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Title: How to clear HBV cccDNA: CRISPR/Cas9 might be promising

Author List and Affiliations: Yan Qiu, Ying Liu, Wen Ren, Jingjing Ren

Yan Qiu: Department of general practice, the first affiliated hospital, Zhejiang University, Hangzhou, Zhejiang, China, 310003; Email: 1513046@zju.edu.cn

ORCID ID: http://orcid.org/0000-0001-7996-4109

Ying Liu: Department of general practice, the first affiliated hospital, Zhejiang University, Hangzhou, Zhejiang, China, 310003; Email: 1511018@zju.edu.cn

ORCID ID: http://orcid.org/0000-0003-2408-3712

Wen Ren: Department of general practice, the first affiliated hospital, Zhejiang University, Hangzhou, Zhejiang, China, 310003; Email: 1512025@zju.edu.cn

ORCID ID: http://orcid.org/0000-0003-0096-3782

Jingjing Ren: Department of general practice, the first affiliated hospital, Zhejiang University, Hangzhou, Zhejiang, China, 310003; Email: 3204092@zju.edu.cn

ORCID ID: http://orcid.org/0000-0001-8732-1875

Correspondence: JingJ Ren, Department of general practice, the first affiliated hospital, Zhejiang University, Hangzhou 310003, China

Email: 3204092@zju.edu.cn

How to clear HBV cccDNA: CRISPR/Cas9 might be promising

ABSTRACT

BACKGROUND: Chronic hepatitis B infected with Hepatitis B virus remains a major health concern worldwide. Despite standard interferon-α and nucleotide analogues have been shown to reduce the deterioration of liver disease among chronic hepatitis B patients, covalently closed circular DNA was still difficult to eradicate.

METHODS: A literature search of Pubmed and Web of science was performed with the following key words: 'CRISPR', 'CRISPR/Cas9', 'hepatitis B', 'HBV', 'chronic hepatitis B' and 'HBV cccDNA'. The information about CRISPR/Cas9 for the treatment of HBV cccDNA or hepatitis B was reviewed.

RESULTS: CRISPR/Cas9 could treat hepatitis B through suppressing or clearing HBV cccDNA with different gRNAs.

CONCLUSION: With the emergence of CRISPR/Cas9 **(**the RNA-guided clustered regulatory interspaced short palindromic repeats, CRISPR**)** editing technology, clearance of hepatitis B virus and better prevention of liver carcinoma seemed to be possible.

KEYWORDS: hepatitis B virus; chronic hepatitis B; cccDNA; CRISPR/Cas9; gene therapy

Introduction

Hepatitis B virus (HBV) infection is still a major public health problem affecting 248 million people worldwide [1]. It has caused 780,000 deaths annually due to

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hepatitis B related diseases such as cirrhosis and hepatocellular carcinoma (HCC) [2, 3]. Up till now, there are seven agents extensively used for the treatment of chronic hepatitis B (CHB) including standard interferon-α (IFN-α) or pegylated interferon-α (PEG-IFN-α) and five nucleotide/ nucleoside analogues (NA). Although both IFN and NA could reduce the progression of liver disease in CHB patients, there are no satisfied needs of existing CHB treatment to clear the covalently closed circular DNA (cccDNA) and better prevention of HCC.

Due to HBV cccDNA, the transcription template for viral mRNA and pregenomic RNA synthesis and secures virus persistence, eradication of HBV infection still poses a great challenge. Fortunately, several strategies to cure HBV are always in research [4-25], including strategies to inhibit the nucleocapsid assembly, HBV RNA, HBV entry receptor, host immune responses, HBV cccDNA and new polymerase inhibitors. As to the strategy to eradicate HBV cccDNA, there are three most common nuclease-initiated genome editing tools: the zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and the RNA-guided clustered regulatory interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) protein endonucleases [26, 27]. In the past few decades, ZFNs and TALENs have attracted extensive attention because of their advantages. Studies found that ZFNs can be used to inhibit viral transcription and replication of duck HBV through blocking the transcription of cccDNA [28] , while TALENs could target the conserved regions of HBV DNA and reduce the cccDNA levels [29]. However, the time-consuming and costly engineering of the target gene-specific

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fusion proteins are prominent disadvantages. Therefore, a novel easy genome editing tool entered the spotlight: the CRISPR/Cas9 technology [30]. This article reviews and discusses the current researches of the novel therapeutic technology-CRISPR/Cas9-to target HBV cccDNA from basic medical perspective.

CRISPR/Cas9 system

CRISPR, a DNA segment with 20-50bp short repetitive sequences, was first discovered in 1987 [31], being found to be associated with a set of genes named CRISPR-associated genes-cas genes [32]. There are three types (I-III) of CRISPR-Cas systems [33]. Among them, the Type II CRISPR-Cas9 system is the first genome editing application in eukaryotic cells [34, 35] and the most widely used for genome editing. It consists of three components: the RNA-guided endonuclease Cas9 [36], the crRNA and the trans-activating crRNA (tracrRNA) [37]. Therefore, it could recognize and cut the target double-stranded DNA using the Cas9 HNH and RuvC-like nuclease domains [38, 39]. For gene targeting applications, either dual-RNA guides or chimeric single-molecule guide RNAs (sgRNAs) [34, 35], [40-46] is indispensable. Several guide RNAs (gRNAs) [13],[47]-[53] to target HBV of genotype A, B, C or D in vitro and in vivo have been designed (table 1).

The experimental models for CRISPR/Cas9 potential treatment of CHB

Cell culture models

With the advantages of being more convenient and faster than in vivo experiments, cell culture are very practical to perform cellular researches in a well-controlled experimental setting. As with the emergence of CRISPR/cas9

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technology for genome editing and HBV therapy, many common in vitro experimental models for human hepatitis B virus infection study were included such as Huh-7 cells, HepAD38 cells, HepG2/NTCP cells, HepARG cells, Hep2 cells and HepG2.2.15 cells[13, 47, 49-58]. Among them, Huh-7 and HepG2 cells with 1.2 or 1.3 copies of HBV genome were more often used for their practices and conveniences. HepG2/NTCP cells were also used for its capability of infecting 1.05 copies of HBV genome in contrast with parental HepG2 cells without NTCP transfection [16]. However, Zhu et al. [59] showed that there were no significant differences of anti-HBV productions by pCas9-2 constructs exists in between Huh-7 and HepG2 hepatoma cells. After transfection with different Cas9/gRNA plasmid vectors, the cells were used for analyzing the effect of CRISPR/Cas9 system on the disruption or suppression of hepatitis B virus.

Animal models

As cultured cells may respond differently to HBV infection and other stimuli than cells in the living organ [59], the capability to express various hepatocyte-specific genes might be lost. Hence, it should consider data discrepancies obtained in vitro and in vivo, and verify the observations from cell cultures in animal models.

According to Maura Dandri [60], there are three types of mouse models of HBV replication up to now. These are transgenic mice, vector-medicated transduction of HBV in mice and hydrodynamic injection of replication competent HBV [61] (figure 1). As mice cannot be infected with HBV but with the advantages of the best

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characterized and most convenient small laboratory animal, genetically modified mice were generated for expressing either viral proteins or the full HBV genome with great efforts of researchers. In 1995, the first HBV-replicating transgenic mice were developed, and the murine hepatocytes could produce infectious HBV virions morphologically indistinguishable from human-derived virions [62].

Since the HBV genome is integrated in the host genome and cccDNA is not build in murine hepatocytes, Maura Dandri thought that viral clearance cannot be achieved. Meanwhie, the mechanisms of cccDNA synthesis and strategies aiming at destabilizing the cccDNA cannot be investigated [63]. Therefore, the targeting of CRISPR/Cas9 nucleases to cccDNA in transgenic mice was all tested combined with hydrodynamic tail-vein injection technology. For instance, Li H [56] and his colleagues delivered gRNA-S4 system to the liver of adult HBV-Tg mice using hydrodynamic tail-vein injection to confirm the effect. Zhu W et al. [55] injected pCas9 constructs into the M-Tg HBV mouse model of HBV by hydrodynamics and found decreased HBsAg of sera and liver HBcAg. Zhen S and his colleagues also used HBV-Tg mice after delivering Cas9 and gRNA-S1+X3 plasmid by hydrodynamics. They showed reduced HBsAg secreted in mouse serum and almost no HBsAg-positive cells in the liver tissue of CRISPR/Cas9-S1+X3-treated mice [50]. Although the HBV-Tg mouse lineage might be produced in different background (C57/BL or BALB/c), the transgene present in these mice consists 1.3 copies of HBV genome and expresses high levels of HBsAg in their serum and has detectable levels of HBV DNA in their serum [61,64,65].

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Apart from the transgenic mouse model, alternative mouse models have been developed. They relied on the transfer of HBV genomes into mice by using viral vector mediated transduction or hydrodynamic injection of HBV genome. However, viral vector mediated transduction combined with the hydrodynamic injection was more used in the application of CRISPR/Cas9 to treat CHB. At present, there were four researches that used both the models [13, 49, 51, 52]. In the research that the CRISPR/Cas9 system facilitates clearance of intrahepatic HBV templates in vivo, pAAV/HBV 1.2 and gRNA/Cas9 dual expression vector were injected into the tail veins of HBV persistent mouse model with C57BL/6 background. In another study that targeting HBV cccDNA by CRISPR/Cas9 nuclease efficiently inhibits viral replication, precccDNA plasmid, pCre plasmid and pX330/pCas9 plasmid were injected into the tail vein of female Balb/c mice. Vyas Ramanan [52] and his colleagues used the hydrodynamic delivery technique to inject 1.3xHBV plasmid and CRISPR expressing plasmid to NRG mice for studying the effect of CRISPR/Cas9 on the suppressing HBV. Similarly, Liu et al. conducted hydrodynamics-based transfection in mice, that is, injecting pHBV1.3 and pSV-β-gal, pX330-U6-HBV gRNA plasmids or pX 330-U6-GFPgRNA plasmid into the tail vein of BALB/c mice [49]. Through all the above mouse models and different gRNA, they demonstrated the inhibition or cleavage effect of CRISPR/Cas9 to target HBV cccDNA and other parameters of viral gene expression and replication.

Remaining Issues and Potential Solutions

Although the advancing CRISPR/Cas9 gene editing technology is now at an

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interesting stage to eradicate the persistent HBV genome, there are still some remaining issues needing to be addressed. Of them, cytotoxic seemed to have been solved in most studies.

First, the efficiency of CRISPR/Cas9 system is low needing to be improved. In some studies [13, 50], serum HBsAg level was reduced but persisted following the CRISPR/Cas9 therapy, potentially leading to rebound viremia and repopulation of HBV in the liver after the cessation of the antiviral therapy. To overcome the hurdle, an efficient delivery system with a high vector, such as adenoviral [67, 68] or adeno-associated virus (AAV) vectors [68, 69], to target cell ratio and achieve continuous and prolonged treatment. Hence, it may be possible for the use of immediate, prolonged treatment and the combination of several treatments.

The second issue is the off-target effect, which is pertinent concerns for all genome-editing strategies and may cause non-specific gene modification events. To utilize the CISPR/Cas9 system for cure of HBV infection, it is best to eliminate this off-target activity. The issue may be solved by designing truncated gRNAs [70], paired nickase gRNAs [71] and gDNAs [72]. Of them, the gDNA guided NAgo system, which has lower off-target effect and does not require any protospacer-adjacent motif (PAM) sequence, has been confirmed suitable for genome editing in human cells.

The third issue is the fraction of cccDNA possibly existing in minichromosomes. Consequently, minichromosomes of cccDNA can still be targeted and cleaved remains unclear, needing further investigation.

Conclusions

Although hepatitis B could be suppressed with drugs, cccDNA could not be fully eradicated. With great efforts of researchers, many new strategies have been used to treat HBV. Of them, CRISPR/Cas9 has been demonstrated to target HBV cccDNA with different gRNAs. Meanwhile, experimental models in vitro and in vivo have been established almost successfully. As to cell culture studies, Huh-7 and HepG2 cells with 1.2 or 1.3 copies of HBV genome were more often used than HepG2/NTCP cells with the capability of infecting 1.05 copies of HBV genome. While involving the animal models, three types of mouse models of HBV replication including transgenic mice, vector-medicated transduction of HBV in mice and hydrodynamic injection of replication competent HBV were gradually improved for CRISPR/Cas9 treatment of HBV. Undoubtedly, there was still some remaining problems related needing to be solved. Hence, further studies should be conducted to perfect the CRISPR/Cas9 system for HBV therapy.

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Author contributions

JingJ Ren and Yan Qiu have made substantial contributions to the conception of the work, Yan Qiu drafted the work, Ying Liu and Wen Ren substantively revised it; and all the authors have approved the submitted version.

Conflicts of Interest

No potential conflict of interest was reported by the authors.

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Table 1 HBV-specific gRNAs to program Cas9

Names	Sequences	PAM	Nucleotide position/target location	References
PS1, PS2, PS3, P1, XCp, eE, PCE and S1	PS1: GGACTTCTCTCAATTTTCTA*GGG* PS2: GGGCTTTCGCAAAATACCTA*TGG* PS3: GGGCCTCAGTCCGTTTCTCT*TGG* P1: GTTTTGCTCGCAGCCGGTCT*GGG* XCp: GGGGGAGGAGATTAGGTTAA*AGG* eE: GCTGTGCCTTGGGTGGCTTT*GGG* PCE: GTCGCAGAAGATCTCAATCT*CGG* S1: GGAGTGGGAGCATTCGGGCC*AGG*	gRNA-P1 genotype A: GGG genotype B: GGA genotype C: GGA gRNA-XCp genotype	PS1 261–283 PS2 621–643 PS3 648–670 P1 1,292–1,314 XCp 1,742–1,764 eE 1,876–1,898 PCE 2,421–2,443 S1 3,028–3,050	Lin SR et al.[13]

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		A+B+C: AGG		
gRNA-P1,P4,P5,P6,S1,S2,X1,X2,X3,C1,C2,C3	P1 CCTCGAGAAGATTGACGATA	P1 AGG	P1 p114	Zhen S et al.[47]
	S1 CAACTTGTCCTGGTTATCGC	S1 TGG	S1 s/p357	
	S2 CAGGTGCAATTTCCGTCCGA	S2 AGG	S2 s/p581	
	P4 GAAAGTATGTCAACGAATTG	P4 TGG	P4 p985	
	P4 AGGTTCCACGCACGCGCTGA	P4 TGG	P4 p1227	
	X1 AAACAAAGGACGTCCCGCGC	X1 AGG	X1 x/p1406	
	X2 CGCCCCGTGGTCGGTCGGAA	X2 CGG	X2 X1506	
	X3 GGTCTCCATGCGACGTGCAG	X3 AGG	X3 X1596	
	C1 TCTAGAAGATCTCGTACTGA	C1 AGG	C1 c1976	
	C2 ACTACTAGGTCTCTAGATGC	C2 TGG	C2 C2138	
	C3 GATTGAGATCTTCTGCGACG	C3 CGG	C3 c/P2414	
	P6 GGCTGGATCCAACTGGTGGT	P6 CGG	P6 p2894	

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sgRNA-1, 2, 3, 4	sgRNA-1F: 5’-caccgGGGGCGCACCTCTCTTTACG-3’ sgRNA-1R: 5’-aaacCGTAAAGAGAGGTGCGCCCCc-3’ sgRNA-2F: 5’-caccgAATGTCAACGACCGACCTTG-3’ sgRNA-2R: 5’-aaacCAAGGTCGGTCGTTGACATTc-3’ sgRNA-3F: 5’-caccgACTACTGTTGTTAGACGACG-3’ sgRNA-3R: 5’-aaacCGTCGTCTAACAACAGTAGTc-3’ sgRNA-4F:	sgRNA-1: CGG sgRNA-2: AGG sgRNA-3:/ sgRNA-4:/	sgRNA-1:1523-1542 sgRNA-2:1681-1700 sgRNA-3:2338-2357 sgRNA-4:2416-2435	Dong C et al.[48]

doi:10.20944/preprints201710.0065.v1

	5’-caccgGATTGAGATCTTCTGCGACG-3’ sgRNA-4R: 5’-aaacCGTCGCAGAAGATCTCAATCc-3’ Targeting sequence			
gRNA1-8	gRNA1caccTACCGCAGAGTCTAGACTCG gRNA2 caccCATTTGTTCAGTGGTTCGTA gRNA3caccGTTGCCGGGCAACGGGGTAA gRNA4caccAAACAAAGGACGTCCCGCGC gRNA5 caccGGTCTCCATGCGACGTGCAG gRNA6 caccGTAGCTCCAAATTCTTTATA gRNA7 caccGACCTTCGTCTGCGAGGCGA gRNA8 caccCCTTCCTGACTGGCGATTGG	/	/	Liu X et al.[49]
sg5, sg6, sg7	sg5: CATTCGGTGGGCGTTCACGG	sg5:2986	sg5: 3,006–2,987	Seeger C et

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	sg6: AATGTCAACGACCGACCTTG sg7: TTTGAAGTATGCCTCAAGGT sg10: GCAGAGGTGAAAAAGTTGCA	sg6:3068 sg10: 1	sg6: 3,048–3,067 sg7: 3,081–3,062 sg10: 21–2	al.[50]
N.S. sgRNA, RT sgRNA, Surface sgRNA, core sgRNA	RT sgRNA: GTTCAGTTATATGGATGATG Surface sgRNA: GCCTGTCCTCCAACTTGTCC core sgRNA: GTACCGCCTCAGCTCTGTAT N.S. sgRNA: GAAATCCTGCAGAAAGACCT	/	/	Kennedy EM et al.[51]
Sg6,13-19,21	/	/	/	Ramanan V et al.[52]
gRNA1-15; dual gRNAs: gRNA1+3,	1 CCTGCTGGTGGCTCCAGTTC 2 GGACCCCTGCTCGTGTTAC 3 GCTGCTATGCCTCATCTTC	1 56-75 2 182-200 3 415-433	Target genotypes 1 A/B/C/D 2 A/B/C/D	Wang J et al.[53]

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gRNA1+4,	4 ATGGGAGTGGGCCTCAGTC	4 640-658	3 A/B/C/D
gRNA 1+13,	5 AGTGTTTGCTGACGCAACC	5 1179-1197	4 A/B/C
gRNA 2+14,	6 GCCAACTGGATCCTGCGC	6 1393-1410	5 A/B/C/D
gRNA 3+5,	7 GGGGCGCACCTCTCTTTACG	7 1521-1540	6 B/C/D
gRNA 4+5,	8 GAGGTGAAGCGAAGTGCACA	8 1578-1597	7 A/B/C/D
gRNA 5+6,	9 CTTCACCTCTGCACGTCGCA	9 1589-1608	8 A/B/C/D
gRNA 5+8,	10AGGAGGCTGTAGGCATAAAT	10 1775-1794	9 B/C/D
gRNA 5+12,	11AGCTTGGAGGCTTGAACAGT	11 1859-1878	10 A/B/C/D
gRNA 8+12,	12CAAGCCTCCAAGCTGTGCCT	12 1865-1884	11 A/B/C/D
gRNA 8+13	13ACTACTGTTGTTAGACGACG	13 2336-2355	12 A/B/C/D
	14CGAGGGAGTTCTTCTTCTAG	14 2367-2386	13 C/D
	15GATTGAGACCTTCGTCTGCG	15 2390-2409	14 A/B/C/D
			15 B/C/D

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sgRNA-S1, sgRNA-S2, sgRNA-X1, sgRNA-X2	S1 GCTCGTGTTACAGGCGGGGT S2 TACCGCAGAGTCTAGACTCG X1 GTCTGTGCCTTCTCATTCTGC X2 CGGGGCGCACCTCTCTTTAC	S1 CCT S2 TGG X1 CGG X2 CCA	/	Karimova M et al.[54]
pCas9-1, pCas9-2	pCas9-1: GCTATGCCTCATCTTCTTGT pCas9-2:CTTCACCCTCTGCACGTCGCA	pCas9-1:/ pCas9-2: TGG	pCas9-1: 420-439 pCas9-2: 1591-1610	Zhu W et al.[55]
gRNA-S3, gRNA-S4, gRNA-S5, gRNA-SP-I, gRNA-SP-II, gRNA-XP, gRNA-CP-BCP, gRNA-CP-URR	gRNA-S3 GGACTTCTCTCAATTTTCTA gRNA-S4 GCTATCGCTGGATGTGTCTG gRNA-S5 CCATTTGTTCAGTGGTTCGT gRNA-SP-I TCGCAGAAGATCTCAATCTC gRNA-SP-II GGGTGGAGCCCTCAGGCTCA gRNA-XP CCTCTGCCGATCCATACTG gRNA-CP-BCP	gRNA-S3 GGG gRNA-S4 CGG gRNA-S5 AGG gRNA-SP-I GGG gRNA-SP-II GGG gRNA-XP CGG	gRNA-S3 263–285 gRNA-S4 368–390 gRNA-S5 688–710 gRNA-SP-I 2418–2440 gRNA-SP-II 3042–3064	Li H et al.[56]

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AAGCCTCCAAGCTGTGCCTT	gRNA-CP-BCP	gRNA-XP 1257–1278
gRNA-CP-URR	GGG	gRNA-CP-BCP
ATGTCAACGACCGACCTTG	gRNA-CP-URR	1868–1890
	AGG	gRNA-CP-URR
		1682–1703

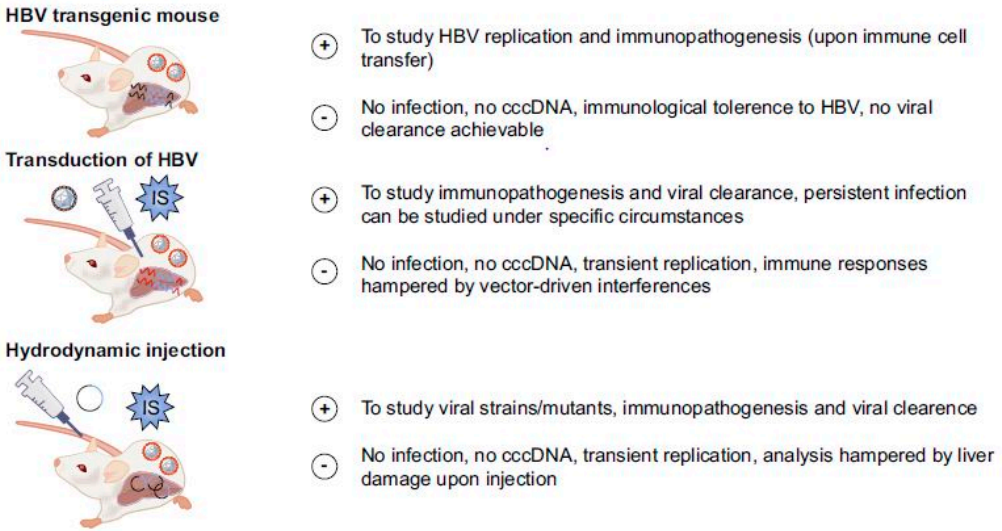

Fig. 1 Mouse models for CRISPR/Cas9 system to treat HBV infection [61]

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