Increased hemoglobin A2 (HbA2; ie, levels > 3.9%) is the most important feature of β-thalassemia carriers. However, it is not uncommon to find persons with borderline HbA2 levels, who pose a relevant screening problem. Several genotypes have been associated with borderline HbA2, but sometimes the reasons for this unusual phenotype are unknown. In this paper, we report, for the first time, that mutations of KLF1 result in HbA2 levels in the borderline range. Six different KLF1 mutations were identified in 52 of 145 subjects with borderline HbA2 and normal mean corpuscular volume and mean corpuscular hemoglobin. Two mutations (T327S and T280_H283del) are here reported for the first time. The prevalent mutation in Sardinians is S270X, which accounts for 80.8% of the total. The frequent discovery of KLF1 mutations in these atypical carriers may contribute significantly to the thalassemia screening programs aimed at identification of at-risk couples. (Blood. 2011;118(16):4454-4458)

Introduction

Accurate determination of the β-thalassemia carrier phenotype is essential for detecting couples at risk for having offspring with thalassemia major. Increased hemoglobin A2 (HbA2) level is considered the most reliable hematologic finding for the identification of β-thalassemia carriers. However, some carriers are difficult to identify because the level of HbA2 is not in the typical carrier range (ie, HbA2 = 3.8%-6.0%). These atypical carriers have borderline HbA2 values (ie, HbA2 levels between normal and β-thalassemia carrier levels, 3.3%-3.8%).1-3 The prevalence of borderline A2 carriers in populations with high frequency of β-thalassemia has been reported in 2.2% to 3.0% in one study and up to 16.7% in another study.2,3 Borderline HbA2 levels associated with reduced mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) are generally the consequence of mild β-thalassemia mutations (ie, HBB c.-92 + 6 T → C), co-inherited δ and β-thalassemia, β-promoter mutations −92 (HBB c.-142 C → T), or coexisting iron deficiency anemia.1,5,6 Borderline HbA2 with normal MCV and MCH may be an outlier value of the normal HbA2 distribution in the noncarrier population or the effect of genetic determinants able to increase HbA2 levels. The genetic determinants so far identified are the triplication of the α-globin genes, β-promoter mutations (HBB c.-151 C → T), and some HBD and HBB gene variants.1-3 However, altogether, these determinants explain only a limited proportion of the borderline HbA2 levels.

Although subjects with borderline HbA2 and reduced MCV and MCH are easily identified with appropriate screening methods, most of the subjects with normal MCV and MCH remain undefined or need a cumbersome laboratory workup (family studies, globin chain synthesis analysis, HBB sequencing) to exclude the presence of globin gene variants, which may interact with β-thalassemia eventually present in the partner.

KLF1 gene mutations have been associated with many different phenotypes both in humans and mice, including hereditary persistence of fetal hemoglobin (HPFH), congenital dyserythrocytopenic anemia, In (Lu) blood group phenotype.5-11 The increased HBG expression determined by mutations of KLF1 prompted us to explore the possibility that the HBD expression and the consequent HbA2 output could also be increased by defects of KLF1. To test this hypothesis, we sequenced KLF1 in a large group of subjects with borderline HbA2 and normal MCV and MCH, and we identified several KLF1 mutations in a consistent proportion of these subjects.

Methods

We have studied 145 subjects with borderline HbA2, defined as HbA2 values between 3.3% and 4.1%, and normal or slightly reduced MCV and MCH. Borderline HbA2 values have been confirmed in all subjects in 3 repeated determinations (twice in the same blood sample at first examination, the third in a second sample after 1-3 years). In these subjects, we had previously excluded the presence of mutations known to be associated with borderline HbA2, including HBB promoter mutations [−101 (HBB c.-151 C → T); −92 (HBB c.-142 C → T),] triplicated α-globin genes, and hemoglobin variants.1 The aforementioned globin gene variants were excluded by appropriate methods (ie, HBB sequence from c.-720 to +137, HBA1 and HBA2 genotyping, and hemoglobin high performance liquid chromatography). We also sequenced the HBD gene, which was found normal from c.-580 to +70 in all but one subject (“DNA analysis”). Eighty normal subjects were used as controls.

The study has been approved by the University of Cagliari Institutional Review Board, and the patients signed the informed consent in accordance with the Declaration of Helsinki.
**Results**

**DNA analysis**

Fifty-two of 145 (35.9%) subjects with borderline HbA2 had heterozygous mutations in the KLF1 gene. Among the KLF1 mutations, the most common was the non-sense KLF1 p.Ser270X mutation, found in 42 subjects (80.8%). Two subjects had the p.Thr280_His283del mutation, 4 the p.Arg319GlufsX34 frameshift mutation, 1 p.Leu326Arg, 2 the Thr327Ser, and 1 the p.Lys332Gln missense mutations. Two mutations (p.Ser270_His283del and Thr327Ser) have never been described before. Complete sequencing of the KLF1 gene did not reveal mutations in the remaining 93 subjects with borderline HbA2.

In all families of subjects with mutated KLF1 available for the study, we confirmed the presence of the proband’s mutation (20 families with p.Ser270X, 1 with p.Arg319GlufsX34, 1 with p.T280_H283del, 1 with p.Leu326Arg, and 1 with p.Thr327Ser) in 1 of the parents and/or in siblings with the same phenotype (supplemental Table 1, available on the Blood Web site; see the Supplemental Materials link at the top of the online article). In one family, both parents had the KLF1 p.Ser270X mutation. It is interesting to note that in this family 2 spontaneous and otherwise unexplained early pregnancy interruptions had occurred. None of the 80 normal HbA2 controls had the KLF1 mutations found in the subjects with borderline HbA2.

In 3 families (1 is reported in Figure 1 and 2 and in supplemental Table 1), we also detected the presence of the codon 39 β-thalassemia non-sense mutation (HBB c.118 C → T p.Gln39X). Overall, 5 subjects were double heterozygotes for β° 39 non-sense mutation and KLF1 mutations. One of the parents of a subject with KLF1 p.Ser270X mutation was also a δ-thalassemia carrier (HBD c.82 G → T p.Ala28ser).18

**Hematologic analysis**

HbA2 and hemoglobin F (HbF) of the probands with KLF1 mutations are reported in Table 1. Other hematologic data and globin chain synthesis analysis are available in supplemental Table 1. Mean HbA2 value was 3.6% ± 0.2% in subjects with the KLF1 p.Ser270X mutation and 3.5% ± 0.2% in those with the frameshift mutation. In our population, mean HbA2 in normal subjects is 2.8% ± 0.2% (range, 2.1%-3.1%) and in β-thalassemia carriers 5.4% ± 0.4% (range, 4.5%-6.2%). HbF was quite variable, ranging from normal (0.2%) to moderately increased levels (5.8%). No correlation was found in these subjects between HbF levels and the known HbF-associated polymorphism Xmn1 in the HBG1 gene, rs9399137 in the HBSil-MYB intergenic region, and rs11886868 in the BCL11A gene.14,15 MCV and MCH were normal in all subjects, except those who coinherited the −3.7-kb α+ thalassemia deletion (supplemental Table 1).
Table 1. HbA2 and HbF in the probands with different KLF1 mutations

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<tbody>
<tr>
<td>No. of subjects</td>
<td>42</td>
<td>2</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>HbA2, %</td>
<td>3.6 ± 0.2 (3.3-4.1)</td>
<td>3.7-3.7</td>
<td>3.5 ± 0.2 (3.3-3.7)</td>
<td>4.0</td>
<td>3.4-3.4</td>
<td>3.8</td>
</tr>
<tr>
<td>HbF, %</td>
<td>2.1 ± 1.2 (0.2-5.8)</td>
<td>1.2-1.3</td>
<td>2.2 ± 0.8 (1.2-3.2)</td>
<td>0.70</td>
<td>1.3-2.3</td>
<td>0.90</td>
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Data are mean ± SD (range).

Discussion

In this study, we report the molecular and hematologic features of a group of persons with borderline HbA2 levels. In 35.9% of the subjects, we found mutations of the KLF1 gene, the most common being the p.Ser270X mutation previously reported associated with increased HbF. The non-sense S270X and the frameshift R319GlufsX34 mutations will ablate the DNA binding domain and hence result in haploinsufficiency of this key erythroid transcription regulator. Among the remaining mutations, T280_H283del will delete cysteine 281, which is essential for Zn coordination, and it is thus predicted to eliminate the Zn finger structure and binding to DNA L326R, T327S, and K332Q missense mutations affects amino acids adjacent to the residues predicted to directly contact DNA, and they might interfere with the binding of Klf1 to DNA. Alternatively, these mutations could impair the interaction of Klf1 with Brg1 and Baf156, previously mapped to the DNA binding domain, thus altering the chromatin remodeling ability of Klf1. Globin gene expression analysis in erythrocyte cultures supported the increased HbA2 phenotype in absence of β-thalassemia mutations. Our results show that the increase of HbA2, associated with KLF1 mutations, is produced at the transcriptional level. In both normal subjects and patients, the δ/β + β-globin mRNA ratios are much higher than the corresponding HbA2/HbA ratio found at the protein level in the peripheral blood. However, with the progress of differentiation from the 9th day to the 11th day of erythrocyte culture, the ratios of δ/β + β expression decrease, suggesting that in the latest stages of maturation the higher output of β-globin mRNA will correct the δ/β imbalance, leading to the slight final increase of HbA2 observed in the mature red blood cells. It is probable that the borderline HbA2 levels found in peripheral blood cells in KLF1 mutants are caused by a delay in the transcriptional switch from the HBD to the HBb gene. Delayed switch should recognize the same mechanism that leads to HPFH in some KLF1 mutant persons and is indirectly supported by the delayed γ- to β-globin switch experimentally found in crosses between heterozygous Klf1 knockout mice and transgenic mice, carrying the full human β-globin gene cluster. By analogy with the mice experiments, the delayed switch occurring in humans could be explained by the competition of the different globin genes for the alternative interaction with the LCR. Because the δ-globin promoter has a highly degenerated CACCC box and does not have any other recognizable Klf1 binding site, it is unlikely that the increased δ-globin transcription results from preferential binding of Klf1 to the δ-globin promoter at reduced Klf1 concentration, as proposed for the increased HbF levels found in the Klf1-related HPFH.

At the phenotypic level, there are no differences among subjects with different KLF1 mutations. In particular, the absence of anemia in this large series of subjects confirms that one functional KLF1 allele is sufficient to sustain normal human erythropoiesis. This is in agreement with previously reported studies, but in contrast with the family described by Arnaud et al in which the presence of
KLF1 haploinsufficiency caused a severe congenital dyserythropoietic anemia. The reasons for this discrepancy could be the presence in the congenital dyserythropoietic anemia patients of undetected mutations in other genes or the variable effects of different KLF1 mutations on erythropoiesis.

Two of the parents with KLF1 mutations and associated iron deficiency anemia or coinherited δ-thalassemia have HbA2 in the low normal range. Both conditions are well-known causes of KLF1 mutations by themselves. In our cohort of KLF1 heterozygous subjects, known additional mutations previously reported associated with borderline HbA2, such as triplicated α-globin genes and/or HBB promoter deletions, were excluded.

In our subjects with borderline HbA2 and KLF1 mutations, HbF varies from normal to moderately increased levels and does not correlate with the presence of the XmnI polymorphism at HBB1 or SNPs, influencing fetal hemoglobin levels at HBS1L-MYB and BCL11A.14,15 Differently from the Maltese HPFH family,7 Sardinian KLF1 heterozygous mutants, even when bearing a comparable non-sense mutation (S270X vs K288X), only rarely cause significant HPFH phenotypes. The discrepancy might be explained by other unknown genetic factors involved in the control of HbF levels.

Interaction of KLF1 mutations with β-thalassemia only results in very high HbA2 levels without any other clinical or hematologic effect. This information is relevant for genetic counseling in couples carrying KLF1 and HBB mutations. The very high levels of HbA2 in these double heterozygotes are probably the result of the cumulative effect of the heterozygous β-thalassemia and KLF1 mutations.

It is interesting to note that the family, in which both parents were heterozygotes for the KLF1 p.Ser270X mutation, had 4 live children and 2 spontaneous early abortions. Hence, the proportion of observed abortions is in good agreement with the mendelian inheritance of a recessive lethal trait, as observed in the Klf1 knockout mice.10 Although this hypothesis is not testable, it is possible that the early pregnancy interruptions were the result of the homozgyosity for the KLF1 p.Ser270X mutation, associated with the total absence of KLF1. This is not in contrast with the simple HPFH phenotype of compound heterozygotes for KLF1 S270X non-sense and K332Q missense mutations9 because the K332Q mutant protein has a reduced but not absent expression with a residual activity.

All KLF1 mutations tested in this study were associated with the In(Lu) blood group. The same blood group phenotype has been reported by Singleton et al9 in subjects with 9 different KLF1 mutations. Thus, the In(Lu) phenotype appears to be a constant feature of KLF1 mutations, suggesting that the amount of KLF1 necessary to regulate Lutheran expression is highly limiting.

The “gray zone” of borderline HbA2 is not an uncommon problem in populations with high frequency of β-thalassemia.1,2,25 Although subjects with borderline HbA2 and reduced MCV/MCH are easily identified because of the presence of well-known HBB and HBD mutations, the presence of HbA2 borderline without hematologic changes (ie, normal MCV/MCH) requires a complex laboratory workup, including the cumbersome and not easily available in vitro globin chain synthesis analysis, to exclude the presence of β-thalassemia mutations. Exclusion of all β-thalassemia carrier states, including carriers of silent β-thalassemia mutations, is essential despite the fact that the interaction of silent HBB mutations with classic β-thalassemia usually results in a mild clinical phenotype.

This is the first report showing that mutations of KLF1 cause activation of the HBD gene and result in increased HbA2 levels, adding a new function to the KLF1 gene. The stimulation of HBD is probably an indirect effect mediated by the impaired looping of the LCR with the HBB gene in favor of the competing HBD gene. A model can be envisaged by which in some subjects, for at present unknown mechanisms, heterozygous KLF1 defects cause preferential interaction of the LCR with the HBD gene, whereas a more pronounced decrease of KLF1 concentration, as observed in compound heterozygotes for KLF1 mutations,9 determines preferential looping with the HBG genes and HPFH. In Sardinians, KLF1 mutations explain more than one-third of the borderline HbA2 phenotype, indicating that this trait is genetically heterogeneous. Moreover, in Sardinia, a common mutation (KLF1 p.Ser270X) accounts for 80.8% of the genetic variability of KLF1 mutations, suggesting the existence of a founder effect similar to that observed in the HBB gene, where the common codon 39 non-sense mutation is responsible for 96% of the HBB mutations.

The identification of KLF1 mutations in subjects with borderline HbA2 and the absence of clinically significant phenotypes in association with the classic HBB mutation should facilitate carrier detection and genetic counseling, significantly contributing to the thalassemia screening programs aimed at identification of at risk couples.

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Authorship

Contribution: L.P., S.S., P.M., F.R.D., and L.M. performed research, analyzed data, and contribute to writing the paper; M.C.S. searched literature, contributed to writing the paper; G.C. and S.S. contributed to data analysis; and R.G. designed research and wrote the paper.

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KLF1 gene mutations cause borderline HbA2

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