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Ten novel mutations in the erythroid transcription factor KLF1 gene associated with increased fetal hemoglobin levels in adults

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Abstract

We investigated whether mutations in the KLF1 gene are associated with increased Hb F levels in ethnically diverse patients referred to our laboratory for haemoglobinopathy investigation. Functionally effective KLF1 mutations were identified in 11 out of 131 adult samples with an elevated Hb F level (1.5-25.0%). Eleven different mutations were identified, 9 of which were previously un-reported. KLF1 mutations were not identified in a matched cohort of 121 samples with normal Hb F levels (<1.0%). A further novel KLF1 mutation was also found in a sickle cell disease patient with a Hb F level of 20.3% who had a particularly mild phenotype. Our results indicate KLF1 mutations could make a significant contribution to Hb F variance in malarial regions where haemoglobinopathies are common. All the mutations identified were heterozygous providing further in-vivo evidence that a single altered KLF1 allele is sufficient to increase Hb F levels.

Introduction

Hereditary persistence of fetal haemoglobin (HPFH) is defined as an increased proportion of fetal haemoglobin which persists beyond infancy. The condition has virtually no adverse clinical effects but is of considerable interest because increased Hb F levels are known to ameliorate the severity of some haemoglobin disorders. HPFH has been identified in a diverse range of ethnic groups\(^1\), however higher frequencies are observed in populations with a high prevalence of haemoglobinopathies and thalassaemias and consequently it is often observed as a co-existing feature of these conditions. It is well established that HPFH can be caused by deletions within the HBB (β-globin) gene cluster on chromosome 11\(^2\) and point mutations in the promoters of the HBG1 and HBG2 (γ-globin genes)\(^3,4\). There are also single nucleotide polymorphisms (SNPs) or oligonucleotide motifs within the β-globin gene cluster which are associated with higher Hb F levels under conditions of erythroid stress such as sickle cell disease or beta thalassaemia. The best known of these is the C-T polymorphism at position -158 of the G-γ promoter which creates an Xmn1 restriction site\(^5\). More recently loci unlinked to the β-globin gene cluster that affect Hb F levels have been identified. Two major sites are the HBS1L-MYB inter-genic region on chromosome 6q23\(^6\) and BCL11A on chromosome 2p16.1\(^7\). Known loci have been shown to account for 50% of the variance seen in Hb F levels indicating that additional loci must be involved\(^8\).

Recently an additional potential locus was identified when point mutations in the KLF1 gene were found to be associated with HPFH in a Maltese\(^9\) family and in a family from Sardinia\(^10\). KLF1 is an essential erythroid transcription factor first identified in 1993 that binds to an important DNA binding site, the CACCC motif in the β-globin gene\(^11\). The gene comprises of a proline rich N-terminal region containing a transactivation domain and a C-terminal region containing three zinc finger domains essential for DNA binding. Recent studies have shown that KLF1 could play a critical role in regulating the switch between fetal and adult haemoglobin expression both by direct activation of β-globin and indirect repression of γ-globin gene expression in adult erythroid progenitors via regulation of BCL11A\(^12\). The finding of HPFH associated with mutations in KLF1 is of significance as it may be in vivo evidence that controlled reduction of KLF1 expression could activate fetal haemoglobin production and therefore provide a potential therapeutic target\(^13\).
This study investigates whether KLF1 mutations are involved in the increased Hb F levels observed in blood samples referred to our laboratory for haemoglobinopathy investigation.

**Design and Methods**

**Study subjects**

*Elevated Hb F level*

131 blood samples referred for haemoglobinopathy investigation (subject age 1-81 years) with an elevated Hb F level (range 1.5 – 25%) were tested for KLF1 mutations and compared against a matched control group of 121 samples that had also been referred for haemoglobinopathy investigation but had normal Hb F levels (<1%). Haemoglobinopathy phenotypes in the study group with an elevated Hb F level consisted of 55 samples with co-existing α-thalassemia trait, 6 carriers for sickle cell trait, 28 β-thalassaemia carriers, 1 case of Hb E Disease and 41 samples with no evidence of any other haemoglobinopathy apart from the elevated Hb F level. Haemoglobinopathy phenotypes in the control group with normal Hb F levels consisted of 41 α-thalassaemia carriers, 9 sickle cell carriers, 29 carriers for a haemoglobin variant, 20 β-thalassemia carriers and 22 samples with no evidence of any haemoglobinopathy.

*Sickle Cell disease*

A cohort of 55 patient samples with sickle cell disease (all homozygous for the sickle cell mutation) were studied to investigate whether KLF1 mutations could be involved in Hb F level variation in this group. Twenty patients had Hb F levels below 10% and 35 had Hb F levels >10%.

**Laboratory Procedures**

Peripheral blood erythrocyte indices were determined using an automated cell counter (Sysmex XE 2100™). Haemoglobin identification and quantifications were carried out using a high performance liquid chromatography system (VARIANT™, Bio-Rad Laboratories, USA) and isoelectric focusing gel electrophoresis (RESOLVE®, PerkinElmer, USA). DNA was extracted from peripheral blood leukocytes by conventional phenol chloroform extraction or on an automated DNA extractor (Chemagen, Baesweiler, Germany). Genomic DNA samples underwent PCR to amplify the human KLF1 gene (NT_086897.1: 4090501-4093981) using previously published primers. PCR products were sequenced using the ABI-PRISM 3100 automated DNA sequencer (Applied Biosystems). Samples containing a mutation in the KLF1 gene had the promoter regions of both the Aγ and Gγ genes amplified using previously published primers and the PCR products sequenced as above. The common 3.7 and 4.2 Kb single α+ thalassaemia globin gene deletion mutations were identified by Gap-PCR and β-globin gene cluster deletions excluded by MLPA in all samples. PolyPhen-2 and SIFT were used to predict the effects of any mutations on protein structure and function.

Approval for this study was provided by the Oxford Research Ethics Committee.
Results and Discussion

Elevated Hb F Subjects

KLF1 mutations which are predicted to effect gene function (PolyPhen-2 and SIFT) were identified in 11 out of 131 (8.4%) subjects with increased Hb F levels (Table 1). Ten had a single heterozygous mutation and one individual was compound heterozygous for two mutations. In total, eleven different KLF1 mutations were identified (Table 1). Beta cluster deletion mutations and mutations in the γ-globin gene promoter sequences were excluded as a cause of the increased Hb F level in all 11 subjects. Functionally effective KLF1 mutations were not identified in the matched cohort of 121 samples with normal Hb F levels. Nine of the 11 mutations identified were previously un-reported.

Eight were mis-sense mutations, one in exon 1 (L51R) and seven in the zinc finger domains (R301C, R301H, W313C, R328H, R328L, T334K and T334R) which would be expected to disrupt DNA binding (Figure 1). Two were frame shift mutations in exon 2, an 11bp deletion (K54PfsX9) producing a new stop codon 8 nucleotides downstream and a 7bp insertion (G176AfsX179) producing a stop codon 178 nucleotides downstream. The latter mutation was identified in two patients, singly and compound heterozygous with the L51R mis-sense mutation. The final mutation identified in this group was a 1bp nucleotide substitution (c.913+1G>A) at the 3’ end of exon 2 which would be predicted to disrupt splicing.

Sickle Cell Anaemia Subjects

Out of the 55 sickle cell disease patients studied one further unreported functionally effective KLF1 mutation was identified (c.914-4_914-1 del CTAG) in a sickle cell disease patient with an elevated Hb F level of 20.3%. Mutations in the γ-globin gene promoter sequences and the Xmn1 polymorphism were excluded as the cause of the increased Hb F in this patient. This KLF1 mutation is a 4bp deletion and is located close to the start of the second zinc finger domain in exon 3 and is likely to result in aberrant splicing.

Tolerated SNPs

Two patients in the HPFH cohort and one patient in the sickle cell disease cohort were found to have SNPs in the coding regions of the KLF1 gene (G5K, G160K, and G250A). PolyPhen-2 and SIFT analyses suggest that they are neutral substitutions which are tolerated and therefore not pathogenic. In support of this G5K and G160K were also found in 2 samples in the normal F level control group.

Recent reports have identified mutations in the KLF1 gene which are associated with a variety of phenotypes in humans. These include the Lutheran blood group, congenital dyserythropoietic anaemia, hereditary spherocytosis, high levels of zinc protoporphyrin, HPFH in two families and most recently borderline increases in Hb A2 levels. The UK population is ethnically diverse and our laboratory receives requests for haemoglobinopathy investigations for individuals who originate from all the malarial regions of the world. Our study identified KLF1 mutations in a significant proportion of these referrals with increased Hb F levels. KLF1 mutations predicted to have an effect were found in 11 out of 131 referrals with increased Hb F levels but KLF1 mutations were not identified in 121 haemoglobinopathy referrals with normal Hb F levels. This strongly suggests that the KLF1 mutations are associated with the observed increased Hb F levels in these patients. All the mutations identified were heterozygous indicating that a single altered KLF1 allele can elevate Hb F.
The increased Hb F levels observed in our KLF1 mutation positive subjects ranged from 1.7% to 14.4%. Well established factors known to cause HPFH (deletions in the β-globin cluster or mutations in the γ-globin gene promoters) were excluded in all cases. An interesting finding was that 4 out of our 11 cases also had borderline increased Hb A2 levels (3.3 – 4.0%). All of these samples had normal β-globin gene sequences, most likely excluding β-thalassaemia as the cause of the elevated Hb A2 level. This finding concurs with a recent report that shows that mutations in the KLF1 gene are associated with elevated HBD (δ-globin) gene expression which gives rise to borderline increased Hb A2 levels. Significantly, a proportion of the cohort in that study had increased Hb F levels as well as increased Hb A2 levels. The proposed mechanism for KLF1 mutations increasing Hb F levels is reduced activation of BCL11A by KLF1 which in turn results in inefficient repression of γ-globin synthesis. The δ-globin gene has no KLF1 binding sites therefore the increase in δ-globin gene expression is most likely to be due to indirect effects. Probably impaired looping of the LCR with the β-globin gene that results in increased expression of the competing δ-globin gene. Whether a KLF1 mutation produces a HPFH phenotype or an increased Hb A2 level (or a combination of both phenotypes) will possibly depend on the balance between these two effects, which in turn will most likely depend on factors specific to a particular KLF1 mutation and other interacting factors.

The majority of our subjects with a KLF1 mutation had hypochromic red cells (MCH <27pg), however this could mostly be explained by the co-existing presence of the extremely common 3.7kb single α-globin gene deletion (Table 1). Exceptions to this were cases 3, 5 and 1. Cases 3 and 5 had an MCH lower than normally observed with a single α-globin gene deletion. Case 1 was of White British descent and had markedly thalassaemic indices but had tested negative for all types of α and β-thalassaemia mutations. The KLF1 mutation (K54PfsX9) identified in this individual would be predicted to be more severe than the other mutations in that it results in the loss of all three zinc finger domains and most of exon 2. It is possible that this could result in severe impairment of the β-globin gene’s association with the LCR resulting in a marked reduction in β-globin expression producing a β-thalassaemia type phenotype. Only one mutation was found more than once (G176AfsX179), in case 10 it was associated with a Hb F level of 1.7% whilst in case 9 where it was found in combination with the L51R mis-sense mutation the Hb F level was 9.5% suggesting that the effects of these mutations may be additive.

A previously unreported KLF1 mutation (c.914-4_914-1 del CTAG) was identified in one of the 55 patients investigated who were homozygous for the sickle cell mutation, which suggests KLF1 mutations are not particularly common in sickle disease. However interestingly, the Nigerian sickle cell disease patient identified with this mutation was completely asymptomatic and maintained a haemoglobin level of 12.7g/dl with a Hb F level of 20.3%. It is possible the KLF1 mutation is ameliorating the phenotype by increasing the Hb F level via reduced γ-globin gene suppression. However the patient also has homozygous α+-thalassaemia, α-thalassaemia is known to have a complex interaction with sickle cell disease but does increase the overall haemoglobin level slightly. It is therefore likely that complex mechanisms including multiple gene interactions are involved in the maintenance of this patient’s robust haemoglobin level and asymptomatic phenotype.
In summary, *KLF1* mutations were found in 8.4% of our elevated Hb F cohort, predominantly in individuals of African, Indian and Southeast Asian descent. This indicates *KLF1* mutations could be a widespread cause of HPFH in malarial regions where haemoglobinopathies are common, possibly making a significant contribution to Hb F variance in these populations. Also, the identification of *KLF1* mutations in individuals with a thalassaemia carrier phenotype and a particularly mild form of sickle cell disease indicates the effects of these mutations are likely to be heterogeneous and complex.

**Authorship and Disclosures**

AEG designed and conducted experiments and wrote the manuscript. SH designed the research and wrote the manuscript. HMPD conducted experiments. JO and AS designed experiments. No conflict of interest to declare.

**References**


Table 1. Haematological parameters and genotypes in subjects with mutations detected in the KLF1 gene.

Cases 1-11 are subjects from the elevated Hb F cohort and case 12 was from the sickle cell anaemia cohort. Only 2 of the 13 mutations detected had been reported previously (marked with an *). The presence of a wild type allele in the KLF1 genotype is indicated by [=].

<table>
<thead>
<tr>
<th>Case</th>
<th>Ethnic group</th>
<th>Hb (g/dl)</th>
<th>RBC (10⁶/mm³)</th>
<th>MCV (fl)</th>
<th>MCH (pg)</th>
<th>HbA₂ (%)</th>
<th>Hb F (%)</th>
<th>β-globin phenotype/genotype</th>
<th>α globin genotype</th>
<th>KLF1 genotype</th>
<th>KLF1 protein change</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>White British</td>
<td>14.3</td>
<td>6.18</td>
<td>69.4</td>
<td>23.1</td>
<td>3.3</td>
<td>3.0</td>
<td>HPFH</td>
<td>αα/αα</td>
<td>c.[159_169 del GAAGTCTGAGG]+ [=]</td>
<td>K54PfsX9</td>
</tr>
<tr>
<td>2</td>
<td>Pakistani</td>
<td>12.7</td>
<td>4.72</td>
<td>79.4</td>
<td>26.9</td>
<td>2.4</td>
<td>14.6</td>
<td>HPFH</td>
<td>αα/αα</td>
<td>c.[901C&gt;T]+ [=]</td>
<td>R301C</td>
</tr>
<tr>
<td>3</td>
<td>Black African</td>
<td>10.6</td>
<td>5.43</td>
<td>61</td>
<td>19</td>
<td>2.4</td>
<td>6.6</td>
<td>HPFH</td>
<td>αα/αα</td>
<td>c.[920G&gt;A]+ [=]</td>
<td>R301H</td>
</tr>
<tr>
<td>4</td>
<td>Black African</td>
<td>12.1</td>
<td>4.74</td>
<td>81.9</td>
<td>25.5</td>
<td>2.8</td>
<td>7.3</td>
<td>HPFH</td>
<td>αα/αα</td>
<td>c.[939G&gt;T]+ [=]</td>
<td>W313C</td>
</tr>
<tr>
<td>5</td>
<td>Thai</td>
<td>12.6</td>
<td>5.51</td>
<td>75.2</td>
<td>22.8</td>
<td>2.4</td>
<td>5.9</td>
<td>HPFH</td>
<td>αα/αα</td>
<td>c.[983G&gt;A]+[+]</td>
<td>R328H*</td>
</tr>
<tr>
<td>6</td>
<td>Slovakian</td>
<td>13.0</td>
<td>4.29</td>
<td>91.7</td>
<td>30.3</td>
<td>3.2</td>
<td>8.7</td>
<td>HPFH</td>
<td>αα/αα</td>
<td>c.[983G&gt;T]+[+]</td>
<td>R328L*</td>
</tr>
<tr>
<td>7</td>
<td>Black African</td>
<td>10.7</td>
<td>4.24</td>
<td>80.4</td>
<td>25.2</td>
<td>3.3</td>
<td>6.8</td>
<td>HPFH</td>
<td>αα/αα</td>
<td>c.[1001C&gt;A]+[+]</td>
<td>T334K</td>
</tr>
<tr>
<td>8</td>
<td>Black African</td>
<td>8.9</td>
<td>-</td>
<td>73.0</td>
<td>23.0</td>
<td>3.8</td>
<td>6.7</td>
<td>HPFH</td>
<td>αα/αα</td>
<td>c.[913+1G&gt;A]+[+]</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Vietnamese</td>
<td>13.7</td>
<td>5.38</td>
<td>77.6</td>
<td>25.4</td>
<td>2.8</td>
<td>9.5</td>
<td>HPFH</td>
<td>αα/αα</td>
<td>c.[526_527Ins CGGCCGCC] [1527&gt;T&gt;G]</td>
<td>G176AfsX179 L51R</td>
</tr>
<tr>
<td>10</td>
<td>Korean</td>
<td>13.8</td>
<td>5.34</td>
<td>78</td>
<td>24.5</td>
<td>4.0</td>
<td>1.7</td>
<td>HPFH</td>
<td>αα/αα</td>
<td>c.[526_527Ins CGGCCGCC]+[+]</td>
<td>G176AfsX179</td>
</tr>
<tr>
<td>11</td>
<td>Thai</td>
<td>11.8</td>
<td>5.80</td>
<td>62.1</td>
<td>20.4</td>
<td>-</td>
<td>11.0</td>
<td>EE</td>
<td>αα/αα</td>
<td>c.[1001C&gt;G]+[+]</td>
<td>T334R</td>
</tr>
<tr>
<td>12</td>
<td>Nigerian</td>
<td>12.7</td>
<td>6.0</td>
<td>67.0</td>
<td>21.0</td>
<td>-</td>
<td>20.3</td>
<td>SS</td>
<td>αα/αα</td>
<td>c.[914-4_914-1 del CTAG]+[+]</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 1. a) Diagram showing the position of the mutations identified in the KLF1 gene. Numbered circles correspond to the case number in Table 1. (untranslated regions = hatched, coding regions = white, zinc finger domains = grey, introns = black. b) Amino acid sequence of the three zinc fingers in KLF1 (NCBI's Homologene) in Homo sapiens. Arrows show the positions of missense mutations within the zinc finger domains.