenous RNA (ceRNA) hypothesis has suggested that noncoding pseudogenes and long noncoding RNAs act as miRNA sponges, which is likely to counteract the effect of miRNAs on the target mRNA transcripts [139]. Therefore, we need to move deeper inside the world of noncoding RNAs in order to prevent and treat diverse cancers.

Besides the miRNAs described in this paper, there are many miRNAs related to cell-cycle regulation and apoptosis [140–142]. However, it is unclear how these miRNAs act additively/synergistically on tumor suppression. Even the longest journey to understand the role of miRNA begins with a single experiment. The next ten years will be more exciting in the quest to see cancer conquered.

Conflict of Interests

The authors declare no competing financial interests.

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Review Article

The Impact of Extracellular Vesicle-Encapsulated Circulating MicroRNAs in Lung Cancer Research

Yu Fujita,^{1,2} Kazuyoshi Kuwano,² Takahiro Ochiya,¹ and Fumitaka Takeshita¹

¹ Division of Molecular and Cellular Medicine, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan

² Division of Respiratory Diseases, Department of Internal Medicine, Jikei University School of Medicine, 3-19-18 Nishi-shinbashi, Minato-ku, Tokyo 105-8471, Japan

Correspondence should be addressed to Fumitaka Takeshita; futakesh@ncc.go.jp

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Lung cancer is the leading cause of cancer-related deaths. Biomarkers for lung cancer have raised great expectations in their clinical applications for early diagnosis, survival, and therapeutic responses. MicroRNAs (miRNAs), a family of short endogenous noncoding RNAs, play critical roles in cell growth, differentiation, and the development of various types of cancers. Current studies have shown that miRNAs are present in the extracellular spaces, packaged into various membrane-bound vesicles. Tumor-specific circulating miRNAs have been developed as early diagnostic biomarkers for lung cancer. Remarkably, some studies have succeeded in discovering circulating miRNAs with prognostic or predictive significance. Extracellular vesicles (EVs), such as exosomes and microvesicles, are recognized as novel tools for cell-cell communication and as biomarkers for various diseases. Their vesicle composition and miRNA content have the ability to transfer biological information to recipient cells and play an important role in cancer metastasis and prognosis. This review provides an in-depth summary of current findings on circulating miRNAs in lung cancer patients used as diagnostic biomarkers. We also discuss the role of EV miRNAs in cell-cell communication and explore the effectiveness of these contents as predictive biomarkers for cancer malignancy.

1. Introduction

Lung cancer remains the leading cause of cancer-related deaths in the world [1]. It is a heterogeneous disorder with two pathological types: non-small-cell lung cancer (NSCLC) and small-cell lung cancer (SCLC). Approximately 85% of all lung cancers are categorized as NSCLC. The most common pathological types of NSCLC are adenocarcinoma (30–50%) and squamous cell carcinoma (30%). Many therapeutic strategies, including surgery, radiotherapy, chemotherapy, and molecular target therapies, are commonly used to treat lung cancer, either alone or in combination. A majority of lung cancer patients are in advanced stages of the disease with limited treatment choices, mainly consisting of cytotoxic chemotherapeutic agents and targeted molecular therapies. Despite the development of novel targeted therapies, the prognosis for lung cancer remains poor due to drug resistance and tumor recurrence. Therefore, one of the major

challenges in lung cancer research is the identification of stable biomarkers that can be routinely measured in samples that are accessible early. Biomarkers for lung cancer have raised great expectations in their clinical applications for early diagnosis, prognosis, and therapeutic responses. However, conventional serum markers, such as carcinoembryonic antigen (CEA) and squamous cell carcinoma antigen (SCC), lack sufficient sensitivity and specificity to facilitate early detection of lung cancer. Disease-driven proteomics based on mass spectrometry have serious technical limitations because of the complexity of the blood proteome. Recently, remarkable attention has been paid to cell-free nucleic acids, such as DNA, mRNA, and microRNA (miRNA), which are present at varying concentrations in the blood of cancer patients [2].

MiRNAs are endogenous, single-stranded, noncoding RNAs 19–22 nucleotides long that regulate translation through their interactions with mRNA transcripts [3]. MiRNAs are first transcribed, for the most part, by RNA polymerase II

as a large primary miRNA (pri-miRNA) then processed by the endonuclease Drosha into a hairpin structure (precursor miRNA) and then further cleaved by the endonuclease Dicer into a single-stranded mature miRNA [4, 5]. The mature miRNA is incorporated into a complex known as the RNA-induced silencing complex (RISC), which contains the Argonaute 2 (AGO2) and glycine-tryptophan 182 kDa proteins (GW182). As a part of this complex, the mature miRNA regulates gene expression by binding to partially complementary sequences in the 3'-untranslated regions (3'UTRs) of the target mRNAs, leading to mRNA degradation or translation inhibition [6]. MiRNAs posttranscriptionally inhibit gene expression through multiple mechanisms, all of which involve base-specific interactions with target mRNA transcripts. A single miRNA may target dozens of mRNAs and one mRNA can be regulated by multiple miRNAs. In mammals, miRNAs are assumed to regulate more than 50% of all protein-coding genes [7]. This evidence indicates that miRNAs are not only important therapeutic targets but also promising biomarkers, as their expression could reflect the information dispersed on thousands of target genes [8]. Recently, dysregulation of miRNA expression was determined to contribute to various physiological and pathological processes, such as development, differentiation, cell proliferation, apoptosis, and homeostasis [9, 10]. A number of researchers have also demonstrated that aberrant miRNA expression is related to cancer development and progression, behaving as tumor suppressors or oncogenes [11]. Some miRNAs are overexpressed or downregulated in certain cancer types. Expression patterns of miRNAs are unique to individual tissue types and differ between cancer and normal samples [12, 13]. Current data support the potential of miRNAs as biomarkers for NSCLC [13–20]. Moreover, assessment of multiple miRNA expression levels can accurately predict prognosis and survival in lung cancer [21].

Over the last few years, recent studies have shown that miRNAs are present in extracellular spaces, such as blood, urine, and saliva [2]. MiRNAs can be secreted via extracellular vesicles (EVs) and by protein-miRNA complexes, such as high-density lipoprotein (HDL) and AGO2, which is part of the RISC [22, 23]. Circulating miRNAs are sensitive to protease treatment of plasma but are protected from plasma RNase digestion [24]. Resistance to the condition has been attributed to the above-described encapsulation and association with protein complexes [25, 26]. Circulating miRNAs are one of the most promising next-generation biomarkers for cancer diagnosis. Moreover, the involvement of EVs in cancer biology is now of great interest. Recent studies have shown that EVs serve as versatile intercellular communication vehicles [27]. These findings have established the novel concept that EV miRNAs have potential not only as putative biomarkers but also for reflecting cancer progression. Here, we provide a perspective on the potential contribution of circulating miRNA and EV research to the development of cancer diagnosis and therapeutics.

2. Mechanism of MicroRNA Release into Extracellular Spaces

Intercellular miRNAs have important functions in many biological processes. Current studies in the field of lung disease have shown that miRNAs are present in extracellular spaces, such as blood, urine, and bronchoalveolar lavage fluid (BALF) [28]. Mitchell et al. firstly provided the direct evidence that circulating miRNAs derived from human prostate cancer xenografts are released into mouse circulation [23]. The release mechanism of circulating miRNAs as an active biological process remains largely unclear. The secretion of miRNAs by cells is related to the microenvironment of cells. Some possible mechanisms have been hypothesized for miRNA release: (1) active secretion of miRNAs in the form of vesicle-containing miRNAs and (2) energy-free passive leakage of cellular miRNAs from broken cells [29]. In fact, circulating miRNAs have been found to be packaged into various membrane-bound vesicles, such as exosomes and microvesicles [26] and to exist in a vesicle-free form associated with protein or high-density lipoprotein complexes [24] (Figure 1). Furthermore, miRNAs can be incorporated into apoptotic bodies [30]. Although the terms *exosomes* and *microvesicles* frequently are used interchangeably, these vesicles differ in their sources and the mechanisms of derivation and have distinct structural and biochemical properties that are likely to affect their roles [31]. Some methods for isolating circulating miRNAs from exosomes and microvesicles in human plasma have been developed, such as ultracentrifugation [32] and the ExoQuick precipitation method [33]. However, the exosomal miRNA profiles are affected by the different extracellular vesicle isolation methods [34]. In addition, it is technically difficult to fully discriminate between exosomes and microvesicles using these collection methods. Therefore, we will use the term extracellular vesicle (EV) in this review, according to the definition of the International Society for Extracellular Vesicles (ISEV), when describing studies using ultracentrifugation to isolate EVs [35]. EVs, especially exosomes, are small membrane vesicles that are released extracellularly after the fusion of multivesicular bodies (MVB) with the cell membrane [27]. On the other hand, microvesicles are vesicles shed by the plasma membrane of healthy cells [36]. The difference between these two terms is based on size of the vesicles: exosomes are in the range of 10–100 nm and microvesicles are in the range of 100–1000 nm [37]. Importantly, it has been suggested that EVs carry communications between cells, allowing for cells to promote biological functions at distant sites [38]. However, they may not be the most prevalent form of circulating miRNAs. Arroyo et al. showed that the majority of serum miRNAs are present as AGO2-miRNA complexes but not within EVs [24]. Significantly, only EV-miRNAs reportedly have a function in communicating between cells and play a role in various biological processes, including immune system regulation, inflammation, and tumor development [28, 31]. Therefore, we consider that EV-miRNAs make it possible to reflect every aspect of human physiological status and provide the advantage of being better biomarkers than other circulating miRNAs.

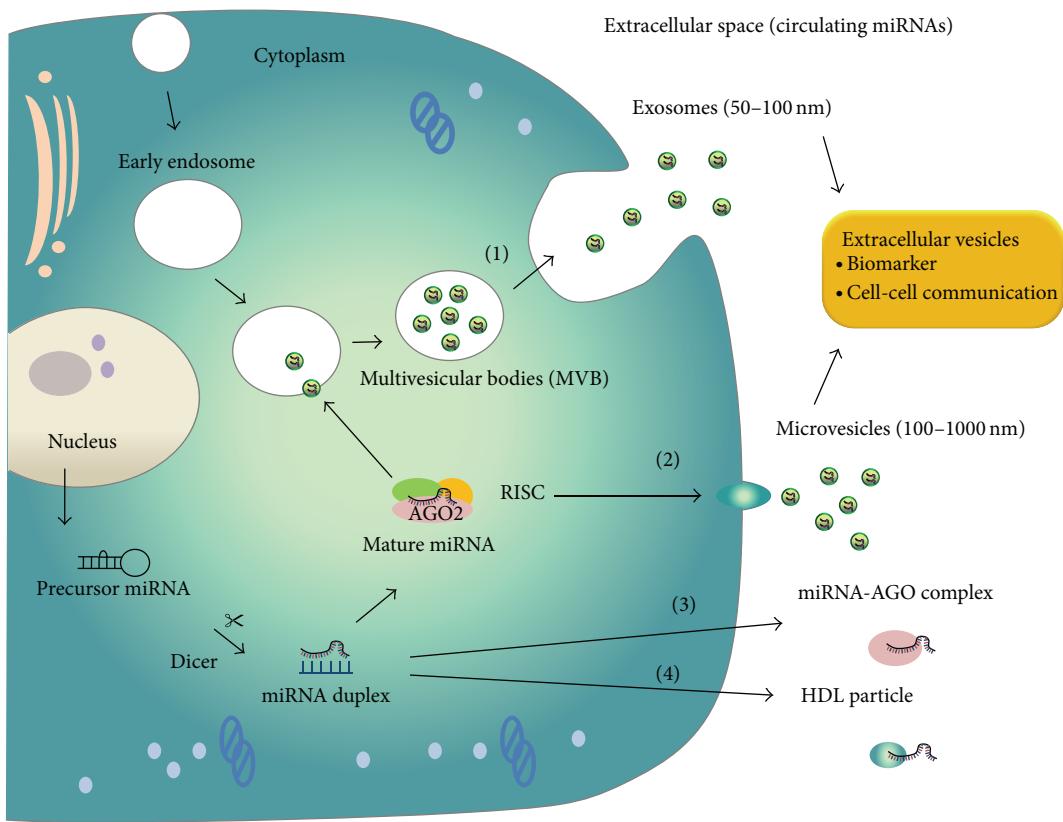


FIGURE 1: MiRNA release mechanisms into extracellular space. Precursor miRNAs are processed by ribonuclease Dicer to mature double-stranded miRNAs (miRNA duplex). One strand of the miRNA duplex is selectively loaded into the RNA-induced silencing complex (RISC), which contains the Argonaute (AGO) family protein as a core component. A fraction of miRNAs are released from living cells into the extracellular environment via the following mechanisms: (1) sorting into multivesicular bodies (MVB) and secretion via exosomes, (2) incorporating into microvesicles that are formed by the outward shedding of the plasma membrane, (3) associating with RNA-binding proteins, such as AGO2 and release of the miRNA-AGO complexes, and (4) exporting and incorporating into high-density lipoprotein (HDL) particles. Extracellular vesicle miRNAs are possibly involved in cell-cell communication.

3. The Potential of MicroRNAs as Circulating Biomarkers in Cancer Patients

Early cancer detection and improved therapeutic response prediction remain the major challenges in cancer research. Currently, tumor cells have been demonstrated to secrete miRNAs into body fluids [2]. With the development of detection technologies, including RT-PCR, microarray, and deep sequencing, we can screen for circulating miRNAs and generate miRNA signatures in body fluids. Circulating miRNAs are novel minimally invasive diagnostic tools for the detection and risk assessment of various types of cancer. Notably, circulating miRNAs levels in serum or plasma could correlate with cancer progression, therapeutic responses, and survival [39]. In general, miRNAs remain stable in the blood circulation. This high stability is due to their resistance to RNase activity, temperatures, extremes of pH, and extended storage in frozen conditions [40]. This evidence suggests that miRNAs could also be used for diagnostic cancer screening or as noninvasive biomarkers for disease monitoring [41]. Blood-based tests would be more reasonable, as they have relatively low cost and can be repeated as well.

Several researches have revealed that circulating miRNA levels are higher in cancer patients than in healthy donors. Tumor-specific miRNAs (miR-21) were first reported in the serum of patients with diffuse large B-cell lymphoma (DLBCL), indicating that circulating miRNAs can be used as biomarkers to monitor the existence of cancer cells [42]. After this research, many studies have analyzed the clinical relevance of circulating miRNAs in blood for diagnosis and survival [23, 43, 44]. To date, differential expression of circulating miRNAs has been reported in cancers of the lung [44], breast [43], liver [45], kidney [46], bladder [47], and prostate [23], among others. Particularly, Mitchell et al. clearly showed that circulating miRNAs originate from tumors and are protected from endogenous RNase activity, suggesting the high potential for using circulating miRNAs as blood biomarkers for cancer [23]. Their most important advantage is the possibility for repeated measurements in a noninvasive manner. Easy access and superior stability in blood plasma and serum also add to their potential as cancer biomarkers [24, 48]. Remarkably, highly expressed circulating miRNAs from cancer patients have been reported

TABLE 1: Circulating miRNAs as a potential biomarker for lung cancer.

Study design	Body fluid type	MicroRNA	Detection method	Reference
NSCLC versus normal	Serum	miR-25 and -223	NGS, qRT-PCR	[44]
NSCLC versus normal	Serum	miR-15b and -27b	qRT-PCR	[52]
NSCLC versus normal	Plasma vesicles	<i>let-7f</i> and miR-30e-3p	qRT-PCR array	[53]
Stage I/II versus IV NSCLC	Serum	miR-126 and -183	qRT-PCR	[54]
NSCLC versus normal	Plasma	miR-21, -126, -210, -486-5p	qRT-PCR	[55]
NSCLC versus normal	Sputum	miR-31, -210	qRT-PCR	[56]
NSCLC versus normal	Sputum	miR-31, -210	qRT-PCR	[57]
NSCLC versus normal (metastasis stage)	Serum	miR-21	qRT-PCR	[60]
Prognosis of NSCLC	Serum	miR-486, -30d, -1, -499	NGS, qRT-PCR	[41]
Prognosis of NSCLC	Plasma	miR-221, -660, -486-5p, -28-3p, -197, -106a, -451, -140-5p, -16	qRT-PCR array	[58]
Prognosis of NSCLC	Serum	miR-21, -141, -200c	qRT-PCR	[59]
Response parameter for chemotherapy	Serum	miR-125b	qRT-PCR	[61]

NGS: next generation Solexa sequencing; qRT-PCR: quantitative real-time polymerase chain reaction; miR: microRNA; NSCLC: non-small-cell lung cancer.

to return to a normal level after tumor resection. Yamamoto et al. have reported that high levels of miR-500 were found in the serum of patients with hepatocellular carcinoma, while the circulating miR-500 returned to normal levels after surgical operation in three of the patients [49]. Serum levels of upregulated miRNAs such as miR-195 and *let-7a* are significantly higher in preoperative plasma from breast cancer patients than after resection [50]. Yamada et al. have reported that surgical removal of urothelial carcinoma coincided with a reduction in urine miR-96 and miR-183 levels [51]. These findings have supported the idea that the level of circulating miRNAs may reflect the expression level of tumor miRNAs.

4. Circulating MicroRNAs in Lung Cancer Patients

Lung cancer is often diagnosed in the late stages with poor prognoses. Therefore, it is important to develop biomarkers to identify early-stage lung cancer and to predict therapeutic responses to chemotherapy or radiotherapy. Circulating miRNAs have been proposed as attractive candidates to be used as cancer biomarkers and are ideal for monitoring by screening. So far, several studies have analyzed the potential diagnostic and prognostic role of circulating miRNAs (Table 1).

Chen et al. used deep sequencing followed by qRT-PCR to identify two NSCLC-specific serum miRNAs (miR-25 and miR-223) [44]. This is the first comprehensive analysis of miRNA profiles in the serum of patients with NSCLC using Solexa sequencing technology. After this study, a lot of research aimed to identify circulating miRNAs with a diagnostic relevance in body fluids such as serum, plasma,

sputum, and pleural effusion [44, 52–57]. Bianchi et al. evaluated a panel of 361 miRNAs in patients from the COSMOS trial to focus on the diagnostic accuracy of circulating miRNAs in NSCLC [52]. This study reports a combination of two differentially expressed miR-15b and miR-27b capable of discriminating NSCLC from healthy donors with high sensitivity and specificity in the training set. This suggests that serum miRNAs have the promising potential to be sensitive and cost-effective biomarkers for the early detection of NSCLC. Although various miRNAs were identified as biomarkers capable of distinguishing between NSCLC patients and healthy donors, different research identified different promising miRNAs.

Recently, several groups have revealed defined serum or plasma miRNA signatures with prognostic and predictive clinical relevance [41, 58, 59]. The most comprehensive study for circulating miRNAs related to prognosis has been conducted by Hu et al. [41]. In a follow-up validation set of 243 NSCLC patients, the serum levels of four miRNAs (miR-486, miR-30d, miR-1, and miR-499) were significantly related to a poor prognosis. When these miRNAs were combined to generate the four miRNA signatures, patients with high-risk signatures had a 9.31-fold increased hazard ratio for cancer death and a shorter median survival. Following computed tomography screening, Boeri et al. showed that 9 circulating miRNAs (miR-221, miR-660, miR-486-5p, miR28-3p, miR-197, miR-106a, miR-451, miR-140-5p, and miR-16) were found to indicate a risk of lung cancer malignancy and poor prognosis [58]. These data suggest that circulating miRNAs can be released into the extracellular spaces even several years before the clinical manifestation of lung cancer. Recently, a significant correlation was reported between serum miR-21 expression and clinicopathological factors of NSCLC patients

[60]. High serum levels of this miRNA were significantly related to lymph node metastasis, advanced clinical staging, and a poor prognosis. Moreover, vesicle-related miRNAs have also been quantified and found to be significant cancer prognosis biomarkers (*let-7f* and *miR-30e-3p*) [53].

In advanced NSCLC patients receiving cisplatin-based chemotherapy, high expression level of circulating miR-125b was a diagnostic and prognostic marker [61]. In addition, the high serum level of miR-125b was shown to be associated with poor sensitivity to neoadjuvant chemotherapy in various types of cancer [61, 62]. This data strongly implied the feasibility of miRNAs as noninvasive or circulating biomarkers for not only the early detection of lung cancer but also its chemoresistance.

Despite promising developments in this research field, circulating miRNAs as biomarkers for cancer need to be sufficiently investigated to validate their potential. Actually, it is unclear from which types of cells the diagnostically relevant miRNAs are derived. It cannot be excluded that circulating miRNAs might be derived from a variety of cell types, such as blood cells, tumor-associated fibroblasts, endothelial cells, and various immune cells residing in the tumor microenvironment. Therefore, we must analyze the miRNA profiles expressed by noncancerous cells, which could reflect specific inflammatory or immune-modulatory processes that occur during carcinogenesis.

5. Extracellular Vesicle-Encapsulated Circulating MicroRNAs in Cancer Progression

EV miRNAs are derived from normal and tumor cells in body fluids as an active secretion mechanism and can mediate cell-cell communication. EVs containing mRNA, miRNAs, and angiogenic proteins released by cancer cells have been revealed [63]. The Swedish group first showed the remarkable discovery that miRNAs are contained inside EVs, which are lipoprotein complexes, including small membrane vesicles of endocytic origin [26]. They demonstrated that mouse and human mast cell-derived EVs contain RNA and miRNA and that RNA from mast cell EVs is transferable to other mouse and human mast cells. In 2010, three groups reported that EV miRNAs are transferred to recipient cells and suppress the expression of target genes [64–66]. Over the last decade, EVs have become the next focus of intensive scientific research as novel mediators of intercellular communication. Recent studies have shown the deep involvement of tumor cell-derived EVs in cancer progression. Moreover, EVs secreted from the surrounding cells have also been reported to regulate tumor pathology [67]. Kosaka et al. reported that EV miRNA-210 released by metastatic breast cancer cells entered endothelial cells and suppressed the expression of its target genes, which resulted in enhanced angiogenesis [68]. Rana et al. reported that EV miR-494 and miR-542-3p derived from

rat adenocarcinoma cells modulate premetastatic organ cells predominantly through transferred miRNA, where miRNA from a metastasizing tumor prepares premetastatic organ stroma cells for tumor cell hosting [69]. Fabbri et al. found that EV miR-21 and miR-29a can induce a Toll-like receptor (TLR)-mediated prometastatic inflammatory response by binding to TLRs regulating for the tumor microenvironment [70]. Recently, Zhou et al. found that EV miR-105 can destroy vascular endothelial barriers to promote brain metastasis [71]. These data suggest the deep involvement of cancer cell-derived EVs in cancer malignancy such as metastasis. Therefore, EV miRNAs have potential not only to be putative biomarkers but also to reflect cancer progression. For this reason, we suggest that EV miRNAs may have the potential of prognostic biomarkers to predict clinical outcome of lung cancer patients. So far, there are few reports that vesicle-free forms associated with protein or HDL complexes can mediate the expansion of cancer malignancy in an autocrine or paracrine manner [25]. The functionality of EV miRNAs in the recipient cells has opened up a new avenue of cell-cell communication in cancer biology, including lung cancer research.

6. Conclusions

Circulating miRNAs are promising lung cancer biomarker candidates (Figure 2). Specific tumor-associated circulating miRNA signatures will have to be developed as early biomarkers for lung cancer. Remarkably, some studies have succeeded in discovering circulating miRNAs with prognostic or predictive significance. In addition, it was reported that some circulating miRNAs are strongly associated with distant metastasis, disease stage, and survival. Regarding this point, we consider that only EV miRNAs may contribute to the management of cancer progression. Therefore, EV miRNAs will be suitable predictive biomarkers for cancer malignancy metastasis and prognosis. We emphasize that it is important to investigate the role of EV miRNAs in cell-cell communication and to explore the effectiveness of these molecules as biomarkers in lung cancer research. As we have shown, circulating miRNAs have been found to be packaged into various membrane-bound vesicles, such as exosomes, microvesicles, and protein complexes. It is technically difficult to completely discriminate between these vesicles using simple collection methods; therefore, we need to collect vesicles using ultracentrifugation when we research the biology of EVs. Gaining deeper understanding of the methods of circulating miRNA detection and analysis will help to clarify the information carried by circulating miRNAs and lead to novel therapeutic approaches targeting the circulating miRNAs.

Conflict of Interests

The authors declare no competing financial interests.

Circulating microRNAs as a promising biomarker platform in lung cancer

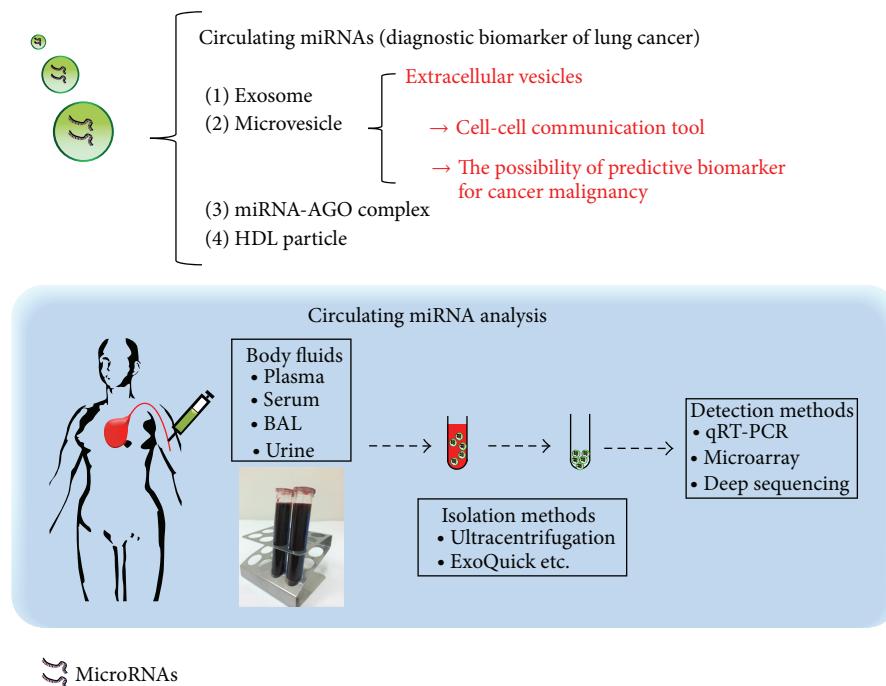


FIGURE 2: Circulating microRNAs as a promising biomarker platform in lung cancer. Circulating miRNAs have been found to be packaged into various membrane-bound vesicles, such as exosomes and microvesicles and to exist in a vesicle-free form associated with protein or high-density lipoprotein complexes. Extracellular vesicles (EVs), such as exosomes and microvesicles, are recognized as novel tools for cell-cell communication and might be predictive biomarkers for cancer malignancy. EVs that circulate in body fluids can be readily recovered using several existing isolation methods and analyzed by various detection methods.

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Review Article

Regulation of MicroRNAs by Natural Agents: New Strategies in Cancer Therapies

Neoh Hun Phuah¹ and Noor Hasima Nagoor^{1,2}

¹ Institute of Biological Science (Genetics and Molecular Biology), Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia

² Centre for Research in Biotechnology for Agriculture (CEBAR), University of Malaya, 50603 Kuala Lumpur, Malaysia

Correspondence should be addressed to Noor Hasima Nagoor; hasima@um.edu.my

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MicroRNAs (miRNAs) are short noncoding RNA which regulate gene expression by messenger RNA (mRNA) degradation or translation repression. The plethora of published reports in recent years demonstrated that they play fundamental roles in many biological processes, such as carcinogenesis, angiogenesis, programmed cell death, cell proliferation, invasion, migration, and differentiation by acting as tumour suppressor or oncogene, and aberrations in their expressions have been linked to onset and progression of various cancers. Furthermore, each miRNA is capable of regulating the expression of many genes, allowing them to simultaneously regulate multiple cellular signalling pathways. Hence, miRNAs have the potential to be used as biomarkers for cancer diagnosis and prognosis as well as therapeutic targets. Recent studies have shown that natural agents such as curcumin, resveratrol, genistein, epigallocatechin-3-gallate, indole-3-carbinol, and 3,3'-diindolylmethane exert their antiproliferative and/or proapoptotic effects through the regulation of one or more miRNAs. Therefore, this review will look at the regulation of miRNAs by natural agents as a means to potentially enhance the efficacy of conventional chemotherapy through combinatorial therapies. It is hoped that this would provide new strategies in cancer therapies to improve overall response and survival outcome in cancer patients.

1. Introduction

According to a report by GLOBOCAN, an estimated 14.1 million new cancer cases and 8.2 million cancer deaths were reported, while 32.6 million people are found to be living with cancer (diagnosed in the past five years) in 2012 worldwide. The same report also projected that the number of new cases would increase to 19.3 million by 2025 due to global population aging. In 2012, the cancers of the lung (1.8 million, 13.0% of the total), breast (1.7 million, 11.9%), and colorectum (1.4 million, 9.7%) were the most commonly diagnosed cancers worldwide, while the most common causes of cancer death were cancers of the lung (1.6 million, 19.4% of the total), liver (0.8 million, 9.1%), and stomach (0.7 million, 8.8%) [1].

Cancer is a group of diseases characterized by the uncontrollable growth and spread of abnormal cells which

can lead to death without timely intervention. Surgery, radiation, and chemotherapy are among the modalities used in cancer treatment, whose goal is to either cure the disease or prolong and improve the patient's quality of life. Although chemotherapy has led to improvement in this manner, drug resistance and toxicities remain major obstacles to improving the overall response and survival of cancer patients [2]. Drug resistance can be divided into two categories: intrinsic (also known as *de novo*) or acquired resistance [3]. Intrinsic resistance results in ineffective therapy from the start due to presence of resistant phenotype in the tumour cells. On the other hand, acquired resistance develops during treatment whereby tumour cells showed initial responsiveness towards anticancer drugs but attained resistant phenotype during the treatment course. This renders subsequent therapy ineffective leading to tumour recurrence and progression [3]. Hence,

there is an urgent need to identify safer but equally effective agents to be used in cancer treatments, which can be found in natural agents.

The use of natural agents is promising because not only do they have minimal toxicity to humans compared to conventional chemotherapies, but also they could target numerous signalling pathways. This is beneficial as malignant transformation and progression are multistage processes caused by gene alterations in more than one signalling pathway. This is one of the most plausible explanations why monomodal therapy typically fails in cancer treatments as the specific inhibitors often target only a single gene in a signalling pathway [4]. Therefore, the impact of natural agents on cancer treatment could be more efficacious, as they can be used alone or as adjuvant in combination chemotherapy to improve therapeutic efficacy by overcoming drug resistance and/or reducing drug-induced toxicities. Hence, many of the anticancer agents currently used in cancer therapies have been developed from natural products such as plants (vincristine, vinblastine, etoposide, paclitaxel, camptothecin, topotecan, and irinotecan), marine organisms (cytarabine), and microorganisms (dactinomycin, bleomycin, and doxorubicin) [5]. Besides these, there are also plant-derived dietary polyphenols such as curcumin [6], resveratrol [7, 8], genistein [9], epigallocatechin-3-gallate [10], indole-3-carbinol, and its derivative 3,3'-diindolylmethane [11, 12] (see Figure 1). A number of studies involving cultured cancer cells and animal models have illustrated the protective role of these dietary polyphenols, and mechanistic studies have demonstrated that they exert their antiproliferative and/or proapoptotic effects to prevent the occurrence and/or spread of various cancers by targeting numerous key elements in intracellular signalling network involved in carcinogenesis [13, 14]. Because of the promising results from these *in vitro* and *in vivo* studies, the efficacies of these natural agents in cancer therapies are being investigated in clinical trials (<http://www.clinicaltrials.gov/>) (see Table 1).

MicroRNAs (miRNAs) are highly conserved, small (~22 nucleotides long) noncoding RNA molecules that regulate genes posttranscriptionally [15]. They are predicted to regulate the expression of around 60% of mammalian genes [16] and are found in abundance in many human cell types [17], making them one of the largest class of gene regulators. Although the miRNA genes are mostly found in the intergenic regions, they can also be present in the exonic and intronic regions, in all human chromosomes except for Y chromosome [18]. They are first transcribed by RNA polymerase II as long primary miRNA molecules (pri-miRNAs) before being processed into short hairpin RNAs of ~70 nucleotides known as pre-miRNA by Drosha (RNase III enzyme) and DGCR8 (DiGeorge syndrome critical region 8) [19]. It is then transported into the cytoplasm in a RanGTP-dependent manner by Exportin-5 to be cleaved by another RNase III enzyme, Dicer, to release a ~22 nucleotides long miRNA duplex [20, 21]. One of the strands is incorporated into RNA-induced silencing complex (RISC) which directs miRNA binding to the 3'-untranslated regions (3' UTR) of target mRNA resulting in mRNA silencing through target cleavage, mRNA degradation, and translation inhibition,

while the complementary miRNA strand is usually rapidly degraded [21, 22] (see Figure 2). However, recent studies showed that they can also bind to other regions such as 5' UTR or coding sequences [23, 24]. Furthermore, they can also upregulate translation of target mRNAs by recruiting proteins complexes to the adenylate-uridylate- (AU-) rich elements of mRNA [25] or interfering with proteins that block the translation of target gene [26]. More interestingly, not only can a single gene be targeted by multiple miRNAs, a single miRNA can also target many genes [27]. miRNAs have been found to be dysregulated in nearly every types of human cancer [28] and various studies have implicated their involvement in a plethora of biological processes, such as tumorigenesis, cell differentiation, proliferation, death, autophagy, metastasis, and drug resistance [29–34]. Hence, identification of these oncogenic or tumour suppressive miRNAs allows for their use as potential targets in cancer therapies [35, 36].

Since natural agents exert their anticancer effects by targeting multiple signalling pathways, and miRNAs regulate diverse biological processes including cell proliferation and programmed cell death, it is thought that miRNAs could play a role in regulating response towards natural agents. Hence, miRNA regulation by natural agents in cancer therapy has been gaining greater attention in recent years. Various studies have reported the dysregulation in the miRNA expression profiles following treatment with natural agents, either as stand-alone or in combination with FDA-approved chemotherapeutic drugs. In one such study, we have previously reported that miRNAs are differentially expressed in response to different treatments, and bioinformatic analysis found their gene targets to be involved in regulating various signalling pathways including apoptosis and cell proliferation [37]. In this review, it was found that most of these dysregulated miRNAs target oncogenes, tumour suppressor genes, and transcription factors (see Tables 2, 3, and 4), and inhibition and/or overexpression of these miRNAs affect cell migration, invasion, proliferation, and apoptosis (see Figure 3). Beside these, there are also studies which found miRNAs to be dysregulated in response to these natural agents, although their targets were not confirmed (these miRNAs are subsequently discussed under “Other miRNAs” section). Therefore, insights from these studies could provide us with a better understanding in the interactions between miRNAs with their specific gene targets and, consequently, help us to delineate the molecular mechanism underlying anticancer drug response.

2. Curcumin

Curcumin (diferuloylmethane) is a natural compound derived from the rhizomes of turmeric (*Curcuma longa*) that inhibits cell proliferation, invasion, migration, angiogenesis, and inflammation and induces cell cycle arrest and apoptosis on various cancers, such as breast, cervical, oral, gastric, melanoma, pancreatic, colon, and prostate [14, 38, 39]. It exhibits its anticancer effects by regulating genes involved in cellular signalling pathways, including nuclear factor-kappa

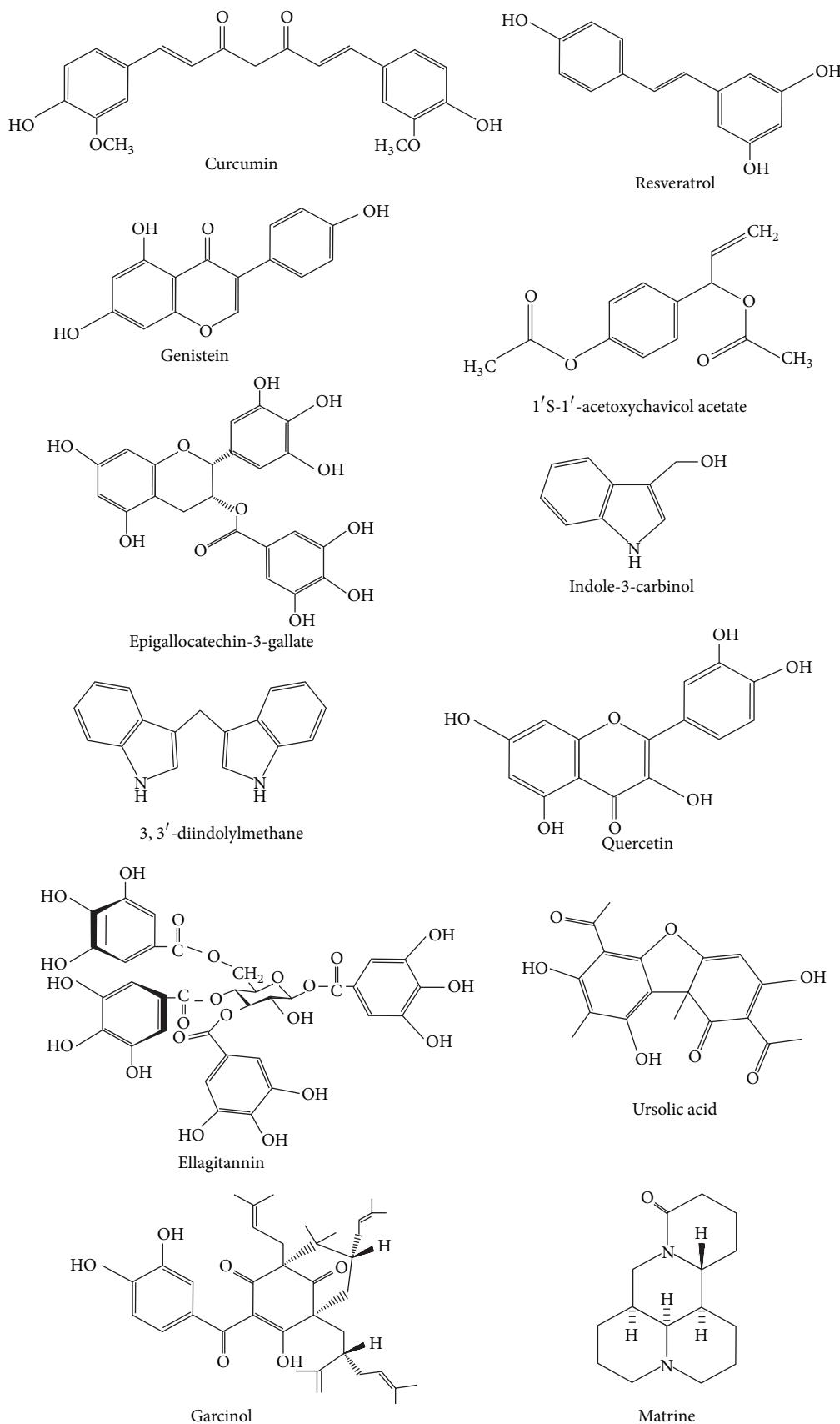


FIGURE 1: Molecular structures of natural agents regulating miRNAs.

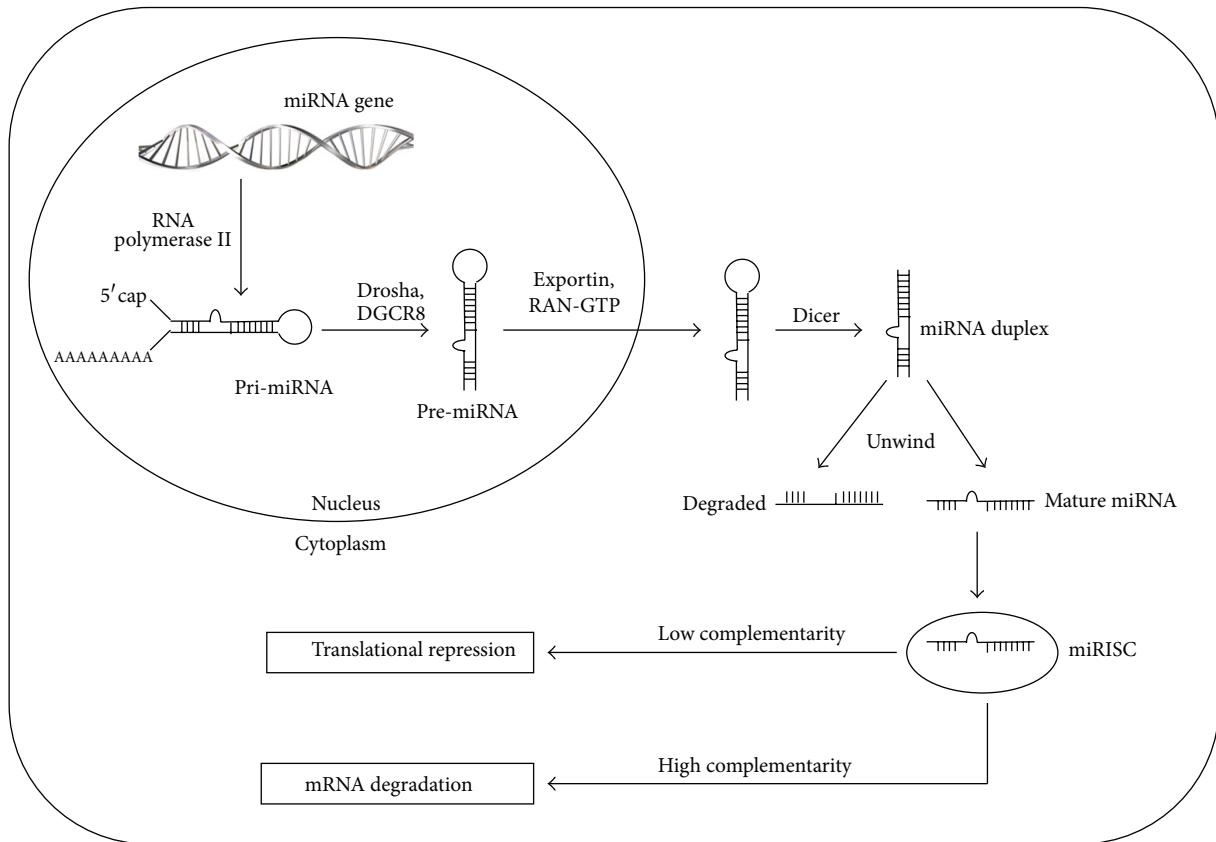


FIGURE 2: Biogenesis of miRNAs.

B (NF- κ B), protein kinase B (Akt), mitogen-activated protein kinase (MAPK), p53, and other pathways [40].

2.1. miRNAs Targeting Oncogenes. Curcumin inhibited proliferation and induced apoptosis through upregulation of miR-181b in MDA-MB-231 breast cancer cells. This miRNA inhibited the expression of matrix metalloproteinases (MMP) by binding to metastases related-cytokines, such as chemokine (C-X-C motif) ligands 1 and 2 (CXCL1 and CXCL2), leading to reduced invasion in both *in vitro* and *in vivo* models. More importantly, it was shown that curcumin upregulated miR-181b and downregulated CXCL1 in tumour cells isolated from primary breast cancers, verifying the clinical significance of their results [41]. Yang et al. reported that curcumin induced apoptosis in MCF-7 breast adenocarcinoma cells through downregulation of B-cell lymphoma 2 (Bcl-2) expression, a key antiapoptotic protein, as well as upregulation of miR-15a and miR-16. Inhibition of these miRNAs restored the expression of Bcl-2 partially and induced cell proliferation [42]. Another study also reported the upregulation of these miRNAs upon treatment in K-562 and HL-60 leukemic cells, and inhibition of these miRNAs partly reversed the downregulation of Wilm's tumour 1 (WT1) induced by curcumin to promote cell growth [43]. A study in 2011 by Saini et al. revealed that curcumin induced hypomethylation of the tumour suppressive miR-203 promoter to increase its expression. This

led to downregulation of its two targets, protein kinase B β (Akt2) and *v-Src* avian sarcoma viral oncogene homolog (Src), culminating in increased apoptosis as well as decreased proliferation, migration, and invasion in T24 bladder cancer cells [44]. A total of 21 miRNAs were found to be dysregulated (5 upregulated and 16 downregulated) in Y26 retinoblastoma cells treated with curcumin. Among the upregulated miRNAs is miR-22, and its overexpression inhibited cell proliferation and migration. The erythoblastic leukemia viral oncogene homolog 3 (Erbb3) was confirmed as its target in the same study [45].

2.2. miRNAs Targeting Tumour Suppressor Genes. Besides that, curcumin suppressed proliferation and induced apoptosis in A549 lung adenocarcinoma cells through downregulation of miR-186*, suggesting its potential role as an oncogenic miRNA. Interestingly, caspase-10 was identified as a direct target of this miRNA [46]. The same group demonstrated similar effects of miR-186* in A549/DDP multidrug-resistant human lung adenocarcinoma cells [47]. In RKO and HCT116 colon cancer cells, the expression of miR-21 which correlated with the inhibition of activator protein-1 (AP-1) binding to its promoter was reduced following treatment with curcumin. Consequently, cell proliferation, tumour growth, invasion, and *in vivo* metastasis were suppressed, while the expression of the tumour suppressor programmed cell death protein 4 (PDCD4), a target of miR-21, was

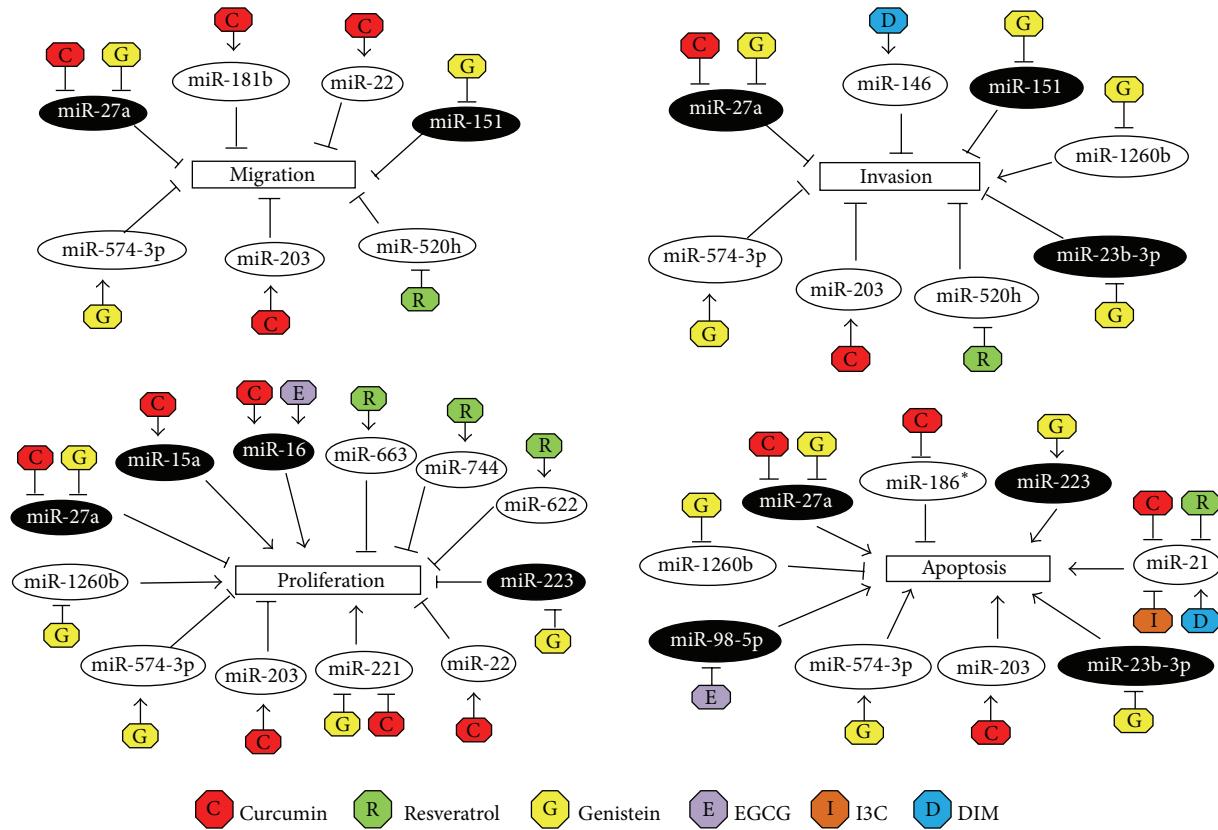


FIGURE 3: Regulation of miRNAs by natural agents and effects of miRNA inhibition (black ellipse) or overexpression (white ellipse) on cell migration, invasion, proliferation, and apoptosis. Inhibitory relationships are denoted as flat arrow heads, whereas positive interactions are denoted as open arrow heads.

upregulated [48]. In another study, curcumin induced cell cycle arrest and apoptosis through downregulation of Notch-1 specific miR-21 and miR-34a, and upregulation of tumour suppressor let-7a, in TE-7 human esophageal cancer cells [49]. Meanwhile, Liang et al. revealed that curcumin induced apoptosis in HepG2 and HepJ5 hepatocellular carcinoma cells by downregulating Bcl-2 level and upregulating Bcl-2-associated death promoter (BAD) level. Their data indicated that overexpression of miR-200a and miR-200b resulted in resistance towards curcumin through reduced apoptotic effect. A decrease in the level of proapoptotic BAD and Bcl-2-associated X protein (BAX) was found in miR-200a- and miR-200b-over-expressed cells, while an increase in Bcl-2 level was only observed in miR-200b, with no changes in miR-200a-over-expressed cells. Taken together, these data suggest that overexpression of these miRNAs conferred resistance towards curcumin through regulation of the Bcl-2 family [50].

2.3. miRNAs Targeting Transcription Factors. Curcumin was found to upregulate 11 and downregulate 18 miRNAs in BxPC-3 human pancreatic adenocarcinoma cells. Of these, the most prominent upregulated miRNA is miR-22 while the most downregulated miRNA is miR-199*. Importantly, miR-22 upregulation decreased the expression of specificity

protein 1 (SP1) and estrogen receptor 1 (ESR1) and vice versa, suggesting that they are targets of miR-22 [78]. The significant relationship between ESR1 gene amplification, which affects the cellular responsiveness to estrogen and antiestrogen, and breast cancer has been reported previously [84, 85]. On the other hand, SP1 proteins play a role in the growth and metastases in various tumour types by regulating cell cycle gene expression and vascular endothelial growth factor receptor [86]. Besides that, curcumin inhibited the growth of RKO and SW480 colon cancer cells through induction of reactive oxygen species (ROS) and repression of specificity proteins (Sp) transcription factors through downregulation of miR-27a, miR-20a, and miR-17-5p. These miRNAs regulate Sp repressors, zinc finger, and BTB domain-containing proteins 4 and 10 (ZBTB4 and ZBTB10) [79]. This has important implications as Sp proteins are transcription factors that regulate genes involved in cell death and angiogenesis and are often overexpressed in tumours [87, 88].

3. Resveratrol

Resveratrol (3,4',5-trihydroxystilbene) is a natural phytoalexin present in several plants, such as grapes, berries, plums, and peanuts. The anticancer effects are mediated

TABLE 1: Clinical trials involving selected natural agents.

Natural agent	Trial identifier	Phase	Cancer type	Status
Curcumin	NCT01042938	2	Breast	Completed
	NCT00094445	2	Pancreatic	Completed
	NCT00192842	2	Pancreatic	Completed
	NCT01333917	1	Colorectal	Completed
	NCT00027495	1	Colorectal	Completed
	NCT01035580	1	Uterine cervical	Completed
	NCT00113841	n.a	Multiple myeloma	Completed
	NCT01975363	n.a	Breast	Active
	NCT01859858	1	Colorectal	Active
	NCT00745134	2	Rectal	Active
	NCT01294072	1	Colon	Active
	NCT01917890	n.a	Prostate	Active
	NCT01160302	0	Head and neck	Active
	NCT01490996	1/2	Colon	Active
	NCT02095717	2	Prostate	Active
	NCT02017353	2	Endometrial	Active
	NCT02064673	2	Prostate	Active
Resveratrol	NCT00641147	n.a	Familial adenomatous Polyposis	Active
	NCT00927485	n.a	Familial adenomatous Polyposis	Active
	NCT01948661	2	Colorectal	Active
	NCT00256334	1	Colon	Completed
Genistein	NCT00433576	1	Colorectal	Completed
	NCT00098969	1	Solid tumours	Completed
	NCT01476592	n.a	Neuroendocrine	Active
	NCT00244933	2	Breast	Completed
EGCG	NCT00118040	2	Bladder	Completed
	NCT00099008	1	Breast	Completed
	NCT00584532	2/3	Prostate	Completed
	NCT00376948	2	Pancreatic	Completed
	NCT00269555	n.a	Prostate	Completed
	NCT00499408	2	Prostate	Completed
	NCT00078923	2	Prostate	Completed
	NCT01985763	1/2	Colorectal	Active
	NCT01325311	2	Prostate	Active
	NCT01126879	2	Prostate	Active
	NCT01628471	1/2	Lung	Active
	NCT00276835	0	Kidney; melanoma	Active
	NCT01182246	1/2	Pancreatic	Active
	NCT00459407	1	Prostate	Completed
Curcumin	NCT00233935	1	Esophageal	Completed
	NCT00573885	2	Lung	Completed
	NCT00303823	2	Cervical	Completed
	NCT01105338	2/3	Prostate	Completed
	NCT00516243	1	Breast	Active
	NCT00942422	2	Multiple myeloma	Active
	NCT00596011	2	Prostate	Active
	NCT01606124	2	Colorectal	Active
	NCT00253643	n.a	Prostate	Active
	NCT00917735	2	Breast	Active
	NCT01360320	2	Colorectal	Active
	NCT00949923	1	Breast	Active

TABLE 1: Continued.

Natural agent	Trial identifier	Phase	Cancer type	Status
I3C	NCT00607932	n.a	Prostate	Completed
	NCT00033345	1	Breast	Completed
	NCT00100958	1	Solid tumours	Completed
DIM	NCT00450229	1	Prostate	Completed
	NCT00305747	1	Prostate	Completed
	NCT00462813	3	Cervical	Completed
	NCT00888654	2	Prostate	Active
	NCT01391689	2/3	Breast	Active

TABLE 2: Selected miRNAs targeting oncogenes.

Natural agent	miRNA	Regulation	Target(s) and function(s)	Cancer type	Reference
Curcumin	miR-181b	Up	Targets CXCL1 and CXCL2; inhibited migration through MMP	Breast	[41]
	miR-15a, miR-16	Up	miRNA inhibition upregulates Bcl-2 and WT1 and induces cell growth	Breast; leukemia	[42, 43]
	miR-203	Up	Targets Akt2 and Src; inhibits cell proliferation, invasion, and migration; and induces cell cycle arrest and apoptosis	Bladder	[44]
	miR-22	Up	Targets Erbb3; inhibits cell proliferation and migration	Pancreatic	[45]
Resveratrol	miR-663, miR-744	Up	Targets eEF1A; inhibits cell proliferation	Breast	[51]
	miR-21	Down	Upregulates Bcl-2; increases apoptosis	Pancreatic	[52]
	miR-622	Up	Targets K-Ras; inhibits proliferation and colony formation <i>in vitro</i> and tumorigenicity <i>in vivo</i>	Lung	[53]
Genistein	miR-34a	Up	Targets HOTAIR	Prostate	[54]
	miR-574-3p	Up	Targets RAC1, EGFR, and EP300; inhibits cell proliferation, migration, and invasion; and induces apoptosis	Prostate	[55]
	miR-1296	Up	miRNA inhibition upregulates MCM2 and vice versa	Prostate	[56]
EGCG	miR-16	Up	Targets Bcl-2	Hepatocellular	[57]
DIM	miR-21	Up	Targets Cdc25A	Pancreatic	[58]
	let-7 family	Up	Downregulates EZH2	Prostate	[59]
	miR-146	Up	Downregulates EGFR, IRAK-1, NF- κ B, and MTA2; inhibits cell invasion	Pancreatic	[60]

through three main mechanisms: inhibition of carcinogenic activation and induction of carcinogen detoxification, induction of growth arrest and apoptosis, and suppression of proinflammatory signalling pathways related to cancer progression [89].

3.1. miRNAs Targeting Oncogenes. Treatment with resveratrol in MCF-7 breast adenocarcinoma cells upregulated miR-663 and miR-774, which were able to retard cell proliferation by inhibiting eukaryotic translation elongation factor 1A2 (eEF1A2) at mRNA and protein levels [51]. Liu et al. also reported downregulation of miR-21 by resveratrol in PANC-1, CFPAC-1, and MIA Paca-2 pancreatic cancer cells, leading to inhibition of Bcl-2 expression. Overexpression of miR-21 was found to reverse downregulation of Bcl-2 and resveratrol-induced apoptosis [52]. In anti-benzo[a]pyrene-7,8-diol-9,10-epoxide-transformed human bronchial epithelial cell line (16HBE-T), resveratrol upregulated miR-622, which targets Kirsten rat sarcoma viral oncogene homolog (K-Ras), to

inhibit cell proliferation, induce cell cycle arrest at G₀ phase, and suppress colonies formation *in vitro* and tumorigenicity in nude mice, while its downregulation impaired growth inhibition. Moreover, it was shown that the binding of miR-622 to K-Ras 3' UTR was affected by resveratrol [53].

3.2. miRNAs Targeting Tumour Suppressor Genes. Resveratrol upregulated miR-663 in SW480 human colon cancer cells, besides downregulating various other miRNAs usually found to be overexpressed in this cancer, such as miR-17, miR-21, miR-25, miR-26a, miR-92a-2, miR-103-1 and -103-2, and miR-181a2. The transforming growth factor beta 1 (TGF β 1) transcript was identified as a target of miR-663 [61]. On the other hand, prostate cancer cells treated with resveratrol displayed downregulation of several miRNAs, including the oncogenic miR-17-92 and miR-106ab clusters. These miRNAs target phosphatase and tensin homologue deleted on chromosome 10 (PTEN), a tumour suppressor protein that is downregulated in nearly all cancers [62].

TABLE 3: Selected miRNAs targeting tumour suppressor genes.

Natural agent	miRNA	Regulation	Target(s) and function(s)	Cancer type	Reference
Curcumin	miR-186*	Down	Targets caspase-10; inhibits apoptosis	Lung	[46, 47]
	miR-21	Down	Targets PDCD4	Colon	[48]
Resveratrol	miR-663	Up	Targets TGF β 1	Colon	[61]
	miR-17-92, miR-10ab	Down	Targets PTEN	Prostate	[62]
	miR-21	Down	Targets PDCD4 and maspin	Prostate	[63, 64]
Genistein	miR-221, miR-222	Down	Targets ARH1	Prostate	[65]
	miR-151	Down	miRNA inhibition suppresses cell migration and invasion; targets N4BP1, CASZ1, IL1RAPL1, SOX17, and ARHGDIA	Prostate	[66]
	miR-23b-3p	Down	Targets PTEN; miRNA inhibition induces apoptosis and inhibits invasion	Renal	[67]
	miR-1260b	Down	Targets sFRP1, Dkk2 and, Smad4; induces cell proliferation and invasion; inhibits apoptosis	Renal; Prostate	[68, 69]
	miR-223	Down	Upregulates Fbw7; miRNA inhibition suppresses cell growth and induces apoptosis	Pancreatic	[70]
	miR-27a	Down	Upregulates ZBTB10 and Sprouty2; miRNA inhibition suppresses cell growth, migration, and invasion and induces apoptosis	Melanoma; Ovarian; Pancreatic	[71–73]
EGCG	miR-98-5p	Down	miRNA inhibition enhances cisplatin-induced apoptosis and increases p53 expression	Lung	[74]
I3C	miR-21	Down	Downregulates PTEN, PDCD4, and RECK	Lung; Pancreatic	[75, 76]
	miR-221	Down	Upregulates PTEN, p27kip1, p57kip2, and PUMA; increases cell proliferation	Pancreatic	[77]

TABLE 4: Selected miRNAs targeting transcription factors.

Natural agent	miRNA	Regulation	Target(s) and function(s)	Cancer type	Reference
Curcumin	miR-22	Up	miRNA inhibition upregulates SP1 and ESR1 expression and vice versa	Pancreatic	[78]
	miR-27a, miR-20a, miR-17-5p	Down	Targets ZBTB4 and ZBTB10	Colon	[79]
Resveratrol	miR-34a	Up	Targets E2F3	Colon	[80]
	miR-520h	Down	Inhibits cell migration and invasion, decreases levels of FOXC2	Lung	[81]
DIM	miR-663	Up	Targets JunB and JunD; decreases miR-155	Leukemia	[82]
	miR-200	Down	Targets ZEB1; downregulates ZEB1, slug, and vimentin; upregulates E-cadherin	Pancreatic	[83]

In another study, resveratrol inhibited cell viability and invasion in highly invasive, androgen independent PC-3 M-MM2 human prostate carcinoma cells, through suppression of Akt. This resulted in the inhibition of miR-21 along with induction of programmed cell death protein 4 (PDCD4) and maspin, targets of miR-21. Similar effects were observed in two other human prostate cancer cells, LNCap and DU145. Additionally, the *in vitro* results were corroborated in severe combined immunodeficient (SCID) mouse xenograft model of prostate cancer, suggesting that resveratrol mediated its effects through inhibition of Akt/miR-21 signalling pathway [63]. Resveratrol also downregulated miR-21 and upregulated PDCD4 in AGS gastric cancer cells. A reduced expression of PDCD4 and overexpression of miR-21 were found in

gastric cancer specimens. Interestingly, there was a significant inverse correlation between miR-21 and PDCD4 protein expression, but not between their mRNA expression, suggesting posttranslational modification [64].

3.3. miRNAs Targeting Transcription Factors. Besides that, it was found that resveratrol upregulated miR-34a in DLD-1 human colon cancer cells. The E2F transcription factor 3 (E2F3), a target of miR-34a, and E2F3's downstream target sirtuin (silent mating type information regulation 2 homolog) 1 (Sirt1) were downregulated following treatment, suggesting that resveratrol exerted its anticancer activity

through miR-34a/E2F3/Sirt1 cascade [80]. In a drug combination study, resveratrol and quercetin induced apoptosis in HT-29 human colon cancer cells by increasing caspase-3 and poly ADP ribose polymerase (PARP) cleavage. The Sp1, Sp3, and Sp4 specificity proteins, together with Sp-regulated antiapoptotic protein, survivin, were reduced at both mRNA and protein levels. Moreover, this combination treatment also decreased miR-27a and induced ZBTB10 [90]. In a study involving CL-5 and A549 lung adenocarcinoma cells, resveratrol downregulated miR-520h and triggered miR-520h-mediated signal cascade, resulting in inhibition of forkhead box C2 (FOXC2) and subsequent suppression of tumour metastasis in both *in vitro* and *in vivo* models. The same study showed association between expression of FOXC2 with epithelial-mesenchymal transition (EMT), cell motility, metastasis, and poorer prognosis in lung cancer patients [81]. Resveratrol upregulated miR-663 in THP-1 human monocytic cells and human blood monocytes. MiR-663 decreased the activity of AP-1 by targeting JunB and JunD transcripts, and its upregulation decreased the levels of miR-155, which has been linked to formation and development of tumours such as breast, gastric, and lung cancers [82, 91].

3.4. Other miRNAs. In A549 human lung adenocarcinoma cells, resveratrol altered miRNA expression profiles, with downregulated miR-92a-2* and upregulated miR-299-5p, miR-194*, miR-338-3p, miR-758, and miR-582-3p exhibiting greater than 20-fold changes. Importantly, identification of their targets through bioinformatics analyses revealed their involvement in regulating apoptosis, cell cycle, cell proliferation, and cell differentiation [92]. In another study, a number of tumour suppressive miRNAs including miR-141 and miR-200c were upregulated by resveratrol, inhibiting the breast cancer stem-like cell characteristics in MDA-MB-231-luc-D3H2LN cells (a luciferase expressing cell line that was derived from MDA-MB-231 human breast adenocarcinoma cells). Repression of miR-141 resulted in increased cell invasion [93]. In p53 wild-type (MCF-7) and p53 mutant-type (MDA-MB-231 and BT-549) breast cancer cells, resveratrol suppressed pAkt and phosphorylation of CCAAT/enhancer binding protein beta (C/EBP- β), which has been identified as a negative regulator of miR-145, leading to upregulation in this miRNA [94]. More recently, it was reported that resveratrol downregulated the expression of DNA methyltransferase 3b (DNMT3b) and upregulated miR-21, miR-129, miR-204, and miR-489 in hormone sensitive mammary tumours, but vice versa in matched normal mammary tissue. An inverse association between DNMT3b and miR-129, miR-204, and miR-489 expression in normal and tumour tissue was also found [95].

4. Genistein

Genistein is a isoflavone naturally found in numerous plants, including fava beans, lupins, and soybeans [96]. Previous reports have shown that it induces cell cycle arrest and apoptosis and inhibits angiogenesis and metastasis in various

cancers including breast, prostate, gastric, lung, pancreatic, melanoma, and renal cancer in both *in vitro* and *in vivo* models. Besides having antioxidant properties, it also inhibits NF- κ B and Akt signalling pathways and antagonizes estrogen- and androgen-mediated signalling pathways [97].

4.1. miRNAs Targeting Oncogenes. In PC-3 and DU145 prostate cancer cells, genistein upregulated miR-34a with HOX transcript antisense RNA (HOXA1) confirmed as its target. Knockdown of this gene decreased cell proliferation, migration, and invasion, and induced apoptosis and cell cycle arrest [54]. Also in the same cells, genistein upregulated miR-574-3p and overexpression of this miRNA inhibited cell proliferation, migration, and invasion *in vitro* and *in vivo*, and apoptosis was induced through reduction of B cell lymphocyte xL (Bcl-xL) and activation of caspase-3 and -9. The Ras-related C3 botulinum toxin substrate 1 (RAC1), epidermal growth factor receptor (EGFR), and E1A binding protein p300 (EP300) were confirmed as targets of miR-574-3p, and inhibition in cell proliferation, migration, and invasion was observed when these three targets were knocked down. The expression of miR-574-3p was found to be significantly lower in PC-3 and DU145 and prostate tumour tissues compared with RWPE-1 normal prostate cells and adjacent normal tissues, respectively. The low expression level of miR-574-3p correlated with advanced tumour stage and higher Gleason score [55]. Also in PC-3 prostate cancer cells, genistein upregulated miR-1296 and suppressed the expression of minichromosome maintenance (MCM) gene family, which is essential for DNA replication and frequently found to be upregulated in various cancers. Inhibition of miR-1296 resulted in upregulation of MCM2 mRNA and protein and vice versa, strongly suggesting MCM2 as its target. Furthermore, MCM2 expression correlated with prostate cancer progression, whereby reduced expression of miR-1296 was observed in prostate carcinoma compared to benign prostate hyperplasia [56].

4.2. miRNAs Targeting Tumour Suppressor Genes. Genistein also downregulated miR-221 and miR-222 in PC-3 prostate cancer cells, leading to upregulation of aplasia ras homolog I (ARH1), a target of these miRNAs. Overexpression of ARH1 led to inhibition of cell proliferation, colony formation, and invasion [65]. It was reported that miR-151 has higher expression in PC-3 and DU145 prostate cancer cells compared to RWPE-1, a nonmalignant epithelial prostate cell line. Genistein downregulated miR-151 and its suppression inhibited cell migration and invasion but not cell proliferation in the prostate cancer cells. Moreover, miR-151 was also shown to bind directly to the 3' UTRs of NEDD4 binding protein 1 (N4BP1), castor zinc finger 1 (CASZ1), interleukin 1 receptor accessory protein-like 1 (IL1RAPL1), SRY (sex determining region Y)-box 17 (SOX17), and Rho GDP dissociation inhibitor (GDI) alpha (ARHGDI). It was found that miR-151 expression is significantly higher in prostate cancer compared to the benign state, although a higher expression did not show significant correlation with lower survival rate [66]. Another miRNA whose expression

is inhibited by genistein is miR-23b-3p, and its knockdown in A-498 and Caki-2 renal cancer cells resulted in significant apoptosis induction and reduction in invasive capabilities, but not cell cycle progression. Furthermore, miR-23b-3p was found to directly target PTEN and its inhibition induced PTEN expression with concomitant reduction in PI3-kinase, total Akt, and IL-32. The expression level of this miRNA was found to be inversely correlated with five-year survival rate in renal cancer patients, and lack of PTEN protein expression was observed in tissue samples with high miR-23b-3p expression [67]. Also in renal cancer cells, genistein significantly decreased the expression of miR-1260b in 786-O and A-498 cells and TCF reporter activity was found to be paralleled to its expression, indicative of its regulation of the β -catenin-dependent pathway. Furthermore, miR-1260b promoted cell proliferation and invasion while apoptosis was reduced in these cells. Tumour suppressors associated with Wnt-signalling such as secreted frizzled-related protein 1 (sFRP1), dickkopf 2 homolog (Dkk2), and mothers against decapentaplegic 4 (Smad4) were confirmed as targets of miR-1260b, and their overexpression decreased cell proliferation and invasion along with increased apoptosis. More pertinently, miR-1260b has higher expression in renal cancer tissues compared to normal kidney tissues and its expression significantly correlates with shorter overall survival [68]. Similar results with miR-1260b were reported in PC-3 and DU-145 prostate cancer cells, suggesting the importance and potential therapeutic benefit of this miRNA in cancer treatment [69]. In a study by Ma et al., it was demonstrated that genistein downregulated miR-223 and upregulated its targets, F-box and WD-40 domain protein 7 (Fbw7). In addition, inhibition of miR-223 retarded cell growth and induced apoptosis in pancreatic cancer cells [70]. Genistein inhibited the expression of miR-27a in C918 uveal melanoma [71], SKOV3 ovarian cancer [72], and pancreatic cancer cells [73]. The expression of ZBTB10 and Sprouty2, targets of miR-27a, was found to be upregulated following treatment, while inhibition of miR-27a suppressed cell growth, migration, and invasion and induced apoptosis in these cells [71–73]. In PC-3 prostate cancer cells, genistein in combination with 5-aza-20-deoxycytidine (5-aza) and trichostatin A (TSA) upregulated miR-145. Loss of miR-145 expression was frequently observed in prostate cancer cell lines and tissue samples compared to normal cells and matched adjacent normal tissues, respectively. This inactivation was seen as a result of DNA methylation in the miR-145 promoter region. Overexpression of miR-145 resulted in decreased cell viability, increased apoptosis, cell cycle arrest, and upregulation of proapoptotic tumour necrosis factor (ligand) superfamily, member 10 (TNFSF10) [98].

4.3. Other miRNAs. Various miRNAs were found to be dysregulated following treatment with genistein in prostate cancer cells: one upregulated (miR548b-3p) and four downregulated miRNAs (miR-15b, miR-125a, miR-125b, and miR-320) in PC-3 cells; five downregulated miRNAs (miR-155, miR-208b, miR-211, miR-376a, and miR-411) in DU-145 cells; one upregulated miRNA (miR-15a), and three downregulated

miRNAs (miR-494, miR-520g, and miR-542) in LNCap cells. These results showed that miRNAs are differentially expressed in different prostate cancer cells treated with genistein, although their roles were not investigated in the same study [99]. Genistein also inhibited miR-21 in A-498 renal cancer cells and tumour xenografts, leading to cell cycle arrest, apoptosis induction, reduced invasion, and migration capabilities as well as increased expression of p21 and p38 MAPK but a reduction in cyclin E2. This study showed that patients with lower miR-21 expression have better five-year survival rate while increased expression correlates with an increased stage of renal cancer, providing clinical relevance in the role of miR-21 in renal cancer [100]. Additionally, genistein upregulated miR-34a in pancreatic cancer cells. Treatment with genistein and overexpression of miR-34a inhibited cell growth and induced apoptosis with concomitant downregulation of Notch-1 signalling pathway [101].

5. Epigallocatechin-3-Gallate (EGCG)

The epigallocatechin-3-gallate (EGCG) is the major polyphenol found in green tea (*Camellia sinensis*) with anticancer effects. Previous reports have shown that it is able to suppress proliferation, induce apoptosis, and inhibit invasion, angiogenesis, and metastasis in various cancer types in both *in vitro* and *in vivo* models by targeting multiple cellular signalling pathways [102, 103].

5.1. miRNAs Targeting Oncogenes. Treatment with EGCG in HepG2 hepatocellular carcinoma cells altered the expression of 61 miRNAs (13 upregulated and 48 downregulated) and among the upregulated miRNAs is miR-16. Its target, Bcl-2, was also downregulated by EGCG. Suppression of miR-16 counteracted the effects of EGCG on apoptosis induction and Bcl-2 downregulation [57].

5.2. miRNAs Targeting Tumour Suppressor Genes. Besides that, it was also reported that EGCG enhanced the effects of cisplatin by downregulating miR-98-5p in A549 lung cancer cells. Inhibition of miR-98-5p enhanced cisplatin-induced cell death and increased expression of p53 [74].

5.3. Other miRNAs. In HepG2 hepatocellular carcinoma cells, five miRNAs (miR-30b*, miR-453, miR-520-e, miR-629, and miR-608) were downregulated following treatment with EGCG. Bioinformatics analysis revealed gene targets of miR-30b* to be involved in regulating various pathways including inflammation, NF- κ B, peroxisome proliferator-activated receptor (PPAR) signalling, insulin signalling, glycolysis and gluconeogenesis, glycerolipid metabolism, mitochondria and oxidative phosphorylation, and glutathione metabolism [104]. EGCG upregulated miR-210, a key component of hypoxia-inducible factor 1 α (HIF-1 α), through the hypoxia-response element found in the promoter region, in both H1299 and H460 non-small-cell lung cancer and CL13 mouse lung adenocarcinoma cell lines. Overexpression of miR-210 reduced cell proliferation rate and anchorage-independent

growth [105]. In a study by Chakrabarti et al., it was reported that EGCG downregulated three oncogenic miRNAs (miR-92, miR-93, and miR-106b) and upregulated three tumour suppressive miRNAs (miR-7-1, miR-34a, and miR-99a), leading to induction of both intrinsic and extrinsic apoptotic pathways in SK-N-BE2 and IMR-32 malignant neuroblastoma cell lines. The alterations in expression of these miRNAs were found to be more significant when treated with EGCG in combination with N-(4-hydroxyphenyl) retinamide (4-HPR), and overexpression of miR-93 and miR-7-1, which exhibited the highest fold-change, decreased and increased efficacy towards EGCG, respectively [106]. The 4-HPR is a synthetic retinoid that has been reported by Reynolds et al., to induce differentiation of neuroblastoma cells and increase survival rates in neuroblastoma patients [107]. Overexpression of miR-93 and miR-7-1, which exhibited the highest fold-change, decreased and increased efficacy towards EGCG, respectively [107]. Similar results were reported on SH-SY5Y and SK-N-DZ malignant neuroblastoma cell lines when used as stand-alone agent [108]. Meanwhile, it was found that oncogenic miR-21 was downregulated while tumour suppressive miR-330 was upregulated in prostate cancer xenograft tissues of EGCG-treated mice [109].

6. Indole-3-Carbinol (I3C) and 3,3'-Diindolylmethane (DIM)

The indole-3-carbinol (I3C) is a naturally occurring glucosinolates found in the *Brassica* vegetables such as cabbage, broccoli, cauliflower, kale, radish, turnip, and brussels sprouts, while 3,3'-diindolylmethane (DIM) is a prominent product obtained when I3C undergoes condensation reactions in the stomach [110]. Both I3C and DIM have been reported to modulate many genes involved in regulating cell cycle, cell proliferation, signal transduction, apoptosis, and other cellular processes [111, 112].

6.1. miRNAs Targeting Oncogenes. Treatment with DIM upregulated miR-21 and downregulated its target, cell division cycle 25 homolog A (Cdc25A), leading to reduced cell proliferation in MCF-7 breast cancer cells. Consequently, repression of miR-21 elevated Cdc25A level, while treatment with DIM partially restored Cdc25A expression and enhanced cell proliferation [58]. It was also reported that DIM upregulated the expression of the let-7 family and reduced the expression of its target, histone-lysine N-methyltransferase (EZH2), to inhibit self-renewal and clonogenic capacity in LNCaP, C4-2B, and PC-3 prostate cancer cell lines. The loss of let-7 family was also found to be inversely correlated with increased expression of EZH2 in prostate cancer tissues compared to adjacent normal prostate tissues [59]. The expression of miR-146a was found to be lower in pancreatic cancer cells compared with normal human pancreatic duct epithelial cells. Treatment with DIM (which increased miR-146a expression) or reexpression of miR-146a downregulated EGFR, interleukin 1 receptor-associated kinase 1 (IRAK-1), NF- κ B and metastasis-associated protein, member 2 (MTA2),

blocking cell invasion in Colo357 and Panc-1 pancreatic cancer cells [60].

6.2. miRNAs Targeting Tumour Suppressor Genes. The upregulation of miR-21, miR-31, miR-130a, miR-146b, and miR-377 observed in vinyl carbamate-induced lung cancer in mice was reversed by I3C. In addition, PTEN, PDCD4, and reversion-inducing-cysteine-rich protein with Kazal motifs (RECK) were identified as potential targets of miR-21 [75]. In another study, it was demonstrated that I3C also downregulated miR-21 in Panc-1 pancreatic carcinoma cells. Overexpression of miR-21 negated I3C-induced sensitivity towards gemcitabine and reduced the expression of its target, PDCD4, which was upregulated by I3C [76]. DIM was also found to downregulate miR-221 and upregulates its targets: PTEN, cyclin-dependent kinase inhibitor 1B (p27^{kip1}), cyclin-dependent kinase inhibitor 1C (p57^{kip2}), and p53 up-regulated modulator of apoptosis (PUMA), leading to suppression of cell proliferation and migration of MiaPaCa-2 and Panc-1 pancreatic cancer cells. MiR-221 was significantly upregulated in pancreatic cancer cell lines and tumour tissues compared to normal pancreatic duct epithelial cells and tissues. Furthermore, patients with high expression of miR-221 had a relatively shorter survival compared to those with lower expression [77].

6.3. miRNAs Targeting Transcription Factors. The expression of miR-200b, miR-200c, let-7b, let-7c, let-7d, and let-7e was found to be downregulated in gemcitabine-resistant MiaPaCa-2 pancreatic cancer cells following treatment with DIM. The zinc finger E-box binding homeobox 1 (ZEB1), target of miR-200, was found to be downregulated following reexpression of miR-200. In addition, slug and vimentin were also downregulated, while E-cadherin was upregulated. These changes were consistent with phenotypic reversal of mesenchymal to epithelial morphology [83]. Besides that, DIM enhanced the effects of Herceptin when used in combination by inhibiting cell growth, colony formation, and inducing apoptosis in SKBR3 and MDA-MB-468 breast cancer cells through upregulation of miR-200. This combination treatment and overexpression of miR-200 resulted in downregulation of FoxM1, which has been implicated in progression of various cancers [113].

6.4. Other miRNAs. In another study, DIM downregulated miR-92a which is known to be associated with receptor activator of nuclear factor- κ B ligand (RANKL) signalling, EMT, and cancer progression to inhibit differentiation of osteoclasts and osteoblasts in prostate cancer metastasis [114].

7. Other Natural Agents

Ellagitannin (1,3-Di-O-galloyl-4,6-(s)-HHDP-b-D-glucopyranose), a polyphenolic compound isolated from *Balanophora japonica*, was reported to upregulate 17 miRNAs and downregulate 8 miRNAs in HepG2 hepatocellular

carcinoma cells [115]. Another study reported that ursolic acid, a pentacyclic triterpene acid found in medicinal herbs such as *Oldenlandia diffusa* and *Radix actinidiae*, induced apoptosis in U251 glioblastoma cells by downregulating miR-21 and inducing the expression of PDCD4, a target of miR-21. Moreover, it was shown that overexpression of miR-21 suppressed the ursolic acid-induced expression of PDCD4 [116]. Garcinol, a polyisoprenylated benzophenone derivative obtained from *Garcinia indica* extracts, was found to reverse EMT in MDA-MB-231 and BT-549 breast cancer cells as well as in xenograft mouse model through upregulation of let-7a, let-7e, let-7f, miR-200b, and miR-200c. Additionally, it was also demonstrated that inhibition of miR-200a, miR-200b, and miR-200c attenuated the garcinol-mediated inhibition of invasion [117]. Besides that, matrine, an alkaloid isolated from *Sophora flavescens*, downregulated miR-21 to induce overexpression of PTEN and inactivate Akt, leading to cell cycle arrest and apoptosis in MCF-7 breast cancer cells [118]. Quercetin, a flavonoid found in onions, apples, tea, and red wine, upregulated miR-142-3p in pancreatic ductal adenocarcinoma cells (MIA PaCa-2, Capan-1, and S2-013). Overexpression of miR-142-3p inhibited cell proliferation and reduced the expression of its target, heat-shock protein 70 (HSP7). It was also shown that miR-143-3p regulated HSP70 expression independent of HSP1 and overexpression of HSP70 rescued miR-143-3p-induced cell death [119]. Furthermore, upregulation of miR-146a, a negative regulator of NF- κ B activation, by quercetin protected CCD-180Co colonic myofibroblast cells against ROS [120]. Recently, we have also reported that a total of 25 miRNAs were found to be significantly differentially expressed following treatment with 1'-S-1'-acetoxychavicol acetate (ACA), a natural compound isolated from the wild ginger, *Alpinia conchigera*, and/or cisplatin on Ca Ski and HeLa cervical carcinoma cells. These include miR-138, miR-210, and miR-744 with their predicted targets involved in signalling pathways regulating apoptosis and cell cycle progression [37]. Another study reported that ACA also downregulated miR-23a in HN4 head and neck squamous cells and its inhibition suppressed cell proliferation and induced apoptosis, with PTEN confirmed as its target [121]. Sulforaphane, an isothiocyanate derived from cruciferous vegetables such as broccoli and broccoli sprouts, upregulated 15 miRNAs (miR-372, miR-342-3p, miR-486-5p, miR-9, miR-9*, miR-145, miR-146a, miR-629, miR-505, miR-758, miR-30a*, miR-27b*, miR-135b*, miR-27b, and miR-23b) and downregulated 3 miRNAs (miR-633, miR-155, and miR-106a*) in NCM460 and NCM356 normal colon epithelial cells [122]. In sulforaphane-treated T24 bladder cancer cells, miR-200c was found to be upregulated, leading to inhibition of EMT and metastasis. Downregulation and overexpression of miR-200c reversed and enhanced ZEB1 repression and E-cadherin induction by sulforaphane, respectively [123]. Besides that, miR-140 was found to be upregulated following sulforaphane treatment in MCF10DCIS and MDA-MB-231 breast cancer cells. Both SOX9 and aldehyde dehydrogenase 1 (ALDH1) were also identified as targets of miR-140, and miR-140 overexpression downregulated the protein levels in both targets [124].

8. Conclusion

Based on current literature, it is increasingly evident that natural agents such as curcumin, resveratrol, genistein, EGCG, I3C, and DIM can alter miRNA expression profiles and target multiple genes at the same time. The pleiotropic effects of natural agents and miRNAs are attractive for cancer therapy since cancer is caused by defects in multiple genes. For this reason, it is proposed that natural agents can be used to downregulate oncogenic miRNAs and upregulate tumour suppressive miRNAs to restore drug sensitivity. Hence, there is a more promising role for natural agents in cancer therapies beyond their current use as chemopreventive agents and supplements. In conclusion, these natural agents which have been tested extensively in clinical trials and found to possess encouraging safety profiles can be used to simultaneously target diverse cellular signalling via specific miRNAs to serve as potential new avenues in cancer treatments. Together, it is hoped that these new strategies would prevent tumour recurrence and resistance towards conventional therapies, leading to improvement in the overall response and survival of cancer patients.

Abbreviations

Ago2:	Argonaute2
Akt:	Protein kinase B
Akt2:	Protein kinase B β
ALDH1:	Aldehyde dehydrogenase 1
AP-1:	Activator protein-1
ARH1:	Aplysia ras homolog 1
ARHGDIa:	Rho GDP dissociation inhibitor (GDI) alpha
BAD:	Bcl-2-associated death promoter
BAX:	Bcl-2-associated X protein
Bcl-2:	B-cell lymphoma 2
Bcl-xL:	B-cell lymphocyte xL
CASZ1:	Castor zinc finger 1
Cdc25A:	Cell division cycle 25 homolog A
C/EBP- β :	CCAAT/enhancer binding protein beta
CXCL:	Chemokine (C-X-C motif)
DNMT3b:	DNA methyltransferase 3b
Dkk2:	Dickkopf 2 homolog
eEF1A:	Eukaryotic translation elongation factor 1A
EGFR:	Epidermal growth factor receptor
EMT:	Epithelial-mesenchymal transition
EP300:	E1A binding protein p300
ErbB3:	Erythoblastic leukemia viral oncogene homolog 3
ESR1:	Estrogen receptor 1
Fbw7:	F-box and WD-40 domain protein 7
FOXC2:	Forkhead box C2
HIF-1 α :	Hypoxia-inducible factor 1 α
HOTAIR:	HOX transcript antisense RNA
HSP70:	Heat-shock protein 70
IL1RAPL1:	Interleukin 1 receptor accessory protein-like 1
IRAK-1:	Interleukin 1 receptor-associated kinase 1

K-Ras:	Kirsten rat sarcoma viral oncogene homolog
MAPK:	Mitogen-activated protein kinase
MCM:	Minichromosome maintenance
MMP:	Matrix metalloproteinases
MTA2:	Metastasis-associated protein, member 2
N4BP1:	NEDD4 binding protein 1
NF- κ B:	Nuclear factor-kappa B
p27 ^{kip1} :	Cyclin-dependent kinase inhibitor 1B
p57 ^{kip2} :	Cyclin-dependent kinase inhibitor 1C
PARP:	Poly ADP ribose polymerase
PDCD4:	Programmed cell death protein 4
PPAR:	Peroxisome proliferator-activated receptor
PTEN:	Phosphatase and tensin homologue deleted on chromosome 10
PSA:	Prostate specific antigen
PUMA:	p53 upregulated modulator of apoptosis
RACI:	Ras-related C3 botulinum toxin substrate 1
RANKL:	Receptor activator of nuclear factor- κ B ligand
RECK:	Reversion-inducing-cysteine-rich protein with Kazal motifs
ROS:	Reactive oxygen species
sFRP1:	Wnt-signalling secreted frizzled-related protein 1
Sirt1:	Sirtuin (silent mating type information regulation 2 homolog) 1
SMAD:	Mothers against decapentaplegic
Sp:	Specificity protein
Src:	V-src avian sarcoma viral oncogene homolog
SOX17:	SRY (sex determining region Y)-box 17
TGF β :	Transforming growth factor beta
TNFSF10:	Tumour necrosis factor (ligand) superfamily, member 10
WT1:	Wilm's tumour 1
ZBTB:	Zinc finger and BTB domain-containing protein
ZEB1:	Zinc finger E-box binding homeobox 1.

Conflict of Interests

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

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Review Article

MicroRNAs: Novel Players in Cancer Diagnosis and Therapies

Aaron L. Oom,¹ Brock A. Humphries,^{1,2} and Chengfeng Yang^{1,2,3}

¹ Department of Physiology, Michigan State University, East Lansing, MI 48824, USA

² Cellular and Molecular Biology Graduate Program, Michigan State University, East Lansing, MI 48824, USA

³ Center for Integrative Toxicology, Michigan State University, East Lansing, MI 48824, USA

Correspondence should be addressed to Chengfeng Yang; yangcf@msu.edu

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First discovered in 1993, microRNAs (miRNAs) have been one of the hottest research areas over the past two decades. Oftentimes, miRNAs levels are found to be dysregulated in cancer patients. The potential use of miRNAs in cancer therapies is an emerging and promising field, with research finding miRNAs to play a role in cancer initiation, tumor growth, and metastasis. Therefore, miRNAs could become an integral part from cancer diagnosis to treatment in future. This review aims to examine current novel research work on the potential roles of miRNAs in cancer therapies, while also discussing several current challenges and needed future research.

1. Introduction

MicroRNAs (miRNAs) were first discovered by Lee et al. in 1993 [1]. In the first decade after their discovery a particular focus was placed on their importance in development as first illustrated by Ambros' group. This developmental focus segued perfectly into cancer applications and has been, for the past decade, the drive for much of the research in miRNA potential use for cancer therapy.

miRNAs are small noncoding RNAs that are produced naturally by the cell. They function by sequence-specific binding of a seed sequence to the 3' end of the untranslated region (UTR) of a target mRNA, causing the mRNA to then be degraded or to be translationally inhibited [2]. miRNAs have been thought to regulate two-thirds of the entire protein coding genome [3]. The expression of miRNAs themselves can also be regulated similarly to that of protein coding genes. Whether through genetic or epigenetic shifts, the expression levels of miRNAs are often altered in many cancers, resulting in abnormal increases or decreases [4]. These alterations have been shown to play a part in almost all facets of cancer development and progression.

Recent research has shown that tumor suppressors, such as phosphatase and tensin homolog (PTEN) [5–10] and p53 [11], can be potential targets of miRNAs. Typically

downregulated in many cancers, the loss of these critical tumor suppressors can greatly increase cell proliferation and tumor progression. Furthermore, the link between some tumor suppressors and survival genes, such as the link between PTEN and the survival effector, AKT (also known as protein kinase B or PKB), may indicate therapeutic means of targeting metastasis, tumor growth, and cancer survival. And indeed, current research is examining these miRNAs that target tumor suppressors both on their own and in combination with traditional therapies, such as cisplatin [9, 10], etoposide, and ionizing radiation [11].

Additionally, certain families of miRNAs have been implicated in epithelial-to-mesenchymal transition (EMT), a critical component of cancer metastasis. EMT is typically marked by changes in morphology and cytoskeletal rearrangement. For example, one emerging family in cancer metastasis is the miR-200 family which is known for directly targeting the E-cadherin suppressors zinc finger E-box binding homeobox 1 and homeobox 2 (ZEB1 and ZEB2) [12, 13]. The loss of miR-200 family members leads to decreased E-cadherin levels. This loss of E-cadherin, an integral cell to cell adhesion protein, correlates with a dramatic increase in cells going through EMT [12, 13].

This review will examine the role that miRNAs could play in cancer therapeutics. To this end, current novel research

work on the cancer therapeutic values of miRNAs will be examined.

2. miRNAs as Potential Markers for Cancer Diagnosis

While some of the current miRNA research work focuses on targeting novel miRNA changes and investigating the mechanism involved, other research is aimed at characterizing the miRNA expression levels for particular cancer types. It is becoming apparent that the severity and stage of cancer can be associated with miRNA levels, as seen below. This is critical, as choosing the most effective treatment option is highly dependent on precisely knowing disease aggressiveness. For example, low grade cancers do not have to be treated as aggressively as high grade cancers.

This strong drive to use miRNA screening for diagnosis serves two particular purposes: as an earlier diagnosis tool and as a more efficient means of diagnosis. Circulating miRNAs have been shown to be one of the very promising biomarkers for cancer earlier diagnosis [14]. It is believed that intracellular miRNAs are packaged into exosomes or microvesicles, which are then expelled from cells and enter the blood stream. Moreover, it has been found that circulating miRNAs are highly stable, which makes them ideal candidates serving as earlier diagnostic biomarkers for cancer [15].

For example, colorectal cancer diagnosis currently utilizes either invasive methods, such as a colonoscopy, or far less precise methods, such as fecal analysis. The often seen reluctance to comply with such measures ultimately results in much later diagnosis in patients [16]. Examining tissue and blood samples from patients of varying stages of colorectal cancer, Yong et al. showed seven miRNAs altered in both, with three of those having strong positive correlations between blood and tissue samples: miR-193a-3p, miR-23a, and miR-338-5p. Interestingly, the levels of each increased as the stage of the cancer progressed, suggesting an important role mechanistically as well as diagnostically for this trio [16].

Much like colorectal cancer, prostate cancer has long suffered from ambiguities and difficulties within diagnosis methodology. The most employed diagnosis method for prostate cancer is the Gleason scale. Tumors are differentiated based on size and histological features. There is a certain gray area when it comes to this method, making it far more difficult to choose a proper course of treatment. The use of miRNAs to categorize prostate cancer subtypes could overhaul the Gleason system and present a more accurate and reliable system. To address this, Wach et al. screened two cohorts of prostate cancer patients. They found that four particular miRNAs—miR-143, miR-145, miR-200c, and miR-375—were the most dramatically altered miRNAs in the two cohorts. Of the four, miR-143, miR-145, and miR-375 were best at distinguishing between malignant and nonmalignant tumors. Considering the three in conjunction, they were able to correctly distinguish between malignant and nonmalignant samples 77.6% of the time [17].

If a cancer type already has a standard method of subtype characterization it is possible for researchers to develop a miRNA screen around the molecular markers used for

diagnosis; this was employed by Leivonen et al. with HER2 positive breast cancer lines and two patient cohorts. The group found a wide variety of miRNAs that downregulated HER2 and found a strong correlation between higher miR-342-5p levels and survival time [18]. While this type of characterization allows for a miRNA signature to be created for various cancer subtypes and thereby offering therapeutic options, it also serves the very practical purpose of diagnosis.

Of the possible benefits that can be derived from miRNA screens, the most substantial is the uniformity. We are currently able to detect miRNA levels from a basic serum sample. The power of taking a single blood sample from a patient and being able to address a wide variety of cancers from one miRNA screen is staggering.

3. miRNAs as Potential Cancer Therapies

Diagnosing from miRNA screens is not the only possible application; there is emerging evidence that it is possible to manipulate miRNA levels to enhance current cancer therapeutics. The miRNA screening process has allowed for the rise of more specialized research that focuses on these significantly dysregulated miRNAs and what they may be targeting. Reintroduction or inhibition of the dysregulated miRNA in conjunction with traditional cancer therapies could make for a more efficient treatment plan. This natural progression of methodology has yielded numerous miRNAs in each cancer type that can also serve as potential targets on their own in various cancers. Many miRNAs have been shown to have therapeutic potentials; in the following sections only a few selected miRNAs and target pathways are discussed to help highlight important and significant areas of the research.

3.1. miRNAs Targeting Receptor Tyrosine Kinases. Receptor tyrosine kinases (RTKs) are well known for their role in upregulating angiogenesis and cell proliferation in many cancers and have therefore served as critical drug targets in cancer treatment [19]. Fasanaro et al. found that miR-210 expression is significantly upregulated under hypoxic conditions. Hypoxic conditions are typical in several cancer microenvironments, such as tumor cores and bone cancers. MicroRNA-210 expression triggered the formation of capillary structures and directly targeted ephrin 3A, a ligand for a member of the RTK subfamily, ephrin receptors [20].

Comparable findings have also been found across various cancers, such as non-small cell lung cancers, breast cancer, and glioblastomas. Oneyama et al. showed that miR-99a expression significantly inhibited tumor growth and cell proliferation in lung cancers. The group showed that miR-99a was targeted by oncogenic proteins such as Ras and epidermal growth factor (EGF). Furthermore, they showed that miR-99a targets the RTK, fibroblast growth factor 3 (FGFR3) [21]. Similarly, Acunzo et al. found that miR-27a targets MET, a well-known oncogene, and epidermal growth factor receptor (EGFR) in non-small cell lung cancers [22]. Mackiewicz et al. found that miR-34a significantly reduces cell migration in breast cancer cell lines by directly targeting the RTK, AXL [23]. Likewise, Feng et al. showed that lin28 expression significantly upregulates HER2 in breast cancer and correlates

with a poorer prognosis in patients when highly expressed. Furthermore, lin28 expression—which is known to target let-7a—significantly increases cell growth *in vitro* [24]. Finally, Rao et al. showed that upregulation of miR-219-5p inhibited cell growth and cell migration in glioblastoma cells via EGFR targeting [25]. Together these findings suggest an alternative method of targeting RTKs across a variety of cancers.

3.2. miRNAs Targeting Bcl-2 Family Members. Bcl-2 family members are either antiapoptotic or proapoptotic, with dysregulation often occurring in both types in many cancers. Kwon et al. found that miR-193a-3p directly targets the antiapoptotic Mcl-1 with stable expression of this miR inhibiting cell growth, inducing apoptosis, and creating DNA damage in the glioblastoma cell line, U-251 [26]. Zhang et al. showed that overexpression of miR-29a significantly inhibited cell proliferation and promoted apoptosis in U2OS and SAOS-2 osteosarcoma cells. This group also showed in a luciferase reporter assay that miR-29a directly inhibits Bcl-2 and Mcl-1, both antiapoptotic family members [27]. Likewise, Ji et al. found that low miR-133a levels corresponded to a poorer prognosis in osteosarcoma patients. When miR-133a was expressed *in vivo*, the group found that tumor volumes were significantly suppressed. MicroRNA-133a was found to target Bcl-xL and Mcl-1 and promoted apoptosis upon overexpression [28]. Similarly in pancreatic cancer Guo et al. showed that restoration of miR-491-5p yielded a slight increase in cell death and a slight decrease in cell proliferation by targeting Bcl-xL and TP53 in SW1990 pancreatic cancer cells [29]. Ji et al. showed that a restoration of miR-34 as well inhibited cell proliferation *in vitro* and tumor growth *in vivo* in MiaPaCa2 pancreatic cancer cells through direct Bcl-2 targeting [30].

Targeting of Bcl-2 was also found in colorectal cancer and leukemia by Zhang and Cimmino. Zhang et al. found that miR-148a significantly induced cell apoptosis by directly targeting Bcl-2 in RKO and Levo colorectal cancer cell lines [31], while Cimmino et al. showed that when miR-15 and miR-16 were reexpressed cell apoptosis was significantly upregulated in MEG-01 leukemia cells by directly targeting Bcl-2 [32]. Lastly, Lin et al. found that upregulation of miR-122 decreased relative cell numbers and significantly increased caspase-3 activity by directly targeting Bcl-w, an antiapoptotic family member, in hepatocellular carcinoma cell lines [33]. With many Bcl-2 family inhibitors still locked in clinical trials, miRNAs that target the family could prove to be another source of Bcl-2 inhibitors.

3.3. miRNAs Targeting the Ras Subfamily. One of the best known oncogene families is the Ras subfamily. The Ras subfamily is critically involved in cell survival, angiogenesis, and proliferation. Upregulation of Ras is found in numerous cancers and causes unchecked growth and invasiveness [34]. Thus, it serves as a valuable target in cancer treatments.

Kasinski and Slack showed that miR-34 inhibits cell proliferation and migration *in vitro* in lung cancer cells by targeting K-Ras in addition to other oncogenes. Additionally, ectopic expression of miR-34 significantly suppressed tumor growth *in vivo* [35]. Similarly, Sun et al. found

that downregulation of miR-31 significantly decreased cell proliferation *in vitro* and showed a significant decrease in tumor size *in vivo* with Caco-2 and HT-29 human colorectal adenocarcinoma cells by directly targeting RAS p21 GTPase activating protein 1 (RASA1), a regulator of the RAS-MAPK pathway [36].

Shin et al. found that expression of miR-181a significantly suppressed cell proliferation and colony formation in squamous cell carcinoma cells. This group also found that miR-181a directly targets K-Ras in squamous cell carcinoma cells [37]. More specifically, Jang et al. showed that restoration of miR-636 by suppression of adenine nucleotide translocase 2 (ANT2) significantly reduces cell proliferation *in vitro* and tumor growth *in vivo* by directly targeting Ras in hepatocellular carcinoma cells [38].

Finally, Wang et al. showed that restoration of miR-451 significantly inhibits cell proliferation and colony formation and induces apoptosis in human non-small cell lung cancer cells. Additionally, expression of miR-451 *in vivo* significantly reduced tumor growth. They showed that miR-451 directly targets Ras-related protein 14 (RAB14) in human non-small cell lung cancer cells [39].

Additional miRNAs having potential therapeutic values are listed in Table 1.

4. miRNAs as Cancer Therapy Resistance Mediators

A large amount of research has also focused on the use of miRNAs in conjunction with traditional therapies and their ability to mediate therapeutic responses. For the purpose of discussion, miRNAs that confer resistance to therapy when highly expressed will be considered resistance mediators. In the interest of space, only a few selected miRNAs are discussed in the following sections.

4.1. miR-21. One prominent example of a resistance mediator is miR-21. Current research suggests that one role of miR-21 in cancer is regulating DNA repair and maintenance in response to treatment, particularly nucleoside analogs. Paik and colleagues found that using a miR-21 inhibitor significantly reduced resistance to gemcitabine in Panc-1, a human pancreatic cancer cell line [78]. Likewise, Hwang and colleagues showed that inhibition of miR-21 dramatically decreased cell growth in PL45 cells, a pancreatic ductal adenocarcinoma cell line, when treated with fluorouracil (5-FU) [79]. Both 5-FU and gemcitabine are chemotherapy agents that act as nucleoside analogs, raising the possibility of a role of miR-21 in DNA repair.

To strengthen this, Wong et al. found that prolonged exposure to temozolomide, an alkylating agent, significantly upregulated miR-21 expression in D54MG glioblastoma cells. Inhibition of miR-21 drastically increased cell death when resistant D54MG cells were treated with temozolomide [81]. Additionally, Griveau et al. showed that when miR-21 was inhibited with a locked nucleic acid in U87MG glioblastoma cells, the cells were far more susceptible to radiation-induced cell death [82]. Deng et al. found that miR-21 also regulates thymidine phosphorylase, dihydropyrimidine

TABLE 1: miRNAs explored as potential cancer therapeutic agents.

miR	Cancer type	Targets
let-7a	Breast [24], endometrial [40]	HER2 and aurora-B
miR-15 and miR-16	Leukemia [32]	Bcl-2
miR-21	Glioblastoma, breast [41]	E-cadherin-ZEB1/2 pathway
miR-21 and miR-181b	Glioma [42]	FOS
miR-22 and miR-200b	Gastric [43]	Wnt-1 pathway
miR-26a	Liver, prostate, skin [44], bladder [45]	Lin28B, Zcchc11, and HMGA1
miR-27a	Lung [22], breast [46]	MET, EGFR, and PI3K-AKT pathway
miR-27b	Colorectal [47]	VEGFC
miR-29a	Osteosarcoma [27]	Bcl-2, Mcl-1
miR-31	Colorectal [36]	RASA1
miR-34	Breast (34a) [23], pancreatic [30], lung [35], lung (34a, c) [48]	AXL, Bcl-2, K-Ras, and PDGFR- α/β
miR-99a	Lung [21]	mTOR/FGFR3
miR-106b-5p	Glioma [49]	RBL1, RBL2, and CASP8
miR-122	Liver [33]	Bcl-w
miR-130b	Pancreatic [50]	STAT3
miR-133a	Osteosarcoma [28]	Bcl-xL and Mcl-1
miR-138	Glioblastoma [51]	EZH2-CDK4/6-pRb-E2F1 pathway
miR-145	Ovarian [52]	p70S6K1 and MUC1
miR-148a	Colorectal [31], liver [53]	Bcl-2 and Met/Snail pathway
miR-150	Lung [54]	p53
miR-155	Breast [46, 55, 56]	VHL, TP53INP1, and PI3K-AKT pathway
miR-181	Squamous cell (181a) [37], breast [57]	K-Ras and ataxia telangiectasia mutated (ATM)
miR-185 and miR-342	Prostate [58]	SREBP pathway
miR-193a-3p	Glioblastoma [26]	Mcl-1
miR-205	Oral [59]	Axin 2
miR-210	Hypoxic conditions [20]	Ephrin-A3
miR-219-5p	Glioblastoma [25]	EGFR
miR-221	Pancreatic [60], breast [61]	PTEN, p27kip1, p57kip2, and PUMA
miR-301a	Breast [62]	PTEN
miR-449a and -449b	Retinoblastoma [63]	E2F transcription factors
miR-451	Lung [39]	RAB14
miR-491-5p	Pancreatic [29]	TP53 and Bcl-xL
miR-494	Glioma [64]	p190B RhoGAP
miR-497	Neuroblastoma [65]	WEE1
miR-636	Liver [38]	Ras
miR-708	Bladder [66]	Caspase-2

dehydrogenase, and human DNA mismatch repair protein Msh2 (hMSH2)—proteins involved in DNA repair and synthesis—in the colon cancer cell line HT-29. When miR-21 was downregulated the cells were significantly resensitized to 5-FU treatment and irradiation [85]. Lastly, Valeri et al. showed that miR-21 was directly targeting hMSH2 and creating 5-FU resistance [86]. All together, these findings strongly suggest that miR-21 is playing an important role in cancer cell DNA repair and maintenance.

Furthermore, evidence is showing that miR-21 could be interacting with apoptosis-regulating proteins and important tumor suppressors, allowing for prolonged cell survival and unchecked growth and proliferation. Li et al. found that when miR-21 was suppressed in U373 MG glioblastoma cells, the cells were significantly resensitized to VM-26, a topoisomerase-II inhibitor. The group found that miR-21 directly targets leucine rich repeat (in FLII) interacting protein 1 (LRRFIP1), a protein with downstream products in

the tumor necrosis factor receptor family [83]. Yang et al. showed that prolonged exposure to cisplatin in SGC7901 cells, a gastric cancer cell line, significantly upregulated miR-21 expression and downregulated PTEN expression. Inhibition of miR-21 resensitized the cisplatin-resistant SGC7901 cells to cisplatin, a platinum-based chemodrug that triggers DNA crosslinking [7]. Based on these findings miR-21 is acting in a wide variety of roles across a variety of cancers. The ubiquitous nature and overexpression of miR-21 in cancers make it a valuable target of great clinical significance.

4.2. miR-125b. The oncomiR miR-125 is of great importance in breast cancer, as there has been a growing body of evidence suggesting that it plays a crucial role in initiation, metastasis, and recurrence. Current research is slightly split on the role of miR-125b with more research suggesting that miR-125b is important in conferring therapy resistant phenotypes in a variety of cancers rather than a therapy sensitive phenotype. Zhou et al. found that inhibition of miR-125b resensitized cells to paclitaxel due to miR-125b directly targeting Bak1, a proapoptotic factor, in a suite of breast cancer cell lines [99]. Similarly, Liu et al. showed that miR-125b was significantly upregulated in taxol-resistant breast cancer cell lines. Further research has elucidated that Snail upregulates miR-125b which directly targets Bak1, thereby reducing chemosensitivity to gemcitabine and taxol [100]. Likewise, Wang et al. showed a negative correlation between circulating miR-125b levels in breast cancer patients and treatment response and a positive correlation between miR-125b levels and disease severity. By directly targeting the cell cycle regulator E2F3 miR-125b has been shown to increase resistance to 5-FU in a variety of breast cancer cell lines [101]. Outside of breast cancer lines, Iida et al. found that miR-125b was upregulated in doxorubicin-resistant derivatives of the Ewing sarcoma lines, WE-68 and VH-64. Once again, miR-125b was targeting Bak1 and p53, thereby decreasing chemosensitivity to doxorubicin [102].

4.3. miRNAs Targeting Tumor Suppressors. As previously mentioned, PTEN and p53 are critical tumor suppressors that are often dysregulated in many cancers. Emerging research suggests that a wide variety of miRNAs target PTEN and p53 allowing for cancer cells to proliferate and grow at an alarming rate. Additionally, the suppression of PTEN and p53 helps to grant a chemoresistant phenotype across several cancers. Fu et al. showed that miR-93 was significantly upregulated in CDDP resistant variations of the ovarian cancer lines OVCAR3 and SKOV3 and in primary tumor samples. Further analysis revealed that miR-93 binds to the 3'UTR of PTEN and regulates its expression [5]. The regulation of PTEN by miRNAs is also found in breast cancer. Liang et al. found that inhibition of miR-19 *in vitro* and *in vivo* decreased cell viability and tumor growth, respectively. Liang et al. found miR-19 to directly target the tumor suppressor, PTEN, in MCF-7 cells [6]. Likewise, Guo et al. showed that when c-Myc is overexpressed there was a significant increase in cell viability, while inhibition of c-Myc significantly reduced cell viability in U87 and U251 glioblastoma multiforme cell lines. The group showed that the

upregulation of c-Myc, a prominent oncogene, upregulates miR-26a, which then directly targets PTEN [8]. Li et al. found that miR-92b was dramatically upregulated in both non-small cell lung cancer tissue samples and cell lines. miR-92b expression was found to significantly resensitize cells to cisplatin by directly targeting PTEN [9]. Zhao et al. found that when they suppressed miR-221, a cisplatin-sensitive phenotype was restored to the osteosarcoma cell lines, SOSP-9607 and MG63. miR-221 was shown to be directly targeting PTEN in the cell lines [10]. Liu et al. found that miR-375 desensitizes the cells to etoposide, a topoisomerase inhibitor, and to ionizing radiation by directly targeting p53 in MCF-7, AGS, and A549 cell lines [11]. The expression of both of these well-known tumor suppressors is typically down in most cancers, but these findings help to elucidate a possible mechanism through which this downregulation occurs and offer clinical targets for slowing tumor progression. This ever expanding and critical overlap between oncomiRs and tumor suppressors may serve to illuminate the most effective targets for supplementing current cancer therapeutics.

5. miRNAs as Cancer Therapy Sensitizers

5.1. let-7 Interactions with Chemotherapy. Research has also uncovered several families of miRNAs that are significantly reduced in most cancers. A wealth of current research strongly suggests that the let-7 family can significantly sensitize cancer cells to therapy, reduce proliferation, reduce invasion, and reduce cell growth. Chen et al. found that the levels of let-7a in patients with acute myeloid leukemia (AML) correlated significantly with better prognosis, and upregulation of let-7a *in vitro* or *in vivo* significantly resensitized AML to cytarabine (also known as Ara-C) treatment. This group found that let-7a is regulated by CXCR4 in acute myeloid leukemia [67]. Lv et al. from the same group as Wang et al. found that lin28 regulation of let-7a also affected chemoresistance in SK-BR-3 cells. Downregulation of lin28 decreased resistance to paclitaxel and high levels of lin28 correlated with metastasis and/or relapse [68]. Bhutia et al. showed that lin28 and SET regulated processing of let-7a in pancreatic cancer cell lines. Furthermore, they found that resistance to gemcitabine correlated with a buildup of unprocessed pre-let-7 along with an increase of RRM2, a potential target of let-7a that is involved in the reduction of ribonucleotides to deoxyribonucleotides [70]. Thus, lin28 may serve as an important therapeutic target, in addition to the use of let-7 mimics.

Guo et al. showed that let-7b reconstitution restored a cisplatin-sensitive phenotype in U251 cells. Additionally, cyclin D1 is a direct target of let-7b, with knockdown of cyclin D1 yielding a phenotype similar to let-7b reconstitution [71]. Sugimura et al. found that let-7c regulates the interleukin-6/STAT3 prosurvival pathway in esophageal cancer. When let-7c was highly expressed, the IC₅₀ for cisplatin was significantly decreased and the overall survival times for patients were increased [72]. Along those lines, Zhao et al. showed that higher levels of let-7c in NSCLC correlated with longer survival times in patients, with carcinoma tissues in patients typically having significantly lower levels of let-7c

than normal tissues. Let-7c was shown to be directly targeting integrin β_3 and MAP4K3. Restoration of let-7c expression inhibited proliferation, invasion, and migration *in vitro* [127]. Cai et al. found that cisplatin treatment downregulated let-7e expression in ovarian cancer cell lines. The group found that enhancer of zeste homolog 2 (EZH2) and cyclin D1, both thought to play a role in drug resistance, are potentially regulated by let-7e. Reexpression of let-7e *in vivo* significantly slowed tumor progression and reduced EZH2 and cyclin D1 expression [73]. The let-7 family is proving to be critical across a wide variety of cancer types, with reexpression often having significant effects, making it an excellent target for future clinical use.

5.2. miR-200 Family Interactions with Chemotherapies. Another emerging metastasis suppressor and therapy sensitizer is the miR-200 family. The miR-200 family, while particularly known for its role in suppressing EMT, is proving to play an additional role in mediating cancer cell response to traditional therapeutic regimens. Much of the current research on the miR-200 family has focused particularly on miR-200a, -200b, and -200c, with far less research looking at miR-141 and miR-429. Soubani et al. showed that the curcumin analog, difluorinated curcumin (CDF), is able to upregulate miR-200a, -200b, and -200c in pancreatic cancer lines. Additionally, it was shown that the expression of CDF upregulated the critical tumor suppressor PTEN [128]. Furthering this, Ali et al. found that CDF-mediated upregulation of miR-200b and miR-200c along with CDF-mediated downregulation of miR-21 elevated PTEN levels and suppressed NF- κ B DNA binding activity. The modulations of these microRNAs significantly resensitized pancreatic cancer cells to gemcitabine [80].

Kopp et al. found that reconstitution of miR-200c in breast cancer significantly sensitized cells to doxorubicin. Furthermore, miR-200c expression was found to be targeting Bmil and TrkB, a potential oncogene and a survival factor, respectively [116]. Cochrane et al. showed that reexpression of miR-200c in ovarian cancer—where expression is typically reduced—significantly resensitized cells to paclitaxel [117]. On the other hand, Prislei et al. showed that miR-200c levels can have varying effects on ovarian cancer. The group showed that TUBB3, a potential target of miR-200c, has both a 200c binding site and a HuR binding site. Depending on the location of HuR, patients with high miR-200c levels had either a better (nuclear HuR) or worse (cytoplasmic HuR) prognosis, with regard to survival time and progression free survival, due to differential interactions of miR-200c with TUBB3 [129]. Lastly, Hur et al. found that restoration of miR-200c to miR-200c deficient colorectal cancer lines yielded a significant increase in cell proliferation. However, there was a marked decrease in cell invasion and migration, suggesting a critical role for miR-200c in suppressing an EMT phenotype. Through luciferase reporter assay the group found that miR-200c targets ZEB1, protein C-ets-1 (ETS1), and vascular endothelial growth factor receptor 1 (FLT1) [130].

5.3. Various miRNA Interactions with Irradiation. While a vast amount of current research focuses on miRNAs in

combination with chemotherapy, a small body of studies examines irradiation treatments in conjunction with miRNA treatment. The alteration of miRNAs involved in DNA damage repair due to radiation would allow for cancer cells to resist radiation treatment [131–133]. To this extent, Huang et al. focused on RAD51 and its paralog RAD51D, two proteins involved in homologous recombination mediated double strand break repair. The group found that miR-107, which directly targeted RAD51 and RAD51D, and miR-103 when upregulated in the human osteosarcoma cell line U2OS sufficiently resensitized cells to irradiation and several chemotherapeutics [96].

Wang et al. showed that overexpression of lin28, a cancer stem cell marker, in SK-BR-3 breast cancer cells, downregulated let-7a. Restoration of let-7a expression significantly resensitized cells to radiation treatment [69]. Wang et al. found that miR-23b expression was down in radiation resistant lines of pancreatic cancer cells. When reexpressed, cells were far more sensitive to radiation treatment both *in vitro* and *in vivo*. The group found that miR-23b targets ATG12, thereby regulating autophagy [88].

Zhang et al. showed that miR-29c is frequently downregulated in nasopharynx cancers. Restoration of typical miR-29c levels significantly resensitized cells to irradiation and cisplatin *in vitro* and significantly reduced tumor growths *in vivo*. It was shown that miR-29c suppresses both Mcl-1 and Bcl-2 [89]. Yang et al. found that miR-145 is typically downregulated in glioblastoma multiforme patients. Reexpression of miR-145 created a temozolomide and irradiation sensitive phenotype *in vitro* and *in vivo*, with an impressively significant reduction in tumor growth. MiR-145 was found to directly target both Oct4 and Sox2 [108]. Liang et al. showed that miR-302 replacement therapy significantly resensitized breast cancer cells to irradiation *in vivo* and *in vitro* by directly targeting AKT1 and RAD52 [120].

A more comprehensive look at miRNA interactions with traditional therapies can be found in Table 2.

6. miRNA Delivery Systems

One of the issues to be confronted with miRNAs in treatment is the system of delivery. A variety of means from nanotechnology to lipids to viruses have been explored, each with its own advantages and setbacks.

6.1. Nanotechnology-Based miRNA Delivery Systems. Much of the current research on miRNA delivery is trending towards the use of nanotechnologies. Ando et al. found that the use of dicetyl phosphate-tetraethylenepentamine-based polycation liposomes was significantly more effective than N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium methylsulfate-based liposomes (DOTAP) for delivering miR-92a to human umbilical vein endothelial cells (HUVECs) and releasing it into the cytoplasm [134]. Biray et al. showed that the use of polyethylene glycol-polyethylenimine nanocomplexes was approximately 80% more effective than control reagents in delivering miR-150 into chronic myeloid leukemia cells [135]. Likewise, Yang et al. found that the use of cationic polyurethane-short branch polyethylenimine for

TABLE 2: miRNAs in combination with traditional cancer therapies.

miR	Cancer type (effect of expression on treatment)	Treatments investigated
let-7a	Leukemia [67] (sensitive), breast [68, 69] (sensitive), pancreatic [70] (sensitive)	Ara-C, irradiation, paclitaxel, gemcitabine
let-7b	Glioblastoma [71] (sensitive)	Cisplatin
let-7c	Esophageal [72] (sensitive)	Cisplatin
let-7e	Ovarian [73] (sensitive)	Cisplatin
miR-9	Glioblastoma [74] (resistant)	Temozolomide
miR-10b	Colon [75] (resistant)	Fluorouracil
miR-15b	Tongue [76] (sensitive)	Gemcitabine
miR-17-5p	Pancreatic [77] (resistant)	Gemcitabine
miR-19a	Breast [6] (resistant)	Taxol, mitoxantrone, etoposide
miR-21	Gastric [7] (resistant), pancreatic [78–80] (resistant), glioblastoma [81–84] (resistant), colorectal [85, 86] (resistant), adenocarcinoma [87] (resistant)	Gemcitabine, fluorouracil, temozolomide, irradiation, cisplatin
miR-23b	Pancreatic [88] (sensitive)	Irradiation
miR-29c	Nasopharynx [89] (sensitive)	Cisplatin, irradiation
miR-30c	Breast [90] (sensitive)	Paclitaxel, doxorubicin
miR-30d, miR-181a and miR-199a-5p	Colorectal, prostate, and leukemia [91] (sensitive)	Trichostatin A
miR-31	Ovarian [92] (sensitive)	Paclitaxel
miR-34a	Breast [93] (sensitive), prostate [94] (sensitive), gastric [95] (miR-34c-5p, sensitive)	Adriamycin, camptothecin, paclitaxel
miR-92b	Lung [9] (resistant)	Cisplatin
miR-93	Ovarian [5] (resistant)	Cisplatin
miR-103 and miR-107	Osteosarcoma, cervical, lung [96] (sensitive)	AZD2281, cisplatin, etoposide, camptothecin, irradiation
miR-106a	Ovarian [97, 98] (sensitive or resistant)	Cisplatin, paclitaxel
miR-125b	Breast [99–101] (resistant), Ewing sarcoma [102] (sensitive)	Paclitaxel, fluorouracil, doxorubicin, gemcitabine
miR-128-2	Lung [103] (resistant)	Cisplatin, doxorubicin, fluorouracil
miR-130a	Liver [104] (resistant)	Cisplatin
miR-140	Osteosarcoma and colon [105] (resistant)	Fluorouracil
miR-141	Ovarian [106] (resistant)	Cisplatin
miR-143 and miR-145	Colon [107] (sensitive)	Fluorouracil, irinotecan, oxaliplatin
miR-145	Glioblastoma [108] (sensitive), cervical [109] (sensitive)	Irradiation, temozolomide, mitomycin
miR-152 and miR-185	Ovarian [110] (sensitive)	Cisplatin
miR-181b	Pancreatic [111, 112] (sensitive or resistant), glioblastoma [113] (sensitive)	Gemcitabine, temozolomide
miR-182	Ovarian [114] (resistant)	Paclitaxel, cisplatin
miR-199a-5p	Liver [115] (sensitive)	Cisplatin
miR-200b	Pancreatic [80] (sensitive), tongue [75] (sensitive)	Gemcitabine, cisplatin
miR-200c	Pancreatic [80] (sensitive), breast [116] (sensitive), ovarian [117] (sensitive)	Gemcitabine, paclitaxel, doxorubicin
miR-223	Liver [118] (sensitive)	Doxorubicin, paclitaxel
miR-298	Breast [119] (sensitive)	Doxorubicin
miR-302	Breast [120] (sensitive)	Irradiation
miR-320	Prostate [121] (sensitive), pancreatic [122] (miR-320c, resistant)	Cisplatin, carboplatin, paclitaxel, gemcitabine

TABLE 2: Continued.

miR	Cancer type (effect of expression on treatment)	Treatments investigated
miR-375	Gastric [11] (resistant), cervical [123] (resistant)	Irradiation, etoposide, paclitaxel
miR-591	Ovarian [97] (sensitive)	Paclitaxel
miR-650	Lung [124] (resistant)	Docetaxel
miR-663	Breast [125] (resistant)	Adriamycin, chlorophosphamide, docetaxel
miR-708	Ewing sarcoma [126] (sensitive)	Etoposide, doxorubicin

miR-145 significantly reduced glioblastoma multiforme tumor sizes *in vivo*. Impressively, the addition of irradiation and temozolomide nearly removed all traces of tumors [108]. Lastly, Cao et al. showed that protamine sulfate-nanodiamond hybrid nanoparticle delivery of miR-203 significantly reduced cell migration and proliferation in the esophageal cancer cell line, Ec-109 [136]. The emergence of nanotechnology as a delivery mechanism, particularly within the last year, is significant for clinical development of miRNA therapies.

6.2. Lipid-Based miRNA Delivery Systems. In the past, cationic lipid and polymer based delivery systems have suffered from cytotoxic side effects *in vivo*, mainly due to their cationic nature. Many of these systems contain common organic structures, creating biochemical consequences in the cell [137]. However, recent advances in the field have yielded promising results. Griveau et al. found that lipid nanocapsule-locked nucleic acid complexes targeting miR-21 in glioblastoma cells significantly resensitized the cells to irradiation at 48 hours after transfection as compared to N-TER Nanoparticle siRNA Transfection System, a current transfection reagent [82]. Similarly, Shi et al. found that solid lipid nanoparticles were effective in delivering anti-miR-21 oligonucleotides to lung cancer cells *in vitro*. This group saw a significant decrease in migration and invasion along with a significant increase in apoptosis of these cancer cells [138]. Moreover, Trang et al. showed that a neutral lipid emulsion containing either miR-34a or let-7b significantly reduced lung tumor growth *in vivo* [139]. Piao et al. showed that lipid-based nanoparticles were effective in delivering pre-miR-107 to neck and squamous cancer cells *in vivo*. Analysis of the tumors revealed a significant reduction in tumor growth and an increase in survival [140]. Current advances may make lipid-based delivery systems viable methods of delivery within the next few years.

6.3. Virus-Based miRNA Delivery Systems. Viral delivery systems, while far more efficient than other mechanisms, frequently elicit immunogenic responses, hindering their overall effectiveness. Because of this significant limitation, viral delivery systems have been primarily limited to *in vitro* work, with retroviruses playing an important role in RNAi research. Current research on the practicality of viral delivery systems for miRNAs is extremely limited and as such is only mentioned for the sake of acknowledgement.

7. Challenges and Perspectives

While miRNAs have a wealth of potential, the field has several challenges that it still needs to address. There are limitations for using circulating miRNAs as potential biomarkers for cancer earlier diagnosis. One of the limitations is the reported inconsistency of circulating miRNA alterations in a particular type of cancer. For example, the levels of a good number of (>30) miRNAs have been found to be significantly changed (increased or decreased) in gastric cancer patients' blood, serum, or plasma samples [141]. Nevertheless, the observed alterations of circulating miRNAs are mostly sporadic with little consensus among different studies. Few circulating miRNAs were found to be similarly and significantly altered in three or more than three studies.

As is usually a problem with most pharmaceuticals, miRNAs in therapy would require an effective delivery system. Much of the current research for this is focusing on various nanotechnologies in hopes of reducing the toxicity seen in some current delivery mechanisms. The selected delivery systems must meet several criteria with no major issues of safety and efficient use. Much of the current research on the safety of RNA interference *in vivo* revolves around shRNAs, but there may still be some applicability in the results. Grimm et al. found that overexpression of over 30 different shRNAs *in vivo* caused liver toxicity and ultimately death in several mice. The group found that miRNAs and shRNAs were competing for cellular processing equipment leading to a buildup of premature miRNAs and shRNAs [142].

Many research groups reintroduce miRNAs through the use of pre-miRNAs. This influx of unprocessed miRNAs could flood the miRNA processing system and lead to toxic, and potentially lethal, side effects. However, Liu et al. showed that while lentiviral delivery of miR-30 to melanomas had comparable effects on cancer progression *in vivo* similar to shRNAs, miR-30 had little incidence of inflammation [143]. So it is possible that miRNAs could attenuate some of the side effects of shRNAs, but this is a gap in knowledge that will need filling as the field moves closer to clinical applications. However, this hurdle of investigating safety and miRNA delivery *in vivo* is just beginning to be met and is the next major challenge to the field on its way to full clinical application.

The field of miRNA use in cancer therapy is most likely heading in a direction that is more oriented towards *in vivo* investigation and translational research. However, presently,

there are some cancers that are far more represented in the literature than others. The current literature is more geared towards cancers such as glioblastomas, breast, ovarian, non-small cell lung, and pancreatic than it is towards cancers like kidney and leukemia. It is the hope of the authors that these gaps in research will soon be filled in, bringing all cancers to a comparable level of understanding. However, the future of the field is a bright one and clinical applications will hopefully come to fruition within the next decade.

Conflict of Interests

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

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