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Promoter region sequence differences in the *A* and *G gamma globin* genes of Brazilian sickle cell anemia patients

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Abstract

Fetal hemoglobin (HbF), encoded by the *HBG2* and *HBG1* genes, is the best-known genetic modulator of sickle cell anemia, varying dramatically in concentration in the blood of these patients. This variation is partially associated with polymorphisms located in the promoter region of the *HBG2* and *HBG1* genes. In order to explore known and unknown polymorphisms in these genes, the sequences of their promoter regions were screened in sickle cell anemia patients and correlated with both their HbF levels and their β^S-globin haplotypes. Additionally, the sequences were compared with genes from 2 healthy groups**,** a reference one (N = 104) and an Afro-descendant one (N = 98), to identify polymorphisms linked to the ethnic background.The reference group was composed by healthy individuals from the general population. Four polymorphisms were identified in the promoter region of *HBG2* and 8 in the promoter region of *HBG1* among the studied groups. Four novel single nucleotide polymorphisms (SNP) located at positions -324, -317, -309 and -307 were identified in the reference group. A deletion located between -396 and -391 in the *HBG2* promoter region and the SNP -271 C→T in the *HBG1* promoter region were associated with the Central African Republic β^S-globin haplotype. In contrast, the -369 C→G and 309 A→G SNPs in the *HBG2* promoter region were correlated to the Benin haplotype. The polymorphisms -396_-391 del *HBG2*, -369 SNP *HBG2* and -271 SNP *HBG1* correlated with HbF levels. Hence, we suggest an important role of *HBG2 and HBG1* gene polymorphisms on the HbF synthesis.

Key words: Fetal hemoglobin; Sickle cell anemia; *HBG1* gene; *HBG2* gene

Introduction

Clinical heterogeneity is an important characteristic of sickle cell anemia (SCA) and other hemoglobinopathies (1). The molecular data regarding diseases comprise the target gene and a large number of known and unknown DNA changes. These alterations concomitantly modify the expression of several genes, including the *gamma globin* genes *(HBG2* and *HBG1)*, responsible for the synthesis of the γ chains present in fetal hemoglobin (HbF) (2-4). Gene expression is modulated by a large number of polymorphisms located in the β-globin gene cluster. Of these, the *HBG2* -158 C→T variation is consistently linked with an increase of *HBG2* expression in *cis*, which are DNA sequences in the vicinity of the structural portion of a gene that are required for gene expression (5). Thus, there are *cis*-acting sequences that behave both positively and negatively in transcriptional regulation. For this reason, many studies have focused on learning about the reactivation of the dormant *HBG* genes during adulthood and in understanding which *cis*-acting elements govern HbF synthesis. The regulatory sequences 5' of *HBG2* and *HBG1* harbor at least eight polymorphic markers. These include the 4-bp deletion (AGCA) at positions -225 to -222 upstream of *HBG1* (2) and the *HBG2* -157 C→T single nucleotide polymorphism (SNP), which has been reported to be associated with high HbF levels (6). Moreover, some of the *HBG2* and *HBG1* polymorphisms are associated with the major β^S-globin

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gene haplotypes, which are named Benin (Ben), Central African Republic (CAR), Senegal (Sen), Arab-Indian and Cameroon (Cam), according to their geographical origin and the ethnic groups in which they are frequently found (1,7). For example, there is a T→A variation in the *HBG1* promoter region (-499) that is associated with the Ben haplotype among Sicilian and North American individuals (8). Because of the wide variability in clinical expression, and the consistent differences in HbF levels among SCA patients with different haplotypes, we have initiated sequence analyses of the *HBG2* and *HBG1* promoter regions in order to identify differences that may be important for understanding the variations in γ-chain synthesis. The 5' promoter regions were chosen because of their potential regulatory role in gene expression. These regions are known to contain both positive (enhancer) and negative (silencer) regulatory elements upstream of the consensus promoter sequences (9). We report here sequence data for these regions of both *HBG2* and *HBG1* from different Brazilian groups, i.e., SCA patients and two healthy groups, a reference one and an Afro-descendant one.

Patients and Methods

Patients and control subjects

Blood samples were collected from 152 SCA patients, 104 apparently healthy individuals randomly chosen from the general population (reference group) and 98 Afro-descendant individuals. The SCA patients have been followed at the Hematology and Hemotherapy Bahia Foundation and were selected between 2004 and 2005. The median age (± SD) of this group of 73 women and 79 men was 10.7 ± 11.2 years.

The reference group consisted of volunteers (employed or their children) with a median age of 9.7 ± 12.2 years, 54 men and 50 women who went to the Faculty of Pharmacy of the Federal University of Bahia for laboratory analyses and who showed an AA hemoglobin profile. The SCA patient group and the reference group are from Bahia, Northeast region of Brazil, which has both the highest frequency of abnormal hemoglobin and the highest rate of race admixture, mainly of individuals of African, European and Amerindian origin (10).

The Afro-descendant group, from the Saracura and Arapema communities, is located in Pará, Brazil. This group consisted of 51 men and 47 women with a median age of 66 ± 14.1 years. In contrast to the SCA and reference groups, the Afro-descendant group derives from a slave community located in Pará, Northern Brazil, where the race admixture is lower, composed basically of individuals of African origin (11).

Individuals who reported any disease were excluded from the reference and the Afro-descendant groups.

The study was approved by the Human Subject Research Ethics Committee of the Oswaldo Cruz Research Foundation in Bahia (process No. 0025.0.0225.000-07) and Amazonia (process No. 209/03). Informed consent was obtained from the parents or guardians.

DNA analyses

Genomic DNA was isolated from peripheral blood leukocytes using the QIAMP DNA Blood Kit (Qiagen, USA) according to manufacturer protocols. The *HBG2* and *HBG1* promoter regions were sequenced (12) with an ABI Prism 3100 DNA sequencer using Kit BigDye 03 Terminator TM Sequencing Standards (Applied Biosystems, USA) and specific sequencing primers: 5'-TGA AAC TgT ggT CTT TAT gAA AAT Tg-3' forward HBG1, 5'-gCA CTg AAA CTg AAA CTg TTg CTT TAT Agg AT-3' forward HBG2, and 5'-ggC gTC Tgg ACT Agg AgC TTA TTg-3' reverse for both genes. The β^S-globin gene haplotypes were identified in the SCA patients by the polymerase chain reaction and by restriction fragment length polymorphism techniques (13).

Statistical analyses

Descriptive analyses included gene and allelic frequencies, odds ratio as an estimate of relative risk and the 95% confidence interval (CI). The chi-square test was used to associate independent categorical variables and to test for the Hardy-Weinberg equilibrium. The level of significance was set at $P < 0.05$. All analyses were carried out using the Epi-Info software, version 6.04.

Results

HBG2 and *HBG1* promoter region polymorphisms

Table 1 shows the frequencies of the *HBG2* and *HBG1* promoter region polymorphisms in the two target groups and in the reference group. Each polymorphism was either a deletion or an SNP, and their frequencies were consistent with the Hardy-Weinberg equilibrium (Table 1) except the -396_-391 del, -369 C→G and -157 T→C of the Afro-descendant group, which was not calculated because there was only one genotype for each polymorphism. We identified four polymorphisms in the *HBG2* promoter, which were located at positions -396 to -391, -369, -309, and -157. Additionally, we identified eight polymorphisms in the *HBG1* promoter, located at -398, -369, -225 to -222, -271, -324, -317, -309, and -307. All polymorphisms showed a more homogeneous distribution pattern in the Afro-descendant group compared with the SCA and reference groups.

When we compared the frequencies of these polymorphisms between the SCA and reference groups, who are supposed to have similar genetic and environmental backgrounds, we found some interesting differences (Table 2). The 6-bp deletion located between -396 and -391 of the *HBG2* promoter region was three times more frequent among SCA patients than in the reference group. Moreover, the other *HBG2* promoter polymorphisms showed a higher frequency of mutant alleles among SCA patients, which were

the SNPs -369 C→G, -309 A→G and -157 T→C.

The frequencies of the 4-bp deletion located at -225 to -221 and of the SNPs -398 G→A and -369 C→G of *HBG1* were similar for the SCA and reference groups. On the other hand, the T allele for the 271 SNP was more frequent in the SCA group.

New polymorphisms identified in the reference group

We identified four new polymorphisms in the *HBG1* promoter region, which were SNPs located at positions -324 (C→T), -317 (A→G), -309 (A→G), and -307 (G→A) (GeneBank Bankit #1186662). Interestingly, they were found together in a heterozygous pattern within three individuals from the reference group.

The polymorphisms -396_-391 del and -369 SNP in the *HBG2* promoter are associated with the -271 *HBG1* SNP and the -309 SNP *HBG2*, respectively

The chance of finding the mutant allele of the -271 *HBG1* SNP in patients who had the *HBG2* -396_-391 deletion was 20 times higher than in patients who did not have it. The same was true when we combined the -369 and -309 *HBG2* SNPs, but the chance decreased approximately 6-fold in this case. The SCA patients who had the

Table 1. Frequencies of polymorphisms in the *HBG2* and *HBG1* promoter regions of patients with sickle cell anemia (SCA), of individuals from the reference group and of Brazilian Afro-descendants.

Position (genotype)	SCA patients (N = 152)	Reference group (N = 104)	Afro-descendant group (N = 98)
HBG2 -396_-391 del			
ND/ND	90 (59.2)	96 (92.3)	98 (100)
ND/D	50 (32.9)	8 (7.7)	0
D/D	12 (7.9)	0	0
HBG2 -369 SNP			
C/C	75 (49.3)	80 (76.9)	98 (100)
G/C	60 (39.5)	23 (22.1)	0
G/G	17 (11.2)	1 (1.0)	0
HBG2 -309 SNP			
A/A	59 (38.8)	79 (76.0)	95 (96.9)
A/G	65 (42.8)	22 (21.2)	3 (3.1)
G/G	28 (18.4)	3 (2.9)	0
HBG2 -157 SNP			
T/T	0	15 (14.4)	0
T/C	4 (2.6)	47 (45.2)	0
C/C	148 (97.4)	42 (40.4)	98 (100)
HBG1 -398 SNP			
G/G	126 (82.9)	98 (94.2)	95 (96.9)
G/A	25 (17.1)	6 (5.8)	3 (3.1)
A/A	0	0	0
HBG1 -369 SNP			
C/C	2 (1.3)	3 (2.9)	0
C/G	26 (17.1)	23 (22.1)	2 (2.0)
G/G	124 (81.6)	78 (75.0)	96 (98.0)
HBG1 -271 SNP			
C/C	98 (64.5)	85 (81.7)	93 (94.9)
C/T	44 (28.9)	18 (17.3)	5 (5.1)
T/T	10 (6.6)	1 (1.0)	0
HBG1 -225_-221 del			
ND/ND	135 (88.8)	82 (78.8)	66 (67.3)
ND/D	17 (11.2)	20 (19.2)	30 (30.6)
D/D	0	2 (1.9)	2 (2.0)

Data are reported as number wiith percent in parentheses. All *HBG1 and HBG2* polymorphisms were in Hardy-Weinberg equilibrium (chi-square test) except -396_-391 del, -369 C→G and -157 T→C in the *HBG2* gene of the Afro-descendant group, which could not be calculated. D = deletion; ND = no deletion; SNP = single nucleotide polymorphism.

-396_-391 deletion within *HBG2* showed a lower chance of having the associated -369 or -309 *HBG2* SNP, with an odds ratio of 0.07 (95%CI = 0.03-0.17) or 0.14 (95%CI = 0.06-0.31), respectively. Similarly, the possibility of finding the -309 *HBG2* and the -271 *HBG1* SNPs together was dramatically lower than the possibility of finding each SNP alone (Table 3).

The polymorphisms *HBG2* -396_-391 del and -271 *HBG1* SNP are associated with CAR β^S-globin gene haplotype

The frequencies of the β^S-globin gene haplotypes were analyzed in 144 patients, and we found 58 (40.3%) with genotype CAR/Ben, 43 (30%) with Ben/Ben, 32 (22.2%) with CAR/CAR, 4 (2.8%) with Ben/Sen, 4 (2.8%) with Ben/Atypical, 2 (1.4%) with Ben/Cam, and 1 (0.7%) with CAR/Cam. The CAR haplotype was strongly associated with the *HBG2* -396_-391 deletion and with the mutant allele for the -271 *HBG1* SNP (Table 4). However, one CAR/CAR patient did not have the mutant allele for the -271 *HBG1* SNP. Among the heterozygous haplotypes, there were only one CAR/Cam and 27 CAR/Ben with the *HBG2* -396_-391 deletion. The mutant allele for the -271 *HBG1* SNP was present in 21 CAR/Ben patients, but not in other heterozygous haplotypes.

The polymorphisms -369 and -309 SNP in *HBG2* are associated with the Ben β^S-globin gene haplotype

The mutant alleles for the -369 and -309 *HBG2* gene SNP were found in patients with the Ben haplotype. Table 3 shows Ben chromosomes in association with these SNP. Among subjects who had the mutant allele for the -369 *HBG2* SNP, 41 were Ben/Ben, 27 CAR/Ben, 2 Ben/Sen, 2 Ben/Atypical, and 1 CAR/Cam. In addition, the mutant allele for the -309 *HBG2* SNP was present in 43 Ben/Ben, 35 CAR/Ben, 4 Ben/Atypical, 3 Ben/Sen, and 2 Ben/Cam subjects. On the other hand, 1 CAR/CAR patient was heterozygous for the -369 *HBG2* SNP.

Table 2. Differences of *HBG2* and *HBG1* polymorphisms between patients with sickle cell anemia and the reference group.

Polymorphism	SCA patients (N = 152)	Reference group (N = 104)	OR (95%CI)
HBG2 -396_-391 del	27*	4	2.98 (1.83-15.9)
HBG2 -369 SNP (allele G)	47**	13	1.08 (1.6-6.15)
HBG2 -309 SNP (allele G)	60**	14	1.39 (2.18-8.03)
HBG2 -157 SNP (allele C)	150**	66	31.8 (10.1-184.0)
HBG1 -398 SNP (allele A)	13*	3	2.05 (0.87-11.3)
HBG1 -369 SNP (allele G)	137	89	0.60 (0.51-3.3)
HBG1 -271 SNP (allele T)	32**	10	1.36 (1.1-1.67)
HBG1 -225_-221 del	8	12	0.2 (0.17-1.08)

SNP = single nucleotide polymorphism. *P < 0.05 compared to reference group (Fisher test); **P < 0.05 compared to reference group (Yates-corrected χ^2 test).

Table 3. Association of polymorphisms in the *HBG2* and *HBG1* promoter region of patients with sickle cell anemia (SCA, N = 152).

	-271 *HBG1* (TC + TT)	-309 *HBG2* (GA + GG)	-369 *HBG2* (CG + GG)
-396_-391 del *HBG2* (D/D + D/ND)	45	17	15
OR/95%CI	23.8 (9.02-65.0)	0.07 (0.03-0.17)	0.14 (0.06-0.31)
P	<0.001*	<0.001*	<0.001*
-369 *HBG2* (CG + GG)	9	63	
OR/95%CI	0.09 (0.03-0.22)	6.75 (3.01-15.3)	
P	<0.001*	<0.001*	
-309 *HBG2* (GA + GG)	13		
OR/95%CI	0.07 (0.03-0.17)		
P	<0.001*		

Data are reported as number. *Yates-corrected χ^2 test.

The polymorphisms -396_-391 del *HBG2*, -369 SNP *HBG2* and -271 SNP *HBG1* are associated with HbF levels

In order to determine a possible relationship of the *HBG2* and *HBG1* polymorphisms with HbF levels, we classified the patients into four groups according to their HbF levels (Table 5). Most of the patients who did not have the -396_-391 *HBG2* deletion showed HbF >5.0%. The presence of the wild-type allele for the -271 *HBG1* SNP and the mutant allele for the -369 *HBG2* SNP was associated with patients who had HbF levels >5.0%. The -309 *HBG1* SNP and the remaining polymorphisms identified did not show any association with HbF levels (data not shown).

Discussion

The present study identified both previously described and new *HBG2* and *HBG1* polymorphisms in three different population groups. It was believed that four of these polymorphisms may be important to SCA patients because of

Table 4. Association of the β^S-globin gene genotypes with the -396_-391 del *HBG2*, -369 *HBG2*, -309 *HBG2*, and -271 *HBG1* polymorphisms in patients with sickle cell anemia (N = 110).

Polymorphism	CAR (N = 62)	OR (95%CI)	P*	Ben (N = 82)	OR (95%CI)	P*
-396_-391 del *HBG2*						
D/D + D/ND	46	14.8 (6.07-37.0)	<0.001	14	0.09 (0.04-0.21)	<0.001
ND/ND	16			68		
-271 *HBG1*						
TC + TT	42	11.7 (4.57-3.08)	<0.001	11	0.11 (0.04-0.25)	<0.001
CC	19			66		
-369 *HBG2*						
CG + GG	15	0.13 (0.06-0.29)	<0.001	57	7.29 (3.30-16.3)	<0.001
CC	47			23		
-309 *HBG2*						
GA + GG	17	0.07 (0.03-0.17)	<0.001	65	11.9 (4.94-29.3)	<0.001
AA	44			12		

CAR = Central African Republic; BEN = Benin. *Yates-corrected χ^2 test.

Table 5. Association of fetal hemoglobin (HbF) levels with the -396_-391 del *HBG2*, -369 *HBG2*, -309 *HBG2*, and -271 *HBG1* polymorphisms in patients with sickle cell anemia (N = 147).

Polymorphism	HbF levels		OR (95%CI)	P*
	≤5.0%	>5.0%		
-396_-391del *HBG2*				
D/D + D/ND	26 (44.1)	33 (55.9)	3.83 (1.79-8.17)	<0.0001
ND/ND	15 (17.0)	73 (83.0)		
-369 *HBG2*				
CG + GG	13 (17.3)	62 (82.7)	0.32 (0.15-0.70)	0.006
CC	28 (38.9)	44 (61.1)		
-309 *HBG2*				
GA + GG	20 (22.0)	71 (78.0)	0.46 (0.22-0.97)	0.07
AA	21 (37.5)	35 (62.5)		
-271 *HBG1*				
TC + TT	25 (49.0)	26 (51.0)	4.80 (2.23-10.3)	<0.0001
CC	16 (16.7)	80 (83.3)		

Data are reported as number with percent in parentheses. *Yates corrected χ^2 test.

their apparent association with HbF levels and with the β^S-globin gene haplotypes. Moreover, all polymorphisms were found to have a more homogeneous pattern of distribution in the Afro-descendant group, which consisted of individuals with a lower rate of racial admixture. This group was formed by descendants of two African slave populations living in Northern Brazil, a different region from that of the other two groups. Historical data suggest that about 90% of the slaves brought to Northern Brazil were from Angola, Congo and Mozambique (14). Both the SCA group and the reference group were from Bahia, Brazil, and both were composed of individuals with high rates of racial admixture. An estimated rate of 97% interethnic panmixia has been shown in Bahia, possibly due to the intensive African slave trade during which more than five million people from several African countries were brought to Brazil between 1550 and 1850, mainly between the 18th and 19th centuries (15). Moreover, Bahia was the only Brazilian State that restricted slave traffic through Ecuador, a fact that explains the correlation between genotype frequencies found in Bahia and Western Africa, principally the Bay of Benin region (10). These facts may explain the different frequencies of all polymorphisms found in the present study.

When we compared the polymorphisms of the *HBG2* and *HBG1* genes between the SCA group and the reference groups we found five of them with a different distribution in their genotypes. Since both groups had similar genetic and environmental backgrounds, it was believed that these differences could be related to the β^S chromosomes. Thus, the -396_-391 deletion and the -369 C→G and -309 A→G SNPs in the *HBG2* promoter might be associated with the polymorphic sites linked to the β^S haplotypes before the occurrence of this mutation. Although the age of the β^S-globin gene mutation is not known with certainty, expansion of the mutation probably occurred in parallel to malaria, a strong selective factor, becoming endemic on the African continent (16). The -157 *HBG2* SNP was recently described in SCA patients from the same region of Brazil, and it was suggested that the C allele at this position may be related to HbF levels (6). In contrast to the report of Adorno et al. (6), we found that 100% of healthy Afro-descendant individuals and about 90% of the SCA and reference group subjects had the variant allele for the -157 *HBG2* SNP. Hence, this SNP seems to be simply an ethnic marker, and may not be related to HbF levels.

The -271 C→T SNP in the *HBG1* promoter has been described in previous reports (2,16) to be exclusively located in β^S chromosomes. However, in the present study, this SNP was also present among the reference and Afro-descendant groups. All 5 Afro-descendant individuals who had the T allele (mutant) were heterozygous for the β^S mutation. On the other hand, 19 β^A individuals from the reference group had this mutant allele instead of the C, which may be a result of a crossing over event between two heterozygous individuals for the β^S mutation. Additionally, the SNP in *HBG1* may be considered a gene conversion of a β^S CAR haplotype because of *HBG2*-like sequences between bases -306 and -271 and extending to bases 25-1107, which have replaced the corresponding *HBG1* sequence (17).

The four new SNPs in the *HBG1* promoter (-324 C→T, -317 A→G, -309 A→G and -307 G→A) identified in the reference group may have origins in Europe or among the Amerindians since they were not found in the two other groups studied. Studies are necessary to confirm this explanation and to determine the genetic impact of these polymorphisms on *HBG1* expression.

When the odds of finding the -396_-391 deletion in *HBG2* together with the -271 *HBG2* SNP were measured, a strong association between these two polymorphisms was identified. There was also an important association between the -369 and -309 SNPs in *HBG2*. These facts may indicate a similar origin of these closely associated polymorphisms or a possible association with the β^S-globin haplotypes.

The major sequence differences in the *HBG2* promoter region are in the β^S chromosomes with the haplotypes CAR (-396_-391 deletion) and Ben (-369 and -309 SNPs). In addition, the -271 SNP in the *HBG1* promoter was also strongly associated with CAR chromosomes. Only one CAR/CAR patient did not show this association, and our results are different from those obtained in previous studies (2,16) in which the investigators did not detect any non-CAR haplotype associated with the mutant allele for the -271 SNP in *HBG1*. This difference may be explained by multiple recombinations between heterozygous CAR and Ben haplotypes.

Finally, we stratified the SCA patients into two groups according to HbF levels to investigate the correlation between these levels and the polymorphisms -396_-391 del *HBG2*, -369 SNP *HBG2*, -309 SNP *HBG2* and -271 SNP *HBG1*, as shown in Table 5. Most of patients who did not have the -396_-391 del in the *HBG2* gene had HbF levels >5.0 ($P < 0.0001$). The absence of the mutant allele (T) for the -271 SNP in the *HBG1* gene also correlated with higher HbF levels ($P < 0.0001$). Conversely, the presence of the mutant allele (G) for the -369 SNP in the *HBG2* gene correlated with higher levels of HbF ($P = 0.006$). The other polymorphisms did not show any relationship with HbF levels. These results are different from those reported by Bouhassira et al. (17) and by Lanclos et al. (2), but both groups of patients previously studied were African-American who had a genetic background different from the Brazilian SCA patients. Moreover, these gene polymorphisms may be part of other molecular events that act synergistically on HbF expression.

Five patients were excluded from these analyses because they were younger than 6 years and this fact may have influenced the results. At birth, HbF represents 80-90% of the total hemoglobin synthesized and gradually decreases to 1% by 10 months in normal infants, but not in SCA children, who may have higher HbF levels up to the

age of 6 years (18).

Further studies will be necessary to clarify the complex interaction between these specific sequences and certain trans-activating factors within specific developmental stages of erythroid cells and its possible impact on the clinical outcome of SCA patients.

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