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Original Research

The relationship between the variations in Gγ and Aγ promotors and the Hereditary Persistence of Fetal Hemoglobin (HPFH)

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Abstract: In the present study, sixty-two samples that have 1.5% and upper level of fetal hemoglobin (HbF), were examined to investigate the relationship between HbF level and non-deletional mutations in both $^{G}\gamma$ (G gamma) globin (HBG2) and $^{A}\gamma$ (A gamma) globin (HBG1) genes. Four variations were observed in the promotor of $^{G}\gamma$ gene, which are -158C/T, -309A/G, -369C/G, and -567T/G. Also, four variations were observed in the 5'-UTR (untranslated regions) and promotor of $^{A}\gamma$ gene, which are +25G/A, -369G/C, -499T/A, and -588G/A. One -222/-225 AGCA del homozygous and six variations as heterozygous in A gamma globin gene promotor region were also observed. The results of the current study suggested that there was a significant relationship between high HbF levels and two variations (-309A/T and -369C/G) in $^{G}\gamma$ gene promotor. Additionally, a significant relationship between two variations (+25G/A and -499T/A) in $^{A}\gamma$ gene promotor was also observed. Furthermore, the persons who carry these variations with high levels of HbF indicated that there might be a haplotype effect between these variations.

Key words: Hemoglobin; A gamma; G gamma; Hereditary fetal hemoglobin; Gγ and Aγ promotors.

Introduction

Hemoglobin (Hb) is a quaternary structure, which is formed from two alpha and two beta chains ($\alpha_2\beta_2$) and contains four heme groups (1-3). Healthy adults have $\alpha_2\beta_2$ structure with a hemoglobin molecule that is called HbA. On the other hand, the hemoglobin formed from two alpha and two gamma ($\alpha_2\gamma_2$) chains is called HbF (4). The amount of HbF is reduced during the first two years of life after birth while the amount of HbA is increased. In healthy adults, the quantity of HbF and the quantity of HbA are estimated to be 1% and 97%, respectively (2).

Hemoglobinopathy is a genetic disorder that leads to a change in the structure of one of the globin chains of the hemoglobin molecule (5). The hemoglobinopathies were characterized by amino acid changes in polypeptide structure or reduction in the synthesis of globin chains. The reduction in the synthesis of the globin chains or the absence of globin proteins causes to thalassemia while the amino acid changes in polypeptide structure causes to abnormal Hb and Hb variants (6,7). Hereditary persistence of fetal hemoglobin (HPFH) is generally inherited to the children when the HbF levels in the adults is increased due to continued γ-globin gene expression after birth. Hereditary persistence of fetal hemoglobin is divided mainly into two classes as deletional and non-deletional HPFH. The deletional HPFH is related to the deletion of $^{G}\gamma$ and $^{A}\gamma$ genes in β globin gene cluster while non-deletional HPFH is characterized with a base substitution or minor deletions in promoters of $^{G}\gamma$ and $^{A}\gamma$ genes. So, these variations affect the binding efficiency of transcription factors (8).

Until today, seventy-nine different variants in $^{G}\gamma$ and $^{A}\gamma$ genes of β-globin gene cluster were identified to be associated with HPFH phenotype. Forty-five of these belong to $^{G}\gamma$ gene, thirty-one belong to $^{A}\gamma$ gene, and the remained three are still un-identified. Seven of the thirty-one $^{A}\gamma$ variants were $^{A}\gamma^{T}$ chain variants, one was a minor homologue deletion, and another one had $^{A}\gamma$-β hybrid chain mutation (9,10). All of the γ variants caused light health problem only, which is mild cyanosis in babies. Incidence in most of the detected γ variants was quite low, although HbF-Sardinia ($^{A}\gamma^{T}$75Ile→Thr) was identified in all societies. Some of these identified variants were HbF-Poole ($^{G\gamma}$130Trp→Gly), HbF Xim Jin ($^{G}\gamma$130Gly→Arg) (unstable), and HbF Onoda ($^{G}\gamma$146His→Tyr) that has high oxygen affinity (5,6,11). None of the thirty-one variants identified in the $^{A}\gamma$ gene had abnormal function and these variants were rare as in the $^{G}\gamma$ gene (6,11).

The substitutions in $^{G}\gamma$-globin gene localized at -202, -175, -161, and -158; the substitutions of $^{A}\gamma$-globin gene localized at -202, -198, -196, -195, -175, -117; and the 4-bp deletion of $^{A}\gamma$-globin gene (-225/-222 AGCA del) were responsible for the permanent expression of the gene. These variations are rarely observed and are associated with an increased HbF value as heterozygous in normal individuals. C→T substitution at -158 position of $^{G}\gamma$-globin gene (called Xmn I-$^{G}\gamma$ polymorphism) was

Table 1. The primers used for amplification of Gγ and Aγ gene promotors.

Gene	Primers	Sequences (5'-3')	PCR product (bp)
G-GAMMA	5-GGl F	5'-AACTGTTGCTTTATAGGATTTTTCA-3'	666
	3-GGl R	5'-GTCTGGACTAGGAGCTTATTGAT-3'	
A-GAMMA	5-AGl F	5'-TCTATTACTGCGCTGAAACTGTG-3'	680
	3-AGl R	5'-GTCTGGACTAGGAGCTTATTGAT-3'	

observed commonly as a polymorphic variant. It also increases the γ-globin gene expression under erythropoietic stress such as β-thalassemia or sickle-cell anemia (12,13).

In Turkey, especially in the Mediterranean region, the β-thalassemia and sickle-cell anemia were often observed; however, the individuals who have high HbF levels are less affected from the disease. The number of studies are rare on the effects of high HbF levels in our country although the prevalence of the thalassemia or sickle-cell anemia are high. Determination of the cause of the increase in amount of HbF value could be contributed to better understand the role of transcription factors in the future, because the high HbF level alleviates the course of the disease in abnormal hemoglobin and β-thalassemia patients. Therefore, in this study, we aimed to investigate the relationship between the non-deletional variations of the volunteers who have 1.5% and upper level of fetal hemoglobin (HbF) in Adiyaman province of Turkey.

Materials and Methods

The ethical approval was obtained from Human Ethics Committee (approval date and number: 16.09.2014/06) of Medical Faculty of Firat University, Elazığ, Turkey. The study was conducted in agreement with the statement on the Declaration of Helsinki.

Complete blood count (CBC), hemoglobin levels, and HbF level were determined using Coulter Counter (Coulter AC.T 5diff AL) and β-thal HPLC systems (Bio-Rad 2 Variant-II Hercules, CA) from the blood samples that were previously collected in EDTA tubes from volunteers. Only the samples with 1.5% and higher level of HbF were included to the study since the amount of HbF is reduced to under %1.5 during the first two years of life after birth (2,3).

DNA isolation was performed using Axygen DNA isolation kit (Axygen Biosciences, CA, USA) as described by manufacturer protocol. The primers, which were used for amplification of the promotors of $^{G}\gamma$ and $^{A}\gamma$ genes for determination of non-deletional gene mutations, are listed in Table 1. The PCR products (amplicons) were 666 bp and 680 bp for $^{G}\gamma$ and $^{A}\gamma$ promotors, respectively. The amplicons were sequenced using Sanger DNA sequencing protocol to detect any non-deletional variation in promotors of both $^{G}\gamma$ and $^{A}\gamma$ genes.

The content of PCR reaction mixture and PCR protocols are presented in Table 2 and Table 3, respectively.

Table 2. Content of PCR mixture.

10 × Buffer ($(NH_4)_2SO_4$)	2.5 µL
25 mM MgCl2	2.5 µL
2 mM dNTPs	2.5 µL
Taq polimerase	0.5 µL
5' Forward primer	1 µL
3' Reverse primer	1 µL
Genomic DNA	5 µL
Sterile distilled water	10 µL
Total volume	25 µL

The genetic variations obtained from this study were compared using GeneBank records for both $^{G}\gamma$ and $^{A}\gamma$ promotors by using BioEdit (BioEdit Sequence Aligment Editor) and nucleotide blast of NCBI GenBank database (GenBank: GU324926 and GU324925) (https://www.ncbi.nlm.nih.gov/genbank/). A statistical package (SPSS, Inc., Chicago, IL, USA, version 16.0) was used for statistical analysis to compare the mean of HbF values and the gene variations.

Results

In total 666 bp was sequenced and four variations were found, which are localized at -158C/T, -309A/G, -369C/G and -567T/G in the promotors of the $^{G}\gamma$ gene (Table 4 and Table 5).

In the present study, four types of substitution variations were observed at different localizations in $^{G}\gamma$ gene promotors. There were significant differences between HbF levels and substitutions at -309A/G and -369C/G localizations. On the other hand, the G/G homozygous genotype was significantly induced the HbF level. Three of four substitutions which have been described in previous studies (-158C/T, -309A/G, -369C/G) were also found in this study; however, the fourth substitution (-567T/G) was observed for the first time (Figure 1). A T/G substitution was observed with an individual while no G/G homozygous was observed at -567 location.

According to the dominant genetic model, HbF value was increased by -309A/G and -369C/G substitutions while HbF was reduced by -158C/T substitution (Table 6). Due to insufficient data the relation with HbF and variation at -567T/G was not analyzed.

Totally 680 bp was sequenced in the promotors of the Aγ gene. Of these sequences, four types of substi-

Table 3. PCR protocol.

Operation	Temperature (°C) for $^{G}\gamma$	Temperature (°C) for $^{A}\gamma$	Time (min.)	Number of Cycles
Initial denaturation	95	95	5	1
Denaturation	95	95	1	30
Annealing	57	63	1	
Extension	72	72	2	
Final extension	72	72	10	1

Table 4. The sequenced $^{G}\gamma$ gene promotor and variations which were found in previous studies and in the current study.

ctgaaactgt tgctttatag gatttttcac			tacactaatg	agaacttaag	aga**t**aatggc
Forward Primer			-590	-580	-570
					**
ctaaaaccac	agagagtata	ttcaaagata	agtatagcac	ttcttatttg	gaaac**c**aatg
-560	-550	-540	-530	-520	-510
					*
cttactaaat	gagactaaga	cgtgtcccat	caaaaatcct	ggacctatgc	ctaaaacaca
-500	-490	-480	-470	-460	-450
tttcacaatc	cctgaacttt	tcaaaaattg	gtacatgctt	taa**ctttaa**a	ctacaggcct
-440	-430	-420	-410	-400	-390
				*	
ca**c**tggagct	a**c**agacaaga	aggtgaaaaa	c**g**gctgacaa	aagaagtcct	ggtatcttct
-380	-370	-360	-350	-340	-330
*	*/**		*		
atggtgggag	a**aga**aaacta	gctaaaggga	agaataaatt	agagaaaaat	tggaatgact
-320	-310	-300	-290	-280	-270
	* *				
gaatc**g**gaac	aaggcaaagg	ctataaaaaa	aattaagcag	cagtatcctc	ttgggggccc
-260	-250	-240	-230	-220	-210
*					
cttccccaca	ctatctcaat	gcaaatatct	gtctgaaacg	gt**c**cctggct	aaactccacc
-200	-190	-180	-170	-160	-150
				*/**	
catgggttgg	ccagccttgc	cttga**ccaat**	agccttgaca	aggcaaactt	ga**ccaat**agt
-140	-130	-120	-110	-100	-90
		***			***
cttagagtat	ccagtgaggc	ca**ggggccgg**	cggctggcta	gggatgaaga	**ataaa**aggaa
-80	-70	-60	-50	-40	-30
		***			***
gcac**c**cttca	gcagttcca**c**	**acactcgctt**	ctggaacgtc	tgaggttatc	aataag**ctcc**
-20	-10 **-1**	**+1 Transcription initiating (5'-UTR)**			**Reverse**
*					
tagtccagac gcc ***ATG*** *GGT CAT TTC ACA GAG GAG GAC AAG GCT ACT ATC ACA*					
Primer	M G H F T E E D K A T I T				
	Exon 1 Amino acids				

*The variations previously determined (GenBank, GU324926.1). **The variations was found in the current study. ***Promotor regions (Langdon and Kaufman, 1998): -26/-30 TATA box, -51/-58 GC box, -84/-88 CAAT box, -111/-115 CAAT box.

Table 5. The statistical significance between HbF value and variations of the $^{G}\gamma$ gene promotor.

Location	Genetic Variation	N	HbF mean	Standard deviation	Standard error	Min HbF	MaxHbF	F	Sig. P
	CC	27	2.2074	1.1358	0.2185	1.50	7.10		
	CT	29	2.0241	0.5054	0.0939	1.50	3.80		
-158 C/T								0.637	0.53
	TT	6	1.8333	0.3669	0.1498	1.50	2.50		
	Total	62	2.0855	0.8319	0.1057	1.50	7.10		
	AA	60	1.9733	0.4762	0.0615	1.50	3.50		
	AG	1	3.8000	-	-	3.80	3.80		
-309 A/G								63.59	0.00
	GG	1	7.1000	-	-	7.10	7.10		
	Total	62	2.0855	0.8319	0.1057	1.50	7.10		
	CC	60	1.9733	0.4762	0.0615	1.50	3.50		
	CG	1	3.8000	-	-	3.80	3.80		
-369 C/G								63.59	0.00
	GG	1	7.1000	-	-	7.10	7.10		
	Total	62	2.0855	0.8319	0.1057	1.50	7.10		
	TT	61	2.0951	0.8354	0.1070	1.50	7.10		
-567 T/G	TG	1	1.5000	-	-	1.50	1.50	0.499	0.48
	Total	62	2.0855	0.8319	0.1057	1.50	7.10		

Table 6. Statistical significance between HbF value and variations of the $^{G}\gamma$ gene promotor using dominant genetic model.

Locations and normal Genotype	Genetic Variations	N	HbF mean	Standard deviation	Standard error	Levene's Test for Equality of Variances	
						F	Significance P
-158 CC	CC	27	2.2074	1.13508	0.21845	4.654	0.035
	CT+TT	35	1.9914	0.48530	0.08203		
-309 AA	AA	60	1.9733	0.47617	0.06147	37.439	0.000
	AG+GG	2	5.4500	2.33345	1.65000		
-369 CC	CC	60	1.9733	0.47617	0.06147	37.439	0.000
	CG+GG	2	5.4500	2.33345	1.65000		
-567 TT	TT	61	2.0951	0.83535	0.10696		
	TG	1	1.5000	-	-		

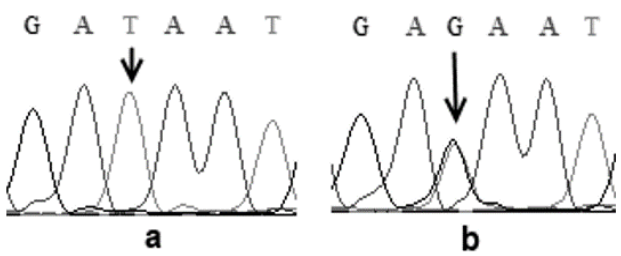

Figure 1. A normal homozygous T/T (a) and a heterozygous T/G (b) variations at -567 localization for $^{G}\gamma$ gene promotor.

tution variations were found, which are localized at +25G/A, -369G/C, -499T/T, and -588G/A. Additionally, a minor AGCA deletion was determined between -222/-225 location (Table 7, 8). A person with AGCA homozygous deletion and six persons with the heterozygous variations were observed (Figure 2). None of the Aγ gene promotor variations was increased the HbF value, except -499T/A variation, which was significantly increased the HbF value in TA heterozygous and AA homozygous genotypes (Table 8). Also, no relationship was found between HbF value and AGCA deletional variation of the Aγ gene promotor.

According to the dominant genetic model results, HbF levels were increased at +25G/A and -499T/A substitutions in promotor of $^{A}\gamma$ gene. However, there was no significant relationship between the HbF value and the variations at -369G/C and -588G/A localizations (Table 9). In addition, there was no significant difference between HbF value and the normal variation and homozygote/heterozygote deletional variations of $^{A}\gamma$ gene.

In this study, the HbF value was measured as 7.1% for only one person who was identified to be five homozygous variations carrier. Of these variations, two were in $^{G}\gamma$ gene (-309G/G, -369G/G) and three in $^{A}\gamma$ gene (+25G/G, -588G/G, -499A/A). On the other hand, the second highest HbF value that measured in this study was 3.8%. It was detected in only one person who has six variations as homozygous or heterozygous in $^{G}\gamma$ and $^{A}\gamma$ genes ($^{G}\gamma$: -158C/T, -309A/G, -369C/G; $^{A}\gamma$: +25G/G, -588G/A, -499T/A).

Discussion

In this study, the promotors of both $^{G}\gamma$ (G gamma) globin (HBG2) and $^{A}\gamma$ (A gamma) globin (HBG1) genes of 62 volunteers who have 1.5% and higher level of HbF were sequenced in order to determine the relationship between HbF level and non-deletional mutations. Four variations in the promotor of $^{G}\gamma$ gene (-158C/T,

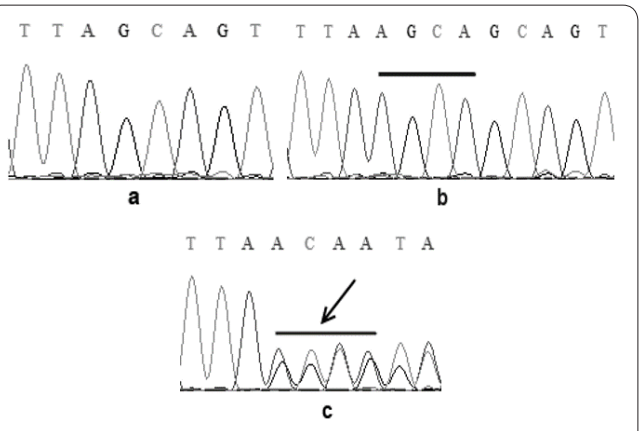

Figure 2. AGCA deletion at -222/-225 location of $^{A}\gamma$ gene. a) Homozygous AGCA deletion; b) Normal; and c) Heterozygous AGCA deletion.

-309A/G, -369C/G, and -567T/G); and four variations in 5'-UTR (untranslated regions) and promotor of $^{A}\gamma$ gene (+25G/A, -369G/C, -499T/A, and -588G/A) were observed. Additionally, an AGCA deletion at -222/-225 location in promotor region of A gamma globin gene was observed. According to the obtained results, there was a significant relationship between high HbF level and two variations in $^{G}\gamma$ gene promotor (-309A/T and -369C/G) as well as between two variations in $^{A}\gamma$ gene promotor (+25G/A and -499T/A). In this case, the persons who carry the mentioned variations with the presence of high levels of HbF indicate that there might be a haplotypic effect among these variations.

Both HBG2 and HBG1 genes transcribe gamma globin mRNA to create the fetal hemoglobin during fetal period of gestation. There is only an amino acid difference between $^{G}\gamma$ and $^{A}\gamma$ globin genes. This difference located at codon 136, while $^{G}\gamma$ has glycine at this location, has alanine (14). The variations that were defined in this study have been identified previously as described in GenBank database (GU324926 and GU324925), except the -567T/G variation in $^{G}\gamma$ globin gene which has been observed and defined for the first time by this study.

Sequences that have effectiveness to initiate the transcription at 5'-promoter regions of both $^{G}\gamma$ and $^{A}\gamma$ globin genes were defined according to the sequencing results (15,16). The identified possible promotor variants for both genes localized at -202 ($^{G}\gamma$ and $^{A}\gamma$), -198 ($^{A}\gamma$), -196 ($^{A}\gamma$),-175 ($^{G}\gamma$ and $^{A}\gamma$), -161($^{G}\gamma$), -158($^{G}\gamma$), -117 ($^{A}\gamma$), and -114 ($^{G}\gamma$ and $^{A}\gamma$) regions. Forget (1998) identified five regions localized between -114 and -202 for $^{G}\gamma$ gene and six regions for $^{A}\gamma$ gene promotors which led to over expression. Langdon and Kaufman (17) reported that cis acting sequences at promotors of

Table 7. The sequenced Aγ gene promotor and variations which were found in previous studies and in the current study.

attaggtctt	atattatgta	acactaatct	a**ttactgcgc** **tgaaactgtg** **g**ctttataga		
			Forward Primer		
aattgttttc	act**g**cactat	tgagaaatta	agagataatg	gcaaaagtca	caaagagtat
-601	-591	-581	-571	-561	-551
	*/**				
attcaaaaag	aagtatagca	ctttttcctt	agaaaccact	gc**t**aactgaa	agagact**a**ag
-541	-531	-521	-511	-501	-491
				*/**	*
atttgtcccg	tcaaaaatcc	tggacctatg	cctaaaacac	atttcacaat	ccctgaactt
-481	-471	-461	-451	-441	-431
ttcaaaaatt	ggtacatgct	tta**g**ctttaa	actacaggcc	tcactggagc	ta**g**agacaag
-421	-411	-401	-391	-381	-371
					*/**
aaggt**a**aaaa	acggctgaca	aaagaagtcc	tggtatc**c**tc	tatg**a**tggga	ga**a**gg**g**aaact
-361	-351	-341	-331	-321	-311
*			*	*	* *
agctaaaggg	aagaataaat	tagagaaaaa	**c**tggaatgac	tgaatcggaa	caaggcaaag
-301	-291	-281	-271	-261	-251
			*		
gctataaaaa	aaatta**agca**	gcagtatcct	cttgggggcc	ccttccccac	actatctcaa
-241	-231	-221	-211	-201	-191
	*/**				
tgcaaatatc	tgtctgaaac	ggtccctggc	taaactccac	ccatgggttg	gccagccttg
-181	-171	-161	-151	-141	-131
ccttga**ccaa**	t**a**gccttgac	aaggcaaact	tga**ccaat**ag	tcttagagta	tccagtgagg
-121	-111	-101	-91	-81	-71
***			***		
cca**ggggccg**	**g**cggctggct	agggatgaag	a**ataaa**agga	agcac**c**cttc	agcagttcca
-61	-51	-41	-31	-21	-11
***			***	*	
c **acactcgct**	tctggaacgt	ctgag**g**ttat	caataag**ctc**	**ctagtccaga**	**cgcc**
-1 +1 Transcription initiating (5'-UTR)			**Reverse Primer**		
ATG *GGT CAT TTC ACA GAG GAG GAC AAG GCT ACT ATC ACA AGC CTG*					
M G H F T E E D K A T I T S L					
Exon 1 Amino acids					

*The variations previously determined (GenBank, GU324925). **The variations was found in our study. ***Promotor regions (Langdon and Kaufman, 1998): -26/-30 TATA box, -51/-58 GC box, -84/-88 CAAT box, -111/-115 CAAT box.

Table 8. The statistical significance between HbF value and variations of the $^{A}\gamma$ gene promotor.

Location	Genetic variations	N	HbF mean	Standard deviation	Standard error	Min HbF	Max HbF	F	Sig. P
+25 G/A*	GG	25	2.2560	1.20142	0.24028	1.50	7.10	1.327	0.275
	GA	19	1.9421	0.32714	0.07505	1.50	2.60		
	AA	7	1.6833	0.17224	0.07032	1.50	2.00		
	Total	51	2.0680	0.88972	0.12583	1.50	7.10		
-369 G/C	GG	57	2.0702	0.86168	0.11413	1.50	3.80	0.528	0.470
	GC	5	2.2600	0.34351	0.15362	1.80	2.60		
	Total	62	2.1651	0.60259	0.13387	1.80	3.80		
-499 T/A	TT	58	1.9776	0.47942	0.06295	1.50	3.50	9.761	,000
	TA	1	3.8000	-	-	3.80	3.80		
	AA	3	3.6000	3.05123	1.76163	1.50	7.10		
	Total	62	2.0855	0.83191	0.10565	1.50	7.10		
-588 G/A*	GG	23	2.1261	1.17598	0.24521	1.50	7.10	0.498	0.611
	GA	23	2.0696	0.50492	0.10528	1.50	3.80		
	AA	5	1.7000	0.18708	0.08367	1.50	2.00		
	Total	51	2.0588	0.85934	0.12033	1.50	7.10		
-222/-225 AGCA del	Normal	55	2.0982	0.86634	0.11682	1.50	7.10	0.055	0.946
	Del homozyg.	1	2.0000	-	-	2.00	2.00		
	Del heterozyg.	6	1.9833	0.56716	0.23154	1.50	2.80		
	Total	62	2.0855	0.83191	0.10565	1.50	7.10		

*Totally 51 DNA specimens were sequenced.

both $^{G}\gamma$ and $^{A}\gamma$ might play a role in the regulation. These researchers also stated that the regulating sequences which are -30ATAAA, -50GGGGCCGG, -85 CCAAT, -112CCAAT, -145CACCC, -170GATA, -190GATA, -180ATGCAAAT, and -200CCCGGG for $^{G}\gamma$ gene. In this study, the cis acting sequences also described as

Table 9. Statistical significance between HbF value and variations of the $^{A}\gamma$ gene promotor using dominant genetic model.

Location and normal genotype	Genetic variations	N	HbF mean	Standard deviation	Standard error	Levene's Test for equality of variances	
						F	Significance P
+25 GG	GG	25	2.2560	1.20142	0.24028	7.809	0.007
	AA+GA	26	1.8800	0.31491	0.06298		
-369 GG	GG	57	2.0702	0.86168	0.11413	0.528	0.470
	GC	5	2.2600	0.34351	0.15362		
-499 TT	TT	58	1.9776	0.47942	0.06295	42.724	0.000
	TA+AA	4	3.6500	2.49332	1.24666		
-588 GG	GG	23	2.1261	1.17598	0.24521	2.366	0.130
	AA+GA	28	2.0036	0.48342	0.09136		
-222/-225 AGCA deletion	Normal	55	2.0982	0.86634	0.11682	0.146	0.704
	Deletional	7	1.9857	0.51778	0.19570		

shown in Table 4 and Table 7 for both $^{G}\gamma$ and $^{A}\gamma$ genes, respectively. Langdon and Kaufman (17) have also reported that the transcription factors interacted with 9 of these regions to affect the transcription rate; however, they noted that there were a TATA box and two CAAT boxes (-30TATA, -85CAAT, and -112 CAAT) localized at basic promotor regions. In the current study, since individuals with high levels of HbF were taken into account no variations have been determined in these regions. A possible variation in the basic promotor regions often reduces the level of genetic expression.

Cui et al. (18) reported that they had detected -225/-222 AGCA deletions in the Aγ gene of a person. Likewise, in the current study, a genotype with homozygous AGCA deletion was detected in the -222/-225 region. The HbF level of this person was measured as 2.0%. Moreover, six individuals with the same mutation in heterozygous state were also found. The mean of their HbF levels was measured as 1.98%. These values are considered as high values for adult individuals, but no statistically significant difference was found between the HbF levels of normal persons who doesn't have mutations. Bouva et al. (13) reported that 4 bp deletions in $^{A}\gamma$-globin gene (AGCA deletion from $^{A}\gamma$ -225 to -222) are responsible for continuous expression of the gene. This is an indication that the identified deletion-type variation is compatible with high HbF values of seven people, in the present study. However, Manca and Masala (10) determined that this type of mutation causes the synthesis of the $^{A}\gamma^{T}$ globin chain variant that reduces the expression level of the mutation by 20%. Therefore, a decrease occurs in the amount of HbF. As it can be seen, different results were obtained among different studies which were done on high HbF levels of the same type variation. These different outcomes may actually be explained by the presence or absence of other variations in the same individuals, which means it is due to the fact that other variations are haplotypically effective (19,20).

Bouva et al. (13) were reported a presence of basal exchange in the $^{G}\gamma$ globin gene promotor ($^{G}\gamma$ −37A→ T) in a Dutch patient, who has 2.3% HbF value. They also reported that this change might be effective in moderate increase of HbF levels. Additionally, the authors determined that the apparent point mutations in the nucleotides -202, -175, -161, and -158 of the $^{G}\gamma$-globin gene, point mutations at -202, -198, -196, -195, -175, and -117 of the $^{A}\gamma$-globin, and 4-bp deletions (AGGA deletion $^{A}\gamma$ from -222 to -225) were responsible for the sustained expression of the gene. The same investigators reported that especially the C → T shift at -158th position of the $^{G}\gamma$-globin gene was a polymorphic variant frequently observed in all populations and increased γ-globin gene expression under erythropoietic stress conditions in health problems such as sickle cell anemia and homozygous β-thalassemia. Other researchers have associated the displacement-type variation at -158th position with high level of HbF (21,22). However, Motum et al. (19) found that the $^{G}\gamma$ -158C/T variant had no association with HbF as a result of studies performed in in vitro, which were done in combination with other types of variants (-198T/C ve -175T/C). Likewise, Tasiopoulou et al. (20) were reported the same results. These investigators determined that two individuals with high HbF levels had the -158C/T type variation, but after further analyzes they reported two other variants (-196C/T and -201C/T) in the same individuals. Therefore, Tasiopoulou et al. (20) have concluded that high level of HbF was related to such variations. In this study, we conclude that $^{G}\gamma$ -158 type variation has no effect on HbF level. This can be explained by the absence of other types of variations mentioned above in individuals with $^{G}\gamma$ -158 type variation. Studies of Motum et al. (19) and Tasiopoulou et al. (20) agree with the current study and supports our conclusion in terms of the effect of $^{G}\gamma$ -158 type variation on HbF level.

In the present study, the two individuals with the highest HbF values were found to have variations in more than one location ($^{G}\gamma$: -309G/G, -369G/G; $^{A}\gamma$: +25G/G, -588G/G, -499A/A). This result indicate to an interactive haplotypic effect of some variations on each other because some of these variations do not increase the level of HbF when it is alone. They appear to increase the level of HbF when they are present together in the same individual. It is important to note that two rare donors, who have both $^{G}\gamma$ -309 and -369 location variations and $^{A}\gamma$ -499 location variations, showing the highest HbF value are confirming the haplotypic effect. It is thought that the $^{G}\gamma$ -309 and -369 location variations effectively contribute to the high HbF level. More importantly, at both of these locations, the HbF value reached to a maximum level when carrying GG type homozygous variation. Since the $^{A}\gamma$ -499 locus variant is TA or AA homozygous type, it only increases the effect of the two variations of the $^{G}\gamma$ gene on HbF. In the two individuals with homozygous AA at $^{A}\gamma$ -499 locus, HbF values did not increase at very high levels due to the absence of variations in the $^{G}\gamma$ -309 and -369 locations.

To date, studies have shown that there are a number of variations in the promotor regions of both $^{G}\gamma$ -globin and $^{A}\gamma$ -globin genes, some of these variations have already been recorded in the GenBank database.

From some of the similar studies, it has been understood that some variations increase the HbF level, while some other studies concluded that these variations can not be related to the HbF level. In this study, many variations were found in the promoter sequences of both genes. Some variations of both $^{G}\gamma$ and $^{A}\gamma$ gene promotors may have not been previously recorded in GenBank database. One of them was identified for the first time in this study. The -567T/G variation was detected for the first time in the present research. Additionally, we also investigated the relationship between HbF and the -309A/T and -369C/G variants of the $^{G}\gamma$ gene and the +25G/A and -499T/A variants of the $^{A}\gamma$ gene for the first time in the current study. Eventually, according to the obtained results it was concluded that these variations are associated with high HbF levels.

In this study, the promotor sequences of both $^{G}\gamma$ and $^{A}\gamma$ genes were sequenced and the relationship between the high HbF value and the variations was studied. In the promotor region of the $^{G}\gamma$ gene, base-change type variations were detected at localizations of -158C/T, -309A/G, -369C/G, and -567T/G. Similarly, base change type variations were found in the 5'-UTR and in the promotor regions of the $^{A}\gamma$ gene at +25G/A, -369G/C, -499T/A, and -588G/A. Only one person represented AGCA deletion on homozygous -228/-225 region variation while six people represented AGCA deletion on heterozygous -222/-225 region variations.

It was determined that the HbF values were statistically higher in the individuals with the -309A/T and -369C/G type variants of the $^{G}\gamma$ gene. The dominant genetic model was also revealed the same result, which referred that the HbF values in these two variants were high, too. Additionally, it was determined that there was a significant increase in HbF levels in the presence of -499T/A variation in the promotor sequences of the $^{A}\gamma$ gene, and the HbF amount increased in both +25G/A and -499T/A variations in the dominant model. Thus, it can be concluded that there was an association between the high levels of HbF and the two variations in the promotor of $^{G}\gamma$ gene and the two variations in the $^{A}\gamma$ gene promotor.

The -567T/G variation, which was detected in the promotor region of the $^{G}\gamma$ gene, has been determined for the first time in the current research. The HbF value of this individual was estimated to be 1.5%. Moreover, the present study is the first study that have been done on determining the relationship between HbF levels and the previously identified type -309A/T and -369C/G variants of the $^{G}\gamma$ gene and of the type +25G/A and -499T/A variants of the $^{A}\gamma$ gene. The presence of variants ($^{G}\gamma$: -309G / G, -369G / G; $^{A}\gamma$: +25 G / G, -588 G / G, -499 A / A) at multiple locations in individuals with high HbF values can be concluded as these variations increase the level of HbF by haplotypic effect.

As a result, it can be said that there are many mechanisms in regulating the expression of $^{G}\gamma$ and $^{A}\gamma$ genes. Among these mechanisms, two could be primarily taken into account; (1) promotor or modular bases located in the higher regions that are defined as enhancers, and (2) regulatory proteins such as transcription factors that are capable of binding to these sequences.

Further research (with a larger samples sizes) from different parts of the country are also needed in order to prove the current study results.

Author's Contribution

Concept: Eyyüp Rencüzoğulları, Ayşe Dalyan, and Muhsin Aydın; Literature review, data collection or processing: Eyyüp Rencüzoğulları, Ayşe Dalyan, Muhsin Aydın, Ahmet Genç, and Süleyman Bayram; Writing: Eyyüp Rencüzoğulları and Muhsin Aydın.

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Conflict of interest statement

The authors report no conflict of interest and are responsible for the content and writing of the paper.

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