

CRISPR-Cas9 potential in treating HIV/AIDS

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Abstract. In this review paper, the mechanism of HIV infection and some of the most efficacious and updated gene therapy methods developed by scientists will be discussed in detail. Different editing strategy using the CRISPR-Cas9 system inhibits different times of the HIV life cycle are involved with all the same target, which is to block the HIV infection pathway as well as its own replication&proliferation pathway. As the most popular and promising way of treating different kinds of diseases which seemed to be incurable in the past, and CRISPR-Cas9 shows high potency under the data of experiments and clinical studies. These give scientists many confidence to run out more research on this new treatment for diseases and at the same time to improve and optimize the function and efficiency of this gene editing toolkit making sure it can used widely in the future as an actual clinical treatment for the HIV/AIDS.

Keywords: Gene editing, HIV/AIDS, CRISPR-Cas9, co-receptors.

1. Introduction

As the most promising gene therapy field which scientists looking forward to utilize, one editing toolkit CRISPR-Cas9 has been discovered by scientists as the most effective and site-specific system for gene editing in the near decades for treating diseases like HIV/AIDS. It can be designed to precisely knockout the gene coded for diseases and so they may be cured completely. As it is named, the system basically contains two components—The CRISPR gene and Cas9 CRISPR associated protein nuclease which was found in streptococcus pyogenes. CRISPR is the abbreviation of clustered regularly interspaced short palindromic repeats, first reported by Yoshizumi Ishino in 1987 when he was testing the sequence of E-coli alkaline phosphatase isoenzyme. After further research, scientists found out that the CRISPR gene seemed to have some more important function as fighting against viral invasion. This was than reported by reverse transcription making the virus cannot change their RNA information to DNA hence to avoid infection. Garneau et al in 2010 when they studied archaea and bacteria to pointed out that the CRIPSR gene may together work with a CRISPR associated protein nuclease(Cas9) made up their natural immune system. They found out in order to fight against the invasion of bacteriophages, bacteria uses CRISPR-Cas9 system as a weapon to break the inserted viral DNA in order to neutralize the virus to stop it from proliferating. Once the virus inserts its DNA into the bacteria genome, it will be sandwiched between the repeated palindromic sequences as pieces called ‘spacer’ and together form a CRISPR array. The Cas9 protein is actually a kind of nuclease protein and it will bind to every spacer and a palindromic repeats, another molecule called tracr-RNA will also bind to the palindromic repeats. This CRISPR array will than under transcription and get cleaved by DNase III to form crRNA and a effector complex molecule, this will than become a strong tool to attack the viral DNA[1].This gives scientists inspiration that instead of using three components in the natural immune system of bacteria, the tracrRNA and crRNA can actually fuse together by a linker to become a single strand RNA(sgRNA), so that means now scientists can synthesize the specific sgRNA for about 10 to 20 nucleotides long they want in laboratory along with Cas9 protein from streptococcus pyogenes to edit any bases of DNA in eukaryotic cells. This is finally proved and tested by Jennifer Doudna and Emmanuelle Charpentier, which they won the Noble price in chemistry in the year of 2020 [2]. At the same time when the HIV virus pandemic seems to become serious and serious around the globe murdering people like an invisible killer. As the previous treating plan for HIV becomes worse and worse for complete cure. By focusing on the infection pathway, scientists start trying to introduce gene-editing and looking forward to see better and different results.

2. HIV background and traditional therapy

2.1. Introduction of HIV

According to World Health Organization (WHO), as causing hematological diseases by infecting helper T cells(CD4+ cells), Human Immunodeficiency Virus or abbreviated as HIV is now still one of the most dangerous and infectious virus over the globe that mainly cause Acquired Immune Deficiency Syndrome(AIDS) [3]. By transferring in blood and other body fluids, they easily pass between person and person if having unprotected sex or using same needles etc. The HIV is a retrovirus which means they infects human body by reverse transcripts its RNA to DNA and combine with human genome for than start duplicating to infect more cells. It mainly infects the helper T lymphocytes to disrupt the immune system and cause other opportunistic infection rate like TB or malaria to rise significantly.

2.2. Traditional Anti Retroviral Treatment(ART)

Before the development of gene editing therapy,the anti retroviral(ARV)drugs like zidovudine are the major way scientists used to fight against the HIV associated syndromes. As it named, the function of them is to inhibit different parts of the HIV life cycle to make them hard to proliferate in order to decrease the infection rate. These includes different kinds of inhibitors such as CD4 binding inhibitor, CCR5 binding inhibitor, membrane fusion inhibitor or reverse transcriptase inhibitor to interfere the virus normal function. For reverse transcriptase inhibitor, it contains two types which are the nucleoside-targeted and nucleotide-targeted. They both works to stop viral RNA from changing to DNA information and thus disrupt the replication step. Other inhibitors all work similarly to block the specific parts of HIV life cycle. However, the problem is the presence of the HIV-1 latent reservoir may have significant effects on resting CD4+ cells and leads to its transcriptional silencing, which makes the ART becoming not sufficient any more to treat the chronic HIV-1 infection as the latent state of the virus significantly inhibits the ART effects. J.Potter et al pointed out in their report showed factors likes drug resistance mutation and toxicity, augmentation of host immune responses builds up obstacles to successful highly active antiretroviral therapy [4]. As a result, the way to inhibit the activation of latent reservoir than become a new direction for scientists to work in and this necessitates scientists to develop alternative therapies , they than turn their hope into the new and promising gene editing toolkit [5]. This first proposed by H-K Liao et al in 2015, they showed the results that the HIV-targeted CRISPR-Cas9 system can be quite efficaciously differentiated into HIV reservoir cell types and gains resistance towards HIV infection, which first verify the feasibility of CRISPR-Cas9 in HIV/AIDS treatment [6].

3. Utilizing CRISPR-Cas9 to Disrupt HIV Infection by Editing Co-receptor Proteins

3.1. HIV Infection Pathway

For the more transmissible type HIV-1, it attacks the host cells by using a glycoprotein gp120 on its surface, it may bind to the CD4 receptors on host cell membrane after undergoing a series of internal rearrangement surrounding the CD4 receptor binding site and after binding it will adopt to a CD4 binding competent state. Another transmembrane glycoprotein, gp41, together with gp120 to form a trimer complex called gp160 on the viral envelope. After binding with CD4, the gp120 will undergo further folding to expose its co-receptor binding sites and this allows the engagement of CCR5 and CXCR4 chemokine co-receptors on host cell membranes. Than an additional structural alteration will take place to expose the gp41 glycoprotein, which inserts into the host cell membrane. Some major structural changes than show in gp41 to fuse the viral membrane and host cell membrane together. The fusion finally allows the viral DNA inserting into the host cells to replicate and proliferate (see figure 1).

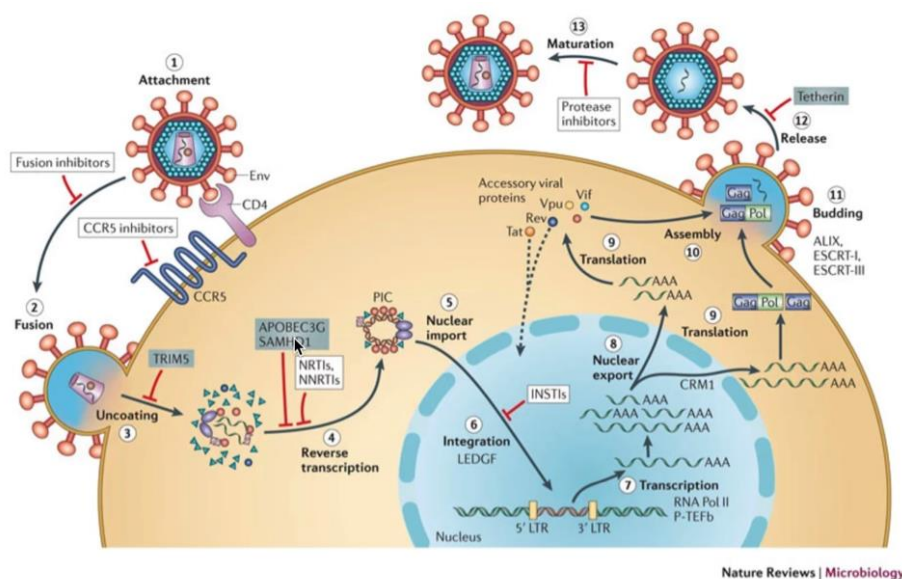


Figure 1 Selected from nature reviews microbiology [7].

The every step in the pathway of the virus is seemed to relate with co-receptors hence it is clear that stop the expression of these genes in host cells may be the way scientists disrupting the HIV function. Before CRISPR, the modifications at co-receptor locus are achieved by using custom endonucleases such as zinc finger nucleases(ZFNs) and transcription activator-like effectors nucleases(TALENs), these have been successfully applied in clinical trials and animal models. However they all shows some defects and low efficiency in editing so scientists than change to choose using the new and more promising CRISPR-Cas9 system to operate. The C-C chemokine receptor type 5, abbreviated as CCR5 is a kind of G protein-coupled receptor(GPCR), it consists seven transmembrane loops and one alpha helix that attaches to the plasma membrane. For gene editing to avoid infection of HIV, the CCR5/CXCR4 protein may choose to be the major part to disrupt glycoprotein gp120/gp41 binding [8]. The general way is to knock out the CCR5/CXCR4 gene and making the virus fails to insert genetic information into host cells for replicate and protect the CD4+ cells from infection(Liu et al 2017) [9]. This is achieve by the typical CRISPR-Cas9 system, initially the gRNA which complimentary with the CCR5 sequence is synthesized in laboratory and the CD4+ T cells needed for editing is extracted from patients body for further processes in vitro. The extracted CD4+ T cells, synthesized guide RNA and human codon-optimized Cas9 protein will than fuse together in the tube to start editing. After the gene been knocked out, the cells will use the natural DNA repair mechanism non-homologous end joining(NHEJ) to directly link two ends of the cleaved DNA together which cause CCR5 loss its function and disrupt the CD4-CCR5 completely to block the HIV invasion. The knock out gene is than under PCR and gel eletrophoresis to test the efficacy before introduce back to patient's body.

3.2. Designing and Optimizing the Efficiency of Gene Editing Treatment

In general, CCR5 and CXCR4 gene knockout using two different gRNA respectively, however another investigation ran out by S Yu et al pointed out that a simultaneously knockout of both CXCR4 and CCR5 genes in an individual CD4+ cells using only one sgRNA may also be available [10]. They found out of a single vector which can express both sgCXCR4 and sgCCR5 gave out ablation which is highly efficient of both CXCR4 and CCR5, which they than replicated this array into primary CD4+ cells and got the similar results. After the confirmation of CRISPR-Cas9 capability, they than transduced Cas9 nuclease and transcribed sgRNAs by electroporation into CD4+ cells and obtained positive results. Another research made by Z Liu et al combined each of the two CXCR4 sgRNA and one CCR5 sgRNA into one vector, which can be used to significantly disrupt both CCR5 and CXCR4

in primary CD4⁺ cells. These both shows the potency that use of CRISPR-Cas9 to simultaneously knockout both CCR5 and CXCR4 is efficient to disrupt the infection of HIV-1, which also have high potential to be applied to the clinical cases for actual therapies. It is their results that gave out a positive prospects for future clinical applications of CCR5 editing therapy.

3.3. Avoiding Off-target Effects

While having break through and successful experiments proving the potential of co-receptors editing, according to Andrew Atkins et al, the off-target effects are still a big barrier stops scientists to move further as it can happen randomly when editing [11]. In order to prevent this happens, some highly sensitive detection method may be used after the operation of CRISPR-cas9. One of which is CIRCLE-seq, reported by Tsai et al, 2017; Lazzarotto et al. 2018 [12,13]. It is worked by using PCR, self-ligation and end-repair to circularize the tested DNA fragments. These circular fragments will than under PCR to proliferate into linear fragments and again using self-ligation to circularize. The 'Double-circularized' fragments will under replication by DNA polymerase and then the sequence will be checked using illumina test to detect if there are off-target effects or not. Apart from this, the HIV immune evasion nature can also be a huge barrier on the way scientists try to eradicate virus infection.

4. Provirus Editing in LTR of HIV-1 Genome

4.1. Potantial of Long Terminal Repeats

Another way scientists looking forward to suppress HIV expression is to edit the HIV integrated proviral DNA by using CRISPR-cas9 which mainly expected to inhibit the latent reservoir of the virus. In case of HIV-1, scientists designed gRNA that targets on the HIV-1 long terminal repeats(LTR) region, tunder the control of a Tatprotein, the guide RNA will be transfected and significantly inhibits the expression driven by LTR region, which will than excise the HIV provirus from the host cell genome and than infection will become unsuccessful [14]. The LTR is a long sequence which usually about 250-600 bp long at the two ends of some retrotransposons and retrovirus genes in eukaryotic genome, first reported by A.P.Czernilofsky and John Shine in 1980 when doing research on gene sequence of avian sacroma leukosis viruses. The LTR sequences usually can code for some reverse transcriptase and integrase which the gene between can used to replicate and inserting themselves into a new location in the genome [15]. In HIV-1 viral genome, its LTR sequence is about 634 bp long and can be seperated into three regions--- U3, R and U5.

In the regulation of HIV-1 gene expression, the Tat protein is a regulatory protein that can drastically enhances the efficiency of transcription, so which means if the trans activation response region(also be abbreviated as TAR region) which it binding to has been genetically modified, the transcription of the proviral DNA will be affected and the infection will not take place. To test the activity of this CRISPR associated way targeting the TAR region, Hirotake Ebina et al ran out an experiment by observing the level of green fluorescent protein under the condition set up to mimic the authentic HIV-1 gene expression, which Roychoudhury et al did the similar experiments targeting the gag and pol gene in order for reservoir deletion and C-H Chung et al did the similar experiments targeting the NF-kB transcription factor binding site on the LTR region [16].

4.2. Use of Green Fluorescent Protein in Specific CellsConfirm the Feasibility of Provirus Editing

For the most well-known experiment ran out by Ebina.et al, under the control of a human U6 polymerase III promoter, they work designed a gRNA vector which targeted the HIV-1 Long terminal repeats region [14]. Result in two gRNA expressing plasmids generated that for targets 5(T5) after the U6 based-transcription took place, which located in the R region. And for targets 6(T6), it located at the binding sequence of NF-kB in the U3 region. Than lentiviral vectors were used to generate out human cells which integrated with HIV-1 provirus to express Tat and green fluorescent protein(GFP).

This process was operated under control of a LTR promoter. Generally it can be simply explained as transfects a strand of specific HIV-1 DNA(provirus) into normal human cells to make it 'infected'. Then they co-transfected T5 and T6 gRNA expression plasmids which mentioned above along with a humanized Cas9 expression plasmids into the LTIG vector-infected cells in vitro ready for testing the result of CRISPR-Cas9 editing. To accurately assess, they prepared 293 T cells and Hela cells and let them get infected by a LTIG vector which has pseudotyped with a envelop protein called VSV-G. Around about a week after the initial transfection, they found out an obvious reduction in mean fluorescence intensity(MFI) and GFP of 293 T cells, which is the cell type they chose, the T5 expression is more effective in reducing green fluorescent protein numbers than T6 expression, the GFP positive cells percentage in T5 is dropped by 25.6%. In another cell type they do research on, which is the Hela cells, this percentage change is quite modest in compare to 293 T which drop only about 10.5%, but at the same time its MFI level dropped much more significant than the 293 T cells.

These reduction gained from results gave out strong evidences to show that the CRISPR-Cas9 system which target on the HIV-1 LTR region successfully stop the HIV-1 gene expression from its provirus LTR. The LTR was then isolated using primer set and then cloned into a plasmid and then the sequences are analyzed and they found out that 18 out of 22 DNA clones contains different types of mutations, including about 1-31 bp lossing or deletion from the end of putative cleavage site or combination of insertion and deletion mutation, which both have non-uniformity in size in compare to the original sequence. These evidence only leads to one natural DNA repair mechanism, the Non-homologous End Joining(NHEJ), which DNA used to repair themselves after gene editing. This leads them to finally gave out a conclusion that T5 CRISPR-Cas9 system can efficiently creates mutations in the HIV-1 TAR region to stop Tat protein binding to enhance transcription, which blocks the infection of HIV-1.

5. Clinical Cases of Editing Stem Cells Through CRISPR-Cas9 System Shows a Promising Future for HIV Patients

In September, 2019, L.Xu et al published a brief clinical report funded by life science department from Beijing university of a patient with HIV/AIDS and acute lymphocytic leukemia on The New England Journal of Medicine [17]. The patient is a man in age around 30 and the infecting HIV type of him was confirmed to be CCR5-tropic at diagnosis. The patient then received Antiretroviral drugs for HIV infection and standard chemotherapy for acute lymphocytic leukemia for six courses to relieve the symptoms. Then he was lucky to accepted an allogeneic hematopoietic stem cell transplantation from a male donor who had fully matched HLA type as the patient. As the donor got unmutated CCR5 gene, the HSPCs(CD34+ cells) derived from the donor were first edited by CRISPR-Cas9 to knock out the expression of CCR5 gene and resulting an indel efficiency around 17% before the operation. Another similar case of a 'Berlin patients' received two allogeneic HSC transplantation from a homologous CCR5 Δ 32/ Δ 32 donor whose cells have about 32-bp deletion to prevent the CCR5 expressio.

Consequently, over about one-and-a-half-year period after the transplantation took place, the ablation efficiency of his CCR5 gene mediated by CRISPR-Cas9 was shown to be raise about 3% in his bone marrow and the CRISPR-mediated CCR5 ablation was also observed to present in multiple hematopoietic lineages including cells like CD4+ and CD8+. Also, because of the non-viral way to introduce Cas9, the introduction of exogenous DNA can be avoided and thus avoided the unexpected off-target indels, this giving the treatment nearly no typical adverse events related to gene editing. The case can be summarized as a successful clinical application of CRISPR-Cas9 for treating HIV/AIDS by editing the fully matched donor's HSPCs and then transplants into patient body. Although some problems and defects may still be observed, this case has verified and confirmed the huge potential of gene editing therapy for HIV infection.

6. Discussion

For the traditional ART to treat HIV, it is clear that it can be more efficient and cheaper in compare with the complicated gene editing therapy for normal people. The anti retroviral drugs are still quite popular in nowadays and it can be much safer as the drugs have been used and tested over a long time. Although a bottle of medicine may not be that cheap, but in compare with the fee needed for experimental gene editing treatment, it is still a better choice for most of the normal people if the situation of patient is not serious. However, one of the main and obvious defects of using anti retroviral treatment is that the patient may have chance need to take the medicine in the rest of his or her life. Once they stop taking these drugs, they may have big chance to get reinfected. And also as I mentioned, because of the presence of provirus latent reservoir, the ART may not work that effectively.

When turn to the gene therapy, at the same time when clinical cases of editing co-receptor proteins are published by scientists to show that HIV/AIDS patient's pain can be abbreviated efficaciously, there are still many limits like off-target effects which can even cause cell mutation to form cancer and HIV immune evasion which cause the gene editing to become invalid. Although these can be prevented using detection method like CIRCLE-seq or nanobody, it is still quite risky and expensive to actual operate for patients, many moral problems may also be discussed. However, the potential of it to treat HIV/AIDS patients completely or to eradicate the virus is still very attractive for people to try and it should be the future direction of HIV treatments. Apart from co-receptors editing, the proviral editing seems to have less moral problems. By inhibiting the transcription of viral DNA, it can also be quite beneficial for those patients who have been diagnosed the HIV/AIDS to effectively alleviate their pain and relatively enhance their immunity as the virus cannot replicate themselves normally and hence the infected leukocytes will reduce significantly. However, unlike the co-receptor CRISPR-Cas9 system targeting human own body cells, the T5 system targeting the virus, which can be more difficult to actually operate in clinical application, so it is now more a theoretical mechanism and its actual use in clinical cases is still remains to be established. Also, because of the main function of T5 system is to block the virus gene expression, it is still possible for patients to get infected when exposing in new viruses outside even after treatment of the old infection in body, these defects all need further discussions. Due to these, in the future the major and most effective therapy against HIV invasion should be a combination of this two gene editing directions. When blocking the provirus reservoir it is important to knockout the co-receptors at the same time to establish a complementary relationship between these two methods. The drawbacks of one can be avoided by another one's advantages, this should be the main direction scientists may develop in the future to finally come out the method that can eradicate the virus and cure HIV/AIDS completely.

7. Conclusion

The use of CRISPR-Cas9 system in targeting HIV to inhibit its infection efficiency can simply be classified as two ways---viral editing and human-cell editing, all targeting to disrupt the normal infection of HIV. In HIV-1 case, for CRISPR-associated human cells editing is to use designed gRNA respectively or simultaneously knockout two important co-receptors CCR5 and CXCR4 in HIV infection pathway and the CCR5 editing has already be applied into the clinical case. Another way of viral editing is to target the provirus LTR region and with NHEJ repair mechanism the transcription factors like Tat or NF- κ B can no longer bind to the original binding site, which efficaciously inhibiting the transcription and thus disrupt the latent reservoir which suppressing the Antiretroviral drugs. From a personal point of view, provirus editing is more likely a theoretical technique in cell level but quite hard to be established into mammals or clinical cases like co-receptor editing. Even if it can be successful applied into clinical cases, another problem of re-infection is also existed. Successfully inhibits the latent provirus reservoir does not means to inhibit the latent viruses elsewhere simultaneously. Thus it is not once and for all as re-infection can be possible because the co-receptors are unaffected and the pathway of infection does not blocked. Hence from my view the

editing of CCR5/CXCR4 can be the more promising therapy and can develop, help more in the clinical field.

Under scientists' efforts, more and more efficient ways to inhibit HIV infection are coming out. As the currently most promising and popular field, gene editing therapy likes utilizing CRISPR-Cas9 definitely will become the major direction to disrupt HIV infection due to its high specificity and less defects. For future developments, it may become possible to edit the HIV gene to inhibit its replication and at the same time editing T cells which HIV mainly infected to make them become more tolerant under the invasion of HIV in order to make the HIV vaccines become possible. Also theoretically it may be possible artificially producing stronger and HIV-targeting macrophages to make our bodies get ability to fight against them in the future. Although in the near 50 years it may be impossible for human to eradicate HIV likes eradicating smallpox, it will become more controllable and less aggressive under the world's efforts.

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