Review

Understanding Hepatitis B Virus (HBV) Persistence: Mechanisms, Consequences and Implications for Cure

Christopher Georgi , Carla S. Coffin , Curtis Cooper *

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Review

Understanding Hepatitis B Virus (HBV) Persistence: Mechanisms, Consequences and Implications for Cure

Running Head: HBV Persistence

Christopher Georgi [1], Carla Osiowy [2] and Curtis Cooper [1,3,*]

[1] Ottawa Hospital Research Institute
[2] University of Manitoba
[3] University of Ottawa- Faculty of Medicine
* Correspondence: ccooper@toh.ca

Abstract: Hepatitis B Virus (HBV) infection carries a significant health burden worldwide. It is a leading cause of acute and chronic liver disease resulting in cirrhosis and hepatocellular carcinoma (HCC). This virus affects millions globally, and despite several medical advancements, research, and efforts to develop curative treatment, the persistence of HBV in infected individuals by cccDNA and integration pose formidable challenges. This review aims to provide a broad-based overview of HBV persistence, integration and the implications for chronic infection and tumorigenesis. By considering these points, we bring forth insights into the advancement of therapies that aim to disrupt the chronicity of HBV infection and improve clinical outcomes.

Keywords: Hepatitis B infection; cccDNA; HBV DNA integration; viral persistence; chronic infection; Hepatocellular Carcinoma

1. Introduction

1.1. Global Health Impact

HBV is a double-stranded DNA virus member of the *Hepadnaviridae* family of viruses.[1] The virus can produce a potentially life-threatening liver infection with both an acute and chronic phase. HBV is transmitted from person to person through blood, semen, and other body fluids. HBV can be horizontally transmitted by needle stick injuries, needle sharing practices, and sexually.[2] HBV can also be transmitted vertically during childbirth resulting in perinatal infection. The World Health Organization reported in 2022 that 254 million people were living with chronic hepatitis B, with 1.2 million new infections occurring annually.[3] The Western Pacific and African regions have the highest prevalence of HBV, with 97 million and 65 million people respectively living with chronic infection. The factors contributing to its high prevalence in these regions include limited access to healthcare services and vaccination coverage as well as suboptimal prevention and control measures. Signs and symptoms that can occur with acute HBV infection include jaundice, fatigue, right upper quadrant abdominal discomfort, pale stools and dark urine which can persist for up to six months. For some patients, HBV establishes chronic infection. HBV caused approximately 1.1 million deaths annually, primarily due to cirrhosis and hepatocellular carcinoma (HCC). HCC related to HBV is the third leading cause of cancer-related death globally in 2020.[4]

1.2. HBV Persistence

HBV is composed of a relaxed circular partially double stranded DNA genome (rcDNA) containing four coding regions.[5, 6] When this virus enters the nucleus of hepatocytes via the sodium-taurocholate-cotransporting polypeptide, a bile acid receptor, the rcDNA is repaired to yield

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a covalently closed circular DNA (cccDNA).[6] Located in the nucleus of the hepatocytes, cccDNA serves as the primary transcriptional template for all viral mRNAs including the pre-genomic RNA (pgRNA) and mRNAs encoding viral proteins.[6, 7] Within the cytoplasm, the viral core protein undergoes self-assembly together with the pgRNA and viral polymerase, forming nucleocapsids.[8] In these nucleocapsids, viral replication takes place through the reverse transcription of the pgRNA. Subsequently, mature viral capsids, now housing rcDNA, are either enveloped with hepatitis B surface antigens (large, middle and small HBsAg) and released from infected hepatocytes through the multivesicular body pathway or directed to the nucleus to sustain the cccDNA pool.[5, 8] Large amounts of sub-viral particles consisting of HBsAg are released from infected hepatocytes which negatively impact HBV-specific immunity.[5] cccDNA originates from both incoming virions and the intracellular recycling of nucleocapsids.[7] This dual origin combined with its long half-life lasting many years, explains why cccDNA concentrations experience minimal decline even after extended treatment with nucleos(t)ide analogues (NA).

The establishment of chronic HBV infection is characterized by the formation of the cccDNA pool in the nucleus of the infected hepatocytes.[9] This establishes a chronic, persistent viral reservoir which is less vulnerable to host immune system strategies attempting to clear infection, leading to a state of chronic infection.[9, 10] The cccDNA serves as a template for viral RNA transcription and viral replication. This ensures the continued production of viral proteins which facilitates chronic infection. In addition, HBV utilizes the reproductive machinery of the infected host cell to replicate thus ensuring a continued presence of viral DNA in daughter cells.[11] Therefore, permanent elimination or silencing of cccDNA is crucial to prevent risk of viral reactivation and makes cccDNA a longstanding challenge to achieving a cure for HBV.[7] However, cccDNA is not the only obstacle to HBV elimination. Targeting integrated HBV DNA in addition to cccDNA may be necessary. Even if not replication competent, integrated HBV is also a source of HBsAg production. Furthermore, HBV DNA integration plays a key role in the development of liver disease and hepatocellular carcinoma.[12] In this review, we aim to discuss the mechanisms, consequences, detection and targeting of both cccDNA and HBV integration.

2. HBV DNA Integration Mechanisms

2.1. Process of HBV DNA Integration in the Host Genome

HBV DNA integration is a complex process influenced by viral and host factors. As discussed, the cccDNA pool serves as the transcriptional template for viral mRNAs and pgRNA. pgRNA is then subsequently reverse transcribed within the newly formed nucleocapsids by a viral polymerase enzyme to yield capsids containing rcDNA. Occasionally, during genome replication, faulty primer translocation to newly synthesized minus-strand DNA results in the production of double-stranded linear DNA (dslDNA) genomes instead of rcDNA genomes, the latter which can then be integrated into the host genome.[8, 13] Recent targeted long-read sequencing has found that while full-length genomes may integrate, this integrated DNA cannot support viral replication, as it lacks precore and core transcripts.[8] Despite a poor understanding of how viral factors affect HBV DNA integration into the host, it is suspected that viral proteins including the HBV polymerase, HBV core and HBV X proteins contribute to the integration process due to their DNA binding activities.[12] The HBV polymerase plays a crucial role in viral reverse transcription and replication.[13] This enzyme converts pgRNA into linear DNA intermediates that are suitable for integration. Furthermore, the HBV X protein enhances HBV replication by stimulating gene expression from the cccDNA template. This protein interacts with host cellular factors involved in DNA repair pathways, such as the DDB1 (Damaged DNA Binding protein 1), promoting the integration of viral DNA into the host cellular genome. Various host factors play keys roles in mediating HBV DNA integration. HBV utilizes host DNA repair machinery to facilitate the integration process. As an example, the virus manipulates the non-homologous end-joining (NHEJ) pathway. Specific components of the NHEJ pathway, which is responsible for repairing double-stranded DNA breaks, such as the Ku70/Ku80 and XRCC4 proteins, are involved in ligating the viral DNA into the host cellular

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genome.[14] Studies indicate that the majority of virus-cell junctions exhibit minimal or no sequence homology with cellular DNA, suggesting they likely utilize the classical NHEJ pathway.[15] Additionally, chromatin structure influences HBV DNA integration, as it interacts with the entire host genome, showing a preference for active chromatin regions.[16] These active chromatin regions can be detected via active histone marks such as H3K4me3, H3K4me2, and H3K27ac.

2.2. Genomic Sites Favored for Integration

In addition to active chromatin regions, the virus preferentially exploits other fragile sites in the genome. For example, sites in the genome that contain double-strand DNA breaks are susceptible to the HBV DNA integration process.[5] Cytosine phosphoguanine (CpG) islands, characterized by a high density of CpG dinucleotide repeats, are favored sites for HBV DNA integration into the cellular genome of hepatocytes.[17, 18] The dinucleotide repeats that make up these CpG islands make them susceptible to viral integration. Telomeres are regions of repetitive DNA sequences at the end of a chromosome responsible for maintaining the genomic stability of the chromosomes and protecting them from degradation and fusion events.[15, 19] Telomeres serve as sites in which HBV DNA integration occurs. During chronic liver injury, there is increased cell turnover, which accelerates telomere shortening, leading to chromosomal instability and cell senescence or apoptosis, which can drive carcinogenesis.[15] HBV integration events have been reported to be enriched in the proximity of telomeres in HCC DNA compared to paired non-tumor tissue. Moreover, human repetitive regions such as long interspersed nuclear elements (LINE), short interspersed nuclear elements (SINE) and simple repeats (microsatellites) are favored sites for HBV DNA integration. HBV DNA integration can lead to deleterious consequences for the host cell function including viral persistence, oncogenesis, altered host signaling pathways and altered gene expression.

2.3. Knowledge Gaps Related to HBV DNA Integration

Despite multiple advancements in our knowledge of HBV many areas require further understanding. Adequate small animal and cell culture models that accurately replicate HBV infection are required.[15] For instance, the mechanism and timing of HBV DNA integration during infection are unclear; whether integration is an early event, continues throughout chronic infection or reaches a stable equilibrium remains to be clarified. Answers to these questions are crucial as they will inform the development of therapies targeting HBV integration events. Moreover, the specific cellular pathways involved in HBV DNA integration remain unidentified, along with the role of other cellular proteins that may be pivotal in this process. There is a lack of clarity regarding viral factors influencing integration such as the potential involvement of viral proteins in the integration process. Addressing these knowledge gaps is key to advancing our understanding of HBV pathogenesis and developing effective therapeutic strategies.

3. HBV Infection Persistence

3.1. HBV DNA Integration Contributes to Persistent Viral Antigen and Host Immune Escape

The formation of the cccDNA pool establishes persistent viral infection with continuous production of viral proteins. Although integrated HBV DNA can result in persistent HBsAg expression, integration of HBV DNA itself is not replicable due to the structure of the integrated dslDNA. However, integrated HBV DNA typically retains full length S gene and transcripts.[7] Integrated viral DNA is, generally, not a source of the viral polymerase, HBeAg, and HBsAg since the integration event separates the promoter regions of these viral proteins from the coding region.[20] In contrast, HBV integrations usually retain all the viral promoter and coding regions needed for HbsAg-encoding transcripts. However, due to the nature of the integration, these transcripts bypass the polyadenylation (polyA) site), resulting in hybrid mRNAs that incorporate host genomic sequences. Therefore, since the polyadenylation signal is required for the appropriate production of HBsAg, HBV DNA integrations are less capable of producing HBsAg compared to cccDNA. Despite this, integrated DNA can still serve as a source of HBsAg expression.[7] Evidence

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indicates that in HBeAg-negative patients, integrated HBV DNA is the main source of HBsAg, whereas in HBeAg-positive patients, cccDNA serves as the primary source. With viral integration, the omnipresence of HBV in the host genome enables escape from recognition by innate immunity.[21] Several strategies are utilized by the virus. HBV replication occurs using a transcriptional template that resembles host chromatin structure. This process produces new virus particles, which are protected by viral capsids. This protection occurs since human hepatocytes lack or have inactive DNA sensing pathways. HBV evades the host immune system by promoting a defective antiviral adaptive immune response. Despite effective T cell responses in acutely infected HBV patients, the CD4 and CD8 T cell responses in chronically infected HBV patients are suboptimal. With chronic infection, anti-HBs levels are generally undetectable. Furthermore, the HBV DNA polymerase can inhibit the translocation of the Poly-ADP Ribose Polymerase 1 (PARP1), an enzyme involved in the repair of chromosomal DNA.[21, 22] The inhibition of this enzyme results in the upregulation of the expression of PD-L1 in tumor cells at the transcriptional level .[21] The upregulation of this ligand results in increased interaction with the PD-1 on the surface of T cells. This interaction leads to T cell inhibition. This immunosuppression leads to disease progression and promotes the development of HCC. HBV can inactivate innate immune system components, such as dendritic cells, natural killer cells, and natural killer T-like cells.[23] By affecting the innate immune system, this compromises adaptive immunity quality, hence ensuring a blunted immune response.

3.2. Role of cccDNA in Viral Latency and Reactivation

Establishment and replenishment of cccDNA contributes to viral latency. Approximately 300 million people are inactive carriers (i.e. hepatitis B core antibody positive and HBsAg negative).[24] Latent HBV can reactivate. Immunosuppressive therapies such as B or T cell depleting agents, biologics, cancer chemotherapeutic agents, corticosteroids, traditional immunosuppressants and emerging novel cell therapy can cause immune dysfunctions and the suppression of anti-HBV immunity.[25] For example, Rituximab, an anti-CD20 monoclonal antibody used in treating autoimmune diseases and lymphomas, binds the CD20 molecule on the surface of B cells resulting in the depletion of B cells. Because it depletes B cells, this class of medication can reduce anti-HBsAg levels with resulting re-emergence of HBsAg and HBV DNA as well as liver enzyme increase with or without clinical signs and symptoms of hepatitis .[26, 27] Immunosuppressants such as corticosteroids can induce direct activation of regulatory elements within HBV genes thereby increasing HBV replication and reactivation.[25] Everolimus, a mTOR inhibitor used to prevent organ transplant rejection and to treat several types of malignancies, can stimulate HBV replication with synthesis of HBsAg which is normally inhibited by mTOR.[28] The phenomenon of viral latency and reactivation due to cccDNA explains how achieving permanent elimination or silencing of cccDNA is crucial to prevent the risk of HBV reactivation, as cccDNA remains a major obstacle in the quest to cure HBV.[7] HBV latency and the phenomenon of reactivation raise important concerns as to the feasibility of HBV elimination strategies (see Clinical Implications Section).

4. Molecular Pathways and Cellular Consequences

4.1. Impact of HBV DNA Integration on Host Signaling Pathways

HBV DNA integration into the host genome can disrupt host signaling pathways. For instance, the X gene, a HBV DNA sequence integrated into the host, can directly activate oncogenes (such as Myc, Ras, Src, and CyclinD1), thus leading to uncontrolled cell growth.[29] The X gene can inhibit the expression of tumor suppressor genes (such as P53 and Rb) hence leading to cellular transformation and oncogenesis. These events contribute to the development of HCC. Moreover, the integrated viral DNA into the host genome can modulate signaling pathways involved in immune recognition and response. Due to its nuclear localization, HBV can escape recognition by the innate and adaptive immune system and thus suppress pro-inflammatory responses during acute HBV infection.[30]

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HBV DNA integration into the host genome contributes to oncogenesis through various mechanisms. Firstly, the inactivation of tumor suppressor genes and the activation of oncogenes can drive cellular transformation and tumor development. In addition, HBV DNA can target actively transcribed chromosomal regions within genes or in the immediate vicinity for integration.[31] During integration, HBV targets gene families involved in cell survival, proliferation and immortalization such as hTERT (telomerase reverse transcriptase) and the PDGF receptor. Hepatocarcinogenesis is frequently associated with the insertion of HBV into the retinoic acid receptor b (RAR-b) and the cyclin A2 genes [30]. Viral integration can cause insertional mutagenesis which can lead to tumorigenesis and HCC.[32] HBV DNA integration can also promote cell transformation by insertional mutagenesis in the host genome.[33] This alteration of the host genome due to viral integration, or "cis-effect", can potentially disrupt host gene function or alter host gene regulation.[31] Insertional mutagenesis is one of the most important mechanisms for the development of HBV-related HCC.[34] HBV DNA integration can also lead to complex structural rearrangements such as gains of chr5p (including *TERT* locus) and chr8q (including *MYC* locus) and losses of chr17p (including *TP53* locus).[33] These structural rearrangements can promote tumorigenesis.

4.2. Mechanisms Underlying HBV-Related Hepatocellular Carcinoma

The HBV viral protein (HBx protein) is vital in HBV-related HCC.[35, 36] In vitro studies have demonstrated that prolonged expression of HBx induces cellular transformation of rodent hepatocytes, while the presence of integrated HBx gene in the chromosomal DNA has been frequently detected in patients diagnosed with HCC.[36] HBx can modulate various hallmarks of HCC. This protein can sustain proliferative signaling via the 5-LOX/FAS mediated positive feedback loop mechanism. HBx protein upregulates the transcription of fatty acid synthase which plays an essential role in tumor cell survival and proliferation. HBx promotes liver cell division by upregulating cyclo-oxygense-2 (COX-2). Moreover, HBx is implicated in resisting cell death. The anti-apoptotic effect of HBx can elevate the expression of many inhibitors of apoptosis including the myeloid cell-leukemia 1 (Mcl-1) and the B cell lymphoma 2 (Bcl-2) that inhibit Caspase 3 and 9. HBx interacts with the apoptosis-inducing factor (AIF) and AIF-homologue mitochondria-associated inducer of death (AMID) thereby inhibiting the translocation of AIF from the mitochondria to the nucleus. HBx enables replicative immortality by maintaining telomere length. Several studies suggest that HBx activates hTERT mRNA expression in hepatocarcinoma and cholangiocarcinoma cells.[29, 36] HBx is involved in initiating angiogenesis by upregulation of VEGF mRNA expression.[36, 37] This viral protein can also induce HIF-1α-related angiogenesis by upregulating metastasis-associated protein 1 (MTA1), a critical gene involved in tumor invasion and metastasis, as well as histone deacetylase (HDAC1).[35, 36] In addition, HBx is implicated in triggering invasion and metastasis. This viral protein is associated with remodeling cell migration, invasion, and extracellular matrix as it upregulates membrane-type matrix metalloproteinases (MMPs).[36, 37] In HCC, the aggressive metastatic behavior of cells is partly induced by HBx. This viral protein disrupts adherence junctions and integrin-associated adhesion to the extracellular matrix, while also promoting the production of MMPs 1, 3 and 9.[36] Integrated viral DNA can evade innate and adaptive immune responses hence escaping elimination by the immune system. Moreover, the HBx protein is implicated in promoting tumor inflammation. For example, elevated levels of HBx in human hepatocytes induce serine/threonine kinases, notably receptor-interacting protein (RIP-1), which facilitates the production of pro-inflammatory cytokines IL-6, IL-8 and CXCL2. Additionally, it leads to the secretion of HMGB1, a cytokine mediator of inflammation. HBx induces genetic instability and defects in chromosomal segregation partly by binding to the HBx-interacting protein (HBXIP) and promoting aneuploidy. HBx drives aneuploidy by disrupting the cell growth stimulating function of HBXIP leading to failed centrosome duplication during prometaphase. HBV deregulates cellular energetics and alter energy metabolism. HBx induces neoplastic traits by downregulating thioredoxin-interacting protein (TXNIP), a crucial regulator of glucose sensing and the reduction–oxidation system.[29, 36] This alteration drives reprogramming of glucose metabolism.[36]

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5. Diagnostic Detection

5.1. Diagnostic Methods for Detecting cccDNA and Integrated HBV DNA

To better understand the pathophysiology of HBV and develop therapeutic strategies targeting cccDNA and integrated HBV, it is critical that these targets and their products can be identified, quantified and discerned from one another. Southern blotting can detect HBV from cccDNA.[38] This technique involves heat digestion and/or restriction endonuclease digestion of cellular DNA to isolate HBV DNA sequence. Gel electrophoresis is then performed, separating the DNA fragments based on their electrophoretic mobility. Competitive PCR (cPCR), another technique used to detect cccDNA involves two templates: a known quantity template and an unknown quantity target template. These templates compete using cccDNA-specific primers in PCR with different product lengths that can be quantified. The equivalence point, where competitor and target quantities are equal, is determined by PCR followed by Southern transfer and analysis using an imaging software. This maximizes the sensitivity compared to direct Southern blotting. Droplet-Digital PCR (ddPCR) can detect and quantify trace amounts of cccDNA which persist in the nucleus of infected hepatocytes post antiviral therapy. Here, a droplet displaying a fluorescent signal indicates the presence of the target sequence (positive result), while the absence of fluorescence indicates a negative event.

Multiple methods have been developed to detect integrated HBV DNA. PCR increases the amount of target DNA making it a suitable method to detect HBV integration from samples that are obtained in small quantity.[39] PCR strategies used for HBV detection include Alu-PCR and inverse-nested PCR (*inv*-PCR). Alu-PCR is a DNA fingerprinting method intended to target Alu elements. These elements have over 1 million copies spread throughout the human genome which is separated into small regions that can be detected using PCR techniques. Inverse PCR is an alternative method utilized for amplifying the unidentified cellular DNA neighboring an integrated sequence. This method utilizes restriction enzymes to cleave and circularize DNA fragments which are then self-ligated and amplified using specific outward-facing primers designated based on the HBV sequence. Next-Generation-Sequencing (NGS) technologies enable rapid sequencing of large amounts of DNA or RNA and are also employed for detecting HBV DNA integration.[39, 40] For example, capture probe NGS is a method used to detect HBV DNA integration sites within the host.[41] Whole Genome Sequencing (WGS) is another technology used for viral integration detection. For this, short-read WGS and long-read WGS are applied. Short-read sequencing allows for more rapid and more cost-effective sequencing of DNA and RNA compared to traditional methods.[42] Short-read WGS techniques such as Illumina, and ThermoFisher Scientific reveal the transcriptional activity and location of HBV integrations.[41, 43] Long-read WGS can produce reads that are much longer, ranging from a few thousand to hundreds of thousands of bases.[42] Long-read WGS methods including PacBio, and Oxford Nanopore are capable of revealing the entire architecture of integrated HBV DNA.[43] However, the high cost of performing population-scale studies with WGS impacts clinical utilization.

Distinguishing between HBV DNA derived from integration sites and cccDNA poses challenges in HBV research.[43] The distinction is crucial to establish a full understanding of viral activity, potentially optimizing treatment and achieving a cure. HBV DNA from cccDNA resides in the nucleus of the hepatocytes and serves as a template for viral transcription and replication. HBV DNA from integration sites is difficult to detect due to variability in integration sites. The two main long-read sequencing techniques developed for this distinction include targeted PacBio and targeted Iso-Seq. These methods allow for detailed resolution of HBV RNA isoforms, distinguishing between transcriptions from cccDNA and those from integrations. The HBV dslDNA, which lacks the DR1-PAS region (the area between direct repeat 1 (DR1) and the polyadenylation signal (PAS)), is suspected to be the precursor for HBV integration. The DR1 PAS region is essential for polyadenylating viral transcripts which is vital for the proper processing and stability of HBV mRNA, and consequently, the production of viral proteins including surface antigens. Given that most HBV DNA integrations occur at the DR1 locus, hence disrupting the DR1-PAS region, these transcripts bypass polyadenylation at the standard HBV PAS site (1935 nt) indicating deficient transcriptional

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activity at this usual site. Consequently, these integrated HBV transcripts exhibit a significant drop in RNA coverage within the DR1-PAS region and show distinct peak breakpoints of chimeric transcripts around DR1.[44] As the four principle cccDNA transcripts all employ a shared 3' polyadenylation signal, the DR1-PAS region mainly represents the transcriptional output of cccDNA. Thus, any transcriptional activity associated with the DR1 PAS region originates primarily from the cccDNA template.[45] Proper function of this region is essential for yielding full length HBV RNAs including those translated to HBsAg. Therefore, by analyzing and distinguishing transcriptions from cccDNA and those from integrations, we can confirm the status of active viral replication in both cccDNA and integrations as well as the primary source of HBsAg in infected patients. This ability is critical for evaluating the residual risks of HBV infection progression, HCC development, as well as viral relapse following HBsAg clearance.[44]

6. Clinical Implications

6.1. Challenges of Targeting cccDNA and Integrated HBV DNA for Therapeutic Intervention

Targeting cccDNA and integrated HBV DNA for therapeutic intervention brings with it multiple challenges. The stability and persistence of integrated HBV DNA in the hepatocytes of infected individuals is a key obstacle. Achieving specificity for viral targets and ensuring efficient and safe delivery of gene editing agents to eliminate integration sites and cccDNA pool from all infected hepatocytes are primary challenges.[46] The wide diversity of HBV genotypes and subtypes (specifically 10 genotypes, identified by the letters A to J) further complicates efforts towards their elimination as antiviral and immune targets are heterogeneous in many cases.[47] The randomness of integrated HBV DNA into the host creates challenges to develop therapies that can target the viral DNA at a multitude of insertion sites. Current therapies based on nucleos(t)ide analogues (NA) and pegylated interferon alpha (IFN) can suppress HBV replication and reduce the risk of complications including cirrhosis and HCC but HBV is not completely eliminated.[48] IFN therapy is capable of regulating the transcription of cccDNA. Treatment with IFN therapy increases expression of the Interferon Induced Protein with Tetratricopeptide Repeats 1 (IFIT1) gene resulting in reduced levels of K4 methylation, K27 acetylation and K122 acetylation on histone H3.[49] These modifications inhibit the transcription of cccDNA. Additionally, epigenetic modifications induced by IFN on cccDNA can lead to the repression of HBV. The transcriptional activity of cccDNA is sustained by histone H3K79 succinylation on the minichromosome. IFN-alpha inhibits the histone acetyltransferase General Control Non-repressed 5 (GCN5) which possesses succinyltransferase activity. This inhibition reduces the succinylation of H3K79 leading to cccDNA clearance. As mentioned, IFN therapy can inhibit HBV replication, thus reducing its levels, potentially at integration sites, and reducing the number of integration events within the host genome.[50] Unfortunately, the use of IFN therapy is limited due to its unfavorable tolerability profile.[48] Moreover, IFN therapy has a relatively low success rate and requires long-duration use which can be challenging for patients; especially in hard-to-reach populations and resource-limited settings where cost and availability of existing antiviral therapies are key barriers to delivering healthcare.[3, 51]

6.2. Emerging Treatment Strategies Aimed at Eradicating cccDNA and Integrated HBV DNA May Lead to Functional Cure in Chronic HBV Infection

Combination therapies are currently considered to represent the future of chronic hepatitis B treatment and cure, potentially involving nucleos(t)ides, other antiviral targeting agents and immune modulating agents.[52] Currently, two distinct therapeutic approaches have been approved for treating CHB patients.[53] IFN-alpha, discussed in the previous section, are multifaceted cytokines categorized under type I IFNs employed in treating patients with certain cancers and viral diseases.[54] Nucleos(t)ide analogs (NA) including tenofovir, entecavir and lamivudine function by inhibiting HBV DNA polymerase activity thereby suppressing viral replication.[55] Commencing NA treatment early may represent a strategy to reduce integration events and HCC risk. There are indications that early viral suppression could improve clinical outcomes.[56] The REVEAL study, a

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long-term prospective cohort study conducted in Taiwan, highlighted the correlation between detectable HBV viral load and long-term HCC risk. Furthermore, HCC development in the context of chronic hepatitis B may begin during the immune-tolerant phase of infection. Introducing NA therapy early may represent a sensible strategy for reducing HCC risk by limiting viral integration into the host cell genome as well as by inhibiting liver fibrosis progression. Diminishing replenishment of cccDNA pools with sustained NA treatment may also contribute to strategies aiming to achieve functional cure.

Multiple other viral and host targeting molecules are in preclinical and clinical development. RNA interference therapies have demonstrated promise by effectively targeting viral replication [48]. This therapy employs small interfering RNA (siRNA) to target viral RNA, effectively inhibiting HBV replication.[57] Antisense Oligonucleotides (ASO) are also tailored to degrade HBV mRNA using the cellular RNase H pathway and can produce HBsAg reductions sufficient to achieve functional cure.[55, 58] Bepirovirsen (GSK3228836), an ASO targeting all HBV mRNAs including HBV mRNA and pgRNA, decreases the level of viral proteins.[59] In a phase 2b clinical trial, Bepirovirsen dosed at 300 mg per week over 24 weeks led to sustained loss of HBsAg and HBV DNA in 9% of individuals with chronic infection. CRISPR/Cas9 technology shows promise in HBV elimination strategies.[60] This modality possesses the ability to reduce viral DNA and cccDNA levels in cell cultures and animal models through targeted viral gene editing. CRISPR-Cas9 can reduce HBV cccDNA pool replenishment by hindering viral replication.[61] Ramanan et al.[62] and Wang et al.[63] utilized CRISPR-Cas9 and RNA interference (RNAi) methods, respectively, to cleave viral DNA and suppress viral replication.[61] In addition to HBV cccDNA, CRISPR-Cas9 technology can also target integrated HBV DNA.[61, 64] Although the CRISPR-Cas9 system is promising, there are challenges and concerns with this strategy. The use of Cas9 to cleave integrated HBV DNA can induce harmful mutations in host DNA, potentially driving genome instability and carcinogenesis.[64] The large size of CRISPR-Cas9 genes complicates their delivery for therapeutic applications in vivo as well.

Drugs targeting specific epigenetic modifications established by viral integration, such as Histone Deacetylase (HDAC) inhibitors, could reverse abnormal epigenetic changes. For example, suberoylanilide hydroxamic acid (SAHA), a HDAC inhibitor, was identified as a chemopreventive agent for patients predisposed to developing HCC.[65]

Immunomodulatory therapies including Toll-like receptor (TLR) agonists, immune checkpoint inhibitors, therapeutic vaccines, and engineered T cells are currently under investigation.[66] Stimulation of TLR7 in plasmacytoid dendritic cells (pDCs) leads to heightened production of IFN and other cytokines, activating natural killer cells and cytotoxic T cells. Immune checkpoint inhibitors aim to reverse T-cell exhaustion, thus enhancing immune responses against HBV-infected cells.

7. Conclusion

The establishment of a cccDNA pool and HBV DNA integration contribute to a persistent formation of viral proteins leading to chronic infection and negative liver-specific outcomes. Viral persistence in the host genome allows the virus to escape immune system recognition thereby contributing to disease progression. HBV DNA integration increases the risk of HCC and represents a site refractory to NA targeting therapies. Detecting and distinguishing between cccDNA and integrated HBV DNA viral products remains a challenge. Currently, DAA and IFN are used to decrease HBV viral load, decrease the risk of liver disease and cancer, and improve clinical outcomes. However, cccDNA and integrated HBV DNA mandate the development of new strategies intended to achieve function cure. Future research efforts dedicated to fully understanding the mechanisms surrounding cccDNA and HBV DNA integration will inform the development of novel curative therapeutic approaches.

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