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Fetal Hemoglobin in Sickle Cell Anemia: Genetic Studies of the Arab-Indian Haplotype

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Abstract

Sickle cell anemia is common in the Middle East and India where the HbS gene is sometimes associated with the Arab-Indian (AI) β -globin gene (*HBB*) cluster haplotype. In this haplotype of sickle cell anemia, fetal hemoglobin (HbF) levels are 3-4 fold higher than those found in patients with HbS haplotypes of African origin. Little is known about the genetic elements that modulate HbF in AI haplotype patients. We therefore studied Saudi HbS homozygotes with the AI haplotype (mean HbF 19.2±7.0%, range 3.6 to 39.6%) and known genotyped cis- and trans-acting elements associated with HbF expression. All cases, regardless of HbF concentration, were homozygous for AI haplotype-specific elements cis to *HBB*. SNPs in *BCL11A* and *HBSIL-MYB* that were associated with HbF in other populations explained only 8.8% of the variation of HbF. *KLF1* polymorphisms associated previously with high HbF were not present in the 44 patients tested. The SNPs and genetic loci we have chosen for this study do not explain the high HbF in sickle cell patients with AI haplotype or its variation among patients with this haplotype. The dispersion of HbF levels among AI haplotype patients suggests that other genetic elements modulate the effects of the known cis- and trans-acting regulators. These regulatory elements, which remain to be

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discovered, might be specific in the Saudi and some other populations where HbF levels are especially high.

Introduction

Fetal hemoglobin (HbF) protects against many of the hematologic and clinical complications of sickle cell anemia [[homozygosity for the sickle hemoglobin (HbS) gene; *HBB* glu6val; reviewed in(1)]. This is dependent on the ability of HbF to hinder deoxyHbS polymerization. HbF level is variable among patients and populations with sickle cell anemia and is highly heritable.(2, 3) HbF expression is regulated by elements linked to the β -globin gene (*HBB*) gene cluster (11p15.5) and other quantitative trait loci (QTL) in trans to *HBB*, two of which are the *HBSIL-MYB* intergenic region on chromosome 6q22-23 and *BCL11A* on chromosome 2p16.1. Together, these QTL accounted for 15 to 30% of HbF variation in sickle cell anemia patients with African origins of the sickle β -globin gene.(2, 4-8)

The HbS gene is also autochthonous to the Middle East and India where it is sometimes on an indigenous Arab-Indian (AI) *HBB* globin gene cluster haplotype.(9-11) This haplotype is marked by an Xmn1 restriction site polymorphism (C>T 158 bp 5' to *HBG2*; rs7482144) and other single nucleotide polymorphisms (SNPs) and insertion-deletion polymorphisms that distinguish it from all African-origin haplotypes, including the Senegal haplotype that also has the Xmn1 restriction site polymorphism. Individuals with sickle cell anemia and the AI haplotype had higher mean HbF levels [average 17%, range 4-32%, reviewed in(11)] than patients with HbS haplotypes of African origin.(12) For example, untreated African Americans had mean HbF levels of 5.8% in males and 7.3% in females with mean ages of about 17 years.(6) Because of their higher HbF, AI haplotype sickle cell anemia patients had milder, albeit not asymptomatic disease when compared with carriers of African *HBB* haplotypes.(13, 14) As the QTL modifying HbF levels in sickle cell anemia patients with the AI haplotype have not been comprehensively studied, we genotyped the major known HbF-modulating QTL in 137 individuals and additional known cis- and trans-acting elements in subsets of these patients to study their association with HbF.

Methods

Patients

Subjects with sickle cell anemia who attended clinics at King Fahd Hospital, Al-Ahsa and King Saud University, Riyadh, Saudi Arabia were selected on the basis of homozygosity for the HbS gene and the AI haplotype, age of at least 10 years; they were not taking hydroxyurea at the time HbF was measured. HbF was measured by high performance liquid chromatography (HPLC).

HbS and the HBB haplotype

Homozygosity for the HbS mutation was confirmed using amplification refractory mutation system analysis (Table S1).(15) The AI haplotype was ascertained by genotyping the *HBG2* Xmn1 C>T restriction site (rs7482144) and a Hinc2 site 5' to *HBE1* (rs3834466), and confirmed by the presence of a C>T polymorphism 68 bp 5' to *HBD*.(16)

Sanger sequencing/TaqMan assay of selected cis- and trans- regions to β^S -globin gene

Polymorphisms in the β -globin gene cluster region were detected by Sanger sequencing or TaqMan assays in HbS homozygote with the Arab Indian haplotype. For many of these assays we selected a smaller group for testing. Selection was based on availability of

samples at time of testing or at random from the subjects we had confirmed as homozygous for HbS and the AI haplotype.

HBB gene cluster regulatory regions (11p15): (Table S1)—Regions selected for sequencing were based on their potential functional role in globin gene regulation and included the *KLF1* binding site in the *HBB* promoter region, an AT motif 530 bp 5' to *HBB*, TTTTA repeats 1412 bp 5' to *HBB*, the *HBD-HBG1* intergenic region, promoters of *HBG2* and *HBG1*, and the cores of hypersensitive sites 2, 3 and 4 (HS-2, 3, 4) of the β -globin gene cluster locus control region (LCR). A -68 *HBD* C>T SNP in the promoter of *HBD* was detected using a custom designed TaqMan assay.

BCL11A, HBS1L-MYB, KLF1, DLX4—We genotyped SNPs in *BCL11A* and *HBS1L-MYB* using either pre-made or custom TaqMan Assays (Applied Biosystems). As *KLF1* is a known regulator of *BCL11A* and globin switching, and has been associated with the phenotype of hereditary persistence of HbF, and as BP1 (*DLX4*) binds to AT motif 530 bp 5' to *HBB* and has a down-regulatory effect on *HBB* expression, we sequenced *KLF1* (n=44) and *BPI* (*DLX4*) (n=23) in randomly selected cases, to exclude polymorphisms in these genes that might be associated with HbF levels.(17)

Statistical analysis

Linear regression was performed on HbF for each genetic locus, adjusting for gender of the subjects. No transformation of the HbF values was necessary as the HbF values of these patients were approximately normally distributed. The analysis was performed using an additive genetic model whereby the total number of minor alleles present was counted for each subject. A 2-sample Kolmogorov-Smirnov test was used to compare the distribution of HbF in patients enrolled in Cooperative Study of Sickle Cell Disease (CSSCD) and in patients with the AI haplotype from Saudi Arabia.(18)

Results

HbF

One hundred and thirty-seven sickle cell anemia patients who met our selection criteria were initially examined (Table 1) and their distribution of HbF concentrations is shown in Figure 1. Mean HbF was $19.2 \pm 7.0\%$. For comparison, African Americans with sickle cell anemia had a mean HbF of $6.6 \pm 5.5\%$. The HbF distribution for African Americans with sickle cell anemia was right skewed, whereas the AI haplotype subjects had a Gaussian or normal distribution. The distributions of HbF in these 2 cohorts were significantly different (p-value $2.2e-16$).

Sanger sequencing/TaqMan assays

BCL11A and HBS1L-MYB—Table 2 shows the associations of polymorphisms in *BCL11A* and the *HBS1L-MYB* intergenic region and HbF in 137 patients. The 2.9 kb region in the 2nd intron of *BCL11A* bounded by rs1427407 and rs4671393 was associated with HbF as suggested previously (4-8), but accounted for only 7.5% of the variation in HbF. Together, *BCL11A* and *HBS1L-MYB* accounted for 8.8% of HbF variance. The remaining polymorphisms we examined were done in subsets of these patients.

HBB-HBD intergenic region—Mutations within *HBB-HBD* region have been linked to elevated HbF.(17, 19) We investigated polymorphisms within this region to further characterize the AI haplotype and to identify candidates that may be associated with elevated HbF. An AT motif, (AC)₂(AT)₉(T)₅, 530 bp 5' to *HBB* is found with the AI

haplotype and postulated to play a role in ameliorating the sickle phenotype due to suppression of *HBB* by binding the repressor protein BP1.(17) In 6 AI haplotype homozygotes that we examined, all had the previously described AI haplotype AT repeat motif. (17)

In 35 patients, the (TTTTA)₆ insertion 1412 bp 5' of *HBB* was present. In previous studies, Benin haplotype cases were reported to have a (TTTTA)₅ insertion (rs61168339; Table 3).

In 6 cases with a mean HbF level of 24.5%, the sequence of the KLF1 binding site in the *HBB* promoter (CCACACCCT) was identical to the reference sequence (GenBank U01317) suggesting a lack of novel polymorphisms that could account for the uniquely elevated HbF found in the AI haplotype.

A C>T SNP 68 bp 5' to *HBD* that we previously found to be a specific marker for the AI haplotype was homozygous in all 137 cases.(16)

***HBG1* and *HBG2* promoters**—Thirty patients were screened for promoter mutations. One polymorphism, a C>G 369 bp 5' to *HBG1* (rs2855040), was identified in the *HBG1* promoter region and a 4 bp (AAGC) insertion at 222 bp 5' to *HBG1* was found. These findings are not unique to sickle patients with the AI haplotype and are therefore unlikely to have a significant role in modulating the elevated HbF that distinguishes the AI haplotypes from other *HBB* haplotypes. The *HBG2* promoter contained the Xmn1 polymorphism, C>T SNP 158 bp 5' to *HBG2* (rs7482144), which is a marker for the AI haplotype.

LCR core regions of HS-2, 3, and 4—Thirty patients were studied. The core regions of HS-3 and HS-4 were identical to the GenBank U01317 reference sequences. In the core of HS-2, the unique TA₁₀(CA)₂(TA)₂CG(TA)₁₂ motif known to be typical of the AI haplotype, was present.

KLF1—Forty-four cases with HbF levels between 9 and 30% were studied. Table 4 shows the HbF levels and the SNPs detected in *KLF1* exons. None of the SNPs in *KLF1* we studied showed an association with elevated HbF in AI haplotype patients and the polymorphisms we detected were “neutral.” SNPs previously associated with increased HbF were not present.(20-22) There was no significant difference in HbF level between the 12 patients, who were heterozygous for the promoter nt -188 C>G polymorphism (rs3817621) and had HbF of 13.2±6.8%, compared with the 10 cases without the same promoter polymorphism with HbF of 16.4±4.5%.

DLX4 (BP1)—Twenty-three patients were studied: 4 were heterozygous for SNP rs58769681 (G>A) in the 5' UTR of *DLX4*; 2 were heterozygous for rs61749026 (A>G; N44S); no mutation was present in the remaining 17 patients.

Discussion

The distribution of HbF concentrations in AI haplotype sickle cell anemia was significantly different than that of patients with African-derived *HBB* haplotypes suggesting differential regulation of *HBG2* and *HBG1* in these genetically distinct populations (Figure 1). We genotyped the known cis- and trans-acting elements associated with HbF regulation to try and explain these differences.

Cis-acting elements

Unique polymorphisms cis to *HBB* and associated with the HbS gene distinguish the AI from other haplotypes. Every patient was homozygous for the cis-acting elements we

examined suggesting that although these elements are linked to the sequences, or are the sequences causing high levels of expression of the γ -globin genes, they alone cannot account for the heterogeneity of HbF among AI haplotype carriers.

We confirmed the presence of the (AC)₂(AT)₉(T)₅ motif in the *HBB-HBD* intergenic region. This is a binding site for BP1 (*DLX4*), which binds more tightly to this region in the AI haplotype compared with other haplotypes, and reduces β^S -globin gene expression.(17, 23) This particular AT repeat motif was associated with the lowest *HBB* mRNA expression in differentiated MEL cells.(24) A similar AT motif was associated with β thalassemia in carriers without a detectable mutation in *HBB*.(25, 26) *HBB* suppression might play a role in the high level of γ -globin gene expression found with the AI haplotype. The (TTTTA)₆ insertion 1412 bp upstream of *HBB* is also seen with the AI haplotype but the functional significance of this insertion/deletion polymorphism is unknown.(27) Functional studies may help to clarify whether the differences in the copy number of this motif can explain the HbF difference between these two *HBB* haplotypes.

The discovery of the Corfu $\delta\beta$ thalassemia deletion first suggested that the *HBD-HBG1* intergenic region had a potential role in activating the γ -globin genes and repressing the downstream β -globin gene.(19) A 3.5 kb region near the 5' portion of *HBD* binds the *HBG* silencer BCL11A, GATA-1 and HDAC1. Its deletion delayed *HBG* to *HBB* switching.(28) A functional C>T SNP 68 bp 5' to *HBD* that is a specific marker for the AI haplotype was previously reported; whether this affects *HBG* expression is unknown.(16)

The -158 C>T SNP (rs7482144) is present in the AI haplotype and is associated with high *HBG2* expression in this haplotype and the African-derived Senegal haplotype. However, the functional role of this polymorphism is ill-defined.(29, 30) Variation in HS-2 of the LCR has been reported to be associated with HbF levels in sickle cell anemia.(31) Further studies are needed to clarify whether or not the HS-2 signature sequence found in the AI haplotype plays a role in γ -globin gene regulation.

Trans-acting elements

QTL on chr6q22-23 (*HBSIL-MYB*)(32, 33) and chr2p16.1 (*BCL11A*) (2, 4, 6, 34) modulate *HBG* expression in all populations studied, albeit with different effect sizes. SNPs in these loci accounted for 15 to 30% of the HbF phenotypic variation in African American sickle cell anemia.(2, 4-7, 35) Among AI haplotype homozygotes, variants in *BCL11A* accounted for 7.5% of HbF variance. SNPs in *HBSIL-MYB* that have been associated with HbF level in other populations were not associated with HbF in the AI haplotype. However the minor allele frequencies of these *HBSIL-MYB* SNPs were about 1% so that a SNP of importance is likely to be rare and have a small effect in this population. It remains possible that AI haplotype patients with high HbF have novel functional in *BCL11A* and *HBSIL-MYB* that are not tracked by the SNPs we genotyped. Alternatively, SNPs in these genes play a lesser role in HbF variance in the AI haplotype than in African haplotypes.

KLF1, a regulator of *BCL11A*, also mediates hemoglobin switching.(22, 36) Its polymorphisms have been associated with the phenotype of hereditary persistence of HbF.(20, 22, 37) We did not find mutations in the *KLF1* zinc finger domains that were previously associated with increased HbF in the 44 cases we studied for these polymorphisms. The SNPs that were present did not show a correlation with HbF in the AI haplotype patients we studied, and were likely to be "neutral." Although a few SNPs were found in *BP1 (DLX4)* in some patients, their role in HbF regulation is unclear.

Rarely, African American HbS homozygotes can have a baseline HbF similar to that associated with the AI haplotype. These individuals more commonly have the minor alleles

of *BCL11A* and the *HBS1L-MYB* intergenic region compared with counterparts with low HbF.(38) In some instances, rare missense mutations in *MYB* have been associated with higher HbF in African Americans with sickle cell anemia.(2) We did not test these specific variants in AI haplotype patients.

Conclusions

To increase our understanding of HbF regulation in AI haplotype HbS homozygotes we compared polymorphisms in selected regions that define this haplotype and also examined known trans acting elements associated with HbF expression. Although all polymorphisms were not studied in all patients, previous studies of the AI haplotype and our results suggest that any variants in these sequences that might be detected if a larger patient sample were to be examined are apt to be rare.

HbF levels are determined by interactions of cis and trans acting elements and are likely to be, modulated by stress erythropoiesis.(39, 40) Carriers of the AI haplotype and sickle cell trait have nearly normal HbF levels(11) suggesting that homozygosity for the AI haplotype and/or the presence of hemolytic anemia is required for the clinical expression of the high HbF phenotype. Some elements cis to *HBB* in the AI haplotype, like the -530 AT motif, down-regulate *HBB*; others, such as the -68 C>T *HBD* promoter SNP might down regulate *HBD* expression.(16, 17) Coupled with continued expression of *HBG2*, this might reduce post-transcriptional competition and enhance the stability of γ -globin RNA.(41, 42) Known trans-acting QTL accounted for only 8.8% of HbF variance in this population and SNPs in *BCL11A* explained only 7.5% of the variance, or about half that found in the largest reported study of African-origin patients. (4) Additional QTL could have evolved in the Arab population and might be discovered by sufficiently powered genome-wide association studies or by whole genome sequencing of highly selected patients. Determining transcription factor binding patterns and histone modification in the *HBB* gene cluster region in AI haplotype cases could provide a better understanding of the influence of different cis acting elements on HbF globin gene expression.

The variation in HbF among patients with the AI haplotype suggests that other genetic elements modulate the effects of the known cis- and trans-acting regulators. These regulatory elements, which remain to be discovered, might be specific for the Saudi and some other populations where HbF levels are especially high.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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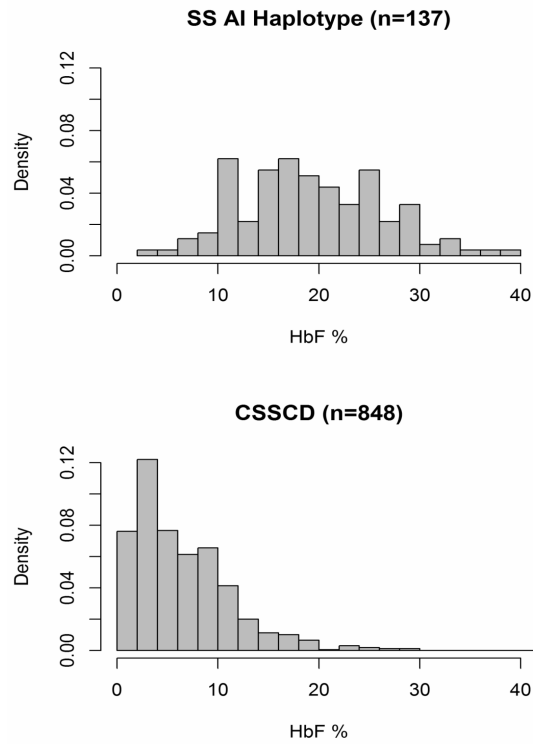


Figure 1. Density plots showing the distribution of HbF in sickle cell anemia with the AI haplotype (SS AI Haplotype)
 For comparison, results from the Cooperative Study of Sickle Cell Disease (CSSCD), an African American sickle cell anemia population are replotted.⁽⁶⁾ All CSSCD subjects were aged ≤ 5 years and none were taking hydroxyurea. These distributions are significantly different ($p=2.2e-16$).

Table 1

A comparison of age and HbF in patients with sickle cell anemia (HbSS) and the Arab-Indian (AI) haplotype.

Category	HbSS AI Haplotype n = 137			CSSCD n = 848			P-value
	Mean	SD	Range	Mean	SD	Range	
Age (yrs)	26.6	10.6	11 - 65	17.2	10.7	5 - 63	<0.001
HbF (%)	19.2	7.0	3.6 – 39.6	6.6	5.5	0.1 – 60	<0.001

Patients with sickle cell anemia from the Cooperative Study of Sickle Cell Database (CSSCD) served as reference in the statistical analysis. Although the CSSCD cohort was slightly younger on average, the HbF difference remains significant as age has an inverse relationship with HbF. Only subjects age 10 or older were included in this analysis as HbF changes are minimal after that age cutoff.

Table 2

Quantitative trait loci (QTLs) and association with HbF in subjects homozygous for HbS with Arab-Indian haplotype.

Gene	SNP	Chr. Position	Major Allele	Minor Allele	SS AI Haplotype (n=137)			
					MAF	Beta	SE	P
<i>BCL11A</i>	rs6732518	60708847	C	T	0.463	1.280	0.827	0.124
<i>BCL11A</i>	rs1427407	60718293	G	T	0.283	2.451	0.891	0.007*
<i>BCL11A</i>	rs766432	60719970	A	C	0.300	2.769	0.864	0.002*
<i>BCL11A</i>	rs11886868	60720496	T	C	0.455	1.773	0.807	0.030*
<i>BCL11A</i>	rs4671393	60721201	G	A	0.301	2.697	0.859	0.002*
<i>BCL11A</i>	rs6729815	60723922	T	C	0.471	-0.161	0.843	0.849
HMIP	rs7775698	135418385	G	A	0.056	-0.991	1.811	0.585
HMIP	rs9399137	135418768	T	C	0.073	1.358	1.621	0.404
HMIP	rs4895441	135426323	A	G	0.090	0.703	1.593	0.660
HMIP	rs9402686	135427567	G	A	0.091	0.699	1.489	0.640
HMIP	rs73555746	135521239	A	C	0.004	5.583	7.085	0.432

QTLs found to be significantly associated with HbF are denoted with *. MAF = minor allele frequency; Beta = effect size on HbF levels; SE=standard error; P= p-value.

Table 3

Polymorphisms in globin gene cluster region detected by Sanger sequencing or TaqMan assays in homozygous sickle cell anemia patients with the Arab Indian haplotype.

Location or Gene (# screened)	Polymorphism or Sequence
<i>HBB-HBD</i> intergenic region	
-530 5' to <i>HBB</i> (6)	(AC) ₂ (AT) ₉ T ₅
-1412 5' to <i>HBB</i> (35)	G(TTTTA) ₆ TTTTG
<i>HBD-HBG1</i> intergenic region	
-68 5' to <i>HBD</i> (137)	C>T
<i>HBG1</i> promoter	
-222 5' to <i>HBG1</i> (30)	AAGC insertion
<i>HBG2</i> promoter	
-158 5' to <i>HBG2</i> (137)	C>T
LCR	
HS-2 Core (30)	(TA) ₁₀ (CA) ₂ (TA) ₂ CG(TA) ₁₂

Table 4*KLF1* polymorphisms in sickle cell anemia.

M39L		S102P		F182L		Number	Gender (M/F)	Age (yrs.) (Mean±SD)	HbF (Mean±SD)
homo	hetero	homo	hetero	homo	hetero				
-	-	+	-	-	-	2	1/1	17.5±2.1	15.3±5.2
-	-	-	+	-	-	17	11/6	26.2±10.4	16.3±7.2
-	+	-	+	-	-	3	1/2	30±10.5	9.3±0.6
-	-	-	+	-	+	2	0/2	37±24	26.1±4.0
-	-	+	-	-	+	1	0/1	25	23
-	-	-	-	-	-	18	11/7	24.8±7.3	15.6±5.1

* S102P: T>C (rs2072597); M39L: A>C (rs112631212); F182L: T>C (rs2072596)