Role of microRNAs in the crosstalk between osteosarcoma cells and the tumour microenvironment

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ABSTRACT

Osteosarcoma (OS) is the most common primary bone tumour, with a peak incidence in adolescents, and the five-year survival rate of patients with metastasis or recurrence is much lower than that of patients without metastasis and recurrence. OS is initiated and develops in a complex tumour microenvironment (TME) that contains many different components, such as osteoblasts, osteoclasts, mesenchymal stem cells, fibroblasts, immune cells, extracellular matrix (ECM), extracellular vesicles, and cytokines. The extensive interaction between OS and the TME underlies OS progression. Therefore, rather than targeting OS cells, targeting the key factors in the TME may yield novel therapeutic approaches. MicroRNAs (miRNAs) play multiple roles in the biological behaviours of OS, and recent studies have implied that miRNAs are involved in mediating the communication between OS cells and the surrounding TME. Here, we review the TME landscape and the miRNA dysregulation of OS, describe the role of the altered TME in OS development and highlight the role of miRNA in the crosstalk between OS cells and the TME.

1. Introduction

Osteosarcoma (OS) is the most common primary bone tumour in adolescents and young adults [1]. The five-year survival rate has remained static, at 70% for non-metastatic patients over the past four decades, but is only 30–40% for patients with recurrence and metastasis [2,3]. The effects of conventional chemotherapy have not improved in recent decades, and emerging targeted therapy and immunotherapy do not offer much promise as successful OS treatments [4]. This treatment dilemma of OS demands further molecular exploration to discover the mechanisms of OS genesis and progression that may enable the design of potential drugs.

The tumour microenvironment (TME) plays a vital role in OS initiation and development [5,6]. As a mixture of cancer cells and their stroma, the features of the TME are abnormal, and the normalization of the TME improves the effectiveness of chemotherapy and immunotherapy in cancers [7,8]. Therefore, investigating the key factors in the TME of OS may reveal potential therapeutic targets.

Recently, microRNAs (miRNAs) have been shown to mediate communication between cancer cells and the TME [9]. miRNAs are short, non-coding RNAs (18–25 nucleotides) that repress gene expression by binding to the targeting mRNA at the 3′-untranslated region to inhibit its translation or induce its degradation [10]. Dysregulated miRNAs have been widely identified in cancers and usually function as onco-genes or tumour suppressors [11]. OS has a markedly different miRNA profile from healthy bone, and these altered miRNAs influence OS behaviour [12]. In this review, we describe the TME landscape and the miRNA profile of OS, most importantly, we summarize the role of miRNAs in the crosstalk between OS cells and the TME.

2. The TME landscape of OS

The TME hosts two major categories of components: 1) acellular components, such as the extracellular matrix (ECM), extracellular vesicles (EVs), cytokines and growth factors; and 2) cellular components, including all nonmalignant cells, such as fibroblasts, endothelial cells, immune cells and adipocytes [13]. In OS, bone cells are also a part of the TME. Fig. 1 illustrates the presence of OS cells in the TME.

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2.1. The ECM

The ECM is primarily composed of fibrous proteins (such as collagens, elastins, fibronectin, and laminins), glycosaminoglycans (GAGs) and proteoglycans, which are locally secreted and form the structural skeleton for most tissues [14].

The downregulation of collagens 1A1, 1A2, 4A1, 5A1 and 12A1 is associated with OS metastasis [15,16]. Matrix metalloproteinases (MMPs) are the major enzymes involved in degrading collagen and remodelling the ECM [17]. OS tissues strongly express MMP-2 and MMP-9, and high MMP-9 expression predicts lung metastasis and poor survival [18,19]. Urokinase-type plasminogen activator (uPA) also degrades the ECM. The activation of uPA contributes to the metastatic phenotype of OS cells, and the uPA inhibitor decreases lung metastasis in mouse models [20]. Lysyl oxidase (LOX) catalyses the cross-linking of collagen and elastin. In the Chinese population, the 22G/C and 473G/A polymorphisms of the LOX gene reportedly increase susceptibility to OS [21].

Fibronectin and laminin are glycoproteins that integrate with adhesion molecules or cell membrane receptors to mediate cell adhesion, proliferation, differentiation, apoptosis, and migration by inducing a number of signalling pathways [22,23]. Upregulated fibronectin-1 and laminin contribute to the doxorubicin resistance of OS cells, and high fibronectin-1 expression is correlated with poor overall survival after surgical resection in OS patients [24,25]. High-grade OS tissues express high levels of fibronectin and its receptor syndecan 4, which are positively correlated with lung metastasis and short overall survival [26].

GAGs, such as chondroitin sulfate, dermanan sulfate, heparan sulfate, keratan sulfate, and hyaluronan, are unbranched polysaccharides that consist of repeated disaccharidic units of uronic acids and hexamine or galactose [27]. GAG expression is significantly higher in OS tissues than in normal tissues [28]. The blockade of heparan sulfate binding to epidermal growth factor receptor 1 (EGFR1) prevents the interaction of fibroblast growth factor 2 (FGF2) with fibroblast growth factor receptor 1 (FGFR1), resulting in the apoptosis of OS cells [29]. The application of 4-methylumbelliferone, an inhibitor of hyaluronan synthesis, has been shown to repress OS proliferation, migration, invasion, and lung metastasis [30].

2.2. Bone cells

Normally, bone homeostasis is in equilibrium due to osteoblast-mediated bone formation and osteoclast-mediated bone resorption. Molecularily, the maturation of osteoclasts is regulated through the binding of the receptor activator of nuclear factor \( \kappa \)B ligand (RANKL) on osteoclasts with its receptor RANK secreted by osteoclasts [31]. In the early stage of OS initiation, bone metabolism is disrupted, generating a vicious circle between OS cells, bone cells, and stromal cells. OS cells secrete factors such as interleukin (IL)-6, IL-11, and parathyroid hormone-related protein (PTHrP) to stimulate osteoclastogenesis by activating osteoclast precursors or upregulating RANKL expression on osteoclasts. Activated osteoclasts release proteases such as cathepsin K to degrade bone. In turn, the destructive bone matrix releases growth factors such as transforming growth factor-\( \beta \) (TGF-\( \beta \)) and fibroblast growth factor (FGF) to promote OS cell growth and metastasis [32,33]. In the late stage of OS initiation, OS cells release factors to suppress osteoclastogenesis [33]. Moreover, the decrease in osteoclasts has been shown to be associated with the lung metastasis of OS [34].

2.3. Mesenchymal stem/stromal cells (MSCs)

MSCs are multipotent cells mainly residing in the bone marrow adipose tissue and placenta [35]. It has been suggested that OS most likely originates from MSCs or MSC-derived cells as OS contains a considerably high number of MSCs [36,37]. Additionally both bone marrow- and adipose-derived MSCs enhance the proliferation and metastasis abilities of OS by activating signal transducer and activator of transcription 3 (STAT3) [38,39]; the activation of STAT3 by MSCs probably leads OS cells to be resistant to doxorubicin and cisplatin [40]. Additionally it has been determined that MSCs are able to promote the progression of OS through signalling pathways such as chemokine (C–C motif) ligand 5 (CCL5) chemokine (C–X–C motif) receptor 4 (CXCR4) and IL-8 [41–43]. Furthermore MSCs isolated from primary OS samples reportedly express markers related to cancer-associated fibroblasts (CAFs) [37] and bone marrow-derived MSCs cultured in conditioned medium derived from OS cells differentiate towards a CAF-like phenotype [44] indicating that MSCs may be the source of CAFs in OS.
2.4. Immune cells

In OS, immune infiltration is frequent and mainly involves T lymphocytes and tumour-associated macrophages (TAMs), accompanying dendritic cells, B lymphocytes, and mast cells [45,46]. The abundance of T lymphocytes, B lymphocytes, and natural killer (NK) cells is higher in non-metastatic OS than in metastatic OS [47]. A study of 102 OS patients showed that a high density of CD8\(^+\) tumour-infiltrating lymphocytes (TILs) and a low density of FOXP3\(^+\) regulatory T-cells (Tregs) are correlated with a favourable survival rate [48].

According to the differentiation process and activities, TAMs are divided into an M1 subtype with antitumour ability and an M2 subtype with pro-tumour ability [45]. TAMs have been demonstrated to exert pro-tumour activities in many tumours by promoting angiogenesis, immunosuppression, cell invasion, and migration [49]. However, the role of TAMs in OS is inconclusive. Buddingh et al. [50] showed that both M1 and M2 TAMs existed in biopsies from OS patients, and the total number of TAMs was positively associated with a low risk of lung metastasis, a good response to chemotherapy and favourable overall survival. Gomez-Brouchet et al. [51] reported that the presence of M2 TAMs was essential for inhibiting OS development. By contrast, Han et al. [52] found that TAMs promoted lung metastasis and induced epithelial-mesenchymal transition (EMT) in OS. These inconsistent findings suggest that the role of TAMs in OS metastasis is complicated and needs further exploration.

2.5. Extracellular vesicles (EVs)

EVs are lipid bilayer-bound vehicles that are released from the cell membrane and contain a diverse array of bioactive cargos, including proteins, nucleic acids (DNA, mRNA, and miRNA) and lipids [53]. Recently, EVs have attracted substantial attention as intercellular communication mediators [54]. Proteomics analysis of EVs from OS cell lines identified more than 1,000 proteins in EVs, of which the most abundant proteins were involved in angiogenesis, cell adhesion, and migration [55]. The metastatic OS cell line 143B secretes EVs containing MMP-1, MMP-13, TGF-β, CD9, and RANKL, all of which participate in bone destruction [56]. EVs from 143B cells also activate MSCs to secrete IL-6, thus stimulating OS progression [57]. MSCs have also been demonstrated to release EVs containing miRNAs, which increase OS migration and apoptosis resistance [58].

3. miRNA dysregulation in OS

Carcinogenesis has long been mainly attributed to gene alterations. Since the first report about the association between chronic lymphocytic leukaemia and miR-15 and miR-16, mounting studies have indicated that miRNAs act as tumour suppressors or oncogenes in many cancers [11,59].

3.1. miRNAs regulate OS progression

Notch signalling pathway dysregulation plays a stimulatory role in OS initiation and development [60]. Jagged1 is a Notch pathway ligand, and the overexpression of miR-26a exerts an anti-OS effect by blocking the Jagged 1/Notch signalling pathway [61]. Notch2 is a crucial receptor in the Notch pathway, and miR-1296-5p represses the proliferation, migration, and invasion of OS cells by targeting Notch2 [62]. The re-expression of miR-34 and miR-200, which are expressed at low levels in OS tissues [63,64], has been shown to reduce the expression of Notch1 [65]. Conversely, miR-10b-5p activates the Notch pathway by downregulating NCOR2, which is a negative regulator of this pathway [66]. Long non-coding RNA (IncRNA) CEBPA-AS1 inhibits the Notch pathway by sponging miR-10b-5p to increase the expression of NCOR2, thereby inhibiting cell growth and stimulating cell apoptosis in OS [66].

Activation of the Wnt signalling pathway contributes to OS progression [67]. miR-377 inactivates the Wnt pathway by targeting histone acetyltransferase 1 (HAT1), thus promoting OS cell apoptosis [68]. miR-425-5p, miR-758, and miR-873 also repress the Wnt pathway to inhibit the malignant phenotype of OS [69–71]. Higher miR-940 expression has been identified in OS tissues and stimulates the Wnt pathway by targeting secreted frizzled-related protein 1 (SFRP1) [72]. The inhibition of miR-940 suppresses the migration and invasion of OS cells while inducing cell apoptosis [72]. Aberrant activation of the PI3K-Akt-mTOR pathway is frequently detected in OS [73], and miR-199a-3p, miR-99a and miR-140 all downregulate mTOR expression [74–76].

3.2. miRNAs affect OS metastasis

 Approximately 20% of OS patients have metastatic lesions at initial diagnosis, and the survival rate among such patients is dismal [3]. The lung is the most frequent metastatic site of OS, and 60%~70% of metastatic patients develop pulmonary metastases [3]. miR-491 is expressed at low levels in OS tissues, and in vitro and in vivo studies showed that overexpression of miR-491 inhibited lung metastasis by targeting α-crystallin, a small heat shock protein [77]. miR-382 exerts a tumour-suppressing function by targeting Y box-binding protein 1, which sustains OS metastasis and relapse [78]. Cadherin-6 is reportedly associated with poor prognosis of OS patients, and miR-223-3p targets cadherin-6 to suppress OS metastasis and progression [79]. Moreover, miR-216a, miR-203a-3p, miR-143, miR-506-3p and miR-627-3p suppress OS metastasis [80-84]. In contrast, miR-19a, miR-20a and miR-135b promote lung metastasis [85-87].

3.3. miRNAs affect OS chemotherapy sensitivity

Chemotherapy is the treatment of choice for OS, but the increasing resistance rate greatly reduces its therapeutic effect. The resistance mechanisms are varied and include reduced drug accumulation, increased detoxification, enhanced abilities of DNA repair or tolerance, and attenuated cell apoptosis [88]. Decreased serum miR-491 expression indicates a poor prognosis in OS patients, and miR-491 inhibits cisplatin resistance by directly targeting α-crystallin [77]. miR-134-5p inhibits malignant brain tumour domain containing 1 (MTBD1), and IncRNA TTN-AS1 can sponge miR-134-5p to upregulate MBTD1 and induce cisplatin resistance [89]. miR-590 expression is low in OS tissues, and the overexpression of miR-590 enhances the cytotoxicity of doxorubicin by targeting wild-type p53-induced phosphatase 1 (WIP1) [90]. miR-15b alleviates doxorubicin resistance by targeting wild-type p53-induced phosphatase 1 (WIP1) [90]. miR-15b alleviates doxorubicin resistance by targeting wild-type p53-induced phosphatase 1 (WIP1) [90]. miR-15b alleviates doxorubicin resistance by targeting wild-type p53-induced phosphatase 1 (WIP1) [90]. miR-15b alleviates doxorubicin resistance by targeting wild-type p53-induced phosphatase 1 (WIP1) [90]. miR-15b alleviates doxorubicin resistance by targeting wild-type p53-induced phosphatase 1 (WIP1) [90]. miR-15b alleviates doxorubicin resistance by targeting wild-type p53-induced phosphatase 1 (WIP1) [90]. miR-15b alleviates doxorubicin resistance by targeting wild-type p53-induced phosphatase 1 (WIP1) [90]. miR-15b alleviates doxorubicin resistance by targeting wild-type p53-induced phosphatase 1 (WIP1) [90].

3.4. miRNAs act as diagnostic and prognostic biomarkers

miRNAs widely exist in the serum. It has been found that the expression levels of miRNAs in body fluids are able to represent the status of tumours. Research has shown that serum miR-9 increased obviously in OS patients compared with healthy controls, and this increase was associated with tumour stage, size, metastasis and outcome [95]. In contrast, serum miR-195 is decreased in OS patients and predicts poor overall survival as an independent prognostic factor [96]. OS patients also have low expression of let-7a and miR-375 in the serum [97,98].

3.5. miRNAs in clinical trials

A summary of anticancer drugs targeting miRNAs in clinical trials is
presented in Table 1. MRX34, a liposomal miR-34a mimic, was found to have antitumour activity in a subset of patients with refractory advanced solid tumours; however, the clinical trial was terminated because of significant immune-related toxicities [99]. In patients with malignant pleural mesothelioma, TargomiRs, a miR-16 mimic, showed an acceptable safety profile and signs of activity [100]. Scientists have also developed nanoparticles, bioengineered prodrugs and EVs to deliver miRNA mimics (miR-199a-3p, let-7a, miR-34a, miR-145, miR-143, and miR-101) to OS cells in vitro and in vivo (Table 2) [101–105].

4. miRNAs in the crosstalk between OS and the TME

4.1. miRNAs target the ECM to suppress OS development

Collagen and fibronectin deposition in the ECM plays a role in chemotherapy resistance [106]. The miR-29 family (miR-29a, miR-29b, and miR-29c) alleviates methotrexate resistance and induces cell apoptosis by targeting COL3A1 [107]. miR-200-3b is upstream of fibronectin-1, and the sponge of miR-200-3b by lncRNA OIP5-AS1 leads to doxorubicin resistance [25]. The degradation of the ECM participates in OS invasion and metastasis. In OS tissues with lung metastasis, miR-3182 expression is significantly lower than that in OS tissues without lung metastasis [108]. MMP-2 is the target of miR-3182, and lncRNA ODRUL sponges to miR-3182 to upregulate MMP-2, thus promoting OS progression [108]. miR-143 acts as a metastasis suppressor by targeting MMP-13 in the OS cell lines HOS and 143B [82]. MMP-16 is the target of miR-145 and miR-328–3, and the inhibition of both miR-145 and miR-328–3 endowed OS cells with a metastatic trait [109,110]. In addition, miR-199a-3p, an antitumour factor in OS, targets genes that are significantly associated with proteinaceous ECM, especially proteoglycans [111].

A microarray analysis conducted between 23 primary OS samples and three osteoblast cell lines identified 33 deregulated miRNAs, 29 of which were confirmed in another 78 OS samples; these 29 deregulated miRNAs were enriched in pathways related to the interaction of ECM and its receptors [112]. Integrins and CD44 are both adhesion molecules that mediate interactions between the ECM and the cell cytoskeleton [113,114]. Integrin α2, integrin α6, and integrin αV are the targets of miR-128, miR-127-3p, and miR-548c-3p, respectively, and the suppression of integrins by these miRNAs blocks OS progression [115–117]. The downregulation of CD44 by miR-34a and miR-199a-3p also suppresses OS metastasis and drug resistance [118,119].

4.2. miRNAs suppress angiogenesis

Angiogenesis, which is the process of creating new blood vessels derived from the pre-existing vascular network, is one of the hallmarks of cancer [120]. The vascular endothelial growth factor (VEGF) superfamily induces angiogenesis by binding to VEGF receptors (VEGFRs), which are predominantly expressed on endothelial cells [121]. Both miR-29 and miR-765 inhibit VEGF to suppress angiogenesis in OS [122,123]. miR-145 targets VEGF to inhibit OS invasion and metastasis [124]. VEGFA is the most crucial member of the VEGF superfamily and binds to VEGFR1 and VEGFR2 [121]. miR-374b targets VEGFA, and chemokine CCL3 downregulates miR-374b to increase VEGFA expression, thus promoting angiogenesis in OS [125]. Both VEGFA and VEGFR1 are targets of miR-134, the overexpression of which significantly attenuates angiogenesis in OS [126]. VEGFA is also targeted by miR-1 [127]. IL-6 is another kind of pro-angiogenic factor. Liu et al. [128] reported that the downregulation of miR-451 on the IL-6 receptor repressed cell growth, migration, and angiogenesis in OS.

4.3. miRNAs inhibit the TGF-β signalling pathway to inhibit OS progression

Although the TGF-β signalling pathway has both pro-
antitumour ability depending on the cancer type, it mainly plays a pro-
tumour role in OS [129]. miR-29 induces cell apoptosis by targeting TGF-β1 [130]. miR-21 inhibits cell proliferation by downregulating TGF-β1 and the protein phosphatase and tensin homologue (PTEN) [131]. TGF-β1 is also a target of miR-339-5p [132], miR-153 represses TGF-β1 and the protein phosphatase and tensin homologue (PTEN) inhibit the activation and function of CD8⁺ T cells by upregulating PD-
immune reactions, such as PD-L1/PD-1 pathway alteration, especially cells [142,143]. Tumour cells usually develop approaches to avoid 133a and miR-200a block the maturation and activation of dendritic genesis and promotes apoptosis[146]. CAFs produce 4.4. miRNAs target the immune system to affect OS growth

Macrophage migration inhibitory factor (MIF) is a pro-inflammatory cytokine expressed by T lymphocytes, macrophages, monocytes, den-
dritic cells and B lymphocytes [139]. MIF expression predicts an increased risk of lung metastasis in OS [140], and the downregulation of MIF by miR-415 suppresses cell proliferation, migration, and angiogenesis and promotes apoptosis [141]. In OS mouse models, both miR-133a and miR-200a block the maturation and activation of dendritic cells [142,143]. Tumour cells usually develop approaches to avoid immune reactions, such as PD-L1/PD-1 pathway alteration, especially under drug treatment. miR-140 has been shown to directly target PD-L1 to suppress OS growth in vitro and in vivo [76]. Conversely, mir-200a inhibits the activation and function of CD8⁺ T cells by upregulating PD-L1 by targeting PTEN [144].

4.5. miRNAs in EVs promote OS metastasis

miRNAs are common cargos in EVs, and the delivery through EVs is more effective in reaching recipient cells [145]. MSCs release EVs containing miR-195, miR-124, and miR-148a to affect migration and the response to doxorubicin in OS cells [58]. CAFs produce EVs with miR-1228 to promote the migration and invasion of OS cells by targeting the protein suppressor of cancer cell invasion (SCAI) [146]. EVs secreted by OS cells have been shown to contain miRNAs associated with cell adhesion and apoptosis, including miR-21-5p, miR-143-3p, miR-148-3p, and miR-181a-5p [147].

5. Conclusions

It is now widely accepted that the interaction between OS and the surrounding TME, but not the tumour itself, plays an essential role in the genesis and development of OS. Recently, a double-blind phase II clinical trial demonstrated that sorafenib, a multi kinase inhibitor of VEGF 1, 2 and 3, doubles the median progression-free survival of patients with metastatic OS compared with that of a placebo group [148]. Increasing evidence has illustrated the potential therapeutic importance of the TME in OS, highlighting the importance of normalizing the TME. However, the high genetic heterogeneity of OS makes the TME much more complex than that of other tumours, and thus, potential TME-normalizing drugs might have multiple targets. Moreover, very few studies consider the TME to be a crucial factor in the development of chemotherapy resistance, and the study of the potential role of the TME in chemotherapy may help to uncover the underlying mechanisms of resistance. The combination of TME-normalizing drugs with chemotherapy will probably alleviate drug resistance and achieve a better therapeutic effect than either treatment alone.

Although an increasing number of miRNAs have been demonstrated to play roles in tumour development, only a few have proceeded into clinical trials. The difficulties of transforming miRNAs from bench to bedside mainly derive from two aspects: 1) it is not easy to choose good miRNA candidates for further study, given the high heterogeneity of OS; 2) the challenge of delivering the potential miRNA mimics or inhibitors to tumour sites accurately and effectively remains. Further studies should focus on the selection of the best candidates among the miRNAs for OS treatment and begin clinical trials on compounds targeting miRNAs. The combination of TME-normalizing drugs, miRNAs, and chemotherapy may offer promise in the treatment of OS.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Table 2

| miRNA mimics in OS studies. | Carrier | Effects | Models | Reference |
|-----------------------------|--------|--------|--------|----------|
| miR-199a-3p                 | Dextran-based nanoparticle | Inhibition of cell proliferation | U-2OS and KHOS cells | [101] |
| miR-145                     | Nanoparticle system | Inhibition of cell proliferation and migration and induction of cell apoptosis | MG-63 cells | [102] |
| miR-34a                     | Bioengineered prodrug | Inhibition of cell apoptosis and cell cycle arrest | MG-63 and 143B cells and xenograft tumour mouse models | [103] |
| miR-143                     | Exosome | Inhibition of migration | 143B cells | [104] |
| miR-101                     | Extracellular vesicle | Inhibition of lung metastasis | SaOS-2, U-2OS, 143B, and SOSP-9607 cells and xenograft tumour mouse models | [105] |
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