Compound heterozygosity for *KLF1* mutations associated with remarkable increase of fetal hemoglobin and red cell protoporphyrin

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ABSTRACT

The persistence of high fetal hemoglobin level in adults may ameliorate the clinical phenotype of beta-thalassemia and sickle cell anemia. Several genetic variants responsible for hereditary persistence of fetal hemoglobin, linked and not linked to the beta globin gene cluster, have been identified in patients and in normal individuals. Monoallelic loss of *KLF1*, a gene with a key role in erythropoiesis, has been recently reported to be responsible for persistence of high levels of fetal hemoglobin. In a Sardinian family, high levels of HbF (22.1-30.9%) were present only in compound heterozygotes for the S270X nonsense and K332Q missense mutations, while the isolated S270X nonsense (haploinsufficiency) or K332Q missense mutation were associated with normal HbF levels (<1.5%). Functionally, the K332Q Klf1 mutation impairs binding to the *BCl11A* gene and activation of the γ- and β- globin promoters. Moreover, we report for the first time the association of *KLF1* mutations with very high levels of zinc protoporphyrin.

Key words: *KLF1*, mutation, heterozygosity, fetal hemoglobin, red cell, protoporphyrin.

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Introduction

Persistent expression of fetal hemoglobin (HbF) is of great clinical relevance given its role in the amelioration of the phenotype of beta-thalassemia and sickle cell anemia. Several studies have identified genes and genetic variants controlling HbF levels in adults (*HBG1/HBG2*, *HBS1-MYB* and *BCL11A*) able to improve the severity of the two major beta-hemoglobinopathies, beta thalassemia and sickle cell anemia.[1-4] Recently a nonsense mutation in the *KLF1* gene, which ablates the DNA binding domain of this key erythroid transcriptional regulator, has been reported in a large Maltese family with hereditary persistence of HbF (HPFH).[5,6] Haploinsufficiency of *KLF1* expression has been considered to be responsible for HPFH.

In the Sardinian family described here, we found a marked increase of HbF only in compound heterozygotes for two *KLF1* mutations and we did not confirm the *KLF1* haploinsufficiency as a cause of HPFH. Moreover, we report, for the first time in humans, very high levels of zinc protoporphyrin associated with *KLF1* mutations.

Design and Methods

We studied a Sardinian family with HPFH. Blood samples were obtained after informed consent. Hematologic and biochemical analyses were performed according to standard procedures. Zinc protoporphyrin in RBC was determined with ZPP hematofluorometer (AVIV Biomedical, Lakewood, NJ, USA) and blood protoporphyrin IX with a fluorometric method.

Genomic DNA was obtained from peripheral blood by standard methods.

Mutation analysis was performed by PCR amplification and DNA sequence analysis of the *KLF1* gene using previously described primers.[6] Genotyping of individual SNPs in the *HBS1L-MYB* (rs9399137) and *BCL11A* (rs11886868) loci was performed using Taqman genotyping assay (Applied Biosystem, Warrington, UK). Alpha globin and bilirubin UDP-glucuronosyltransferase (*UGT1A1*) gene genotyping was carried out as previously described.[7,8]

The site directed mutation in K332Q in the *KLF1* cDNA was obtained with the QuickChange Mutagenesis Kit (Stratagene, La Jolla, CA, USA).

Band shift, supershift, Western blots and transactivation analysis

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were performed as previously described.[9] The intensities of the *KLF1* shifted bands were determined with the ImageQuant software after gel autoradiography on phosphoscreen and acquisition with PhosphoImager Storm 840 (GE Healthcare).

The study was approved by the institutional review board of the hospital (ASL8 Ethics Commitee).

Results and Discussion

Hematologic phenotype (Table 1)

The propositus (II-1) presented moderate, normochromic, normocytic anemia, reticulocytes in the upper normal values (0.97×10^6/μL), highly increased HbF (30.9%), normal HbA_2 and unbalanced alpha/beta globin chain synthesis ratio (alpha/beta =1.8). The G-gamma/A-gamma ratio was of fetal type with high prevalence of the G-gamma globin 80%). He also showed increased unconjugated bilirubin levels (34.2 μmol/L), almost absent haptoglobin, high serum ferritin (490 μg/mL) and very elevated red blood cell zinc protoporphyrin (306 μg/dL; normal values less than 35 μg/dL). Increase in red cell protoporphyrin was confirmed by direct determination of protoporphyrin IX (270 μg/dL; normal values less than 50 μg/dL). Blood lead levels were normal. The osmotic fragility test was normal.

The brother (II-2) had similar hematologic phenotype, including very high RBC zinc protoporphyrin, but he presented microcytosis, hypochromia mild anisopoikilocytosis, lower HbF level (22.1%) and a less unbalanced globin chain synthesis ratio (alpha/beta=1.33).

Both parents and the third brother (II-3) have normal hematologic phenotype, normal HbF and normal red blood cell zinc protoporhyrin levels. Subjects with the S270X mutation (see below) have the In(Lu) blood-group phenotype.[10]

DNA analysis (Figure 1)

KLF1 gene sequencing of the proband and brother (II-2-) revealed a genetic compound condition for a nonsense mutation (p. S270X) at exon 2, inherited from the father and a missense mutation (p. K332Q) at exon 3 inherited from the mother. The brother (II-3) had only the K332Q missense mutation.

We also genotyped two individual SNPs in the *HBS1L-cMYB* loci and *BCL11A* loci, previously associated with increased HbF levels (Table 1). XmnI *Gγ* promoter polymorphism was absent in all family members. Analysis of the alpha globin gene cluster revealed the 3.7 Kb deletion in I-1 and II-2 (genotype –alpha/ alpha alpha). The S270X nonsense mutation here reported is predicted to completely ablate the zinc finger domain and the ability of KLF1 to

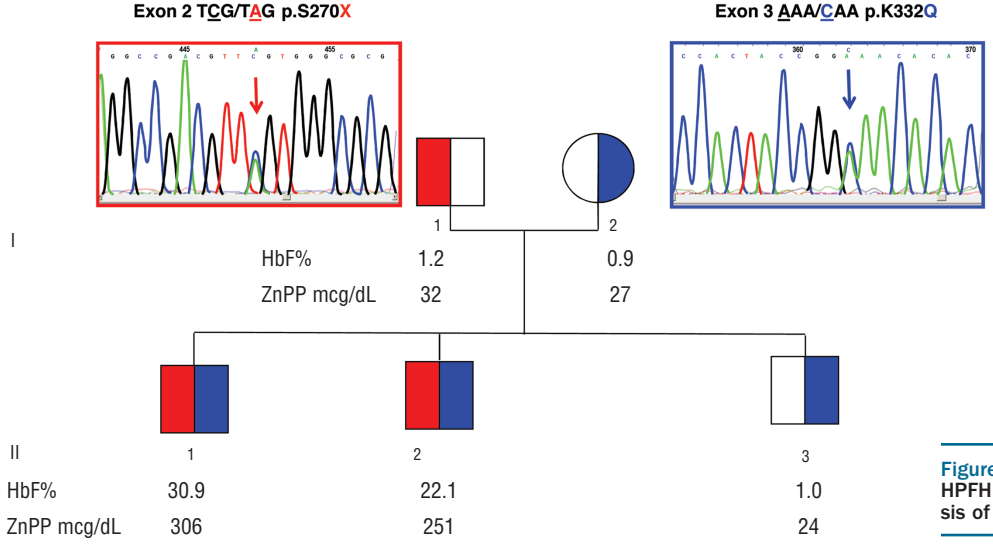

Figure 1. Pedigree of the Sardinian HPFH family and sequence analysis of the *KLF1* gene.

Table 1. Hematologic parameters and genotypes of the Sardinian HPFH family.

Case	Age y	Hb (g/dL)	MCV (fl)	MCH (pg)	HbA2 (%)	HbF[1] (%)	Globin synthesis ratio α/β	Globin synthesis ratio α/β+γ	ZnPP2 (mcg/dL)	Bilirubin3 (μmol/L)	Hapto globin 4 (mg/dL)	Serum ferritin5 (ng/mL)	*KLF1* S270X	*KLF1* K332Q	α globin genotype	*BCL11A* Rs11886868	*HBS1L-cMYB* Rs9399137
I-1	57	12.9	82	27.3	3.2	1.2	1.1	-	32	8.5	45	220	+/-	-/-	αα/αα	T/C	T/C
I-2	53	12.3	85	28.2	2.9	0.9	1.0	-	27	8.4	55	nd	-/-	+/-	–α/αα	T/C	T/T
II-1	31	11.5	82	27.6	2.8	30.9	1.8	1.3	306	34.2	<5.8	490	+/-	+/-	αα/αα	T/C	T/C
II-2	30	12.1	73	24.3	3.3	22.1	1.3	1.1	251	30.8	<5.8	550	+/-	+/-	–α/αα	C/C	T/C
II-3	26	15.4	93	32.2	3.2	1.0	-	-	24	9.2	60	nd	-/-	+/-	αα/αα	T/T	T/C

[1]Mean HbF level (%) in control subjects 0.4±0.4 (range 0.1-1.4); [2]Zinc protoporphyrin normal values < 35 mcg/dL; [3]Unconjugated; [4]Haptoglobin normal values 40.0 - 200.0 mg/dl; [5]Serum ferritin normal values 25.0 - 280.0.

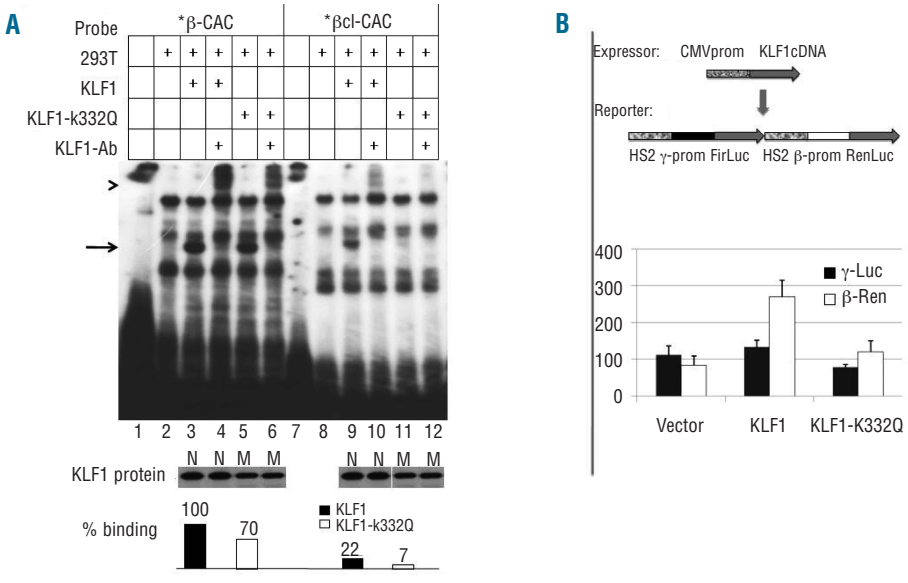

Figure 2. (A) **Top: band shift and supershift analysis of wild-type KLF1 and mutant KLF1K332Q. *Probes used were the beta-globin proximal CAC box (β-CAC, lanes 1-6) and the BCL11A KLF1 site found at -325 from the putative cap site (Bcl-CAC, lanes 7-12). All extracts derived from HEK-293T cells not transfected (lanes 1, 7) or transfected with the indicated KLF1 proteins. KLF1 specific antibody was added in lanes 4, 6, 10 and 12. The arrow points at the KLF1 specific complexes that are supershifted (arrowhead) by KLF1 antibody (lanes 4, 6, 10, 12). Middle: Western blot analysis showing the relative amounts of KLF1 (N) and KLF1K332Q (M) protein loaded in each lane. Bottom: band intensities of KLF1 specific complexes from lanes 3, 5, 9 and 11 determined by PhosphoImager volumetric analysis and expressed as percentage of KLF1 binding to the β-globin CAC box in lane 3.**

(B) Transactivation analysis of γ- and β-globin promoters in a dual luciferase reporter construct in MEL cells. Top: scheme of the expressor and reporter constructs (HS2 is the 5' hypersensitive site 2 derived from the β-globin locus control region, FirLuc and RenLuc indicate the firefly and renillaluciferase genes). Bottom: transactivation analysis. Histograms represent the luciferase activities of the β- and γ-promoters expressed as percentage of the value of the mock transfected MEL cells

interact with DNA. The missense K332Q mutation lies in the second KLF1 zinc finger domain and in combination with the S270X nonsense, further reduces KLF1 function.

The *KLF1* gene encodes a key transcription factor regulating the developmental switch from fetal to adult globin. Based on previous and recent experimental data it has been hypothesized that after birth high levels of KLF1 activate the *HBB* gene and *BCL11A* expression, which in turn suppresses *HBG1/HBG2* expression, while in the fetus reduced KLF1 levels result in very low *HBB* and *BCL11A* gene expression and therefore in low beta and high gamma globin levels.[6] It is interesting to note that subjects II-1 and II-2, with genetic compound for the two KLF1 mutations, have unbalanced alpha/beta globin chain synthesis ratio (i.e. in the beta-thalassemia carrier range), despite having normal beta globin gene sequence and not increased HbA2 levels. The reduced beta globin production and the excess of G-gamma chains partly resembles a late fetal or newborn condition, consistent with the key role of KLF1 in the globin switching. The milder imbalance in II-2 as compared to II-1 is due to the coinheritance of deletion alpha-thalassemia. Globin chain synthesis ratio is normal (alpha/beta=1.1) in the carrier of isolated *KLF1* nonsense mutation.

In subject II-2, homozygote for the C variant at *BCL11A* rs 11886868 which is strongly associated with high HbF levels,[2-4] HbF is lower than in his brother II-1 who is heterozygote (T/C) at the same rs. This unexpected finding could be the result of the coinheritance of alpha thalassemia that, by reducing the amount of alpha globin chains, could decrease the assembling into HbF tetramers ($alpha_2gamma_2$).

Borg *et al.*[5] very recently described in a Maltese family with high very variable levels of HbF (range 3.3-19.5%) two linked mutations in the *KLF1* gene: a nonsense mutation at exon 2 (p.K288X) that, by removing the two amino-terminal zinc fingers, will completely abrogate the DNA binding domain of the mutated protein and a missense mutation (p.M39L) at exon 2, considered a neutral substitution. Expression profiling and functional assays on primary erythroid progenitors from the Maltese individuals with HPFH and on *KLF1* knockdown cells suggested that diminished KLF1 activity results in decreased expression of *BCL11A* gene, which is a stage-specific repressor of *HBG1/HBG2* genes and HbF production.[5,11] Consistent with the results of Borg *et al.*,[5] Zhou *et al.*[12] found that BCL11A levels were dramatically down-regulated in a *KLF1* mouse model. Overall, the observations from the Maltese family are in agreement with the hypothesis that the effects of KLF1 haploinsufficiency are the cause of HPFH. Results in our family are different. Only individuals with two *in trans KLF1* mutations have HPFH, while the monoallelic loss of *KLF1* expression in subject I-1 is associated with normal HbF levels, even though produced by an upstream stop codon that predicts a smaller protein as compared to the Maltese mutation. The concurrent presence of the two *in trans KLF1* mutations is the only possible explanation for the higher HbF levels found in Sardinian HPFH subjects. Another relevant difference is that the subjects with the two *KLF1* mutations in our family have a more severe hematologic phenotype with higher HbF levels, (22.1-30.9% in our family *vs.* 3.3-19.5% in the Maltese family), mild hemolysis and very high levels of red blood cell protoporphyrin. It has been previously reported that, beside beta and gamma globin chains, *KLF1* regulates several enzymes in the heme biosynthetic pathway. This role may explain the dramatic increase of the zinc protoporphyrins[13-15] observed in the *Nan* mutant mouse, which is caused by a mutation in a crucial residue (E339D) of the central Zn finger that alters the DNA binding specificity of KLF1. The mild hemolysis (probably due to RBC membrane destabilization despite the normal

osmotic fragility test) and the (TA) dinucleotide insertion in the TATA-box of the *UGT1A1* promoter in subjects II-1 and II-2 [(TA)7/(TA)6], are responsible for the slight increase in bilirubin level. It should be pointed out that in this family the blood group Lu phenotype is dominant, whereas the increased Hb F and ZnPP levels are recessive. The apparent discordance may be explained by the fact that target erythroid genes are variably regulated by KLF1,[14] hence the concentration of KLF1 may be limiting for some genes and partially redundant for others which will be activated or suppressed at lower KLF1 concentrations.

Functional analysis

To exclude the possibility that the K332Q mutation, which is a partially conservative amino acid substitution, could be a silent protein variation, we analyzed functionally promoter binding and transactivation potential of the K332Q mutant compared to wild-type KLF1. In electrophoretic mobility shift assays, we showed that binding of KLF1 K332Q mutant to the beta globin proximal CACC box is reduced to 70%, whereas binding to the *BCL11A* promoter is only 32% of wild-type protein binding (Figure 2A). Moreover, in transactivation assays in murine erythroleukemia cell lines (MEL) we confirmed that the reduced binding to the beta globin CACC box of K332Q KLF1 translates into a reduced transactivation potential on both gamma and beta-globin genes (Figure 2B). Hence, the activation of the gamma globin gene observed *in vivo* is not the result of an altered tropism of KLF1-K332Q toward preferential binding and activation of the gamma globin *versus* the beta globin gene. Our combined results are consistent with the HPFH being mediated by the released *BCL11A* repression of the gamma globin gene. Further elements in favor of K332Q being a causal mutation are the evolutionary conservation of the mutated lysine residue and, most importantly, the otherwise unexplained altered globin biosynthetic ratio in the compound heterozygous relative to the simple KLF1 heterozygous defect.

In conclusion, our observations do not confirm that monoallelic loss of *KLF1* is sufficient to cause HPFH. Moreover, we report for the first time in humans that *KLF1* mutations are associated with very high zinc protoporphyrin levels, confirming in humans the relevance of KLF1 in the control of the erythropoietic pathway leading to heme biosynthesis.

Authorship and Disclosures

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