Gamma reactivation using the spongy effect of KLF1-binding site sequence: an approach in gene therapy for beta-thalassemia

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ARTICLE INFO	ABSTRACT
	Objective(s): β-thalassemia is one of the most common genetic disorders in the world. As one of the promising treatment strategies, fetal hemoglobin (Hb F) can be induced. The present study was an attempt to reactivate the γ-globin gene by introducing a gene construct containing KLF1 binding sites to the K562 cell line. ***Materials and Methods:*** A plasmid containing a 192 bp sequence with two repeats of KLF1 binding sites on β-globin and BCL11A promoters was constructed and used to transfect the K562 cell line. Positive selection was performed under treatment with 150 μg/ml hygromycin B. The remaining cells were expanded and harvested on day 28, and genomic DNA was extracted. The PCR was carried out to verify insertion of DNA fragment to the genome of K562 cells. The cells were differentiated with 15 μg/ml cisplatin. Flowcytometry was performed to identify erythroid differentiation by detection of CD235a+ cells. Real-time RT-PCR was performed to evaluate γ-globin expression in the transfected cells. ***Results:*** A 1700 bp fragment was observed on agarose gel as expected and insertion of DNA fragment to the genome of K562 cells was verified. Totally, 84% of cells were differentiated. The transfected cells significantly increased γ-globin expression after differentiation compared to untransfected ones. ***Conclusion:*** The findings demonstrate that the spongy effect of KLF1-binding site on BCL11A and β-globin promoters can induce γ-globin expression in K562 cells. This novel strategy can be promising for the treatment of β-thalassemia and sickle cell disease.

Introduction

B-thalassemia is one of the most common genetic disorders worldwide. Annually, a number of children are born affected with β-thalassemia (1). Deficient production of the β-globin chains leads to the clinical harshness of β-thalassemia resulting in a surplus of unpaired α-globin molecules that can precipitate within erythroid precursors and bring about membrane injury; therefore, these cells will face toxicity and death consequently leading to ineffective erythropoiesis and the related clinical features of the disease (2). At the present time, regular transfusion of RBCs is regarded as the main treatments for sickle cell disease (SCD) and β-thalassemia, and iron chelation therapy is used to eliminate the iron introduced in excess with transfusions (1). In spite of using stringent iron chelation regimens, many people still suffer from the problems caused by iron overload (3). Two therapeutic strategies available for

treatment of these diseases are bone marrow transplantation (BMT) and gene therapy (2). A series of problems has constrained BMT including GVHD and lack of suitable donors (4). Furthermore, as clinical evidence shows, the severity of β-thalassemia could be alleviated by induction of HbF production (5). HbF continues to be expressed in adult life due to a variety of linked and unlinked mutations in a phenomenon represented by hereditary persistence of fetal Hb (HPFH) (5, 6). There are patients with compound heterozygosity for SCD and β-thalassemia alleles coinheriting the HPFH allele. These patients would have milder phenotype associated with increased HbF levels (5, 7).

A number of agents such as 5-azacytidine, Hydroxyurea and hydroxybutyrate have been identified to cause improvement of HbF production. However, all of these have carcinogenic effects, Hydroxyurea is less effective in β-thalassemia patients (4, 8–10). Although

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Figure 1. A 192 bp sequence that contains KLF1 binding sites on β-globin and BCL11A promoter

preliminary trials about hydroxybutyrate as a histone deacetylase (HDAC) inhibitor were promising, it has not been effective clinically (1, 11).

As observed above, the reactivation of γ-globin expression is a well-established therapeutic method for treatment of SCD and β-thalassemia. This has motivated scientists to understand the mechanism regulating hemoglobin switching in relation to clinical importance (6, 12). A number of regulatory elements and nuclear factors including KLF1, BCL11A, SOX6, GATA1, FOG1, and NuRD complex (HDAC1 & HDAC2) have contributed to the hemoglobin switching process (13). BCL11A has a pivotal role in fetal to adult Hb switch. It is also deemed to be an indispensable factor in silencing γ-globin binding to LCR (locus control region) and δ-γ globins intergenic region (12, 14). Presenting siRNA and shRNA to knockdown BCL11A has led to increased level of γ-globin chain production in erythroid progenitors (15).

Several lines of evidence show that transcription of BCL11A is activated by a factor known as KLF1 (ErythroidKruppel-like Factor) (16). KLF1 regulates adult Hb through binding to the sequence of CACCC (13, 17-19). Some studies indicate that KLF1 represses gamma globin via binding to the promoter of BCL11A (2, 13, 16, 17).

Evidence shows that knockdown of KLF1 could result in significant improvement of gamma globin production and strict inhibition of BCL11A expression. KLF1 can be considered as an important target for the treatment of the beta globin disorders. This transcription factor makes a strong connection to the CCTCCCACCCCCTGCC sequence of BCL11A promoter (17) as well as the CCACACCCT element (20–22) which is at -90 of the β-globin promoter (22) and actively transcribes the genes.

The present study was launched to design a plasmid cassette containing multiple sequences of KLF1 binding sites on β-globin and BCL11A promoters. It was transfected to the K562 cell line and the spongy effect of KLF1 ectopic binding was evaluated on elevation of HbF levels.

Materials and Methods
Plasmid construction
A 192 bp sequence was designed containing two identical repeats of KLF1 binding sites on β-globin promoter –CACACCCT- and BCL11A promoter – CCCACCCCT– with the portion of their upstream and downstream flank with a *MluI* restriction site located on both sides (Figure 1). The sequence was made by the Gene Cust Company in pUC57 plasmid (named as pUC57-Seq). *Escherichia coli* strain *TOP10F′*was obtained from Pasteur Institute of Iran and transformed by pUC57-Seq according to the chemical method of Higa and Mandel protocol. Transformed bacteria were cultured in LB agar medium containing 100 µg ampicillin/ml. The resulting colonies were assessed using colony PCR I (forward M13 primer: 5′-TTGTAAAACGACGGCCAGT-3′ and reverse M13 primer: 5′-ACAGGAAACAGCTATGACCATG- 3′). The PCR product was digested with *MluI.* The products were run on 3% agarose gel. The target products were extracted from the gel using DNA extraction kit (Bioneer, Korea) according to the manufacturer's instructions.

Endotrail lentiviral transfer vector that contained a hygromycin resistant cassette was digested with *XbaI* and *SpeI* to remove endostatin and trail CDS to close upstream promoter to the hygromycin gene. The product was run on 1% agarose gel and the plasmid backbone was extracted from the gel. Digested product was self-ligated using T4 DNA ligase (p-Lenti). The self-ligated pLenti plasmid was digested with *MluI*. The linearized plasmids were then treated with calf intestinal alkaline phosphatase (CIAP) (Thermo Scientific,USA), to prevent self-ligation and extracted from agarose gel using the DNA extraction kit.

The ligation reaction between the pLenti and the 192 bp fragment was performed (p-lenti-192) and ligation product was used to transform the competent *E. coli* Top10 F′. The resulting clones were evaluated using colony PCR II (forward primer within the transfer vector backbone: 5′TAGTGAACGGATCTCGACGG 3′ and reverse primer within the hygromycin gene: 5′GACGTCGCGGTGAGTTCAG 3′). The p-lenti-192 plasmid was digested and linearized with *NheI*.

The K562 cell line
The human myeloid leukemia cell line, K562, was provided by Pasteur Institute of Iran. The cells were grown in Roswell Park Memorial Institute (RPMI) 1640 (Sigma-Aldrich Co) medium supplemented with 100 IU penicillin/ml, 100 µg streptomycin/ml, and 10% fetal bovine serum (FBS) in 5% CO_2 humidified atmosphere. To confirm the K562 cell line, total RNA of about 10^6 cells were extracted using

TRIZOL reagent (Invitrogen, Life Technologies, USA) following the manufacturer's instructions. First strand cDNA was synthesized using first strand cDNA synthesis kit (Fermentas, USA). The reverse-transcribed products were used as template DNA for real-time RT-PCR using Real quality RS-BCR-ABL p210-RQ-S53-48/96 kit (AB Analytica, Italy) in a real-time instrument (The Applied Biosystems® StepOne™ Real-Time PCR Systems, Life Technologies).

Transfection and verification of insertion

The K562 cell line was transfected with the linearized p-lenti-192 using Lipofectamine@ LTX and Plus™ reagent (Invitrogen, North America), following manufacturer's instructions.

Transfected K562 cells were cultured in a T25 flask with 150 μg hygromycin B/ml (Roche, Germany) beginning 48 hr after transfection. After drug selection, cells were harvested on day 28, and genomic DNA was isolated by genomic DNA extraction kit(Genetbio, Korea). The PCR was carried out in order to verify insertion of the fragment to k562 genome using cppt forward primers 5′ GTGCAGGGGAAAGAATAGTAG 3′ and hygromycin reverse primers 5′GCGTCTGCTGCT CCATAC 3′).

Differentiation and flowcytometry

To induce erythroid differentiation, transfected and untransfected K562 cells were centrifuged at 800 rpm for 5 min and were seeded in FBS-free RPMI 1640 plus 15 μg cisplatin/ml (23) (Sigma-Aldrich Co, USA). The cells were incubated at 37 °C, 5% CO_2 for 7 days. The expression of the erythroid-specific surface antigen, CD235a, was determined by direct immunofluorescent staining with FITC- conjugated mouse monoclonal antibody against human CD235a (Biolegend, USA). FITC conjugated mouse IgG1 was used as the isotype antibody. About 5×10^4 of both kinds of K562 cells were suspended in 200 μl RPMI and the FITC conjugated mouse antibodies were added and incubated at 4 °C for 30 min. Cells were washed with PBS and assayed by flowcytometry (BectonDickinson, Germany). The CellQuest software was used for flowcytometry analysis. Total events of 10000 cells were counted per tube. Gating was performed based on forward-scattered light (FSC) and side-scattered light (SSC).

Relative quantitation of γ-globin expression

Gamma globin mRNA was detected by real-time RT-PCR method. We used beta-actin as an endogenous control. The RNA extraction and cDNA synthesis from differentiated (KD) and transfected-differentiated (KTD) K562 cells were performed. First strand cDNAs were used as templates for real-time PCR. The primer sequences for beta-actin were FBAP 5′ TTCGAGCAAGAGATGGCCA 3' and RBAP 5′ CACAGGACTCCATGCCCAG 3'. The primers for γ–

globin were FGGP 5′ GGACAAGGCTACTATCACAA 3' and RGGP 5′CAGTGGTATCTGGAGGACAG 3'. The cycling program was as follows: initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 sec, annealing and extension at 60°C for 1 min.

Results

p-lenti-192 construction

After colony PCR I on pUC57-Seq, a DNA fragment of 300 bp was observed (Figure 2-A). After digesting the 300 bp fragment with *MluI*, three DNA fragments including one 192 and two 50 bp are produced (Figure 2-B). The 192 bp fragment was extracted from the gel.
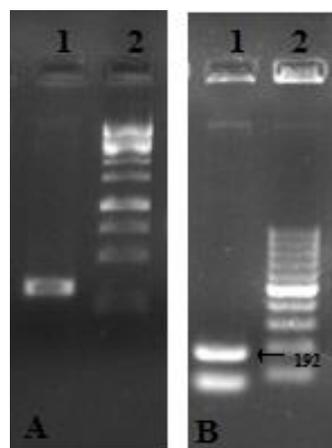

Figure 2. Isolation 192 bp fragments from pUC57-Seq. (A) The product of colony PCR I on pUC57-Seq. (1) Colony PCR I product, 300 bp, (2) 1 kb DNA Ladder. **(B)** Colony PCR I product after digestion with *Mlu I* enzyme. (1) Digested product, (2) 100 bp DNA Ladder
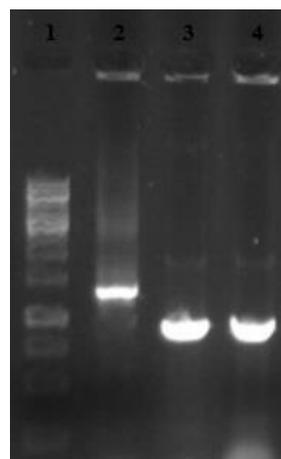

Figure 3. The product of colony PCR II for confirmation of accuracy of ligation. (1) 1 kb DNA Ladder, (2) 1156 bp, ligation product of two 192 bp fragments together into a vector during ligation reaction, (3,4) 964 bp, ligation product of 192 bp fragments into a vector during ligation reaction
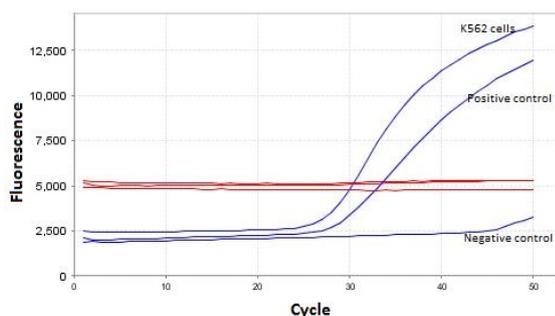
Figure 4. Confirmation of the K562 cell line by Taq Man real-time PCR for the BCR-ABL fusion gene (multicomponent plot). The graphs for positive and negative controls are shown. The studied cells carried p210 BCR-ABL translocation, thereby confirming the authenticity of K562 cell line

The results of colony PCR II on plenti-192 colonies confirmed the accuracy of ligation between a 192 bp fragment and pLenti. We expected to see only the 964 bp band on agarose gel, but surprisingly, an 1156 bp band was observed, too (3). This was due to the ligation of two 192 bp fragments together into a vector during the ligation reaction by reconstruction of the *MluI* enzymatic site. We selected and grew the clone containing 1156 bp fragment for achieving a higher efficiency. The recombinant plasmid was named p-lenti-(192)2.

Confirmation of the K562 cell line
The Taq-man real-time RT-PCR technique confirmed BCR-ABL expression in the studied K562 cell line (Figure 4). Expression of BCR-ABL confirmed the identity of the K562 cell line.

Transfection and verification of insertion
To verify insertion of pLenti-(192)2 into K562 cell's genome, PCR was performed, and the 1700 bp fragment was observed on agarose gel as expected (Figure 5).

Differentiation and flowcytometry
In the flowcytometry assay, after differentiation, 92% of untransfected cells and 84% of transfected cells expressed CD235a marker on their surface (Figure 6).

Relative quantitation of γ-globin expression
Quantitative relative real-time RT-PCR for γ-globin expression showed that the transfected cells with p-lenti-(192)2 significantly increased γ-globin expression (about fourfold) in comparison to untransfected cells after differentiation (Figure 7).

Discussion
β-hemoglobinopathies are the most common genetic disorders. Understanding the γ to β hemoglobin switching process and the reactivation of the HbF expression in adults could provide a
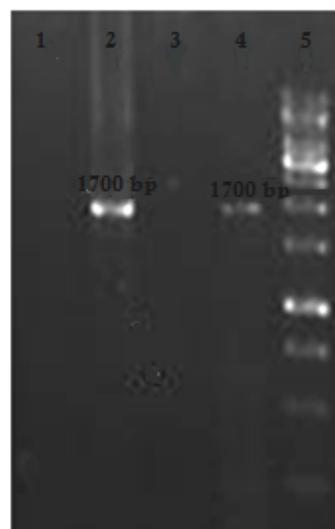

Figure 5. The PCR product to verify insertion of the interested fragment to K562 cells. (1) Negative control, (2) Positive control (plenti-192 (2)), (3) Untransfected K562, (4) Transfected K562, (5) 1 kb DNA Ladder

therapeutic approach for β-thalassemia (7). KLF1 plays a pivotal role in activation of the β-globin gene through dual mechanisms: direct activation of the β-globin gene through binding to its promoter and repression of γ-globin indirectly through interaction with the *BCL11A* promoter (24). The present study was an attempt to reactivate γ-globin gene by introducing a gene construct containing KLF1 binding sites to the K562 cell line.

Among many studies performed for treatment of beta hemoglobinopathies, transferring a working copy of beta globin gene was an option; however, many obstacles and side effects were encountered which restricted the therapeutic approach. One of the restrictions was the failure in carrying large amounts of genetic material, β-globin gene, and locus control region regulatory element. To treat beta hemoglobinopathies, lentiviral vectors (LV) were introduced; these vectors may still have limited efficacy due to transgene silencing led by DNA methylation or by heterochromatinization as the result of chromosomal positional effects. Due to the semi-random pattern of integration of these vectors, the effects of chromosomal position on gene expression have a serious influence on LV-based gene therapy (25).

An alternative option is correcting the mutation in the beta globin gene of the induced pluripotent stem (iPS) cell via gene targeting accompanied by differentiating the corrected cells to HSCs and returning the cells to the patient. Yet, some impediments are needed to be removed before taking advantage of the iPS treatment of ß thalassemia. The removal of the transcription factors when they become useless is considered to be one of the most challenging problems. Secondly, reestablishing the correct re-programming is essential so that it would not be possible for the iPS cells to develop into tumors (26).
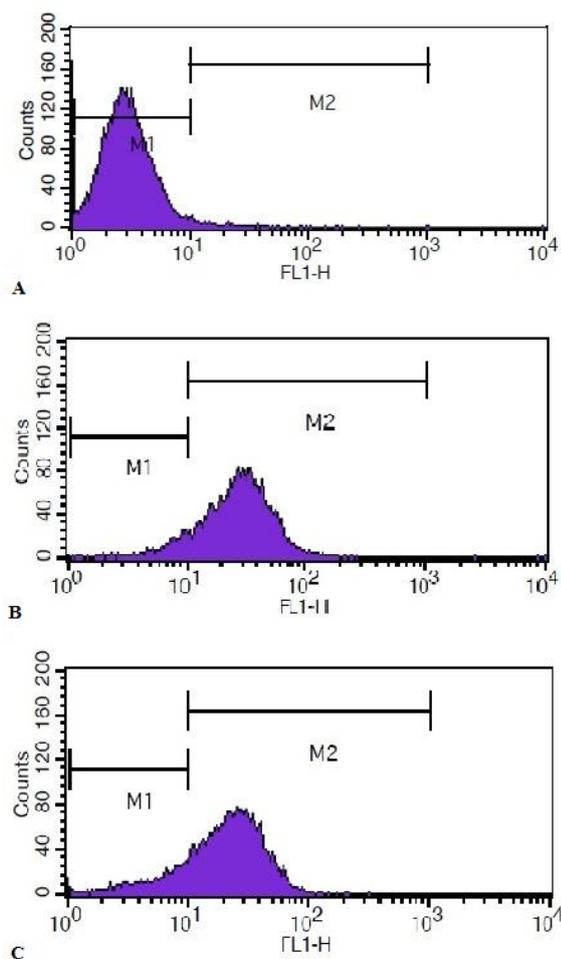
Figure 6. Result of CD235a positive cells by flowcytometry.
(A) Undifferentiated K562, **(B)** Untransfected K562 cells were differentiated and 92 % of the cells were differentiated, (C) Transfected K562 cells were differentiated and about 84% of the cells were differentiated

Figure 7. Relative quantitation of γ-globin expression. KD: Differentiated K562. The K562 cells were differentiated under 15 µg/ml of cisplatin. KTD: Differentiated -transfected K562. The expression of γ-globin was compared between KD and KTD groups by quantitation comparative Ct real-time PCR. ***P-value < 0.001, KD vs. KTD. (RQ: Relative Quantification)

on γ-globin promoter) induction was performed via adding Tert-butylhydroquinone (tBHQ) in the K562 cell line (27).

Studies have indicated that knockdown of the *BCL11A* gene through introducing siRNA results in γ-globin increase in human primary erythroid cells (15); however, iRNA use has some limitations such as targeted delivery of RNAi to desired cells, off-target effects, and cytotoxicity (28).

In other study, disruption was made in the KLF1 gene using CRISPR/cas9 to inhibit process of switching in order to increase γ-globin (8-fold) (29).

Conclusion

In the present research, to elevate HbF, we used a novel strategy with no need for introducing large genetic material or using chemical drugs necessary in other strategies. Two spongial sites that are the binding sites of KLF1 on the promoters of β-globin (CCACACCCT) and BCL11A (CCTCCCACCCCCTGCC), were inserted into the genome in duplicate repeats (total length of 192 bp). As the results of Q-RT-PCR showed, in comparison with the differentiated non-transfected cell line, the level of γ-globin expression was significantly (four folds) improved in differentiated- transfected K562 cells. The findings support the existence of these competitive sites (spongial sites) in the cells that compete with the main sites for gaining and capturing the KLF1 protein. Therefore, occupation of these spongial sites by KLF1 effectively reduces the number that can bind to main sites on β-globin and BCL11A promoter in β-cluster, leading to decreased *BCL11A* expression, which has a central role in silencing the γ-globin gene and increased levels of fetal hemoglobin. This strategy has another advantage theoretically, it also seems that the β-globin expression level would reach a minimum amount but K562 does not express beta globin and there was no possibility to examine this occurrence (7). A disadvantage of our strategy is the

Principally, treating beta thalassemia and SCD by induction of γ-globin using different approaches has many advantages over the transfer of normal β-globin gene. Hydroxyurea, 5-azacytidine, myleran, and butyrate as chemotherapeutic agents have been utilized for treating β-thalassemia through stimulation of HbF synthesis. On the other hand, the clinical application of these agents in β-thalassemia and SCD treatment has been limited by their cytotoxicity, growth-inhibitory effect, fear of long-term carcinogenesis, and only modest HbF-inducing activity (7). HbF-inducing agents would also cause rapid cellular apoptosis of erythroid progenitors in β-thalassemia that overstimulate the cell stress signaling pathway that might be the cause of irreversible cellular apoptosis before γ-globin gene expression and HbF synthesis (7).

Research on the molecular pathways that are involved in γ-globin induction usually lead to finding drug target molecules. For example, NRF2-ARE signaling pathway (the factor binding to ARE region
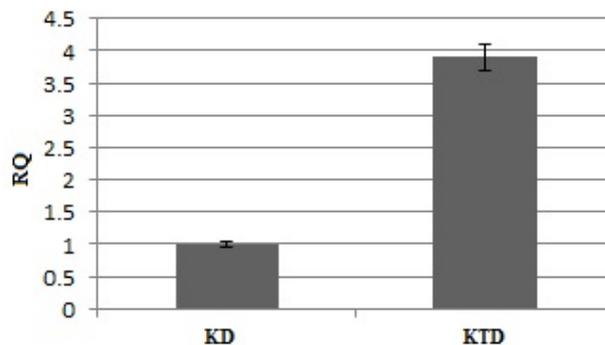
random integration of the construct into the genome, which may interrupt the operation of cellular genes, activation of oncogene and inactivation of tumor suppressors which may lead to cancer.

In the present research, we introduced a novel strategy for induction of HbF, which could be examined in future research for treatment of β-thalassemia and SCD.

Acknowledgment

Conflict of interest

This article does not contain any studies with human participants or animals performed by any of the authors.

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