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Progress and development of CRISPR-Cas tool in the treatment of thalassemia

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Abstract. β-thalassemia is the world's major hemoglobin disorders. It is a hereditary chronic hemolytic anemia caused by a break in the β-globin peptide chain genes of hemoglobin, resulting in reduced or complete absence of synthesis of the β-globin peptide chains. Patients with β-thalassemia rely on blood transfusions for survival, which exacerbates the disease burden on national economies and the financial burden on patients in developing countries. Repeated blood transfusions can also lead to organ damage. The fetus's protein genes are usually silenced early and then replaced by adult protein genes. But mutations that cause gammaglobin to persist in the fetus can improve the electrocution of globin. Therefore, reactivating the protein genes of the fetus is the core of research. CRISPR/Cas9 is the common way to reduce the effects of disease by correcting disease-causing mutations, increasing or disrupting protein expression. CRISPR/Cas9 and ·considered effective new methods for generating hemoglobin. Hence, the main focus of this review is to explore the details of CRISPR/Cas9 inβ-thalassemia treatment in the context of Mediterranean β-thalassemia.

Keywords: Genetic Therapy, Thalassemia, Genome Editing.

1. Introduction

Thalassemia, also known as hemoglobinosis, is primarily prevalent in single-inherited genetic disorders. Thalassemia is generally considered a rare disease; however, its prevalence exhibits geographical disparities. While it is uncommon in northern China, it is more prevalent in regions such as Guangdong, Guangxi, and Sichuan, with a particularly high incidence in Guangdong. On average, approximately 1 in 9 individuals in Guangdong province carries the genetic mutation responsible for thalassemia. Thalassemia results from mutations in the DNA, which are responsible for oxygen transport by the body, constituting hemoglobin. These mutations associated with thalassemia are inherited from parents and can affect both the alpha and beta chains of the hemoglobin molecule. When there is a reduction in the production of these chains, thalassemia develops. The severity of thalassemia in affected individuals depends on the number of mutated genes inherited from their parents, with a greater number of mutations resulting in more severe forms of the disease. The severity of the condition is also determined by the specific hemoglobin molecules affected [1].

CRISPR-Cas9 is an adaptive immune defense system that has evolved primarily serving to combat viral and foreign DNA invasions. In the context of gene therapy, CRISPR-Cas9's gene-editing

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technology holds great promise for addressing a wide range of genetic diseases, including AIDS, eye disorders, tumors, and blood-related conditions, which are the main focus of this paper. This technology has already demonstrated successful genome modifications in mammalian, fish, and human cells, as well as various bacteria. When prokaryotic organisms encounter viral threats, they can extract a fragment of the viral DNA and store it in a designated region within their genome, known as the CRISPR storage. Upon subsequent viral invasions, prokaryotes can recognize the virus by referencing the stored DNA fragment and effectively neutralize the virus by cleaving its DNA. Capitalizing on the unique features of the system, scientists have transformed it into a highly effective genome editing tool [2].

While dyspoietic blastoblastic transplantation is one of the treatments for thalassemia, it is expensive and finding a suitable donor can be challenging. Another approach involves providing life-sustaining measures, which not only impose significant financial burdens on families but also consume substantial blood resources, resulting in a lower quality of life for patients without addressing the underlying cause of the disease. However, through CRISPR-Cas9 gene editing, it is possible to edit the BCL11A enhancer site in the hematopoietic cells of patients with beta-thalassemia and sickle cell anemia. Subsequently, transplanting the edited hematopoietic stem cells can yield a high proportion of normally functioning hemoglobin. This breakthrough suggests that gene editing technology holds the potential to eradicate a range of genetic diseases caused by protein mutations [2]. The CRISPR-Cas9 technology achieves this by transforming mutated genes into normal ones through genome insertion, deletion, or displacement. This approach can reactivate the production of fetal gamma proteins, offering an alternative to defective proteins and potentially serving as a means to alleviate or treat thalassemia [2].

This paper aims to provide researchers utilizing gene editing technology for thalassemia treatment with the latest developments, promote its advancement, and explore the challenges and potential solutions of CRISPR-Cas9 technology in gene therapy. The paper will comprehensively synthesize the principles of CRISPR-Cas9 genetic editing, serving as a reference for research on genetic therapy for blood-related diseases.

2. The three clinical phenotypes of thalassemia

Thalassemia is a type of hemoglobin disorder and represents the most prevalent hereditary hemoglobinopathy. Normal adult hemoglobin molecules, referred to as Hb A, consist of two pairs of peptide chains, α and β. Thalassemia arises from an imbalance in hemoglobin synthesis, primarily stemming from reduced production of at least one of the globin peptide chains [1].

β-thalassemia arises when a break occurs in the β-globin gene, leading to diminished production of the β-peptide chain and impaired synthesis of hemoglobin A. These mutations or deletions can result in either partial or complete loss of function of the β-globin gene. As there are two β-globin genes in an individual's genome, patients can exhibit heterozygous, homozygous, or compound heterozygous mutations [3].

Within the context of β-thalassemia, the clinical phenotype can be categorized into three classes based on the extent of impairment in β-globin production. Mild β-thalassemia occurs in heterozygotes and typically remains asymptomatic, characterized by mild to moderate microcytic anemia. Individuals with mild thalassemia who are carriers generally do not face significant health implications and do not require treatment. However, if carriers with mild thalassemia procreate with each other, their offspring may potentially develop severe β-thalassemia. Intermediate β-thalassemia presents clinical manifestations that fall between the mild and severe forms and is the result of inheriting two β-thalassemia alleles. Severe β-thalassemia form manifests in homozygotes or severe compound heterozygotes, resulting in a profound deficiency of β-globin. Individuals with severe β-thalassemia experience severe anemia and exhibit highly active bone marrow hyperplasia [4]. Symptoms may emerge as early as 1 to 2 years of age and include severe anemia, transfusional iron overload, and excessive iron absorption. Patients may develop jaundice, leg ulcers, and gallstones. Splenomegaly (enlarged spleen) is common and can lead to accelerated destruction of transfused normal red blood cells due to splenic sequestration. Active bone marrow hyperplasia can cause thickening of the skull bones

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and prominence of the cheeks. Long bones are susceptible to pathological fractures, impacting growth and development and potentially delaying or hindering the onset of puberty.

3. The current status of thalassemia treatments

Standardized blood transfusion and iron chelation therapy are pivotal components in the management of thalassemia patients. Hematopoietic stem cell transplantation (HSCT) represents the sole established curative intervention in current clinical practice, whereas gene therapy and novel pharmaceutical agents present emerging prospects in the realm of thalassemia therapeutics.

3.1. Blood Transfusion Therapy

The primary objective of blood transfusion therapy is to sustain hemoglobin concentrations within the vicinity of normal levels. This intervention serves to mitigate compensatory bone marrow hyperplasia, curtail extramedullary hematopoiesis, diminish intestinal iron absorption, counteract chronic hypoxia, foster proper growth and development, and enhance the overall quality of life [5].

3.2. Iron Chelation Therapy

In light of recurrent blood transfusions and chronic anemia, thalassemia patients often grapple with secondary iron overload arising from excessive absorption of iron within the gastrointestinal tract and other contributing factors. The accumulation of excess iron can implicate multiple organ systems, including the heart, liver, spleen, kidneys, pancreas, precipitating cellular damage and organ dysfunction. Clinical manifestations may encompass growth retardation, infertility, liver cirrhosis, and congestive heart failure. Notably, congestive heart failure constitutes the most prevalent cause of mortality in thalassemia, underscoring the critical importance of vigilant monitoring and precise assessment of iron overload. Various conventional methods are employed for evaluating iron metabolism, encompassing the measurement of SF levels and MRI for the quantification of iron concentrations within the liver and heart. Timely implementation of iron chelation therapy, involving agents such as deferoxamine, deferasirox, and deferiprone, serves as a pivotal safeguard against complications arising from iron overload. The selection of an appropriate iron chelator necessitates careful consideration of variables such as the specific thalassemia variant, the extent of iron deposition, patient compliance, tolerability, and economic factors [6].

3.3. Hematopoietic Stem Cell Transplantation (HSCT)

HSCT presently stands as the sole curative modality for thalassemia, with clinical remission rates ranging from 80% to 90%. It represents the preferred therapeutic avenue for severe forms of thalassemia and should ideally be conducted expeditiously, ideally between the ages of 2 and 7. Moreover, HSCT should be contemplated in instances of deteriorating intermediate thalassemia [6].

3.4. Pharmaceutical Interventions

Pharmacological interventions hold promise for intermediate thalassemia and certain severe cases of β-thalassemia, aiming to augment the γ-globin gene expression, enhance fetal hemoglobin (HbF) synthesis, restore equilibrium in the α- to non-α-globin chain ratio, and ameliorate anemia symptoms. Commonly employed medications encompass hydroxyurea, erythropoietin (EPO), and traditional Chinese medicine formulations like Yimei Shengxue Granules. Recent research has unveiled additional agents, including 5-az, decitabine, short-chain fatty acids, and sildenafil, which have demonstrated the potential to activate the γ-globin gene, elevate HbF expression, and ameliorate anemia symptoms.

3.5. Splenectomy

Thalassemia patients exhibit a shortened lifespan of red blood cells, resulting in their sequestration and hemolysis within the spleen. Splenectomy can mitigate red blood cell destruction, thus enhancing anemia and elevating hemoglobin (HGB) levels. However, this procedure is not devoid of risks, as it may engender heightened susceptibility to infections, thrombotic events, and pulmonary hypertension.

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The decision to undertake splenectomy necessitates a judicious appraisal of potential advantages and drawbacks [7].

4. The development and mechanism of CRISPR technology

Gene editing is a technology that facilitates precise modifications to an organism's genome by altering its DNA sequence. The development of gene editing techniques has evolved through distinct phases.

Zinc Finger Nucleases (ZFNs) are synthetically engineered proteins designed to selectively bind to specific DNA sequences, guiding a nuclease-like cleavage activity. This cleavage activity induces double-strand breaks at precise gene locations, thereby initiating the cellular repair mechanisms [8]. Through this repair process, precise and deliberate modifications to the genome can be accomplished. However, it's worth noting that while ZFNs were pioneering gene editing tools, they are relatively intricate to design and construct compared to the subsequent CRISPR-Cas9 technology, demanding more time and resources [9].

Similar to ZFNs, TALENs are tools capable of directing the cleavage of specific DNA sequences. TALENs employ a transcription activation-like effector nuclease mechanism. These TALENs draw inspiration from transcription activator-like effector nucleases found in the bacterium, a plant pathogenic bacterium that interacts with plant cells during infections. These enzymes possess the capacity to recognize precise DNA base sequences. TALENs comprise two primary components: transcription activator-like effectors (TALEs) and a nuclease domain. Each TALE consists of a series of repeat units, with each unit corresponding to a specific base pair in the DNA sequence. The amino acid residues within these units determine the specificity of TALEs' binding to DNA sequences. By designing these repeat units according to the target DNA sequence's base composition, TALENs can be accurately directed to bind the target sequence [10].

CRISPR technology, on the other hand, represents a groundbreaking gene editing tool with contemporary applications. Its widespread adoption began following the research conducted by Jennifer Doudna and Emmanuelle Marie Charpentier.

Cas9, which stands for "CRISPR-associated protein 9," is an enzyme that, when guided by a specific RNA sequence known as guide RNA (gRNA), can precisely cleave a targeted DNA sequence. This precision allows for the accurate editing, addition, or removal of genes within the genome [9][10].

In comparison to ZFNs and TALENs, the CRISPR-Cas9 technique offers significant advantages in terms of ease of design and operation, enabling researchers to conduct gene editing with greater speed, precision, and cost-effectiveness.

5. Thalassemia treatment strategy with CRISPR/Cas9

5.1. In situ repair of mutant genes

5.1.1. Repair of Exonic Point Mutations/Frameshift Mutations Mediated. Exonic point mutations or frameshift mutations can lead to abnormal protein structure or synthesis, as seen in β-thalassemia [11]. In such cases, precise repair is necessary to restore gene function. The commonly used method is gene homologous recombination repair pathway. However, the efficiency and stability of homologous recombination are low in HSCs and iPSCs. This has led to the development of an alternative precise repair method using CRISPR-Cas9 to repair mutations in the genome. Cross-over homologous recombination technology exhibits higher efficiency and stability.

5.1.2. Repair of Intronic Point Mutations Mediated by CRISPR/Cas9. Intronic point mutations can lead to abnormal mRNA splicing, affecting protein translation and leading to diseases. For instance, the β-thalassemia-causing β-globin gene IVS2-654 (C>T) mutation results in abnormal splicing of β-globin mRNA precursor, producing an aberrant mRNA (254 bp) with an additional 73 bp intron sequence compared to the normal mRNA (181 bp), consequently impacting β-globin synthesis [12]. Since introns do not encode proteins, as long as intronic mutations do not affect mRNA precursor splicing, gene

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function remains unaffected. Therefore, precise repair is unnecessary, and can restore the function of genes with intronic mutations [13-15]. Repairing DNA double-strand breaks near intronic mutations through NHEJ efficiently deletes intronic mutations (achieving up to 98% efficiency), restoring gene function [14, 15]. However, the heterogeneity of insertions or deletions resulting from non-homologous end joining raises the question of whether these mutations introduce new abnormalities in gene expression and protein function, necessitating further research.

5.1.3. In Situ Repair Mediated by Single Nucleotide Editing Technology. For hereditary blood disorders caused by single nucleotide mutations, in addition to the aforementioned in situ repair using CRISPR/Cas9 technology, precise repair of mutations can also be achieved through single nucleotide editing technology. Editing the -28(A>G) mutation in the β-globin gene using single nucleotide editing demonstrated that in patient-derived hematopoietic stem cells, 36.4% of mutations were accurately repaired (C>T), 56.4% were erroneously edited (C>G/A), and 3.6% were not edited [16]. This suggests that the efficiency of precise repair mediated by single nucleotide editing technology in patient hematopoietic stem cells needs improvement. Additionally, single nucleotide editing technology has issues with high off-target rates. Studies have shown that single nucleotide editing systems can lead to extensive off-target effects. In the HEK293T cell line, the single nucleotide editing system BE3 caused nearly 100% RNA single nucleotide variations [17]. Introducing point mutations into deaminases may be an effective method to reduce off-target effects [17].

5.2. Regulation of Gene Expression

The imbalance between α and β chains is a direct cause of thalassemia. Reduced or absent synthesis of β chains in β-thalassemia patients leads to a relative excess of α chains. Reducing α chain synthesis can alleviate the symptoms of thalassemia. Gene editing of the α-globin cis-regulatory elements can modulate α-globin expression. Research shows that knocking out the core elements of the α-globin gene enhancer MCS-R2 in β-thalassemia patient hematopoietic stem cells using CRISPR-Cas9 can decrease α chain synthesis levels.

β thalassaemia is a complex and diverse disorder caused by mutations located on chromosome 11. The type of mutation in the hexabromobiphenyl gene determines the degree of inhibition of β-globin synthesis, thereby affecting the severity of clinical symptoms.

γ Chain is a component of fetal hemoglobin HbF (α2γ2), highly expressed during fetal development. After birth, γ chains are gradually replaced by β chains, transitioning the predominant adult hemoglobin HbA (α2β2). Reactivating the γ-globin gene leads to the production of γ chains that combine with α chains to form HbF. This can substitute for HbA and alleviate symptoms caused by relative excess of α chains in thalassemia and abnormal β chain structure in sickle cell anemia [18, 19]. γ-Globin gene expression is regulated by various elements, including transcription factors, promoters, and enhancers. Transcriptional repressor BCL11A significantly influences low γ-globin gene expression in adult erythroid cells [20]. BCL11A binds to the proximal promoter of the γ-globin gene and suppresses its expression. While deleting BCL11A in hematopoietic stem cells significantly increases γ-globin gene expression in differentiating erythroid cells, it affects lymphocyte maturation [21]. Further research identified a erythroid-specific enhancer of BCL11A. Deletion of this enhancer's DNA enzyme I hypersensitive site (DHS) +58 in hematopoietic stem and progenitor cells specifically downregulates BCL11A expression in erythroid cells, without affecting other cell types [20]. The use of CRISPR/Cas9 removes this site in patients with β thalassemia and sickle cell anemia, where hematopoietic stem cells result in the formation of fetal hemoglobin with erythrocyte formation. Clinical trials establishing this concept are ongoing.

6. Limitations and future development

There are several important milestones in the future prospects and drawbacks of CRISPR technology, according to public reports, CRIDPR gene editing-based therapeutics have successfully reached the clinical development stage, CRISPR Therapeutics and Vertex have begun to submit a rolling marketing

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application for exa-cel to the FDA, and are expected to complete the submission. If exa-cel is approved, it will be the first CRISPR gene-editing therapy approved for marketing. Exa-cel, is a one-time therapy being developed for sickle cell anemia and transfusion-dependent β thalassemia. CRISPR gene-editing therapies have the potential to cure currently difficult cases, such as cancer. CRISPR works by modifying T cells or other immune cells so that they can recognize and destroy tumor cells. In this article's discussion of the treatment of transfusion type β thalassemia, CRISPR can be used to repair mutations in the hemoglobin gene, thereby curing hereditary anemias.

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Application of CRISPR in medicine is still very limited, the main thing is that it has three drawbacks. The first is that it will be interfered by a variety of different factors, and editing efficiency will be affected; The second is that CRISPR has a higher risk of off-targeting. The so-called off-target effect is the possibility of accidental changes of the genome in the region of the target during editing, which may lead to genetic mutation or cytotoxicity. The third and final drawback is that gene-editing technology still has moral and social concerns, which are manifested in whether changing the genes of human embryos is correct, whether CRISPR technology will cause genetic contamination or biosecurity risks, whether it will increase unequal social relations, and whether it will lead to new social discrimination problems. There are still some questions about the long-term safety and efficacy of CRISPR technology, and more research is needed to support it before CRISPR can actually be pushed into clinical treatment.

Authors Contribution

Fiona Jun Lu and Zuzhe Xu contributed equally and they are co first authors, their names were listed in alphabetical order; Wei Fen is the second author.

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