KLF1 gene mutations cause borderline HbA₂

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Abstract
Increased hemoglobin A2 (i.e. levels higher than 3.9%) is the most important feature of beta thalassemia carriers. However, it is not uncommon to find individuals with borderline hemoglobin A2 (i.e. HbA2 levels between 3.3 and 3.8%), who pose a relevant screening problem. Several genotypes have been associated with borderline hemoglobin A2, 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 hemoglobin A2 levels in the borderline range. Six different KLF1 mutations were identified in 52 out of 145 subjects with borderline HbA2 and normal MCV and MCH. 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.

Introduction
Accurate determination of the beta thalassemia carrier phenotype is essential for detecting couples at risk for having offspring with thalassemia major. Increased HbA2 level is considered the most reliable haematological finding for the identification of beta thalassemia carriers. However, some carriers are difficult to identify because the level of HbA2 is not in the typical carrier range (i.e. HbA2 =3.8 to 6.0%). These atypical carriers have borderline HbA2 values, i.e. HbA2 levels between normal and beta thalassemia carrier levels (HbA2=3.3-3.8%)1-3. The prevalence of borderline A2 carriers in populations with high frequency of beta thalassemia has been reported in 2.2 to 3.0% in one study and up to 16.7% in another study2-3. Borderline HbA2 levels associated with reduced MCV and MCH are generally the consequence of mild beta+-thalassemia mutations (i.e. HBB c.92 +6 T→C), co-inherited delta and beta thalassemia, beta promoter mutations -92 (HBB c.-142 C → T) or coexisting iron deficiency anemia1-5. Borderline HbA2 with normal MCV and MCH may be an outlier value of the normal HbA2 distribution in the non-carrier population or the effect of genetic determinants able to increase HbA2 levels. The genetic determinants so far identified, are the triplication of the alpha globin genes, beta promoter mutations (HBB c.-151 C → T) and some HBD and HBB gene variants1-3. However, all together these determinants explain only a limited proportion of the borderline HbA2 levels.

While 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 work-up (family studies, globin chain synthesis analysis, HBB
sequencing) to exclude the presence of globin gene variants, that may interact with beta 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 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.

**Materials and Methods**

We have studied 145 subjects with borderline HbA2, defined as HbA2 values between 3.3 to 4.1 %, and normal or slightly reduced mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH). Borderline HbA2 values have been confirmed in all subjects in three repeated determinations (twice in the same blood sample at first examination, the third in a second sample after 1 to 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 alpha globin genes and hemoglobin variants. The above mentioned hemoglobin gene variants were excluded by appropriate methods (i.e. *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 (see results). Eighty normal subjects were used as controls.

The study has been approved by the University of Cagliari IRB and the patients signed the informed consent in accordance with the Declaration of Helsinki.

Venous peripheral blood was used for hematological, 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 Inc. Miami, FL). Types and amounts of hemoglobin were determined by HPLC (Bio-Rad Variant II analyzer, Bio-Rad, Segrate Mi, Italy). 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.

Genomic DNA was obtained with standard methods. The *KLF1* gene was sequenced using previously described primers. The common single nucleotide polymorphism (SNP) C→T at position -158 of the *HBG2* promoter (XmnI site) (rs7482144) was detected by direct digestion of
PCR amplified DNA with XmnI restriction enzyme. Genotyping of individual SNPs in the HBS1L-MYB (rs9399137) and BCL11A (rs 11886868) loci was performed using TaqMan genotyping assay (Applied Biosystem, Warrington, UK).

The two-step liquid erythroid cultures were obtained from peripheral blood with the procedure developed by Fibach et al. Real time RT-PCR quantification of mRNA expression was carried out using TaqMan RNA Assay kits according to the manufacturer’s protocol (Applied Biosystem, Foster City, CA USA).

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

**Results**

**DNA analysis**

Fifty-two out 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, four the p.Arg319GlufsX34 frameshift mutation, one the p.Leu326Arg, two the Thr327Ser and one the p.Lys332Gln missense mutations. Two mutations (p.Ser270X and Thr280_His283del) lie in exon 2 and four (p.Arg319GlufsX34, p.Leu326Arg, p.Thr327Ser, p.Lys332Gln) in exon 3. All mutations, except Thr280_His283del which has been found in two unrelated Philipino subjects, have been identified in Sardinian individuals. 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 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, one with p.Arg319GlufsX34, one with p.T280_H283del, one with p.Leu326Arg, and one with p.Thr327Ser) in one of the parents and/or in siblings with the same phenotype (see Supplementary Table 1). In one family both parents had the KLF1 p.Ser270X mutation. It is interesting to note that in this family two 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 three families (one is reported in Fig 1 and two in Supplementary Table 1), we detected also the presence of the codon 39 beta thalassemia nonsense mutation (HBB c.118 C→T p.Gln39X). Overall five subjects were double heterozygotes for beta° 39 non-sense mutation and KLF1 mutations. One
of the parents of a subject with *KLF1* p.Ser270X mutation was also delta thalassemia carrier (*HBD* c.82 G→T p.Ala28ser)\(^{18}\).

**Hematological analysis**

HbA\(_2\) and HbF of the probands with *KLF1* mutations are reported in Table 1. Other hematological data and globin chain synthesis analysis are available in Supplementary 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 beta 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 co-inherited the -3.7 kb alpha\(^+\) thalassemia deletion (see Supplementary Table 1).

Alpha/beta globin chain synthesis ratios were in the normal range (0.96±0.04) in 11 evaluated subjects with normal alpha globin genotype and in the alpha thalassemia carrier range (<0.8) in 5 subjects with co-inherited alpha thalassemia. (see Supplementary Table 1)

Similar hematological data were found in the parents or relatives (belonging to 24 available families) with different *KLF1* mutations, except two who had HbA\(_2\) levels in the low normal range, due to associated iron deficiency anemia (HbA\(_2\)= 2.1 %) or co-inherited delta thalassemia (HbA\(_2\)=2.2%) (see Supplementary Figure 1). Five subjects, double heterozygotes for *KLF1* and *HBB* thalassemia mutations, had quite high HbA\(_2\) levels (7.0 and 7.8%), outside of the beta thalassemia carrier range.

Blood group phenotyping randomly performed in four 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 qRT-PCR at the 9\(^{th}\) and 11\(^{th}\) 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 3-7% up to the highest value of 18% (Fig. 2). The delta/delta+beta mRNA globin chain ratios were globally reduced at the 11\(^{th}\) day of culture suggesting that erythroid differentiation is accompanied by a partial delta- to beta-globin switch.
Hence, with advancing maturation the ratios of delta/delta+beta mRNA expression tend toward the lower values observed at the protein level. The increase of the delta-globin mRNA among the three patients carrying the same S270X mutation was variable.

**Discussion**

In this study we report the molecular and hematological features of a group of individuals with borderline HbA2 levels. In 35.9% of the subjects we found mutations of KLF1 gene, the most common being the p.Ser270X mutation previously reported associated with increased HbF. 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 affect 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 remodelling ability of Klf1. Globin gene expression analysis in erythroid cultures supported the increased HbA2 phenotype in absence of beta 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 delta/delta+beta 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 to the 11th day of erythroid culture, the ratios of delta/delta+beta expression decrease, suggesting that in the latest stages of maturation the higher output of beta-globin mRNA will correct the delta/beta imbalance leading to the slight final increase of HbA2 observed in the mature red blood cells. It is likely that the border-line 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 individuals, and is indirectly supported by the delayed gamma- to beta-globin switch experimentally found in crosses between heterozygous Klf1 knock out mice and transgenic mice, carrying the full human beta-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. Since the delta-globin promoter has a highly degenerated CACCC box and does not have any other recognizable Klf1 binding site, it is unlikely that the increased delta-globin transcription results from preferential binding of Klf1 to the delta-globin promoter at
reduced Klf1 concentration, as proposed for the increased HbF levels found in the Klf1 related HPFH\textsuperscript{24}.

At the phenotypical 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\textsuperscript{6,7,9}, but in contrast with the family described by Arnaud et al \textsuperscript{8} in which the presence of KLF1 haploinsufficiency caused a severe congenital dyserythropoietic anemia (CDA). The reasons for this discrepancy could be the presence in the CDA 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 co-inherited delta thalassemia have Hba\textsubscript{2} in the low normal range. Both conditions are well known causes of reduced Hba\textsubscript{2} levels\textsuperscript{4,5}. Thus, the normal values of Hba\textsubscript{2} are the resultant of factors acting in opposite directions and are only apparent exceptions to the increased Hba\textsubscript{2} levels produced by KLF1 mutations. In our cohort of KLF1 heterozygous subjects, known additional mutations previously reported associated with borderline Hba\textsubscript{2}, such as triplicated alpha-globin genes and/or HBB promoter deletions were excluded.

In our subjects with borderline Hba\textsubscript{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 influencing fetal hemoglobin levels at HBS1L-MYB and BCL11A\textsuperscript{14,15}. Differently from the Maltese HPFH family\textsuperscript{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 beta thalassemia only results in very high Hba\textsubscript{2} levels without any other clinical or hematological effect. This information is relevant for genetic counseling in couples carrying KLF1 and HBB mutations. The very high levels of Hba\textsubscript{2} in these double heterozygotes, are likely the result of the cumulative effect of the heterozygous beta 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 four live children and two 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 knock out mice\textsuperscript{10}. Although this hypothesis is not testable, it is possible that the early pregnancy interruptions were due to the homozygosity for the KLF1 p.Ser270X mutation, associated with 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, since 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 in subjects with nine 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 beta thalassemia. While 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 hematological changes (i.e. normal MCV/MCH) requires a complex laboratory work-up, including the cumbersome and not easily available in vitro globin chain synthesis analysis, to exclude the presence of beta thalassemia mutations. Exclusion of all beta thalassemia carrier states, including carriers of silent beta thalassemia mutations, is essential despite the fact that the interaction of silent HBB mutations with classical beta thalassemia usually results in a mild clinical phenotype.

This is the first report to showing that mutations of KLF1 cause activation of the HBD gene and result in increased HbA2 levels, adding a new function to KLF1 gene. The stimulation of HBD is likely an indirect effect mediated by the impaired looping of the LCR with the HBB gene in favour 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, determines preferential looping with the HBG genes and HPFH. In Sardinians, KLF1 mutations explain over 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 classical HBB mutation, should facilitate carrier detection and genetic counselling significantly contributing to the thalassemia screening programs aimed at identification of at risk couples.
Authorship:

P.L., S.S., P.M., D.FR. and M.L. performed research, analyzed data and contribute to writing the paper; S.MC. and B.S. collected clinical data; A.C. analyzed data and reviewed the manuscript; G.R. designed research and wrote the paper.

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Conflict of interest: No conflict of interest to declare

References

Table 1. HbA₂ and HbF mean ± standard deviation and range ( ) in the probands with different \textit{KLF1} mutations

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**Fig. 1.** Pedigree of a family showing independent segregation of heterozygous beta thalassemia and KLF1 mutation. The arrow indicates the proband: head-arrows indicate double heterozygotes for HBB and KLF1 mutations who have outlier levels of HbA2.

**Fig. 2.** Expression of the delta-globin relative to the expression of the sum of delta- and beta-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.
Fig. 2

The bar chart depicts the delta/delta+beta mRNA ratio for various KLF1 mutations. The y-axis represents different mutations: Normal, S270X, K332Q, R319Efs, S270X, and S270X. The x-axis represents the ratio ranging from 0.00 to 0.20.

- Normal: The bar shows a lower ratio on the 11th day compared to the 9th day.
- S270X: The ratio is higher on the 11th day compared to the 9th day.
- K332Q: The ratio is higher on the 11th day compared to the 9th day.
- R319Efs: The ratio is significantly higher on the 11th day compared to the 9th day.
- S270X: The ratio is higher on the 11th day compared to the 9th day.
- S270X: The ratio is higher on the 11th day compared to the 9th day.

The chart indicates that the mutations S270X and K332Q show an increase in the delta/delta+beta mRNA ratio on the 11th day, while the mutation R319Efs shows a much higher ratio on the 11th day.
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