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Step-Wise Approach for In Silico CRISPR-Cas9 Gene Editing of IVS I-110 and IVS I-6 Beta-Thalassemia-Causing Variants

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Abstract

β-thalassemia is a common monogenic blood disorder caused by disease-causing variants, mostly point mutations in *HBB* gene. In Egypt, it is the most common hemoglobinopathy with minor allele frequency of 0.03 and a carrier rate varying between 5.3 to more than 9%) creating a major social and financial burden for the patients, their family, and the society in general. The current study aims to design a guide RNA to target the most common mutations identified among B Thalassemia patients. By using the human variation viewer database, two mutations within the *HBB* gene have been selected. By computational tools, two guide RNA have been designed and showed a high efficiency score towards the desired mutations. The selected two gRNA were successfully cloned into synthetic plasmid (PX459). The data successfully established a stepwise in-silico approach for designing CRISPR/Cas9 gene editing model for correction of IVS I-110 (G>A, rs35004220) and IVS I-6 (T>C, rs35724775) variants in *HBB* gene.

Keywords: CRISPER cas9 , β-thalassemia, insilico study.

1. Introduction

Correction of disease-causing genetic variants via gene editing approaches has been used in many rare disorders with variable levels of success **[1,2].** Several attempts have aimed to develop efficiently reliable methodologies to make precisely targeted changes to the genome of living cells. Over the past years, manipulation of gene function via RNA interference (RNAi) and homologous recombination have shown disadvantages, primarily mutagenesis through transit and off-target effects **[3].** DNA double-stranded breaks (DSBs) can be introduced at or close to the site of the mutation using bio-engineered nucleases such as zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), thus DNA repair via homologous recombination can be manipulated to correct/edit the mutated target. However, both bio-engineered nucleases were challenged by the cost and time consumption **[4].**

One of the most promising gene editing tools is formulated using bacterial Clustered Regularly Interspaced Short Palindromic repeats (CRISPR). The CRISPR utilized by *Streptococcuspyogenes* as an immune defence mechanism relies on protein called cas-9 nuclease **[5].** Originally, the viral and

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plasmid DNA were found to be degraded by the host bacterium into short repeats and incorporated into a CRISPR locus. The locus is then transcribed and processed to generate small RNAs (crRNA-CRISPR RNA) which serve as a bacterial “memory” or adaptive immunity system to guide effector endonucleases to induce double strand breaks (DSB) in order to dysfunction the invading DNA based on sequence complementarity **[5,6,7].**

The CRISPR/Cas9 system is now known to be valuable to edit/repair non-functional genes and disease-causing variants in mammalian cells. Using a 20-nucleotide short guide RNA (gRNA) of 20-nucleotide incorporated into CRISPR locus cassette, the *Streptococcuspyogenes* cas9 targets a trinucleotide (NGG) protospacer adjacent motif (PAM) in the target mammalian DNA inducing DSB in proximity. In return, cellular repair mechanism is induced, particularly homologous directed repair (HDR) events which rely on template strand for repair. In gene editing scenario, the template repair strand can be used to change/correct a targeted base, hence, the correction of a disease-causing base-change **[8,9,10].** The ease and flexibility of the system generated tremendous excitement due to its possible clinical applications. Here in, the study aimed to establish CRISPR/Cas9 gene editing technique to be applied into patient-derived primary cell culture, e.g., hematopoietic stem cells (HSCs) or skin fibroblasts for correction of beta-thalassemia disease-causing variants. The most severe form of beta thalassemia is beta thalassemia major. It involves two beta-globin genes that are either incorrect. Those who have beta thalassemia major need blood transfusions for the rest of their lives. While Beta thalassemia minor, also known as beta thalassemia trait, frequently manifests as moderate anemia. One faulty or absent beta-globin gene is involved. Some individuals with mild beta thalassemia show no symptoms at all.Our approach targets two intronic variants: IVS I-110 (G>A, rs35004220) and IVS I-6 (T>C, rs35724775) in the human hemoglobin beta (*HBB*) gene on chromosome 11, which were identified as the most common mutations among Egyptian β-thalassemia patients **[11].** The ideal approach for curing a genetic disease such as β-thalassemia is to correct the disease-causing mutations. To date, HSC transplantation is the only cure despite being limited by obtainability of compatible donors. Gene therapy has been used successfully to treat monogenic recessive hematopoietic disorders via integration of functional genes into progenitor HSCs **[12,13].** The workflow developed herein relies on the in-silico design of gRNA targeting IVS I-110 (G>A, rs35004220) and IVS I-6(T>C, rs35724775), and its incorporation into vector plasmid harbouring CRISPR/Cas9 cassette.

2. Methodology/Experiment:

A- Molecular Study for HBB gene sequencing

Thalassemia patients were subjected to complete clinical including pedigree analysis for at least 3 generations with emphasis on consanguinity, and family members having similar or different genetic abnormalities. The study was approved by Ethics committee of National Research Centre. Routine haematological investigations including CBC and homolytic profile assessment. Genomic DNA was extracted from peripheral blood leukocytes of all patients using QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacture instructions. Amplified Beta globin gene (*HBB*) fragments were subjected to direct sequencing in both directions. Samples were run on 2% agarose gel. Purified samples were subjected to cycle sequencing using Big Dye Terminator v3. 1 Kit. The collected sequencing results were blasted on the reference gene on the NCBI gene bank. This step is essential to identify any single nucleotide polymorphisms (SNPs) around IVS I-110 and IVS I-6 which could interfere with the function gRNA while targeting the exact complementary sequence upstream IVS I-110 and IVS I-6.

Using the human variation viewer database (https://www.ncbi.nlm.nih.gov/variation/view/) was used to assess the variations around both mutations that affect gRNA design for gene editing experiment, **Fig (3 & 4)**.

B- Designing gRNAs

Optimal gRNA design is critical for successful gene editing experiments, so our aim is how to choose the best tool for sgRNA design to minimize the off-targets effects._First, we designed it

manually, according to the previous data published. We found the following essentials of CRISPR gRNA design **[14,15].** The target region or gene: Around each mutation, 500 base pair (bp) upstream and downstream were downloaded according to human genome assembly: *Homo sapiens(hg38)*, chr11:5226350-5227242 coordinates.

C- In silico prediction tools for CRISPR/Cas9 off target effects

We used the computational tool CRISPOR (ucsc.edu)to search for matching sgRNAs by gene name or input the target sequence, provide options to use alternate PAM sequences, provide options to predict off-targets.

D- Design of repair templates

SNAP gene software (SnapGene | Software for everyday molecular biology) was used for visualization of the experimental design. ssODN repair templates were designed to be 160bp and have homology arms flanking the deletion site to facilitate HDR. One ssODN template was ordered for each allele. It is essential to mention that CRISPR/Cas9 introduces double strand breaks (DSB) guided by the designed gRNA. DSB activates the main cellular repair main pathways: non-homologous end joining (NHEJ), and homology-directed repair (HDR). ssODN facilitates HDR which uses the ssODN homologous DNA repair template to precisely correct the DSB through genomic recombination. This allows for the correction of specific mutations into the genome **[16].**

E- Molecular cloning

sgRNAs (for each IVSI.110 and f IVSI.6) were purchased as single-stranded DNA oligos (25nmoles standard desalted, IDT) with BbsI sticky ends and cloned in pairs into pX459 (Addgene #62988) using a one-step cloning reaction as described by the **[17].** A cloning mixture volume of 5µL or 10µL was then transformed into 25µL or 50µL, respectively, of One Shot TOP10 Chemically Competent E.Coli (ThermoFisher #C404003) following the manufacturer's protocol. Transformed bacteria were plated on carbenicillin or ampicillin LB agar plates and incubated at 37℃ overnight. Individual colonies were picked into 3-5mL of LB broth and cultured at 37℃ overnight. Isolation of the plasmid DNA from bacterial clones was conducted using Zymo or Qiagen mini prep and maxi prep kits (Qiagen #27104, Zymo #D4208T).

3. Results

A- Molecular Study for HBB gene sequencing

The sequencing chromatograms of IVS I-110 (G>A, rs35004220) and IVS I-6 (T>C, rs35724775) sequencing chromatograms are shown in **Fig (1 & 2).**

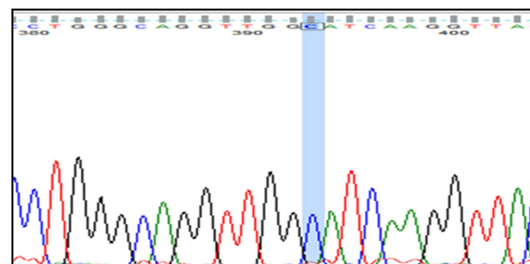

Figure 1 : Homozygous mutation in IVSI.6 (T>C, rs35724775)

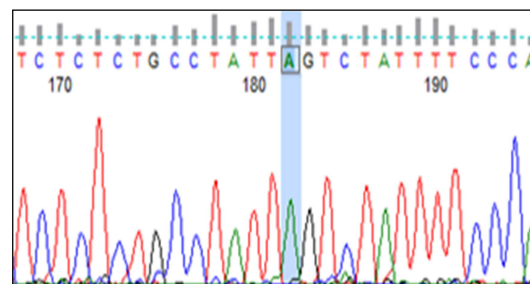

Figure 2: Homozygous mutation in IVSI.110 (G>A, rs35004220)

The human variation viewer database (https://www.ncbi.nlm.nih.gov/variation/view/) was used to assess the variations around both mutations that affect gRNA design for gene editing experiment **Fig (3 & 4).**

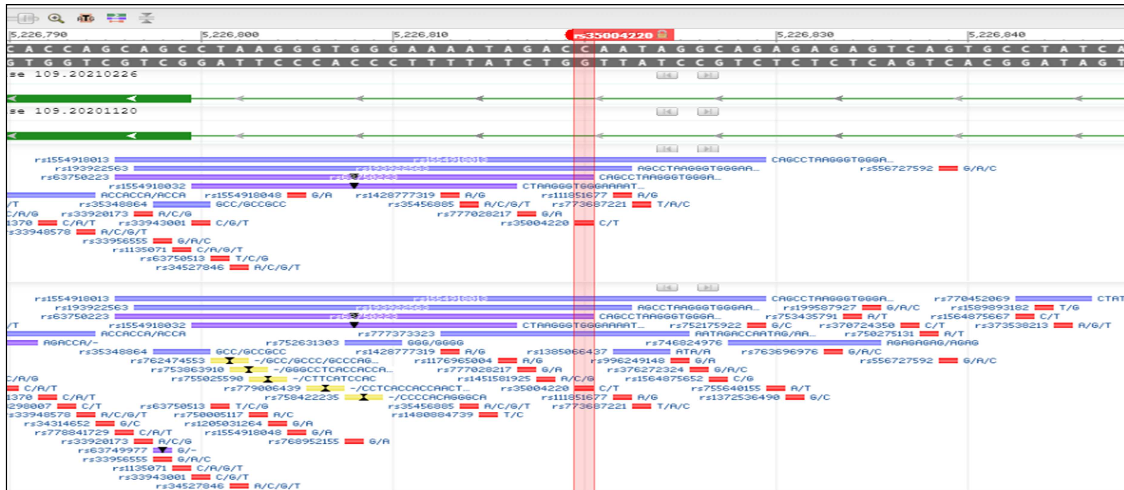

Figure 3: Variations flanking the IVS I-110(G>A, rs35004220) mutation.

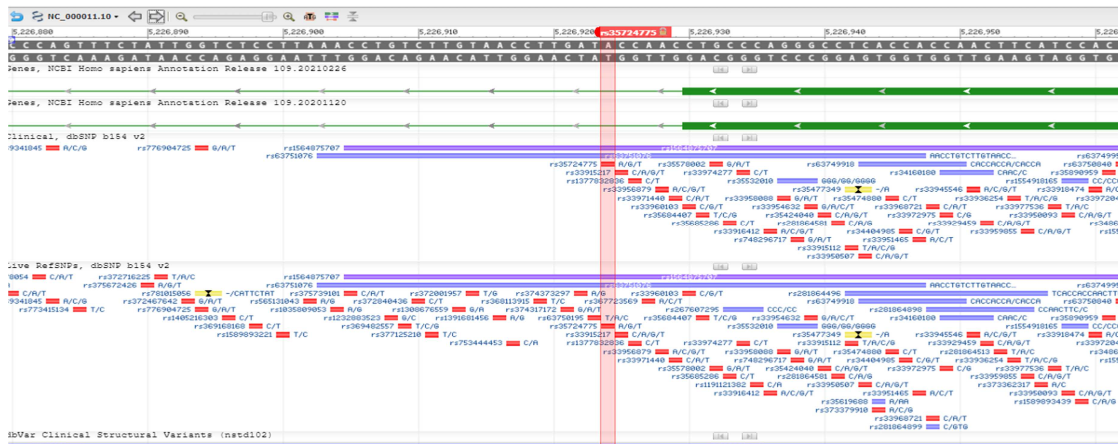

Figure 4: Variations flanking the IVS I-6(T>C, rs35724775) mutation.

B- Designing gRNAs

The target sequences around IVSI.110 and IVSI.6 mutations (mutations are shown in red).

- **Target of IVSI.110**: 3'-CACTGACTCTCTCTGCCTATTGGTCTATTTTCCA-5'
- **Target of IVSI.6:** 3'- GCCCTGGGCAGGTTGGTATCAAGGTTACAAGACAGG

Identifying potential PAM (NGG) sequence in the target DNA:

- **Target of IVSI.110**: 3'-CACTGACTCTCTCTGCCTATTGGTCTATTTTCCA-5'
- **Target of IVSI.6**: 3'- GGCCCTGGGCAGGTTGGTATCAAGGTTACAAGACAGG -5'

Target PAM (NGG) sequences may be ideal for conventional Cas9. Determine the 5 start for gRNA by counting 20 bp upstream of the PAM sequence:

- **Sequence of gRNA for IVSI.110:** 3'-CACTGACTCTCTCTGCCTAT-5' PAM: TGG
- **Sequence of gRNA for IVSI.6:** 3'-TGGTATCAAGGTTACAAGAC-5' PAM: AGG

C- In silico prediction tools for CRISPR/Cas9 off target effects

Using CRISPOR (ucsc.edu), as shown in Table 1, two gRNA were chosen. gRNA110 targets IVS I-110 with high efficiency score above 75% and its mismatches score indicate that the gRNA sequence is unique to its target in the human genome since introducing 2 mismatches in the gRNA is required for an off-target effect to occur in three different genomic locations while introducing 3 mismatches in the gRNA is required for an off-target effect to occur in 27 different genomic locations, and 4 mismatches in the gRNA is required for an off-target effect to occur in 207 different genomic locations indicated by (0-0-3-27-207) score in the mismatches column. Similarly, gRNA6, targeting IVS I-6, had high efficiency score above 75% and its mismatches score indicate that the gRNA sequence is unique to its target in the human genome evident by (0-0-3-10-115) score in the mismatches column.

Table 1: CRISPOR tool score for gRNA110 and gRNA6

gRNA	Sequence	specificity	efficiency	Mismatches	Off targets
470/rev gRNA 110	**CACTGACTCTCTCTGCCTAT TGG G**	**67**	**77**	**0-0-3-27-207 0-0-1-4-4 237 offtargets**	**3: intergenic:RP11-577G20.2 4: intergenic:RP11-21J7.1-AL 4:intron:ZSWIM6 Show all**
556/rev gRNA 6	**TGGTATCAAGGTTACAAGAC AGG**	**78**	**76**	**0-0-3-10-115 0-0-0-2-1 128 offtargets**	**4:intron RIN2 4:intron PARK2 4:intron AC009480-3 Show all**

D- Design of repair templates

The visualization of the design can be shown below via SNAP gene software (SnapGene | Software for everyday molecular biology) which allows the visualization of the sequence for the repair template which is a single stranded DNA (ssODN, yellow box) of the forward/upper strand, see **Fig(5 & 6).**

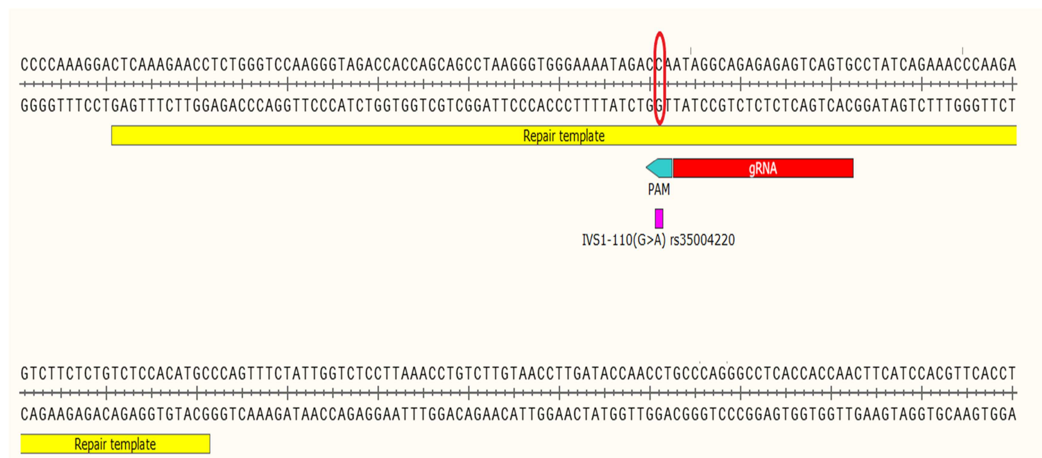

Figure 5: gRNA110 design for introduction of IVS I-110(G>A, rs35004220) mutation.
The figure shows IVS I-110(G>A, rs35004220) mutation site (red circle around G (wild type) allele), the change from G to A will be introduced via the repair template which is a single stranded DNA (ssODN, yellow box). gRNA is shown as a red bar preceded by PAM site TGG on the reverse strand (green arrow)

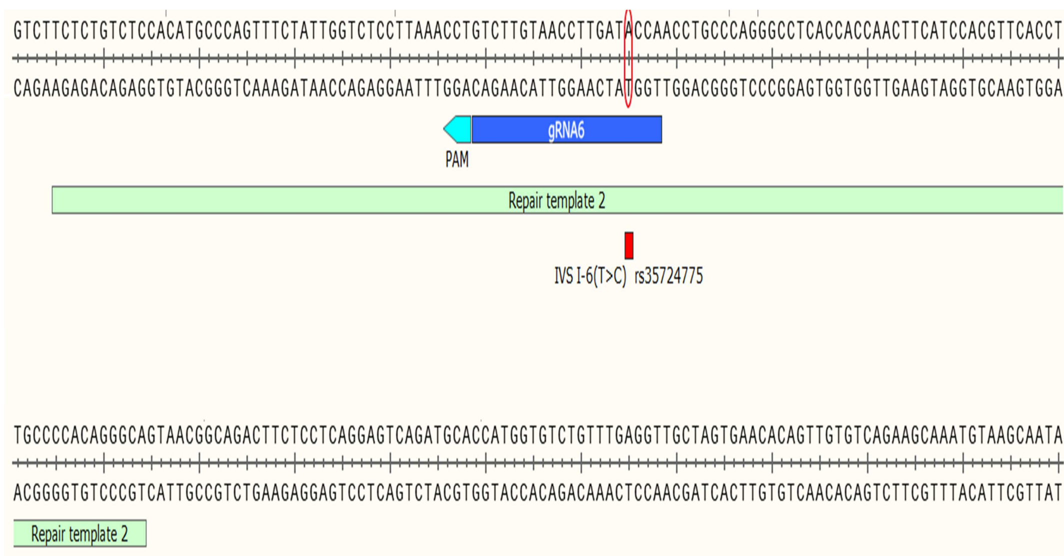

Figure 6: gRNA6 design for introduction of IVS I-6(T>C, rs35724775) mutation.
The figure shows the IVS I-6(T>C, rs35724775) mutation (red circle around T (wild type) allele), the change from T to C will be introduced via the repair template which is a single stranded DNA (ssODN, light green box). gRNA is shown as a blue bar preceded by PAM site TGG on the reverse strand (light blue arrow).

E- Molecular cloning

Single sgRNAs (for each IVSI.110 and IVSI.6) were successfully cloned in PX459. This was confirmed by loss of restriction site of BbSI enzyme. Figure 7 below shows cloned PX459 with IVSI.110 (Plasmid1-3 (P1-3)) and IVSI.6 (P4&5).

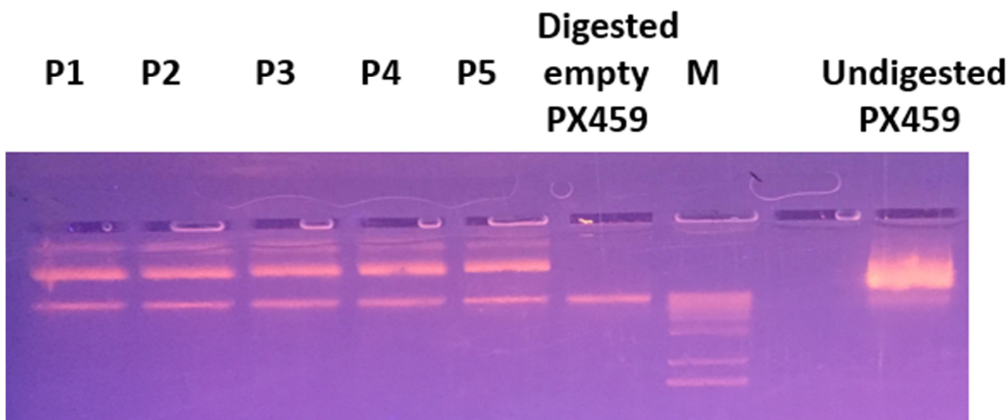

Figure 7: Digestion of cloned PX459 preparations (cloned with gRNA for correction of IVSI.110 (prep1-3) and IVSI.6 (prep4&5) by *BbSI* enzyme.

4. Discussion

β-thalassemia is a common monogenic blood disorder caused by disease-causing variants, mostly point mutations in *HBB* gene [8,18].

In Egypt, it is the most common hemoglobinopathy with minor allele frequency of 0.03 and a carrier rate varying between 5.3 to more than 9% [19,20] creating a major social and financial burden for the patients, their family, and the society in general [21]. Clinical trials of CRISPR technology in β-thalassemia treatment has been approved in Europe [22]. In fact, the promising results of the CTX001 medicine, developed by CRISPR Therapeutics and Vertex Pharmaceuticals, have reignited interest in using gene editing to treat beta thalassemia patients. Since gene-editing techniques are at a new revolutionary phase in the treatment of the hereditary disorder, our aim was to establish a workflow for using the CRISPR/Cas9 system in future diagnostic and therapeutic settings for beta-thalassemia.

In the current study, we have successfully developed a stepwise in-silico approach for designing CRISPR/Cas9 gene editing model for correction of IVS I-110 (G>A, rs35004220) and IVS I-6 (T>C, rs35724775) variants in *HBB* gene; these are the most common mutations among Egyptian β-thalassemia patients **[11].** gRNA110 and gRNA6 were successfully incorporated into PX459 plasmid which could be used to transfect patient-derived HSCs as a therapeutic approach or as a model for disease correction via CRISPR/Cas9 gene editing. Treatment for patients with thalassemia includes lifelong blood transfusions to maintain the haemoglobin levels with normal growth and development in order to relieve symptoms and avoid severe anaemia **[23].** Despite chronic blood transfusion regimens, excess iron is produced leading to iron overload that may cause mortality through iron associated heart and liver toxicity and iron chelation regimens are usually initiated at an early age **[24].** Despite improvements in current treatment, quality of life is poor with overall survival until age of 30 years being only 55% **[25].** For these reasons, gene editing for the most common mutations identified in HBB gene among Egyptian thalassaemic patients is of paramount important aim to be applied in Egypt and our study aimed to establish CRISPR gene editing technique as a first step to be applied in thalassemia and other genetic diseases.

Mostly, gene editing reports using CRISPR/Cas9 technique targeted HBB gene codon 6 mutation responsible for sickle cell anaemia disease (SCD), these reports utilized hematopoietic stem and progenitor cells obtained from peripheral blood and bone marrow of patients with SCD **[26,27].** In addition, other studies utilized CRISPR/Cas9-mediated genome editing to target the γ-globin repressor binding site in patient-derived hematopoietic stem and progenitor cells in order to restore fetal haemoglobin synthesis and correct the SCD phenotype **[28,29].** Another study was performed to activate the fetal haemoglobin in erythroid cell lineage using CRISPR/Cas9 technology to derepress γ-globin genes, HBG1 and HBG2 **[30].**

Although many studies have been conducted using CRISPR technology in gene editing, there are still many challenges to consider, and delivery has always been one of the most important issues in the field of gene therapy. Importantly, HDR and even knockout efficiency are currently low in different tissues, therefore, higher conduction efficiency is needed to compensate **[31,32,33].** interestingly, in silico methods play a crucial role in advancing CRISPR/Cas technology for gene editing that enhance experimental workflows, making CRISPR/Cas more accessible and continuously of optimizing computational tool technology help to address challenges and improve accuracy.

5. **Conclusions**

These breakthroughs signal a new era in healthcare, providing hope for cures and enhanced treatments for previously untreatable conditions. As CRISPR technology continues to evolve, it is anticipated to yield even more transformative advantages for patients worldwide. In silico analysis of CRISPR technology remains crucial for efficient gene editing, with researchers continuously refining these methods to unlock the full potential of CRISPR/Cas systems. The limitation of the current study is that although we performed a comprehensive in silico on target and off target analysis to select the best gRNAs , we didn't perform an *in vitro* study on clinical samples.

6. Conflicts of interest

The authors state that they have no known competing financial interests or personal relationships that could have influenced the work presented in this study.

7. Funding sources

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8. Acknowledgments

N/A

9. References

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