

Progress of CRISPR-Cas9 in Treatment of β -thalassemia

Shenru Dai^{1,*}

¹ School of Life Science, Jilin University, Changchun, China

* Corresponding Author Email: daisr1320@jlu.edu.cn

Abstract. β -thalassemia is a common genetic blood disorder, including three clinical and hemtological conditions: the thalassemia intermedia, β -thalassemia carrier state, and thalassemia major. It is widely distributed in the tropical, subtropical and mediterranean regions, and It has grown to be a significant global public health issue. Currently, the sole curative treatment for β -thalassemia is hematopoietic stem cell transplantation (HSCT), but the development of the HSCT is limited by the lack of an immunomatched donor and the high cost. Considering the advancement of gene editing technology in recent years, it is possible to cure the β -thalassemia with low cost and higher chances of surviving. CRISPR-Cas9 system, since its characteristics of simple operation, targeting specificity and low price, has been widely used in the field of life science and medical science. Currently, the study of CRISPR-Cas9 technology in the treatment of β -thalassemia has made a partial progress. This review systematically introduces the application of CRISPR-Cas9 technology in the treatment of β -thalassemia.

Keywords: β -thalassaemia; CRISPR-Cas9; Gene Editing.

1. Introduction

Blood disorders are primary diseases in the hematopoietic system, and the common ones are thalassemia, hemophilia, and sickle cell disorders. According to the WHO Global Hemoglobinopathy Epidemiology Report in 2008, hemoglobin disorders (including sickle cell disorders and thalassemia) are a major cause of death among children under five in 71% of the world's 229 countries [1]. These diseases do great harm to people's health. Traditional treatment primarily mainly relies on iron-chelation therapy, blood transfusions, and, in some rare situations, splenectomy [2]. However, there are many side effects by using the above methods, such as the blood-borne infection, the secondary-iron overload, and easy to relapse. A small minority of patients can be treated with the only existing curative treatment for thalassemia: haemopoietic stem cell transplantation. Today, with this therapy, more than 90% of patients survive, with an approximate 80% disease-free survival rate. Nevertheless, the shortage of donors, immunological rejection, and high cost limit the acceptability of this treatment method [3]. In recent years, studies have found that thalassemia is caused by the changes of genes [3]. Gene-editing technology emerging in the 1980s has made gene therapy possible, and the genetic blood disorders are expected to be cured.

Nowadays, the common tools of gene editing include clustered regularly interspaced short palindromic repeats-associated systems (CRISPR-Cas), transcription activator like effector nuclease (TALEN), and zinc finger nuclease (ZFN). Compared with the other two generations of gene-editing techniques, CRISPR-Cas technology has many advantages, such as simplicity of operator, low cytotoxicity and low cost, etc. In 2013, CRISPR-Cas9 was successfully applied to mammalian cell gene editing [4]. Since then, it has been widely used in the research of various diseases, including thalassemia.

2. CRISPR-Cas9 system

The adaptive immune system called CRISPR-Cas developed during the lengthy evolution of bacteria and archaea. It can be used to fight against viruses and exogenous DNA. CRISPR array and Cas operon make up the CRISPR-Cas9.

The three primary phases of CRISPR-mediated adaptive immunity are adaption, expression, and interference [5]. During the adaptation step, the bacteria will captures a fragment from the foreign

DNA and inserts this nucleotide fragment into the CRISPR array. In the expression step, prior to being processed into mature CRISPR RNAs (crRNAs), the CRISPR array will be translated into precursor CRISPR RNAs (pre-crRNAs). The crRNAs and Cas proteins are subsequently combined to form the CRISPR ribonucleoprotein (crRNP) complexes. During the interference step, crRNA guides the crRNP complexes to its complementary DNA array. Then, the Cas proteins cut off the DNA.

CRISPR-Cas are primarily classified into two classification, class1 and class2, on the basis of the variations in the Cas protein [6]. In class1 the effector complex is made up of a number of different subunits., while in class2 the effector complex is a single protein with multiple functional domains. Only one Cas9 protein is required for the CRISPR-Cas9, which makes it be derived from class2. Under the guidance of a single-guide RNA (sgRNA), the Cas9 protein may uniquely recognize the protospacer adjacent motif (PAM) array of the target gene, and may then cut off double-stranded DNA to cause a double-strand break (DSB). After that, the non-homologous end joining (NHEJ) or homologous recombination (HDR) repair mechanism is triggered to repair the above DNA. With an exogenous DNA template, the HDR achieves precise repair at the target site, whereas the NHEJ produces nucleotide insertions or deletions, which typically disables the gene [7].

3. β -thalassemia

Hemoglobin is a special protein that transports oxygen, which is composed of heme and globin. The globin is a tetramer consisting of two different pairs of polypeptide chains (α chain and β chain). A deletion or point mutation on chromosome 11's hemoglobin subunit beta (HBB) gene causes the β -thalassemia. The mutation or deletion results in complete failure of β chain (β^0) or partial synthesis (β^+), and the proportion of α chain and β chain is imbalanced [8]. The redundant α chain is unstable. The free α chain will accumulate, degenerate and degrade, and then produce cytotoxic active oxides *in vivo*, which can damage the maturation and viability of red blood cells, and destroy the erythrocyte membrane, and eventually lead to hemolytic anemia. The degree of chain synthesis inhibition is determined by the type of HBB gene mutation, and in turn decides the severity of clinical symptoms. The severe case of β -thalassemia can be β^0 or β^+ homozygote (β^0/β^0 , β^+/β^+), or β^0 and β^+ heterozygote (β^0/β^+). The intermediate cases of β -thalassemia mostly are β^0/β^+ heterozygote or β^+ homozygote (β^+/β^+). The mild symptoms of β -thalassemia almost are β^0 or β^+ with normal genotype heterozygote (β^0/β , β^+/β) [9]. Currently, the following three categories can be used to categorize CRISPR-Cas9-based therapies.

3.1. Correction of mutations in the HBB gene based on HDR

When the Cas9 protein produces the distrand DNA, HDR can be used to edit the gene. The HDR is template-dependent. The study found that single-stranded DNA oligodeoxynucleotides (ssODNs) was more efficient than double-stranded DNA (dsDNA). Niu et al. [10] designed the CRISPR-Cas9 that pointed at the HBB gene's CD41/42 mutation. It was proved that ssODNs combined with CRISPR-Cas9 could correct the HBB gene's CD41/42 mutation in induced pluripotent stem cells (IPSCs). In this study, according to the whole-exome sequencing, high mutation load wasn't detected in the exons of the corrected IPSCs after CRISPR/Cas9 targeting. This method provides a safe and effective strategy for correcting HBB gene mutations in β -thalassemia IPSCs.

3.2. Restraining the expression of α chain gene

The main reason of β -thalassemia symptoms is the unbalanced of α chains and β chains. When α -thalassemia combines with β -thalassemia, the quantity of free α chains will greatly decreases, and the clinical symptoms of patients can be relieved [11]. Mettananda et al. edited the gene in the hematopoietic stem cells (HSCs) of primary human with the CRISPR-Cas9 [12]. They deleted the enhancer of the MCS-R2 α -globin enhancer, which caused the α -thalassemia globin. As anticipated, the expression was seen that the expression of α chains was reduced and the pathological globin chain

imbalances were corrected in the cells of beta-thalassemia patients when the altered CD34⁺ cells were transformed into erythroid cells. This approach has the potential to translate into a treatment for β -thalassemia. However, one of the disadvantages of this approach is that it can only be applied to patients with partial expression of β chains.

3.3. Regulating the expression of HBG

HBG is a hemoglobin gene that abundantly expresses in the fetal period. It can encode generative γ -globin chains. Fetal hemoglobin (Hb F) is a tetramer that is consisting of two γ chains and two α chains. After the birth of fetus, the expression of HBG is inhibited, and the synthesis of γ chains is reduced. After that the Adult hemoglobin (Hb A) that contains two α chains and two β chains eventually replaces the Hb F. If the expression of HBG is restored, the synthesis of γ chain can be increased, and the missing function of β chain can be replaced by the γ chain. It will relieve the clinical symptoms of β -thalassemia [13].

During the conversion process of fetal-to-adult hemoglobin, there are some important transcription factors such as BCL11A, ZBTB7A, KLF1, GATA1, NF-E2, and TAL1 [14]. Among these factors, it has been shown that BCL11A is one of the major regulators of silencing HBG in mouse models and human cells [15]. BCL11A inhibits Hb F's expression by binding to other known γ -globin inhibitors. Therefore, knocking out BCL11A is a potentially effective way to treat the β -thalassemia. BCL11A plays an essential role in hemopoietic stem cells' (HSCs) self-renewal, the growth of the central nervous system, and the maturation of B lymphocyte [16]. The expression of BCL11A must be specifically inhibited in erythroid cells, which is extremely difficult. The issue is resolved by the identification of an erythroid enhancer sequence. Only the erythroid lineage cells were affected by the deactivation of this enhancer, which resulted in BCL11A being repressed and γ -globin being induced [15].

CRISPR-Cas9 was used in Frangoul's study [17] to specifically target progenitor cells and CD34⁺ hematopoietic stem's BCL11A erythroid enhancer. Two patients reported increased amounts of allele editing in their blood and bone marrow a year after starting treatment. The content of Hb F was increased. Both of the patients were independent infusion, and eliminated the onset of vascular occlusion. But the patients developed some adverse effects, such as neutropenic pneumonia, sepsis, abdominal pain, etc. The study involved only a few patients. It is still a question whether the treatment is universal.

4. Conclusion

At present, the CRISPR-Cas9 has obtained some research results in the treatment of β -thalassemia, but it still has some problems to be solved.

(1) Off-target effect. Off-target effect is mutations that generate in non-target regions. Now, one of the main issues with CRISPR-Cas9 is the off-target effect, and it is difficult to avoid. Because of the permanence of the genetic modifications, if mutations occur at some important loci, they can lead to serious consequences, such as adverse reactions, the emergence of new disease, and even death. The studies have found that the off-target rate of the CRISPR system can reach 5.6%~125% of the targeted editing efficiency [18]. Additionally, the Cas9 protein may cut off DNA several times, which may cause deletion in large segments of chromosome ends at the megabase level.

(2) Delivery mode. The main delivery methods of the CRISPR-Cas9 include microinjection, electroporation, AuNP, and adeno associated virus (AAV) and so on [19]. The microinjection requires precise instrumentation and operation, and it is difficult to perform in large batches. The electroporation do harm to the phospholipid bilayer on the cell membrane, so it is more suitable for *in vitro* experiments. The AuNP contains gold atoms, which may easily causes irreversible damage to the cells. Compared to the previous methods, the AAV has lower toxic and less immunogenic. It is a safer delivery method, that is suitable for *in vivo* application. There are still some disadvantages of AAV. For example, the packaging size for the AAV genome is limited to the maximal length of

~4.7 kbp, while the Cas9 gene has a length of 4.3 kbp. Consequently, two distinct AAV vectors must be used to deliver the Cas9 gene and sgRNA independently [20].

As an emerging technology, the CRISPR-Cas9 technology offers benefits and drawbacks. In the future, it will be constantly improved and perfected, and eventually become mature. It must make more remarkable achievements, and become a powerful therapy to conquer blood diseases, tumors and even more diseases.

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