Cell. Mol. Biol. (ISSN: 1165-158X)

Journal Homepage: www.cellmolbiol.org

Cellular and Molecular Biology

Original Article

Genome editing in K562 cells suggests a functional role for the XmnI Gγ polymorphism: a widely used genetic marker in β-thalassemia and sickle cell disease patients

Azadeh Ahmadifard[1], Nahal Maroofi[2], Maryam Maleki Tehrani[1], Tahere Dabestani[1], Masoumeh Sadat Mousavi Maleki[2], Sepideh Bayrami[2], Mehdi Banan[1,2*]

[1] *Genetics Research Center, University of Social Welfare and Rehabilitation Sciences, Tehran, Iran*
[2] *Gene Therapy and Regenerative Medicine Research Center, Hope Generation Foundation, University of Social Welfare and Rehabilitation Sciences, Tehran, Iran*

Article Info

Article history:

Received: March 12, 2024
Accepted: April 07, 2024
Published: July 31, 2024

Use your device to scan and read the article online

Abstract

The XmnI Gγ -158 C/T polymorphism has been widely associated with fetal hemoglobin (HbF) levels, the severity of disease, and the response to the drug hydroxyurea (HU) in both β-thalassemia (β-thal) and sickle cell disease (SCD) patients. However, the functional significance of this single nucleotide polymorphism (SNP) remains unclear. To gain insight, green fluorescence protein (GFP) cassettes harboring the XmnI C or T alleles in their left homology arms (i.e. Gγ promoters) were knocked into the Gγ gene(s) of K562 cells via CRISPR/Cas9. Subsequently, the GFP fluorescence levels were compared in the ensuing cell populations and isolated clones. In both instances, median fluorescence intensities (MFI) of the knockin cells having the inserted XmnI T allele were higher than those having the XmnI C allele. Our results suggest that the XmnI T allele can increase Gγ expression in K562 cells. The possible functional significance of the XmnI Gγ -158 C/T polymorphism provides a rationale for the aforementioned associations. Furthermore, the XmnI polymorphism as a functional SNP substantiates its importance as a prognostic marker.

Keywords: β-thalassemia; CRISPR/Cas9, Knockin, Sickle cell disorder, XmnI polymorphism.

1. Introduction

The hemoglobin molecule is composed of two α-globin chains and two β-like chains. In humans, the composition of the fetal hemoglobin (HbF) is $\alpha_2\gamma_2$, whereas after birth, the main hemoglobin is the adult hemoglobin (HbA) or $\alpha_2\beta_2$. The hemoglobin disorders β-thal and SCD are both caused by mutations in the β-globin gene. In β-thal, homozygous or compound heterozygous mutations (>300 identified to date) in the β-globin gene lead to the elimination or reduction in HbA levels [1]. The pathophysiology of the β-thal disorder is anemia, red blood cell (RBC) lysis, and iron overload in various bodily organs [2]. In SCD patients, a single mutation in the β-globin gene leads to expression of the sickled hemoglobin (HbS). The HbS polymerizes under low oxygen concentrations, causing the sickling of RBCs and clinical complications such as vaso-occlusion and hemolytic anemia [3].

Increased HbF levels in adults can reduce the severity of the diseases caused by HbS production or a lack of HbA expression in SCD and β-thal patients, respectively. To date, three quantitative trait loci (QTLs) have been identified which associated with HbF levels [4]. One of these QTLs is a single nucleotide polymorphism (SNP) residing in the promoter of the Gγ gene (-158 C/T) called the *XmnI* polymorphism (rs782144). In a genome-wide association study (GWAS), the *XmnI* polymorphism was identified as a key SNP associated with HbF levels in SCD patients [5]. These findings have been supported by numerous other studies, in which the *XmnI* T allele has been associated with higher HbF levels and the amelioration of disease severity in both β-thal and SCD patients [6-10]. It is important to note, however, that these associations have only been observed in patients with hemoglobin disorders, in which stress erythropoiesis causes a significant increase in the HbF-producing F-cells.

The *XmnI* Gγ SNP may also act as a genetic marker for predicting the response of β-thal and SCD patients to the drug hydroxyurea (HU) [11]. HU acts by inducing HbF expression in the patients under treatment. However, only a subset of patients responds to HU i.e. a reduction in blood transfusions or painful crises in β-thal and SCD patients, respectively. In a number of association studies,

* Corresponding author.
E-mail address: mbbanan@yahoo.com (Mehdi Banan).
Doi: http://dx.doi.org/10.14715/cmb/2024.70.7.33

the *XmnI* T allele has been correlated with the response to HU in both β-thal and SCD patients. As suggested, this correlation may be a consequence of higher baseline HbF levels being present in the HU-responder patients as compared to the non-responders [12].

Despite its wide use as a genetic marker, however, no function has been identified for the *XmnI* Gγ C/T SNP. As a result, it has been suggested that other variations in linkage disequilibrium (LD) with the *XmnI* polymorphism may be the functional SNPs influencing HbF expression [1]. Several such candidate SNPs have also been reported [13].

More recently, CRISPR/Cas9 genome editing studies in HUDEP-2 cells and hematopoietic stem/progenitor cells (HSPCs) isolated from SCD patients have shown a functional role for the γ-globin promoter -158 region [14]. In particular, insertions/deletions (indels) produced in this region have resulted in the induction of γ-globin (Gγ and Aγ) or HbF expression in the SCD HSPCs and HUDEP-2 cells post-differentiation. These results, however, do not directly assess the influence of the *XmnI* G -158 C/T polymorphism on expression.

To further investigate this premise, green fluorescent protein (GFP) cassettes bearing the -158 C or the T alleles in their left homology arms were knocked into the Gγ gene of K562 cells. Fluorescence levels of the resulting cell populations and isolated clones were then compared.

2. Material and methods

2.1. Cell culture

The K562 cells were a kind gift from Prof. Frank Grosveld (Erasmus MC, Rotterdam, Netherlands). The K562 cells were cultured in Roswell Park Memorial Institute Medium (RPMI), 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin (Capricorn Scientific, Ebsdorfergrund, Germany) in a CO_2 incubator (Memmert, Büchenbach, Germany) at concentrations below 2×10^5 cells/ml. Single-cell cloning was performed by using the serial dilution method in 96-well plates. Cells were grown in 100 µl media containing RPMI, 10% FBS, and 30% conditioned media. After one week, wells containing a single cluster of cells were spotted and transferred to 12-well plates for further expansion.

2.2. Karyotyping

Culturing and harvesting of K562 cells and preparation of slides were performed according to standard cytogenetic procedures [15]. Slide preparations were allowed to age at 170°C for 16 hours. After Giemsa staining, chromosome analysis was performed on the G-banded metaphases. The karyotyping of metaphases was performed by using a Nikon Eclipse E400 microscope (Tokyo, Japan) and karyotyping software (Onyxlab, Tehran, Iran).

2.3. Plasmid constructs

The CRISPR plasmid was constructed by cloning the γ-globin-specific small guide RNA (sgRNA) template into the PX459 vector (Addgene, Cambridge, MA, USA), as previously described [16]. The previously constructed donor plasmid contains a GFP.(IRES).NeoR.pA cassette [16]. This cassette is flanked by an 822 nt. Gγ-specific left homology arm (LHA) with 91.97% sequence homology to Aγ, and an 857 nt. Aγ-specific right homology arm (RHA) with 98.02% homology to Gγ [GFP= green fluorescence protein; IRES=internal ribosome entry site; Neo^R=neomycin resistance gene; pA= polyadenylation signal]. The γ-specific LHA and RHA fragments had been amplified by polymerase chain reactions (PCR) from K562 genomic DNA (gDNA), which contains the C allele at the Gγ -158 position (data not shown).

2.4. PCR mutagenesis by overlap extension

In order to create an analogous donor plasmid containing the *XmnI* T allele in the LHA, PCR mutagenesis by overlap extension was utilized by using the above donor plasmid as template [17,18]. The following primers were used for the overlap PCR reactions: Outer forward (OF) primer: 5′-TTTGAGCTCTCATCGGGTGCCTACATACATA-3′; Inner forward (IF) primer: 5′-GTCTGAAACGGTTCCTGGCTA-3′; Inner reverse (IR) primer: 5′-TAGCCAGGAACCGTTTCAGAC-3′; Outer reverse (OR) primer: 5′-TTTGAATTCGTCTGGACTAGGAGCTTATTGA-3′ [the underlined sequences in the OF and OR primers are SacI and EcoRI recognition sites, respectively; the underlined nucleotides (nt.) in the IF and IR primers constitute the *XmnI* T allele]. The final PCR product was digested with SacI and EcoRI. The LHA having the Gγ -158 C allele was removed from the original donor plasmid following SacI/EcoRI digestion, and the new LHA harboring the *XmnI* T allele was ligated and substituted. The composition of the new donor plasmid was verified by restriction enzyme mapping and Sanger sequencing.

2.5. Transfection and antibiotic selection

The CRISPR (PX459-sgRNA) and donor plasmids were co-transfected into K562 cells by using Lipofectamine 2000 (Thermo Fisher Scientific, Cambridge, MA, USA). Transfection efficiency was monitored by transfecting, in parallel, the pSV-β-Galactosidase Control Vector (Promega, Madison, WI, USA) into K562 cells. The latter cells were then stained by using the β-Gal Staining Set (Roche Applied Science, Mannheim, Germany). Cells co-transfected with the CRISPR and donor vectors were selected by using a two-step selection process [16,19]. After 48 hours, the cells were put under puromycin selection ($Puro^R$ is in the PX459 plasmid) for two days. Upon washing and expansion, the cells were put under G418 selection for three weeks (Neo^R is in the donor plasmid). Antibiotic levels were determined by using death curve analyses.

2.6. Screening for knock-ins

Genomic DNA was isolated from the edited cell populations by using the salting out method (Miller et al, 1988). Knock-in of the GFP cassettes into the Gγ DNA break-site was verified by using PCR, with one primer falling in the transgene and the other outside of the homology arm (either LHA or RHA). The primers used for PCR were: P1: 5'-CCAATATGTCAGAAACAGCACTG-3'; P2: 5'-GTGGTGCAGATGAACTTCAGG-3'; P3: 5'-CATCACGATGGCCGCAATAA-3'; and P4: 5'-CTGGACATACTTTGCCCCCA-3'. Identity of the PCR products was verified by using Sanger sequencing.

2.7. Transgene copy number

The copy number of the integrated GFP cassettes was determined by using quantitative PCR (qPCR) and the $2^{\Delta Ct}$

calculation method. The *BCL11A* gene located on chromosome 2 was used as the internal control (i.e. three copies in K562 cells). The real-time PCR reactions were run in duplicate by using the RealQ Plus 2x Master Mix Green (Ampliqon, Odense, Denmark) on an Applied Biosystems 7500 platform.

2.8. Genomic edits

The frequencies of indels produced in the non-recombinant γ-globin (Aγ and Gγ) copies (i.e. those lacking an integrated cassette) were performed by using a polyacrylamide gel electrophoresis (PAGE)-based genotyping assay, as previously described [20,21]. The presence of the 4.9 Kb Gγ-Aγ deletions was detected by using gap-PCR, with a forward primer binding to the Gγ upstream region and an Aγ-specific Amplification Refractory Mutation System (ARMS) reverse primer [22]. The frequency of indels was determined by using the ImageJ Gel Analysis software.

2.9. Flow cytometry

GFP fluorescence levels were detected by using a Partec PAS flow cytometry system (Sysmex Partec, Gorlitz, Germany). The data was analyzed by using the FlowJo® v7.6 software (FlowJo, LLC, USA).

2.10. Statistical analysis

Statistical analysis was performed by using Microsoft Excel (Microsoft, Bellevue, WA, USA). Significance was determined by using a two-tailed Student's t-test. $P<0.05$ was considered as significant.

3. Results

3.1. Strategy for assessing the functional significance of the XmnI polymorphism

K562 is an erythroleukemia cell line with high γ-globin expression levels. As such, it has been widely used as a model system for the HbF-producing F-cells [23]. K562 cells may have three normal copies of chromosome 11, where the β-locus is located [15]. In support, karyotyping of the K562 cell line at hand showed the presence of three normal copies of chromosome 11 (Fig. S1).

In order to evaluate the functional significance of the *XmnI* polymorphism, we sought to compare the fluorescence levels of K562 GFP→Gγ knockin cells having either the -158 C or the T allele in the left homology arms (LHA) of the inserted cassettes (i.e. in their Gγ promoters). We reasoned that such an approach would 1) overcome the need to edit all six copies of the γ-globin genes (3Gγ, 3Aγ), and 2) allow for assessing the effect of the -158 C/T SNP on Gγ expression only (Note: The Gγ and Aγ promoters diverge in their >200 bp distal region). Moreover, we postulated that by using this approach, indels produced in the γ-globin copies lacking an integrated cassette would not confound our results.

3.2. The GFP cassettes are inserted into the Gγ gene

Previously we had knocked-in a GFP cassette into the 5′-untranslated region (5′-UTR) of the Gγ gene in K562 cells by using the CRISPR/Cas9 system [16]. The utilized CRISPR plasmid expressed a sgRNA that created double-strand (ds)-DNA breaks just upstream of the γ-globin start codon. Furthermore, the donor plasmid harbored a GFP.(IRES).Neo^R.pA cassette. This cassette, designed with ~800 bp homology arms, is intended for insertion of the cassette upstream of the Gγ gene start codon and expression of the GFP and Neo^R proteins (the GFP via its start codon and the Neo^R gene via the IRES) (Fig. 1). The homology arms had been amplified from K562 genomic DNA, which carries the C allele at the Gγ -158 position (data not shown). As a result, the edited cell population and isolated clones that were obtained contained the *XmnI* C allele upstream of the inserted GFP cassette.

Here, we sought to create an equivalent edited K562 cell population with a substituted *XmnI* T allele. To this end, an analogous donor plasmid having the -158 *XmnI* T allele in its left homology arm was first created via overlap PCR mutagenesis. Subsequently, the new donor plasmid and CRISPR vector were co-transfected into K562 cells. Following antibiotic selection of the cells, genomic DNA was extracted and knockin of the GFP.Neo^R cassette into the Gγ gene was verified by PCR of either arm—with one primer falling in the transgene and the other outside of the homology arm (Fig. 2a). Sanger sequencing of the amplicons suggested that the GFP.Neo^R cassette was inserted specifically into the Gγ 5′-UTR (Fig. 2b and Fig. S2). Moreover, the *XmnI* T allele was retained in the left homology arm post-recombination (Fig. 2c).

Next, we examined whether integration of the GFP cassette had resulted in a concurrent replacement of the Gγ-Aγ intergenic region in the edited cell populations. To this end, PCR reactions were deployed by using a transgene-specific forward primer and Gγ- or Aγ-specific reverse ARMS primers. The utilized ARMS primers had previously been validated for functionality and specificity [22].

Here, genomic replacements were detected in both the *XmnI* C and *XmnI* T KI populations (Fig. S3). We reasoned, however, that these replacements would not interfere with the transcriptional regulation of the GFP cassette mediated via the Gγ promoter and the locus control region (LCR)—given that no known transcriptional regulatory elements were deleted in the process [24].

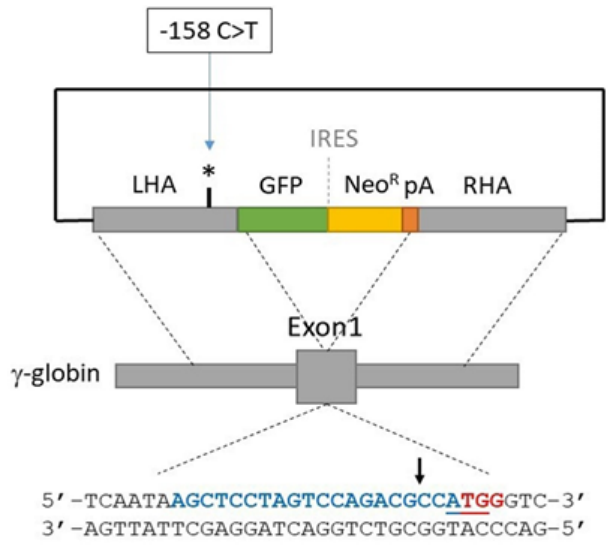

Fig. 1. Schematic of the GFP knockin construct and its Gγ gene insertion site. The utilized sgRNA sequence is shown in blue and its protospacer adjacent motif (PAM) in red. The start codon (ATG) of the γ-globin gene is underlined. [LHA=left homology arm; GFP=green fluorescence protein; IRES=internal ribosome entry site; Neo^R=neomycin resistance gene; pA=polyadenylation signal sequence; RHA=right homology arm].

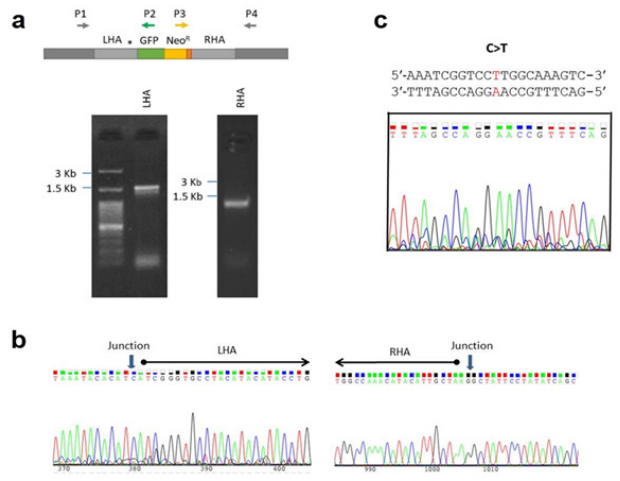

Fig. 2. Verifying knockin of the GFP/*XmnI* T cassette into the γ-globin gene(s) of K562 cells. (a) PCR reactions of the left and right homology arms (LHA, RHA) and (b) Sanger sequencing of the resulting amplicons confirmed insertion of the GFP cassette into the γ-globin gene. (c) Retention of the *XmnI* T allele post-recombination was also established via Sanger sequencing.

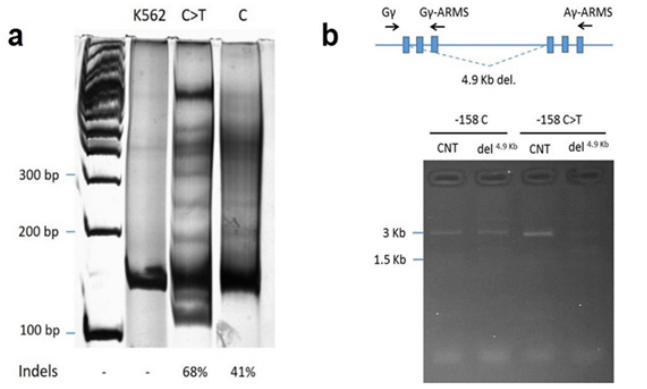

Fig. 3. Genomic edits in the γ-globin copies lacking an inserted GFP cassette. (a) Indels in the 5′-UTRs of the Aγ and Gγ genes were detected via PAGE-genotyping in the edited K562 cell populations. (b) Presence of the Gγ-Aγ 4.9 Kb deletion in these cell populations was detected by using gap-PCR. A control (CNT) PCR reaction was also carried out by using a Gγ promoter-specific F-primer and a Gγ-ARMS R-primer.

3.3. Validating the precision of insertions in cellular clones

The precision of the GFP→Gγ knockin had previously been validated in cellular clones isolated from the Gγ -158 C KI population [16]. Besides one clone, the GFP cassette had been accurately inserted into the Gγ gene of K562 cells i.e. without any indels at the break junction.

In line, accuracy of the transgene insertions was hereby analyzed in cellular clones isolated from the Gγ -158 T KI population (Table 1). In 8/10 of the isolated clones, the GFP cassette was accurately inserted into the Gγ 5′-UTR. Furthermore, in all of the latter clones, the *XmnI* T allele was detected upstream of the transgene. In addition, a correlation between transgene copy number and fluorescence levels was not detected in cellular clones isolated from either population.

3.4. Genomic edits in the γ-globin copies lacking an integrated transgene

We assumed that genome edits in the non-recombinant γ-globin copies (i.e. those lacking an integrated cassette) would not affect GFP expression levels in *cis* or in *trans*. Nevertheless, the nature and frequency of the genome edits in the non-recombinant γ-globin copies of the two populations were compared. In the *XmnI* C and T KI populations, indels were detected in 41% and 68% of the non-recombinant γ-globin (Gγ and Aγ) 5′-UTRs, respectively (Fig. 3a). Furthermore, presence of the 4.9 Kb Gγ-Aγ deletion, created by the simultaneous ds-DNA breaks in the Gγ and Aγ genes, was detected in both populations (Fig. 3b).

3.5. The XmnI polymorphism increases GFP expression

Subsequently, GFP fluorescence levels in the two edited cell populations were compared. In the Gγ -158 C and T KI populations, 82.8% and 99.3% of the cells were GFP^+, respectively (Fig. 4a). In the former population, the GFP^- cells persisted even after prolonged antibiotic selection. Previously, two GFP^- clones had been isolated from this population [16]. One turned out to be G418 sensitive ($G418^S$). The other seemed to have indels at the CRISPR/Cas9 break entry site. Presumably, these indels had da-

Table 1. Data related to the *XmnI* T knock-in clones are summarized. In clone 10, the LHA and RHA could not be PCR amplified as described in Materials and Methods. In contrast, amplicons were obtained when using PCR primer sets within the GFP cassette (data not shown). Thus, in this clone, GFP expression had presumably resulted from the insertion of the cassette into a non-targeted locus.

EGFP clones	Relative fluorescence	Median fluorescence intensity (MFI)	Estimated GFP copy number	Precision in LHA junction	Precision in RHA junction	Gγ or Aγ 5'-UTR insertion	-158 C or C>T
K562	N/A	0.476	N/A	N/A	N/A	N/A	N/A
1	Low	2.50	1	Yes	Yes	Gγ	C>T
2	Low	2.59	2	Yes	Yes	Gγ	C>T
3	Low	2.73	2	Yes	Yes	Gγ	C>T
4	Medium	4.10	3	Yes	Yes	Gγ	C>T
5	Medium	4.71	1	Yes	Yes	Gγ	C>T
6	High	5.05	3	Yes	Yes	Gγ	C>T
7	High	5.20	3	Yes	Yes	Gγ	C>T
8	High	5.80	2	Yes	Yes	Gγ	C>T
9	High	7.75	N/A	No	No	N/A	N/A
10	High	14.0	N/A	N/A	N/A	N/A	N/A

(N/A=not applicable).

maged the ribosome binding site of the GFP gene, but not the IRES of the Neo^R gene—resulting in the creation of a GFP$^-$G418^R subpopulation. To gain insight, we sought to isolate additional GFP$^-$ clones from this population. Here, two more GFP$^-$ clones were isolated, both of which turned out to be G418^S.

In the GFP$^+$ subpopulations, on the other hand, the median fluorescence intensities (MFI) of the Gγ -158 C and T-bearing GFP$^+$ cells were 4.50 and 6.75, respectively. This was evident by a rightward shift in the fluorescence curve of the GFP$^+$ *XmnI* T population relative to that of the *XmnI* C population (Fig. 4a). As a result, 30% of the GFP$^+$ cells having the *XmnI* T allele displayed higher fluorescence levels than those having the C allele.

The fluorescence intensities were also compared in cellular clones isolated from the two populations i.e. only those containing accurate transgene insertions. Here, the average MFI was increased from 2.60±1.31 in clones isolated from the *XmnI* C population to 4.09±1.31 in those isolated from the *XmnI* T population (P<0.05) (Fig. 4b). Collectively, these results suggest that presence of the *XmnI* T allele could upregulate Gγ expression levels in K562 cells.

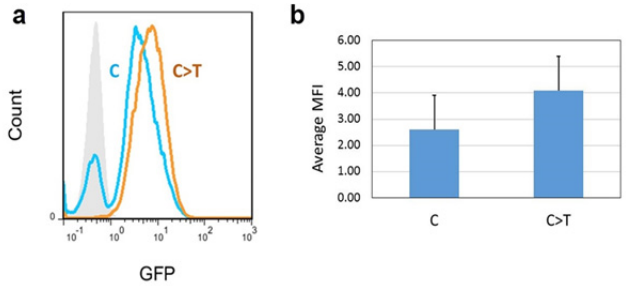

Fig. 4. Presence of the the *XmnI* T allele increases GFP fluorescence levels in the edited K562 cells. (a) Fluorescence levels were compared in the two edited cell populations and (b) the isolated GFP$^+$ clones (*XmnI* C n=9; *XmnI* C>T n=8). The MFI mean ± s.d. of the isolated clones is plotted (Two-tailed Student's t-test P<0.05).

4. Discussion

The *XmnI* polymorphism is a well-known genetic marker that has been widely associated with HbF levels, disease severity, and the response to HU in both β-thal and SCD patients. This SNP is associated strongly with the amelioration of disease severity in SCD and β-thal patients (i.e. β-thal major vs. β-thal intermedia). It also correlates with the blood transfusion intervals in transfusion-dependent β-thal (TDT) patients [25,26]. As such, it is a valuable prognostic marker for predicting the course of the disease and its management in patients by clinicians. Furthermore, association of the XmnI polymorphism with the response to HU (a drug with adverse side effects) renders it a useful marker for personalized medicine.

To the best of our knowledge, however, no definitive function has been associated with this SNP. In earlier transient transfection assays performed in K562 cells, expression constructs containing the *XmnI* T allele had failed to increase reporter gene activity [27]. However, in those experiments, the effect of the *XmnI* T allele was not assayed in its genomic context. In this study, we took advantage of the CRISPR/Cas9 technology to assess the effect of the *XmnI* polymorphism in the context of the entire β-locus. In summary, our results suggest that the Gγ -158 C/T polymorphism may possibly be a functional SNP.

The current study was performed by using K562 cells—a cell line that is considered to be a model for F-cells. In support, association of the *XmnI* T allele with increased HbF levels has been reported exclusively in patients with hemoglobin disorders, in which F-cell numbers are amplified [1,28]. In that regard it would be interesting to replicate these experiments in cord blood (CB)-derived HSPCs which mainly produce γ-globin and HbF post-differentiation [29]. However, performing similar knockin experiments in CB-derived HSPCs may be technically challenging [30].

Our results in K562 cells suggest that the inducing effect of this SNP on γ-globin expression may be limited; since only 30% of the edited cells having the *XmnI* T allele in their KI cassettes showed higher GFP fluorescence levels than those having the C allele. This is consistent with a number of clinical data, in which the *XmnI* T/T, rather than the *XmnI* T/C, genotype had shown an association with the amelioration of disease severity in patients [28]. For example, in an earlier cohort study of 306 Iranian β-thal patients, we detected an association between the *XmnI* T/T genotype along with the *BCL11A* QTL with the milder β-thal intermedia disease-type [31]. Likewise, we detected a correlation between the *XmnI* T/T genotype with the response to HU in a cohort of 81 transfusion-dependent β-thal patients [32].

To date, a few potentially functional genetic determinants in LD with the *XmnI* polymorphism have been reported. In particular, an SNP in the LCR and another variation in the Gγ -588 region have been linked to the *XmnI* polymorphism in Iranian β-thal patients most of whom were homozygous for the IVS-II-1 (C>A) mutation [13,33]. Furthermore, a β-locus haplotype encompassing the *XmnI* T allele has been reported in Indian β-thal intermedia patients, most of whom had the IVS-I-5 (G>C) mutation [34]. In addition, GWAS fine-mapping has been used to identify an SNP in the β-locus which is associated more strongly than rs7482144-*XmnI* with HbF levels in African American SCD patients [35]. These associations, unlike those involving the *XmnI* polymorphism, have only been detected in singular cohorts. Nevertheless, the functional significance of these other β-locus SNPs cannot be ruled out.

The present results support those previously obtained by Weber *et al.*, which suggested a functional role for the γ-globin -158 region. There, disruption of the -158 site resulted in a slight upregulation of γ-globin levels in differentiated HUDEP-2 cells and HSPCs obtained from SCD patients. Likewise, it would be interesting to determine the effect of these indels in K562 cells.

Notably, the HUDEP-2 cells and SCD-HSPCs represent adult erythroid cells—whereas K562 cells represent end-stage fetal-like erythroid cells (in that these cells express γ-globin but lack γ- to-β globin switching). As such, K562 cells serve as an alternative model for functional studies involving the XmnI polymorphism—especially in light of the fact that correlations of the XmnI polymorphism with HbF levels have only been detected in SCD and β-thal patients, in which F cell levels are amplified.

Weber et al. proposed that the -158 region may act as a binding site for a transcriptional repressor. However, to date, no such repressor has been identified. Therefore, the mechanism by which the -158 region, and more spe-

cifically the *XmnI* Gγ -158 C/T SNP, may act on γ-globin transcription in either fetal- or adult-like erythroid cells remains to be elucidated.

Abbreviation

GFP: green fluorescence protein; **HbA**: adult hemoglobin; **HbF**: fetal hemoglobin; **HU**: hydroxyurea; **IRES**: internal ribosome entry site; **LHA**: left homology arm; **MFI**: median fluorescence intensity; **Neo^R**: neomycin resistance gene; **pA**: polyadenylation signal; **QTL**: quantitative trait loci; **RHA**: right homology arm; **SCD**: sickle cell disease; **SNP**: single nucleotide polymorphism; **β-thal**: β-thalassemia

Conflict of interest

The authors report no conflicts of interest.

Consent for publications

All authors read and approved the final manuscript for publication.

Authors' contributions

AA: Investigation and formal analysis; NM: Investigation and formal analysis; MMT: Investigation and formal analysis; TD: Investigation and formal analysis; MSMM: Investigation; SB: Investigation; MB: Conceptualization, funding acquisition, formal analysis, supervision, and writing.

Funding

This work was funded by Grant No. 64032 from the Genetics Research Center, University of Social Welfare and Rehabilitation Sciences to Mehdi Banan, PhD.

Acknowledgements

The authors gratefully acknowledge the Genetics Research Center of the University of Social Welfare and Rehabilitation Sciences. This work was supported by the Genetics Research Center (University of Social Welfare and Rehabilitation Sciences, Tehran, Iran) grant 64032 to Mehdi Banan.

References

1. Thein SL (2018) Molecular basis of beta thalassemia and potential therapeutic targets. Blood Cells Mol Dis 70: 54-65. doi: 10.1016/j.bcmd.2017.06.001
2. Taher AT, Musallam KM, Cappellini MD (2021) β-Thalassemias. N Engl J Med 384 (8): 727-743. doi: 10.1056/NEJMra2021838
3. Piel FB, Steinberg MH, Rees DC (2017) Sickle cell disease. N Engl J Med 376 (16): 1561-1573. doi: 10.1056/NEJMra1510865
4. Thein SL, Menzel S, Lathrop M, Garner C (2009) Control of fetal hemoglobin: new insights emerging from genomics and clinical implications. Hum Mol Genet 18 (R2): R216-223. doi: 10.1093/hmg/ddp401
5. Lettre G, Sankaran VG, Bezerra MAC, Araújo AS, Uda M, Sanna S, Cao A, Schlessinger D, Costa FF, Hirschhorn JN (2008) DNA polymorphisms at the BCL11A, HBS1L-MYB, and β-globin loci associate with fetal hemoglobin levels and pain crises in sickle cell disease. Proc Natl Acad Sci U S A 105 (33): 11869-11874. doi: 10.1073/pnas.0804799105
6. Badens C, Joly P, Agouti I, Thuret I, Gonnet K, Fattoum S, Francina A, Simeoni M-C, Loundou A, Pissard S (2011) Variants in genetic modifiers of β-thalassemia can help to predict the major or intermedia type of the disease. Haematologica 96 (11): 1712. doi: 10.3324/haematol.2011.046748
7. El-Ghamrawy M, Yassa ME, Tousson AM, El-hady MA, Mikhaeil E, Mohamed NB, Khorshied MM (2020) Association between BCL11A, HSB1L-MYB, and XmnI γG-158 (C/T) gene polymorphism and hemoglobin F level in Egyptian sickle cell disease patients. Ann Hematol 99: 2279-2288. doi: 10.1007/s00277-020-04187-z
8. Pereira C, Relvas L, Bento C, Abade A, Ribeiro ML, Manco L (2015) Polymorphic variations influencing fetal hemoglobin levels: association study in beta-thalassemia carriers and in normal individuals of Portuguese origin. Blood Cells Mol Dis 54 (4): 315-320. doi: 10.1016/j.bcmd.2015.02.001
9. Rujito L, Basalamah M, Siswandari W, Setyono J, Wulandari G, Mulatsih S, Sofro AS, Sadewa AH, Sutaryo S (2016) Modifying effect of XmnI, BCL11A, and HBS1L-MYB on clinical appearances: A study on beta-thalassemia and hemoglobin E/beta-thalassemia patients in Indonesia. Hematol Oncol Stem Cell Ther 9 (2): 55-63. doi: 10.1016/j.hemonc.2016.02.003
10. Sajadpour Z, Amini-Farsani Z, Motovali-Bashi M, Yadollahi M, Khosravi-Farsani N (2020) Association between Different Polymorphic Markers and β-Thalassemia Intermedia in Central Iran. Hemoglobin 44 (1): 27-30. doi: 10.1080/03630269.2019.1709204
11. Ghosh D, Panja A, Saha D, Banerjee U, Datta AK, Basu A (2021) Drug Repurposing: Hydroxyurea Therapy Improves the Transfusion-Free Interval in HbE/Beta-Thalassemia—Major Patients with the Xmn I Polymorphism. Genet Test Mol Biomarkers 25 (8): 563-570. doi: 10.1089/gtmb.2021.0031
12. Ronchi A, Ottolenghi S (2013) To respond or not to respond to hydroxyurea in thalassemia: a matter of stress adaptation? Haematologica 98 (5): 657-659. doi: 10.3324/haematol.2013.084392
13. Neishabury M, Zamani S, Azarkeivan A, Abedini SS, Darvish H, Zamani F, Najmabadi H (2012) The modifying effect of Xmn1-HBG2 on thalassemic phenotype is associated with its linked elements in the beta globin locus control region, including the palindromic site at 5′ HS4. Blood Cells Mol Dis 48 (1): 1-5. doi: 10.1016/j.bcmd.2011.10.001
14. Weber L, Frati G, Felix T, Hardouin G, Casini A, Wollenschlaeger C, Meneghini V, Masson C, De Cian A, Chalumeau A, Mavilio F, Amendola M, Andre-Schmutz I, Cereseto A, El Nemer W, Concordet JP, Giovannangeli C, Cavazzana M, Miccio A (2020) Editing a gamma-globin repressor binding site restores fetal hemoglobin synthesis and corrects the sickle cell disease phenotype. Sci Adv 6 (7): eaay9392. doi: 10.1126/sciadv.aay9392
15. Naumann S, Reutzel D, Speicher M, Decker HJ (2001) Complete karyotype characterization of the K562 cell line by combined application of G-banding, multiplex-fluorescence in situ hybridization, fluorescence in situ hybridization, and comparative genomic hybridization. Leuk Res 25 (4): 313-322. doi: 10.1016/s0145-2126(00)00125-9
16. Jafari H, Hesami S, Safi M, Ghasemi F, Banan M (2019) Expression and hydroxyurea-triggered induction of EGFP upon CRISPR/Cas9-mediated integration into the γ-globin gene of K562 cells. Biotechnol lett 41: 691-700. doi: 10.1007/s10529-019-02671-9
17. Heckman KL, Pease LR (2007) Gene splicing and mutagenesis by PCR-driven overlap extension. Nat Protoc 2 (4): 924-932. doi: 10.1038/nprot.2007.132
18. Vallejo AN, Pogulis RJ, Pease LR (2008) PCR Mutagenesis by Overlap Extension and Gene SOE. CSH Protoc 2008: pdb prot4861. doi: 10.1101/pdb.prot4861
19. Banan M (2020) Recent advances in CRISPR/Cas9-mediated knock-ins in mammalian cells. J Biotechnol 308: 1-9. doi: 10.1016/j.jbiotec.2019.11.010
20. Maroofi N, Azarkeivan A, Banihashemi S, Mohammadparast S,

Aghajanirefah A, Banan M (2017) An enhancer haplotype may influence BCL11A expression levels and the response to hydroxyurea in beta-thalassemia patients. Pharmacogenomics 18 (10): 995-967. doi: 10.2217/pgs-2017-0019

21. Zhu X, Xu Y, Yu S, Lu L, Ding M, Cheng J, Song G, Gao X, Yao L, Fan D, Meng S, Zhang X, Hu S, Tian Y (2014) An efficient genotyping method for genome-modified animals and human cells generated with CRISPR/Cas9 system. Sci Rep 4 (1): 6420. doi: 10.1038/srep06420
22. Banan M, Esmaeilzadeh-Gharehdaghi E, Nezami M, Deilami Z, Farashi S, Philipsen S, Esteghamat F, Pourfarzad F, Ali Imam A, Najmabadi H (2012) c AMP response element-binding protein 1 is required for hydroxyurea-mediated induction of γ-globin expression in K 562 cells. Clin Exp Pharmacol Physiol 39 (6): 510-517. doi: 10.1111/j.1440-1681.2012.05702.x
23. Drexler HG, Matsuo Y, MacLeod RA (2004) Malignant hematopoietic cell lines: in vitro models for the study of erythroleukemia. Leuk Res 28 (12): 1243-1251. doi: 10.1016/j.leukres.2004.03.022
24. Lu HY, Orkin SH, Sankaran VG (2023) Fetal Hemoglobin Regulation in Beta-Thalassemia. Hematol Oncol Clin North Am 37 (2): 301-312. doi: 10.1016/j.hoc.2022.12.002
25. Maryami F, Azarkeivan A, Fallah MS, Zeinali S (2015) A Large Cohort Study of Genotype and Phenotype Correlations of Beta-Thalassemia in Iranian Population. Int J Hematol Oncol Stem Cell Res 9 (4): 198-202.
26. Winichagoon P, Fucharoen S, Chen P, Wasi P (2000) Genetic factors affecting clinical severity in beta-thalassemia syndromes. J Pediatr Hematol Oncol 22 (6): 573-580. doi: 10.1097/00043426-200011000-00026
27. Motum PI, Lindeman R, Harvey MP, Trent RJ (1993) Comparative studies of nondeletional HPFH gamma-globin gene promoters. Exp Hematol 21 (7): 852-858.
28. Mohammad SNNAi, Iberahim S, Wan Ab Rahman WS, Hassan MN, Edinur HA, Azlan M, Zulkafli Z (2022) Single Nucleotide Polymorphisms in XMN1-HBG2, HBS1L-MYB, and BCL11A and Their Relation to High Fetal Hemoglobin Levels That Alleviate Anemia. Diagnostics 12 (6): 1374. doi: 10.3390/diagnostics12061374
29. Jin H, Kim HS, Kim S, Kim HO (2014) Erythropoietic potential of CD34+ hematopoietic stem cells from human cord blood and G-CSF-mobilized peripheral blood. Biomed Res Int 2014: 435215. doi: 10.1155/2014/435215
30. Bak RO, Dever DP, Porteus MH (2018) CRISPR/Cas9 genome editing in human hematopoietic stem cells. Nat Protoc 13 (2): 358-376. doi: 10.1038/nprot.2017.143
31. Banan M, Bayat H, Namdar-Aligoodarzi P, Azarkeivan A, Kamali K, Daneshmand P, Zaker-Kandjani B, Najmabadi H (2013) Utility of the multivariate approach in predicting beta-thalassemia intermedia or beta-thalassemia major types In Iranian patients. Hemoglobin 37 (5): 413-422. doi: 10.3109/03630269.2013.805418
32. Banan M, Bayat H, Azarkeivan A, Mohammadparast S, Kamali K, Farashi S, Bayat N, Khani MH, Neishabury M, Najmabadi H (2012) The X mn I and BCL11A single nucleotide polymorphisms may help predict hydroxyurea response in Iranian β-thalassemia patients. Hemoglobin 36 (4): 371-380. doi: 10.3109/03630269.2012.691147
33. Hamid M, Mahjoubi F, Akbari MT, Arab A, Zeinali S, Karimipoor M (2009) Molecular analysis of gamma-globin promoters, HS-111 and 3'HS1, in beta-thalassemia intermedia patients associated with high levels of Hb F. Hemoglobin 33 (6): 428-438. doi: 10.3109/03630260903336479
34. Dabke P, Colah RB, Ghosh K, Nadkarni A (2013) Effect of a group of genetic markers around the 5' regulatory regions of the beta globin gene cluster linked to high HbF on the clinical severity of beta thalassemia. Blood Cells Mol Dis 50 (3): 156-160. doi: 10.1016/j.bcmd.2012.11.003
35. Galarneau G, Palmer CD, Sankaran VG, Orkin SH, Hirschhorn JN, Lettre G (2010) Fine-mapping at three loci known to affect fetal hemoglobin levels explains additional genetic variation. Nat Genet 42 (12): 1049-1051. doi: 10.1038/ng.707