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Review Article

Tumor-associated Exosomes Are Involved in Hepatocellular Carcinoma Tumorigenesis, Diagnosis, and Treatment

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Abstract

Hepatocellular carcinoma (HCC) has become a challenging disease worldwide. There are still limitations in the diagnosis and treatment of HCC, and its high metastatic capacity and high recurrence rate are the main reasons for its poor prognosis. The ability of extracellular vesicles (EVs) to transfer functionally-active substances and their widespread presence in almost all body fluids suggest their unprecedented potential in the study of various cancers. The unique physicochemical properties of EVs determine their potential as antitumor vaccines and drug carriers. In the last decade, the study of EVs in HCC has evolved from a single hot topic to a system with considerable scale. This paper summarizes the role of EVs, especially exosomes, in the occurrence, metastasis and tumor immunity of HCC, reviews their applications in tumor diagnosis, prognosis and treatment, describes the pros and cons of these studies, and looks forward towards the future research directions of EVs in HCC.

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Keywords: Extracellular vesicles; Exosome; Hepatocellular carcinoma; Tumor formation and progression.

Abbreviations: AFP, alpha-fetoprotein; Akt, activating protein kinase B; AMSCs, adipose tissue-derived mesenchymal stem cells; CAFs, cancer-associated fibroblasts; CH, chronic hepatitis; CTCs, circulating tumor cells; DC, dendritic cell; Dex, dendritic cell-derived exosomes; ECM, extracellular matrix; EMT, epithelial to mesenchymal transition; ESCRT, endosomal sorting complex required for transport; EVs, extracellular vesicles; HCC, hepatocellular carcinoma; HSCs, hepatic stellate cells; HUVEC, human umbilical vein endothelial cells; lncRNA, long non-coding RNA; miRNA, microRNA; MVBs, multivesicular bodies; OS, overall survival; ROS, reactive oxygen species; TACE, transarterial chemoembolization; TAMs, tumor-associated macrophages; TMEs, tumor microenvironments; TETs, Tetmethylcytosine dioxygenases; Treg, regulatory T cell.

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world and the third leading cause of cancer death worldwide, with 840,000 new cases and at least 780,000 deaths each year. Due to the insidious onset of HCC and the limited effectiveness of current treatments, the prognosis of HCC is unsatisfactory.[1] Because the sensitivity and specificity of the classic blood diagnostic marker alpha-fetoprotein (AFP) are not satisfactory, new diagnostic markers have been proposed for the early diagnosis of liver cancer in recent decades.[2] Despite the limited benefit, hepatic resection, liver transplantation or the application of chemotherapy for unresectable HCC remain the main options to treat HCC. Moreover, surgical resection is more suitable for patients with isolated tumors, and limitations and drawbacks emerge when the number of tumors is high.[3] According to a recently published cancer statistics report, the survival rate of most common cancers has improved to some extent over the past four decades, but the prognosis of liver cancer is still poor. Due to its high metastatic capacity and recurrence rate, the 5-year survival rate has reached only 18%.[4] Therefore, there is an urgent need to identify effective, noninvasive, and specific biomarkers that can provide early identification of HCC. In addition, there is an urgent need to explore more biological therapies to provide new ideas for the diagnosis and treatment of HCC that will allow us to better understand disease progression and find better approaches to treat HCC.

Exosomes first came to researchers' attention in 1983, when it was discovered that reticulocyte-releasing exosomes could carry transferrin receptors into the extracellular space.[5] In the last 30 years, exosomes have gone from being initially thought of as a process by which cells dispose of waste products to being considered a new mechanism of intercellular communication, changing our view of the molecular mechanisms of intercellular exchange and disease progression. In recent years, the concept of exosomes has been further expanded. Many vesicles with similar functions and properties secreted by cells (including exosomes, microvesicles and apoptotic vesicles, etc.) are collectively referred to as extracellular vesicles (EVs). The increasing interest in the ability of EVs to alter the local and distant microenvironments during HCC progression has led to new perspectives on intercellular communication involving vari-

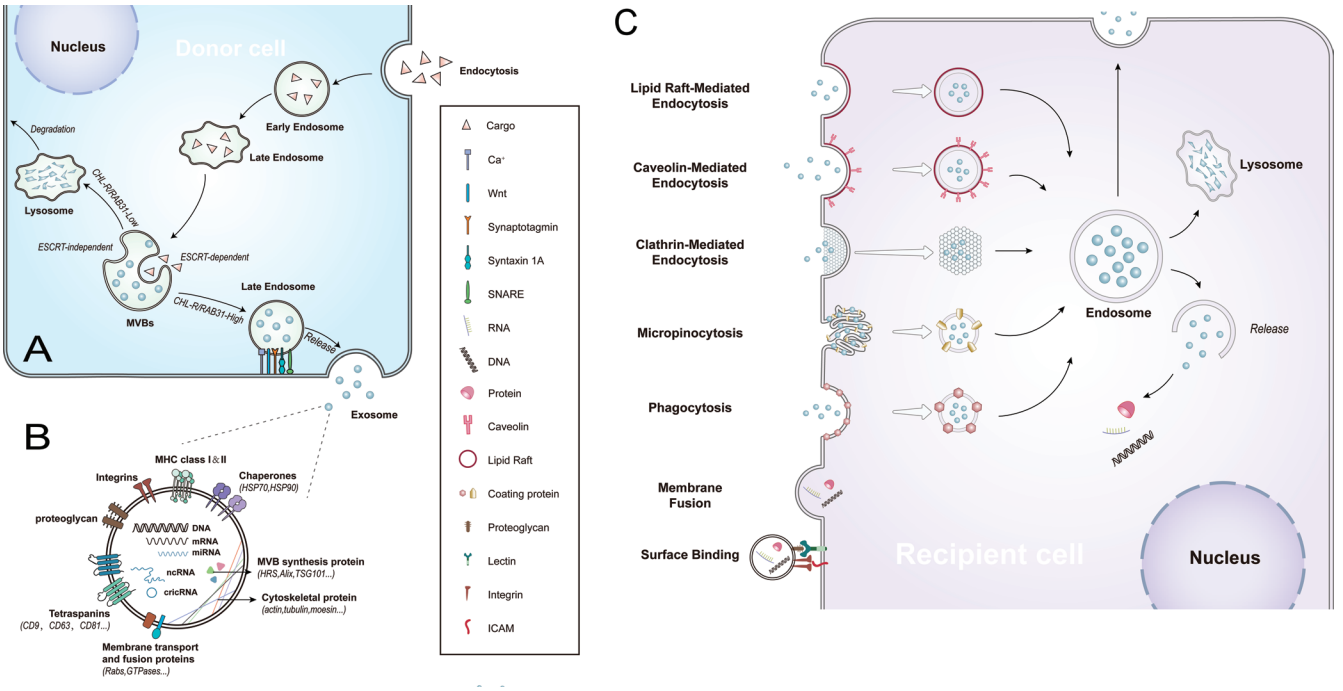

Fig. 1. Schematic diagram of the main mechanisms of exosome production, secretion and uptake. (A) Exosome formation. Endocytosis wraps material to form early endosomes; early endosomes continue to mature to form late endosomes; endosomal plasma membrane buds inward to form MVBs; sorting and intracellular transport of exosomes depend on the classical ESCRT-dependent pathway or ESCRT-independent pathway; MVB either binds to lysosomes to digest their contents of exosomes or binds to cells to release their contents of exosomes. (B) Major components and inclusions of exosomes. (C) Binding and uptake of exosomes. Exosomes recognize and bind to receptor cells to deliver specific signals by membrane surface binding or membrane fusion; exosomes enter target cells via macropinocytosis or phagocytosis pathways; and exosomes enter target cells via clathrin-, caveolae-, or lipid raft-mediated endocytosis.

ous biological functions and disease progression, thus enabling us to effectively address the ensuing clinical challenges.

Overview of exosomes

Exosomes are a class of EVs that are released by almost all human cells.[6] Humans circulate quadrillions of exosomes at all times. We can isolate exosomes from biological fluids, including plasma,[7] saliva,[8] urine,[9] and ascites.[10] The structure of exosomes was first described by Johnstone *et al*.[11] in 1989. Exosomes are disk- or cup-shaped, membrane-structured vesicles with a diameter of approximately 30–150 nm and having an electron microscopic density of 1.10–1.21 g/mL.[12] EVs include exosomes, microvesicles and apoptotic vesicles, and exosomes are the most widely studied among them. There are many substances that can be carried inside the exosome: proteins, lipids, nucleic acids, and inorganic salt ions.

Exosomes were first identified in 1983 in the supernatant of sheep erythrocytes cultured *in vitro*.[5] Initially, exosomes were thought to be excess membrane proteins released during cell maturation to regulate membrane function and to serve as organelles for the removal of cellular debris and the elimination of cell surface molecules. In 1996, Raposo *et al*.[13] discovered that exosomes secreted by B lymphocytes could present antigens to activate T lymphocytes, and since then, scientists have continued to recognize the function of exosomes.

The production, screening, secretion, release, and uptake of exosomes are regulated by specific signaling pathways.[14] Functionally, exosomes are considered key players in different biological processes in both physiological and pathological contexts. In recent years, an increasing number of studies have found that exosomes play a critical role in the regulation of tumor cell signaling pathways, early tumor molecular markers, prognostic factors, etc., and may even serve as carriers of genes and drugs.[15] These findings have provided new ideas and directions for the diagnosis and treatment of malignant tumors such as liver cancer.

The formation and secretion of exosomes involves several stages. (1) The cell membrane invaginates to form endocytic vesicles, and multiple endocytic vesicles fuse with each other to form early endosomes. (2) Early endosomes invaginate again and encapsulate intracellular material to form multiple intraluminal vesicles, which are further transformed into late endosomes or multivesicular bodies (MVBs). (3) After specific signaling pathway screening, MVBs either fuse with the cell membrane, releasing intraluminal vesicles into the extracellular space to form exosomes, or fuse with lysosomes, leading to digestion and degradation of contents in MVBs.[16,17]

Exosomes of different origins preferentially interact with specific types of receptor cells, resulting in exosomes of different tumor cell origins that can specifically accumulate in organs or tissues.[18] Exosomes are taken up by recipient cells in several ways. 1. Exosomes can recognize and remain on the surface of the recipient cell membrane and deliver specific signals.[19] 2. Exosomes enter recipient cells through endocytosis, phagocytosis and pinocytosis and bind to the endoplasmic reticulum or nuclear membrane to deliver informative material and perform important biological functions.[20,21] 3. Exosomes fuse with the plasma membrane of recipient cells and release their contents released directly into the cytoplasmic lysate. The mechanisms of exosome formation, secretion and uptake are described in Figure 1.

The sorting, intracellular translocation, release, and rec-

ognition of bound target cells of exosomal contents are a series of finely-regulated processes that require the involvement of many proteins. First, exosome formation and content sorting involve a series of endosomal sorting complexes required for transport (ESCRT) and Vps4; second, intracellular transport of exosomes involves numerous molecular switches, including RAB GTPase proteins and cytoskeletal proteins. These proteins determine whether the next destination of the MVB fuses with the cell membrane to release extracellular material or is digested by lysosomes.[22,23] Third, exosome binding to the target site requires binding recognition of specific proteins involving four tetraspanin proteins (CD9, CD63, CD81, CD151, etc.)[24] that are abundantly expressed on the exosome surface. Much of the exosome targeting to specific cell lines is mediated through protein receptors and adhesion molecules (tetraspanin, integrins, proteoglycans and lectins) enriched on the exosome surface.[25] Additional proteins that are labeled for use in the detection of exosomes include SNARE, apoptosis-linked gene 2-interacting protein X (also known as Alix), tumor susceptibility gene 101 (also known as TSG101),[26] and heat shock proteins (such as HSP90). In addition to a specific protein composition, exosomes also have a specific lipid composition. The exosome membrane is rich in cholesterol, ceramide and sphingomyelin, which are also involved in the formation and secretion of exosomes.[27] When we identify exosomes, we usually need to detect at least one positive transmembrane/lipid binding protein (CD9, CD63, CD83 or integrin) and one cytosolic protein recovered in EVs (ALIX, TSG101, syntenin or HSP70) and at least one negative protein (albumin, lipoprotein and ribosomal protein) level.[28]

The recognition, binding and uptake of exosomes are the basis for the accurate performance of their functions. Systematic analysis of the mechanism of exosome-cellular action will help to identify the function of specific exosomes in physiological and pathological settings and provide a more solid theoretical basis for exosomes as therapeutic targets and diagnostic markers for HCC.

Role of exosomes in HCC

HCC is characterized by dysregulation or dysfunction of multiple signaling pathways that mediate tumor behavior, local spread, and propensity for multifocal tumor growth. Numerous experiments have confirmed that exosomes could represent a contributory mechanism to liver carcinogenesis and promote the metastasis and progression of HCC by regulating the tissue microenvironment and multiple signaling pathways in cancer and normal cells. In addition, exosomes are directly involved in information sharing between tumor cells. Oncogenic molecules from tumor cells at the primary site can be transmitted via exosomes to different tumor cell subtypes in adjacent or even distant organs. In HCC, this mechanism of information sharing among tumor cells can often directly promote cancer cell proliferation or control cell death.

We summarize all studies related to exosomes in the development of HCC in Table 1.[29–58] The pattern of HCC-associated exosomes involved in various intercellular molecular interactions in the HCC tumor microenvironment (TME) is shown in Figure 2.

Cell growth

PTEN is an important oncogene, and its expression is generally decreased in tumors such as HCC. miR-21 downregulates PTENp1 expression by regulating the expression of TETs. Experiments showed that uptake of exosomal miR-21 derived from HCC cells by other HCC cells could significantly affect cell growth and promote HCC cell proliferation.[29] The exosomal long non-coding RNA (lncRNA) FAL1 was upregulated in the serum of HCC patients and transferred to HCC cells, which accelerated HCC cell proliferation and metastasis through competitive binding to miR-1236.[30] Golgi membrane protein 1 (also known as GOLM1/GP73) is a serum marker for HCC. Uptake of exosomal GOLM1 by HCC cells activates the GSK-3β/MMP signaling pathway, accelerating cell proliferation and promoting the progression of HCC.[31] Exosomes produced by HCC cells are transferred to surrounding adipocytes, activating the NF-κB pathway, inducing an inflammatory phenotype in adipocytes, increasing the synthesis of inflammatory mediators (such as IL-6, IL-8), and promoting the proliferation of HCC cells.[59] Adipose tissue release of exosomal circRNA targets deubiquitination-associated USP7 in HCC, inhibits miR-34a, promotes HCC proliferation, and stabilizes cellular DNA damage.[60]

Linc-RoR is a hypoxia-responsive lncRNA, aberrantly expressed in tumor cells. HCC cells deliver exosomal Linc-RoR to neighboring HCC cells under hypoxic conditions to neutralize miR-145 expression, thereby increasing the expression of HIF-1α mRNA. HIF-1a targets PDK1, which regulates mitochondrial function during hypoxia and thus resists hypoxia to allow cancer cell survival.[32]

Cell death

Some exosomes secreted by HCC cells also inhibit malignant biological behavior. HCC cells secrete specific types of exosomes to reach target cells, where cell death occurs by regulating the cell cycle and activating apoptosis. miR-122 is a micro RNA (miRNA) that can specifically inhibit liver cancer growth. Adipose tissue-derived mesenchymal stem cells (commonly referred to as AMSCs) produce exosomal miR-122, which is transduced into HCC cells and induces cell cycle G0/G1 arrest and apoptosis.[61] MiR-122 is released from the exosomes of HCC cells and can be taken up by other HCC cells lacking miR-122, inhibiting HCC growth by suppressing cell cycle progression. Meanwhile, recipient HCC cells also secrete insulin-like growth factor 1 (also known as IGF-1), which counteracts the expression of miR-122 in donor cells to ensure their own proliferation.[33] Cancer-associated fibroblasts (CAFs) secrete exosomal miR-320a that metastasizes to HCC, inhibits the activation of the MAPK pathway, and suppresses the cell cycle progression of HCC cells by binding PBX3.[34]

Neutral sphingomyelinase 1 (also known as NSMase1) is an enzyme that converts sphingomyelin (also known as SM) to ceramide (also known as Cer). Exosomal NSMase1 secreted by HCC cells can reduce the SM/Cer ratio of target cells, induce apoptosis through activation of the caspase-3 signaling pathway, and inhibit the growth of HCC.[35] Circ-0051443 upregulates BAK1 expression in HCC cells by competitively binding miR-331-3p, promoting apoptosis and arresting the cell cycle. Therefore, liver cancer exosome circ-0051443 can be used as a predictor and potential therapeutic target for HCC.[36]

Metastasis capability

In addition to directly promoting tumor volume, more metastatic HCC cells can also confer the ability to migrate and invade through exosomes to those cells with lower or no metastatic potential. CircPTGR1 is an exosomal circRNA specifically expressed in high metastatic potential hepatoma cells (i.e. LM3) and transferred via serum to pairs of nonmetastatic or low metastatic potential hepatoma cells.

Table 1. Cell biological behavioral changes and signaling pathways mediated by HCC-related exosomes

Molecular	Type	Source	Recipient cell	Type of function	Mechanism	Ref
miR-210	miRNA	Hepatoma cells	Endothelial cells	Angiogenesis	Inhibit SMAD4 and STAT6	50
miR-155	miRNA	Hepatoma cells	Endothelial cells	Angiogenesis	Not mentioned	51
lncRNA-H19	lncRNA	Hepatoma cells	Endothelial cells	Angiogenesis	Increase VEGF and ICAM1	52
NKG2D, HSP70	Protein	Hepatoma cells	Endothelial cells	Angiogenesis	Not mentioned	53
Vasorin	Protein	Hepatoma cells	Endothelial cells	Angiogenesis	Not mentioned	54
CLEC3B	Protein	Hepatoma cells	Endothelial cells	Angiogenesis	Activation of AMPK signal pathway	55
ANGPT2	Protein	Hepatoma cells	Endothelial cells	Angiogenesis	Up-regulate Akt/eNOS and Akt/ β-catenin pathways	56
miR-200b-3p	miRNA	Hepatoma cells	Endothelial cells	Inhibits angiogenesis	Upregulate ERG	57
CXCR4	Protein	Hepatoma cells	Lymphatic endothelial cells	Lymphangiogenesis	Enhance the secretions of MMP-9, MMP-2 and VEGF-C	58
miR-103	miRNA	Hepatoma cells	Endothelial cells	Vascular permeability	Inhibit VE-Cad, p120 and ZO-1	45
circRNA-100338	circRNA	Hepatoma cells	Endothelial cells	Vascular permeability	Decrease VE-cadherin and ZO-1 expression	47
miR-1247-3p	miRNA	Hepatoma cells	Fibroblast	CAFs	Downregulate B4GALT3 and activate β1-integrin/NF-κB axis	48
miR-21	miRNA	Hepatoma cells	Fibroblast	CAFs	Depress PTEN, upregulate PDK1/Akt pathway	49
linc-ROR	lncRNA	Hepatoma cells	Hepatoma cells	Antihypoxia	Neutralize miR-145 and activate linc-RoR–miR145–HIF-1a axis	32
NSMase1	Protein	Hepatoma cells	Hepatoma cells	Apoptosis	Decrease the ratio of sphingomyelin/ceramide	35
miR-122	miRNA	Hepatoma cells	Hepatoma cells	Cell cycle arrest	Not mentioned	33
miR-320a	miRNA	CAFs	Hepatoma cells	Cell cycle arrest	Binding of PBX3 inhibits MAPK pathway activation	34
circRNA-0051443	circRNA	Hepatoma cells	Hepatoma cells	Cell cycle arrest Apoptosis	UpregulatesBAK1 expression	36
circRNA-0004277	circRNA	Hepatoma cells	Hepatoma cells	EMT	Inhibition of ZO-1	39
circ-MMP2	circRNA	Hepatoma cells	Hepatoma cells	EMT	Sponging miR-136-5p	40
miR-32-5p	miRNA	Hepatoma cells	Hepatoma cells	EMT/angiogenesis	Suppress PTEN and activate PI3K/Akt pathway	41
circ-PTGR1	circRNA	Hepatoma cells	Hepatoma cells	Metastasis	Activate MET via interacting with miR-449a	37
S100A4	Protein	Hepatoma cells	Hepatoma cells	Metastasis	Activate STAT3	44
miR-21	miRNA	Hepatoma cells	Hepatoma cells	Proliferation	Suppress the TETs/PTENp1/PTEN pathway	29
lncRNA-FAL1	lncRNA	Hepatoma cells	Hepatoma cells	Proliferation metastasis	Suppress miR-1236 and upregulate ZEB1 and AFP	30
GOLM1	Protein	Hepatoma cells	Hepatoma cells	Proliferation metastasis	Activate GSK-3β/MMPs signaling axis	31
p120-catenin	protein	Hepatoma cells	Hepatoma cells	Proliferation metastasis	Inhibit STAT3 signaling	38
SMAD3	Protein mRNA	Hepatoma cells	Hepatoma cells	Promoted adhesion	Enhance TGF-β-SMAD3-ROS signal	42
miR-25-5p	miRNA	Hepatoma cells	Hepatoma cells	Enhanced invasive ability	Inhibit LRRC7 expression	46
LOXL4	Protein	Hepatoma cells	Hepatoma +endothelial cells	Promoted adhesion	Down-regulate PTEN and up-regulate Akt/Snail signaling pathway	43

ERG, erythroblast transformation-specific related gene.

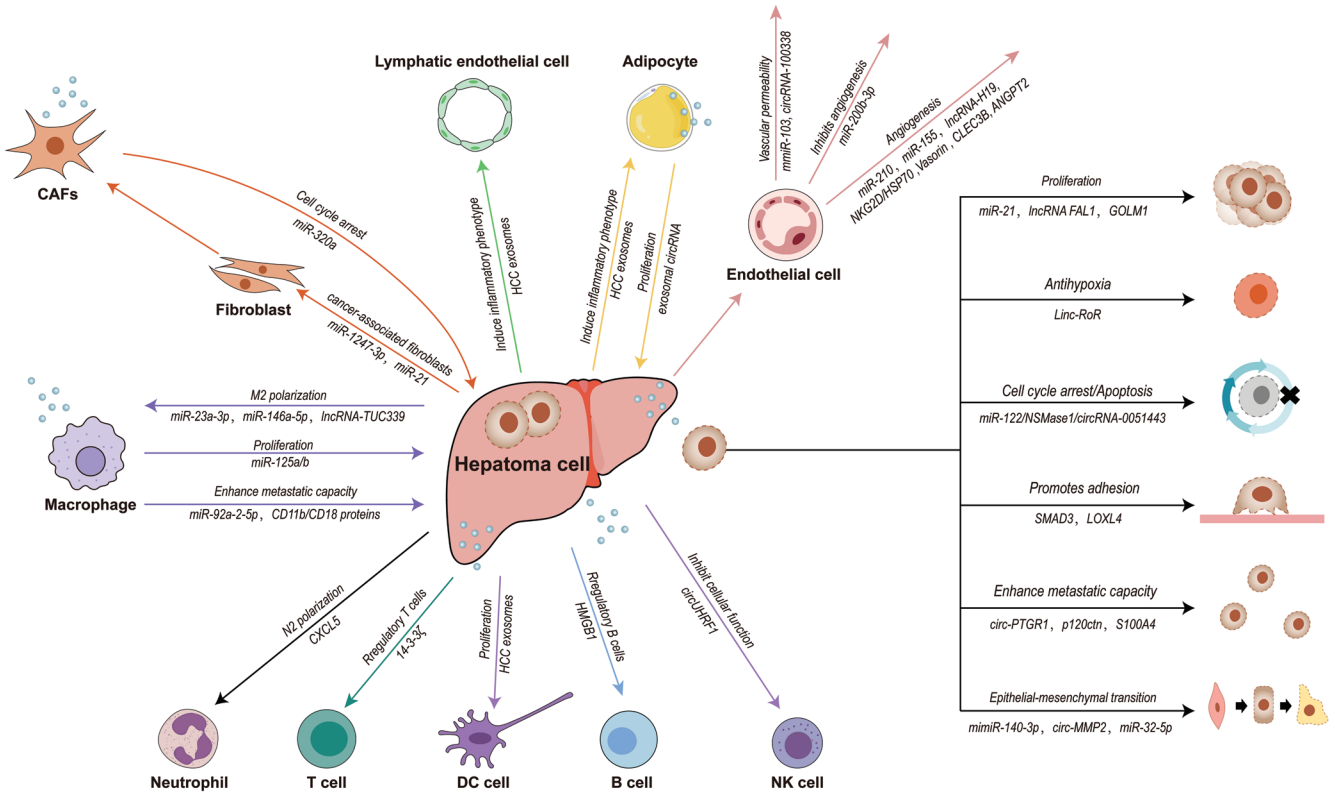

Fig. 2. Exosome network in the HCC microenvironment.

CircPTGR1 in recipient cells competes with MET (a receptor for hepatocyte growth factor) for targeting miR-449a to enable the migration and invasion of cells.[37] P120-Catenin (also known as p120ctn) is a member of the family of armadillo proteins that operate in cell adhesion and signal transduction. Exosome p120ctn secreted by HCC cells can inhibit the metastasis of HCC cells and the expansion of hepatic stem cells (HSCs) by inhibiting STAT3 signaling.[38]

EMT

Epithelial-mesenchymal transition (EMT) is a cell transformation biological process defined by the loss of epithelial characteristics and acquisition of a mesenchymal phenotype, giving cells the ability to metastasize and invade, and is involved in tumor progression and metastasis.[62] The loss of E-cadherin and the increase in vimentin indicate activation of EMT in human cancers, suggesting an association with tumor progression.[63] Exosomes circ-0004277 from HCC cells stimulate EMT in peripheral cells via cellular communication to further promote HCC invasion into normal surrounding tissues.[39] HCC cells secrete exosomal circ-MMP2 by acting as a molecular sponge for miR-136-5p, thereby promoting the development of EMT and metastasis in adjacent HCC cells.[40] In liver cancer cell lines and patient tissues, exosomal miR-32-5p activates the PI3K/activating protein kinase B (Akt) pathway by suppressing PTEN, leading to EMT and angiogenesis.[41] As a result, upregulation of miR-32-5p leads to a decrease in E-cadherin and an increase in vimentin, promoting EMT; however, in contrast, upregulation of miR-140-3p reverses EMT in HCC. Vps4A contributes to the sorting of proteins into exosomes. One study found that Vps4A regulates the exosomal sorting of β-catenin, thereby reducing β-catenin signaling and inhibiting EMT and metastasis of HCC.[64] Although Vps4A does not act directly on recipient cells, it exerts tumor-suppressive effects by affecting exosomal miRNA production and sorting in hepatoma cells. These findings provide new ideas for investigating the role of exosomes in HCC.

Premetastatic niches

In the more than 100 years since Stephen Paget's "seed and soil" hypothesis was formulated, much progress has been made in deciphering the mechanisms of organ-specific metastasis of tumor cells. Tumor cells secrete molecule-containing exosomes that are transported to specific distal organs and reconfigure their microenvironment upon arrival, achieving increased vascular permeability and adhesion of tumor cells and organ-specific implantation.

HCC cells released exosomes containing SMAD family member 3 (also known as SMAD3) protein and mRNA, activating the TGF-β-SMAD3-reactive oxygen species (ROS) signaling pathway in receptor tumor cells. ROS promote adhesion and distant colonization of CTCs by regulating adhesion molecules.[42] The exosomal LOXL4 produced by HCC enables tumor cells to form stable adhesions to the ECM by activating the FAK/Src pathway. These studies provide new evidence for the formation of premetastatic niches in HCC.[43] Integrins on tumor exosomes determine the organoleptic nature of metastasis and activate Src phosphorylation and S100 gene expression in receptors to stimulate prosurvival pathways and establish an inflammatory environment. The establishment of an inflammatory environment in specific distant organs contributes to the specific colonization of tumor cells. Exosomal S100A4 was secreted by highly meta-

static hepatoma cells, which significantly enhanced the *in vitro* invasion and *in vivo* metastasis of hepatoma cells with low metastasis. Exosomal S100A4 upregulates OPN expression through activation of STAT3 phosphorylation.[44]

Some exosomes make it easier for tumor cells to break through the vascular endothelium by affecting vascular permeability or make it easier for circulating tumor cells (CTCs) to be implanted in distant organs. Therefore, increased vascular permeability is also an important part of the premetastatic niches. Exosomal miRNA-103 secreted by hepatoma cells increases vascular permeability. Mechanistic studies have revealed that miR-103 secreted by hepatoma cells can be delivered to endothelial cells via exosomes, which then attenuate the integrity of the endothelial junction by inhibiting the expression of VE-cadherin (also known as VE-Cad), p120-catenin (also known as p120), and zonula occludens 1 (also known as ZO-1).[45] Exosomal miR-25-5p enhances the movement of HCC cells into endothelial cells by inhibiting the expression of leucine-rich repeat-containing protein 7 (also known as LRRC7), a protein associated with cell adhesion and migration. These tumor cells enter the blood circulation and become CTCs. Tumor self-seeding occurs when circulating malignant cells reinfiltrate the original tumor. This process may give rise to more aggressive tumor cells, which may contribute to the progression of cancer.[46] HCC cells secrete exosomal circRNA-100338 for delivery to vascular endothelial cells, which decreases VE-cadherin and ZO-1 expression in vascular endothelial cells, thereby disrupting the tight junctions between cells, increasing the permeability of vascular endothelial cells, and promoting hematogenous metastasis of HCC.[47]

CAFs

Intercellular crosstalk between tumor cells and fibroblasts can be mediated by HCC-derived exosomes. We found that HCC cells can transform normal HSCs into CAFs. CAFs create a suitable "soil" for tumor origination and secrete numerous growth factors promoting tumor growth and angiogenic factors promoting tumor angiogenesis. In addition, CAFs attract numerous inflammatory cytokines and secrete a great number of soluble products promoting tumor cell invasion and metastasis. HCC cells secrete exosomal miR-1247-3p, which directly targets B4GALT3 and leads to activation of β1-integrin-NF-κB signaling in fibroblasts. Activated CAFs further promote the progression of HCC lung metastasis by secreting proinflammatory cytokines, including IL-6 and IL-8.[48] Exosomal miRNA-21 secreted by HCC cells directly targets PTEN, resulting in activation of PDK1/Akt signaling in HSCs. Activated CAFs further promote cancer progression by secreting angiogenic cytokines, including VEGF, MMP2, MMP9, bFGF, and TGF-β.[49]

Angiogenesis

During the progression of tumor development, tumor cells can affect different changes in vascular endothelial cells through the exosomal pathway. This process involves many biological events, such as ECM degradation, vascular endothelial cell proliferation and migration, lumen formation, and increased permeability. With the rapid increase in tumor size and insufficient blood supply to the tumor, cells inside HCC are often in a hypoxic state. Stimulated by the hypoxic emergency, tumor cells promote angiogenesis by secreting exosomes to activate epithelial signaling pathways in response to hypoxic stress.[65]

Exosomal miR-32-5p is overexpressed in HCC tissues, and targeting endothelial cells to inhibit PTEN expression activates the PI3K/Akt pathway, thereby inducing angiogenesis.[41] HCC cell-secreted exosomal miR-210 may be transferred into endothelial cells and thereby promote tumor angiogenesis by inhibiting the expression of SMAD4 and STAT6.[50] The secretion of exosomal miR-155 by HCC cells under hypoxic conditions promotes the microvascular formation of human umbilical vein endothelial cells (HUVECs), and exosomal miR-155 may affect angiogenic activity in HCC.[51] Exosomal lncRNA H19 released from CD90+ hepatoma cells acts on endothelial cells to upregulate VEGF expression and promote angiogenic phenotypes and intercellular adhesion.[52] Exosomal HSP70 and NKG2D produced by HCC cell lines induce endothelial HUVECs to form vascular lumens.[53] Vasorin (also known as VASN) is a type I transmembrane protein that plays an important role in tumor development and angiogenesis, and HepG2-derived VASN can be transferred to HUVECs through receptor-mediated exocytosis to promote their proliferation and thus enhance neovascularization.[54] The downregulation of CLEC3B in exosomes suppresses VEGF secretion in both HCC cells and ECs and eventually inhibits angiogenesis. Mechanistically, CLEC3B-mediated VEGF expression in tumor cells and ECs depends on the activation of the AMPK signaling pathway.[55] The similarly secreted exosomal ANGPT2 by HCC cells can induce vascular endothelial cell proliferation and tumor angiogenesis.[56] Transfer of exosomal miR-200b-3p from hepatocytes to vascular endothelial cells inhibits the expression of erythroblast transformation-specific related genes and reduces tumor angiogenesis. The expression of miR-200b-3p and secretion of exosomal miR-200b-3p are usually downregulated in HCC tissues, and studies on miR-200b-3p may be a new target against tumor angiogenesis.[57]

In addition to angiogenesis, some studies have found that exosomes can also promote lymphatic tract proliferation, making liver cancer cells more susceptible to lymphatic pathway metastasis. Exosomal CXCR4 from HCC cells increased the lymphatic endothelial cell proliferative rate and lymphatic tube formation ability and was shown to promote lymphatic metastasis of liver cancer cells by increasing the secretion of MMP-9, MMP-2, and VEGF-C.[58]

Immune regulation

In HCC, exosomes are closely associated with the tumor immune microenvironment in addition to their powerful ability to regulate tumor metastasis. The inherent immune tolerance properties of normal liver and the loss of immune surveillance function in the TME of HCC are key reasons for the high malignancy and low survival rate of HCC. Liver cancer cells reshape the TME through various mechanisms to evade immune surveillance and eventually promote tumor proliferation and metastasis. This tumor cell immune escape pathway can be achieved either by exosomes to activate a specific protumor immune response or by direct immune suppression (Table 2).[66–76]

The protumor immune response is the activation and promotion of regulatory T cell (Treg) function by exosomes derived from HCC cells. Tregs also block antitumor immune responses, resulting in immunosuppression. The exosome 14-3-3ζ protein is secreted by HCC cells that is taken up by tumor-infiltrating T lymphocytes (TILs), resulting in a shift in the direction of primary T cell differentiation from effector T cells to Tregs. Tregs suppress excessive immune responses by expressing CTLA4 and secreting IL-10 and TGF-β, and this immunosuppressive property coincides with the promotion of tumor cell immune escape.[66]

B cells can also be activated by internalization of tumor-derived exosomes. Regulatory B cells (also known as Bregs) accumulate in the tumor environment to express IL-10 in

Table 2. Biological roles of HCC-related exosomes among immune cells

Molecular	Type	Source	Recipient cell	Type of function	Mechanism	Ref
14-3-3ζ	Protein	Hepatoma cells	T cells	Tregs	Not mentioned	66
HMGB1	Protein	Hepatoma cells	B cells	Bregs	Activate TLR-MAPK pathway	67
miR-23a-3p	miRNA	Hepatoma cells	Macrophage	TAMs	Inhibit PTEN expression and active Akt	68
miR-146a-5p	miRNA	Hepatoma cells	Macrophage	TAMs	Activate NF-κB signaling	69
TUC339	lncRNA	Hepatoma cells	Macrophage	TAMs	Not mentioned	70
miR-92a-2-5p	miRNA	Macrophage	Hepatoma cells	TAMs	Suppress the PHLPP/p-Akt/β-catenin axis	71
αMβ2-integrin	Protein	macrophage	Hepatoma cells	TAMs	Activate the MMP-9 signaling pathway	72
miR-125a/b	miRNA	macrophage	Hepatoma cells	TAMs	Decrease CD90 expression	73
CXCL5	Protein	Hepatoma cells	Neutrophils	TANs	Not mentioned	74
circUHRF1	circRNA	Hepatoma cells	NK cell	Immunosuppression	Decrease the expression of mir-449c-5p inhibit IFN- γ and TNF- α secretion	76
miR-92b	miRNA	Hepatoma cells	NK cell	Suppress cytotoxicity	Downregulate CD69	75

NK, natural killer; TAN, tumor-associated neutrophil.

large numbers, and thus immunosuppression of tumors occurs. The HCC-derived exosome HMGB1 activates the TLR-MAPK pathway in B cells, promotes TIM-1(+) B cell expansion, suppresses CD8(+) T cell activity, and increases the expression of the immunosuppressive cytokine IL-10.[67]

In addition to Tregs, tumor cell-derived exosomes also stimulate the differentiation of tumor-associated macrophages (TAMs) toward the M2 phenotype. As an important component of the tumor stroma, M2-type TAMs induce immunosuppression and promote tumor cell growth, metastasis, tumor angiogenesis, and stabilization. HCC cells can release exosomes under conditions of endoplasmic reticulum stress. Some of these exosomes are taken up by TAMs and contribute to macrophage M2 polarization through different pathways. For example, exosomal miR-23a-3p increases the expression of PD-L1 and inflammatory cytokines by inhibiting PTEN expression and Akt, thereby inducing the conversion of macrophages to the M2 type.[68] Exosomal miR-146a-5p remodels macrophages by activating NF-κB signaling and inducing proinflammatory factors, leading to M2-polarized TAMs.[69] Lastly, HCC-derived exosomal lncRNA-TUC339 targets macrophages near tumors in the environment and promotes macrophage activation and M2 polarization.[70] Simultaneously, exosomes derived from M2-type TAMs also accelerated the progression of HCC. Macrophage secretion of exosomal miR-92a-2-5p transferred to HCC cells targets the androgen receptor, inhibits androgen receptor translation, alters the PHLPP/p-Akt/β-catenin signaling pathway, and increases the invasiveness of HCC cells.[71] Exosome-mediated transfer of functional CD11b/CD18 proteins from TAMs to tumor cells may contribute to the migration potential of HCC cells.[72] TAM exosomes with low levels of miR-125a/b may promote HCC cell growth and stem cell properties.[73] The discovery of a new communication mechanism between TAMs and HCC cells provides a new target to treat HCC.

Similar to macrophages, tumor-associated neutrophils exert protumorigenic effects in the TME. HCC-derived exosomes promote neutrophils to undergo pretumorigenic N2 polarization. TGF-β-positive HCC cells increased the secretion of exosomal CXCL5, inducing the infiltration of N2 neutrophils and further stimulating the proliferation of HCC cells.[74]

HCC cells release exosomes carrying multiple antigens for presentation to dendritic cells (DCs). Using liver cancer cell exosome pulses to stimulate DCs resulted in the proliferation of DCs, an increased number of T lymphocytes at the tumor site, increased levels of IFN-γ, and decreased levels of IL-10 and TGF-β, achieving tumor growth inhibition.[77]

NKG2D is an activating receptor expressed mainly on the surface of natural killer cells and plays an important role in cancer immunosurveillance. Exosomes secreted by HCC cells can induce downregulation of NKG2D on the surface of natural killer cells, leading to impaired cytotoxic function and favoring immune escape and progression of HCC.[78] Exosomal miR-92b produced by HCC cells enhances the metastatic ability of HCC by inhibiting CD69 on natural killer cells.[75] The expression of circUHRF1 was higher in HCC cell tissues than in paraneoplastic tissues. Exosomal circUHRF1 secreted by HCC cells inhibits natural killer cell secretion of IFN-γ and TNF-α, leading to natural killer cell dysfunction. Degradation of miR-449c-5p upregulates TIM-3 expression to inhibit natural killer cell function, driving resistance to anti-PD1 immunotherapy in HCC patients.[76]

Potential of exosomes as biomarkers for HCC

Biomarkers are a class of indicators found in blood, body fluids, and tissues that can reflect physiological processes, pathological processes, or therapeutic interventions by drugs. Biomarkers are widely used in disease screening, diagnosis, prognosis, and treatment monitoring. Due to the lack of early symptoms of HCC, the low sensitivity and specificity of existing laboratory tests, such as AFP for HCC screening,[79] and the difficulty in detecting early tumors by imaging, HCC, as a tumor with a high degree of malignancy, rapid progression and poor prognosis, patients often miss the optimal treatment period before diagnosis. Exosomes are widely found in blood, urine, saliva and other body fluids. The ease of extraction and the large amounts extracted suggest that exosomes are suitable as a noninvasive biomarker for cancer detection. The level of molecules contained in exosomes often varies among different tumors,

Table 3. Exosomes as a biological marker for HCC

Biomarker	Type	Biomarker applications	Function type	Ref
miR-21↑	miRNA	Significantly and positively correlated with tumor stage	Diagnosis	81
miR-93↑	miRNA	Significantly correlated with HCC tumor stage, size, and patient OS	Diagnosis Prognosis	82
miR-665↑	miRNA	Overexpression is associated with short survival	Diagnosis Prognosis	83
miR-92b ↑	miRNA	Predictor of HCC recurrence	Monitoring	75
miR-718↓	miRNA	Suppresses cell proliferation, predictor of HCC recurrence	Monitoring	84
miR-122↓	miRNA	Evaluation of treatment effect indicators	Predictive	85
miR-638↓	miRNA	Predicting OS	Prognosis	86
miR-125b↓	miRNA	Prognostic biomarker for HCC	Prognosis	87
miR-9-3p↓	miRNA	Contribute to early HCC detection and diagnosis	Screening	88
miR-10b-5p↑	miRNA	Potential biomarker for early-stage HCC	Screening	89
lncRNA-HEIH↑	lncRNA	Potential biomarker for early-stage HCC	Screening	90
lnc-FAM72D-3↑ lnc-EPC1-4↓	lncRNA	Potential biomarkers for HCC diagnosis	Diagnosis	91
LINC00161↑	lncRNA	Promote tumor migration and invasion	Diagnosis Prognosis	92
ENSG00000258332.1↑ LINC000635↑	lncRNA	Elevation related to metastasis and worse OS	Prognosis	93
miR-21↑ lncRNA-ATB↑	lncRNA	Faster progress and shorter OS of HCC	Prognosis	94
lncRNA-FAL1↑	lncRNA	Promote proliferation and migration	Prognosis	30
ENSG00000248932.1↑ ENST00000440688.1↑ ENST00000457302.2↑	lncRNA	Diagnosis of HCC and dynamic monitoring of HCC metastasis	Diagnosis Monitoring	95
circPTGR1↑	circRNA	Positively correlated with tumor stage, indicating a poor prognosis	Prognosis	37
mRNA-hnRNPH1↑	mRNA	Diagnosis of HCC, Child-Pugh classification, metastasis, TNM stage and OS	Diagnosis Prognosis	96
LG3BP↑ PIGR↑	protein	Diagnosis between intrahepatic CCA and HCC	Diagnosis	97
CAP1	protein	Monitoring metastasis and recurrence of liver cancer	Monitoring	98
miR-140-3p↓ miR-30d-5p↓ miR-29b-3p↓	miRNA	Biomarkers for predicting HCC cell migration and prognosis	Prognosis	99

CCA, cholangiocarcinoma.

and this variation is more pronounced when compared to that in healthy people. This difference can also be found in different stages of development of the same tumor disease. In addition, the environment inside the exosome is relatively simple and stable compared to the complex conditions of tissues and cells, and the exosome can deliver various biological effector molecules to the desired target through the blood.[80] With the rapid development of research at the exosomal molecular level, diagnostic applications of exosomes in HCC have an optimistic future. This review summarizes the various exosomes as relevant biological markers in HCC (Table 3).[30,37,75,81–99]

Due to the widespread presence of miRNAs in exosomes that are stable and difficult to degrade, miRNAs are most likely to be potential HCC biomarkers. The literature shows that serum exosome miR-21 levels are significantly higher in patients with HCC than in patients with chronic hepatitis (CH) B and liver cirrhosis and that elevated serum exosome miR-21 levels are positively correlated with tumor stage. In addition, the sensitivity of the serum miR-21 level assay was much lower than that of serum exosomal miR-21.[81] Therefore, serum exosomal miR-21 can be used as a potential biomarker for the diagnosis of HCC. The levels of serum exosomal miR-93[82] and miRNA-665[83] were significantly higher in patients with HCC than in healthy subjects, suggesting a positive correlation with tumor size, clinical stage, local invasion and metastasis. In addition, overexpression of exosomal miR-93 and miRNA-665 uniformly showed shorter survival times in patients with HCC, suggesting that serum exosomal miRNAs can be used both for diagnosis and as an independent indicator of liver cancer prognosis.

Some exosomal miRNAs can be used for postoperative monitoring for HCC recurrence due to significant differences in expression in patients with tumor recurrence. Serum exosomal miR-92b was significantly elevated in patients with HCC. The level of miR-92b will decrease after living donor

liver transplantation. If miR-92b is maintained at a higher level at 1 month after living donor liver transplantation, post-transplant HCC recurrence has occurred. Therefore, miR-92b has great potential as a predictor of HCC recurrence.[75] Exosomal miR-718 can also be used as a monitoring indicator of liver cancer recurrence posttransplantation. Decreased serum exosomal miR-718 in patients after liver transplantation often indicates liver cancer recurrence. HOXB8 is a target gene of miR-718, as has been shown in HCC patients;[84] suppression of miR-718 resulted in upregulation of HOXB8 expression and a poor prognosis for HCC patients. A study confirmed that miR-122 after/before transarterial chemoembolization (commonly referred to as TACE) (miR-122 ratio) in liver cirrhosis patients was significantly associated with patient prognosis. The higher the miR-122 ratio, the longer the survival of the patient. Alterations in exosomal miR-122 levels may represent a predictive biomarker for cirrhotic patients treated with TACE.[85] Some exosomal miRNAs often help predict disease outcomes before treatment. Low expression of miR-638 in serum exosomes of patients with HCC before treatment usually indicates a short overall survival (OS).[86] Due to the stable presence of exosomal miRNAs in the blood, exosomal diagnostic markers have an advantage over even the classical serum marker AFP. Several studies have confirmed that the sensitivity and specificity of exosomal miR-125b,[87] miR-9-3p,[88] and miR-10b-5p[89] for the diagnosis of HCC patients are significantly better than those of conventional serum AFP.

After exosomal miRNAs, a number of exosomal lncRNAs have been found to have potential value as biological markers. The expression of lncRNA-HEIH was significantly increased in hepatitis C virus-associated HCC compared with CH C patients,[90] suggesting that indicators such as exosomal lncRNA-HEIH and serum exosome ratio are better biomarkers for early screening of HCC. Lnc-FAM72D-3 was highly expressed in HCC, and in contrast, lnc-EPC1-4 functioned as an oncogene repressor. Statistical analysis revealed significant differences in the expression of lnc-FAM72D-3 and lnc-EPC1-4 in HCC development, which may help identify potential diagnostic biomarkers for HCC.[91] A series of studies found that serum exosomes LINC00161,[92] ENSG00000258332.1, LINC000635,[93] lncRNA-ATB,[94] and lncRNA-FAL1[30] were significantly elevated in HCC patients compared with CH patients. The expression of these exosomal molecules was negatively correlated with patient OS. Therefore, future studies of exosomal lncRNAs as independent biomarkers for the diagnosis and prognosis of HCC are warranted. The expression of ENSG0000248932.1, ENST0000440688.1 and ENST0000457302.2 was higher in HCC than in CH patients and cancer-free controls. The three lncRNAs combined with AFP values had higher predictive sensitivity and specificity for the development of HCC and metastasis of HCC.[95] Therefore, an increasing number of recent studies prefer to combine several exosomes or even traditional serum diagnostic markers to improve the accuracy and sensitivity of the diagnosis.

In recent years, an increasing number of studies have shown that exosomal circRNAs, mRNAs, DNA, and proteins released into serum play an important role in the development and subsequent treatment of HCC and have also been used as molecular markers for the early diagnosis, therapeutic evaluation, and prognosis of HCC. Serum expression of exosomal circPTGR1 was significantly increased in HCC patients and positively correlated with tumor stage, indicating a poor prognosis.[37] The combination of exosomal mRNA-hnRNPH1 and AFP further improves the differentiation of HCC patients in Child-Pugh staging, portal vein tumor emboli, lymph node metastasis, TNM staging, and OS.[96] Exosomal proteins may be used in the differential diagnosis of liver cancer and related diseases. In HCC, both exosomal proteins LG3BP and PIGR showed higher diagnostic capacity than AFP, and the AUG values of serum exosomal LG3BP (AUG, 0.904) and PIGR (AUG, 0.837) were higher than those of serum AFP(AUG,0.802). In addition, elevated exosomal LG3BP is significantly different in patients with cholangiocarcinoma and HCC and can be used to differentiate HCC from related diseases.[97] In addition, a study used protein profiling to identify 129 proteins present in HCC exosomes, many of which are significantly differentially expressed in different phenotypes associated with HCC. Among them, adenylate cyclase-associated protein 1 (also known as CAP1) is widely present in the exosomes of HCC cells. HCC cells with high metastatic capacity produce exosomal CAP1 with significantly increased expression, so investigators believe that exosomal CAP1 may predict metastasis and recurrence of HCC.[98]

In summary, as an important part of "liquid biopsy," exosomes have great application prospects in the precise diagnosis and treatment of diseases. Exosomes are carriers of macromolecular substances and play an important physiological role in the process of information exchange and signal transduction in cells of the body. The advantages of high stability, rich content, noninvasiveness and rapid detection make EVs promising as novel circulating biomarkers with potential applications for clinical disease adjuvant diagnosis. In cancer diagnosis, the application of exosome biomarkers requires high sensitivity and specificity. Many exosomal biomarkers with extracellular miRNAs or proteins are considered potential biomarkers, but these candidates are not as sensitive and specific as classical serum biomarkers and most of them cannot predict prognosis. Marker development has the qualities of a long development cycle and a low success rate. If we can obtain a large number of potential exosome markers by using advanced high-throughput technology screening, learn from the experience of existing marker development, improve the reproducibility of exosome marker research results, and establish a laboratory quality management system for exosome markers, we will effectively improve the diagnostic accuracy and greatly promote the efficiency of clinical translation of exosome diagnostic markers.

Exosome involvement in drug resistance in liver cancer

Sorafenib is a first-line targeted drug for the treatment of HCC and is effective in prolonging the survival of patients with advanced HCC. However, HCC is highly resistant to chemotherapy, which poses a great challenge to the pharmacological treatment of liver cancer. In two ways, exosomes are involved in the study of drug resistance in HCC. Exosomes released from tumor cells can help cells excrete cytotoxic drugs and thus participate in the inhibition of apoptosis; drug-sensitive cells can also develop drug resistance by absorbing exosomes from drug-resistant cells.

First, exosomes produced by HCC cells can activate the HGF/c-Met/Akt signaling pathway in hepatocytes, inhibiting sorafenib-induced apoptosis, thereby leading to drug resistance.[100] The chemotherapeutic drug sorafenib increases exosomal linc-ROR expression in HCC cells. TGF-β selectively enriches exosomal linc-ROR and inhibits p53 expression, thereby reducing apoptosis and decreasing the sensitivity of HCC cells to sorafenib. These findings implicate extracellular vesicular lncRNA as a mediator of the chemotherapeutic response and support targeting linc-ROR to improve chemosensitivity in HCC.[101] Exosome circRNA-SORE is transported between HCC cells and plays an important role in sorafenib resistance by binding to the oncogenic protein YBX1 and preventing YBX1 degradation.[102] Second, specific pumping of anticancer drugs from tumor cells also contributes

to multidrug resistance. Expression of exosomal linc-VLDLR was increased in HCC cells in response to sorafenib. Uptake of exosomal linc-VLDLR by neighboring cells increased the expression of ABCG2. This protein is a member of the ATP-binding cassette (also known as ABC) transporter superfamily, which is involved in drug export and can lead to specific excretion of chemotherapeutic drugs and reduced sorafenib-induced cell death in HCC cells.[103] These findings provide new insights into the role of EVs and the lncRNA-mediated chemostress response during chemotherapeutic drug treatment in HCC and could be a target for HCC resistance studies.

Prospects of exosomes as a treatment for liver cancer

Some cell-to-cell transmissions of exosomes exert tumor-suppressive effects. Many studies have found that exosomes exhibit more potent tumor-suppressive effects than cellular lysates, and exosomes are even used as biovehicles in clinical cancer treatment, including liver cancer. In response to the role and mechanism of exosomes in tumor cells, several strategies for exosome involvement in the treatment of HCC are described below, and the potential value and practical value of exosome treatment for HCC are discussed.

Such applications of exosomes as novel drug delivery vehicles have been extensively investigated. MiR-122 expression in HCC cells inhibits tumor cell growth, invasion, and tumor formation and can make these cells sensitive to chemotherapeutic agents, such as adriamycin and sorafenib.[104] Application of the miR-122 plasmid transfected with AMSCs for 48 h resulted in the production of large amounts of exosomal miR-122. Intratumor injection of exosomal miR-122 significantly enhanced the sensitivity of HCC cells to sorafenib.[61] A study conducted in rats found that the proliferation, migration and metastasis of HCC cells were significantly inhibited after tail vein injection of miR-320a-containing exosomes. The confirmation of *in vivo* and *in vitro* experiments increases the prospect of using exosomal miR-320a for the treatment of HCC.[34] MiR-335-5p inhibits the growth and invasion of HCC cells in both *ex vivo* and *in vivo* experiments. In one study, the authors injected exosomes containing miR-335-5p into HCC tumors and found that such an approach induced tumor growth arrest.[105] Intratumor injection of exosomes is similar to TACE and is a safe and effective drug delivery model along with complete intravenous administration. Exosomes have many advantages as novel carriers of therapeutic drugs; for example, they are stable, bypassing the hepatic immune environment and remaining stable in the circulatory system, they can penetrate tissue membranes and even the blood-brain barrier (BBB), and they have better targeting and thus higher efficacy.

Several other studies have found that secretion of miRNA-carrying exosomes by HCC cells can promote tumor progression. They designed a nanoparticle containing small interfering RNA to downregulate sphingosine kinase 2 (also known as Sphk2). Nanoparticle-induced Sphk2 gene silencing in HCC cells could reduce the secretion of exosomal miRNA-21, thus contributing to the inhibition of tumor cell migration and the tumorigenic function of exosomes on normal hepatocytes.[106] Melatonin is a well-known hormone with certain cytotoxic and immunomodulatory effects that inhibit tumor function. The application of melatonin-treated HCC cell-derived exosomes (termed in that study as Exo-MT) downregulated the expression of PD-L1 and attenuated the secretion of inflammatory cytokines, such as IL-6, IL-10, IL-1β, and TNF-α. These findings provide a new avenue by which to study the altered immunosuppressive state of macrophages.[107] Vps4 is a key regulator in exosome genesis and sorting. Vps4A selectively encapsulates oncogenic miR-27b-3p and miR-92a-3p into exosomes, secretes them out of cells, and accumulates oncogenic miR-193a-3p, miR320a and miR-132-3p in HCC cells. Experiments have confirmed that overexpression of Vps4A leads to inactivation of the PI3K-Akt pathway and thus inhibits cell growth, migration and invasion.[108] The design of therapeutic drugs for HCC from the perspective of exosome synthesis, sorting and uptake mechanisms is also a valuable research direction.

Dendritic cell-derived exosomes (Dex) has shown comparable efficacy to mature DCs in stimulating antigen-specific T cell activation *in vivo*, and therefore many studies have identified Dex as the most promising vaccine candidate for tumor-associated exosomes. Research on Dex tumor vaccines has developed rapidly in recent years and the first generation of therapeutic vaccines designed and developed using Dex have shown good tumor suppression and biosafety in mouse models, as well as low adverse effects.[109] Even this vaccine has been used in clinical trials in patients with advanced non-small cell lung cancer.[110] In addition to Dex, the researchers used ascites from colorectal cancer patients to isolate and extract exosomes for clinical trials of immunotherapy with autologous-derived exosomes. The researchers found that some patients showed a significant increase in the activity of natural killer cells in their bodies after treatment, suggesting the feasibility of special cell-derived exosomes modified as immunotherapeutic agents.[111] The application of exosomes to liver cancer tumor vaccines holds great promise for future research.

Conclusions, questions and future prospects

Exosomes are widely distributed in the body and carry various secretory cell-derived bioinformatically-predicted molecules, which can be circulating markers with clinical diagnostic value. Compared with histopathological examination, blood and fluid-based exosomal assays are highly acceptable to patients, easily monitored, and more reflective of the overall disease state. Compared to traditional serum-free nucleic acid and protein markers, exosomes have the advantages of significant targeting, encapsulation of a larger amount of information, easy preservation, and low interference from the test matrix, making them a highly promising biomarker for rapid research development. Today, exosome-based diagnostic reagents have moved from the laboratory to the clinic, and urine exosome-based diagnostic kits from Exosome Diagnostics Company (Waltham, the United States) have greatly improved the efficacy of multicenter differential diagnosis of prostate cancer from benign lesions.[112]

The clinical application of exosomal markers still has many problems to be solved. First, a rapid, simple, stable, high-recovery and clinically-operable purification method for clinical exosome specimens still needs to be further explored. In addition, because almost all cells in the body can secrete exosomes, it is important to screen for tissue-specific exosome markers or to isolate and identify exosomes of tissue-specific origin. Research on exosomes as biological markers is still emerging, and there are still many issues that need further resolution.

Reviewing the 35-year history of exosome research, our understanding of exosomes has increased rapidly. Exosomes have a broad impact on each step of the biological process of tumor progression and metastasis. Studies related to exosomes in HCC are even more evident in the following aspects: 1. Exosomes carry various biofunctional cytokines to nearby tumor cells, share information about their malignant proliferation, and induce changes in recipi-

ent cells, such as proliferation, inhibition of apoptosis, EMT, invasion or drug resistance; 2. Inducing the formation of EMT and CAFs contributes to the establishment of the TME, attracts multiple inflammatory factors and secretes numerous soluble products to promote tumor cell invasion and metastasis; 3. Inducing immunosuppression by activating specific types of immune cells (TAMs, Tregs, etc.) to inhibit the body's recognition and attack of tumor cells; 4. Endothelial cell proliferation and neovascularization are promoted by carrying metastatic, soluble E-cadherin, DLL4 or miRNAs5, eliminating tight junctions of vascular endothelial cells by carrying relevant miRNAs, leading to tumor progression and metastasis; 6. Excretion of intracellular toxic drugs through the exosomal pathway, leading to tumor cell drug resistance; and 7. Use of exosomes to alter the microenvironment of distant organ locations, increasing metastatic cell adhesion capacity and vascular permeability, thus establishing a premetastatic niche in distant organs.

The value of exosomes in the treatment of HCC has broad application prospects. Exosomes, as natural endogenous transport carriers, have the advantages of low toxicity, non-immunogenicity and good permeability. Precise and effective delivery of the load without activating the innate or acquired immune system prevents patients from acquiring immunity to the delivery vehicle after the first treatment. In addition, stimulation of the antitumor immune response by editing specific antigens on the membrane surface of exosomes provides a new therapeutic strategy for exosomes to be used as immune vaccines against liver cancer in the future. Exosomes produced by erythrocytes, after encapsulation of drugs with anticancer effects and intravenous infusion into the circulatory system, can be selectively enriched in the liver,[113] providing unique conditions for drug-targeted therapy for liver cancer. Although the details are not yet fully understood, essentially, nature has designed this mechanism over millions of years to allow efficient and safe exchange of RNA and various proteins between cells, which is a fundamental advantage.

However, there are still many difficulties to overcome in the design of exosomes as mediating therapeutic strategies in clinical cancer treatment, including liver cancer. First, it is difficult to purify a single species of exosomes. Exosomes are a mixture of numerous EVs produced by cells. Only specific types of exosome molecules have therapeutic effects on tumors, while other types of exosomes contain molecular substances that may have no therapeutic effect or may even promote tumor progression. Second, current methods for applying ultracentrifugation to extract exosomes are limited and inefficient. It is important to ensure that the number of exosomes is sufficient to elicit effective tumor therapeutic benefit. Therefore, it is necessary to establish more cost-effective and time-saving isolation and purification methods. Third, the integration of miRNAs or drugs producing therapeutic effects into exosomes remains a challenge. As mentioned above, neither the isolation of exosomes from donor cells overexpressing miRNAs nor the application of electroporation to transfer miRNAs into isolated exosomes meet the needs of clinical applications. More effective methods are needed to improve the efficiency of integration. Although the road to applying exosomes for liver cancer is challenging, the road is not hopeless. We have seen the morning sun at the end of the road, and all we need to do is to walk toward it and embrace it. We believe that exosomes will be widely used in the clinical treatment of liver cancer.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

All authors contributed to the study conception and design. Conception of the study (YC, YX), performed the literature search and data analysis (HW), and drafted and/or critically revised the work (LY, PH, YZ, WZ, NM, RH). The first draft of the manuscript was written by Hang Wang and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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