Brief report

Amelioration of Sardinian β^0 thalassemia by genetic modifiers

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Sardinian β-thalassemia patients all are homozygotes for the same null allele in the β-globin gene, but the clinical manifestations are extremely variable in severity. Previous studies have shown that the coinheritance of α-thalassemia or the presence of genetic variants that sustain fetal hemoglobin production has a strong impact on ameliorating the clinical phenotype. Here we evaluate the contribution of variants in the *BCL11A*, and *HBS1L-MYB* genes, implicated in the regulation of fetal hemoglobin, and of α-thalassemia coinheritance in 50 thalassemia intermedia and 75 thalassemia major patients. We confirm that α-thalassemia and allele C of single nucleotide polymorphism rs-11886868 in *BCL11A* were selectively represented in thalassemia intermedia patients. Moreover, allele G at single nucleotide polymorphism rs9389268 in the *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. (Blood. 2009; 114:3935-3937)

Introduction

The clinical manifestations of β-thalassemia are extremely variable in severity, ranging from the transfusion-dependent form of thalassemia major to the 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 β-thalassemia is directly related to the degree of imbalance between α- and β- and/or γ-globin chains, any factor that can reduce the degree of imbalance (by reducing α- or increasing β- and/or γ-globin chains) may ameliorate the clinical phenotype. In the β-globin gene itself, more than 200 mutations have been characterized, including many that retain partial β-gene function.

Recently, genetic variants that modulate HbF levels but fall outside of the hemoglobin genes have been identified, at the *BCL11A* locus and in the *HBS1L-MYB* intergenic region.[3-5] The BCL11A protein has been further reported to affect globin gene regulation by interacting with specific sequences in the β-globin gene cluster.[6,7] Although the precise variants in the genes involved have not been identified, *BCL11A* gene variation has also been shown to moderate the phenotype of homozygous β-thalassemia.[5] Here we have evaluated the relative contributions of *BCL11A* variation, *HBS1L-MYB* variation, and coinheritance of α-thalassemia in ameliorating the severity of homozygous β-thalassemia in Sardinian persons.

Methods

Sample description

We studied 50 patients (24 males and 26 females) with mild non–tranfusion-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] whereas thalassemia intermedia patients (mean age, 41.0 ± 9.8 years) have never been transfused or sporadically transfused during infections or surgery (< 10 blood units in total). All patients were homozygous for the β^0 39 nonsense C→T mutation and were negative for the Xmn I-158 Gγ polymorphism, which may increase γ chain production.[9]

Genotyping and statistical analysis

α-Thalassemia was detected by the gap polymerase chain reaction technique (deletion defects) or restriction enzyme digestion (nondeletion defects).[10] The α-globin genotype was classified as 0, 1, or 2 according to the number of mutated copies of the *HBA* gene. Persons with nondeletion α-thalassemia affecting the α2 gene have been grouped with the corresponding α-thalassemia deletion phenotype (ie, α α/α^{HphI} α with -α/α α and -α/α^{NcoI}α with -α/-α). A variable was defined for each of the single nucleotide polymorphisms (SNPs) rs11886868 and rs9389268, 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). 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 3 loci, we masked the known phenotype status for all persons and calculated the predicted probability to be affected by thalassemia intermedia given the estimated OR from the full logit model (thalassemia status: intercept, rs11886868, rs9389268, number of α-mutated copies). If the predicted probability was 0.5 or greater, we assigned to the person 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,

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Table 1. Summary of the association with the amelioration of β^0-thalassemia

SNP/locus	Allele	Frequency of thalassemia major	Frequency of thalassemia intermedia	*P*	OR (95% confidence interval)
rs11886868/*BCL11A*	C	0.21	0.48	< .001	5.15 (2.46-12.06)
rs9389268/*HBS1L-MYB*	G	0.15	0.35	< .001	4.61 (2.18-10.76)
*HBA**	−	0.25	0.52	< .001	3.32 (1.75-6.80)

For each marker, we reported the minor allele with the correspondent frequency on the 2 groups of patients, the *P* value for allelic differences, and the odds ratio. For the *HBA* locus, genotypes have been coded as 0, 1, or 2 according to the number of mutated copies of the *HBA* gene. Because of small counts, persons with $-\alpha/\alpha^{HphI}\alpha$ and $-\alpha/\alpha^{NcoI}\alpha$ have been grouped with $-\alpha/-\alpha$, and $\alpha^{NcoI}\alpha/\alpha\alpha$ with $-\alpha/\alpha\alpha$.

*The allele − indicates one mutated copy.

given this cutoff point of 0.5. This correct count divided by the total number of persons 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 person, we also considered the number of mutated copies of the α-globin gene (*HBA*). SNPs in *BCL11A* and *HBS1L-MYB* were selected based on a recent genome-wide association 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 α-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 ($P < .001$, 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 with thalassemia major patients ($P < .001$, Table 1). In addition, we confirmed that those persons carrying at least one mutated copy of the α-globin gene were, for the most part, patients with thalassemia intermedia ($P < .001$, 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 coinheritance of α-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 3 loci are able to correctly predict 75% of the phenotypes ("Statistical analysis"), although this estimate may be reevaluated 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 α-globin locus ($P = .04$).

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 (ie, those associated with the amelioration of the phenotype): allele C at rs118886868, allele G at SNP rs9389268, and a mutated copy of the α-globin gene (Figure 1). Ninety-two percent of the thalassemia intermedia patients carry at least 2 positive alleles, whereas 65% of thalassemia major carry one or no copies. Interestingly, we did not observe persons homozygous at all 3 loci, probably because of the small sample size. We further assessed whether *BCL11A, HBS1L-MYB*, or the coinheritance of α-thalassemia also modulates total Hb levels in

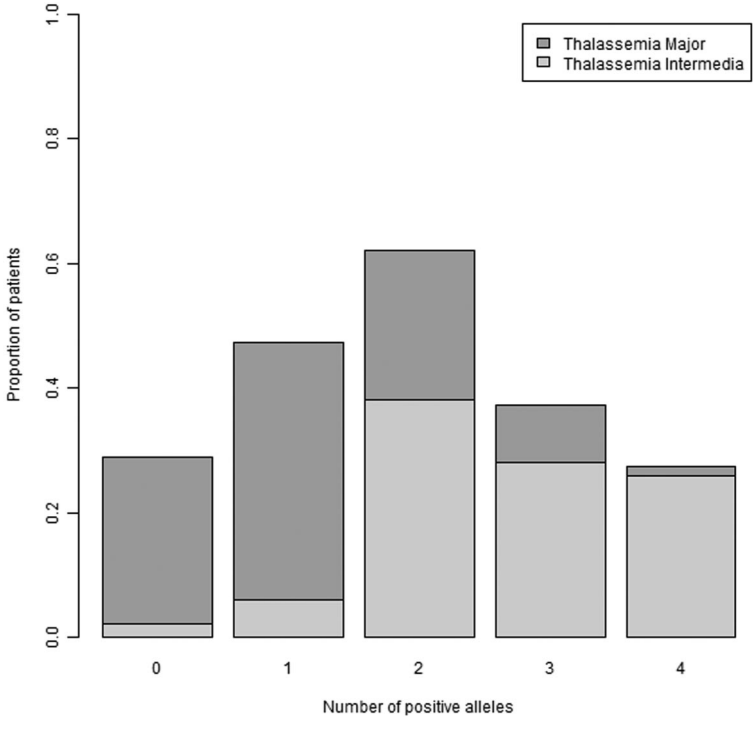

Figure 1. Proportion of patients and number of ameliorating alleles in the 2 groups. The figure shows the proportion of thalassemia major and thalassemia intermedia patients carrying 0, 1, or more of the alleles considered to be responsible for the amelioration of the clinical expression of the phenotype (positive alleles). Persons carrying more than 4 positive alleles were not observed.

thalassemia intermedia patients or correlates with the age at which the patient underwent splenectomy. Some trends toward association were observed, but they were not statistically significant ($P > .5$, data not shown).

For decades genetic studies have made it clear that thalassemia patients carrying the same β-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 β^0-thalassemia patients, and we find that the 3 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 coinheritance of α-thalassemia. This is in agreement with previous observations that identified the 2p15 locus as the major modifier of HbF levels in healthy populations.[4,5] Interestingly, our results are apparently at variance with what was observed in Europeans for F-cell production, where the *HBS1L-MYB* locus has a stronger effect than *BCL11A*.[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 3 loci is clear when we consider the number of positive (ie, 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 hemoglobin 6.4 g/dL, occasional red blood cell transfusions). The only patient classified as having thalassemia major but bearing 4 positive alleles is similarly at the edge of that group: he started transfusions when hemoglobin 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 3 modifying factors would reduce the imbalance of α- versus non–α-hemoglobin chains, the determinant of clinical severity of β-thalassemia. The reduced globin chain imbalance permits selective survival of the erythroid precursors, resulting in a reduction of unproductive erythropoiesis.[11,12]

Recently, variants in the *BCL11A*, *HBS1L-MYB*, and *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.[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 counseling.

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Authorship

Contribution: R.G., S. Sanna, M.U., and A.C. designed the research, analyzed data, and wrote the paper; L.P., M.C.S., S. Satta, and G.U. performed the research; M.E.L. and S.B. collected the clinical data; and G.R.A. and S. Sanna performed statistical analysis.

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