## Review



## Exosomes in Hepatitis B Virus Transmission and Related Immune Response

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The chronicity of Hepatitis B virus (HBV) infection relates to both viral factors and host factors. HBV could result in persistent infection and even serious liver disease, including chronic hepatitis B (CHB), cirrhosis and hepatocellular carcinoma (HCC). Although the HBV vaccine can effectively prevent HBV infection, chronic HBV infection still endangers human health and results in a large social burden. Moreover, the mechanisms underlying the HBV-mediated imbalance of the immune response and persistent infection are not fully understood. Exosomes are extracellular vesicles (EVs) 40-160 nm in size that are released from many cells and transfer specific functional RNAs, proteins, lipids and viral components from donor to recipient cells. These exosome nanovesicles are associated with various biological processes, such as cellular homeostasis, immune response and cancer progression. Besides, previous studies on exosomes have shown that they take part in viral pathogenicity due to the similarity in structure and function between exosomes and enveloped viruses. Moreover, exosome as a novel immunomodulatory carrier plays a significant role in viral immunology. In this review, we focus on the latest progress in understanding the role of exosomes in HBV transmission as well as their vital roles in immune regulation during HBV infection. Furthermore, we discuss the potential clinical applications of exosomes in hepatitis B infection, including the use of exosomes in the auxiliary diagnosis and treatment of hepatitis B.

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### Introduction

In 1963, hepatitis B virus (HBV) was first discovered in a patient's blood by Dr. Baruch Blumberg (2002); later, the infectious virion was determined by microscopy (Dane et al. 1970). HBV is the pathogen that causes hepatitis B. Infection with HBV could result in impaired liver function. Long-term, persistent HBV infection increases the risk of liver cirrhosis and hepatocellular carcinoma (HCC) (Fattovich et al. 2004; Yuan et al. 2019). According to the World Health Organization (WHO) Global Hepatitis Report 2017, about 260 million individuals are chronically infected with HBV worldwide, and HBV caused an estimated 887,000 deaths in 2015. At present, antiviral therapies are the main means to control HBV replication, and are mainly limited to pegylated interferon (PEG-IFN) and nucleos/tide analogs (NAs) (Likhitsup and Lok 2019). Most subjects with antiviral therapy barely achieve hepatitis B surface antigen (HBsAg) clearance, therefore many patients need NAs life-long treatment by controlling the proliferation and spread of the virus (Likhitsup and Lok 2019). Thus, further research is urgently required to meet the WHO goal of eliminating viral hepatitis by 2030.

Exosomes are extracellular vesicles (EVs) 40-160 nm in size that were first discovered in studies of mammalian reticulocyte maturation (Johnstone et al. 1987). Exosomes can be secreted by almost all cells. They are widely and stably present in biological fluids such as blood (Caby et al. 2005), urine (McKiernan et al. 2016), saliva (Zlotogorski-Hurvitz et al. 2016), cerebrospinal fluid (CSF) (Stuendl et al. 2016), breast milk (Admyre et al. 2007), seminal plasma (Vojtech et al. 2014), amniotic fluid (Dixon et al. 2018) and

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ascites (Hu et al. 2019). Exosomes carry proteins, lipids and nucleic acids that mediate regulatory effects on cell-tocell communication. To date, it has been found that exosomes are involved in a variety of biological functions, including immune regulation (Mittelbrunn et al. 2011; Zhang et al. 2018), cell regeneration (Tan et al. 2014), viral transmission (Ramakrishnaiah et al. 2013) and tumor metastasis (Wortzel et al. 2019).

In this review, we summarize the role of exosomes in HBV transmission as well as their roles in immune regulation during HBV infection, thereby providing new perspectives on persistent HBV infection. Furthermore, we discuss the potential clinical applications of exosomes during HBV infection; such applications may contribute to the auxiliary diagnosis and treatment of hepatitis B infection.

## Interaction between HBV Transmission and Exosome Biogenesis

### Process of HBV replication

HBV belongs to the family *Hepadnaviridae* and possesses a partially double-stranded, circular DNA genome (Summers et al. 1975). The genome has four major open reading frames (OFRs), namely, surface (S), polymerase (P), core (C) and X (Blum et al. 1989). The S region encodes the three HBV envelope proteins preS1, preS2, and hepatitis B surface antigen (HBsAg) (Seeger and Mason 2000). The C region encodes hepatitis B core protein (HBcAg) and hepatitis B e antigen (HBeAg) (Seeger and Mason 2000). The P region and the X region encode DNA polymerase (DNAP) and hepatitis x antigen (HBx), respectively (Seeger and Mason 2000). These five proteins are important for HBV replication, viral particle formation, and transmission (Seeger and Mason 2000). The synthesis of HBV DNA occurs simultaneously with virus packaging and replication (Schädler and Hildt 2009) (Fig. 1). Generally, HBV enters hepatocytes through endocytosis in which HBV envelope proteins interact with receptors on the hepatocytes (Schulze et al. 2010; Yan et al. 2012; Verrier et al. 2016), and the HBV virion is encapsulated in an endosomal vesicle (Blondot et al. 2016). HBcAg forms the nucleocapsid that envelopes the relaxed circular DNA (rc DNA), which is covalently linked to the DNAP (Summers et al. 1975;



#### Fig. 1. HBV hijacks exosomes for transmission.

HBV enters hepatocytes through endocytosis with the assistance of receptors and is released by endosomal vesicles. In the hepatocyte nucleus, rc DNA is converted to cccDNA. Later, cccDNA is transcribed into pgRNA and HBV mRNAs. Further, HBV mRNAs are transported to the cytoplasm for translation. HBV-encoded proteins, including HBsAg, HB-cAg, HBeAg and HBx proteins, are synthesized through the ER/Golgi complex. pgRNA is converted into ss DNA under the action of reverse transcriptase. The mature HBV nucleocapsid formed as the double-stranded DNA partially circularized. HBs subviral particles are secreted as monomeric secretory proteins. Mature HBV virions may bud into MVBs as they regress or hijacks the biogenesis of exosomes. Furthermore, HBV-associated exosomes can transfer HBV components to naïve hepatocytes independently of the presence of classic receptors.

rc DNA, relaxed circular DNA; cccDNA, covalently closed circular DNA; pgRNA, pregenomic RNA; HBsAg, hepatitis B surface antigen; HBc, hepatitis B core protein; HBeAg, hepatitis B e antigen; HBx, hepatitis B x antigen; MVB, multivesicular bodies; ER, endoplasmic reticulum; Golgi, Golgi apparatus.



Fig. 2. The biogenesis and release of exosomes.

Exosomes are generated by membrane invagination and membrane fusion. The first invagination of the cell membrane forms intracellular endosomes that fuse to form early sorting endosomes; the second membrane invagination in late sorting endosomes (LSEs) give rise to intraluminal vesicles (ILVs). Subsequently, LSEs form multivesicular bodies (MVBs). Eventually, ILVs are secreted as exosomes into the extracellular environment through the fusion of membranes. In the extracellular milieu, exosomes can exploit ligand-receptor binding or directly fuse with the cell membrane to transfer their cargoes to recipient cells. ER, endoplasmic reticulum; Golgi, Golgi apparatus.

Schädler and Hildt 2009). In hepatocytes, the nucleocapsid is released from endosomal vesicles (Blondot et al. 2016). Later, rc DNA is transported into the hepatocyte nucleus and converted to covalently closed circular DNA (cccDNA) (Schädler and Hildt 2009). The cccDNA subsequently serves as a template and is transcribed into HBV mRNAs and HBV replication intermediates (HBV-RI), including pregenomic RNA (pgRNA), single-stranded DNA (ss DNA) and double-stranded linear DNA (DL-DNA) (Schädler and Hildt 2009). HBV-RI, together with core proteins, DNAP and heat shock proteins, forms the immature progeny nucleocapsids (Schädler and Hildt 2009). Finally, the double-stranded DNA is partially circularized to complete the replication of HBV DNA, and mature nucleocapsids are formed (Blondot et al. 2016). The mature HBV virion is infectious and can be transported into the extracellular environment and infect more hepatocytes (Blondot et al. 2016). However, the precise machinery of HBV budding and egress is still obscure. As reported previously, HBV budding depends on the multivesicular bodies (MVBs) pathway (Lambert et al. 2007; Watanabe et al. 2007) (Fig. 1).

## HBV hijacks exosome biogenesis for transmission

Exosomes and viruses have several common charac-

teristics, including their biophysical properties and their capacity to transport bioactive materials among cells (Meckes and Raab-Traub 2011; van Dongen et al. 2016). Previous studies have shown that the biogenesis and release of viruses are similar to those of exosomes, suggesting that viruses may hijack the exosomal pathway (Gould et al. 2003; Nguyen et al. 2003). The generation of exosomes is a dynamic process that involves the invagination and fusion of plasma membranes (Kalluri and LeBleu 2020) (Fig. 2). Initially, the inward budding of the plasma membrane forms intracellular endosomes that fuse to form early sorting endosomes (ESEs) (Huotari and Helenius 2011). Then, the invagination of the membrane in the late sorting endosomes (LSEs) leads to the generation of intraluminal vesicles (ILVs), and the LSEs eventually give rise to MVBs. Finally, ILVs are secreted as exosomes into the extracellular environment through the fusion of the MVBs with the plasma membrane (Mathieu et al. 2019). The generation of ILVs and MVBs are necessary steps in the biogenesis of exosomes (Hessvik and Llorente 2018). It is widely accepted that the endosomal sorting complexes required for transport (ESCRT) pathway and the ceramide-dependent pathway play a critical role in the formation of ILVs and MVBs (Huotari and Helenius 2011). Interestingly, HBV virions and nucleocapsids were found to engage the ESCRT

Viral components		Exosome source (treatment)	Reference
HBV DNA	rc DNA	Serum of CHB patients	(Yang et al. 2017b)
	cccDNA	HepG2 cells (co-cultured with HBV-positive serum)	(Liu et al. 2019)
HBV RNA	HBx	Serum of CHB patients	(Yang et al. 2017b)
		HepG2.2.15 cells	(Yang et al. 2017b)
	HBs/p	Serum of CHB patients	(Yang et al. 2017b)
HBV proteins	HBsAg	Serum of CHB patients	(Yang et al. 2017b)
	HBeAg		
	Core protein	HepAD38 cells (HBV-induced)	(Jia et al. 2017)
	Large envelope protein		
	Protein p		
	HBx protein	Huh-7 cells (transfected with pGFP-HBx plasmid)	(Kapoor et al. 2017)
HBV-miRNA	HBV-miR-3	Serum of CHB patients	(Yang et al. 2017a)

Table 1. HBV components present in exosomes derived from HBV-infected cells and CHB patients.

rc DNA, relaxed circular DNA; cccDNA, covalently closed circular DNA; HBsAg, hepatitis B surface antigen; HBeAg, hepatitis B e antigen; HBx, hepatitis B x; P, HBV polymerase.

complex to assist their egress from infected cells (Prange 2012; Votteler and Sundquist 2013; Stieler and Prange 2014), and some studies have shown that HBV DNA can be transported into exosomes via the ceramide-dependent pathway (Sanada et al. 2017). Therefore, HBV and exosomes seem to share a common mechanism for budding and release from cells. Several studies of HBV-associated exosomes showed that exosomes contain HBV components, including HBV DNA (Yang et al. 2017b; Liu et al. 2019), HBV RNA (Kapoor et al. 2017; Yang et al. 2017b), HBV proteins (Jia et al. 2017; Kapoor et al. 2017; Yang et al. 2017b) and even HBV-miR-3 (Yang et al. 2017b) (Table 1). Accordingly, it is likely that HBV buds into MVBs and is delivered via an exosome-dependent pathway. In recent years, increasing evidence has shown that HBV-associated exosomes can transfer HBV components including cccDNA to naïve hepatocytes (Kapoor et al. 2017; Sanada et al. 2017; Yang et al. 2017b). Thus, it is clear that HBV could utilize exosomes to greatly improve its transmission efficiency, although the precise mechanism by which HBV enters exosomes has not yet been fully described. Furthermore, how HBV-associated exosomes enter recipient cells is still unclear. They may directly fusing with the plasma membrane (PM) or attaching to the PM through receptors to release HBV components into naïve hepatocytes.

### Exosome Regulates Immune Response in HBV Infection

### *HBV-associated exosomes suppress the immune response*

In general, innate immune systems rapidly recognize and respond to exogenous pathogens. Pattern-recognition receptors (PRRs), including Toll-like receptors (TLRs) and retinoic acid-inducible gene-I (RIG-I), which can recognize viral components and promote the production of interferons (IFNs) by triggering signaling cascades (Takeuchi and Akira 2010). TLRs other than TLR 3 recruit myeloid differentiation factor 88 (MyD88) for induction of type I IFNs, while TLR 3 promotes the production of type I IFNs through a MyD88-independent pathway that requires Toll/ interleukin (IL)-1 receptor (TIR) domain-containing adapter-inducing interferon- $\beta$  (TRIF) (Brown et al. 2011). RIG-I recognizes double-stranded RNA (ds RNA) and induces the production of type-I IFNs and pro-inflammatory cytokines (Yoneyama et al. 2004). It exerts antiviral effects by interacting with the adaptor protein mitochondrial antiviral signaling (MAVS) and activating transcription factors such as IFN regulatory factors 3 (IRF3) and nuclear factor- $\kappa$ B (NF- $\kappa$ B) (Yoneyama et al. 2004; Liu et al. 2015). IFNs can induce HBV DNA degradation by activating the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway (Belloni et al. 2012). In addition to IFNs, many antiviral materials such as protein kinase RNA-activated (PKR), apolipoprotein B messenger RNAediting enzyme catalytic polypeptide-like 3G (APOBEC3G or A3G) and some pro-inflammatory cytokines play a significant role in the immune response (Kim et al. 2018; Ren et al. 2020). Nevertheless, previous studies showed that HBV weakens the efficacy of these components of the DNA-sensing machinery and further disturbs the IFN response (Wu et al. 2009). Additionally, in the context of HBV infection, the levels of some immunosuppressive cytokines such as IL-10 and transforming growth factor- $\beta$ (TGF- $\beta$ ) have been reported to increase (Wu et al. 2010; Sun et al. 2012). These anti-inflammatory cytokines can further impair the production of IFNs by natural killer (NK) cells and dendritic cells (DCs) (Busch and Thimme 2015; Li et al. 2019).

HBV components may induce dysfunctions of immune cells with the assistance of exosomes. It has been reported that exosomes from the serum of CHB patients can mediate HBV transmission into NK cells with the aid of TGF- $\beta$ 



Fig. 3. Exosomes involved in immune regulation during HBV infection.
A. Exosomes suppress the antiviral immune responses. Exosomes mediate dysfunction of immune cells, including NK cells, PD-1-positive T cells and DCs, and inhibit the antiviral effects of HBV-neutralizing antibodies.
B. Exosomes promote the antiviral immune responses. Exosomes can activate macrophages and transfer antiviral materials to hepatocytes to enhance the immune response.
NK cell, natural killer cell; DCs, dendritic cells; IFN, interferon; IL-6, interleukin-6; APOBEC3G, apolipoprotein B

messenger RNA-editing enzyme catalytic polypeptide-like 3G; PD-1, programmed-death protein 1; PD-L1, programmed death-ligand 1; NKG2DL, NKG2D ligands; rc DNA, relaxed circular DNA.

(Yang et al. 2017b). Later, HBV-associated exosomes lead to weakened NK cell proliferation and viability (Yang et al. 2017b). Inhibition of IFN- $\gamma$  production and of the cytolytic activity of NK cells was also observed when the cells were incubated with HBV-associated exosomes (Yang et al. 2017b). Moreover, the researchers found the down-regulation of RIG-I in NK cells as well as decreased levels of NF- $\kappa$ B and p38 mitogen-activated protein kinase (MAPK) both in CHB patients and in co-culture systems of HBVassociated exosomes and primary NK cells from healthy donors (Yang et al. 2017b). In addition, exosomes secreted from HBV-infected cells are endocytosed by monocytes and induce programmed-death ligand-1 (PD-L1) expression, concomitant with the downregulation of CD69 (Kakizaki et al. 2018). PD-L1 is a negative immunomodulatory molecule that suppresses T cells expressing PD-1, and CD69 is a marker of activated immune cells (Keir et al. 2008; Notario et al. 2019). Therefore, depletion and inactivation of T cells in chronic HBV infection may result from increased expression of PD-L1 by monocytes induced by HBV-associated exosomes. These results suggest that HBV-associated exosomes play a role in regulating the quality and quantity of immune cells (Fig. 3A).

Notably, HBV-associated exosomes not only cause dysfunctions of immune cells but also resist the effects of

antiviral substances. Anti-HBs antibody, one of HBVspecific neutralizing antibodies, is derived from B cells, and it targets circulating HBV envelope proteins and thereby inhibits HBV transmission (Rehermann 2003; Neumann-Haefelin and Thimme 2018). However, neutralizing antibodies showed a low response to HBV-DNA in the presence of HBcAg+CD81+ exosomes (Sanada et al. 2017). And similar phenomena were observed in CD81+ hepatitis C virus (HCV)-associated exosomes. As reported previously, exosomes were found to attenuate the antiviral effects of HCV-neutralizing antibodies (Deng et al. 2019), and CD81+ exosomes may play a major role in HCV immune evasion (Ashraf Malik et al. 2019). Therefore, it is presumed that CD81+ exosomes are involved in resistance to antibody neutralization and result in immune evasion of HBV. Moreover, A3G is an antiviral substance that mediates HBV hypermutation, resulting in inhibition of HBV replication (Noguchi et al. 2005). It is reported that HBx protein promotes A3G export from HBx-expressing cells via exosomes and decreases the intracellular concentration of A3G (Chen et al. 2017). Low levels of A3G in cells might weaken the ability of A3G to inhibit HBV replication (Chen et al. 2017). Furthermore, one recent study suggested that IFN in exosomes derived from Huh-7 cells may induce the transfer of transmembrane protein 2 to dendritic cells (DCs), thereby suppressing endogenous IFN- $\alpha$  synthesis and blocking the anti-HBV efficacy of exogenous IFN- $\alpha$  (Shi et al. 2019) (Fig. 3A).

## *HBV-associated exosomes promote antiviral immune response*

In contrast to the foregoing results, several studies have demonstrated that HBV-associated exosomes can also promote immune responses during HBV infection (Fig. 3B). Exosomes released from HBV-infected hepatocytes led to increased expression of NKG2D ligands in macrophages, an effect that may further induce NK cells to produce IFN- $\gamma$  in the early stage of HBV infection (Kouwaki et al. 2016). Interestingly, HBV-miR-3 is an HBV-encoded miRNA that shows anti-HBV effects. This molecule was found in HBV-infected hepatocytes and in the serum exosomes of patients with CHB. In hepatocytes, HBV-miR-3 targets a unique site of the HBV 3.5-kb transcript to specifically reduce the expression of HBV-RI and HBc protein, thereby suppressing HBV virion production (Yang et al. 2017a). Besides, this miRNA can also activate the JAK/ STAT signaling pathway by downregulating SOCS5 (a suppressor of cytokine signaling), thereby enhancing the antiviral effect of IFN (Zhao et al. 2020). In exosomes, HBVmiR-3 can facilitate the M1 polarization of macrophages and increase IL-6 secretion (Zhao et al. 2020). Additionally, Li et al. (2013) reported that IFN- $\alpha$ -treated liver nonparenchymal cells (LNPCs) showed increased expression of antiviral proteins such as A3G, MyD88 and PKR in macrophages and liver sinusoidal endothelial cells (LSECs), which can inhibit HBV infection (Li et al. 2013). More importantly, exosomes from IFN-a-treated LNPCs showed enrichment of IFN- $\alpha$  as well as A3G (Li et al. 2013). These antiviral materials in exosomes can be internalized by hepatocytes and can directly mediate HBV inhibition both in vitro and in vivo (Li et al. 2013). Another study by this group clarified that macrophage-derived exosomes exploit T cell immunoglobulin and mucin receptor 1

(TIM-1) to efficiently deliver IFN- $\alpha$  to hepatocytes (Yao et al. 2018) (Fig. 3B).

Therefore, HBV-associated exosomes recognize recipient cells and deliver biologically active substances to them, resulting in pro- or anti-inflammatory effects. Overall, these host and viral molecules play dual roles in regulating both immune cells and cells in the liver, leading to imbalanced immune system function. Accordingly, HBV-associated exosomes may create a persistent microenvironment that favors HBV survival and replication.

# Roles of exosomal miRNAs and proteins during HBV infection

Exosomes are rich in proteins and miRNAs (Mathivanan and Simpson 2009). They carry these cargoes to recipient cells and perform a variety of biological functions under both physiological and pathological conditions (van Niel et al. 2006). By analyzing these bioactive substances in HBV-associated exosomes, it is possible to develop a further understanding of the chronic infection caused by HBV.

MiRNAs are a class of small, noncoding RNAs approximately 22 nucleotides in length that target mRNAs for translational repression (Bartel 2004; Ha and Kim 2014). Valadi et al. (2007) found that mast cell-derived exosomes contain functional miRNAs that play important roles in genetic communication between cells. Recently, studies of exosomal miRNAs have shown that their profiles are altered in HBV-infected cells, as are their roles in regulating the production of antiviral interleukins and directly mediating HBV replication (Table 2). It is reported that HBV increased the expression levels of miR-21, miR-29a and other immunosuppressive miRNAs in exosomes; these miRNAs can be transferred to THP-1 macrophages to suppress the expression of IL-12 subunits and finally impair antiviral activity (Kouwaki et al. 2016). Similarly, several exosomal miRNAs, such as miR-21, miR-192, miR-215, miR-221, and miR-222, were found to directly target the

Table 2. Functions of Exosomal miRNAs in HBV-infected cells and in patients with CHB.

microRNA	Expression	Functions	Reference
miR-21	Up	Suppression of IL-12 and IL-21 expression	(Kouwaki et al. 2016; Enomoto et al. 2017)
		Potential biomarkers for CHB	(Li et al. 2015)
miR-25	Up	Suppression of HBV DNA and HBsAg levels	(Wu et al. 2020)
miR-29a	Up	Suppression of IL-12 expression	(Kouwaki et al. 2016)
miR-192	Up	Suppression of IL-21, IL-12 and IL-15 expression	(Enomoto et al. 2017)
miR-193a	Up	Suppression of cccDNA and HBsAg levels	(Wu et al. 2020)
miR-215	Up	Suppression of IL-21, IL-12 and IL-15 expression	(Enomoto et al. 2017)
miR-221	Up	Suppression of IL-21 expression	(Enomoto et al. 2017)
miR-222	Up	Suppression of IL-21 expression	(Enomoto et al. 2017)
miR-574	Up	Suppression of HBV DNA, cccDNA, pgRNA, and HBV DNA polymerase levels	(Wu et al. 2020)

IL-12, interleukin-12; IL15, interleukin-15; IL-21, interleukin-21; HBsAg, hepatitis B surface antigen; pgRNA, pregenomic RNA; cccDNA, covalently closed circular DNA; CHB, chronic hepatitis B.

long 3' untranslated region (UTR) of human IL-21 mRNA and repress the expression of IL-21 in human T helper 2 cells (Enomoto et al. 2017). Therefore, it is presumed that some of the miRNAs that are enriched in exosomes can impair the innate immune response by targeting the proinflammatory cytokines degradation in the context of HBV infection. However, a recent study showed that some exosomal miRNAs are increased both in CHB patients who are IFN responders and in THP-1 macrophages (Wu et al. 2020). These IFN- $\alpha$ -related exosomal miRNAs, including miR-25, miR-193 and miR-574, can suppress HBV replication by degrading HBV DNA. Importantly, no change in three IFN-a-related miRNAs was observed in IFN nonresponders (Wu et al. 2020) (Table 2). These results suggest that some exosomal miRNAs may be involved in the induction of abnormal immunity by HBV, while some promote the antiviral effects of IFN- $\alpha$ .

In addition, several studies have shown that exosomal protein profiles are altered in HBV-infected cells. These exosomal proteins are closely related to immune regulation. One study reported that subunit proteins of the proteasome complex can be selectively packaged into exosomes derived from cells in which HBV is replicating and that they enhance the proteasomal activity of recipient cells (Jia et al. 2017). These exosomal proteasome proteins might further modulate the production of cytokines such as IL-6 by recipient monocytes (Jia et al. 2017). In a separate study, the levels of some immunoregulatory proteins, including alpha-2-macroglobulin (A2M) and lactotransferrin (LTF), were found to be significantly decreased in exosomes released from HBV-infected cells (Zhao et al. 2014). Intriguingly, the author found that the level of valosin-containing protein (VCP) was increased both in exosomes secreted from HBVinfected cells and in exosomes obtained from the serum of patients with CHB (Zhao et al. 2014). As previously reported, VCP was necessary for maintaining protein homeostasis and associated with HCC development (Chan et al. 2006; Zhang et al. 2017). Therefore, HBV may induce infected cells to produce exosomes with negative immune regulatory effects that can promote the transformation of hepatocytes into cancer cells (Zhao et al. 2014). Additionally, some exosomal proteins are specific to hepatocytes and are involved in drug detoxification, energy metabolism, and cell proliferation (Zhao et al. 2014). Overall, these exosomal proteins may participate in maintaining the physiological function of the liver (Jia et al. 2017).

In general, most of the increased level of exosomal miRNAs and proteins that have been observed during HBV infection play a role in suppressing immune responses, whereas there is a decrease in the levels of bioactive substances with antiviral effects in the context of HBV infection. This is consistent with the previously observed host immune tolerance caused by HBV infection (Hong and Bertoletti 2017). These bioactive materials are likely induced by HBV. Clarification of how HBV regulates the

expression of biologically active substances in exosomes may contribute to the development of a new anti-HBV treatment.

## **Potential Applications of Exosomes in HBV Infection**

Exosomal miRNAs as new biomarkers for HBV infection

Exosomal miRNAs in the extracellular environment are protected from degradation by exosomes (Köberle et al. 2013). Due to their advantages in terms of stability, quality, quantity and wide distribution in body fluids, miRNAs may become novel diagnostic biomarkers for disease (Nik Mohamed Kamal and Shahidan 2020). The expression levels of exosomal miRNAs in patients with CHB were shown to be correlated with the patients' HBeAg and alanine aminotransferase (ALT) levels (van der Ree et al. 2017). Moreover, some evidence suggests that exosomal miRNAs are more stable and sensitive to reflect liver inflammation activity than ALT (Cheng et al. 2018a). High-throughput sequencing analysis of the expression profiles of plasma exosomal miRNAs of 10 patients with CHB with persistently normal ALT (PNALT) showed that the differential expression of exosomal miRNAs is associated with liver inflammation grade (Metavir inflammation score) (Li et al. 2018). Six exosomal miRNAs (miR-25-3p, miR-221-3p, miR-122-3p, miR-146b-5p, miR-425-5p and miR-148a-3p) were strongly upregulated, and three exosomal miRNAs (miR-372-3p, miR-5585-3p and miR-374c-3p) were significantly downregulated in 5 patients with liver tissue inflammation grades  $\geq$  A2 compared with the remaining 5 patients, who had scores < A2 (Li et al. 2018). Furthermore, ALT cannot accurately reflect liver inflammation, but exosomal miRNAs are valuable in auxiliary diagnosis of liver disease (Bala et al. 2012a). MiR-155 is considered an inflammation-related marker that is upregulated in chronic hepatitis C (CHC) patients (Bala et al. 2012a, b). Serum exosomal miR-192 and miR-30a were significantly increased in patients with alcoholic hepatitis (AH) (Momen-Heravi et al. 2015). In addition, exosomal miRNAs have been found to be related to diseases caused by HBV. Exosomal miR-212 is upregulated in HBV-infection HCC compared to non-HBV-infection HCC (Zhang et al. 2019). At the same time, the expression of exosomal miR-212 was observed to be closely related to HBV-infection cirrhosis with ascites (Zhang et al. 2019). Additionally, serum exosomal miR-21 may represent an optimal biomarker of liver injury. Several studies have reported that miR-21 is enriched in serum exosomes (Wang et al. 2014) and that it is highly expressed in patients with CHB compared to healthy controls (HCs) (Wang et al. 2014; Li et al. 2015). Furthermore, serum exosomal miR-21 may distinguish CHB from HCC. In Wang et al. (2014), exosomal miR-21 was substantially downregulated in patients with CHB compared to HCC patients (30 samples in each group). However, in another study, serum exosomal miR-21 was found highly expressed in patients with CHB compared to HCC patients (18 samples in each group) by using a combination of several endogenous control genes to normalize the expression of serum exosomal miR-21 (Li et al. 2015). These results indicate that exosomal miRNAs may become potential diagnostic and prognostic tools for HBVassociated diseases in the future, but many questions still need to be addressed. First, to accurately compare the levels of exosomal miRNA transcripts, a suitable reference gene is needed as a normalization factor, but there are no recognized endogenous control genes for normalizing the expression of serum miRNAs (Li et al. 2015). Second, there are no unified standards for sample size, isolation protocol or analytical approach (microarray versus RT-qPCR) to exosome isolation and miRNA analysis (Nik Mohamed Kamal and Shahidan 2020). Third, none of the exosomal miRNAs found to date is specific to the auxiliary diagnosis of CHB. For example, miR-21 has potential as a biomarker both of non-HCC tumors and of non-oncologic diseases such as colorectal cancer (Tsukamoto et al. 2017), breast cancer (Hannafon et al. 2016) and type 1 diabetes (Lakhter et al. 2018). Finally, the sources of exosomal miRNAs in serum and plasma are still unclear. Overall, elucidation of the mechanisms underlying the interaction between exosomal miRNAs and HBV-associated diseases would improve our understanding of the pathogenesis of HBV infection.

### Exosomes as novel therapies for HBV infection

At present, medication is an essential but limited treatment for chronic HBV infection (Likhitsup and Lok 2019). NAs focus on inhibiting HBV replication rather than helping the immune system implement an immune strategy against HBV (Fanning et al. 2019). Exosomes can be considered substances that are exogenous to recipient cells and deliver cargoes between cells (Cheng et al. 2018b). These features of exosomes suggest that we can modify exosomes for use in HBV treatment. It has been reported that exosomes derived from stimulated cells can promote immune responses and lead to inflammation (Alexander et al. 2015). Researchers found that exosomes isolated from lipopolysaccharide (LPS)-stimulated THP-1 cells evoke a proinflammatory profile in spleen cells of healthy mice by induction of cytokines (Jesus et al. 2018). Exosomes derived from IFN-a-treated macrophages deliver antiviral proteins and restore the impaired hepatocyte antiviral response (Yao et al. 2019). Additionally, DNA vectors can be utilized to generate engineered immunogenic exosomes that can induce a cytotoxic T lymphocyte (CTL) immune response against HBV antigens (Ferrantelli et al. 2018). Furthermore, the clustered regularly interspaced short palindromic repeats (CRISPR)/associated nuclease (Cas) protein 9 system is a tool that makes it possible to perform efficient modification of specific genes for degradation (Hryhorowicz et al. 2017). The CRISPER/Cas9 system is a novel therapy for cccDNA clearance in CHB patients (Yang and Chen 2018; Kostyushev et al. 2019). Recent studies have shown that exosomes isolated from CRISPR/Cas9

expressing cells contained functional single-stranded guided RNA (gRNA) and Cas9 protein (Chen et al. 2019). These materials can be transferred to surrounding cells and further lead to the destruction of the HBV genome in cells (Chen et al. 2019; Wang et al. 2019). These results provide us with a new perspective on engineering exosomes to activate the host immune response or directly induce HBV repression. In addition, exosomes released from active CD4+ T cells can promote B cell activation and further enhance the efficiency of the HBsAg vaccine (Lu et al. 2019). In a separate study, researchers found that exosomes combined with hepatitis B recombinant antigen induced a humoral immune response similar to the response to HBsAg solution in mice (Jesus et al. 2018). Accordingly, exosomes have efficient antigen presentation functions and can be used as novel vaccines or as adjuvants for inducing antiviral effects.

In recent years, studies have been reported that exosomes can be modified with drugs for cancer therapeutic interventions (Xiao et al. 2017; Wei et al. 2019). Thus, we may exploit exosome-loaded drugs against HBV in the future. Although clinical evidence for exosome therapy in hepatitis B is lacking, exosomes combined with exogenous and endogenous therapeutic biomolecules show promise for potential anti-HBV applications.

### Conclusions

Exosomes, as one type of extracellular vesicle, transfer cargoes between cells and play vital roles in immune regulation (Zhang et al. 2018), virus infection (Ramakrishnaiah et al. 2013) and tumorigenesis (Fu et al. 2019). The present review summarizes the roles of exosomes in the pathogenesis of HBV and shows their potential applications in controlling HBV infection. The budding of exosomes and HBV are extremely similar, and the presence of HBV components in exosomes suggests that HBV can utilize exosomes for its transmission (Meckes and Raab-Traub 2011; van Dongen et al. 2016). However, further research is needed to clarify the mechanism through which HBV enters exosomes. On the one hand, HBV-associated exosomes protect HBV from nuclease degradation and promote the spread of HBV (Kapoor et al. 2017). On the other hand, HBV-associated exosomes are extremely complex independent structures that undergo dynamic changes that promote virus adaptation, resulting in an imbalance of pro- and antiinflammatory effects during the progression of HBV infection. Moreover, multiple bioactive substances such as proteins and miRNAs have been observed in HBV-associated exosomes, the roles of these cargoes need to be fully elucidated during HBV infection. Furthermore, considering the limitations imposed by experimental conditions, whether in vitro experiments actually reflect the in vivo conditions remains to be determined. In conclusion, although reports have shown that exosomes play a vital role in viral infection, research on the roles of exosomes in HBV replication and hepatitis B infection is still in an early stage. A more extensive and in-depth understanding of the interplay of exosomes and HBV is urgently needed.

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### **Author Contributions**

Ju Wang wrote the manuscript and prepared figures and tables; Jiezuan Yang and Dan Cao provided expert comments and edits. All authors have read and agreed to submission of the manuscript.

### **Conflict of Interest**

The authors declare no conflicts of interest.

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