Progress of CRISPR/Cas9 in the treatment of sickle cell disease

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Abstract. Sickle cell disease (SCD) is a hereditary monogenic disease, which is characterized by the substitution of glutamic acid at the sixth position of β-peptide chain by valine, resulting in insufficient hemoglobin synthesis and a serious threats to human health. SCD causes about 80% of mortality and morbidity worldwide. Patients with SCD who are homozygous usually die before the age of 30. Patients with SCD who are heterozygous usually have a better prognosis because of their low intraerythrocytic hemoglobin S. However, FDA-approved drugs such as hydroxyurea and L-glutamine can reduce the severity of the disease, and there is no definite cure for all patients with SCD. At this stage, many scholars use creation and correction methods to induce pluripotent stem cells (IPSCs) to treat sickle cell disease. Gene editing is currently considered to be one of the most potential methods for the treatment of SCD. Among them CRISPR/Cas9 gene editing engineering technology has already made an abyss contribution to the transformation of genome engineering and its application in clinical or medical experiments. This review systematically introduces the gene therapy of CRISPR/Cas9, and summarizes the challenges and prospects of this technique in the treatment of sickle cell anemia.

Keywords: CRISPR/Cas9; SCD; gene editing; sgRNA.

1. Introduction

SCD is a typical recessive genetic disease. In the case of sickle red blood cells, their oxygen-carrying capacity is 50% of that of normal red blood cells. Studies have shown that amino acid mutations in hemoglobin peptides can lead to sickle cell anemia. That is, an "A-T" on the double strand of DNA becomes "T-A", resulting in the change of the base sequence on the corresponding mRNA from GAA to GUA. The former is a codon for encoding glutamic acid, and the latter GUA is a codon for encoding valine, leading to the abnormal synthetic hemoglobin[1]. Since the deformability of these cells is very weak, they are prone to stasis and destruction in microcirculation, and hemolytic anemia is prone to occur. Patients usually have jaundice, anemia, hepatosplenomegaly and poor development within 3-4 months of birth. Sickle-shaped cells blocking microcirculation can further cause organ dysfunction, mainly manifested as abdominal pain, shortness of breath, prerenal pain and hematuria. Studies have found that sickle cell anaemia is more common among blacks in Africa and India, which is usually inherited by autosomal dominance. It is more severe and has a poor prognosis. The incidence of the disease is about one in ten thousand, however, it usually focuses on specific ethnic groups. For example, the incidence of African Americans is as high as one in six hundred, while that of Hispanic Americans is only about one in one thousand [2].

In clinical practice, there is no specific treatment for SCD. It is generally recommended to prevent infection and hypoxia at ordinary times. Oxygen supply, fluid infusion and blood transfusion are provided when hemolytic attack occurs. In the past, sickle cell anemia was usually treated by blood transfusion or hydroxyl urea, but it could not be cured and the improvement effect was not obvious. In recent years, researchers have used Clustered Regularly Interspersed Short Palindromic Repeats (CRISPR/Cas9) to study the treatment of genetic diseases. In this way, it seems that CRISPR/Cas9 gene editing technology has gradually become a hot trend, and it is bound to bring new hope for the treatment of sickle cell anemia.

2. CRISPR/Cas9 system

Until 2012, Jinek et al. proposed a shocking genome editing technique called CRISPR-Cas9, which uses specific guided RNA sequences to identify the target DNA region and successfully guide the effector CaS protein to edit [3]. Once the target cells are introduced, CRISPR/Cas9-mediated double strand breaks (DSB) will promote the repair of damaged DNA. The above process involves the repair mechanism of DNA, which leads to some insertions / deletions, which usually deprive the gene sequence of its own specific gene functions, or if Homology Directed Repair (HDR) id activated, homologous stands will be carried to repair DNA breaks. From this point of view, by editing of effectively inhibiting the chromosome region of SCD mutation expression, CRISPR/Cas9 technology can provide unprecedented help in directionally correcting SCD mutation or inducing fetal hemoglobin expression.

CRISPR/Cas9 gene editing technology can be divided into sgRNA and Cas9 nuclease, which is a gene editing system for bacterial immunity. Tis mechanism can be understood to be due to the cleavage of bacteriophage or plasmid DNA, which makes specific DNA site editable. CRISPR/Cas9-mediated sequence-specific target recognition must be able to recognize the PAM sequence, on this basis, the 20 nucleotides of sgRNA can be hybridized with the target site of DNA, and double strand breaks can be produced at the target site. The DSB produced by CRISPR/Cas9 will trigger various repair of intracellular DNA. Among them, Non-Homologous End-Joining (NHEJ)-metated DNA repair technology can quickly connect DSB and produce a small number of insertion and deletion mutaions at the target site. And the mutations inserted at these targets can help us disrupt or remove target genes. Through investigation, it is found that HDR-mediated error-free DNA repair is much more complex than NHEJ-mediated repair, especially the repair requires the use of a homologous donor DNA sequence as a repair template [4-5]. This technique achieves the purpose of treating patients with sickle cell anemia by editing and correcting induced pluripotent stem cell genes. The edited IPSCs can be normally used for hematopoietic differentiation and restore the correct gene expression, or by reactivating the expression of γ-globin gene to alleviate the clinical symptoms and signs of patients, providing a potential choice for the treatment of patients.

3. Treatment of SCD with CRISPR/CAS9

In the first three months of pregnancy, HBF was the main globin type, until the sixth month, the main type was replaced by HBA, and both protein types were retained at 11. Hba, like Hbf, remains on chromosome 11, and the transition from HBF to adult globin is governed primarily by a site control region (LCR) controlled by an enhancer upstream of the gene that does not activate expression until it circulates to each globin promoter, otherwise it will not be expressed [6]. After switching to HBA, it was found that HBF, which was not completely inhibited, was obviously unevenly distributed in erythrocytes. Through a large number of studies, it can be inferred that HBF may be largely concentrated in specific cells of f-cells. Due to the high blood level of infants, the improvement of adult HBF level needs to be paid more attention to [7]. The increase of HBS can promote the protective effect of SCD. It was found that HBF increased significantly in patients with asymptomatic SCD [8]. In accordance with previous findings, HPFH-like phenotypes were obtained from the deletion or inversion of 13.6kb chromosomal regions in heat shock cells of SCD patients, which led to an increase in erythrocyte HBF levels, thus improving the results of in vitro sickle test [9]. Similarly, Wang (2008) used CRISPR/Cas9 point mutations in 115,200 clusters of gamma globin promoters to inhibit the binding of HBF transcriptional suppressors BCL11A and LRF, thus inhibiting the expression of HBF [10]. To demonstrate the applicability of these methods, animal models need to be established for in vivo evaluation before human studies can be conducted. Li et al. solved the defect that red blood cells could not be provide in mice by using human β-globin site transgenic (β-yac) mice, thus effectively studied the machine-processed of the destruction of inhibitor binding region in γ-globin promoter on the mechanism *in vivo* [11]. In secondary transplantation experiments, there was a clear shift from human beta to gamma globin expression in adult mouse erythrocytes with

significant target distribution. Studies have found that, in fact, gene transcriptional regulators can also effectively control the expression of HBF, such as SCA/TAL1, GATA1 and KLF1[12]. While all of these factors can be considered as potential candidates, it is challenging to directly target these factors for HBF induction. Frangoul et al. [13] edited the sickle cell disease gene using CRISPR/CAS9. The results show that α-globin and γ-globin are the main components of fetal hemoglobin, in which γ-globin decreases with the production of β-globin and adult hemoglobin after delivery. We know that BCL11A is a gene that inhibits the expression of γ-globin in red blood cells. In this way, the content of hemoglobin can be increased by down-regulating the expression of BCL11A and activating γ-globin. This is why many researchers began to use CPRSPR-Cas9 technology to edit autologous CD34+ cells, specifically for silencing BCL11A gene, to reactivate fetal hemoglobin production, thus verifying that CRISPR/Cas9 gene editing technology can effectively solve sickle cell disease.

4. Challenges of CRISPR technology in the treatment of SCD

Gene editing is a mainstream tool for correcting gene sequences. However, due to the high cost and the consumption of manpower and brainpower, the traditional gene editing methods are not recommended. CRISPR/Cas9 genome editing system can solve all the shortcomings of traditional gene editing engineering, and has been widely used. Not only because it is easy to use and cost-effective, but also supports the establishment of a genome-wide screening library to identify or circle therapeutic gene / chromosome regions that directly affect the target phenotype. However, it also has some shortcomings that need to be improved, such as the efficiency of gene editing, specificity and transmission mode. Before CRISPR technology can be used in clinical practice, many conditions must be met, and the adverse events caused by permanent unexpected variations should be minimized.

4.1. Off-target effect

The biggest challenge of CRISPR/Cas9 is the off-target effect, that is, the generation of unnecessary mutations at non-target locations in the genome. It has been shown that the efficiency of gene knockdown in cells can be significantly improved by altering their length, secondary structure, or chemical composition, prolonging the length of double strands to change the structure of sgRNA, and mutating the fourth thymine in thymine continuum to cytosine or guanine. In recent years, Cas9 proteins have been developed, such as Cas9 nicking enzyme or Fok1 fused to degraded sgRNA to perform ssDNA fragmentation. As a result, the missed target effect of WT Cas9 mutants can be significantly reduced [14]. And it can also increase the targeting ability of CRISPR/Cas9 nuclease by changing the PAM recognition site of CRISPR/Cas9 nuclease without losing the targeting specificity, which can change the characteristics of different variants of PAM-targeted Cas9 nuclease. For example, VQR and VRER variants can show stronger specificity in human cells, while another variant, xCas9, is a variant that can discriminate a wider range of PAM sequences than natural Cas9. Compared with typical SpCas9, it has stronger DNA specificity and lower genome-wide missed target effect [15].

4.2. Delivery efficiency

Although the study of CRISPR/Cas9 has become an important way to treat anemia, the technology is not yet mature and has not been popularized in clinic. How to make Cas9 protein enter the cytoplasm and nucleus is the main obstacle to the application of CRISPR system. At present, CRISPR/Cas9 technology has made remarkable achievements in many aspects the study of plasmid DNA, mRNA or functional protein complex. Studies have shown that adeno-associated virus (AAV) is a commonly used delivery vehicle in vivo, providing higher delivery efficiency, thus allowing tissue/organ targeting. The immune response caused by AAV may limit its application. However, because of its long-term expression, there may be a high risk of off-target mutations. The most challenging aspect of in vitro-based editing is its transitivity, specificity, and effectiveness. In vitro methods can be performed in a controlled environment and easily designed using multiple methods.

However, the in vivo method is too uncontrollable and will face the challenge of uncontrolled miss events. If the strategy of continuous expression of Cas9 is adopted, the experiment may be at the risk of gene editing for a long time, thus making the experiment at the risk of missing the target for a long time. In this context, the selection of appropriate delivery tools, highly specific gene mapping tools, is a potential way to prevent unintended mutations and reduce potential immune responses.

4.3. Gene editing efficiency

Application of CRISPR/CAS9 controlled by target flanking PAM sequence. For example, studies on genetic correction of characteristic chromosomal regions lead to a limited range of RNA selections for other specific CAS proteins. Therefore, in order to identify a large number of different PAM sequences, scientists have devoted themselves to studying and designing various Cas effect proteins for a long time. However, although different CAS effector proteins recognize different PAM, thus expanding the targeting of genomic location, the effectiveness and safety of CRISPR have not been well studied. Therefore, mature CAS is the first choice for researchers. We know that the recognition ability of SpCas9 to PAM is 5'NGG 30, but Cas9 homologues need longer PAM site. Although longer PAM can show the advantages of efficient delivery, it also has a lot of shi'yong. For example, studies have shown that smaller Cas effector proteins, such as Cas9 (SaCas9) derived from Staphylococcus aureus, have the advantage of efficient virus transmission because the 'NNGRRT' PAM site. Through the targeted mutagenesis of the residues near the double strands of DNA in PAM, the preference of PAM was successfully changed, thus the targeting range of Cas9 protein was expanded. Results of the Streptococcus canis Cas9 (ScCas9) study showed 50-NNG-30PAM, with a reported sequence similarity of 89.2% to SpCas9[16]. Studies have shown that it is critical and promising to improve the targets of various Cas effector proteins on the genome. However, while improving efficiency, we also need to ensure its security.

5. Conclusion

SCD is a monogenic disease that imposes a huge public health burden on the public. Therefore, a large number of scientists have began to study ways to treat the disease to help patients get rid of the disease. In recent years, CRISPR/Cas9 technology gives scientists new ideas and provides benefits for patients with sickle cell anemia, it can help patients relieve symptoms, and effectively treat the disease to some extent. Its main mode of action is:

(1) The increase of Hb F can be induced by inducing small fragment deletion or point mutation in the upstream promoter of γ-globin gene and deleting the promoter of β-globin gene, so as to alleviate the clinical symptoms of anemia;

(2) Replacement of loss of function in sickle cell anemia by induction of gamma-globin expression through regulation of fetal hemoglobin transcription factors or cis-acting elements such as BCL11a, ZBTB7a, KLF1, DNMT1, MYB, HRI, etc.;

(3) Producing specific DSB and HDR at specific sites to correct specific gene mutations;

Although CRISPR/CAS9 gene editing technology has many advantages, such as simplicity, efficiency, more studies are needed to improve its off-target effect and delivery efficiency. With the continuous development of gene editing technology, more exploration of targets and editing methods will continue to optimize gene editing strategies, thus bringing the best therapeutic effect for SCD.

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