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Investigating The Correction of IVS II-1 (G> A) Mutation in *HBB* Gene in TLS-12 Cell Line Using CRISPR/Cas9 System

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Abstract

Objective: Beta-thalassemia is a group of inherited hematologic. The most *HBB* gene variant among Iranian beta-thalassemia patients is related to two mutations of IVSII-1 (G>A) and IVSI-5 (G>C). Therefore, our aim of this study is to use the knock in capability of CRISPR Cas9 system to investigate the correction of IVSII-1 (G>A) variant in Iran.

Materials and Methods: In this experimental study, following bioinformatics studies, the vector containing Puromycin resistant gene (PX459) was cloned individually by designed RNA-guided nucleases (gRNAs), and cloning was confirmed by sequencing. Proliferation of TLS-12 was done. Then, the transfect was set up by the vector with GFP marker (PX458). The PX459 vectors carrying the designed gRNAs together with Single-stranded oligodeoxynucleotides (ssODNs) as healthy DNA pattern were transfected into TLS-12 cells. After taking the single cell clones, molecular evaluations were performed on single clones. Sanger sequencing was then performed to investigate homology directed repair (HDR).

Results: The sequencing results confirmed that all three gRNAs were successfully cloned into PX459 vector. In the transfection phase, The TLS-12 containing PX459-gRNA/ssODN was selected. Molecular evaluations showed that the *HBB* gene was cleaved by the CRISPR/Cas9 system, that indicates that the performance of non-homologous end joining (NHEJ) repair system. Sequencing in some clones cleaved by the T7E1 enzyme showed that HDR was not confirmed in these clones.

Conclusion: IVS-II-1 (G> A) mutation, which is the most common thalassemia mutation especially in Iran, the CRISPR/Cas9 system was able to specifically target the *HBB* gene sequence. This could even lead to a correction in the mutation and efficiency of the HDR repair system in future research.

Keywords: Beta-Thalassemia, Gene Therapy, Homology Directed Repair, Non-Homologous End Joining

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Introduction

Beta thalassemia, one of the most common hereditary hematological diseases in the world, may be a point mutation or small deletion in the beta globin gene (1-3). In Iran, the most common mutation related to beta thalassemia is IVS II-1 (G>A). The first place in the world in terms of prevalence of IVS II-1 (G>A) mutation is Iran, and this mutation is the most common beta-thalassemia mutation in all regions of Iran (1-4). In this mutation, a single nucleotide changes in the first nucleotide of the second intron, which leads to the same sequence change at the exon-intron junction and affects normal mRNA transcription (5). Transplantation of allogeneic hematopoietic stem cells is considered as a definitive treatment in thalassemia patients, although it is limited by limitations such as the lack of HLA-matched donors and the risk of post-transplantation complications (6, 7). Recently, gene therapy has made great strides in the therapeutic outlook for thalassemia (8-10). DNA editing based on interspaced short palindromic repeats (CRISPR) has emerged to correct different genetic abnormalities such as thalassemia (11-14). CRISPR-associated protein-9 nuclease (Cas9) is an RNA-guided endonuclease that utilizes RNA-DNA base pairing to spot target genomic DNA (15-17). Bound to

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its target via the guide RNA (gRNA), Cas9 generates DNA double-strand breaks (DSBs) at the pre-specified genomic sites that instantaneously activate the endogenous DNA repair mechanisms (17). DSB repair is accomplished by either non-homologous end joining (NHEJ) or homology-directed repair (HDR) (18). The HDR repair mechanism requires a healthy gene pattern that has a very high homology with the downstream and upstream of the gene site to be repaired. The single-stranded oligonucleotide pattern (ssODN) is used for this purpose (19-21). The aim of this study is to evaluate the use of CRISPR/Cas9 technology in correcting the IVS II-1 (G> A) mutation of the *HBB* gene in a TLS-12 cell line that was taken from a beta-thalassemia patient with this mutation.

Materials and Methods

Bioinformatics studies

In this experimental study, diagram of human *HBB* locus (IthaNet: https://www.ithanet.eu/), showed three exons and two introns that introns were named IVS 1 and 2 (Fig.1). Primer designing tool and Primer-BLAST section was used to design primers (22). To design guide RNA (gRNA), online designing tools, Chop chop version 3.0.0, Crispor version 5.01 and Dharmacon version 2.0, were used. Then, 3 to 5 gRNA candidates were tested for cut efficiency by a DNA mismatch detection method (https://dharmacon.horizondiscovery.com/applications/gene-editing/hdr-single-strand-dna-donor-oligos/). Possible off-targets were investigated using OligoAnalyzer Tool from Integrated DNA Technologies (IDT) online software. Then, three 20 bp gRNAs were selected in terms of cutting efficiency and preventing from off-targets (Table 1). Single-stranded oligodeoxynucleotides (ssODN) as a healthy DNA pattern with 121 nucleotides was designed using the Dharmacon version 2.0 online software (https://dharmacon.horizondiscovery.com/applications/gene-editing/hdr-single-strand-dna-donor-oligos/) (Table 2).

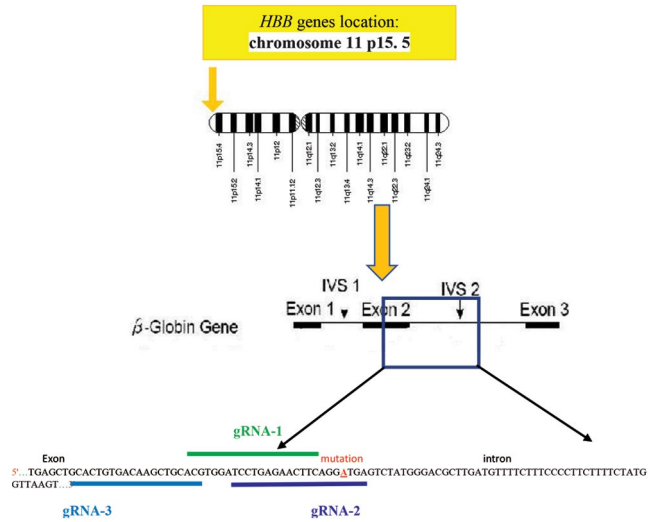

Fig.1: Diagram of human *HBB* gene locus.

Preparation of PX458 and PX459 vector for cloning

We used vector PX459 (pSpCas9-2A-Puro V2.0; Addgene, Cambridge, USA) and vector PX458 [pSpCas9 (BB) -2A-GFP; Addgene, Cambridge, USA]. The PX459 vector enables the expression of SpCas9, a guide RNA (gRNA), and a Puromycin resistance gene. The PX458 vector contains SpCas9, a guide RNA (gRNA) and the GFP gene (23). PX459 vectors were digested with BbsI (ER1011., Thermo Fisher Scientific, USA) and the linearized vectors were gel purified, forward and reverse oligonucleotides for each 3 gRNAs were annealed, phosphorylated, and ligated into pX459 BbsI cloning sites downstream of the U6 promoter.

Table 1: Three gRNA targeting sites were selected and represented for the *HBB* gene (g1, g2 and g3)

Type of gRNA	Target sequence (5′-3′)	Genome location of *HBB*
gRNA-1	F: CACCGACGTGGATCCTGAGAACTTC	474-494
	R: CTGCACCTAGGACTCTTGAAGCAAA	
gRNA-2	F: CACCGGAACTTCAGGATGAGTCTAT	486-506
	R: CCTTGAAGTCCTACTCAGATACAAA	
gRNA-3	F: CACCGGCACTGTGACAAGCTGCACG	456-476
	R: CCGTGACACTGTTCGACGTGCCAAA	

Table 2: Design of the single-stranded oligonucleotide (ssODN) sequence

Temple	Primer sequence (5′-3′)	Genome length (bp)
ssODN	TTTGCCACACTGAGTGAGCTGCACTGTGACAAGCTGCACGTCGATCCTGAGAACTTCAGAG TGAGTCTATGCGACCCTTGATGTTTTCTTTCCCCTTCTTTTCTATGGTTAAGTTCATGTC	121

Polymerase chain reaction

Colony polymerase chain reaction (PCR) was performed on a peqSTAR 96X Thermal Cycler system (Peqlab, Erlangen, Germany). The PCR reaction consisted of 9.5 µl ddH_2O, 12.5 µl Super PCR Mastermix 2X (YT1501, Yekta Tajhiz, IRAN), 1 µl of DNA sample 5-100 ng/reaction, 1 µl of forward primer10 µM (5′-TCCTTTGGGGATCTGTCCACTC-3′), 1 µl of reverse primer 10 µM (3′-CTGTTTCCCATTCTAAACTGTACCC-5′) (Yekta Tajhiz, IRAN) in a total volume of 25 µl. The PCR was performed after an initial denaturation at 94˚C for 5 minutes followed by 35 cycles of denaturation (94˚C for 30 seconds), annealing (55˚C for 55 seconds) and extension (72˚C for 30 seconds), and a final extension step (72˚C for 7 minutes).

gRNA cloning inside PX459 vector

DH5α strain from *E. coli* were selected as susceptible cells for vector transformation. Then, 3 PX459 vectors containing 3 gRNA based on Addgene transformation protocol transformed into bacteria separately. Bacterial culture containing PX459 vector and gRNA in plate was done. Positive colonies were selected by use of Ampicillin (A9518, Sigma, USA) in the bacterial culture medium (10855021, Thermo Fisher Scientific, USA). Then, PX459 plasmid containing gRNA was extracted from positive colonies with a Yekta Tajhiz extraction kit (FAPDE050., Iran). Then, PCR reactions were performed to verify successful insertion. To confirm the sequence of the cloned fragment of gRNAs 1, 2 and 3 in the PX459 vector, the plasmids were extracted from the two clones. Using gRNAs forward primers, plasmids were sequenced by Tehran University Sequencing Center. In order to prepare a stock of DH5α bacteria containing PX459 vector plasmids containing gRNAs, the bacteria were poured into a vial with 60% glycerol (49781, Sigma, USA) and transferred to a freezer at -80˚C.

Cell culture

Totally, 10 cc Blood sample was obtained under sterile conditions from a thalassemia affected, with the IVS-II-1 (G> A) variant in the *HBB* gene. The lymphoblastoid cells Using Epstein–Barr viral vectors, an immortal cell line of them was developed in the human and animal cell bank, National Center for Genetic and Biological Resources, Tehran, Iran. These cells were named TLS-12 cell lines. TLS-12 cells were cultured in RPMI1640 medium (R8758., Sigma, USA) with 10% FBS (Sigma, USA) and 2 mM L-Glutamine (Sigma, USA).

This project was found to be in accordance to the ethical principles and the national norms and standards for conducting medical Research in Iran. The research Ethics evaluated by Tarbiat Modares University (IR.MODARES.REC.1397.081).

ssODN Transfection with vector containing both the Cas9 and gRNA target cassettes to the TLS-12 cells

In order to determine the lowest lethal dose of Puromycin (A1113802., Thermo Fisher Scientific, USA) on the TLS-12 cells, 100,000 TLS-12 cells were cultured in a 24-well plate (in 6 groups with three replicates). They were treated with different concentrations of the Puromycin 2, 1.5, 1, 0.75, 0.5 and 0 g/ml. The survival percentage of TLS-12 cells was checked after 24 and 48 hours. A dose lower than the first Puromycin dose that kills all cells is the appropriate dose to select a population of Puromycin-resistant PX459 vector cells. PX458 vector containing GFP gene (Addgene, Cambridge, USA) was used to evaluate the efficiency of transfections The efficiency of ssODN and PX458 vector transfection to the TLS-12 cells after 24, 36 and 48 hours was investigated by immunofluorescence microscopy. To transfect, PX459 vector containing Cas9 coding sequence and inserted gRNA along with ssODN template was transfected into TLS-12 cells in 5 groups using X-tremeGENE Hp (Sigma, USA). First, 200,000 TLS-12 cells were incubated at 37˚C in a 6-well plate with RPMI medium and a concentration of 2 mM L-glutamine (serum-free medium) for 2 hours. Then, 400 pmol ssODN, 2 µg PX459 vector and 6 µl of X-Terem Hp were added to the TLS-12 cells. After 8 hours, 10% FBS was added to the medium. Also, 24 hours after transfection, a 0.75 µg of Puromycin was added to the culture medium. After 36 hours, Puromycin was removed from the medium.

Single-clone proliferation of TLS-12

To obtain and propagate single-cell clones, 100 µl of cell suspension with a cell count of 200,000 cells was added to 5 ml of complete medium in a plate. Under the microscope, single clones were isolated and transferred to a 96-well cell pellet containing 100 µl of culture medium. After the proliferation of single clones, each of the single-cell clones was transferred to a 24 well plate. Also single-cell clones were transferred to a 6 well plate and then to a T12.5 flasks. Then single cell clones reached a population of 4,000,000 cells, and were frozen in two vials.

Genome editing

DNA was extracted from homogeneous cells with a Yekta Tajhiz DNA extraction kit (FABGK001, Iran). Then, the *HBB* gene region of each of the TLS-12 single clones was amplified with PCR reaction. In the next step, 100 ng of TLS-12 genome without genetic manipulation was annealed with 100ng of each of the homogeneously manipulated TLS-12 genomes. Then, 0.25 µl of the T7E1 enzyme (LE101-01., TransGen Biotech, Chinese) was added to the annealing product and kept at 37˚C for 3 hours. The T7E1 is a structure-selective enzyme that detects structural abnormalities in heteroduplex DNA. In this method, the target genomic DNA is amplified by PCR to detect gene editing generated by the CRISPR/Cas9

after cell transfection. The transfected cell PCR product was then denatured and annealed with the target cell PCR product (Wild type, WT). If the NHEJ cleavage occurred by the CRISPR/Cas9, a heteroduplex would be formed from amplitudes of different lengths (e.g., mutant and WT amplitudes) leading to the DNA aberration detected by the T7E1.

The enzymatic digestion product was then examined on agarose gel. Confirming genome editing, amplicon of gRNA3 and gRNA mix was Sanger sequenced by the Sequencing Center of Tehran University, Tehran, Iran. Data processing from sequencing was done by the BioEdit (version 7.2, informer technologies) software.

Results

gRNAs cloning in the pX459 for Targeting *HBB* gene via CRISPR/Cas9 system

Using online software tools, three gRNAs and an ssODN 121 bp as CRISPR/Cas9 system repair tools targeting single mutant nucleotide in the second intron of the *HBB* gene were designed. They were generated by Yekta Tajhiz company. All gRNAs were cloned into a PX459 vector that contained an ampicillin resistance gene and positive clones, Ampicillin resistant, were selected (Fig.2A, B). Sequencing results confirmed successful cloning and gRNA sequences (Fig.2C).

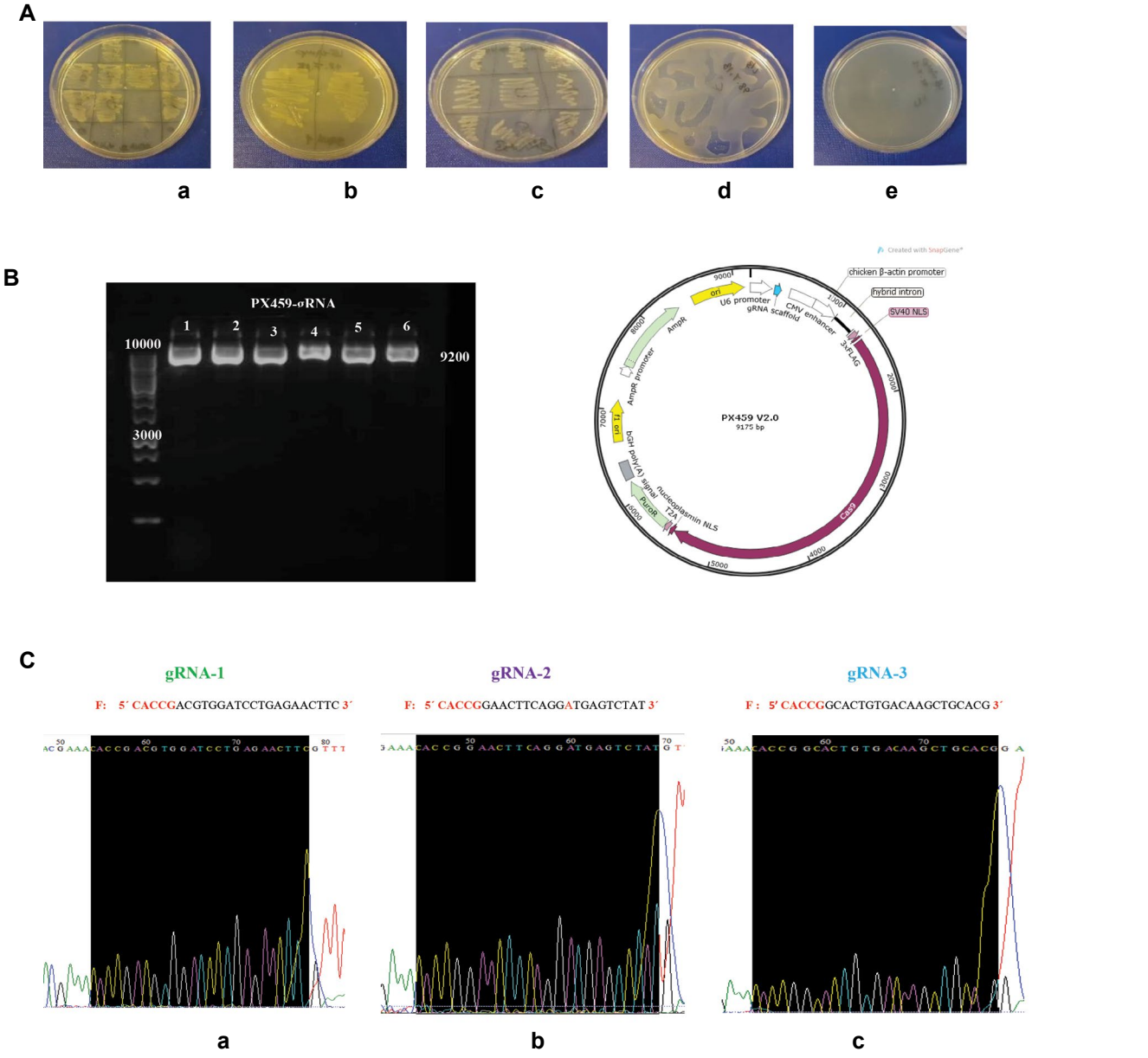

Fig.2: gRNAs cloning in pX459 for Targeting HBB gene via CRISPR/Cas9 system. **A.** Confirmation of transformation by pX459 vector containing gRNA in susceptible cells, bacterial culture with vector containing gRNA1 in plate containing LB and ampicillin culture medium (a), bacterial culture with vector containing gRNA2 in plate containing LB and ampicillin medium (b), bacterial culture with vector containing gRNA3 in plate containing LB and ampicillin medium (c), positive control: culture of susceptible bacteria in the LB culture medium without ampicillin (d), negative control: culture of susceptible bacteria in LB medium with ampicillin (e), **B.** Recombinant plasmid electrophoresis of pX459 - gRNA on agarose gel. **C.** Sanger sequencing of constructed pX459 – gRNA1 in positive colonies (a), Sanger sequencing of constructed pX459 – gRNA2 in positive colonies (b), Sanger sequencing of constructed pX459 – gRNA3 in positive colonies (c).

The efficiency of transfection by the X-tremeGENE Hp in the TLS-12 cell line

To evaluate the transfect efficiency of ssODN and PX458 vector in the TLS-12 cell line, gene transfect to these cells with ssODN and vector PX458 containing GFP gene was examined by immunofluorescence microscopy at intervals of 24, 36 and 48. Fluorescent light was detected at 36 and 48 hours with very low intensity (Fig.3A).

Determining the lethal dose of Puromycin on the TLS-12 cell line

We observed that the 1 µg/ml Puromycin causes 100% cell death. Due to the fact that the TLS-12 cells were destroyed despite receiving PX459 vector (containing gRNA sequence) in the presence of 1 µg Puromycin; therefore, it was decided to continue the work by reducing the concentration of the Puromycin, 0.75 µg (Fig.3B).

Transfect of ssODN and PX459 vector containing gRNA and Cas9 sequences by X-tremeGENE Hp in TLS-12 cell line

For genomic editing of the IVS-II-1 (G> A) mutation in the *HBB* gene by CRISPR/Cas9, PX459-gRNA1, PX459-gRNA2, PX459-gRNA3 and PX459-gRNA mix (a combination of all three PX459-gRNA) as well as ssODN in 5 groups of TLS-12 cells, use of X-tremeGENE Hp was transfected. After removing Puromycin, the cells transfected with PX459-gRNA1 and PX459-gRNA2 were killed. To obtain a homogeneous population of cells, TLS-12 cell lines transfected with PX459-gRNA mix and PX459-gRNA3 were used (Fig.3C), so single cell proliferation was used, that 10 cell clones were obtained from TLS-12 cell line transfected by PX459-gRNA3 and 10 cell clones from TLS-12 cell line transfected by PX459-gRNA mix.

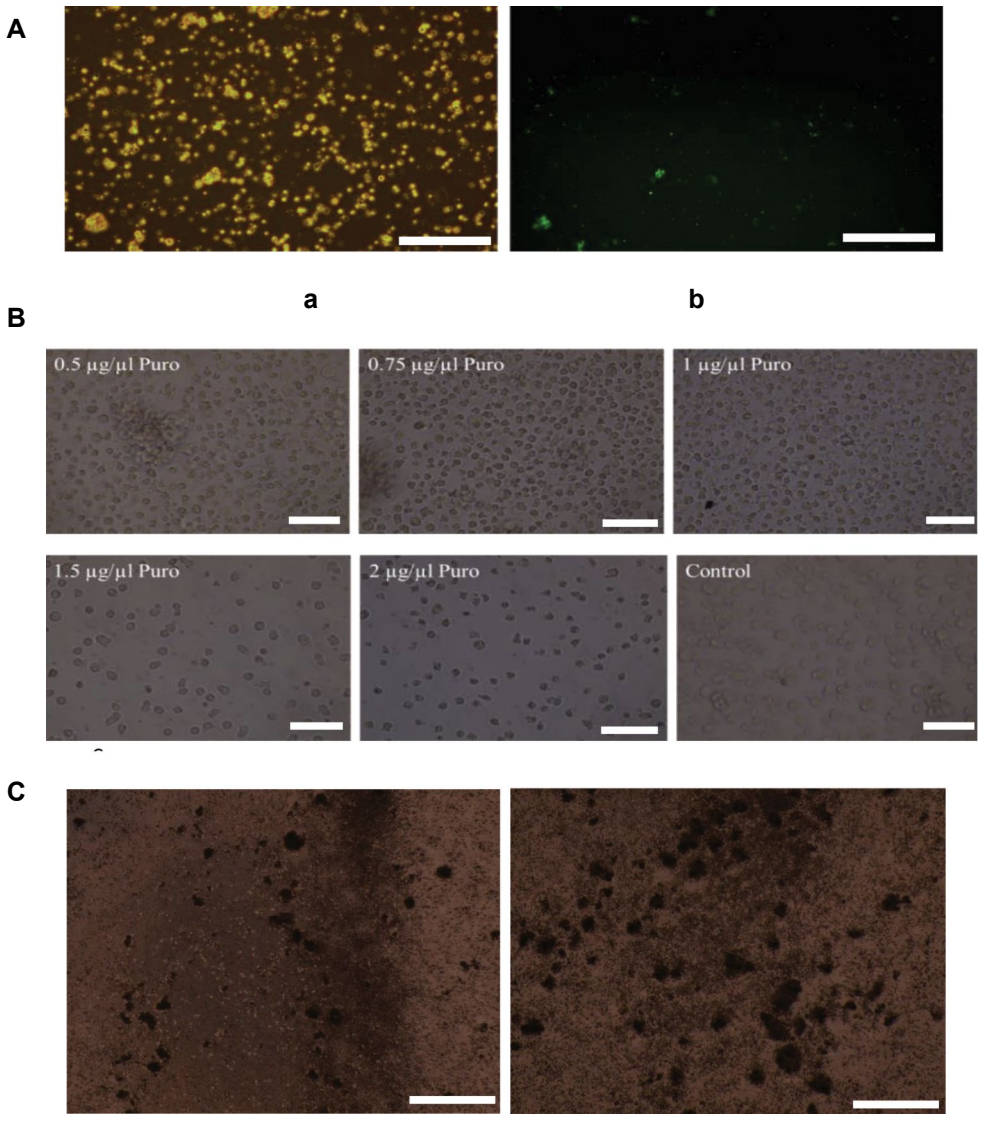

Fig.3: Transfect of ssODN and PX458 and PX459 vector to the TLS-12 cells. **A.** The efficiency of post-GFP- PX458 transfection in TLS-12 cells. 48 hours - bright field microscopy (a), 48 hours - fluorescence microscopy (b) (scale bar: 200 µm). **B.** TLS-12 cells were exposed to different doses of Prumycin (0 to 2 µg/ml) for 48 hours (scale bar: 50 µm). **C.** TLS-12 cells transfected by PX459-gRNA3 and PX459-gRNAmix after treatment with the Prumycin (scale bar: 200 µm).

Following Cas9/gRNA transfection, *HBB* genotyping

T7 assays were done on extracted DNA from 10 single colonies of TLS-12 transfected by PX459-gRNAmix (Fig.4Aa) and 10 single colonies of the TLS-12 transfected by the PX459-gRNA3 vector (Fig.4Ba, 4Ca). Amplification of the target region of each 10 TLS-12 cell clones transfected with a gRNA-mix showed PCR product with length of 290 bp. After denaturation and re-annealing of the PCR product of manipulated cell lines with PX459-gRNA mix vector and effect of 0.25 µL T7EI enzyme for 3 hours at 37˚C were observed at least two amplicons with lengths of 220 and 70 bp It meant that in all 10 cell clones transfected with gRNA mix, the cuts were made by Cas9 and the NHEJ (non-homologous end joining) repair pathway happened in these cells (Fig.4Ab).

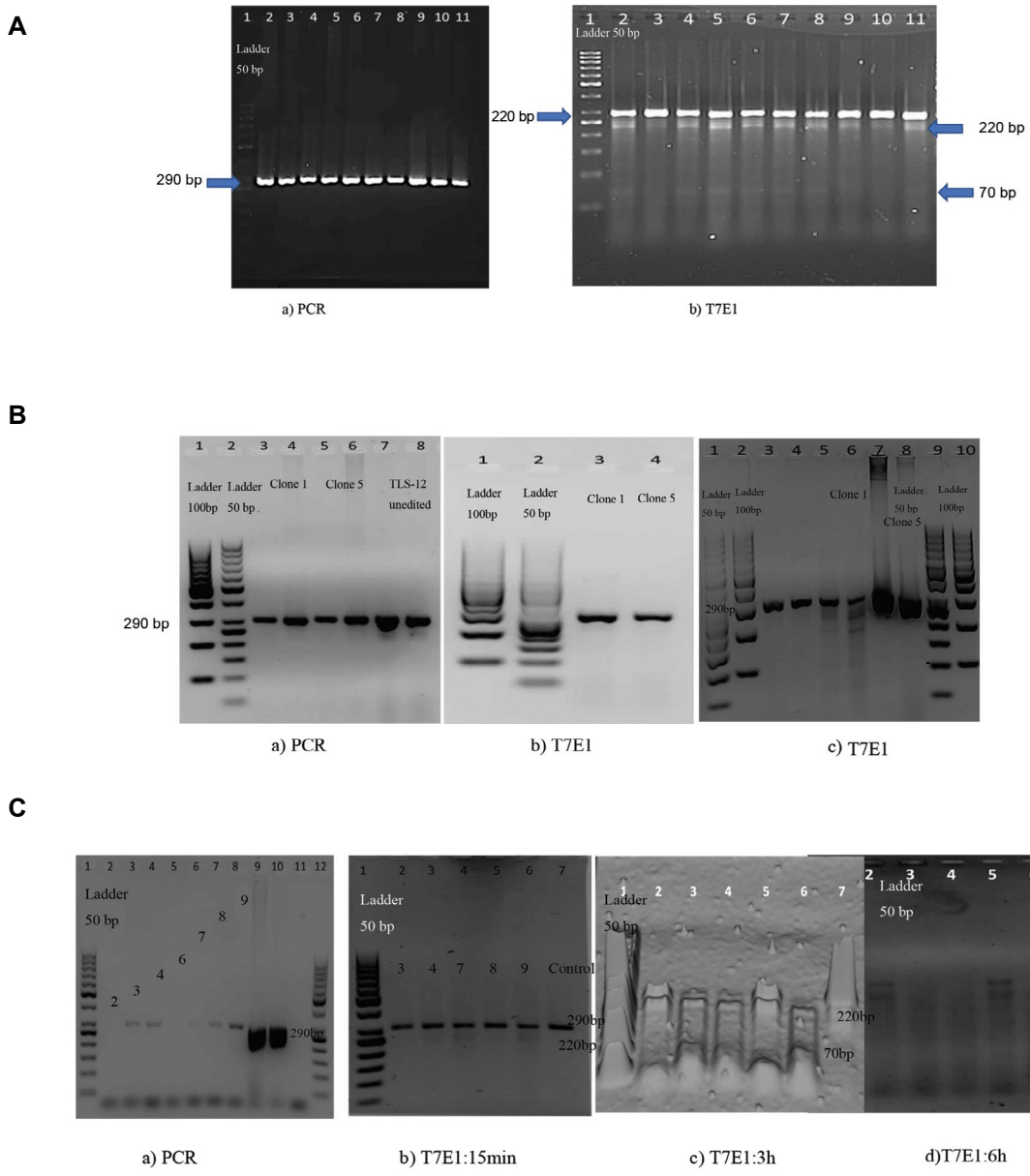

Fig.4: Molecular testing of *HBB* gene following transfection of PX459-gRNA. **A.** Genetic correction of IVSII-1 G> A mutation in 10 clones of TLS-12 cells transfected by PX459-gRNAmix. The PCR reaction product showed one band (290 bp) (10 cell clones) (respectively from 2-11) (a). Affected by the enzymatic reaction of 0/25 µl T7EI enzyme (10 cell clones) (respectively from 2-11) (b). All studied clones showed at least two clear bands with lengths of 70 bp and 220 bp, which indicated the cuts created by CRISPR/Cas9 on the studied groups. **B.** Genetic correction of IVSII-1 G> A mutation in 2 clones, clone No.1 and No.5, of TLS-12 cells transfected by PX459-gRNA3. The PCR reaction product showed one band (290 bp) (a), Affected by the enzymatic reaction of 1 µl T7EI enzyme (clones No.1 and No.5) (b). In the two studied clones indicated that no cleavage had occurred in these two groups. Well No. 3 showed PCR product from ADM-SC cell genome (as a healthy genome in terms of *HBB* gene). Well No.4 showed one band (290 bp) PCR product from TLS-12 cells (c). Also, wells, No.5 and No.6, that were shown as positive control treatment with 1 µl of T7 enzyme and Treatment with 0.25 µl T7 enzyme respectively. Then, Wells, No. 7 and No. 8, affected by the enzymatic reaction of 0.25 µl T7EI enzyme (clones No.1 and No.5). **C.** Genetic correction and changes in T7E1 enzymatic activity over time (15 minutes, 3 hours and 6 hours) in 7 clones (clones number: 2, 3, 4, 6, 7, 8 and 9) of TLS-12 cells transfected by PX459-gRNA3. The PCR reaction product showed one band (290 bp) (clones Number: 3, 4, 7, 8 and 9), Clone 2 and 6 did not show any bands (a-c). Then, changes in T7E1 enzymatic activity over time (15 minutes, 3 h and 6 hours) in 5 clones (clone Number: 3, 4, 7, 8 and 9) showed clones number: 4, 7 and 9 were cut by CRISPR/Cas9 after treatment with T7E1 enzyme, And the best enzyme activity is three hours after treatment.

After the effect of 1 µl T7EI enzyme on the annealed product and incubated for 3 hours at 37˚C of two cell clones transfected with PX459-gRNA3 vector, the mismatch was not created by CRISPR/cas9 (Fig.4Bb). Therefore, the repair pathway of non-homologous end-joining (NHEJ) has not happened in these cells. Then, 0.25 µl of the T7EI enzyme was applied to the same annealed product of two cell clones transfected with gRNA3 and incubated for 3 hours at 37˚C. The results showed that the system was not created on those two cell clones (Fig.4Bc).

Five clones from TLS-12 cell line transfected with PX459-gRNA3 vector were showed PCR product with a length of 290 bp. Using restriction enzyme, the size (bp) of the target amplicon fragments were of 220 and 70. Therefore, the repair pathway of NHEJ has happened in these cells. To investigate the changes in enzyme activity over time, the enzyme digestion product was analyzed on an agarose gel after 15 minutes, 3 hours and 6 hours at 37˚C. The clearest result was detected 3 hours after the treatment (Fig.4Cb-d). Finally, to check the G>A mutation correction in the TLS-12 cell line in which the targeted by T7E1 enzyme was clearly detectable, the efficiency of the homology directed repair (HDR) system was not proven in these clones by Sanger sequencing.

Discussion

Beta thalassemia is one of the most common genetic disorders that affects the most people worldwide every year (24). Common treatments include blood transfusions and prescription iron chelators (25). Bone marrow (BM) allogeneic transplantation is another limited option because of matching HLA typing BM donors limited and the potential for (graft versus host disease) GVHD (26). The five most common mutations in the Iranian population are IVS II-1 (G> A), IVS I-5 (G> C), FSC 8/9 (+ G), FSC36 / 37 (-T), IVS I-110 (G> A) (27). The application of CRISPR/Cas9 technology has not been used in the world to investigate the modification of IVS-II-1 (G>A) mutation in *β-globin* gene. Therefore, this study is the first study in the field of using the knockin capability of this CRISPR/Cas9 system in the treatment of diseases in Iran. One of the most controversial studies in thalassemia genetic modification was the use of the CRISPR Cas9 system in the human embryonic cells in 2015 by Liang et al. (28). The researchers injected a complex of oligos gRNA, Cas9, RNA and ssODNA into different concentrations into tripronuclear zygotes. The researchers concluded that the CRISPR Cas9 system could effectively cleave the endogenous *β-globin* gene, but that HDR efficiency for the *β-globin* gene was low.

In the present study, we have used a PX458 vector that its transfection efficiency was defined 60 to 80%. At first, Wattanapanitch et al. (29) used this method. They examined genetic correction in high-capacity cells induced from a patient with E/β thalassemia by the CRISPR Cas9 system. The PX459 plasmid was used for gRNA construction, and the PX458 was used as a positive control. They obtained approximately 30% transfection efficiency in the Eβ-iPSC2 cells. The corrected clones were differentiated into hematopoietic progenitor cells. These progenitor cells transformed into the erythroid cells that expressed the mature *HBB* gene and HBB protein. Gabr et al. (30) in a study close to ours in 2020 investigate the use of CRISPR/Cas9 technology in correcting the *HBB* gene mutations in CD34+ cells collected from β-thalassemia patients with IVS-1-110 (G→A) mutation and successfully mutation-corrected CD34+ cells subjected to erythroid differentiation. In this study, the most important goal of which was to investigate the performance of NHEJ and HDR repair systems after cutting the genome by endonuclease CAS9, the NHEJ repair system was completely performed in 15 clones, but HDR efficiency was low in the examined clones. Among the reasons for the failure of the HDR repair pathway is the impossibility of designing a high-performance gRNA in the IVS-II-1 mutation region (G> A) corresponding to the PAM region corresponding to the PX459 vector. Although, three gRNAs were designed, we probably did not have the gRNA that could correct the desired single nucleotide mutation. Another reason for the failure of the HDR repair pathway is the PX459 vector. This vector has two versions. In version 1, the necessary inefficiency for the Promycin resistance gene has been reported, which causes the non-selective cells receiving the vector and decreases the percentage of gene transfer efficiency. So, we speculated that version 1 of the vector PX459 had been used because of the failure to correct the mutation. In addition, another reason for the failure of the HDR repair pathway was the achievement of fewer single cell clones. Only 10 single-cell clones were obtained from each gRNA, but in the case of gRNA2, no cell was raised that we could get a single clone, and the cells were destroyed after the transfection stage. The last reason is that more clones were not examined in sequencing. One of the suggestions to improve the method of this study is the use of a vector with a different selection gene and high efficiency .For example ,in the next steps ,it is better to use the vector PX458 and isolate the recipient cells with the Fluorescence activated cell sorting (FACS) technique based on the expression of GFP. Also, in the next steps, it is better to use a method that can isolate a larger number of homogeneous cell clones.

Conclusion

The CRISPR/Cas9 system is considered a tool for the future thalassemia gene editing therapy. In this study, which is the first study in the world on IVS-II-1 (G> A) mutation, the CRISPR/Cas9 system was able to specifically target the HBB gene sequence and the NHEJ repair system was done successfully. This could even lead to a correction in the mutation and efficiency of the HDR repair system in the future.

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Authors 'Contributions

N.S., S.A., S.A.Sh.F.; Conceptualization, Methodology, Resources, Visualization, Supervision, and Funding acquisition. M.S.; Validation and Investigation. All authors read and approved the final manuscript.

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