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The XmnI $^{G}\gamma$ polymorphism influences hemoglobin F synthesis contrary to *BCL11A* and *HBS1L-MYB* SNPs in a cohort of 57 β-thalassemia intermedia patients

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ABSTRACT

The HbF level is a quantitative trait influenced by many loci inside or outside the β-globin gene cluster. The aim of this study was to analyze in 57 β-thalassemia intermedia patients with very various genotypes the effects on fetal hemoglobin levels of SNPs lying in three genes or chromosome regions which include the *Xmn*I $^{G}\gamma$ polymorphism at position −158 of the *HBG2* promoter (rs7482144), two SNPs located in the *BCL11A* region (rs4671393 and rs11886868) and three SNPs located in the *HBS1L-MYB* region (rs28384513, rs9399137 and rs4895441). Our study shows a strong correlation between the *Xmn*I $^{G}\gamma$ polymorphism and the fetal hemoglobin expression in this patient population ($p = 0.002$). Unfortunately, although recent studies clearly showed a role of SNPs in *BCL11A* and a *HBS1L-MYB* region on either clinical expression or fetal hemoglobin levels of β-hemoglobinopathies such as sickle cell disease and β-thalassemia, SNPs in *BCL11A* and the *HBS1L-MYB* region did not show statistically significant correlations with fetal hemoglobin levels. This suggests that the *BCL11A* and *HBS1L-MYB* loci have a minor effect on HbF level compared to the *Xmn*I QTL in β-thalassemia intermedia patients.

Introduction

β-thalassemia is one of the most common human monogenic disorder in the world [1,2]. Its clinical phenotype varies from an asymptomatic state, called thalassemia minor, to a transfusion-dependent form, called thalassemia major. Thalassemia intermedia lies between these two extremes with two main categories of patients. Among those who are affected mildly, the hemoglobin level varies between 7 and 10 g/dL and no clinical signs are encountered until adult life. The second category of patients presents a more severe anemia which may affect growth development, even if regular blood transfusions are not necessary [3]. Most of this clinical variability depends on the β-globin genotype but other important genetic modifiers have also been identified: a relative excess of α-globin chains increases the α/β imbalance and the hemolysis while a persistence of HbF synthesis partially compensates for the lack of HbA and leads to a milder disease progression [4].

In adulthood, the residual HbF level is a quantitative trait which depends on many genetic loci [5]. Pioneering works have demonstrated that the common single nucleotide polymorphism (SNP) (C/T) at position −158 of the *HBG2* promoter (*Xmn*I site) (rs7482144) represents a major quantitative trait locus (QTL) for the regulation of HbF level ([6–8]. More recently, large family and genome-wide association studies have shown that regions outside of the β-globin gene cluster (2p16, 6q23, 8q and Xp 22.2–22.3) are also implicated in HbF regulation [9,10]. Two of them have been particularly studied: it has been demonstrated that the 6q23 SNPs that influence HbF level reside in a nearly contiguous segment of 79 kb in the *HBS1L-MYB* intergenic region. In Caucasian populations, these SNPs are distributed in 3 linkage disequilibrium (LD) blocks and genetic variants with the strongest effects are concentrated in a 24 kb region referred as block 2 [11,12]. As for the 2p16 region, it has been showed that HbF modulating SNPs reside in a unique 14 kb LD block, within the intron 2 of the *BCL11A* gene [13,14].

The link between HbF level and particular tag-SNPs in these regions has already been validated in independent cohorts of patients suffering from β-haemoglobinopathies. SNP rs28384513, rs9399137, rs4895441 (*HBS1L-MYB*) and rs4671393 (*BCL11A*) were identified in sickle cell disease patients [15], while rs11886868 (*BCL11A*) was

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found to be a major factor for the amelioration of the phenotype of β-thalassemia on Sardinian patients homozygous for the β^0 C>T mutation at codon 39 [16,17]. But, up to now, no such study has focused on β-thalassemia intermedia patients with very various genotypes. This was the aim of our work.

Materials and methods

Patients, hematological data and DNA extraction

This retrospective study was conducted on 57 patients (sex ratio M/F = 25/32), mostly originating from the Mediterranean area and diagnosed as β-thalassemia intermedia by the physician hematologist. Eight patients (19, 20, 22, 25, 34, 35, 36 and 54) presented a more severe form of β-thalassemia intermedia as they received many or sporadic transfusions and/or a hydroxyurea treatment during the course of their disease. However, for each of them, the blood sample used for the present study was withdrawn at the time of diagnosis, before any treatment (Supplementary Data Table 1).

For each individual, an EDTA blood sample was collected during a routine medical examination and divided into two aliquots. The first one was intended to DNA analysis while the second one was used for the determination of the HbF percentage by a cation-exchange liquid chromatography (CE-LC) method (Variant I, Bio-Rad, Hercules, CA, USA) using the "β-Thalassemia Short Program". The system was calibrated with the Bio-Rad Variant Hemoglobin A_2/F calibrator. The limit of quantification of the HbF percentage with this apparatus was estimated at 0.5% by the manufacturer, a value which was independently verified [18]. The HbF percentage was multiplied by the total hemoglobin level (determined during the same clinical examination with a blood cell counter) to obtain the HbF level (g/L). After obtention of a signed agreement for genetic studies, genomic DNA was extracted from 200 µL of the previously collected EDTA blood sample with the QIAmp DNA extraction kit (Qiagen, Strasbourg, France), either manually or with the QIAcube® device (Qiagen).

α and β-globin genotyping

Our α-globin genotyping assays comprised: (i) a multiplex gap-PCR reaction to identify the main α-globin gene deletions (−3.7 kb; −4.2 kb; −20.5 kb; SEA and MED) [19], (ii) the α-Globin StripAssay™ kit (ViennaLab, Austria), (iii) the direct sequencing of the α_1- and α_2-globin genes on ABI Prism 3130 XL (Applied Biosystems, Courtaboeuf, France) [20] and (iv) another gap-PCR reaction to identify the $\alpha\alpha\alpha^{anti3.7}$ and $\alpha\alpha\alpha^{anti4.2}$ triplication alleles [21]. Our β-globin genotyping assays comprised (i) the β-Globin StripAssay MED™ kit (ViennaLab, Austria), (ii) the direct sequencing of the β-globin gene and (iii) a semi-quantitative PCR method when a large deletion of the β-globin gene cluster was suspected [22]. When the obtained α and β-globin genotypes were not in accordance with a β-thalassemia intermedia phenotype, a multiplex ligation-dependent probe amplification (MLPA) reaction was carried out on the α- and/or the β-globin gene cluster (MRC Holland, Amsterdam, The Netherlands) [23] to search for additional genetic abnormalities.

XmnI, HBS1L-MYB and BCL11A genotyping

After PCR amplification of the $^G\gamma$ promoter, the detection of the SNP (C>T) at nucleotide −158 was carried out using *Xmn*I restriction-enzyme digestion [24,25]. To genotype the five tag-SNPs previously identified on sickle cell disease (SCD) and heterozygous β-thalassemia patients, in-house developed high-resolution melting (HRM) methods were performed on the Rotor-Gene 6000 (Qiagen, Courtaboeuf, France). The 20 µL reaction mixture contained 1× PCR buffer, 1 mM $MgCl_2$ (1.5 mM for rs11886868), 0.2 µM of each primer, 100 ng of genomic DNA, 10 nM of dNTPs, 5 µM of the DNA intercalating dye Syto® 9 (Invitrogen, Carlsbad, CA, USA), and 2 U of Taq polymerase Platinum® (Invitrogen). Primers and PCR/HRM protocols are shown in Supplementary Data, Tables 2 and 3. As expected, heterozygous samples were clearly identifiable but we preferred to confirm them by direct sequencing to ensure that the change in the melting curve shape was not due to an unexpected SNP. As wild-type and mutant homozygous patients were very difficult to distinguish from each other, we secondly performed the so-called 'spiking-method' with a known wild-type homozygous sample to differentiate them [26].

Population genetics and statistical analysis

For each SNP studied, we compared the observed genotype frequencies with those based on the Hardy–Weinberg equilibrium (HWE) [27]. The most likely haplotypes of the *BCL11A* and *HBS1L-MYB* regions were also determined by the Phase 2.1.1. software (available from: http://stephenslab.uchicago.edu/software.html) [28]. Associations with the HbF level (g/L) were searched using the Kruskal–Wallis (KW) test (XLStat statistical software v. 2009.4.03; Addinsoft, Paris, France). This non-parametric test was used because the HbF levels did not follow a normal distribution in our cohort (Fig. 1).

Results

α and β-globin genotypes

The α and β-globin genotyping results confirmed the diagnosis of β-thalassemia intermedia for all patients, except for patients 39 and 54 for which only a heterozygous β-thalassemia mutation could be identified (Supplementary Data, Table 1). However, for these two patients, β-thalassemia intermedia was phenotypically and clinically obvious. The most frequently found abnormalities were the β^0 C>T mutation at codon 39 ($n = 14$), the $\alpha\alpha\alpha^{anti-3.7}$ allele ($n = 7$ patients) and the β^+ IVSI-110 G>A mutation ($n = 4$ patients). It is noted that β-thalassemia mutations involving the β-globin gene promoter have been identified for 12 patients.

For two patients, the MLPA analysis was necessary to complete the diagnosis: patient 8 presented a large unknown deletion of the β-globin gene cluster whose breakpoints remain to be characterized, while patient 45 had a segmental duplication of the α_1 and α_2-globin genes. This type of duplication, involving the α-globin gene cluster and leading to a thalassemia intermedia phenotype, has been previously described elsewhere [29]. Two patients presented a very atypical β-thalassemia intermedia genotype: β^0 C>T mutation at codon 39 associated with a loss of heterozygosity in *trans* (patient 55) [30] ; β^+ −30 T>A mutation associated with the $\delta^0\beta^+$-Senegalese deletion in *trans* (patient 22) [31].

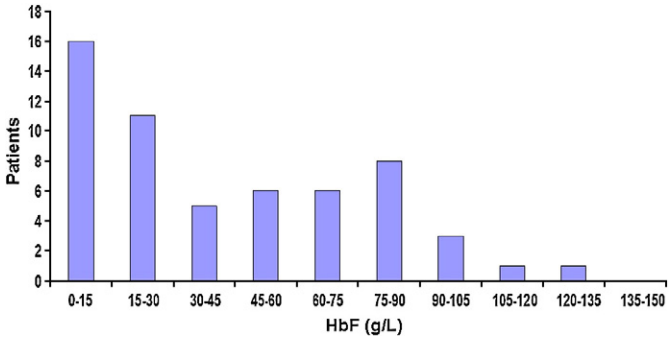

Fig. 1. Histogram of the HbF levels (expressed in g/L) in a cohort of 57 patients suffering from β-thalassemia intermedia.