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INHIBITIONS OF HEPATITIS C VIRUS REPLICATION WITH THE HELP OF CRISPR/CAS 9 TECHNOLOGY

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Abstract:
Hepatitis c infection caused by Hepatitis c virus (HBV) is a major world-wide health problem. Current therapeutic strategies rarely eradicate HBV infections and fail to attain complete cure. There is urgent need to develop advanced treatment strategies to successfully remove HBV infection and eliminate hidden reservoirs of virus. Recently, the establishment of a novel RNA-guided gene editing tool known as the clustered regularly interspaced short palindromic repeats/CRISPR-associated nuclease 9 (CRISPR/Cas9) system, has significantly enabled site-specific mutagenesis and characterizes a very beneficial possible therapeutic means for diseases which includes extermination of invasive pathogens e.g. HBV. This review highlights the recent developments in the use of CRISPR/Cas9 to specifically target HBV DNA sequences for inhibition of replication of HBV and to bring mutations in viral genome, animal models. Benefits, restrictions and viable solutions, and proposed guidelines for forthcoming study in CRISPR/Cas9 are described to highlight the chances and challenges for curative therapy of chronic hepatitis B infection.

Keywords: *Hepatitis c virus (HBV), covalently closed circular DNA (cccDNA), CRISPR/Cas9, inhibition of HBV replication, antiviral therapy.*

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INTRODUCTION:

350 million persons are affected with hepatitis c virus (HBV) globally. Near about 10% of patients have chronic type of hepatitis c. Chronic hepatitis c (CHB) is a severe condition that may lead towards more critical symptoms like cirrhosis and hepatocellular carcinoma(Kennedy et al., 2015b). According to estimation 700 thousand patients infected with HBV develop chronic infection and died each year. From previous 20 years many antiviral therapies are in practice to suppress HBV infection but these are not effective to eliminate the infection(Dienstag, 2008). HBV is member of Hepadnaviridae virus family and replicates inside the hepatocytes of human. The HBV genome contains DNA which is partially double-stranded(Gebbing et al., 2015). It comprises of a complete coding strand and other is incomplete non-coding strand known as relaxed circular DNA (rcDNA)(Ayoub and Keeffe, 2011). When virus infects hepatocytes, rcDNA from virus is transported towards the nucleus(Mehvish and Qadir, 2011). Where it is transformed into covalently closed circular DNA (cDNA). cDNA works as the template for transcription of viral pregenomic RNA (pgRNA) and protein coding mRNAs(Kennedy et al., 2015b). After transcription viral transcripts are moved into the cytoplasm where these are translated into viral proteins. Then, the which consists of viral core and polymerase protein, reverse transcribe and form a new viral rcDNA(Li et al., 2017). Enveloped nucleocapsids which contains DNA secreted into cytoplasm as progeny virus or sometimes transported back to the nucleus for amplification of the cccDNA pool(Peng et al., 2015).

Present drugs against HBV include interferon-α (IFN-α), nucleotide/nucleoside analogues used to adjust the immune response between host and virus(Perz et al., 2006). Due to limited efficiency IFN-α cannot give in high dosage because patients cannot tolerate high dose. Although nucleoside analogues are used to control the infection but they cannot functionally cure HBV(Schädler and Hildt, 2009). cccDNA is persistent, so long-term treatment required for patients, which is expensive and develop drug resistance in patients(Niaz et al., 2010). HBV is a retrovirus, it can cause mutations during the reverse transcription which enhances their chance to be safe from removal by traditional antiviral drug treatment(Block et al., 2015). cccDNA have amazing stability and cannot disrupt by current antiviral therapies. More efficient and non-toxic methods are required for clearance of the cccDNA(Kennedy et al., 2015a). Recently, numerous newgenome-editing methods have been developed, which based on sequence-specific endonucleases like zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and the most recent CRISPR (clustered regularly interspaced short palindromic repeats)/Cas9 (CRISPR associated 9) system(Li et al., 2017). The CRISPR/Cas9 system is basically natural defence mechanism of bacteria and archaea against virus. The Cas9 protein of type II CRISPR/Cas system has ability to cleave DNA at specific site with the help of CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA)(Lin et al., 2015). PAM sequence (NGG) necessary for Cas9 protein to cleave the DNA. PAM sequence present downstream of the target DNA. It produces DNA breaks in double stranded DNA known as double strand breaks (DSBs). These DSBs are repaired by homologous direct repair mechanism or by non-homologous end-joining (NHEJ) repair mechanism(Liu et al., 2015). The NHEJ mechanism is error-prone and can create insertions/deletions or other mutations during repair which cause frameshift mutations and ultimately gene knockout(Qadir, 2011). Moreover, there is also a chance that the crRNA–tracrRNA complex can be merged into a single guide RNA (gRNA)(Mei et al., 2016). For cleavage CRISPR/Cas9 system needs a single strand that matches with the target DNA of virus. CRISPR/Cas9 system is convenient as compared to ZFN- and TALEN genome editing methods. That is recent advances in genome editing are based on the CRIPSR/Cas9 system(Moyo et al., 2017).

The gRNA-guided CRISPR/Cas9 (gRNA/Cas9) system has been effectively used in numerous areas of biological and medical sciences. This technique also has application in virus research, for example for the genome editing of Epstein–Barrvirus and herpes simplex virus, and the abolition of human immunodeficiency virus (HIV) infection. This review provides the information that the gRNA/Cas9 system could greatly inhibit replication of HBV(Moyo et al., 2017). Inhibition can be in vitro and in vivo in which the viral genome directly disrupts by HBV specific gRNAs. Furthermore, HBV-specific gRNA/Cas9 systems can prevent the replications of HBV of different strains in cells. A single gRNA/Cas9 system can significantly reduce the viral genome and combination of different gRNA/Cas9 systems used to eliminate the persistent viral genome(Seeger and Mason, 2015).

This review demonstrates the prospect to inhibit replication of virus and clearance of persistent cDNA of HBV that is used for functional anti-HBV therapy, reduce the drug resistance which occur due to mutations during replication(Zhu etal., 2015) One of

the major advantage of CRISPR/Cas9 over other approaches for gene editing is that its capacity of multiplex targeting which provides a way for several disruptions, insertions, and deletions with greater efficiency and less cost. Thus, for improved destruction efficiency in the genome of HBV for multiple genomic editing, the blend of diverse site specific sgRNAs that can target several sequences will be perfect scheme(Seeger and Sohn, 2014).

MATERIALS AND METHODS:

Table 1: Questionnaire to evaluate awareness about etiology of Hepatitis C

Hepatitis C is a	**Yes**	**No**
1. Viral disease		
2. Bacterial disease		
3. Fungal disease		
4. Genetic disease		
5. Metabolic disease		
Ever suffered from Hepatitis C		
6. You		
7. Your family		
8. Your relative		
9. Your neighbor		
10. Your friend		
Hepatitis C is transmitted by		
11. Contacts or blood transfusion		
12. From parents to offspring		
Hepatitis C may be treated by		
13. Medicines		
14. Surgery		
15. Do not worry, it is easily curable		

Table 1: Questionnaire to evaluate awareness about etiology of Hepatitis A

Hepatitis C is a	**Yes**	**No**
1. Viral disease		
2. Bacterial disease		
3. Fungal disease		
4. Genetic disease		
5. Metabolic disease		

Table 2: Questionnaire to evaluate views about prevalence of Hepatitis C

Ever suffered from Hepatitis C	**Yes**	**No**
1. You		
2. Your family		
3. Your relative		
4. Your neighbor		
5. Your friend		

3: Questionnaire to evaluate views about transmission of Hepatitis C

Hepatitis A is transmitted by	**Yes**	**No**
1. Contacts Table or blood transfusion		

Table 4: Questionnaire to evaluate views about Hope for Hepatitis C

Hepatitis C may be treated by	**Yes**	**No**
1. Medicines		
2. Surgery		
3. Do not worry, it is easily curable		

RESULTS:

Table 5: Awareness about etiology of Hepatitis C: Views of Postgraduate Biology Students

Questions	**Male**		**Female**		**Total**	
	Yes	**No**	**Yes**	**No**	**Yes**	**No**
1. Viral disease	**92%**	**8%**	**96%**	**4%**	**96%**	**4%**
2. Bacterial disease	**41%**	**59%**	**15%**	**85%**	**30%**	**70%**
3. Fungal disease	**70%**	**30%**	**73%**	**27%**	**30%**	**70%**
4. Genetic disease	**37%**	**63%**	**70%**	**30%**	**34%**	**74%**
5. Metabolic disease	**63%**	**37%**	**80%**	**20%**	**90%**	**10%**

DISCUSSION:

Role of CRISPR/Cas9 to Target cDNA and Inhibition of HBV Replication:

Genome of the HBV has four long open reading frames that are surface, core, polymerase, and X protein. These are translated into only seven proteins which are very important for replication of virus. For suppression of viral gene expression and replication, targeting any 1 of 7 proteins is enough. Many examples are present in which HBV genome is targeted with CRISPR/Cas9 system. A scientist reported that the CRISPR/Cas9 system can disrupt the viral genome both in vitro and in vivo(Saayman et al., 2015). Cas9/sgRNA specific for HBV can significantly decrease the construction of HBV core and surface antigen, when a complex of Cas9 and HBV expression plasmid transfected into carcinoma cells derived from Huh7 hepatocyte(Schiffer et al., 2012). Moreover, CRISPR/Cas9 system efficiently decrease the intrahepatic HBV-expressing vectors level and surface antigen serum levels in an HBV infected mouse model(Zhen et al., 2015). Transduction of Cas9 and HBV-specific gRNAs by using lentiviral, effective inhibition of HBV DNA and cccDNA is observed(Schweitzer et al., 2015). According to a study levels of total HBV viral DNA decline up to ~1000-fold by the CRISPR/Cas9 and cccDNA level declines up to ~10-folds. According to Seeger and Sohn HBV contaminations can be repressed up to eightfold by HBV-specific guide RNAs in NTCP expressing HepG2 cells. In alternative study, Liu et al. stated that HBV-specific gRNA/Cas9 can prevent the

HBV replication of diverse genotypes equally in vitro and in vivo, it is due to removal and fallible repair of viral DNA templates(Seeger and Sohn, 2014). This system can efficiently impede levels of HBV DNA and HBsAg countenance in liver of mouse. According to Dong et al. the CRISPR/Cas system can be used for impeding intracellular cccDNA. Ramanan et al. showed that sgRNAs targeting conserved regions of HBV cause strong inhibition of virus replication both in vitro and in vivo, and extended this antiviral activity to virus isolated from patients(Kennedy et al., 2015c). Continuous expression of Cas9/sgRNA shows rapid decline of persistent cccDNA and HBV proteins in a denovo infection model. Recently, Karimova et al. demonstrated that an advanced CRISPR/Cas9 nickase system has capability to disturb both HBV cccDNA and fused HBV sequences in HeLa and HEK293 cell lines(Shalem et al., 2014). In short, these findings demonstrate the successfully use of the CRISPR/Cas9 system in destroying HBV cDNA both in vitro and in vivo, and shown the therapeutic approach of CRISPR/Cas9 in both acute and chronic HBV infection(Shen et al., 2014).

Challenges for the CRISPR/Cas9 system as a Novel HBV therapeutic approach:

Before clinical application of CRISPR/Cas9 systems for HBV treatment, significant problems are needed to be addressed. Major concern is related to capability of extermination of all viruses(Hai et al., 2014). Yet, by Ramanan et al. achieved the best result for cleavage of HBV via CRISPR/Cas9. The suppression of cDNA is about 92% in cultured cells. One of the major challenge is presence of HBV DNA in different tissues external the liver and replication of HBV in different cell lines(Kennedy and Cullen, 2015). To eradicate HBV, it is important that nucleases transported to every single infected cell in liver and outside the liver reservoirs of virus. For sustained in vivo anti-HBV action, an effective vehicle for delivery must be used(Schweitzer et al., 2015).

Recombinant adeno-associated viral vectors (rAAVs) are considered as best delivering vehicle for CRISPR/Cas9 due to the less immune response, non incorporating nature, and high infection efficacy(Yin et al., 2014). Packaging difficulty can be resolved by enchanting benefit of a split-Cas9 system or by means of a smaller Cas9 orthologs e.g. SaCas9 from Staphylococcus aureus, which is shorter than 1 kb(Seeger and Sohn, 2014). The second issue of CRISPR/Cas9 system is the probable off-target effects. Previously published data show that the rate of off-target activity of CRISPR/Cas9 system is high, even in case of sgRNAs which have almost five nucleotides mismatches(Shalem et al., 2014). Numerous methods have been established to decrease off-target effects.

One approach is “paired nicking”. It reduces the off-target delivery by using two spaced gRNAs and a mutated Cas9 (Cas9n). Both can cleave single-strand DNA and instead of producing DSBs a nick is formed on both DNA strands. Nicks further repaired accurately by the cell repair mechanism. Paired nicking reduced off-target effect by 50–1,000 fold in cell lines comparing to the wild-type Cas9.

2nd strategy is the use of Truncate sgRNAs at the end of the complementary targeting sequence. Fu et al. described that a short sgRNAs of 17 or 18 nucleotides may increase specificity for target sequence by more than 5000-fold
without decreasing the on-target activity. fCas9 is another modified Cas9 which designed by fusing of catalytically inactive Cas9 and Fok I nuclease. Specificity of fCas9 system increased by140-fold than normal Cas9 by increasing targeting sequences in human cells(Xu et al., 2014). But, these methods remain less effective. Selection of appropriate target sites in the genome of HBV has greater concern. The main issue of Nucleoside/Nucleotide analogue therapy is the occurrence of HB variants which are therapy-resistant. CRISPR/Cas9 system provides specific sequence recognition(Shen et al., 2014). In case of high viral, the CRISPR/Cas9 provide a pool of therapy-resistant viruses which can reestablish infection. So, overlapping of targeting sequence with regions like (Y)-methionine (M)-aspartic acid (D)-aspartic acid (D) (YMDD) motif should avoid.

Importantly, due to high sequence deviation, genome of HBV is classified into eight genotypes A–H(Yang and Kao, 2014). Use of multiple CRISPR/Cas9 constructs and targeting of highly conserved regions between diverse viral genotypes are good approaches for reducing the issue of current and de novo mutations(Komatsu, 2014). Persistent incorporated linearized viral DNA, cleaves by the CRISPR/Cas9 system. The incorporation of sub genomic viral DNA parts into genome of host is common in hepatocellular carcinoma or chronically infected patients. Host gene function can be disrupted by cleavage of incorporated viral DNA which lead to insertion/deletion mutation in host genome. The undesirable side effects must be evaluated carefully(Kennedy et al., 2015c).

Potential Target for HBV in Gene Editing Therapy with CRISPR/Cas9:

Different genome editing strategies have been currently used as tool against numerous human viruses. In curative HIV research field, studies presented that CD4+ T cells with chemokine (CC motif) receptor 5 (CCR5)-disturbed by nucleases show resistance against HIV-1 infection. ZFNs which disrupt CCR5 is under phase I clinical trials, is breakthrough(Wyman and Kanaar, 2006). Recently Sodium taurocholate co-transporting polypeptide identified as an entrance receptor of HBV and HDV which facilitates the transportation of bile acids and other molecules from portal circulation. One class of the indirect anti-HBV agents in progress are entrance inhibitors, which target the HBV receptor NTCP(Yan et al., 2013). With the inspiring result of nucleases based CCR5 gene editing therapy, it stances to reason behind use of CRISPR/Cas9 for disruption of NTCP expression block the entrance of HBV and stop the spread of viral infection(Yang et al., 2013). Yet again, although no reports of dangerous diseases linked with faults in the NTCP gene have been available yet, the possible modification of function of gene and NTCP disruption toxicity must be assessed(Komatsu, 2014).

CONCLUSION:

The CRISPR/Cas9 technology has enormously advanced our capability to edit the and provides a new era of gene therapies for treatment of diseases.

Although it still in a clinical trial stage, CRISPR/Cas9 system used both in vitro and in vivo way for inhibition of HBV replication and gene expression, and may establish a new beneficial strategy for HBV infection. Currently use of combined CRISPR/Cas9 therapy and RT inhibitors may attain the maximum rates of viral response. Still many difficulties remain to evaluate including safety and effective delivery of the system, CRISPR/Cas9 strategies provide immense potential to cure chronic HBV infection. Experimental research has revealed that CRISPR/Cas9 cleaves episomal cccDNA, decreases pgRNA, and inhibit viral proteins without any cytotoxic effects, providing considerable evidence that CRISPR/Cas9 strategy is a promising tool with the possibility to work as an efficient HBV infections treatment and to exterminate cccDNA. But it is significant to be careful around the challenges that reduce the practicability and security of these innovative methods. Many problems should be resolved. Many improvements are required to achieve effective and safer therapeutic techniques to use CRISPR/Cas9 technology for eradication of cccDNA and inhibition of HBV.

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