# Role of exosomal miR-21 in the tumor microenvironment and osteosarcoma tumorigenesis and progression (Review)

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**Abstract.** Osteosarcoma is the most common bone tumor affecting both adolescents and children. Early detection is critical for the effective treatment of the disease. Derived from cancer cells, miR-21 contained within exosomes in the tumor microenvironment may act on both cancer cells and the surrounding tumor microenvironment (TME), including immune cells, endothelial cells and fibroblasts. In human serum and plasm, the level of exosomal miR-21 between osteosarcoma patients and healthy controls differs, supporting the role of miR-21 as a biomarker for osteosarcoma. The involvement of a number of miR-21 target genes in tumor progression suggests that miR-21 may significantly affect the plasticity of cancer cells, leading to tumor progression, metastasis, angiogenesis and immune escape in osteosarcoma. Understanding the biogenesis and functions of exosomal miR-21 is of great value for the diagnosis and therapy of cancer, including osteosarcoma. The present review discusses the role of miR-21 in the tumor microenvironment, and in the development and progression of osteosarcoma, with an aim to summarize the functions of this miRNA in cancer.

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*Abbreviations:* TME, tumor microenvironment; MVEs, multivesicular endosomes; 3' UTR, 3' untranslated region; PTEN, phosphatase and tensin homolog; PI3K/AKT, phosphoinositide 3-kinase/RAC-α serine/threonine-protein kinase; EMT, epithelial-to-mesenchymal transition; VEGF, vascular endothelial growth factor; CAFs, cancer-associated fibroblasts; FAP, fibroblast-activated protein; α-SMA, α-smooth muscle actin; SDF-1, stromal-derived factor 1; TLR-8, Toll like receptor-8; MDSCs, myeloid-derived suppressor cells; ESCRT, endosomal sorting complex required for transport; RISC, RNA-induced silencing complex; TSAP6, tumor suppressor-activated pathway 6; NF1A, nuclear factor 1 A-type

*Key words:* miR-21, exosomes, tumor microenvironment, osteosarcoma, tumorigenesis, progression

**Contents**



## 1. Osteosarcoma

Osteosarcoma is the most common human primary malignant bone tumor affecting children and young adults (1), and is usually located in the distal femur, the proximal tibia, or the proximal humerus (2). The tumor is evolved from mesenchymal cells and is characterized by spindle cells and aberrant osteoid formation pathologically (3). The survival of patients with localized disease is increased with aggressive, multi-agent, neo-adjuvant chemotherapy and limb-salvage surgery. However, despite aggressive therapies, the long-term survival rate of patients with metastatic or recurrent disease however is <20% (2,4,5). Although several genes have been reported to be involved in osteosarcoma tumorigenesis and therapeutic resistance (6-11), the precise molecular mechanisms involved in these processes remain unclear. The identification of clinically relevant diagnostic and prognostic biomarkers, such as circulating or cellular/tissue biomarkers, is, therefore, urgently required.

## 2. Exosomes

Exosomes are lipid bilayer membrane bound, nano-sized extracellular vesicles that are 40-100 nm in diameter (12-14). The exosomes are formed by pinching off of multivesicular

endosomes (MVEs) on the membrane in the form of small intraluminal vesicles within the MVE and packing with cytoplasmic contents, including proteins, mRNAs and microRNAs (miRNAs or miRs) (15,16). The secretion of exosomes occurs when MVEs fuse with the plasma membrane, and thus release the contents in exosomes into the extracellular environment. According to the exosome content database, ExoCarta, 9,769 proteins, 1,116 lipids, 3,408 mRNAs and 2,838 miRNAs have been identified in exosomes of different cell types in multiple organisms (17,18).

Exosomes were first described as vesicles released from reticulocyte MVEs for the removal of obsolete transferrin receptors (19,20). However, in recent years, exosomes have been considered to be important mediators of cellular communication in both normal physiological processes, and in the development and progression of diseases, such as cancer. Exosomes have been identified in the majority of bodily fluids, including the serum, urine, amniotic fluid, saliva, breastmilk, cerebrospinal fluid and nasal secretions (21,22). Importantly, cancer cells secrete a greater number of exosomes compared to healthy cells (23), indicating their potential for use as diagnostic biomarkers.

## 3. Exosomal miRNAs and cancer

miRNAs are a class of small non-coding endogenous RNAs (18-24 nucleotides in length). miRNAs can act as post-transcriptional gene regulators by pairing with complementary sequences in the 3' untranslated region (3' UTR) of target mRNAs, leading to mRNA degradation or translational repression (24,25). miRNAs regulate a variety of cellular processes associated with carcinogenesis, such as cell proliferation, cell cycle, apoptosis, angiogenesis, invasion and metastasis (26-31).

The expression of miRNAs is altered in a variety of cancer types and is associated with the disease stage in some cases. Some specific miRNAs may contribute to tumor growth, progression, metastasis and drug resistance (32-35). Among these, miR-21 is most notable, since it has been extensively studied in various types of cancer. The majority of miRNAs detectable in serum and saliva is concentrated within exosomes (23). The level of miRNAs is similar in both circulating exosomes from cancer patients and tumor cells (22,36). In a number of studies, the high expression of circulating miR-21 has been used to differentiate cancer patients from healthy individuals and predict disease outcomes (37 and refs. therein), suggesting that circulating exosome miRNAs, such as miR-21 can be utilized as for liquid biopsy miRNA profiling.

## 4. Exosomal miR-21 and osteosarcoma

hsa-mir-21 or miR-21 is an abundantly expressed miRNA in different types of mammalian cells (38-40), indicating its importance among miRNAs. miR-21 regulates biological processes, such as osteoclastogenesis, osteoclast differentiation, etc. Thus, miR-21 plays an essential role in the development of diseases, such as cancer, cardiovascular diseases and inflammation (41-43) The *hsa-miR-21* gene is located on chromosome 17q23.2. Pri-miR-21 (primary transcript containing miR-21) is located within the intronic region of the *tmem49* gene. Even though pri-miR-21 and *tmem49* genes overlap in the same direction of transcription, pri-miR-21 is transcribed by its own promoter and is terminated with its own poly(A) tail. The pri-miR-21 transcript is subsequently processed into mature miR-21 (44,45).

The expression of miR-21 has been found to be increased in the majority of cancer types analyzed, rendering it an established oncogenic miRNA (44-54). Functional analyses in epithelial-, hepatocyte- and glial cell-derived cell lines support the regulatory role of miR-21 in cell growth, migration and invasion (46,47,55-57). Moreover, miR-21 and its associated pathways play a critical role in the pathogenesis of osteosarcoma and act as a therapeutic target for this tumor type (58). miR-21 exhibits a significantly higher expression in osteosarcoma tissues compared to adjacent normal tissues (59-61). miRNA expression has been shown to be positively associated with Enneking clinical staging and lung metastasis (62). Moreover, serum miR-21 has been reported to be a biomarker for chemosensitivity and the prognosis of human osteosarcoma (63). Additionally, circulating miR-21 levels are higher in patients with osteosarcoma than in healthy individuals. Studies have demonstrated that the detection of plasma miR-21 together with miR-143 and miR-199a-3p in patients with osteosarcoma can discriminate between the presence or absence of this tumor (62,64). The evaluation of tumor metastasis and histopathological subtype from tumors of patients with osteosarcoma has revealed a higher level of miR-21 in patients with metastatic compared to non-metastatic disease (62).

## 5. Exosomal miR-21 and tumor microenvironment

The tumor microenvironment (TME) differs from that of normal tissues. The TME is composed of cellular and extracellular components. The cellular components of the TME involve cancer-associated fibroblasts (CAFs), myofibroblasts, adipocytes, endothelial cells, epithelial cells and immune inflammatory cells, such as T lymphocytes, B lymphocytes, natural killer cells and natural killer T-cells, and tumor-associated macrophages (65). Cells in the TME are in constant autocrine and paracrine communication, which contributes to tumor development, progression, drug resistance and metastasis (66-70).

Exosomes provide a unique method of information transfer both locally and globally by releasing their contents into the target cell, e.g., miRNAs. By releasing exosomes, tumor cells reprogram their surroundings in the TME into a tumor-permissive or tumor-promoting environment (71-74). mir-21 present in osteosarcoma cell-derived exosomes may affect the TME (75,76), mediating the crosstalk between cancer cells, endothelial cells, immune cells, and fibroblasts in the TME to promote osteosarcoma development by i) the stimulation of tumor angiogenesis; ii) the inhibition of the immune response by acting directly on effector cells; iii) interfering with the regulation of stromal cell activation; and iv) the promotion of cancer progression by preparing the metastatic niche (Fig. 1).

*miR-21 promotes cancer progression and metastasis (crosstalk among cancer cells).* Cancer is a highly heterogeneous disease. A single tumor is composed of groups of genetically clonal cells

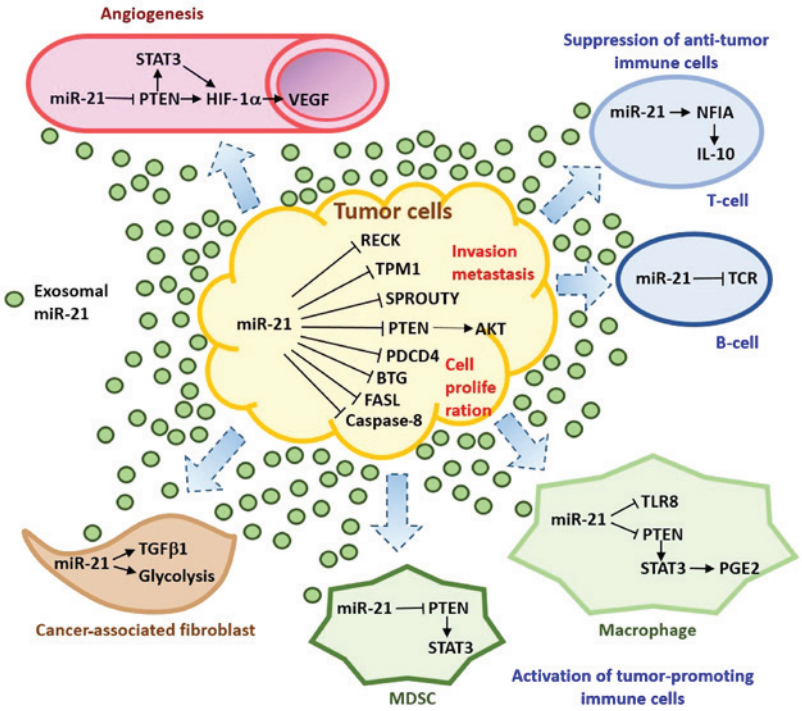


Figure 1. Exosomal miR-21 regulates the tumor microenvironment in osteosarcoma by targeting specific molecules in tumor cells, endothelial cells, cancer-associated fibroblasts and immune cells. The pathways depicted herein were deduced from studies focusing only on a specific cell type (75,76,83,85,86), and miR-21 is originated from other sources in addition to osteosarcoma. AKT, RAC-alpha serine/threonine-protein kinase; BTG, B-cell translocation gene; FASL, Fas ligand; NF1A, nuclear factor 1 A-type; PDCD4, programmed cell death 4; PTEN, phosphatase and tensin homolog; HIF-1α, hypoxia-inducible factor 1α; RECK, reversion-inducing-cysteine-rich protein with Kazal motifs; STAT3, signal transducer and activator of transcription 3; TCR: T-cell receptor; PGE2, prostaglandin E2; TLR8; Toll-like receptor 8; TPM1, tropomyosin 1; VEGF, vascular endothelial growth factor.

that have different growth rates, metastatic potential, invasion potential and sensitivities to chemotherapy and radiotherapy. Individual malignant tumor cells can affect the invasion and migration of surrounding tumor cells by releasing exosomal miRNAs. In contrast to exosomes released from healthy cells, exosomes derived from patients with osteosarcoma have been shown to significantly increase the adhesion, migration and viability of MG63 human osteosarcoma cells (77). Synthetic miR-143 introduced into osteosarcoma cells is released via exosomes. The delivery of exosome-contained miR-143 significantly reduces the migration of osteosarcoma cells, suggesting that miRNAs in exosomes regulate the function of cancer cells (78).

miR-21 is suggested to function as an oncogene, since it promotes the growth and development of osteosarcoma and other cancer types. In MG-63 cells, the downregulation of miR-21 results in decreased proliferation and invasion. By contrast, the elevation of miR-21 by using a mimic increases cell proliferation and invasion (79). The phosphoinositide 3-kinase (PI3K)/RAC-α serine/threonine-protein kinase (AKT)/mammalian target of rapamycin (mTOR) signaling pathway is one of the important pathways dysregulated in osteosarcoma (80-82). miR-21 may regulate the expression of the tumor suppressor phosphatase and tensin homolog (PTEN). The downregulation or deletion of PTEN activates the PI3K/AKT (phosphoinositide 3-kinase/RAC-α serine/threonine protein kinase) signaling pathway, leading to cancer development, invasion and metastasis (83,84).

In addition to PTEN, miR-21 is suggested to target the TGF-β1 signaling pathway to promote cell proliferation in osteosarcoma. miR-21 knockdown inhibits the proliferation of osteosarcoma and promotes the expression of PTEN and TGF-β1 proteins in MG63 and U2OS human osteosarcoma cell lines (85). Moreover, it has been demonstrated that treatment with a TGF-β1 inhibitor countered the inhibitory effects of miR-21 knockdown on osteosarcoma cell proliferation (86).

Caspase-8 is a direct target of miR-21; miRNA negatively regulates the expression of caspase-8. The overexpression of miR-21 has been shown to enhance cell viability and survival, whereas it suppresses the apoptosis of the human osteosarcoma cell line, SAOS-2. In a subsequent study, miR-21 suppression was shown to increase caspase-8 expression and decrease apoptosis (60).

miR-21 is a regulator of drug resistance in osteosarcoma. The suppression of miR-21 activity has been shown to enhance the resistance of U2OS cells to cisplatin, while the ectopic expression of miR-21 in MG-63 cells reduces the resistance. Elevated miR-21 levels suppress the expression of Sprouty2. On the other hand, the ectopic expression of Sprouty2 has been shown to rescue the miR-21-mediated suppression of resistance to cisplatin, but not doxorubicin or methotrexate in osteosarcoma cells (87).

The process of metastasis is considered to be initiated in the majority, if not all, by an epithelial-to-mesenchymal transition (EMT) of the tumor cells. This allows them to migrate to and enter the vascular or lymphatic vessels, resulting in either local or distant metastasis (88,89). miR-21 has been revealed to be an important miRNA associated with cancer invasion and metastasis. The study by Yan *et al* (90) demonstrated that miR-21 enhanced the invasion and migration of human

breast cancer MCF7 and MDA-MB-231 cells, whereas cancer cell invasion was inhibited by the knockdown of miR-21. The mechanism of metastasis regulation by miR-21 is highly complex. miR-21 targets PDCD4, tropomyosin and PTEN that are known to modulate cancer cell invasion and metastasis (91-94). Moreover, miR-21 can inhibit PTEN expression and activate AKT signaling, inducing EMT, and promoting the invasion and migration of cancer cells (95-97). Furthermore, miR-21 can trigger the interleukin (IL)-6/signal transducer and activator of transcription (STAT)3/nuclear factor (NF)-κB-mediated signaling loop for the maintenance of EMT in cancer cells (98).

*miR-21 induces tumor angiogenesis (crosstalk between tumor cells and endothelial cells).* Tumor angiogenesis refers to the ability of tumor cells to recruit their own vasculature, which is critical for cancer progression. Several studies have demonstrated that exosome-encapsulated miRNAs secreted from tumor cells are able to induce angiogenesis in different cancer types (99-102). Exosomal miR-21 released by transformed lung cancer cells has been shown to induce vascular endothelial growth factor (VEGF) production and angiogenesis in nearby normal bronchial cells in a STAT3-dependent manner (99). In another study, the overexpression of miR-21 in the human prostate cancer cell line, DU145, was shown to increase the expression of Hypoxia-inducible factor (HIF)-1α and VEGF, and induce tumor angiogenesis. The miR-21-mediated activation of the AKT and extracellular signal-regulated kinase (ERK)1/2 signaling pathways enhances HIF-1α and VEGF expression (103).

*miR-21 activates cancer-associated fibroblasts (CAFs; crosstalk between tumor cells and fibroblasts).* CAFs become 'activated' during the neoplastic process. CAFs are capable of accelerating the growth and promoting the invasion of tumor cells (104-109). Cancer exosomes trigger the transformation of fibroblasts through the transforming growth factor (TGF) β/Smad pathway and elicit unique effects from soluble TGFβ. The depletion of miR-21 blocks TGF-β1-induced CAF formation, whereas the overexpression of miR-21 promotes CAF induction, independent of TGF-β1. These findings clearly demonstrate that miR-21 is a critical regulator of TGF-β1 signaling induction of CAF formation (110). The conditioned medium from human lung cancer A549 cells has been shown to increase miR-21 expression and thus, through the TGF-β pathway, induce migration, CAF-like morphology and CAF markers [periostin, α-smooth muscle actin (α-SMA) and podoplanin] in human lung fibroblast MRC-5 and IMR-90 cells (111).

Exosomes from the multiple myeloma cell line, OPM2, have been showon to contain high levels of miR-21. The Co-culture of OPM2 exosomes with CAFs has also been shown to significantly increase the proliferation of and induce the CAF transformation of mesenchymal stem cells, demonstrated by the increased expression levels of fibroblast-activated protein (FAP), α-SMA and stromal-derived factor 1 (SDF-1), and the secretion of IL-6 (112). In another study, miR-21 was demonstrated to induce the metabolic alteration of CAFs and to affect the development of the human pancreatic cancer cell line, BxPC-3. Compared to normal fibroblasts, CAFs exhibited an increased miR-21 expression, glucose uptake and lactic acid production. Treatment with a miR-21 inhibitor reduced glycolysis in CAFs. The co-culture of BxPC-3 cells with CAFs treated with the miR-21 inhibitor reduced oxidative phosphorylation and the invasion of BxPC-3 cells (113).

*Exosomal miR-21 suppresses the immune system (crosstalk between tumor cells and immune cells).* Exosomes secreted by tumor cells are able to inhibit the immune system by acting directly on effector cells or indirectly through their regulation (114-120). Tumor cells secrete miR-21 together with miR-29a to communicate with macrophages, eliciting a pro-inflammatory pro-metastatic response. miR-21 is a paracrine agonist of Toll-like receptor-8 (TLR-8). The activation of TLR-8 in immune cells triggers a pro-inflammatory response, that may lead to tumor growth and metastasis (121). miR-21 induces tumor-associated macrophage reprogramming through STAT3, and thereby facilitates growth, intravasation and the spread of tumor cells (122,123).

Myeloid-derived suppressor cells (MDSCs) are the major myeloid cells responsible for the immune evasion of cancer. They are composed of a heterogeneous population of immature myeloid cell progenitors, macrophage precursors, granulocytes and dendritic cells. MDSCs can promote tumor growth by the promotion of angiogenesis or the suppression of innate and adaptive immune responses. miR-21 has been shown to be upregulated in bone marrow-derived and splenic MDSCs, and increases MDSC survival and proliferation by targeting PTEN and thus, STAT3 activation (112,124).

miR-21 expression is higher in effector and memory $CD8^+$ T-cells than in naïve $CD8^+$ T-cells. Memory $CD8^+$ T-cells are emerging as a key player in tumor immunosurveillance (125), and recent evidence suggests that they can control tumor growth. miR-21 negatively regulates TCR signal transduction to modulate the sensitivity of memory T-cells (118,119). Moreover, miR-21 may be involved in tumor-mediated immunosuppression (120,126). miR-21 from C666-1 nasopharyngeal cancer cells has been shown to activate nuclear factor 1 A-type (NF1-A) and induce IL-10 in B cells, which inhibits cytotoxic $CD8^+$ T-cells (127).

## 6. Perspectives

*Biogenesis of exosomal miR-21: Loading, release, and uptake.* The majority of serum miRNAs is concentrated in exosomes. The loading of miRNAs into exosomes is dependent mainly on the surface molecules of exosome membranes, the endosomal sorting complex required for transport (ESCRT), and the specific binding motifs of the miRNAs themselves. A previous study demonstrated that miRNAs in exosomes are released through a ceramide-dependent secretory mechanism (128). Notably, exosomes secreted by cancer cells contain miRNAs associated with the miRNA-processing machinery RNA-induced silencing complex (RISC), and therefore, have the ability to process precursor miRNAs into mature miRNAs in a cell-independent manner, resulting in efficient and rapid reprogramming of transcriptome of the target cells (129).

Certain miRNAs are secreted selectively into exosomes. For example, the let-7 miRNA family in certain metastatic

gastric cancer cell lines is secreted selectively to extracellular environment via exosomes (130). Breast cancer cell lines release the majority of miR-451 and miR-1246 selectively via exosomes, whereas both miRNAs are retained inside the non-malignant mammary epithelial cells and normal fibroblasts (131). Additionally, the TME may influence exosome release and uptake. The acidic microenvironment of tumors increases the rate of exosomal release and uptake by cancer cells (132). Furthermore, the aberrant exosome biogenesis and secretion in cancer cells, as compared with normal cells, may cause the differences in receptor recycling/degradation, plasma membrane remodeling, and the ability of endosomes to function as a signaling entity (133). In a number of types of cancer, aberrant p53 activity may result in the overexpression of the tumor suppressor-activated pathway 6 (TSAP6), through which it increases exosome production (134,135). Furthermore, heparanase, an enzyme upregulated in numerous cancer cell lines, has been shown to regulate exosome secretion in cancer cells (136).

The level of exosomal miR-21 in serum is higher in patients with osteosarcoma than in healthy individuals, which is also reflected by the high levels of miR-21 found in tumor tissue (61-64). The molecular mechanisms of exosome sorting and release have been studied extensively recently. However, the differences in miRNA content between cancer- and healthy cell-derived exosomes, the differences in exosome biogenesis between cancer and healthy cells, and the reasons why cancer cells release more exosomes with distinct content, such as miR-21, remain unclear. Additionally, whether the release of miR-21 in exosomes occurs through a distinct secretary pathway remains to be elucidated. Further investigation into the distinct exosome cargo loading mechanisms for miR-21 and other miRNAs in osteosarcoma and other cancers may aid in the identification of specific therapeutic targets related to exosome biogenesis. Further research may facilitate the use of exosomes as delivery vehicles for drugs, antigens, etc., for cancer treatment in the future.

*Exosomal miR-21 as a cancer biomarker.* Exosomal miR-21 is detected in human serum and plasma. Differences in miR-21 expression have been found in serum or plasma from patients with osteosarcoma and in healthy controls, supporting the role of miR-21 as a biomarker for osteosarcoma (61-64). The advantages of using exosomal miR-21 as a non-invasive biomarker are that miRNAs in blood are persistent and are highly stable against destruction by ribonucleases (137,138). The current methods used for exosome isolation include ultracentrifugation, size exclusion or precipitating with reagents (139). Ultracentrifugation is the commonly used method for exosome isolation. However, it is a lengthy process, which requires a large volume of samples and costly reagents and equipment. Thus, the method is impractical for clinical diagnosis. The size exclusion method uses columns to separate extracellular vesicles based on size and requires only a small amount of samples. However, the exosomes collected have heterogeneous sizes, curtailing the use for cancer diagnosis. The precipitation of exosomes with reagents not only pulls down miRNAs, but also protein aggregates that complicate the analysis for disease-specific markers.

In order to implement miR-21 as a biomarker for osteosarcoma, along with more effective methods for the isolation of cancer exosomes and exosomal miR-21, additional clinical trials are required to validate its use in the diagnosis of osteosarcoma and other types of cancer. Most importantly, miR-21 needs to be profiled together with other miRNAs or biomarkers specific to osteosarcoma to improve the reliability and specificity of miR-21 as a biomarker.

## 7. Conclusions

Exosomal miR-21 is detected in human serum and plasma. miR-21 expression in serum or plasma from patients with osteosarcoma differs from that in healthy controls, supporting the use of miR-21 as a biomarker for osteosarcoma. Exosomal miR-21 promotes cancer progression and development by mediating the crosstalk among cells in the TME. Larger-scale studies are warranted to further validate the sensitivity, specificity and applicability of circulating miR-21 as a biomarker for osteosarcoma in the future. Furthermore, extensive efforts need to be made to identify the specific cargo-loading mechanisms for exosomal miR-21. These studies will be critical for targeting miR-21 in cancer therapy to improve early-stage exosome- and miRNA-based therapeutics.

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## Availability of data and materials

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## Authors' contributions

SW and TL wrote the manuscript. SW performed the literature search for this review article. FM and YF revised and corrected the manuscript. FM and YF assisted in the literature search for this review article and also contributed to the conception and design of the study. FM contributed to processing of the figure. TL and SH conceived and designed the study. All authors read and approved the final manuscript.

## Ethics approval and consent to participate

Not applicable.

## Patient consent for publication

Not applicable.

## Competing interests

The authors confirm they have competing interests.

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