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MicroRNAs of tumor extracellular vesicles: secretion mechanisms and function

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Abstract: Extracellular vesicles (EVs) are heterogeneous membranous vesicles released from many cell types into the extracellular space. EVs containing microRNAs (EV-miRNAs) are selectively secreted in response to various pathological and physiological conditions, serving as novel mediators for intercellular crosstalk. EV-miRNAs play significant roles in diverse physiologies and diseases. Especially, accumulating evidence supports the role of tumor-derived EV-miRNAs in tumorigenesis. EV-miRNAs mediate the communications between tumor cells and non-malignant cells in the tumor microenvironment (TME), thereby affecting angiogenesis, invasion, metastasis, drug resistance and immunity evasion. In this review, we summarize the evidence on the selectivity of miRNAs secretion and underlying mechanisms. We also describe the pathological functions of tumor-derived EV-miRNAs on tumorigenesis and secretion. Then, we discuss the potential applications of EV-miRNAs as biomarkers in cancer diagnosis and prognosis.

Keywords: extracellular vesicles; extracellular miRNAs; miRNAs secretion mechanisms; tumor microenvironment; cancer

1. Introduction

Extracellular vesicles (EVs) are bilayer vesicles secreted by all cells under both physiological and pathological conditions. They consist of small EVs (with a diameter ranging from 50 to150 nm, also known as exosomes) and large EVs fraction (with a diameter ranging from 50 to 1000 nm, also known as microvesicles) [1]. The lipid bilayer membrane of EVs is derived from the Golgi apparatus or endosomes. Large EVs are directly yielded by an

outward budding of the plasma membrane. Meanwhile, small EVs start from early endosomes formed by endocytosis of the cell membrane, and then develop into late endosomes, which form intraluminal vesicles (ILVs) by budding inward. Late endosomes containing multiple luminal vesicles form multivesicular bodies (MVBs). Exosomes are released when the MVBs membrane fused with plasma membrane [2]. According to the aggregation of exosomal substances and the process of membrane invagination, the formation mechanism of exosomes can be divided into two types: endosomal sorting complex required for transport (ESCRT) dependent and ESCRT independent. Apoptosis-linked gene 2-interacting protein X (Alix), Vacuolar protein sorting-associated protein 4 (Vps4), and Tumor susceptibility gene 101 protein (Tsg101) help the ESCRT machinery in exosome biogenesis. The release of EVs mainly include transport, anchoring on the membrane, and fusion with the plasma membrane. Until now, a family of small GTP-binding proteins (GTPases) have been found to participate in the transport of MVBs to the cell membrane and the secretion of EVs, including the RAB protein family (RAB5, RAB11, RAB27, RAB35, *etc*.) [3] and the Rho protein family (RhoA, RAC1, CDC42) [4]. Subcellular localization of MVBs depends on the interaction of the RAB proteins with actin and microtubule cytoskeletons, which are also regulated by RAB proteins and their effector kinetin, promoting vesicle movement along the actin and/or microtubule cytoskeleton. In various human tumor cells, RhoA is necessary for the release of microvesicles and prevents carcinogenic transformation in tumor growth [5]. It has also been reported that CDC42 is a key node of multiple regulatory signals in the biogenesis of microvesicles, and CDC42 can indirectly promote the production of microvesicles by inhibiting the internalization of cell surface proteins [6]. In addition, soluble N-ethylmaleimide-sensitive factor attachment protein receptors (SNAREs) family are also involved in the fusion process between MVBs and the cell membrane [7]. In different cell types, synaptosome-associated proteins [8], vesicle-associated membrane proteins 7 [9] of the SNAREs family are involved in the regulation of MVBs fusion with the plasma membrane. These findings reveal the complex regulatory mechanism of the production and secretion of EVs, and provide a strategy for cancer treatment.

EVs contain many molecular substances from donor cells, including proteins, lipids, nucleic acids, and metabolites [2]. EVs can be taken up by recipient cells and release the molecular substances, thus regulating the biological function of the recipient cell [10]. MicroRNA (miRNA) is a widely studied EV-containing molecule. MiRNAs in extracellular vesicles (EV-miRNAs) mediate intercellular communications and play important roles in physiological and pathological processes, including metabolism [11], tumorigenesis [12], neuron disease [13], immunity [14] and cardiovascular disease [15]. The tumor microenvironment (TME) includes surrounding immune cells, vascular endothelial cells, fibroblasts, extracellular matrix and signaling molecules. The interactions between malignant and non-malignant cells shape the microenvironment and thus affect the development and progression of tumor. Tumor cells in the TME directly and indirectly interact with surrounding cells through the secretion of signaling molecules and autocrine and paracrine modes, forming a unique “soil” suitable for tumor cell growth. Tumor-derived EV-miRNAs

are important modulators during tumorigenesis. Tumor cells secreted more abundant EVs, in which pathology-related miRNAs are selectively encapsulated. EVs facilitate the information relay between tumor cells and various non-malignant cells in the TME, thereby leading to promote tumor growth, angiogenesis, migration, metastasis, suppressed immune response, drug resistance, and reprogrammed metabolism [16,17]. Of note, EV-miRNAs are selectively packaged into EVs rather than randomly released. This is evidenced by the different abundance and expression signature between EVs and their parental cells, and tailored EV-miRNAs profile in response to different physiological or pathological stimuli [18]. These findings suggest that EV-miRNAs secretion is a highly regulated process.

Here, we review the findings that support the selectivity of EV-miRNAs secretion, and highlight their significant roles in tumorigenesis and potential candidates as cancer diagnosis and therapeutics.

2. Mechanisms underlying regulated secretion of EV-miRNAs

Plenty of studies have shown that miRNAs are not randomly integrated into EVs, some miRNAs are enriched in EVs, while others are nearly absent. For example, serum EV-miR-21 levels were higher in patients with glioblastoma than in healthy individuals [19]. More than 90% of miR-451 is secreted into the extracellular matrix, and this mechanism of active secretion from cells may contribute to cancer progression and metastasis [20]. For another example, analysis of serum EV-miRNAs in patients with colorectal cancer (CRC) showed up-regulated expressions of miR-150, miR-1229, miR-1246, miR-223, miR-23a, let-7a and miR-21, which is consistent with the upregulation and their carcinogenic role reported in CRC tissues [21,22]. Other studies found that miR-486-5p and miR-3180-5p in serum EVs were upregulated and miR-548c-5p, miR-638, miR-5787, miR-6869-5p and miR-8075 were down-regulated in CRC patients, and these miRNAs were associated with infiltration of tumor cells [23]. These results suggest that there are certain mechanisms that regulate the active sorting of specific miRNAs into EVs, which can be influenced by multiple factors.

2.1. Effect of RNA-binding proteins and sequence characteristics on EV-miRNAs secretion

MiRNAs with specific sequence motifs (EXO motifs) are preferentially loaded into EVs. EXO motifs (GCCG, UGAC, UCCG, GGAC, GGCG and UGCC, *etc.*) may enhance the binding of miRNAs with lipid draft on the membrane of MVBs [24]. RNA binding proteins (RBPs) are reported to recognize these motifs and facilitate the importing of miRNAs into EVs by bind with lipid draft with high affinity on MVBs membrane [25]. For example, heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1) with a high affinity with ceramide, mediated the sorting of miR-198 and miR-601 into EVs via binding with the motif GGAG/UGCA [26]. Research has shown that some viral miRNAs may also have EXO motifs, such as CCCT or CCCG, thus affecting EVs secretion of host cells [27]. Meanwhile, synaptotagmin-binding cytoplasmic RNA-interaction protein (SYNCRIP) facilitated miR-3470a and miR-194-2-3p into EVs by binding to GGCU, and SYNCRIP knockout disrupted the loading of these miRNAs into EVs [28]. What's more, Annexin A2 is another protein

that is involved in miRNAs sorting given its ability to bind with specific miRNAs and its abundance in EVs [29]. Y-Box Binding Protein 1 (YBX-1) selectively packaged miR-223 into exosomes of HEK293T cells via a specific RNA binding domain [30]. An overview of the mechanisms and RNA-binding proteins is presented in Table 1.

Table 1. miRNAs secretion mechanisms regulated by RBPs.

RBPs	Mechanism	Reference
Heterogeneous nuclear ribonucleoprotein A2B1	Binds miR-198 and miR-601 via GGAG/UGCA motifs	[26]
Synaptotagmin-binding cytoplasmic RNA-interaction protein	Binds miR-3470a and miR-194-2-3p via GGCU motif	[28]
Argonaute 2	Binds let-7a, miR-100, miR-223, miR-320a into EVs	[31]
Y-Box Binding Protein 1	Binds miR-133, miR-223 into exosomes	[30,32]
MEX3C	Binds miR-451a into EVs	[33]
Major Vault Protein	Binds miR-193a into EVs	[34]
La protein	Binds miR-122 into EVs	[35]
Annexin A2	Binds miR-16, miR-21, miR-24, miR-29a, miR-100 into EVs	[36]
Apoptosis-linked gene 2-interacting protein X	Binds miR-24, miR-31, miR-99b, miR-221, miR-16, miR-451 into EVs	[37]
Human antigen R	Binds miR-126, miR-92a, miR-200b, miR-132 into EVs	[38]

In addition to motif characteristics, end base type also determines miRNAs encapsulation into EVs. Endogenous miRNAs with 3'-end adenylate were preferentially intracellular in B lymphocytes, while miRNAs with 3'-end uridylated were enriched in EVs, suggesting a key sorting signal on the miRNAs sequence at the 3' end [39]. Meanwhile, hydrophobic modification of miRNAs can also affect the affinity of miRNAs molecules with membranes. It has been reported that some mammalian miRNAs could be methylated, thereby affecting their binding to the lipid raft region of the MVBs membrane [40]. In conclusion, different sequence characteristics and modifications of miRNAs may affect not only the binding with specific RBPs, but also the interaction with MVBs plasma membrane, thereby regulating miRNAs secretion into EVs.

2.2. Effect of MVBs membrane lipid raft compositions on EV-miRNAs secretion

Lipids and proteins containing in the lipid raft region of the MVBs membrane also mediate the sorting of miRNAs. Ceramide is enriched in the lipid raft region of the membrane and promotes the secretion of EVs [41]. Studies have reported that the release of EVs was reduced after the inhibition of Neutral Sphingomyelinase 2 (nSMase2), which produces ceramide molecules [42]. Overexpression of nSMase2 increased the expression levels of miR-16 and miR-146a in exosomes, but had no effect on miRNA levels in cells [43]. Furthermore, Ceramide is metabolized into sphingosine and sphingosine 1-phosphate through ceramidase and sphingosine kinase. The sphingosine molecules in the MVBs membrane may increase

the affinity of miRNAs with the membrane [44]. Caveolin-1 (Cav-1) locates in the pits of the plasma membrane and plays a key role in regulating membrane transport. Heedoo *et al.* reported that Cav-1 protein was identified in EVs secreted by pulmonary epithelium, which further strengthened the important role of Cav-1 in the selective transport of miRNAs to EVs [45]. Meanwhile, Cav-1 had a synergistic effect with hnRNPA2B1 to promote the secretion of miR-17 and miR-93 in EVs [46]. The Vps4A is required for normal endosomal transport and MVBs fusion. Hepatocellular carcinoma (HCC) cells overexpressing Vps4A promoted the secretion of miR-27b-3p, miR-92a-3p, miR-193a-3, miR-320a and miR-132-3p in EVs [47]. Decreasing the expression level of Vps4A in HEK293T cells inhibited the secretion of miR-92a and miR-150 in EVs [48].

Lipid rafts of MVBs is a cholesterol and sphingomyelin-rich microdomain in the plasma membrane, which can serve as "targets" for miRNAs and can protect miRNAs degradation [10]. It has been reported that the expression of miRNAs was 13 times higher than that of Argonaute 2 (Ago2) protein in HeLa cells, and excess miRNAs were available for binding to the lipid raft of MVBs in addition to Ago2 proteins [49]. It has also been reported that many of the high abundance of side-chain miRNAs in EVs may be derived from this protective property of lipid rafts, suggesting that there may be a transport mechanism for side-chain miRNAs to enter EVs [50].

2.3. Effect of miRNA-mediated silencing complex on EV-miRNAs secretion

The relationship between miRNA-induced silencing complex (miRISC) and miRNAs secretion promotes the bidirectional redistribution of miRNAs from the cytoplasmic P-body to MVBs, thereby regulating the physiological homeostasis of miRNA-mRNA. This bidirectional redistribution is reflected in the following aspects. On the one hand, because the main components of miRISC are co-located with MVBs, blocking the transport of MVBs to lysosomes may lead to excessive accumulation of miRISC, while blocking the formation of MVBs leads to the loss of miRISC [51]. On the other hand, changes in miRNA target mRNA levels may alter miRNAs secretion into EVs, mainly through a balance between miRNAs activity and MVBs [52].

Ago2 and phosphorylation levels control the secretion of EV-miRNAs. Knockout of Ago2 reduced the type or abundance of secreted miRNAs such as miR-451, miR-150 and miR-142-3p [20]. KRAS-MEK-ERK signaling pathway could promote Ago2 phosphorylation, which inhibited the interaction between miRNAs and endosomes, thereby impairing miRNAs secretion[31]. MiRNAs that bind with Ago2 instead of interacting with target mRNA are subjected degradation, while miRNAs that interact with target mRNA are protected [53]. Notably, it has recently been reported that precursor miRNAs (pre-miRNAs) associated with complexes (such as Ago2, Dicer) were processed into mature miRNAs in EVs secreted by breast cancer, and a new method for integrating pre-miRNAs into EVs processing has been found [54].

2.4. Effect of physiological or pathological conditions changed on EV-miRNAs secretion

The miRNA expression profile of EVs secreted by tumor cells and normal cells is different, and the changes of the physiological and pathological conditions of donor cells affect the sorting of miRNA into EVs. Therefore, EV-miRNAs can be used as potential molecular diagnostic biomarkers to track disease progression for early diagnosis and treatment. However, tumor cells release large numbers of EVs due to their inherent cancer-related mutations, but the situation is further complicated by the presence of populations of EVs from different cells that dilute the actual expression of miRNAs produced by tumor cells [55]. Different physiological or pathological stimuli can alter miRNA expression levels in EVs. For example, one study found that EVs from CRC cell lines before and after treatment with cetuximab were also present in approximately 90% of intracellular miRNAs. The miRNAs with potential tumor suppressive properties (such as miR-142-5p, miR-150, miR-223 and miR-433) appeared in the EVs of CRC cells after treatment [56]. Another report showed that blood cells and cultured mononuclear macrophages can actively and selectively package miRNAs into microvesicles when they are subjected to various stimuli such as lipopolysaccharide, hydrogen peroxide (H_2O_2), advanced glycation end products (AGE) and oleic acid/palmitic acid (OA/PA). In addition, miRNAs expression in microvesicles also changed under different stimulus conditions. MiR-26b and miR-222 reduced secretion to EVs in H_2O_2 stimulation, but promoted secretion to microvesicles in AGE and OA/PA stimulation [12].

MiRNA secretion is also influenced by changes of energy levels in donor cell. MiRNAs can secrete against the concentration gradient, suggesting that EV-miRNAs secretion may be an ATP-dependent process. The formation of EVs is associated with membrane fusion and rupture, and this process is ATP dependent [57]. The results of microarray analysis showed that 218 mature miRNAs were positive in both ovarian tumor cells and secreted EVs, including 12 miRNAs with high levels in cells and 31 miRNAs with high levels in EVs [58]. Wang *et al.* found that intracellular ATP levels affected the number of extracellular miRNAs, except miR-671-3p and miR-943, possibly because these miRNAs were passively leaked from ruptured cells [57].

3. The function of EV-miRNAs in tumor microenvironment

The TME is mainly composed of tumor cells, epithelial cells, vascular endothelial cells, fibroblasts, mesenchymal stem cells, immune cells, and a variety of inflammatory factors [59]. The development of tumor is a complex, multi-step and dynamic biological process. EV-miRNAs from tumor cells mediate the crosstalk between tumor cells and non-malignant cells in the TME, constructing a permissive surrounding environment to their growth. In contrast to this “domestication” of tumor cells, non-tumor cells also influence the occurrence and development of cancer by secreting miRNAs [60]. The interaction between tumor cells and surrounding cells in the TME mediated by miRNAs secretion provides favorable conditions for tumor development.

3.1. EV-miRNAs mediated interaction between tumor cells and fibroblasts

The fibroblasts within tumor stroma, which are also termed cancer-associated fibroblasts (CAFs), have heterogeneous populations. CAFs are vital constituents of the TME, and their interactions with tumor cells play a pivotal role in cancer development and progression. Recently, an increasing number of reports have demonstrated that EV-miRNAs compose the communication networks between tumor cells and CAFs to modulate cancer progression. For example, CRC cells promoted the transformation of normal fibroblasts into CAFs by miR-200 [61]. Previous studies have shown that highly metastatic HCC cells secrete exosomal miR-1247-3p, leading to activation of β1-integrin-NF-kB signaling in fibroblasts cells [62]. It has also reported that the secretion of miR-155 from tumor cells induced peri-tumor fibroblasts and mesenchymal stem cells to form CAF-like phenotypes [63]. Conversely, the relationship between EV-miRNAs and CAFs activation is unlikely to be unidirectional. While cancer-derived exosomal miRNAs can promote CAF differentiation, exosomal miRNAs released by CAFs in the TME play an important role in therapy resistance. CAFs also affect tumor cells growth by secreting miRNAs. For example, CAFs influenced the invasion, proliferation, metastasis, drug resistance and metabolic properties of tumor cells by transferring miR-21 [64], miR-409 [65], miR-500a-5p [66]. The miRNAs expression profile in CAFs and their EVs reflected the progress of cancer. It was reported that miR-320a overexpression in CAFs could inhibit HCC tumorigenesis, suggesting that CAF-mediated HCC tumor progression is partially related to the deletion of anti-tumor miR-320a in the exosomes of CAFs, and the transfer of exosomal miR-320a derived from stromal cells might be a potential treatment to inhibit HCC progression [67]. Understanding EV-miRNAs communication networks in tumor-fibroblast interaction will enable the development of therapeutic strategies and may provide novel functional markers of CAFs [68].

3.2. EV-miRNAs mediated interaction between tumor cells and endothelial cells

Tumor-derived EV-miRNAs promote the angiogenesis by regulating endothelial cells in the TME and modulates tumor metastasis [69]. For example, tumor cells secreted EVs containing miR-210, promoting endothelial cell migration and angiogenic activity [70] MiR-25-3p could be transferred from CRC cells to endothelial cells via exosomes, consequently promoted vascular permeability, angiogenesis and CRC metastasis [71]. Researchers have also found that miR-21 in EVs increased vascular endothelial growth factor levels in recipient cells and led to angiogenesis and malignant transformation of human bronchial epithelial cells [72]. Conversely, the endothelial cell proliferation, migration, germination, branching and tubular formation promote tumor angiogenesis. The normal epithelial cells influence tumor cells by secreting miRNAs. For example, EVs secreted by non-abnormal epithelial cells transferred miR-143 into tumor cells and inhibited tumor growth [36]. However, the exact contribution of EV-miRNAs during angiogenesis and cancer metastasis remains unclear. Understanding these relationships could help the development of cancer therapy.

3.3. EV-miRNAs mediated interaction between tumor cells and immune cells

The major function of the immune system is not only to protect against various infectious pathogens but also to promote cancer progression and evasion from immune surveillance [73]. Many investigations indicate that the immune systems play a dual role in cancer progression and can both suppress or support the tumor cells. Immune evasion is a big challenge for cancer therapy. EV-miRNAs play important roles in regulating immune envision. By secreting miRNAs, tumor-derived EV-miRNAs affect immune cells and promote immune evasion. For example, miR-21 and miR-203 secreted from tumor cells could "do what in immune cell" to promote immune escape [74,75]. MiR-214 secreted by tumor cells entered $CD4^+$ T cells to inhibit immunity and promoted tumor growth by promoting the proliferation of Treg cells [76]. Conversely, EV-miRNAs derived from immune cells broadly modulate antigen presentation and T cells function by playing immune-stimulatory or immune-suppressive roles, leading to highly efficient anti-tumor immunity or tumor immune tolerance [77]. For example, macrophage-derived miR-223 stimulated aggressive activity of tumor cells [78]. MiR-126 was secreted by M2 macrophages expressing IL-13, which promoted tumor angiogenesis and metastasis [79]. It was reported that monocytes transferred miR-155 to neuroblastoma via EVs and induced drug resistance of neuroblastoma by down-regulating the expression of telomere repeat sequence binding factor 1 [74]. Further understanding of EV-miRNAs between tumor cells and immune cells communication networks will provide several avenues for cancer immunotherapy and diagnosis.

3.4 EV-miRNAs mediated interaction between tumor cells and cancer stem cells

Cancer stem cells (CSCs) are the self-renewing population in the TME that exert resistance to anticancer drugs and radiotherapy [80]. There is a strong connection between CSCs and tumor proliferation, metastasis, and recurrence. Recent studies have demonstrated that exosomal miRNAs secreted by CSCs interact with other surrounding TME and cancer cells, thereby promoting cancer progression and inhibit the pro-apoptotic property [81,82]. Although exosomal miRNAs from cancer cells and CSCs display different profiles, they contribute to the malignant phenotype in many types of tumors. For example, miR-155 transferred by CSCs-derived exosomes enhanced the resistance of breast cancer cells to doxorubicin and paclitaxel treatment [83]. The high levels of miR-210 derived from gemcitabine-resistant pancreatic CSCs EVs transferred the resistant phenotype to gemcitabine-sensitive pancreatic cancer cells, thereby inhibiting apoptosis and promoting proliferation [84]. Collectively, miRNAs from CSCs-derived exosomes are capable of transferring drug resistance to sensitive cancer cells.

3.5. EV-miRNAs mediated interaction between tumor cells and other tumor cells

As mentioned above, tumor-derived EV-miRNAs affected surrounding stromal cells in the TME to promote cancer progression. However, tumor cells can also affect other tumor cells via EV-miRNAs. Less invasive tumor cells can take up miRNAs delivered from invasive

tumor cells, which may prompt worsening of a primary tumor. For example, proto-oncogenic EVs conferred a malignant phenotype on other tumor cells and epithelial cells by transferring miR-200 [61]. The breast cancer cells-derived exosomes could be specifically internalized by non-small cell lung cancer cells via a specific interaction between overexpressed integrin β4 (on exosomes) and surfactant protein C on the cancer cells [85]. Tumor cells can secrete EV-miRNAs to facilitate the transfer of drug-resistance property between tumor cells. For example, drug-resistant breast cancer cells induced the transformation of sensitive cells into drug-resistant cells by secreting miR-155 [83]. Prostate cancer treatment with paclitaxel often fails due to the development of chemo-resistance caused by miR-34a downregulation, which has been suggested to be a predictive biomarker for response to docetaxel with clinical relevance to prostate cancer progression by regulating the anti-apoptotic gene *BCL-2* [86]. Furthermore, there are the crosstalk between various tumor-derived EV-miRNAs and the TME. What are the effects of EV-miRNAs on some highly aggressive tumors? The metastatic breast cancer promoted cell invasion via release of miR-10b by the primary tumor into the culture environment of surrounding cells [87]. Highly metastatic CRC cells-derived EVs rich in miR-181a-5p could activate hepatic stellate cell, thereby facilitating liver metastasis in CRC patients [88]. The diagnosis of pancreatic cancer has been advanced, and is prone to metastatic drug resistance after chemotherapy and radiotherapy, which are leading to high mortality. MiR-17‑5p levels of serum EVs from pancreatic cancer patients were higher than those in non-pancreatic cancer patients and healthy participants. High expression of miR-17-5p suggests advanced tumor metastasis in patients with pancreatic cancer [89]. The significant upregulation of miR-4525, miR-191, miR‑451a and miR‑21 in EVs from patients with pancreatic cancer may be biomarkers of early diagnosis, recurrence and poor survival [90,91]. Other studies have found that miR-18a, miR-27a, miR-20b, miR-221 were highly expressed in lung metastasis of HCC, which may be used as a clinical tool to evaluate the prognosis of HCC [92]. Collectively, tumor-derived EV-miRNAs play a crucial role in the acquisition and transfer of the malignant trait by monitoring and regulating tumor resistance.

4. Conclusion

4.1 The therapy potential of EV-miRNAs

Researches into EVs have expanded rapidly in recent years, and have good application prospects in the prevention, diagnosis, treatment and prognosis of various diseases. EVs have unique advantages such as low adaptive immunogenicity and toxicity, good stability, high transport efficiency, strong biocompatibility and ability to cross the blood-brain barrier. Therefore, these properties of EVs are suitable for *in vivo* delivery of various therapeutic agents, including proteins, nucleic acid drugs, targeted drugs, gene editing drugs, *etc*. EVs have emerged as new cell-free therapeutic strategies for treating a variety of diseases.

MiRNAs-coated EVs delivered by donor cells to recipient cells can mediate intercellular communication, regulate the expression of target genes in recipient cells. Researchers have explored the values of EVs in delivering miRNAs for cancer therapy. The miRNAs content in EVs play key roles in this cell-to-cell communication and influence the fate of recipient

cells. Cells can selectively load specific miRNAs into EVs, but not randomly. It shows that the abundance and expression of miRNAs in EVs are different from those in parent cells. Moreover, tumor cells can change the expression of secreted miRNAs under different physiological and pathological conditions, which makes EV-miRNAs become a secretion regulatory factor in the TME. EV-miRNAs mediate the crosstalk between tumor cells and non-malignant cells in the TME, regulating tumor immunity, tumor proliferation, tumor angiogenesis, tumor metastasis, and drug resistance. Therefore, changes in tumor-derived EV-miRNAs expression reflect disease progression, further supporting EV-miRNAs as non-invasive biomarkers in molecular detection, which complement the therapeutic strategies of circulating tumor cells, and have good prospects of clinical diagnosis, treatment and prognosis. Furthermore, the nano-drugs, including therapeutic miRNA and other nucleic acid drugs, can be delivered to tumor cells through the modification of EVs membrane, which have good anti-tumor effects and provide a new idea for targeted tumor therapy. For example, *in vivo* self-assembled exosomes can package therapy small RNAs to treat various diseases, including EGFR/KRAS in lung cancer, EGFR/TNC in glioblastoma, PTP1B in obesity, and HTT in Huntington's disease [13,93] , which represent a next generation RNAi therapeutics. In addition, based on the therapy potential, EV-miRNAs can judge drug resistance efficacy and disease prognosis, and measure the effects of clinical treatment of tumors.

4.2 The challenges in EV-miRNAs based therapy

Although we have outlined some of the mechanisms involved in miRNAs secretion, the details of these mechanisms remain largely unknown. There are currently no more efficient assesses to track each cell's ability to secrete miRNA. In addition, existing studies have not been able to cover all the secretion mechanisms of EV-miRNAs, and the secretion mechanisms of some EV-miRNAs with important physiological and pathological functions are still unclear. To best propel this field forward, future research and more investigations should be directed towards strengthening. Nonetheless, the most exciting but challenging application will be to utilize EVs and their cargo as a clinical tool to diagnose and monitor disease, perhaps even for gene therapy. Superlative EVs-based therapy could combine with other anti-tumor treatments, which will be widespread therapeutic potential applications, but much work remains to achieve this goal. Another challenge is to generate large-scale production of EVs for clinical application. Moreover, there are still some limitations regarding encapsulated miRNAs: how to load the desired cargo and what are the most suitable cells for producing clinical-grade EVs remains to be further investigated. Researches have reported that MSCs and HEK293T cells are two commonly used cell types for producing miRNA-loaded EVs. However, the quantification process for EV-miRNAs requires the development of a series of standard steps. There is also a need to evaluate the relationship between tumors and EV-miRNAs and to systematically develop biomarkers for cancer diagnosis and prognosis. Therefore, more and more widespread therapeutic applications should be proposed.

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Conflicts of Interests

The authors declare no competing interests.

Authors' Contribution

Conceptualization, J.L.; methodology, J.L. and Y.Y.; software, Y.Y.; validation, Y.Y., J.F. and Y.W.; formal analysis, Y.Y.; investigation, Y.Y.; resources, Y.Y.; data curation, Y.Y.; writing—original draft preparation, Y.Y.; writing—review and editing, Y.Y., J.F. and Y.W.; visualization, Y.Y.; supervision, J.L. and X.X.; project administration, X.X.; funding acquisition, J.L. and X.X. All authors have read and agreed to the published version of the manuscript.

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