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Genetic modifiers of beta-thalassemia and clinical severity as assessed by age at first transfusion

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*These two authors contributed equally to this work

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Acknowledgments
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Key words: beta-thalassemia, genetic modifiers, fetal hemoglobin, thalassemia major, thalassemia intermedia.
ABSTRACT

Background

Clinical and hematological features of beta-thalassemia are modulated by different factors resulting in a wide range of clinical severity that mainly include the type of disease-causing mutation, alpha-globin and gamma-globin chain production ability. In the present study we investigate the respective contribution of known modifiers to the prediction of beta-thalassemia clinical severity as assessed by the age at first transfusion of patients.

Design and Methods

We studied the effect of seven loci in a cohort of 316 beta0-thalassemia Sardinian patients. In addition to characterizing the beta-globin gene mutations, alpha-globin gene defects and HBG2:g.-158C>T polymorphism, we genotyped two different markers at the BCL11A gene and three in the HBS1L-MYB intergenic region using SNP microarrays, imputation and direct genotyping. We performed Cox proportional hazard analysis of the time to first transfusion.

Results

According to the resulting model, we were able to explain phenotype severity to a large extent (Harrell’s C-index=0.72; Cox & Snell $R^2=0.394$) and demonstrated that most of the model discriminatory ability is attributable to the genetic variants affecting fetal hemoglobin production (HBG2:g.-158C>T, BCL11A and HBS1L-MYB loci: C-index=0.68, $R^2=0.272$), while the remaining is due to alpha-globin gene defects and gender. Consequently, significantly distinct survival curves can be described in our population.

Conclusions

This detailed analysis clarifies the impact of genetic modifiers on clinical severity of the disease measured by time to first transfusion by determining their relative contributions in a homogeneous cohort of beta0-thalassemia patients. It may also support clinical decisions regarding the beginning of transfusion therapy in patients with beta-thalassemia.
INTRODUCTION

Beta-thalassemia is characterized by a decreased or absent beta-globin chain synthesis due to a variety of mutations that result in an excess of alpha-globin chains, which precipitate in red blood cells’ precursors in the bone marrow, causing their premature death. (1) In Sardinia, the most common type is a nonsense mutation at codon 39 of the beta-globin gene (HBB:c118C>T). (2)

The majority of patients manifest a severe form of anemia (thalassemia major) and are transfusion dependent from the first years of life. When performed regularly, red blood cell transfusion eliminates anemia-related complications and compensatory marrow expansion, and extend survival of patients. Approximately five to ten percent of patients live without requiring periodic blood transfusion and are said to have thalassemia intermedia. (3) These two forms of the disease are the extreme tails of a large clinical variability: patients might need to start transfusions after days, months or even years of life, proof of a great variation in disease severity.

This remarkable phenotypic diversity of thalassemia patients is associated with a great variety of genotypes including presence of mild/silent beta-thalassemia alleles, coinheritance of alpha-thalassemia or presence of genetic determinants associated with increased production of gamma-globin chains and consequent ability to produce functional fetal hemoglobin (Hb F) in adult life. (4) All these conditions reduce alpha/beta-globin chain imbalance and ineffective erythropoiesis. Hb F level is regulated by three major loci: HBG2:g.-158C>T on 11p15.4, HBS1L-MYB intergenic region on 6q23.3, and BCL11A on 2p16.1. These three loci together are responsible for 20 to 50% of the Hb F trait variance in patients with beta-thalassemia or sickle cell disease, and in healthy Europeans. (5–10)

Here we evaluated the effect of the HBG2:g.-158C>T, BCL11A, and HBS1L-MYB variants, together with coinherence of alpha-thalassemia and gender, on beta-thalassemia phenotype severity in Sardinian patients, measured through time at first transfusion.
DESIGN AND METHODS

Studied sample and phenotypic assessment

We retrospectively studied 316 beta0-thalassemia patients from Sardinia, all followed at the Ospedale Microcitemico of Cagliari (168 males and 148 females), with thalassemia major (266 subjects - median age 33 years [5th percentile=13, 95th percentile =38]) or thalassemia intermedia (50 subjects - median age 43 years [5th percentile=17, 95th percentile=61]), including 125 patients enrolled in a previous study on phenotype amelioration. (11) Thalassemia intermedia patients are defined as patients that have never been transfused, or have only been transfused sporadically during infections or surgery (< 10 blood units in total). (3) Beta-thalassemia mutations were of HBB:c118C>T/HBB:c118C>T type for 92.4% and of HBB:c118C>T/HBB:c.20delA type for 6.3% of the studied sample; remaining mutations are reported in Table 1. The continuous phenotypic severity distribution among thalassemia patients was measured by the time at which they started transfusion therapy. Criteria to start transfusion were persistent (i.e. more than 2 weeks) hemoglobin level lower than 7 g/dl in absence of infections, moderate to severe spleen enlargement and poor growth. The time to event was calculated as the time between birth and the first red blood cell transfusion or between birth and the last follow-up (January 2011) for patients who were not on transfusion therapy. Age at first transfusion was retrospectively collected through the WebTHAL computerized clinical records database (http://www.thalassemia.it), in use for the daily management of patients in our center.

This retrospective study was conducted in accordance with the Declaration of Helsinki and the patients gave informed consent for the DNA analysis.

SNPs selection

We selected five SNPs from the HBS1L-MYB intergenic region and the BCL11A locus known to be associated with Hb F levels, as follow (see Table 2):

rs1427407: the most significant SNP associated with Hb F levels within BCL11A, as reported in Menzel et al. 2007. (12) This SNP is in high linkage disequilibrium (LD) with rs766432 (r²=0.98) in our sample, and with rs4671393 (r²=0.88 / D’=1) in the CEU samples based on 1000 Genomes Project pilot phase 1 (CEU.1kG), for which effects on Hb F levels are also well-documented. (13,14)
rs10189857: within BCL11A, documented to have an independent effect on Hb F levels. (15)

rs9399137: the most significant SNP for Hb F levels within the HBS1L-MYB intergenic region in different population (5,12), in complete LD with a 3-bp deletion located in close proximity to four erythropoiesis-related transcription factors binding sites. (15,16)

rs4895441: a SNP within the HBS1L-MYB intergenic region, widely reported to be associated with Hb F levels (5,17) and in complete LD with rs9402686 ($r^2=1 / D'=1$ from CEU.1kG data), also reported to be independently associated with Hb F levels. (15)

rs6904897: within the HBS1L-MYB intergenic region, this SNP is in complete LD with rs28384513 ($r^2=1 / D'=1$ from CEU.1kG data), reported to be independently associated with Hb F levels. (15)

**Genotyping**

DNA was extracted from venous peripheral blood with standard methods.

Mutation analysis of the beta-globin gene was performed by direct DNA sequencing.

The HBG2:g.-158C>T polymorphism was determined as described. (18) Alpha-globin gene defects were determined using GAP-PCR or restriction enzyme digestion for deletional and non-deletional defects, respectively. (19)

SNPs were directly genotyped except for rs4895441 that was genotyped using the Affymetrix Genome-Wide Human SNP Array 6.0 according to the manufacturer's protocol and rs6904897 that was imputed with the MACH software, version 1.0.16, using a combined panel of Utah Residents of Northern and Western European Ancestry (CEU) and Tuscan samples (TSI) from the International Hapmap consortium as reference samples. (20)

Sixteen samples (from patients selected for being positive for the HBG2:g.-158C>T polymorphism) for which SNP array data were not available, were genotyped using TaqMan SNP genotyping assay (Applied Biosystems, Warrington, UK) for each of the five SNPs.
Quality controls

Microarrays data underwent quality control procedures on samples including: sample call rate (CR) (exclusion when CR<95%), cryptic relatedness (exclusion of first degree relatives), inbreeding coefficient (exclusion if negative with CR<98%) and reported gender versus heterozygosity of X chromosome SNPs (exclusion if discordant). Principal component analysis as implemented in EIGENSTRAT was performed for outliers detection. (21)

Quality controls attributes for the SNPs used in the present study are described in Table 2.

Statistical analysis

All genome-wide quality control measures were performed using the PLINK software package, version 1.07 (22) while the SPSS statistical software package, version 18.00 (SPSS, IBM, Somers, NY, USA), was used for subsequent analysis.

All markers selected for the present study were entered in a backward stepwise Cox proportional hazard model to characterize their effect on time to first transfusion, together with gender, alpha-globin gene defects and status for HBG2:g.-158C>T polymorphism (only -/- and +/- genotypes were observed for this SNP). A variable was defined for each SNP with values 0, 1, or 2 according to the number of copies of the less frequent allele, except for rs6904897: showing no difference between G/G and G/T genotypes survival curves it was codified 0 in these cases and 1 otherwise. Alpha-globin gene defects were classified as 0, 1, or 2 according to the number of deleted or mutated copies of the HBA gene (see Table 1 for details). Gender was codified 0 if female and 1 if male. Covariates were excluded from the model when their p-value was greater than 0.10.

Patients were considered uncensored when blood transfusion occurred during the study and censored when blood transfusion did not occur. We report Cox and Snell $R^2$ as well as Harrell’s concordance index (C-index) to assess how well the model performed.
**RESULTS**

Results from the stepwise Cox proportional hazard model are presented in Table 3. We refer below to predictors’ values for later time to transfusion as positive and predictors’ values for earlier time to transfusion as negative.

The HBG2:g.-158C>T polymorphism demonstrated the strongest effect on beta-thalassemia phenotype severity (Hazard Ratio (HR)=0.08, p<0.001), followed by rs1427407 [BCL11A] (HR=2.37, p<0.001), alpha-globin gene defects (HR=0.52, p<0.001), rs4895441 [HBS1L-MYB] (HR=1.94, p<0.001), rs10189857 [BCL11A] (HR=1.31, p=0.004), rs6904897 [HBS1L-MYB] (HR=0.79, p=0.047) and gender (HR=0.73, p=0.013). The SNP rs9399137 [HBS1L-MYB], in high LD with rs4895441 (r²=0.90 from CEU.1kG data), was the only predictor removed from the model (HR=1.29, p=0.298). Among all two-way interactions tested, the only significant one was between rs1427407 and rs10189857 (HR=1.66, p=0.036).

Discriminatory power of the model was high (C-index=0.72; R²=0.394) and most of it was attributable to Hb F production modulators (HBG2:g.-158C>T, BCL11A and HBS1L-MYB loci: C-index=0.68, R²=0.272), while the remaining was attributable to alpha-globin gene defects and gender.

According to our model prediction, 50% of patients with all negative predictors would undergo their first transfusion within the first 100 days of life and 99% of them would need regular transfusion before the first year of life. On the other hand, with all positive predictors, the probability to undergo transfusion at 10 years is still only 6‰.

We evaluated survival curves for time to first transfusion for four groups defined by the quartiles of the distribution of the linear predictor score (i.e. the sum of the product between covariate values and their corresponding parameter estimates). Lower values (first quartile) correspond to different combinations of mostly positive predictors (82 cases - linear predictor score values below 1.45), while higher values (fourth quartile) correspond to different combinations of mostly negative predictors (76 cases - linear predictor score values above 2.70). Intermediate groups include 78 cases with linear predictor score values between 1.45 and 2.05 (second quartile) and 80 cases with linear predictor score values between 2.05 and 2.70 (third quartile).
Following this classification, 50% of patients in the fourth quartile group underwent their first transfusion within six months of life, whereas only 3% of patients in the first quartile group had started transfusions by the same age. In this group it took more than six years to reach 50% of patients that started transfusion, whereas by the same age all patients had undergone their first transfusion in the fourth quartile group. In the first quartile group, 47% of patients never started red blood cells' transfusion (see Figure 1).

All survival curves were significantly different from each other (p<0.01, Breslow’s test). In particular, the third quartile group was significantly different from the fourth quartile group (p<0.001) and the second quartile group was significantly different from both the first and third quartile risk-groups (p<0.001 and p=0.007 respectively).
DISCUSSION

The present study aimed at measuring the influence of known genetic modifiers of beta0-thalassemia on phenotype severity assessed as time to first transfusion. To this aim, SNPs in the BCL11A gene and HBS1L-MYB intergenic region were selected based on previous studies and genotyped in a group of 316 patients, as well as alpha-globin gene defects and HBG2:g.-158C>T polymorphism. All these variables, together with gender, were included in a Cox proportional hazard model for time at first transfusion, and their respective effects were measured. Results showed a substantial impact of Hb F production variants and alpha-globin gene defects on beta-thalassemia phenotype severity, allowing to predict the risk of patients to start transfusion at different times of their life.

In this study we assumed that the time to first transfusion accurately reflects variations in beta-thalassemia phenotype severity. Such hypothesis seems to be supported by our results, as all variables and the hierarchy of their effects agree with previous studies on genetic modifiers of both Hb F levels and beta-thalassemia clinical severity, even though other unknown genetic factors and clinical conditions might be co-responsible for need of transfusion. (3, 11, 15, 23, 24)

To the best of our knowledge, the present study is the first to analyze the severity of beta-thalassemia in a quantitatively defined manner and to account for such complete set of known predictors. In a previous study, Galanello et al. (11) studied the effect of two SNPs (rs11886868 in BCL11A and rs9389268 in HBS1L-MYB intergenic region) and alpha-globin genes defects on the amelioration of Sardinian beta0-thalassemia, defined as major versus intermedia status. A recent study by Badens et al. (24) further extended this analysis accounting for HBG2:g.-158C>T polymorphism and beta0/beta+ status, in addition to the previously mentioned markers, in a heterogeneous cohort of 106 patients with 30 different beta-globin gene mutations. The present analysis expands these results by including the effect of different independent predictors in each gene, selected to be the strongest reported to date, in a homogeneous cohort of beta0-thalassemia patients, that we believe allows to better define the respective effect of each predictor. Above all this work relates genetic modifiers to time at first transfusion, a key event that characterizes disease severity regardless of patients’ major or intermedia phenotype, thus notably increasing our knowledge on the specific effects of genetic modifiers of beta-thalassemia clinical severity.
While it is likely that future whole genome sequencing studies will better define the genetic polymorphisms that modulate the effect of the *BCL11A* and *HBS1L-MYB* loci, results from the present study could already be of support in clinical settings, by providing clear probabilities for the need to start transfusion at different ages as function of the personal genetic background of patients.
AUTORSHIP AND DISCLOSURES

Franco Anni and Fabrice Danjou took primary responsibility and drafted the paper. Franco Anni, Lucia Perseu and Stefania Satta performed experimental study and produced experimental data. Carlo Dessi collected clinical data and provided clinical support. Fabrice Danjou and Marcella Devoto performed statistical analysis. Paolo Fortina provided support for the microarray-based experiments and genetic analysis. Eliana Lai provided access to study material. Renzo Galanello coordinated the study. All authors contributed to the final version of the paper.

The authors report no potential conflict of interest.
REFERENCES


Table 1. Genotypic frequencies of genetic markers and clinical characteristics.

<table>
<thead>
<tr>
<th>β⁰ Genotype</th>
<th>Cases (%)</th>
<th>Median time to first transfusion in months (5th-95th %)</th>
<th>Thalassemia intermedia patients (% per row)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBB:c118C&gt;T/HBB:c118C&gt;T</td>
<td>292 (92.4)</td>
<td>9 (3-53)</td>
<td>13.7</td>
</tr>
<tr>
<td>HBB:c118C&gt;T/HBB:c.20delA</td>
<td>20 (6.3)</td>
<td>32 (8-83)</td>
<td>50.0</td>
</tr>
<tr>
<td>HBB:c118C&gt;T/HBB:c.230delC</td>
<td>3 (0.9)</td>
<td>18 (14-91)</td>
<td>0.0</td>
</tr>
<tr>
<td>HBB:c118C&gt;T/HBB:c.315+1G&gt;A</td>
<td>1 (0.3)</td>
<td>7 (7-7)</td>
<td>0.0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HBG2: g.-158C&gt;T</th>
<th>Cases (%)</th>
<th>Median time to first transfusion in months (5th-95th %)</th>
<th>Thalassemia intermedia patients (% per row)</th>
</tr>
</thead>
<tbody>
<tr>
<td>- / -</td>
<td>300 (94.9)</td>
<td>9 (3-57)</td>
<td>12.3</td>
</tr>
<tr>
<td>+ / -</td>
<td>16 (5.1)</td>
<td>13 (9-63)</td>
<td>81.3</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>α gene defects</th>
<th>Cases (%)</th>
<th>Median time to first transfusion in months (5th-95th %)</th>
<th>Thalassemia intermedia patients (% per row)</th>
</tr>
</thead>
<tbody>
<tr>
<td>class 0</td>
<td>αa/αa</td>
<td>169 (53.5)</td>
<td>7 (2-49)</td>
</tr>
<tr>
<td>class 1</td>
<td>α/aα</td>
<td>94 (29.7)</td>
<td>11 (3-50)</td>
</tr>
<tr>
<td>class 2</td>
<td>α/aα</td>
<td>30 (9.5)</td>
<td>34 (8-80)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>BCL11A</th>
<th>Cases (%)</th>
<th>Median time to first transfusion in months (5th-95th %)</th>
<th>Thalassemia intermedia patients (% per row)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs1427407</td>
<td>T / T</td>
<td>7 (2.2)</td>
<td>11 (10-25)</td>
</tr>
<tr>
<td>rs10189857</td>
<td>G / G</td>
<td>93 (29.4)</td>
<td>16 (3-77)</td>
</tr>
<tr>
<td>rs9399137</td>
<td>G / G</td>
<td>216 (68.4)</td>
<td>7 (2-57)</td>
</tr>
<tr>
<td>rs4895441</td>
<td>A / G</td>
<td>154 (48.7)</td>
<td>9 (3-66)</td>
</tr>
<tr>
<td>rs6904897</td>
<td>A / A</td>
<td>112 (35.4)</td>
<td>8 (2-49)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HBS1L-MYB intergenic region</th>
<th>Cases (%)</th>
<th>Median time to first transfusion in months (5th-95th %)</th>
<th>Thalassemia intermedia patients (% per row)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs9399137</td>
<td>C / C</td>
<td>6 (1.9)</td>
<td>28 (3-32)</td>
</tr>
<tr>
<td>rs4895441</td>
<td>G / G</td>
<td>17 (5.4)</td>
<td>9 (3-80)</td>
</tr>
<tr>
<td>rs6904897</td>
<td>G / G</td>
<td>106 (33.5)</td>
<td>14 (3-63)</td>
</tr>
</tbody>
</table>

1 overall 15.8% of patients have intermedia form of the disease (50/316)
2 55% of HBB:c118C>T/HBB:c.20delA patients are +/- for the HBG2:g.-158C>T polymorphism
3 αHphI refers to the HBA2:c.95+2_95+6delTGAGG whereas αNcol refers to the HBA2:c.2T>C mutation; -α³⁷ and -α⁴² refer to the commonly denominated 3.7-kb rightward deletion and 4.2-kb leftward deletion of the alpha gene.
Table 2. Characteristics of single nucleotide polymorphisms used in the study.

<table>
<thead>
<tr>
<th>Locus</th>
<th>SNP</th>
<th>Chr.</th>
<th>Position (GRCh37)</th>
<th>Call Rate / r²</th>
<th>p-value for Hardy-Weinberg Equilibrium test</th>
<th>Minor Allele Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BCL11A</strong></td>
<td>rs1427407</td>
<td>2</td>
<td>60718043</td>
<td>CR=1.00 ¹</td>
<td>0.41</td>
<td>0.17</td>
</tr>
<tr>
<td></td>
<td>rs10189857</td>
<td>2</td>
<td>60713235</td>
<td>CR=1.00 ¹</td>
<td>0.81</td>
<td>0.40</td>
</tr>
<tr>
<td><strong>HBS1L-MYB</strong></td>
<td>rs9399137</td>
<td>6</td>
<td>135419018</td>
<td>CR=1.00 ¹</td>
<td>0.13</td>
<td>0.18</td>
</tr>
<tr>
<td>intergenic region</td>
<td>rs4895441</td>
<td>6</td>
<td>135426573</td>
<td>CR=1.00</td>
<td>0.63</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>rs6904897</td>
<td>6</td>
<td>135382980</td>
<td>r²=0.99 ²</td>
<td>0.05</td>
<td>0.22</td>
</tr>
</tbody>
</table>

¹ direct genotyping
² squared correlation between imputed and true genotypes
Table 3. Cox proportional hazards model results.

<table>
<thead>
<tr>
<th>Locus</th>
<th>p</th>
<th>Hazards Ratio</th>
<th>Harrell's C-index</th>
<th>Predictor for later transfusion start</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBG2:g.-58C&gt;T</td>
<td>&lt;0.001</td>
<td>0.081</td>
<td>0.54</td>
<td>+/-</td>
</tr>
<tr>
<td>α gene defects</td>
<td>&lt;0.001</td>
<td>0.514</td>
<td>0.61</td>
<td>class 2 (^1)</td>
</tr>
<tr>
<td>BCL11A</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs1427407</td>
<td>&lt;0.001</td>
<td>2.391</td>
<td>0.63</td>
<td>T allele</td>
</tr>
<tr>
<td>rs10189857</td>
<td>0.005</td>
<td>1.312</td>
<td></td>
<td>G allele</td>
</tr>
<tr>
<td>HBS1L/MYB</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs4895441</td>
<td>&lt;0.001</td>
<td>1.979</td>
<td>0.57</td>
<td>G allele</td>
</tr>
<tr>
<td>rs6904897</td>
<td>0.020</td>
<td>0.697</td>
<td></td>
<td>TT genotype</td>
</tr>
<tr>
<td>Gender</td>
<td>0.016</td>
<td>0.738</td>
<td>0.52</td>
<td>Male</td>
</tr>
</tbody>
</table>

\(^1\) classes’ definitions are reported in table 1
Legend

Figure 1. Kaplan-Meier survival curves for patient with different combinations of predictors for later or earlier transfusion need.
1 statistical tests of differences between the curves are reported in the text.