Disrupting HBV Persistence: Genetic Engineering Approach to Break the Oncogenic HBx Gene

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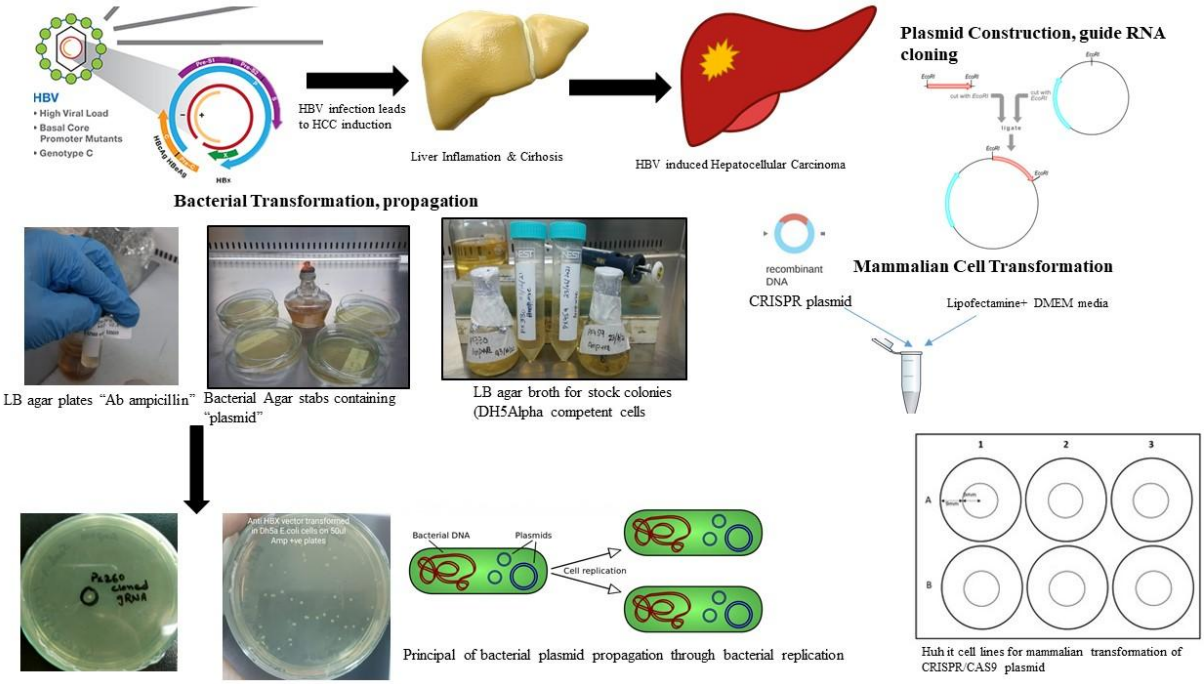
Abstract

Chronic Hepatitis B virus (HBV) infection can cause liver cirrhosis or cancer, making it a persistent worldwide health concern. Nucleoside analogs used in current antiviral treatments can prevent HBV from replicating, but they do not break the covalently closed circular DNA that HBV currently possesses. This study presents the state-of-art-technology as an alternative treatment option to control the disease. Using the program CRISPR RGEN (http://www.rgenome.net/) the gRNAs were designed and cloned insilco in mammalian expression (vector px260, Addgene) and were later sent for commercial synthesis to be synthesized and cloned in the px260 vector. Propagated in E. coli DH5 alpha cells, the custom cloned vector construct was isolated and visualized using gel electrophoresis. Cell culture-based testing performed at the Hepatitis Laboratory (University Airrlanga Indonesia). Huhit cells were utilized for lipofectamine mediated transfection. However, after successful transfection, confirmation was done through PCR and Sanger sequencing. An efficient plasmid based CRISPR/CAS9 vector has been designed and constructed successfully to target HBx gene in liver cells. Plasmid´s, cloning propagation, isolation and confirmation were done in bacterial cells (selected through antibiotic). The initial phase of testing on Huhit cells was performed. However further validation experiments on HepG2 cells and mice models are the prospects based on positive outcome of previous testing. The goal of this planned study is to use CRISPR/cas9 genome editing technology therapeutically targeting oncogenic Hbx gene of HBV.Which could speed up the development of anti-HBV CRISPR/Cas 9 based therapies in conjunction with currently available treatments (NAs).

Keywords: CRISPR/Cas9, HBV, HBX, IFN, Anti-HB.



Graphical Abstract

Introduction

Across the world, the leading cause of liver disease is chronic HBV infection. In the World Health Organization's 2018 report on worldwide cancer statistics, liver cancer ranked sixth worldwide and is the fourth-leading cause of cancer-related fatalities[1]. Despite the development of effective vaccines, HBV infection is still a major issue of global public health. A recent survey estimated that 240 million persons are chronically infected by HBV, a sizable portion of whom require antiviral therapy to prevent the long-term detrimental consequences, including cirrhosis and HCC [2, 3]. Currently, two types of antiviral therapy, interferon-alpha (IFN- α) and nucleos(t)ide analogue (NA), are available for chronic hepatitis B (CHB) [3]. IFN acts through both direct antiviral and indirect immunomodulatory effects, while NA suppresses viral replication by inhibiting the viral polymerase. Although effective, neither of them has satisfactory sustained off-therapy responses. Most patients suffer from rebound viremia and relapsing hepatitis after the cessation of antiviral therapy and often must receive life-long treatment. Long-term antiviral therapy not only has a potential risk for the emergence of drug resistance but also imposes a high socio-economic burden in HBV endemic countries. As a result, a novel therapy for curing chronic HBV infection is still in pressing need.[4]

Methodology

In-silico guide RNA Design

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An online webtool CRISPR RGEN (http://www.rgenome.net/) was used to design gRNAs against *HBV X* gene (HBV ayw strain). Table 1 shown below gives the sequence of 2 guide RNA sequences, designed and used in the study, along with the PAM regions.

Table 1. Sequence of guide RNAs.

Sr. No	Name	Sequence
1	gRNA 1	5'AAACAAAGGACGTCCCGCGCAG G3'
2	gRNA 2	5'CGCCGACGGGACGTAAACAAAG G3'

Sequence in red=PAM sequence

The plasmids were received in the form of agar stabs (figure 1). Following the Add gene instructions, those were temporarily stored at 4 ºC. Bacterial inoculum utilized for liquid cultures are shown below in Figure 2.

Plasmid-Design & Construction of anti-HBV CRISPR/CAS9 system

Gene sequence of *HBX* (Gene ID: 944566) and HBV genome sequence (NCBI Reference Sequence: NC_003977.2) were downloaded from NCBI. *HBX* gene sequence was selected for finding suitable CRISPR for which potential 20-base sequences on the HBV were searched by using CRISPR RGEN web tool which is suitable and most used tool used for this purpose. Two suitable targets **(20 nt followed by a PAM sequence)** were selected and it was ensured that these have fewest potential off-target matches throughout the selected host genome [5].

In-silico cloning of gRNA in PX260 (empty Vector)

Both the selected gRNAs were cloned in empty vector PX260. The *in-silico* software "snapgene" was used to clone gRNA (figure 3). The empty vector sequence was opened in the snapgene, Restriction cloning option was selected then gRNA was inserted in the given space and cloned at Bbs1 sites right after the U6 promoter. The cloned sequence was retrieved shown in Figure 4. The sequence was then sent for commercial synthesis to get cloned vector for further experiments.

DNA-custom cloning

Plasmid sequence construct with cloned guide RNA sequence (*in-silico* cloning) was sent for synthesis to "DNA custom cloning" company (USA).

Bacterial Culturing on Agar Plates

The plasmid received was cloned in the bacteria spread on agar plates. Single colony of those bacteria was selected and inoculated in 1.5 mL of LB(liquid) media containing 50 µL ampicillin. The culture was incubated overnight at 37℃ in a shaking incubator (figure 4**).** The LB agar plates having ampicillin selection were prepared. Overnight grown culture was taken and spread on ampicillin positive LB agar plates with the help of a sterilized loop. The streaking was done in the form of 4-quadrant. The streaked plate was incubated overnight culturing at 37ºC.

Plasmid Propagation in E. coli (DH5alpha)

Bacterial Transformation

The concentrated plasmid was then propagated in bacterial cells. Heat-shock transformation method was used to transform the competent cells.

Heat-shock Transformation

The already prepared competent cells (50 µL) were taken from the repository within a 1.5 mL tube. Total 5 µL plasmid was taken to transform those competent cells and was mixed gently in the competent cells followed by incubation on ice for 40 mins. Subsequently the mixture was subjected to heat shock, given at 42 ºC for 90 secs and was again placed on ice for 5 min. Later, 800 µL of LB broth was added to it, mixed well and incubated at 37C for an hour. Finally, 200 µL of transformed cells were spread on LB agar plate (Amp +ve). The ampicillin negative LB agar plates were used as control. The plates were then left for Incubation at 37 ºC overnight. Next day, a few isolated colonies were observed on the agar plate as shown in Figure 5. Some of those were selected randomly and used for further confirmation analysis.

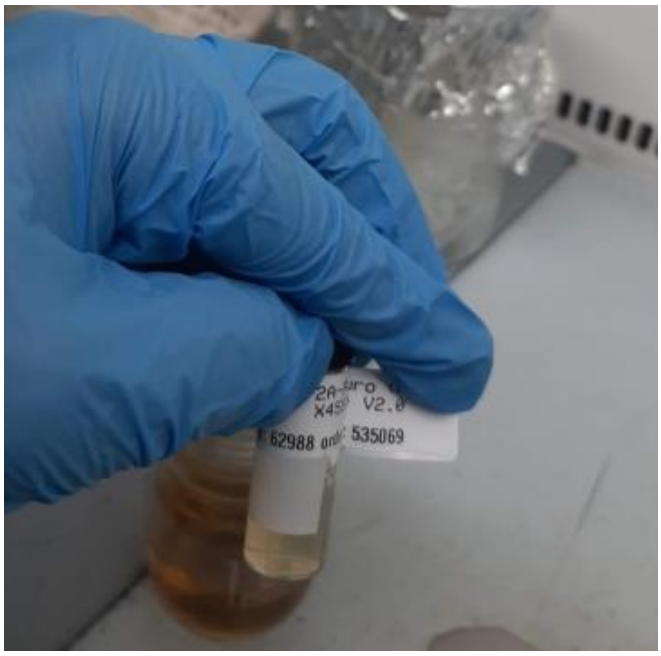

Figure 1. Plasmids received in transformed DH5alpha (Agar Stabs).

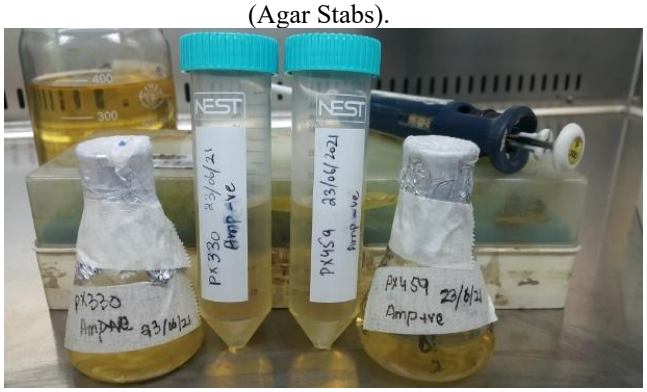

Figure 2. Bacterial Inoculum in LB Broth.

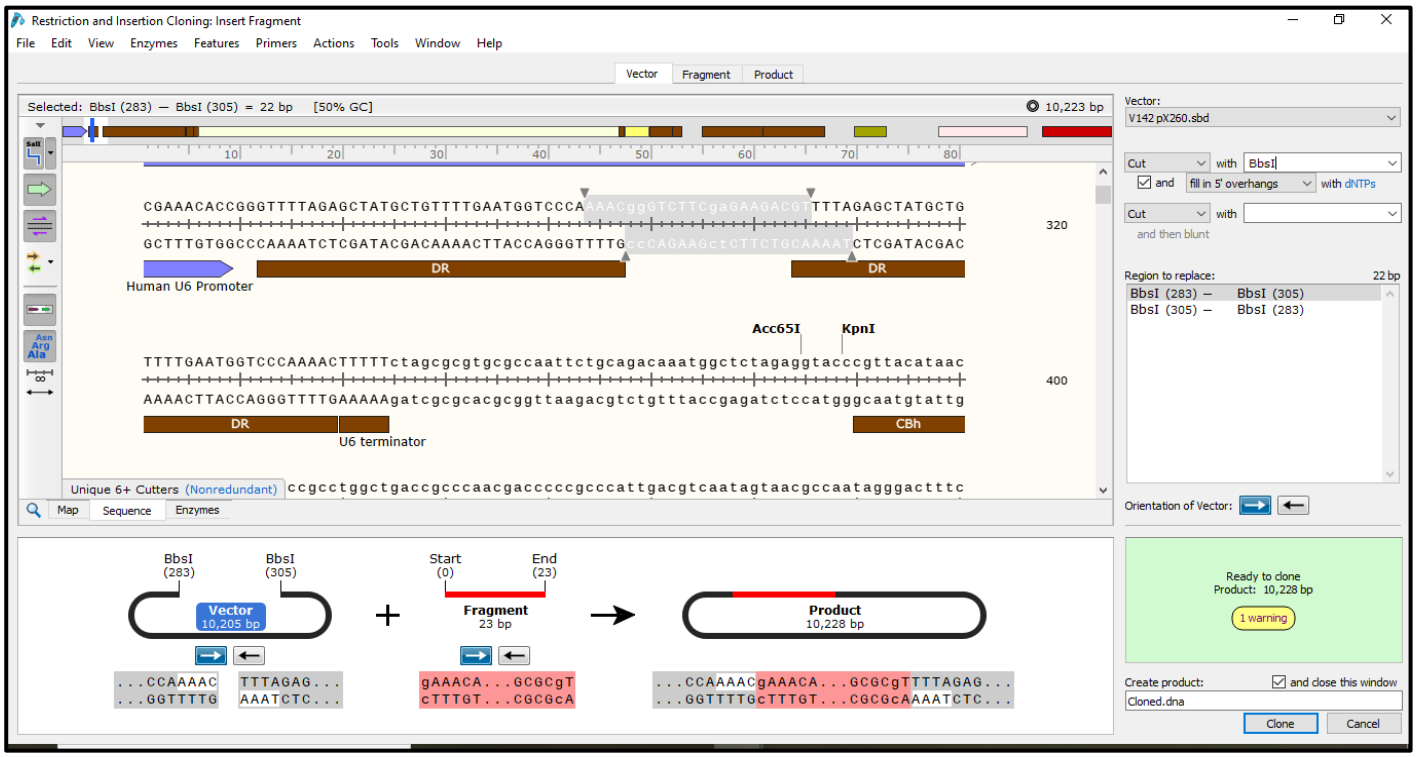

Figure 3. *In-silico* cloning reaction performed by restriction digestion at BbsI site and cloning of 20bp guide RNA.

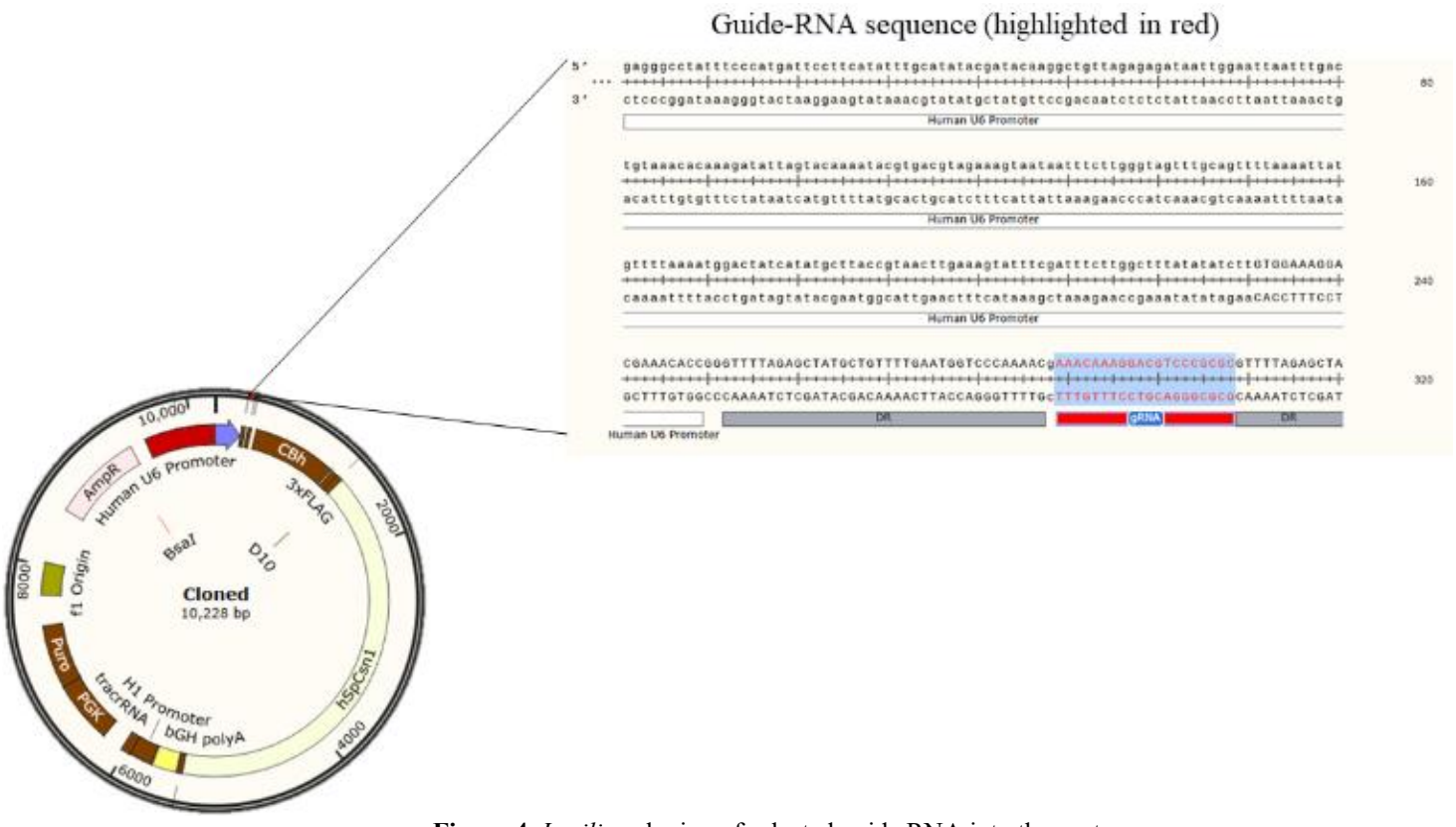

Figure 4. *In silico* cloning of selected guide RNA into the vector.

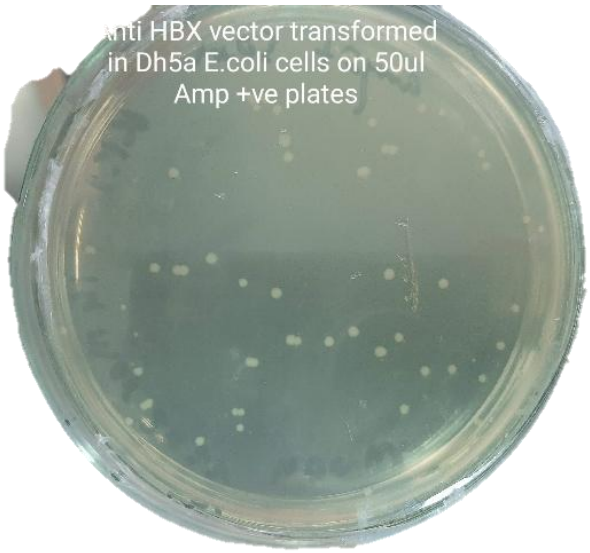

Figure 5. Bacterial colonies recovered on agar plates (ampicillin +ve).

Cell culture testing on Huh-7it cells

Huhit cells cultured in 5 mL media containing DMEM media, 10% FBS (50ml), 1% essential amino acid, antibiotic (streptomycin). After 24 hours the cultures were observed continuously for confluency until 80-90% confluent culture was obtained. For cell splitting, the media was discarded and the cells were washed with PBS (10 mL) twice. Then 1 mL of trypsin was added and mixture was incubated for 4 min at 37 ºC. DMEM (8mL) was added and again subjected to centrifugation at 1200 rpm for 4 min. After cells transfection, supernatant was collected at 24hr and 48hr stage and the media was changed. The supernatant was collected to determine the protein expression by the transformed cells.

Lipofectamine mediated Transfection (Protocol)

Plasmid transfections in Huh7it cells were seeded in the 24 well plate (without antibiotic) at $6x10^4$ cells/ml, left in incubator for 12-24hr. The wells were washed with 1xPBS (500 µL) and about 500 µL fresh serum free media (Opti MEM) was added. The plasmid (1 µL) was diluted in 250 µL Opti MEM media. (At the same time 2 µL of lipofectamine was diluted by addition of 250 µL Opti MEM). The mixture was incubated at room temperature for 5 mins. Mix both the dilutions (plasmid+lipofectamine diluted in OptiMEM) drop by drop. Later, 500 µL DMEM media was added to this mixture for transfection purpose drop wise to the cell. The mixture was shaken well by hand (swirl) before subjecting to overnight incubation. The media was changed the next day (after 24 hr). The cells were observed for any damaging effects or stress induced by transfection under microscope.

Sequence Analysis by Sanger sequencing

After plasmid extraction, sanger sequencing was performed using guide RNA specific primers (forward and reverse) for confirming the plasmid transfection into Huhit7 cells. Applied Bio system (AB sequencing) was used utilized for this purpose.

Results

Plasmid Isolation and Confirmation

The extracted plasmid was then run on agarose gel. The gel was visualized under gel doc (UV Illuminator) (Figure 6).

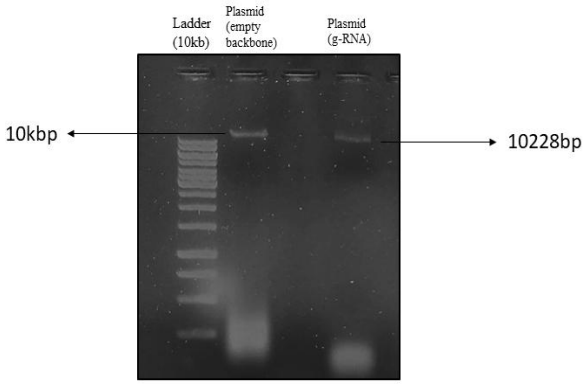

Figure 6. Gel electrophoresis image of Isolated plasmid PX260 (cloned vector), size 10,228bp shown with 10kb ladder.

Plasmid Quantification through Nano drop

The concentration of the plasmid was checked using nano-drop. About 1 µL of TE buffer was used as a control. 1 µL of extracted sample was poured on the optical fibers of the nano-drop machine. The concentrations of the plasmid are as shown in Table 2

Table 2: Nanodrop values for plasmid.

Plasmid	Concentration (ng / µL)	A260/280	A260/230
PX260 (Empty Vector)	36.59	1.80	1.92
PX260 (Cloned Vector)	62.85	1.88	1.65

Potential Off-Target Analysis

The potential off-target analysis performed using (www.rgenome.net/). There was no off target obtained. The guide RNA sequence was used without PAM sequence, for off target prediction The screen-shot of the analysis shown below in Figure 7.

Lipofectamine mediated Transfection

Lipofectamine mediated transfection was carried out in Huhit cells in six well plate seeded. While Figure 8a, b gives the image for cell counting done on a cell counter grid

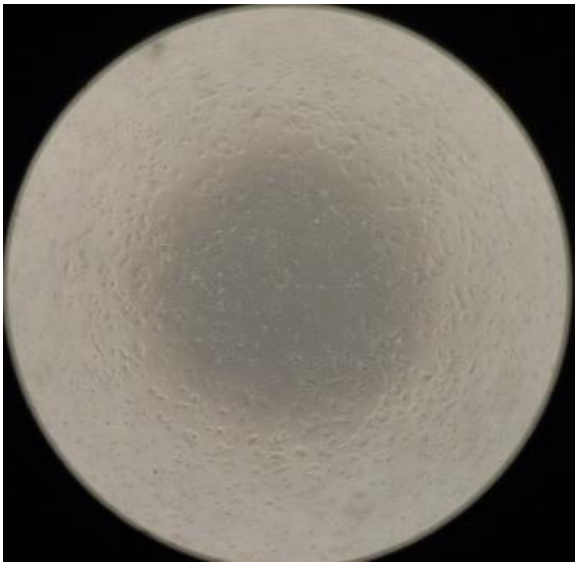

Figure 8(a). Microscopic image of Huh7 it cells

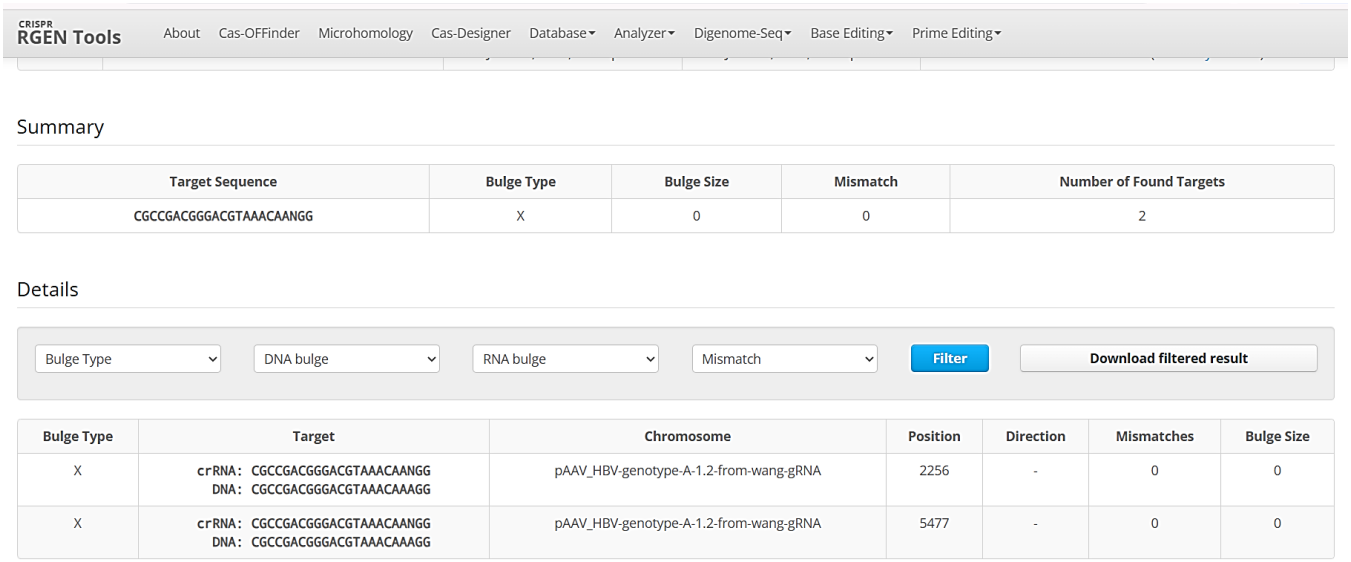

Figure 7. Potential off targets obtained, after RGEN tool for CRISPR-guide RNA analysis.

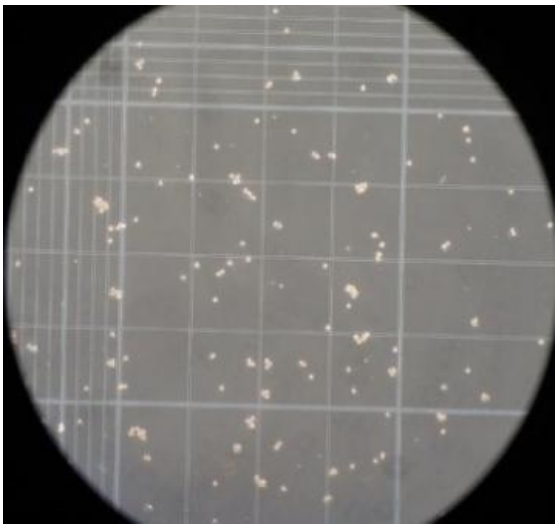

Figure 8(b). Huh7it cells counting (seeded six well) of cells in 6 well plate.

RNA extraction from supernatant

Total RNA extraction was performed using total RNA extraction kit (RNeasy Kit QIAGE, Cat no. 74104). The extracted RNA proceeded for cDNA synthesis using random primers. Figure 10 below gel electrophoresis image. For monitoring the quantity and quality of RNA extracted from cells nanodrop values are given below in table 3. However, figure 9 below indicates the gel electrophoresis image after c-DNA synthesis (table 4) PCR reaction mix are provided as follows.

Table 3. Nanodrop values for the quantity of RNA extracted from transfected cells

Vector	Quantity	A260/280	A260
KKV(cloned vector)	73.9ng/ul	2.56	1.84
KKV(cloned vector)	25ng/ul	2.55	0.68
VO (empty vector)	31.6ng/ul	2.49	0.79
Control (1)	72.2ng/ul	2.70	1.8
Control (2)	42.9ng/ul	2.23	1.07

Table 4. cDNA synthesis PCR reaction mix along with quantities.

Reagents	Quantity
DNA remover	2ul
Random primers	1ul
RT mastermix	2ul
Sample	7ul
Total reaction volume	12ul

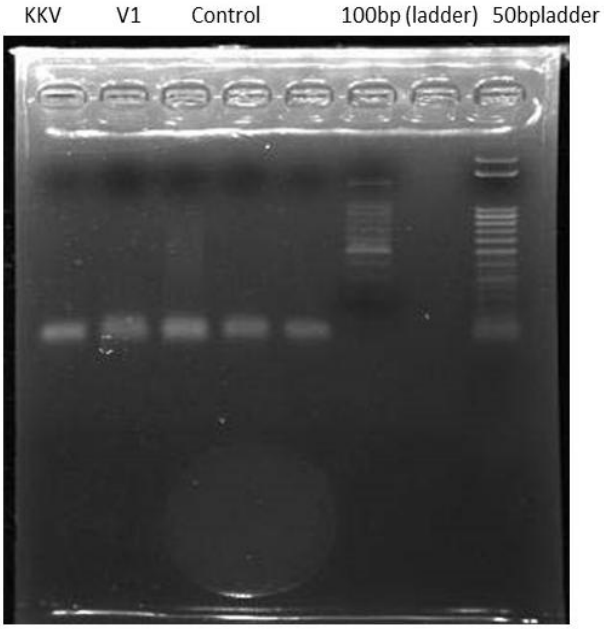

Figure 9. Gel electrophoresis image of cDNA synthesis PCR product (1%agrose gel).

Sequencing Confirmation- (Sanger sequencing)

The sequencing was performed with specific g-RNA primers in order to confirm the successful transfection of plasmids inside mammalian cells. The transfection confirmation is done through plasmid isolation after 48hr of Lipofectamine mediated transfection and further pursued with sequencing analysis shown below (Figure 10)

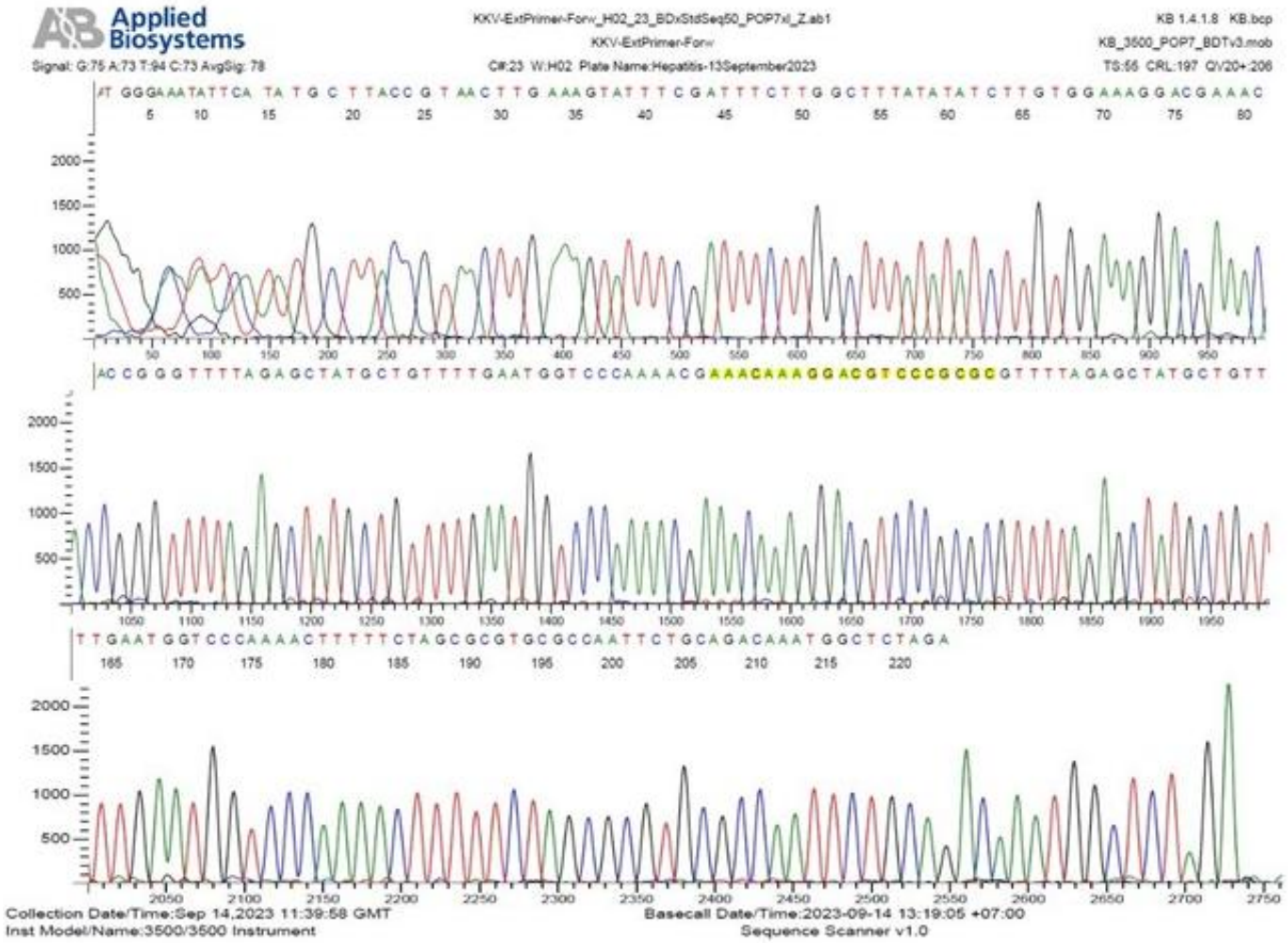

Figure 10. Sanger sequence analysis performed for plasmid guide RNA sequence confirmation. The highlighted sequence is the guide RNA.

Discussion

Over 350 million individuals are chronically infected with HBV, and about 2 billion people have had an HBV infection in the past. Population has been divided according to three endemicity levels; low (greater than 2%), intermediate (from 2% to 7%), and high endemicity (less than equal to 8%).[6] In 2015, approximately, new 1.75 million HCV cases were reported [7]. With 9 million people affected, hepatitis B virus infection is widely reported in Pakistan. Lack of sufficient healthcare facilities and public knowledge are to blame for its rise in Pakistan. In Pakistan, the carrier rate is 3% to 5%. Pakistan has a prevalence of roughly 63.71% of genotype D [8]. Currently, there are two antiviral treatment options for chronic hepatitis B (CHB): nucleos(t)ide analogue (NA) and interferon-alpha (IFN-α) [3]. Despite their effectiveness, neither of them has long-lasting, satisfying off-therapy reactions. However more efficient delivery system like nanoparticles based delivery of therapies are the key area to be explored. Viral vectors have been widely used in recent years to transport CRISPR cassettes both in vitro and in vivo,[9] primarily because of their high gene delivery efficiency and steady transgene expression over the long term[10-12]. The varied tissue tropism of AAV serotypes, in particular, allows AAV to be flexibly built to target particular organs or tissues [13, 14]. Ramanan et al, in 2015 demonstrated that two of the sgRNA/Cas9 combinations showed decreased production of pgRNA and HBV viral antigens. They infected the cultured NTCP-expressing HepG2 cells with patient-derived HBV. The effect of two pairs of sgRNA and cas9 was observed and the results showed reduced productive infection and HBV replication. After hydrodynamic injection of mice with the pairs, robust reduction in circulating viral particles by up to 95% of the controls were observed. Production of viral mRNA and secretion of HBeAg was also observed to be reduced by more than 50% [15].

Acknowledgments

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References

1. Lv, W., et al., The Application of the CRISPR/Cas9 System in the Treatment of Hepatitis B Liver Cancer. Technology in Cancer Research & Treatment, 2021. 20: p. 15330338211045206.
2. Schweitzer, A., et al., Estimations of worldwide prevalence of chronic hepatitis B virus infection: a systematic review of data published between 1965 and 2013. The Lancet, 2015. 386(10003): p. 1546-1555.

3. Yang, H.-C. and J.-H. Kao, Revisiting the natural history of chronic HBV infection. Current Hepatology Reports, 2016. 15(3): p. 141-149.
4. Yang, H.-C. and P.-J. Chen, The potential and challenges of CRISPR-Cas in eradication of hepatitis B virus covalently closed circular DNA. Virus research, 2018. 244: p. 304-310.
5. Hsu, P.D., E.S. Lander, and F. Zhang, Development and applications of CRISPR-Cas9 for genome engineering. Cell, 2014. 157(6): p. 1262-1278.
6. Te, H.S. and D.M. Jensen, Epidemiology of hepatitis B and C viruses: a global overview. Clinics in liver disease, 2010. 14(1): p. 1-21.
7. Blach, S., et al., Global prevalence and genotype distribution of hepatitis C virus infection in 2015: a modelling study. The lancet Gastroenterology & hepatology, 2017. 2(3): p. 161-176.
8. Idrees, M., S. Khan, and S. Riazuddin, Common genotypes of hepatitis B virus. Journal of the College of Physicians and Surgeons--pakistan: JCPSP, 2004. 14(6): p. 344-347.
9. Li, L., S. Hu, and X. Chen, Non-viral delivery systems for CRISPR/Cas9-based genome editing: Challenges and opportunities. Biomaterials, 2018. 171: p. 207-218.
10. Heckl, D., et al., Generation of mouse models of myeloid malignancy with combinatorial genetic lesions using CRISPR-Cas9 genome editing. Nature biotechnology, 2014. 32(9): p. 941-946.
11. Yu, W., et al., Nrl knockdown by AAV-delivered CRISPR/Cas9 prevents retinal degeneration in mice. Nature communications, 2017. 8(1): p. 14716.
12. Shalem, O., et al., Genome-scale CRISPR-Cas9 knockout screening in human cells. Science, 2014. 343(6166): p. 84-87.
13. Zincarelli, C., et al., Analysis of AAV serotypes 1–9 mediated gene expression and tropism in mice after systemic injection. Molecular therapy, 2008. 16(6): p. 1073-1080.
14. JM, G.G.V.L.W., New recombinant serotypes of AAV vectorsCurr. Gene Ther, 2005: p. 5285-297.
15. Ramanan, V., et al., CRISPR/Cas9 cleavage of viral DNA efficiently suppresses hepatitis B virus. Scientific reports, 2015. 5(1): p. 1-9.