Amelioration of Sardinian beta-zero thalassemia by genetic modifiers

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Abstract

Sardinian beta-thalassemia patients all are homozygotes for the same null allele in the beta-globin gene, but the clinical manifestations are extremely variable in severity. Previous studies have shown that the co-inheritance of alpha-thalassemia or the presence of genetic variants that sustain HbF production have a strong impact on ameliorating the clinical phenotype. Here we evaluate the contribution of variants in the \textit{BCL11A}, and \textit{HBS1L-MYB} genes, implicated in the regulation of HbF, and of alpha-thalassemia co-inheritance in 50 thalassemia intermedia and 75 thalassemia major patients. We confirm that alpha-thalassemia and allele C of SNP rs-11886868 in \textit{BCL11A} were selectively represented in thalassemia intermedia patients. Moreover, allele G at SNP rs9389268 in the \textit{HBS1L-MYB} locus was significantly more frequent in the thalassemia intermedia patients. This trio of genetic factors can account for 75% of the variation differences in phenotype severity.
Introduction

The clinical manifestations of beta-thalassemia are extremely variable in severity, ranging from the transfusion-dependent form of thalassemia major to an asymptomatic carrier state. Between the two extremes are thalassemia intermedia patients, who show a wide spectrum of phenotypes but are not transfusion dependent. The remarkable clinical diversity is associated with a great variety of genotypes.\(^1,2\) Because the severity of homozygous beta-thalassemia is directly related to the degree of imbalance between alpha and beta and/or gamma globin chains, any factor that can reduce the degree of imbalance -- by reducing alpha or increasing beta and/or gamma chains -- may ameliorate the clinical phenotype. In the beta-globin gene itself, more than 200 mutations have been characterized including many that retain partial beta-gene function.

Recently, genetic variants that modulate Hb F levels but fall outside of the hemoglobin genes, have been identified, at the \textit{BCL11A} locus and in the \textit{HBS1L-MYB} intergenic region.\(^3\) The \textit{BCL11A} protein has been further reported to affect globin gene regulation by interacting with specific sequences in the beta-globin gene cluster.\(^6,7\) Though the precise variants in the genes involved have not been identified, \textit{BCL11A} gene variation has also been shown to moderate the phenotype of homozygous beta-thalassemia.\(^5\) Here we have evaluated the relative contributions of \textit{BCL11A} variation, \textit{HBS1L-MYB} variation, and coinheritance of alpha-thalassemia in ameliorating the severity of homozygous beta-thalassemia in Sardinian individuals.

Materials and Methods

We studied 50 patients (24 males and 26 females) with mild non-transfusion dependent thalassemia intermedia and 75 patients (29 males and 46 females) with severe transfusion-dependent thalassemia major. The study was approved by the hospital Ethic Committee of ASL8 Cagliari and the patients gave informed consent in accordance with the Declaration of Helsinki. Thalassemia major patients (mean age 24.4 +/- 7.1 years) were
regularly transfused from their first year of life\(^8\), while thalassemia intermedia patients (mean age 41.0 +/- 9.8) have never been transfused or sporadically transfused during infections or surgery (less than 10 blood units in total). All patients were homozygous for the beta\(^{39}\) non-sense C→T mutation and were negative for the Xmn I -158 G\(\gamma\) polymorphism [which may increase gamma chain production.]\(^9\) alpha thalassemia was detected by the GAP-PCR technique (deletion defects) or restriction enzyme digestion (non-deletion defects).\(^{10}\) The alpha - globin genotype was classified as 0, 1 or 2 according to the number of mutated copies of the HBA gene. Individuals with non-deletion alpha - thalassemia affecting the alpha 2-gene have been grouped with the corresponding alpha-thalassemia deletion phenotype (i.e. alpha alpha/alpha\(^{39}\) alpha with -alpha/alpha alpha and -alpha/alpha\(^{39}\) alpha with -alpha/-alpha). A variable was defined for each of the SNPs, rs11886868 and rs2389268, with values 0,1 or 2 according to the number of copies of the less frequent allele (C and G, respectively). Genotyping of HbF modulating genetic variants was performed using a TaqMan® SNP genotyping assay (Applied Biosystems, Warrington, UK). Evaluation of the associations, odds ratio (OR), and tests for interaction were performed fitting a logistic regression in R statistical software (http://www.R-project.org/ ). To evaluate the number of phenotypes that could be correctly predicted by looking at the genotypes at the three loci, we masked the known phenotype status for all individuals and calculated the predicted probability to be affected by thalassemia Intermedia given the estimated odds ratio from the full logit model (Thalassemia status ~ intercept, rs11886868, rs2389268, number of alpha-mutated copies). If the predicted probability was 0.5 or greater, we assigned to the individual an outcome of 1 (Thalassemia Intermedia), and an outcome of 0 (Thalassemia Major) if less than 0.5. Then, we counted the phenotypes that were correctly predicted, given this cutoff point of 0.5. This correct count divided by the total number of individuals is known as “R-square count”.
Results and discussion

In this study, we genotyped 50 patients with thalassemia intermedia and 75 patients with thalassemia major at SNP rs11886868, in BCL11A and at SNPs rs9389268 in the HBS1L-MYB. For each individual we also considered the number of mutated copies of the alpha-globin gene (HBA). SNPs in BCL11A and HBS1L-MYB were selected based on a recent G-WA scan in Sardinian samples\(^5\). Thirty-nine of 50 thalassemia intermedia patients, carry at least one HbF-associated allele at BCL11A, 29 at least one HbF-associated allele at HBS1L-MYB, and 38 at least one mutated copy of alpha-globin gene.

As observed previously, the frequency of the minor allele C of SNP rs11886868 in the BCL11A gene is correlated with an increase in the production of fetal hemoglobin, significantly higher in the thalassemia intermedia patients (p=2.05x10\(^{-5}\), Table 1). Similarly, the frequency of the minor allele G at SNP rs9389268 in the intergenic region HBS1L-MYB was more represented in this group compared to thalassemia major patients (p=8.11x10\(^{-4}\), Table 1). In addition, we confirmed that those individuals carrying at least one mutated copy of the alpha-globin gene were for the most part patients with thalassemia intermedia (p=9.9x10\(^{-5}\), Table 1).

Interestingly, the contribution of BCL11A variation appeared to be greater than that of the HBS1L-MYB locus variants (OR= 5.15 and 4.61, respectively), whereas both were appreciably larger than the effect attributable to the co-inheritance of alpha-thalassemia (OR= 3.32, Table 1). A more definitive estimate should be possible once the causative variants at those loci are identified. Taken together, the three loci are able to correctly predict 75% of the phenotypes (see methods), although this estimate may be re-evaluated when the causative variants at BCL11A and HBS1L-MYB loci will be detected.
We also tested whether any epistatic effect was detectable, but only one barely significant interaction was observed between SNP rs9389268 in the HBS1L-MYB intergenic region and the alpha-globin locus (p=0.04).

In addition, to assess the cumulative effect of the tested loci, we considered a score variable defined as the number of positive alleles carried by each patient (i.e. those associated with the amelioration of the phenotype: allele C at rs118886868, allele G at SNP rs9389269, and a mutated copy of the alpha globin gene) (Figure 1). Ninety-two percent of the thalassemia intermedia patients, carry at least two positive alleles, while 65% of thalassemia major carry one or no copies. Interestingly, we did not observe individuals homozygotes at all three loci, probably due to the small sample size. We further assessed whether BCL11A, HBS1L-MYB or the co-inheritance of alpha-thalassemia also modulate total Hb levels in thalassemia intermedia patients or correlate with the age at which the patient underwent splenectomy. Some trends toward association was observed, but they were not statistically significant (p> 0.5, data not shown).

For decades genetic studies have made it clear that thalassemia patients carrying the same beta-globin genotype can show remarkable phenotypic diversity. Much of this variability can now be understood based on the response of HbF levels to variants at these 3 loci.

To our knowledge, this is the first study reporting a contribution of genetic markers in HBS1L-MYB in beta° thalassemia patients, and we find that the three loci act in an additive fashion, with each copy of the modulating allele at each locus contributing to the amelioration of the phenotype expression. Furthermore the interaction terms were generally insignificant; with only one barely significant interaction observed. Larger studies are necessary to confirm this epistatic effect. The SNP variant in the BCL11A gene contributed more strongly than the HBS1L-MYB locus or the co-inheritance of alpha-thalassemia. This is in agreement with previous observations that identified the
2p15 locus as the major modifier of HbF levels in healthy populations\textsuperscript{4,5}. Interestingly, our results are apparently in variance with what observed in Europeans for F-cell production, where the \textit{HBS1L-MYB} locus has a stronger effect than \textit{BCL11A}\textsuperscript{4}. However, it has to be considered that although highly correlated, the phenotypes may be influenced in a different fashion by those genes. Furthermore, the different amount of variance explained by each marker also reflects the heterogeneity of the allele frequencies among populations of different ethnicity. The impact of the three loci is clear when we consider the number of positive (i.e, ameliorating) alleles in each patient. We observed that only one patient with thalassemia intermedia carried no positive allele, and that patient is the most severely affected among the group (splenectomized at 2 years, mean Hb 6.4 g/dl, occasional RBC transfusions). The only patient classified as having thalassemia major but bearing 4 positive alleles is similarly at the edge of that group, he has started transfusions, when Hb levels dropped during an episode of infection and his transfusion needs might be transient. These patients may have additional modifying factors, either environmental or perhaps at one of the genetic loci, contributing to the as yet unexplained 25\% of variability.

In keeping with traditional views, all three modifying factors would reduce the imbalance of alpha versus non alpha haemoglobin chains, the determinant of clinical severity of beta thalassemia. The reduced globin chain imbalance permits selective survival of the erythroid precursors, resulting in a reduction of unproductive erythropoiesis\textsuperscript{11,12}.

Recently, variants in the \textit{BCL11A}, \textit{HBS1L-MYB} and \textit{HBB} loci have been shown to be associated with HbF levels and the moderation of pain crisis rate in sickle cell disease patients as well.\textsuperscript{13} Thus, the HbF-associated SNPs, increasing the production of fetal hemoglobin over the lifetime of a patient, may be considered as an innate therapy for several hemoglobin disorders. Genotyping of these variants may eventually help to predict the severity risk in newborns, and accordingly improve genetic counselling.
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Author Contribution: R.G., S.Se., M.U. and A.C. designed the research, analyzed data and wrote the paper. L.P., M.C.S., S.Sa. and G.U. performed the research. M.E.L. and S.B. collected the clinical data. R.G.A. and S.Se. performed statistical analysis. The authors have no conflict of interest to declare.

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References


Figures and Tables

Table 1. Summary of the association with the amelioration of beta-thalassemia.

For each marker we reported the minor allele with the correspondent frequency on the two group of patients, the p-value for allelic differences and the Odd Ratio. For the \textit{HBA} locus, genotypes have been coded as 0, 1 or 2 according to the number of mutated copies of the \textit{HBA} gene. Due to small counts, individuals with -a/HPHi\textsubscript{a} and -a/NCOI\textsubscript{a} have been grouped to -a/-a, and NCOI\textsubscript{a}/aa with -a/aa.

<table>
<thead>
<tr>
<th>SNP/Locus</th>
<th>Allele</th>
<th>Freq T.Major</th>
<th>Freq T.Intermedia</th>
<th>p-value</th>
<th>OR (95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs11886868/BCL11A</td>
<td>C</td>
<td>0.21</td>
<td>0.48</td>
<td>$2.05 \times 10^{-5}$</td>
<td>5.15 (2.46 - 12.06)</td>
</tr>
<tr>
<td>rs9389268/HBS1L-MYB</td>
<td>G</td>
<td>0.15</td>
<td>0.35</td>
<td>$8.1 \times 10^{-4}$</td>
<td>4.61 (2.18 – 10.76)</td>
</tr>
<tr>
<td>\textit{HBA}*</td>
<td>-</td>
<td>0.25</td>
<td>0.52</td>
<td>$9.9 \times 10^{-5}$</td>
<td>3.32 (1.75 - 6.80)</td>
</tr>
</tbody>
</table>

* The allele “-“ indicates one mutated copy.
Figure 1. Proportion of patients and number of ameliorating alleles in the two groups. The figure shows the proportion of thalassemia Major and thalassemia Intermedia patients carrying 0, 1 or more alleles considered to be responsible for the amelioration of the clinical expression of the phenotype (positive alleles). Individuals carrying >4 positive alleles were not observed.
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