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ORIGINAL ARTICLE

The Hellenic type of nondeletional hereditary persistence of fetal hemoglobin results from a novel mutation (g.-109G>T) in the *HBG2* gene promoter

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Abstract Nondeletional hereditary persistence of fetal hemoglobin (nd-HPFH), a rare hereditary condition resulting in elevated levels of fetal hemoglobin (Hb F) in adults, is associated with promoter mutations in the human fetal globin (*HBG1* and *HBG2*) genes. In this paper, we report a novel type of nd-HPFH due to a *HBG2* gene promoter mutation (*HBG2*:g.-109G>T). This mutation, located at the 3′ end of the *HBG2* distal CCAAT box, was initially identified in an adult female subject of Central Greek origin and results in elevated Hb F levels (4.1%) and significantly increased Gγ-globin chain production (79.2%). Family studies and DNA analysis revealed that the *HBG2*:g.-109G>T mutation is also found in the family members in compound heterozygosity with the *HBG2*:g.-158C>T single nucleotide polymorphism or the silent *HBB*:g.-101C>T β-thalassemia mutation, resulting in the latter case in significantly elevated Hb F levels (14.3%). Electrophoretic mobility shift analysis revealed that the *HBG2*:g.-109G>T mutation abolishes a transcription factor binding site, consistent with previous observations using DNA footprinting analysis, suggesting that guanine at position *HBG2/1*:g.-109 is critical for NF-E3 binding. These data suggest that the *HBG2*:g-109G>T mutation has a functional role in increasing *HBG2* transcription and is responsible for the HPFH phenotype observed in our index cases.

Marios Phylactides and Farzin Pourfarzad contributed equally to this work.

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Keywords Nondeletional hereditary persistence of fetal hemoglobin · β-thalassemia · Fetal globin genes · *HBG2* · Mutation · Regulatory element · Transcription

Introduction

Hereditary persistence of fetal hemoglobin (HPFH) is a genetic condition characterized by persistent elevated levels of fetal hemoglobin (Hb F) in the adult erythroid stage [1]. HPFH heterozygotes have a normal clinical phenotype and hematological indices, except for the lower levels of hemoglobin A_2 (Hb A_2) that constitutes the hallmark of HPFH. The reason for that is the increased frequency of interactions between the locus control region (LCR) and the mutated γ-globin gene promoter compared to the normal situation. Subsequently, this shifts the transcriptional balance in favor to the otherwise silent, fetal globin genes, resulting in lower *HBD* gene transcription and Hb A_2 levels [2].

The various forms of HPFH are distinguished into two main categories, namely, deletional and nondeletional HPFH (nd-HPFH), due to large deletions 3′ to the fetal globin genes or nucleotide substitutions in the fetal globin gene promoters, respectively [3]. The current explanation for the functional role of the latter HPFH category is the destabilization of a yet unknown silencing protein complex that permits LCR–gene interactions and recruitment of the basal transcriptional machinery to the mutated γ-globin gene promoter, leading to reactivation of fetal globin gene transcription [4].

To date, there are few γ-globin promoter mutations, leading to nd-HPFH clustered on or in close proximity to γ-globin gene *cis*-regulatory elements [5]. The most important nd-HPFH is the Greek type (*HBG1*:g.-117G>A [6]), whose functional role is also demonstrated in transgenic mice [7]. This mutation is located at the 5′ end of the distal γ-globin gene CCAAT box where important erythroid-specific transcription factors bind. Other mutations cluster approximately 200 bp upstream to the γ-globin genes transcription initiation site where a GC-rich region is located. Although clinically silent, the mutations leading to nd-HPFH provide valuable insights into the transcriptional regulation of the human fetal globin genes, which in turn can enable design of novel strategies for β-thalassemia therapeutics.

In this communication, we report the Hellenic type of nd-HPFH due to a novel G>T transversion in the *HBG2* gene promoter, located at the 3′ end of the distal *HBG2* CCAAT box.

Materials and methods

Case selection

For the needs of this study, the mother and two sisters of the index case were also recruited. Unfortunately, the father of the index case was deceased, but, nevertheless, his sister was available for study (Fig. 1a). Also, 31 heterozygotes for the silent *HBB*:g.-101C>T β-thalassemia mutation have been included (Table 1), as well as 89 normal (non-thalassemic) individuals. The study was previously approved by the hospital's ethics committee.

Hemoglobin studies

Blood samples were collected, with consent, in vacutainers with ethylenediaminetetraacetic acid as anticoagulant. Hematological indices were measured with an automated cell counter and Hb A_2 and Hb F levels were quantitated, using cation exchange high-performance liquid chromatography (HPLC; VARIANT™, BioRad, Hercules, CA, USA). Globin chain quantitation was performed using reverse-phase HPLC, using the RP-18 column (Pharmacia, Uppsala, Sweden).

DNA analysis

Total genomic DNA isolation and γ-globin gene promoters' amplification was done as previously described [8]. Mutation screening in the human γ-globin gene promoters was performed using denaturing gradient gel electrophoresis (DGGE) with a 40–70% linear gradient of denaturing agents (urea and formamide; 100% denaturant: 7 M urea, 40% formamide), as previously described [9]. *HBB* and *HBD* gene mutation screening was done as described in Losekoot and coworkers [10] and Papadakis and coworkers [11], respectively. Automated DNA sequencing was performed directly on the polymerase chain reaction (PCR) products. Screening for *HBA2/1* gene deletions was done according to the multiplex gap-PCR strategy of Tan and coworkers [12], while a PCR-restriction fragment length polymorphism (RFLP) approach was used to screen for the commonest point mutations and small indels in the *HBA2/1* genes, leading to nondeletional α-thalassemia in the Hellenic population [13].

Electrophoretic mobility shift assays

Electrophoretic mobility shift assays (EMSA) analysis was performed using total nuclear protein extracts from mouse erythroleukemia (MEL) and human K562 cells [14]. In brief, 5 μg nuclear extracts were used per reaction and competitions were done using 100-fold molar excess of the indicated double-stranded oligonucleotides before addition of the nuclear extract. Nucleotide sequence of the oligonucleotides used in these studies is available upon request. Supershift analysis was performed using a home-brewed GATA-1 antibody [15, 16].

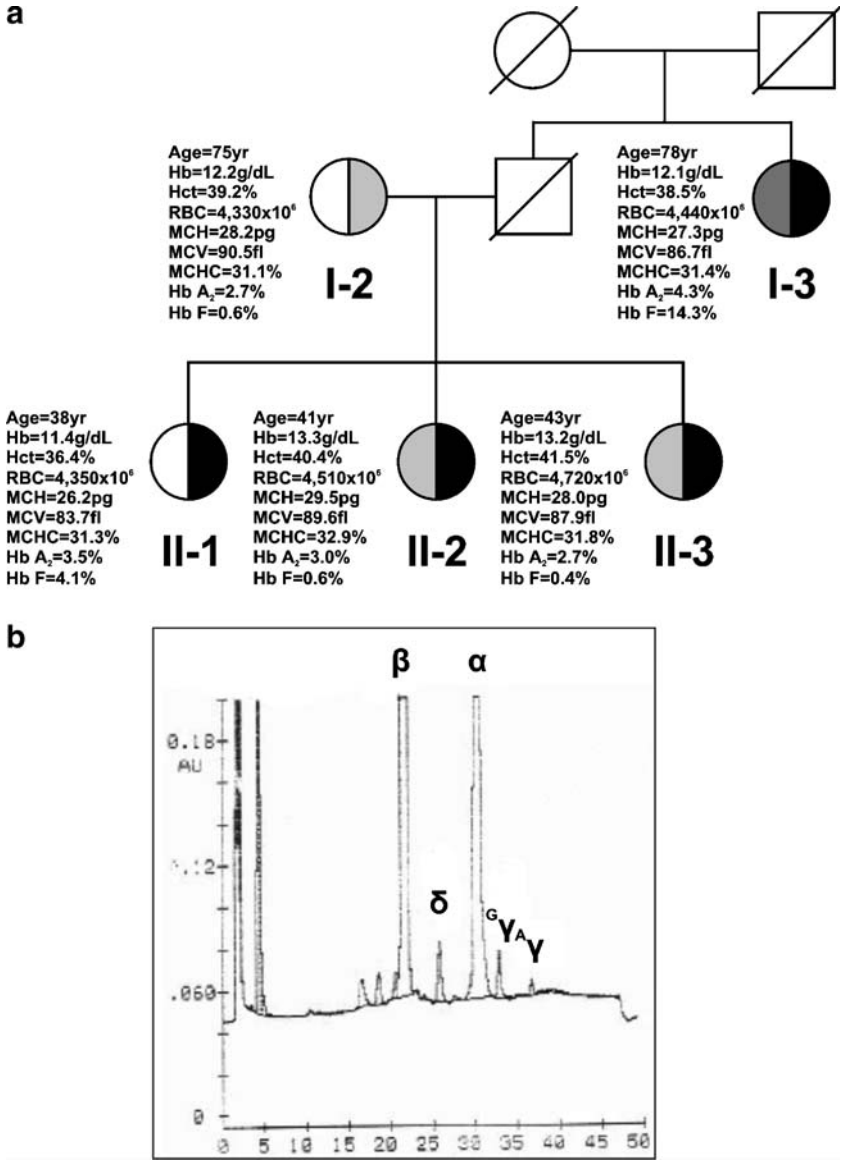

Fig. 1 **a** Family tree showing the heterozygous and compound heterozygous cases for the Hellenic type of nd-HPFH, bearing the novel *HBG2*:g.-109G>T promoter mutation (depicted in *black*) and the *HBG2*:g.-158C>T polymorphism (depicted in *light gray*) or the silent *HBB*:g.-101C>T β-thalassemia nonsense mutation (depicted in *dark gray*), along with the corresponding hematological indices. **b** Chromatogram from RP-HPLC from the index case showing the significant increase in the Gγ/Aγ-globin chain ratio

Table 1 Comparison of the hematological indices, Hb A_2 and Hb F levels among the compound heterozygous for the novel *HBG2*:g.-109G>T mutation and the silent *HBB*:g.-101C>T β-thalassemia mutation (I-3; see also Fig. 1a), and heterozygotes for the silent *HBB*:g.-101C>T β-thalassemia mutation (note the markedly increased Hb F levels in case I-3)

Hematological indices	*HBB*:g.-101C>T		
	HBG2:g.-109G>T (I-3)	Wild-type Females (n=18)	Wild-type Males (n=13)
Age (years)/sex	78/F	Adults	Adults
Hb (g/dL)	12.1	12.4±1.22	14.5±0.84
Hct (%)	38.5	37.1±2.76	43.1±2.30
RBC ($\times 10^6$)	4,440	4,382±446	5,144±265
MCH (pg)	27.3	28.4±1.54	28.5±0.88
MCV (fl)	86.7	85.5±4.03	84.3±2.69
MCHC (%)	31.4	32.7±1.04	33.9±0.64
Hb A_2 (%)	4.3	3.79±0.24	3.92±0.37
Hb F (%)	14.3	2.53±1.8	1.60±1.17

Results

During routine carrier screening for β-thalassemia, an adult female subject was identified with moderately elevated Hb F levels (4.1%) and significantly increased Gγ/Aγ-globin chain ratio (Fig. 1b). An aberrant electrophoretic pattern was identified during mutation screening in the *HBG2* promoter by DGGE analysis (Fig. 2a). DNA sequencing of the proximal *HBG2* promoter region revealed a novel sequence variation, namely, a G>T transversion at position −109 relative to the gene's transcription initiation site (*HBG2*:g.-109G>T; Fig. 2b). DNA sequencing did not reveal any other variant nucleotide in either one of the γ-globin genes promoters and distal 5′ regulatory regions between positions −672 and +25. Neither the *HBG2*:g.-158C>T (XmnI) nor the *HBG1*:g.-225-222(AGCA)del polymorphisms, which are often correlated with moderately increased HbF levels, were found in the index case.

Hemoglobin studies and DNA analysis of the available family members indicated that the propositus mother (I-2) was a carrier of the *HBG2*:g.-158C>T (XmnI) polymorphism, while both sisters (II-2, II-3) were compound heterozygotes for the *HBG2*:g.-158C>T polymorphism and the novel *HBG2*:g.-109G>T mutation. Surprisingly, in the latter cases, Hb F levels were normal. Although the propositus father was deceased, his sister, who was available for study, presented with significantly increased Hb F levels (14.3%; Table 1, Fig. 1a). DNA analysis revealed that the propositus aunt (I-3) was also compound heterozygous for the *HBG2*:g.-109G>T promoter mutation and the silent *HBB*:g.-101C>T β-thalassemia mutation. The *HBG2*:g.-109G>T base substitution was neither identified in 31 β-thalassemic chromosomes, bearing the silent *HBB*:g.-101C>T mutation nor in 209 normal (nonthalassemic) chromosomes, suggesting that the novel *HBG2*:g.-109G>T variation is not a frequent polymorphism. Mutation screening in the *HBD* gene in the index case and her family members, using DGGE analysis [11], failed to identify any *HBD* mutations (data not shown). In addition, all family members were not carriers either of known *HBA2/1* gene deletions or the commonest point mutations and small indels in the *HBA2/1* genes, determined by gap-PCR and PCR-RFLP analysis, respectively.

In order to verify the functional significance of the novel *HBG2*:g.-109G>T mutation, we measured Gγ-globin chain levels, using reverse-phase HPLC. Our results showed that the Gγ/Aγ-globin chain ratio was significantly increased, compared to normal (nonthalassemic) individuals and carriers for the *HBB*:g.-101C>T mutation (79.2/20.8 versus 40/60, respectively) [17]. Also, we performed EMSA analysis, using nuclear extracts from both MEL cells and human K562 cells, expressing the adult and fetal erythroid program, respectively, in order to assess whether the

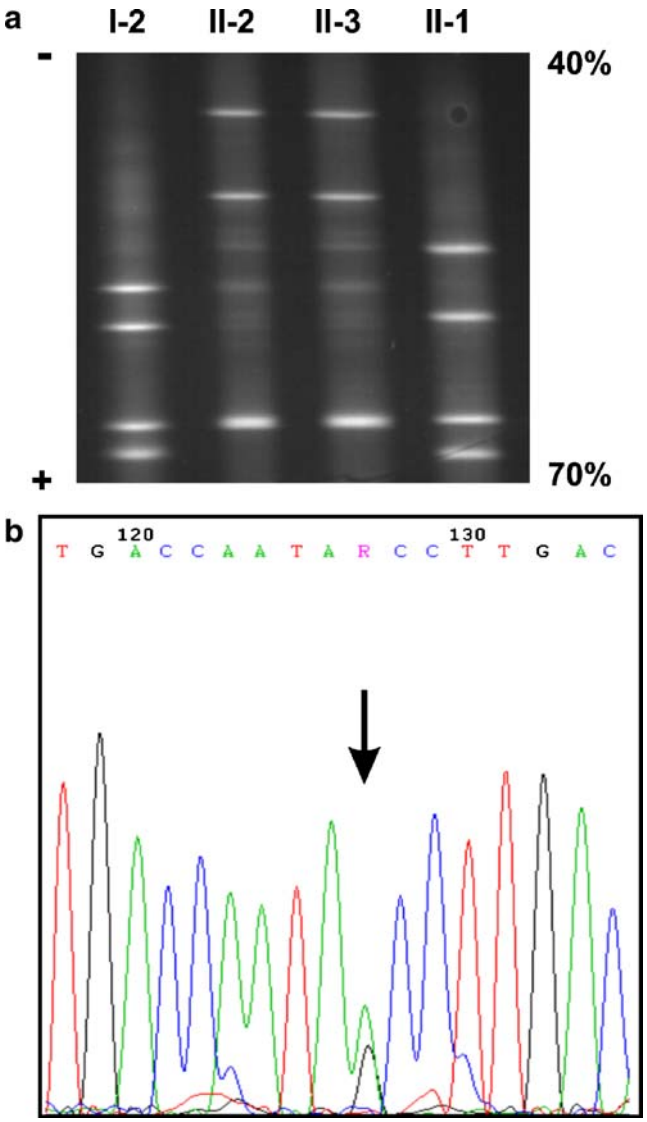

Fig. 2 **a** DGGE analysis of the promoter region of the *HBG2* gene. Numbering correlates with the family tree in Fig. 1a. Note the mutant *HBG2*:g.-158 T (*lower band*) and *HBG2*:g.-109 T homoduplexes (*upper band*) that comigrate in *lanes II-2* and *II-3*, compared to *lanes I-2* and *II-1*, respectively. The 40–70% denaturing gradient corresponds to the *top* and *bottom* of the gel, respectively. **b** DNA sequencing analysis, performed in the forward and reverse (not shown) orientation, revealing a G>T transition at position −109 of the *HBG2* gene promoter (*arrow*)

HBG2:g.-109G>T mutation affects protein binding in vitro. Indeed, EMSA analysis using a 40-bp oligonucleotide bearing the wild-type sequence (G) at position *HBG2*:g.-109 showed a double-band electrophoretic pattern, suggesting that two proteins and/or protein complexes bind to the oligonucleotide used. Addition of nonradioactive GATA-1 oligonucleotides, both from *HBG2* and *HBB* promoters [15], resulted only in the lower band being efficiently competed, contrary to the upper band that remained visible (Fig. 3a), indicating that the lower band most likely

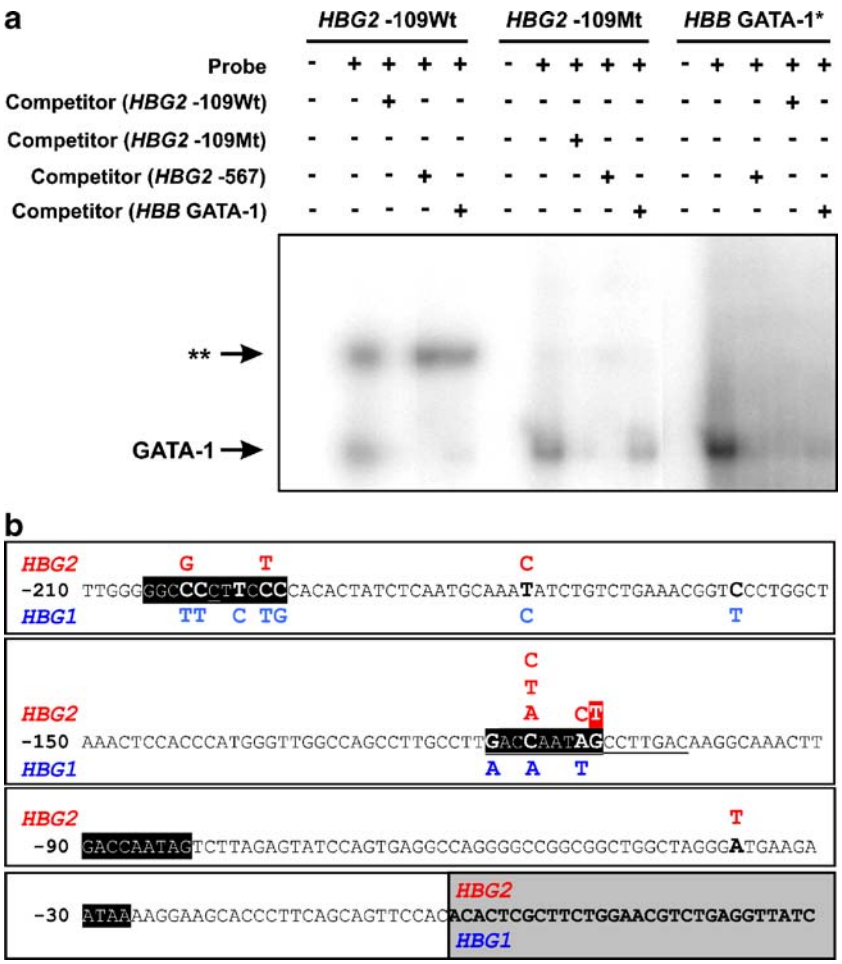

Fig. 3 **a** Electrophoretic mobility shift assays of the oligonucleotides containing either the wild-type (*Wt*; *HBG2*:g-109 G) or mutant (*Mt*; *HBG2*:g-109 T) sequence using nuclear protein extracts prepared from dimethyl sulfoxide-induced MEL cells. The *HBG2* −109Mt oligonucleotide most likely abolishes a NF-E3 binding site in vitro (depicted as *two asterisks*). Competition with nonlabeled *HBG2* −109Wt oligonucleotide results in the disappearance of GATA-1 and NF-E3 band shifts, suggesting that both proteins bind to the *HBG2* −109Wt oligonucleotide. Competition with nonlabeled *HBG2* −567 or *HBB* GATA-1 oligonucleotides [15] results in the disappearance only of the GATA-1 (*lower*) band. Competition with nonlabeled *HBG2* −567 or *HBB* GATA-1 oligonucleotides suggests that the affinity of GATA-1 is stronger to the former oligonucleotide. Use of nuclear protein extracts prepared from K562 cells yielded identical electrophoretic mobility shifts (not shown). The *asterisk* indicates the oligonucleotide sequence located in the *HBB* gene promoter region. **b** Schematic representation of the various nd-HPFH mutations reported to date for the *HBG2* (in *red*) and *HBG1* (in *blue*) globin genes (*underlined* sequences depict the two small deletions also leading to nd-HPFH). The novel nd-HPFH mutation reported in this study is highlighted in *red*. *Gray box* represents the sequences downstream to the transcription initiation site and sequences in *solid black boxes* represent phylogenetically conserved fetal globin genes *cis*-regulatory elements

represents GATA-1 binding. This finding was confirmed using GATA-1 antibody, resulting in a supershift of the lower (GATA-1) band (data not shown). EMSA analysis using a 40-bp oligonucleotide bearing the mutant sequence (T) at position *HBG2*:g.-109 revealed that binding of the protein (or protein complex) in the upper band was abolished. These data indicate that the upper band shift represent protein (or protein complex) binding only to the wild-type sequence at position *HBG2*:g.-109 (G) and that the *HBG2*:g.-109G>T transversion inhibits protein binding in vitro. Consistent with previous footprinting experiments [18], these data suggest that the *HBG2*:g.-109G>T mutation is responsible for the resulting nd-HPFH phenotype.

Discussion

In this paper, we report the Hellenic type of nd-HPFH, resulting from a novel G>T transversion in the promoter region of the *HBG2* gene. In the Hellenic population, the dominant nd-HPFH is the Greek type (*HBG1*:g.-117G>A [6]), accounting for almost 90% of the HPFH chromosomes [9]. The Cretan nd-HPFH (*HBG1*:g.-158C>T [9]) is the second most frequent type, followed by the *HBG1*:g.-201C>T and the *HBG2*:g.-196C>T mutations [19]. No deletional HPFH has even been reported for the Hellenic population.

The *HBG2*:g.-109G>T mutation was found both in heterozygosity as well as in compound heterozygosity with

the *HBG2*:g.-158C>T (XmnI) polymorphism and the silent *HBB*:g.-101C>T β-thalassemia mutation, the latter being one of the most common *HBB* mutation leading to β-thalassemia in the Hellenic population [20, 21]. Interestingly, however, the resulting Hb F levels varied in each case. First of all, heterozygosity for the *HBG2*:g.-109G>T mutation resulted in moderately increased Hb F levels in the index case (4.1%). Also, compound heterozygosity with the silent *HBB*:g.-101C>T β-thalassemia mutation resulted in substantially increased Hb F levels (14.3%), as expected for compound heterozygous cases for nd-HPFH and β-thalassemia [22]. There are very few examples of nd-HPFH/β-thalassemia compound heterozygous cases, which are extremely rare and only occasionally reported in the literature [22, 23]. On the contrary, compound heterozygosity for the *HBG2*:g.-109G>T mutation and the *HBG2*:g.-158C>T polymorphism has no effect in the observed Hb F levels (Fig. 1a). There are two possible explanations for this observation: (a) the *HBG2*:g.-158C>T polymorphism itself exerts a silencing effect on the *HBG2*:g.-109G>T mutation or (b) another yet unidentified *cis*-regulatory element suppresses Hb F production in the sisters of the index case. The latter assumption resembles our recent findings [16] and reaffirms that the regulation of γ-globin gene expression and Hb F production is multifactorial and likely combinatorial and that many genetic factors can putatively impact Hb F production in adults.

The *HBG2*:g.-109G>T mutation resides at the 3′ end of the distal *HBG2* CCAAT box where the CP1 and NF-E3 transcription factors bind [18]. On the basis of in vitro binding studies, GATA-1 and NF-E3 have both been implicated as candidate suppressors of γ-globin gene transcription that would act by binding to the region [18, 24]. In particular, guanosine at position *HBG2*:g.-109 has been previously shown to be a strong contact point for NF-E3 [24], suggesting that the *HBG2*:g.-109G>T transversion activates Hb F production most likely by abolishing NF-E3 binding. This assumption is in accordance with our EMSA analysis data indicating that the *HBG2*:g.-109G>T transversion results in abolishing protein binding in vitro (Fig. 3a). Taking into consideration that the Gγ-globin chain levels were doubled in the index case (I-1; Fig. 1a), these data strongly suggest that the *HBG2*:g.-109G>T mutation is responsible for the resulting HPFH phenotype.

The novel nd-HPFH mutation described in this paper is located at the 3′ end of the *HBG2* gene's distal CCAAT box, a phylogenetically conserved *cis*-regulatory element that has been shown to harbor many ubiquitous and erythroid-specific transcription factor binding sites [1]. Nine out of 21 nd-HPFH mutations have been identified in this region (Fig. 3b), suggesting that this regulatory element is critical for γ-globin gene transcription. Contrary, however, to the majority of these nd-HPFH mutations that have a substantial effect in Hb F production, the *HBG2*:g.-109G>T nd-HPFH mutation per se results in only moderate increase in HbF levels. In addition, the neighboring *HBG2*:g.-110A>C mutation, leading to the Czech nd-HPFH, also has a negligible effect in HbF production [25]. This is unexpected since the *HBG1*:g.-117G>A nd-HPFH mutation, residing at the 5′ end of the distal CCAAT box, leads to a substantial increase in γ-globin gene transcription. This can be explained by the fact that (a) different mutations in the distal CCAAT box abolish different transcription factors binding [24], which would exert a differential effect over γ-globin gene reactivation in the adult stage, (b) the *HBG1*:g.-117G>A and *HBG2*:g.-109G>T reside on different genes, hence their relative distance from the LCR, being directly proportional to the frequency of LCR/globin gene promoter interactions [26], varies. It is noteworthy that not all nd-HPFH mutations give a reproducible HPFH phenotype in transgenic mice (unpublished data).

Altogether, these data emphasize that γ-globin gene silencing should be the result of a gradual change in the transcription factor environment affecting the transcriptional balance between the *HBG1*/*HBG2* and *HBB* genes.

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