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Transient Expression Assay of $^{A}\gamma$-588 (A/G) Mutations in the K562 Cell Line

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ABSTRACT

Background: In the previous study, we have shown that the presence of A allele at position -588 in $^{A}\gamma$-globin gene was highly frequent and closely associated with fetal hemoglobin elevation among β-thalassemia intermedia patients. Therefore, we decided to investigate whether this allele (A allele at -588) could result in an increase in $^{A}\gamma$-globin gene expression to ameliorate the severity of the disease in thalassemia patients. **Methods:** Three constructs containing μ locus control region, $^{A}\gamma$-globin and β-globin genes were designed and employed in the transient expression assay. The difference among constructs was in the promoter region of $^{A}\gamma$-globin gene (A and G alleles at -588). A construct with T to C base substitution at -175 of $^{A}\gamma$-globin, created by site-directed mutagenesis, was selected as positive control. The K562 cell line was transfected with the above constructs. Subsequently, the expression of $^{A}\gamma$-globin gene was determined by quantitative real-time reverse transcription-PCR. **Results:** There was not a significant increase in the expression of $^{A}\gamma$-globin gene in the construct containing A allele comparing the one with G allele at -588. **Conclusions:** -588 (A>G) mutation does not play a major role in regulation of $^{A}\gamma$-globin gene, suggesting that other factors may be involved. *Iran. Biomed. J. 15 (1 & 2): 15-21, 2011*

Keywords: β-thalassemia, $^{A}\gamma$-globin, K562 cells

INTRODUCTION

The human β-globin cluster contains five developmentally regulated genes (5'-ε-Gγ-Aγ-δ-β-3') on the short arm of chromosome 11. The Gγ and Aγ genes are normally expressed at high levels only during fetal development, followed by switching to adult (δ, β) during the perinatal period. Due to switching, fetal hemoglobin (HbF) comprises only 1% to 2% of the total hemoglobin in healthy adults [1, 2].

The genes of β-globin cluster are regulated by locus control region (LCR), which is composed of five major DNase I hypersensitive sites (HS1-HS5) located far upstream of the genes [3]. It seems the LCR activates β-globin gene expression through a direct interaction with the promoter region and make an open chromosomal region more accessible to *trans*-acting factors [4]. Many studies have investigated the roles of individual HS in various expression assays [5, 6]. HS2 acts as a strong enhancer in both transient expression assays and transgenic mice experiments. HS3 was also shown to enhance the expression of β-globin gene. HS4 does not enhance solely, but can increase expression in cooperation with other HSs [5]. In contrast to normal switching, the individuals with hereditary persistence of fetal hemoglobin (HPFH) have constantly high levels of HbF during adult life. This elevation is mainly due to large deletions within β-globin cluster in HPFH and δβ-thalassemia or in some cases of non-deletional HPFH (nd-HPFH) by mutations in the promoter region of γ-globin genes [7, 8]. Several nd-HPFH mutations have been

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Table 1. $^{A}\gamma$-globin oligonucleotide primers used for PCR and real-time experiments.

Primer	Location	Sequence (5′>3′)	Amplicon (bp)
AγF	-883 to -860	accggtCACAGTACCTGCCAAAGAACATTC[1]	2788
AγR	+1885 to +1905	gcggccgcAATCAATCCAGCCCCCAGGTC[2]	
AγF	-883 to -860	accggtCACAGTACCTGCCAAAGAACATTC[1]	720
R1 site direct	-183 to -160	CCGTTTCAGACAGATGTTTGCATT[3]	
R2 site direct	-183 to -160	AATGCAAACATCTGTCTGAAACGG[3]	1840
R2 Aγ R	+1633 to +1657	atggcggccgcACACATACACACTTCCCTCAATATA[2]	
Real AγF		ATGTGGATCCTGAGAACTTCAAGCT	136
Real AγR	+1451 to +1469	GACAGGGCACTGGCCACTG	

Underlined bases are related to [1]*AgeI* site, [2]*Not I* site and [3]underlined bases are mutated to produce -175(T>C) base substitution

reported in the promoter region of either the $^{G}\gamma$- or $^{A}\gamma$-globin genes [9]. Most of these mutations occur in transcription factor binding sites, creating the new factor binding motifs or disrupting the existing ones [9]. The regulatory sequences 5' to the $^{A}\gamma$-globin gene harbor some polymorphic markers, including -588 A>G, -521 C>G, -500 C>T, -499 T>A, -202 C>T, -198 T>C, -196 C>T, -195 C>G, -175 T>C, -117 G>A, and -114 C>T. The most important mutations are -175 T>C and -117 G>A that their functional roles have been demonstrated in transgenic mice [8, 10-13].

The level of HbF may also be influenced by some other genetic determinants linked to the β-globin gene cluster such as polymorphisms in the regulatory region of $^{G}\gamma$-globin gene. The presence or absence of the -158 C>T $^{G}\gamma$-*Xmn*I polymorphism has been identified to be associated with the high level of HbF in thalassemia intermedia (TI) patients [3]. We have previously shown that the presence of A allele at -588 was highly frequent and closely associated with HbF elevation in TI patients [3].

Therefore, we decided to investigate whether this allele (A allele at -588) could result in an increase in $^{A}\gamma$-globin gene expression. For this purpose, three constructs containing μLCR, $^{A}\gamma$-globin and β-globin genes were designed for exploiting in the transient expression assay in K562 erythroid cell line.

MATERIALS AND METHODS

Plasmid DNA constructions. The genomic DNA was obtained from TI patients with A and G alleles at -588 of $^{A}\gamma$-globin [3]. The primers AγF and AγR were designed to amplify the sequences from -883 to +1905 of $^{A}\gamma$-globin gene (Table 1). These primers also were engineered to contain *AgeI* (forward primer) and *NotI* (reverse primer) at their 5' ends (Table 1) and the fragments cloned into the T/A vector (Fermentase, Hanover, MD, USA). Intact core sequences of HS4 (442 bp), HS3 (2004 bp) and HS2 (1468 bp) designated μLCR (unpublished data) were cloned into pBGGT plasmid [14]. In order to clone $^{A}\gamma$-globin fragment, two complementary oligonucleotides containing *NotI* and *SalI* sequences with *XhoI* ends were annealed together and directly cloned into the unique *XhoI* site of the pBGGT construct. The 3.9-kb μLCR fragment was released by *NheI* and *AgeI* from pBGGT construct and inserted into pCDNA3.1 plasmid (Invitrogen, Paisley, UK). Subsequently, the fragments of $^{A}\gamma$-globin gene (*AgeI-NotI*) with A allele at -588 and β-globin gene (*NotI-PmeI*) subcloned in pCDNA3.1 μLCR construct, generating plasmid construct pCDNA3.1 μLCR$^{A}\gamma^{-588(A)}\beta$. PCR mutagenesis was used to generate a 2560-bp fragment of the PCR products containing the T>C mutation at position -175 in three stages. Stage 3 was modified to include primers AγF and R2 AγR (Table 1), engineered to contain an *AgeI* (forward primer) and *NotI* sites (reverse primer) at their 5' ends. The presence of mutation was confirmed by DNA sequencing. Similar strategies were also adopted to create constructs pCDNA3.1 μLCR$^{A}\gamma^{-588(G)}\beta$ and pCDNA3.1 μLCR$^{A}\gamma^{-175(C)}\beta$ (Fig. 1).

Cell growth and transfection. Human erythroleukemia K562 was cultured in DMEM (GIBCO-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (GIBCO-BRL, Grand Island, NY, USA), penicillin/streptomycin (BioSera, Ringmer, East Sussex, UK) in a humidified 5% CO_2 environment. Transient DNA transfections were performed by electroporation using a Bio-Rad Gene Pulser (Bio-Rad Laboratories, Hercules, CA, USA)

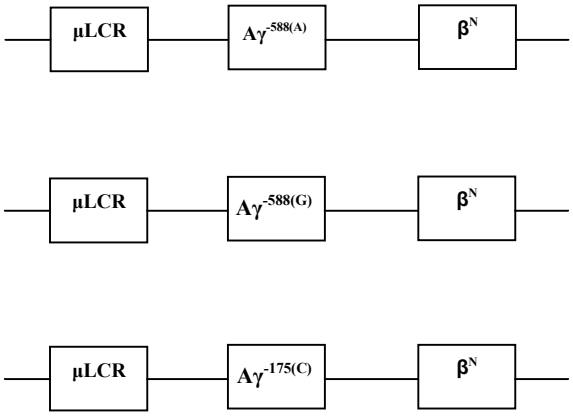

Fig. 1. Schematic representation of the expression constructs and their individual components.

and Lipofectamine™ LTX (Invitrogen Corp., Carlsbad, CA, USA) in triplicate form. Approximately, 2.5×10^6 cells of K562 were electroporated with 50 μg/ml of the constructs and mock plasmid (pCDNA3.1). Lipofectamine™ LTX was prepared according to the manufacturer's protocol.

Efficiency of electroporation and transfection. The rate of transfection in K562 cell line was determined by analysis of green fluorescent protein (GFP) expression of transfected pEGFP-N1 plasmid (Clontech, Palo Alto, CA, USA) by flow cytometry (Partec, Germany). Tranfection efficiency was determined 48 h after transfection. Transfection and electroporation experiments were optimized based on Table 2. For electroporation, the cells were suspended in DMEM without FBS and transferred to a sterile electroporation cuvette (Bio-Rad Gene Pulser cuvette, 0.4 cm), in the presence of 50 μg /ml of pEGFP-N1 plasmid.

Total RNA extraction and cDNA synthesis. Total RNA was extracted from K562 cell line using Trizol reagents (Sigma- Aldrich, Germany) following the manufacturer's instructions and resuspended in 30 μl of DEPC-treated water. One microgram of total RNA was reverse transcribed with Oligo(dT) primers and Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The concentration of cDNA was determined by a Nanodrop ND-1000 Spectrophotometer (Nanodrop Technologies, Rockland, DE) at 260/280 nm.

Real-time PCR. Real-time reverse transcription-PCR was performed in final reaction volumes of 25 μl, consisting of 12.5 μl SYBR Green I master mix (Applied Biosystems, Warrington, UK), 500 nm of forward and reverse primers and 5 ng of cDNA. Thermal cycling was performed on the ABI 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) using the following cycling conditions: 10 min at 95°C as first denaturation step, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. Each complete amplification stage was followed by a dissociation stage; at 95°C for 15 s, 60°C for 30 s and 95°C for 15 s. The samples were analyzed in triplicate for each studied construct. PCR primers (Real AγF and Real AγR) were designed using the primer express software v.3.0 (Applied Biosystems, Foster City, CA, USA) to specifically amplify mRNA from the $^{A}\gamma$-globin gene (Table 1). To avoid amplification of contaminating genomic DNA, exon-exon junction forward primer was designed.

Table 2. Electroporation and transfection experiments in K562 cell line.

Transfection condition	Lipofectamine™ LTX kit			DNA (μg/ml)	Electroporation voltage (V)
	DNA (μg)	TR (μL)	PR (μL)		
1	0.2	0.50	0.2		
2	0.2	0.70	0.2	50	270
3	0.2	1.10	0.2		
4	0.5	1.25	0.5		
5	0.5	2.00	0.5	50	300
6	0.5	2.75	0.5		
7	1.0	2.50	1.0		
8	1.0	3.50	1.0	50	220
9	1.0	5.50	1.0		
10	2.5	6.25	2.5		
11	2.5	10.50	2.5	50	500
12	2.5	13.75	2.5		

DNA, pEGFP-N1 plasmid; TR, transfection reagent; PR, plus reagent

Table 3. Efficiency of transfection by Lipofectamine™ LTX kit and electroporation in K562 cell line.

Transfection condition	Lipofectamine™ LTX kit (%)	Electroporation (%)		
			Cell viability (%)	
			PBS	DMEM
1-3	5.30 ± 0.80	23.15 ± 2.1	15	68
4-6	8.74 ± 0.45	26.13 ± 2.8	16	75
7- 9	5.39 ± 1.30	19.00 ± 1.9	13	78
10-12	0.65 ± 1.50	8.96 ± 3.2	17	65

Gene expression was calculated according to Livak and Schmittgen [15] using β-actin as an internal reference gene. The PCR efficiency of $^{A}\gamma$-globin gene was calculated according to the slope of the standard curve and the following equation: Efficiency = $[10^{(-1/slop)}]$-1 [16]. Statistical analysis including mean, standard deviation and correlation coefficients were carried out using SPSS for windows, version 12 (SPSS Inc., Chicago, IL, USA).

Statistical analysis. Quantitative variables were expressed as means ± S.D. while qualitative variables were expressed as percentages. Fisher's exact test was used for comparing the qualitative variables. *P* values lower than 0.05 were considered as statistically significant.

RESULTS

Constructs. The three constructs included pCDNA3.1μLCR$^{A}\gamma^{-588(G)}\beta$, pCDNA3.1μLCR$^{A}\gamma^{-588(A)}\beta$ and pCDNA3.1μLCR$^{A}\gamma^{-175(T>C)}\beta$ were created and confirmed by sequencing. These constructs contain about 4 kb μLCR, 3 kb $^{A}\gamma$-globin gene and 2 kb β-globin gene. We assumed that the μLCR would have a local effect on the promoters of $^{A}\gamma$- and β-globin genes [1].

Optimization of transfection. Transfection experiments by Lipofectamine™ LTX kit (Invitrogen, USA) were optimized according to the suppliers' instruction with variable amounts of plasmid DNA and the volumes of transfection reagents. For each transfection, twelve different conditions were examined, and every experiment was repeated three times. The results of transfection yield for each set of transfection are shown in Table 3. The highest mean rate of transfection by Lipofectamine™ LTX kit was 8.74 ± 0.45 (Fig. 2 and Table 3). In addition, to optimize DNA electroporation, different electroporation conditions were tested (Table 2), including variations in electric field and capacitance. The best results, represented by 26.13 ± 2.8 GFP- positive cells, were achieved in a voltage of 300 V combined with a capacitance of 950 μF (Fig. 2). As shown in Table 3, cell viability was greatly reduced in phosphate buffered saline (PBS); therefore, we used DMEM as electroporation buffer which resulted in much higher (75%) cell viability.

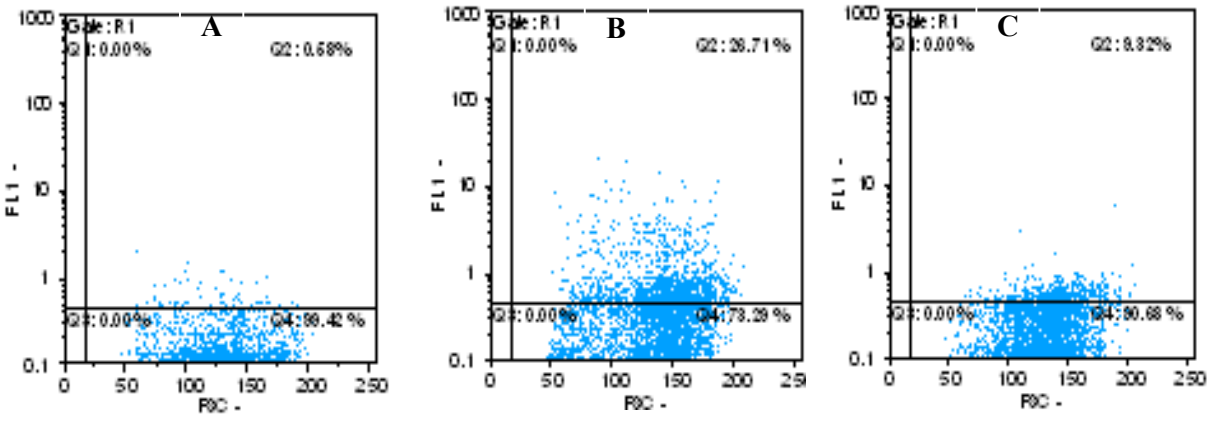

Fig. 2. A flow cytometric assay to determine the percentage of GFP-positive cells in K562 cell line. **(A)** non-transfected negative control cell; **(B)** transfected cell by electroporation; **(C)** transfected cell by Lipofectamine™ LTX kit.

Table 4. The mRNA levels of $^{A}\gamma$-globin gene in different constructs transfected to K562 cells, compared to mock plasmid.

Constructs	Avg. $^{A}\gamma$ C_T	Avg.β-actin C_T	ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta CT}$
Transfection by electroporation					
pCDNA3,1 (Mock plasmid)	20.8	21.1	-0.3	0.00 ± 0.12	1.0 (0.9- 1.1)
pCDNA3.1 μLCR$^{A}\gamma$-588(A)β	22.0	24.5	-2.5	-2.20 ± 1.60	4.6 (1.5-13.9)
pCDNA3.1μLCR$^{A}\gamma$-588(G)β	23.4	25.8	-2.4	-2.10 ± 0.40	4.3 (3.2-5.6)
pCDNA3.1μLCR$^{A}\gamma$-175(T>C)β	20.5	25.3	-4.8	-4.50 ± 2.00	22.6 (6.9-111.4)
Transfection by lipofectamine™ LTX kit					
pCDNA3,1 (Mock plasmid)	24.4	25.4	-1.0	0.00 ± 0.80	1.0 (0.2-1.8)
pCDNA3.1 μLCR$^{A}\gamma$-588(A)β	24.3	26.5	-2.2	-1.20 ± 1.00	2.3 (1.3-4.6)
pCDNA3.1μLCR$^{A}\gamma$-588(G)β	24.0	26.0	-2.0	-1.00 ± 0.40	2.0 (1.5-2.6)
pCDNA3.1μLCR$^{A}\gamma$-175(T>C)β	20.8	25.0	-4.2	-3.20 ± 2.20	9.2 (2-42.2)

Expression studies in K562 cells. The K562 cells were transfected with the created constructs by determined Lipofectamine™ LTX kit transfection conditions and electroporated with 300 V and 950 μF in triplicate form. All transfections were performed at least in triplicate and repeated several times. To determine the relative amount of the $^{A}\gamma$ transcripts in transfected K562 cells, qRT-PCR assay was employed using synthesized cDNA from the total RNA isolated from the transfected K562 cells. The data of transient expression experiments are summarized in Table 4. In comparison to the Mock plasmid (pCDNA3.1), the -175 (T>C) mutation increased the expression of the $^{A}\gamma$-globin gene by 9.2 and 22.6 folds based on Lipofectamine LTX kit and electroporation, respectively. The expression of $^{A}\gamma$-globin gene in two other constructs, including A and G allele at -588 were (4.6 and 2.3) and (4.3 and 2.0) times, respectively. The variation of expression results might be related to transfection methods. Nevertheless, the expression level of constructs containing A allele was 1.15 fold higher than constructs with G allele. However, the difference between the expression levels was not significant.

DISCUSSION

In order to determine the effect of any mutations in the regulatory regions of the $^{A}\gamma$-globin gene, the human β-globin gene has to have a normal expression. This depends on many types of regulatory elements residing within the β-globin cluster including the LCR, globin enhancer elements, the individual globin gene promoter and upstream regions. In addition, the promoter of β-globin gene itself may compete with the γ-globin gene for interaction with the LCR. The LCR also interacts with the minimal γ-gene promoters and sequences of the upstream promoter [2, 5, 13,17]. The linking of the LCR sequences to the γ-globin gene in the expression constructs is critical to detect the impact of any mutations in regulatory elements of the $^{A}\gamma$-globin gene expression [18]. It has been also presumed that regulation occurs through physical interactions between factors interacting with these elements, which are located at considerable distances from each other [2, 5, 13,17].

In the present study, we have developed a functional assay to compare the possible effect of Aγ -588 (A/G) mutations on its gene expression in the K562 cell line. The constructs elements were designed in a way to mimic their *in vivo* transcriptional orientations except the distance between different elements in the construct. The construct included the following elements: the μLCR, $^{A}\gamma$-globin gene with mutant and normal promoters and a normal β-globin gene. For the functional study, the rate of transfection in K562 cells was determined by analysis GFP expression using flow cytometry. We found that the yield of transfection was very low, imposing important limitations on functional studies. To overcome these limitations, we optimized and compared different conditions for the transfection and electroporation assays (Table 2). The highest mean rate of transfection by Lipofectamine™ LTX kit and electroporation was achieved by 8.74 ± 0.45 and 26.13 ± 2.8 in GFP- positive cells, respectively. The other studies, reported different rate of electroporation, achieved transfection efficiency of about 84.4% and 40% [19, 20]. This rate of transfection is higher than our yield (26.13 ± 2.8), but cell viability in the present study is higher than that previously reported [19, 20]. These main

differences may be due to the use of DMEM as an electroporation buffer in the electroporation assay.

We have previously shown that, the presence of A allele at -588 was highly frequent and closely associated with HbF elevation in β-TI patients, especially those who had IVSII-1/IVSII-1 genotype [3]. Therefore, we aimed to determine the effect of -588 (A/G) mutations in the $^{A}\gamma$-globin gene expression in the K562 cell line. It has been reported that the $^{A}\gamma$ -175 T>C mutation increases the $^{A}\gamma$-globin expression and hence, causing nd-HPFH [18]. Thus, we applied the -175 T>C site-directed mutation in the $^{A}\gamma$-globin promoter in order to determine the ability of our construct model in up-regulating the $^{A}\gamma$-globin gene *in vitro* system. In the most transient expression assays up-regulation of the -175 (T>C) mutation in *in vitro* system could result in a maximum 20-fold increase in γ-globin promoter strength by reporter gene assay [21-24]. In *in vivo* assay, the -175 mutation also leads to 50 to 100 fold up-regulation in γ-gene in adult erythroid cells [22, 24, 25].

Our data showed that the construct containing the -175 T>C mutation (pCDNA3.1μLCR$^{A}\gamma^{-175(C)}$ β) is able to increase the expression up to 9.2 to 22.6 folds, compared to the Mock plasmid (Table 4). This increase is greater than previously reported *in vitro*. In the pCDNA3.1μLCR$^{A}\gamma^{-588(A)}$ β and pCDNA3.1μLCR$^{A}\gamma^{-588(G)}$ β constructs, the expression of $^{A}\gamma$-globin gene were (4.6-2.3) and (4.3-2.0) times, respectively when compared to the mock plasmid. However, there was not a significant difference between the expression of $^{A}\gamma$-globin gene between the construct containing A and the one with G allele at -588. Our result is close to the previously published data indicating that the $^{A}\gamma$-588 A>G had no effect on $^{A}\gamma$-globin expression [26]. In addition, transient expression assays showed 2.6 fold decrease in chloramphenicol acetyl transferase protein levels, due to the presence of the $^{A}\gamma$-588 A>G mutation, compared with the normal $^{A}\gamma$ promoter [11].

In conclusion, our results indicate that -588 A allele does not play a major role in overexpression of $^{A}\gamma$-globin gene, suggesting that other factors are involved. Therefore, this mutation may not relate to increased promoter function of $^{A\gamma}$-globin gene. However, it is worth mentioning that the up-regulation of $^{A}\gamma$-globin gene might be dependent on DNA structure modifications, which is not occurred in this *in vitro* system.

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