CRISPR/Cas9 시스템을 이용한 B형 간염 바이러스 치료

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Application of CRISPR/Cas9 in Treating Hepatitis B Virus

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The advent of oral antiviral agents has revolutionized hepatitis B treatment. It has led to decreased incidence and mortality related to hepatocellular carcinoma. However, although nucleos(t)ide analogs (NA) are fast and potent in inhibiting hepatitis B virus (HBV) polymerase and reverse transcriptase activity, complete cure of the virus is not possible. The complete eradication of HBV requires the covalently-closed-circular DNA (cccDNA) to be eliminated. Novel gene editing methods, such as zinc finger nucleases, transcription activator-like effector nucleases, and the clustered regularly interspaced short palindromic repeats/Cas9 (CRISPR/Cas9) system, designed to target specific DNA sequences has great potential for therapeutic application. Among these, the CRISPR/Cas9 system may be the most feasible approach to eradicate HBV cccDNA. Further studies are needed to develop a more efficient and safer method of delivery using the CRISPR/Cas9 system to achieve complete cure of chronic hepatitis B. (*J Liver Cancer* 2017;17:111-116)

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INTRODUCTION

Hepatitis B virus (HBV) is a major health problem worldwide despite the availability of highly effective vaccines since 1982. There are still more than 350 million chronic carriers with an increased risk of developing hepatic decompensation, cirrhosis, and hepatocellular carcinoma.¹ The estimated worldwide mortality related to HBV is 0.5-1.2 million deaths per year, with hepatocellular carcinoma accounting for 320,000 deaths a year.² The advent of oral antiviral agents has

revolutionized hepatitis B treatment and decreased incidence and mortality related to hepatocellular carcinoma after the application of oral antiviral agents is evident.³⁻⁵ However, although nucleos(t)ide analogs (NA) have fast and potent inhibitory effects on hepatitis B virus polymerase and reverse transcriptase activity, they do not completely eliminate HBV and cause viral relapse after stopping treatment. Additionally, long-term use of NA results in resistance development. Interferon- α can clear HBV but is limited by adverse systemic effects which is not tolerated well by most patients.⁶

The complete eradication of HBV requires the covalently-closed-circular DNA (cccDNA) to be eliminated.⁷ Novel nuclease-based gene editing methods, such as zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and clustered regularly interspaced short palin-

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dromic repeats (CRISPR)/Cas9 system have been harnessed to disrupt HBV genome.⁸⁻¹³ Among these, the function and application of the CRISPR/Cas9 system with its highly efficient and simple method will be reviewed as a novel therapy for HBV.

1. HBV infection and the cccDNA

HBV belongs to the Hepadnaviridae family and is a DNA virus with a 3.2 kb-long partially double-stranded relaxed circular DNA (rcDNA) genome. It has four long open reading frames (surface, core, polymerase, and X protein) which are translated into protein important for viral replication.¹⁴ HBV enters the cell through irreversible binding of the virion to the Na⁺-taurocholate cotransporting polypeptide (NTCP).¹⁵ Upon infection, rcDNA is translocated to the nuclei of infected hepatocytes where it is converted to cccDNA. HBV cccDNA is the transcriptional template which generates all viral gene products including the pregenomic RNAs (pgRNA) and

messenger RNAs (mRNAs) for viral proteins. Cytoplasmic pgRNA and the P protein (viral reverse transcriptase) are co-packaged into viral core proteins and undergoes reverse transcription to rcDNA. Nucleocapsids containing rcDNA are either converted to the cccDNA in the nucleus or enveloped by viral surface proteins in the endoplasmic reticulum and secreted as virions. The complete eradication of HBV infection (i.e. complete cure) requires fully eliminating the cccDNA (Fig. 1).⁷

NAs achieve “functional cure” by directly inhibiting reverse transcription through competitive binding with endogenous substrates or incorporation into the viral DNA to act as chain terminators.¹⁶ Although highly potent in suppressing the viral DNA, it has minimal effect on inhibiting or eliminating the transcription of cccDNA.^{7,17} And rebound viremia is always possible even when only a single cccDNA remains after stopping NAs. Therefore, the cccDNA is a major obstacle in eradicating HBV and novel therapeutic approaches are needed.

Figure 1. Lifecycle of hepatitis B virus (HBV). HBV enters the hepatocyte and is uncoated in the cytoplasm. The relaxed-circular DNA (rcDNA) is released from the nucleocapsid into the nucleus and is converted to the covalently closed circular DNA (cccDNA). The cccDNA acts as a template for the transcription of pregenomic RNA (pgRNA) which is transferred into the cytoplasm. HBV pgRNA acts as the viral protein mRNA and is reverse transcribed into rcDNA. Nucleocapsids containing rcDNA are secreted from the host cells as virions.

2. Basic application of the CRISPR/Cas9 system

Genome editing offers the advantage of sequence-specific DNA targeting via inducing double strand breaks (DSB) at the lesion of interest. When DSBs are made, the lesion is corrected by one of two major repair methods: homology-directed repair (HDR) or nonhomologous end joining (NHEJ). NHEJ does not require a repair template, but forms short insertions or deletions (indels) in the target sequence. The resultant indels can cause frameshift mutations which leads to the production of nonfunctional, incomplete proteins, or to micro-RNA degradation. ZFNs and TALENs, protein-based genome-editing tools, have been utilized for the specific destruction or inactivation of HBV genome (Table 1).^{8,9} However, both programmable nucleases require a pair of site-specific nucleases for each target and customizing the DNA-binding domains requires substantial effort. In contrast, the CRISPR/Cas9 system is more easily customizable and can be redirected to target any DNA sequence by redesigning the sequence of guide RNA (gRNA).¹⁸⁻²⁰

CRISPR is derived from the prokaryotic adaptive immune system.²¹ It was originally recognized in *Escherichia coli* in 1987 and later found to include barcode-like sequences of viral or plasmid origin, termed spacers.²²⁻²⁵ In 2007, the role of the repeats in adaptive defense was confirmed by spacer integration into the bacterial genome following phage challenge and resistance specificity determined by spacer-phage sequence similarity.²¹ CRISPR works in sync with the Cas gene to cleave DNA or RNA sequences targeted by a small guide RNA.²⁶⁻²⁸ Among the six CRISPR systems, only the bacterial

type II CRISPR/cas9 system has been utilized for RNA-guided engineering nucleases.^{29,30} Cas9, a single endonuclease, of the CRISPR/Cas9 system acts in concert with two guide RNAs: CRISPR RNA (crRNA) and trans-acting CRISPR RNA (TracrRNA). Scientists designed a linker loop to engineer a dual tracrRNA:crRNA called single guide RNA (sgRNA) participating in sequence-specific DNA cleavage with Cas9 to simplify the process.¹⁹ Short 2 to 5 bp conserved sequences known as protospacer adjacent motifs (PAM) located on the opposite side of the RNA-DNA hybridization are required for Cas9-DNA recognition. After recognition, double stranded DNA cleavage is performed by two Cas9 domains. Programmed nucleases with customized sgRNA can cleave genomic DNA at specific loci which enables precise genome editing. Based on these key findings, the CRISPR/Cas9 system has been rapidly developed as a novel genome editing tool (Fig. 2).

3. Targeting HBV cccDNA using the CRISPR/Cas9 system

The application of CRISPR/Cas9 system for cleavage and elimination of HBV cccDNAs both *in vitro* and *in vivo* are being shown in several studies. Kennedy et al. first demonstrated inhibition of HBV DNA production and cccDNA accumulation for *in vitro* models of both chronic HBV infection (HepAD38 cells) and *de novo* infection (HepARG cells).¹⁰ The CRISPR/Cas9 system suppressed total HBV viral DNA levels by up to ~1,000-fold and cccDNA levels by up to ~10-fold. The CRISPR/Cas9 system targeting the conserved re-

Table 1. Comparison of programmable nucleases

	ZFN	TALEN	CRISPR
DNA-binding moiety	Zinc finger protein	Protein	sgRNA
Target site size, bp	18-36	30-40	20
Nuclease	FokI	FokI	Cas
Efficiency	Low and variable	High and variable	High and variable
Off-target effects	High	moderate	Variable
Ease of manipulation	Low	Low	High
Origin of discovery	Eukaryotes	Plant pathogen	Prokaryotes

ZFN, zinc finger nuclease; TALEN, transcription activator-like effector nuclease; CRISPR, clustered regularly interspace short palindromic repeats.

Figure 2. Overview of the clustered regularly interspace short palindromic repeats (CRISPR)/Cas9 system. The targeted gene of interest is selected and a guide RNA is designed forming a gRNA:Cas9 endonuclease complex. The gRNA:Cas9 complex recognizes the protospacer adjacent motif (PAM) and the protospacer complementary to the gRNA which results in a blunt dsDNA break. The dsDNA break is repaired via two pathways; (i) the error-prone non-homologous end-joining or (ii) homology-directed repair. The homology-directed repair enables specific gene editing of the target in interest.

gions of HBV genome to have a broad antiviral activity against different genotypes have been demonstrated by Liu et al. The group reported that HBV-specific gRNA/Cas9 targeting the conserved regions of different HBV genotypes could inhibit the replication of HBV both *in vitro* and *in vivo*.³¹ Ramanan et al. demonstrated that sgRNAs targeting conserved regions of HBV cause strong inhibition of virus replication

both *in vitro* and *in vivo* which could potentially lead to a viable antiviral therapy.³² Other studies also claimed that the CRISPR/Cas9 system could inactivate HBV cccDNAs and integrated HBV genomes.^{10,11,33,34} Karimova et al. also successfully inhibited HBsAg expression in both chronically and *de novo* infected human hepatoma cell lines by targeting the S- or X-open reading frames.³⁴ In summary, the studies have demonstrated the effectiveness of the CRISPR/Cas9 system in inhibiting HBV cccDNA both *in vitro* and *in vivo* and mark the potential of CRISPR/Cas9 system in treating chronic HBV infection.

4. Current limitations of the CRISPR/Cas9 system

There are still several key limitations to overcome before clinical application. First, current *in vitro* researches on CRISPR/Cas9 is limited by a lack of a reliable measure method in quantifying cccDNA. The real-time polymerase chain reaction amplification and Southern blotting assays used currently must be further refined to be more reliable and convenient. Secondly, the greatest efficacy reported to date is about 92% cccDNA reduction in cultured cell.³² As noted above, to achieve not just functional cure but complete cure, elimination of cccDNA in hepatic and extrahepatic viral reservoirs is necessary. Thirdly, potential off-target effects with CRISPR/Cas9 system is a major obstacle before achieving clinical use. As demonstrated by Fu et al. using a human cell-based reporter assay, significant off-target activity of CRISPR/Cas9 system occurs at a high rate.³⁵ Several approaches have been developed to reduce the off-target effect. The use of paired nickases, truncated gRNAs, genetically engineered fusion of catalytically inactive Cas9 and FokI nuclease has shown greater specificity for the target sites.³⁶⁻³⁸ Furthermore, the cleavage of HBV integrates in human genome may lead to indels in the host genome and disrupt the gene function.³⁹ The resulting effects may lead to genomic instability and promotion of carcinogenesis. The fourth concern is the selection of the proper gRNA targeting the HBV genome. In clinical practice, most of the chronic hepatitis B patients at indication of therapy are being treated with NAs. As the CRISPR/Cas9 system relies on a precise sequence recognition, the selection of HBV

with mutations by NAs suppressing the wild type virus will cause loss of the CRISPR/Cas9 recognition site.³⁹ This will cause replication of treatment-resistant viruses re-establishing viremia in the patients. The last concern is contemplating the delivery of the CRISPR/Cas9 system. The *in vivo* delivery system relies on viral and nonviral vectors. Among current viral vectors, the adeno-associated virus (AAV) vectors are promising due to the low pathogenicity, immune stimulation and nonintegrating nature. AAVs also show a marked hepatotropism and recent AAV variants with even more highly hepatotropism have been reported.⁴⁰ Recent studies utilizing non-viral vectors to deliver Cas9 mRNA and gRNAs with reduced HBV DNA have also been reported.⁴¹

CONCLUSION

The advent of NAs has greatly decreased the risk of progression to cirrhosis and the development of hepatocellular carcinoma. However, to achieve complete eradication of HBV infections, a new therapeutic method targeting the cccDNA is needed. Novel genome editing methods that allow alteration of target specific DNA sequences are expected to have great therapeutic potential. Although further studies to increase the efficiency and delivery of the CRISPR/Cas9 system is needed, it is a viable method for targeting HBV cccDNA. The eradication of cccDNA through these technologies may lead to complete cure of chronic hepatitis B and stop the progression of the disease to hepatocellular carcinoma.

Conflicts of Interest

The authors have no conflicts to disclose.

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