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Optimization for the therapeutic application of CRISPR/Cas 19 technologies for CHB

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Abstract: Hepatitis-B Virus (HBV) is able to escape the immune system by converting HBV DNA into covalently closed circular DNA (cccDNA). This allows an acute Hepatitis B infection to evolve into a chronic Hepatitis B infection which may lead to life-long illness. After the discovery of the CRISPR/Cas9 system, different trials have been done to eliminate the cccDNA but none of them have been very effective. Based on a previous study, I propose a new methodology by which the CRISPR administration by replacing dual Adeno-Associate virus (AAV) vector and add another single-guide RNA (sgRNA) and accorded promoter to intensify the affect.

1. Introduction

The hepatitis B virus (HBV)is a DNA virus that replicates in human liver cells. HBV usually causes acute infections and sometimes can lead to chronic infections. In the global burden of disease study conducted in 2010, HBV was ranked as the prioritized public health issue in the world by WHO and as of 2021 there is still no cure for HBV [1]. In 2019,the WHO estimated that there were 296 million people living with chronic HBV and approximately 1.5 million new acute HBV infections that occur globally each year. The estimated worldwide mortality for HBV- infected individuals is more than 780,000 each year [2].

In 2016, the WHO estimated that there were 120 million HBV infections in China, which consisted of one-third of the chronic infections worldwide. Among them, 30 million people in China were estimated to have chronic HBV. According to WHO, the reported cases of new HBV infections in China have been decreasing due to the emergence of effective vaccinations in 2006. Before vaccinations became widely available, approximately 300,000 people died annually in China due to hepatitis B-related diseases [3]. From 2015-2016, the mortality rate for hepatitis B-related diseases remained relatively stable for females and decreased for males. However,in 2019, the mortality rate for HBV in China still reached 11.4 per 100,000/people with an estimated population of 1.4 billion [4].

HBV is transmitted from human to human primarily in adults via horizontal transmission through exposure to infected blood. HBV can be vertically transmitted from mother to baby either in utero or at birth. There is no evidence that HBV is a zoonotic virus because no known animal reservoirs have been found.

HBV primarily infects hepatocytes in the liver. HBV can enter the nucleus of the host cells and integrate into the host cell DNA which is unusual for a virus. After the HBV DNA is integrated into the nucleus of host cells, the immune system cannot eliminate the HBV and these cells result in a latent infection which can be the main mechanism of chronic hepatitis B [5].

Chronic hepatitis B (CHB)can cause a life-long illness possibly leading to liver cirrhosis,liver cancer or liver failure. Patients who showed acute inflammation symptoms during acute HBV infection may not notice that the HBV infection has gradually developed into chronic HBV until related diseases are activated. When symptoms appear, chronic HBV patients can experience similar symptoms of acute HBV that include fever, fatigue, loss of appetite, nausea, vomiting and abdominal pain [6].

After the infection of HBV,virus-specific CD8+T cells in the adaptive immune response play a critical role in fighting the infection. Scientists found a vigorous CD8+ T cell response in patients with acute HBV infection that are able to resolve their HBV infection. However, HBV-specific CD8+ T cell responses were found to be weak, almost undetectable in patients with chronic HBV infection [7]. It is very difficult for the immune system to eliminate HBV acute infection without clearing the covalently closed circular DNA (cccDNA) in the nucleus of infected liver cells. Despite the fact that HBV DNA has the ability to integrate into the host DNA,the main replicating template in the nucleus is the cccDNA of HBV [8]. Thus,although patients may maintain protective immunity for the rest of their lives, the CTL immune response is still insufficient to eliminate all the cccDNA in chronic infections [9]. Antibodies to HBV are made and help with limiting the virus during acute and chronic infection but do not eliminate a chronic infection. However, antibodies that are critical for HBV protection will be elicited to prevent HBV by the vaccine.

Currently, recombinant protein vaccines are used to prevent HBV worldwide. The Recombivax HB vaccine was the first vaccine for hepatitis B to be produced using recombinant DNA technology and is given with 3 shots over a 6-month period. It is very effective at preventing HBV acute infection, but the vaccine will not work to eliminate chronic HBV infection. Because the first vaccine for HBV was invented in 1986, adults 40-49 years of age have the highest rate of HBV infection due to low vaccination coverage and the passage of time regarding younger generations who live in countries that have developed a high herd immunity for HBV.

However, in 2019, the WHO announced that the global vaccination coverage for doses given at birth was still only 42% of the world. In 2016, the vaccine coverage for new born reached 95% in China [10]. If the global vaccination coverage can reach the level of herd immunity,it is possible to eradicate the disease completely. Therefore, it is probably impossible to eliminate the HBV infection within the next few years.

There are many drugs invented to treat HBV. Monotherapy with nucleotide analogue is the current treatment for HBV. Drugs like entecavir (ETV), lamivudine, adefovir, tenofovir disoproxil fumarate (TDF), Besifovir dipivoxil maleate (BSV) and tenofovir alafenamide (TAF) are used as

therapy to reduce the number of HBV. However, none of them have cured chronic HBV because no drugs have been effective in preventing the reactivation of HBV latently-infected cells.

2. Discussion

Although multiple drugs and inhibitors have been developed that reduce the viral DNA by inhibiting the integration, scientists have no tools to detect covalently closed circular DNA (cccDNA) in the nucleus that was converted from HBV DNA form the virion. The template of HBV transcription, covalently closed circular DNA, plays a critical role in the reproduction cycle of the virus and permits the persistence of infection. They transcribe 4 critical groups of viral mRNAs. Those mRNAs then will be translated into either viral protein in the cytoplasm or converted back into viral DNA and form new virus. Because HBV can replicate in human nucleus, the immune system cannot detect the antigens, and this allows the virus escape the CTL immune mechanisms. In that case, HBV have enough time to replicate through the cell cycle and reactivate the disease.

In order to eradicate all latent and chronic infections of HBV, new treatments need to target the elimination of the cccDNA. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)has been proposed to serve as a potential solution for chronic hepatitis B. CRISPR is an adaptive defense system that originally came from bacteria. CRISPR can be used to directly target and cut off in specific locus in genome. Therefore, based on its high efficiency of gene editing ability, it's a promising tool for genetic editing treatment. After Jinek et al. [11] discovered the principle of CRISPR, scientists started to try to modify CRISPR in use of human to treat different kind of genetic disorders and even cure other diseases caused by integrated virus. With the help of Cas9,an enzyme,this system may be able to cut the HBV cccDNA and develop spacers to prevent further integrations.

Selecting a suitable vector is very essential to deliver the CRSIPR-Cas 9 into the right position where hepatocytes located. Currently, several types of virus, including retrovirus, adenovirus, adeno-associated virus (AAV), and herpes simplex virus, have been modified in the laboratory for use in gene therapy applications in animal models. [12] The most popular vector in animal trials belongs to AAVs vector. First, AAVs have already been approved by numerous human trials in gene therapy due to their high safety index and their therapeutic potential. Second, AAVs are significantly less immunogenic than other virus that may cause fewer side effects after the therapy. A recent trial used to test the efficiency of CRSIPR/Cas9 to treat HBV was done. In March 2021, Stone et al. [13] evaluated the use of CRSIPR-Cas9 with adeno-associated virus (AAV)vectors as a therapy to treat chronic HBV in humanized mice, which showed the feasibility of using SaCas9 to eliminate HBV in humanized mice with the support of entecavir. They used AAV vectors that construct AAV-EFM-SaCas9-SV40pA-sgRNA-hU6-sgRNA for the trial. Entecavir was used prior to AAV administration. The result, however, showed the scanty of numerous SaCas9 in the vectors before administration that resulted in insufficient elimination of HBV. The IU/ml of HBV decreased in short-term after AAV administration but gradually increased for unknown reason.

Based on the result of humanized rice trial, I think there are several potential solutions to improve the efficiency of CRISPR. I proposed that the design of AAV need to be improved so that CRISPR can be better exhibited to eliminate more HBV DNA. First, I proposed to use the dual AAV vectors instead of the single strand AAV that used in previous treatment.[13] because the maximum

capacity of single strand AAV genome only give up to less than 4 kb, [12] some more suitable promoters cannot be used due to the limitation of the coding capacity. In the study, Stone et al.[13] choose the short EF1a promoter for expression of SaCas9 instead of smCBA promoter which owns higher efficiency but is too large for the vector. Therefore, in order to optimize the efficiency of CRSIPR/Cas9 in further human trials, dual AAV vector that have larger capacity for transgenes is a potential administration for future therapeutic treatment.

Second, the numbers of single guide RNA (sgRNA) in the vector is insufficient. I proposed to design one more sgRNA by obtaining different sequences of our target genes on the cccDNA that meet the requirement of PAMs. sgRNA is the key to the specific lock on the DNA sequence. CRISPR will bind to the specific sequence that correspond to specific sgRNA. In the study that done by Stone et al.[13], two sgRNAs had been included into the construction of AAV administration due to the limited capacity of AAV vector. The previous administration did not result well. CRSIPR/Cas9 are able to locate HBV but the numbers of sgRNA in the vector are not enough to eradicate every hepatitis B virus in hepatocytes. I believe that this is the reason hepatitis B virus reactivated after the AAV administration. Therefore, I suggest designing another sgRNA to locate another sequence on the cccDNA after changing from single strand AAV vector to dual AAV vector.

I believe that locating specific PAMs that corresponded with Cas9 is the first step to design sgRNA as Cas9 protein starts binding DNA by binding to PAMs in the sequence. In this case, the

PAMs for that Cas9 protein is NGG showed on coding strand and opposing strand or CCN showed on opposing strand and coding strand where N in the coding strand represent “A”. After locating the PAMs, we need to copy 20 bases that on the 5 prime ends of the PAMs. If we are using CCN, we need to locate 20 bases to the right while to the left if we are using NGG as our guide RNA that do not include the PAMs sequence. [14] After including one more sgRNA, another promoter is needed to express the sgRNA in order to minimize repeat sequence and potential recombination in the AAV vector backbone. In previous humanized mice trial done by Stone et al. [13], hH1 and hU6 was used to express the two sgRNA. As optimization for humanized mice trial, using tRNAgln pol III as the third promoter to transcribe tRNA with other non-coding RNAs and express corresponding sgRNA.

Into a nutshell, new experiments to test dual AAV vectors with AAV-EFM-SaCas9-SV40pA-sgRNA-hU6-sgRNA- tRNA gln pol III-sgRNA in humanized mice is an optimization for potential treatment of CRISPR. Currently, because of the lack of current understanding about CRSIPR-Cas9, I believe advanced human trails should wait until the high feasibility in animal are proved and the technology of CRSIPR-Cas 9 grow into relatively matured.

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