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Chapter 5

Exosomes as the Promising Biomarker for Epstein-Barr Virus (EBV)-Associated Cancers

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Abstract

Exosomes are microvesicles with sizes ranging from 50 to 150 nm. These small vesicles are known to morphologically and functionally resemble virus particles from human immunodeficiency virus type I (HIV-I) and human T-lymphotropic virus type I (HTLV-I). The function of exosomes is to mainly mediate cell-to-cell communication by exchanging various macromolecules including proteins, lipids and nucleic acids in diverse cellular processes. Due to its size and structural simplicity, the transfer of pathogenic or virulent cellular factors across the cells mediated by exosomes is more efficient, hence facilitating the dissemination of viral infections and cancer diseases. The pathogenic role of exosomes in various cancers such as lung and breast, and their potentials as biomarkers have been previously studied, yet limited information is known for Epstein-Barr virus (EBV)-associated cancers. In this chapter, we discuss current evidences that support the pathogenic roles of exosomes in EBV-related cancers and their potentials as biomarkers in cancer diagnostics and therapy response. Here, we also highlight the potential challenges in the development of exosome-based biomarkers for clinical application.

Keywords: EBV, exosomes, cancer, biomarker, nasopharyngeal carcinoma, diagnostics, therapy

1. Introduction

Exosomes are microvesicles that play major roles in cell-to-cell communication. These small vesicles have nano-scaled size, resembling the size of HIV-1 particles. Indeed, several reports have highlighted the similarity between exosomes and HIV-1 particles in terms of structure and functions [1, 2]. Biological roles of exosomes in HIV-1 pathogenesis have been extensively reviewed and the implication of the pathogenic exosomes depends highly on the contents that they carry [3, 4]. The functions of exosomes in other viral infections, such as hepatitis

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C virus (HCV), herpes simplex virus (HSV) and so on, have also been demonstrated [5, 6]. In addition to viral diseases, exosomes have also been reported to play critical roles in cancer pathogenesis, including those in glioma, lymphoma, colorectal carcinoma, melanoma, ovarian and breast cancers [7, 8]. However, little is known for the function of exosomes derived from tumour viruses or oncoviruses such as HTLV-1, EBV and human papilloma viruses (HPV) in virus spreading as well as oncovirus-driven tumour development and dissemination. Among all, EBV is the most common infection and it infects more than 90% of human adult population globally [9]. Cumulative findings demonstrated that EBV infection is associated with various lymphoid and epithelial malignancies, including nasopharyngeal carcinoma (NPC), Burkitt's lymphoma (BL), gastric carcinoma (GC), Hodgkin's lymphoma (HL) and Non-Hodgkin's lymphoma [9, 10]. Similarly, EBV has also been reported to contribute to breast and cervical cancers [11, 12]. **Figure 1** summarizes the cancers that are associated with EBV infection, and percent association with EBV is depicted in a form of pyramid.

Exosomes are ubiquitously present in almost all biological fluids, including urine, plasma, saliva, ascites, breast milk, semen, bronchoalveolar lavage liquid, amniotic fluid and cerebrospinal fluid [4–7]. They are secreted from various cell types such as dendritic cells (DCs), macrophages, T cells, B cells and cancerous cells [4–7]. The omnipresence of exosomes makes them the ideal targets for cancer diagnostics and anti-cancer therapy. However, it remains to be seen whether exosomes from different sources present similar pathogenic profiles and can be interchangeably targeted for diagnostics and therapeutic purposes. Exosomes generally have a density of 1.13–1.21 g/mL [13]. They are surrounded by a lipid bilayer and they are enriched with macromolecules such as lipids (e.g. cholesterol and glycosphingolipids), carbohydrates (e.g. high mannose and complex N-linked glycans), proteins (e.g. tetraspanins CD9, CD63 and CD81, MHC molecules, Rabs, actin, alix, HSP70 and TSG101) and nucleic acids (e.g. DNAs, mRNAs and miRNAs) [14, 15]. While EBV infection is highly associated

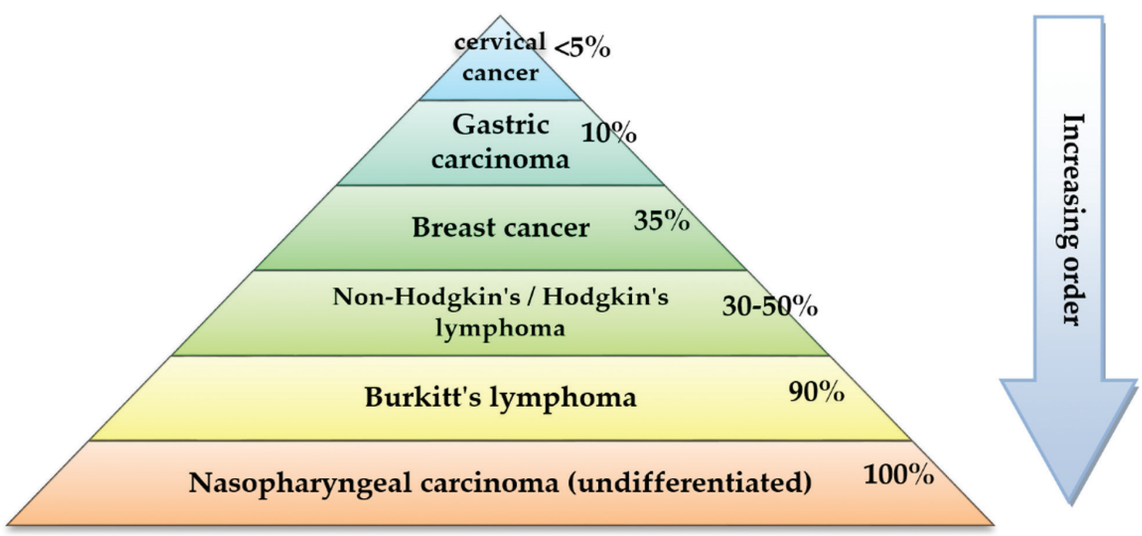


Figure 1. Association of EBV infection with virus-associated cancers. The association of EBV and cancers is represented by the above pyramid in a rising order from top to bottom. Almost 100% of undifferentiated NPC cases are associated with EBV infection while cervical cancer has been reported to be linked with EBV infection, but to the least extent with about less than 5% of total cases.

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with a plethora of cancer diseases, it is strikingly surprising that only a few studies have been carried out to investigate the role of EBV-derived exosomes in cancer development and progression. This chapter aims to summarize current findings that demonstrate the biological functions of EBV-derived exosomes in the cancer pathogenesis. We also attempt to discuss the potentials of using EBV-derived exosomes as diagnostic biomarkers and to target these exosomes in anti-cancer therapy, while reviewing the challenges entailed in the above efforts.

2. Pathogenic roles of exosomes in EBV-associated cancers

Exosomes are originated from cellular endosomes, whereby the inward budding takes place on endosomal multivesicular bodies (MVBs) to form intraluminal vesicles (ILVs) [5, 6]. The subsequent molecular event then determines whether ILVs enter lysosomal degradation pathway or are being secreted out from the producer cells in the form of exosomes upon the fusion of MVB membrane with the plasma membrane [5, 6]. **Figure 2** illustrates these processes that involve the budding of endosomes, formation of EBV pathogenic factors-loaded exosomes and the delivery of the pathogenic exosomes to the target cells. The biogenesis of exosomes has been previously reviewed in great depth [14, 15], and these findings are important to enhance our understandings towards the function of exosomes in multiple cellular processes especially in cancer pathogenesis. The role of EBV-derived exosomes in EBV-associated cancers has been partly discussed in previous reviews [5, 8], but the proteins or genes involved and the underlying mechanisms have not been clearly illustrated. In this section, we will discuss the pathogenic roles of the contents in EBV-derived exosomes such as latent membrane proteins (LMPs), mRNAs and miRNAs in contributing to the EBV-associated cancers.

2.1. Exosomal latent membrane proteins (LMPs)

Latent membrane proteins (LMPs) are oncogenic proteins that are highly associated with cancer pathogenesis particularly in HL and NPC. There are two types of LMPs: LMP1, and LMP2 that consists of LMP2A and LMP2B. Each of these oncogenic proteins has distinct function in EBV-related human cancers [16, 17]. For instances, LMP1, which is a viral mimic of tumour necrosis factor receptor (TNFR) family member CD40, has been reported to activate a cascade of oncogenic signalling pathways such as nuclear factor kappa B (NF-κB), phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K) and c-Jun N-terminal kinases (JNKs) in EBV-associated cancers [10, 16, 17]. On the other hand, LMP2 plays central roles in maintaining viral latency in EBV-infected B cells as well as inducing transformation and migration of EBV-infected cells [10, 16, 17]. Whether or not the functions of cell-associated LMPs are fully retained in the exosomal LMPs, it remains to be proven. The development of the EBV-related cancers is associated with three EBV latency types based on the expression of EBV proteins (i.e. LMP1, LMP2 and Epstein-Barr virus nuclear antigen-1, EBNA1). Latency type I, which is usually observed in BL and GC, consistently displays strong expression of EBNA1 [17, 18]. Latency type II, on the other hand, results in the expression of LMP1, LMP2 and EBNA1 as seen in HL and NPC, whereas in latency type III, all latent proteins/antigens are expressed in the course of acquired immune deficiency syndrome (AIDS)-related lymphomas and lymphoblastoid

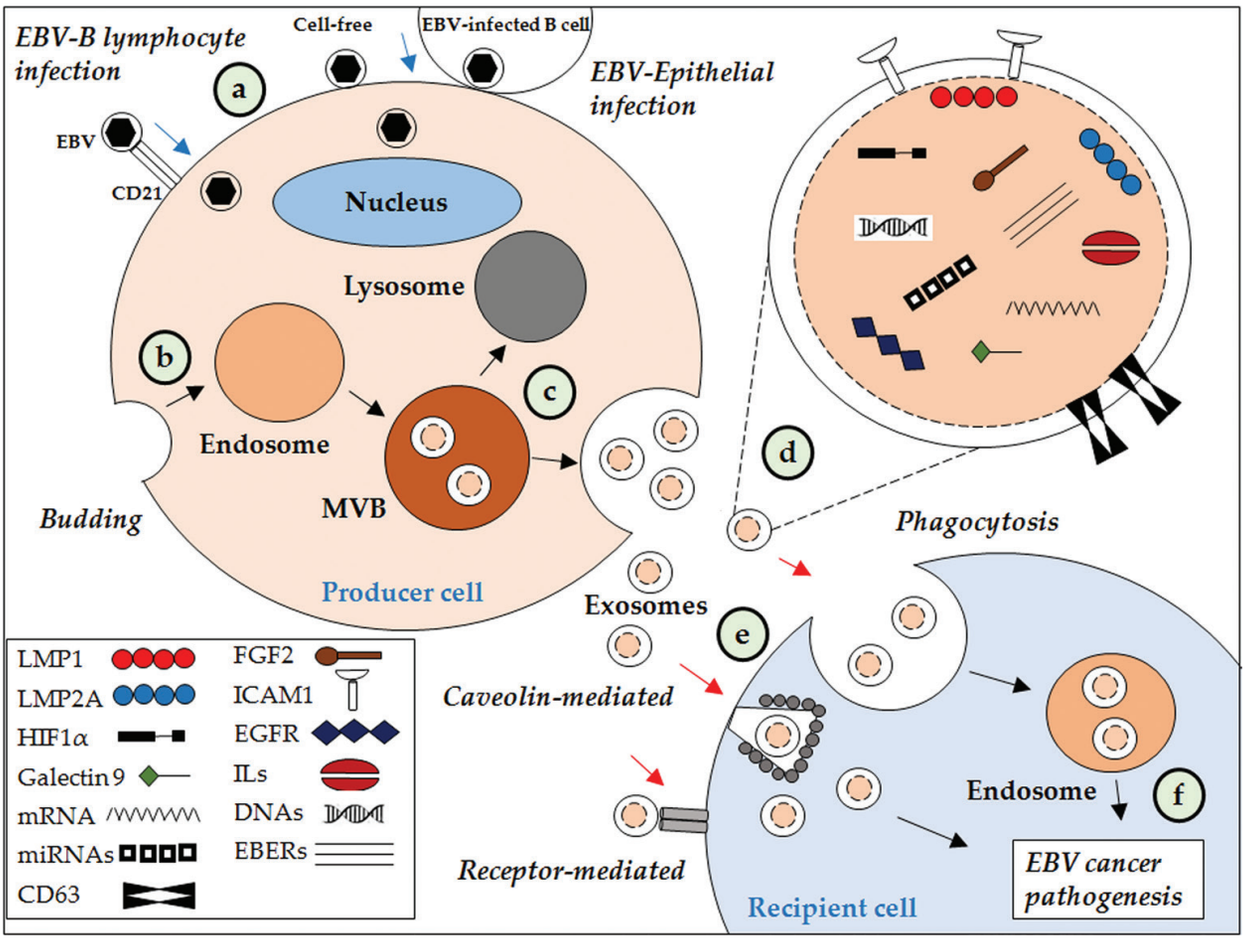


Figure 2. Generation of exosomes in EBV-infected cells and delivery of EBV pathogenic factors-enriched exosomes to recipient cells. (a) EBV infects B cells through viral glycoproteins/CD21 interaction, whereas EBV could infect epithelial cells either through cell-free manner or cell-to-cell contact mediated by infected B cells. (b) Exosomes are generated from the inward budding of endosomes, which then forms MVBs followed by ILVs. (c) ILVs are then either degraded by lysosomes or released from the producer cells extracellularly as exosomes. (d) Owing to the overexpression of EBV proteins/genes in the infected cell, exosomes are loaded with these pathogenic factors. (e) These exosomes are then delivered to the neighbouring cells via phagocytosis, receptor- or caveolin-mediated endocytosis. (f) The pathogenic factors are released from the EBV-derived exosomes, which then contribute to the cancer pathogenesis.

cell lines (LCLs) [17, 18]. Of note, the LMPs-carrying exosomes have been previously shown to be secreted by EBV-infected cells particularly the NPC cells [19–21].

So far, only a few studies have shown the involvement of exosomal LMP1 in activating various oncogenic pathways in EBV-associated cancers unlike those in the case of cell-derived LMP1 [16, 17]. Current findings suggest that exosome-derived LMP-1 plays central roles in attenuating the immune response in both EBV infection and the development and progression of EBV cancers [20, 22, 23]. For instances, the LMP1- and galectin 9-containing exosomes derived from EBV-infected LCLs and NPC cells have been found to inhibit infiltrating T-lymphocyte activation and proliferation through an action mediated by the conserved trans-membrane domain of LMP1 [20]. This consequently resulted in the immune escape of tumour cells, and hence promoting the cancer growth and progression [24]. Exosomes containing LMP1 and HIFα have also been demonstrated to induce tumour invasion in NPC [19]. Moreover, LMP1

was shown to promote the expression of fibroblasts growth factor 2 (FGF2) and along with the LMP1, FGF2 was excreted from the tumour cells via Na(+)/K(+)-ATPase-dependent exosomal pathway [25]. Notably, FGF2 is an important angiogenic factor in tumour invasion and it has been recently shown that FGF2 can be targeted by miR-16 to inhibit in cell proliferation and invasion in NPC [26]. However, whether or not the LMP1-induced exosome-derived FGF2 secretion is implicated in the EBV-associated cancers, particularly in the aspect of tumour metastasis warrants further investigations.

On top of the immunosuppressive and potentially invasive roles of exosomal LMP1, expression of intercellular adhesion molecule 1 (ICAM1) or CD54 was also up-regulated by exosomal LMP1 [21]. Notably, overexpression of ICAM1 is generally seen in various types of EBV-related cancers such as NPC, GC and NHL [27–29]. Similarly, exosomal LMP1 also induced epidermal growth factor receptor (EGFR) expression [30] which is another important receptor that modulates a cascade of oncogenic signalling pathways such as mitogen-activated protein kinase (MAPK), c-Jun N terminal kinase (JNK), phosphatidylinositol 3 kinase (PI3K) and nuclear factor kappa-beta (NF-κB) in EBV cancers [31, 32]. In line with of the role of exosomal LMP1 in EGFR-related pathways, Meckes and co-workers also demonstrated that exosomal LMP1 activated Extracellular signal-regulated kinase (ERK) and v-Akt murine thymoma viral oncogene homolog (AKT) signalling pathways in the target cells [30]. This finding suggests that the transfer of oncogenic LMP1 via exosomal pathway may modulate the growth of neighbouring cells, hence contributing to the cellular transformation of cancer cells.

Interestingly, exosomal LMP1 is also found to interact with tetraspanin CD63, a common marker of exosomes. This interaction facilitates the former to escape lysosomal degradation [33] which may result in enhanced oncogenicity of exosomal LMP1. In addition, the level of LMP1 is highly correlated with CD63 expression [19]. This suggests that LMP1 may upregulate the exosomal secretion, hence promoting the EBV-associated cancers [19]. As summarized in **Table 1**, cumulative findings suggest that exosomal LMP1 promotes the growth and progression of EBV-associated cancers. While the cell-associated or intracellular LMP1 plays multiple roles in EBV latent infection and cancer pathogenesis particularly by modulating NF-κB, PI3K/AKT, MAPK and JAK/STAT pathways [31, 32], future investigations are warranted to demonstrate whether the exosomal LMP1 similarly carries these tumorigenic functions and pathogenic effects in EBV-associated cancers.

As compared with LMP1, the role and function of exosomal LMP2A/2B in EBV-associated cancers are less understood. Incorporation of LMP2 into exosomes has been previously observed and these exosomes were released and taken by recipient cells [34, 35–37]. Several studies have provided insights on the mechanism of exosomal LMP2 secretion. Ikeda and Longnecker demonstrated that cholesterol depletion via methyl-beta-cyclodextrin (MCD) depletion can increase exosomal secretion of LMP2A, indicating the inverse dependency between the release of LMP2A-carrying exosomes and cholesterol level [35]. In another study, the interaction between LMP2A and endocytic adapter proteins, Amphiphysin 1 and 2 was found to be essential in order for LMP2A to accumulate into exosomes [38]. However, no further study has been done to investigate the pathogenic role of Amphiphysin proteins on EBV-associated cancers. Future investigations are required to uncover the pathogenic role of exosomal LMP2 in these cancers.

Exosomal content	Pathogenic effect	Reference
LMP1 & HIF1α	Promotion of tumour invasion	[19]
LMP1 & Galectin 9	Immunosuppression of T lymphocytes	[20]
LMP1 & ICAM1	Induction of ICAM1 expression by LMP1 Modulation of multiple oncogenic pathways	[21]
LMP1 & FGF2	Induction of FGF2 expression by LMP1 Potential driver of tumour invasion	[25]
LMP1 & EGFR	Induction of EGFR expression by LMP1 Modulation of multiple oncogenic pathways	[30]
LMP1 & CD63	Increased secretion of exosomal LMP1	[33]
LMP2A & Amphiphysin	Increased secretion of exosomal LMP2A	[38]
BHRF1-3 miRNA	Detected in the EBV-infected cells and transferable via exosomes	[43]
miR-BART15-3p	Detected in EBV-infected cells and transferable via exosomes to corresponding cells	[44]
hsa-miR-24-3p hsa-miR-891p hsa-miR-106a-5p hsa-miR-20a-5p hsa-miR-1908	Inhibition of T-cell proliferation and differentiation Induction of Treg cells Increased pro-inflammatory cytokine expression Regulation of MARK1 signaling pathway	[45]
EBV latent phase mRNAs	Potential expression of LMP1, LMP2, EBNA1, and EBNA2 in recipient cells upon taking up these exosomes	[46]
EBER1 and EBER2	Detected in the EBV-infected cells and transferable via exosomes	[47]
CCL20	Enhanced Treg recruitment and expansion	[22]
dUTPase	Induction of NK-κB activation and pro-inflammatory cytokine secretion	[48]
IFI16, caspase-1, IL-1β, IL-18, IL-33	Enrichment of caspase-1 resulted in the secretion of active immune-modulatory cytokines	[49]

Table 1. Potential pathogenic roles of exosomes in EBV-associated cancers.

2.2. Exosomal RNAs

Cancer-derived exosomes carry RNAs particularly miRNAs that are implicated in cancer pathogenesis such as in breast and colorectal cancers [39, 40]. In EBV-related cancers, the pathogenic role of RNAs such as miRNAs, mRNAs and Epstein-Barr virus-encoded small RNAs (EBERs) has drawn considerable attention in the past few years [17, 41]. miRNAs are non-coding RNAs that modulate multiple cellular processes, including the promotion of tumourigenesis via a cascade of signalling pathways [41]. They are small molecules with an approximate size of 22 nucleotides [41]. The mature miRNA functions by interacting with the target mRNA and block their activities by repressing the translation. The effects of miRNAs have been implicated in various EBV-associated cancers such as NPC, GC and BL [17, 41, 42]. However, the tumorigenic role of exosomal miRNAs is underexplored in EBV-related cancers as opposed to the cellular miRNAs.

There are substantial findings supporting the notion that oncogenic miRNA-carrying exosomes may play pathogenic roles in EBV-associated cancers [21, 30, 43]. For instances, BHRF1-3 miRNA has been shown to be secreted from the EBV-infected cells and they retained their cellular function upon delivery to the recipient cells [43]. Choi and colleagues demonstrated that the miR-BART15-3p could be detected in EBV-associated exosomes and its expression level was 2 to 16-fold higher in the exosomes compared with the cellular level in GC cells [44], hence suggesting its potential tumorigenic role. On the other hand, Ye and group showed that the exosomal miRNAs promoted the tumour progression by modulating multiple cellular processes in NPC [45] (**Table 1**). Further studies are required to investigate and validate their roles in promoting EBV-derived cancers. The occurrence of exosomes carrying mRNAs encoding for oncogenic EBV proteins such as LMP1, LMP2, EBNA1 and EBNA2 [46, 47] as well as EBERs has also been documented (**Figure 1**). While the functions of these RNAs in the EBV-infected cancer cells are well-described, whether or not the exosomal RNAs are transferable to the recipient cells and exert their tumorigenic effects remains a question.

2.3. Other exosomal pathogenic factors

In addition to EBV-associated proteins/genes such as LMPs, EBERs, EBV-related miRNAs and mRNAs, exosomes related to EBV-associated cancers may also contain other endogenous proteins that potentially promote cancer progression such as transcription factor Galectin-9 [20], EGFR [30], HIF1α [19], ICAM1 [21], FGF2 [25], chemokine (C-C motif) ligand 20 (CCL20) [22], dUTPase [48] and interleukins (ILs)/caspase 1/interferon-inducible protein 16 (IFI16) [49] (**Table 1**).

Mrizak and co-workers demonstrated that CCL20-containing exosomes recruited the CD25+FOXP3+ Treg cells and enhanced their expansion in NPC [22]. The involvement of these exosomes in the Treg interaction may therefore support immune evasion in NPC. In the case of dUTPase enzyme, up-regulated expression of this enzyme has been observed in the exosomes derived from the EBV-positive Burkitt's lymphoma cell line, Raji. These enzymes are found to induce the cytokine release from DCs and PBMCs, which may activate the NF-κB pathway [48].

Interestingly, exosomes derived from the Raji cells and other EBV-infected cell lines are also enriched with various immune modulators such as IFI16, cleaved caspase-1, IL-1β, IL-18 and IL-33 [49]. The presence of these proteins in these exosomes may suggest that EBV utilizes the host exosome pathway in immune escape of tumour hence contributing to the EBV-associated cancer progression.

3. Exosomes as biomarkers

We have discussed the tumorigenic role of exosomes in EBV-associated cancers in previous section. Since these exosomes carry a great variety of pathogenic molecules (**Figure 2**), they can be potentially used for diagnostic and/or prognostic markers in the EBV-related cancers. Indeed, several reports have highlighted the potentials of employing exosomes as

the biomarkers in various cancers [50, 51], and the exosome-containing miRNAs are the most popular cancer diagnostic markers out of all [52, 53]. There have been several lines of evidences suggesting that EBV oncoproteins can be targeted for cancer diagnostics. For instances, Houali and colleagues showed that both EBV oncoproteins, LMP1 and BARF1, could be detected in serum and saliva of NPC patients, and the secreted LMP1 was highly associated with exosome-like vesicles [54]. Both EBV oncoproteins were presented with high mitogenic activity that supported their implication in oncogenic development of NPC. The fact that exosomes are abundantly expressed in patient serum and saliva further support the potential of using the oncoprotein-enriched exosomes for cancer diagnostics [3–5]. Similarly, Mao and co-workers also highlighted the potential of LMP1 and LMP2A as the prognostic markers for extranodal NK/T-cell lymphoma (ENKTL) patients [55]. LMP1 and LMP2A were overexpressed in ENKTL tumours and they significantly correlated with the patients' overall survival. However, whether or not the exosomal LMP1 and LMP2A have the similar values for prognosis remain to be seen.

In addition, other pathogenic factors that are enriched in EBV exosomes such as EBV DNAs, EBV mRNAs and EBV miRNAs have also been shown to be potential biomarkers for EBV cancer diagnosis [56–59] (**Table 2**). Using quantitative PCR (qPCR), Yip and colleagues showed that high-EBV DNAs could be detected in plasma/serum of NPC patients at late stages of disease and the viral loads were associated with poor survival or frequent relapse in NPC patients [56, 59]. On the other hand, Stevens and colleagues demonstrated that the EBV DNA load measurement might have limited value as the diagnostic marker for NPC as the viral load did not reflect the intact tumour cells [56, 59]. Hence, they recommended to combine circulating EBV DNA measurement and EBV serology to increase the diagnostic sensitivity. The same group also showed that the combination of EBV DNA and BARF1 mRNAs detection from patients' nasopharyngeal brushings could be a good non-invasive method for

Exosomal target	Source/sample	Cancer type	Reference
LMP1	Serum, saliva, tumour	Lymphoma, NPC	[54, 55]
LMP2A	Tumour	Lymphoma	[55]
BARF1	Serum, saliva	NPC	[54]
EBV DNAs	Cell lines, tumour, NP brushing, serum/plasma	NPC	[56, 57, 59]
EBV mRNAs	Cell lines, tumour, NP brushing, serum	NPC	[57, 59]
EBV miRNAs	Cell lines, plasma	NPC	[58]
Galectin	Tumour	NPC	[65]
EGFR	Tumour	Prostate cancer*	[61]
HIF1α	Tumour	Breast cancer	[60]

*Not EBV-associated cancer.

Table 2. Potential exosomal target for prognostic/diagnostic biomarker development in EBV cancers.

NPC diagnosis [57]. On the other hand, Zhang and co-workers showed that EBV miRNAs (miR-BART7 and miR-BART13) can serve as important biomarkers for NPC diagnosis and prediction of treatment efficacy [58]. The potential prognostic value of EGFR and HIF1α has been previously demonstrated in breast and prostate cancers [60, 61]. It would be interesting if these proteins can also be used as prognostic markers for EBV-related cancers. However, this requires further investigations. While circulating EBV DNAs, mRNAs, miRNAs and other components in serum/plasma is useful for EBV-related cancer diagnostics, more efforts should be focused in discriminating the diagnostics values of cell- and exosome-derived nucleic acids.

More interestingly, exosomes have shown to protect their cargoes from degradation. For instance, it has been shown that the mRNAs and miRNAs encapsulated in exosomes are protected from RNAses [62]. They are more stable and can be stably employed as diagnostic biomarkers. Recent advances in methods have also made the exosome isolation from various biofluids simpler, more straightforward, and with better quality and yield [63, 64]. The EBV proteins/genes that can potentially be developed into diagnostic markers are summarized in **Table 2**.

4. Exosomes as predictive markers for therapy response

Pathogenic exosomes have been previously linked to the treatment failure of cancers [65, 66]. As exosomes exhibit pathogenic effects on tumour formation and progression, the oncogenic activity of exosomes can be intervened by blocking the production/release of the exosomes or the specific exosomal proteins/genes. **Figure 2** summarizes the pathogenic and tumorigenic factors derived from the EBV exosomes and the potential targets for anti-cancer therapy development. In fact, there have been studies showing that EBV-associated proteins and nucleic acids (e.g. DNA, mRNA and miRNA) can be targeted for anti-cancer therapy development particularly in NPC [67–70]. For instance, various strategies, such as cell-based immunotherapy, antibody-based and drug-based therapies, have been developed against EBV LMPs in NPC [68]. Similarly, Cao and colleagues also showed that DNAzyme resulted in significant tumour regression by targeting and cleaving off the LMP1 mRNA from NPC patients [67]. Other non-EBV tumour-promoting but LMP-associated proteins that could be targeted in NPC are EGFR [71, 72] and vascular endothelial growth factor (VEGF) [73, 74]. In addition, targeting the whole exosome has also been shown to be a potent therapeutic strategy for cancer therapy [66, 75]. However, the potential of developing these pathogenic proteins derived from tumour-associated exosomes into the therapy is unclear and further investigations are required.

As abovementioned, exosomes also contribute to the immune evasion of cancer cells [20, 22, 23]. For example, the galectin-9-containing exosomes have been shown to inhibit the proliferation and induce apoptosis of EBV-specific T cell, hence preventing the T cell-mediated recognition and killing of these cancer cells [20, 23]. Therefore, blocking these exosomes may restore the functions of immune cells to act and kill the cancer cells together with a plethora of other active tumour-killing activities. It can be envisioned that the development of a therapeutic strategy blocking the galectin-9 or other proteins from oncogenic exosomes may restore the

immune surveillance. Over the past few years, considerable work has also been done on targeting the whole exosomes rather than targeting the specific proteins [76, 77]. The exosomal removal using a modified kidney dialysis system has also been proposed to bring this therapeutic approach to the clinics [76]. These findings suggest that diminishing or eliminating the tumorigenic exosomes may be a good therapeutic approach to reverse the exosome-mediated cancer progression, particularly in the aspect of immune dysregulation.

5. Challenges and limitations

The facts that exosomes are ubiquitous and can be detected in most of the biofluids give advantages to the development of diagnostics biomarkers. Several studies have also demonstrated that biologically functional and intact exosomes could be isolated from human plasma/serum [63, 64]. Moreover, the isolation/purification method of high-quality and quantity exosomes from body fluids has greatly improved in the past few years [63, 64]. Hence, the development of exosome-targeting diagnostic biomarker has high potential and can be developed into an important liquid biopsy-based diagnostic test for cancers in future. However, several considerations need to be taken into account to ensure the success of biomarker and therapy development.

Sensitivity and specificity are important criteria for cancer biomarker development. While exosomes containing EBV-associated contents (e.g. mRNAs, miRNAs, LMPs, galectin 9 etc.) have tumour-promoting and pathogenic properties and are expressed during the disease development (**Table 1**), they are not specific to particular type of cancer of which they could contribute to such as NPC, GC, BL and so on (**Figure 1**). Before a specific exosomal target is discovered for each EBV-related cancer, other non-invasive tests such as cancer antigen screening and magnetic resonance imaging (MRI) can be carried out simultaneously to enhance the diagnostic outcome in terms of cancer specificity. Furthermore, the expression of some target protein/gene in the patients may highly depend on the disease state. The expression may be too low to be detected or undetected at all during the early stage of cancer, hence the sensitivity may be the issue. On top of that, the quality and quantity of the pathogenic exosomes can be a challenge for diagnostic biomarker development even though it has been shown that high quality of exosomes could be detected from cancer patients [63, 64]. This will highly rely on the method used for the exosome isolation/purification for the diagnostic purpose, and it is extremely important to ensure the high consistency and reproducibility of the test.

As an important therapeutic target, EBV proteins/nucleic acids can be targeted for tumour regression as described in the previous section. Exosomes play important roles in cell-cell communication mainly by regulating cellular processes, hence complete removal of exosomes is not a feasible therapeutic strategy as it will affect the well-being of other normal cells or cellular processes under a normal condition [7, 14]. Hence, it is important to specifically target only the exosomes enriched with the pathogenic factors without affecting the biological activities of existing exosomes. In addition, the exosomal contents may largely vary and are heterogeneous depending on the sources or origins [7, 14]. This may be another challenge especially the targeted exosomes are from the patient's body fluids that may be derived from a diverse range of cells. Furthermore,

Purpose	Potential issue	Note
Diagnostic biomarker development	Specificity	Not specific to particular cancer type
	Sensitivity	No/low expression in early-stage cancer
	Quality	Inactive and not functional exosomal content
	Quantity	Low yield of the pathogenic exosome or protein/gene
Anti-cancer therapy development	Toxicity	Off-target effect against all functional exosomes
	Heterogeneity	Contain multiple types of functional protein/gene
	Dose and course	Wide in range due to the personalized differences
	Delivery	Therapeutics may not reach the ubiquitous exosomes
	Bioavailability	Therapeutics may be degraded before reaching to the targets

Table 3. Potential challenges for the development of exosome-targeted cancer diagnostics and anti-cancer therapy.

some potential targets may be scarcely expressed or not at all in the targeted exosomes which will hinder the efficiency to target exosomes for any anti-cancer therapy. Other considerations include the dosage of exosome-targeting drugs that may vary from one to another, the delivery system for targeting exosomes, the bioavailability/stability of the delivered therapeutic molecules and the treatment course. Further works are required to evaluate the clinical safety of exosome-targeting treatment strategy. **Table 3** summarizes the potential challenges during the development of diagnostic marker and therapy by targeting the exosomes in EBV cancers.

6. Conclusion

EBV-derived exosomes play seminal roles in the pathogenesis of EBV-associated cancers especially in NPC. Cumulative findings suggest that EBV-exosomes may be ideal targets for the development of diagnostic/prognostic markers and anti-cancer therapy. However, several issues need to be taken into account during the development as abovementioned. As limited studies have been carried out, more investigations are required to further validate the feasibility of targeting the pathogenic EBV exosomes for clinical diagnosis of EBV cancers. Current findings also suggest that the targeted exosomes could be developed into vaccines for EBV infections to reduce the EBV-induced cancers.

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