New insights into Epstein-Barr virus-associated tumors: Exosomes (Review)

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Abstract. Epstein-Barr virus (EBV) is endemic worldwide and is associated with a number of human tumors. EBV-associated tumors have unique mechanisms of tumorigenesis. EBV encodes multiple oncogenic molecules that can be loaded into exosomes released by EBV$^+$ tumor cells to mediate intercellular communication. Moreover, different EBV$^+$ tumor cells secrete exosomes that act on various target cells with various biological functions. In addition to oncogenicity, EBV$^+$ exosomes have potential immunosuppressive effects. Investigating EBV$^+$ exosomes could identify the role of EBV in tumorigenesis and progression. The present review summarized advances in studies focusing on exosomes and the functions of EBV$^+$ exosomes derived from different EBV-associated tumors. EBV$^+$ exosomes are expected to become a new biomarker for disease diagnosis and prognosis. Therefore, exosome-targeted therapy displays potential.

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1. Introduction

Epstein-Barr virus (EBV), which was first discovered in 1964 (1), is endemic worldwide and is associated with a number of human tumors, including nasopharyngeal carcinoma (NPC), EBV-associated gastric cancer (EBVaGC) and certain types of lymphoma (2-4). Previous studies have demonstrated that EBV encodes multiple viral proteins and nucleic acids that have complex effects in suppressing tumor cell apoptosis promoting tumor angiogenesis (5) and promoting tumorigenesis (6). Moreover, EBV-related viral proteins and nucleic acids also induce epithelial-mesenchymal transition (EMT) (7) and promote tumor metastasis (8). However, the mechanism by which EBV causes tumorigenesis is not completely understood. Notably, the morbidity of EBV-associated tumors, such as NPC, does not match the prevalence of EBV (9). In addition, EBV-associated tumors have different prognoses; some patients live with the disease for several years, while others progress quickly (9). Classical parameters for disease diagnosis and monitoring, such as serum EBV antibody titers or EBV-DNA loads, display certain clinical limitations (10). To date, no superior EBV-specific biomarkers compared with the classical parameters have been identified. Therefore, it is important to study the biomechanisms of EBV-associated tumors and to identify specific biomarkers for these tumors. In recent decades, exosomes have become the focus of cancer research due to their intercellular communication ability. Exosomes are 40-100 nm-diameter vesicles that are released by cells (11). Almost all normal cells can secrete exosomes, and tumor cells appear to release more exosomes than normal cells (12). Recent studies have revealed that EBV-infected tumor cells can persistently release exosomes loaded with viral proteins or nucleic acids (13,14). As an important component of the tumor microenvironment (TME), exosomes are vectors by which tumor cells, including EBV-associated tumor cells, can transfer oncogenic cargo that can act on target cells (15). To date, a few studies have addressed exosomes derived from EBV$^+$ tumors, and these studies suggest that the oncogenic molecules encoded by EBV not only display tumorigenic effects on uninfected cells via transfer through exosomes, but also exert potential immunosuppressive effects (14,16,17). Moreover, these exosomes can enter circulating body fluids and be transported throughout the body (10). Exosome separation technology is gradually advancing, and exosomes have been used as new drug delivery carriers in molecular targeted

tumor therapy (18). EBV$^+$ exosomes are expected to become a new biomarker or therapeutic target for EBV-associated tumors. The present review summarizes the important roles of exosomes in EBV-associated tumors to provide insight into the biomechanisms of these diseases from a new perspective.

2. Exosomes

Loading, release and uptake of exosomes. The formation of exosomes is a complex process, and numerous studies have described it in detail. The term exosome describes 40-100 nm-diameter vesicles that contain complex RNA and proteins. Exosomes form via the following axis: endosome-multivesicular body (MVB)-intraluminal vesicle (ILV). When MVBs fuse with the cell membrane, exosomes are released from parental cells (11). The formation of ILVs is the main process by which cargo, including proteins, lipids and nucleic acids, is loaded into vesicles. The endosomal sorting complex required for transport (ESCRT) machinery serves a critical role in the sorting of proteins into exosomes in parental cells (19). The RNA cargo of exosomes is enriched in small RNAs, especially microRNAs (miRNAs/miRs) (20). There are some other essential mechanisms of miRNA cargo loading in addition to the ESCRT machinery. Neutral sphingomyelinase 2 is the rate-limiting enzyme in the synthesis of ceramides, which could influence the loading of miRNAs into exosomes (21). Heterogeneous nuclear ribonucleoproteins (hnRNPs) are a family of conserved nuclear proteins that bind to nascent RNA polymerase II transcripts to produce hnRNP granules. Several hnRNPs, especially hnRNP A2B1 and hnRNP Q, are implicated in miRNA packaging into exosomes (22). hnRNPA2B1 can recognize and bind to specific motifs in the 3' untranslated regions (3'UTRs) of miRNAs and then transport miRNAs into exosomes (23).

The intracellular movement of MVBs involves the microtubule network, and localization of MVBs to the plasma membrane requires kinesin-dependent movement toward microtubule plus ends (24). The release of exosomes depends on the forward motion of MVBs to fuse with the plasma membrane (25). Rab GTPases, a subfamily of proteins in the Ras superfamily of GTPases (26), and soluble N-ethylmaleimide-sensitive fusion protein-attachment protein receptor proteins can interact to induce exosome release (27).

Previous studies indicate that there are three main mechanisms underlying exosome uptake by recipient cells: i) Direct interaction (28); ii) fusion with the plasma membrane (29); and iii) internalization (30). In immune cells, major histocompatibility complex (MHC)-T cell receptor interactions can also facilitate the uptake of mutual exosomes (28). Once exosomes enter recipient cells, they trigger a series of biological effects through multiple pathways, including Erk1/2, Jak/STAT, NF-κB and PI3K/Akt signaling pathways (31).

Role of EBV in the biogenesis of exosomes. The mechanisms of exosome formation are briefly summarized in Fig. 1. Notably, some molecules encoded by EBV can participate in the loading processes. In B cell-derived exosomes, the 3' ends of miRNAs are uridylated, and miRNAs from the parental cells share adenylated 3' ends, suggesting that 3' end modification of miRNAs may be another mechanism for sorting miRNAs into exosomes (32). Nkosi *et al* (33) revealed that the viral protein latent membrane protein 1 (LMP1) can interact with the ESCRT pathway and associated proteins, including CD63, Syntenin-1, programmed cell death 6 interacting protein, tumor susceptibility 101, human growth factor-regulated tyrosine kinase substrate (Hrs) and charged multivesicular body proteins (CHMPs). Moreover, the study demonstrated that the LMP1-interacting proteins Hrs and Syntenin-1 serve major roles in directing LMP1 into EVs for packaging and secretion (33).

Exosomes in the TME. The TME is a complex interactome between tumor cells, adjacent cells (including adipocytes, fibroblasts, lymphocytes and dendritic cells) and the intercellular matrix. Cancer progression and metastasis are closely related to alterations in the TME; in particular, the characteristics of tumors, such as sustained proliferation, avoidance of immune surveillance, and activation of invasion and metastatic cascades, are influenced by the TME (34). In turn, cancer cells synthesize and secrete biomolecules to reprogram the surrounding cells and remodel the TME to be suitable for survival (35). The TME modulates numerous types of cell-cell communication through diverse signaling networks, including juxtacrine and paracrine interactions. Regarding paracrine signaling interactions, exosomes are an important and emerging mechanism of cell-cell communication (36). The TME also regulates the secretion of exosomes (37), and interactions of exosomes with the TME benefit the growth of the tumor. For example, exosomes derived from leukemia cells have been shown to accelerate cancer-associated fibroblast activation to remodel the TME to a more cancer-permissive state (38). Stress conditions, such as extracellular acidity and hypoxia, are common in the TME. On the one hand, the accumulation of lactic acid or H^+ ions is a common characteristic of the TME, but TME acidity increases the release of tumor-derived exosomes (TEXs) (39). On the other hand, under hypoxic stress, tumor cells remodel the TME and facilitate angiogenesis by inducing the secretion of exosome-containing proteins associated with vascular endothelial growth factor (VEGF) signaling (40). Hypoxia can enhance miR-23a loading into lung cancer-derived exosomes. Endothelial cells take up exosomal miR-23a, which targets prolyl hydroxylase 1/2, resulting in the induction of hypoxia-inducible factor-1α (HIF1α) accumulation. Through this pathway, lung cancer cells remodel the TME and enhance tumor angiogenesis (41). Moreover, hypoxia-mediated enhancement of TEX release has also been observed in breast (42), bladder (43), prostate (44) and ovarian cancer (45) through multiple pathways, such as TGF-β2, TNF1α, IL-6, tumor susceptibility 101, Akt, integrin linked kinase 1 and β-catenin pathways.

3. EBV expresses multiple oncogenic molecules

EBV infection is gradually being recognized as endemic worldwide. It has taken several years to gain a clear understanding of the relationship between EBV and different types of human cancer, including Burkitt lymphoma, EBV$^+$ diffuse large B cell lymphomas (DLBCLs), Hodgkin lymphoma (HL), NPC, EBVaGC, post-transplant lymphoproliferative disease (PTLD), natural killer (NK)/T cell lymphoproliferative disease (NK/T-LPD) and lymphoma (2-4).

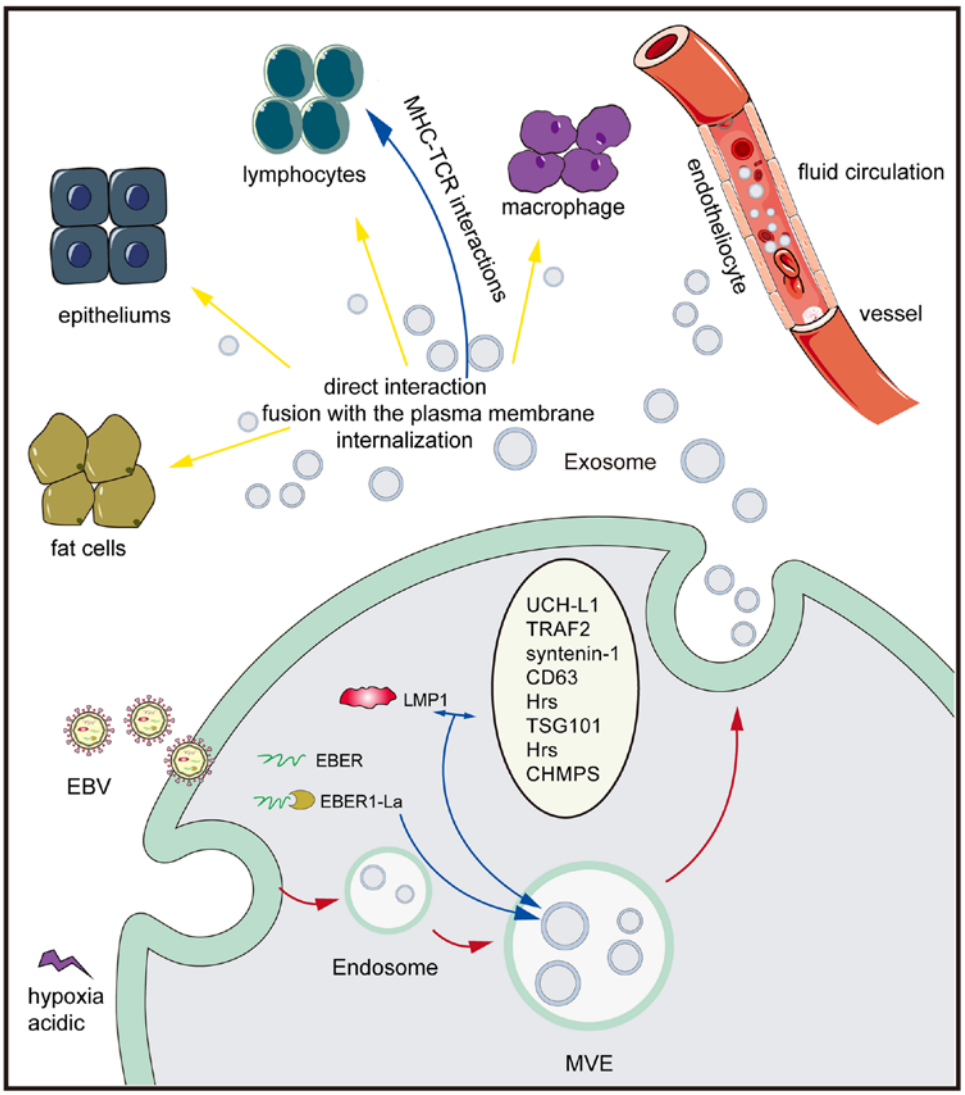

Figure 1. Overview of formation of exosomes and exosomes in the TME. EBV-encoded molecules, such as EBER and LMP-1, are loaded into exosomes and regulate the formation of exosomes. The endosomal sorting complex required for transport pathway and associated proteins, including CD63, Syntenin-1, Alix, TSG101 and Hrs, interact with LMP-1, inducing LMP-1 loading in exosomes. Tumor-derived exosomes target surrounding cells or enter the fluid circulation. Stress from the TME, such as hypoxia and acidic microenvironment, stimulates the synthesis and secretion of exosomes. TME, tumor microenvironment; EBV, Epstein-Barr virus; EBER, EBV-encoded RNA; LMP, latent membrane protein; Alix, programmed cell death 6 interacting protein; TSG101, tumor susceptibility 101; Hrs, human growth factor-regulated tyrosine kinase substrate; UCH-L1, ubiquitin C-terminal hydrolase L1; TRAF2, TNF receptor associated factor 2; CHMPs, charged multivesicular body proteins; MVE, multivesicular endosome; La, Lupus antigen; MHC, major histocompatibility complex; TCR, T cell receptor.

EBV has a relatively large double-stranded DNA genome and expresses ~80 proteins and 46 functional small untranslated RNAs (46). EBV was the first human virus known to encode miRNAs (47), and EBV-miRNAs have recently become a research hotspot. As small non-coding RNAs that are 19-25 nucleotides in length and display partial homology to sequences in their target mRNAs, miRNAs can modulate gene expression in numerous species. Loading of an miRNA onto the 3'UTR of its target mRNA by the RNA-induced silencing complex results in either translational repression or degradation of the mRNA, ultimately leading to reduced protein synthesis (48). EBV expresses 25 different pre-miRNAs and at least 44 mature miRNAs. As one of the eight known human herpesviruses, EBV has two life cycle phases. Primary EBV infection occurs primarily in the epithelial cells of the host pharynx and is followed by infection of B lymphocytes (49). Primary infection usually occurs in the first years of life and does not produce symptoms. Subsequently, the virus is transmitted in saliva, and if primary infection is delayed until later in life, infectious mononucleosis may occur (50). As B lymphocytes carrying EBV enter the blood circulation, systemic EBV infection can occur (51,52). The virus then enters the second life cycle phase, known as latency. According to the latent genes expressed by EBV in host cells, latent infection in hosts can be classified into four types: 0, I, II and III) (53,54). The latency patterns of EBV gene expression in different infections are summarized in Table I.

The EBV genome has been confirmed to encode viral proteins and nucleic acids associated with a variety of tumors. Moreover, the EBV-DNA load could be a prognostic factor in NPC (55,56), HL (57) and PTLD-DLBCL (58). However, some studies have indicated that in hydroa vacciniforme-like

Table I. Patterns of latent gene expression in EBV-infected cells.

A, Type 0			
Gene	Function	Host cell	(Refs.)
EBER	Promote cell proliferation, inhibit apoptosis and transform cells.	Infected dormant memory B cells	(122)
B, Type I			
Gene	Function	Host cell	(Refs.)
EBNA1	Ensure the persistence of the viral genome in cells as they multiply.	BL cells	(5,7,8,99,123-126)
EBV-miR-BART (*Bam*HI-A rightward transcripts)	Tumorigenesis: Promote angiogenesis, suppress apoptosis and promote host cell survival. Tumor metastasis: EMT.		
EBER	Promote cell proliferation, inhibit apoptosis and transform cells.		
C, Type II	Function	Host cell	(Refs.)
EBNA1	Ensure the persistence of the viral genome in cells as they multiply.	HL, NPC, DLBCL, EBVaGC and chronic lymphocytic leukemia cells	(127-131)
EBER	Promote cell proliferation, inhibit apoptosis and transform cells.		
EBV-miRs-BART	Tumorigenesis: Promote angiogenesis, suppress apoptosis and promote host cell survival. Tumor metastasis: EMT.		
LMP			
LMP1	Act as a strongly oncogenic protein that can interact with numerous signaling molecules.		
LMP2A			
C, Type II	Function	Host cell	(Refs.)
EBNA		Immunoblastic lymphoma cells, DLBCL cells and EBV-LCLs	(128,132-134)
EBNA1	Ensure the persistence of the viral genome in cells as they multiply.		
EBNA2	Act as a transcription factor that leads to the expression of viral LMP genes and ~300 host cell genes.		
EBNA3			
EBNA3B			
EBNA3C			
EBNA-LP			
LMP			
LMP1	Act as a strongly oncogenic protein that can interact with numerous signaling molecules.		
LMP2A			

Table I. Continued.

D, Type III	Function	Host cell	(Refs.)
EBER			
EBER1			
EBER2			
EBV-miR-BART			
EBV-miR-BHRF-1 (*Bam*HI fragment H rightward open reading frame-1 miRNA)			

EBER, EBV-encoded RNA; EBNA, Epstein-Barr nuclear antigen 1; EBV, Epstein-Barr virus; miR, microRNA; BART, *Bam*HI-A rightward transcripts; EMT, epithelial-mesenchymal transition; LMP, latent membrane protein; LP, leader protein; BHRF-1, *Bam*HI fragment H rightward open reading frame-1; BL, Burkitt lymphoma; HL, Hodgkin lymphoma; NPC, nasopharyngeal carcinoma; DLBCL, diffuse large B cell lymphoma; EBVaGC, EBV-associated gastric cancer; LCL, lymphoblastoid cell line.

lymphoproliferative disorder (an EBV-associated NK/T cell lymphoproliferative disorder), the EBV-DNA load is not significantly correlated with patient prognosis (59,60). Therefore, the use of EBV-DNA load as a prognostic biomarker varies across tumors (61). The mechanisms by which the latent virus reactivates and influences NK/T cells, B cells and other cells requires further investigation. In addition, as aforementioned, the high prevalence of EBV does not match the incidence of EBV-associated tumors. Thus, EBV infection may not be the key mechanism underlying EBV-associated tumorigenesis. The proteins or nucleic acids encoded by EBV may serve pivotal roles in tumorigenesis and tumor development as tumor regulators. In addition, exosomes serve essential roles in the transfer of oncogenic molecules, as well as in the tumorigenesis and tumor metastasis of EBV-associated tumors (62,63).

4. Different EBV$^+$ tumor cells secrete exosomes acting on various target cells

The type of latent infection varies among individuals, although type 0 is the most widespread, resulting in the expression of various genes corresponding to latency types. Interestingly, the amount of exosomes secreted by EBV$^+$ cells differs among the latency patterns, and cells with type III latency secrete the most exosomes (13). In addition, EBV-associated tumors exhibit different latency patterns and TMEs. Therefore, exosomes from different tumor sources have different functions due to their different parental cells and target cells (46). The functions of exosomes derived from cells of different EBV$^+$ tumors are summarized in this chapter.

Functions of exosomes derived from EBV$^+$ B cells. Once EBV infection occurs, the viral genome is disassembled and integrated into the host genome. Thus, even in latency, EBV$^+$ B cells could persistently express viral proteins and nucleic acids (64). LMP1 is a major oncoprotein of EBV, and numerous studies have demonstrated that it can be loaded on the membrane of exosomes (14,33,65,66). LMP1 also exists in exosomes derived from LMP1-transfected DG75 cells (a Burkitt lymphoma cell line). DG75 exosomes can be taken up by isolated B cells within the peripheral blood mononuclear cell population, which leads to enhanced proliferation and induces B cell differentiation toward a plasmablast-like phenotype via induction of activation-induced cytidine deaminase and the production of circle and germline transcripts for IgG1 in B cells (62). *In vitro*, after infection of B cells, LMP1 induces immortalization and aberrant proliferation, leading to the development of lymphoblastoid cell lines (LCLs) (67). The N-terminus and transmembrane domain 1 are sufficient for targeting LMP1 to extracellular vesicles (EVs) (68). Kobayashi *et al* (14) reported that ubiquitin C-terminal hydrolase-L1 and C-terminal farnesylation, a post-translational lipid modification, contribute to the direction of LMP1 to exosomes. Moreover, Rialland *et al* (69) found that the B cell receptor can modulate the protein content of exosomes upon stimulation and target its bound antigen to these vesicles. On the other hand, exosomes derived from LCLs carry an EBV glycoprotein, gp350, and preferentially target B cells via the interaction of this glycoprotein with its ligand, CD21 (70). In addition, these exosomes contain high levels of MHC-II molecules, which induce homogeneous antigen-specific T cell responses (66). The transmembrane freedom of exosomes is important for cellular interactions and this property might become the basis of exosome-targeted therapy.

Exosomes released from EBV$^+$ B cells are internalized by recipient cells primarily via caveolin-dependent endocytosis (71). When exosomes derived from EBV$^+$ B cells enter the TME, they can create an immunosuppressive microenvironment that affects T cell immune responses to ensure the proliferation of tumor cells. Cells with latent EBV infection can continuously produce EBV-encoded RNAs (EBERs), which elicit proinflammatory responses after sensing by pathogen recognition receptors (72,73). Lupus antigen (La) is an abundant RNA binding protein in the nucleus of latently infected B cells that binds nascent viral Pol III transcripts, protecting the 3' ends from degradation by exonucleases (74). EBERs are transported from the nucleus to the cytoplasm and shed in exosomes by binding to La (16). Based on these studies, Baglio *et al* (75) proposed that by interacting with La and loading into exosomes, EBV nuclear 5'pppEBER1 escapes cytosolic detection in cells with established latent infection. Then, the viral cargo loads are internalized by

plasmacytoid dendritic cells (pDCs), triggering antiviral immunity through exosomes. Another study showed that exosomes secreted from P3HR1 cells (an EBV$^+$ Burkitt lymphoma B cell line) can increase the production of indoleamine 2,3-dioxygenase (IDO), TNF-α and interleukin (IL)-6 in human monocyte-derived macrophages (MDMs) via the retinoic acid-inducible gene I pathway. Moreover, EBER-1-activated IDO in MDMs suppresses the proliferation of T lymphocytes and diminishes the cytolytic activity of CD8$^+$ T cells (76). Thus, EBER1$^+$ exosomes derived from EBV$^+$ B cells might promote tumorigenesis by inhibiting cellular immunity in the TME.

EBV-miRNAs are loaded into exosomes to induce a series of downstream effects (77). Higuchi *et al* (78) showed that exosomes derived from EBV$^+$ lymphoma cells can regulate the activity of macrophages and induce the immunoregulatory phenotype *in vitro*. In this process, the expression levels of TNF-α, IL-10 and ARG1 are partially regulated by EBV-*Bam*HI A rightward transcripts (BART)-miRNAs. Ito *et al* (63) observed that a phosphatidylserine-exposing subset of EVs secreted from lymphoma cells transformed with the EBV strain Akata converted surrounding phagocytes into tumor-associated macrophages (TAMs) by inducing an inflammatory response partially mediated by EBV-miRNAs. Using mass spectrometric analysis, the study indicated that several immunomodulatory proteins, especially integrin αLβ2 and fibroblast growth factor 2 (FGF2), are key factors in the TAM-inducing ability of EVs. Moreover, in the clinic, higher loads of BART miRNAs correlate with worse outcomes in elderly patients with EBV$^+$ DLBCL. Furthermore, EBV-BART-miRNAs might be the link between EBV$^+$ B cells and uninfected T cells or NK cells. Haneklaus *et al* (79) showed that exosomal EBV-miR-BART15 released from EBV$^+$ B cells can enter uninfected T cells, targeting the miR-223 binding site in the NLR family pyrin domain containing 3 3'UTR to inhibit inflammasome-mediated IL-1β production, which was consistent with previous interpretations (80,81). T cells can be suppressed by LMP1$^+$ exosomes (66); thus, EBV$^+$ exosomes display immunosuppressive effects. Extranodal NK/T cell lymphoma, nasal type (ENKTCL) is a rare EBV-associated non-Hodgkin lymphoma (82). However, the mechanism of EBV entry into NK cells remains unknown. Lee *et al* (83) reported that EBV mRNAs and CD21 RNA can be transferred into NK cells from B cells by exosomes. However, this transfer is not sufficient to maintain EBV persistence or allow EBV entry into NK cells. Therefore, whether EBV genomic components can affect NK/T cells via exosomes requires further investigation.

These studies indicate that exosomes derived from EBV$^+$ B cells can affect uninfected cells, and highlight the immunomodulatory function and oncogenic effect of exosomes. EBV hijacks host cells to build an immunosuppressive TME by secreting exosomes. Moreover, Ahmed *et al* (84) reported that exosomes derived from cells with both type I and III latent EBV infection induce apoptosis in B cells, T cells and epithelial cells via the Fas cell surface death receptor (Fas)/Fas ligand (FasL) pathway. As the majority of studies of exosomes derived from EBV$^+$ B cells have been conducted *in vitro*, further studies, especially *in vivo* studies, are needed to clarify the complete roles of exosomes.

Functions of exosomes derived from nasopharyngeal epithelial cells. NPC is a malignant tumor derived from nasopharyngeal epithelial cells that has a high incidence in southern China, Mediterranean Africa and some regions of the Middle East (85). The role of EBV in NPC has been studied for decades. Virtually all NPCs are EBV$^+$ (64); however, the complete mechanisms of EBV in the development and progression of NPC remain unclear. Viral proteins or nucleic acids might be involved in these processes. In recent years, researchers have focused on the roles of exosomes, and these studies are summarized to highlight the role of EBV in NPC.

Although the oncogenicity of LMP1 is known, there are no effective strategies to target this oncoprotein. With further studies, researchers have begun to focus on EBV$^+$ exosomes. In 2006, Keryer-Bibens *et al* (86) first demonstrated that NPC cells can release HLA class II$^+$ exosomes containing galectin 9 and/or LMP1. Galectin-9 is a ligand of the membrane receptor T cell immunoglobulin and mucin domain-containing protein 3, which is able to induce apoptosis in mature Th1 lymphocytes, weakening immunological surveillance (87). A previous study demonstrated that LMP1 can target the epidermal growth factor receptor (EGFR), inducing cell proliferation (88). Moreover, LMP1 increases the loading of EGFR into exosomes secreted by NPC cells, and exosomes containing LMP1 and EGFR are taken up by epithelial cells, endothelial cells and fibroblasts, which activates the ERK and PI3K/Akt pathways to affect cell proliferation (89). On the other hand, HIF1α regulates numerous key aspects of tumor development and progression by promoting increases in proliferation, invasiveness and neoangiogenesis (90). Aga *et al* (91) demonstrated that LMP1$^+$ exosomes secreted by NPC cell lines contain HIF1α. Furthermore, LMP1$^+$ exosomes and HIF1α can counterbalance the levels of E-cadherin and N-cadherin in recipient cells, consistent with EMT-associated changes. FGF-2, a member of the FGF family, is active in embryogenesis and morphogenesis, and serves a key role as an angiogenic factor involved in tumor progression and invasion (92). Ceccarelli *et al* (93) demonstrated that FGF-2 is packaged into exosomes and LMP1 selectively promotes this secretory process. LMP1 can interact with nucleic acids in addition to human proteins. For instance, miR-203 functions as a tumor suppressor in NPC and can be downregulated by LMP1 (94). A further study demonstrated that aspirin can reverse EMT and exosomal LMP1 might serve a pivotal role in this process. Exosomal LMP1 exhibited potential EMT-inducing ability, aspirin suppressed exosomal LMP1 secretion from EBV$^+$ cells by influencing NF-κB, and a decrease in exosomal LMP1 uptake alleviated the inhibition of miR-203 by LMP1. The aforementioned results were further confirmed in an *in vivo* study; thus, aspirin could inhibit NPC lung metastasis by reversing the LMP1/NF-kB/exosomal LMP1/miR-203 axis in nude mice (17).

Weakening or evading the immune surveillance function of lymphocytes is beneficial to the proliferation of tumor cells. Mrizak *et al* (95) showed that NPC cell-derived exosomes (NPC-Exos) express CCL20 on their surface, which prioritizes the infiltration of regulatory T cells (Tregs) into the tumor. In addition, NPC-Exos recruit CD4$^+$ T cells and induce their conversion into suppressive Tregs. Under induction by NPC-Exos, Tregs change their phenotype and their

immunosuppressive ability is enhanced. These findings show that EBV$^+$ exosomes can exert immunosuppressive effects not only by affecting pDCs, MDMs and CD8$^+$ T cells, but also by recruiting Tregs.

EBV-BART-miRNAs have been found in exosomes secreted from infected epithelial cells, and these miRNAs can affect mitochondrial respiration in exosome recipient cells to modify the TME to support the growth of infected cells, thereby contributing to viral fitness (96). In another report, Meckes *et al* (89) showed that EBV-BART-miRNAs (including miR-BART-1, 4, 5, 7, 9, 11, 12, 13, and 16) are also selectively loaded into exosomes secreted from cultured EBV$^+$ NPC cells. These viral miRNAs have been found to have biological functions and to be involved in molecular mechanisms associated with tumor immune evasion, proliferation, apoptosis resistance, invasion and metastasis (97). EBV-miR-BART3 can target the tumor suppressor integrator complex subunit 6 to promote cell proliferation and transformation in NPC (98). In a nude mouse model, EBV-BART3-3p was found to directly target the tumor suppressor gene tumor protein 53, which led to inhibition of senescence in GC cells, suppression of NK cells and macrophage infiltration into tumors (99), which are post-transcriptional regulation processes independent of genetic mutations. However, few studies have addressed whether EBV-miRNAs can mediate these changes through exosomes, and the specific mechanism remains unclear. Tumor occurrence results from the accumulation of a number of different factors. As the functions of EBV$^+$ exosomes in NPC have been gradually explored, it can be speculated that viral oncogenic cargo might be transferred to uninfected cells through exosomes, resulting in the occurrence or progression of NPC.

Similarly, exosomes derived from NPC cells share analogous immunosuppressive functions like those of exosomes from EBV$^+$ B cells. Importantly, exosomes from NPC cells could also induce the angiogenesis, EMT and metastasis of tumors (17,95). Therefore, we speculate that targeting EBV$^+$ exosomes is of significance for the treatment of NPC, and might be able to reverse the progression of tumors by blocking tumor cell immune escape and inhibiting tumor angiogenesis and EMT at the same time.

Exosomes derived from EBV-associated gastric cancer cells. EBVaGC accounts for ~10% of GC cases worldwide, with variable frequencies among geographic regions (100). In 1990, a possible association with EBV was first reported in a case of gastric carcinoma (101). To date, studies investigating EBV$^+$ exosomes in EBVaGC are limited. In 2013, Choi *et al* (102) reported that miR-BART15-3p is enriched in exosomes derived from EBVaGC cells and can induce apoptosis partially by inhibiting the translation of the apoptosis inhibitor baculovirus inhibitor of apoptosis repeat-containing ubiquitin-conjugating enzyme. However, DCs are pivotal to tumor immunity (103), and Hinata *et al* (104) found that the maturation of DCs is suppressed by exosomes derived from EBV$^+$ epithelial cells, which weakens tumor immunity. The aforementioned studies show the paradoxical functions of EBV$^+$ exosomes, including inhibition of host immunity while promoting EBV$^+$ cell apoptosis, which might explain why the prognosis of EBVaGC is favorable compared with that of other types of GC (105).

Although there are a number of different kinds of EBV-associated tumors, they primarily include B cell lymphoma, NPC and EBVaGC. These three different tumors have different TMEs but share an analogous immunosuppressive characteristic, which might be induced by EBV$^+$ exosomes (63,95,104). The known mechanisms of different EBV$^+$ tumor cell exosomes acting on various target cells are summarized in Fig. 2.

5. Circulating EBV$^+$ exosomes act as a biomarker for EBV-associated tumor diagnosis

As viral oncoproteins and nucleic acids are contained in exosomes, these EBV$^+$ exosome-derived targets could be helpful diagnostic markers. Moreover, exosomes are present and can be detected in almost all biological fluids, including serum, plasma, semen, breast milk, cerebrospinal fluid, urine, saliva, ascites, amniotic fluid and bronchoalveolar lavage fluid (10), which may overcome the limitations of the specificity of classical EBV infection detection methods. For example, EBER *in situ* hybridization is performed primarily with biopsy tissues, whereas enzyme immunoassays used to determine EBV antibody titers and polymerase chain reaction used to determine EBV-DNA loads are usually performed with plasma (10). Moreover, as the gold standard, the sensitivity of EBER ISH varies across tissues.

In 2005, Caby *et al* (106) first identified exosomes in blood, indicating that exosomes could travel throughout the body and modulate targeted cells via the circulatory system. Subsequently, Houali *et al* (107) detected LMP1 and EBV *Bam*HI-A Rightward Frame 1 in serum and saliva from North African and Chinese patients with NPC. Other studies have further suggested that EBV$^+$ exosomes could be used as biomarkers not only for the diagnosis of EBV infection, but also for predicting the prognosis of patients with EBV-associated tumors. A previous study on ENKTCL showed elevated expression levels of LMP1 and LMP2A in tumor cells. High LMP1 expression was associated with positive B symptoms, and the expression of both LMP1 and LMP2A showed a significant correlation with overall patient survival (108). Due to the noninvasiveness of obtaining exosomes from body fluids, LMP1$^+$ and LMP2A$^+$ exosomes might be promising biomarkers of ENKTCL in the clinic. Cyclophilin A (CYPA) is a member of the immunophilin family (109). Liu *et al* (110) demonstrated that both the serum and exosomal CYPA levels of patients with NPC were significantly higher compared with those of normal cases. Moreover, the level of CYPA was positively correlated with LMP1 in NPC exosomes. Therefore, the authors hypothesized that CYPA combined with EBV-viral capsid antigen-IgA may be a more discriminatory biomarker panel in the diagnosis of NPC. However, whether LMP1$^+$ and LMP2A$^+$ exosomes have prognostic value requires further investigation.

In addition to viral proteins, EBV-miRs have been shown to be potential diagnostic biomarkers for EBV-associated cancer. Zhang *et al* (111) demonstrated that EBV-miR-BART7 and EBV-miR-BART13 can serve as important biomarkers for NPC diagnosis and the prediction of treatment efficacy. Similarly, Wardana *et al* (112) found that overexpression of circulating EBV miR-BART7 correlated with positive regional

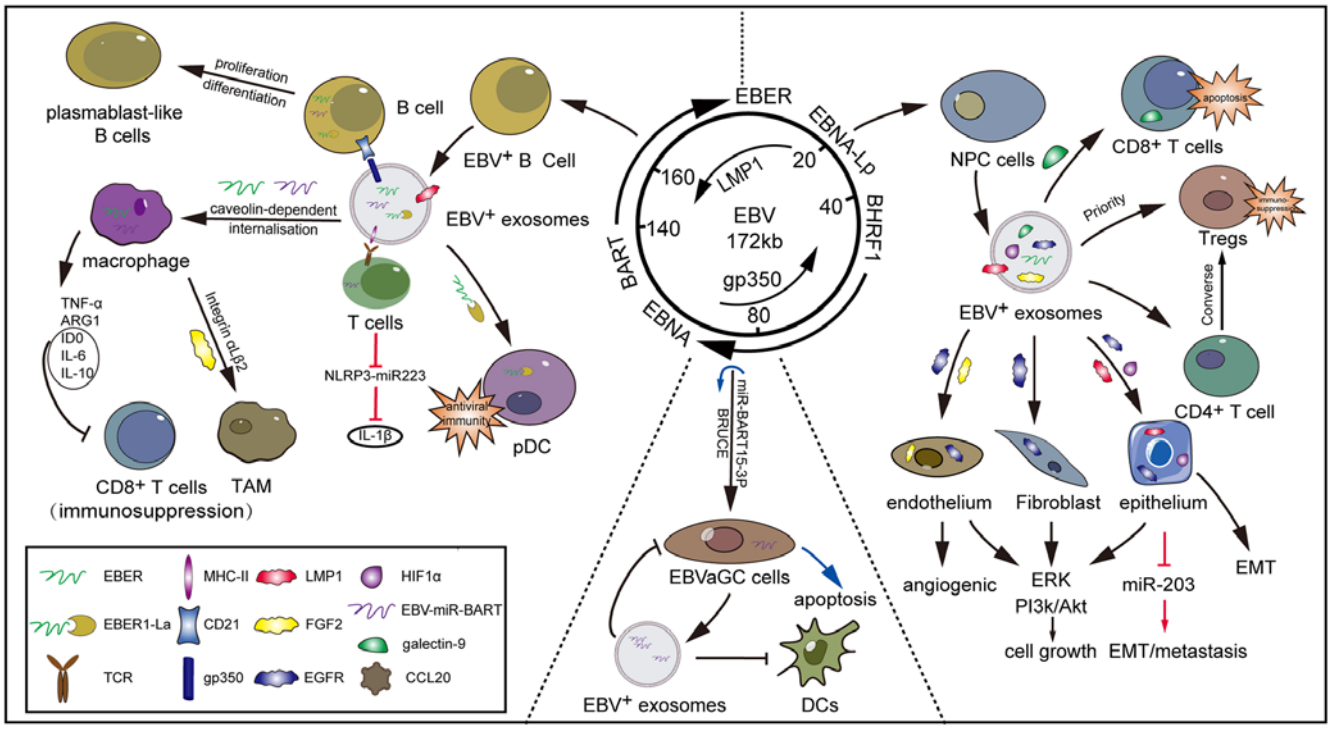

Figure 2. Role of EBV⁺ exosomes in the TME of B cell lymphoma, NPC and EBVaGC. For B cell lymphoma, EBV⁺ exosomes primarily act on lymphocytes with immunomodulatory functions. The EBV⁺ exosomes induce the proliferation and differentiation of naive B cells into plasmablast-like B cells. EBV⁺ exosomes also target macrophages, leading to transformation into TAMs and stimulating the secretion of immunosuppressive cytokines to inhibit CD8⁺ T cells. Moreover, EBV⁺ exosomes inhibit T cell synthesis of IL-1β. In the TME of NPC, EBV⁺ exosomes exert a strong immunosuppressive function, which is induced by recruiting Tregs and promoting CD8⁺ T cell apoptosis. In addition, EBV⁺ exosomes from NPC target endothelium, fibroblasts and epithelium to stimulate cell growth by delivering EGFR. EBV⁺ exosomes from NPC also stimulate EMT, promoting tumor metastasis and angiogenesis. The immunosuppressive effects of EBV⁺ exosomes from EBVaGC are primarily achieved via inhibiting pDCs. miR-BART15-3p is enriched in exosomes from EBVaGC cells and can induce the apoptosis of target cells, including tumor cells. EBV, Epstein-Barr virus; TME, tumor microenvironment; NPC, nasopharyngeal carcinoma; EBVaGC, EBV-associated gastric cancer; TAM, tumor-associated macrophages; EGFR, epidermal growth factor receptor; EMT, epithelial-mesenchymal transition; pDC, plasmacytoid dendritic cells; miR, microRNA; BART, *Bam*HI-A rightward transcripts; NLRP, NLR family pyrin domain; ARG1, arginase 1; IL, interleukin; EBER, EBV-encoded RNA; TCR, T cell receptor; MHC, major histocompatibility complex; LMP, latent membrane protein; FGF, fibroblast growth factor; EGFR, epidermal growth factor receptor; HIF, hypoxia-inducible factor; CCL, C-C motif chemokine ligand; EBNA, Epstein-Barr nuclear antigen; La, lupus antigen; BHFR, *Bam*HI fragment H rightward open reading frame; BRUCE, baculovirus inhibitor of apoptosis repeat-containing ubiquitin-conjugating enzyme; DC, dendritic cell.

lymph node status, highlighting the diagnostic and prognostic values of circulating miR-BART7 for patients with NPC. A number of the 49 EBV-encoded miRNAs have been proven to serve key roles in the development of NPC (113). miRNAs encapsulated in exosomes are protected from degradation by RNases. Thus, exosome encapsulation is more conducive to the detection of viral miRNAs and can reduce the number of false negatives caused by degradation during specimen transportation (114). In the early years, the separation technology of exosomes was immature and the extraction cost was high, which hindered the study of exosomes (115). With the development of separation technologies for isolating exosomes from body fluids, the detection of exosomes as biomarkers for diseases may become a mainstream approach.

6. Future perspectives: Exosomes could be utilized as a new therapeutic method

As aforementioned, exosomes synthesized by different parental cells load different molecules and target different cells. Specifically, exosomes are ideal tools for miRNA-based interactions between tumor cells. In turn, tumor cells persistently secrete exosomes and act on peritumoral cells, remodeling the TME to facilitate the growth of tumor cells. Therefore, modification of exosomes, especially the loaded miRNAs, may serve as a novel therapeutic strategy for cancer. For example, loss or downregulation of miR-122 is closely related to poor prognosis and metastasis in hepatocellular carcinoma (HCC) (116). Based on this finding, Lou *et al* (117) constructed an miR-122 expression plasmid, which was transfected into adipose tissue-derived mesenchymal stem cells (AMSCs). Then, AMSC-derived exosomes were harvested and added to recipient HCC cells. Interestingly, miR-122-transfected AMSCs effectively packaged miR-122 into secreted exosomes and provided HCC cells with sensitivity to chemotherapeutic agents through altering miR-122-target gene expression. Moreover, Wang *et al* (118) overexpressed EBV-miR-BART10-5p and hsa-miR-18a, which strongly induced angiogenesis *in vitro* and *in vivo*. Mechanistically, the association of viral miRNAs with human miRNAs was found to promote cancer angiogenesis and to involve the concordant downregulation of sprouty RTK signaling antagonist 3 (Spry3; a tumor suppressor) expression, activating Spry3/MEK/Erk-dependent pathways

and regulating the expression of VEGF and HIF1a in a Spry3-dependent manner. Moreover, iRGD-tagged exosomes containing antagomiR-BART10-5p and antagomiR-18a were utilized to suppress the angiogenesis and growth of NPC.

An immunosuppressive TME exists in most EBV-associated tumors, which impedes the efficacy of immunotherapies (119). The majority of previous studies have confirmed that exosomes derived from EBV$^+$ tumor cells serve a role in inhibiting body immunity and promoting tumor cell immune evasion in the process of tumor progression (63,95,104). Therefore, targeting EBV$^+$ exosomes is considered as a promising new treatment for EBV$^+$ tumors. Wang *et al* (120) found that exosomes derived from phosphoantigen-expanded Vδ2-T (Vδ2-T-Exos) cells could promotes EBV antigen-specific CD4 and CD8 T cell expansion and kill EBV-associated tumor cells through FasL/TNF-related apoptosis-inducing ligand pathways. These findings suggested a strategy for the treatment of EBV-associated tumors using Vδ2-T-Exos.

The application of targeting EBV$^+$ exosomes for antitumor therapy requires further investigation. However, the aforementioned findings warrant future studies to investigate the potential of exosomes as anti-EBV-associated tumor-specific therapeutic targets. For example, preventing EBV-encoded molecules from loading into exosomes or inhibiting the release of EBV$^+$ exosomes might be serve as novel strategies. Given the recent increased interest in the use of exosomes as vectors for targeted therapy with nanomaterials (121), this therapeutic approach may offer clinical potential.

7. Conclusion

EBV has been studied for decades. EBV is closely related to numerous kinds of tumors in terms of both epidemiology and molecular biology. However, the key pathogenic mechanisms of the virus may not be the viral particles themselves but instead the molecules encoded by the virus, indicating that antiviral drugs may not be useful. Exosomes act as perfect carriers for these viral molecules to protect them from degradation by host enzymes and transport them to other cells with the continuous influence of target cells. In different TMEs, EBV$^+$ tumor exosomes target different cells but share similar immunosuppressive functions. We speculated that the viral molecules loaded in the exosomes served a pivotal role. To date, therapies for EBV$^+$ tumors have displayed limited effectiveness, and a vaccine for EBV has not yet been produced. In the future, the treatment and prevention of EBV$^+$ tumors may focus on EBV$^+$ exosomes, and exosome-based disease monitoring and treatment, as well as viral vaccines may have potential, but these approaches require further exploration.

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Authors' contributions

WC drafted, proofread and revised the manuscript. YX edited and revised the manuscript. TW edited the manuscript. TW and LW provided the project financial support. LW supervised and oversaw the production of this review. All authors have read and approved the final manuscript. Data authentication is not applicable.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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