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The Biological Role and Clinical Implication of MicroRNAs in Osteosarcoma

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Abstract

The main causes of death in osteosarcoma (OS) patients are the development of distant metastasis and resistance to chemotherapy. Clarification of the pathophysiological molecular mechanisms that contribute to the malignant phenotype in OS and identification of a molecular target, such as a diagnostic marker, prognostic predictor, or chemosensitivity sensor, are strongly desired to develop therapeutics for OS patients. Accumulating evidence has demonstrated that microRNAs (miRNAs), small endogenous single-stranded noncoding RNAs, play critical roles not only in biological but also pathological processes such as cancer. miRNAs can function as oncogenes or tumor-suppressive genes depending on the mRNA they target. They are strongly associated with OS invasion, metastasis, and chemoresistance as well as OS cancer stemness. Furthermore, miRNAs are associated with commonly altered genes, such as TP53 and RB1. Additionally, recent global microRNA expression analyses have identified specific miRNAs correlated with the clinical stage and the response to chemotherapy. In this chapter, we summarize the current understanding of the pathological roles of miRNAs as well as their potential utility as OS biomarkers.

Keywords: microRNA, metastasis, chemoresistance, cancer stem cells, therapy

1. Introduction

Osteosarcoma (OS) is the most common primary malignant bone tumor. Before the 1970s, treatment generally included only surgical resection. However, because approximately 80% of patients have developed pulmonary metastases by the initial diagnosis, the 5-year survival rate was 10–15% [1, 2]. Due to the development of multidrug chemotherapy, surgical wide

resection, and reconstruction with tumor prosthesis, the prognosis has gradually improved over the past 30 years [3]. Despite advances in multimodality treatment, the prognosis is still poor in patients with metastasis and/or acquisition of anticancer drug resistance. Because the critical molecular mechanisms contributing to the development of distant metastases and acquisition of chemoresistance in OS remain largely unknown, elucidation of the detailed pathophysiological molecular mechanisms is strongly desired to develop the novel tools for OS diagnosis, prognostic prediction, and treatment against OS.

microRNAs (miRNAs) are endogenous single-stranded, noncoding RNAs with approximately 22 nucleotides in length that regulate gene expression by cleavage or translational repression at the post-transcriptional level by base pairing with the 3′ untranslated region (UTR) of their target mRNAs. To date, 2588 mature miRNAs have been identified, and they regulate the expression of more than a half of all human genes [4, 5]. Emerging evidence has demonstrated that miRNAs not only regulate biological processes such as development, differentiation, apoptosis, and proliferation but also modulate pathological conditions [6]. Genetic or epigenetic alterations, dysregulation of transcription factors, and abnormal microRNA biogenesis can alter the dysregulation of microRNA expression [7]. As a result, the misexpressed microRNAs contribute to many types of human diseases, including cancer [6–8]. miRNAs can function as either oncogenes or tumor suppressors depending on their individual target mRNAs, and abnormal miRNA expression has been observed in various solid and hematopoietic tumors in relation to the initiation and progression of tumors including growth, metastasis, and drug resistance. Furthermore, miRNA expression profiling of human tumors has identified signatures associated with diagnosis, staging, progression, prognosis, and response to treatment.

After the first study examining the association between the microRNAs and OS pathogenesis in 2009 [9], numerous studies have reported miRNA expression profiles from clinical OS samples and cell lines, and the association between miRNAs and malignant phenotypes. The altered gene expression previously reported in OS patients is closely association with altered miRNA expression. There is growing evidence that miRNAs play critical roles in various pathological processes, such as tumorigenesis, invasion, metastasis, chemoresistance, and cancer stem cell maintenance in OS [10, 11]. Therefore, altered miRNA expressions could be a useful diagnostic and prognostic tool for OS patients [10, 11].

Here, we summarize the pathological roles of miRNAs in OS and their potential value as diagnostic and prognostic biomarkers for OS patients.

2. Biological machinery and miRNA function

miRNAs are small, noncoding, single-stranded RNAs 18–25 nucleotides long that regulate gene expression at the post-transcriptional level. miRNAs are mainly transcribed by RNA polymerase II to generate primary-miRNAs (pri-miRNAs), which are usually 3–4 kb long and characterized by hairpin structures. In the nucleus, these pri-miRNAs are cleaved into 70–100 nucleotide precursor-miRNAs (pre-miRNAs) by Drosha and DGCR8 (DiGeorge Syndrome Critical Region Gene-8). Pre-miRNAs are transferred to the cytoplasm by Exportin-5 and

cleaved to form a miRNA duplex by Dicer and TRBP (transactivating response RNA-binding protein). The two miRNA strands of the duplex are processed into two different mature miR-NAs (-3p or -5p). Mature miRNAs are incorporated into the RNA-induced silencing complex (RISC), which contains Argonaute 2 (Ago2) and GW182. As a part of this complex, mature miRNAs suppress gene expression by binding to the 3′UTR of target mRNAs, which are recognized by 6–8 nucleotides at the miRNA 5′-terminus called seed sequence, leading to mRNA degradation or translation inhibition depending on the complementarity between the miRNA seed sequence and the 3′UTR of the mRNA (**Figure 1**) [7, 12].
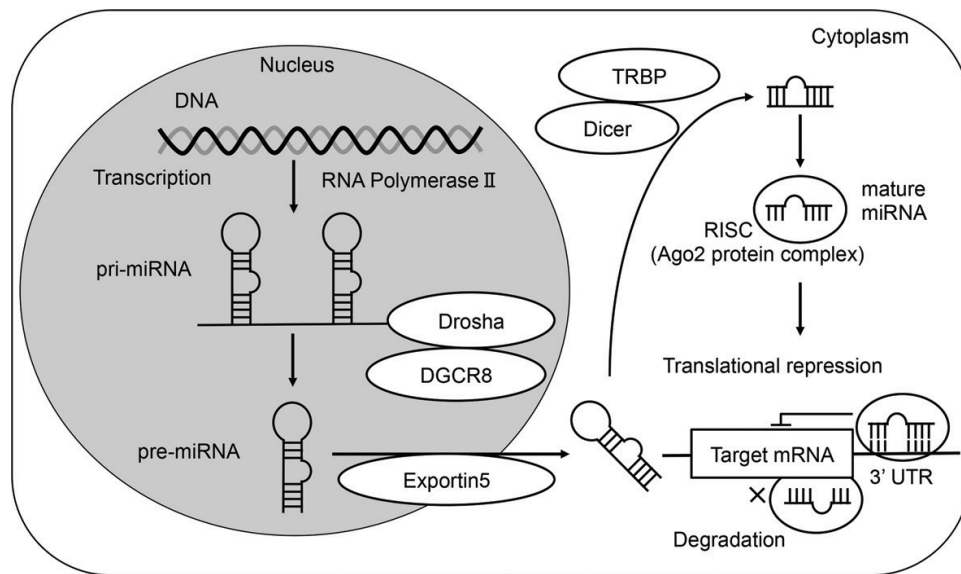
Figure 1. MicroRNA biological machinery. miRNAs are mainly transcribed by RNA polymerase-II to generate pri-miRNAs. In the nucleus, pri-miRNAs are cleaved into 70–100 nucleotide pre-miRNAs by Drosha and DGCR8. Pre-miRNAs are transferred to the cytoplasm by Exportin-5 and cleaved to form a miRNA duplex by Dicer and TRBP. The two miRNA strands of the duplex are processed into two different mature miRNAs (-3p or -5p). Mature miRNAs are incorporated into the RISC, which contains Ago2. As a part of this complex, mature miRNAs suppress gene expression by binding to the 3′UTR of target mRNAs, which are recognized by 6–8 nucleotides at the miRNA 5′-terminus called seed sequence, leading to mRNA degradation or translation inhibition.

3. miRNAs in cancer

The relationship between cancer and miRNAs was first reported in 2002. Calin et al. demonstrated that miR-15 and miR-16 at chromosome 13q14 were deleted or downregulated in the majority of chronic lymphocytic leukemia (CLL) cases and that these miRs induced apoptosis by direct suppression of Bcl-2 (B cell lymphoma 2) in CLL cells [13, 14]. Genetic or epigenetic changes, dysregulation of transcription factors, and abnormal microRNA biogenesis can alter microRNA expression [7]. Accumulating evidence suggests that dysregulated miRNAs induce cancer initiation and progression [6], and aberrant miRNAs can function as oncogenes or tumor suppressor genes depending on their target genes.

4. Dysregulation of microRNAs in OS

The relationship between OS and miRNA expression has been reported in over 400 publications to date. Aberrantly expressed miRNAs have been shown to play essential roles in the biological processes of OS pathogenesis through the regulation of numerous protein-coding genes and signaling pathways (**Tables 1** and **2**).

miRNA	Target gene	Function	Reference
miR-34a	Surviving	Proliferation, apoptosis, chemoresistance to CDDP	[21]
	mTOR, MET, MDM4	Proliferation, apoptosis	[17]
	Eag1	Proliferation, tumor growth *in vivo*	[16]
	CD44	Migration, invasion	[22]
	c-Met	Proliferation, migration, invasion, tumor growth and metastasis *in vivo*	[15]
miR-143	Bcl-2	Migration, invasion, apoptosis	[20]
	ATG2B, Bcl-2, LC-1,2	Proliferation, chemoresistance to DOX, autophagy, tumor growth *in vivo*	[23]
	VCAN	Migration, invasion	[24]
	MMP13	Invasion, metastasis	[18]
	Bcl-2	Proliferation, apoptosis, tumorigenicity	[19]
miR-144	ROCK1, ROCK2	Proliferation, invasion, tumorigenesis and metastasis *in vivo*	[25]
	ROCK1	Proliferation, migration, invasion	[26]
	Ezrin	Invasion, metastasis	[27]
	TAGLN	Proliferation, invasion	[28]
miR-145	FLI-1	Proliferation, migration, apoptosis, tumor growth *in vivo*	[29]
	ROCK1	Proliferation, migration, invasion	[30]
	ROCK1	Proliferation, invasion	[31]
	VEGF	Invasion, angiogenesis	[32]
miR-451	LRH-1	Proliferation, cell cycle	[33]
	CXCL16	Proliferation, invasion	[34]
	PGE2, CCND1	Proliferation, cell cycle, apoptosis	[35]

Table 1. Tumor suppressive microRNAs and targets in osteosarcoma.

miRNA	Target gene	Function	Reference
miR-20a	ERG2	Proliferation, cell cycle	[37]
	Fas	Metastasis	[36]
miR-21	PTEN	Proliferation, invasion, apoptosis	[39]
	SPRY2	Migration, invasion	[41]
	Bcl-2	Chemoresistance to CDDP	[38]
	RECK	Invasion, migration	[40]
miR-135b	FOXO1	Proliferation, invasion	[42]
	Myocardin	Proliferation, migration, invasion	[43]
miR-155	HBP1	Proliferation, cell cycle, tumor growth *in vivo*	[44]
	–	Proliferation, migration, invasion	[45]
	–	Autophagy, chemoresistance to DOX and CDDP	[46]
miR-214	PTEN	Proliferation, migration, invasion	[47]
	PTEN	Proliferation, apoptosis, tumorigenicity	[48]
	LZTS1	Proliferation, invasion, tumor growth *in vivo*	[49]

Table 2. Ongogenic microRNA and targets in osteosarcoma.

miR-34a: The overexpression of miR-34a inhibited the proliferation, migration, and invasion of OS cell lines (SOSP-9607 and Saos-2) *in vitro* and decreased tumor growth and pulmonary metastasis of SOSP-9607 cells *in vivo* by directly targeting c-Met [15]. Based on a bioinformatics analysis, they demonstrated that miR-34a had multiple putative targets associated with proliferation and metastasis, including members of the Wnt and Notch signaling pathways. Wu et al. demonstrated that miR-34a was significantly downregulated in clinical OS tissues and cell lines. Overexpression of miR-34a inhibited the proliferation of OS cells (MG-63 and Saos-2) *in vitro* and tumor growth *in vivo* by decreasing the expression of Ether à go-go 1 (Eag1) [16]. Furthermore, Tian et al. demonstrated that miR-34a inhibited proliferation and induced apoptosis in MG-63 cells through the p53 signaling pathway [17].

miR-143: Osaki et al. compared HOS and 143B OS cells (highly metastatic variant of HOS transformed by v-Ki-ras) using a miRNA microarray analysis [18]. miR-143 was significantly downregulated in 143B cells, and transfection of miR-143 decreased cell invasiveness by directly targeting matrix metalloproteinase 13 (MMP13). Furthermore, the systemic admin-

istration of miR-143/atelocollagen complexes significantly suppressed the lung metastasis of 143B *in vivo*. Zhang et al. demonstrated that the restoring miR-143 expression reduced OS cell (MG-63 and U-2OS) viability, promoted apoptosis *in vitro*, and suppressed tumorigenicity *in vivo* [19], and they identified Bcl-2, an important antiapoptotic protein, as a direct target of miR-143. Li et al. also showed that miR-143 promoted apoptosis in OS cells (MG-63 and U-2OS) through caspase-3 activation by targeting Bcl-2 [20]. Moreover, miR-143 overexpression significantly suppressed cell migration and invasion.

miR-20a: Huang et al. found the higher miR-20a expression in metastatic Saos-2 cells compared with original Saos-2 cells [36]. The metastatic cells expressed low levels of Fas, which is inversely correlated to lung metastasis, and miR-20a directly regulated the expression levels of Fas. The inhibition of miR-20a expression decreased the occurrence of metastasis *in vivo*. Ectopic expression of miR-20a promotes the proliferation and cell cycle progression of Saos-2 cells by directly suppressing early growth response 2 (EGR2), a key regulator of proliferation and the cell cycle [37].

miR-21: miR-21 was significantly overexpressed in OS tissues compared with matched normal bone tissues [38], and miR-21 knockdown reduced migration and invasion in MG-63 cells by directly regulating RECK (reversion-inducing-cysteine-rich protein with kazal motifs), a tumor suppressor gene. Lv et al. demonstrated that PTEN might be a potential target of miR-21 [39]. The miR-21 expression level was significantly higher in MG-63 cells than in a human fetal osteoblastic cell line, hFOB1. 19, and miR-21 overexpression increased proliferative and invasive abilities and reduced apoptosis in MG-63 cells. The authors suggested that miR-21 activates the PI3K/Akt pathway by suppressing PTEN expression. In addition, miR-21 regulates the MAPK signaling pathway by targeting SPRY2 (protein sprout homolog 2), an antagonist of MEK1/2, as an oncogenic miRNA that increases cell proliferation, cell cycle progression, and inhibits apoptosis [40].

5. miRNAs associated with dysregulated genes in OS

OS exhibits a broad range of genetic and molecular alterations, such as the gains, losses, or rearrangements of chromosomal regions that result in inactivation of tumor suppressor genes and the misregulation of major signaling pathways [50].

5.1. TP53-associated miRNAs

TP53, located in 17q13.1, is a tumor suppressor gene that is mutated in more than 20% of human OS patients, which drives OS initiation and progression of OS [51]. Recent studies have demonstrated an association between TP53 and miRNAs. He et al. demonstrated that miR-34s (miR-34a, b, and c), which was decreased OS tissues and regulated by p53, affected the expression of CDK6, E2F3, Cyclin E2, and Bcl-2, and induced G1 arrest and apoptosis partially in a p53-dependent manner [9]. Novello et al. demonstrated that miR-34a demethylation by p53 was important for etoposide sensitivity [52]. They demonstrated that U2-OS

cells either with the wild-type p53 or a dominant-negative form of p53 both of which were expressing increased levels of unmethylated miR-34awere more sensitive to etoposide than p53-deficient OS cells (MG-63 and Saos-2).

5.2. RB1-associated miRNAs

RB1 on 13q14 is one of the most commonly inactivated genes in sporadic OS [53]. Poos et al. performed the miRNA expression analysis of OS cell lines based on their proliferative activity to generate a coregulatory network between miRNA and transcription factor. As a result, they found that downregulation of miR-9-5p, miR-138, and miR-214 was correlated to a strong proliferative phenotype in OS cells through their effect on NFKB and RB1 signaling pathways and focal adhesion molecules [54].

5.3. RUNX2-associated miRNA

The chromosomal region 6p 12–21 is commonly amplified and DNA gains occur in 40–50% of tumors. This region contains RUNX2 which promotes terminal osteoblast differentiation and is elevated in conventional OS [53]. van der Deen et al. demonstrated that miR-34c which is elevated by p53 and targets RUNX2, is absent in OS tissue [55]. This p53-miR-34c-RUNX2 pathway controls osteoblast growth and its alteration may impact on OS pathogenesis.

These data indicate that miRNAs play critical roles in OS pathogenesis by regulation of and interaction with commonly aberrant genes.

6. Cancer stem cell-associated miRNAs

It is widely considered that cancer stem cell (CSC) populations possibly drive the refractory nature of cancer, especially multidrug resistance and distant metastasis. OS stem cells have been isolated and identified by using cell sorting methods based on specific cell surface markers such as CD133, Hoechst dye side population assay, and sphere colony formation assays. Several groups have demonstrated that miRNAs are involved in the maintenance and stimulation of CSC population in various cancers, including OS [56]. miR-29b-1 expression was decreased in 3AB-OS cells, a CSC line selected from MG-63 cells, and its overexpression causes cell proliferation, self-renewal, and chemoresistance. This is accompanied by the downregulation of stem cell markers (Oct3/4, Sox2, Nanog, CD133, and N-Myc), cell cycle-related markers (CCND2, E2F1, E2F2), and antiapoptotic markers (Bcl-2 and IAP-2) [57]. Xu et al. demonstrated a relationship between miR-382 and CSCs in OS [58]. miR-382 expression was significantly lower in highly metastatic OS cell lines and relapsed OS clinical samples. Likewise, the overexpression of miR-382 decreased the CSC population defined by CD133 and ALDH1 expression and osteosphere capacity. *In vivo* experiments showed that miR-382 overexpression inhibited CSC-induced tumor formation. However, the association between miRNAs and CSCs is still under investigation, thus further research will be required to develop the novel therapeutic strategies targeting CSCs in OS.

7. Chemoresistance-associated miRNAs

Advances in chemotherapy have contributed to the dramatic improvement to OS patient outcomes. Most OS patients receive multidrug chemotherapy that consists of doxorubicin (DOX), cisplatin (CDDP), methotrexate (MTX), and ifosfamide (IFO), but certain population of OS patients exhibit chemoresistance. The molecular mechanisms driving poor response to chemotherapy remain largely unclear, and there are no biomarkers to discriminate between good and poor responders before chemotherapy.

DOX: miR-301a expression promoted HMGCR expression by targeting AMP-activated protein kinase alpha 1 and enhanced the resistance of OS cells (U2OS, MG-63) to DOX [59]. Chang et al. have demonstrated that miR-101 overexpression dramatically reduces DOX-induced autophagosome formation by suppressing autophagy 4 (Atg4) in U2-OS cells, thereby enhancing the sensitization of tumor cells to DOX [60]. miR-184 expression was induced by DOX, and overexpression or silencing of miR-184 reduced or enhanced DOX-induced apoptosis by targeting Bcl-2-like protein 1 (BCL2L1) [61].

CDDP: Overexpression of miR-224, which is downregulated in OS cell lines and tissues, contributed to the increased sensitivity of MG-63 cells to CDDP by targeting Rac1 [62]. miR-33a was upregulated in chemoresistant OS tissues and promoted resistance to CDDP in OS cell (MG-63, Saos-2) by downregulating TWIST [63]. Downregulation of miR-497 induced CDDP resistance through the PI3K/Akt pathway by directly targeting VEGFA in human OS [64], and miR-138 functions as a tumor suppressor by enhancing sensitivity to CDDP in OS via direct downregulation of EZH2 [65].

MTX: Decreased miR-126 reduced the sensitivity to MTX and promoted apoptosis in OS cells (MG-63 and U-2OS) [66]. According to high-throughput miRNA expression analysis, Song et al. demonstrated that miR-140 expression was associated with chemosensitivity in OS tumor xenografts [67]. The authors showed that miR-140 overexpression induced MTX resistance by targeting HDAC4 possibly through p53-dependent manner. Furthermore, they proved that miR-215 caused G2 arrest by suppressing DTL expression, and that led to chemoresistance against MTX [68].

IFO: Five miRNAs (miR-92a, miR-99b, miR-132, miR-193-5p, and miR-442a), which inhibit the TGF-β and Wnt pathways by *in silico* analysis, discriminate good from poor IFO responders against IFO [69].

These reports suggest that miRNAs associated with an anticancer drug might be potential chemosensitivity biomarkers and promising therapeutic targets for OS patients.

8. Detection of miRNA in blood samples

There are few biomarkers for the diagnosis and prognosis prediction of OS patients other than alkaline phosphatase (ALP). Meta-analysis has demonstrated that high ALP level is significantly

associated with a poor overall survival and event-free survival rate and the presence of metastasis at diagnosis [70]. However, predictors of poor outcome are mainly clinical parameters, such as proximal extremity or axial skeleton involvement, large size/volume, detectable metastases at diagnosis, and poor response to preoperative chemotherapy [53]. Recently, growing evidence indicates the clinical usefulness of miRNAs as biomarkers, and numerous candidate miRNAs in blood samples have been reported in OS patients (**Table 3**).

miRNA	Blood sample	Reference
Upregulated miRNAs		
miR-221	Serum	[71]
miR-191	Serum	[73]
miR-199a-5p	Serum	[78]
miR-27a	Serum	[72]
miR-195-5p, 199a-3p, 320a, 374a-5p	Plasma	[77]
miR-155	Serum	[44]
miR-9	Serum	[83]
miR-196a, 196b	Serum	[76]
miR-21	Serum	[74]
	Plasma	[75]
Downregulated miRNAs		
miR-223	Serum	[79]
miR-106a-5p, 16-5p, 20a-5p, 425-5p, 451a, 25-3p, 139-5p	Serum	[84]
miR-195	Serum	[80]
miR-133b, 206	Serum	[82]
miR-34b	Plasma	[81]
miR-199a-3p, 143	Plasma	[75]

Table 3. Dysregulated miRNAs in blood samples of OS patients.

Upregulated miRNAs in OS: The expression level of miR-221 [71], miR-27a [72], miR-191 [73], and miR-21 [74, 75] in blood samples was increased in OS patients compared with healthy controls, and compared to preoperation and postoperation state, the expression levels of miR-199a-5p [76] and four miRNAs (miR-195-5p, miR-199a-3p, miR-320a, and miR-374a-5p; [77]) were decreased in postoperative compared to preoperative blood samples. It has been suggested that upregulations of these miRNAs were significant predictors for poor overall and disease-free survival. The expression levels of these miRNA were related to clinical stage [71–74], tumor size [73], distant metastasis [71–73, 75], and chemoresistance [50], the upregulations of these miRNAs were significant predictors for poor overall and disease-free survival.

In addition, the combination of dysregulated miRNAs was more accurate than the individual expression level of each miRNA. The coexpression of miR-196a/miR-196b [78] and the combination of four miRNAs (miR-195-5p, miR-199a-3p, miR-320a, and miR-374-5p) [77] were superior predictors to any of the miRNAs alone.

Downregulated miRNAs in OS: In contrast, the expression level of miR-223 [79], miR-195 [80], and miR-34b [81] in blood samples was significantly decreased in OS patients compared to healthy controls. These miRNAs levels were associated with clinical stage [79, 80] and distant metastasis [79–81], and the decreased expression of these miRNAs was associated with shorter overall survival and disease-free survival. In addition, the coexpression of miR-133b/miR-206 was a prognostic factor for overall survival and disease-free survival [82].

These data indicate the potential of miRNAs in blood samples as diagnostic markers, prognotic predictors, and chemosensitivity sensors.

9. Conclusion

Dysregulated miRNAs contribute to the initiation and progression of human OS in several pathobiological aspects. The detection of aberrant miRNAs could be a versatile tool for diagnosis, prognosis and chemosensitivity judgment, and inhibition of oncogenic miRNAs and/or restoration of tumor-suppressing miRNAs could be a novel strategy for treatment of OS.

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