Roles of small extracellular vesicles in the development, diagnosis and possible treatment strategies for hepatocellular carcinoma (Review)

SHUYUE YANG*, JIAXIN WANG*, SHIDONG WANG, ANNI ZHOU, GUIPING ZHAO and PENG LI

Department of Gastroenterology, Beijing Friendship Hospital, Capital Medical University, Beijing 100050, P.R. China

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Abstract. Hepatocellular carcinoma (HCC) is the most common malignancy of hepatocytes accounting for 75-85% of primary hepatic carcinoma cases. Small extracellular vesicles (sEVs), previously known as exosomes with a diameter of 30-200 nm, can transport a variety of biological molecules between cells, and have been proposed to function in physiological and pathological processes. Recent studies have indicated that the cargos of sEVs are implicated in intercellular crosstalk among HCC cells, paratumor cells and the tumor microenvironment. sEV-encapsulated substances (including DNA, RNA, proteins and lipids) regulate signal transduction pathways in recipient cells and contribute to cancer initiation and progression in HCC. In addition, the differential expression of sEV cargos between patients facilitates the potential utility of sEVs in the diagnosis and prognosis of patients with HCC. Furthermore, the intrinsic properties of low immunogenicity and high stability render sEVs ideal vehicles for targeted drug delivery in the treatment of HCC. The present review article summarizes the carcinogenic and anti-neoplastic capacities of sEVs and discusses the potential and prospective diagnostic and therapeutic applications of sEVs in HCC.

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Correspondence to: Dr Peng Li or Dr Guiping Zhao, Department of Gastroenterology, Beijing Friendship Hospital, Capital Medical University, No. 95 Yong'an Road, Xicheng, Beijing 100050, P.R. China
E-mail: lipeng@ccum.edu.cn
E-mail: zhaoguiping@ccmu.edu.cn

*Contributed equally

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1. Introduction

Liver cancer is ranked as the sixth most prevalent malignancy worldwide, and was the third highest cause of cancer-associated mortality worldwide in 2020 with ~905,677 new cases and 830,180 cancer-associated mortalities annually (1). Hepatocellular carcinoma (HCC) is the predominant subtype of hepatic carcinoma, and accounts for 75-85% of all primary liver cancer cases (2). Infection with hepatitis B or C viruses (HBV or HCV, respectively) causes chronic liver injury and has recently been reported to play a pivotal place in the carcinogenesis and development of HCC (3). Due to the vaccination against HBV, the prevalence of HBV and the incidence rate of HCC have markedly decreased in numerous high-risk regions, such as China (4). However, the current situation is far from satisfactory in numerous low- and middle-income countries, due to the shortage of HBV vaccines, and the lack of improved sanitation and regular screening (5). Thus, the 5-year overall survival rate of patients with HCC remains low (<25%), and large-scale efforts are urgently required to elucidate the mechanisms underlying the development of neoplasia and to improve the preliminary diagnostic rate of HCC (6,7).

Small extracellular vesicles (sEVs), which were previously known as exosomes, have a diameter of 30-200 nm and are a subset of EVs that were first described by Johnstone *et al* (8) in the 1980s. Following several decades of research, it was observed that sEVs not only function in cellular waste disposal, but also serve as an excellent vehicle for cell-cell communications. sEVs contain complex and diverse materials, including DNAs, RNAs, proteins, lipids and metabolites, and they shuttle these bioactive molecules between cells (9). The cargos of sEVs can be internalized by recipient cells, thus mediating the metabolic activities of recipient cells and consequently participating in both normal physiology and acquired abnormalities, such as immune responses, mammalian reproduction and development, central nervous system-related diseases and cancers (10). In HCC, accumulated evidence has indicated that sEVs play an essential role in carcinogenesis and in the remodeling of the tumor microenvironment (TME), as well as in proliferation, metastasis, angiogenesis and drug

resistance (11). The profiles of sEV cargos are origin-specific, and the distinct expression of sEV cargos between patients with HCC and healthy subjects renders sEVs a potential diagnostic biomarker for HCC (12). Furthermore, certain sEV RNAs may serve as molecular markers for the early detection, TNM staging, prognostic evaluation and recurrence monitoring in HCC, which may contribute more effective to diagnosis and treatment options (13). Considering the intrinsic property of sEVs of transferring information and altering the biological response of recipient cells, recent studies have highlighted their potential utility values in the therapeutic fields of several diseases, including cardiovascular diseases and cancers (14,15).

The present review article summarizes the biogenesis of sEVs, as well as the role of sEVs in the tumorigenesis and progression of HCC. In addition, the potential and emerging clinical applications of sEVs in the diagnosis and treatment of HCC are discussed (Fig. 1).

2. Biology of sEVs

'EV' is a heterogeneous collective term for phospholipid bilayer membrane-encapsulated nano or microvesicles. Traditionally, EVs were broadly categorized into cytoplasmic membrane-derived ectosomes and exosomes of endosome-origin (16). However, without optimal isolation methods and real-time imaging technologies to visualize the process of release or specific markers of different subtypes of EVs, the differentiation between exosomes and small ectosomes is unlikely due to their analogous intrinsic properties and the overlapping size. Thus, the latest guideline of Minimal Information for Studies of Extracellular Vesicles 2018 (MISEV2018) proposed the use of standard terminologies for EV subtypes followed by physical characteristics, biochemical composition and the condition of progenitor cells (17). In the present review article, the term 'EV' encompasses a heterogeneous population of both exosomes and nano-scaled ectosomes with a diameter <200 nm.

Ectosomes, which are microvesicles and microparticles with a diameter ranging from 50 to 1,000 nm, are vesicles produced directly by the outward budding of the plasma membrane (18). By contrast, the process of the synthesis and release of exosomes is a more complicated and intricate sequence of multiple fusion events, budding of the plasma membrane and releasing of specific payloads (Fig. 2). The first inward invagination of a lipid bilayers contributes to the generation of early-sorting endosomes (ESEs) (19). ESEs can mature towards late-sorting endosomes (LSEs), which is followed by the formation of multivesicular bodies (MVBs) through the second intraluminal budding of the endosomal membrane, during which, specific bioactive compounds such as nucleic acids, proteins, and lipids are gradually enriched in intraluminal vesicles (ILVs) (20). Although the mechanisms underlying the formation of ILVs and specific bioactive compound sorting system have not yet been well elucidated, the majority of oncologists hypothesize that endosomal sorting complex required for transport (ESCRT) facilitates exosome budding. Of note, an ESCRT-independent mechanism may play a role in the biogenesis of exosomes, since no notable decrease in the release of exosomes was observed following the inhibition of ESCRT family activity (21). These two pathways may not be completely separated, although they function synergistically in the synthesis of exosomes (22). MVBs mainly have two endings: i) These mature MVBs may be incorporated into autophagosomes or lysosomes for hydrolysis of vesicular contents; or ii) they can be incorporated into the cellular plasma membrane and be subsequently expelled into the extracellular space as exosomes (23). When arriving to their recipient cells, sEVs are recognized and assimilated into cells via the following mechanisms: donor-acceptor interaction, membrane fusion, phagocytosis, and clathrin-independent and clathrin-dependent endocytosis, depending on their physical and biological properties (24,25). For example, angiopoietin-2 (ANGPT2)-bearing sEVs derived from HCC cells are transferred into human umbilical vein endothelial cells (HUVECs) via endocytosis (26). The processes of formation, secretion and uptake of exosomes are depicted in Fig. 2, as reported in a previous study by the authors (27).

sEVs can be excreted by almost all cells types and are abundant in the human body, existing in biological fluids, such as plasma, urine, tears, plasma and breast milk (28). sEVs can be isolated from cell culture conditioned media, multiple biofluids, or tissue using several methods. The separation of sEVs principally involves five approaches, including differential ultracentrifugation, sucrose and iodixanol density ultracentrifugation, polyethylene glycol precipitation, size exclusion chromatography (SEC) and immunoaffinity capture (29). However, it is extremely difficult to identify a single separation strategy with both a high recovery rate and high specificity. The present study aimed to systematically review the recent, cutting-edge research on sEVs and HCC, focusing on high-quality studies using differential ultracentrifugation or density gradient centrifugation as the separation methods of sEVs, with an intermediate recovery rate and purity according to MISEV2018 (17). Notably, all these aforementioned approaches have their own advantages and disadvantages; thus, a combined method, such as differential ultracentrifugation followed by SEC is scalable for future sEVs-based studies (30). Other articles (31-68) discussing methods of isolation of sEVs involving only ultracentrifugation or commercial kits are cited Table SI. Further investigations on a more effective and reproducible approach for separating sEVs are urgently required.

3. Roles of sEVs in HCC tumor formation and progression

As aforementioned, sEVs encapsulate a series of cargos, including nucleic acids, proteins and lipids, and sEV-related research has mainly focused on the ability of sEVs to exchange of these cargos between cells (69,70). Previous studies on the roles of sEV cargos in cancer have demonstrated that sEVs are involved in almost all hallmarks of cancers, including tumor initiation and formation (71-75), in the remodeling of the TME (76,77), apoptosis (50), angiogenesis (78,79), metastasis (80-83), immune escape (52), and drug resistance (84). The present review article summarizes the literature that highlights the significance of sEV cargos in the carcinogenesis and development of HCC (Fig. 3), as presented in Table I.

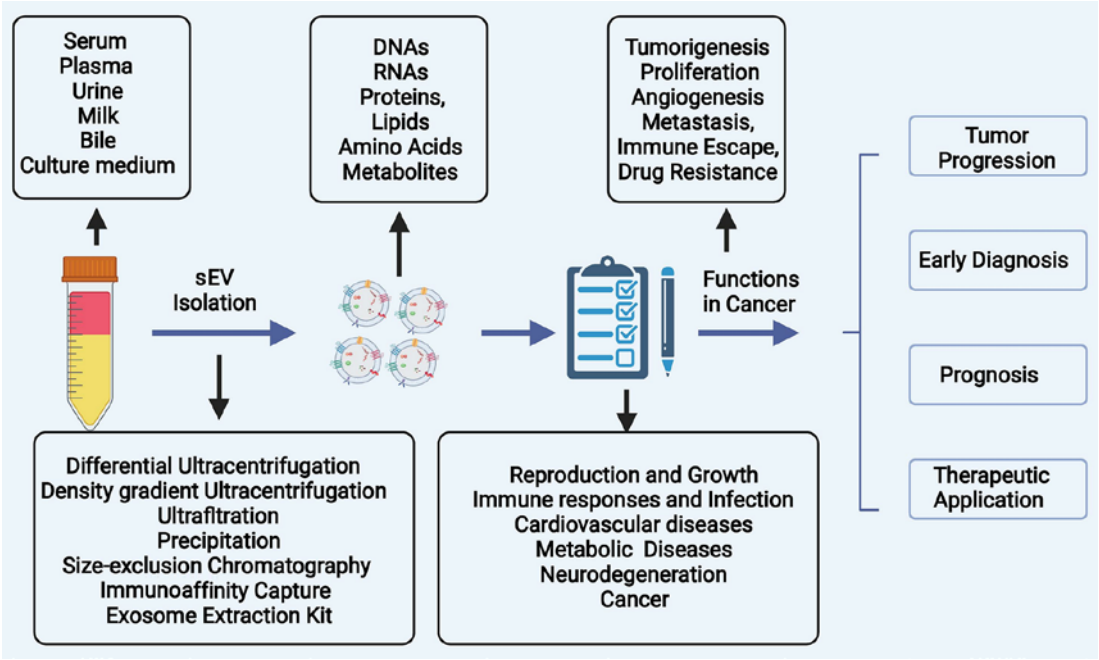

Figure 1. The fundamental purpose of the present review was to introduce the biogenesis of sEVs, generalize the role of sEVs payloads in the initiation and development of HCC, and dialectically discuss the clinical applications of sEVs in the diagnosis and possible treatment applications of HCC. sEVs, small extracellular vesicles; HCC, hepatocellular carcinoma.

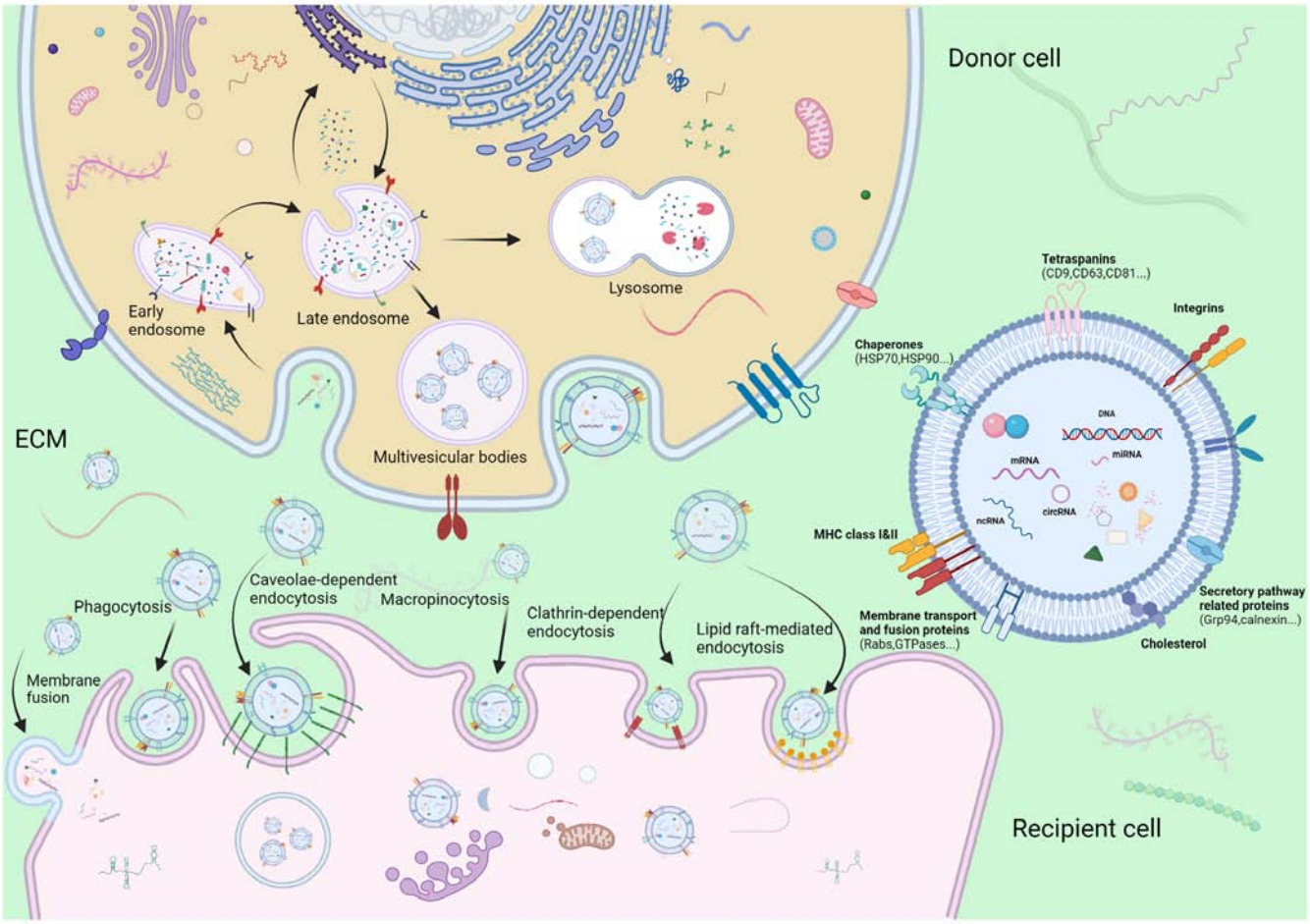

Figure 2. Schematic diagram illustrating the process of formation, secretion and uptake of sEVs. Inward budding of the cellular plasma membrane forms the early-sorting endosomes. Subsequently, intraluminal budding of endosomes generates MVBs encapsulating intraluminal vesicles. sEVs are ultimately liberated by incorporating of MVBs to plasma membrane and the exocytosis of intraluminal vesicles. The mechanism of sEV uptake includes donor-acceptor interaction, membrane fusion, phagocytosis, and clathrin-independent and -dependent endocytosis. sEVs, small extracellular vesicles; MVBs, multivesicular bodies; ECM, extracellular matrix.

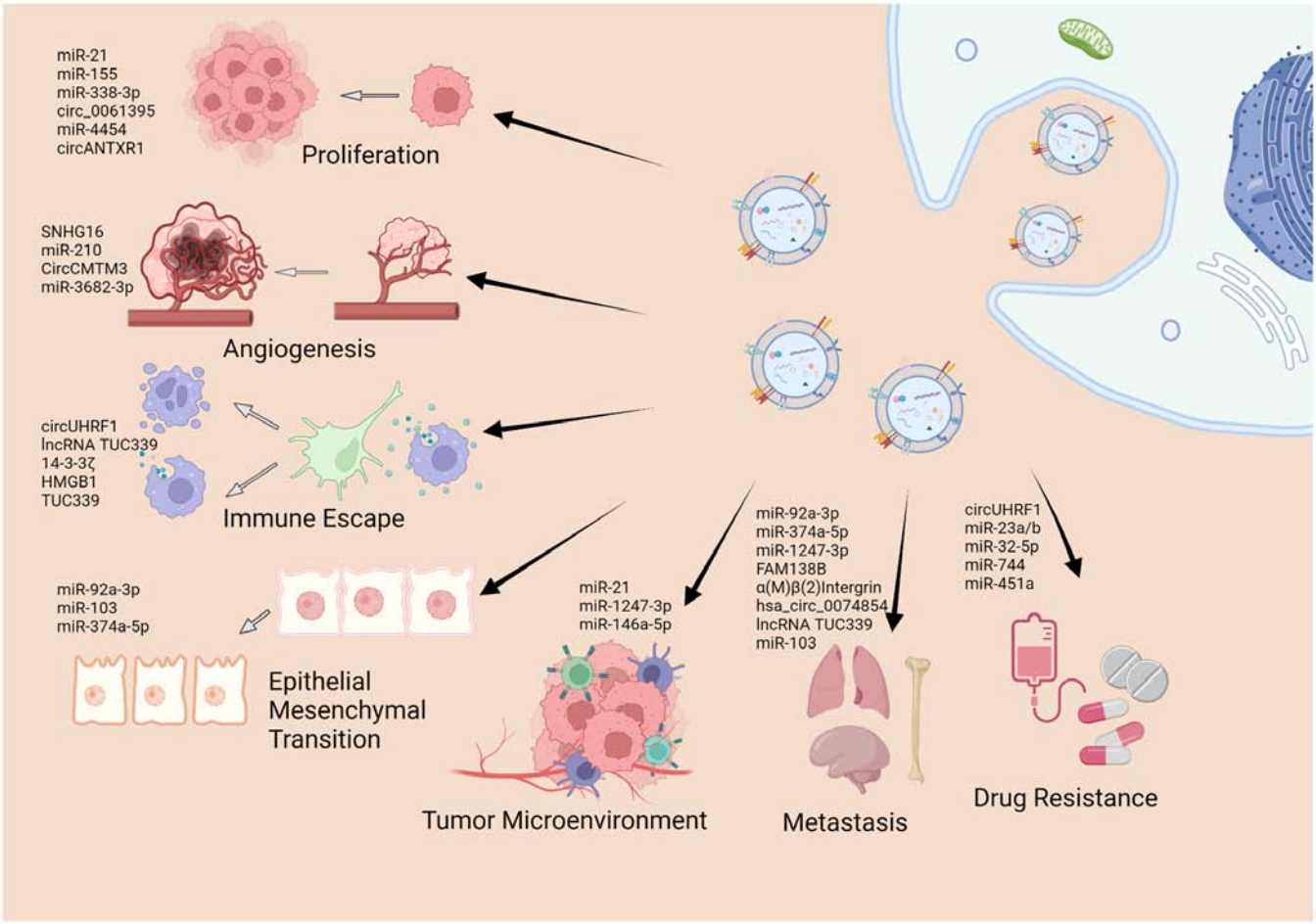

Figure 3. Biological function of sEV cargos in development of HCC. sEVs cargos are involved in numerous hallmarks of HCC, including proliferation, angiogenesis, epithelial-mesenchymal transition, metastasis, immune escape, drug resistance as well as remolding of the tumor microenvironment. The figure was created using Biorender (https://biorender.com/). sEVs, small extracellular vesicles; HCC, hepatocellular carcinoma.

TME. Tumorigenesis is not a single-step event, but a consequence of long-term alteration of mutations of genes and functional changes in the TME (85). Emerging evidence suggests that sEVs participate in the initiation, formation and remodeling of the TME in HCC (11). Chronic hepatitis B (CHB) remains a main factor responsible for HCC development, and sEVs are implicated in the spread, immune regulation and antiviral response of HBV infections (86). For example, exosomes from macrophages can deliver IFN-α-related microRNAs (miRNAs or miRs) to HBV-infected hepatocytes, and activate the antiviral response to suppress HBV replication and expression (87). The exosomal long non-coding RNA (lncRNA) HOTTIP has been shown to play a role in mediating the antiviral effect of tenofovir alafenamide following HBV infection (88). It has been demonstrated that sEVs from $CD4^+$ T-cells can enhance B cell responses and potentiate the efficacy of the hepatitis B surface antigen vaccine (89). These studies indicate that sEVs can mediate immune regulation and antiviral response in HBV infection. A previous study also indicated that sEVs may exert negative immune regulatory effects, and that they are indispensable in the transformation from liver cirrhosis (LC) to liver cancer (90). The interplay between cancer cells and the TME is an essential activity that supports or prevents tumor development and progression. In HCC, tumor cells co-exist with other non-cancerous cells that constitute the TME and enhance tumor growth via various mechanisms. sEVs can exert an effect on the information and remodeling of the TME. For instance, exosomal miR-21 derived from HCC has been found to promote the conversion of hepatic stellate cells into cancer-associated fibroblasts (CAFs), and to facilitate the formation of the TME (91). Exosomal-miR-1247-3p from HCC cells has been shown to reduce the expression of B4GALT3 in CAFs and stabilize β1-integrin, leading to the activation of fibroblasts via the NF-κB signaling pathway (92). To summarize, sEVs play an essential role in the pathogenesis of HBV-related hepatic diseases, transformation from precancerous diseases to HCC and in the formation of the TME to confer tumorigenesis in HCC.

Proliferation and apoptosis. The development of HCC can be partly attributed to the rapid proliferation and uncontrolled expansion of tumor cells, which also accounts for tumor progression and resistance to therapy. sEVs mediate tumor growth and expansion by affecting the cell cycle, proliferation rate and apoptosis of HCC cells (93-95). Cao *et al* (71) suggested that exosomal miR-21 can influence HCC by altering the expression of the tumor suppressor genes, PTEN and PTEN pseudogene 1. Sun *et al* (96) indicated that exosome-specific miR-155 targeted PTEN and consequently stimulated the proliferation of HCC cells. On the contrary, certain

Table I. Roles of sEVs in the initiation and development of hepatocellular carcinoma.

Type	Molecule	Function	Signaling/target	Year of publication	(Refs.)
miRNA	miR-21↑	Promotes proliferation, migration, angiogenesis and invasion; inhibits apoptosis	PTEN/E-cadherin	2019, 2018	(71,80,91)
	miR-10b↑	Promotes proliferation, migration and invasion	PTEN/E-cadherin	2019	(80)
	miR-23a/b↑	Promotes proliferation, migration and chemoresistance to 5-Fu	VHL-HIF-1α	2019	(115)
	miR-15a↓	Inhibits proliferation, migration and invasion	SALL4	2021	(76)
	miR-25↑	Promotes proliferation and migration	Wnt/β-catenin	2021	(72)
	miR-155↑	Promotes proliferation	PTEN	2019	(96)
	miR-451a↑	Inhibits resistance to paclitaxel, proliferation, migration and invasion	ADAM10	2021	(117)
	miR-3682-3p↑	Inhibits angiogenesis	RAS-MEK1/2-ERK1/2	2021	(101)
	miR-374a-5p↑	Promotes EMT, migration and invasion	GADD45A	2020	(105)
	miR-210↑	Promotes angiogenesis	SMAD4 and STAT6	2018	(100)
	miR-92a-3p↑	Promotes EMT and migration	PTEN/Akt/Snail	2020	(103)
	miR-92a-2-5p↑	Promotes invasion	AR/PHLPP/p-AKT/β-catenin	2020	(82)
	miR-146a-5p↑	Remodels the TME	STAT3/SALL4	2019	(77)
	miR-30a-3p↑	Inhibit migration and invasion	SNAP23	2021	(83)
	miR-1247-3p↑	Promotes migration	β1-integrin-NF-κB	2018	(92)
	miR-25-5p↑	Promotes migration and invasion	LRRC7	2018	(81)
	miR-4454↑	Promotes proliferation, migration, invasion, and angiogenesis, and inhibits cycle arrest, apoptosis	Vps4A, Rab27A	2021	(95)
	miR-338-3p↑	Inhibits proliferation, invasion and migration, and induce apoptosis,	ETS1	2021	(97)
	miR-744↓	Promotes proliferation and inhibits chemosensitivity to sorafenib	PAX2	2019	(118)
lncRNA	lncRNA H19↑	Promotes angiogenesis and adhesion of endothelial cell	VEGF, ICAM1	2015	(78)
	FAM138B↑	Inhibits proliferation, migration and invasion	miRNA-765	2020	(106)
	SNHG16↑	Promotes angiogenesis	PI3K/Akt/mTOR	2021	(99)
	lncRNA 85↑	Promotes proliferation and migration and polarization	miRNA-324-5p	2020	(73)
	TUC339↑	Regulation of macrophage activation	IL-1β and TNF-α	2018	(109)
circRNA	hsa_circ_0074854↑	Promotes migration and invasion	HuR	2021	(108)
	circFBLIM1↑	Promote progression and glycolysis	miRNA-338/LRP	2020	(75)
	circRNA-100338↑	Promotes angiogenesis and invasion	mTOR	2020	(102)
Protein	14-3-3ζ↑	Impairs the function, proliferation and activation of TILs	AXL or TGF-β/ERK	2018	(112)
	NSMase1↑	Inhibits proliferation and induce apoptosis	JNK	2018	(94)
	ENO1↑	Promotes proliferation and metastasis	FAK/Src-p38MAPK	2020	(74)
	LOXL4↑	Promotes migration and angiogenesis	FAK/Src	2019	(79)

Table I. Continued.

Type	Molecule	Function	Signaling/target	Year of publication	(Refs.)
	α(M) β(2) Integrin↑	Promotes metastasis	MMP9	2021	(107)
	HMGB1	Promotes immune evasion	TLR 2/4, MAPK	2018	(113)
	VEGF↑	Induces acquired resistance to AATs	-	2019	(84)

Upward arrows (↑) indicate upregulation and downward arrows (↓) indicate downregulation. EMT, epithelial-mesenchymal transition; TME, tumor microenvironment; TILs, tumor-infiltrating T-lymphocytes; AATs, anti-angiogenic therapies; PTEN, phosphatase and tensin homolog; VHL-HIF-1α, von Hippel-Lindau/hypoxia-inducible factor; SALL4, spalt-like transcription factor 4; ADAM10, a disintegrin and metalloprotease 10; MEK1/2, mitogen-activated proteinkinase kinase 1/2; ERK1/2, extracellular regulated protein kinases 1/2; GADD45A, growth arrest and DNA damage 45-alpha; SMAD4, mothers against decapentaplegic homolog 4; STAT, signal transducer and activator of transcription; AR, androgen receptor; PHLPP, PH domain leucine-rich repeat protein phosphatase; SALL4, transcription factor Sal-like protein-4; SNAP23, synaptosome-associated protein 23; LRRC7, leucine-rich repeat-containing 7; Vps4A, vacuolar protein sorting 4 homolog A; ETS1, E26 transformation specific-1; PAX2, paired box gene 2; VEGF, vascular endothelial growth factor; ICAM1, intercellular adhesion molecule 1; FAM138B, family with sequence similarity 138 member B; SNHG16, small nucleolar RNA host gene 16; PI3K, phosphatidylin-ositol-3-kinase; mTOR, mechanistic target of rapamycin; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α; HuR, human antigen; circFBLIM1, circRNA filamin binding LIM protein 1; LRP6, lipoprotein receptor-related protein 6; AXL, activity of the receptor tyrosine kinase; NSMase1, neutral sphingomyelinase 1; TGF-β, transforming growth factor-β; JNK, c-Jun N-terminal kinase; ENO1, alpha-enolase; FAK, focal adhesion kinase; LOXL4, lysyl oxidase-like 4; MAPK, mitogen-activated protein kinase; MMP9, matrix metallopeptidase 9; HMGB1, high mobility group box 1; TLR, Toll like receptor.

sEV-encapsulated cargos, such as miR-338-3p can inhibit cell proliferation, induce cell apoptosis and consequently repress the progression of HCC (97). In addition, another study demonstrated that the proliferative and migratory abilities of HCC cell lines were potentiated, while their apoptosis was counteracted via the enforced expression of the exosomal lncRNA H19 (98). Furthermore, sEV constituents may intervene in the cell cycle to regulate the progression of HCC. It has been corroborated that circ_0061395 silencing can trigger cell cycle arrest and apoptosis, and suppress the proliferation of HCC *in vitro*, as well as inhibit tumor growth (56). Similarly, miR-4454 inhibitor-mediated exosomes can substantially exacerbate cycle arrest, apoptosis and the formation of reactive oxygen species in HCC (95). Of note, the progression of HCC is a result of the accumulation of several time-intersecting steps, including invasion, migration, angiogenesis, immune escape and metastasis, and sEV cargos may also function via several mechanisms. Huang *et al* (62) suggested that the silencing of circANTXR1 can suppress HCC progression, not only by inhibiting the proliferative ability of HCC, but also by hampering the migration, invasion and metastasis of tumor cells. The roles of sEVs in other hallmarks of tumor progression will be further discussed in the following section.

Angiogenesis. Angiogenesis refers to the formation of new blood vessels from pre-existing ones. It is a complex, multistep process involving extracellular matrix remodeling, endothelial cell migration and ultimately generation of microvessels. Angiogenesis not only provides sufficient oxygen and nutrition for cancer cells, but is also essential for HCC proliferation, local invasion and distant metastasis. The significance of sEVs in cancer angiogenesis has been widely explored and documented recently (98). In HCC, exosomal SNHG16 can sponge miR-4500 and activate angiogenesis in HUVECs by regulating polypeptide N-acetylgalactosaminyltransferase 1 via the PI3K/Akt/mTOR pathway (99). Lin *et al* (100) reported that tumor-derived exosomes (TDEs) containing miR-210 could target SMAD4 and STAT6 in endothelial cells, and thereby promote the angiogenesis of HCC. The functions of these sEV cargos are multifaceted, and they alter the gene expression of the recipient cells, which become more aggressive and exhibit malignant characteristics. Apart from regulating angiogenesis, they may also control phenotypic changes, such as the proliferative or migratory abilities of cancer cells. A previous study demonstrated that circCMTM3-bearing sEVs can drive the angiogenesis of HUVECs, as well as their viability, migration and invasion (58). Certain studies have found that serval sEV cargos may play the opposite role and suppress angiogenesis in HCC (34,101). For instance, HCC-derived exosomes containing miR-3682-3p have been shown to attenuate angiogenesis via targeting ANGPT1, which is dependent on the RAS-MEK1/2-ERK1/2 pathway (101). Taken together, these results indicate that angiogenesis is a complex process that is orchestrated by multiple biological factors, and the treatment of angiogenesis may provide a novel prospective therapeutic approach for HCC.

Epithelial-mesenchymal transition (EMT) and metastasis. Widespread metastasis in patients with HCC remains a major challenge for treatment, and a main reason for treatment failure,

as well as one of the leading-causes of cancer-associated mortality (102). Metastasis is a multistep process involving EMT, invasion into vessels, intravascular transport and organ-specific seeding. The most common mode of metastasis in HCC is intrahepatic metastasis, followed by lymphatic metastasis and distant metastasis to the lungs. sEVs are involved in multiple steps of HCC metastasis, and the importance of sEVs in HCC metastasis has recently been widely reported. Firstly, sEVs contribute to the EMT of HCC cells. Yang *et al* (103) demonstrated that exosomal miR-92a-3p from high-metastatic HCC cell lines can potentiate EMT and metastasis by inactivating PTEN and activating Akt/Snail signaling. Similarly, Chen *et al* (104) suggested that TDEs from HCC cells can accelerate EMT, and induce HCC progression and recurrence by activating the MAPK/ERK signaling pathway; however, those studies did not clarify the specific sEVs-carrying cargo that is involved in this process. sEVs exacerbate the migratory and invasive abilities of HCC, which may promote the metastasis of HCC. It has been observed that miR-374a-5p in exosomes potentiates the migration and invasion of HCC by regulating growth arrest and DNA damage inducible alpha (105). In addition, sEVs can orchestrate the organotropic metastasis of HCC by converting the pre-metastatic microenvironment into a tumor cell-friendly site. A previous study demonstrated that Exo-miR-1247-3p derived from HCC can trigger β1-integrin-NF-κB signaling in fibroblasts in the lungs, and is positively associated with several pro-inflammatory cytokines, such as IL-8 and IL-6, which promote the lung metastasis of HCC (92). Recent studies have identified numerous sEV cargos that are involved in the metastasis of HCC, including FAM138B (106), α(M) β(2) integrin (107), hsa_circ_0074854 (108) and lncRNA TUC339 (109). It should be noted that the aforementioned sEV cargos may not only participate in one step of metastasis, but may play multifaceted roles in the whole process of metastasis. For example, Fang *et al* (42) indicated that, apart from promoting EMT and enhancing tumor motility *in vitro*, HCC cells can secrete sEV-encapsulated miR-103, which also potentiates vascular permeability and lung metastasis in mouse models.

Immune response and therapeutic resistance. The immune system plays a paramount role in recognizing and eliminating malignant cells and foreign invaders. In the processes of tumor initiation and progression, aberrant proliferation and gene alteration in cancer cells can generate abnormally expressed antigens, which should be adequately presented, recognized and eliminated by the immune system (110). During their fight against the immune system, cancer cells also evolve, and may acquire the ability to evade immunosurveillance via various mechanisms. Among the immune escape effects, that contribute to cancer progression and drug resistance, engagement of the attenuation or abrogation of immunocytes is worth mentioning, and sEVs play a pivotal role in this process (111). Recent studies have suggested that HCC-derived sEVs can impair the function of natural killer (NK) and T-cells, as well as activate immuno-suppressive cells such as M2 macrophages. For instance, exosomal circUHRF1 from HCC triggers the exhaustion of NK cells and subsequently induces resistance to therapy (52). Exo-lncRNA TUC339 has also been shown to be internalized by macrophages, and to modulate M1/M2 polarization and suppress the antitumor immune response in HCC (109). Apart from diminishing the activity of the innate immune system, previous studies have indicated that TDEs from HCC can also impede the activation and function of specific immunocytes such as T- and B-cells (32,46,52). Wang *et al* (112) found that exosomal 14-3-3ζ released by HCC cells suppressed the antineoplastic characteristics of tumor-infiltrating T-lymphocytes. Tumor-derived exosomal HMGB1 can enhance the expansion the T-cell Ig and mucin domain (TIM)-1 (+) regulatory B-cells and facilitate HCC immune evasion (113). Collectively, sEVs play multiple roles in the communication of HCC and immune cells, and are critical for the immune escape of HCC cells and tumor progression. Thus, sEVs may serve as ideal therapeutic targets for HCC, although further investigations into this matter are warranted.

HCC is one of the most aggressive cancer types, and hepatic resection remains the gold standard of treatment for HCC if the patients can withstand surgery. For patients who experience HCC recurrence or cannot tolerate surgery, targeted therapies involving the use of sorafenib, a multi-kinase inhibitor compound, and chemotherapy including paclitaxel and 5-fluorouracil (5-FU) are first-line treatments. Resistance to drugs remains a main obstacle to the effective treatment of these patients. The mechanisms underlying drug resistance remain complex and elusive; however, but the roles of sEVs in this process is emerging and have captured the interest of researchers. For instance, a previous study found that transfection with GRP78 small interfering RNA into bone marrow-derived mesenchymal stem cells could yield sEVs containing siGRP78, thus mediating targeted RNA silencing, which increased the sensitivity of drug-resistant cancer cells to sorafenib and improved the drug resistance reversion (114). Furthermore, as previously demonstrated, sEV-encapsulated miR-23a/b derived from adipocytes was transferred to neighboring HCC cells, which enhanced their chemoresistance to 5-FU by targeting the VHL/HIF axis (115). The upregulation of miR-32-5p-bearing sEVs has been shown to induce multidrug resistance by potentiating EMT and angiogenesis via targeting the PI3K/PTEN/Akt signaling pathway (34). Another study demonstrated that sEVs secreted from cancer stem cells induced regorafenib insensitivity by upregulating Nanog expression (116). These findings highlight the significance of sEVs in the drug resistance of HCC, which results from sEVs directly suppressing drug efficacy against tumor cells, or from sEVs regulating the gene expression of recipient cells to facilitate cancer survival. Since sEVs can alter the drug sensitivity of HCC cells, it is tempting to engineer a sEV-derived vehicle to deliver specific agents for HCC treatment (117). Studies on this topic are currently underway, and the preliminary results are promising. Wang *et al* (118) indicated that the downregulation of miR-744 in HCC tissue and cell lines was implicated in the chemoresistance to sorafenib, and HCC cell lines treated with miR-744-upregulating sEVs were more sensitive to sorafenib, which provides a potential approach to reduce the occurrence of drug resistance.

4. Clinical applications of sEVs in HCC

sEVs as diagnostic and prognostic biomarkers in HCC. Despite the advances in the diagnosis of HCC, the number of new cases and cancer mortalities associated with HCC remain

high (1). The diagnosis of HCC relies heavily on imaging analyses, such as magnetic resonance imaging or computed tomography; however, the diagnosis of the majority of patients is confirmed at an advanced stage, and thus these patients miss the optimal treatment period (119). Immense efforts have been made as regards the early diagnosis of HCC, although success has been limited. Alpha-fetoprotein (AFP) is a traditional HCC marker with a low specificity, which has a limited value in the differential diagnosis between HCC and other liver diseases (120,121). As regards other biomarkers, such as golgi glycoprotein 73, AFP-L3, phosphatidylinositol proteoglycan 3 and decarboxylated prothrombin, they do not provide any obvious advantage in the early diagnosis of HCC compared with AFP (122,123). Therefore, a non-invasive method with a high diagnostic sensitivity and specificity is urgently required. Recently, liquid biopsies and particularly circulating tumor cells, have attracted extensive interest for the diagnosis and monitoring of HCC (124). Studies on other tumor-derived components, such as circulating tumor DNA, sEVs and serum miRNAs are also increasing (125-127).

sEVs have the following advantages: i) Due to being protected by the sEV membrane, sEV cargos have a high stability and cannot easily degraded by lysosomes; ii) since the secretion of sEVs is a normal physiological event for tumor cells, sEVs can be detected in the majority of fluids, and their extraction is relatively non-invasive; iii) compared with plasma biomarkers, bioactive molecules from sEVs contain less interference of plasma; and iv) markedly, cargos of sEVs have extensive homology with recipient cells, which can confer sEVs superior sensitivity and specificity than traditional methods (128-130). Differential ultracentrifugation is the most common method used to separate sEVs from the cell culture medium. However, each biological fluid presents specific biophysical and chemical characteristics that render it different from culture conditioned medium. Despite current mainstream commercial kits are based on precipitation, which may result in EV populations bound to or mixed with introduced components, such as antibodies, beads or polymers; the majority of studies on the potential clinical applications of sEVs use this method as it is user-friendly, cost-effective and has potential for scale-up production (17). Furthermore, the efficiency and repeatability of sEVs separated using the ExoQuick™ kit have been demonstrated to be comparable with those of differential ultracentrifugation (131). Therefore, the articles cited in the current section include those that using commercial kits to isolate sEVs.

Numerous studies on the role of sEVs as HCC promising biomarkers have been conducted (132-136). The importance of sEVs as HCC biomarkers is reflected in numerous aspects, including the fact that sEV cargos may serve as biomarkers for the early detection of HCC; among these sEV cargos, miRNAs are the most extensively investigated ones. For instance, the expression of miR-21 and miR-10b in sEVs is markedly increased in patients with HCC compared with that of healthy individuals and patients with CHB, indicating that sEVs-carrying miR-21 and miR-10b may be used as early diagnostic biomarkers for HCC (80). Similarly, by comparison with that of patients with LC, the expression of miR-221, miR-192 and miR-146a in exosomes was increased in patients with HCC, and Fründt *et al* (137) indicated that sEVs carrying miR-146a could distinguish patients with HCC from patients with LC with an area under the curve value of 0.80±0.14 in a logistic regression model, and miR-96, miR-122, miR-200a had similar effect (138-140). Other sEV cargos, such as proteins and other non-coding RNAs (ncRNAs), including lncRNAs and circRNAs, may also play a role in the preliminary diagnosis of HCC, and it has been observed that LINC00161, circRNA 0006602, LDHC, sphingosines, dilysocardiolipins, lysophosphatidylserines, and (O-acyl)-1-hydroxy fatty acids are early diagnostic biomarker candidates (48,121,141-143).

Apart from their early diagnostic value, sEV cargos may be involved in the prediction of tumor staging and metastasis (144-147). Exo-miR-1307-5p expression in plasma has been found to be positively associated with tumor stage and progression, while sEVs carrying miR-125b have been shown to possess anti-metastatic features and are indicators of early metastasis in HCC (148-150). Other sEV cargos can play a similar role in tumor staging or metastasis prediction, and the function of lncRNA ATB, hnRNPH1 and ASMTL-AS1 in this regard has been reported (151-153). In addition, certain sEV cargos may serve as prognostic indicators and may predict the prognosis of patients with HCC. It has been corroborated that miR-638, miR-150-3p, lncRNA CRNDE and circAKT3 in the sEVs of patients with HCC are implicated in overall survival and disease-free survival and may serve as independent indicators of a poor prognosis (31,154-157). It should be noted that the abnormal expression of certain sEV cargos, such as miR-718, miR-125b and miR-92b is not only an effective tool to evaluate survival, but is also a potential marker to predict the recurrence of HCC (32,149,158). Notably, sEVs-carrying miR-122, hsa-circRNA-G004213 and DANCR are also potential markers to evaluate the efficacy of HCC surgical and interventional treatment (153,159-161). In addition to the above, a panel of tumor specific biochemical indicators has been proposed for a higher sensitivity and specificity compared with single one (162-166). For example, Sorop *et al* (167) established exosomal miR HCC Score including serum AFP and the level of plasma sEVs-carrying miR-21-5p and miR-92a-3p with a great diagnostic ability of HCC (AUC=0.85). Taken together, previous studies have demonstrated that certain sEV cargos, including ncRNAs, mRNAs, lipids and proteins, may serve as potential HCC diagnostic and prognostic biomarkers. The potential HCC biomarkers are summarized in Table II and the efficacy of these candidates warrants further validation.

Therapeutic potential of sEVs in the treatment of HCC. Liver cancer is ranked as the third leading cause of cancer-associated mortality worldwide due to resistance to traditional drugs, as well as diagnosis in the late stage (1,168). The identification of novel drugs for targeted therapy is imperative for patients with HCC (169). As aforementioned, the distinctive property of sEVs in delivering functional molecules and altering the biological behavior of recipient cells highlights their potential application as ideal therapeutic vehicles in cancer therapy, both at the theoretical and practical level. The development of engineered sEVs, with a purpose of acting as alternatives to chemotherapeutic and targeted agents, is currently ongoing. sEVs have several advantages compared with previous drug carriers, such as liposomes: First, sEVs achieve highly efficient drug delivery due to their facility of penetrating biological barriers.

Table II. sEVs carrying cargos as biomarkers for HCC.

Type of sEVs contents	Molecules	Origin	Potential functions	Year of publication	(Refs.)
miRNA	miR-21	Serum	Differentiates HCC patients from healthy control, and predict survival	2019	(80,126)
	miR-10b	Serum	Differentiates E-HCC patients from healthy control, and predict DFS	2019	(80)
	miR-638	Serum	Is negatively associated with survival and predicts recurrence	2018, 2021	(154,157)
	miR-122	Serum	The ratio of miR-122 predicts DSS for HCC patients with liver cirrhosis treated with TACE, and miR-122 differentiate HCC patients from healthy controls	2018	(140,159)
	miR-212	Serum	Differentiates HBV-infection HCC patients from non-HBV-infection HCC patients, and predict survival	2019	(120)
	miR-455-5p, miR-30c-5p	Plasma	Predicts survival	2020	(127)
	miR-150-3p	Plasma	Is negatively associated with survival	2021	(31)
	miR-1307-5p	Serum/Plasma	Predicts metastasis	2020	(148)
	miR-92b	Serum	Predicts recurrence for HCC patients after LDLT	2019	(32)
	miR-125b	Serum	Predicts early metastasis, survival and recurrence	2017, 2021	(149,150)
	miR-146a	Plasma	Differentiate HCC patients from cirrhosis patients	2021	(137)
	miR-192	Plasma	Predicts survival	2021	(137)
	miR-320d	Serum	Differentiates HCC patients from healthy controls, and predicts TNM stage, lymph node metastasis, and survival and survival	2020	(144)
	miR-4661-5p	Serum	Differentiates HCC patients at early stage and predict prognosis	2020	(135)
	miR-93	Serum	Differentiates HCC patients from healthy controls, predict TNM stage, and survival	2018	(36)
	miR-665	Serum	Differentiates HCC patients from healthy controls, predicts TNM stage, local metastasis, and survival	2017	(145)
	miR-718	Serum	Predicts recurrence for HCC patients after liver transplantation	2015	(158)
	miR-224	Serum	Differentiates HCC from healthy controls, and predict survival	2019	(136)
lncRNA	lncRNA ATB	Serum	Predicts TNM stage and survival	2019	(151)
	DANCR	Serum	Predicts recurrence for HCC patients with HCV after curative HCC resection	2021	(160)
	ASMTL-AS1	Serum	Predicts recurrence and metastasis for HCC patients after insufficient RFA	2020	(153)
	LINC00161	Serum	Differentiates HCC patients from healthy controls	2018	(48)
	LINC00853	Serum	Differentiates E-HCC from non-HCC	2020	(142)
	lncRNA-HEIH	Serum	Differentiates HCC from HCV patients	2018	(133)
	CRNDE	Serum	Predicts TNM stage, OS and DFS	2021	(155)
circRNA	circRNA 0006602	Plasma	Differentiates HCC from non-HCC	2021	(121)
	hsa_circ_0070396	Plasma	Differentiates HCC patients from healthy controls	2021	(134)
	circAKT3	Serum	Predict recurrence and OS	2020	(156)
	hsa-circRNA-G004213	Plasma	Predict efficacy of TACE	2021	(161)

Table II. Continued.

Type of sEVs contents	Molecules	Origin	Potential functions	Year of publication	(Refs.)
mRNA	hnRNPH1	Serum	Differentiates HCC patients from healthy controls and CHB patients, and predicts lymph node metastasis, TNM stage and OS	2018	(152)
	LDHC	Serum	Differentiates E-HCC patients from healthy controls, and predicts efficacy, recurrence	2020	(141)
Protein	ENO1	Serum	Predicts TNM stage, and metastasis	2020	(74)
Lipid	Sphingosines, dilysocardiolipins, lysophosphatidylserines, and (O-acyl)-1-hydroxy fatty acids	Plasma	Differentiates E-HCC patients from non-HCC	2021	(143)
Panel	miR-10b, miR-21, miR-122 and miR-200a	Serum	Differentiates E-HCC patients from healthy controls and cirrhosis patients	2015	(139)
	miR-21-5p, and miR-92a-3p	Plasma	Together with serum AFP establishing Exosomal miR HCC score, and differentiates HCC patients from healthy controls and cirrhosis patients	2020	(167)
	miR-18a, miR-20b, and miR-221	Plasma	Predicts metastasis	2021	(165)
	miR-122, miR-21, miR-96	Serum	Differentiates HCC patients from cirrhosis patients	2020	(138)
	miR-10b-5p, miR-221-3p, miR-223-3p, and miR-21-5p	Plasma	Differentiates HCC patients from non-HCC	2020	(162)
	miR-122, and miR-148a	Serum	Together with serum AFP, differentiates E-HCC patients from cirrhosis patients	2018	(140)
	miR-4661-5p, and miR-4746-5p	Serum	Differentiate E-HCC patients from non-HCC	2020	(135)
	miR-4718, miR-642a-5p, miR-6826-3p, and miR-762	Serum	Differentiates HCC patients from HCV patients, and predicts recurrence for SVR-HCC with DAA	2019	(166)
	hsa_circ_0004001, hsa_circ_0004123, hsa_circ_0075792	Serum	Differentiates HCC patients from healthy controls	2020	(163)
	MALAT1 and SNHG1, DLEU2 and AFP	Serum	Differentiates E-HCC patients from non-HCC	2021	(164)

Table II. Continued.

Type of sEVs contents	Molecules	Origin	Potential functions	Year of publication	(Refs.)
	ENSG00000258332.1 LINC00635	Serum	Predicts TNM stage, lymph node metastasis, and OS. Together with AFP, differentiates HCC patients from non-HCC	2018	(146)
	Cofilin-1 and CCT8	Serum	Predict vascular invasion, TNM stage, survival. Together with AFP, differentiates HCC patients from non-HCC	2021	(147)

HCC, hepatocellular carcinoma; E-HCC, early-stage hepatocellular carcinoma; HCV, hepatitis C viruses; CHB, chronic hepatitis B; TACE, transarterial chemoembolization; LDLT, living donor liver transplantation; DAA, direct-acting antiviral therapy; SVR, sustained viral response; DFS, disease-free survival; DSS, disease-specific survival; OS, overall survival; AFP, alpha-fetoprotein; TNM, tumor, node, metastasis.

In addition, the cellular origin of sEVs makes them well tolerated, and they can easily escape immune clearance, which also reduces drug dose and toxicity (111,170,171); Furthermore, the heterogeneity of proteins on the sEVs membrane facilitates the targeting abilities of sEVs. In addition, sEVs are more biocompatible, safe and stable than liposomes (172,173). Taken together, sEVs have a great potential to serve as nano-carriers in the treatment field. The present section mainly focuses on the advancements made in sEV research as regards their application in therapy.

To achieve a better understanding of sEVs as vehicles for therapeutic agents, methods of sEV preparation, the engineering of sEVs and the selection of cargos are under investigation, and the preliminary results are promising. Multiple therapeutic agents, including chemotherapeutic drugs and nucleic acids or their inhibitors, can be loaded. For example, in a previous study, the subcutaneous injection of sEVs containing miR-let-7a into a breast cancer mouse model exhibited an antitumor ability by targeting EGFR (174). In HCC, recent research has validated the importance of sEVs in the delivery of EV-packaged drugs (175). A previous study provided a prospective approach for generating sEV-associated adeno-associated virus containing inducible caspase 9 (iCasp9) suicide gene (Vexo-AAV6-iCasp9). The engineered sEVs possessed a low immunogenicity and toxicity, and were readily absorbed by HCC cells, consequently increasing HCC regression in an *in vivo* xenograft model (176). Another study encapsulated erastin (a typical ferroptosis inducer) and rose bengal (RB, a well-known photosensitizer) into sEVs and engineered CD47 on the surface of sEVs to protect the designed sEVs from phagocytosis by macrophages. The sEVs induced obvious ferroptosis in HCC, with minimized toxicity in the liver and kidneys (177). Apart from packing antitumor payloads into sEVs, previous studies have developed nanoparticles targeting specific adhesion or receptor proteins on the surface of sEVs membranes for targeted delivery. Tian *et al* (80) designed a nano-drug based on the PDCM system by targeting sEVs shuttling miR-21 and miR-10b, which markedly decreased HCC growth and the numbers of metastatic lung nodules. TDEs not only assist in exacerbating tumor progression, but also increase the resistance of cancer cells to antitumor treatments (178). Sorafenib and transarterial chemoembolization have been considered optional treatments for terminal-stage HCC for numerous years. Due to acquired drug resistance to commonly used chemotherapeutic agents, the clinical outcome and overall survival of patients with HCC remain unsatisfactory (179). The expression of programmed cell death protein 1 (PD-1) in HCC tissues from patients with HCC who accepted sorafenib treatment was upregulated and induced T-cell apoptosis (180). Therefore, immune checkpoint inhibitors, such as commonly used PD-1 antibodies and PD-L1 antibodies have been introduced into medical practice as part of the HCC regimen; however, the efficacy of the combination of sorafenib and immunotherapy has not yet been fully elucidated. Shi *et al* (180) treated mouse models of HCC with the triple treatment of sorafenib, PD-1 antibodies and DC-derived exosomes (DEXs), which markedly prolonged the survival time mice with HCC by comparison with the mice treated with sorafenib alone, DEXs alone, or the

combination of DEXs and sorafenib. Taken together, these preclinical studies offer encouragement for the application of sEVs as vehicles for HCC treatment.

Despite the rapid development of advanced techniques, major limitations remain in the current understanding of sEVs vs. ideal treatment scenarios: i) For sEV technology to play a role as a drug delivery vector, it is necessary to ensure high purity and adequate production. There are still several obstacles which hamper the efficacy of current methods, such as being time consuming, having a high cost and generating polluting by-products. Oncologists increase the total yield of sEVs by intracellular calcium production, external stress, cytoskeletal blocking, drug stimulation and the induction of gene expression factors. Furthermore, sEVs usually represent heterogeneous populations from different cell sources, and no standard separation process has been established to date to achieve product consistency (181); ii) the efficient incorporation of external antitumor agents and molecules is another demanding challenge that needs to be optimized. The high drug-loading content in sEVs must be sufficient to obtain a therapeutic response. Several approaches, including transfection, electroporation and sonication (182,183), can be applied to upload the desired biomolecules into sEVs. However, it is difficult to ensure the integrity and biostability of the plasma membrane and the function of sEVs; iii) the current evidence for the ability of sEVs to deliver specific messages derives from cell culture studies. The biodistribution and tissue or cellular tropism *in vivo* will determine the application of therapeutic sEVs in clinical practice. At present, there is insufficient evidence for *in vivo* and clinical applications, which is a critical topic for future research in this area. Due to being subjected to elimination by the mononuclear phagocyte system, the half-life of sEVs in the systemic circulation is relatively short (184). Thus, further studies on the balance between prolonged circulation time and increased risk of toxicity on major organs are warranted; and iv) currently, sEVs need to be stored under-20 and -80˚C in phosphate buffer saline (185). Therefore, identifying a suitable storage method is one of the barriers to be overcome.

In summary, while the application of sEVs as a therapeutic drug delivery system remains in its infancy, the deeper understanding of the aforementioned obstacles will provide a new orientation for cancer nanomedicine and immunotherapy.

5. Conclusions and future perspectives

sEVs can trigger the alteration of gene expression and induce aggressive behaviors in HCC cells; however, whether such observations can be replicated *in vivo* needs to be further investigated, since the precise isolation and high concentration of cell culture-derived sEVs could not be achieved in the majority of *in vivo* studies published thus far. Paradigm-shifting findings in the field of HCC diagnosis have resulted in new avenues for research on HCC biomarkers. Their easy availability, vesicle-tethering stability and high donor-homology confer sEVs an unparalleled advantage as HCC biomarkers compared with traditional biomarkers. However, the clinical value of sEVs as HCC biomarkers is still limited due to the absence of clinical research with large sample sizes. Extensive efforts are currently being made to identify sEVs biomarkers with high specificity and sensitivity, and apply them to clinical practice. The role of sEVs in cancer therapy has been studied extensively in recent years; however, research on sEVs for HCC remains limited. Before applying them in clinical practice, it is important to validate the purity, safety and effectiveness of sEVs-encapsulated agents. Further research is warranted to guarantee the homogeneity of sEVs, improve the efficiency of their isolation methods and reduce the associated side-effects. The targeting of sEVs is another issue that needs to be resolved. Surface modification is a typical approach to harvest targeted sEVs by modifying the proteins or peptides that specifically expressed on the cell membrane through gene transfection. Engineered sEVs can be selectively delivered to target cells and reach the standard in terms of yield and targeted therapy. However, the safety, mutagenesis and time-consuming limit their clinical applications. Currently, aptamers, also known as chemical antibodies, have attracted the attention of oncologists. The majority of aptamers have been utilized to guide nanoparticles, therapeutic and imaging agents to target locations in several promising anticancer preclinical studies, whereby they are able to modulate tumor retention and biodistribution. However, all these issues cannot be solved in a short period of time, and as the number of clinical studies increases, more patients will gain clinical benefit from research in sEVs.

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Authors' contributions

GZ and PL conceptualized the study. SY and JW were involved in the writing and preparation of the original draft. SW and AZ were involved in the writing, reviewing and editing of the manuscript. All authors have read and approved the final manuscript. Data authentication is not applicable.

Ethics approval and consent to participate

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Competing interests

The authors declare that they have no competing interests.

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