Ⅲ．研究成果の刊行に関する一覧

発表者氏名	論文タイトル名	発表誌名	巻名	ページ	出版年
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Ⅳ．研究成果の刊行物・別刷り

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Regular Article: Red Cells, Iron and Erythropoiesis

Mutations in Krüppel-Like Factor 1 Cause Transfusion-Dependent Hemolytic Anemia and Persistence of Embryonic Globin Gene Expression

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Short title for running head: *KLF1* mutations cause congenital hemolytic anemia

Keywords: Krüppel-Like Factor 1, chronic hemolytic anemia, thalassemia, *trans*-acting factor mutations, pyruvate kinase deficiency

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Key Points

- KLF1 mutations cause severe congenital hemolytic anemia associated with deficiency of red cell Pyruvate Kinase
- Severe deficiency of KLF1 causes hereditary persistence of embryonic globin synthesis

Abstract

In this study we report eight compound heterozygotes for mutations in the key erythroid transcription factor Krüppel-like factor 1, (KLF1) who presented with severe, transfusion-dependent hemolytic anemia. In most cases the red cells were hypochromic and microcytic consistent with abnormalities in hemoglobin synthesis. In addition, in many cases, the red cells resembled those seen in patients with membrane defects or enzymopathies; known as chronic non-spherocytic hemolytic anemia (CNSHA). Analysis of RNA and protein in primary erythroid cells from these individuals provided evidence of abnormal globin synthesis, with persistent expression of fetal hemoglobin and, most remarkably, expression of large quantities of embryonic globins in post-natal life. The red cell membranes were abnormal, most notably expressing reduced amounts of CD44 and consequently manifesting the rare In(Lu) blood group. Finally, all tested patients showed abnormally low levels of the red cell enzyme pyruvate kinase; a known cause of CNSHA. These patients define a new type of severe, transfusion dependent CNSHA caused by mutations in a *trans*-acting factor (KLF1) and reveal an important pathway regulating embryonic globin gene expression in adult humans.

Introduction

Anemia is a major public health problem affecting over 1.5 billion people worldwide.[1] The major causes of anemia include malnutrition, particularly iron and folate deficiency, and infections including malaria and HIV. However, a substantial proportion of patients with anemia have inherited defects of red cells, with mutations found in genes encoding red cell enzymes, membrane proteins, heme and globins. For example, every year over 300,000 affected individuals are born with severe anemia resulting from inherited hemoglobinopathies.[2] However, following screening for mutations in genes currently known to underlie red cell defects, there are many cases of inherited anemia in which either the patient's clinical and hematological phenotype is not consistent with their described genotype, or in whom the cause of the anemia remains completely unknown.

Here we report 8 unrelated patients who displayed severe, transfusion-dependent neonatal anemia with red cell abnormalities ranging from a thalassemia-like morphology (with hypochromic microcytic red cells) to that usually associated with chronic non-spherocytic hemolytic anemias (CNSHA) with anisopoikilocytosis, fragmented cells and a high reticulocyte count. In addition, all of the probands had a remarkable persistence of fetal and embryonic globin synthesis. We found that the parents of these children also had significantly raised levels of fetal hemoglobin (HbF) consistent with previous observations in heterozygotes for *KLF1* mutations. In the light of emerging evidence that mutations in *KLF1*, the gene encoding erythroid Krüppel-like factor (EKLF), give rise to persistent postnatal γ-globin expression in humans[3-7] and embryonic globin expression in mice [8,9] we sequenced this gene in the probands. We discovered that all of them are compound heterozygotes for mutations in *KLF1*. Only three compound heterozygotes for KLF1 mutations have been previously reported and their phenotypes only partially described [6-7]. The transcription factor KLF1 is a master regulator of terminal erythroid differentiation controlling expression of many key pathways and structures including cell division, the cell membrane and cytoskeleton, iron metabolism, heme- and globin-synthesis [5]. Here, we show that individuals with mutations affecting both alleles of *KLF1* exhibit severe anemia in which one or more of these pathways is significantly disrupted

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giving rise to a wide spectrum of hematological phenotypes. The syndromes described here add to an increasing list of *trans*-acting mutations which cause unusual forms of inherited anemia. [10-13] Most importantly all such patients show persistent expression of substantial amounts of embryonic hemoglobins in adult life. Despite extensive characterization of the patterns of globin expression in millions of patients in the last 30 years persistent expression of embryonic globins in adults has very rarely been reported.

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Materials and Methods

Blood samples

Blood was collected from all patients and parents after signing a written informed consent. Patients on regular blood transfusion had a period of at least 10 weeks without blood transfusions before sample collection. This study was approved by the local ethical committees at Siriraj hospital, Bangkok, Thailand and at the John Radcliffe Hospital, Oxford, UK. This study was conducted in accordance with the Declaration of Helsinki.

Hematology and blood serum factors

Routine hematology was determined using an automated red blood cell counter (Sysmex F280, Kobe, Japan). Hemoglobin was analyzed by liquid chromatography using an automated hemoglobin analyzer (HB GOLD; Cumbria, Burrow-in-Furness, UK) and by isoelectric focusing (RESOLVE®, PerkinElmer, USA). Abnormal hemoglobins were excised from the IEF gel, digested with trypsin and analyzed using matrix-assisted laser desorption/ionization time-of flight (MALDI-TOF/TOF) mass spectrometer (Ultralex Bruker Daltonics, Coventry, UK). Routine biochemical parameters were measured using standard techniques.

Red cell phenotype analysis

Samples were tested for the Lutheran blood group using gel card (DiaMed, Cressier sur Morat, Switzerland) with other additional blood group antigens including P1, Le^a, Le^b, k, Kp^a, Kp^b, Jk^a, Jk^b, M, N, S, s, Fy^a and Fy^b as per manufacturer's instructions. The presence of Band-3 (AE-1 anion exchanger) on the red blood cell surface was estimated by the eosin-5-maleimide (EMA) binding assay. [14]

Red blood cell enzyme activities for glucose-6-phosphate dehydrogenase (G6PD), 6-phosphogluconate dehydrogenase (6PGD), pyruvate kinase (PK), hexokinase (HK), glucose phosphate isomerase (GPI), phosphofructokinase (PFK), aldolase (ALD) and phosphoglycerate kinase (PGK) were assessed as described previously. [15] Since

reticulocytes contain higher amounts of these enzymes than mature red blood cells, a calculation for the net activities of each enzyme by correction for the reticulocyte number was performed in order to compare with our standard reference range.[15]

Flow cytometry to assess the proportions of Hb F, [16] Hb Bart's(γ4), Hb zeta (ζ) and CD44 was performed on mature erythrocytes (CD71-) and circulating erythroblasts (CD71+) of patients and parents. In addition, leukocytes were stained with CD44 and CD45 after lysis of erythrocytes with FACs lysis solution (BD Biosciences, San Jose, CA, USA). The antibodies used were anti-Hb F directly conjugated to FITC (Caltag, Burlingame, CA, USA), anti-Hb Bart's (γ4), anti-Hb zeta (ζ), CD44 PE, CD45 Per CP and CD71 PE-Cy5 (BD Biosciences, San Jose, CA,USA) and isotype controls (mouse immunoglobulin G FITC and FITC-conjugated F(ab')2 anti-mouse globulin (Dako, Cambridge,UK).

Molecular characterization of the globin, KLF1 and PKLR genes

We performed multiplex GAP-PCR[17] and ARMS-PCR for detection of common α-thalassemia mutations. [18] Sequence analysis was performed on 1.3 kb of the α2 and α1, 1.81 kb of the ζ, 2.2 kb of the ε and 2.85 kb of the β globin genes, hypersensitive site-2 (HS2) of the β globin locus control region (β-LCR; 630 bp), all promoters of the $^G\gamma$ (807 bp), $^A\gamma$ (802 bp) and β (1044 bp) globin genes using standard techniques. All primer sequences are summarized in Supplementary Table 6. A Multiplex Ligation- dependent Probe Amplification (MLPA) assay was performed to exclude large rearrangements of the β globin cluster. [19] The *KLF1* and *PKLR* genes were amplified and sequenced as previously described. [20,21]

Primary erythroid cell culture and expression analysis

Erythroid cells were obtained using a two-phase culture system [22] and harvested once they were double positive for CD71 and Glycophorin A. RNA was extracted, reverse transcribed to generate double stranded cDNA (Superscript Double Stranded cDNA Synthesis Kit, Invitrogen, Paisley, UK) and labeled with dCTP-Cy3 as the test sample while 500 ng. of sonicated genomic DNA was labeled with dCTP-Cy5 as the input

(Bioprime DNA Labeling System, Invitrogen, Paisley, UK). Both test and input samples were applied to a custom designed Agilent expression array, as described. [23] cDNA was analyzed by Real-time PCR assays obtained from Applied Biosystem's Assay-On-Demand resource. Expression was calculated relative to a reference gene (PABPC1) and normal Thai subjects (n=6) as controls using the Pfaffl method. [24]

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Results

Clinical Presentation

All eight patients reported here presented with neonatal jaundice requiring phototherapy and, in five cases, blood transfusion. Most patients were anemic at presentation with marked hepato-splenomegaly. By one year of age, all patients had received at least one blood transfusion and subsequently all required regular transfusion during the first years of life. Three patients required splenectomy due to hypersplenism. A summary of the clinical presentations and disease severity of the eight probands is presented in Supplementary Table 1 and associated footnotes.

Hematological Evaluation

Full blood counts were determined for all eight index patients, their parents and available siblings (Table 1). Despite transfusion, the levels of Hb in the probands (range 3.0 g/dl to 8.3 g/dl) were low in some cases (Table 1). With one exception (P-1), all of the patients had hypochromic red cells (MCH 20.1-24.8 pg), suggesting abnormalities in the heme and/or globin synthesis pathways. All of the patients showed raised numbers of reticulocytes (5-16%) and many nucleated red blood cells in the peripheral blood, suggesting a significant hemolytic component to the anemia. The morphology of the peripheral blood was variable. In some patients (P-1, Figure 1A, and P-2-4, Supplementary Figure 1), the peripheral blood resembled that seen in patients with thalassemia, with marked hypochromasia, microcytosis, anisopoikilocytosis, target cells and fragmented red cells. In those patients who co-inherited globin gene defects the severity of the changes was much greater than normally associated with these genotypes. By contrast, the peripheral blood of others (P5, Figure 1B, and P6-8, Supplementary Figure 1) resembled that seen in non-spherocytic hemolytic anemia, with numerous fragmented red blood cells, schistocytes and acanthocytes.

The parents and siblings of the eight affected individuals were not anemic (Hb 11.4-15.6 g/dl) although many of them had abnormal red cell indices because they are carriers for

common forms of thalassemia in Thailand (see below). In the six individuals (highlighted in grey in Table 1) who were not carriers of hemoglobinopathies ($\alpha\alpha/\alpha\alpha$: β/β) the red cell indices were either unremarkable or marginally hypochromic and microcytic (average MCH; 27.1 + 1.71 pg, range 25.1-29.3 pg and MCV; 81.9 + 5.57 fL, range 75.4-89.5 fL).

Analysis of Hemoglobin

Hemoglobin analysis revealed abnormally high levels of HbF (average 38%, range 2.6-54.6%) in all patients, consistent with increased F cells by flow cytometry (heterocellular pattern, Supplementary Table 2). Two unanticipated hemoglobins present at levels of up to 18% were observed in all patients. Using, IEF and MALDI-TOF mass spectrometry (Figure 1B, Supplementary Figure 2 and Table 1), these hemoglobins were identified as the γ chain tetramer Hb Bart's (γ_4) and the embryonic hemoglobin Portland I ($\zeta_2\gamma_2$). The presence of both Hbs was further confirmed by flow cytometry (Supplementary Table 2). Hb Gower 2 ($\alpha_2\varepsilon_2$) was not detected at significant amounts and other minor bands (which may correspond to Hb Gower 1, $\zeta_2\varepsilon_2$) were not examined further. Increased levels of embryonic ζ and ε globin mRNA expression were observed by analysis of cDNA extracted from the peripheral blood of patient P-2 using a tiled microarray (Figure 2A, B) and in other five patients using real-time PCR assays (Figure 2C). Interestingly, significantly increased proportions of HbF was observed in 14 of 15 parents from the index families (range 0.2-4.7 %, average (SD); 2.34 + 1.34%, Table 1) but none of the parents expressed Hb Bart's or embryonic globins as assessed by chromatography and IEF (Table 1). Detection of F-cells, anti-ζ and anti-Bart's using flow cytometry was variable in these parents and siblings (Supplementary Table 2).

Analysis of Structural Proteins Commonly Mutated in Red Cell Disorders

Only two of the probands (P-1 and P-5) were found to have entirely structurally normal α- and β-globin genes. The other patients were single or double heterozygotes for common mutations of the globin genes. However, their clinical severity far exceeded that seen in their relatives or other carriers of thalassemia. All patients, and many of the parents, had unexplained, increased levels of HbF, which could have been linked to deletion(s)

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involving the β-globin locus.[25] However, we excluded the possibility of mutations lying within the *cis*-regulatory regions involved in γ-globin gene expression using multiplex-ligation probe assays and sequencing analysis (Supplementary Figure 3 and Supplementary Table 3).

Although the hematological profiles in these patients were complex, four (P-5 to P-8) were initially diagnosed with red cell enzymopathies.[26] These patients were tested for eight common red cell enzyme activities and were found to have significant pyruvate kinase deficiency (<50% of normal after reticulocyte count correction). Based on these findings we evaluated the PK status of the remaining four patients (P-1 to P-4) in whom we did not initially suspect any enzymopathy. All had significantly reduced levels of PK activity (Supplementary Table 2). However, DNA sequencing of the coding region including the intron/exon boundaries of the *PKLR gene*, encoding the red cell pyruvate kinase enzyme, failed to identify any causative mutations.

We next evaluated expression of proteins present on the red-cell membrane and in the cytoskeleton. Extensive minor blood group analysis in five index families is summarized in Supplementary Table 4. All patients had the rare In(Lu) blood group phenotype (a^-/b^-). In addition, all parents and siblings, except one (the father of P-2), also expressed this rare blood group phenotype. The In(Lu) phenotype results from the suppression of two cell surface glycoproteins BCAM and CD44 which carry the Lutheran blood group antigens.[21] It has previously been shown that carriers of KLF1 mutations have the In(Lu) phenotype and expression of CD44 was significantly reduced in the erythrocytes of the majority of patients tested (Supplementary Table 2) but not in their leukocytes, whereas expression of the cytoskeletal protein Band 3 evaluated by EMA binding assay, appeared unchanged compared to control samples (Supplementary Table 2).

Identification of mutations in the KLF1 genes

Although the levels of embryonic (ζ and ε) and fetal (γ) globin RNA and protein are significantly elevated in all probands, analysis of their globin gene haplotypes showed that they have inherited quite different combinations of α and β globin haplotypes (supplementary Figures 6 and 7). Given that elevations in embryonic globins due to *cis*-acting mutations have never been described previously, it seemed very unlikely that these patients had inherited a variety of *cis*-mutations causing persistent embryonic gene expression. To analyze this in further detail the embryonic ζ and ε genes, and the fetal γ genes were sequenced in all patients but no changes (other than common single nucleotide polymorphisms) were found. These observations led us to hypothesize that the genetic lesion in these patients may lie in a *trans*-acting factor involved in erythroid differentiation and maturation. We therefore DNA sequenced the coding region and intron/exon boundaries of two key erythroid transcription factors, *GATA1* and *KLF1*, in one proband (P-2). No likely pathogenic changes were identified in the *GATA1* gene, however, we found two changes in *KLF1*, a c.902G>A transition leading to a p.R301H substitution and a novel 7 bp insertion (c.525_526InsCGGCGCC) resulting in a frameshift (p.G176RfsX179) and disruption of the protein prior to the zinc finger DNA binding domain (Figure 3 and Table 1). Each of these sequence changes has a separate parental origin. The c.902G>A change has been previously reported to underlie elevated levels of HbF as has a similar 7bp insertion (leading to p.G176AfsX179).[6] To gain further genetic evidence that compound heterozygosity for deleterious *KLF1* alleles may underlie the abnormalities reported here we DNA sequenced this gene in the seven remaining patients. Surprisingly, we found all seven to be compound heterozygotes for likely pathogenic *KLF1* changes (Figure 3, Table 1 and Supplementary Figure 4). Four previously unreported coding changes were identified: a c.172C>T transition causing a premature truncation at position 58 in the protein (p.Q58X) prior to the zinc finger domains, a c.892G>C transversion predicted to alter alanine in the first zinc finger (p.A298P), a c.991C>T transition leading to a p.R331W amino acid change in the second zinc finger and a c.1003G>A change leading to a p.G335R substitution which alters a

glycine residue just outside the second zinc finger domain. All three novel missense changes identified alter residues that are highly conserved throughout evolution (Figure 3). We also identified a C>T change 154 bp 5□ of the initiating ATG codon (-154 C>T) in a single patient (P3). This region has previously been reported to include the promoter region of *KLF1*[20] and the change is predicted to alter the binding of transcription factors P53, Pax5 and ETF (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3). This change was absent in 100 population-matched control individuals and is also absent from dbSNP (http://www.ncbi.nlm.nih.gov/SNP/) suggesting it to be rare. These genetic data, taken together with the patient's phenotypic abnormalities, strongly suggest this change disrupts expression of the associated *KLF1* transcript as seen in one patient (P-1) tested (Figure 2C).

All parents and some siblings studied were heterozygotes for the *KLF1* changes present in probands. The four coding changes are extremely rare and are not listed in dbSNP136 (http://www.ncbi.nlm.nih.gov/projects/SNP/) nor in ~13,000 alleles from African and European Americans listed in the Exome Variant Server (EVS) (http://evs.gs.washington.edu/EVS/). Additionally, both of these *KLF1* changes were absent from 296 normal chromosomes from population-matched control individuals. The p.Q58X change is predicted to prematurely truncate the KLF1 protein, while the three novel missense changes alter invariant residues in the first and second zinc finger regions (Figure 3) and may disrupt DNA binding. Altered binding of KLF1 *in vivo* may underlie the observed PKLR deficiency in these patients as the WT KLF1 protein binds the promoter region of the *PKLR* gene in human erythroid progenitor cells (Figure 4).

KLF1 Target genes

The predominant hematological phenotypes in these compound heterozygotes appear to arise from the effects of KLF1 on the globin genes (causing abnormal globin synthesis) and the genes encoding CD44 (causing the In(Lu) phenotype) and pyruvate kinase (causing CNSHA). Of interest, based on re-analysis of previously published data

(Supplementary Table 5), these erythroid genes are amongst the most sensitive to changes in the levels of KLF1. Others have previously analyzed the binding of KLF1 to *cis*-elements surrounding the globin genes and the CD44 gene.[27] Here we have analyzed binding of KLF1 to the PKLR gene (based on data from reference[28] showing that it specifically binds the erythroid promoter of this gene (Figure 4). This is clearly consistent with mutations in the *KLF1* gene causing severe down-regulation of the levels of PK resulting in CNSHA. It was of interest that all patients with the CNSHA phenotype and PK deficiency (P-5-P-8) have exactly the same combination of mutations (p.G176RfsX179;p.A298P –Table 1). Finally, it was of interest that in a single case (P-2) that could be analyzed, the level of *BCL11A* mRNA was reduced (Supplementary Figure 5) when both alleles of the *KLF1* gene are mutated. This is consistent with previous observations showing that this gene is a direct target of KLF1 and is normally involved in the silencing of γ globin synthesis.[3,4] Following on from observations of the effect of BCL11A deficiency on embryonic globin expression in the mouse [29], and the anecdotal observation that embryonic expression is increased in a patient with congenital dyserythropoietic anemia type IV associated with a dominantly acting KLF1 mutation (E325K). [30] It seems plausible that when KLF1 is downregulated to a critical level, it also has a similar effect on human embryonic globin expression.

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Discussion

We have defined a new cause of severe anemia in pediatric patients who are compound heterozygotes for mutations in the key erythroid transcription factor KLF1. The cardinal features of this condition are transfusion dependent anemia associated with abnormalities in red cell enzymes (e.g. PK deficiency), red cell membranes (In(Lu) phenotype), and an abnormal pattern of globin synthesis with very high levels of HbF and readily detectable levels of embryonic globins in adult life.

It appears that *KLF1* mutations may cause a wide spectrum of phenotypes and this may have been expected from its pleiotropic role in erythropoiesis. The principles emerging from current studies suggest that both the levels of KLF1 and the type of mutated protein produced may exert different effects on KLF1 target genes and consequently cause a wide range of red cell phenotypes. The nonsense mutation G176RfsX179 was identified in five of the families reported here and is similar to the G176AfsX179 reported in two individuals from Korea and Vietnam, [6] but no patients have inherited two nonsense mutations. It seems likely that this would lead to embryonic or early fetal lethality due to severe *in utero* anemia as occurs in null mice. [31] This warrants further studies of KLF1 in families with unexplained, recurrent hydrops fetalis or fetal loss, which cannot be accounted for by other conditions.

It has previously been shown that a mutation (E325K) in the second zinc finger domain of KLF1 causes a significant alteration in DNA binding especially at the β-globin genes resulting in a severe red cell and clinical phenotype (congenital dyserythropoietic anemia, CDA) even when only one allele is mutated. [29] It is thought that this mutation acts as a dominant negative, gain of function mutation. By contrast, the patients described here have newly defined phenotypes (severe thalassemia or CNSHA) associated with KLF1 mutations. It is of interest that all patients with CNSHA had exactly the same genotype (Table 1, Family E-H) suggesting that the phenotype is largely driven by the *KLF1* genotype rather than by epistatic effects. Of particular interest the A298P mutation lies in

a key residue of the first zinc finger of KLF1 which determines the binding specificity of the protein.[32]

Given the widespread effects of KLF1 in erythropoiesis summarized here, it seems unlikely that this study and that of Satta et al [7] have captured the full spectrum of these inherited anemias. Despite the apparent relationship between genotype and phenotype, it is interesting that all three previously described compound heterozygotes for *KLF1* mutations (p.S270X/p.K332Q)[7] and (G176Afs179X;L51R)[6] all had similarly raised levels of HbF (22, 31 and 9.5%) but rather milder anemia (Hb 12.1, 11.5 and 13.7 g/dl) than the patients described here. However, two of these were adults (aged over 30 years)[7] when analyzed and also harbored a mutation (p.K322Q) which, *in vitro,* had only a mildly decreased DNA binding affinity to the promoters tested (~2 fold) and therefore may represent a relatively mild hypomorphic allele. [33] The age of the third patient is not reported. We have noticed (anecdotally) that the anemia in our patients has improved with age, requiring progressively less transfusion. It will be important to follow up all patients with *KLF1* mutations to determine whether severity may be truly age dependent.

Over the past few years the role played by KLF1 in globin gene regulation has become clearer. [5,34] Almost since its discovery, it has been known that KLF1 is an activator of β globin expression, but more recently it has been shown to play a role in silencing γ globin expression probably by regulating BCL11A and its interaction with Sox6 at the γ globin gene.[3,35,36] Mutations in *KLF1* therefore reduce β globin expression (causing β thalassemia) [31] and de-repress γ globin expression thereby increasing the synthesis of HbF. [4] However, the relationship between these effects and the mutations that down regulate *KLF1* are not straightforward; not all heterozygotes for *KLF1* mutations produce increased levels of HbF[37] and, in those who do, the level can be very different even in the presence of the same mutation, demonstrating variable penetrance. [7,36,37] These observations have led to speculation about the role of KLF in regulating the levels of HbF.[35] The data presented here clarify the situation since all reported compound heterozygotes produce substantial amounts of HbF putting beyond doubt that KLF1 plays a major role in normally repressing γ globin synthesis in humans *in vivo.* Reactivation of

γ globin synthesis by down-regulating KLF1 might provide a way to ameliorate the effect of severe β-hemoglobinopathies. [3,38]

In mice, which do not have a fetal stage of erythropoiesis, the KLF1/BCL11A circuit affects embryonic rather than fetal gene expression. [39] This pathway normally suppresses embryonic globin synthesis in definitive erythroid cells, but a knockout of *Klf1* appears to have no effect on embryonic globins in primitive erythropoiesis, when they are normally expressed. In this model definitive cells are not produced to determine whether or not the embryonic genes are correctly suppressed later in development.[5] It was reported anecdotally that a patient with CDA type IV and the E325K mutation had raised levels of embryonic globins. Here we show that in compound *KLF1* heterozygotes, human embryonic (ζ and ε) globin expression persists in adults. This indicates that KLF1 also plays a role in repressing embryonic globin expression in humans. This finding is striking since reactivation of embryonic globin is virtually never seen in other erythroid disorders. At present the pathway underlying embryonic globin repression is poorly understood. However it has recently been shown that two members of the KLF family (KLF3 and KLF8) may be involved in regulating embryonic globin expression, and a knockout of these two transcription factors in erythroid cells de-represses embryonic globin synthesis. [40] Since expression of these two factors is driven by KLF1, it is possible that mutations in this protein might reduce KLF3 and KLF8 leading to derepression of embryonic globins. Further analysis of this pathway is warranted since preliminary studies in mice suggest that persistence of embryonic globin expression could ameliorate severe hemoglobinothathies. [41]

In conclusion, we have shown that a wide spectrum of unexplained forms of severe inherited anemia may be caused by *trans* acting mutations in the key erythroid transcription factor KLF1. Most remarkably in every case where the downregulation is sufficiently severe to cause anemia, there is an associated persistence of embryonic globin expression, revealing a new pathway by which globin gene expression might be manipulated to ameliorate other inherited hemoglobinopathies.

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Authorship contributions

V.V. served as the principle investigator of this study and contributed to case identification, patient care, study design, conducting the study, analysis of the data and drafting the manuscript. S. E., S. R., N. C., C. F., K. L., H. K., S. B. and J. S. performed laboratory and DNA analysis; M. N., K. S. and V.S.T. took care of patients and collected samples; C.B and D.S. prepared the manuscript; R.G., S.P. and D.R.H. provided support, research direction, study plan, prepared the manuscript and provided mentorship. All authors contributed to the data review and provided their comments on the manuscript.

Disclosure of Conflicts of Interest

All authors declared no conflict of interest.

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Family E																				
P5	M	18*	3.4	11.1	1.37	81.0	24.8	30.6	25.4	10.1	113	62.7	29.0	4.6	10.2	6.0	αα/αα	β/β	G176RfsX 179	A298P
		23**	8.2	27.4	3.38	81.1	24.2	29.9	22.4	6.0	86									
Father	M	58	14.4	43.7	5.4	81.0	26.6	33.0	14.6	1.0	0	82.8	0.9	3.8	0	0	$-\alpha^{3.7}/\alpha\alpha$	β/β	W	A298P
Mother	F	55	14.1	42.7	4.85	88.0	29.1	33.1	13.9	1.0	0	82.8	3.1	2.8	0	0	αα/αα	β/β	G176RfsX 179	W
Family F																				
P-6	F	0.9**	6.6	21.0	2.94	71.0	22.3	31.3	23.8	10.0	8	33.3	49.8	2.4	13.7	0.8	$\alpha^{CS}\alpha/\alpha\alpha$	β/β	G176RfsX 179	A298P
		5.7***	8.3	27.1	3.82	70.9	21.6	30.5	23.4	10.0	13	45.0	50.0	3.0	+	+				
Father	M	34	13.2	40.1	5.5	73.0	24.0	32.9	14.4	6.0	0	83.0	0.2	2.7	0	0	$\alpha^{CS}\alpha/\alpha\alpha$	β/β	W	A298P
Mother	F	30	13.9	41.5	5.2	80.0	26.6	33.5	14.4	2.0	0	94.8	2.4	2.8	0	0	αα/αα	β/β	G176RfsX 179	W
Brother 1	M	11	11.8	36.7	5.2	70.4	22.7	32.3	15.1	1.0	0	96.4	0.5	3.1	0	0	$\alpha^{CS}\alpha/\alpha\alpha$	β/β	G176RfsX 179	W
Brother 2	M	8	11.7	35.1	4.7	75.4	25.1	33.3	15.1	1.0	0	94.9	2.4	2.7	0	0	αα/αα	β/β	W	A298P
Family G																				
P-7	M	0.2**	5.7	17.8	2.66	67.0	21.2	31.9	21.9	11.0	146	33.9	54.6	2.2	14.3	0.3	$-\alpha^{3.7}/\alpha\alpha$	β/β	G176RfsX 179	A298P
Mother	F	22	11.9	36.4	4.59	79	26.0	32.8	14.4	1.0	0	85.8	0	2.6	0	0	$-\alpha^{3.7}/\alpha\alpha$	β/β	G176RfsX 179	W
Family H																				
P-8	M	4**	5.4	16.3	2.18	75.0	24.7	33.1	20.4	10	26	69.0	19.0	10.0	+	+	αα/αα	β^E/β	G176RfsX 179	A298P
Father	M	32	13.5	45.2	5.1	66.0	19.9	29.9	16.1	2.0	0	95.9	0.9	3.2	0	0	$--^{SEA}/\alpha\alpha$	β/β	W	A298P
Mother	F	30	12.7	40.7	5.53	74.0	23.0	31.5	15.1	2.0	0	66.6	4.7	28.7	0	0	αα/αα	β^E/β	G176RfsX 179	W

Table 1: Hematological parameters, globin and *KLF1* genotypes in Thai families with unusual forms of hereditary hemolytic anemia.

Cases	Sex	Age (yrs. mths)	Hb (g/dL)	Hct (%)	RBC ($x10^6/\mu L$)	MCV (fL)	MCH (pg)	MCHC (g/dL)	RDW (%)	Retic. (%)	NRBC (/100WC)	Hemoglobin typing (%)					Globin genotypes		*KLF1* genotypes	
												Hb A	Hb F	HbA_2/E	Hb Portland-1	Hb Barts	αα/αα	β/β	allele-1	allele-2
Family A																				
P-1	F	4*	3.0	8.5	1.06	80.1	28.1	35.1	16.2	6.0	248	70.1	16.2	3.1	ND	ND	αα/αα	β/β	R331W	G335R
		6.2**	6.9	25.1	2.36	106.2	29.1	27.4	19.0	16.0	1510	38.5	51.8	2.0	4	2				
Father	M	37	13.4	40.8	4.56	89.5	29.3	32.7	13.3	2.0	0	82.3	1.5	2.7	0	0	αα/αα	β/β	W	G335R
Mother	F	34	12.2	37.4	4.69	79.7	26.0	32.6	13.5	1.0	0	82.5	3.0	2.7	0	0	αα/αα	β/β	R331W	W
Family B																				
P-2	M	0.6*	5.9	18.2	2.76	65.9	21.4	32.4	NA	5.0	26	72.3#	16.6	3	8.1&	0	$--^{SEA}/\alpha\alpha$	β^E/β	G176RfsX 179	R301H
		12**	7.5	25.7	3.72	69.1	20.1	29.1	17.8	9.0	181	16.9	39.1	4.3	16.2	14.7				
Father	M	36	15.6	46.3	5.88	78.8	26.5	33.6	13.6	0.5	0	65.1	3.6	2.0	0	0	αα/αα	β/β	W	R301H
Mother	F	23$	11.4	37	6.41	57.1	17.8	30.9	ND	ND	0	74.9	3.4	21.7	0	0	$--^{SEA}/\alpha\alpha$	β^E/β	G176RfsX 179	W
Brother	M	10	12.5	37.8	5.67	66.7	22.1	33.1	17.4	1.0	0	54.0	6.9	26.9	0	0	αα/αα	β^E/β	G176RfsX 179	W
Family C																				
P-3	M	0.2	5.2	19.0	NA	74.0	21.0	28.0	26.0	11.0	124	ND	ND	ND	ND	ND	αα/αα	β^E/β^E	-154C/T	A298P
		1.10**	7.7	24.5	3.35	73.1	23.0	31.4	26.2	14.0	57	21.0	52.8	22.7	+	+				
Father	M	31	14.0	42.1	5.45	77.3	25.7	33.3	14.5	1.0	0	68.4	0.5#	31.1	0	0	αα/αα	β^E/β	W	A298P
Mother	F	32	11.6	35.1	5.0	70.4	23.2	33.0	14.4	0.5	0	72.0	4.3#	23.5	0	0	$\alpha^{CS}\alpha/\alpha\alpha$	β^E/β	-154C/T	W
Family D																				
P4	M	1.2*	6.5	21.1	2.98	70.6	21.7	30.7	37.1	10.0	100	78.7	5.5	8.3	7.3	0.2	$--^{SEA}/-\alpha^{3.7}$	β^E/β	Q58X	A298P
		5.4**	6.6	21.5	3.09	69.5	21.4	30.9	24.3	5.0	49	70	2.6	9.5	17.6	0.3				
Father	F	28	14.0	42.5	6.98	60.9	20.1	32.9	17.2	1.5	0	75	2.6	22.4	0	0	$--^{SEA}/\alpha\alpha$	β^E/β	Q58X	W
Mother	M	27	12.1	34.6	4.81	72.0	25.2	35	15.5	1.0	0	70.2	1.8	28	0	0	$\alpha\alpha/-\alpha^{3.7}$	β^E/β	W	A298P

Legends to Table

Family A: * This study was done when this patient was first referred to our hospital and 2 months after the last blood transfusion. **This study was performed 1 year after splenectomy and the patient was free from blood transfusion. Of note, P-1 is the only patient with a normal MCH (28 pg) suggesting that the co-inheritance of thalassemia makes a significant contribution to the defects in Hb synthesis seen in our patients.

Family B: *This study was performed when the patient (P-2) was first seen at our hospital in 1997 (6 month old). #The patient received less than 1 month of blood transfusion before this study and he has been on regular transfusion since. &At first diagnosis, this abnormal hemoglobin was identified as Hb Bart's. ** This study was performed 6 months after splenectomy. $The mother of P-2 died of lung cancer on 18/2/2004.

Family C: **The patient was withheld from regular blood transfusion for 4 months before this study. #HbF measurements were variable even within the same individual; at 2 year earlier of this study, Hb F level in the mother and the father were 1.5 and 1.2% respectively by LPLC (same laboratory). Moreover, Hb F levels measured were different based on the techniques used; in the mother Hb F were 4.3%, 3.4% and 3.3% and the father were 0.5%, 0.9% and 0 % based on LPLC, HPLC and capillary electrophoresis (CE) respectively. Using the CE, we were able to discriminate between Hb E and HbA_2, interestingly, the levels of HbA_2 in the mother was 3.8% (with 20.8% Hb E) and 5.4% (with 26.1% Hb E) in the father. In addition, 0.2% of Hb Constant Spring (Hb CS) eluted at the RT 305 seconds was identified in the mother.

Family D: *This study was done 2 months after blood transfusion when this patient was first referred. **This study was performed 3 months after the regular blood transfusion was held.

Family E: *This study was performed when this patient was withheld from blood transfusion for 10 weeks. ** This study was performed 6 months after splenectomy.

Family F: **This study was performed when this patient was first presented at our hospital. ***This study was performed after the patient was free from blood transfusion for 2 years.

Family G: **This study was performed when this patient first presented at 2 month-old.

Family H: **This evaluation was done when the patient was first referred to our center and the last blood transfusion was around 12 weeks before this study.

NA, not available; ND, not determined; MCV, mean corpuscular volume; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; RDW, red cell distribution width; Retic, reticulocyte count; P= patient, M= male, F= female. Data of hemoglobin analyses

showed here derived from liquid chromatography in which HbA_2 and Hb E were eluted at the same window and could not separate based on this methodology. W= wide type for *KLF1* gene sequences. Hb Portland-1 ($\zeta_2\gamma_2$) and Hb Bart's (γ_4), have not been quantified in every patients, see text. Individuals without globin gene mutations are highlighted with grey colour.

Figure Legends.

Figure 1

A-B: Wright stained peripheral blood pictures from two index patients (A; P-1, B; P-5) who had normal α- and β-globin genes. In P-1, a marked hypochromic microcytosis with anisopoikiocytosis, acanthocytes and numerous nucleated red blood cells was observed. While in P-5, a blood picture with numerous fragmented red blood cells with schistocytes and numerous nucleated red blood cells was seen and similar to non-spherocytic hemolytic anemia (NSHA). Both smears were performed after splenectomy in both patients and free from blood transfusion. Peripheral blood features in other patients are available in Supplementary data-2. **C: Isoelectric focusing study of embryonic globins identified in a patient with *KLF1* mutations.** Comparing hemoglobin profiles from the patient, P-2 with control human embryonic stem cell (hES) derived hematopoietic cells reveals three distinct abnormal hemoglobin protein bands separated at a more cathodic position than Hb A and Hb F. These hemoglobin species were similar to those of patients (P-2, in triplication) as they were separated to the same isoelectric points. These hemoglobin bands were subsequently identified by mass spectrometry to be Hb Portland-1 ($\zeta_2\gamma_2$) and Hb Bart's (γ_4). Of note, a different level of embryonic protein expression during erythroid differentiation from embryonic to fetal erythropoiesis in hES cells from day 6 to day 10 was observed (right hand panel). Moreover, a fast moving hemoglobin specie of Hb Gower 2 ($\alpha_2\varepsilon_2$) was identified in this erythroid cell model but not from the patient. The standard hemoglobin controls are shown on the far left lane.

Figure 2

Expression profiles of the primary erythroid culture cells from patients with *KLF1* mutations. An expression profiling of the α(A) and β(B) globin gene clusters using Agilent tiled oligonucleotide array was performed in P-2 erythroid cells compared with normal control. **A:** A distinctive higher peak of ζ globin gene expression (dark arrow with grey box highlighted) was observed in the patient (lower panel) compared to a normal cDNA from control sample (upper panel). **B:** Two unique up-regulated expression peaks of γ (arrow 1) and ε (arrow 2) globin genes in the patient was shown. Another up-regulating expression of non-coding mRNA (arrow 3) was observed at the β globin locus control region (β-LCR, hypersensitive site-2, HS-2), all with dark arrows and grey box highlighted. However the meaning of this observation on the down-stream globin gene regulation and hereditary persistent of

embryonic globin in the patient remains unclear. There was no significant change on expression of other nearby genes on both clusters. **C:** Expression profile of erythroid specific genes from primary erythroid cell samples from five patients (P) with *KLF1* mutations and normal controls (n= 6) using qPCR and Taqman probe hybridization showing a marked increase of fetal and embryonic globin mRNA expression (HBZ; ζ globin gene, HBE; ε globin gene, HBG; γ globin gene) in all patients with relatively normal expression of *KLF1*. This suggests that, although, most patients carry one nonsense mutation, that could result in haplo-insufficiency, a missense mutation on the other allele might produce stable transcripts that could be detected at similar levels to normal. Alternatively, our mRNA analysis used might not be sensitive enough to demonstrate a minor perturbation in the level of *KLF1* expression. With the exception of BCL11A (B-cell CLL/lymphoma 11A (zinc finger protein) from one patient (P-2), there was no change in expression compared to normal controls of other erythroid specific genes including; CD71, SOX6, ERAF, GYPA; glycophorin A and EPOR; erythropoietin receptor (Supplementary Figure 5).

Figure 3

***KLF1* gene structure, identification of variants in cases reported in this study and their location within conserved domains.** The upper panel shows a schematic representation of *KLF1*, exons are shown to scale with the coding sequence in black and untranslated regions in white, and the locations of nucleotide changes identified here are shown. Below this is a diagram representing the KLF1 protein with the previously annotated transactivating domain and the three zinc finger domains encoded by exons 2 and 3. The locations of all mutations identified in this study are shown; all changes are novel except for the previously reported R301H substitution (asterisk). The lower panel shows positions and conservation of amino acid residues found to be changed in the probands reported here (arrows). The entire first and second zinc finger sequences are shown with interspecies conservation, the extent of each zinc finger domain is shown above each region. The cysteine and histidine residues involved in the coordination of zinc are denoted by the # symbol and the residues that contact specific bases in DNA are marked with a + symbol.

Figure 4

Binding of KLF1 to the *PKLR* gene. The upper panel shows a representation of the *PKLR* gene structure including the start of the erythroid (E) specific transcript (dark grey) and the liver (L) specific first exon in light grey. The lower panels show KLF1 binding and DNase I

hypersensitive sites in cultured human erythroid progenitors (EP), K562; erythroleukemia cell line, HepG2; liver cells line as indicated.

Figure 1

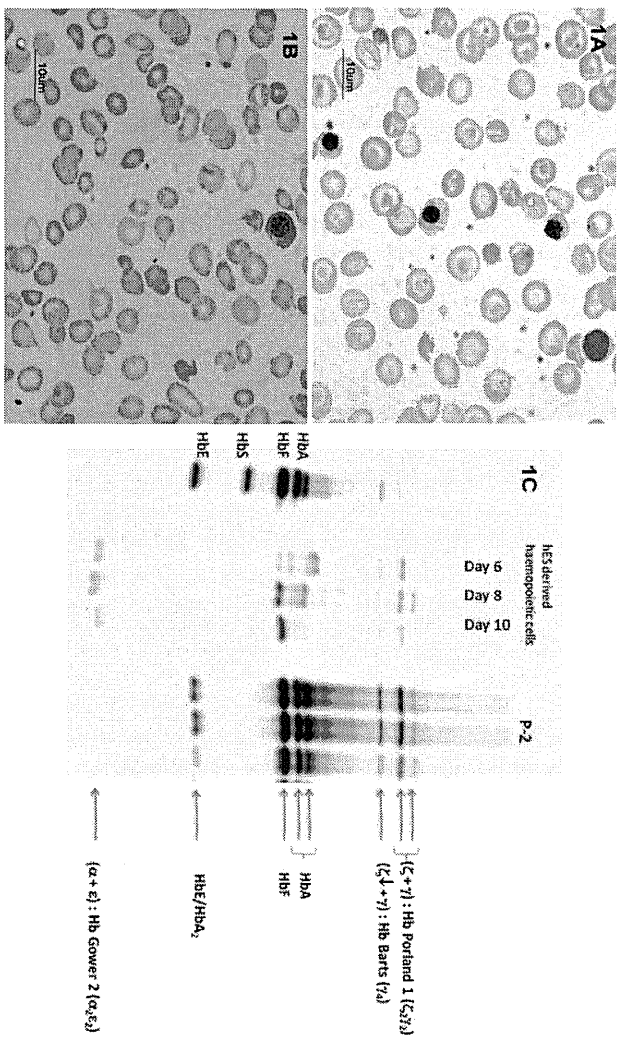
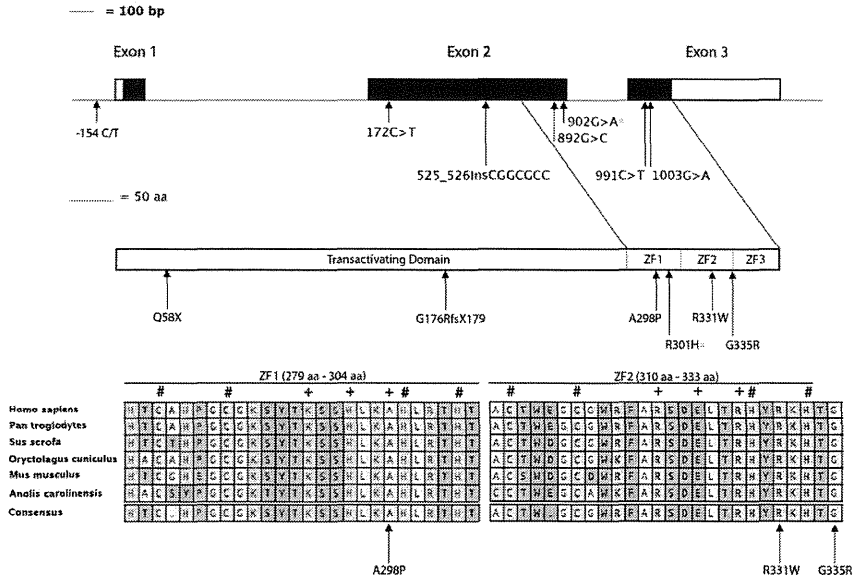
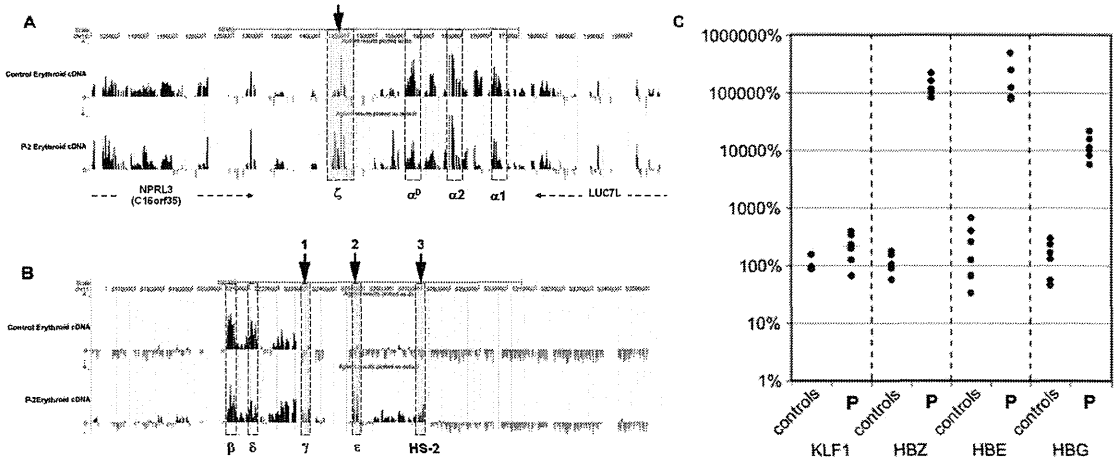


Figure 3



30

Figure 2



29