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Review Article

Extracellular vesicles in cancer immunotherapy: Therapeutic, challenges and clinical progress

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ABSTRACT

Cancer is a major global concern due to its high mortality rate. Tumor immunotherapy has revolutionized cancer treatment. However, low response rates and immune-related complications remain challenges. Extracellular vesicles (EVs), including exosomes, have emerged as promising therapeutic tools for various pathological conditions, especially cancer. Evidence indicates that changes in the quantity and composition of EVs can influence the immunosuppressive tumor microenvironment, potentially affecting the effectiveness of immunotherapy. Exploiting EVs for immune sensitization has generated significant clinical interest. This review provides an in-depth understanding of the origin of EVs, their therapeutic applications (such as drug delivery nanoplatforms and cancer immunotherapies, including vaccines), diagnostic potential as tumor biomarkers, ongoing EV-based clinical trials, and the challenges encountered in EV-based cancer immunotherapy.

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1. Introduction

Cancer is the leading cause of death worldwide, which has reduced the quality of life [1]. The Global Cancer Observatory (GLOBOCAN) 2022 revealed about 20 million new cancer cases and nearly 10 million cancer fatalities [2]. The main cancer treatments are chemotherapy, radiation therapy, cryoablation, and surgical procedures. Pioneering treatments like precision therapy and immunotherapy target tumor cells with minimal side effects without affecting non-cancerous cells [3]. Cancer immunotherapy majorly modifies the tumor immune microenvironment (TIME), which enables immune cells to combat cancer cells [4]. Therefore, understanding the mechanisms of how the immune system evades cancer cells is crucial in recent cancer treatment. Some immunotherapy approaches employed in cancer treatment are immune checkpoint inhibitors (ICI), oncolytic virotherapy, adoptive cell therapy, cancer vaccines, and pericyte therapy [5,6].

Cancer immunomodulation has recently advanced cancer research using exovesicles or extracellular vesicles (EVs). EVs are a collection of particles enclosed in a phospholipid bilayer membrane, released by cells into the intercellular space [7]. Prokaryotic and eukaryotic cells shed EVs. EVs are classified based on their sources, size, characteristics, formation, and functions, namely exosomes, microvesicles (MVs), and apoptotic bodies (apoptotic EVs) [8–10]. These vesicles facilitate cellular interactions in standard and pathological conditions by transferring various biological materials, such as nucleic acids, lipids, and proteins from different cell types [7,8,11,12]. Furthermore, EVs emitted from cancer cells impede the effectiveness of immunotherapy and undermine immune responses against cancer. On the contrary, EVs from immunocytes trigger anticancer immune responses by transporting cancer-specific antigens and different stress proteins. However, EVs can either boost or hinder anticancer immunity within the tumor microenvironment (TME) [7].

EV-based therapies have been developed to counter immune evasion due to the protumorigenic effect stimulated by the TME. These approaches focus on inhibiting the production of EVs by cancer cells or utilizing EVs released naturally by immune cells [7]. EVs can also serve as drug carriers in cancer treatment by modifying their content or surface with therapeutic agents. This allows them to target cancer and immune cells, producing potent anticancer immune responses [13]. Preliminary studies have shown that engineered EVs can activate T lymphocytes and macrophage responses, reducing cancer progression [7]. This review explores the role of EVs in immunotherapy and as cargo in cancer immunoregulation. It also provides an overview of EV biology and its functions. In conclusion, we examine the potential role of EVs in immunotherapy and the challenges in clinical practice.

2. EVs: definition and origins

Recently, there has been increasing interest in understanding EVs' generation, role, and potential, although their metabolic byproducts have often led to limited interest [14]. To improve the isolation and classification of different EV types, the International Society for Extracellular Vesicles (ISEV) introduced the Minimal Information for Studies of Extracellular Vesicles (MISEV) guidelines in 2014 and subsequently updated in 2018 and 2023. EV production can be affected by external stimuli, regulation of EV biogenesis, and innovative culture systems. Hence, optimizing the culture conditions and combining EVs with synthetic liposomes could improve the yield [15].

2.1. *Exosome (Exo)*

In the 1980s, Exos were identified as substances secreted by various cultured cells, typically ranging in size from 30 to 150 nm [16–20]. This secretion occurs when multivesicular bodies (MVBs) fuse through the inward budding of the plasma membrane, releasing contents that include enzymes to break down 5′-ribonucleotides into ribonucleosides and orthophosphate, resulting in the development of intraluminal vesicles (ILVs) (Fig. 1). After this process, ILVs fuse with the cell membrane, enabling the release of Exos into the extracellular environment [21,22]. However, in the late 1980s, smaller vesicles with 30 to 100 nm diameter were from endosomes and released during the maturation of reticulocytes [22]. Later in the 20th century, studies showed that B lymphocytes and dendritic cells (DCs) also release similar vesicles originating from endosomes [21]. This demonstrated the role of Exos in immune surveillance and anti-tumor immunomodulation [23]. The membrane of Exos is composed of various phospholipids and proteins derived from the parent cell, including carrier proteins, stress proteins, and tetraspanins [24,25]. It also possesses a globular or cup-like morphology [8,24]. Recent advancements in cytoplasmic membrane research uncovered that Exos play roles beyond simple waste transport [14]. The endosomal sorting complex required for transport (ESCRT)-dependent mechanisms and alternative pathways are used to generate Exos [26,27].

2.1.1. *ESCRT-dependent Exo biogenesis*

ESCRT-dependent pathway is the primary mechanism for Exo production. This pathway involves ESCRT-0, -I, -II and -III, as well as endosomal membrane deubiquitinases [26]. For instance, studies have shown that HGS (ESCRT-0) is essential for generating Exos. Meanwhile, GPR143 is crucial in regulating the ESCRT-dependent Exo biogenesis pathway. It impacts selective protein sorting and influences cancer cell motility and metastasis via the integrin/FAK/Src pathway, highlighting its importance in cancer progression [28].

2.1.2. *Alternative pathways of Exo biogenesis*

Exos can be formed through G-proteins like the Ras superfamily, ADP ribosylation factor 6 (ARF6), the enzyme phospholipase D2 (PLD2), and the syndecan-binding protein syntenin processes [29]. Recent studies indicate that alternative pathways, including autophagy-dependent secretome and amphisome formation, are essential for Exo biogenesis. These pathways present potential therapeutic

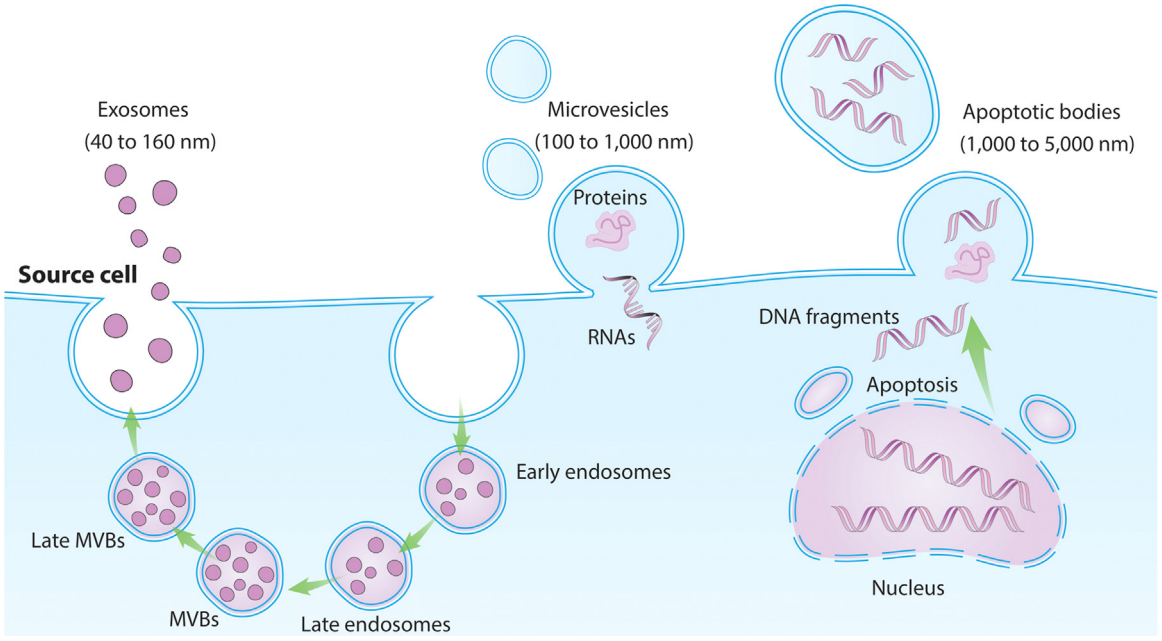

Fig. 1 – Process of EV formation and release from a source cell. Exos (40 to 160 nm) are released when MVBs fuse with the plasma membrane. MVs (100 to 1,000 nm) form directly from the plasma membrane, containing proteins and RNAs. Apoptotic bodies (1,000 to 5,000 nm) are produced during apoptosis and contain DNA fragments.

targets for altering cellular secretion profiles in various diseases [30]. Additionally, some Exos can be released through budding at the cell surface membrane without involving the Rab family [31].

2.2. *Microvesicle*

Microvesicles (MVs), also known as platelet dust, are platelet-derived particles found in healthy blood plasma and serum [23]. They are membrane-bound vesicles that blebbed from the cell membrane before being released into the extracellular space (Fig. 1). The diameter of MVs ranges from 100 nm to 1,000 nm, whereas oncosomes or oncogenic EVs can reach sizes of up to 10,000 nm [22,24]. MVs are found in various biofluids such as circulatory fluid, renal excretion, joint fluid, and bodily fluids in healthy and diseased states [24]. The generation of MVs involves phospholipid rearrangement, including phosphatidylserine (PS) externalization and activation of the contractile apparatus, while their shedding is stimulated by external factors [31]. For example, the biogenesis of MVs is triggered by the influx of calcium ions (Ca^{2+}) and an increase in cytosolic Ca^{2+} levels [31,32]. It activates the phospholipid transporter scramblase and the cysteine proteinase calpain [32]. Meanwhile, scramblase activity causes the translocation of phospholipids within the cytoplasmic membrane, while intracellular Ca^{2+}-dependent calpain activity leads to the breakdown of various proteins, further facilitating MVs blebbing [31,32]. This results in the fusion of proteins from the cytoplasmic membrane to the protruding MVs while the molecules on the cell membrane become encapsulated within the MVs [33].

2.3. *Apoptotic body*

Apoptotic body (Apo-EVs), a subcategory of EVs, are 1,000 to 5,000 nm in size [32]. These vesicles are produced during apoptosis and may contain fragmented intracellular organelles and nuclear components (Fig. 1) [33]. The membrane composition of Apo-EVs reflects the changes that occur on the surface of the parent cells during apoptosis [32]. Macrophages clear most Apo-EVs. Previous studies suggest that those escaping phagocytosis can transmit information to nearby or distant cells [33].

2.4. *EV cargo*

EVs encapsulate and transport components, including proteins, lipids, nucleic acids, metabolites, and non-coding RNAs. The composition and quantity of these contents influence EV formation and secretion [22]. Notably, EVs contain proteins that govern their biosynthesis process, proteins like ESCRT-related proteins such as tumor susceptibility gene 101 (TSG101) and apoptosis-linked gene 2-interacting protein X (ALIX) (Fig. 2) [31]. Meanwhile, EV generation and secretion proteins, including small GTP-binding proteins like Ras-related protein Rab-27A (Rab27A), Ras-related protein Rab-11B (Rab11B) and ARF^{3} [31]. In addition, cell membrane proteins, specifically tetraspanins like tetraspanin 30, tetraspanin 28 and tetraspanin 29, along with cell-signaling proteins [*e.g.*, epidermal growth factor receptor (EGFR), major histocompatibility complex class I/II (MHC I/II)], and other membrane-associated proteins like CD107a (Lysosome-associated membrane glycoprotein 1) and CD71(Transferrin receptor protein 1), are commonly found in

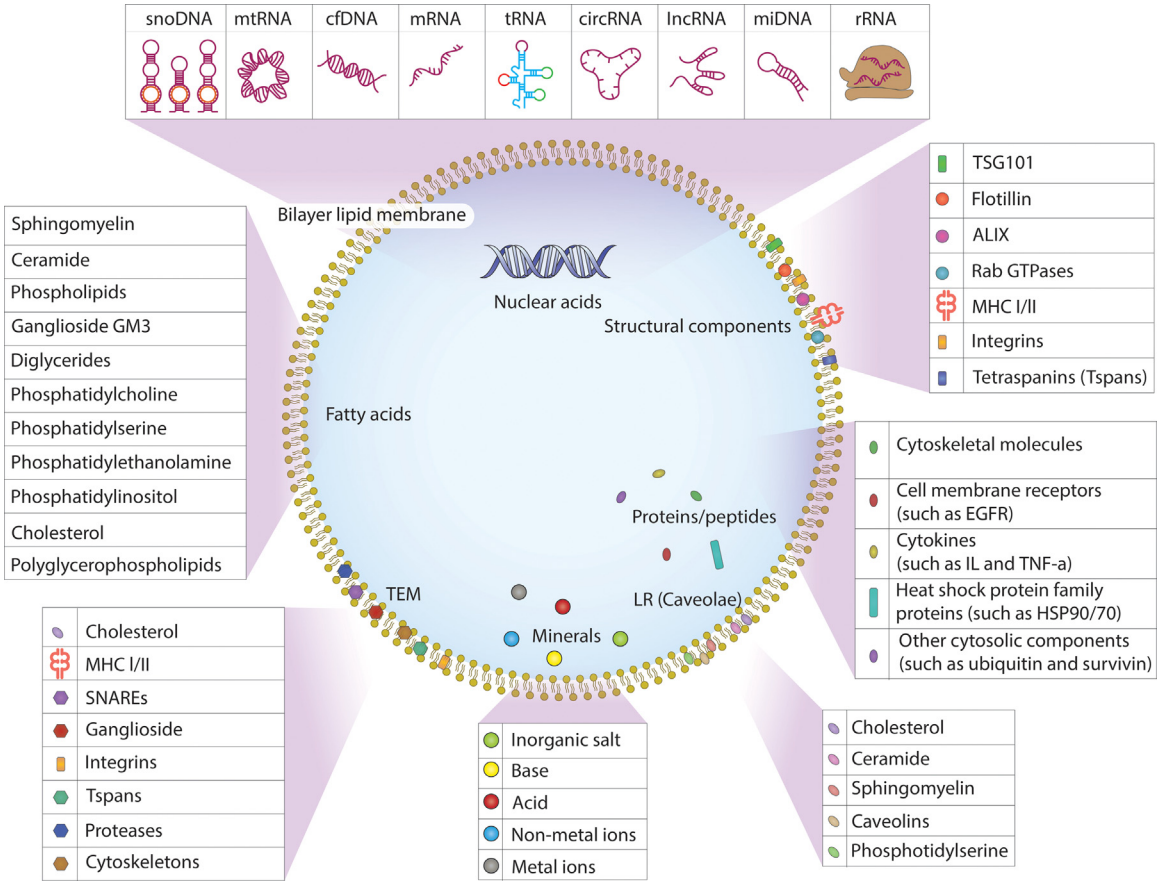

Fig. 2 – Composition and structural components of EVs. EVs have various components: nuclear acids such as snoDNA, mtRNA, cfDNA, mRNA, tRNA, lncRNA, miDNA and rRNA; a bilayer lipid membrane comprising sphingomyelin, ceramide, phospholipids, ganglioside GM3, diglycerides, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, phosphatidylinositol, cholesterol and polyglycerophospholipids. Structural components include proteins/peptides, cytoskeletal molecules, and minerals. It also features signaling proteins like EGFR and cytokines such as ILs and TNF-α, with key proteins including TSG101, Flotillin, ALIX, Rab GTPases and HSP90/70.

EV [31,34]. The lipid composition of EVs closely resembles the parent cells in which they are derived. However, prior studies have suggested that different categories of EVs are uniquely associated with specific lipids. Lipids such as sphingomyelin, cholesterol, hematoside, unsaturated fatty acids, PS, and ceramide are in high concentrations within EVs [31]. EVs also contain a diverse array of genetic materials, including nuclear DNA, mitochondrial DNA (mtDNA), ribosomal RNAs, transfer RNAs, messenger RNAs (mRNAs), microRNAs (miRNAs), small non-coding RNAs, long and small untranslated RNAs, piRNAs, vtRNA and Y RNA [31].

2.5. *Functions*

EVs play a vital role in biological processes by delivering proteins and lipids that activate receptors on target cell membranes, facilitating fusion with the target cell's cytoplasmic membrane. This process enables the transfer of various elements, including sequence-specific DNA-binding factors, transforming genes, long and small untranslated RNAs, mRNAs, and infectious agents into the recipient cells [35]. Through these mechanisms, EVs play a role in maintaining normal physiological functions, including stem cell retention, tissue repair, immune surveillance, physiological neovascularization, and natural hemostatic processes [32,35]. EVs can be considered signalosomes because of their intricate signaling entities that govern critical cellular and bodily processes. In addition, EVs contribute to cancer development due to their pivotal role in regulating biological phenomena. These signaling effects of EVs imply that they may inherently offer therapeutic potential, particularly in immunotherapy, because of their wide range of mechanisms [35].

3. EVs isolation and characterization

3.1. *EVs isolation*

Despite extensive studies on EVs, standardized procedures for their collection, separation, and preservation are still lacking. Several strategies exist for isolating EVs, but the diminutive diameters complicate the purification procedure. EV separation methods exhibit inherent tendencies to specific EV subpopulations or risk contamination with co-isolated materials [36]. For instance, because thrombocytes and

Table 1 – The pros and cons of each EV isolation methods.

Isolation method	Advantages	Disadvantages	Ref.
Differential ultracentrifugation	High purity	Time-intensive Low yield of EVs Requirement specialized ultracentrifuge Induction of physical damage to EVs	[36,41]
Polymer-based precipitation	User friendly Cost-efficient High EVs recovery Straightforward implementation	Low purity Difficulty of eliminatingpreparationreagents from the final preparation	[36,47]
Immunoaffinity capture	Isolation of EV subpopulations with remarkable specificity	Elimination of EVs that do not exhibit the specific marker Challenging in the removal of bound antibodies Expensive Low yield of EVs Time-intensive	[36,38,41,47]
Size exclusion chromatography	Preservation of EV function and structure Narrower size distribution of isolated EVs Short duration of sample processing High EVs recovery Good purity	Requirement for manual collection Dilution of the purified sample There is no risk of contamination from particles with similar densities. Long isolation duration Selection of EVs of specific sizes over others	[41,45]

lipoproteins in plasma and serum display similar EV sizes and densities, there are no techniques to completely isolate all EVs or their subpopulations without contaminants [37]. Moreover, there is a significant difference between these techniques based on duration, complexity, and necessity for specific instruments and expert skills [37]. Therefore, various methods produce varying outcomes regarding yield, purification, and compatibility for future usage. The physical and biochemical factors used in isolating EVs are diameter, density, charge, and specific biomarkers [36]. Different techniques for EV isolation have been employed, such as differential ultracentrifugation, Polymer-Based Precipitation, Immunoaffinity capture, and size exclusion chromatography (Table 1) [32,38].

3.1.1. *Differential ultracentrifugation*

Differential ultracentrifugation represents the initial technique employed to isolate EVs from biological fluids and cell culture medium [38]. This technique is based on the isolated EVs' density, size and shape. The main principle of this method is that larger and denser particles sediment out first, enabling the effective fractionation and purification of the target EV populations [39,40]. This technique entails a series of centrifugation steps using different centrifugal forces to facilitate the isolation of particles [38]. Since centrifugation relies on density as the fundamental principle for particle isolation, the pellet obtained from this process contains EVs and contaminations exhibiting equivalent densities. The contaminations may include viruses, proteins, lipoproteins, and cell residue removed by low-speed pre-centrifugation. Followed by vesicle isolation using centrifugal forces between 19,000 and 100,000 $\times g$ [38]. This approach is inadequate for recovering EVs from highly viscous biological fluids like plasma [41].

3.1.2. *Polymer-based precipitation*

The polymeric precipitation process is a phase separation via ultracentrifugation based on the interaction between the samples and the reagents, where the less soluble elements aggregate to form sediments. However, the high centrifugal forces of ~1,500 g could negatively impact the integrity of EVs. On the other hand, the co-isolation of EVs with unwanted proteins during polymer-based precipitation could result in impurities from the pellets [42]. For instance, coagulation factor I, commonly referred to as fibrinogen, is a plasma component that can compromise the purity of isolated EVs from plasma. However, adding thrombin, followed by centrifugation, can alleviate impurities [43]. In addition, polymer-based precipitation techniques have led to the detection of serum albumin and Apoproteins E in isolated EVs [44].

3.1.3. *Immunoaffinity capture*

The isolation method based on immune capture relies on the EV surface antigen [39]. This technique fundamentally employs antibodies for identifying EV receptors or biomarkers, including tetraspanins (*e.g.*, tetraspanin 29, tetraspanin 30 and tetraspanin 28), stress proteins, and MHC proteins. This involves the binding of antibodies to specific surface antigens. For example, to improve blood EV purity, the immune-depletion approach removes serum, plasma proteins, and lipoproteins [41]. The significant advantage of this method is the potential to isolate EVs from a particular source. In clinical conditions such as heart diseases or cancers, the immunoaffinity capture technique can separate targeted cells/tissue EVs by identifying specialized markers on those cells or tissues. Assays like enzyme-linked immunosorbent assay (ELISA) and magneto-immunoprecipitation are used [36].

3.1.4. *Size exclusion chromatography*

Size exclusion chromatography is a method that isolates EVs according to their size, eliminating the need for ultracentrifugation [45]. Size-based isolation techniques use ultrafiltration, Exo isolation kits, chromatography, and

hydrostatic filtration dialysis [46]. During this procedure, EVs traverse a column containing a porous stationary phase that captures smaller components. Consequently, this configuration enables more prominent entities like EVs to exit more quickly than smaller constitutes. This technique preserves EVs while facilitating EVs' separation from a substantial protein background [45]. Size exclusion chromatography offers a more rapid and straightforward process than differential ultracentrifugation [47]. Additionally, Size exclusion chromatography has fewer co-separated contaminants than precipitation-based techniques, primarily eliminating high-density lipoprotein (HDL) and big multiprotein complexes that are not EVs [38,47]. Nevertheless, instances of minimal amounts of albumin and various lipoproteins comparable in size to EVs (such as low-density lipoproteins (LDLs), intermediate-density lipoproteins (IDLs), very-low-density lipoproteins (VLDLs), lipoprotein(a) (Lp(a)), and chylomicrons) within blood EV isolates have been documented [38].

3.2. *EV characterization*

The characterization of EVs involves physical and biochemical approaches.

3.2.1. *Physical characterization*

Nanoparticle tracking analysis (NTA) measures the size and concentration of EVs using an optical microscope. This device irradiates the sample with laser light, and the light scattering from EV movement correlates with EV size. Modern NTA devices can measure particle motion in an electric field, enabling the calculation of zeta potential (an indicator of particle surface charge) [48]. Another approach for EV characterization is single particle interferometric reflectance imaging (SP-IRIS), a new optics-based approach. Measurements produced by SP-IRIS are more reliable than NTA. Furthermore, resistive pulse sensing (RPS) is also a method for characterizing EVs through size, count, and surface charge, while flow cytometry (FCM) enhances our understanding of EV biology.

3.2.2. *Biochemical characterization*

Biochemical characterization involves antibody affinity labeling, nucleic acid sequencing (NA-Seq), and mass spectrometry (MS). NA-seq methods characterize EVs in the context of RNA transport by identifying and quantifying RNA species packaged in EVs. Meanwhile, MS analyses characterize lipid and protein components of EVs. Antibody affinity labeling analyzes EV content and cell-derived EVs [49].

4. Unique properties of EVs in cancer immunity

The function of EVs in cancer immunology is complex and dynamic, and it involves cancer-specific antigens and the activation of anti-cancer immune responses. Given the complexity of the TME, EVs can either promote or suppress tumor progression, depending on their specific roles [50]. For instance, targeting EV mechanisms or their components could be a promising strategy for developing anti-tumor therapies due to the higher EV released by tumor cells than non-tumor cells. EVs can suppress tumor progression by regulating immune cells in the TME. EVs also enable leukocyte communication by regulating immune responses against tumors [51]. Furthermore, Exos from mast cells indirectly stimulate B and T lymphocytes by influencing the differentiation of DCs [50]. Meanwhile, EV dysregulation promotes tumor content release, mediating communication between cancer cells, the TME, and distant metastatic sites [51].

Exos play a crucial role in regulating anti-cancer immune responses. EVs can be anti-cancer vaccines developed from immune cells like DCs that activate immune responses against tumors [52]. Cancer-derived Exos contain heat shock protein 70 kDa (HSP70), which regulates natural killer (NK) cells, resulting in DC activation [50]. Previous studies have shown that DCs and T lymphocytes are targets for cancer-derived EVs [51]. Conversely, cancer-derived EVs inhibit DC activity [51]. Reports have shown that Exos suppress DC development, facilitating immune evasion by increasing the expression of B-cell stimulatory factor-2 (BSF-2/IL-6) in bone marrow precursor cells of DCs [53]. EVs can also modulate T lymphocyte activity by impeding the function of other leukocyte populations, particularly DCs [54]. For example, T lymphocytes expressing Fas undergo apoptosis when exposed to the CD178 antigen found on tumor cell-derived Exos [55]. Previous studies have demonstrated that cancer-derived EVs can alter T lymphocyte behavior, especially by enhancing the proliferation, differentiation, and activation of regulatory T (Treg) lymphocytes, which suppress immune responses [51]. For instance, transforming growth factor-beta1 (TGFβ1) expression in Exos from malignant cells is associated with Tregs activation [56].

EVs can facilitate cancer progression, as studies have shown that Exos released by tumor cells can suppress the proliferation and cytotoxic activity of NK cells by downregulating NK cell group 2 member D (NKG2D) [57]. For instance, EVs activate nuclear factor kappa B (NFκB) signaling, leading to the secretion of inflammatory cytokines and triggering proinflammatory responses. This stimulates tumor-promoting immune cells like macrophages [51]. Gaining a deeper understanding of the immunological functions of Exos in cancer could pave the way for developing innovative and effective tumor-fighting strategies. A summary of EVs used in anti-cancer immune responses or pro-cancer immune responses is shown in Table 2.

5. Heterogenous EVs with diverse functions in cancer

EVs exhibit heterogeneity in size, composition, and biological function. Furthermore, they display various biophysical properties, including diameters, density, charge, and content makeup, resulting in heterogeneity of the EVs released into the intercellular milieu [97]. Our main emphasis here will be on the implications of various types of EVs in their diverse functions within cancer settings (Fig. 3).

Table 2 – Role of EVs in Cancer immunity.

Source of EVs	Cancer type	Cargo of EVs	Pro-tumor effects	Anti-tumor effects	Role in immune evasion	Type of study	Ref.
Tumor-derived Exos	NSCLC	PD-L1	Promotes tumor growth through immune escape	N/A	Suppresses T cell killing of cancer cells	Experimental	[58]
Glioblastoma stem cell-derived Exos	GBM	PD-L1	Induces M2 macrophages and increases PD-L1 expression in monocytes	N/A	Enhances immune evasion via macrophage polarization	Experimental	[59]
Tumor-derived microparticles	Triple-negative breast cancer	PD-L1	Promotes immune suppression via PD-L1-associated pathways	N/A	Facilitates immune evasion via macrophages	Experimental	[60]
Tumor-derived Exos	HCC	HMGB1	Promotes regulatory B cell expansion	N/A	Fosters immune evasion by increasing TIM-1+ regulatory B cells	Experimental	[61]
CAFs -derived Exos	HCC	PD-L1, IL6, STAT3	Induces PD-L1^{+} neutrophils, fostering immune suppression	N/A	Immune suppression via the IL6-STAT3 pathway	Experimental	[62]
Tumor-derived Exos	GC	PD-L1	Increases neutrophil expression of PD-L1, suppressing T cell immunity	N/A	Enhances immune evasion by neutrophils	Experimental	[63]
Tumor-derived Exos	Melanoma	PD-L1	Drives immunosuppressive macrophages in pre-metastatic niches	N/A	Induces systemic immune suppression and promotes macrophage-mediated immune evasion	Experimental	[64]
Bone marrow-derived cell Exos	Tumor-bearing mice	PD-L1	Suppresses anti-tumor immunity	N/A	Inhibits antitumor immunity through PD-L1+ Exos	Experimental	[65]
Glioblastoma-derived EVs	GBM	LGALS9	Suppresses DC antigen presentation	N/A	Immune evasion through LGALS9-mediated suppression of T cell responses	Experimental	[66]
TEVs	HCC	circGSE1	Promotes Treg cell expansion	N/A	Induces immune escape through expansion of Treg cells	Experimental	[67]
HCC	HCC	miR-146a-5p	Drives T-cell exhaustion through M2 macrophage activation	N/A	Promotes immune evasion via T-cell exhaustion	*In vivo/in vitro*	[68]
HCC	HCC	14–3–3ζ protein	Impairs anti-tumor function of tumor-infiltrating T lymphocytes	N/A	Reduces T-cell cytotoxicity by interfering with immune recognition	*In vivo/in vitro*	[69]
Prostate tumor	Prostate cancer	Unknown	Reduce NKG2D expression on NK and CD8^{+} *T* cells with low cytotoxic activity	N/A	Facilitates immune evasion with low cytotoxic lymphocyte activity	*In vivo/in vitro*	[70]
GC	GC	miRNA-107	MDSCs, which suppress immune response	N/A	Supports immune suppression by increasing MDSC population	*In vivo/in vitro*	[71]
Breast cancer cells	Breast cancer	Unknown	N/A	Gallic acid inhibits EV secretion, potentially hindering tumor growth	Prevent immune evasion through reduced EV secretion	*In vitro*	[72]
Glioblastoma cells	Glioblastoma	Unknown	Exo secretion leading to therapy resistance	N/A	Immune evasion and drug resistance	*In vitro*	[73]

(*continued on next page*)

Table 2 (*continued*)

Source of EVs	Cancer type	Cargo of EVs	Pro-tumor effects	Anti-tumor effects	Role in immune evasion	Type of study	Ref.
HCC	HCC	PD-L1	Exosomal PD-L1 suppresses T-cell activity, promoting tumor growth	Nanounit strategy reverses PD-L1 mediated suppression, leading to enhanced ferroptosis	Blocks T-cell activation via exosomal PD-L1	*In vivo*	[74]
DEXs	NSCLC	DC antigens	N/A	Boost immune response after chemotherapy	Reduce recurrence of immune evasion by sustaining immune memory	Clinical study	[75]
Engineered EVs	Various cancers	Anti-PDL1 proteins and chemotherapeutics	N/A	Effective chemotherapy and immunotherapy by targeting tumor cells	Overcomes PD-L1 immune evasion in the TME	Preclinical	[76]
Pancreatic cancer	Pancreatic cancer	Unknown	N/A	Enhances immunotherapy and reprograms the TME by targeting tumor cells	Mitigates immune suppression within the TME	*In vivo/in vitro*	[77]
HCC	HCC	Iron oxide nanoparticles and exosomal content	N/A	Polarizes macrophages to an M1 phenotype, promoting an immune response against the tumor	Shifts macrophage polarization to favor anti-tumor immunity	*In vivo/in vitro*	[78]
Pancreatic cancer cell Exos	Pancreatic cancer	miRNA-155 and miRNA-125b2	Reprograms macrophages to promote tumor growth	N/A	Promotes immune evasion by reprogramming macrophages	*In vivo/in vitro*	[79]
Glioblastoma tumor	Glioblastoma	Checkpoint inhibitors	N/A	High efficacy of checkpoint inhibitors when combined with radiation therapy	Blocks immune evasion by priming immune response before therapy	Preclinical	[80]
Engineered immune cells	Various cancers	Radiotherapy-enhancing proteins	N/A	Enhances radiotherapy by making cancer cells more vulnerable to immune attack	Prevents immune evasion by enhancing immune cell recognition	*In vivo*	[81]
M1 macrophage-derived Exos	Various cancers	IL-4 receptor antagonists	N/A	Reprograms TAMs to promote an immune response against the tumor	Overcomes immune suppression by promoting M1 macrophage polarization	Preclinical	[82]
Tumor cells	Lung cancer	IL-12 mRNA	None reported	Promotes systemic immunity	None reported	Preclinical	[83]
Tumor cells	NSCLC	TGF-β	Immune suppression, poor response to ICI	None reported	Predicts resistance to ICI therapy	Clinical	[84]

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Table 2 (*continued*)

Source of EVs	Cancer type	Cargo of EVs	Pro-tumor effects	Anti-tumor effects	Role in immune evasion	Type of study	Ref.
Tumor cells	NSCLC	PD-L1, B7-H3, B7-H4	Enhances immune evasion	None reported	Suppresses T-cell activity	Clinical	[85]
Breast cancer cells	Breast cancer	EV-associated factors	Immune cell suppression	None reported	Differential T-cell regulation	Pilot study	[86]
Serum-derived EVs	Breast cancer	EV cargo	Promotes immune evasion	None reported	Regulates T-cell escape mechanisms	Clinical	[87]
Tumor cells	GC	YTHDF1-targeting m6A modulators	Epigenetic immune suppression	Enhances chemotherapy	Reduces immune recognition	Preclinical	[88]
Tumor cells	GC	YTHDF1	Epigenetic immune regulation	Enhances immune response to therapy	Reduces immune recognition	Preclinical	[89]
Tumor cells	Breast cancer	Multifunctional cargo	Lymph node metastasis	Enhances immunotherapy	None reported	Preclinical	[90]
Genetically engineered EVs	Diiferent cancer	Immune-modulating factors	None reported	Enhances anti-tumor immunity	None reported	Preclinical	[91]
Tumor cells	Different cancer	STING agonist	None reported	Suppresses tumor growth	Enhances immune response	Preclinical	[92]
Tumor cells	Various cancers	CD300a	Suppresses T cell function	None reported	Regulates Treg cells in tumors	Preclinical	[93]
Tumor cells	Various cancers	PD-L1	Immune suppression	None reported	Evades immune checkpoint inhibition	Clinical	[94]
Stem cells	Various cancers	Immunoregulatory molecules	None reported	Immune cell activation	Enhances immune modulation	Preclinical	[95]
Treg cells	Various cancers	EVs from Tregs	Immune suppression	None reported	Modifies DCs	Preclinical	[96]

NSCLC, Non-small cell lung cancer; GBM, glioblastoma multiforme; PD-L1, programmed death-ligand 1; HCC, hepatocellular carcinoma; GC, gastric cancer; MDSCs, expands myeloid-derived suppressor cells; M1, Pro-inflammatory macrophages; M2, Anti-inflammatory macrophages; TAMs, tumor-associated macrophages; CAFs, cancer-associated fibroblasts; DEXs, DC-derived exosomes. A summary of how EVs modulate immune responses, impacting both pro-tumor and anti-tumor mechanisms across various cancer types.

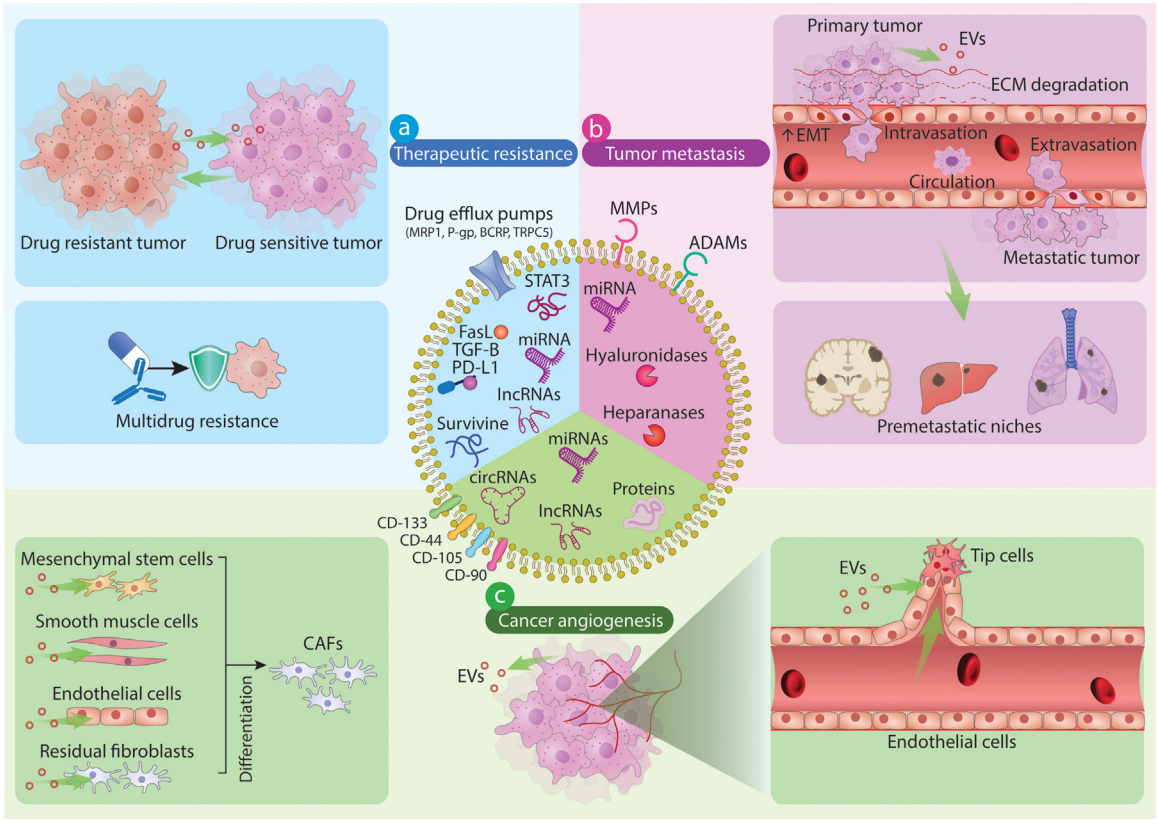

Fig. 3 – Mechanisms of EVs in cancer progression and therapeutic resistance. (a) Comparison between drug-resistant and drug-sensitive tumors in therapeutic resistance. (b) Depiction of the metastatic cascade in Tumor Metastasis. (c) Illustration of tumor-associated blood vessel formation in cancer angiogenesis.

The heterogeneity of EVs could be observed based on the composition. For example, the differences in protein profiles between MVs and Exos in the neuroblastoma cell line (SH-SY5Y) [98]. This variation suggests new perspectives in identifying potential biological markers for tumors [99].

5.1. *Heterogenous EVs and their different roles in cancer growth and metastasis*

Exos derived from SH-SY5Y cells promote *in vitro* growth and motility of SK-N-BE2 human neuroblastoma cells compared to MVs [99]. In contrast, NIH3T3 fibroblast cells respond more invasively to LIM 1863 colonic carcinoma cells-derived MVs than Exos [98]. Understanding these underlying differences could offer valuable insights into cancer treatment and contribute to developing more precise EV-based diagnostic and therapeutic strategies. For instance, N2a cells, a mouse neuroblastoma cell line, release diverse subpopulations of Exos with or without CD63 [100]. Exos with tetraspanin exhibit non-specific attachment to neurons and neuroglia, while Exos with CD63 tend to attach to the neuron's dendrons [100]. Previous studies have shown that B16F10 mouse melanoma cell lines release two Exo subsets, low-density and high-density Exo, with different gene expressions [101]. For instance, glutamine transporter SLC38A1 is upregulated in endothelial cells (ECs) when exposed to low-density Exos. Glutamine is essential in malignant cells, supporting energy production, redox balance, macromolecule synthesis, and signaling pathways [101,102]. These attributes enhance the glutamine metabolic process for innovative clinical approaches designed for detecting, observing, and treating tumors [102]. The differentiation of exosomal subsets promotes exosomal biological mechanisms and functionality and facilitates the advancement of Exo-related diagnosis and treatment approaches [101]. Several studies have also indicated that integrins from cancer-derived Exos play a crucial role in disseminating cancerous cells to particular remote sites, a phenomenon known as organotropic metastasis. Furthermore, Exos from tumor surface proteins TSP-180 (α6β4 integrin) and VLA-6 (α6β1 integrin) promote lung metastases by preferentially binding to lung fibroblasts and epithelial cells. In contrast, Exo adhesion to liver-resident macrophages (Kupffer cells) is mediated by αVβ5 integrin, contributing to liver metastases. This indicates that integrins found in Exos may serve as indicators for metastatic organotropism [103].

5.2. *Heterogenous EVs and their different roles in coagulation activity and cancer immunity*

EVs from B16-F1 mouse melanoma cells showed that Apo-EVs had more significant hemostatic activity than MVs and Exos

after *in vitro* coagulation tests. Additionally, immunization studies in melanoma mouse models demonstrated that Apo-EVs offered the most effective anti-cancer immune defense [104]. These could influence the choice of tumor treatment strategies. Specific approaches, such as cytoablation regimens, can induce the secretion of tumor-derived Apo-EVs, elevating the thrombosis danger and enhancing tumor immunity associated with T lymphocytes and NK cells [98,104]. Exo interactions are driven by integrin αLβ2 and B220 on DC surfaces, while tetraspanin-29, TAPA1, and the vitronectin receptor on Exo surfaces enable their targeting and uptake by DCs. Therefore, targeted cells can recognize and absorb Exos, affecting tetraspanins or integrins expression [98].

6. Clinical trials and therapeutic potential of EVs

EVs hold therapeutic potential due to their involvement in treating various diseases [22]. One remarkable feature of EVs is their capacity to transport molecular signatures from their parent cells, enabling precise targeting and interaction with recipient cells [105]. These vesicles can encapsulate a wide array of molecules, including proteins, RNAs, and lipids, making them versatile carriers for delivering a range of therapeutic agents, from small molecules to large nucleic acids [106]. EVs are biomarkers derived from cancer cells that can provide insights into tumor biology and aid early detection. In contrast, engineered EVs can deliver anti-cancer drugs or genetic material to tumor sites [107]. It provides genetic material, such as miRNAs and mRNAs, to recipient cells, influencing their behavior and function. This capability is explored for therapeutic purposes, like silencing oncogenes or upregulating tumor suppressor genes in cancer treatment [108]. The ability to transfer functional RNA makes EVs promising for precision medicine, tailoring therapies to individual genetic profiles. EVs, particularly those from mesenchymal stem cells (MSC), are also being investigated for tissue repair and regeneration. They've shown potential in promoting healing and reducing inflammation, with applications in treating chronic wounds, bone fractures, and other regenerative needs [109,110].

Clinical applications of EVs have advanced, offering new treatment possibilities for various diseases [106]. EVs have shown significant potential due to their role in intercellular communication and ability to transport bioactive molecules such as proteins, RNAs, and drugs. This has led to multiple clinical trials, particularly for cancer treatment and biomarker identification [107,111]. Recent clinical trials have focused on EVs derived from human and plant cells for drug delivery, cancer vaccines, and diagnostics [112]. Their stability in body fluids and ability to protect biomolecules from degradation make them ideal for non-invasive diagnostics, with EV-based proteins and miRNAs promising as cancer biomarkers [113]. EVs are also utilized for targeted drug delivery in conditions like colon cancer, COVID-19, and degenerative disorders. They are considered promising candidates for targeted drug delivery because of their intricate structure, composed of lipids, surface proteins, and receptors that facilitate precise tissue and cell targeting [112].

EVs function as biomarkers for antitumor vaccines and drug delivery systems. EV-based vaccines contain active molecules like MHC and costimulatory molecules, which help stimulate immune cells to trigger anti-tumor responses. Clinical trials have explored tumor-derived EV (TEV) components, such as DNA, miRNA, long noncoding RNA (lncRNA), and specific tumor antigens, as biomarkers for cancer diagnosis [114]. EVs move through the bloodstream because of the membrane composition and nanoscale size, which enhance therapeutic efficiency. Preclinical and clinical trials have highlighted the ability of Exos to trigger anti-tumor immune responses. EVs from stem cells could promote tissue repair, while Exo-based vaccines could be used in regenerative medicine. For instance, DEXs developed as vaccines have enhanced the efficacy and safety in aggressive cancers like glioblastoma when combined with other therapies. Furthermore, combining DCs-Exo vaccines with NK cell therapies offers a synergistic strategy to enhance anti-tumor immune responses [112,115].

Additionally, Exos derived from B-cell Non-Hodgkin Lymphoma serve as diagnostic markers due to their high CD20 expression, allowing them to evade the immune system [116]. Clinical trials have utilized Exos as predictive biomarkers for immunotherapy responses in renal and HCC [117,118]. Despite the progress made, further research is required to clarify EV-mediated mechanisms, address their heterogeneity, and optimize storage for clinical use. Enhancing EV yield and quality through advanced isolation and production techniques is vital for their clinical success. Some clinical applications of EVs are highlighted in Table 3.

7. Potentials of EVs in cancer immunotherapy

EVs can deliver functional molecules to target cells that influence the immune microenvironment, which could promote effective cancer immunotherapy strategies. Immune cell-derived EVs coordinate the complex interaction between the immune system and the cancer anti-tumor immunity landscape [114]. These EVs are derived from immune cells such as T cells, B cells, NK cells, DCs and macrophages, exhibiting unique characteristics for effective cancer immunotherapy [125]. They release surface receptors and effector molecules that enable them to modulate immune responses. They also boost antitumor immunity due to their cell-homing and targeting abilities, thus minimizing off-target effects and enhancing the therapeutic potential of their cargo. Additionally, immune-stimulatory molecules can activate immune cells or inhibit checkpoints to counteract tumor-induced immunosuppression [126].

EVs trigger immune cells to combat various diseases like cancer. For instance, NK cells combat cancer by utilizing EVs as molecular tools to target and destroy transformed or infected cells. These EVs contain a range of cytotoxic proteins, including perforin, granzymes, and tumor necrosis factor (TNF)-related apoptosis-inducing ligands (TRAIL), which promote apoptosis in cancer cells. Additionally, NK cell-derived EVs are rich in tumor-suppressor microRNAs, further enhancing their cytotoxic effects. By distributing

Table 3 – Therapeutic and diagnostic applications of EVs.

Disease	Intervention / Treatment	Therapeutic potential	Study design	Study start	Ref/NCT
Colon cancer	Plant Exos	Effective delivery of curcumin to colon tumors	Phase I	2011	[119]
Pancreas cancer	Exos	MSC-derived Exo with siRNA to target metastatic pancreatic cancer	Phase I	2021	[120]
Osteosarcoma	Circulating Exo in Blood samples	Exo-derived RNA as a diagnostic biomarker for osteosarcoma	Pilot study	2017	[121]
Cholangiocarcinoma	Circulating Exos in Blood samples	Exo-derived RNA as a diagnostic biomarker for cholangiocarcinoma	Pre-clinical	2017	[122]
GC	Circulating Exos in blood samples	Exos as predictive biomarkers for GC	Observational case-control	2013	[123]
Lung cancer	Circulating Exos in blood samples	Exos as biomarkers to manage and improve lung cancer	Observational cohort	2018	NCT04315753[a]
AML	UCMSC-Exo	UCMSC Exos for AML patient's treatment	Phase 1	2024	NCT06245746[a]
DLBCL	Exos in blood samples	Exos used in immunotherapy escape	Not applicable	2019	NCT03985696
Breast cancer	EVs isolated from the plasma of breast cancer	EVs serve as diagnostic and predictive biomarkers in breast cancer	Observational case-control	2020	[124]
CRC	Circulating Exos	EVs act as biomarkers in CRC	Observational cohort	2020	NCT04523389[a]
Thyroid cancer	EVs	EVs serve as biomarkers in thyroid cancer	Observational cohort	2020	NCT04742608 [a]
Malignant pleural effusion	Drug-packaging microparticles	Delivery of chemotherapy with tumor-derived microparticles for malignant pleural effusion treatment	Phase 2	2013	NCT01854866[a]
GC	Exo-based liquid biopsy	Diagnostic for GC	Observational cohort	2023	NCT06342427[a]
Prostate cancer	Exosomal microRNA	Diagnostic for aggressiveness of prostate cancer in Chinese patients	Observational cohort	2018	NCT03911999[a]
Liver cancer	Exo-based liquid biopsy	Diagnostic for liver cancer	Observational case-only	2024	NCT06342414
Lung cancer	Serum exosomal lncRNAs	Biomarker for the diagnosis of lung cancer	Observational case-control	2017	NCT03830619[a]
CRC	Exosomal microRNAs (cf-miRNA and exo-miRNA, respectively)	Early detection of CRC	Observational case-control	2023	NCT06342401[a]
ICC	Exo-based liquid biopsy	Lymph node metastasis detection	Observational case-control	2023	NCT06381648
Pancreatic cancer	Serum exosomal microRNAs	Early detection of pancreatic cancer	Observational case-control	2023	NCT06388967[a]
Advanced adenomas and CRC	Serum exosomal circulating microRNAs	Detection of CRC and adenomas	Observational case-control	2020	NCT06342440[a]
Sarcoma	Exosomal proteins and nucleic acids	Monitoring disease progression	Observational cohort	2018	NCT03800121[a]
Lung squamous Carcinoma	Serum Exosomal miRNA	Predicting therapeutic efficiency in lung SCC	Observational cohort	2022	NCT05854030[a]

AML, acute myeloid leukemia; UCMSC-Exo, Umbilical cord-derived MSCs exosomes; DLBCL, diffuse large B-cell lymphomas; CRC, colorectal cancer; ICC, intrahepatic cholangiocarcinoma.

[a] The relevant information obtained from the website: https://clinicaltrials.gov/.

these potent effector molecules, NK cell-derived EVs boost immune surveillance, averting tumor progression, metastasis, and the detrimental effects of cancer [127]. Meanwhile, DC-derived EVs (DCEVs) have immunostimulatory properties and drug delivery potentials that promote effective immune cell attacks on cancer cells. DCEVs activate the immune system and prime T lymphocytes, particularly cytotoxic T lymphocytes (CTLs), for antitumor activity [128,129].

EVs released from macrophages harbor multifaceted functions in the context of tumor immunity because macrophages polarize to pro-inflammatory (M1) or anti-inflammatory (M2). Macrophage-derived EVs play a role in antigen presentation and aid the adaptive immune response against cancer cells. Depending on signals from the TME, EVs derived from macrophages can either promote tumor growth and metastasis or trigger antitumor immune responses, highlighting their dual role in regulating tumor immunity [130–132]. Meanwhile, classical neutrophils release neutrophil-derived trails (NDTRs) and neutrophil-derived MVs (NDMVs). NDTRs recruit and activate immune cells, while NDMVs have immunosuppressive effects. Neutrophil-derived EVs offer pathogen defense and are used as drug carriers due to their short lifespan [133].

EVs derived from $CD4^+$ and $CD8^+$ T cells have diverse functions that promote immune responses against tumors. These EVs carry miRNAs, antigens and cytokines that regulate immune cell activation, proliferation, and function. T cell-derived EVs affect the balance between immune surveillance and tumor evasion in TME [134]. Meanwhile, B cells-derived EVs act as antigen-presenting vehicles that activate CTLs to enhance antitumor responses. B cell-derived EVs may also have immunosuppressive effects, triggering T cell apoptosis or altering immune cell function within the TME. Understanding this complex interaction is essential for developing targeted immunotherapeutic strategies against cancer [135,136]. Exploiting such inherent EV properties made it possible to engineer these nano-vesicles to target a particular cell type or deliver the therapeutic payload at the tumor site [134].

TEVs are essential in intercellular communication, spreading immunosuppressive signals that enable them to evade immune surveillance and promote tumor growth. These carry TEVs containing immunosuppressive molecules, such as TGF-β, PD-L1 and Fas ligand, suppressing immune cells in the TME [114]. Previous studies have demonstrated that TEVs trigger robust $CD8^+$ T cell responses and reduce Treg cells, positioning them as promising candidates for cancer vaccines [107,137]. TEVs contain immunogenic components, such as MHC-I molecules and HSP70, which activate immune responses targeting cancer cells [128,138]. In 2008, the first clinical trial using colorectal carcinoma-derived TEVs with granulocyte-macrophage colony-stimulating factor (GM-CSF) showed limited efficacy and safety [139]. Additionally, bacterial EVs have shown promise as cancer immunotherapy without side effects [128]. Furthermore, TEVs can enhance the transport of antitumor drugs and nanomaterials. Engineered immune cell-derived EVs represent a new frontier in immunotherapy tailored for the delivery of cancer patient therapy. EVs can be modified for specific functions, enhancing their therapeutic potential through genetic engineering, membrane engineering, and cargo delivery strategies. EVs are essential mediators between cells in different physiological and pathological processes, including immune responses against tumors [13,82,89,106] (Fig. 4). This suggests that immune cell-derived EVs offer improved safety and functionality, positioning them as a promising new strategy for cancer therapy.

8. EVs and their roles as cancer vaccine

Vaccines serve different purposes: traditional vaccines are preventive, while cancer vaccines primarily treat existing cancers. However, preventive cancer vaccines exist but are limited to virus-related cancers. For example, viruses such as human papillomavirus (HPV), associated with genital cancers, and hepatitis B and C viruses, linked to liver cancer, are key risk factors [140]. The main goal of therapeutic vaccines is to stimulate strong T cell responses, particularly from $CD8^+$ CTLs, by using adjuvants to enhance the immune system's reaction [141]. One prevalent method involves the injection of DCs, which initiate and direct immune responses against specific antigens. Upon maturation, DCs display increased co-stimulatory factors, active MHC peptide complexes, and adhesion molecules to engage immune cells. Furthermore, DCEVs transport functional molecules involved in antigen presentation, enhancing the potential for immune cell-mediated tumor rejection. Recent studies demonstrate that DCEVs are the earliest cancer vaccines to achieve clinical application [142,143]. However, DC-based immunotherapy has yielded inconclusive results, involves high costs, faces challenges in production standards, and loses effectiveness with prolonged storage [140]. Previous studies have demonstrated that DEXs and MVs possess immunostimulatory effects, activating $CD8^+$ T cell responses against specific antigens and significantly enhancing the relative proportion of germinal center B cells (Fig. 4) [144]. DEXs are cancer vaccines with high stability attributed to their lipid composition and abundance of MHC class I/II molecules compared to DCs [142,145]. A phase II clinical trial that used DEXs indicated an increased proportion of tetraspanins (CD82, CD81, CD63), HSP70, TSG101 and HLA-DR, and a peptide elution strategy was employed to enhance affinity with MHC I proteins [145]. In a phase I clinical trial, DEXs were used as cell-free anticancer vaccines in lung cancer patients [146]. These Exos demonstrate increased resistance to immunosuppressive signals within the TME [145]. Besse et al. [75] and Munich et al. [147] demonstrated that DEXs can boost antitumor responses in T cells and NK cells via TNF and interferon gamma (IFN-γ) superfamily ligands. Some modifications involve tailoring EV membranes to improve tumor targeting and optimizing their cargo to maximize antitumor effectiveness [148].

TEVs could serve as therapeutic cancer vaccines. They are lipid bilayer particles from tumor cells that have characteristics and functions of their origin recipient cells [114,115]. TEVs play a crucial role in communication between immune and tumor cells, carrying both tumor-associated antigens (TAAs) and tumor-specific antigens (TSAs) from parent tumor cells, including PD-L1, gp100, MHC I, MHC II, TYRP, and HSP proteins like HSP70 and HSP90 [149,150]. These vesicles naturally contain TAAs, which aid in antigen presentation and can be engineered to carry immune cell activators, thereby boosting the vaccine's effectiveness [114]. TEVs also facilitate tumor growth and immune suppression by converting monocytes into MDSCs, which inhibit effector T cells. They also induce anergy in natural killer T (NKT) cells, promote M2-like macrophage polarization, and expand Tregs

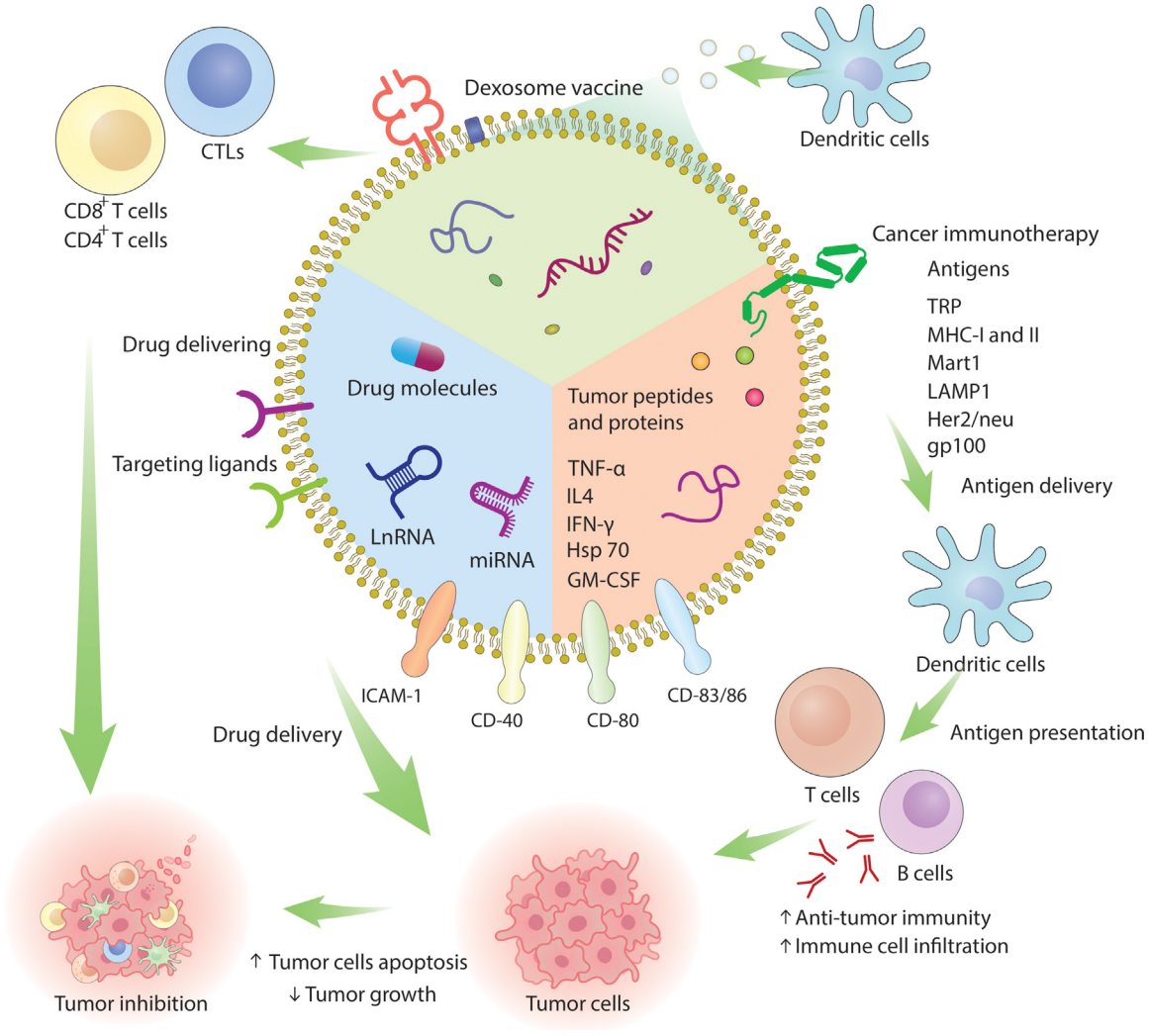

Fig. 4 – Exo-based cancer immunotherapy and drug delivery. EVs act as versatile platforms for enhancing cancer treatment by combining immunotherapy and targeted drug delivery strategies.

[151–153]. TEVs activate the immune system in early-stage cancer by delivering tumor antigens, such as MHC-I molecules and HSPs to DCs, triggering $CD8^+$ T cell responses. In late-stage cancer, TEVs promote immune evasion [61,154,155]. TEVs can also contribute to immune suppression and tumor growth [114]. When transferred to DCs, TEVs can initiate antigen-specific CTL responses, leading to adequate protection against autologous tumors than irradiated tumor cells, apoptotic bodies, or tumor lysates *in vitro* and *in vivo* models [107,126]. Sometimes, it is necessary to pair cancer vaccines with adjuvants to boost immune responses and strategies, further neutralizing tumor-promoting effects [141]. Conversely, TEVs can promote cancer progression, drug resistance, immune evasion, and metastasis. Consequently, the utilization of TEVs directly in cancer therapy raises safety concerns, thereby impeding their potential as safe cellular cancer vaccines [148].

Different cells secrete EVs, which does not affect their role as vaccines. For instance, Yaddanapudi et al. [156] demonstrated that human embryonic stem cells secrete EVs that express GM-CSF, which can be used effectively as a prophylactic cancer vaccine. Additionally, cytotoxic and helper T cells secrete EVs to mediate cellular interactions and induce functional modifications. Qiu et al. [157] also demonstrated that activated T cells release Exos that contain anti-programmed cell death 1 (PD-1), which triggers PD-L1 internalization within Exos or from the surface of cells. This mechanism aids in re-establishing tumor immune surveillance and mitigating immune evasion mediated by PD-L1 in triple-negative breast cancer. Furthermore, EVs derived from NK cells carry cytotoxic proteins like perforin and FasL, which induce cell death in multiple types of tumor cells via activating immune responses [158]. Zhu et al. [159] also demonstrated that TNF-α containing EVs derived from NK-92 cells influence melanoma cell growth and apoptosis. Additionally, the ability of TAMs to exhibit distinct phenotypes like M1 with tumor-suppressive properties and M2 with tumor-promoting properties has made nanovesicles (NVs) essential in the TME. NVs from M1-polarized macrophages can reprogram M2 TAMs into M1 macrophages, further

stimulating the secretion of pro-inflammatory cytokines thereby initiating antitumor immune reactions [160].

Outer membrane vesicles (OMVs) are tumor vaccines secreted by Gram-negative bacteria known to contain various bacterial components, such as lipopolysaccharide (LPS), peptidoglycan, proteins, nucleic acids, and toxins [161]. These vesicles form through several mechanisms: cell lysis, loss of cross-linking proteins between the outer phospholipid bilayer and peptidoglycan layer, accumulation of peptidoglycan or misfolded proteins, and insertion of foreign signaling molecules into the outer membrane. OMVs play vital roles in bacterial communication and host interactions. OMVs use the circulatory system to move to different organs, facilitating long-distance signaling between bacteria and host tissues in their host [161]. Previous studies have demonstrated the accumulation of OMV in the TME due to the enhanced permeability and retention (EPR) effect, which promotes vesicle retention at tumor sites. OMVs are an important vaccine due to the unique physiological features that trigger immune responses even without direct bacterial contact. OMVs carry LPS and lipoproteins, which can induce programmed cell death in host cells upon interaction [161,162]. Developing OMV-based anti-tumor vaccines involves using genetic engineering to incorporate foreign proteins into or onto the vesicles, eliciting targeted immune responses with low immunogenicity and minimal side effects. Another approach is the fusion of an antigen with an OMV protein, like CytolysinA (ClyA) or hemoglobin protein (Hbp), to form chimeric proteins expressed on the OMV surface [162]. For instance, the fusion of ClyA-GFP protein enhances immune responses by producing increased antibody titers compared to the controls in mice [162]. Grandi et al. [163] also demonstrated that engineered OMVs carry FAT1 antigen that is overexpressed in various tumor cells, which led to anti-tumor responses and tumor regression in mice with colon cancer.

9. Roles of EVs in cancer drug delivery

EVs are promising candidates for optimizing drug delivery in cancer therapy due to their natural targeting abilities and lower immunogenicity than synthetic nanoparticles [164,165]. They can efficiently transport drugs to tumor sites, navigate biological barriers like the blood-brain barrier, and evade phagocytosis [166]. Cytomembrane-targeted photodynamic priming induces EV storm for efficient drug delivery and tumor destruction via bioorthogonal reaction, improving therapeutic efficacy [167]. EVs are stable during circulation and use endogenous cargo delivery mechanisms. They effectively target and disseminate cancer cells, potentially preventing tumor progression and relapses [165,168]. Engineered EVs can improve tumor specificity, reduce systemic toxicity, and even present T-cell antigens to enhance immune responses against cancer (Fig. 4) [169]. Previous studies have shown that chemotherapeutic drugs delivered by EVs are more stable and effective, with less toxicity than conventional therapies. Their ability to carry nucleic acids and proteins makes them ideal for gene therapy and targeting cancer cells [165]. EVs can serve in allogeneic and autologous treatments that include immune cells, MSCs, cancer cells, and cell lines [170]. TEVs express integrins and adhesion molecules, which enhance selective binding to target cells in metastatic sites, improving therapeutic efficacy while minimizing off-target effects [171].

EVs loading uses electroporation, sonication, and extrusion for small molecules, proteins, and nucleic acids. At the same time, the genetic engineering of donor cells can enhance specific targeting without damaging the EV membrane [172]. On the contrary, post-isolation loading techniques preserve the integrity of EV membrane [173]. For instance, hydrophobic drugs like paclitaxel (PTX) loaded in MSCs produced EVs with high cytotoxic effects and increased drug solubility and stability [174]. Alternatively, previous studies have shown that EV cargo can be modified by transfection in breast cancer cells engineered to contain miR-134, resulting in effective anti-HSP90 treatments [175,176]. In a clinical trial, curcumin-loaded EVs demonstrated effective tumor-targeted anti-inflammatory therapy [177]. Artificial EVs, either semi-synthetic or fully synthetic EV mimetics, offer promising drug delivery by combining liposomes with specific EV membrane proteins [177]. They can also improve targeting and immune evasion by using PEG to extend circulation time or incorporating pH-sensitive elements for controlled drug release in the acidic TME. Previous studies have shown that engineered EVs delivered drugs by effectively targeting leukemia, improving survival rates [178].

Preclinical studies have demonstrated the potential of EV-based drug delivery systems in cancer therapy. For example, Exos loaded with the chemotherapeutic agent PTX have shown increased efficacy in reducing tumor growth in a mouse lung cancer model [179]. Despite their promising potential, translating EV-based drug delivery systems into clinical use faces several challenges. Previous studies have shown that cell source influences the composition and function of EVs, a major challenge affecting the therapeutic efficacy and safety [180]. Meanwhile, advanced characterization techniques like NTA, dynamic light scattering (DLS), and high-resolution FCM could tackle EV heterogeneity, offering insights into their biological functions [181].

EVs are biocompatible because they trigger immune responses based on their origin and composition [182]. Comprehensive preclinical studies are essential to evaluate the immunogenicity, toxicity, and bodily distribution [183]. Addressing these concerns is vital for regulatory approval and patient safety, impacting the clinical development of EV-based drug delivery systems [184]. Currently, there are no standardized regulatory guidelines for EV-based therapies precisely. The regulatory framework for EVs is still evolving, with agencies like the FDA and EMA working to establish guidelines for their development, characterization, and clinical testing. Furthermore, establishing clear criteria for EV purity, potency, and safety, along with standardized manufacturing and quality control protocols, can address challenges related to EV selection, source, isolation, characterization, storage, immunogenicity, and regulatory approval, unlocking their full potential for drug delivery [169,181,185–187].

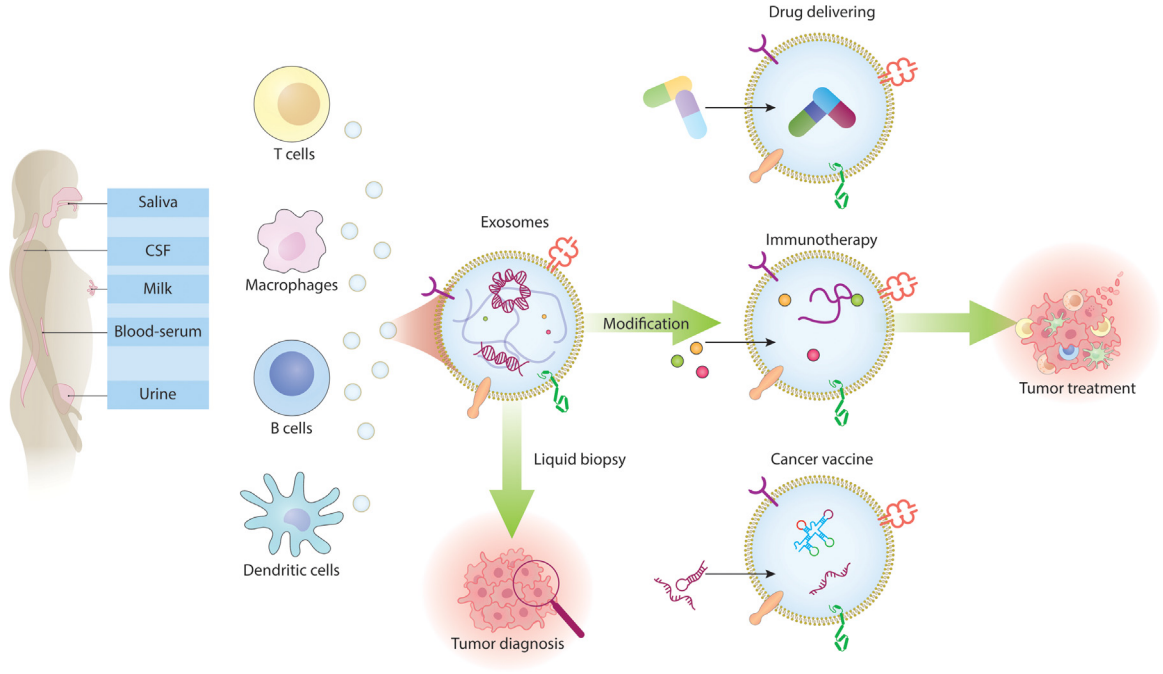

Fig. 5 – Roles of EVs in cancer diagnosis and treatment. EVs, derived from various cells such as T cells, macrophages, B cells and DCs, can be isolated from bodily fluids, including saliva, CSF, milk, blood serum and urine. These EVs can be modified for various applications: liquid biopsy for tumor diagnosis, drug delivery systems targeting tumors, immunotherapy to enhance immune response, and cancer vaccines to stimulate immunity.

10. Roles of EVs as tumor biomarkers

EV-based biomarkers offer significant benefits over traditional diagnostic methods. They are abundant in various body fluids like saliva, blood, urine, semen, breast milk and bronchoalveolar fluid, enabling minimally invasive sampling (Fig. 5) and making them a non-invasive diagnostic tool. They carry various biomolecules that can be measured, potentially enhancing diagnostic sensitivity and specificity [188]. Tissue sampling remains a significant challenge, specifically in cancers such as central nervous system tumors [189]. Aside from EVs, biomarkers like cell-free DNA (cfDNA) and circulating tumor cells (CTCs) are targets that offer novel insights and expand the horizons of cancer management. cfDNA are short fragments of nucleic acids present in bodily fluids such as blood and urine, primarily released during apoptosis, while circulating tumor DNA (ctDNA) is from cancer cells and provides insights into the genetic and epigenetic alterations of the primary tumor. Ev's vesicular lipid membrane shields their cargo from degradation, enhancing stability compared to cfDNA and soluble proteins in bodily fluids. Due to this protective feature and their abundance, they often outperform cfDNA and soluble proteins as tumor indicators [190].

EV-based liquid biopsies have been used for clinical studies, especially for early cancer detection and tracking minimal residual disease in patients after therapy [191,192]. For instance, ExoDx Prostate IntelliScore, a urine EV-based assay, effectively distinguishes higher-grade prostate cancers (Gleason score 7 or higher) from lower-grade cancers and benign conditions [192]. Previous studies have shown that mutations common in pancreatic cancer, such as KRASG12D and TRP53R273H, are detectable in DNA-derived EVs released into the bloodstream of affected patients [193]. Bulk analysis of heterogeneous EV populations results in concentration and expression patterns variability. This variability arises from the diverse cellular origins of EVs and the absence of standardized protocols for their isolation and characterization. Pre-analytical factors further contribute to technical inconsistencies, including storage duration, freeze-thaw cycles, and transport conditions. Moreover, small patient sample sizes in clinical studies limit the reliability of findings. Advanced single-particle analysis techniques, such as imaging FCM and fluorescence-based NTA, could enhance EV classification accuracy, thereby reducing biological variability [190].

EVs serve as clinical indicators for therapy response prediction, disease progression, tumor burden, and monitoring of treatment efficacy [191,194,195]. Sun et al. [196] demonstrated that the plasma levels of vesicular copine-3 (CPNE3) in patients with CRC are positively related to the tissue expression level of CPNE3 in tumor samples. This indicated that the concentration of EVs and their molecular content in liquid biopsies from cancer patients are essential indicators of clinical parameters and can function as tumor markers. Previous studies on CRC and breast cancer have shown elevated levels of EVs in cancer patients associated with drug resistance, disease progression, and reduced overall survival [194,195]. Following surgical removal of the primary tumor, EV levels in plasma decreased significantly, indicating that the tumor mass is a significant factor driving increased

EVs released [191]. Monitoring plasma EV concentrations in post-surgical conditions serves as an indicator of tumor burden in cancer patients. Additionally, chemotherapy and radiotherapy have been shown to stimulate the release of EVs in patients with various types of cancer [197,198]. These findings suggest that measuring EV levels could potentially enhance the evaluation of therapeutic responses in clinical settings.

EV-related nucleic acids such as DNA, RNA, lncRNAs, miRNAs and mRNAs can be biomarkers. Nucleic acids like RNAs and proteins have emerged as potential tumor diagnosis indicators (Table 4) [188]. In the context of cancer diagnosis, using EVs is essential in highlighting the characteristics of TEVs, which could offer avenues for early disease detection. For instance, Hornick et al. [199] showed that EVs associated with AML were present in the bloodstream before leukemic blasts became detectable. Melo et al. [200] also showed a cell surface proteoglycan, glypican-1 (GPC1), enhances the levels of EVs before the tumor becomes visible through standard imaging methods in an *in vivo* model of pancreatic cancer.

TEVs are pivotal in transferring specific types of proteins and miRNA from drug-resistant cells to drug-sensitive cells (Fig. 3). This transfer process has been observed across different cancer treatments, including tamoxifen resistance in breast cancer [234]. For another example, EVs carrying HER2 can bind to trastuzumab, reducing drug efficacy. This interaction highlights a specific pathway through which TEVs can influence the response to trastuzumab treatment in breast cancer patients [235]. Most EV biomarkers associated with chemoresistance have demonstrated elevated plasma levels in non-responsive cancer patients, as evidenced by various protein markers and RNA-based molecules [236,237]. Conversely, some studies have demonstrated a reduction in EV-derived miRNA levels in the blood of patients who did not respond to chemotherapy or radiotherapy [238].

TEV biomarkers can monitor therapy effectiveness by analyzing changes in their expression levels in patient samples after treatment compared to before treatment. The levels of RNAs and proteins in plasma EVs decreased following chemotherapy or tumor removal, such as extracellular matrix metalloproteinase inducer (EMMPRIN) in CRC and prostate-specific membrane antigen (PSMA) in prostate cancer [190,239]. Additionally, RNA molecules associated with EVs, such as miR-155 and the lncRNAs HOTAIR, have emerged as effective indicators for tracking responses to surgery and chemotherapy in patients with breast cancer [240,241]. Some biomarkers are highly expressed in circulating EVs after favorable treatment responses. For example, higher serum levels of EpCAM-positive EVs during chemotherapy were linked to prolonged progression-free survival among patients diagnosed with pancreatic ductal adenocarcinoma (PDAC), potentially indicating a stress response induced by the treatment in the tumor [242]. Moreover, during PD-1 immunotherapy, elevated PD-L1 expression on small EVs in plasma has been correlated with enhanced treatment response in melanoma patients [243].

Regarding predicting disease state and disease progression, the complete mRNA profile of plasma-derived EVs has been associated with metastasis in osteosarcoma [121].Also, high levels of miR-17-5p and miR-92a-3p in EVs from CRC is linked to the cancer stage [244]. These findings emphasize the critical role of EV biomarkers in predicting disease states. Several bodily fluids in different cancers are also biomarkers. For instance, EV-based blood biopsies are used for diagnosis, prognosis, and monitoring of cancer patients, while salivary gland fluid is a potential source for EV-based liquid biopsies in oral cancer despite contamination with bacterial EVs [245]. Additionally, urine-derived EVs are evaluated in urological malignancies, such as prostate [246], bladder, and kidney cancers [190]. Previous studies have also identified cancer-derived EVs as biomarkers in bodily fluids, specifically by detecting miR-21 in EVs from the cerebrospinal fluid (CSF) of patients with GBM [247]. In addition, bile-derived EVs contain high levels of claudin-3 in cholangiocarcinoma [248], and DNA methylation markers were identified in EVs derived from gastric juice in GC [245]. Plasma-derived EVs DNA (EV-DNA) can detect mutations in the Kirsten rat sarcoma (KRAS) gene in PDAC patients [249].

11. Challenges and future directions in EV-based cancer immunotherapy

The major challenge involved in EV-based cancer immunology is the isolation of Exos and the effective usage of these vesicles in immunotherapy. Johnstone RM et al. first identified Exos in 1983 [250]. Later, in 1996, Raposo et al. [251] clarified that Exos play a crucial role in presenting antigens from B cells and initiating T cell responses. Since then, Exos have attracted significant interest as a critical players in cellular communication, diagnosis, progression, and treatment of tumors. Thery and colleagues were the first to introduce Exos in cancer therapies [250]. Exos play a crucial role in regulating the microenvironment by modulating the immune system and escape, resulting in increased metastasis, tumorigenesis and drug resistance [252]. Notably, the functions of Exos can differ widely depending on the cells derived and the specific biological context. The purposes and mechanisms underlying the physiological release of Exos remain unclear and require further investigation [250,251].

Meanwhile, the structure of Exos, such as the lipid bilayer, shields them from degradation and removal in the bloodstream, making them a promising tool for drug delivery in different diseases like cancer. Despite significant progress, the biological functions of Exos remain incompletely understood, and developing new treatments continues to encounter several challenges [250,251]. The varying isolation methods of Evs based on the heterogeneity and the nanoscale size make it difficult to isolate them efficiently [36,47]. Separating Exos from different body fluids and cell cultures also lacks standardization [128]. Hence, developing standardized and efficient methodologies to distinguish between the various types of EVs is essential. Addressing this issue is critical for our understanding and fully harnessing the potential of these dynamic vesicular structures. Hence, characterizing EVs involves DLS, size and concentration measurement, electron microscopy, immunoblotting, and proteomic analysis [253]. In addition, limited data on Exo composition and quantities, interactions between tumor cells and immune cell-derived EVs, the impact of clinical

Table 4 – Highlights on EV biomarker types, their sources, and their roles in various cancers.

Biomarker type	Biomarker name	Source of EVs	Cancer Type	Role	Expression	Ref
microRNA	miR-7641	Cancer cells	Breast cancer	Promotes progression, metastasis	↑	[65]
	miR-1290	Circulating Exos	Epithelial ovarian cancer	Diagnostic biomarker	↑	[201]
	miRNA-720	Serum-derived EVs	HCC	Diagnostic performance marker	↑	[202]
	miR-205	Plasma-derived EVs	Ovarian cancer	Early diagnosis, staging indicator	↑	[203]
	miR–1910–3p	Cancer-derived EVs	Breast cancer	Promotes proliferation, metastasis, autophagy	↑	[204]
	miR-20b-5p	Serum-derived EVs	NSCLC	Early diagnostic marker	↑	[205]
	miR-382	Serum-derived EVs	NSCLC	Prognostic biomarker	↓	[206]
	miR-3937	Tumor-origin EVs	CRC	Early minimally invasive biomarker	↑	[207]
	miR-1269a	Serum-derived EVs	NSCLC	Diagnostic biomarker	↑	[63]
	miR-92b	Serum-derived EVs	Breast cancer	Diagnostic biomarker	↑	[208]
	miR-1470	Serum-derived EVs	CRC	Promotes cell proliferation, metastasis	↑	[209]
	miR–4732–5p	Plasma-derived EVs	Epithelial ovarian cancer	Non-invasive diagnostic biomarker	↑	[210]
	miR-1269a	Serum-derived EVs	NSCLC	Diagnostic marker plays an oncogenic role	↑	[63]
	miR–3187–5p	Serum-derived EVs	NSCLC	Early-stage diagnostic biomarker	↑	[204]
	miR-3937	TEVs	CRC	Early diagnostic biomarker for liquid biopsy	↑	[207]
	miR-92b	Serum-derived EVs	Breast cancer	Diagnostic biomarker promoting tumorigenesis	↑	[208]
lncRNA	LINC00355	Exos	GC	Promotes malignant progression	↑	[211]
	NNT-AS1	Serum Exos	CRC	Potential biomarker, oncogene via miR-496/RAP2C axis	↑	[212]
	TTN-AS1	CTCs	Cholangiocarcinoma	Promotes proliferation and migration	↑	[213]
	MALAT1	Exos	Wilms' tumor	Candidate biomarker for liquid biopsy monitoring	↑	[214]
	FOXD2-AS1, NRIR, XLOC_009459	Serum Exos	CRC	Diagnostic biomarkers	↑	[215]
	Various lncRNAs	Tumor-derived Exos	NSCLC	Expression analysis for potential biomarkers	Varying levels of expression in tumors and Exos	[81]
	LncRNA signature	Exos	Ovarian cancer	Associated with prognosis and immunotherapy biomarkers	–	[216]
	RP5–977B1	Exos	NSCLC	Novel minimally invasive biomarker for diagnosis and prognosis	↑	[217]
	THEMIS2–211	Circulating Exos	HCC	Promotes growth and metastasis	↑	[218]
	SNHG16	Urinary Exos	Bladder cancer	Diagnostic biomarker	↑	[218]
	Various lncRNAs	Urinary Exos	Bladder cancer	Noninvasive biomarkers for diagnosis	↑	[219]
	FGD5-AS1	Exos	Pancreatic cancer	Promotes TAM M2 polarization, enhancing proliferation	↑	[76]

(*continued on next page*)

Table 4 (*continued*)

Biomarker type	Biomarker name	Source of EVs	Cancer Type	Role	Expression	Ref
circRNAs	CircRNA ACTN4	Exos	ICC	Promotes tumor progression by recruiting YBX1 to initiate FZD7 transcription	↑	[220]
	CircACTN4	Exos	Breast cancer	Promotes tumorigenesis and progression by regulating MYC expression	↑	[74]
	Circ_0000735	Exos	NSCLC	Contributes to malignant progression	↑	[221]
	CircATP8A1	Exos	GC	Induces macrophage M2 polarization via miR–1–3p/STAT6 axis	↑	[222]
	CircRNAs	Exos	GC	Noninvasive liquid biopsy biomarker for early detection	↑	[223]
	CircRNAs	Exos	Papillary thyroid cancer	Diagnostic tool for distinguishing papillary thyroid cancer	↑	[205]
	CircRNA panel	Plasma exosomal RNA	Hepatitis B virus-related HCC	Large-scale diagnostic tool for HCC	↑	[224]
	CircRUNX1	Exos	Esophageal squamous cell carcinoma (ESCC)	Facilitates growth and metastasis via miR-449b-5p/FOXP3 axis	↑	[225]
	CircLPAR1	Exos	CRC	Functions in diagnosis and suppresses tumorigenesis	↓	[226]
Protein	Integrin αVβ3	M2-like macrophage-derived EVs	NSCLC	Facilitates metastasis	↑	[14]
	CD97	T EVs	Gastric carcinoma	Promotes lymphatic metastasis	↑	[227]
	CD9	CAFs EVs	Malignant melanoma	Inhibits proliferation of melanoma cells	↑	[228]
	Rab27b	TEVs	GC	Associated with peritoneal metastases	↑	[229]
	B7-H4	Irradiated TEVs	Glioblastoma	Increases FoxP3 expression in Th1 cells	↑	[230]
	PSM-E	Prostate TEVs	Prostate cancer	Inhibits M2 macrophage polarization	↓	[231]
	ADAM17	TEVs	CRC	Promotes pre-metastatic niche formation	↑	[232]
	ITGB6	Dormant TEVs	Lung adenocarcinoma	Activates CAFs s	↑	[233]
	Glypican-1	Cancer-cell-derived Exos	Pancreatic cancer	Aid early detection of pancreatic cancer	↑	[200]

characteristics on Exos, and a lack of *in vivo* studies make Exos challenging to work with [128,254]. There is also no information on Exo distribution, half-life, blood concentrations and urine clearance, and the capture of Exos by macrophages during circulation is also a challenge. Strategies to enhance Exo stability, prolong half-life during circulation and minimizing immune clearance have been considered [181].

Another challenge is that therapeutic Exo storage, manufacturing, and biosafety still need to be addressed, as well as practical and logistical issues [255]. Following purification, EVs must be stored under appropriate conditions to preserve stability. Hence, optimizing the storage conditions for various types of EVs is essential for successful clinical translation. The commonly utilized storage is −80 °C, while methods like lyophilization with cryoprotectants, such as trehalose, serve as alternatives [256]. Furthermore, optimizing the manufacturing processes to achieve scalable mass production and multiple iterations could improve the yield needed. The preclinical pharmacokinetics, pharmacodynamics, and biosafety of the drug and EV should be studied to prevent side effects. This is because drug delivery by EVs results in liver and spleen accumulation. Hence, EV engineering involving immunological, physical, and chemical modifications can mitigate this issue by reducing tissue toxicity and accumulation and facilitating targeted therapy. Extensive clinical trials are needed to confirm the safety and effectiveness of Exos as a treatment option [257]. Preclinical

studies have shown the effect of EV-based therapies and drug delivery systems, but there are insufficient reports on the toxic effects [128]. Despite considerable progress in understanding the biological functions of EVs, their potential as engineered tools for advanced cancer immunotherapy remains largely unexplored.

12. Conclusion

EVs have become a crucial focus in cancer research, providing valuable insights and new opportunities for therapeutic advancements. This review underscores the diverse roles of EVs, from their fundamental properties to their transformative potential in cancer treatment. With their unique characteristics, EVs show great promise in cancer therapy, especially immunotherapy, where they can boost immune responses against tumors. Their use as drug delivery systems enhances targeted treatment, minimizing off-target effects. Additionally, EVs are valuable biomarkers, offering a non-invasive method to monitor tumor progression and treatment response. Developing EV-based cancer vaccines is also a groundbreaking strategy that could improve vaccine effectiveness and patient outcomes.

However, several challenges must be addressed to fully harness the potential of EVs therapeutically. Some significant problems include the lack of standardized methods for isolating EVs, leading to their quality and composition variability. The heterogeneity of EVs and the absence of a universal standard for isolation and characterization complicate the interpretation of EVs study. Furthermore, the biological roles of EVs are not fully understood, while their interactions with various cell types and clinical settings add further complexity. Additional challenges include scaling up EV production, ensuring their stability *in vivo*, and addressing potential risks such as immunogenicity or toxicity, all of which must be resolved before EV-based therapies can be broadly adopted in clinical practice.

Conflicts of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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