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KLF1 gene mutations cause borderline HbA_2

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Increased hemoglobin A_2 (HbA_2; ie, levels > 3.9%) is the most important feature of β-thalassemia carriers. However, it is not uncommon to find persons with borderline HbA_2 (levels, 3.3%-3.8%), who pose a relevant screening problem. Several genotypes have been associated with borderline HbA_2, 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 HbA_2 levels in the borderline range. Six different *KLF1* mutations were identified in 52 of 145 subjects with borderline HbA_2 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)

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Introduction

Accurate determination of the β-thalassemia carrier phenotype is essential for detecting couples at risk for having offspring with thalassemia major. Increased hemoglobin A_2 (HbA_2) 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 HbA_2 is not in the typical carrier range (ie, HbA_2 = 3.8%-6.0%). These atypical carriers have borderline HbA_2 values (ie, HbA_2 levels between normal and β-thalassemia carrier levels, 3.3%-3.8%).[1-3] The prevalence of borderline A_2 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 HbA_2 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), coinherited δ and β-thalassemia, β-promoter mutations −92 (*HBB* c.-142 C→T), or coexisting iron deficiency anemia.[1-5] Borderline HbA_2 with normal MCV and MCH may be an outlier value of the normal HbA_2 distribution in the noncarrier population or the effect of genetic determinants able to increase HbA_2 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 HbA_2 levels.

Although subjects with borderline HbA_2 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 dyserythropoietic anemia, In (Lu) blood group phenotype.[6-11] The increased *HBG* expression determined by mutations of *KLF1* prompted us to explore the possibility that the *HBD* expression and the consequent HbA_2 output could also be increased by defects of KLF1. To test this hypothesis, we sequenced *KLF1* in a large group of subjects with borderline HbA_2 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 HbA_2, defined as HbA_2 values between 3.3% and 4.1%, and normal or slightly reduced MCV and MCH. Borderline HbA_2 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 HbA_2, 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 hemoglobin 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.

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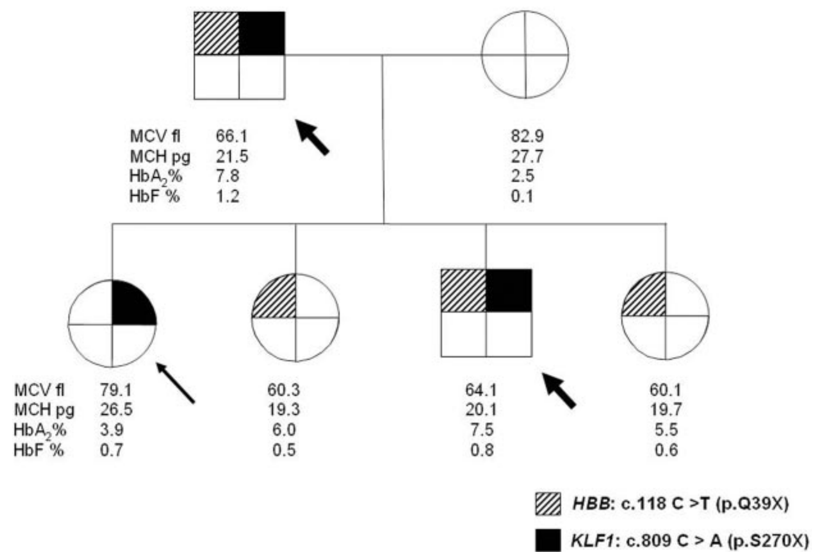

Figure 1. Pedigree of a family showing independent segregation of heterozygous β-thalassemia and *KLF1* mutation. The arrow indicates the proband: head-arrows indicate double heterozygotes for *HBB* and *KLF1* mutations who have outlier levels of HbA_2.

Venous peripheral blood was used for hematologic, hemoglobin, DNA, and expression studies

Complete whole blood cell count was obtained in all subjects, by electronic cell counters (Gen-S and LH750 Hematology Analyzer, Beckman Coulter). Types and amounts of hemoglobin were determined by high performance liquid chromatography (Bio-Rad Variant II analyzer, Bio-Rad). Two-level calibration of the instrument and sample analysis were carried out according to the manufacturer's recommendations. In some subjects, globin chain synthesis analysis was carried out as previously reported.[12]

Genomic DNA was obtained with standard methods. The *KLF1* gene was sequenced using previously described primers.[6] The common single nucleotide polymorphism C → T at position −158 of the *HBG2* promoter (XmnI site; rs7482144) was detected by direct digestion of polymerase chain reaction amplified DNA with XmnI restriction enzyme.[13] Genotyping of individual single nucleotide polymorphisms in the *HBS1L-MYB* (rs9399137) and *BCL11A* (rs 11886868)[14,15] loci was performed using TaqMan genotyping assay (Applied Biosystems).

The 2-step liquid erythroid cultures were obtained from peripheral blood with the procedure developed by Fibach et al.[16] Real-time RT-PCR quantification of mRNA expression was carried out using TaqMan RNA Assay kits according to the manufacturer's protocol (Applied Biosystems).

The amounts of mRNA relative to the endogenous 18S RNA were calculated on day 9 and day 11 of the second phase of liquid culture growth using the comparative cycle threshold (C_t) method ($2^{-\Delta\Delta Ct}$).[17] The experiments were carried out in triplicate.

Results

DNA analysis

Fifty-two of 145 (35.9%) subjects with borderline HbA_2 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 the p.Leu326Arg, 2 the Thr327Ser, and 1 the p.Lys332Gln missense mutations. Two mutations (p.Ser270X and Thr280_His283del) lie in exon 2 and 4 (p.Arg319GlufsX34, p.Leu326Arg, p.Thr327Ser, and p.Lys332Gln) in exon 3. All mutations, except Thr280_His283del, which has been found in 2 unrelated Filipino subjects, have been identified in Sardinian persons. Two mutations (Thr280_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 HbA_2.

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 HbA_2 controls had the *KLF1* mutations found in the subjects with borderline HbA_2.

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

HbA_2 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 HbA_2 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 HbA_2 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 XmnI in the *HBG1* gene, rs9399137 in the *HBS1L-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).

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Table 1. HbA_2 and HbF in the probands with different *KLF1* mutations

Mutation	p.Ser270X	p.Thr280_His283del	p.Arg319GlufsX34	p.Leu326Arg	p.Thr327Ser	p.Lys332Gln
No. of subjects	42	2	4	1	2	1
HbA_2, %	3.6 ± 0.2 (3.3-4.1)	3.7-3.7	3.5 ± 0.2 (3.3-3.7)	4.0	3.4-3.4	3.8
HbF, %	2.1 ± 1.2 (0.2-5.8)	1.2-1.3	2.2 ± 0.8 (1.2-3.2)	0.70	1.3-2.3	0.90

Data are mean ± SD (range).

α/β-globin chain synthesis ratios were in the normal range (0.96 ± 0.04) in 11 evaluated subjects with normal α-globin genotype and in the α-thalassemia carrier range (< 0.8) in 5 subjects with coinherited α-thalassemia (supplemental Table 1).

Similar hematologic data were found in the parents or relatives (belonging to 24 available families) with different *KLF1* mutations, except 2 who had HbA_2 levels in the low normal range, because of associated iron deficiency anemia (HbA_2 = 2.1%) or coinherited δ-thalassemia (HbA_2 = 2.2%; supplemental Figure 1). Five subjects, double heterozygotes for *KLF1* and *HBB* thalassemia mutations, had quite high HbA_2 levels (7.0% to 7.8%), outside of the β-thalassemia carrier range.

Blood group phenotyping randomly performed in 4 subjects with p.Ser270X, one with the p.Arg319GlufsX34, one with the p.Leu326Arg, and one with p.Lys332Gln, showed in all the In(Lu) blood group.

Expression studies

Time point analysis of gene expression by quantitative reverse-transcribed polymerase chain reaction at the 9th day and 11th day of erythroid culture in 5 different carriers with *KLF1* gene defects (3 with S270X, one with R319EfsX34, and one with K332Q mutation) showed an overall increase in the expression of the *HBD* globin gene relative to the *HBB* globin gene, from the normal mRNA value of 3% to 7% up to the highest value of 18% (Figure 2). The δ/δ + β mRNA globin chain ratios were globally reduced at the 11th day of culture, suggesting that erythroid differentiation is accompanied by a partial δ- to β-globin switch. Hence, with advancing maturation, the ratios of δ/δ + β mRNA expression tend toward the lower values observed at the protein level. The increase of the δ-globin mRNA among the 3 patients carrying the same S270X mutation was variable.

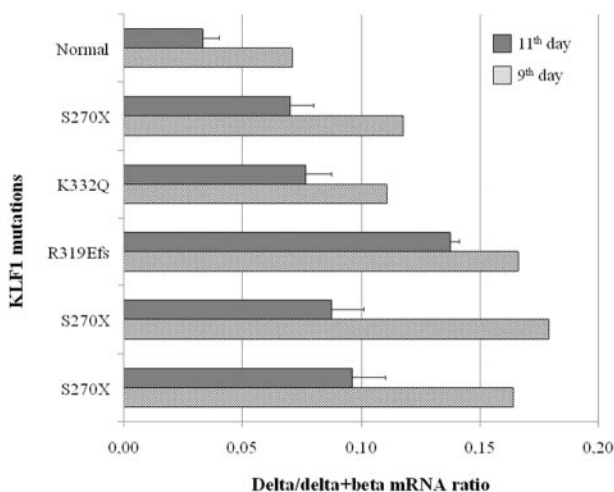

Figure 2. Expression of the δ-globin relative to the expression of the sum of δ- and β-globin genes in erythroid progenitors at 9th and 11th days of liquid culture. All subjects, except the normal control, are heterozygous mutants for the indicated mutations of the *KLF1* gene.

Discussion

In this study, we report the molecular and hematologic features of a group of persons with borderline HbA_2 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.[7] The non-sense S270X and the frameshift R319EfsX34 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,[19] thus altering the chromatin remodeling ability of Klf1. Globin gene expression analysis in erythroid cultures supported the increased HbA_2 phenotype in absence of β-thalassemia mutations. Our results show that the increase of HbA_2, associated with *KLF1* mutations, is produced at the transcriptional level. In both normal subjects and patients, the δ/δ + β-globin mRNA ratios are much higher than the corresponding HbA_2/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 erythroid culture, the ratios of δ/δ + 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 HbA_2 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.[20,21] 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,[22,23] 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.[24]

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,[6,7,9] but in contrast with the family described by Arnaud et al[8] in which the presence of

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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 HbA_2 in the low normal range. Both conditions are well-known causes of reduced HbA_2 levels.[4,5] Thus, the normal values of HbA_2 are the resultant of factors acting in opposite directions and are only apparent exceptions to the increased HbA2 levels produced by *KLF1* mutations. 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 HbA_2 and *KLF1* mutations, HbF varies from normal to moderately increased levels and does not correlate with the presence of the XmnI polymorphism at *HBG1* or $SNPs_s$ 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 HbA_2 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 HbA_2 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 homozygosity 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 mutations[9] 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 al[6] 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 HbA_2 is not an uncommon problem in populations with high frequency of β-thalassemia.[1,2,25] Although subjects with borderline HbA_2 and reduced MCV/MCH are easily identified because of the presence of well-known *HBB* and *HBD* mutations, the presence of HbA_2 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 HbA_2 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 HbA_2 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 HbA_2 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. and S.B. collected clinical data; A.C. analyzed data and reviewed the manuscript; and R.G. designed research and wrote the paper.

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