Review Article
Exosomes in osteosarcoma research and preclinical practice

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Abstract: Osteosarcoma (OS) is a rare soft-tissue malignant tumor with high lung metastasis and mortality rates. Preoperative chemotherapy, surgical resection of the lesion and postoperative chemotherapy are still the main treatments for osteosarcoma. The prognosis, however, is poor for patients with nonresectable, primary metastatic or relapsed disease. Recent studies have shown that targeted therapy for OS based on the characteristics of exosomes is very attractive. Exosomes are nanosized extracellular vesicles (EVs) that participate in cell-to-cell communication by transporting biologically active cargo molecules, causing changes in OS cell function and playing important roles in OS disease progression. With the characteristics of secretory cells, exosomes transport cargo (e.g., microRNAs) that can be used to detect the progress of a disease and can serve as markers and/or therapeutic targets for clinical diagnosis of OS. In this review, the roles of exosomes in OS pathogenesis, invasion, metastasis, drug resistance, diagnosis and treatment are summarized. In addition, this article elaborates a series of challenges to overcome before exosomes are applied in clinical practice and provides suggestions based on current evidence for the direction of future research.

**Keywords:** Osteosarcoma (OS), exosomes, microRNA (miRNA), challenges

Introduction to exosomes

Exosomes are EVs with a size of 30 to 150 nm. The exosome production and release process in the cell is shown in **Figure 1**. Exosomes were originally discovered by Johnstone et al. [1] during in vitro culture of sheep reticulocytes and were considered "cell dust", thus not attracting the attention of researchers. Later, in 2007, Valadi et al. [2] confirmed for the first time that exosomes contain both functional mRNA and microRNA (miRNA), called "exosome shuttle RNA", which can be transferred to other cells and function in a new location. Since then, exosome research has attracted widespread attention.

Exosomes are marked by aggregated small RNAs, including messenger RNA (mRNA), miRNA, transfer RNA (tRNA) and long noncoding RNA (lncRNA) [3, 4]. These RNA molecules, together with proteins, act as genetic materials and play a vital roles in cell-cell communication through exosome transfer [5]. Exosomes act on the recipient cell in three different ways [6, 7] (**Figure 2**). It carries lipids and proteins similar to those in the cell of origin, which interact with recipient cells to trigger cargo release or signal transduction cascade induction, ultimately leading to changes in cell activity or function [8].

Compared with stem cells, exosomes have the advantage of being stable, easy to store and access, and transformable, and they are nonimmunogenic. Thus, as carriers of specific genes or drugs to treat disease, exosomes are appropriate choices for use in cell-free targeted therapy [9]. Exosomes are present in various body fluids, such as serum, saliva and urine.
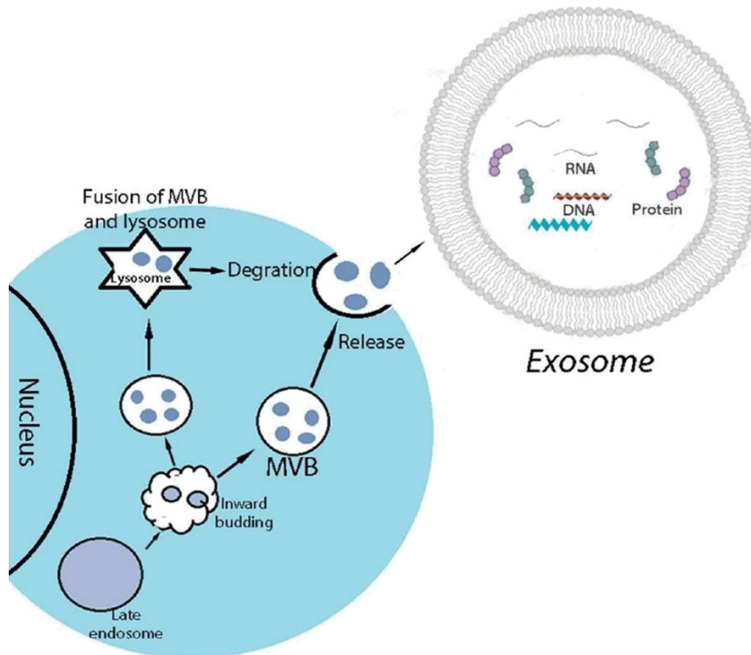
Figure 1. Exosome biogenesis begins with invagination of the endosomal membrane to form intracellular vesicles. In late endosomes, inward protrusion of the endosomal membrane leads to accumulation of small vesicles, thereby transforming the endosome into multiple multivesicular bodies (MVBs). MVBs can enter the degradation pathway upon maturation into lysosomes or can be released as exosomes into the extracellular system upon fusion with the plasma membrane.
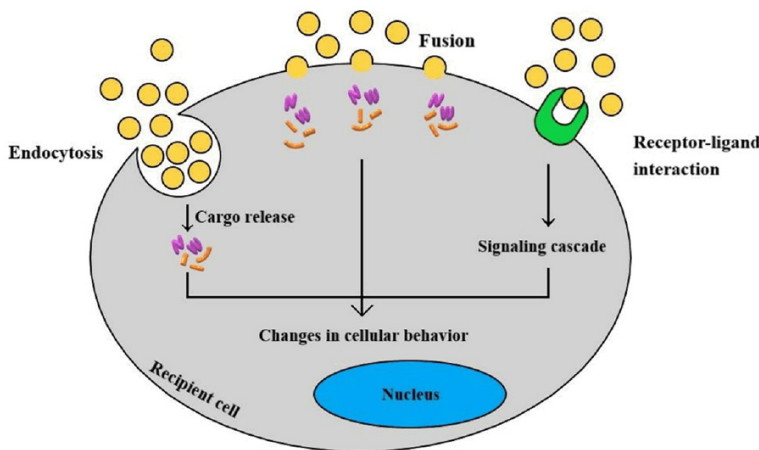
Figure 2. Exosomes interact with receptor cells through three mechanisms: 1) release of their cargo after entry into the cell via endocytosis; 2) direct fusion with the plasma membrane to nonselectively release the protein and small RNA molecules contained within; and 3) interaction with exosome ligands and receptor proteins on the cell membrane surface.

Blood samples can be easily collected from patients for separation and then used to identify specific RNA molecules or proteins in exosomes, which is an ideal noninvasive diagno-

sis/prognosis technique [10, 11]. Currently, exosomes have many potential uses in medicine, and research on exosomes is widely concentrated in the fields of cancer, drug delivery and regenerative medicine [12]. With advances in biomedicine, exosomes are rapidly being developed for use in new tumor treatment methods and can be used for clinical diagnosis (markers and liquid biopsy tests) [13], as drug carriers [14, 15], disease targets [16] and in prognosis monitoring [17].
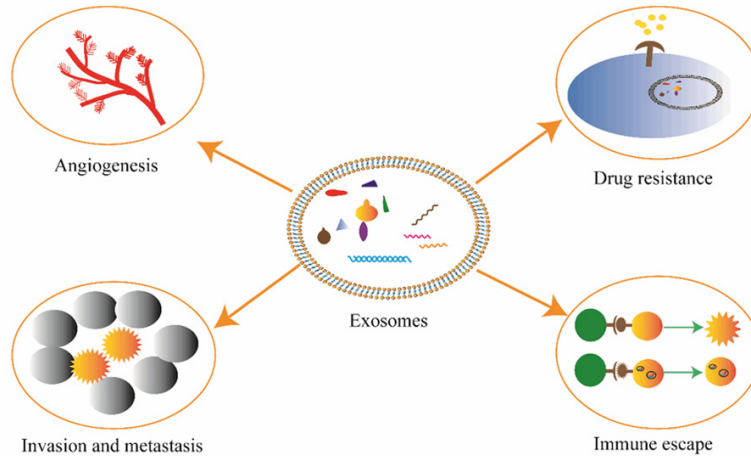
Osteosarcoma

Pathogenesis

Osteosarcoma (OS) is the most common primary bone cancer, with 4 or 5 cases per million people per year. OS mainly affects children and adolescents from 5 to 20 years old and adults in their 70s [18, 19]. The most common site of OS is the metaphysis of long bones [20]. High-grade OS usually spreads to the lungs, followed by distant bones [21]. Secondary lung cancer is the main cause of death in OS patients [22]. To date, the factors and pathways that regulate the OS metastasis process are unclear.

In recent years, the pathogenesis of OS has been extensively studied, and the main focus has been on the origin and signaling pathways of OS cells. According to reports, OS may be derived from immature stromal spindle cells [23]. The most important pathways in OS pathogenesis are the Wnt, Notch, NF-κB, p53, PI3K/Akt and MAPK pathways [24-26]. The balance between cell survival and apoptosis depends on the Wnt and NF-κB pathways

Figure 3. Exosomes carrying genetic materials participate in the development of osteosarcoma by promoting angiogenesis, invasion and metastasis, immune escape and drug resistance.

and on the ratio of MAPK activity to PI3K/Akt pathway activity [26].

According to a bioinformatics analysis, TP53, MAPK1, ESR1, NOTCH3 and CASP1 may play roles in OS development [27]. miRNAs (such as miR-21, miR-34a, miR-143, miR-148a, miR-195a, miR-199a-3p, and miR-382) [26] participate in OS pathogenesis by regulating multiple target genes and signaling pathways. Although research into the molecular mechanisms continues to deepen, no evidence is available to verify the most fundamental and important aspects of OS pathogenesis, and identification of its etiology is crucial for finding new therapeutic targets. In addition, due to the existence of unknown factors, such as chromosome, karyotype and genome mutations, effective OS treatment and determining a complete treatment strategy are difficult.

Treatment

Surgery and systemic chemotherapy are the main treatment options for OS, but their efficacy is not satisfactory. According to statistics, the 5-year survival rate using a multidisciplinary approach varies from 60 to 70% [28]. Patients with OS diagnosed with distant metastases usually have the primary tumor surgically removed, which significantly prolongs the survival of patients with OS originating in the extremities. However, for patients with pelvic/spine OS, primary tumor surgery does not improve survival [29].

High-dose methotrexate (HD-MTX), Adriamycin, cisplatin and ifosfamide have antitumor activity against OS and are currently recognized as first-line chemotherapy drugs [30-32]. In the past 30 years, the available chemotherapy regimen and therefore the survival rate have not changed significantly [33]. Chemotherapy-induced tumor necrosis is still the gold standard [34]. High-grade OS often recurs, and the pattern of recurrence differs in biological significance. Compared with patients with systemic relapse, for whom chemotherapy had no effect, patients with local recurrence have shown significantly improved survival rates after chemotherapy [35]. In summary, simple surgery and chemotherapy cannot achieve the desired OS treatment results. In recent years, comprehensive treatment of OS has also led to the development of molecular targeted therapies, immunotherapy, gene therapy, embolization, radiofrequency ablation and stem cell therapy [36], which may become the mainstream treatments sometime in the future.

Roles of exosomes in osteosarcoma progression

Promotion of angiogenesis

Exosomes carrying cargo cause changes in the activity and function of receptor cells, such as promoting osteoclast differentiation and bone resorption activity and enhancing blood vessel formation and endothelial cell growth, thus upregulating the expression of angiogenesis markers [37] (**Figure 3**).

According to research, miR-21 can significantly affect the plasticity of cancer cells, leading to tumor metastasis and angiogenesis, and is also involved in tumor immune regulation [38]. Exosomes secreted by OS cells and carrying miR-148a and miR-21-5p as cargo can participate in establishment of the tumor microenvironment (TME) and stimulate endothelial cells to secrete more angiogenic factors and organize into tube-like structures. These effects

may be important for changes in the osteoclast phenotype and endothelial cell activity [37]. Sex-determining region-related high-mobility group box 4 (SOX4) has been identified as a direct target gene of miR-25-3p. miR-25-3p can inhibit OS cell proliferation, migration and invasion by targeting SOX4 expression in bone tissue and thus plays a role in cancer suppression [39]. Moreover, miR-25-3p embedded in OS cell-derived exosomes can promote capillary formation and vascular endothelial cell invasion [40].

Promotion of growth and invasion

In the TME, exosomes derived from OS and bone marrow cells (BMCs) can specifically stimulate migration of OS cells in vitro and in vivo through the urokinase plasminogen activator (uPA)-dependent signaling pathway [41]. Exosomes secreted by tumor cells promote tumor growth, metastasis, and angiogenesis by regulating the TME and can escape host immune surveillance [42, 43]. In addition, stroma exosomes can induce OS cells to acquire a cancerous phenotype; for example, exosomes from cancer-associated cells in multidimensional cultures can affect the proliferation, metastasis, drug resistance and epithelial-mesenchymal transition of OS cells [44]. Studies have shown that exosomes secreted by cancer-associated fibroblasts (CAFs) carrying miR-1228 as molecular cargo are transferred to OS cells and promote migration and invasion of these OS cells [45].

Exosomes secreted by tumor cells contribute not only to the formation of a suitable environment for metastasis or premetastasis but also to tumor-like transformation of the resident cells (mesenchymal cells, MSCs) in metastatic organs [46, 47]. When stem cells are recruited to the tumor stroma, tumor exosomes enter the stem cells to induce acquisition of a malignant phenotype, and in turn, the stem cells secrete exosomes to promote OS cell proliferation, migration and invasion. Zhao et al. [48] demonstrated that bone marrow mesenchymal stem cell exosomes (BMSC-exos) encapsulate plasmacytoma variant translocation 1 (PVT1, a carcinogen) and transport it to OS cells. Transported PVT1 inhibits ERG degradation and ubiquitination in OS cells and sponges miR-183-5p to promote tumor growth and metastasis. Human BMSC-exos (hBMSC-exos) can pro-

mote OS cell proliferation, migration and invasion by promoting oncogenic autophagy in OS. Silencing of autophagy-related gene 5 (ATG5) in OS cells can eliminate the tumor-promoting effect of hBMSC-exos [49]. The mechanism by which MSC-exos promote OS cell growth and metastasis may involve activation of the hedgehog signaling pathway [50] and IL-6/STAT3 signaling pathway [51]. In conclusion, exosomes in the TME are key factors in OS growth and metastasis (**Figure 3**).

Exosomal miRNA participation in growth and invasion

OS cell lines selectively package miRNAs as molecular cargo for EVs. These miRNAs can act as paracrine drugs to regulate the TME, including immune cells, endothelial cells and fibroblasts [38]. Qin et al. [52] found that miR-208a promotes OS cell proliferation, migration and invasion through downregulation of PDCD4 and activation of the ERK1/2 pathway. miR-675 was significantly upregulated in patients with OS lung metastasis. In contrast to exosomes derived from nonmetastatic MG63 cells, exosomes derived from a metastatic MG63 OS cell line induce an increase in the ability of recipient osteoblasts to migrate and invade. miR-675 affects the invasion and metastasis of OS tumor cells by inhibiting the expression of CANL1 in recipient cells [53]. Jerez et al. [54] used next-generation miRNA sequencing technology to examine miRNAs isolated from microvesicle-depleted EVs. These EVs were derived from six human OS or osteoblast cell lines with different metastatic potential (i.e., SAOS2, MG63, HOS, 143B, U2OS and hFOB 1.19 cells). The most prominent miRNAs are miR-21-5p, miR-143-3p, miR-148a-3p and miR-181a-5p, which are enriched between 3- and 100-fold and are relatively abundant in the EVs derived from metastatic SAOS2 cells compared to the level in nonmetastatic MG63 cells. Gene Ontology analysis of predictive targets indicates that miRNAs in EVs may regulate OS cell lines by potentially inhibiting apoptosis and/or cell-related gene networks (e.g., MAPK1, NRAS, FRS2, PRCKE, BCL2, and QKI) and cell transfer potential and adhesion [54].

Participation in immune escape

Immune escape is the key mechanism of tumor progression. Cancer exosomes directly or indi-

rectly (via MSCs) affect innate or adaptive immune components and participate in tumor immune escape (**Figure 3**). Tumor-derived exosomes (TEMs) carry immunosuppressive molecules and factors known to interfere with the function of immune cells. The development, maturation and antitumor activity of an immune cell can be directly or indirectly affected upon exosome delivery of an inhibitory protein similar to protein in the parent tumor cell. Exosomes also deliver genomic DNA, mRNA and miRNA to immune cells, thereby reprogramming the function of these cells to promote tumor progression [55, 56]. Metastatic OS cell exosomes promote the expression of M2 macrophage markers and induce immunosuppression by producing TGFB2 (found in key tumor-mediated immunosuppression-related signaling pathways) [57].

TEMs incubated with T cells induce apoptosis of activated CD8+ cells via the Fas ligand pathway [58]. TEMs induce immune suppression by promoting T regulatory cell expansion and inactivation of antitumor CD8(+) effector T cells, thus contributing to tumor escape [58]. Inhibition of the cytotoxic killing capacity of natural killer (NK) cells was correlated with the expression of MHC 1 short chains in malignant exosomes. Incubation of NK cells with exosomes downregulated NKG2D expression and decreased NK cell functionality [59]. Compared with normal osteoblasts, OS-derived exosomes contain immunomodulatory substances, which can reduce the proliferation rate of T cells and promote the T-regulated phenotype [60]. Although TEMs can express tumor antigens and are therefore proposed to be of use in OS vaccines, they can also inhibit T cell signaling molecules and induce apoptosis, which makes TEM-based OS vaccines difficult to manufacture on a large scale.

Induction of drug resistance

The greatest problem with chemotherapy in OS patients is drug resistance, which may cause a rapid increase in metastasis [61]. Previous studies have shown that acquired multidrug resistance (MDR) is mediated by exosomes released by drug-resistant cells [62]. Exosomes are the main mechanism by which drug resistance is transferred (**Figure 3**). The exosomes of doxorubicin-resistant OS cells can be absorbed by surrounding cells to induce a doxorubicin-resistant cell phenotype, which may be the reason why exosomes with MDR-1 mRNA and its product P-glycoprotein enhance the ability to resist the action of doxorubicin in previously sensitive cells [63]. Exosomes can not only induce TME cells, tumor metastasis and tumorigenic phenotype acquisition but can also induce drug resistance in OS cells. Exosomes produced by resistant cancer cells and/or TME cells confer resistance to anticancer drugs to other cells, apparently through a variety of mechanisms [64]. According to reports, tumor resistance is related to upregulation of miR-25-3p in OS exosomes [40].

Roles of exosomes in osteosarcoma treatment

Exosomes are a double-edged sword; they not only play an important role in tumorigenesis, angiogenesis and metastasis but can also inhibit tumor progression [65] (**Table 2**). Various exosome components provide emerging diagnostic and therapeutic methods for fighting OS and have attracted substantial attention in the field of liquid biopsy and biomarker determination. Exosomes are used as diagnostic biomarkers of many cancer types (e.g., colon cancer [66] and breast cancer [67]). By taking advantage of exosomes in the circulation system, cancer can be detected early. In addition, exosomes provide indications of disease progression and the response of cancer patients to treatment [68, 69]. Studies have reported that exosomal integrins can be used to predict organ-specific metastasis [70]. The miRNA in exosomes can also regulate the drug sensitivity of cancer cells [71]. Exosomes secreted by immune cells may be used in cancer diagnosis and immunotherapy and are being developed for vaccination and chemical drug delivery [72].

OS cell-derived exosomes may become potential targets in cancer therapy [16]. The specific RNA molecules contained in exosomes are powerful tumor indicators because they reflect the current state of the tumor. Therefore, we can design drugs that target these RNA molecules to develop personalized drug treatments. Previous evidence has shown that miRNA molecules in the serum of OS patients can be potential targets [73, 74]. However, the specific mechanisms of these RNA molecules and their

Table 1. Summary of studies on exosomal miRNAs in osteosarcoma

Secretory cells	Receptor cell	Cargo (miRNA)	Influences	References
OS cell lines	Osteoclast and endothelial cells	miR-21-5p and miR-148a	Osteoclastogenesis, bone resorption and tumor angiogenesis	[37]
OS cell lines	Immune cells, endothelial cells and fibroblasts	miR-21	Angiogenesis, metastasis and immune escape of osteosarcoma cells	[38]
OS cell lines	N/A	miR-25-3p	Promotion of capillary formation and vascular endothelial cell invasion; relationship to drug resistance	[40]
CAFs	OS cell lines	miR-1228	Promotion of OS cell migration and invasion	[45]
BMSCs	OS cell lines	miR-208a	Promotion of cell proliferation, migration and invasion	[52]
OS cell lines	Osteoblasts	miR-675	Promotion of cell migration and invasion by targeting CALN1	[53]
OS or osteoblast cell lines	N/A	miR-21-5p, miR-143-3p, miR-148a-3p and 181a-5p	Regulation of osteosarcoma cells by potentially inhibiting apoptosis and/or cell-related gene networks	[54]
OS cell lines	N/A	miR-195-3 p	Upregulation of osteosarcoma cells to promote cell proliferation and invasion	[81]
MSCs	OS cell lines	miR-143	Suppression of osteosarcoma cell migration	[94]

N/A: Not Applicable.

Table 2. Exosomes applications

Application prospect	Purpose	Molecular	References
Biomarkers	Diagnose the disease and determine the stage	miRNA ncRNA	[77-82, 83]
Liquid biopsy	Diagnose and confirm the disease under non-invasive conditions	cell-free DNA (cfDNA), lncRNAs and proteins	[85]
Drug carriers	Targeted therapy	miRNA, proteins and chemical drugs	[93-96]
Prognostic indicators	Clinical prognosis monitoring	miRNA and mRNA	[17, 40, 82, 99]

expression in exosomes must be further clarified to better implement intervention measures and prevent the influence of irrelevant proteins or harmful RNA molecules. Next, the current relevant research on the use of exosomes in OS diagnosis, treatment and prognosis determination is introduced.

Exosomal miRNAs as biomarkers

miRNAs are single-stranded, noncoding RNA molecules comprising 18-24 nucleotides. miRNAs have activity in various organisms and are involved in posttranscriptional regulation of gene expression [75] (**Table 1**). The expression of miRNAs is fine-tuned and very specific [76]. Abnormal miRNA expression is found in nearly all tumor types and has the potential to be used as a biomarker in a variety of cancer types [77]. OS cell lines can selectively package miRNAs as EV molecular cargoes that act as paracrine drugs to regulate the TME. The mechanism by which miRNA is incorporated into exo-

somes is very specific because it is based on a specific sorting mechanism [78]. Exosomal miRNA profiles are markers of tumor cell types and reflect parental cell characteristics [79, 80]. miRNAs that reflect the characteristics of OS cells can signify differences in OS patients and can be used as biomarkers.

The serum and plasma levels of exosomal miR-21 differ between OS patients and healthy controls, which supports the contention that miR-21 has a role as an OS biomarker [38]. Through high-throughput sequencing analysis, Ye et al. [81] found that miR-92a-3p, miR-130a-3p, miR-195-3p, miR-335-5p, and let-7i-3p expression levels in the exosomes of OS patients were higher than in healthy patients. In vitro and in vivo studies have indicated that secretions from 143B OS cells exhibited miR-195-3 upregulation, which promoted cell proliferation and invasion. The results of the experiment verified that miRNAs derived from the exosomes of OS cells in plasma can be used as new diagnostic

biomarkers and might provide treatment options for OS [81]. Fujiwara et al. [82] successfully verified that serum exosomal miR-25-3p can be used as a noninvasive blood-based biomarker for tumor monitoring in OS patients. Clinical development of this methodology as a noninvasive diagnostic or monitoring strategy may be promising for use in patients with malignant diseases. In addition to miRNA, noncoding RNA (ncRNA) can be used as a new potential biomarker or disease-targeting agent in many diseases. Exosomes used as carriers to effectively transfer ncRNA to receptor cells can also play key roles in OS disease treatments [83].

Liquid biopsy

Accurate diagnosis of OS and disease staging are prerequisites for effective surgical and medical treatment. Tissue biopsy and imaging are still commonly used disease sampling and evaluation methods, respectively. However, tissue biopsy samples are sometimes difficult to obtain, and it is impossible to accurately assess a situation of nonobvious tumor metastasis. A surgical margin may need to be reestablished several times [84]. In contrast, liquid biopsy is used to obtain samples for analysis of therapeutic circulating tumor cell (CTC) targets, cell-free circulating tumor DNA (ctDNA) released from metastatic remnants into the peripheral blood and gene mutations that confer drug resistance. Liquid biopsy enables a focused examination on cell-free DNA (cfDNA), lncRNAs and proteins in EVs and thus is a potential diagnostic tool for determining new therapeutic targets in patients with viral hepatitis and fatty liver [85].

Liquid biopsy is a noninvasive and time-saving method for obtaining samples that can be evaluated to obtain accurate information regarding early and midterm OS, which is helpful in making the best treatment decisions, assessing the response to treatment and evaluating new drugs or drug combinations for future trials [86, 87]. Zang et al. [88] established an OS biobank that provides samples for research for the purpose of promoting clinical and basic scientific research using exosomes to dynamically monitor tumors. Brady et al. [89] confirmed that serum-derived exosomes contain unique protein characteristics, which were tracked throughout the canine OS disease process.

Distinctive serum exosomal proteins can be obtained through noninvasive liquid biopsy and used to diagnose and provide real-time information on disease progression, similar to the method used for evaluating human malignancies [90, 91]. As a new diagnostic and therapeutic tool for OS clinical management, exosomes have shown great application prospects.

Drug carriers

Currently, miRNA is considered a potential anticancer drug; however, the conventional methods of delivering miRNAs, proteins and chemical drugs do not usually produce the desired effect. Exogenous miRNA is easily degraded in the body, exogenous protein cannot perform the required function due to a lack of natural conformation, and administered chemical drugs are lethal to normal cells. However, use of exosomes as carriers can solve these problems [92].

Exosomes have recently emerged as promising drug delivery systems with low immunogenicity, high biocompatibility, and high delivery efficacy [93]. Shimbo et al. [94] showed that exosomes can deliver synthetic miR-143 into OS cells, greatly reducing OS cell migration. Wei et al. [95] developed a nanodrug composed of Adriamycin and exosomes derived from MSCs. Compared with free Adriamycin, the prepared nanodrug showed enhanced cell uptake efficiency and antitumor effects in the MG-63 OS cell line and effectively killed OS cells. Chemotherapy is the main adjuvant therapy for OS, but serious systemic chemotherapy side effects cannot be prevented. Exosomes can suppress tumors by delivering appropriate amounts of chemical drugs, which greatly reduces systemic harm [93]. Compared with other artificial carriers, exosomes, as natural carriers of chemicals, can also prevent phagocytosis by macrophages and prolong the half-life of the chemicals [96].

MSC-exos have an inherent homing ability similar to that of the parent cells, can protect cargo from extracellular degradation, and deliver genetic material, immunomodulatory proteins, enzymes, and growth factors directly to recipient cells [97]. Abello et al. [98] injected human umbilical cord mesenchymal stem cell (HUC-MSC) exosomes into OS mice, and within

Table 3. Preclinical challenges of exosomes

Exosome	Details	Challenges	References
Origin	Almost all body fluids	Mostly from serum	[101]
Isolation and Purification	Ultracentrifugation, size exclusion chromatography, ExoQuick and total exosome isolation	Low yield, high protein contamination rate, ununified extraction standards	[104-107]
Storage conditions	-80°C in phosphate buffer	Changes in EV size and loss of biological function	[111]
Targeted regulation	There is no "one-to-one" connection between miRNA and target mRNA	The therapeutic dose, treatment plan and method of administration have not been determined. The interference of "harmful" RNA	[97, 117]

24-48 hours of injection, the HUC-MSC exosomes continuously accumulated in tumors. Exosomes can be used as natural carriers of anticancer drug molecules at lower doses to induce therapeutic effects and reduce side effects.

Prognostic indicators

By monitoring differences in miRNA and mRNA expression in the exosomes secreted by OS cells, cell responses to chemotherapy can be predicted [17]. In OS patients with adverse reactions to chemotherapy, a significant correlation was found between the loss of control of miRNAs in exosomes, especially a decrease in miR-124, miR-133a, miR-199a-3p and miR-385 and overexpression of miR-135b, miR-148a, miR-27a and miR-9. These exosomal RNA molecules are reliable biomarkers in classifying OS with different chemotherapy sensitivities [17]. miR-1258 expression is significantly reduced in OS tissues and OS cell lines and is associated with malignant clinical manifestations and poor clinical prognoses of patients with OS [99]. Upregulation of miR-1258 significantly inhibited cell proliferation and promoted cell cycle arrest in G0/G1. AKT3 has been identified as a direct target of miR-1258, which binds to the 3'-UTR of AKT3 mRNA. Therefore, the miR-1258-AKT3 axis may be a promising prognostic marker and therapeutic target in human OS [99].

A miR-25-3p imbalance in human OS cells is negatively correlated with clinical prognosis, while the Dkk3 expression level is positively correlated with clinical prognosis [40]. The expression of miR-25-3p was found to be upregulated in exosomes from OS cell lines, and when added to human umbilical vein endothelial cells (HUVECs), exosomal miR-25-3p promoted the formation of capillaries. The sensitivity of serum miR-25-3p levels as an indica-

tor of the prognosis of patients is greater than that of serum alkaline phosphatase (ALP, a known serum-based OS tumor marker) [82]. In summary, exosomal miRNAs differentially expressed in OS patients before and after treatment have the potential to be biomarkers of OS prognosis.

Preclinical challenges of exosome application

Despite the great potential of exosome-based cancer treatment methods, many problems must be solved before they are used in the clinic. Monolayer-cultured tumor cells are still the main source of cancer exosomes for research purposes, and these exosomes may differ in size distribution compared with the exosomes produced by tumor patients. Establishing tumor models through bioengineering for OS exosome research may lead to effective diagnostic improvements [100]. Most current research is based on extraction of exosomes from serum [101], although many researchers have begun to concentrate on noninvasively obtaining bioavailable specimens, such as saliva [102] and urine [103]. However, this method is still limited, and new types of tests for noninvasively obtained samples, such as vaginal discharge, stool or tears, should be developed.

To date, no consensus has been reached on the technical standards for production and isolation of exosomes [104] (**Table 3**). Therefore, exosomes used for biomarker identification and delivery of targeted cargo molecules are not subjected to technical standardization during purification or analysis processes [105]. Exosome yields are low, and commercially prepared exosomes are not suitable for clinical treatment due to profound protein contamination and aggregation [106]. Although exosome production is reported to be feasible on a small scale, many deficiencies have limited large-scale production efforts [107]. The effects of

various isolation procedures on exosome size, integrity, recovery and RNA and protein content are unclear; in other words, different separation methods may lead to differences in exosome concentration, purity, and size. A consistent separation technology should be used for exosome production, and the same separation method should be applied in each study.

Storage temperature is an important factor for maintaining EV activity [108]. Previous studies have shown that storage at high temperatures reduces the number of exosomes retained and the exosome content, while storage at -80°C causes fewer changes [109, 110]. Therefore, the common storage conditions are -80°C in phosphate buffer. Recent studies have indicated that low temperature can affect the stability of the EV lipid membrane, and ice crystals formed at low temperature can cause mechanical damage, even damaging the lipid membrane and resulting in loss of content and corresponding biological function [111] (**Table 3**). When stored at +20°C or +4°C for 1 day, the antibacterial effect of EVs is significantly decreased. Storage at -20°C for 28 days causes changes in EV size and loss of antibacterial function [112]. Although storage at -80°C has a significant effect on the number and size of EVs, it partially preserves antibacterial function for as many as 28 days but greatly changes the physical and functional characteristics of EVs [112]. Freeze-drying or spray-drying is a newly developed storage method for exosomes and is a possible alternative to refrigerated maintenance of EVs; however, this approach has not been widely applied in experimental studies [113, 114]. Furthermore, different EV sources and sample preparation processes affect the quality and stability of EVs. For example, an increase in the number of freeze-thaw cycles will lead to a reduction in the number of EV particles and rapid degradation of the contents. Thus, appropriately reducing the number of freeze-thaw cycles during exosome use is necessary [115, 116].

The molecular genetic basis of carcinogenesis and cancer progression is complex, and there is not a "one to one" connection between miRNAs and target mRNAs. An average miRNA can have more than 100 targets, and one mRNA can be regulated by a variety of miRNAs. Therefore, the potential regulatory circuit affected by a miRNA may be enormous. Currently, the research on miRNA targets is not perfect, and the roles of some of these potential targets in OS carcinogenesis and progression are still unknown. In the future, meaningful work needs to be carried out to determine the targets of miRNAs and the full range of their roles in OS. Biological nanoparticles have great application value in the field of cancer vaccines, but there is a lack of in-depth research focused on identification of the molecules, including membrane components, molecular signals and pathways, that are critical for the biological functions that lead to the release of biological nanoparticles [117] (**Table 3**). Further experimental studies should identify the exact disease-specific molecules that promote tissue repair and regeneration and prevent the interference of "harmful" RNA in exosomes. Information on the targeting ability of exosomes for gene therapy is lacking. Can bioengineering and cell modification techniques be used to modify the surface of MSC-exos to enhance their cell-targeting ability? In addition, it is necessary to determine the disease-specific treatment dose, the appropriate treatment plan and the best method of MSC-exo administration [97].

Summary and prospects

Existing evidence indicates that exosomes are very promising for use in targeted OS therapy. As intercellular communication molecules, exosomes play important roles in OS pathogenesis and treatment, but to date, the specific functions and mechanisms of exosomes are not fully understood, and the long-term safety of exosome therapy cannot be predicted. The understanding of exosome effectiveness, intrinsic components and mechanisms will continue to be expanded through future research. In addition, before applying exosomes in the clinic, their productivity and storage conditions must be improved to prevent loss of exosome function, which is the key to their basic research and therapeutic applications. With the gradual gains in understanding of the nature of exosomes, the corresponding diagnostic and therapeutic techniques are constantly being improved. Future research may devote more energy to in vivo models and clinical applications to help clarify the issues currently limiting the use of exosomes.

Acknowledgements

Disclosure of conflict of interest

None.

Abbreviations

OS, Osteosarcoma; miRNA, MicroRNA; EVs, Extracellular vesicles; MVBs, Multiple multivesicular bodies; mRNA, Messenger RNA; tRNA, Transfer RNA; lncRNA, Long noncoding RNA; NF-κB, Nuclear Factor-kappa B; PI3K/Akt, Phosphoinositide 3-kinase/Protein kinase B; MAPK, Mitogen-activated protein kinase; HD-MTX, High-dose methotrexate; TME, Tumor microenvironment; SOX4, Sex-determining region-related high-mobility group box 4; uPA, Urokinase plasminogen activator; uPAR, uPA receptor; BMCs, Bone marrow cells; CAFs, Cancer-associated fibroblasts; MSCs, Mesenchymal cells; BMSC-exos, Bone marrow mesenchymal stem cell exosomes; PVT1, Plasmacytoma variant translocation 1; ATG5, Autophagy-related gene 5; hBMSC-exos, Human bone marrow mesenchymal stem cell exosomes; TEMs, Tumor-derived exosomes; NK cell, Natural killer cell; MDR, Multidrug resistance; N/A, Not Applicable; ncRNA, Noncoding RNA; CTCs, Circulating tumor cells; ctDNA, Circulating tumor DNA; cfDNA, Cell-free DNA; HUC-MSC, Human umbilical cord mesenchymal stem cell; HUVECs, Human umbilical vein endothelial cells; ALP, Alkaline phosphatase.

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