

## Circulating MicroRNAs in Sarcoma: Potential Biomarkers for Diagnosis and Targets for Therapy

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### Abstract

The importance of microRNAs (miRNAs) in tumor biology has been recognized over the past several years. Recently, evidence of circulating miRNAs in both healthy and unhealthy individuals has been accumulated, and is accelerating their potential to transform clinical diagnostics and therapeutics. Since there is a lack of useful biomarkers for bone and soft tissue sarcomas, the discovery of novel biomarkers that can be used at early disease stages to detect tumors or predict tumor response to chemotherapy or the chance of survival is one of the most important challenges in sarcoma management. Further more highly, sensitive and specific biomarkers might help diagnostic classification, since some cases are unclassifiable using modern diagnostic modalities. In this review, we summarize the emerging evidence of circulating miRNAs in sarcoma and discuss their potential as novel biomarkers and therapeutics.

### Sarcoma Needs Novel Biomarkers

Sarcomas are malignant neoplasms originating from transformed cells of mesenchymal origin and are different from carcinomas that are malignant neoplasms originating from epithelial cells. The word "sarcoma" is derived from the Greek word *sarkoma* meaning "fleshy outgrowth," and present as either a bone sarcoma or a soft tissue sarcoma [1]. Malignant primary bone sarcomas constitute 0.2% of all malignancies in adults and approximately 5% of childhood malignancies, for which data were obtained in one large series [2]. Cancer registry data with histological stratification indicate that osteosarcoma is the most common primary malignant bone tumor, accounting for approximately 35% of all cases, followed by chondro sarcoma (25%), Ewing sarcoma (16%), and chordoma (8%) [3]. Soft tissue sarcomas constitute fewer than 1% of all malignancies, 50 per million population [2,4]. According to the results of the Surveillance, Epidemiology, and End Results study (<http://seer.cancer.gov/data/>), which included 26,758 cases from 1978 to 2001, leiomyosarcoma was the most common sarcoma, accounting for 23.9% of all cases. Other major histological types included malignant fibrous histiocytoma (MFH; 17.1%), liposarcoma (11.5%), dermatofibrosarcoma (10.5%), rhabdomyosarcoma (RMS; 4.6%), and malignant peripheral nerve sheath tumor (MPNST; 4.0%) [5]. Although MFH was the second most common sarcoma in this series, the diagnostic term MFH is now replaced for pleomorphic sarcomas without defined differentiation. Therefore, the incidence rates of MFH will be updated in future studies based on changes in diagnostic criteria that parallel advancements in the understanding of MFH etiology.

According to histological type, treatment options for most patients with sarcoma include surgical resection followed by limb or trunk reconstruction, and pre-operative (neoadjuvant) and/or post-operative (adjuvant) chemotherapy and radiotherapy. Although surgical resection is the mainstay of treatment for musculoskeletal sarcomas, chemotherapy also has a proven role in the primary therapy of certain types of bone sarcomas and a potential role in some patients with soft tissue sarcomas [6]. Despite the development of combined modality treatments, a significant proportion of patients with sarcoma respond poorly to chemotherapy, leading to local relapse or distant metastasis. The main cause of death due to sarcoma is lung metastasis, for which prognosis is extremely poor [7,8]. Therefore, early detection of recurrent or metastatic diseases or early decision-making according

to tumor response to chemotherapy could improve patient prognosis. However, there are currently no effective biomarkers in such situations, thus imaging methods, such as X-ray, computed tomography (CT), positron emission tomography-CT, magnetic resonance imaging, and scintigraphy, are mostly used to detect or monitor tumor development. Indeed, only few studies have reported the usefulness of serological markers such as alkaline phosphatase (ALP) [9], lactic dehydrogenase (LDH) [10,11], and CA125 [12] in the patients with osteosarcoma, Ewing sarcoma, and epithelioid sarcoma, respectively. Therefore, the discovery of novel biomarkers to detect tumors or predict drug sensitivity is one of the most important challenges in sarcoma management.

### Circulating MicroRNAs (miRNAs) As Potential Biomarkers and Treatment Targets

miRNAs are small non-coding RNA molecules that modulate the expression of their multiple target genes and play important roles in various physiological and pathological processes, such as development, differentiation, cell proliferation, apoptosis, organogenesis, and homeostasis [13-15]. A variety of miRNAs have been investigated in various human cancers over the past several years [16]. Aberrant miRNA expression has been shown to contribute to cancer development through various mechanisms, including deletions, amplifications, and mutations involving miRNA loci, epigenetic silencing, dysregulation of transcription factors that target specific miRNAs, or the inhibition of miRNA processing [17,18]. Growing evidence has revealed that miRNAs are frequently upregulated or downregulated in various

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tumors and indicated that miRNAs act as either an oncogenes or a tumor suppressors [18,19].

Recently, tumor cells have been shown to secrete miRNAs into the circulation [20]. Therefore, analysis of circulating miRNA levels in serum or plasma presents a novel approach for diagnostic cancer screening. For example, Lawrie et al. [21] were the first to report that tumor-associated miRNA levels in the serum of patients with cancer were higher than those in healthy individuals, indicating that circulating miRNAs can be used as biomarkers to monitor the existence of cancer cells. This group also demonstrated that high miR-21 expression was associated with relapse-free survival in patients with large B-cell lymphomas [21]. Expression of other circulating serum or plasma miRNAs has been widely reported by other investigators. To date, differential expression of circulating miRNA has been reported in cancers of the breast [22], lung [23], stomach [24], liver [25], kidney [26], bladder [27], prostate [28], and ovaries [20], among others. However, it is possible that measuring these miRNAs in the serum or plasma of cancer patients may yield false-positive results because tumor cells may also change the profile of miRNAs of other circulating cells. Validation studies based on more and larger patient sets would be necessary to focus on key miRNAs with high sensitivity and specificity.

The main issues that remain unresolved in measurement of circulating miRNAs include the normalization, amplification, and contamination [29]. There is no consensus on suitable small RNA reference genes for use as internal controls. Current protocols need correction for technical variability using spiked-in synthetic non-human (*Caenorhabditis elegans*) miRNA as a normalizing control [28-30]. Moreover, there is a higher risk of cellular contamination when preparing plasma as the supernatant is pipetted away from the cellular pellet. Profiling of miRNA by qRT-PCR is also dependent on the type of anticoagulant used, where EDTA and citrate are acceptable, but heparin impedes the qRT-PCR reaction [29]. Given these uncertainties surrounding miRNA analysis, further studies to establish consensus protocols could resolve these issues and accelerate this novel method toward clinical application as a novel approach to monitor or detect tumor development.

## Circulating miRNAs in Patients with Sarcoma

The first report of circulating miRNAs as potential diagnostic markers was presented in 2010 by Miyachi et al. [31] who analyzed the expression levels of muscle-specific miRNAs in the sera of rhabdomyosarcoma patients and healthy controls [31]. To date, the evidence is restricted to only three types of sarcomas, i.e., osteosarcoma, rhabdomyosarcoma, and malignant peripheral nerve sheath tumor, as summarized in Table 1.

## Osteosarcoma

Osteosarcoma is the most common primary malignancy of the bone and accounts for 60% of all childhood bone malignancies [32,33]. The most common primary sites of osteosarcoma are the distal femur, proximal tibia, and proximal humerus, with approximately 50% of cases originating in the vicinity of the knee. The WHO classification recognizes additional histological variants in addition to the conventional osteosarcomas (osteoblastic, chondroblastic, and fibroblastic types); telangiectatic osteosarcoma, small cell osteosarcoma, low-grade central osteosarcoma, secondary osteosarcoma, parosteal osteosarcoma, periosteal osteosarcoma, and high-grade surface osteosarcoma [34]. Standard treatment of patients with conventional osteosarcoma consists of neoadjuvant chemotherapy, surgical resection, and

adjuvant chemotherapy [35]. With this combined treatment, the 5-year overall survival for patients with no metastatic disease at diagnosis is 60%–80% [36-41]. However, a significant proportion of patients with osteosarcoma still respond poorly to chemotherapy and have a greater risk of local relapse or distant metastasis even after curative resection of the primary tumor. Indeed, outcomes are far worse for patients who present with metastatic disease, since the 5-year overall survival is less than 30% [42], and has shown little improvement over the past two decades despite multiple clinical trials with increased intensity. Therefore, the discovery of sensitive and specific minimally invasive biomarkers that could detect osteosarcoma at an early stage would be one of the most important challenges. Moreover, it would be helpful if these biomarkers could predict the chance of survival or response to chemotherapy, especially during early treatment stages before surgery.

Four miRNAs (miR-21, miR-34b, miR-143, and miR-199-3p) have been reported as potential osteosarcoma biomarkers. Yuan et al. [43] investigated serum miR-21 expression levels in 65 patients with osteosarcoma and 30 healthy controls by qRT-PCR and found that serum miR-21 expression levels were significantly higher in patients with osteosarcoma than in the controls [43]. Moreover, increased serum miR-21 levels were significantly correlated with Enneking stage and chemotherapeutic resistance. The mean  $\Delta C_t$  value of miR-21 in the responder group was significantly higher than that in the nonresponder group. Notably, the upregulation of miR-21 was an independent unfavorable prognostic factor for overall survival [43]. Indeed, it has been reported that miR-21 is aberrantly overexpressed in various cancers and is involved in the pathogenesis of cancers [44,45]. The effects of miR-21 on proliferation, migration, invasion, and apoptosis have already been elucidated in cancers of the breast, liver, and colon [46-48]. In osteosarcoma, Ziyang et al. [49] reported that miR-21 was significantly overexpressed in osteosarcoma tissues, and its knockdown decreased cell invasion and migration of osteosarcoma MG-63 cell line. *RECK* (reversion-inducing-cysteine-rich protein with kazal motifs), a tumor suppressor gene, was found to be a direct target that was negatively regulated by miR-21 in an osteosarcoma cell line and human osteosarcoma specimens [49].

Ouyang et al. [50] evaluated the expression levels of six miRNAs (miR-34, miR-21, miR-199-3p, miR-143, miR-140, and miR-132) that had been reported as aberrantly expressed in osteosarcoma using plasma from 40 patients with osteosarcoma and 40 matched healthy controls by qRT-PCR [50]. They found that plasma miR-21 levels were significantly higher in patients with osteosarcoma than in controls, whereas miR-199a-3p and miR-143 were decreased. Furthermore, plasma miR-21 and miR-143 levels were correlated with metastasis and histological subtype, whereas plasma miR-199a-3p correlated with histological subtype. Interestingly, the area under the curve (AUC) value of the combined signature of three miRNAs (miR-21, miR199-3p, and miR-143) was higher than that of bone-specific alkaline phosphatase (0.953 and 0.922, respectively), and the sensitivities and specificities of the combined miRNAs were 90.5 and 93.8%, respectively. The aberrant expression of miR-199-3p in osteosarcoma was first reported by Duan et al. [51] who found that miR-199a-3p, miR-127-3p, and miR-376 were significantly downregulated in osteosarcoma cell lines compared to osteoblasts [51]. Overexpression of miR-199a-3p in osteosarcoma cell lines significantly decreased cell growth and migration. In addition, they identified that miR-199a-3p suppressed the expression of the oncogenic and antiapoptotic proteins mTOR and STAT3. Osaki et al. [52] were the first to demonstrate that the expression of miR-143 was decreased in metastatic osteosarcoma cells. They profiled the miRNA expression in a parental HOS cell line and its

Sarcoma entity	Promising circulating miRNAs		Study design	Sample type	Sample size	Technology	Circulating miRNAs examined	Normali- zation	Other related clinical factors	Reference
	Upregulated	Downregulated								
Osteosarcoma	miR-21		OS vs normal	Serum	65 patients vs 30 healthy controls	qRT-PCR	1	snRNA U6	①Enneking stage ②Drug resistance ③Prognosis	Yuan et al. [43]
Osteosarcoma		miR-34b	OS vs normal, tissue and plasma	Plasma	133 patients vs 133 healthy controls	qRT-PCR	2	cel- miR-39	Metastasis	Tian et al. [53]
Osteosarcoma	miR-21	miR-143, miR-199a-3p	OS vs normal	Plasma	40 patients vs 40 healthy controls	qRT-PCR	6	cel- miR-39	①Metastasis (miR-21, 143) ②Histological subtype	Ouyang et al. [50]
Rhabdomyosarcoma	miR-206		RMS vs non-RMS vs normal, tissue and plasma	Serum	8 RMS patients vs 23 non-RMS patients vs 17 healthy controls	qRT-PCR	4	miR-16	N.A.	Miyachi et al. [31]
Malignant peripheral nerve sheath tumor	miR-24, miR-801, miR-214		Sporadic MPNST vs NF1 MPNST vs NF1	Serum	Screening: 10 sporadic MPNST vs 10 NF1 MPNST vs 10 NF1 Validation: 83 sporadic MPNST vs 61 NF1 MPNST vs 90 NF1	Solexa sequencing, qRT-PCR		Genome- wide profiling by Solexa sequencing cel- miR-39	N.A.	Weng et al. [61]

Abbreviations: OS: Osteosarcoma; RMS: Rhabdomyosarcoma; MPNST: Malignant peripheral nerve sheath tumor; NF1: Neurofibromatosis type 1; N.A: not available

**Table 1:** Differential expression of circulating miRNAs in patients with bone and soft tissue sarcoma.

sub-clone 143B metastatic osteosarcoma cell line, and found that miR-143 was the most downregulated miRNA in 143B cells [52]. Significant inhibition of cell invasion was observed in miR-143-transfected 143B cells. Several genes were identified as probable candidates of miR-143 targets by a comprehensive collection system to detect miRNA-target mRNA. Among them, matrix metalloprotease-13 was one of the most probable targets of miR-143, which was positive in clinical specimens of metastatic cases by immunohistochemistry, but negative in those of at least three cases showing higher miR-143 expression levels in the nonmetastatic group [52].

Tian et al. [53] investigated the associations between plasma miR-34b/c expression levels in osteosarcoma, and found that plasma miR-34b level was significantly lower in osteosarcoma patients than in controls and related with its expression in osteosarcoma tissues [53]. Furthermore, plasma miR-34b expression levels were significantly decreased in patients with metastatic disease compared to patients with nonmetastatic disease, while no significant difference in miR-34b levels was observed between patients with osteoblastic and nonosteoblastic diseases [53]. Indeed, the miR-34 family, which is a direct target of the p53 tumor suppressor gene, are composed of three homologous miRNAs (miR-34a, miR-34b, and miR-34c), and are associated with the tumor growth and metastasis of various human cancers. Previous reports from He et al. [54] and Yan et al. [55] have demonstrated the association of miR-34a with osteosarcoma. They identified decreased miR-34a expression levels in tumor samples and found that miR-34a overexpression could inhibit the tumor growth and metastasis by downregulation of the proto-oncogene *c-Met* [54,55].

### Rhabdomyosarcoma

Rhabdomyosarcoma (RMS) is the most common soft tissue sarcoma in childhood, representing 5%–8% of all pediatric malignancies [56]. Histopathologically, RMS is classified into embryonal (eRMS), alveolar (aRMS), and pleomorphic types. Depending on the size and location

of the primary tumor, most cases are treated with a combination of chemotherapy, radiation therapy, and surgery. Adult patients with a complete response to chemotherapy had a 5-year survival rate of 57% compared to only 7% for poor responders [57].

Miyachi et al. [31] were the first to suggest use of circulating miRNAs for sarcoma diagnosis. They focused on muscle-specific miRNAs (miR-1, miR-133a, miR-133b, and miR-206) that were shown to be more abundantly expressed in myogenic tumors. Expression levels of these muscle-specific miRNAs were confirmed to be higher in RMS cell lines and culture supernatants than in other cell lines. In their analysis of muscle-specific miRNA serum levels in RMS patients, normalized serum miR-206 showed the highest sensitivity and specificity among muscle-specific miRNAs [31]. Importantly, miR-206 expression decreased after treatment of RMS [31]. In the analysis of miR-206 expression levels with RMS cells, Missiaglia et al. [58] found that muscle-specific miRNA levels were lower in RMS than in skeletal muscles, but generally higher than that in other normal tissues [58]. Moreover, low miR-206 expression correlated with poor overall survival in patients with RMS, and increased miR-206 expression in cell lines inhibited cell growth and migration and induced apoptosis [58]. Similar results were reported by Tauli et al. [59] who showed that increased miR-206 expression caused a major switch in the global expression profile toward mature muscle, rescued differentiation of both eRMS and aRMS, and blocked tumor growth [59]. Therefore, serum miR-206 expression may be used as a predictive biomarker of tumor aggressiveness and patient prognosis, but further studies with larger patient cohorts are needed to confirm this supposition.

### Malignant Peripheral Nerve Sheath Tumor

Malignant peripheral nerve sheath tumors (MPNSTs) are highly aggressive soft tissue sarcomas that account for 3%–10% of all soft tissue sarcomas [60]. These tumors typically originate from cells constituting the nerve sheath, such as Schwann and perineural cells.



Approximately half of MPNSTs occur sporadically, with the remaining originating in patients with the autosomal dominant genetic disorder neurofibromatosis type 1 (NF1). Individuals with NF1 have high lifetime risk of developing MPNST. However, screening for malignant transformation in patients with NF1 is difficult because of the large number and diverse anatomical sites of neurofibromas that occur in these patients as well as the lack of useful biomarkers for differential diagnosis.

Weng et al. [61] investigated the role of serum miRNAs to distinguish MPNST patients with and without NF1. They applied Solexa sequencing to screen for differentially expressed miRNA in pooled serum from 10 patients with NF1, 10 patients with sporadic MPNST, and 10 patients with NF1 MPNST patients [61]. As a result, miR-801 and miR-214 showed higher expression levels in sporadic MPNST patients and NF1 MPNST patients than NF1 patients [61]. Moreover, miR-24 was significantly upregulated in NF1 MPNST patients. Therefore, they concluded that the combination of the three miRNAs (miR-801, miR-214, and miR-24) could be used to distinguish NF1 MPNST patients from NF1 patients [61]. A previous report from Subramanian et al. [62] also demonstrated that miR-214 was relatively upregulated in MPNSTs compared to benign tumors[62]. They considered that high expression of *TWIST1* in the majority of MPNSTs might be involved in miR-214 expression in MPNSTs, since *TWIST1* has been known to induce miR-214 expression in mouse neural cells[62].

### miRNAs as Potential Treatment Targets

Analysis of miRNA expression in serum and tumor tissue involved in sarcomagenesis may be useful to identify novel targets for miRNA-based therapy. Among the miRNAs discussed as potential biomarkers of sarcoma (Table 1), miR-143 has already been investigated for therapeutic potential *in vivo*. Based on the evidence that miR-143 was downregulated in metastatic 143B osteosarcoma cells compared to non-metastatic HOS cells, Osaki et al. [52] assessed the therapeutic potential of miR-143 against spontaneous lung metastasis mouse model using 143B osteosarcoma cells by systemic administration of a miR-143 mimic and miR-negative control (NC). Experimentally, 50 µg of miR-143 mimic or miR-NC was mixed with atelocollagen and administered intravenously into mice in groups of 10 at 1, 4, 7, 10, 13, 16, and 19 days after inoculation of 143B cells [52]. The results showed that, at 3 weeks after inoculation, six of eight mice exhibited lung metastasis on *in vivo* imaging system and the other two mice died due to lung metastasis following miR-NC/atelocollagen treatment, whereas only two of the 10 mice in the miR-143/atelocollagen-treated group showed lung metastasis. This preclinical trial has shed light on the therapeutic potential of miRNAs against osteosarcoma. However, the toxicity of miRNA therapy should be considered, since miRNA can simultaneously regulate multiple target mRNAs. Thus, a large series to study the safety of miRNA-based therapy is necessary. On the other hand, development of a drug delivery system (DDS) would be an important step toward the clinical application of miRNA-based therapy. While Atelocollagen has been shown to be effective against osteosarcoma in an *in vivo* study; there is little consensus regarding the standard use of DDS. Further investigations for key miRNAs for each type of sarcoma and toxicological testing of miRNA mimics, along with development of DDS, would accelerate the therapeutic possibility of targeting miRNAs as novel treatment options for sarcomas.

### Conclusions

There is a growing amount of evidence of miRNA profiling in bone and soft tissue sarcoma not only in tumor cells and tissues but also patient serum and plasma samples. Despite some exceptions, most

of these findings have shown that aberrant expression of circulating miRNAs correlated with that of tumor cells and tissues, indicating that serum or plasma miRNA expression could serve as a novel biomarker for sarcoma. To date, there are few useful biomarkers to monitor sarcoma. Although some issues remain unresolved regarding the measurement of circulating miRNA levels, we believe that a novel noninvasive miRNA-based assay with high sensitivity and specificity for and its therapeutic use will be available for clinical applications in the near future.

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