Advances in the mechanisms of extracellular vesicles and circulating tumor cells in hepatocellular carcinoma metastasis (Review)

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Abstract. Metastasis of hepatocellular carcinoma (HCC) is a key factor contributing to poor patient prognosis, with extracellular vesicles (EVs) and circulating tumor cells (CTCs) playing a marked synergistic role in this process. Current research suggests that EVs, acting as essential mediators of intercellular communication, facilitate CTC-driven HCC recurrence and metastasis by transporting bioactive molecules, including nucleic acids, proteins and lipids. CTCs are regarded as the initiators of tumor metastasis, while the bioactive cargo carried by EVs functions as critical factors that promote their outgrowth. EVs not only remodel the microenvironment of target organs by forming a 'pre-metastatic niche', but also establish a permissive microenvironment for CTC colonization. This tripartite interplay establishes a cascade model that enhances metastatic dissemination. The present manuscript reviewed the recent advancements and clinical value in understanding the roles of EVs and CTCs in HCC metastasis and recurrence, as well as the mechanisms by which EVs mediate CTC-driven liver cancer spread, aiming to promote further in-depth research into HCC metastasis mechanisms.

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1. Introduction

Liver cancer ranks as the sixth most prevalent malignancy, with hepatocellular carcinoma (HCC) comprising ~90% of cases (1,2). Projections suggest that by 2030, HCC will be responsible for >1 million deaths globally, positioning it among the deadliest cancers (3). Notably, numerous patients are diagnosed at an advanced stage, highlighting the need for reliable methods for early detection and monitoring of HCC metastasis and recurrence. Advancements in early diagnosis and management of HCC have been substantial, with molecular detection techniques for early detection gaining marked attention and moving toward clinical application (4). Liquid biopsy, an emerging non-invasive diagnostic approach, involves collecting liquid samples to detect tumor-associated molecular markers, providing insights into the tumor phenotype, genetics and transcriptome (5). Key components include circulating tumor cells (CTCs), extracellular vesicles (EVs), circulating tumor DNA and RNA (6). Among these, CTCs and EVs have garnered marked interest due to their stability and sensitivity, making them the most promising liquid biopsy markers. CTCs are malignant cells shed into the bloodstream from primary or metastatic tumors, capable of invading distant organs *via* the circulatory system, earning them the term disseminators of malignancy (7,8). Some researchers propose that CTCs have robust prognostic value, serving as tools for monitoring HCC progression and guiding treatment post-resection of the primary lesion (9,10). Based on their biological mechanisms and size, EVs are typically categorized into exosomes, microvesicles and apoptotic bodies, all playing pivotal roles in tumorigenesis, metastasis and invasion. Among them, exosomes are nanoscale vesicles with a diameter of 30-150 nm; they originate from the endosomal system and are released into the extracellular space upon fusion of multivesicular bodies with the plasma membrane. Microvesicles have a size range of 100-1,000 nm and are directly formed by budding off from the plasma membrane. Apoptotic bodies are the largest EV subtype, with a diameter typically ranging from 50 to

5,000 nm; they are formed at the end stage of programmed cell death (apoptosis) and are generated by budding and rupture of the apoptotic cell membrane (11,12); in this context, they are collectively referred to as EVs. Tumor-derived EVs, detectable in the blood of patients with early-stage HCC, regulate recipient cell functions by reprogramming signaling pathways. Cancer cell-derived EVs notably alter the tumor microenvironment (TME) and facilitate the formation of pre-metastatic niches (PMNs) (13). Furthermore, EVs have been shown to protect CTCs during the metastatic process. Molecules carried by EVs, such as nucleic acids, proteins and microRNAs (miRNAs), contribute to the recurrence and metastasis of HCC *via* CTCs, where CTCs are likened to seeds, and the cargo within EVs acts as fertilizer, together influencing cancer cell behavior and promoting metastasis. The present manuscript reviews the mechanisms underlying EV and CTC-mediated HCC metastasis, as well as the role of EVs in enhancing CTC-driven metastasis, to provide a comprehensive understanding of HCC metastasis, recurrence and prognosis (14) (Fig. 1).

2. Mechanism of EVs in HCC metastasis

EVs play a complex yet pivotal role in HCC metastasis. Due to their high heterogeneity, distinct subpopulations of EVs can precisely and collaboratively drive the metastasis process. Their mechanisms encompass various aspects, including PMN formation, modulation of the tumor immune microenvironment (TIME), immune regulation and angiogenesis. The present section summarizes the involvement of EVs in HCC recurrence and metastasis (15-24) (Table I).

PMN. The establishment of a tumor-induced microenvironment in distant organs is needed for the colonization, adaptation and survival of metastatic cells. This metastatic niche, located in distant organs, is termed the PMN. The initial event in HCC metastasis is the formation of the PMN, which may even occur during the early stages of HCC development (25). EVs, secreted by various cell types, serve as intercellular communication carriers; the unique receptors and specific intercellular adhesion molecules on their surfaces enable targeted recognition of recipient cells and facilitate signal transduction. Subpopulations of EVs derived from different cellular sources play distinct roles and collaborate in this process. Specifically, integrins on the surface of tumor-derived EVs are essential for their interactions with distant organs during PMN formation (26); therefore, EVs act as key mediators in the formation of PMNs.

A hallmark of aggressive malignancies is excessive angiogenesis. Neovascularization is essential for providing oxygen, nutrients and energy, all of which support cancer cell growth, metastasis and the development of multidrug resistance (27). Remodeling of the microvasculature is a prerequisite for PMN formation, including multi-step collaborative processes such as increased vascular permeability, neovascularization and functional reprogramming of endothelial cells (28,29). The heterogeneity of EVs is reflected in their precision in regulating endothelial cells. Small EV subpopulations derived from HCC, rich in miRNA cargo, play a central role in regulating endothelial cell function; for instance, an EV subpopulation enriched with miR-183-5p activates the PI3K/AKT pathway by downregulating SIK1, thereby enhancing endothelial cell proliferation, migration, angiogenesis and vascular permeability, ultimately promoting metastasis *in vivo* (30). By contrast, large EVs or apoptotic body subpopulations may be more inclined to carry and deliver intact protein growth factors, such as vascular endothelial growth factor (VEGF) and fibroblast growth factor, directly providing strong pro-proliferative signals to endothelial cells. For instance, Golgi protein 73 secreted by EVs derived from HCC can enhance VEGF production in HCC cells and bolster mitogenic signaling in vascular endothelial cells, thereby promoting angiogenesis in the TME (31). Studies by Xu *et al* (15) and Yokota *et al* (16) established the key paradigm of HCC-derived EVs disrupting vascular barriers, remodeling angiogenesis and initiating PMN by delivering effector molecules such as basigin and miR-638, while downregulating critical vascular endothelial junction proteins (such as VE-cadherin, ZO-1). These findings are notable but focusing solely on these functions risks oversimplifying a dynamic and heterogeneous biological process. At the mechanistic level, while individual effector molecules have been identified, little is known about their upstream regulation; which tumor cell subpopulations selectively load these cargos and under what conditions? In addition, increased vascular permeability is only the initial step in PMN formation. Furthermore, could the damaged vascular endothelium release specific EVs or factors that feedback and enhance the invasiveness of the primary tumor? In the context of hepatocirrhosis, where HCC is already associated with inflammation and vascular abnormalities, do tumor-derived EVs exacerbate this 'fragile foundation' or initiate a novel destructive program? Differentiating between these two scenarios is essential for understanding the specificity of HCC metastasis.

In the metastatic niche, cancer-associated fibroblasts (CAFs) play an active role in tumor metastasis progression (32). Fang *et al* (17) showed that high-metastatic HCC cells have a greater ability to convert normal fibroblasts into CAFs compared with low-metastatic HCC cells during PMN formation. Fibroblasts induced by EVs from high-metastatic HCC cells exhibit elevated expression of pro-inflammatory genes such as interleukin-1β, transforming growth factor β (TGF-β) and collagen types I, III and IV, which are critical for modulating the TME and promoting carcinogenesis. Exosome microarray detection confirmed the notable role of HCC-derived EVs in converting fibroblasts to CAFs by activating the β1-integrin-NF-κB signaling within the PMN. This research revealed that specific subpopulations of EVs released by highly metastatic HCC cells express unique integrin combinations on their surface, which dictate their targeting to specific organs such as the liver and lungs. Upon uptake by resident cells in these target organs, EVs release their cargo, such as miR-638, which activates relevant signaling pathways, promoting angiogenesis, inflammatory factor release and initiating PMN formation.

TIME. The TIME encompasses various immune cells, extracellular immune factors, endothelial cells and CAFs, all contributing to tumor survival (33). The recurrence and metastasis of HCC is a multi-step process in which tumor cells not only evade apoptosis and immune responses but also rely on immune cell communication within the TIME (34). Immune

Table I. Role of EVs in hepatocellular carcinoma recurrence and metastasis.

Cargo	Pathway	Mechanisms	(Refs.)
EV-CLTA	Up regulation of BSG	Remodeling the microvascular niche	(15)
miRNA-638	Down regulation of vascular endothelial-cadherin and ZO-1	Remodeling the microvascular niche	(16)
miRNA-1247-3p	Activation of β1-integrin-NF-κB	Promote CAF transformation	(17)
miRNA-92b	Inhibition of CD69	Inhibition of NK cell activity	(18)
lncRNA PART1	Up regulation of TLR4	Promote macrophage M2 polarization	(19)
miRNA-15b	Inhibition of Hippo	Promote macrophage M2 polarization	(20)
EV-S100A10	Up regulation of EGFR	Promote EMT	(21)
miRNA-21	Activation of PDK1/AKT	Activation of CAFs and promote angiogenesis	(22)
miRNA-21-5p	Activation of YAP/β-catenin	CD8+T cell depletion	(23)
miRNA-92a-2-5p	Activation of PHLPP/p-AKT/β-catenin	Regulation of tumor immune microenvironment	(24)

CLTA, clathrin light chain A; BSG, basigin; ZO-1, zonula occludens-1; CAFs, cancer-associated fibroblasts; TLR4, toll-like receptor 4; S100A10, S100 calcium binding protein A10; EGFR, epidermal growth factor receptor; EMT, epithelial-mesenchymal transition; EV, extracellular vesicle.

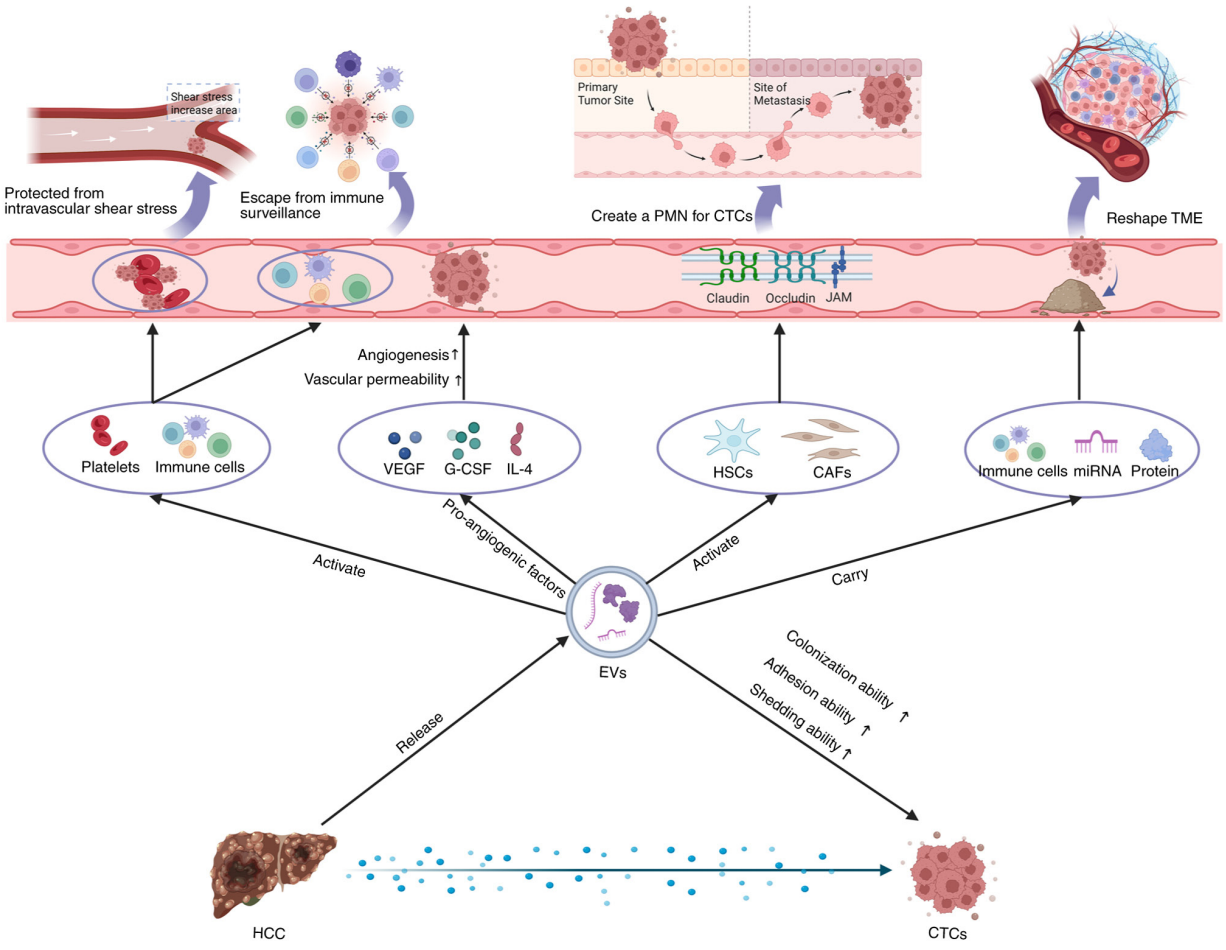

Figure 1. Mechanism of EVs-mediated CTCs promoting HCC metastasis. EVs protect CTCs by reducing intravascular shear stress and evading immune surveillance, while releasing cytokines to increase vascular permeability and enhance the shedding ability of CTCs. Additionally, EVs can create pre-metastatic niches for CTCs and remodel the TME, promoting the colonization ability of CTCs. CTC, circulating tumor cells; TME, tumor microenvironment; HSC, hepatic stellate cell; EVs, extracellular vesicles; PMN, pre-metastatic niches; miRNA, microRNA; CAFs, cancer associated fibroblasts; HCC, hepatocellular carcinoma.

cells, including tumor-associated macrophages (TAMs) and regulatory T cells (Tregs), accumulate within the TIME and facilitate the establishment of an immunosuppressive environment. Conversely, immune cells such as natural killer (NK) cells, $CD8^+$ T cells, and $CD4^+$ T cells work together to counteract tumor-promoting effects (35). EVs are abundant

in the immune microenvironment of HCC, acting as mediators of intercellular communication. The transfer of RNA or proteins *via* EVs between cells can influence the composition and homeostasis of the TIME, contributing to immune escape and promoting HCC metastasis and recurrence (36,37).

Numerous studies have confirmed that EV-mediated crosstalk regulates immune cell activity within the HCC TIME. The functional impairment of NK cells is recognized as a key mechanism for tumor cells to evade immune surveillance. Liu *et al* (38) demonstrated in an HCC mouse model that EVs secreted by hepatocytes lacking fructose-1,6-bisphosphatase 1 specifically target and infiltrate NK cells, suppressing NK-mediated tumor surveillance and promoting immune remodeling within the HCC TME. Exosomes secreted by HCC cells, which carry major histocompatibility complex class I-related chain A, may inhibit NK cell function by competitively suppressing agonistic NKG2D receptor signaling, thereby reducing NK cell cytotoxicity against HCC *in vivo* (39,40). Additionally, microRNA carried by EVs derived from HCC can further contribute to NK cell dysfunction and immune evasion. For instance, miR-92b, mediated by HCC-EVs, downregulates the activation marker CD69 on NK cells, impairing their activity (18).

Macrophages, notable players in the innate immune response of the liver, are also involved in HCC proliferation, metastasis, invasion and angiogenesis (41). TAMs are increasingly recognized as essential components of the HCC TIME (42,43). Tumor-derived factors can polarize macrophages into either the M1 phenotype, which exhibits anti-tumor activity, or the M2 phenotype, which promotes tumor growth (44). EVs derived from HCC cells promote macrophage activation and M2 polarization through various mechanisms, enabling tumors to evade immune surveillance (45,46). For example, EVs secreted by HCC deliver long non-coding (lnc)RNA PART1 to TAMs, upregulating TLR4 expression and thus inducing M2 polarization. Polarized M2 macrophages in turn promote HCC cell proliferation, metastasis and tumor growth *in vivo* (19). In another study, M2 polarization induced by HCC-EVs released exosomes that transferred miR-15b to HCC cells, inhibiting the Hippo pathway and promoting HCC proliferation, invasion and metastasis by targeting large tumor suppressor kinase 1 (20). The T cell-mediated immune response is central to cancer immunity, critical for immune surveillance and tumor cell clearance (47). In the TIME, T cell regeneration often leads to the deterioration of T cell responses and HCC progression. EVs may modulate the function and activity of $CD4^+$ T cells, $CD8^+$ T cells and Tregs, influencing the homeostasis of TIME and contributing to immune suppression, HCC progression and metastasis (19,48). Gong *et al* (49) demonstrated that norcholic acid increases programmed death ligand 1 (PD-L1) levels on the surface of HCC and its exosomes, notably inhibiting $CD4^+$ T cell function and inducing an immunosuppressive microenvironment that promotes HCC proliferation, invasion and metastasis. Furthermore, EVs from other immune cells in HCC contribute to the depletion of $CD8^+$ T cells, another mechanism of immune evasion. For example, HCC-EVs carrying miR-146a-5p induce macrophage polarization towards the $CD206^+PD\text{-}L1^+CD80^+$ M2 phenotype, and these EVs-induced macrophages impair $CD3^+$ T cell function by upregulating expression of inhibitory receptors (such as programmed cell death-1 and cytotoxic T-lymphocyte associated protein 4) on co-cultured T cells (50). Another mechanism for creating an immunosuppressive microenvironment in HCC is the recruitment of Tregs to TIME. The acidic microenvironment in HCC promotes the upregulation of miR-21 expression in EVs; miR-21 enhances Treg proliferation and recruitment to TIME while reducing the proportion of Th17 cells. The resulting imbalance between Th17 and Treg cells leads to immune suppression, fostering HCC invasion and metastasis (51,52).

CAFs are key components of the TIME, with increasing evidence supporting their role in promoting HCC recurrence and metastasis through immune suppression (53,54). EVs carrying miR-20a-5p, released by CAFs, activate the Wnt/β-catenin signaling pathway by downregulating LIM domain and actin binding 1, thereby facilitating immune evasion and enhancing HCC metastasis and invasion (55,56). Under hypoxic conditions, CAFs inhibit the activity and cytotoxicity of $CD8^+$ T cells *via* an EV-dependent mechanism, secreting EVs rich in circHIF1A and binding to HuR, upregulating PD-L1 expression, which in turn promotes HCC cell proliferation, migration and invasion. These findings suggest that the interaction between CAFs and the TIME, mediated by EVs, is a notable factor driving HCC progression (57). Further investigation into the activation of CAFs by HCC-derived EVs may offer new insights into the mechanisms underlying recurrence and metastasis, as well as potential therapeutic strategies.

In conclusion, EVs do not promote HCC metastasis in a general or indiscriminate manner. Their high heterogeneity, reflected in the diversity of cellular origins, sizes and molecular cargos, results in functionally specialized subpopulations. These subpopulations collaborate with high precision in both space and time; some prepare the environment by directing remote organs to form the PMN, others remodel microvessels to facilitate transport and others destroy the immune surveillance system along the way, enabling immune evasion. Consequently, future research into liver cancer metastasis and the development of EV-based liquid biopsy and treatments must focus on identifying, isolating and analyzing key pathogenic EV subpopulations, rather than considering EVs as a homogenous entity.

3. Mechanism of CTCs in HCC metastasis

Epithelial-mesenchymal transition (EMT). EMT is a process in which epithelial cells lose their adhesive properties and acquire mesenchymal traits, including enhanced migration and invasion capabilities (58). This mechanism plays a marked role in the metastasis of advanced HCC. EMT influences tumor cell proliferation and metastasis through various mechanisms, including the active release of CTCs from primary tumors, facilitating distant metastasis and promoting HCC recurrence (59,60). The activation of EMT in CTCs primarily occurs within the bloodstream, involving dynamic processes related to immune evasion and autophagy (61,62).

During EMT-mediated metastasis in HCC, CTCs contribute to the degradation of the basement membrane and extracellular matrix (ECM). Under hypoxic conditions and through paracrine signaling, CTCs activate transcription

factors, miRNAs and other regulatory elements, leading to the disruption of tight and adhesive cell connections and reorganization of the cytoskeleton. This upregulates mesenchymal markers and downregulates epithelial markers, enabling tumor cells to migrate through the matrix and enter the bloodstream (63,64). CTCs can be classified into three phenotypes based on EMT markers: Epithelial, mesenchymal and mixed CTCs (65). A study of 33 patients with HCC found that the number of epithelial CTCs was positively correlated with tumor size, the number of mixed CTCs was positively correlated with tumor number and the number of mesenchymal CTCs (M-CTCs) was positively correlated with metastasis. Mixed CTCs are key players in the EMT transition of HCC and contribute notably to intrahepatic metastasis, whereas M-CTCs may serve as predictors of extrahepatic metastasis (66). Both experimental and clinical studies highlight the marked role of EMT-associated CTCs in HCC metastasis and recurrence (67,68). A study of 112 patients with HCC showed that elevated CTC counts and a higher percentage of M-CTCs preceded the clinical detection of HCC recurrence. Notably, BCAT1, a gene markedly upregulated in these patients, may trigger the EMT process (69). Therefore, classifying CTCs based on EMT characteristics could serve as an early predictor of HCC recurrence, metastasis and survival (70). Furthermore, Lu *et al* (71) demonstrated that platelets, by binding to CTCs, induce autophagy *via* the AMPK/mTOR signaling pathway, which promotes EMT and enhances the motility of HCC cells.

The activation of EMT in CTCs may also be linked to immune responses. Huang *et al* (72) confirmed through *in vitro* experiments that there is a significant positive correlation between the number of Treg cells, CTC count and vascular infiltration, suggesting that Treg cells may serve as a key regulator of CTC release. Treg cells can enhance the invasive potential of HCC cells by secreting TGF-β1, thus triggering EMT. Therefore, targeting Treg cells within the TME, for example, through the depletion of Tregs *via* CCR8 targeting (73), or by inhibiting Treg-derived TGF-β1 signaling to suppress EMT (72), may represent a promising therapeutic strategy to prevent CTC release and, consequently, HCC metastasis and recurrence. Another study found that lncRNA FOXD1-AS1 promotes HCC immune evasion *via* PD-L1 and affects CTC production and metastasis in HCC mouse models by upregulating the PI3K/AKT signaling pathway, thereby regulating EMT and ultimately driving HCC proliferation, invasion, and metastasis (74).

Formation of microvascular invasion (MVI). MVI, also known as microvascular tumor thrombus, is characterized by the infiltration of malignant cells into the microvessels surrounding the liver tumor. It is one of the notable pathological features in HCC, strongly associated with poor prognosis following surgical resection and transplantation, early recurrence and intrahepatic metastasis (75). Studies suggest that CTCs released by HCC can form vascular thrombi from individual cells, serving as key components that further promote HCC recurrence and metastasis. Preoperative CTC counting may serve as a predictive tool for MVI and provide dynamic monitoring of HCC prognosis (9). Several mechanisms are thought to be involved in this process.

Firstly, CTCs derived from HCC trigger a coagulation response in peripheral blood, forming circulating tumor microemboli (CTM), which facilitates CTC migration across the endothelial barrier and metastasis both within and outside the liver. Studies have shown that tissue factor is overexpressed in the peripheral blood of patients with HCC and is closely associated with extrahepatic metastasis, lymphatic spread and portal vein thrombosis (76). Tissue factor, notably expressed in cancer cells and CTCs, promotes the binding of CTCs with coagulation factors, leading to the formation of clumps composed of both tumor and non-tumor cell components, such as platelets and fibrin. This coagulation response ultimately facilitates the aggregation and adhesion of CTCs. When tissue factor enters the bloodstream, it activates coagulation factor VII and initiates the extrinsic coagulation pathway, leading to the formation of CTMs in circulation (77). Moreover, once CTMs are formed, tissue factor can regulate the proliferation and growth of HCC cells by activating protease-activated receptors, making it a key factor in reshaping the TME and promoting the formation and progression of MVI.

Secondly, CTCs facilitate the dissemination and colonization of HCC tumor cells by activating platelets, thereby creating a favorable environment for tumor growth. Growing evidence suggests that platelets play a pivotal role in promoting the invasion and metastasis of HCC cells through multiple mechanisms. The ability of platelets to protect tumor cells in circulation is key to their support of hematogenous dissemination (78). CTCs induce platelet activation and aggregation, with activated platelets binding to CTCs, shielding them from the shear forces exerted by blood flow and vessel walls. This protection is essential for the survival of CTCs and their subsequent adhesion to the vascular endothelium at metastatic sites (79). Moreover, CTC-induced platelet activation also aids HCC cells in evading immune surveillance. Sun *et al* (80) demonstrated that CTC-platelet adhesion helps cancer cells escape NK cell-mediated innate immune responses by upregulating the immune checkpoint CD155-TIGIT, thereby promoting distant metastasis. Activated platelets also secrete numerous growth factors, including TGF-β, VEGF and platelet-derived growth factor. VEGF, secreted by CTCs, enhances microvascular permeability by enlarging the spaces between adjacent endothelial cells. The interaction between CTCs and platelets markedly increases the likelihood of MVI (81). In summary, platelets activated by CTCs not only protect the CTCs but also contribute to the formation of new blood vessels in HCC by transporting cytokines that provide nutrients essential for tumor cell growth (82,83). Additionally, platelets regulate the integrity of tumor vasculature, promoting CTC adhesion and transendothelial migration, which leads to HCC metastasis and colonization. While the role of platelets in HCC metastasis is well-documented, further research is needed to elucidate the specific mechanisms underlying the activation and maintenance of the CTC-platelet interaction.

Tumor cells consisting of two or more CTCs are referred to as CTC clusters or CTMs. CTC clusters have been proposed as indicators of tumor metastasis. Although the number of CTC clusters in circulation is smaller than that of single CTCs, their metastatic potential is notably higher, increasing by 23-50 times (84). Understanding the formation mechanisms of CTC clusters is therefore critical for elucidating the metastasis of

HCC. Currently, two widely accepted mechanisms explain the formation of CTC clusters; the first involves tumor cells directly forming cluster-like structures that invade microvessels after detaching from the primary tumor (85). Aceto *et al* (86) were the first to demonstrate that CTC clusters originate from oligoclonal tumor cell populations rather than from cell aggregation within blood vessels. The authors also identified plakoglobin-dependent intercellular adhesion as critical for CTC cluster formation. The second mechanism suggests that tumor cells detach from the primary tumor due to shear forces from blood flow, diffuse into blood vessels and aggregate into clusters (87). Sun *et al* (62) prospectively measured CTCs in five key vascular sites in patients with HCC and detected CTCs in tumor-feeding vessels in approximately half of the patients. Notably, CTCs were found in the hepatic veins of 15 patients but not in the surrounding arteries, and CTC recurrence was observed in peripheral veins in five of these patients. This suggests that individual CTCs may aggregate again in the bloodstream. While this contradicts conclusions made by Aceto *et al* (86), it warrants further investigation. The formation of CTC clusters is a multistage and dynamic process, with mechanisms exhibiting spatiotemporal heterogeneity. The two main pathways currently recognized are collective shedding from the primary tumor and single-cell re-aggregation in circulation, which likely to coexist and complement each other. CTC clusters undergo a complex life cycle, beginning with clonal collective shedding from the primary tumor, followed by dynamic structural evolution in the challenging environment of circulation, partially disintegrating and partially reassembling. Ultimately, the metastasis efficiency may depend on the ability of certain cell populations to maintain an aggregated state or effectively reorganize within distant blood vessels. The unique hemodynamics of the portal venous system in HCC, the abundance of immune cells in the liver and the altered vascular structure in cirrhosis may make the dynamic evolution of CTC clusters particularly active in the intrahepatic circulation. This may play a key role in the intrahepatic dissemination of HCC.

4. Mechanisms of EV-CTC interactions promoting HCC metastasis

EVs enhance the shedding ability of CTCs. At the onset of metastasis, EVs enhance CTC shedding by promoting EMT and ECM remodeling, stimulating angiogenesis, increasing vascular permeability and strengthening the adhesion ability of CTCs. In advanced HCC, target organ metastasis is common, with EMT activation playing a marked role in initiating metastasis (88). Studies have demonstrated that EVs are pivotal in the metastatic process of HCC, inducing EMT activation and weakening the adhesion of tumor cells, thus facilitating the shedding of tumor cells to form CTCs (89). EVs regulate key genes (for example, PRDM16 and LHX6) in the EMT pathway, activating the Wnt/β-catenin, Notch and MAPK/ERK signaling pathways, among others, to promote the detachment of CTCs from the primary tumor site and enhance their shedding. Chen *et al* (90) observed that HCC cells treated with EVs showed elevated expression of α-SMA and vimentin, along with reduced expression of E-cadherin, promoting mesenchymal marker expression. Western blot analysis confirmed that these processes were mediated through the MAPK/ERK pathway, facilitating tumor cell migration, chemotaxis and invasion. Further research has revealed that EVs derived from hepatic stellate cells (HSCs) activate the Notch signaling pathway in HCC cells *via* PRDM16, leading to increased proliferation, migration, invasion and EMT progression (91). Hypoxia-induced EVs in HCC activate EMT through the Wnt/β-catenin signaling pathway, promoting HCC cell proliferation, migration and invasion (92). Moreover, tumor-derived EVs can directly or indirectly promote angiogenesis and increase vascular permeability by releasing pro-angiogenic factors (for example, VEGF and IL-8), which play a pivotal role in the formation of shed CTCs at the primary tumor site (93). Hypoxic HCC cell-derived EVs co-cultured with M2 macrophages secrete higher levels of VEGF, granulocyte colony-stimulating factor and IL-4, which further stimulate angiogenesis and increase vascular permeability, enabling tumor cells to shed into circulation as CTCs, thereby enhancing metastasis (94). Disruption of vascular barriers is a pivotal step in CTC-mediated HCC metastasis, requiring the breakdown of tight junctions and the integrity of vascular endothelial cells (95). Tumor-derived EVs enhance vascular permeability and promote CTC production and shedding by transferring their contents to endothelial cells. The protein nidogen 1, secreted by HCC-derived EVs, increases the permeability of lung endothelial cells by disrupting the integrity of the endothelial barrier and promoting angiogenesis, raises tumor necrosis factor receptor 1 expression and facilitates the formation of the PMN in the lungs (96).

In summary, EVs are key initiators driving HCC metastasis, initiating EMT to internally transform tumor cells and endow them with migratory potential. Simultaneously, EVs directly target and downregulate key junction proteins (such as VE-cadherin and p120-catenin) in endothelial cells by delivering specific cargoes, such as miR-103. This disruption of tight junctions compromises the vascular endothelial barrier, thereby reducing the physical obstacles to CTC intravasation into the circulatory system. This dual approach facilitates CTC generation and shedding, driving HCC metastasis.

EVs protect CTCs and enhance their adhesion ability. CTCs are notable drivers of distant metastasis in HCC. The survival of CTCs in circulation faces notable challenges, including the need to withstand intravascular shear stress and evade immune surveillance. EVs can interact with various hematopoietic and immune cells. For example, EVs induce platelet activation by delivering CD97. Activated platelets can protect CTCs from hemodynamic shear forces, thereby enhancing CTC survival in the bloodstream. Additionally, EVs can induce neutrophils to form neutrophil extracellular traps, which bind to CTCs and promote their adhesion to blood vessels, helping CTCs survive and initiate metastasis (97,98). EVs carry molecules that interact with circulating immune cells, inducing immunosuppression and further protecting CTCs from immune system attacks. Research has demonstrated that PD-L1 is markedly expressed on CTCs, and EVs promote immune escape by upregulating PD-L1 expression on CTCs, thereby hindering $CD8^+$ T cell-mediated immune responses in HCC (99-101). The delivery of immune checkpoint molecules such as PD-L1 by EVs is a recognized immune evasion mechanism in various

solid tumors. In HCC, however, this process occurs within a unique liver immune microenvironment. In the specific context of chronic inflammation caused by HBV/HCV infection or non-alcoholic fatty liver disease and alcoholic liver disease, Kupffer cells undergo marked functional remodeling and spatial repositioning, while being in a 'pre-activated' immune regulatory state, characterized by upregulation of immune checkpoint molecules such as PD-L1 (102,103). This pre-existing immune tolerant microenvironment in the liver, shaped by chronic inflammation, makes Kupffer cells more sensitive to EVs from tumor sources. Therefore, the EVs in HCC not only directly inhibit immune effector cells, but also may synergistically amplify the immunosuppressive effect by acting on these already sensitized Kupffer cells, thereby achieving dual protection against CTCs; however, to the best of our knowledge, there is currently no direct evidence that reveals whether the specific mechanism of EV-mediated CTC survival specifically occurs in HCC with different etiological backgrounds such as HBV, HCV, non-alcoholic fatty liver disease or alcoholic liver disease. Therefore, this hypothesis still requires targeted verification through the design of rigorous experiments. EVs can enhance CTC adhesion, facilitating their colonization in specific organ microenvironments, highlighting the importance of the PMN.

EVs reshape TME to facilitate the colonization ability of CTCs. The TME of HCC is dynamic and complex, comprising cancer cells, stromal cells, blood vessels, nerve fibers and related decellularized components (104). EVs have emerged as key mediators of intercellular communication within the TME, driving tumor progression (105). Accumulating evidence suggests that EVs transfer functional biological factors to recipient cells, reshaping the TME by reprogramming both cancer cell and stromal cell metabolism (106). Fang *et al* (17) demonstrated that EVs released by metastatic HCC cells carry miR-1247-3p, which directly targets B4GALT3 in fibroblasts. This activates the β1-integrin-NF-κB signaling pathway, inducing the transformation of fibroblasts into CAFs, and promotes lung metastasis in patients with HCC.

Furthermore, EVs contribute to the formation of a PMN for CTCs, creating a favorable foundation for their colonization. Tumor-derived EVs serve as key intercellular messengers that recruit inhibitory immune cells (such as TAMs and Tregs) to distant organs by carrying cytokines (such as TGF-β1 and IL-10). These recruited immune cells suppress antitumor immunity through functional reprogramming, including M2 polarization of macrophages and enhancement of Treg-mediated suppression. Concurrently, EVs promote the establishment of a PMN in distant organs by increasing vascular permeability, activating fibroblasts and remodeling the extracellular matrix. This coordinated remodeling of the immunosuppressive and stromal microenvironment facilitates the colonization and outgrowth of CTCs at distant sites (107). EVs released by HCC cells form a liver PMN rich in fibrotic stroma and immunosuppressive cells by activating HSCs and recruiting myeloid-derived suppressor cells; this creates an environment that promotes CTC colonization. For example, TGF-β1-enriched EVs induce HSCs to secrete fibronectin, thereby promoting CTC adhesion and invasion (108). Conversely, EVs released by CTCs can further recruit immunosuppressive cells (such as Tregs) and remodel the liver TME, forming a positive feedback loop. For instance, CCL5 secreted by CTCs is transmitted to Kupffer cells through EVs, stimulating the release of tumor necrosis factor-α, exacerbating liver inflammation and fibrosis and accelerating HCC metastasis (61).

In summary, EVs support CTCs by constructing a dynamic, multi-layered protective network. In circulation, platelet-derived EVs encapsulate CTCs, forming clusters that shield them from shear stress, aiding their survival in the bloodstream. Before colonization, EVs act as messengers, modifying the target organ microenvironment and creating niches for CTCs. However, most current research is based on *in vitro* models, and whether the complex *in vivo* microenvironment fully aligns with this protective model remains uncertain. Future research should focus on longitudinal clinical cohort studies to dynamically monitor changes in EV cargo and CTC characteristics in patients with HCC at various stages, before and after treatment, to establish causal relationships. Additionally, novel liquid biopsy strategies targeting specific molecular markers of 'CTC-protective EVs' should be developed to enable early and functional prediction of metastatic risk.

5. Current status, challenges and clinical value of EVs and CTCs in liver cancer detection

Tumor-derived EVs are increasingly recognized as a promising liquid biopsy tool, with studies highlighting their potential in detecting high-risk patients with HCC, yielding promising results (109,110). However, the inherent heterogeneity of EVs presents both a source of valuable information and a major challenge for clinical application. The complex origins of EVs in the blood of patients, coupled with signals from liver cancer being potentially obscured by EVs from normal cells, complicate their clinical translation. Consequently, researchers have focused on developing more efficient methods for isolating EVs, aiming to enhance their clinical utility. Despite these advancements, a standardized protocol for EV isolation is still lacking, and isolating specific EV subpopulations with high purity remains a challenge, resulting in mixtures, signal dilution and interference, thus limiting their clinical applicability (111).

Microfluidic separation technology, known for its low sample consumption, high throughput and rapid reaction speed, has emerged as a promising method for EV isolation in recent years (112). This technology enables the simultaneous sorting of different EV subpopulations based on immune affinity and/or physical properties, showing promise for the specific and efficient enrichment of HCC-derived EVs from the blood of patients with cirrhosis, which is critical for distinguishing tumor signals from benign inflammatory backgrounds. Such advancements could markedly improve early diagnosis and monitoring of minimal residual lesions (113). Sun *et al* (114) developed an EV click chip that achieves high purification efficiency (90.2%) and recovery rate (82.7%) for HCC-derived EVs. This method integrates: i) A multi-labeled antibody cocktail targeting HCC-derived EVs; ii) a nanostructured substrate to increase surface area for EV interaction; and iii) a reversible capture/release system mediated by click chemistry. Combined with reverse transcription droplet

digital PCR, this platform shows promising potential for early detection of HCC. Wu *et al* (115) quantified Fascin-1-positive EVs in patient plasma using nanoflow cytometry, finding that the levels of Fascin-1-positive EVs in the plasma of patients with HCC were notably higher than in healthy controls, with a positive association with HCC staging. This demonstrates the potential of Fascin-1-positive EVs as a novel liquid biopsy marker for distinguishing early-stage HCC from healthy individuals. Mechanistically, Fascin-1 may be selectively packaged into EVs by HCC cells, where it can upregulate F-actin levels, promoting cancer cell migration. Another research group developed a detection method based on surface proteins of HCC-derived EVs to quantify three subpopulations: $EpCAM^+$ $CD63^+$, $CD147^+$ $CD63^+$ and $GPC3^+$ $CD63^+$ HCC-EVs. The HCC-derived EV score, calculated from these subpopulations, is used to detect early HCC, showing a sensitivity of 91% and specificity of 90% in distinguishing early HCC from cirrhosis (116). Thus, while EVs hold potential in screening high-risk HCC populations, they should not replace existing detection methods but rather complement them by providing dynamic molecular insights. Moving forward, there is a need for the validation of marker combinations in large prospective cohorts and the development of standardized, high-throughput detection techniques suitable for mass screening.

The heterogeneity of CTCs poses a marked challenge for their reliable detection in bodily fluids, limiting the clinical application of CTC-based assays. Technologies that capture CTCs based on epithelial markers may overlook invasive subpopulations that have undergone EMT, missing key targets and complicating clinical detection. In patients with HCC, CTCs are often present in low quantities, with only 0-86 CTCs detectable per 5 ml of peripheral blood (68). Consequently, there is a need for more specific and sensitive CTC capture technologies for further analysis.

Currently, no FDA-approved platform exists for detecting HCC-related CTCs. Most CTC detection methods rely on biological characteristics (antibody capture, such as EpCAM), physical properties (such as size and density) or non-enriched techniques, followed by immunocytochemical staining for CTC characterization. Platforms focused on positive or negative enrichment based on immune affinity can be further categorized into magnetic and microfluidic devices. For instance, the CellSearch platform uses magnetic beads to isolate EpCAM-labeled cells (117); however, this technology has notable limitations for HCC applications. The primary issue is the mismatch between the epithelial markers it relies on and the high heterogeneity and metastatic nature of HCC. Only 20-35% of HCC tumor tissues express EpCAM (118), leading to the missed detection of a large fraction of EpCAM-negative or low-expressing cells. A study by Morris *et al* (119) demonstrated that the Isolation by Size of Epithelial Tumor Cells platform achieved a 100% CTC detection rate in patients with HCC, whereas CellSearch detected only 28% of CTCs. Additionally, CellSearch may fail to detect more aggressive M-CTCs, resulting in false negatives, especially in early-stage HCC or in post-treatment patients with low CTC counts. Single-cell RNA sequencing (scRNA-seq) offers a powerful approach to uncover spatiotemporal transcriptional dynamics related to tumor cell cycles and immune evasion signals. Sun *et al* (61) used scRNA-seq to profile the transcriptome of 113 single CTCs from four different vascular sites in 10 patients with HCC. The authors identified chemokine CCL5 as a key mediator of CTC immune evasion. The overexpression of CCL5 in CTCs is regulated by the p38-MAX signaling pathway, which recruits Tregs to promote immune evasion and facilitate CTC metastasis and seeding. This highlights the potential of using scRNA-seq in the future to develop a comprehensive molecular profile of HCC-related CTCs. By identifying genes or protein markers, it may be possible to create a standardized, clinically applicable multi-marker detection panel.

Studies have highlighted the potential of CTCs in predicting treatment efficacy and monitoring disease progression in HCC, especially after liver resection. CanPatrol™, a technology based on biophysical characteristics, employs a microfiltration system to isolate and classify various CTC phenotypes. In a cohort study of 115 patients with HCC, CTCs were isolated using the CanPatrol enrichment method and classified *via in situ* hybridization. The findings showed that M-CTCs were associated with high Ki67 expression, both correlating with poor prognosis in patients with HCC. The combination of M-CTCs and Ki67 expression may serve as a critical prognostic marker for predicting overall survival following liver resection (120). Since EMT is frequently linked with tumor aggressiveness, understanding the biological connection between EMT and metastasis warrants further mechanistic investigation. Additionally, Chen *et al* (67) used the CanPatrol method to detect total CTCs, M-CTCs and CTC-white blood cell (WBC) clusters in 136 patients with HCC. The simulated receiver operating characteristic curve revealed an area under the curve of 0.821 for CTC-WBC clusters (sensitivity 90.0%, specificity 93.7%), outperforming both total CTCs (0.718) and M-CTCs (0.716). Furthermore, patients with positive CTC-WBC clusters had a higher recurrence rate, with levels rising 10 months before clinical detection of recurrence. While these studies establish statistical associations between CTC subtypes and HCC prognosis, the molecular mechanisms by which these subtypes promote tumor proliferation, immune evasion and metastasis remain insufficiently understood. Future research must include expanded clinical validation, as well as the integration of models such as organoids and *in vivo* imaging to explore the biological roles of these CTC subpopulations. This will enable the development of targeted clinical intervention strategies, advancing from mere prognostic markers to actionable therapeutic guidance tools.

6. Conclusions

The present manuscript reviews advances in understanding the roles of EVs and CTCs in HCC metastasis and recurrence. EVs enhance CTC shedding during metastasis and protect CTCs from immune system attack. The bioactive molecules carried by EVs promote CTC-mediated HCC recurrence and metastasis, with CTCs acting as the initiators of metastatic spread and EVs facilitating their colonization by remodeling the microenvironment.

In conclusion, EVs and CTCs work synergistically to promote HCC metastasis and invasion through a multifaceted mechanism. EVs act as messengers, paving the way for CTC colonization by forming PMNs, remodeling the TME, transmitting molecular signals and regulating immune responses. CTCs, as the executors

of metastasis, enable distant spread through processes such as EMT, MVI and adaptive heterogeneity. Despite the marked progress, several questions remain to be addressed. For instance, it is still unclear whether CTCs release novel subpopulations of EVs after colonizing distant sites, potentially remodeling established metastatic foci. Further research is needed to explore this aspect. Additionally, future therapeutic strategies could focus on combining immune checkpoint inhibitors with EV/CTC-targeted therapies, offering precise interventions to manage HCC, prevent recurrence, and curb metastasis. These advancements will deepen the understanding of liver cancer metastasis, ultimately improving clinical outcomes for patients with HCC.

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Authors' contributions

All authors contributed to the study conception and design. The study was conceived by ZW, while SY conducted the literature search and data analysis. The manuscript was drafted and/or critically revised by SY, WL and ZW. The first draft of the manuscript was written by SY. Data authentication is not applicable. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

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Competing interests

The authors declare that they have no competing interests.

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