Article

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CRISPR/Cas9 System with Dual gRNAs Synergically Inhibit Hepatitis B Virus Replication

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Background: In recent years, a gene-editing technology known as clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 has been developed and is progressively advancing into clinical trials. While current antiviral therapies are unable to eliminate the Hepatitis B virus (HBV), it stands as a prime target for the CRISPR/Cas9 technology. The objective of this study was to enhance the efficacy of CRISPR/Cas9 in suppressing HBV replication, lowering HBsAg and HBeAg levels, and eliminating covalently closed circular DNA (cccDNA).

Methods: To enhance the anti-HBV effectiveness of CRISPR/Cas9, our study delved into a dual-guide RNA (gRNA) strategy. After evaluating the antiviral activities of multiple gRNAs that effectively impeded HBV replication, we identified three specific gRNAs—namely 10, 4, and 21. These gRNAs were selected for their targeting of distinct yet conserved regions within the HBV genome.

Results: In HBV-stable cell lines, namely HepAD38, and HBV infection models of HepG2-NTCP cells, our investigation revealed that the co-application of gRNA-10 with either gRNA-4 or gRNA-21 within the CRISPR/Cas9 system demonstrated heightened efficacy in impeding HBV replication, reducing the levels of HBsAg, HBeAg, and cccDNA levels, along with a more pronounced promotion of HBsAg clearance when compared to the use of a single gRNA.

Conclusions: The CRISPR/Cas9 system employing dual gRNAs has proven highly effective in both suppressing HBV replication and facilitating HBsAg clearance. This promising outcome suggests that it holds potential to emerge as a novel approach for achieving the functional cure of patients with HBV infection.

Keywords: CRISPR/Cas9 system; gRNA; antiviral therapy; Hepatitis B virus; chronic hepatitis B

Introduction

Hepatitis B virus (HBV) impacts nearly 300 million individuals worldwide and stands as a primary cause of cirrhosis and liver cancer in China [1]. Current therapies, encompassing nucleoside analogues (NAs) and pegylated interferon (PEG-IFN), effectively manage the progression of HBV-induced diseases [2–4]. However, complete viral elimination remains elusive with these treatments due to the persistent presence of covalently closed circular DNA (cccDNA) [5]. Although cccDNA is not currently targeted by existing therapies, its endurance poses a substantial barrier to achieving a cure for chronic hepatitis B (CHB) [6].

The gene-editing technology, clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9, holds the potential to treat diseases by either disrupting disease-relevant genes or correcting deleterious base mutations [7,8]. Within the CRISPR/Cas9 system, the design of a guide RNA (gRNA) plays a crucial role, guiding the Cas9 protein to a specific genomic site. Upon binding to the target sequence of the gene, the Cas9 protein induces a specific double-strand break (DSB), potentially causing frameshift and/or premature termination of codons, effectively disrupting the open reading frame (ORF) of the target gene [9].

Research has extensively explored the efficacy of CRISPR/Cas9 in antiviral therapy by targeting viral genomes, including human papillomavirus [10], herpes simplex virus, and human immunodeficiency virus [11]. Specifically regarding HBV, studies confirm that CRISPR/Cas9 can significantly reduce HBV replication in models such as the HBV replicon transfection model [12–14]. Notably, CRISPR/Cas9 has been reported to target cccDNA, leading to a significant reduction in viral gene expression in stable cell lines like HepG2.2.15 and HepAD38 [14,15]. Scott *et al.* [16] demonstrated the effectiveness of CRISPR/Cas9 in reducing HBV replication by targeting cccDNA in a HepG2-NTCP cell infection model.

In vivo experiments further support the potential of CRISPR/Cas9, revealing its ability to significantly suppress HBV replication and antigen expression in mouse models [17,18]. Collectively, these studies underscore the promising prospect of CRISPR/Cas9 as a potential cure for CHB.

Nevertheless, the capability of CRISPR/Cas9 to effectively inhibit HBV and disrupt cccDNA remains restricted, particularly in challenging models like the humanized chimeric mouse infection [19,20]. In an effort to enhance the anti-HBV efficacy of CRISPR/Cas9, we devised a system incorporating ten gRNAs, each targeting diverse regions of the HBV genome. Through a comparative efficacy study, we identified three candidate gRNAs, subsequently employing them in combination. Ultimately, we assessed the advantages of Cas9 with dual HBV-specific gRNAs in comparison to Cas9 with a single gRNA, focusing on their effectiveness in inhibiting HBV replication, reducing HBsAg and HBeAg levels, and clearing cccDNA *in vitro*.

Materials and Methods

Design and Cloning of HBV-Specific gRNA

The sequences of gRNA-4, 10, 11, 21, 60, 69, 159, 178, and 231 were adapted from previously published studies [13,14,18,19,21,22], while the sequence for gRNA-55 was designed using the online Benching [23]. The CRISPR/Cas9 expression vector, lentiCRISPRv2, was sourced from Addgene (Cambridge, MA, USA), and its construction method was detailed in a previous study [24]. For the examination of the antiviral effect of CRISPR/Cas9 with dual gRNAs, lentiviral vectors expressing CRISPR/Cas9 systems targeting gRNA-4, gRNA-10, gRNA-21, and antibiotic resistance blasticidin were individually constructed.

Cell Culture and Transfection

The HepAD38 cells were procured from Shanghai Yubo Biotechnology Company under the catalog number AE-312, while the HepG2 cells were obtained from Shanghai Xige Biotechnology Company with the catalog number XG-X3336. Mycoplasma testing confirmed the absence of mycoplasma contamination, and Short Tandem Repeats (STR) analysis verified the absence of misidentified or cross-contaminated cells.

Human embryonic kidney 293, HepG2, and HepAD38 cells were cultured in complete Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum, 2 µM L-glutamine, high glucose, and 1% penicillin/streptomycin. HepG2-NTCP cell lines were sustained in a medium supplemented with 2 µg/mL puromycin.

For experimental procedures, HepG2 cells were seeded in a 48-well plate at a density of 5×10^5 cells/well. The prcccDNA, pCMV-Cre (encoding Cre recombinase), and EGFP plasmids were co-transfected into HepG2 cells with the gRNA/Cas9 expression vector at a ratio of 1:1:3. Transfection of all cells was carried out according to the manufacturer's instructions using EZ Cell Transfection Reagent (Life-iLab, Beijing, China).

Lentivirus Production and Transduction

The lentiCRISPRv2 plasmids, catalog number MZ-1280, were obtained from Shanghai Qiming Biotechnology Company (Shanghai, China). For co-transfection, lentiCRISPRv2 plasmids, psPAX2 (encoding Gag and Pol/Addgene plasmid 12260), and pMD2.G (encoding VSV-G/Addgene plasmid 12259) were combined in a 4:3:1 ratio and introduced into HEK 293T cells. Three days post-transfection, the supernatant was collected, and centrifugation was employed to remove cell debris. Lentivirus was concentrated using EZ lentivirus concentrated solution (Life-iLab, Beijing, China). HepAD38 cells were inoculated into 24-well plates at a density of 10^5 cells/well, and transfected with lentivirus at a multiplicity of infection (MOI) of 10. The 2 µg/mL puromycin or 10 µg/mL blasticidin were added to the medium 36 hours after lentiviral transfection and incubated for 12 hours to eliminate untransduced cells.

HBV Infection of HepG2-NTCP Cells

HepG2-NTCP cells were plated in a 48-well plate at a density of 10^5 cells per well using complete DMEM medium. For infection, the cells were exposed to supernatants obtained from HBV-replicating HepAD38 cells. The culture supernatants were filtered through a 0.45-µm filter and precipitated with 10% PEG-8000 (Sigma-Aldrich, Dover, DE, USA). Detection of HBV DNA copies was conducted using real-time polymerase chain reaction (PCR). HBV infection was initiated at a MOI of 500, employing a DMEM complete medium supplemented with 2% DMSO and 4% PEG-8000. Following 16–24 hours of HBV infection, cells underwent triple washing with PBS. Subsequently, the culture medium was refreshed every other day, and culture supernatants were collected for determining HBV DNA copy numbers. Once HBV infection was established (at 4 days post-infection), HepG2-NTCP cells were transduced with Cas9/gRNAs lentivirus.

Detection of HBsAg and HBeAg

Cell culture supernatants were collected and refreshed at 3-day intervals, and the presence of HBsAg and HBeAg was assessed using the enzyme-linked immunosorbent assay (ELISA), following the manufacturer's instructions (KHB, Shanghai, China). Optical density readings for antigen levels were measured at 450 nm using a microplate spectrophotometer.

Quantification of cccDNA

The isolation of HBV cccDNA, or recombinant covalently closed circular DNA (rcccDNA), involved a slightly modified Hirt procedure [25]. Briefly, the sample was diluted in a 10-mM Tris-HCl/10 mM EDTA buffer. SDS was added, and the mixture was stirred slowly at room temperature for 30 minutes to achieve cell cleavage. Subsequently, 1M NaCl was introduced, and the lysate was incubated at 4 °C overnight, followed by centrifugation at 4 °C and 14,500 g for 30 minutes. DNA in the supernatant underwent two extractions with phenol, one with phenol/chloroform/isoamyl alcohol (25:24:1), and then was precipitated overnight with ethanol. The DNA was centrifugally precipitated, washed with 70% ethanol, and dissolved in 10 mM Tris-HCl/1 mM EDTA buffer. Next, the extracted DNA underwent pre-digestion with Exo I and Exo III (NEB, California, USA). The resulting products were quantified using the TB Green® Premix Ex Taq™ II kit (Tli RNaseH Plus) (Takara, Kyoto, Japan).

For HepAD38 cccDNA, the forward primer was P5: 5′-TGCACTTCGCTTCACCT-3′, and the reverse primer was P6: 5′-AGGGGCATTTGGTGGTC-3′. The cccDNA amplification (735 bp) involved denaturation at 95 °C for 5 minutes, followed by 45 cycles of denaturation at 95 °C for 30 seconds, annealing at 62 °C for 25 seconds, and extension at 72 °C for 45 seconds. The relative cccDNA amount was determined using the $2^{-\Delta\Delta Cq}$ method, normalized to mitochondrial DNA COX-1 as an internal control.

In the rcccDNA model, primers P3: 5′-CAAGACAGGTTTAAGGAGAC-3′ and P4: 5′-GAGAGAAAGGCAAAGTGGAT-3′ were derived from the loxP-chimeric intron. The relative amount of rcccDNA in HepG2 cells was determined by the $2^{-\Delta\Delta Cq}$ method, normalized to the co-transfected EGFP plasmid.

Extraction and Reverse Transcription of HBV RNA

Total cellular RNA was extracted using TRIzol reagent (Invitrogen, California, USA). To achieve this, 1 mL of TRIzol Reagent was directly added to the 6-well plate containing the cells for cell lysis. After a 5-minute incubation, 0.2 mL of chloroform per 1 mL of TRIzol Reagent was added. The HBV RNA was quantified using a Nanodrop2000 (Thermo Fisher Scientific, Los Angeles, MA, USA), and subsequently, reverse transcription was performed using a Transcriptor First Strand cDNA Synthesis Kit (Takara, Kyoto, Japan).

Quantification of HBV RNA and HBV DNA

Following the manufacturer's guidelines, cDNA quantification was performed using the TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) kit (Takara, Kyoto, Japan). The PCR primers for HBV 3.5 kb RNA were HBV2268F: 5′-GAGTGTGGATTCGCACTCC-3′ and HBV2372R: 5′-GAGGCGAGGGAGTTCTTCT-3′. For HBV total RNA, the specific primers used were HBV1803F: 5′-TCACCAGCACCATGCAAC-3′ and HBV1872R: 5′-AAGCCACCCAAGGCACAG-3′.

The cDNA amplification protocols for HBV 3.5kb RNA and HBV total RNA involved denaturation at 95 °C for 30 seconds, followed by 40 cycles of denaturation at 95 °C for 5 seconds and 60 °C annealing for 30 seconds. Gene expression levels were determined using the $2^{-\Delta\Delta Cq}$ method, with values normalized to GAPDH expression as an internal control. For HBV DNA extracted from the supernatant, a TIANGEN DNA Blood Mini Kit (TIANGEN, BeiJing, China) was used, and qualification was carried out using the TB Green® Premix Ex Taq™ II (Tli RNaseH Plus) kit (Takara, Japan). The specific primers used for HBV DNA were HBV DNA-F: 5′-GAGTGTGGATTCGCACTCC-3′ and HBV DNA-R: 5′-GAGGCGAGGGAGTTCTTCT-3′.

Cell Counting Kit-8 Assay

For monitoring cell viability, the Cell Counting Kit-8 (CCK-8) from Dojindo Laboratories, Japan, was employed. Eight days post gRNA/Cas9 lentivirus infection, the culture medium was refreshed, and each well received 10 µL of the CCK-8 solution. The plate was then incubated at 37 °C for 4 hours, followed by measuring the absorbance at 450 nm using a microplate spectrophotometer.

Design of HBV-Targeting CRISPR/Cas9 System

Ten targeting sequences were carefully selected and incorporated into the lentiCRISPRv2 vector. These sequences were designed to maximize conservation across various HBV genotypes while minimizing homology to the human genome. Through rigorous screening, these sequences were demonstrated to be more efficient than other candidate gRNAs. The sequences and locations of the HBV-specific gRNAs are presented in Table 1 (Ref. [13,14,18–22]) and visually represented in Fig. 1a.

Table 1. Sequences of HBV-specific gRNAs.

Name	Targeting ORF	gRNA sequence (5′-3′)	Location
4 [20]	S/Pol	TATCGCTGGATGTGTCTG	368-385
10 [19]	PreC	GCTTGGAGGCTTGAACAGT	1859-1877
11 [19]	X/cp	GGAGGCTGTAGGCATAAAT	1776-1794
21 [14]	Pol/Xp	CTCTGCCGATCCATACTG	1256-1273
55	X/Pol	GCCGACGGGACGTAAACAA	1421-1439
60 [21]	PreC	AGCCTCCAAGCTGTGCCTT	1867-1885
69 [21]	Core	TTGACCCGTATAAAGAATT	1908-1926
159 [22]	X/Pol	GCCGTTCCGACCGACCACG	1503-1521
178 [13]	X/Pol	GGGCGCACCTCTCTTTACG	1521-1540
231 [18]	X/Pol	GGTCTCCATGCGACGTGCAG	1597-1616
GFP		ATGTGATCGCGCTTCTCGT	

The names of gRNAs were consistent with the original articles.
ORF, open reading frame; GFP, green fluorescent protein.

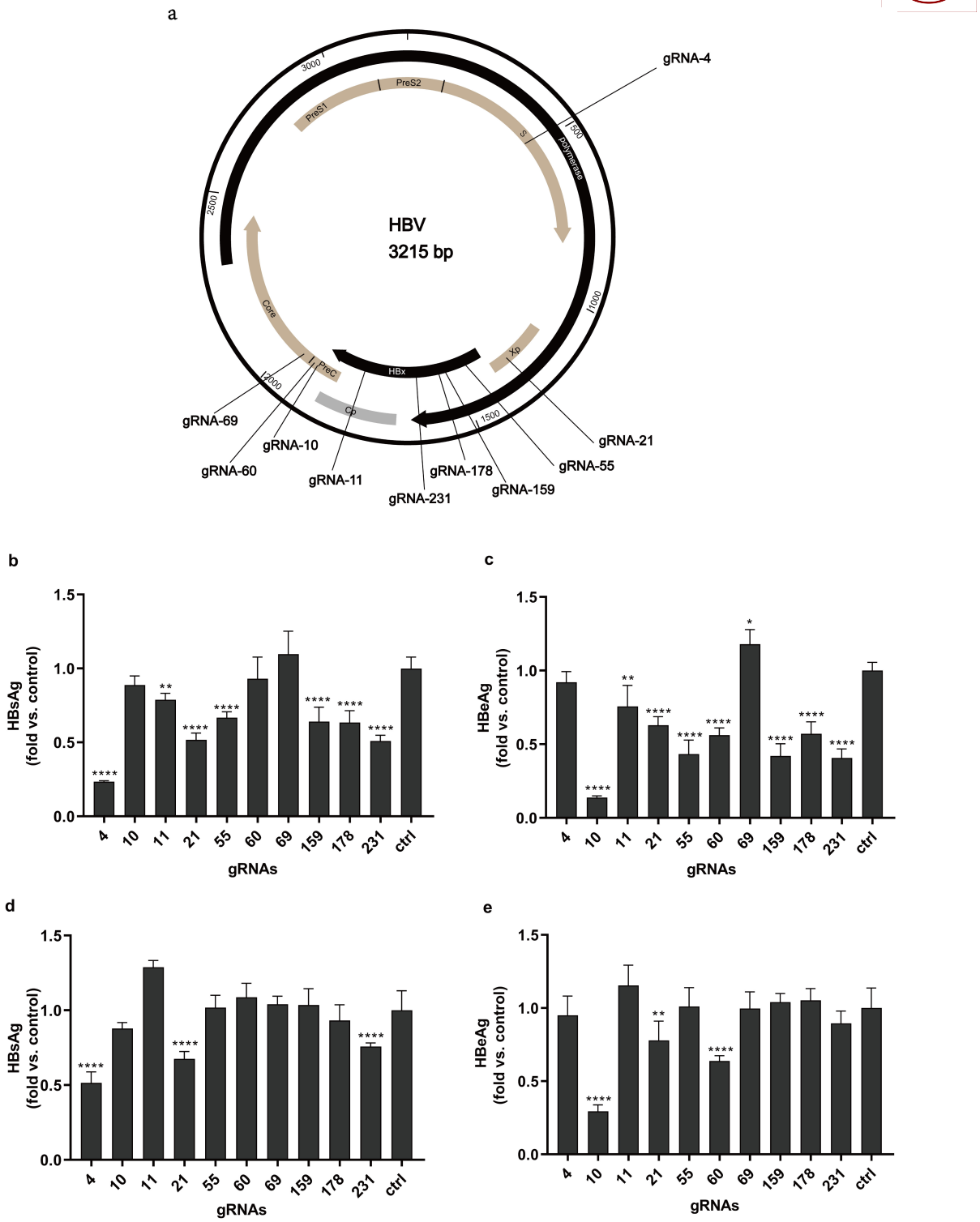

Fig. 1. Ability of clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 with Hepatitis B virus (HBV)-specific gRNAs to inhibit the HBV antigen. (a) Schematic illustration of the guide RNA (gRNA)-targeted sequences located in the HBV genome. (b) Specific gRNAs could reduce HBsAg levels in HepAD38 cells. (c) Specific gRNAs could reduce HBeAg levels in HepAD38 cells. (d) Specific gRNAs could reduce HBsAg levels in the rcccDNA model. (e) Specific gRNAs could reduce HBeAg levels in the rcccDNA model. The 10 gRNAs in the experimental group targeted different regions of the HBV genome. The gRNA of the control group was a non-HBV-related gRNA sequence, which did not target HBV. The same gRNA was used as a control group in Fig. 1b–e. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$.

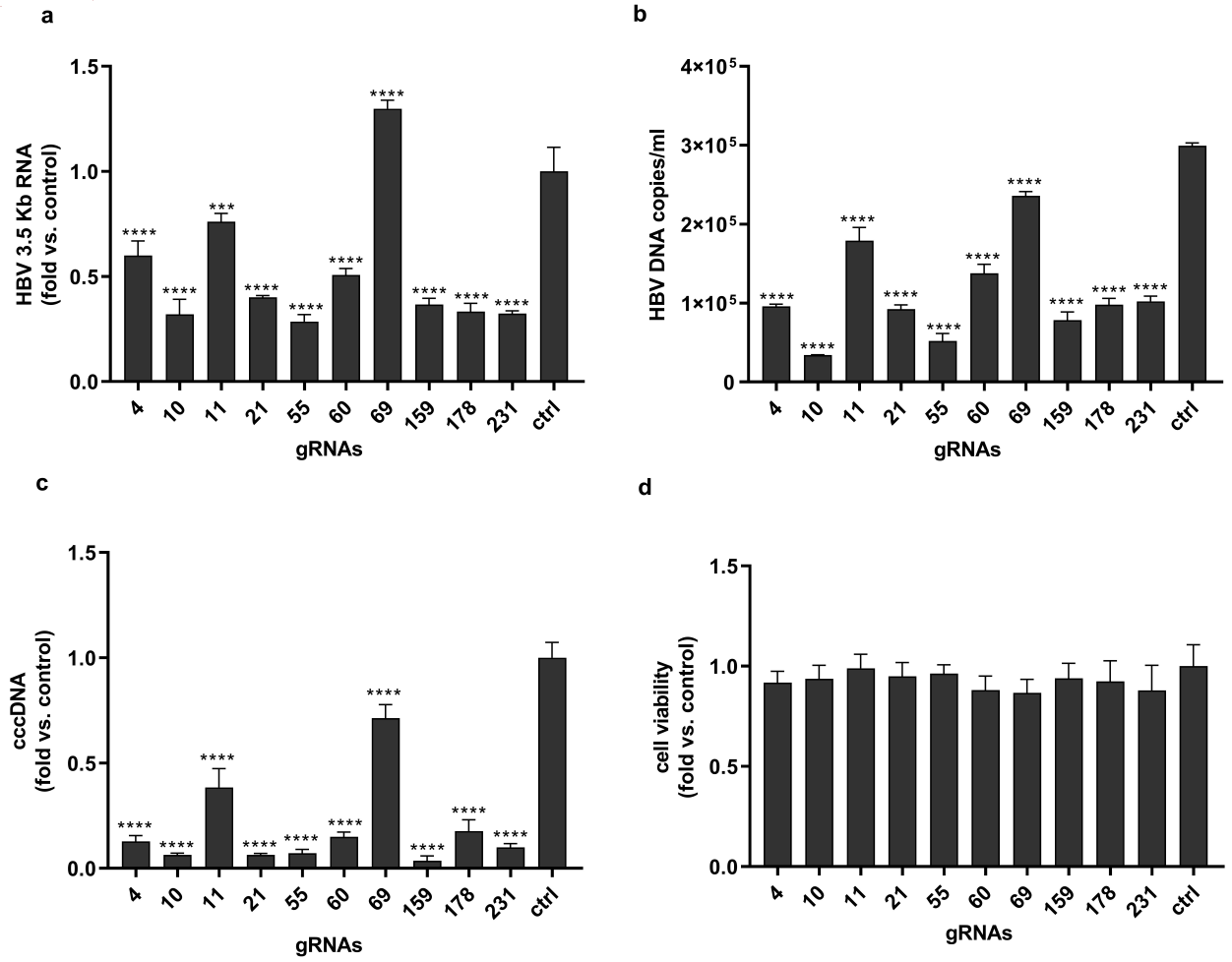

Fig. 2. Ability of clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 with HBV-specific gRNAs to inhibit HBV replication and reduce covalently closed circular DNA (cccDNA). (a) Specific gRNAs could reduce HBV 3.5 kb RNA levels in HepAD38 cells. (b) Specific gRNAs could reduce HBV DNA levels in HepAD38 cells. (c) Specific gRNAs could reduce cccDNA levels in HepAD38 cells. (d) CRISPR/Cas9 system with gRNAs had no significant effects on cellular proliferation. ***$p < 0.001$, ****$p < 0.0001$.

Recognizing the pivotal role of the X protein in sustaining HBV replication, six gRNAs were directed towards the X protein, overlapping the polymerase gene, or Cp promoter. Additionally, four gRNAs were targeted towards the PreC/Core ORF and preS1/S2/S ORF, respectively.

Statistical Analysis

All data are expressed as mean ± SD. Statistical analysis was performed using GraphPad Prism 7.04 (San Diego, CA, USA) software for a two-tailed independent Student's *T*-test. $p < 0.05$ was considered statistically significant.

Results

HBV-Specific CRISPR/Cas9 could Suppress the Expression of HBV Antigens

We assessed the anti-HBV effects of the reconstructed CRISPR/Cas9 in HepAD38 cells replicating HBV under doxycycline conditions. Upon removing doxycycline from the culture medium, the cells expressed viral pgRNA and formed cccDNA. To determine the ability of HBV-specific CRISPR/Cas9 to suppress the expression of HBV antigens, ELISA assays were performed using the supernatant of HepAD38 cells transduced with CRISPR/Cas9 lentivirus.

As depicted in Fig. 1b,c, six specific gRNAs (gRNA-11, 21, 55, 159, 178, and 231) exhibited the capacity to simultaneously reduce both HBsAg and HBeAg. Notably, gRNA-4 displayed the most significant effect in suppressing HBsAg, while not suppressing HBeAg. Conversely, gRNA-10 demonstrated the most pronounced reduction in HBeAg but had no effect on decreasing HBsAg. This discrepancy may be attributed to variations in the functional regions targeted by these HBV-specific gRNAs.

For the rcccDNA model, where rcccDNA served as a natural cccDNA surrogate, and HBsAg was expressed solely from rcccDNA produced by Cre/loxP-mediated recombination and subsequent RNA splicing [26], gRNAs-4, 21, and 231 were observed to significantly reduce HB-

sAg levels (Fig. 1d). In the same model, gRNAs-10, 21, and 60 demonstrated significant reduction in HBeAg levels (Fig. 1e).

CRISPR/Cas9 System with gRNAs could Inhibit HBV Replication

HBV 3.5kb RNA and HBV DNA serve as reliable indicators for HBV replication in HepAD38 cells. Therefore, we assessed the production of HBV 3.5kb RNA and HBV DNA in the supernatant of HepAD38 cells transduced with CRISPR/Cas9 lentivirus. Nine gRNAs (4, 10, 11, 21, 55, 60, 159, 178, and 231) demonstrated a significant reduction in HBV 3.5 kb RNA levels (Fig. 2a). While the difference was statistically significant, the ability of gRNA-11 to reduce HBV 3.5 kb RNA was weaker compared to the other eight gRNAs (4, 10, 21, 55, 60, 159, 178, and 231) (Fig. 2a).

All gRNAs (4, 10, 11, 21, 55, 60, 69, 159, 178, and 231) led to a significant decrease in HBV DNA levels in HepAD38 cells (Fig. 2b). Similarly, although the differences were statistically significant, gRNA-11 and gRNA-69 exhibited less ability to reduce HBV DNA levels compared to the other eight gRNAs (4, 10, 21, 55, 60, 159, 178, and 231) in HepAD38 cells (Fig. 2b).

All gRNAs (4, 10, 11, 21, 55, 60, 69, 159, 178, and 231) resulted in a significant decrease in cccDNA levels in HepAD38 cells (Fig. 2c). The CCK-8 assay indicated that the CRISPR/Cas9 system with gRNAs affected viral production but had no significant effects on cellular proliferation (Fig. 2d).

Conserveness Evaluation of HBV-Specific gRNAs Targeting Sequences

To assess the conserveness of HBV-specific gRNAs targeting sequences across known HBV variants, 9777 full-length HBV sequences spanning genotypes A~H and recombinant genotypes were retrieved from the HBV database [27]. As depicted in Fig. 3, gRNA-60 targeted the most highly conserved region of the HBV genome across all HBV genotypes. Similarly, gRNA-10 and gRNA-21 targeted highly conserved regions of the HBV genome. The conserveness of gRNA-10 targeting HBV sequences exceeded 92% among different HBV genotypes, except for a slightly lower conserveness (75.7%) in genotype A. For gRNA-21, the conserveness targeting HBV sequences was over 81.4% in all HBV genotypes and recombinant HBV strains. In addition to genotype F and genotype H, gRNA-4 and gRNA-178 also targeted highly conserved regions of the HBV genome (Fig. 3). In summary, five gRNAs targeting relatively highly conserved regions of the HBV genome were identified, namely gRNA-60, 10, 21, 4, and 178, indicating that the corresponding Cas9/gRNAs had a wide range of reactivity.

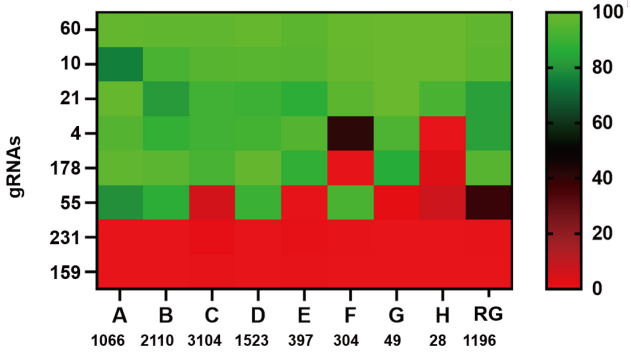

Fig. 3. Conserveness of the targeting sequences of HBV-specific gRNAs. Correspondingly, intensities of red and green indicate low and high rates of conserveness. The numbers at the bottom of the picture represent the number of HBV sequences for different genotypes.

Anti-HBV Effect of CRISPR/Cas9 with Dual gRNAs in HepAD38 Cells

As illustrated in Fig. 4a, the combination of gRNA-4 and gRNA-10 effectively reduced HBsAg to very low levels, even though CRISPR/Cas9 with gRNA-10 alone showed no significant effect on HBsAg. Moreover, the combination of gRNA-10 and gRNA-21 exhibited a synergistic effect on reducing HBsAg levels, significantly outperforming the individual use of these two gRNAs. Additionally, the combination of gRNA-4 and gRNA-21 (gRNA-4+gRNA-21) led to a greater reduction in HBsAg compared to gRNA-4 alone, although the difference was not statistically significant (Fig. 4a).

Similarly, gRNA-4+gRNA-21 demonstrated a synergistic effect in inhibiting HBeAg production (Fig. 4b). The optimal impact on inhibiting HBeAg production was achieved when combining gRNA-10 with gRNA-21, a result not attainable with the use of gRNA-4 or gRNA-21 alone (Fig. 4b).

In Fig. 4c, the synergistic effect of dual gRNAs on suppressing HBV replication was observed. The combination of gRNA-4 and gRNA-10 (gRNA-4+gRNA-10) synergistically reduced the level of HBV DNA in the supernatant of HepAD38 cells. Although the combination of gRNAs did not exhibit a synergistic effect in reducing cccDNA compared to gRNA-10 alone, the combination of gRNA-4+gRNA-10 and gRNA-10+gRNA-21 surpassed the efficacy of gRNA-4 or gRNA-21 used alone in reducing cccDNA (Fig. 4d). Fig. 4e,f demonstrated that the effect of gRNA-10 combined with other gRNAs was comparable to that of gRNA-10 used alone in inhibiting HBV total RNA and 3.5kb RNA.

Anti-HBV Effect of CRISPR/Cas9 with Dual gRNAs in HBV Infection HepG2-NTCP Cells

As depicted in Fig. 5a,b, both HBsAg and HBeAg decreased compared to the control group, indicating the

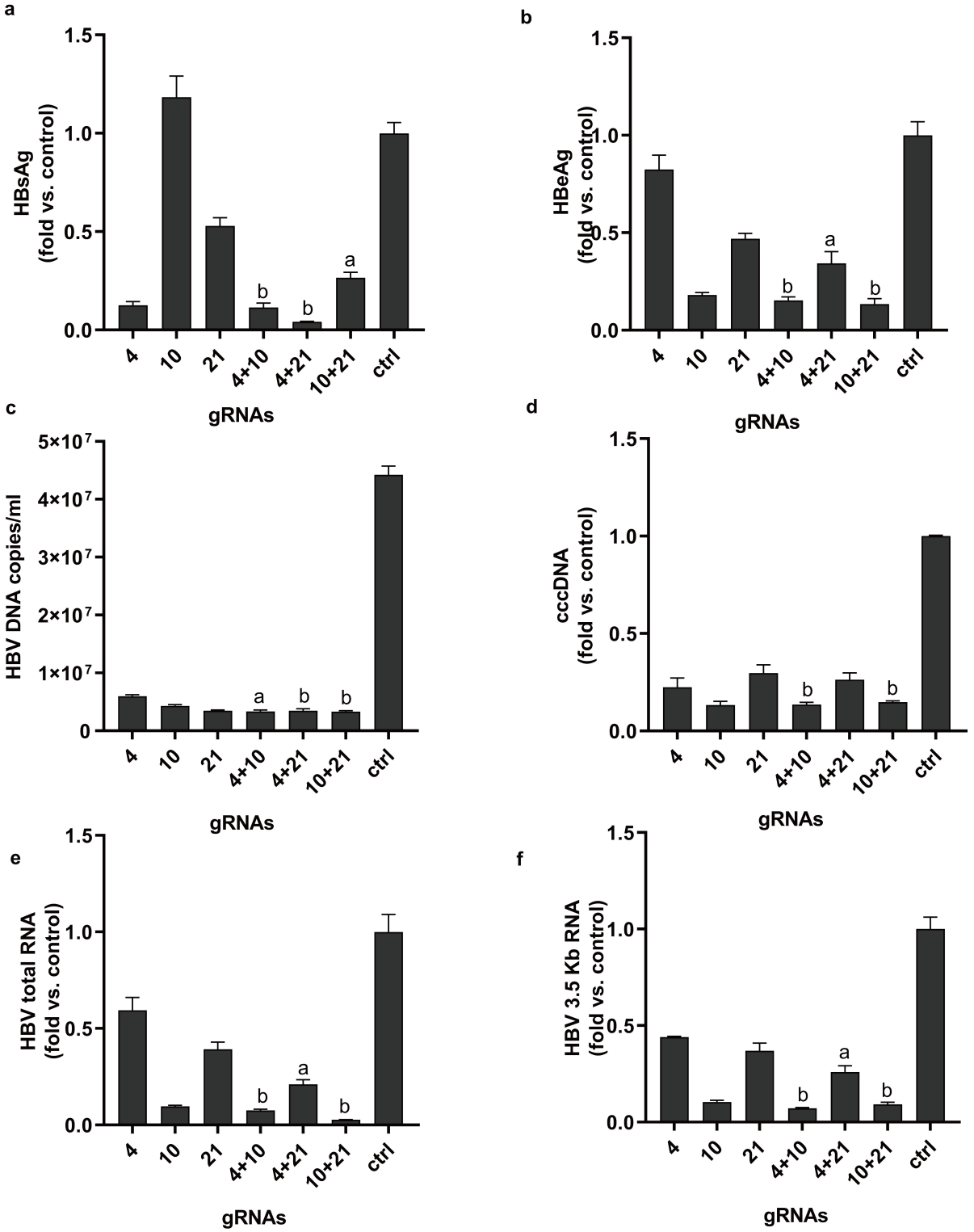

Fig. 4. Anti-HBV effect of CRISPR/Cas9 with dual gRNAs in HepAD38 cells. (a) The combination of two gRNAs had a synergistic effect on reducing HBsAg levels. (b) The combination of two gRNAs had a synergistic effect on reducing HBeAg levels. (c) The combination of two gRNAs had a synergistic effect on suppressing HBV replication. (d) The combination of two gRNAs had a synergistic effect on reducing cccDNA levels. The effect of gRNA-10 combined with other gRNA is the same as that of gRNA-10 used alone in inhibiting HBV total RNA (e) and HBV 3.5kb RNA (f). $^{a}p < 0.05$, $^{b}p < 0.01$. ^{a}p showed that the combination of two gRNAs had a significantly higher inhibition efficiency than that of a single gRNA, named a synergistic effect. ^{b}p showed that the combination of two gRNAs did not produce a synergistic effect.

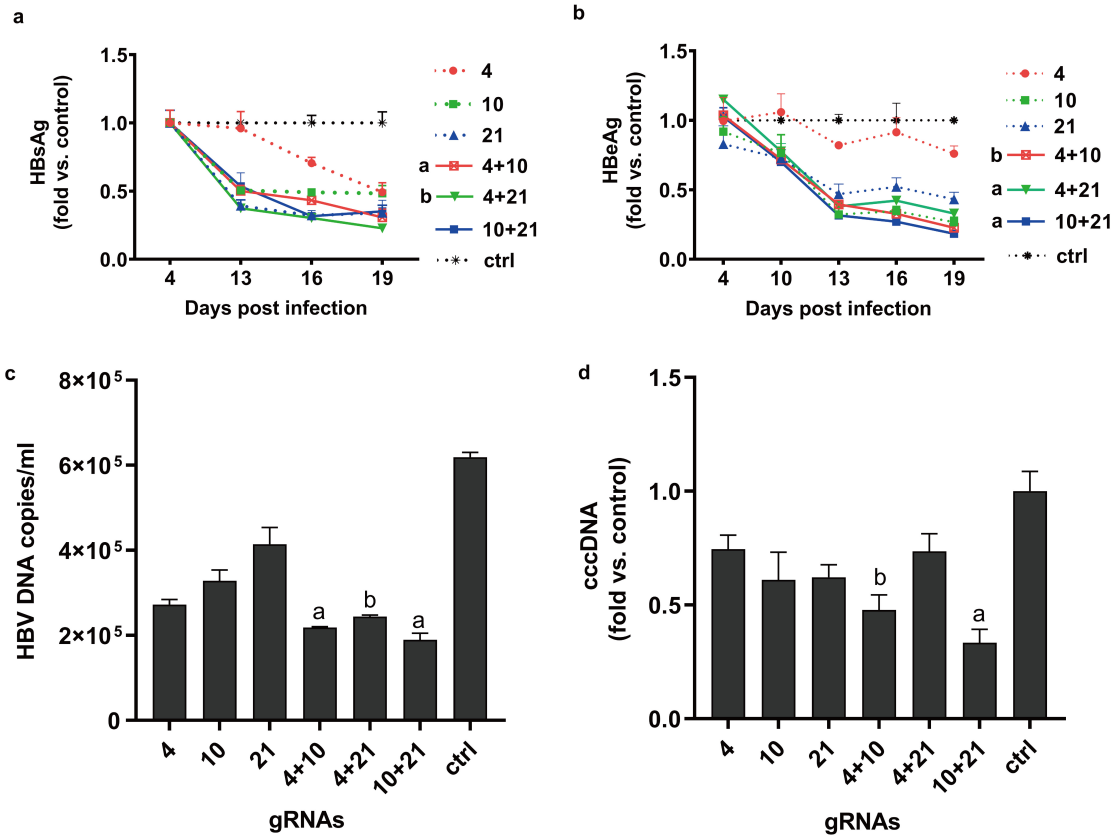

Fig. 5. Anti-HBV effect of CRISPR/Cas9 with dual gRNAs in HBV infection HepG2-NTCP cells. (a) The combination of two gRNAs displayed a synergistic effect on reducing HBsAg levels. (b) T combination of two gRNAs displayed a synergistic effect on reducing HBeAg levels. (c) The combination of two gRNAs displayed a synergistic effect on reducing HBV DNA levels. (d) The combination of two gRNAs displayed a synergistic effect on reducing cccDNA levels. ^{a}p showed that the combination of two gRNAs had a significantly higher inhibition efficiency than that of a single gRNA, named a synergistic effect. ^{b}p showed that the combination of two gRNAs did not produce a synergistic effect.

anti-HBV effect of CRISPR/Cas9. The combination of gRNA-4 and gRNA-10 exhibited a synergistic effect on reducing HBsAg levels compared to gRNA-4, gRNA-10, or gRNA-21 used alone (Fig. 5a). Similarly, the combination of gRNA-10 and gRNA-21 displayed a synergistic effect on reducing HBeAg levels compared to gRNA-10 or gRNA-21 used alone (Fig. 5b). In line with the results from the HepAD38 cell and the rcccDNA models, gRNA-10 demonstrated the most potent inhibition effect on HBeAg in the HBV infection HepG2-NTCP cell system. Although the combination of gRNA-4 and gRNA-10 did not show a synergistic effect in reducing HBeAg levels compared to gRNA-10, it was superior to gRNA-4 used alone (Fig. 5b).

Furthermore, we observed that the combination of gRNA-10 with either gRNA-4 or gRNA-21 resulted in a more significant decline in HBV DNA levels than a single gRNA (Fig. 5c). Additionally, the combination of gRNA-10 and gRNA-21 led to an almost 50% reduction in cccDNA compared to gRNA-10 or gRNA-21 used alone (Fig. 5d).

Discussion

Chronic HBV infection continues to pose a global health challenge. In recent years, therapeutic genome editing has emerged as a potential cure strategy for HBV infection. The development of CRISPR/Cas9 technology for antiviral therapies has garnered significant attention [28–30]. While CRISPR/Cas9 has demonstrated effective cleavage of HBV both *in vitro* and *in vivo*, further advancements are necessary to enhance its ability to inhibit HBV replication, reduce HBsAg and HBeAg, and clear cccDNA.

While gRNA-11 has shown significant effectiveness in reducing HBsAg and HBeAg in the HepAD38 cell line (Fig. 1b,c), its impact on reducing HBsAg and HBeAg in the rcccDNA model is not as pronounced (Fig. 1d,e). Consequently, gRNA-11 was not selected for combination. In the rcccDNA model, gRNA-21 demonstrated the ability to simultaneously reduce HBsAg and HBeAg levels (Fig. 1d,e). On the other hand, gRNA-4 and gRNA-231 could only reduce HBsAg levels, while gRNA-10 and gRNA-60 could solely reduce HBeAg levels (Fig. 1d,e). Therefore, gRNA-21 was chosen for combination.

In our exploration of strategies to enhance the antiviral effects of CRISPR/Cas9, we screened several gRNAs targeting different regions of the HBV genome, aiming to identify those that effectively inhibit HBV replication or reduce HBV antigen levels. Our findings revealed that gRNA-10, targeting the preCore ORF, demonstrated optimal effects in reducing cccDNA, inhibiting HBV replication, and decreasing HBeAg levels, albeit with no significant impact on reducing HBsAg levels. On the other hand, gRNA-4 exhibited the most pronounced inhibitory effect on HBsAg but proved ineffective in inhibiting HBV replication and reducing HBeAg levels. Notably, gRNA-21 showed a moderate impact on cccDNA, HBV replication, and antigen expression.

The observed differences in inhibitory effects could be attributed to two potential reasons: (1) The cleavage of cccDNA by the CRISPR/Cas9 system may lead to its inability to be repaired by non-homologous end joining (NHEJ), resulting in the destruction of cccDNA integrity [31]. (2) Some cccDNAs might undergo mutations at the cleavage site during NHEJ repair, potentially influencing transcription or specific antigen expression. Consequently, the inhibitory efficiency of different gRNAs varies depending on the functional regions they target.

A crucial factor in the development of CRISPR/Cas9-based anti-HBV therapeutics is the conservation of target regions within the HBV genome. In our study, the targeted areas of the five selected gRNAs (60, 21, 10, 4, 178) were found to be highly conserved across HBV genotypes A–H (Fig. 3). Beyond genotypic variations, the absence of proofreading functionality in the viral reverse transcriptase and a high replication rate contribute to the presence of genetically distinct yet closely related variants within individual HBV populations [32].

These variants may introduce mismatches between designed gRNAs and the targeted HBV sequences. However, our study revealed that gRNA-4, gRNA-10, and gRNA-21 could effectively recognize nearly all HBV variants tested. Furthermore, the combination of these gRNAs could potentially broaden the range of HBV sequences targeted by the CRISPR/Cas9 system with dual gRNAs. This approach may help mitigate the emergence of HBV quasispecies that could evade the CRISPR/Cas9 treatment.

Several studies have explored the potential of CRISPR/Cas9 in combating HBV infection, showcasing various strategies to enhance its effectiveness. Chen *et al*. [33] demonstrated that gRNA and Cas9 could be transported via endogenous exosomes from CRISPR/Cas9-expressing cells, acting as vectors to deliver functional Cas9 and HBV-specific gRNA to cleave HBV DNA in HuH7 cells. Zhang *et al*. [34] showed that gRNA5 and/or gRNA9 RNPs significantly reduced HBV cccDNA, HBV total DNA, pre-genomic RNA, HBsAg, and HBeAg levels. Additionally, Wang *et al*. [19] reported the greater inhibition efficacy of dual gRNAs on HBsAg and HBeAg compared to single gRNA alone. Consistent with these findings, our study also demonstrated the superior effectiveness of CRISPR/Cas9 with dual gRNAs compared to single gRNA. Specifically, the combination of gRNA-4 and gRNA-10 achieved a simultaneous reduction of approximately 85% in HBsAg and HBeAg levels, synergistically decreased HBV DNA levels, and significantly reduced cccDNA levels. In contrast, gRNA-4 or gRNA-10 alone could not achieve the same reduction in all these virological indicators. In the HepG2-NTCP cell infection model, the combination of gRNA-10 and gRNA-21 exhibited a significant synergistic effect on reducing HBV indicators, particularly cccDNA. This synergistic effect is likely attributed to the cumulative anti-HBV effects of gRNA-10 and gRNA-21.

Ensuring the safety of the CRISPR/Cas9 system requires consideration of potential off-target effects. A study conducted whole-genome sequencing on liver genomic DNA from chimeric mice treated with AAV2-Cas9/gRNA10, revealing no specific insertion/deletion variations [35]. Similarly, next-generation sequencing demonstrated the absence of indel formations at eight gRNA-21 off-target sites after the constitutive expression of CRISPR/Cas9 and gRNA-21 in HepG2.2.15 cells for over four weeks [14]. Although no evidence of off-target effects has been observed, conducting a comprehensive genome-wide analysis of potential off-target effects for the CRISPR/Cas9 system with dual gRNAs is essential. This analysis is particularly important due to the low homology between viral and human genomic Cas9 targets.

This study has some limitations that warrant acknowledgment.

(1) While our results indicate that CRISPR/Cas9 with dual gRNAs may reduce cccDNA levels, it is crucial to delve further into the fate of the HBV mini-chromosome post-gene editing. A study reported that dual gRNAs could cleave cccDNA into two fragments, giving rise to two novel viral episomes that remain transcriptionally active [36]. Further investigation is needed to understand the dynamics and consequences of cccDNA editing in greater detail. (2) The delivery of CRISPR/Cas9 with dual gRNAs could be explored using different modalities, such as RNPs or mRNA delivery methods [37,38]. This avenue is worth exploring in primary human hepatocytes and liver-humanized mice models [39] to better understand the applicability and efficiency of the approach in more complex and relevant biological systems.

Conclusions

To summarize, our study demonstrated that CRISPR/Cas9 with dual gRNAs effectively inhibited HBV replication and reduced levels of HBsAg, HBeAg, and cccDNA in both HBV-stable cell lines HepAD38 and HBV infection models of HepG2-NTCP cells. Specifically, combining gRNA-10 with either gRNA-4 or gRNA-21 in the CRISPR/Cas9 system showed enhanced efficacy in inhibiting HBV infection, reducing HBsAg and HBeAg

levels, and promoting HBsAg clearance. This suggests that the CRISPR/Cas9 system with dual gRNAs holds promise as a valuable tool for eradicating HBV, offering a potential new method for achieving the functional cure of patients with CHB.

Availability of Data and Materials

We declared that materials described in the manuscript, including all relevant raw data, will be freely available to any scientist wishing to use them for non-commercial purposes, without breaching participant confidentiality. The supporting data can be accessed from the corresponding authors.

Author Contributions

Study concept and design: YH, QL, ST, and LC. LF, QY, and SS executed experiments, analyzed data, and drafted the manuscript. Critical revision of the manuscript: QL, ST, and LC. All authors contributed to significant editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

The Clinical Research Ethics Committee of Shanghai Public Health Clinical Center has confirmed that no ethical approval is required for this study. This study did not involve any human subjects, and there was no need to provide consent to participate.

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Conflict of Interest

The authors declare no conflict of interest.

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