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REVIEW

Biological functions and clinical applications of exosomal non-coding RNAs in hepatocellular carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, with a high mortality rate. Its dismal prognosis is attributed to late diagnosis, high risk of recurrence and drug resistance. To improve the survival of patients with HCC, new approaches are required for early diagnosis, real-time monitoring and effective treatment. Exosomes are small membranous vesicles released by most cells that contain biological molecules and play a great role in intercellular communication under physiological or pathological conditions. In cancer, exosomes from tumor cells or non-tumor cells can be taken up by neighboring or distant target cells, and the cargoes in exosomes are functional to modulate the behaviors of tumors or reshape tumor microenvironment (TME). As essential components, non-coding RNAs (ncRNAs) are selectively enriched in exosomes, and exosomal ncRNAs participate in regulating specific aspects of tumor development, including tumorigenesis, tumor metastasis, angiogenesis, immunomodulation and drug resistance. Besides, dysregulated exosomal ncRNAs have emerged as potential biomarkers, and exosomes can serve as natural vehicles to deliver tumor-suppressed ncRNAs for treatment. In this review, we briefly summarize the biology of exosomes, the functions of exosomal ncRNAs in HCC development and their potential clinical applications, including as biomarkers and therapeutic tools.

Keywords Hepatocellular carcinoma · Exosome · Exosomal non-coding RNA · Tumor biology · Biomarker · Delivery vehicle

Introduction

Liver cancer is the sixth common malignancy and the fourth leading cause of cancer-related death worldwide in 2018 [1]. Hepatocellular carcinoma (HCC) accounts for 80% of liver cancer, and it mainly occurs in the context of chronic inflammation and fibrosis that is caused by virus hepatitis, alcohol and non-alcohol fatty disease (NAFLD) [2–4]. The 5-year survival rate of HCC is lower than 20% [5], primarily attributed to difficulty of early diagnosis via current biomarkers [6]. Consequently, most HCC patients are diagnosed at an advanced stage, accompanied with intrahepatic or distant metastasis. When diagnosed, the patients are not eligible for curative treatments (resection or transplantation) while palliative treatments are the exclusive choices [7, 8]. For example, the multi-target kinase inhibitor sorafenib can prolong the survival of patients with advanced HCC for approximately 3 months. However, the response rate of sorafenib is low (~ 10%) and most patients develop disease progression due to drug resistance [9, 10]. Moreover, even for those receiving surgical resection, recurrence is a severe problem and half of the patients with recurrence die within 1 year [11, 12]. Thus, there is an urgent need to develop new approaches of early diagnosis, real-time monitoring, and effective treatment for HCC.

Liquid biopsy is a minimally invasive technology for detecting tumor cells or nucleic acids from body fluids, including circulating tumor cell (CTC), circulating tumor DNA (ctDNA) and exosome, which help diagnose cancer, detect disease progression or therapeutic response [13]. However, the numbers of CTCs are few and half-life of

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ctDNA is short. Thus, increasing studies have focused on exosomes, which are abundant in biofluids and keep stable for a long time. Exosomes are small extracellular vesicle (EVs) secreted by most cells and contain bioactive cargoes (proteins, nucleic acids, lipids). In cancer, exosomes from tumor cells or non-tumor cells can transfer the cargoes to recipient cells to modify their phenotypes and reshape tumor microenvironment (TME), affecting tumor development [14]. Thus, exosomes are essential messengers in tumor progression. Moreover, exosomes are widespread in diverse biofluids, e.g., blood, urine, saliva. The concentration of exosomes in cancer patients is higher than healthy individuals, and cargoes in exosomes can reflect the origin cells and real-time diseases states. Thus, exosomes and their cargoes are under investigation as potential biomarkers contributing to diagnosis [15], prognosis [16] and treatments [17] in cancer. What's more, since exosomes are stable and with low immunogenicity, they are exploited as vehicles for carrying drugs and anti-tumor nucleic acids to treat cancer.

Non-coding RNAs (ncRNAs) are a class of RNAs without protein-coding capability, such as microRNAs (miRNAs), long non-coding RNAs (lncRNAs) and circular RNAs (circRNAs). By interacting with DNAs, RNAs or proteins, ncRNAs can regulate gene expression on transcription, post-transcription, epigenetic levels and modulate cellular processes [18]. Emerging evidence suggests that various ncRNAs are dysregulated in cancer and participate in complex network of biological regulation, e.g., cell proliferation, migration, apoptosis [19]. Interestingly, ncRNAs are selectively enriched in exosomes, and exosomal ncRNAs can exert biological functions in recipient cells to affect the critical processes of tumor development, e.g., tumorigenesis, tumor metastasis, angiogenesis, immunomodulation and drug resistance [20]. Besides, due to aberrantly expressed, exosomal ncRNAs represent a powerful source of biomarkers [21, 22] and novel therapeutic targets. In this review, we mainly overview the biology of exosomes, the functions of exosomal ncRNAs in HCC development and their potential clinical applications, including as biomarkers or therapeutic tools.

The characteristics and biogenesis of exosomes

Exosomes are often referred to EVs, but they should not be confused. Different from other EVs, exosomes are 30–100 nm membranous vesicles of endocytic origin that are released by live cells while apoptosis bodies are secreted during apoptosis and microvesicles (MVs) are shedding from plasma membrane directly [23]. Although exosomes were considered as cellular garbage bins to remove hazardous substance before, recent studies have identified them as important means of intercellular communication [24]. Exosomes can mediate bulk of physiological or pathological processes, e.g., antigen presentation [25], injury repair [26], and tumor metastasis [27]. The functions of exosomes depend on their contents. An array of biomacromolecules is contained in exosomes, including proteins (heat shock proteins, tetraspanin), nucleic acids (mRNAs, ncRNAs, DNAs), as well as lipids (cholesterol). In Exocarta (www.exocarta.org), 9769 proteins, 3408 mRNAs, 2838 miRNAs and 1116 lipids were identified in exosomes in multiple organisms. Besides, exRNA Atlas (exrna-atlas.org/), a data repository of the Extracellular RNA Communication Consortium (ERCC), includes 5309 small RNA sequencing and qPCR-derived exRNA profiles from human and mouse biofluids in 19 studies. And the expression of long RNA species, including 15,501 lncRNAs, 18,333 mRNAs and 58,330 circRNAs, in human blood exosomes were collected from 92 samples in exoRbase (www.exorbase.org/). The cargoes are cell-type specific, e.g., exosomes derived from B-lymphocytes expressed MHC I/II molecules and the B cell marker CD20 [28]. However, the cargoes in exosomes are not identical to those in parent cells and are often influenced by extracellular stimuli, e.g., hypoxia and low pH could induce the biogenesis of exosomes and change the contents [29, 30]. It illustrates that the biogenesis of exosomes and sorting of the cargoes is precisely regulated.

In brief, exosomes are generated as intraluminal vesicles (ILVs) that are formed by inward budding of endosomal membrane during the maturation of multivesicular bodies (MVBs), and secreted upon fusion of MVBs with plasma membrane (Fig. 1). The mechanisms of exosomal biogenesis involve multiple factors [31], and the most known regulator is endosomal sorting complex required for transport (ESCRT). There are four types of ESCRT, including ESCRT 0-III. ESCRT 0 and ESCRT I cluster-specific cargoes and target them to endosomal membrane, then ESCRT I-ESCRT II complex are involved in buddings of endosomal membrane and they recruit ESCRT III to perform scission of ILVs. Apart from ESCRT, there are also other regulators in exosomal biogenesis, e.g., tetraspanins (CD9, CD63, CD81) and some accessory proteins (ALIX, VPS4). The motility of MVBs to plasma membrane is regulated by GTPase, e.g., Rab 27a/b.

After secreted into extracellular space, exosomes can naturally target to neighboring or distant recipient cells. The exosomes may activate downstream signal pathways in recipient cells by ligand-receptor interaction [32]. Additionally, exosomes can be internalized through fusion with plasma membrane, phagocytosis or receptor-mediated endocytosis (clathrin dependent and independent) to deliver the cargoes into cytoplasm. The cargoes are functional in recipient cells and change the phenotypes of recipient cells, e.g., mRNAs can be translated to proteins, and miRNAs can

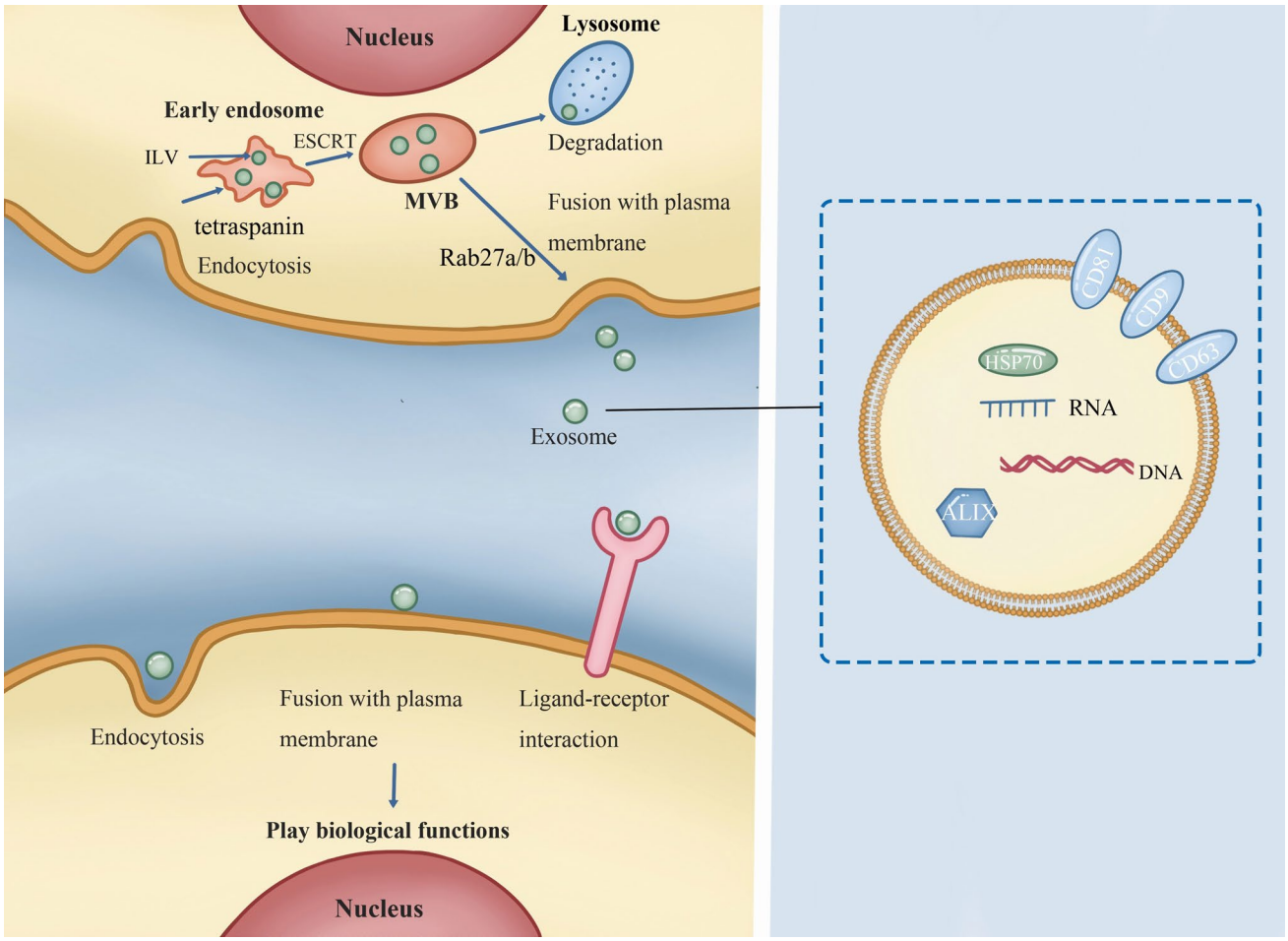

Fig. 1 The biogenesis, secretion and uptake of exosomes. Early endosomes are formed by endocytosis. With the assistance of endosomal sorting complex required for transport (ESCRT), intraluminal vesicles (ILVs) are generated while early endosomes develop to multivesicular bodies (MVBs). Some MVBs fuse with lysosome to be degraded while other MVBs are trafficked to fuse with the plasma membrane (it is regulated by GTPase, e.g., Rab27a/b) to release ILVs to extracellular milieu, which are called exosomes. Exosomes contain bioactive cargoes derived from parent cells, including DNAs, RNAs, proteins, as well as lipids. Exosomes can be ingested by recipient cells by endocytosis or fusing with plasma membrane, or through ligand–receptor interaction to exert diverse biological functions

regulate gene expression by binding to target mRNAs in recipient cells [33]. In cancer, it has been reported that tumor cells release more exosomes than normal cells, and tumor-derived exosomes (TDEs) participate in the initiation and progression of cancer while normal cell-derived exosomes can inhibit tumorigenesis, due to differential components.

Separation and characterization of exosomes and exosomal ncRNAs

To investigate the functions of exosomes in cancer biology, we need to interrogate exosomal contents. In this review, we mainly focus on ncRNAs in exosomes since they are the most abundant and crucial biomolecules derived from exosomes. First, we need to isolate exosomes from samples, e.g., conditioned culture medium, ground tissues or biofluids. Up to now, there is no standard method of exosome isolation, and five categories of exosome isolation techniques have been developed, including differential ultracentrifugation-based techniques, size-based techniques, immunocapture-based techniques, precipitation, and microfluidic-based techniques [34]. According to a worldwide survey of International Society for Extracellular Vesicles (ISEV), differential centrifugation is the most commonly used method for separation (about 56%), and density gradient centrifugation, filtration, size-exclusion chromatography (SEC), immuno-isolation, precipitation are used by 5–20% of respondents each [35]. Moreover, additional techniques that involve a wide variety of microfluidic device have been developed, such as field-flow fractionation (FFF), asymmetric flow field-flow fractionation (AFFF), and field-free viscoelastic flow. However, absolute purification or complete isolation of exosomes is an unrealistic goal yet.

Current methods, including centrifugation protocols or commercial kits all cannot separate exosomes specifically and completely. Thus, the separation products are actually mixture of different EVs and other substances, e.g., MVs and lipoprotein, which may affect downstream analysis. Thus, appropriate methods should be selected depending on downstream applications and scientific question. Highly purified exosomes are needed to attribute a function or biomarker to exosomes while less pure exosomes may be acceptable when a biomarker is useful without enrichment.

To further the promise of EVs including exosomes as biomarkers or for treatment, The ISEV board members have published a minimal information for studies of extracellular vesicles (MISEV) in 2014 and update in 2018 [36]. For separation, 93% of MISEV2018 survey respondents agreed with the categorization of techniques by recovery and specificity. For example, the methods with high recovery and low specificity include precipitation and low-molecular weight filters, the methods with intermediate recovery and specificity include SEC, differential centrifugation, high-molecular weight filters and membrane-affinity columns, the methods with low recovery and high specificity include density gradient centrifugation and immuno-isolation. And there is no method with high recovery and high specificity. Combinations of these methods outperform single-method approaches. Any newly developed technique for isolation must indicate to which of the 4 recovery/specificity options. More importantly, 98% of MISEV2018 Survey respondents agreed that all the experimental details, e.g., centrifugation (g-force, rotor, ultracentrifuge, adjusted k-factor, tube type, adaptor, time, temperature, and brake), should be deposited on the EV-TRACK (http://evtrack.org/index.php) to allow reproducibility.

Moreover, characterization of exosomes is important to show the success of isolation and demonstrate that biomarkers or functions are associated with exosomes [37]. According to MISEV 2018, the sources of EVs, e.g., number of secreting cells, volume of biofluid, should be described, and abundance of EVs should be characterized by total particle number or protein or lipid content, although none of them are exclusively associated to EVs. The purity of EVs can be measured by ratios of proteins/particles, lipids/particles or lipids/proteins. For general characterization, at least three positive protein markers of specific subtypes of EVs, including at least one transmembrane/lipid-bound protein/cytosolic protein must be evaluated, at least one negative protein marker and the presence of co-isolated components, e.g., lipoprotein or albumin, should be tested. Even more, two different but complementary techniques should be used for characterization of single vesicles, for example, images of single EVs at high resolution by electron microscopy (EM), scanning-probe microscopy (SPM), atomic-force microscopy (AFM) or super-resolution microscopy, size measured by resistive pulse sensing (RPS), or light-scattering properties, e.g., nanoparticle tracking analysis (NTA). As for exosomes, exosomes usually have cup-shaped morphologies under EM, express the markers associated with biogenesis (e.g., Tsg101, Alix, CD9/63/81, HSC70) and absence of ER markers (Calnexin), and the size of exosomes vary from 30 to 200 nm. However, many studies do not provide the adequate information of characterization yet and it needs to be improved in the future.

After separation of exosomes, we can extract exosomal ncRNAs from exosomes by RNA extracting kits or Trizol. The protocols of separation can be obtained from exRNA (http://exrna.org). Due to low concentration of exosomal RNAs, conventional methods for RNA evaluation are unavailable. For example, the results of concentration measured by Nanodrop or Qubit are inaccurate due to limitation of detection threshold. Instead, Agilent 2100 bioanalyzer can be used to evaluate the quality and quantity of exosomal RNAs. The results show that most RNA cargoes in exosomes are small RNAs which are 20–200 nucleotides in length and there is little 18S/28S rRNA. Deep sequencing reveals that the most abundant exosomal RNAs are miRNAs, and there are also fragments of mRNAs, lncRNAs or circRNAs and other types of ncRNAs, e.g., tRNAs, which are rarely explored yet [38, 39]. To identify dysregulated exosomal ncRNAs, quantitative analysis is required, and next-generation sequencing (NGS) [40], microarray [41], qPCR and digital PCR [42] can be used. A great number of exosomal ncRNAs have been reported to be dysregulated in HCC, and we will discuss their biological functions and clinical applications below.

The effects of exosomal ncRNAs on fibrosis/cirrhosis

HCC mainly occurs in a context of liver cirrhosis (LC) caused by chronic infection of hepatitis B virus (HBV) or hepatitis C virus (HCV) [2]. In the process of cancerization, ncRNA-carrying exosomes can help virus spread and disturb host immunity, leading to persistent viral infection, causing DNA damage and HCC occurrence. For example, miR-122 was enriched in serum exosomes from HCV patients, and exosomal miR-122 could help virus RNA transmission and replication between HCV-infected hepatocytes and normal hepatocytes via AGO2-miR-122-HSP90 complex [43]. Besides, serum exosomes from HCV patients could inhibit the function of natural killing (NK) cells and this effect was correlated with miR-122-5p (reduced expression of granzyme B, IGF1 receptor) and miR-222-3p (repressed expression of STAT5B) [44]. Similar situations also occur in HBV patients, caused by exosomal miR-21 and miR-29a, which suppressed the IL-12 expression in macrophages and

prevented the activation of NK cells [45]. Chronic virus infection will activate hepatic stellate cells (HSCs), resulting in sustained inflammation and fibrosis. The study from Devhare PB demonstrated that HCV-infected hepatocytes could transfer exosomal miR-19a to HSCs, and miR-19a could target SOCS3 in HSC, which activated the STAT3-mediated transforming growth factor β (TGF-β) signaling pathway and promote fibrosis, which may favor the HCC tumorigenesis [46].

Additionally, NAFLD or non-alcohol steatohepatitis (NASH) has become another increasingly important disease leading to HCC. ncRNA-carrying exosomes from hepatocytes and non-hepatocytes both participate in progression of NAFLD. For example, in diet-induced steatohepatitis, the levels of exosomes increased early and reflected changes in liver histopathology. The levels of miR-122 and miR-192, two microRNAs abundant in hepatocyte increased in circulating exosomes. Thus, circulating exosomal ncRNAs may be biomarkers reflecting liver injury [47]. The elevated circulating miR-122 was associated with decreased liver miR-122, which resulted in upregulation of modulators of tissue remodel (HIF-1α, vimentin) and induced liver fibrosis [48]. Interestingly, circulating miR-122 may not be exclusively from liver. Baranova A et al. reported that adipose might actively release tumor-suppressing, anti-fibrotic miR-122 into circulation via exosomes, circulating miR-122 might infuse to liver to delay NAFLD progression and inhibit tumorigenesis [49]. Thus, detecting the levels of exosomal ncRNAs may help monitor chronic liver disease progression and evaluate the risks of HCC.

Exosomal ncRNAs and HCC progression

HCC is caused by genetic mutation and epigenetic dysregulation, involved with complex signal network. The malignant behaviors of tumor cells not only depend on themselves, but are also modulated by the interaction between tumor cells and their TME, which comprises of endothelial cells, fibroblasts, immune cells, and extracellular matrix (ECM) [50]. Apart from cell–cell contact and soluble factors, ncRNA-carrying exosomes, as signal carriers between different cells, can affect many aspects of HCC progression, including tumor metastasis, tumor angiogenesis, tumor immunity and drug resistance (Fig. 2). Understanding the roles of exosomal ncRNAs in cancer biology help develop novel anti-tumor strategy.

Exosomal ncRNAs and HCC metastasis

Metastasis, the leading cause of death in cancer, is a multistep process, including tumor cells detach from primary lesion, invade the ECM, invade into circulation, survive in blood stream, extravasate from vessels, invade into secondary organs, and form metastases in target organs [51]. Exosomal ncRNAs have been demonstrated to participate in modulating sequential processes of HCC metastasis, including initiation of epithelial–mesenchymal transformation (EMT), regulation of tumor growth and invasion, increased vascular permeability, formation of pre-metastatic niches, and tumor seeding.

EMT is a conservative process that tumor cells lose their epithelial phenotypes and acquire mesenchymal phenotypes, resulting in enhanced motility and decreased cell–cell adhesion. Evidence suggests that EMT is related to initiation of metastasis and malignant transformation [52]. Both exosomal ncRNAs from tumor cells and stroma cells are potential mediators of EMT by activating relevant pathways. For example, the expression of Vimentin (mesenchymal marker) was increased while E-cadherin (epithelial marker) was decreased in HCC cells after treatment with HCC-derived exosome. lncRNA FAL1 was upregulated in HCC-derived exosomes, and lncRNA FAL1 could act as sponger of miR-1236 to increase the expression of ZEB1, which was the key transcription factors in EMT, and promote tumor metastasis [53]. Additionally, cancer-associated fibroblasts (CAFs) are known to play a great role in HCC progression, including inducing EMT [54]. The study from Zhang Z revealed that CAF-derived exosomes could induce EMT in HCC cells by activating MAPK pathways and upregulate MMP2 expression to remodel the ECM, facilitating HCC metastasis, which was associated with loss of miR-320a in CAF-derived exosomes [55].

On another hand, tumor cells can transfer oncogenic ncRNAs to recipient cells via exosome to promote tumor growth and invasion in autocrine or paracrine manners. For instance, TAK1, belonging to MAP3K subfamily, is a vital component of tumorigenesis. Selective miRNAs (e.g., miR-584) enriched in HCC-derived exosomes could be internalized by recipient HCC cells, subsequently modulated TAK1 expression to enhance HCC growth and invasion [56]. Similarly, miR-93 was upregulated in HCC patients' serum exosomes and medium of HCC cell line, and exosomal miR-93 could stimulate the proliferation and invasion of HCC cells by targeting TIMP2/TP53INP1/CDKN1A [57]. In another study, lncRNA TUC339 was identified to be enriched in HCC-derived exosomes, and exosomal lncRNA TUC339 could promote cell proliferation and reduce cells adhesion to ECM, resulting in HCC spread [58]. Interestingly, the oncogenic effect of exosomes has been shown to not only occur by TDEs, but also non-TDEs. For example, adipose tissue could affect tumorigenesis by secreting adipokines [59]. The study from Zhang H demonstrated that plasma exosome circ-deubiquitination (circ-DB) was upregulated in HCC patients with higher body fat ratios, and exosome circ-DB derived from adipocytes could suppress miR-34a

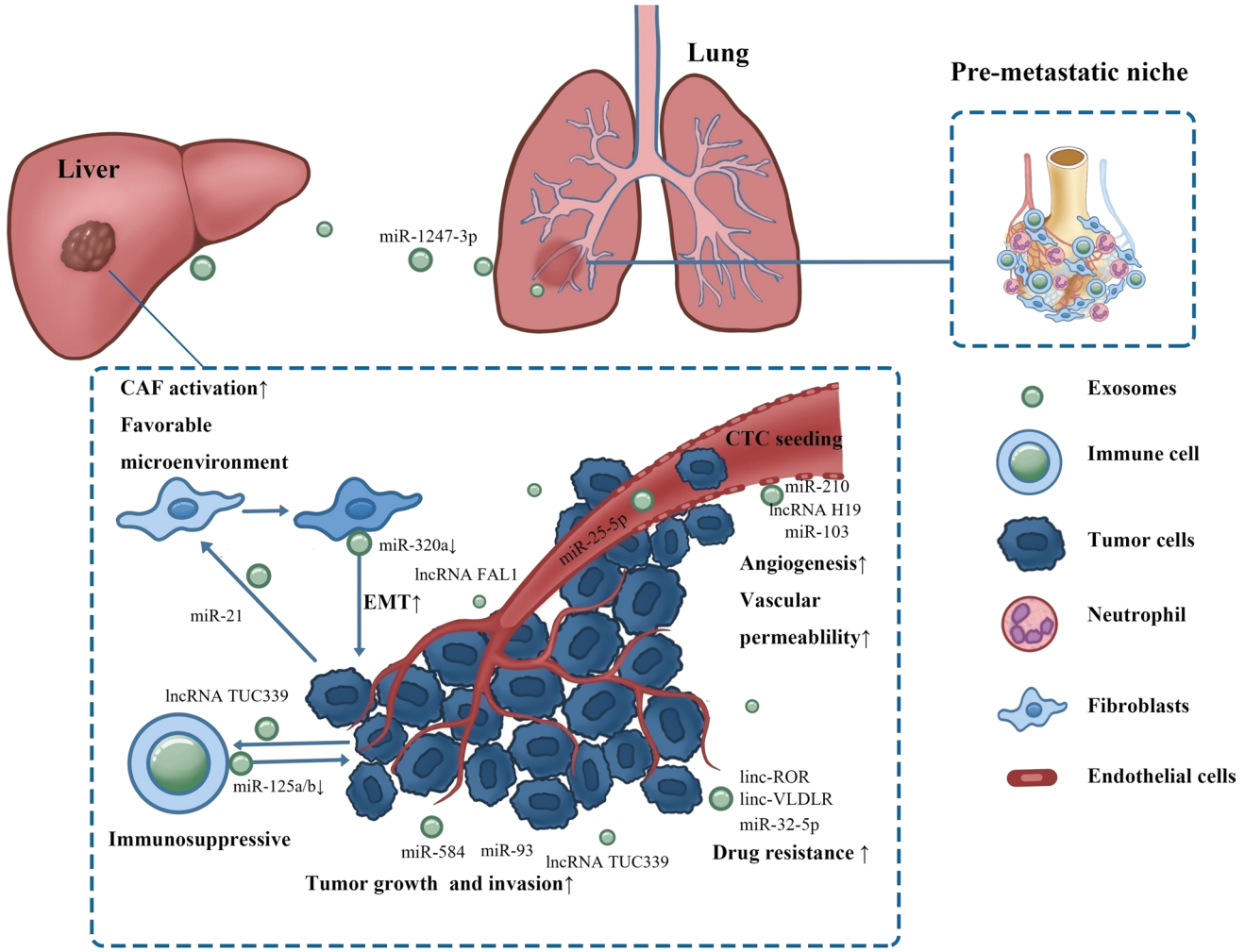

Fig. 2 The interaction between HCC cells and microenvironment via exosomal ncRNAs. As functional contents, non-coding RNAs (ncRNAs) in both tumor-derived exosomes (TDEs) and non-TDEs participate in the communication between HCC cells and tumor microenvironment to promote HCC progression. Exosomal ncRNAs could promote tumor growth and invasion (miR-584, miR-93, lncRNA TUC339, decreased miR-125a/b), initiate the epithelial–mesenchymal transition (EMT) (lncRNA FAL1, decreased miR-320a), increase vascular permeability (miR-103), activate CAFs to form the pre-metastatic niche (miR-21, miR-1247-3p), affect circulating tumor cells (CTCs) seeding (miR-25-5p), promote tumor angiogenesis (miR-210, lncRNA H19), induce immunosuppressive (lncRNA TUC339), mediate drug resistance (linc-ROR, linc-VLDLR, miR-32-5p), etc.

and activate the USP7/Cyclin A2 signaling pathway in HCC cells to promote HCC growth [60].

The occurrence of metastasis not only requires the enhancement of growth and invasion of tumor cells, also need tumor cells traverse the endothelial barrier. The vessels in tumor lesions have aberrant structures with increased permeability, which favor tumor metastasis. The crosstalk between cancer cells and endothelial cells, mediated by exosomal ncRNAs, takes part in impairing the vascular integrity to assist tumor cells to invade into circulation [61]. One study revealed that higher level of serum exosomal miR-103 was associated with higher metastasis potential, higher recurrence risks and shorter survival in the patients with HCC. The exosomal miR-103 secreted by HCC cells could act on endothelial cells to attenuate the endothelial junction integrity and increase vascular permeability by targeting endothelial adherens junction proteins, such as VE-Cadherin, p120-catenin, zonula occludens-1 (ZO-1), resulting in increased transendothelial invasion of HCC cells and metastasis occurrence [62].

In addition, before the implantation of tumor cells, primary tumors can establish a favorable microenvironment in target organs to support metastases growth, which is named pre-metastatic niche. The pre-metastatic niche, with the characteristics of inflammation, immunosuppressive and angiogenesis/vascular permeability [63], can be created by TDEs that remodel stromal contents, such as activation of CAFs and recruitment of myeloid-derived suppressor cells

(MDSCs) [64]. Lung is the most common site of HCC distant metastasis. The study from Fang T demonstrated the crosstalk between tumor cells and fibroblasts mediated by TDE-influenced lung metastasis of HCC. They found that the levels of serum exosomal miR-1247-3p were higher in HCC patients with lung metastasis than those without. High metastatic HCC cells could secrete exosomal miR-1247-3p to activate CAFs via targeting B4GALT3, leading to activation of β1-integrin-NF-κB signaling in fibroblasts, and activated CAFs released inflammatory cytokines (IL-6, IL-8) to form an inflammatory microenvironment, fostering lung metastasis [65].

In the final step of metastasis, CTCs will seed in the target organ and grow to form metastases. It was reported that integrin in exosomes determines organ-specific metastasis [66], and exosomal ncRNAs also affect tumor seeding. Tumor self-seeding is a process that CTCs re-infiltrate the original tumor, in which primary tumor-derived cytokines act as CTC attractants and vascular leakage favor the infiltration of CTCs [67]. The study from Liu H demonstrated that exosomes from primary HCC could be transferred to CTCs to enhance their migratory and invasive abilities, resulting in tumor self-seeding, which may breed more aggressive tumor cells and contribute to HCC progression, this effect was associated with horizon transfer of exosomal miR-25-5p by targeting leucine rich repeat containing 7 (LRRC7) [68]. It illustrated the crosstalk between primary tumor and CTCs affected HCC progression.

Exosomal ncRNAs promote angiogenesis

Active angiogenesis, supplying adequate nutrition and oxygen to tumor cells, is a critical step for tumor growth and metastasis. Due to intratumor hypoxia, tumor cells can release soluble factors, e.g., vascular endothelial growth factor (VEGF), as well as exosomes to promote pathological angiogenesis [69]. Associated exosomal ncRNAs have been investigated in HCC. For example, the levels of serum exosomal miR-210 were elevated in HCC patients and associated with microvessel density (MVD). By targeting SMAD4 and STAT6 in endothelial cells, exosomal miR-210 derived from HCC cells could promote proliferation, migration and tube formation of endothelial cells, which are essential for angiogenesis [70]. In another study, hypoxia could stimulate HCC cells to secret exosomes containing miR-155 to promote angiogenesis, and the levels of exosomal miR-155 were positively associated with the expression of VEGF and HIF-1α in HCC samples. Increased exosomal miR-155 in preoperative plasma was significantly correlated with early recurrence and poor prognosis [71]. HCC cells can also promote angiogenesis indirectly through CAFs. For example, Zhou et al. reported that HCC cells secreted exosomal miR-21 that directly targeted phosphatase and tensin homolog (PTEN) to convert HSCs to CAFs, and CAFs could promote angiogenesis by releasing angiogenic cytokine, e.g., VEGF, MMP2, MMP9, bFGF and TGF-β [72]. Additionally, the crosstalk between cancer stem cell (CSC) and endothelial cells was reported to drive tumor angiogenesis. $CD90^+$ liver cancer stem cells released exosomes containing lncRNA H19, which could be internalized by endothelial cells, subsequently upregulated the expression of VEGF and its receptor VEGFR1 in endothelial cells, inducing angiogenesis [73]. Blocking the transfer of exosomal ncRNAs abrogates these effects, providing novel strategies for inhibiting tumor progression.

Exosomal ncRNAs modulate tumor immunity

The role of immune microenvironment cannot be ignored for tumor progression. Tumor cells can escape from immune surveillance and induce immune tolerance by multiple ways, including releasing exosomes [74]. Both ncRNAs-carrying exosomes from tumor cells and immune cells could influence immune responses [75]. On one hand, tumor cells could transfer oncogenic ncRNAs to immune cells through exosomes to induce the expansion or differentiation of immunosuppressive cells, e.g., regulator T cells (Treg), and promote apoptosis or suppress the activity of effector cells, e.g., cytotoxic T lymphocytes (CTLs) [76], leading to local or systemic immunosuppression. For example, under ER stress, HCC-derived exosomal miR-23a-3p could upregulate the expression of PD-L1 in macrophages through PTEN-PI3K/AKT pathway, which subsequently inhibited T cell function [77]. In another study, HCC-derived exosomal lncRNA TUC339 could educate the macrophages, leading to reduced pro-inflammatory cytokine production (IL-1β,TNF-α), compromised phagocytosis (CD86) and drove M2 polarization, which promoted HCC progression. By bioinformatic analysis, lncRNA TUC339 were found to be involved in regulation of CXCR chemokine receptor binding, cytokine signaling pathway, an immune system defense response [78]. Interestingly, TDEs could also activate anti-tumor immunity by presenting tumor-associated antigen (TAA) [79]. The balance between both sides of TDEs is uncertain. On another hand, ncRNA-carrying exosomes from immune cells also affect tumor progression and the effects depend on the types of immune cells. For example, exosomes from DC cells could present TAAs to CTL to activate anti-tumor immunity [80]. Exosomal miR-142 and miR-223 from primary macrophages could inhibit HCC growth by decreasing the expression of stathmin-1 (STMN1) and insulin-like growth factor-1 receptor (IGF-1R) [81]. Conversely, tumor-associated macrophages (TAMs), the most abundant immune cells in TME, released exosomes to promote HCC growth and stem cell properties by targeting CD90, associated with decreased levels of miR-125a/b

[82]. In another study, exosomes derived from functional exhausted $CD8^+$ T cells led to exhaustion of normal $CD8^+$ T cells, and 257 dysregulated lncRNAs were identified that actively participated in the regulation of diverse process of $CD8^+$ T cells, e.g., metabolism, biosynthetic process [83]. Thus, therapeutic intervention based on exosomal ncRNAs in immune regulation will help remodel anti-tumor immunity.

Exosomal ncRNAs mediate drug resistance

Drug resistance, an essential factor for poor prognosis, is a significant clinical problem [84]. As a research hotspot, exosomal ncRNAs have been found to be involved with drug resistance. For example, when exposed to anticancer agents, such as sorafenib, camptothecin and doxorubicin, the expression of lincRNA-VLDLR (linc-VLDLR) was increased in both HCC cells and TDEs. When incubated with TDEs, the levels of linc-VLDLR in recipient HCC cells were elevated, and linc-VLDLR could increase the expression of ATP-binding cassette, subfamily G member 2 (ABC-G2), which involved in export of chemotherapeutic agents to reduce drug-induced cell apoptosis. [85]. CSCs are also involved in drug resistance. TGF-β could enrich lincRNA-ROR (linc-ROR) within TDEs, leading to increased number of $CD133^+$ CSCs and reduced chemotherapy-induced cell death through repression of p53, resulting in drug resistance [86]. Moreover, the drug-resistant cells could deliver exosomal ncRNAs (miR-32-5p) horizontally to drug-sensitive cells to induce EMT and drug resistance by inhibiting PTEN and activating PI3K/AKT pathway in recipient cells [87]. What's more, exosomes from stromal cells, e.g., CAFs or TAMs also play a great role in drug resistance in other cancers although there is no study in HCC yet. These findings all support targeting exosomal ncRNAs to enhance chemosensitivity in HCC.

Exosomal ncRNAs as biomarkers for HCC

As part of liquid biopsy, exosomes are widespread in various body fluids, and ncRNAs in TDEs can reflect the information of tumors and real-time disease progression. Thus, exosomal ncRNAs are regarded as a powerful source of non-invasive biomarkers for HCC. Blood is the most frequently used source in the researches of biomarkers. The standardization of sample collection, isolation and analysis were reviewed elsewhere [88]. Compared with other forms of ncRNAs, e.g., ncRNAs in tissues or circulating free ncRNAs, circulating exosomal ncRNAs have advantages of non-invasive and stable (the lipid bilayer of exosomes can protect ncRNAs from degradation), raising great interest of researchers. Table 1 summarizes the circulating exosomal ncRNAs that serve as biomarkers in HCC.

Exosomal ncRNAs as diagnostic biomarkers

Early diagnosis can improve clinical outcomes of patients with HCC since curative resection or liver transplantation is available for early HCC and 5-year survival rate can be higher than 50% [89]. Thus, screening in high-risk population, e.g., the patients with cirrhosis, chronic hepatitis B (CHB) or chronic hepatitis C (CHC), may be beneficial [90, 91]. However, due to unsatisfactory sensitivity and specificity, the efficacy of current screening tools [ultrasound and serum α-fetal protein (AFP)] was suboptimal [92]. Thus, we need new biomarkers alone or combined, with a high sensitivity and specificity, to improve the accuracy of early diagnosis.

A great number of studies suggested that circulating exosomal miRNAs could serve as potential diagnostic biomarkers, which differentiated HCC from non-HCC individuals, such as healthy control, as well as the patients with LC, CHB or CHC. For example, the levels of serum exosomal miR-93 in HCC patients were significantly higher than healthy controls (diagnostic efficacy: area under the curve, AUC = 0.825) [57]. Serum exosomes from patients with HCC contained lower levels of miR-9-3p than those from healthy controls [93]. The levels of miR-21 were increased in serum exosomes from patients with HCC than in CHB or healthy volunteers, and it was correlated with cirrhosis and tumor stages [21]. In another study, eight exosomal miRNAs (miR-122, miR-125b, miR-145, miR-192, miR-194, miR-29a, miR-17-5p, and miR-106a) had significant differences between HCC and normal serum samples. The AUC of these eight exosomal miRNAs as diagnostic biomarkers varied from 0.535 to 0.850 [94].

Apart from exosomal miRNAs, recent studies have investigated the roles of serum exosomal lncRNAs as diagnostic biomarkers for HCC. For example, one study demonstrated that the levels of serum exosomal lncRNA ENSG00000258332.1 and LINC00635 were higher in HCC patients than in patients with CHB (AUC: 0.719 and 0.750, respectively) [95]. Similarly, the increased levels of serum exosomal lncRNA HEIH were found to be able to discriminate HCV-related HCC patients from patients with HCV-induced cirrhosis or CHC [22].

Although single exosomal ncRNAs can diagnose HCC, combining panels of biomarkers will enable a more accurate prediction of HCC than single biomarker for high-risk individuals. For example, Liu H et al. detected the levels of four most dysregulated miRNAs (miR-10b, miR-21, miR-122 and miR-200a) at different stages during HCC development in rat models. The four miRNAs in serum and serum exosomes obtained more remarkable alternation than serum AFP at early HCC stages. Different combinations of AFP, exosomes and exosomal miRNAs had stronger power in predicting HCC than serum AFP alone (AUC, 0.943 vs 0.826)

Table 1 Exosomal ncRNAs as biomarkers in HCC

Function	NcRNA (expression)	Source	Method	Cohort	Year [refs.]
Diagnosis	miR-93 (↑)	Serum	qPCR	85 HCC vs 23 healthy control	2018 [57]
	miR-9-3p(↓)	Serum	qPCR	30 HCC vs 10 healthy control	2018 [93]
	miR-21 (↑)	Serum	qPCR	30 HCC vs 30 CHB, 30 healthy control	2014 [21]
	miR-122 (↑) miR-125b (↑) miR-145 (↑) miR-192 (↑) miR-194 (↑) miR-29a (↑) miR-17-5p (↑) miR-106a (↑)	Serum	qPCR	80 HCC vs 30 healthy control	2019 [94]
	ENSG00000258332.1(↑) LINC00635(↑)	Serum	qPCR	60 HCC vs 85 LC vs 96 CHB vs 60 healthy control	2018 [95]
	LncRNA HEIH (↑)	Serum	qPCR	10 HCC vs 22 LC vs 25 CHC	2018 [22]
	miR-10b (↑) miR-21 (↑) miR-122 (↓) miR-200a (↓)	Serum (rat)	qPCR	Normal, cirrhosis, early stage and late stage of HCC	2015 [96]
	miR-18a (↑) miR-221(↑) miR-222(↑) miR-224(↑) miR-101(↓) miR-106b (↓) miR-122(↓) miR-195(↓)	Serum	qPCR	20 HCC vs 20 LC vs 20 CHB	2015 [97]
Predicting poor prognosis	miR-638 (↓)	Serum	qPCR	126 HCC (retrospective)	2018 [99]
	miR-125b (↓)	Serum	qPCR	128 HCC (retrospective)	2017 [100]
	miR-665 (↑)	Serum	qPCR	30 HCC (retrospective)	2017 [101]
	miR-21 (↑) lncRNA ATB (↑)	Serum	qPCR	79 HCC (prospective)	2018 [102]
Predicting recurrence/metastasis	miR-103 (↑)	Serum	qPCR	85 HCC	2018 [62]
	miR-1247-3p (↑)	Serum	qPCR	110 HCC	2018 [65]
	miR-155 (↑)	Plasma	qPCR	40 HCC	2018 [71]
	miR-718 (↓)	Serum	Microarray	6 HCC	2015 [103]

[96]. Moreover, ncRNAs are selectively enriched within exosomes, increasing the sensitivity of detection. The study from Sohn W demonstrated that the levels of serum exosomal miR-18a, miR-221, miR-222 and miR-224 were higher in HCC than in CHB or LC while the levels of exosomal miR-101, miR-106b, miR-122 and miR-195 were lower in HCC than in CHB. Unlike exosomal miRNAs, the levels of serum circulating miRNAs showed a smaller difference between HCC and CHB [97].

From all these studies, we can conclude that exosomal ncRNAs can be used as novel diagnostic biomarkers for HCC. However, the results among different studies may be contradictory. It might be caused by patient selection, variable etiology, sample number and detection methods. Therefore, multi-center, large-sample clinical researches are needed in the future. Even more, the development of a standard isolation method to obtain sufficient exosomes of high purity is a challenge. The present gold standard ultracentrifugation is time-consuming, equipment dependent and has low yields. The cost of commercial kits is high and the purity is variable due to different principles as said before. Recently, microfluidic systems have been developed with advantages of rapid separation and small sample requirement (even a drop of blood) [98], providing a bright future in exosome field.

Exosomal ncRNAs as prognostic and predictive biomarkers

Except for diagnosis, exosomal ncRNAs have been introduced to assess HCC prognosis. From several retrospective studies, circulating exosomal ncRNAs have been suggested as effective prognostic markers for HCC. For example, negative association of serum exosomal miR-638 with tumor

size, vascular infiltration and TNM stage was observed. Decreased levels of serum exosomal miR-638 predicted poor prognosis in HCC. [99]. In another study, the levels of miR-125b were associated with tumor number, encapsulation and TNM stage. The patients with lower serum exosomal miR-125b levels showed reduced time to recurrence (TTR) and overall survival (OS). [100]. And the survival time of the exosomal miR-665 high-expression group was significantly shorter than that of the low-expression group [101]. However, the results of retrospective studies may be influenced by selection bias, and prospective studies are required. In a recent prospective study, Lee et al. reported that the OS and progression-free survival (PFS) were significantly lower in HCC patients with higher levels of exosomal miRNA-21 and lncRNA-ATB [102].

Since tumor metastasis and recurrence are crucial factors for poor prognosis, researchers may use exosomal ncRNAs to evaluate metastasis potential of tumors or predict recurrence to improve the management of HCC. For example, higher level of serum exosomal miR-103 was associated with higher metastasis potential, higher recurrence risks and shorter survival in the patients with HCC [62]. Increased level of serum exosomal miR-1247-3p was related to lung metastasis of HCC [65]. High expression of exosomal miR-155 in preoperative plasma was significantly correlated with early recurrence [71]. And the levels of preoperative serum exosomal miR-718 were lower in the patients with recurrence after liver transplantation compared with those without recurrence [103]. By detecting these circulating exosomal ncRNAs, we can learn more information about tumor biology and monitor tumor progression of high-risk patients carefully.

Another reason for poor prognosis is drug resistance. As mentioned above, linc-VLDLR, linc-ROR, and miR-32-5p in exosomes were reported to mediate the occurrence of drug resistance in vitro. We may detect circulating exosomal ncRNAs of HCC patients to predict therapeutic efficacy of anticancer drugs, although there is no such study yet. Further, researchers can dynamically monitor the levels of circulating exosomal ncRNAs at different time spots (before treatment, after treatment, disease progression) to evaluate the efficacy of treatments and real-time progression.

Therapeutic functions of exosomal ncRNAs in HCC

As TDEs can carry oncogenic ncRNAs to recipient cells to promote tumor progression, researchers may inhibit tumorigenesis by blocking the biogenesis, secretion and uptake of exosomes. However, normal cells also release exosomes to maintain physiological functions [104]. Indiscriminate suppression of exosomes biogenesis may lead to side effects. Thus, it is better to affect TDEs specifically or sort of oncogenic ncRNAs into exosomes. For example, sphingosine kinase 2 (Sphk2) is an important regulator in exosome biogenesis. Using Sphk2 siRNA-loaded nanoparticles to treat HCC cells can reduce miRNA-21 sorting into exosomes, contributing to the inhibition of tumorigenic function of exosomes [105]. The synthetic nanoparticles can be replaced by exosomes, which are regarded as natural nanoparticles, with advantages of low biotoxicity, low immunogenicity and better biocompatibility. Thus, exosomes are potential options for new therapeutic approaches in cancer.

Unmodified ncRNA-carrying exosomes from normal stem cell

It was reported that normal cells could secrete exosomal miRNAs to inhibit tumor growth [106]. Recently, researches have focused on the roles of normal stem cell and their exosomes in cancer therapy. For example, mesenchymal stem cell (MSC) was reported to migrate to TME and could affect hepatocarcinogenesis and HCC progression [107]. Evidence suggests that MSC-derived exosomes could inhibit HCC progression, which was associated with delivering tumor-suppressed ncRNAs [108, 109]. However, some studies also reported that the exosomes from MSC may promote tumor progression [110]. Thus, whether it is safe to use MSC-derived exosomes to treat cancer is uncertain. Besides, exosomal ncRNAs from another stem cells, human adult liver stem cell (HLSC) can also influence the growth of hepatoma. For example, HLSC-derived exosomes could inhibit hepatoma growth by transferring anti-tumor miRNAs to downregulate the genes involved in cell proliferation, e.g., Cyclin D1 targeted by miR-223, E2F-2 targeted by miR-31, MDR1, MIF, RAB14 targeted by miR-451, DHFR targeted by miR-24 [111]. In another study, exosomal miR-15a, miR-181b, miR-320c and miR-874 from HLSCs could decrease the expression of ITGB3, FGF1, EPHB4 and PLAU to inhibit tumor angiogenesis [112]. Thus, the anticancer therapy based on exosomes derived from normal stem cells may be one more choice.

Engineered exosomes with tumor-suppressed ncRNAs: target tumor cells or TME

Tumor-suppressed ncRNAs are downregulated in cancer, and overexpressing tumor-suppressed ncRNAs in tumor cells inhibit tumor progression. We can package desired tumor-suppressed ncRNAs into exosomes by electroporation or transfecting the exosome-producing cells (Fig. 3), and then use the engineered exosomes to transport tumor-suppressed ncRNAs to HCC cells to reverse their malignant phenotypes. Some positive results have been obtained. For example, Lou G et al. reported that miR-122-transfected

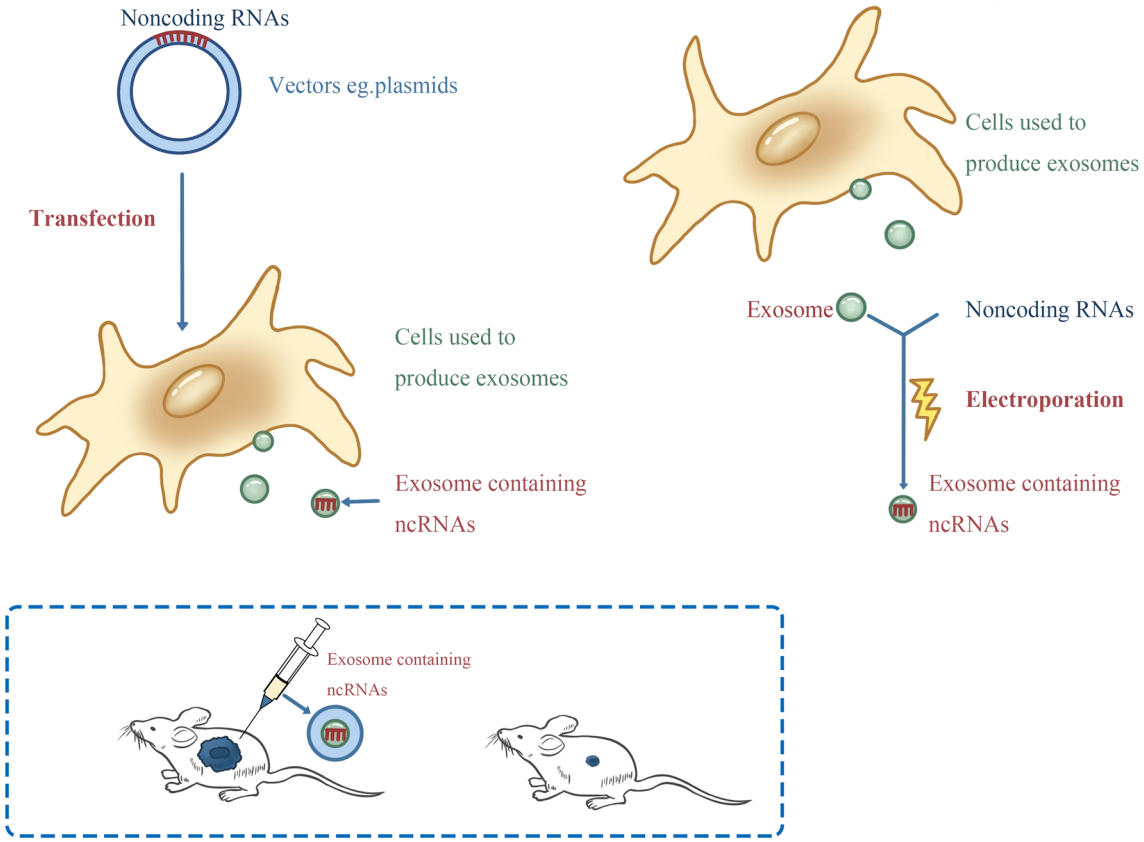

Fig. 3 The two strategies of loading ncRNAs into exosomes. It is promising to use engineered exosomes to deliver anti-tumor ncRNAs to treat HCC. Mesenchymal stem cells (MSC) are most frequently used cells to produce exosomes in the studies. We can use vectors that contain ncRNAs to transfect MSCs, and MSCs can package the ncRNAs into secreted exosomes. Additionally, we can also use electroporation to actively load ncRNAs into MSC-derived exosomes. In assays, researches have observed that the engineered exosome-containing anti-tumor ncRNAs can inhibit tumor growth

adipose mesenchymal stem cells (AMSCs) could effectively package miR-122 into secreted exosomes, and miR-122-loaded exosomes made HCC cells more sensitive to chemotherapeutic agents by repressing CCNG1, ADAM10 and IGF1R [113]. Liang G et al. used electroporation to actively load miR-26a to HEK-293T cell-derived exosomes, and miR-26a-loaded exosomes could inhibit HCC growth and invasion by inhibiting CCND2, CCNE2, CDK6 [114]. In another study, HSC-derived exosomes were loaded with miR-335-5p, and miR-335-5p-loaded exosomes could be taken up by HCC cells and inhibited HCC cell proliferation and invasion by targeting CDC42, CDK2, CSNK1G2, EIF2C2, EIF5, LIMaK1, NRG1, PLK2, TCF3, THBS1, YBX1, and ZMYND8 [115]. Moreover, it is feasible to use siRNA-loaded exosomes to treat cancer. For example, GRP78 is associated with sorafenib resistance, using the exosomes that encapsulated siRNA against GRP78 could suppress sorafenib resistance in HCC [116].

Since TME is essential for tumor progression, targeting the stromal cells, e.g., CAFs, endothelial cells and immune cells, to remodel TME help treat cancer. For example, overexpressing miR-320a in CAFs can repress HCC metastasis by targeting PBX3 [55]. TDEs and DC-derived exosomes (DEXs) are reported to present TAAs and elicit anti-tumor immunity, forming a new class of vaccines for cancer immunotherapy [79, 80, 117]. NcRNAs are critical regulators in the immune system, influencing the differentiation, maturation, expansion and function of immune cells [118]. Thus, ncRNA-modified exosomes can be used for immunotherapy in HCC. For example, specific ncRNAs (miR-155, miR-142, and let-7i) modified TDEs could target IL-6, IL-17, IL-1b, TGFβ, SOCS1, KLRK1, IFNγ, and TLR4 to induce DC maturation and enhance their immune stimulation ability [119]. These results all suggest a novel therapeutic approach for tumor treatment.

Conclusions and perspectives

In this review, we mainly summarized recent literatures on the biological functions of exosomal ncRNAs in HCC development and their clinical applications. Exosomes are small EVs

from most cell types and mediate signal transduction between different cells by transporting cargoes. NcRNA-carrying exosomes from tumor cells and stroma cells can modulate the interaction between HCC cells and their microenvironments, contributing to tumor progression by regulating specific aspects, including tumor metastasis, tumor angiogenesis, tumor immunity and drug resistance. It provides us a novel horizon of tumorigenesis and potential therapeutic targets. Due to aberrant expression and ability of reflecting disease state, circulating exosomal ncRNAs can serve as diagnostic or prognostic biomarkers for HCC. Moreover, it is a promising strategy to exploit exosomes as natural nanoparticle to deliver tumor suppressor ncRNAs to target HCC cells or TME for treatment.

However, there are still some difficulties remain to be overcome. First, for liquid biopsy, we need a standard method to isolate exosomes quickly, easily and specifically, and detect the dysregulated exosomal ncRNA rapidly and cheaply. And there is no proven biomarker in large sample and multi-center researches yet. Second, for studying biological functions, since most experiments are done in vitro and culture environment cannot imitate actual conditions, maybe the cargoes and their concentration are not identical to those in our body, thus, it is uncertain whether exosomal ncRNAs really have regulator functions in vivo as in vitro. Third, for therapy, the exosome-producing cells should be selected carefully to ensure the safety of treatment. Red blood cells (RBCs) were reported as potential exosome-producing cells since they were readily available in blood banks and devoid of DNA [120]. Further, we need to learn more mechanisms about exosomal biogenesis and design an optimal system to produce more exosomes to meet therapeutic demands. Moreover, a high-efficacy and safe technology of loading ncRNAs into exosomes needs to be developed. Last but not least, targeting strategies of exosomes need to be studied further to achieve efficient delivery and avoid side effects. In all, as our understanding of exosomes improves, using exosomes in the management of HCC will become a reality in some day.

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Compliance with ethical standards

Conflict of interest The authors have declared no conflicts of interest.

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