Ann Hematol (2013) 92:53–58
DOI 10.1007/s00277-012-1625-9

ORIGINAL ARTICLE

Functional analysis of a novel *KLF1* gene promoter variation associated with hereditary persistence of fetal hemoglobin

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Received: 31 July 2012 / Accepted: 5 November 2012 / Published online: 18 November 2012
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Abstract Hereditary persistence of fetal hemoglobin (HPFH) is a rare hereditary condition resulting in elevated levels of fetal hemoglobin (HbF) in adults. Typical HPFH is associated with promoter mutations or large deletions affecting the human fetal globin (*HBG1* and *HBG2*) genes, while genetic defects in other genes involved in human erythropoiesis, e.g. *KLF1*, also result in atypical HPFH. Here, we report the first *KLF1* gene promoter mutation (*KLF1*:g.-148G>A) that is associated with increased HbF level. This mutation was shown to result in drastically reduced CAT reporter gene expression in K562 cells, compared to the wild-type sequence ($p=0.009$) and also in reduced *KLF1* gene expression in vivo. Furthermore, consistent with in silico analysis, electrophoretic mobility shift analysis showed that the *KLF1*:g.-148G>A mutation resides in a Sp1 binding site and further that this mutation leads to the ablation of Sp1 binding in vitro. These data suggest that the *KLF1*:g-148G>A mutation could play a role in increasing HbF levels in adults and further underlines the role of KLF1 as one of the key transcription factors involved in human fetal globin gene switching.

Keywords Hereditary persistence of fetal hemoglobin · Fetal hemoglobin · *KLF1* gene · Promoter mutation · Transcription · Sp1

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Introduction

Hereditary persistence of fetal hemoglobin (HPFH) is a rare inherited condition that results in increased fetal hemoglobin (HbF) levels in adult life [1]. Typical HPFH results from either promoter mutations (non-deletional HPFH) or large deletions (deletional HPFH), affecting the human fetal globin (*HBG1* and *HBG2*) genes; these genetic defects result in high HbF levels and reciprocally by reduced HbA_2 levels, which are one of the key features of typical HPFH [1]. Recent experimental evidence suggests that genetic defects in genes outside the human β-globin gene cluster also result in HPFH. For example, *KLF1* gene mutations have been recently demonstrated to result in persistent HbF levels in adulthood [2–5]. Although clinically benign, these conditions provide valuable insights into the molecular mechanism that governs the transcriptional regulation of the human fetal globin genes, which in turn can enable design of novel strategies for β-thalassemia therapeutics. Here, we report the first promoter mutation in the *KLF1* gene and further provide functional evidence suggesting that this mutation results in decreased *KLF1* gene transcription mediated by the alteration of a Sp1 transcription factor binding site.

Materials and methods

Hemoglobin studies and DNA analysis

Blood samples were collected, with consent, in vacutainers with Na citrate as anticoagulant. Hematological indices were

measured with an automated cell counter, and HbA_2 and HbF levels were detected by standard methods (cellulose chromatography and alkali denaturation, respectively). The study was previously approved by the hospital's ethics committee.

Total genomic DNA isolation and γ-globin gene promoters' amplification were done as previously described [6]. The entire coding and promoter region of the *KLF1* gene was amplified and resequenced using amplification primers shown in Table 1. Amplification conditions were 30 cycles of 60 s denaturation at 95 °C, 60 s annealing at 60 °C, and finally 60 s elongation at 72 °C. Amplification of *BCL11A* gene to screen for the rs11886868 single nucleotide polymorphism (SNP), previously shown to be associated with high HbF levels [7], was performed using 20 pmol of each primer (BCL11A-FW and BCL11A-RV; Table 1), 300 ng of genomic DNA, 200 μmol/l of each dNTP (Fermentas, ON, Canada), 2.75 mM $MgCl_2$, and 1 U HotStart Taq DNA polymerase (Qiagen). Amplification conditions were 35 cycles of 60 s denaturation at 95 °C, 60 s annealing at 56 °C, and 60 s elongation at 72 °C. Subsequently, the PCR fragment was resequenced using BigDyeTM Terminators V3.1 Ready Reaction Kit (Applied Biosystems) using a 3130 Genetic Analyzer (Applied Biosystems) according to the manufacturer's instructions and the same primers used for the PCR amplification step.

Functional assays

KLF1 gene promoter fragments, spanning from positions −231 to +44, relative to the transcription initiation site, were amplified using forward and reverse primers (Table 1) containing *Hin*dIII and *Xba*I restriction enzyme sites at their 5′ end and subcloned into pBLCAT5 vector (Promega). *KLF1* gene promoter regions were amplified from the subject bearing the *KLF1* gene promoter mutation. After digestion, PCR products were subcloned into unique cloning sites (*Hin*dIII and *Xba*I) of the pBLCAT5 reporter vector (Promega). All constructs were sequenced in both orientations to confirm that no other mutations were incorporated during the PCR amplification step.

Cell culture, transfection, and functional CAT assays

K562 cells were maintained in complete MEM growth medium suplemented with 10 % bovine serum at 37 °C in 10 % CO_2 atmosphere. For each transfection, a total of 2×10^6 K562 cells were placed into a 10-cm dish in MEM medium without antibiotics, and 6 μg of each pCAT construct was co-transfected with 2 μg of pCH110 (Amersham Pharmacia Biotech) using Lipofectamine 2000 (Invitrogen). The pCH110 vector bearing the β-galactosidase gene was used as an internal control to normalize for transfection efficiency. Twenty four hours post-transfection, β-gal assays were performed with a β-galactosidase enzyme assay system (Promega), and CAT activities were determined using CAT enzyme-linked immunosorbent assay (Roche). The normalized CAT activities were evaluated as a percentage of pBLCAT5 vector activity (CAT5) (*tk* promoter driving the *cat* gene) used as a positive control, which was set as 100 % and are presented as the means±standard deviation of at least three independent experiments. Statistical significance was determined by Student's *t* test, with two-tailed, paired samples, and a difference of $p<0.05$ was considered significant.

Electrophoretic mobility shift assay

The oligonucleotides used in electrophoretic mobility shift assay (EMSA) and supershift assays are provided in Table 1.

Table 1 Nucleotide sequence of the primers used for the amplification of the *KLF1* gene promoter and coding regions for resequencing, reporter construct production, and EMSA analysis

Primer name	Primer sequence (5′>3′)	Fragment size (bp)
Gene amplification		
KLF1_ex1_FW	GCTTTGGACACAGGGTTAGT	400
KLF1_ex1_RV	TCAGGTCAAGATGCAGGTCT	
KLF1_ex2a_FW	TTCCAAAGCCTCTGCGTCAG	570
KLF1_ex2a_RV	ACGCCGCAGGCACTGAAAG	
KLF1_ex2b_FW	GGGAGGAAGAGGACGATGA	985
KLF1_ex2b_RV	GGACAAGGAAGCCATAAGC	
KLF1_ex3_FW	AGGCTGAGTAAAGGGGTGTG	650
KLF1_ex3_RV	ACCTTCAGGAGCCGCTTTCT	
BCL11A_FW	TTGAACTCTCCAGGGAATGG	459
BCL11A_RV	CCTTCCCTAACCCTCTGACC	
Subcloning		
KLF1pr_FW	CCCAAGCTTGGGTGGCTTTGGACACAGGGTTAGTCT	295
KLF1pr_RV	GCTCTAGAGCTCAGTGTGCTGATGGAGGGCAA	
EMSA analysis		
KLF1:g.-148G	AAACAGTGCCCCCCCGCCGCCTTGCCTTGC	30
KLF1:g.-148A	AAACAGTGCCCCCCCACCGCCTTGCCTTGC	

Amplification conditions are available upon request

Probes were 5′ end-labeled with [γ-^{32}P]ATP, and nuclear extracts from K562 cells were prepared according to Zukic and coworkers [8]. For EMSA analysis, 5 μg of nuclear extract prepared from K562 cells was incubated for 30 min at 37 °C, with 2 ng of ^{32}P-labeled oligonucleotide probe in a binding buffer consisting of 50 mM Tris pH 8.0, 250 mM NaCl, 5 mM DTT, 5 mM EDTA, and 50 % glycerol in a total volume of 25 μl. In competition assays, 100-fold molar excess of unlabeled competitor was included in the binding reaction. In the supershift assays, 0.2 μg of anti-Sp1 mouse monoclonal Ab (Santa Cruz Biotechnology) was added to reaction mixtures before addition of the nuclear extract.

Quantitative real-time PCR analysis

Peripheral blood mononuclear cells were isolated by Ficoll density-gradient centrifugation. Total RNA was extracted using TRI reagent (Sigma-Aldrich) and reverse-transcribed using RevertAid M-MuLV Reverse Transcriptase (Fermentas) and random hexamer primers, according to the manufacturer's instructions. *KLF1* gene expression levels were quantified by quantitative reverse-transcriptase polymerase chain reaction using SYBR Green chemistry in 7500 Real-Time PCR system (Applied Biosystems) and RT2qPCR Primer Assay-SYBR Green Human KLF1 kit (SABiosciences). *GAPDH* gene served as an internal control. Relative quantification analysis was performed using comparative ddCt method, using RNA isolated from individuals with normal *KLF1* gene sequence, as negative control.

Results

DNA analysis

During screening for β-thalassemia, an adult female subject of Serbian origin was identified with elevated HbF levels (HbF=11 %) and the following hematological indices: MCH=22.6 pg, Hb=12.8 g/dL, and HbA_2=2.5 %. DNA resequencing of the *KLF1* gene revealed a novel promoter mutation (*KLF1*:g-148G>A; Fig. 1), as well as a common genomic variation affecting exon 2 of *KLF1* gene (p.S102P). We failed to identify any mutation in either one of the γ-globin genes promoters, while the rs11886868 SNP in intron 2 of the *BCL11A* gene, previously shown to be associated with high HbF levels [7], was also absent from the index case. We did not screen for deletional mutants leading to deletional HPFH or δβ-thalassemia. However, the hematological indices of the index case are not indicative of any of these syndromes [1].

DNA resequencing of the *KLF1* gene promoter in 128 chromosomes from the general population in Serbia revealed two other alleles bearing the *KLF1*:g-148G>A

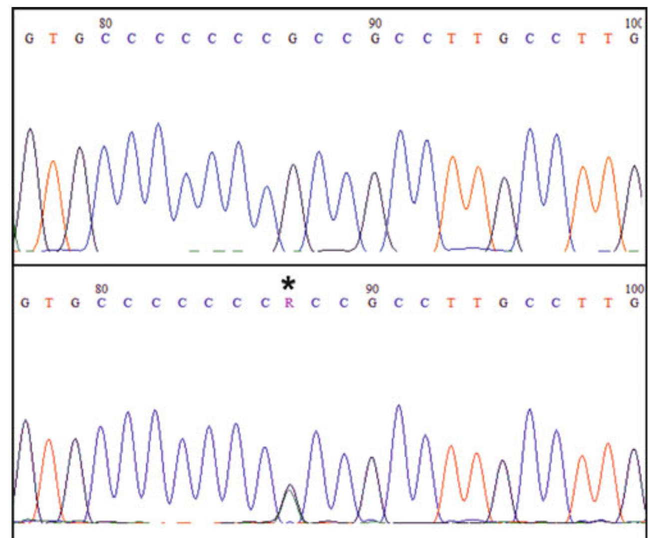

Fig. 1 DNA resequencing, performed in the forward and reverse (not shown) orientation, showing the G>A transition at position g.-148 of the *KLF1* gene promoter (*asterisk*, *lower panel*)

variation. Unfortunately, hematological indices of these control cases were not available for comparison. Also, the *KLF1*:g-148G>A variation was completely absent in 100 normal (non-thalassemic) chromosomes of Greek origin, with normal hematological indices and low HbF levels (<2 %). These data suggest that the *KLF1*:g-148G>A variation is extremely rare in the general population.

Functional and expression studies

It was previously shown that decreased KLF1 levels are correlated with increased HbF levels [2]. We have therefore attempted to demonstrate whether the *KLF1*:g-148G>A promoter variant results in decreased reporter gene transcription in reporter assays, which may explain the high HbF levels in the index case. A *KLF1* gene promoter fragment from −231 to +44 relative to *KLF1* gene transcription initiation site, bearing the wild-type and variant sequence at position *KLF1*:g.-148, was amplified and subcloned into the *Hin*dIII and *Xba*I restriction enzyme sites of the pBLCAT5 vector (Promega, Madison, WI, USA; Fig. 2a) and subsequently transfected into 2×10^6 K562 cells, as previously described [8]. Reporter gene experiments showed a drastically reduced reporter gene expression in the *KLF1*:g.-148A construct, compared to the one bearing the wild-type sequence (*KLF1*:g.-148G; p=0.009; Fig. 2b). Furthermore, results of quantitative RT-PCR analysis showed lower *KLF1* gene expression by 25–30 % as a result of the *KLF1*:g.-148G>A variation, compared to *KLF1* gene expression of healthy volunteers with normal *KLF1* gene sequence that served as negative controls (Fig. 3). Also, since DNA resequencing also revealed the presence of the common *KLF1*:p.S102P variant in the second exon,

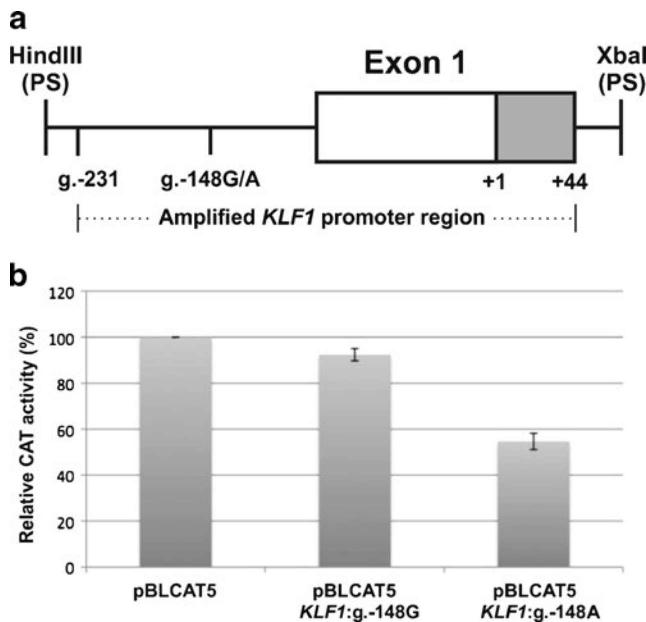

Fig. 2 **a** Schematic drawing indicating the amplified *KLF1* promoter region subcloned between the *Hin*dIII and *Xba*I restriction sites of the pBLCAT5 vector polycloning site (PS). *White and grey boxes* depict the non-coding and coding regions of exon 1, respectively. **b** Functional analysis of the *KLF1* promoter constructs bearing the wild-type (G) and variant (A) nucleotide at position *KLF1*:g.-148. The normalized CAT activities were evaluated as a percentage of pBLCAT5 vector activity (CAT5; *tk* promoter driving the *cat* gene) used as a positive control, which was set as 100 % and are presented as the means± standard deviation of at least three independent experiments

quantitative RT-PCR was also performed using total RNA isolated from an individual who was homozygote for the *KLF1*:p.S102P variant. Our data indicate that *KLF1* gene expression of the *KLF1*:p.S102P homozygote was lower by approximately 15 % compared to negative controls (Fig. 3), suggesting that the *KLF1*:g.-148G>A variation indeed has a significant role in reducing *KLF1* gene expression levels. Subsequently, in silico analysis using the MatInspector algorithm [9] indicated that this novel promoter variant resides in a tentative Sp1 transcription factor binding site. Electrophoretic mobility shift assay was performed using 5 μg of total nuclear protein extracts from human K562 cells and ^{32}P-labelled oligonucleotides bearing the wild-type and variant *KLF1* promoter sequence. EMSA analysis using a Sp1 antibody resulted in a slower migration of the protein–DNA complex formed with the *KLF1*:g.-148G probe but not in the *KLF1*:g.-148A probe in which no protein–DNA complex was formed. These data confirm the in silico analysis prediction that the *KLF1*:g.-148G>A mutation resides in a Sp1 binding site and further that this mutation leads to the ablation of Sp1 binding in vitro (Fig. 4). Overall, these data suggest that the *KLF1*:g-148G>A mutation most likely leads to decreased *KLF1* gene transcription and as such, could have a role in increasing HbF levels in adults.

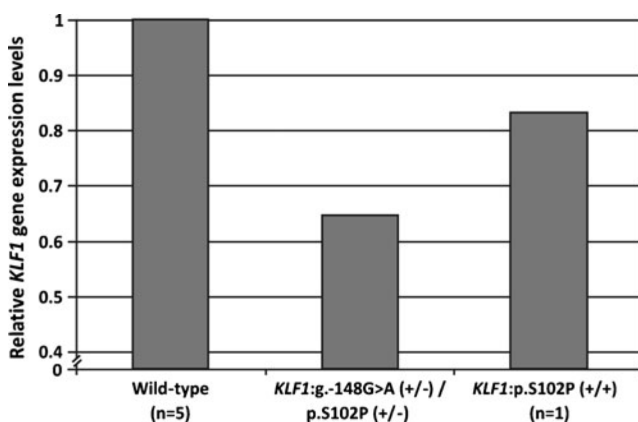

Fig. 3 Quantitative RT-PCR analysis indicating a reduction of the relative *KLF1* gene expression levels in the index case compared to negative controls (n=5) and a homozygous *KLF1*:p.S102P case

Discussion

The genetic etiology of typical HPFH are deletions within the human β-globin gene cluster on chromosome 11 and point mutations in the promoter regions of the *HBG1* and *HBG2* (γ-globin) genes [1]. Also, SNPs, such as the *HBG2*: g.-158C/T genomic variation or variable number of tandem repeats within the β-globin gene cluster, are often associated with high HbF levels under erythropoietic stress [1]. Recently, genomic loci residing outside the human chromosome 11 have been shown to affect HbF levels. Such loci are *BCL11A* on chromosome 2p [7], *KLF10* on chromosome 8q [10] and genes, such as *HBS1L-MYB*, residing on chromosome 6q [11]. Interestingly, *KLF1* was recently identified as an additional potential genomic locus to be associated with HPFH mainly in Maltese, Sardinian families but also in other individuals from various origins, namely Africa, India, and Southeast Asia [2–5]. KLF1 is a fundamental erythroid

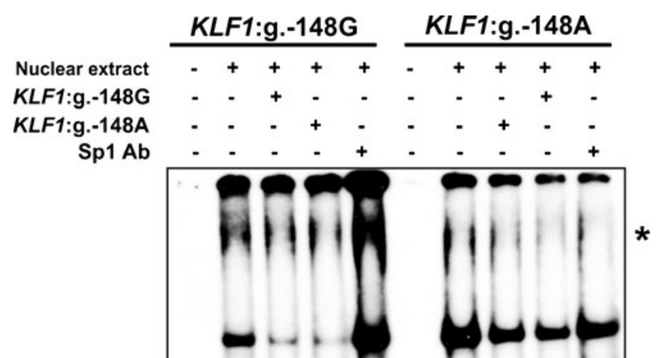

Fig. 4 EMSA analysis using *KLF1*:g.-148G and *KLF1*:g.-148A probes, and anti-Sp1 antibody and nuclear extracts isolated from K562 cells. Supershift due to the binding of the Sp1 transcription factor to the KLF1:g.-148G but not the KLF1:g.-148A oligonucleotide is depicted with an *asterisk*

transcription factor, consisting of a proline-rich N-terminal region that includes the transactivation domain that binds to the *HBB* gene promoter CACCC DNA binding site through its C-terminal region containing three zinc finger domains [12]. Recent experimental evidence suggests that *KLF1* has a dual regulatory role in the human fetal-to-adult globin gene switching both by direct activation of *HBB* and indirect repression of γ-globin gene expression in adult erythroid progenitors via regulation of *BCL11A* [2, 13].

In this paper, we report the first *KLF1* gene promoter mutant that could be associated with increased HbF levels and provide functional evidence that this mutant results in decreased reporter gene expression mediated presumably by the loss of Sp1 binding. Mutations in the *KLF1* gene are associated with a plethora of phenotypes and clinical conditions, including the congenital dyserythropoietic anemia, hereditary spherocytosis, high levels of zinc protoporphyrin, Lutheran blood group, on top of atypical HPFH [14]. These *KLF1* mutations and their accompanying clinical conditions are documented in the HbVar database [3, 15].

The *KLF1*:g.-148G>A mutation resides inside a Sp1 binding site in the promoter, according to our in silico analysis. Previous results suggest that Sp1 has an instrumental role in the activation of the human β-globin locus [16]. Therefore, it seems plausible that the loss of an Sp1 binding site may result in the decrease of *KLF1* gene transcription. This assumption is in concordance with our EMSA and CAT reporter gene assays indicating that the *KLF1*:g.-148G>A mutation results in abolishing Sp1 binding in vitro and a reciprocal decrease of CAT reporter gene expression levels by almost twofold (Fig. 2b) and *KLF1* gene expression levels by roughly 25–30 % (Fig. 3). These data suggest that the *KLF1*:g.-148G>A mutation leads to reduced *KLF1* gene transcription, which could explain, at least in part, the observed HPFH phenotype in the index case, further underlining the significant role of KLF1 on human fetal globin genes switching.

Acknowledgments This work was partly supported by grants from the Research Promotion Foundation (Cyprus, ΠΙΔΕ046_02) and the European Commission [International Thalassemia Network (ITHANET) Coordination action 026539] to GPP and by grant no. III41004 from Ministry of Education and Science, Republic of Serbia to SP.

Conflict of interest The authors declare that they have no conflict of interest.

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