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Polymorphic variations influencing fetal hemoglobin levels: Association study in beta-thalassemia carriers and in normal individuals of Portuguese origin

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ABSTRACT

Three major loci have been associated with HbF levels, including −158C/T (XmnI) at *HBG2* promoter region, and several polymorphisms at *BCL11A* intron-2 and *HBS1L-MYB* (HMIP) intergenic region. Mutations in the KLF1 gene were recently associated with increased HbF levels.

This study aims to evaluate whether genetic variability at these loci influences HbF levels in β-thalassemia carriers and in normal individuals of Portuguese origin.

Sixty five β-thalassemia carriers, HbF levels ranging from 0.2% to 9.5%, and 60 individuals with normal hematological parameters, HbF levels ranging from 0.2% to 7.4%, were selected for this study.

In β-thal carriers linear regression models revealed a strong statistical significant association for *HBG2* (XmnI) rs7482144 ($\beta = 0.455$; $P = 5.858 \times 10^{-7}$), and nominal significance for *BCL11A* rs766432 ($\beta = 0.215$; $P = 0.029$) and *HMIP* rs9399137 ($\beta = 0.209$; $P = 0.011$). In normal individuals, a case (HbF > 2%; n = 15) *vs.* control (HbF < 1.7%; n = 45) model, showed nominal significant associations for *BCL11A* SNPs rs11886868 (OR = 4; P = 0.001), rs766432 (OR = 3.7; P = 0.002) and rs7606173 (OR = 0.36; P = 0.032). *KLF1* rs3817621 was not found associated with HbF levels.

Our results suggest that in Portuguese β-thal carriers the *HBG2* XmnI polymorphism is strongly associated with HbF levels. In normal individuals, *BCL11A* polymorphisms, but not *HMIP* or *HBG2* (XmnI) *loci*, are nominally associated with HbF expression.

1. Introduction

Fetal hemoglobin (HbF, $\alpha 2\gamma 2$) levels vary among individuals as a consequence of genetic variation. The identification of modulators responsible for the reactivation of gamma-globin genes has been extensively studied, since high levels of HbF contribute to ameliorate the clinical manifestations and severity of sickle cell anemia (SCA) and β-thalassemia (β-thal) [1,2]. Thus, the understanding of these modulation factors and how the fetal to adult (γ to β) globin chain switch occurs in the course of human ontogeny could be important to identify targets for the development of new therapies to improve the treatment of these hemoglobinopathies [1,2].

Recent genome wide association studies (GWAS), conducted in both healthy nonanemic populations and in patients with sickle cell disease or beta thalassemia of European, African or Asian descent, have identified several single nucleotide polymorphism (SNP) variants along the genome associated with HbF levels (or the highly correlated F-cells number). The three important HbF level loci determinants identified were: i) SNPs at promoter nucleotide of *HBG2* on chromosome 11p15, including the −158 C/T (rs7482144) variant also known as XmnI; ii) SNP variants located at the *HBS1L-MYB* intergenic region (HMIP) on chromosome 6q23, and iii) SNPs located at the *BCL11A* gene on chromosome 2p16 [1,3–5]. The erythroid transcription factor KLF1 activates BCL11A and assists in coordinating the switch from fetal to adult hemoglobin [6]. Individuals with loss-of-function KLF1 mutations were known to have persistently elevated HbF [7–9].

A modest elevation of HbF (1–5%), called heterocellular hereditary persistence of fetal hemoglobin (HPFH) or Swiss type HPFH, is observed in 10–15% of apparently normal individuals in the absence of any hematological disorder [10]. This condition had been regarded as a multifactorial quantitative trait linked to polymorphic variations in regulatory sequences of the γ- and β-globin genes, especially the −158 C > T polymorphism [11–13], or in introns of γ-genes, but also with other loci mapping elsewhere in the genome (Xp 22.2–22.3 region, 6q23 and 8q) [14–16].

In beta-thalassemia, the synthesis of the β-globin polypeptide is reduced as a result of mutations in this gene, leading to an excess of alpha-

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globin chains which precipitate in red blood cell precursors, contributing to dyserythropoiesis and premature cells death [17]. Ineffective production of mature red blood cells in homozygotes or compound heterozygotes for β-globin mutations leads to anemia with severely clinical manifestations (β-thalassemia major and β-thalassemia intermedia) [17]. Increased production of γ-globin chains can modulate the disease severity by reducing the number of free α-chains, which decreases the dyserythropoiesis [18,19]. Carriers of β-thalassemia (β-thal minor) have a mild asymptomatic anemia with no clinical symptoms. Several genetic association studies have been performed in β-thal carriers across different populations and have identified sequence variants associated with the HbF levels [20–23].

Nevertheless, results regarding the genetic association basis for both conditions, β-thal carriers and HPFH, are not entirely clear, and some loci previously identified in patients with severe hemoglobinopathies have not yet been assessed in such individuals. Thus, the aim of this study was to evaluate whether genetic variability in *loci BCL11A*, *HMIP*, *HBG2* and *KLF1* are associated with HbF levels in normal subjects with common forms of HPFH and in β-thal carriers of Portuguese origin.

2. Material and methods

2.1. Study population

A total of 125 unrelated subjects of Portuguese origin were recruited for this study, including heterozygous for beta thalassemia mutations and *HBB* wild-type.

Sixty five (35 females and 30 males) were beta thalassemia carriers, aged 2–77 years (mean age 34.3 y; median age 35 y), with HbF levels ranging from 0.2% to 9.5% and HbA2 levels raised between 3.4% and 6.8% (mean 4.78%). These subjects classified as β-thal *minor* were heterozygous for one of the following mutations in the *HBB* gene [c.118C>T (p.Glu40term); c.48G>A (p.Trp16term); c.92+6T>C; c.126_129delCTTT; c.92+1G>A; cd.92+110G>A].

A second population group of 60 subjects (34 females; 26 males), aged 2–75 years (mean age 29.87 y; median age 30 y), with normal hematological parameters, HbF levels ranging from 0.2% to 7.4% and normal HbA2, was selected: a control group of randomly recruited subjects (n = 35) with normal HbF levels ranging from 0.2% to 1.6%, and 15 subjects with HbF levels >2%, classified as HPFH.

Informed consent was provided by all the participants. HbF and HbA2 levels were determined by high performance liquid chromatography (HPLC) using Variant 2 (Bio-Rad, CA, USA).

2.2. Genotyping

Seven SNPs were chosen for this study based on the recent publications in PubMed reporting genetic variants most strongly associated with increased HbF levels: loci *BCL11A* (rs11886868, rs766432 and rs7606173), HBS1L-MYB (*HMIP*) (rs9399137 and rs6934903), *HBG2* (rs7482144), and *KLF1* (rs3817621). SNPs rs11886868, rs7606173, rs9399137 were genotyped by TaqMan assay using pre-designed probes (Applied Biosystems, Foster City, USA). Allelic discrimination was performed according to the manufacturer's instruction on a BioRad RT-PCR system (MiniOpticon, BioRad, CA, USA). SNPs rs766432, rs6934903, rs7482144 and rs3817621 were genotyped by PCR-RFLP using the restriction enzymes AccI, HpyCH4III, XmnI, and AciI, respectively. To assess genotyping reproducibility, 10% of random samples were re-genotyped for all SNPs with 100% concordance.

A commercially available kit (SALSA MLPA kit P102-B2 HBB), using multiplex ligation-dependent probe amplification (MLPA), was used to screen for deletions in the beta-globin cluster for individuals with HbF levels >5%.

2.3. Statistical analysis

The allelic and genotypic frequencies of all polymorphisms were estimated by direct counting and Hardy–Weinberg equilibrium probability values were achieved using an exact test. Associations of SNPs with HbF levels, after logarithmic transformation to near normalize the quantitative trait distribution, were performed: i) in the β-thal *minor* group by linear regression under an additive genetic model; ii) in the normal subjects by a case-control model (subjects with HPFH *vs.* subjects with normal HbF), using 2% HbF as cutoff-point, estimating P values, odds ratio (OR), and 95% confidence intervals (CI), crude and with age and sex as covariates. All these statistical analyses were done using the set-based tests implemented on PLINK software v.1.07 (http://pngu.mgh.harvard.edu/purcell/plink/) [24].

Graphical analyses, normality of the data assessed by the Kolmogorov–Smirnov test and comparisons of HbF levels between genotypes by using non-parametric (Mann–Whitney U for β-thal carriers) or parametric (one way ANOVA, followed by post hoc Bonferroni test for normal individuals) tests according to the distribution of data, were performed with the SPSS software, version 20.

A significant P-value was considered below 7.1×10^{-3} (0.05/7) by applying a Bonferroni correction for multiple testing, and a P-value below 0.05 was considered significant for individual SNPs.

3. Results

The demographic characteristics and hematological parameters of the populations under investigation are shown in Table 1.

3.1. Beta thalassemia carriers

Regarding the β-thal *minor* group, total genotyping rate was 0.942. The minor allele frequencies (MAF) observed for the seven polymorphisms in the total sample were displayed in Table 2. The genotype distributions were in agreement with the Hardy–Weinberg equilibrium (P > 0.05). Deletional mutations in the β-genes cluster were excluded in individuals with HbF levels >5%.

Using a dominant model (genotypes homozygous for the ancestral allele *versus* homozygous and heterozygous for the derived allele), we analyzed the distribution of log transformed HbF values according to SNP genotypes by the Mann–Whitney U test (Table S1). Significant differences were observed for SNPs *BCL11A* rs766432 (P = 0.034), HMIP

Table 1
Description of demographic and hematological data (mean ± SD) in the two population groups studied: beta-thalassemia carriers and subjects with normal hematological parameters.

Characteristics	β-thal minor	Normal individuals
Age (mean ± SD) (years old)	33.25 (±20.6)	29.87 (±20.8)
Age (range) (years old)	2–77	2–75
Males (n)	30	26
Females (n)	35	34
Hematological parameters		
HbF (%)	1.35 (±1.02)	1.36 (±1.61)
HbF (range; %)	0.2–9.5	0.2–7.4
HbA2 (%)	4.76 (±0.76)	2.65 (±0.29)
HbA2 (range; %)	3.4–6.8	2.0–3.5
RBC ($\times 10^{12}$)*	5.54 (±0.74)	4.79 (±0.75)
Hb (g/dL)*	11.8 (±1.46)	13.03(±2.00)
MCV (fL)*	67.54 (±6.77)	84.92 (±9.30)
MCH (pg)*	21.44 (±2.26)	27.53 (±3.86)
MCHC (g/dL)*	31.78 (±1.86)	32.37 (±2.62)
RDW (CV %)*	15.78 (±2.82)	14.40 (±1.98)
Reticulocytes (% RBC)**	2.09 (±1.25)	1.97 (±1.55)
WBC ($\times 10^{3}$)*	8.08 (±3.22)	7.23 (±2.53)
Platelets ($\times 10^{6}$)*	283.28 (±121.71)	286.58 (±105.91)

Hematological parameters on 53 (*) and 39 (**) subjects with β-thal *minor*, and 29 (*) and 22 (**) normal subjects.