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Gene Editing with CRISPR-Cas9 Technology for Hepatitis B Virus: A Literature Review

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ABSTRACT

Hepatitis B Virus (HBV) is a global problem with a significant impact on morbidity and mortality rates that are expected to continue to increase if no further treatment is carried out. HBV is caused by the Hepatitis virus type B, which until now has no medical therapy that can eliminate HBV. The latest developments referring to gene editing for genetic and non-genetic diseases are clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9. CRISPR-Cas9 appears with a promising approach with solutions to various disease disorders. CRISPR-Cas9 with DNA editing by Cas9 endonuclease, which is guided by the RNA sequence is able to pair with the target base sequence. The purpose of this paper is to examine the effectiveness and potential of the gene editing method with CRISPR-Cas9 as a therapy for Hepatitis B disease. The writing method is a Literature Review with databases obtained through Google Scholar, PubMed, and ScienceDirect. Various studies and methods have been developed to improve the success of CRISPR-Cas9. Off-target effects are detected to reduce the risk of CRISPR-Cas9. The results show that CRISPR-Cas9 can inhibit HBV replication in vitro and in vivo. Therefore, the technology shows much-needed potential for eradicating the hepatitis B virus. However, further studies are needed to determine its safety and effectiveness as a hepatitis B therapy.

Key words: *Hepatitis B; CRISPR-Cas9; cccDNA; Gene Editing*

INTRODUCTION

The transmission of diseases caused by viruses is one of the global problems affecting human health (Usmar et al., 2021; Alberts et al., 2022; Howell et al., 2023). Hepatitis B virus (HBV) causes hepatitis type B disease. HBV infection contributes to significant health care (Veracruz et al., 2022). This viral infection is the leading cause of liver cancer, which has a significant impact on morbidity and mortality worldwide (Campbell et al., 2021; Hu et al., 2022). In 2015, an estimated 887,220 people died from HBV infection, consisting of 87,076 from acute hepatitis, 462,690 from cirrhosis, and 337,454 from HCC. In 2016, 27 million people were infected with HBV, with 10.5% of those infected with hepatitis B aware of their infection, while only 4.5 million (16.7%) of those diagnosed were undergoing treatment (Pattyn et al., 2021). The death rate from HBV is expected to increase by 39% from 2015 to 2030 if no further action is taken (Hsu et al., 2023; Zhang et al., 2022).

Medical therapy that can eradicate the hepatitis B virus is not yet available. Therapy is carried out over the long term to suppress viral replication and development (Putri NAS et al., 2022). Interferon was the initial therapy for hepatitis B, followed by nucleoside analogues (NA). Subsequently, adefovir, entecavir, adefovir, Peg-IFN, adefovir, and tenofovir were approved for use as hepatitis B virus therapies (Kurniawan, 2021). Hepatitis B virus therapy cannot eliminate hepatitis B surface antigen (HBsAg). Therefore, new therapies targeting HBsAg that are no longer detectable after therapy is discontinued, becoming the goal of ongoing research (Lim et al., 2023). RNA-guided gene editing technology, known as clustered regularly interspaced short palindromic repeats or CRISPR-associated nuclease 9 (CRISPR-Cas9), has been discovered (Noor et al., 2020). This approach can be used to treat viral infections (Usmar et al., 2021). The development of this technology for the destruction of HBV is expected to be a solution in the future (Noor et al., 2020; Ansori et al., 2023).

Many bacteria and archaea have developed adaptive immune systems guided by RNA and encoded by CRISPR loci. CRISPR/Cas-related genes provide immunity acquired from bacteriophage infection and plasmid transfer. CRISPR–Cas9 is a technology capable of manipulating the genomes of various organisms due to its simple design, ease of use, and high efficiency (Jiang & Doudna, 2017).

CRISPR-Cas9 technology with DNA editing by Cas9 endonuclease, guided by RNA sequences capable of pairing with target base sequences (Usmar et al., 2021; Rasheed et al., 2021). This technique has successfully suppressed viruses and cccDNA in cells through viral gene editing (Cai et al., 2023). The persistence of cccDNA viruses is a major challenge in achieving HBV cure. cccDNA is a molecular reservoir of HBV and serves as the sole template for transcription of all viral RNAs, including pregenomic RNA (pgRNA) (Martinez et al., 2021). Similarly, cccDNA is a major component of the HBV life cycle, serving as the transcription template for all HBV transcripts (He et al., 2024).

CRISPR-Cas9 technology is a revolutionary breakthrough in genetic engineering. This technology shows great promise in improving the treatment of diseases caused by viral infections and genetic disorders due to its simple design and high success rate (Zhuo et al., 2021). Using this technology, researchers are able to visualize and eliminate MRE RNA in human cells (Lv et al., 2021; Batra et al., 2017).

The purpose of this paper is to examine the effectiveness and potential of CRISPR-Cas9 gene editing as a therapy for hepatitis B. CRISPR-Cas9 gene editing has exceptional target specificity. Although this type of technology is developing rapidly, it is still in the clinical trial phase, due to various safety concerns (Feng et al., 2024). However, one of the challenges in this field is off-target effects (Singh et al., 2024; Singh et al., 2018). Various studies have been conducted to detect and measure off-target effects caused by CRISPR (Yan et al., 2021; Manghwar et al., 2020). This technology continues to be engineered to reduce off-target effects and improve efficiency for treatment (Pausch et al., 2020; Zhang et al., 2020).

METHODS

This study used the Literature Review method by identifying literature on the use of CRISPR-Cas9 technology with gene modification for the treatment of Hepatitis B and the increase in HBV replication after CRISPR-Cas9 treatment. A total of 10 articles were selected and further analyzed to answer the research objectives. The databases used in this study were found through Google Scholar, PubMed, and ScienceDirect using relevant keywords, namely Hepatitis B, CRISPR/Cas9 technique, and genome editing. The inclusion criteria for this study were articles published in the last 5 years, articles in English, and articles that were fully accessible (full paper). Excluded articles are those that are not available in their entirety and those that are duplicates. Articles that met the criteria were then analyzed based on their relevance to the research topic. The data obtained were presented in the form of tables and narratives.

RESULTS AND DISCUSSION

The permanent loss of HBsAg along with the undetectable HBV DNA is the goal of anti-HBV therapy. With the current treatment options available, this therapeutic goal is still rarely achieved (Salpini et al., 2022). However, the CRISPR-Cas9 technology developed by researchers worldwide holds great potential (**Table 1**), particularly in

addressing infectious diseases (Christian et al., 2024). CRISPR-Cas9 technology has opened new avenues in the treatment of infectious diseases (Sari & Kasasiah, 2024).

Table 1. CRISPR-Cas9 Technology Gene Editing for Hepatitis B Virus

No	Author	Article Title	Method	Results
1	Stone et al., 2021	CRISPR-Cas9 gene editing of hepatitis B virus in chronically infected humanized mice	This study utilized adeno-associated virus (AAV) vectors and CRISPR-Staphylococcus aureus (Sa)Cas9 for HBV genome editing in humanized mice. Two single guide RNAs (sgRNAs) with HBV specificity in FRG mouse livers were used	The results of this study show that mice with HBV gene editing exhibit higher levels of SaCas9 delivery to human HBV hepatocytes compared to untreated mice. HBV-specific AAV-SaCas9 therapy significantly improves the survival of human hepatocytes.
2	Martinez et al., 2022	CRISPR-Cas9 Targeting of Hepatitis B Virus Covalently Closed Circular DNA Generates Transcriptionally Active Episomal Variants	Utilizing CRISPR-Cas9 with HBV gRNA using HepG2- cells. NTCP. CRISPR-Cas9 targets cccDNA, causing indel formation. Southern blotting (SB), target DNA sequencing, and transcriptomic sequencing (RNA-seq) show that dual gRNA treatment causes the formation of new HBV DNA variants that remain transcriptionally active.	CRISPR-Cas9 can damage the HBV gene, thereby reducing viral replication. Dual gRNAs can form new transcriptionally active species after CRISPR-Cas9 cleavage.
3	Wang et al., 2022	CRISPR/Cas9 delivery by NIR-responsive biomimetic nanoparticles for targeted HBV therapy	Gene editing in HBV-infected cells and HBV-Tg mice using NIR (near-infrared). PC Biotin-NHS Ester (PCB) that can be photolyzed was used for UCNPs-Cas9. Cas9/sgRNA was released under NIR, entered the nucleus with the help of a nuclear	Through this study, HBV was found to be inhibited. In HBV-Tg mice, there was no significant cytotoxicity and minimal target DNA damage.

			localization sequence (NLS), and then inhibited viral replication.	
4	Smekalova et al., 2024	Cytosine base editing inhibits hepatitis B virus replication and reduces HBsAg expression *in vitro* and *in vivo*	Inactivating cccDNA and HBV DNA using a combination of guide RNA (gRNA) and CBE in HBV-infected cells such as HepG2-NTCP, primary human hepatocytes (PHH), and HepG2 cell lines	This base editing can cause mutations in cccDNA and HBV DNA that inhibit HBV replication.
5	Gorsuch et al., 2022	Targeting hepatitis B cccDNA with a sequence-specific ARCUS nuclease to eliminate hepatitis B virus *in vivo*	Development of adeno-associated virus (AAV) episomal and non-human primate (NHP) mouse models to assess the activity of ARCUS-POL nuclease against cccDNA *in vivo*	In mice and NHPs, a decrease in AAV quantity and a high frequency of indels were observed. In mice expressing HBsAG, a permanent reduction of 96% was observed.
6	Walther et al., 2024	Comparative analysis of lipid nanoparticle-mediated delivery of CRISPR-Cas9 RNP versus mRNA/sgRNA for gene editing *in vitro* and *in vivo*	Comparing lipid nanoparticles (LNP) containing mRNA Cas9, sgRNA, and homologous templates via homology-directed repair (HDR) with LNP containing Cas9-RNP and HDR templates. This test was conducted on mice.	Gene editing in mice showed that LNP delivered mRNA Cas9 and sgRNA. LNP deleted the hepatocin gene by 60%. Delivery of mRNA Cas9 was superior to Cas9-RNP for CRISPR-Cas9 applications for gene editing *in vitro* and *in vivo*.
7	Wang et al., 2023	Interferon-stimulated immune profile changes in a humanized mouse model of HBV infection	Humanized IFN type I receptor (huIFNAR) mice were created using CRISPR-Cas9 knock-in technology, and changes in gene expression profiles in response to human IFNα were characterized *in vivo*. Subsequently, the antiviral effects of human interferon were	FNAR mice that can mimic human IFN responses *in vivo*. Long-term treatment with human IFNα can reduce or even eliminate HBsAg.

			tested using an AAV-HBV model.	
8	Murai et al., 2022	Inhibition of nonhomologous end joining-mediated DNA repair enhances anti-HBV CRISPR therapy	Primary human hepatocytes (PHH) were obtained from the livers of NOG-TKm30 humanized chimeric mice using a two-step collagenase-pronase liver perfusion method. The antiviral effect of the CRISPR-Cas9 technique was enhanced by inhibiting adenosine diphosphate ribose polymerase (PARP) to target cccDNA.	CRISPR targeting HBV cccDNA can provide antiviral effects with significant reduction of cccDNA in HBV-infected cells.
9	Zeng et al., 2024	Engineered extracellular vesicles for delivering functional Cas9/gRNA to eliminate hepatitis B virus cccDNA and integration	Light-induced heterodimerization is combined with protein acylation to increase the efficiency of loading Cas9 protein into extracellular vesicles (EV). Vesicular stomatitis virus-glycoprotein (VSV-G) is incorporated into the EV membrane, which can release Cas9 protein from endosomes and increase gene editing in recipient cells.	Engineered EVs containing Cas9/gRNA and VSV-G demonstrate effective results in reducing viral antigens and cccDNA in HBV-infected and replicating cells *in vitro* and *in vivo*.
10	Kostyushev et al., 2023	Depleting hepatitis B virus relaxed circular DNA is necessary for resolution of infection by CRISPR-Cas9	Using CRISPR-Cas9 technology to suppress HBV cccDNA and prevent viral reactivation by reducing the genomic form of HBV rcDNA (relaxed circular DNA) using reverse transcriptase inhibitors.	Inactivation of HBV cccDNA using the CRISPR-Cas9 technique is not sufficient to cure the infection. DNA methylation can significantly reduce nucleic acid activity with a higher RNP ratio. Disruption of HBV DNA integration in infected cells can lead to

	mutations, chromosome loss, and cell death. The worst case scenario is carcinogenesis.

CRISPR is an adaptive immune system commonly found in archaea and bacteria. CRISPR can recognize specific targets by targeting DNA or RNA sequences in target cells. Cas enzymes form guide RNA (crRNA) and tracrRNA to cut the target DNA or RNA sequence (Christian et al., 2024). Nuclease damages the double strand in the target gene sequence, then through the DNA repair mechanism, precise and versatile genetic manipulation is possible (Yu et al., 2019). Deleting or inserting the desired sequence into the gene can be done (Vaghari-Tabari et al., 2022). Cas9 targeting is specifically controlled by 20-nt of sgRNA and the presence of Protospacer Adjacent Motif (PAM) close to the target genome sequence (Zhang et al., 2015). The memory of bacteria from viruses that have infected them can enhance the bacterial immune system (Christian et al., 2024).

According to research conducted by Stone et al. (2021), gene editing in five of the eight treated animals showed a very significant increase in human hepatocyte survival compared to control animals. Mice treated with SaCas9 showed a decrease in liver cccDNA levels, although this did not reach statistical significance. The AAV-SaCas9 vector expresses two sgRNAs targeting different parts of the HBV coding sequence. This occurs because the HBV gene often undergoes two independent cleavages that can divide the molecule into two parts. However, it is still unclear whether the decrease in cccDNA levels is caused by Cas9 or not.

Research conducted by Murai et al. (2022), HBV-CRISPR directly cuts cccDNA and suppresses cccDNA in HepG2-hNTCP-C4 cells susceptible to HBV expressing Cas9 (HepG2-hNTCP-C4-iCas9) or primary human hepatocytes (PHH) expressing Cas9. As demonstrated by the research conducted by Martinez et al. (2022), hepatitis B virus treated with CRISPR-Cas9 successfully suppressed viral replication. The results of research conducted by Stone et al. (2021), indicate that Cas9 can cleave cccDNA and integrate HBV DNA into the host genome, although the integration rate is low. This method is promising for treating chronic diseases, but further research is needed.

Similar results were also shown by research conducted by Wang et al. (2022), where HBV replication can be inhibited *in vivo* and *in vitro*. Treatment with UCNPs-Cas9@CM+NIR can significantly reduce 3.5 kb HBV RNA, intracellular HBV DNA, HBV cccDNA, and HBV viral antigens after incubation compared to the control. Research conducted by Smekalova et al. (2024) also showed that cytosine base editing can efficiently suppress cccDNA and HBV DNA *in vitro* and *in vivo*. According to Gorsuch et al. (2022), ARCUS-POL can reduce HBsAg by targeting cccDNA and HBV DNA *in vitro*. *In vivo* efficacy in NSG mice showed a 96% reduction in HBsAg. However, this therapy does not eliminate cccDNA, so viral reactivation can still occur.

CRISPR-Cas9 has emerged as a highly promising gene editing technique, offering solutions for various genetic disorders. However, the application of CRISPR-Cas9 still faces significant challenges, such as off-target effects, which reduce the therapeutic potential of CRISPR-Cas9 (Mengstie et al., 2024). Clinical applications of CRISPR-Cas9 can be considered if the risk of off-target cutting is close to zero (Kostyushev et al., 2019). CRISPR-Cas9 in the form of RNP is safer and provides rapid editing, which can then eliminate HBV cccDNA from infected cells (Kostyushev et al., 2023).

According to Walther et al. (2024), all mice treated with LNP Cas9-RNP delivering the HDR template (pLNP-HDR) died within 20 hours after tail vein injection, while mice treated with mRNA Cas9 (mLNP-HDR) remained alive and showed no signs of illness. The death of these mice was likely due to particle aggregation or contamination by Cas9 protein and endotoxins. Gene deletion with CRISPR-Cas9 via mLNP was detected in hepatocytes at a rate of 60%.

Research conducted by Wang et al. (2023) shows that huIFNAR mice with HBsAg suppression by human PEG-IFNα2 require a relatively long time. The loss of HBsAg often requires several months after IFNα treatment in CHB patients. However, wild-type C57BL/6J AAV-HBV mice treated with IFN for 2 weeks can suppress HBsAg19. This is due to the lack of the human immune system.

Zeng et al. (2024) conducted research by engineering extracellular vesicles (EV) loaded with Cas9/gRNA and VSV-G, which yielded good results where the engineered EV effectively reduced viral antigens and cccDNA levels in HBV-infected and replicating cells. This study also showed that *in vitro* and *in vivo* engineered EV gene editing and antiviral agents have the potential to eliminate cccDNA and carcinogenic DNA integration. However, the cells used in this study were unable to observe the long-term effects of engineered EV treatment on the virus.

CRISPR-Cas9 technology has triggered major changes in genome editing in various fields, including biotechnology and health (Aljabali et al., 2024). CRISPR-Cas9 improves gene editing results compared to older techniques. However, this technique has off-target issues that are currently a concern. Off-target effects are unexpected, unwanted, and even harmful changes to the genome (Guo et al., 2024). To improve gene editing, researchers have developed modified Cas9 variants and new gene targeting methods with fewer side effects (Mengstie et al., 2024).

Gene editing must be fast and precise so that toxicity and the possibility of off-target effects can be limited (Martinez et al., 2022). Off-target effects can occur in DNA sequences with 3-5 base pairs at the PAM site of the sgRNA guide sequence (Zhang et al., 2015). sgRNA creates off-target mutations that affect other regions similar to the target gene sequence, thereby disrupting normal gene function. To reduce off-target effects, sgRNA must be designed appropriately, such as using Cas9 with the D10A mutation, where D10A is capable of damaging single strands (Vaghari-Tabari et al., 2022).

According to Kostyushev et al. (2023), DNA methylation can inhibit the antiviral properties of CRISPR-Cas9 RNP and reduce nucleolytic activity at the target. This can be neutralized by increasing the RNP dose. Off-target activity of StCas9 was not detected. However, sustained expression of CRISPR/Cas9 in suppressing HBV may increase the risk of off-target effects and toxicity. HBV cccDNA undergoes significant degradation but is then followed by the reformation of HBV cccDNA and viral replication. Therefore, RNA interference (RNAi)-based therapies for eliminating HBV are unlikely to be monotherapies. RNAi therapy combined with traditional nucleotide analogs, peg-interferon-α, and viral entry inhibitors is being studied to achieve complete cure or control of HBV replication after more stable treatment. This study demonstrates that CRISPR-Cas9 RNP targeting HBV provides a strategy to overcome HBV infection with a single dose of CRISPR-Cas9 RNP.This study demonstrates that CRISPR-Cas9 RNP targeting HBV provides a strategy to overcome HBV infection with a single dose of CRISPR-Cas9 RNP.

CONCLUSION

Gene editing with CRISPR-Cas9 technology shows great potential for treating hepatitis B virus. Various studies have demonstrated the success of this technology in editing alleles with extraordinary target specificity. Using humanized mice, HBV can be eliminated *in vitro* and *in vivo*. This technology is also capable of reducing cccDNA levels, which are currently a major obstacle, although the results are not yet significant. This opens up great opportunities for HBV therapy to permanently eliminate the virus. However, the application of CRISPR-Cas9 still faces important challenges, including the risk of off-target effects due to imperfect DNA cutting. This is an issue that needs to be addressed.

Overall, CRISPR-Cas9 has the potential to become a therapy for the treatment of HBV. Further research is needed to ensure that this technology can be used in safe and effective clinical applications for eliminating HBV.

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