Fetal Hemoglobin in Tunisian Sick Cell Disease Patient: Relationship with Polymorphic Sequences *Cis* to the β -Globin Gene

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Abstract Fetal hemoglobin (HbF) plays a dominant role in ameliorating morbidity and mortality of hemoglobinopathies. We evaluated the effects of polymorphic markers within the β -globin gene cluster to identify the genetic mechanisms that influence HbF on Tunisian sickling patients ($n = 242$). Haplotype analysis was carried out by polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) and the framework polymorphism was established by PCR-sequencing, four independent regions of interest were identified: the 5' region of β -LCR-HS2 site, the intervening sequence II (IVSII) region of two fetal ($G\gamma$ and $A\gamma$) genes and the 5' region of β -globin gene. The correlation of these various Haplotypes and SNPs with HbF expression and clinical data was studied. Our data showed that among the various polymorphic markers analyzed, only the sequence (AT) x N12(AT) y in LCR HS2 region was significantly associated ($p < 0.05$) with increased HbF levels, suggesting that the β -globin gene cluster exerts a significant effect on HbF in sickle cell patients. This study can improve understanding of the physiopathology of the disease and aid to increase our ability to predict clinical severity.

Keywords Fetal hemoglobin (HbF) · Microsatellite repeats · Sickle cell anemia

Introduction

Fetal hemoglobin (HbF, $\alpha_2\gamma_2$) is the predominant hemoglobin in fetal life. After birth HbF is gradually replaced by adult hemoglobin (HbA, $\alpha_2\beta_2$), due to the switch from γ to β -globin gene expression. In normal subjects HbF constitutes less than 1 % of the total hemoglobin (Hb) by the end of the first year of life. The synthesis of HbF is restricted to a subpopulation of red cells, known as F-cells (FC) [1, 2], and the HbF levels are directly correlated to the number of FC [3, 4]. DNA mutation may lead to a persistent expression in γ -globin gene down-regulation. HbF levels, which are regulated by multiple genes with influence of an environmental component, play a dominant role in ameliorating morbidity and mortality of the principal congenital Hb disorders such as sickle cell disease (SCD) [5, 6]. The high degree of HbF is a well characterized diagnostic feature and correlates with reducing morbidity and mortality in patients with these blood disorders. Identification of genes and genetic variants that contribute to augmentation of HbF can perhaps lead to strategies for effective control of globin genes expression and ultimately new therapeutic approaches.

However, this variability of HbF levels is genetically heterogeneous and seems to result from complex interactions between varieties of genetic determinants, some of which are linked to the β -globin gene cluster. Indeed, the γ -globin genes are close to β -globin gene, the question arose to whether the variability of gene environment of the sickle cell mutation could contribute to the variability of HbF expression? Several studies have shown the role of polymorphisms in this variability. Some correlation has been found in particular regarding the presence of an XmnI site and high expression of the $G\gamma$ -globin gene [7–10]. More recently, another set of polymorphisms has been demonstrated: the microsatellite

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Fig. 1 Map of the β -globin gene cluster and the polymorphic

repetitions which could be the binding site of the transactivators factors involved in chromatin structure of the β -globin locus [11]. Several of these polymorphisms were identified in the β -LCR region, the intervening sequence II (IVSII) region of two fetal ($G\gamma$ and $A\gamma$) genes and the 5' region of β -globin gene. In others Studies have mapped some of the determinants to chromosomes Xp22 [12], 6q23 and 8 [13, 14], but the precise loci involved have not yet been identified.

The aim of this study was to determine the effect of these putative genetic markers on HbF expression on Tunisian sickling patients. We studied seven polymorphic regions of the β -globin gene cluster, including four repeat sequences, and evaluated their association with HbF expression in SCD Tunisian patients.

Materials and Methods

Patients

The study was performed on 242 non-consanguineous sickle cell patients from the pediatrics services of the university hospital of Tunis. All samples have been obtained during the 1st time of diagnosis. All are homozygotes and the HbF values were determined before HU treatment. Blood samples were collected in EDTA as anticoagulant. All the patients were from Tunisia. Mean patient age was 13.17 years. The homozygous SS state was confirmed by family studies and in some cases by the direct detection of the β^S mutation using the restriction enzyme *DdeI*.

Hematological Analysis

Hb pattern was performed by cation exchange HPLC using the VARIANT D-10TM Hb Analyzer (D-10TM Hb Testing System, Bio-Rad Laboratories, Marnes la Coquette, France) according to the procedure recommended by the manufacturer [15] and the presence of HbS was confirmed by sickling and solubility tests. All samples were obtained at the time of diagnosis, before hydroxyurea (HU) treatment was initiated.

DNA Sequence Analysis

Genomic DNA was isolated from peripheral blood leukocytes by phenol–chloroform extraction method [16]. The

following framework polymorphisms were investigated by Polymerase chain reaction (PCR) followed by direct sequencing: (AT)xN12(AT)y repeat configurations within the 5'HS2 region of β -LCR site (rs72452842) [17, 18], (TG)n (CG)m configurations in the IVSII region of fetal globin gene ($G\gamma$ (rs141297974) and $A\gamma$ (rs61080176)) [19] and (AT)xTy repeat configuration in the 5' region of β -globin gene (rs372547761) [20] (Fig. 1). Tunisian β S restriction Haplotypes (HR) were investigated by RFLP protocol as described previously in Moumni et al. and the results were adopted in this study [21].

Statistical Data

The EPI Info (version 6.04) and Statistical Package for the Social Sciences (SPSS[®], version 18) programs were used for statistical analyses. The means of HbF levels were determined by the counting method and submitted to χ^2 test and $p < 0.05$ was considered statistically significant. The relationship between HbF levels and genetic markers was investigated by use of the Student's t test: two groups HbF were established, the first group contained individuals with low HbF levels ($<15\%$) and the second group contained individuals with high HbF levels ($>15\%$). The level of significance was set at $p < 0.05$ in all analyses.

Results

HbF Levels in the Population

We studied 242 homozygous sickle cell patients (SS). The mean and standard deviation for HbF were $15.25 \pm 1.3\%$ and the range was 2.3–34.3 %. 160 subjects (66 %) were from first group of HbF $< 15\%$ and 82 subjects were from second group of HbF $> 15\%$.

Markers Analysis

Molecular data and allele frequencies concerning the restriction Haplotypes and microsatellite configurations, observed in β^S chromosomes, are described in Tables 1 and 2. The (AT)xN12(AT)y configurations of 5'HS2 region of β -LCR were named L1 to L13, the (TG)n (CG)m

Table 1 Molecular sequences and allele frequencies of the microsatellite configurations of β -LCR-*HS2* region, *IVSII* of fetal gene ($G\gamma$ and $A\gamma$) and 5' of β -*Globin* gene region

β -LCR- <i>HS2</i> (AT) x N ₁₂ (AT) y	%	$G\gamma$ (TG) n (CG) m	%	$A\gamma$ (TG) n (CG) m	%	5' β (AT) x T y	%
L1-(AT)10N12(AT)11	–	G1-(TC)1(TG)11(CG)3	1.83	A1-(TC)1(TG)11(CG)3	–	B1-(AT)7T7	6.61
L2-(AT)6N12GT(AT)8	0.53	G2- (TC)2(TG)9(AG)(TG)2(CG)2	29.36	A2(TC)2(TG)9(CG)5(TG)7	18.69	B2-(AT)9T4	49.25
L3-(AT)7N12GT(AT)9	1.58	G3-(TC)1(TG)13(CG)2	5.50	A3- (TC)1(TG)9(CG)2CACG(TG)7	1.87	B3-(AT)9T5	5.41
L4-(AT)7N12GT(AT)7	0.53	G4-(TC)1(TG)17(CG)5	2.75	A4- (TC)1(TG)10(CG)2CACG(TG)7	1.87	B4-(AT)8T6	1.80
L5-(AT)7N12GT(AT)8	0.53	G5-(TC)1(TG)19(CG)4	2.75	A5- (TC)2(TG)9(CG)2CACG(TG)7	26.17	B5-(AT)8T5	3.90
L6-(AT)8N12GT(AT)7	62.11	G6-(TC)2(TG)11(CG)3	0.92	A6-(TC)1(TG)9(CG)5(TG)7	0.93	B6-(AT)8T4	3.30
L7-(AT)8N12GT(AT)10	0.53	G7-(TC)2(TG)15(CG)4	0.92	A7-(TC)2(TG)7(CG)5(TG)9	1.87	B7-(AT)6T9	1.80
L8-(AT)9N12GT(AT)7	0.53	–	–	–	–	B8- (AT)10T5	1.50
L9-(AT)8N12(AT)10	2.11	–	–	–	–	–	–
L10- (AT)10N12GT(AT)11	1.05	–	–	–	–	–	–
L11- (AT)8N12GT(AT)11	2.11	–	–	–	–	–	–
L12-(AT)9N12(AT)10	0.53	–	–	–	–	–	–
L13-(AT)9N12(AT)11	0.53	–	–	–	–	–	–

The configurations of β -LCR-*HS2* were named L1 to L13 (N12: ACACATATACGT, N12GT: ACACATATACGTGT), those of *IVSII*- $G\gamma$ were named G1 to G7, those of *IVSII*- $A\gamma$ were named A1 and A7 and those 5' of β -*Globin* gene were named B1 to B8. L1, G1, A1 and B1 are the reference sequence configurations from the HUMHBB*

* The nucleotides are numbered according to the HUMHBB 73,308 bp GenBank, Version: U01317.1 GI:455025

Table 2 Frequencies of restriction Haplotypes of β -globin cluster and HbF levels with SS Tunisia patients

Haplotypes	5' $G\gamma$ XmnI	$G\gamma$ HindIII	$A\gamma$ HindIII	$\psi\beta$ HincII	3' $\psi\beta$ HincII	IVS2 β AvaII	3' β HinfI	Frequency %	HbF %
Bénin	–	–	–	–	+	+	+	60.54	12.74
Bantu	–	+	–	–	–	+	+	2.71	10.36
Atypical									
A	+	–	–	–	–	+	+	8.43	17.26
A1	–	–	–	–	–	+	+	23.49	10.8
A2	–	–	–	–	–	–	–	3.31	6.25
B1	–	–	+	–	–	+	+	1.5	27.04

configurations of *IVSII* region of $G\gamma$ gene were named G1–G7, the (TG) n (CG) m configurations of *IVSII* region of $A\gamma$ gene were named A1–A7 and the (AT) x T y configurations 5' region of β -*globin* gene were named B1–B8.

Tunisian β^S HR were investigated by RFLP protocol as described previously in Moumni et al. and the results were adopted in this study [21] describing six Haplotypes: Benin is the most common, bantu and four atypical Haplotypes A, A1, A2 and B1 (Table 2).

Table 1 shows the existence of several variations in the 5' β -LCR *HS-2* for β^S chromosomes and the L6

(AT) $_8$ N $_{12}$ GT(AT) $_7$ configuration was predominant in the studied SCD population with 62.11 % of the total alleles.

Seven different microsatellite configurations of the (TG) n (CG) m motif were found among β^S chromosomes in the *IVSII* region of $G\gamma$ -*globin* gene (Table 1). One novel sequence configurations G2 (TC)2(TG)9(AG)(TG)2(CG)2 was found and it was predominant with 29.36 % of total alleles. In the *IVSII* region of $A\gamma$ -*globin* gene, seven different microsatellite configurations of the (TG) n (CG) m motif were found among Tunisian β^S chromosomes (Table 1). Two novel configurations: A3 (TC)1(TG)9(CG)2CACG(TG)7 and A5

Table 3 HbF values for each polymorphic markers of the β -LCR-*HS2* region, *IVSII* of fetal gene ($G\gamma$ and $A\gamma$) and 5' of β -*Globin* gene region

β -LCR <i>HS2</i> (AT)xN12(AT)Ty	HbF %	$G\gamma$ (TG)n (CG)m	HbF %	$A\gamma$ (TG)n (CG)m	HbF (%)	5' β (AT)xTy	HbF (%)
L1	7.23	G1	14,49	A1	–	B1	12,55
L2	34.35	G2	11,23	A2	14,60	B2	13,29
L3	2.9	G3	14,21	A3	10.2	B3	7,33
L4	31.92	G4	6,85	A4	11,18	B4	18,96
L5	19.6	G5	–	A5	11,14	B5	10,25
L6	12.11	G6	–	A6	11,44	B6	7,96
L7	4.1	G7	25,56	A7	4,29	B7	9,07
L8	11.54	–	–	–	–	B8	12,95
L9	8.04	–	–	–	–	–	–
L10	7	–	–	–	–	–	–
L11	5.3	–	–	–	–	–	–
L12	8.27	–	–	–	–	–	–
L13	2.39	–	–	–	–	–	–

(TC)2(TG)9(CG)2CACG(TG)7 were found and the A5 was predominant with 26.17 % of total alleles which shows specificity to Tunisian β S chromosomes. The (AC)1(TG)11 (CG)3 sequence as a reference configuration correlates with a normal chromosome in both $G\gamma$ and $A\gamma$ -globin gene named G1 and A1.

Eight microsatellite configurations of the (AT)xTy motif in the 5' β -globin gene studied among β S chromosomes were shown. The most frequent configuration was B2 (AT)9T4, it is specific to Tunisian β S chromosomes with an allele frequency of 49.25 % (Table 1).

The average of HbF levels for each marker was calculated using SPSS software (SPSS18) (Table 3). It gives that, the highest amounts were associated with the L2 (34.35 %) in 5' β -LCR *HS-2*, G7 (25.56 %) and A2 (14.6 %) respectively in the *IVSII* region of $G\gamma$ and $A\gamma$ -globin gene and B4 (18.96 %) in the 5' β -globin gene (Table 3).

Markers/HbF Association

The statistical test was performed by “SPSS” software using student's *t* test to deduce the significance as p-value, in order to find an association between the markers polymorphisms and the HbF expression. However, two groups HbF were establishing according to their HbF levels: high (>15 %) and low (<15 %). The results revealed that the p-value of RFLP-Haplotypes ($p = 0.623$), *IVSII* $G\gamma$ region ($p = 0.864$), *IVSII* $A\gamma$ region ($p = 0.539$) and 5' β -globin region ($p = 0.298$) were not significant. In contrast, only the p-value in the 5' β -LCR *HS-2* region was significant ($p = 0.005$) compared to other regions.

Discussion

Sickle cell anemia (SCA) is a monogenic disease with widely heterogeneous phenotypes. Its severity is moderated by high Fetal Hb levels (HbF > 15 %). Mechanisms responsible for HbF production in adults are not fully comprehended. However, several studies have linked variations in HbF to polymorphisms in β -globin cluster. Taking this aim into consideration, we studied restriction Haplotypes and for polymorphic markers and assessed their association with HbF expression.

In Tunisia, SCA Haplotype was reported for the first time by Abbes and al. [22] and later by Moumni et al. [21] describing six Haplotypes: Benin is the most common, bantu and four atypical Haplotypes [21].

The sequencing results revealed a great molecular heterogeneity of microsatellites repetitions with the predominance of some motifs which are specific to β S chromosome such as the following configurations L6 (AT)8N12GT(AT)7 within the 5' *HS2* region of β -LCR site, the two newest configurations G2 (TC)(TG)9(AG)(TG)2(CG)2 and A5 (TC)2(TG)9(CG)2CACG(TG)7 within *IVSII* region of the fetal-globin genes ($G\gamma$ and $A\gamma$) respectively and the configuration (AT)9T4 with the 5' region of β -globin gene, confirming the diversity of Tunisian β S chromosome.

The Student's *t* test shows that significant association between the markers polymorphisms in the 5' β -LCR *HS-2* region and the HbF expression, suggesting that the polymorphism at the β -LCR could play an important role in the expression of the fetal γ -globin gene.

In fact, the real expression regulation of the γ -globin gene is presumably under the control of other factors yet to be discovered, as mentioned by different authors [21, 23].

Over expression of the γ gene is responsible of the synthesis of a high level of HbF that plays the role of protector against HbS polymerization. Consequently, a high level of HbF (15 %) is a decisive factor in the modulation of SCD severity [24, 25].

It is interesting to note that the β -LCR-HS2 contains several sequence elements specific to the binding site of ubiquitous or erythroid factors [24, 25], suggesting that mutations in these marker polymorphisms can increase the affinity of these factors when finding their consensus mutated sequence and can thus modulate the severity of the SCD [26]. However, The variation of the polymorphic microsatellites in regulator *cis* elements could alter the binding of two transcriptional factors homeobox protein B2 (Hox-B2) [27] and β protein 1 (BP1) by activating or repressing the transcription of certain β -globin genes [28, 29].

This study highlights that the β -LCR-HS2 region plays an important role in gene expression and regulation, thus it improves understanding of the physiopathology of the disease and aid to increase our ability to predict clinical severity.

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