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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 employed targeted genotyping of polymorphic sites to explore cis- and trans- acting elements associated with high HbF expression. We also described sequences which appear to be unique to the AI haplotype for which future functional studies are needed to further define their role in HbF modulation. All cases, regardless of HbF concentration, were homozygous for AI haplotype-specific elements cis to *HBB*. SNPs in *BCL11A* and *HBS1L-MYB* that were associated with HbF in other populations explained only 8.8% of the variation in HbF. *KLF1* polymorphisms associated previously with high HbF were not present in the 44 patients tested. More than 90% of the HbF variance in sickle cell patients with the AI haplotype remains unexplained by the genetic loci that we studied. 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 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 (QTLs) in trans to *HBB*, two of which are the *HBS1L-MYB* intergenic region on chromosome 6q22-23 and *BCL11A* on chromosome 2p16.1. Together, these QTLs 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

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[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 QTLs modifying HbF levels in sickle cell anemia patients with the AI haplotype have not been comprehensively studied, we genotyped the major known HbF-modulating QTLs 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 Fahad 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 5 years. These patients 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 homozygotes 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 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 an AT motif 530 bp 5′ to *HBB* and has a down-regulatory effect on *HBB* expression, we sequenced *KLF1* (n = 44) and *BP1* (*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 Fig. 1. Mean HbF was 19.2 ± 7.0%. For comparison, African Americans with sickle cell anemia had a mean HbF of 6.6 ± 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 with AI haplotype, a $(TTTTA)_6$ insertion 1412 bp 5′ of *HBB* was present. For comparison, we sequenced 8 Benin haplotype cases and found these patients had 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