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Original Article

Level of Hemoglobin F and ^{G}g Gene Expression in Sickle Cell Disease and Their Association with Haplotype and XmnI Polymorphic Site in South of Iran

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Abstract

Background: Molecular genetic factors regulating hemoglobin F (Hb F) expression are important modifiers of the severity of sickle cell anemia (SS).

Methods: The prevalence of XmnI polymorphic site, the $^{G}\gamma$:$^{A}\gamma$ ratio and the Hb F level were determined using PCR-RFLP procedure, HPLC and alkaline denaturation method, respectively, in various haplotypes of 52 patients with SS, 18 patients with sickle/β-thalassemia (S/Thal), 17 with sickle cell trait (AS) and 53 normal subjects from Fars and Khuzestan provinces who attended the Hematology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran during 2002–03.

Results: The prevalence of XmnI (+/+) site in patients with SS was 53.8% which was higher than that for S/Thal (23.5%), AS (22.2%) and normal individuals (7.5%). There was a correlation between the presence of XmnI site and high $^{G}\gamma$:$^{A}\gamma$ ratio in SS and S/Thal patients with Arab-Indian homozygous or heterozygous haplotypes (contingency coefficient=0.43, P=0.002). In the present study, the Hb F level was significantly higher in SS patients with one or two Arab-Indian haplotypes as compared to Bantu, Benin and Cameroon haplotypes. However, the Hb F level was significantly higher in patients with S/Thal having two XmnI sites carrying Arab-Indian and Senegal haplotypes as compared to Bantu, Benin and Cameroon haplotypes. The increasing effect of presence XmnI site on Hb F level appears only when hemolytic stress is present as in SS and S/Thal patients (contingency coefficient=0.35, P=0.01).

Conclusion: The presence of XmnI polymorphic site in haplotype backgrounds of Arab-Indian and Senegal in sickle cell anemia is correlated with high level of Hb F and $^{G}\gamma$:$^{A}\gamma$ ratio.
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Keywords ● Hemoglobin F ● gamma chain ● haplotype ● sickle cell disease ● thalassemia ● hemoglobinopathy

Introduction

Molecular genetic factors regulating hemoglobin F (Hb F) expression are important modifiers of the severity of sickle cell anemia (SS). High levels of Hb F interfere

with Hb S polymerization, increase the solubility of Hb S and thus reduce the sickling phenomena. Therefore, high Hb F level is correlated with a milder clinical picture in patients with SS. An inverse correlation between the level of Hb F and painful crisis of SS has been established.[1]

Two non-α chains of Hb F are coded by $^{G}\gamma$ and $^{A}\gamma$ genes of the β-globin gene cluster. After birth, a developmental switching occurs and the ratio of $^{G}\gamma$:$^{A}\gamma$ reduces from 7:3 to 4:6.[2] In some patients with SS in whom the sickle gene is linked to the Senegal and Arab-Indian haplotypes, this switching does not occur; in these patients, the content of $^{G}\gamma$ chain in Hb F remains high.[3,4]

It has been suggested that the substitution of C **à** T in the position -158 of the $^{G}\gamma$ gene (recognized by the XmnI restriction enzyme) is related to a higher level of $^{G}\gamma$ expression,[5] higher levels of Hb F, delayed switching from fetal to adult hemoglobin,[6] and milder presentation of SS.[7,8] Several studies revealed a strong correlation between the presence of XmnI site 5´ to the $^{G}\gamma$ gene and high $^{G}\gamma$:$^{A}\gamma$ ratio of the Hb F.

In two separate studies on Senegal,[9] and Bantu,[10] haplotypes, Nagel, et al, found high levels of $^{G}\gamma$ chain (>60%) in Hb F of SS patients who had at least one chromosome with the Senegal haplotype; the $^{G}\gamma$ chain level in SS patients with Bantu haplotype was 40.4%. Moreover, Ballas, et al,[11] showed the presence of XmnI site in all patients with SS who had Senegal haplotype. They also showed the correlation between the presence of XmnI site and high $^{G}\gamma$:$^{A}\gamma$ ratio in Hb F.
Miller, et al,[4] reported the presence of C **à** T substitution 5´ to the $^{G}\gamma$ gene and a high percentage of $^{G}\gamma$ chain (>70%) in patients with SS from eastern provinces of Saudi Arabia.

Despite the strong evidence of the influence of XmnI polymorphic site on $^{G}\gamma$ gene expression, there is little information available on the correlation between the percentage of Hb F and the -158 C **à** T in SS. Nagel, et al,[10] found a direct correlation between percentage of $^{G}\gamma$ and percentage of Hb F in patients with SS who had Bantu haplotype. Peri, et al,[6] reported a positive correlation between this polymorphism and the degree of Hb F synthesis. Sampietro, et al,[12] in a study on the Hb F level and F-cell numbers from normal individuals found a positive correlation between these parameters and the presence of XmnI site 5´ to the $^{G}\gamma$ gene.

On the other hand, some other studies showed a lack of increase in Hb F level in the presence of the XmnI polymorphism in sickle cell trait (AS), normal (AA) individuals,[4] or in those with atypical haplotypes,[11] and proposed other autosomal genetic determinants or probable contribution of X-chromosome in this process.[13]

To find whether presence of XmnI site may affect the Hb F levels and $^{G}\gamma$:$^{A}\gamma$ ratio of the Hb F in patients with SS, we studied this site in normal subjects and patients with SS from the Fars and Khuzestan provinces during 2002–03.

Patients and Methods

All patients with SS, (87) who attended the Hematology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran, between 2002 and 2003 were included in this study. Blood samples were obtained from 52 patients with SS, aged 5–41 years (25 females and 27 males); 18 patients (10 females and eight males) with sickle/β-thalassemia (S/Thal), aged 6–46 years; 17 individuals with AS, aged 9–58 years (eight females and nine males) and 53 normal control subjects, aged 18–49 years (13 females and 40 males). Because of the influence of age on the Hb F level, patients younger than five years were excluded from the study. All patients and controls were from Fars and Khuzestan provinces. The study protocol was approved by the Research Ethics Committee of Shiraz University of Medical Sciences. All subjects were informed about the study's objectives and gave written informed consents.

Cellulose acetate electrophoresis at alkaline, citrate agar gel electrophoresis at acid pH, and solubility test were used for the diagnosis of SS.[14] DNA was extracted from white blood cells by phenol-chloroform.[15] A 650-bp fragment 5′ to the $^{G}\gamma$ gene was amplified using the primers 5′–aac tgt tgc ttt ata gga ttt t–3′ and 5′–agg agc tta ttg ata act cag ac–3′ as described by Old.[16] Amplification conditions included 94 °C for five min followed by 30 cycles of 94 °C for one min, 55 °C for one min, 72 °C for 1½ min, with a final extension period of five min at 72 °C. Ten to 15 µL of the amplified PCR product was digested with five units of XmnI restriction enzyme and electrophoresed on a 3% agarose gel. In the presence of T allele, two fragments of 450- and 200-bp were produced. The presence of the normal allele C loses cleavage site for XmnI and thus an intact 650-bp fragment was produced.

The polymorphic restriction sites studied were, 5′ to the ε gene by HindII, 5′ to the $^{G}\gamma$ gene by XmnI, within IVS-2 of the $^{G}\gamma$ and $^{A}\gamma$ genes by HindIII, 5′ to the $^{A}\gamma$ by TaqI, 3′ to the ψβ by HindII, IVS-2 of the β-gene by AvaII, and 3′ to the β-gene by HinfI.[16]

The Hb F was measured by alkaline denaturation method.[17] The $^{G}\gamma$ and $^{A}\gamma$ contents of Hb F were determined by high performance liquid chromatography (HPLC) using acetoni-

trile and 0.1% trifluoroacetic acid (TFA) in water and an increasing gradient of acetonitrile.[18] The chain separation was performed on a preparative C_{18} column.

Statistical analysis

SPSS v 11.5 was used for the statistical analyses. The allelic frequencies were calculated by the gene counting method. Categorical variables, such as allelic frequencies were analyzed by χ^2 test. Two-tailed Student's t test was used to compare means of two quantitative variables. Pearson's correlation coefficient was used to examine the correlation between the presence of XmnI polymorphic site and haplotypes with other variables. P<0.05 was considered as statistically significant.

Results

The studied polymorphic restriction sites in the β-globin gene cluster and five typical haplotypes of Arab-Indian, Senegal, Cameroon, Benin and Bantu and atypical haplotypes are shown in table 1.

The prevalence of XmnI polymorphic site in patients and controls is depicted in table 2. Fifty-three and eight-tenth percent of patients with SS had two XmnI sites; 32.7% of the patients had one chromosome bearing this site. Most (53%) people with AS had one XmnI site. XmnI (-/-) was the predominant state (44.4%) in patients with S/Thal. Almost 49% of normal individuals had the XmnI (+/-) polymorphism similar to AS subjects (table 2).

The status of XmnI polymorphic site and the percentage of Hb F in all patients are given in table 3. In patients with SS, the Hb F level was significantly higher in those who had one (P=0.04) or two (P=0.01) XmnI sites as compared to those with the site absent (table 3). In patients with S/Thal, only the presence of two XmnI sites (+/+) compared to the absence of the site (-/-) was associated with a significant increase in the Hb F percentage (P=0.009). A positive correlation was observed between the Hb F level and the presence of XmnI site in SS and S/Thal groups (contingency coefficient=0.35, P=0.01). Among AS individuals, the presence or absence of XmnI site did not affect the Hb F level (table 3). Normal individuals with or without XmnI site had an Hb F levels of around 0.5% (data not presented).

The $^{G}\gamma$:$^{A}\gamma$ ratios of Hb F in SS, S/Thal, and AS

Table 1: Polymorphic restriction sites in the β-globin gene cluster in various typical and atypical haplotypes which were analyzed in sickle cell patients.

Haplotype	HcII5′ε	XmnI5′$^{G}\gamma$	HdIII,IVSII$^{G}\gamma$	TaqI 5′$^{A}\gamma$	HdIII,IVSII$^{A}\gamma$	HcII3′ψβ	AvaII,IVSIIβ	HfI3′β
Arab-Indian	+	+	+	+	-	+	+	-
Arab-Indian A	+	-	+	+	-	+	+	-
Senegal	-	+	+	+	-	+	+	+
Senegal A	-	+	+	+	-	+	-	+
Cameroon	-	-	+	+	+	+	+	-
Benin	-	-	-	-	-	+	+	+
Bantu	-	-	+	+	-	-	+	+
Bantu A1	-	-	-	+	-	-	+	+
Bantu A2	+	-	-	+	-	-	+	+
Bantu A2a	+	-	-	+	-	-	-	+
BantuA4	-	-	+	+	-	+	+	+
Bantu A6	-	-	+	+	+	-	+	+

Table 2: The prevalence of XmnI polymorphic site in SS, AS, S/Thal groups and normal individuals.

Individuals (n)	XmnI Site		
	+/+	+/-	-/-
	n (%)		
SS (52)	27 (53.8)	17 (32.7)	7 (13.5)
AS (17)	4 (23.5)	9 (53)	4 (23.5)
S/Thal (18)	4 (22.2)	6 (33.4)	8 (44.4)
Normal (53)	4 (7.5)	26 (49.1)	23 (43.4)

Table 3: The Hb F percentage and XmnI status in SS, AS, S/Thal groups.

XmnI	SS		S/Thal		AS	
	n	Hb F% (Mean ±SD)	n	Hb F% (Mean ±SD)	n	Hb F% (Mean ±SD)
(+/+)	28	16.8 ±9*	3	16.8 ±4.9**	4	1.5 ±0.76
(+/-)	14	15.2 ±9.1*	6	12.7 ±3.6	9	1.5 ±1.1
(-/-)	7	7.0 ±4.5	8	8.2 ±4.4	4	0.83 ±0.1

*Significantly different comparing XmnI (+/+) with XmnI (-/-) (P=0.01) or comparing XmnI (+/-) with XmnI (-/-) (P=0.04).
**Significantly different comparing XmnI (+/+) with (-/-) (P=0.009).
Levels of significance determined by ANOVA test.

groups, in the presence and absence of XmnI site are shown in table 4. In patients with SS, the $^{G}\gamma$:$^{A}\gamma$ ratio was significantly higher in those with one or two XmnI sites as compared to those who did not have this site (P<0.001). The presence of one or two XmnI sites in patients with S/Thal caused a significantly higher $^{G}\gamma$:$^{A}\gamma$ ratio as compared to those who did not have this site (P<0.007 and P<0.001, respectively). Among patients with SS and S/Thal, there was a positive correlation between the $^{G}\gamma$:$^{A}\gamma$ ratio and the presence of XmnI site (contingency coefficient=0.43, P=0.002). The $^{G}\gamma$:$^{A}\gamma$ ratio did not change significantly with the presence or absence of the XmnI site in AS individuals.

In patients with SS, the percentage of Hb F was significantly higher in those with homozygous (P=0.007) and heterozygous (P=0.04) Arab-Indian haplotype as compared to all African haplotypes (excluding Senegal). Furthermore, in SS group, the $^{G}\gamma$:$^{A}\gamma$ ratio was significantly higher in those homozygous for Arab-Indian haplotype (P<0.001) or those heterozygous for Arab-Indian with one of Bantu, Benin, or Cameroon haplotypes (P<0.001) as compared to African haplotypes (except Senegal). In patients with S/Thal only heterozygous Arab-Indian haplotype existed. In these patients, the Hb F level and $^{G}\gamma$:$^{A}\gamma$ ratio was significantly higher (P=0.004 and P=0.001, respectively) in those heterozygous for Arab-Indian haplotype combined with Senegal haplotype than those with combinations of African haplotypes (Bantu, Benin, Cameroon). The $^{G}\gamma$:$^{A}\gamma$ ratio was significantly higher (P=0.007) in those heterozygous for Arab-Indian haplotype combined with one of the African haplotypes (except Senegal) than those with Bantu, Benin, and Cameroon haplotypes (table 5).

Discussion

Gilman and Huisman,[5] suggested that -158 C **à** T substitution in the $^{G}\gamma$ promoter which is recognized by XmnI restriction enzyme is associated with a higher expression of $^{G}\gamma$ gene both in patients with SS and in Afro-Americans heterozygous for β-thalassemia from Georgia. In the present study, we found higher ratios of $^{G}\gamma$:$^{A}\gamma$ (>2.1) in all patients (table 4) with one or two XmnI sites. In patients with SS and S/Thal, the Hb F level was the highest in XmnI (+/+), moderate in XmnI (+/-) and the lowest in XmnI (-/-). The same pattern was observed for $^{G}\gamma$:$^{A}\gamma$ ratios in these patients (table 4). For the limited number of AS individuals studied, the comparison between $^{G}\gamma$:$^{A}\gamma$ ratios in three states of XmnI was not possible and the difference in Hb F levels was not statistically significant.

There was a significant positive correlation between the presence of XmnI polymorphic site and $^{G}\gamma$:$^{A}\gamma$ ratio (contingency coefficient=0.43, P=0.002) in patients with SS and S/Thal. Although there was a correlation between the presence of XmnI site and the high $^{G}\gamma$:$^{A}\gamma$ ratio, there was a single S/Thal patient with the absence of XmnI site and high $^{G}\gamma$:$^{A}\gamma$ ratio (3.3). In

Table 4: The $^{G}\gamma$:$^{A}\gamma$ ratio and XmnI status in patients.

XmnI	SS		S/Thal		AS	
	n	$^{G}\gamma$:$^{A}\gamma$ ratio (Mean ±SD)	n	$^{G}\gamma$:$^{A}\gamma$ ratio (Mean ±SD)	n	$^{G}\gamma$:$^{A}\gamma$ ratio (Mean ±SD)
(+/+)	25	2.6±0.34*	3	3.3±0.40**	2	4.9±3.3
(+/-)	12	2.4±0.92*	6	2.4±0.75**	6	2.2±0.8
(-/-)	4	0.5±0.13	8	0.9±0.99	1	3.5

*Significantly different comparing XmnI (+/+) with XmnI (-/-) or comparing XmnI (+/-) with XmnI (-/-) (P<0.001).
**Significantly different comparing XmnI (+/+) with XmnI (-/-) (P=0.001) or comparing XmnI (+/-) with XmnI (-/-) (P=0.007). Levels of significance determined by ANOVA test.

Table 5: Level of Hb F and $^{G}\gamma$:$^{A}\gamma$ ratio in various haplotypes of patients.

Haplotype	Hb F% Mean ±SD (n)			$^{G}\gamma$:$^{A}\gamma$ ratio Mean ±SD (n)		
	SS	S/Thal	AS	SS	S/Thal	AS
A-I/A-I	17.3±9.1 4.1-44.2(26)	-	-	2.6±0.34 1.9-3.2 (24)	-	-
A-I/Bantu[+] /Benin /Cameroon	15.5±9.5 3.7-33.8(13)	12.7±3.6 7.2-18.1(6)	1.5±1.1 0.47-3.8(9)	2.5±0.94 1.6-4.6(11)	2.4±0.8 1.8-3.8(6)	2.1±0.73 1-3.4(7)
A-I/Senegal	13.2±1.2 12.3-14.1(2)	18.5±5.2 12.1-23.5(4)	1.5±0.76 0.75-2.6(4)	2.2±0.6 1.8-2.6(2)	3.3±0.4 3-3.7(3)	4.9±3.3 2.6-7.2(2)
A-IA/Senegal	4.4(1)	-	-	-	-	-
Senegal/Senegal	6.2(1)	-	-	-	-	-
African/African[++]	7±4.5 2.3-13.3(7)	8.2±4.4 3.7-16(8)	0.83±0.1 0.74-0.98(4)	0.5±0.13 0.33-0.65(4)	0.9±0.99 0.33-3.3(8)	3.5(1) -

[+]Including typical and atypical Bantu haplotype, [++]Including Bantu, Benin, and Cameroon haplotypes.
*See the text for significance.

agreement with our results, Labie, *et al*,[19] found a high $^{G}\gamma$ expression in β-thalassemia associated with haplotype II lacking the XmnI site and suggested the possible involvement of several other substitutions in regions 5′ to the γ gene.

The mild presentation of SS has been described in the eastern provinces of Saudi Arabia and Bahrain along with frequently encountered XmnI polymorphic site.[7] XmnI site was present in nearly all of the patients with SS from Saudi Arabia,[4] and patients with Senegal haplotype.[3,20] In Saudi Arabian patients.[21] the $^{G}\gamma$:$^{A}\gamma$ ratio was found to be 3.2. The mild clinical presentation of SS associated with high Hb F levels in Iran has long been known without any understandings of its molecular basis.[22] The results of the present study indicated that in sickle cell patients of southern Iran, the XmnI site was present in all individuals with haplotypes of Arab-Indian or Senegal except for one patient with atypical Arab-Indian A-Senegal haplotype who lacked the XmnI site on the chromosome linked to the Arab-Indian A haplotype. The presence of this site in SS patients homozygous for Arab-Indian haplotype sharply increased the Hb F levels (17.3%) and $^{G}\gamma$:$^{A}\gamma$ ratios (2.6) (table 5). Similar effects were observed with heterozygous form of Arab-Indian haplotype with one of the typical or atypical African (except for Senegal) haplotypes (15.5% Hb F, and $^{G}\gamma$:$^{A}\gamma$ ratio of 2.5). The combination of Arab-Indian haplotype with Senegal haplotype produced similar results. Since the most predominant haplotype in patients from Fars and Khuzestan provinces is the Arab-Indian haplotype,[23] which is linked to the presence of XmnI site, the mild phenotype of SS in Iran is well-explained. The Hb F level and $^{G}\gamma$:$^{A}\gamma$ ratio in various combinations of African haplotypes including Bantu, Benin, and Cameroon (except for Senegal haplotype) were around 7%, and 0.5, respectively (table 5). Previously, we reported,[22] that SS patients from Southwest of Iran with homozygous Arab-Indian or heterozygous Arab-Indian/Bantu A_2 haplotype had a higher percentage of Hb F and $^{G}\gamma$:$^{A}\gamma$ ratio. However, in that study, it was not possible to compare the percentage of Hb F and $^{G}\gamma$:$^{A}\gamma$ ratio among various haplotypes in patients with SS, for the small sample size studied for each haplotype. Among patients with S/Thal, no homozygous Arab-Indian haplotype was encountered, but the combination of Arab-Indian with Senegal haplotype caused the highest level of Hb F, and $^{G}\gamma$:$^{A}\gamma$ ratio (18.5% and 3.3, respectively). The combination of Arab-Indian with Bantu, Benin or Cameroon haplotypes resulted in high Hb F percentage (12.7%) and $^{G}\gamma$:$^{A}\gamma$ ratio (2.4) (table 5). In patients with S/Thal the $^{G}\gamma$:$^{A}\gamma$ ratio for all non-Senegal African haplotypes was about 0.9. Within AS subjects, no relationship was found between the presence of XmnI site and $^{G}\gamma$:$^{A}\gamma$ ratio.

We therefore, concluded that the presence of C **à** T substitution at -158 of $^{G}\gamma$ gene, which is observed only in Arab-Indian and Senegal haplotypes, is the strong evidence for predicting high $^{G}\gamma$ gene expression, particularly in the presence of hemolytic stress. Nagel, *et al*,[20] suggested that erythropoietic stress, the most likely (but not necessarily the only) factor involved in thalassemia, is accompanied by an increase in $^{G}\gamma$ expression—a phenomenon which is partially determined by the β-like gene cluster haplotype. Moreover, a mild form of β^0/β^0-thalassemia has been reported,[24] in an Algerian patient which was related to the high level of Hb F due to the presence of homozygous state of XmnI (+/+).

While, our findings indicated that the presence of at least one chromosome with Arab-Indian haplotype would cause a significantly higher Hb F level than the presence of one Bantu, Benin, or Cameroon haplotype, there was a wide variation in the Hb F levels among each haplotype, which might indicate the presence of diverse mechanisms including *cis* or *trans* acting enhancers or silencers, and a possible role of some *trans* acting factors in modulation of Hb F expression. Nagel, *et al*,[20] in a study on Benin and Senegal haplotypes, encountered differences in the percentage of Hb F among patients with SS bearing these haplotypes which led them to conclude that the correlation between Hb F levels and haplotype is not a tight one. Furthermore, in a population of Maltese homozygotes for β-thalassemia, the XmnI site was found to be associated with variations in Hb F and $^{G}\gamma$-globin levels.[25]

Moreover, it has been proposed,[26] that XmnI site is involved in the expression of the $^{G}\gamma$-globin gene through its interactions with transcription factors, and that polymorphisms in the transcription factors could influence Hb F expression, conditional upon the XmnI-$^{G}\gamma$ site.

Conclusion

We found that the presence of XmnI polymorphic site in the haplotype background of Arab-Indian and Senegal in SS and S/Thal patients results in higher level of Hb F and $^{G}\gamma$:$^{A}\gamma$ ratios which ameliorate the clinical severity and hematological presentations of patients with SS.

Acknowledgments

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