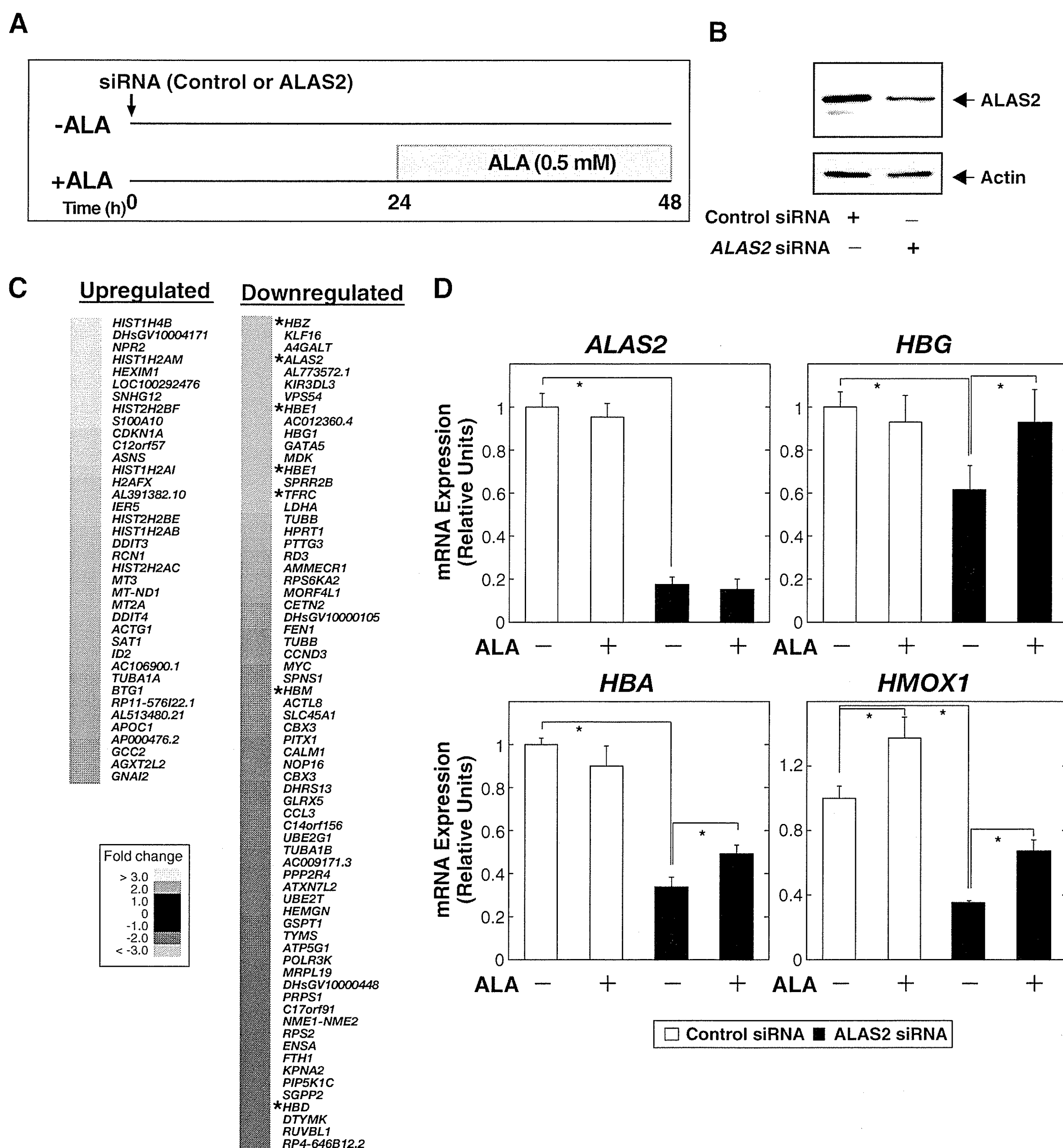

Fig. 4. ALA restores defects in ALAS2 deficiency in HiDEP cells. (A) Experimental protocol for *ALAS2* knockdown and *ALA* rescue in HiDEP cells. (B) siRNA-mediated ALAS2 knockdown in HiDEP cells. Anti-ALAS2 Western blot of whole cells extracts. Actin was a loading control. (C) The heat map depicts the fold change resulting from *ALAS2* knockdown in HiDEP cells. Genes displaying >2-fold changes are shown. Asterisk indicates globin genes, *ALAS2*, and *TFRC*. (D) Quantitative RT-PCR analysis of *ALAS2*, *HBG*, *HBA*, and *HMOX1* in HiDEP cells following the experimental protocol of (A). The data are expressed as means ± SE ($n = 4$). $^*p < 0.05$.

required to clarify the mechanism by which ALA is transported into erythroid cells.

3.4. ALA restores defects in ALAS2 deficiency in human iPS cell-derived erythroblasts

To recapitulate the phenotype of XLSA, we performed siRNA-mediated knockdown of *ALAS2* in HiDEP cells, which were subsequently treated with ALA (Fig. 4A). We first performed expression profiling based in *ALAS2*-knockdown HiDEP cells, which was confirmed by Western blotting (Fig. 4B). The analysis revealed >2-fold upregulation and downregulation of 38 and 68 genes caused by *ALAS2* knockdown, respectively (Fig. 4C and Supplementary Table 2). The downregulated gene ensemble included *ALAS2*, globins (*HBZ*, *HBG*, *HBE*, *HBD*, and *HBM*) in addition to genes involved in iron metabolism (ferritin heavy chain 1 and transferrin receptor (*TFRC*)). Gene ontology analysis revealed significant enrichment of cellular iron ion homeostasis ($p = 0.000076$), cell division ($p = 0.00062$), DNA repair ($p = 0.0006$), and translation ($p = 0.018$), implying that heme was involved in various biological processes in erythroid cells. The downregulation of *TFRC* may imply iron overload due to decreased porphyrin synthesis, which diminishes *TFRC* mRNA stability through the inactivation of iron regulatory proteins [27]. However, ringed sideroblasts were not observed in HiDEP cells with *ALAS2* knockdown (data not shown). Noticeably, ALA treatment significantly improved the effects of *ALAS2*

knockdown-mediated downregulation of *HBA*, *HBG*, and *HMOX1* (Fig. 4D), suggesting that ALA could restore defects of ALAS2 depletion in human erythroblasts.

Ideally, it would have been desirable to test the effects of ALA in a mice model of XLSA. Unfortunately, the mice are not available for further testing, because *Alas2*-knockout mice die by day 11.5 *in utero* [28]. In addition, although ringed sideroblast formation is observed after transgenic rescue of *ALAS2* in *Alas2*-knockout mice, the mice die soon after birth [29]. Thus, there is a need for the development of a CSA mouse model. Nevertheless, ALA is an endogenous amino acid that has been shown to be safe in clinical settings [1]; therefore, it could be administered in patients with XLSA who are refractory to PLP supplementation. In conclusion, our data suggests that ALA could serve as a novel therapeutic option for CSA with *ALAS2* mutations.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.10.050.

References

[1] M. Ishizuka, F. Abe, Y. Sano, et al., Novel development of 5-aminolevurinic acid (ALA) in cancer diagnoses and therapy, Int. Immunopharmacol. 11 (2011) 358–365.
[2] K. Furuyama, K. Kaneko, P.D. Vargas, Heme as a magnificent molecule with multiple missions: heme determines its own fate and governs cellular homeostasis, Tohoku J. Exp. Med. 213 (2007) 1–16.
[3] R.S. Ajioka, J.D. Phillips, J.P. Kushner, Biosynthesis of heme in mammals, Biochim. Biophys. Acta 1763 (2006) 723–736.
[4] H.A. Dailey, P.N. Meissner, Erythroid heme biosynthesis and its disorders, Cold Spring Harb. Perspect. Med. 3 (2013) a011676.
[5] R. Ohba, K. Furuyama, K. Yoshida, et al., Clinical and genetic characteristics of congenital sideroblastic anemia: comparison with myelodysplastic syndrome with ring sideroblast (MDS-RS), Ann. Hematol. 92 (2013) 1–9.
[6] H. Harigae, K. Furuyama, Hereditary sideroblastic anemia: pathophysiology and gene mutations, Int. J. Hematol. 92 (2010) 425–431.
[7] A.K. Bergmann, D.R. Campagna, E.M. McLoughlin, et al., Systematic molecular genetic analysis of congenital sideroblastic anemia: evidence for genetic heterogeneity and identification of novel mutations, Pediatr. Blood Cancer 54 (2010) 273–278.
[8] S. Bekri, A. May, P.D. Cotter, et al., A promoter mutation in the erythroid-specific 5-aminolevulinate synthase (ALAS2) gene causes X-linked sideroblastic anemia, Blood 102 (2003) 698–704.
[9] K. Kaneko, K. Furuyama, T. Fujiwara, et al., Identification of a novel erythroid-specific enhancer for the ALAS2 gene and its loss-of-function mutation which is associated with congenital sideroblastic anemia, Haematologica 99 (2014) 252–261.
[10] R. Kurita, N. Suda, K. Sudo, et al., Establishment of immortalized human erythroid progenitor cell lines able to produce enucleated red blood cells, PLoS ONE 8 (2013) e59890.
[11] T. Fujiwara, H. Harigae, S. Takahashi, et al., Differential gene expression profiling between wild-type and ALAS2-null erythroblasts: identification of novel heme-regulated genes, Biochem. Biophys. Res. Commun. 340 (2006) 105–110.
[12] M.C. Cooper, J. Levy, L.N. Cantor, et al., The effect of erythropoietin on colonial growth of erythroid precursor cells in vitro, Proc. Natl. Acad. Sci. U.S.A. 71 (1974) 1677–1680.
[13] T. Fujiwara, H. Saitoh, A. Inoue, et al., 3-Deazaneplanocin A (DZNep), an inhibitor of S-adenosylmethionine-dependent methyltransferase, promotes erythroid differentiation, J. Biol. Chem. 289 (2014) 8121–8134.
[14] T. Nagai, H. Harigae, K. Furuyama, et al., 5-Aminolevulinate synthase expression and hemoglobin synthesis in a human myelogenous leukemia cell line, J. Biochem. 121 (1997) 487–495.
[15] J. Sun, M. Brand, Y. Zenke, et al., Heme regulates the dynamic exchange of Bach1 and NF-E2-related factors in the Maf transcription factor network, Proc. Natl. Acad. Sci. U.S.A. 101 (2004) 1461–1466.
[16] T. Tahara, J. Sun, K. Igarashi, et al., Heme-dependent up-regulation of the alpha-globin gene expression by transcriptional repressor Bach1 in erythroid cells, Biochem. Biophys. Res. Commun. 324 (2004) 77–85.
[17] T. Tahara, J. Sun, K. Nakanishi, et al., Heme positively regulates the expression of beta-globin at the locus control region via the transcriptional factor Bach1 in erythroid cells, J. Biol. Chem. 279 (2004) 5480–5487.
[18] K. Ogawa, M. Tashima, T. Toi, et al., Inhibition of erythroid differentiation by stem cell factor in K562 cells expressing the c-kit gene, Exp. Hematol. 22 (1994) 45–51.
[19] T. Rutherford, J.B. Clegg, D.R. Higgs, et al., Embryonic erythroid differentiation in the human leukemic cell line K562, Proc. Natl. Acad. Sci. U.S.A. 78 (1981) 348–352.
[20] G. Berkovitch-Luria, S. Yakobovitch, M. Weitman, et al., A multifunctional 5-aminolevulinic acid derivative induces erythroid differentiation of K562 human erythroleukemic cells, Eur. J. Pharm. Sci. 47 (2012) 206–214.
[21] G. Ahlin, C. Hilgendorf, J. Karlsson, et al., Endogenous gene and protein expression of drug-transporting proteins in cell lines routinely used in drug discovery programs, Drug Metab. Dispos. 37 (2009) 2275–2283.
[22] S.A. Ingersoll, S. Ayyadurai, M.A. Charania, et al., The role and pathophysiological relevance of membrane transporter PepT1 in intestinal inflammation and inflammatory bowel disease, Am. J. Physiol. Gastrointest. Liver Physiol. 302 (2012) G484–G492.
[23] S. Frølund, O.C. Marquez, M. Larsen, et al., Delta-aminolevulinic acid is a substrate for the amino acid transporter SLC36A1 (hPAT1), Br. J. Pharmacol. 159 (2010) 1339–1353.
[24] M. Bermúdez Moretti, S. Correa Garcia, C. Perotti, et al., Delta-Aminolevulinic acid transport in murine mammary adenocarcinoma cells is mediated by beta transporters, Br. J. Cancer 87 (2002) 471–474.
[25] T. Fujiwara, H. O'Geen, S. Keles, et al., Discovering hematopoietic mechanisms through genome-wide analysis of GATA factor chromatin occupancy, Mol. Cell 36 (2009) 667–681.
[26] D.T. Thwaites, L. Basterfield, P.M. McCleave, et al., Gamma-aminobutyric acid (GABA) transport across human intestinal epithelial (Caco-2) cell monolayers, Br. J. Pharmacol. 129 (2000) 457–464.
[27] S. Taketani, Acquisition, mobilization and utilization of cellular iron and heme: endless findings and growing evidence of tight regulation, Tohoku J. Exp. Med. 205 (2005) 297–318.
[28] O. Nakajima, S. Takahashi, H. Harigae, et al., Heme deficiency in erythroid lineage causes differentiation arrest and cytoplasmic iron overload, EMBO J. 18 (1999) 6282–6289.
[29] O. Nakajima, S. Okano, H. Harada, et al., Transgenic rescue of erythroid 5-aminolevulinate synthase-deficient mice results in the formation of ring sideroblasts and siderocytes, Genes Cells 11 (2006) 685–700.

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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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Key Points

- KLF1 mutations cause severe congenital hemolytic anemia associated with a deficiency of red cell pyruvate kinase.
- A severe KLF1 deficiency causes hereditary persistence of embryonic globin synthesis.

In this study, we report on 8 compound heterozygotes for mutations in the key erythroid transcription factor Krüppel-like factor 1 in patients 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 nonspherocytic 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 postnatal 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 (Krüppel-like factor 1) and reveal an important pathway regulating embryonic globin gene expression in adult humans. (*Blood*. 2014;123(10):1586-1595)

Introduction

Anemia is a major public health problem affecting more than 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 red cell defects, with mutations found in genes encoding red cell enzymes, membrane proteins, heme, and globins. For example, every year more than 300 000 affected individuals are born with severe anemia resulting from inherited hemoglobinopathies.[2] However, after 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 the cause of the anemia remains completely unknown.

Here we report on 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 nonspherocytic 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 3 compound heterozygotes for KLF1 mutations have been previously reported, and their phenotypes have been 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, and heme and globin synthesis.[5] Here, we show that individuals with mutations affecting both alleles of *KLF1* exhibit severe anemia, in which 1 or more of these pathways is significantly disrupted, giving rise to a wide spectrum of hematological phenotypes. The syndromes described here add to an increasing

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list of *trans*-acting mutations that cause unusual forms of inherited anemia.[10-13] Most important, 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 during the last 30 years, persistent expression of embryonic globins in adults has very rarely been reported.

Materials and methods

Blood samples

Blood was collected from all patients and parents after they signed a written informed consent. Patients receiving 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, United Kingdom. This study also 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). Hemoglobin was analyzed by liquid chromatography, using an automated hemoglobin analyzer (HB Gold; Cumbria) and by isoelectric focusing (IEF) (Resolve; PerkinElmer). Abnormal hemoglobins were excised from the IEF gel, digested with trypsin and analyzed using a matrix-assisted laser desorption/ionization time-of flight (MALDI-TOF/TOF) mass spectrometer (Ultralex Bruker Daltonics). Routine biochemical parameters were measured using standard techniques.

Red cell phenotype analysis

Samples were tested for the Lutheran blood group using a gel card (DiaMed) 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 binding assay.[14]

Red blood cell enzyme activities for glucose-6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase, pyruvate kinase (PK), hexokinase, glucose phosphate isomerase, phosphofructokinase, aldolase, and phosphoglycerate kinase were assessed as described previously.[15] Because 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 to compare the results with our standard reference range.[15]

Flow cytometry to assess the proportions of HbF,[16] Hb Bart's (γ4), Hb ζ (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 fluorescence-activated cell sorter lysis solution (BD Biosciences). The antibodies used were anti-HbF directly conjugated to fluorescein isothiocyanate (FITC; Caltag), anti-Hb Bart's (γ4), anti-Hb ζ (ζ), CD44 PE, CD45 Per CP, and CD71 PE-Cy5 (BD Biosciences), as well as isotype controls (mouse immunoglobulin G FITC and FITC-conjugated F[ab′]2 anti-mouse globulin; Dako).

Molecular characterization of the globin, KLF1 and PKLR genes

We performed multiplex Gap-PCR[17] and amplification-refractory mutation system–PCR assays 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 and hypersensitive site 2 of the β-globin locus control region (630 bp), all promoters of the $^G\gamma$- (807 bp), $^A\gamma$- (802 bp), and β-globin (1044 bp) genes, using standard techniques. All primer sequences are summarized in supplemental Table 6, available on the *Blood* Web site. A multiplex ligation-dependent probe amplification 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 2-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), and labeled with dCTP-Cy3 as the test sample; 500 ng sonicated genomic DNA was labeled with dCTP-Cy5 as the input (Bioprime DNA Labeling System; Invitrogen). 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 Offal method.[24]

Results

Clinical presentation

All 8 patients reported here presented with neonatal jaundice requiring phototherapy and, in 5 cases, blood transfusion. Most patients were anemic at presentation with marked hepatosplenomegaly. By 1 year of age, all patients had received at least a single blood transfusion, and subsequently, all of them required regular transfusion during the first years of life. Three patients required splenectomy caused by hypersplenism. A summary of the clinical presentations and disease severity of the 8 probands is presented in supplemental Table 1 and associated footnotes.

Hematological evaluation

Full blood counts were determined for all 8 index patients and their parents and available siblings (Table 1). Despite transfusion, the levels of Hb in the probands (range, 3.0–8.3 g/dL) were low in some cases (Table 1). With a single exception (P1), all of the patients had hypochromic red cells (mean cell hemoglobin [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 (P1, Figure 1A; P2–P4, supplemental 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 coinherited globin gene defects, the severity of the changes was much greater than normally associated with these genotypes. In contrast, the peripheral blood of others (P5, Figure 1B; P6–P8, supplemental Figure 1) resembled that seen in nonspherocytic hemolytic anemia, with numerous fragmented red blood cells, schistocytes, and acanthocytes.

The parents and siblings of the 8 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 following). In the 6 individuals (highlighted in gray in Table 1) who were not carriers of hemoglobinopathies (αα/αα: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]; average mean corpuscular volume, 81.9 + 5.57 fL [range, 75.4–89.5 fL]).

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

												Hemoglobin typing (%)					Globin genotypes		*KLF1* genotypes	
Cases	Sex	Age (y, mo)	Hb (g/dL)	Hct (%)	RBC (×106/μL)	MCV (fL)	MCH (pg)	MCHC (g/dL)	RDW (%)	Retic. (%)	NRBC (/100WC)	Hb A	Hb F	HbA2/E	Hb Portland-1	Hb Bart's	αα/αα	β/β	Allele 1	Allele 2
Family A																				
Pi	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																				
P2	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/αα	βE/β	G176RfsX179	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/αα	βE/β	G176RfsX179	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	βE/β	G176RfsX179	W
Family C																				
P3	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	βE/	−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	+	+		βE		
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	βE/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	αCSα/αα	βE/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/-	βE/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	α3.7			

Data of hemoglobin (Hb) 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. Hb Portland-1 ($\zeta_2\gamma_2$) and Hb Bart's (γ_4) have not been quantified in every patient (supplemental Table 1 and associated clinical text). Individuals without globin gene mutations are highlighted in gray.

F, female; M, male; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; MCV, mean corpuscular volume; NA, not available; ND, not determined; RDW, red cell distribution width; P, patient; Retic., reticulocyte count; W, wild type for *KLF1* gene sequences.

*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 when the patient was free from blood transfusion. Of note, P1 is the only patient with a normal MCH (28 pg), suggesting that the coinheritance of thalassemia makes a significant contribution to the defects in Hb synthesis seen in our patients.

‡This study was performed when the patient (P2) was first seen at our hospital in 1997 at age 6 months.

¶The patient received less than 1 month of blood transfusion before this study and 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 P2 died of lung cancer on February 18, 2004.

††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 years before this study, the Hb F levels in the mother and the father were 1.5% and 1.2%, respectively by low-pressure liquid chromatography (LPLC; same laboratory). Moreover, the Hb F levels measured were different based on the techniques used; in the mother, Hb F levels were 4.3%, 3.4%, and 3.3%, and those of the father were 0.5%, 0.9%, and 0%, based on LPLC, high-performance liquid chromatography, and capillary electrophoresis, respectively. Using capillary electrophoresis, we were able to discriminate between Hb E and HbA_2; interestingly, the levels of HbA_2 in the mother were 3.8% (with 20.8% Hb E) compared with 5.4% (with 26.1% Hb E) in the father. In addition, 0.2% of Hb Constant Spring eluted at retention time 305 s was identified in the mother.

¶¶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.

‖‖This study was performed when this patient was withheld from blood transfusion for 10 weeks.

***This study was performed 6 months after splenectomy.

†††This study was performed when this patient first presented at our hospital.

‡‡‡This study was performed after the patient was free from blood transfusion for 2 years.

¶¶¶This study was performed when this patient first presented at age 2 months.

§§§This evaluation was done when the patient was first referred to our center, and the last blood transfusion was performed around 12 weeks before this study.

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Table 1. (continued)

Cases	Sex	Age (y, mo)	Hb (g/dL)	Hct (%)	RBC (×106/μL)	MCV (fL)	MCH (pg)	MCHC (g/dL)	RDW (%)	Retic. (%)	NRBC (/100WC)	Hemoglobin typing (%)					Globin genotypes		*KLF1* genotypes	
												Hb A	Hb F	HbA2/E	Hb Portland-1	Hb Bart's	αα/αα	β/β	Allele 1	Allele 2
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/αα	βE/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	αα/-α3.7	βE/β	W	A298P
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	G176RfsX179	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	−α3.7/αα	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	G176RfsX179	W
Family F																				
P6	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	αCSα/αα	β/β	G176RfsX179	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	αCSα/αα	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	G176RfsX179	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	αCSα/αα	β/β	G176RfsX179	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																				
P7	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	−α3.7/αα	β/β	G176RfsX179	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	−α3.7/αα	β/β	G176RfsX179	W
Family H																				
P8	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	βE/β	G176RfsX179	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/αα	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	βE/β	G176RfsX179	W

Data of hemoglobin (Hb) 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. Hb Portland-1 ($\zeta_2\gamma_2$) and Hb Bart's (γ_4) have not been quantified in every patient (supplemental Table 1 and associated clinical text). Individuals without globin gene mutations are highlighted in gray.

F, female; M, male; MCH, mean cell hemoglobin; MCHC, mean cell hemoglobin concentration; MCV, mean corpuscular volume; NA, not available; ND, not determined; RDW, red cell distribution width; P, patient; Retic., reticulocyte count; W, wild type for *KLF1* gene sequences.

*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 when the patient was free from blood transfusion. Of note, P1 is the only patient with a normal MCH (28 pg), suggesting that the coinheritance of thalassemia makes a significant contribution to the defects in Hb synthesis seen in our patients.

‡This study was performed when the patient (P2) was first seen at our hospital in 1997 at age 6 months.

¶The patient received less than 1 month of blood transfusion before this study and 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 P2 died of lung cancer on February 18, 2004.

††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 years before this study, the Hb F levels in the mother and the father were 1.5% and 1.2%, respectively by low-pressure liquid chromatography (LPLC; same laboratory). Moreover, the Hb F levels measured were different based on the techniques used; in the mother, Hb F levels were 4.3%, 3.4%, and 3.3%, and those of the father were 0.5%, 0.9%, and 0%, based on LPLC, high-performance liquid chromatography, and capillary electrophoresis, respectively. Using capillary electrophoresis, we were able to discriminate between Hb E and HbA_2; interestingly, the levels of HbA_2 in the mother were 3.8% (with 20.8% Hb E) compared with 5.4% (with 26.1% Hb E) in the father. In addition, 0.2% of Hb Constant Spring eluted at retention time 305 s was identified in the mother.

¶¶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.

‖‖This study was performed when this patient was withheld from blood transfusion for 10 weeks.

***This study was performed 6 months after splenectomy.

†††This study was performed when this patient first presented at our hospital.

‡‡‡This study was performed after the patient was free from blood transfusion for 2 years.

¶¶¶This study was performed when this patient first presented at age 2 months.

§§§This evaluation was done when the patient was first referred to our center, and the last blood transfusion was performed around 12 weeks before this study.

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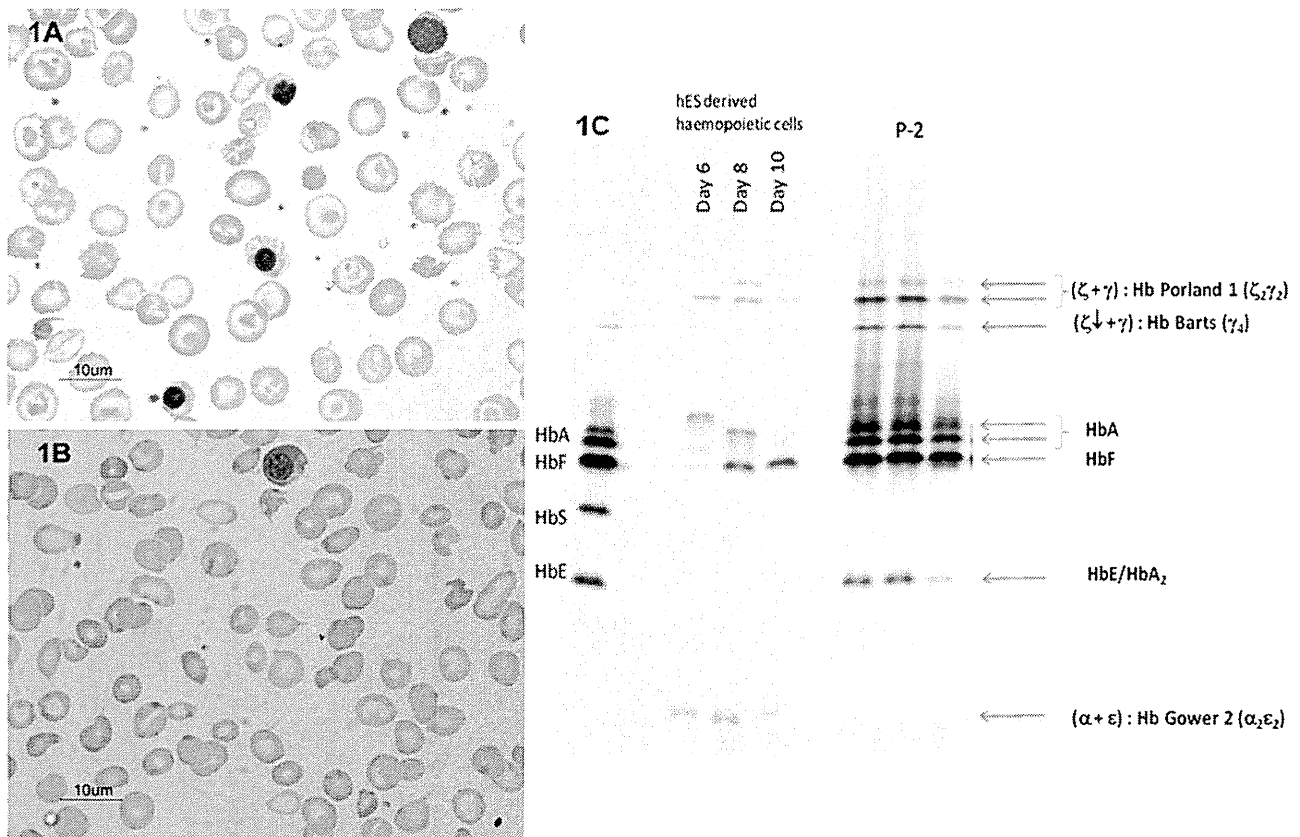

Figure 1. Wright stained peripheral blood pictures from 2 index patients who had normal α- and β-globin genes. In P1 (A), a marked hypochromic microcytosis with anisopoikilocytosis, acanthocytes, and numerous nucleated red blood cells was observed; in P5 (B), a blood picture with numerous fragmented red blood cells with schistocytes and numerous nucleated red blood cells was seen, similar to nonspherocytic hemolytic anemia. Both smears were performed after splenectomy in both patients and were free from blood transfusion. Peripheral blood features in other patients are available in supplemental Figure 1. (C) IEF study of embryonic globins identified in a patient with *KLF1* mutations. Comparing hemoglobin profiles from the patient, P2, with control human embryonic stem cell (hES)-derived hematopoietic cells reveals 3 distinct abnormal hemoglobin protein bands separated at a more cathodic position than HbA and HbF. These hemoglobin species were similar to those of patients (P2, 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*). 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.

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; supplemental 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, supplemental 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 P2, using a tiled microarray (Figure 2A-B), as well as in other 5 patients using real-time PCR assays (Figure 2C). Interestingly, significantly increased proportions of HbF were 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 (supplemental Table 2).

Analysis of structural proteins commonly mutated in red cell disorders

Only 2 of the probands (P1 and P5) 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 a deletion or deletions 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 hematologic profiles in these patients were complex, 4 patients (P5–P8) were initially diagnosed with red cell enzymopathies.[26] These patients were tested for 8 common red cell enzyme activities and were found to have significant PK deficiency (<50% of normal after reticulocyte count correction). On the basis of these findings, we evaluated the PK status of the remaining 4 patients (P1–P4), 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*,

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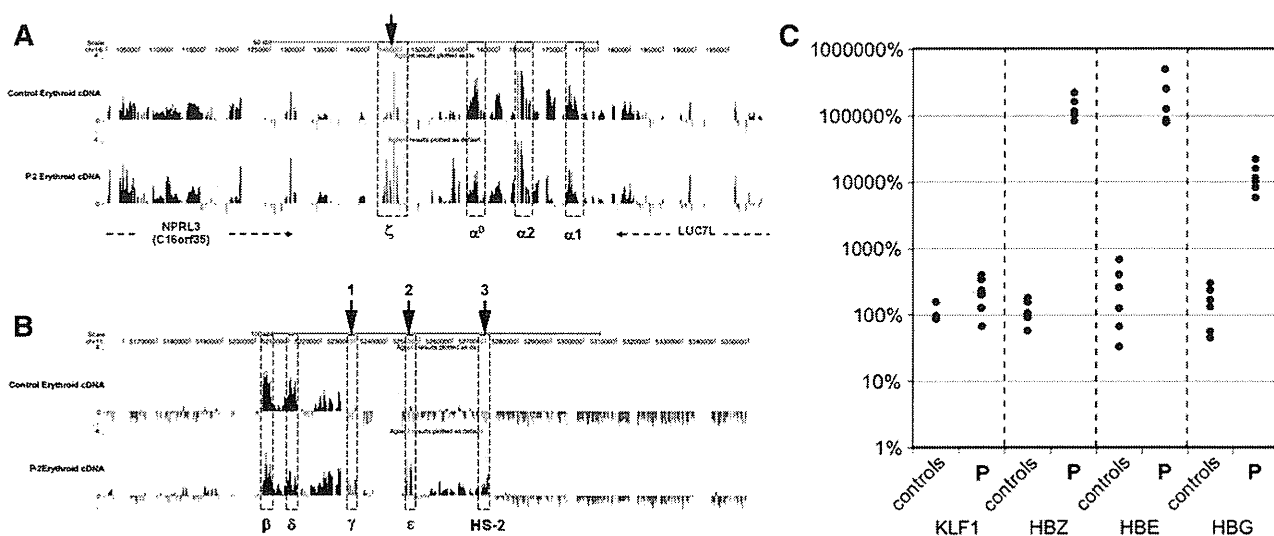

Figure 2. Expression profiles of the primary erythroid culture cells from patients with *KLF1* mutations. An expression profiling of the α(A) and β(B) globin gene clusters using Agilent tiled oligonucleotide array was performed in P2 erythroid cells compared with normal control. (A) A distinctive higher peak of ζ globin gene expression (dark arrow with gray box highlighted) was observed in the patient (bottom) compared with a normal cDNA from a control sample (top). (B) Two unique upregulated expression peaks of γ-globin (arrow 1) and ε-globin (arrow 2) genes in the patient were shown. Another upregulating expression of noncoding mRNA (arrow 3) was observed at the β-globin locus control region (β-globin locus control region, hypersensitive site 2 [HS-2]), all with dark arrows and gray boxes highlighted. However, the meaning of this observation on the downstream globin gene regulation and hereditary persistence 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 5 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 a single nonsense mutation, which 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 1 patient [P2]), there was no change in expression compared with normal controls of other erythroid-specific genes, including CD71, SOX6, ERAF, GYPA, glycophorin A, and EPOR (erythropoietin receptor; supplementary Figure 5).

encoding the red cell PK 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 5 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 P2), also expressed this rare blood group phenotype. The In(Lu) phenotype results from the suppression of 2 cell surface glycoproteins, BCAM and CD44, which carry the Lutheran and Indian blood group antigens, respectively.[21,27] It has previously been shown that carriers of KLF1 mutations have the In(Lu) phenotype and that 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 integral protein Band 3 evaluated by eosin-5-maleimide binding assay appeared unchanged compared with 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 (supplemental Figure 6 and supplemental Figure 7). Given that elevations in embryonic globins resulting from *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. Therefore, we DNA sequenced the coding region and intron/exon boundaries of 2 key erythroid transcription factors, *GATA1* and *KLF1*, in a single proband (P2). No likely pathogenic changes were identified in the *GATA1* gene; however, we found 2 changes in *KLF1*, a c.902G>A transition leading to a p.R301H substitution and a 7 bp insertion (c.525_526InsCGGCGCC), resulting in a frameshift (p.G176RfsX179) and disruption of the protein before 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 7 remaining patients. Surprisingly, we found all 7 to be compound heterozygotes for likely pathogenic *KLF1* changes (Figure 3, Table 1, and supplemental 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) before 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 that alters a glycine residue just outside the second zinc finger domain. All 3 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

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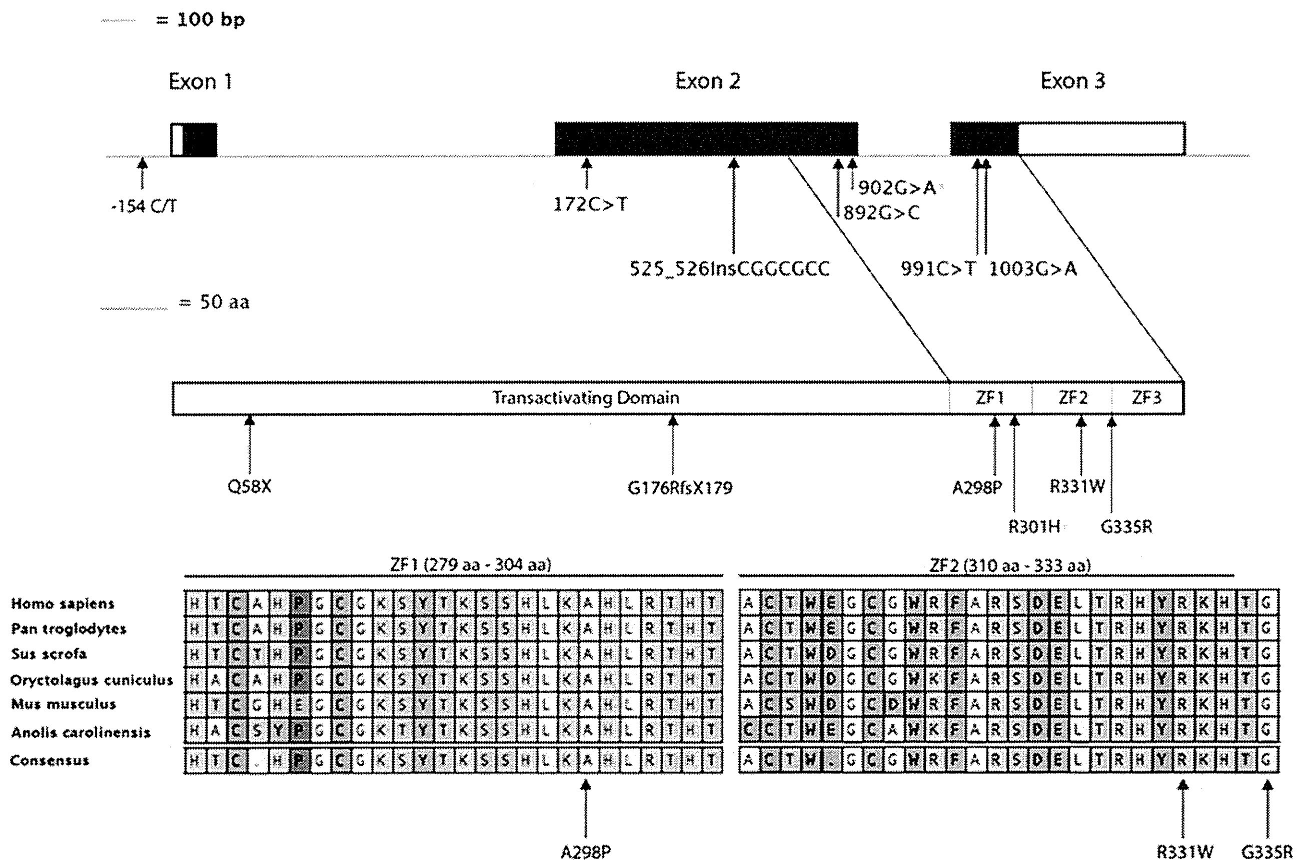

Figure 3. *KLF1* gene structure, identification of variants in cases reported in this study, and their location within conserved domains. (top) Schematic representation of *KLF1*; exons are shown to scale, with the coding sequence in black and untranslated regions in white, and the locations of nucleotide changes identified here. Below this is a diagram representing the KLF1 protein with the previously annotated transactivating domain and the 3 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). (bottom) A position 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, and 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.

(−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 tumor suppressor p53, paired box 5, and epidermal growth factor receptor–specific transcription factor (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 single nucleotide polymorphism database (http://www.ncbi.nlm.nih.gov/SNP/), suggesting it is 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 a single patient (P1) tested (Figure 2C).

All parents and some siblings studied were heterozygotes for the *KLF1* changes present in probands. The 4 coding changes are extremely rare and are listed neither 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 (http://evs.gs.washington.edu/EVS/). In addition, 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, and the 3 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 hematologic 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 PK (causing CNSHA). Of interest, on the basis of reanalysis of previously published data (supplemental Table 5), these erythroid genes are among 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.[28] Here we have analyzed binding of KLF1 to the PKLR gene (based on data from Su et al[29] 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 downregulation of the levels of PK resulting in CNSHA. It was of interest that all patients with the CNSHA phenotype and PK deficiency (P5–P8) have exactly the same combination of mutations (p.G176RfsX179;p.A298P; Table 1). Finally, it was of interest that in a single case (P2) 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

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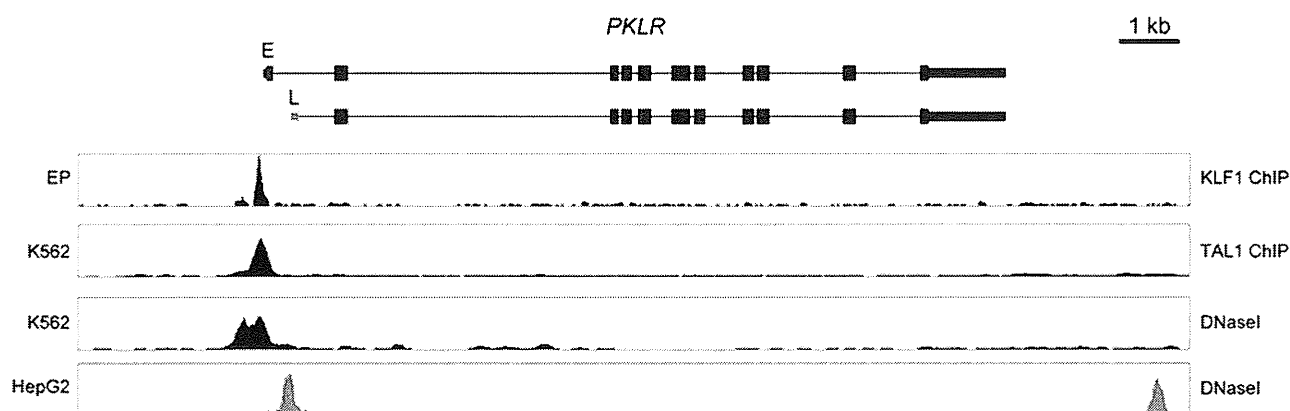

Figure 4. Binding of KLF1 to the *PKLR* gene. (top) a representation of the *PKLR* gene structure including the start of the erythroid (E)-specific transcript (dark gray) and the liver (L)-specific first exon in light gray. (bottom) KLF1 binding and DNase I hypersensitive sites in cultured human erythroid progenitors (EP), K562; erythroleukemia cell line, HepG2; and liver cells line, as indicated.

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[30] 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.

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 (eg, 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, which 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 5 of the families reported here and is similar to the G176AfsX179 reported in 2 individuals from Korea and Vietnam,[6] but no patients have inherited 2 nonsense mutations. It seems likely that this would lead to embryonic or early fetal lethality resulting from severe in utero anemia, as occurs in null mice.[32] 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), even when only a single allele is mutated.[30] It is thought that this mutation acts as a dominant negative, gain-of-function mutation. In 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 is that the A298P mutation lies in a key residue of the first zinc finger of KLF1, which determines the binding specificity of the protein.[33]

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 3 previously described compound heterozygotes for *KLF1* mutations (p.S270X/p.K332Q)[7] and (G176AfsI79X; L51R)[6] 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, 2 of these patients were adults (older than 30 years)[7] when analyzed and also harbored a mutation (p.K322Q) that, 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.[34] The age of the third patient is not reported. We have noticed (anecdotally) that 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.

During the last few years, the role played by KLF1 in globin gene regulation has become clearer.[5,35] Almost since its discovery, it has been known that KLF1 is an activator of β-globin expression, but more recently, it also 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.[3,36,37] Mutations in *KLF1* therefore reduce β-globin expression (causing β thalassemia)[32] and de-repress γ-globin expression, thereby increasing the synthesis of HbF.[4] However, the relationship between these effects and the mutations that downregulate *KLF1* are not straightforward: Not all heterozygotes for *KLF1* mutations produce increased levels of HbF,[38] and in those who do, the level can be very different, even in the presence of the same mutation, demonstrating variable penetrance.[7,37,38] These observations have led to speculation about the role of KLF in regulating the levels of HbF.[36] The data presented here clarify the situation because all report that 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 downregulating KLF1 might provide a way to ameliorate the effect of severe β-hemoglobinopathies.[3,39]

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In mice, which do not have a fetal stage of erythropoiesis, the KLF1/BCL11A circuit affects embryonic rather than fetal gene expression.[40] 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 congenital dyserythropoietic anemia type 4 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 because 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 2 members of the KLF family (KLF3 and KLF8) may be involved in regulating embryonic globin expression, and a knockout of these 2 transcription factors in erythroid cells de-represses embryonic globin synthesis.[41] Because expression of these 2 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 because preliminary studies in mice suggest that persistence of embryonic globin expression could ameliorate severe hemoglobinothathies.[42]

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

Contribution: 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.J., K.S., and V.S.T. took care of patients and collected samples; C.B. and D.S. prepared the manuscript; R.J.G., S.P., and D.R.H. provided support, research direction, and study plan and prepared the manuscript and provided mentorship; and all authors contributed to the data review and provided their comments on the manuscript.

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References

1. McLean E, Cogswell M, Egli I, Wojdyla D, de Benoist B. Worldwide prevalence of anaemia, WHO Vitamin and Mineral Nutrition Information System, 1993-2005. *Public Health Nutr.* 2009; 12(4):444-454.
2. Modell B, Darlison M. Global epidemiology of haemoglobin disorders and derived service indicators. *Bull World Health Organ.* 2008;86(6): 480-487.
3. Borg J, Papadopoulos P, Georgitsi M, et al. Haploinsufficiency for the erythroid transcription factor KLF1 causes hereditary persistence of fetal hemoglobin. *Nat Genet.* 2010;42(9):801-805.
4. Zhou D, Liu K, Sun CW, Pawlik KM, Townes TM. KLF1 regulates BCL11A expression and gamma-to beta-globin gene switching. *Nat Genet.* 2010; 42(9):742-744.
5. Siatecka M, Bieker JJ. The multifunctional role of EKLF/KLF1 during erythropoiesis. *Blood.* 2011; 118(8):2044-2054.
6. Gallienne AE, Dréau HM, Schuh A, Old JM, Henderson S. Ten novel mutations in the erythroid transcription factor KLF1 gene associated with increased fetal hemoglobin levels in adults. *Haematologica.* 2012;97(3):340-343.
7. Satta S, Perseu L, Moi P, et al. Compound heterozygosity for KLF1 mutations associated with remarkable increase of fetal hemoglobin and red cell protoporphyrin. *Haematologica.* 2011; 96(5):767-770.
8. Pilon AM, Arcasoy MO, Dressman HK, et al. Failure of terminal erythroid differentiation in EKLF-deficient mice is associated with cell cycle perturbation and reduced expression of E2F2. *Mol Cell Biol.* 2008;28(24):7394-7401.
9. Siatecka M, Sahr KE, Andersen SG, Mezei M, Bieker JJ, Peters LL. Severe anemia in the Nan mutant mouse caused by sequence-selective disruption of erythroid Kruppel-like factor. *Proc Natl Acad Sci USA.* 2010;107(34):15151-15156.
10. Babbs C, Roberts NA, Sanchez-Pulido L, et al; WGS500 Consortium. Homozygous mutations in a predicted endonuclease are a novel cause of congenital dyserythropoietic anemia type I. *Haematologica.* 2013;98(9):1383-1387.
11. Dgany O, Avidan N, Delaunay J, et al. Congenital dyserythropoietic anemia type I is caused by mutations in codanin-1. *Am J Hum Genet.* 2002; 71(6):1467-1474.
12. Nichols KE, Crispino JD, Poncz M, et al. Familial dyserythropoietic anaemia and thrombocytopenia due to an inherited mutation in GATA1. *Nat Genet.* 2000;24(3):266-270.
13. Sankaran VG, Ghazvinian R, Do R, et al. Exome sequencing identifies GATA1 mutations resulting in Diamond-Blackfan anemia. *J Clin Invest.* 2012; 122(7):2439-2443.
14. Tachavanich K, Tanphaichitr VS, Utto W, Viprakasit V. Rapid flow cytometric test using eosin-5-maleimide for diagnosis of red blood cell membrane disorders. *Southeast Asian J Trop Med Public Health.* 2009;40(3):570-575.
15. Tachavanich K, Viprakasit V, Pung-Amritt P, Veerakul G, Chansing K, Tanphaichitr VS. Development of a comprehensive red blood cell enzymopathy laboratory in Thailand: the study of normal activity in eight erythroenzymes in Thais. *Southeast Asian J Trop Med Public Health.* 2009; 40(2):317-326.
16. Mundee Y, Bigelow NC, Davis BH, Porter JB. Simplified flow cytometric method for fetal hemoglobin containing red blood cells. *Cytometry.* 2000;42(6):389-393.

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17. Chong SS, Boehm CD, Higgs DR, Cutting GR. Single-tube multiplex-PCR screen for common deletional determinants of alpha-thalassemia. *Blood.* 2000;95(1):360-362.
18. Chan AY, So CC, Ma ES, Chan LC. A laboratory strategy for genotyping haemoglobin H disease in the Chinese. *J Clin Pathol.* 2007;60(8):931-934.
19. Harteveld CL, Voskamp A, Phylipsen M, et al. Nine unknown rearrangements in 16p13.3 and 11p15.4 causing alpha- and beta-thalassaemia characterised by high resolution multiplex ligation-dependent probe amplification. *J Med Genet.* 2005;42(12):922-931.
20. Kanno H, Fujii H, Hirono A, Miwa S. cDNA cloning of human R-type pyruvate kinase and identification of a single amino acid substitution (Thr384——Met) affecting enzymatic stability in a pyruvate kinase variant (PK Tokyo) associated with hereditary hemolytic anemia. *Proc Natl Acad Sci USA.* 1991;88(18):8218-8221.
21. Singleton BK, Burton NM, Green C, Brady RL, Anstee DJ. Mutations in EKLF/KLF1 form the molecular basis of the rare blood group In(Lu) phenotype. *Blood.* 2008;112(5):2081-2088.
22. Fibach E. Techniques for studying stimulation of fetal hemoglobin production in human erythroid cultures. *Hemoglobin.* 1998;22(5-6):445-458.
23. De Gobbi M, Anguita E, Hughes J, et al. Tissue-specific histone modification and transcription factor binding in α globin gene expression. *Blood.* 2007;110(13):4503-4510.
24. Pfaffl MW. The ongoing evolution of qPCR. *Methods.* 2010;50(4):215-216.
25. Thein SL, Wood WG. In: Steinberg MH, Forget BG, Higgs DR, Weatherall DJ, eds. *The molecular basis of β thalassemia, δβ thalassemia and hereditary persistence of fetal hemoglobin. Disorders of hemoglobin.* 2nd ed. Cambridge: Cambridge University Press; 2009:323-356.
26. Prchal JT, Gregg XT. Red cell enzymes. *Hematology (Am Soc Hematol Educ Program).* 2005;19-23.
27. Helias V, Saison C, Peyrard T, et al. Molecular analysis of the rare in(Lu) blood type: toward decoding the phenotypic outcome of haploinsufficiency for the transcription factor KLF1. *Hum Mutat.* 2013;34(1):221-228.
28. Tallack MR, Whitington T, Yuen WS, et al. A global role for KLF1 in erythropoiesis revealed by ChIP-seq in primary erythroid cells. *Genome Res.* 2010;20(8):1052-1063.
29. Su MY, Steiner LA, Bogardus H, et al. Identification of biologically relevant enhancers in human erythroid cells. *J Biol Chem.* 2013;288(12): 8433-8444.
30. Esteghamat F, Gillemans N, Bilic I, et al. Erythropoiesis and globin switching in compound Klf1:Bcl11a mutant mice. *Blood.* 2013;121(13): 2553-2562.
31. Arnaud L, Saison C, Helias V, et al. A dominant mutation in the gene encoding the erythroid transcription factor KLF1 causes a congenital dyserythropoietic anemia. *Am J Hum Genet.* 2010;87(5):721-727.
32. Perkins AC, Sharpe AH, Orkin SH. Lethal beta-thalassaemia in mice lacking the erythroid CACCC-transcription factor EKLF. *Nature.* 1995; 375(6529):318-322.
33. Turner J, Crossley M. Mammalian Krüppel-like transcription factors: more than just a pretty finger. *Trends Biochem Sci.* 1999;24(6):236-240.
34. Singleton BK, Lau W, Fairweather VS, et al. Mutations in the second zinc finger of human EKLF reduce promoter affinity but give rise to benign and disease phenotypes. *Blood.* 2011; 118(11):3137-3145.
35. Borg J, Patrinos GP, Felice AE, Philipsen S. Erythroid phenotypes associated with KLF1 mutations. *Haematologica.* 2011;96(5):635-638.
36. Sankaran VG. Targeted therapeutic strategies for fetal hemoglobin induction. *Hematology (Am Soc Hematol Educ Program).* 2011;2011:459-465.
37. Satta S, Perseu L, Maccioni L, Giagu N, Galanello R. Delayed fetal hemoglobin switching in subjects with KLF1 gene mutation. *Blood Cells Mol Dis.* 2012;48(1):22-24.
38. Perseu L, Satta S, Moi P, et al. KLF1 gene mutations cause borderline HbA(2). *Blood.* 2011; 118(16):4454-4458.
39. Sankaran VG, Nathan DG. Reversing the hemoglobin switch. *N Engl J Med.* 2010;363(23): 2258-2260.
40. Sankaran VG, Xu J, Ragoczy T, et al. Developmental and species-divergent globin switching are driven by BCL11A. *Nature.* 2009; 460(7259):1093-1097.
41. Funnell AP, Mak KS, Twine NA, et al. Generation of mice deficient in both KLF3/BKLF and KLF8 reveals a genetic interaction and a role for these factors in embryonic globin gene silencing. *Mol Cell Biol.* 2013;33(15):2976-2987.
42. Russell JE, Liebhaber SA. Reversal of lethal alpha- and beta-thalassemias in mice by expression of human embryonic globins. *Blood.* 1998;92(9):3057-3063.

First-line treatment for severe aplastic anemia in children: bone marrow transplantation from a matched family donor *versus* immunosuppressive therapy

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ABSTRACT

The current treatment approach for severe aplastic anemia in children is based on studies performed in the 1980s, and updated evidence is required. We retrospectively compared the outcomes of children with acquired severe aplastic anemia who received immunosuppressive therapy within prospective trials conducted by the Japanese Childhood Aplastic Anemia Study Group or who underwent bone marrow transplantation from an HLA-matched family donor registered in the Japanese Society for Hematopoietic Cell Transplantation Registry. Between 1992 and 2009, 599 children (younger than 17 years) with severe aplastic anemia received a bone marrow transplant from an HLA-matched family donor (n=213) or immunosuppressive therapy (n=386) as first-line treatment. While the overall survival did not differ between patients treated with immunosuppressive therapy or bone marrow transplantation [88% (95% confidence interval: 86-90) *versus* 92% (90-94)], failure-free survival was significantly inferior in patients receiving immunosuppressive therapy than in those undergoing bone marrow transplantation [56% (54-59) *versus* 87% (85-90); *P*<0.0001]. There was no significant improvement in outcomes over the two time periods (1992-1999 *versus* 2000-2009). In multivariate analysis, age <10 years was identified as a favorable factor for overall survival (*P*=0.007), and choice of first-line immunosuppressive therapy was the only unfavorable factor for failure-free survival (*P*<0.0001). These support the current algorithm for treatment decisions, which recommends bone marrow transplantation when an HLA-matched family donor is available in pediatric severe aplastic anemia.

Introduction

Aplastic anemia is defined as peripheral blood pancytopenia caused by bone marrow failure; the pathogenesis of this disease is thought to involve autoimmune processes.[1-3] The principal interventions responsible for improved survival in aplastic anemia are bone marrow transplantation (BMT) and immunosuppressive therapy (IST). In children, BMT from an HLA-matched family donor (MFD) is the treatment of choice for severe aplastic anemia (SAA).[1,4-6] For children lacking an MFD, IST with a combination of antithymocyte globulin and cyclosporine has been used as a therapeutic option.[6-10] However, this treatment approach is based on the results of comparative studies between these therapies that were conducted mainly in the 1980s, and there have been few recent studies that compare the outcome of BMT recipients with comparable patients receiving IST.

The largest pediatric series in previous studies was reported by the European Group for Blood and Marrow Transplantation (EBMT) and included 304 children treated from 1970 to 1988; that study indicated survival was better following first-line BMT than after first-line IST (63% *versus* 48%; *P*=0.002) but did not compare failure-free survival after the two therapies.[6] Our previous analysis showed a significant advantage for patients receiving BMT from an MFD as first-line treatment in a study of 100 children with SAA who were treated between 1984 and 1998.[1] In patients who received first-line IST, 10-year overall and failure-free survival rates were 55% and 40%, respectively, both of which were markedly inferior to the rates in patients who initially underwent BMT, which was associated with 10-year overall survival and failure-free survival rates greater than 90%. Since the 1980s, the outcomes of both BMT and IST have improved, likely due to better supportive care and advanced treatment and transplantation protocols. A recently published Cochrane review regarding BMT from an MFD and IST as first-line treatment also pointed out that all studies included in the analysis

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had a high risk of bias due to their study design and were conducted more than 10 years ago and may not be applicable to the standard of care of today.[11] Updated evidence to aid treatment decisions in pediatric SAA is, therefore, required.

In children, the choice of an appropriate treatment is particularly influenced by the long-term sequelae of the disease and its therapy. Thus, failure-free survival is much more important than survival alone when analyzing the long-term outcomes of children with aplastic anemia. Lack of response, relapse, and clonal evolution are problematic in the IST setting, whereas graft failure, acute and chronic graft-*versus*-host disease (GVHD), and infectious complications limit the success of BMT. In the present study, we compared the outcomes of children with SAA who received IST or BMT from an MFD as first-line treatment using data from nationwide IST and BMT registries.

Methods

Patients

Between 1992 and 2009, a total of 599 consecutive children (younger than 17 years) with acquired SAA underwent BMT from an MFD or received IST as first-line treatment in Japan; 213 patients with an MFD underwent BMT and were registered in the Transplant Registry Unified Management Program (TRUMP) conducted by the Japanese Society for Hematopoietic Cell Transplantation, and 386 patients without an MFD were enrolled in two consecutive prospective multicenter trials (AA-92/97) conducted by the Japanese Childhood Aplastic Anemia Study Group and were initially treated with IST (Table 1). The disease severities were defined as previously reported.[12,13] Underlying inherited marrow failure disorders were excluded clinically and by chromosome fragility testing. Marrow cytogenetic studies were performed for all patients, and patients with clonal cytogenetic abnormalities were excluded from this study. Patients with paroxysmal nocturnal hemoglobinuria with clinical symptoms and positive findings on the Ham test/sucrose test were also excluded from this analysis. All treatments were performed after obtaining written informed consent from patients or their parents in accordance with the Declaration of Helsinki.

Immunosuppressive therapy and bone marrow transplantation procedures

The characteristics of the treatment procedures are detailed in Table 2. Three hundred and eighty-six patients were enrolled in the AA-92 (n=84) and AA-97 (n=302) trials, and all the patients were initially treated with a combination of antithymocyte globulin and cyclosporine A. Response to IST and disease relapse were evaluated as previously reported.[12] Transplantation data were collected with the use of standardized forms provided by the TRUMP. A total of 213 patients underwent BMT from an MFD as first-line treatment following the local protocols for conditioning regimens and GVHD prophylaxis. Patients who did not reach neutrophil counts $>0.5\times10^9/L$ for 3 consecutive days after transplantation were considered to have had primary graft failure. Patients with initial engraftment in whom absolute neutrophil counts subsequently declined to $<0.5\times10^9/L$ were considered to have had secondary graft failure. Acute and chronic GVHD were evaluated according to standard criteria.[14-16] More details on methods are provided in the *Online Supplementary Methods section.*

Statistical analyses

The date of analysis was July 30, 2012. Survival probabilities were estimated by the Kaplan-Meier method and compared between different groups of patients using the log-rank test. The influence of potential risk factors on overall survival and failure-free survival was assessed according to first-line treatment (BMT or IST), time period of treatment (1992-1999 or 2000-2009), age and other variables related to each treatment. Overall survival was defined as the time from diagnosis to death or last follow-up. Failure-free survival was defined as survival with treatment response. Death, primary or secondary graft failure, and secondary malignancy in the BMT group, and death, relapse, disease progression requiring stem cell transplantation (SCT) from an alternative donor or second IST, clonal evolution and evolution to paroxysmal nocturnal hemoglobinuria in the IST group were consid-

Table 1. Patients' characteristics.

	First-line treatment		
	BMT n=213	IST n=386	*P*
Age at diagnosis, year, median (range)	10 (0-16)	9 (0-16)	NS
Age at treatment, year, median (range)	11 (0-16)	9 (0-16)	NS
Gender			
Male / female	119/94	217/169	NS
Etiology, n. of patients (%)			
Idiopathic	204 (96)	312 (81)	<0.0001
Hepatitis	7 (3)	67 (17)	
Others	2 (1)	7 (2)	
Severity, n. of patients (%)			
Very severe aplastic anemia	–	227 (59)	–
Severe aplastic anemia	–	159 (41)	
Interval diagnosis-treatment, days, median (range)	84 (14-4605)	15 (1-180)	<0.0001
Time periods of treatment, n. of patients (%)			
1992-1999	121 (57)	155 (40)	0.0001
2000-2009	92 (43)	231 (60)	

BMT: bone marrow transplantation; IST: immunosuppressive therapy; NS: not significant.

ered treatment failures. For multivariate analyses, the Cox proportional hazard regression model was used. *P* values less than 0.05 were considered statistically significant. This study was approved by the institutional ethics committee of the Japanese Red Cross Nagoya First Hospital.

Results

Patients' characteristics

The characteristics of the 599 children are detailed in Table 1. The groups treated first-line with BMT (n=213) or IST (n=386) were similar with regards to age at diagnosis, age at treatment and male/female ratio. The majority of patients in both groups had a diagnosis of idiopathic disease, although the proportion of patients with non-idiopathic disease was higher in the IST group. Seven patients (3%) in the BMT group and 67 patients (17%) in the IST group suffered from hepatitis-associated disease. Nine patients had drug-induced or virus-associated disease. Information on the proportion of very severe disease was not available for 141 patients who underwent BMT because the severity of the SAA was not a required item for the registry. The clinical features of these patients were similar to those of the remaining patients. In the IST group, details regarding the severity of disease were provided for all patients: 227 (59%) had very severe disease and 159 (41%) suffered from severe disease. As expected, the time to treatment was significantly longer in the BMT group; the median interval between diagnosis and treatment was 15 days (range, 1-180 days) and 84 days (range, 14-4605 days) for those treated with IST and BMT, respectively. In accordance with decisions taken by the patients and the parents, ten patients underwent BMT more than 5 years after diagnosis. None of the patients who received IST before BMT from an MFD were included in the BMT group.

Immunosuppressive therapy

Response to IST at 6 months was not evaluable in 11 patients for the following reasons: early death (n=7) or BMT from an alternative donor within 6 months of IST (n=4). The causes of the early deaths were sepsis (n=3), interstitial pneumonia (n=2), hemolysis of unknown cause (n=1) and accidental ingestion (n=1). Of the patients who underwent BMT from an alternative donor within 6 months, two patients died of graft failure or cardiac toxicity related to the preconditioning regimen. Overall, 238 of the 375 evaluable patients (63%) improved with first-line IST and achieved a partial response (n=151) or complete response (n=87) at 6 months. All of these patients achieved transfusion independence.

For all 386 patients who received IST initially, the 10-year overall survival rate was 88% [95% confidence interval (CI): 86-90], as shown in Figure 1A, and the median follow-up time for living patients was 106 months (range, 22-224 months). In contrast to the high rate of overall survival, the result regarding survival with response was unsatisfactory, the 10-year failure-free survival rate being 56% (95%

Table 2. Treatment characteristics.

Bone marrow transplantation	213
Conditioning regimen, n.	
High-dose CY (200 mg/kg) -based	158
CY ± low-dose irradiation	86
CY + ATG ± low-dose irradiation	72
FLU + CY (100-120 mg/kg) -based	44
FLU + CY ± low-dose irradiation	29
FLU + CY + ATG ± low-dose irradiation	15
Myeloablative	11
CY + TBI (10-12 Gy)	7
BU + CY	4
GVHD prophylaxis, n.	
CyA + MTX	174
CyA alone	23
Tacrolimus + MTX	6
Others	10
Immunosuppressive therapy	**386**
IST trial, n.	
AA-92	84
AA-97	302
IST regimen, n.	
CyA + ATG	140
CyA + ATG + G-CSF	246

CY: cyclophosphamide; ATG: antithymocyte globulin; FLU: fludarabine; TBI: total body irradiation; BU: busulfan; CyA: cyclosporine; MTX: methotrexate; G-CSF: granulocyte colony-stimulating factor.

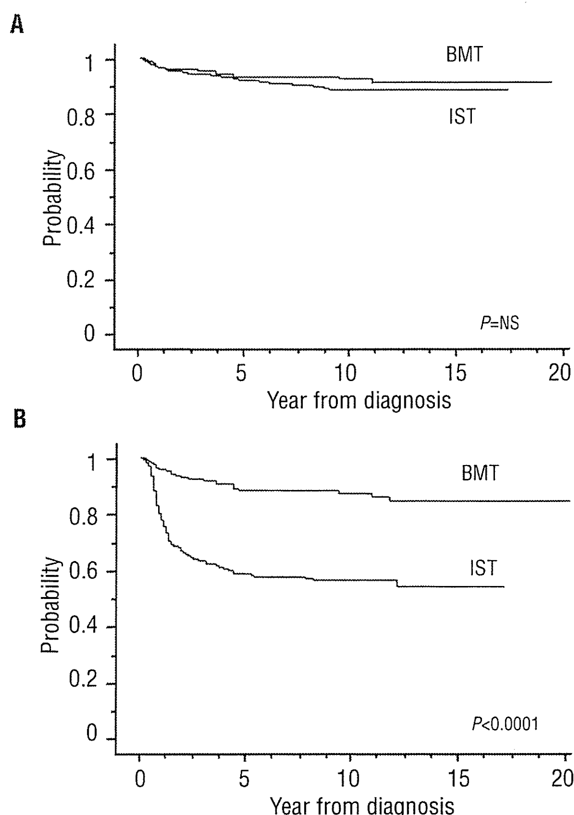

Figure 1. Survival of 599 children with severe aplastic anemia according to first-line treatments with immunosuppressive therapy (IST) (n=386) or bone marrow transplantation (BMT) (n=213). (A) Overall survival. The 10-year overall survival was 88% (95% CI: 86-90) in the IST group and 92% (95% CI: 90-94) in MFD BMT recipients (*P*=NS). (B) Failure-free survival. The 10-year failure-free survival was 56% (95% CI: 54-59) in the IST group and 87% (95% CI: 85-90) in the BMT group (*P*<0.0001).

CI: 54-59) (Figure 1B). The cause of treatment failure included death in 12 patients [due to intracranial hemorrhage (n=2), pneumonia (n=1), traffic accident (n=1) and sudden death (n=1) in addition to the seven early deaths], relapse in 23 patients, disease progression requiring second-line treatment in 109 patients, evolution to myelodysplastic syndrome in 15 patients, and appearance of paroxysmal nocturnal hemoglobinuria in two patients. After failed IST, a total of 113 patients underwent SCT from an alternative donor as second- or third-line treatment. The 10-year overall survival of these patients who received a transplant after failed IST was 79% (95% CI: 75-83) with a median of 435 days from diagnosis and SCT. We then analyzed the influence of potential risk factors for survival in the IST group. The prognostic significance of the clinical parameters is shown in Table 3. In the univariate analysis, age younger than 10 years at diagnosis was associated with a favorable overall survival rate [93% (95% CI: 91-95) *versus* 82% (95% CI: 78-86); *P*=0.012], and this was confirmed in a multivariate model. However, the rate of failure-free survival did not differ between patients in the two age groups. No other variables were significantly associated with survival after IST in either univariate or multivariate analyses.

Bone marrow transplantation

In the BMT group, 209 patients (98%) achieved primary engraftment at a median of 16 days after transplantation. As shown in Figure 1A and 1B, the 10-year overall survival and failure-free survival rates for all 213 patients who were treated initially with BMT from an MFD were 92% (95% CI: 90-94) and 87% (95% CI: 85-90), respectively. When the analysis was applied to the patients who underwent BMT within 180 days from diagnosis, similar results were observed; the 10-year overall survival and failure-free survival rates were 94% (95% CI: 92-96) and 89% (95% CI: 86-92), respectively. The median follow-up time for living patients was 101 months (range, 18-213 months). The cause of treatment failure included primary graft failure in two patients, secondary graft failure in ten patients, second malignancy in one patient, and death due to other complications in 12 patients. Although both patients without primary engraftment died, nine of the ten patients with secondary graft failure remain alive; eight were saved by a second transplant, and one recovered spontaneously. Twenty-five of 209 patients (12%) who had achieved primary engraftment developed grade II to IV acute GVHD, and extensive chronic GVHD was observed in 13 of 209 patients (6%) alive 100 days after BMT.

The prognostic significance of the clinical parameters, including variables related to transplantation, was then assessed. We found no association between age, gender, etiology, interval between diagnosis and BMT, or time period of treatment and treatment outcome (Table 3). Of particular interest with regards to conditioning regimens is the fact that the addition of antithymocyte globulin produced an improvement of overall survival [96% (95% CI: 92-99) *versus* 87% (95% CI: 84-91); *P*=0.021], whereas the rate of failure-free survival was comparable. A fludarabine-based regimen did not affect outcome after BMT from an

Table 3. Univariate analysis of 10-year overall survival (OS) and failure-free survival (FFS), according to first-line treatment.

	IST					BMT				
		OS		FFS			OS		FFS	
Variable	N. of patients	% (95% CI)	P	% (95% CI)	P	N. of patients	% (95% CI)	P	% (95% CI)	P
Age at diagnosis										
Younger than 10 years	219	93 (91-95)	0.012	57 (54-61)	0.754	89	95 (93-98)	0.163	92 (89-95)	0.200
10 years or older	167	82 (78-86)		55 (51-59)		124	90 (87-93)		84 (81-88)	
Gender										
Male	217	87 (84-90)	0.628	60 (56-64)	0.089	119	91 (87-94)	0.383	87 (83-90)	0.679
Female	169	90 (87-92)		52 (48-56)		94	94 (91-97)		88 (84-91)	
Etiology										
Idiopathic	312	88 (86-91)	0.661	54 (51-57)	0.185	204	92 (90-95)	0.568	87 (85-90)	0.934
Other	74	87 (83-92)		66 (60-71)		9	88 (76-99)		88 (76-99)	
Severity										
Very severe	227	90 (88-92)	0.600	57 (53-60)	0.965		–		–	
Severe	159	85 (82-89)		56 (52-60)						
Interval diagnosis-treatment										
Less than median days	187	91 (88-93)	0.537	60 (57-64)	0.170	105	95 (92-97)	0.322	91 (88-94)	0.362
Median days or more	199	86 (83-89)		53 (49-56)		108	90 (87-94)		85 (82-89)	
Time periods of treatment										
1992-1999	155	85 (82-88)	0.119	54 (50-58)	0.545	121	91 (89-94)	0.510	87 (84-90)	0.801
2000-2009	231	92 (90-94)		59 (56-63)		92	95 (93-98)		89 (85-93)	
Conditioning regimen										
With ATG		–		–		87	96 (92-99)	0.021	86 (83-90)	0.648
Without ATG						126	87 (84-91)		85 (82-89)	
GVHD prophylaxis										
CyA + MTX		–		–		174	93 (90-95)	0.924	88 (85-91)	0.809
Others						39	93 (88-98)		86 (80-93)	

ATG: antithymocyte globulin; CyA, cyclosporine; MTX, methotrexate.

MFD, although the number of patients treated with such regimens was too small to draw any conclusions. Multivariate analysis showed that none of the variables significantly influenced survival.

Survival and prognostic factors

The overall outcomes of the 599 children with SAA, stratified according to their first-line treatment, are shown in Figure 1A and 1B. Our data clearly showed a significant advantage for children receiving BMT from an MFD as first-line treatment; the failure-free survival was significantly superior in patients treated with BMT than in those in whom IST was used ($P<0.0001$), whereas the overall survival of patients in these two treatment groups did not differ. Figure 2A and 2B show survival curves in all patients treated in the two sequential time periods, 1992-1999 and 2000-2009: results were comparable over time [10-year overall survival: 88% (95% CI: 86-90) *versus* 93% (95% CI: 91-95); 10-year failure-free survival: 67% (95% CI: 65-70) *versus* 68% (95% CI: 66-71)], indicating no significant improvement in the last two decades. When age groups were considered, overall survival at 10 years in the younger group (<10 years old) was significantly better than that in the other age groups [93% (95% CI: 92-95) *versus* 85% (95% CI: 83-88); $P=0.007$], although no difference in failure-free survival was observed (Figure 3A and 3B). The favorable overall survival in the younger group may be mostly due to that observed in the first-line IST group. In multivariate analysis, age younger than 10 years at diagnosis was identified as a favorable factor for overall survival ($P=0.007$), and choice of first-line BMT from an MFD was confirmed as an independent favorable factor for failure-free survival ($P<0.0001$), as shown in Table 4.

Discussion

For children with SAA, BMT and IST have been accepted as standard treatments during the past three decades. The current guideline recommends BMT from an MFD as the treatment of choice for pediatric SAA[17-19] based on the results of comparative studies performed in the 1980s.[1,5,6,20,21] On the other hand, recent prospective studies with intensified IST for pediatric SAA have resulted in dramatic improvements in survival.[22,23] For example, a study from the EBMT showed a 100% overall survival rate at 6 years after first-line IST in 31 SAA patients younger than 20 years treated from 2002 to 2008.[22] These excellent overall survival results after IST have led to discussion about

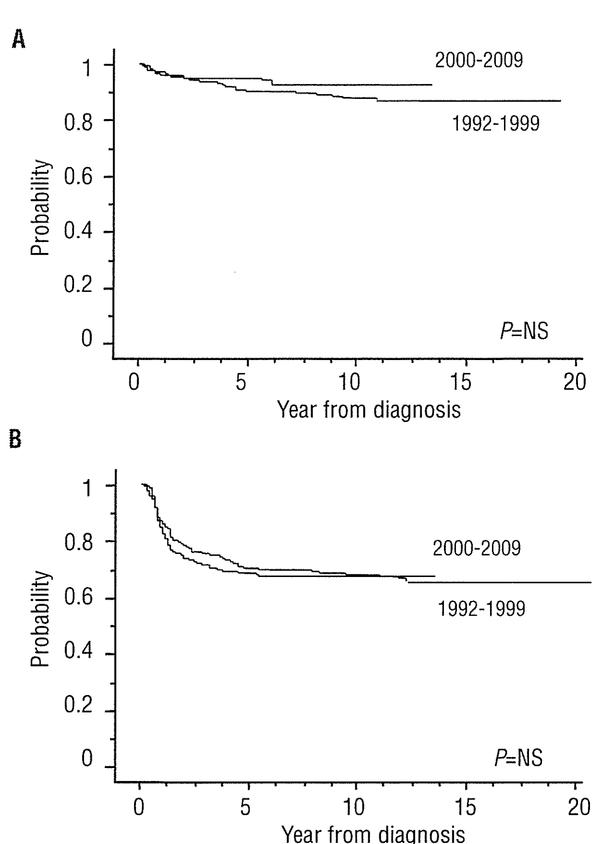

Figure 2. Survival of patients according to time periods of treatment: 1992-1999 (n=276) or 2000-2009 (n=323). (A) Overall survival. The 10-year overall survival was 88% (95% CI: 86-90) in 1992-1999 *vs.* 93% (95% CI: 91-95) in 2000-2009. (B) Failure-free survival. The 10-year failure-free survival was 67% (95% CI: 65-70) in 1992-1999 *vs.* 68% (95% CI: 66-71) in 2000-2009.

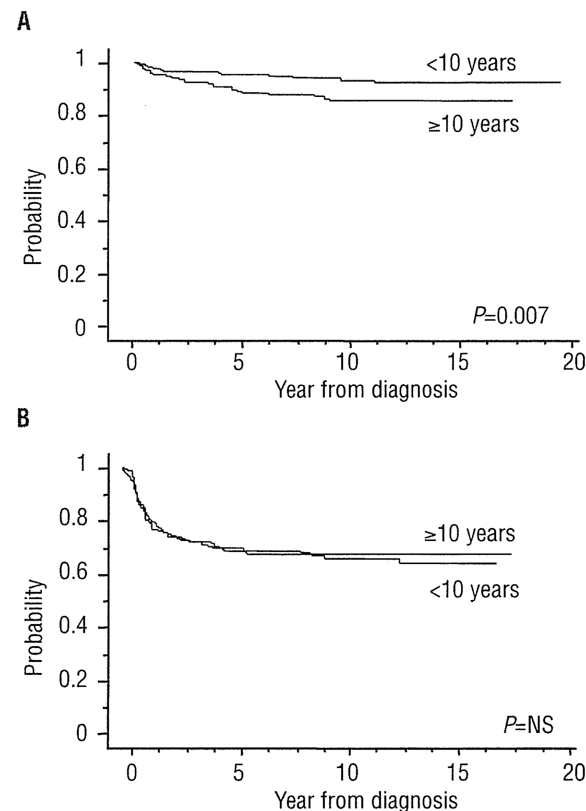

Figure 3. Survival of patients according to age at diagnosis: <10 years (n=308) or ≥10 years (n=291). (A) Overall survival. The 10-year overall survival in the younger group (<10 years) was significantly better than that in the other group [93% (95% CI: 92-95) *vs.* 85% (95% CI: 83-88); $P=0.007$]. (B) Failure-free survival. No difference in failure-free survival at 10 years was observed [67% (95% CI: 65-70) *vs.* 63% (95% CI: 59-67)].

the first-line treatment in children with SAA. To obtain solid evidence on which to base treatment decisions, ideally, a randomized controlled trial is required. However, because of the rarity of the disease, no randomized controlled trials comparing IST with BMT from an MFD as first-line treatment for SAA exist, and only retrospective studies using data from registries or relatively small cohorts of patients are available. Following the previous report of 304 children treated from 1970 to 1988,[6] the EBMT SAA Working Party (SAAWP) reported a consecutive study of 911 children younger than 16 years initially treated with IST (n=304) or BMT (n=607) between 1991 and 2002, which indicated that first-line IST gave an overall survival rate comparable to that of first-line BMT (81% *versus* 79%).[10] Unfortunately, the analyses had several limitations, because the drugs used for IST varied (e.g., antithymocyte globulin only, cyclosporine A only, or a combination of antithymocyte globulin and cyclosporine A) and the donor types used for BMT were not consistent (15% of the donors were mismatched family donors or matched/mismatched unrelated donors, although the majority of those were MFD). In addition, neither EBMT study provided results on failure-free survival,[6,10] which seems to be much more important than survival alone. Recent advances in supportive care and salvage therapies have effectively rescued non-responders to IST.[24] On the other hand, relapse, clonal evolution in the IST group and secondary graft failure and late malignancy in the BMT group are serious problems in long-term survivors. That is the reason why overall survival is no longer the only endpoint to determine optimal first-line treatment in children with SAA. In Japan, we have conducted consecutive prospective trials with a unified IST regimen consisting of antithymocyte globulin and cyclosporine A since 1992, enrolling 386 SAA patients younger than 17 years. During the same period, 213 SAA patients younger than 17 years underwent BMT from an MFD and were registered into the TRUMP, which provided a unique opportunity to investigate updated evidence for treatment decisions in pediatric SAA, although this study also had limitations due to its retrospective nature.

This study confirmed the excellent outcomes obtained in Japanese children with SAA treated with BMT from an MFD or IST. Consistent with the EBMT studies,[10,22] the survival of children with SAA initially treated with IST has improved markedly since the 1980s, when first-line IST gave greatly inferior survival (with overall survival rates of around 40-50%) when compared with first-line BMT[1,5,6,20,21]; in the current analyses, the probability of overall survival at 10 years in the patients treated first-line with IST reached 88%, which was comparable to that of the group treated first-line with BMT. Recent significant advances in second-line SCT, especially with a matched unrelated donor, may contribute to this marked improvement in survival after first-line IST.[25-27] In our series, a certain number of patients underwent SCT from an alternative donor after failed IST as a second- or third-line treatment. When patients were subdivided into three groups (first-line BMT from an MFD, IST only, and SCT after failed IST groups), the 10-year overall survival rates in these groups were 91%, 93% and 79%, respectively (P<0.0001), confirming that, in the case of failure of IST, SCT from an alternative donor is a very good salvage option, whereas MFD BMT and IST are excellent first-line treatments for children with SAA.

Regarding survival with response after first-line treatment, we found that the failure-free survival rate in

Table 4. Multivariate analysis of favorable factors for survival in all 599 patients with SAA.

Overall survival	Hazard ratio	95% CI	P
First-line treatment: BMT	1.619	0.881-2.977	NS
Treatment period: 2000-2009	1.536	0.556-2.753	NS
Age: <10 years	2.207	1.240-3.927	0.007
Failure-free survival	**Hazard ratio**	**95% CI**	**P**
First-line treatment: BMT	4.497	2.935-6.891	<0.0001
Treatment period: 2000-2009	1.090	0.812-1.464	NS
Age: <10 years	1.113	0.833-1.488	NS

BMT: bone marrow transplantation; NS: not significant.

patients treated with IST plateaued over the past two decades after having slightly improved since the 1980s (from 40% in the 1980s to 56% currently).[1] Thus, unlike the overall survival results, failure-free survival in the IST group was significantly inferior to that in the MFD BMT group. Consistent with our observations, the EBMT group also demonstrated no significant improvement in outcomes in response to IST since the 1990s.[10] This may suggest that the IST regimen has not improved over time. Over the past decade, with the hypothesis that more intense IST might produce better outcomes, the addition of newer immunosuppressive agents, such as mycophenolate mofetil and sirolimus to antithymocyte globulin and cyclosporine A, has been tested, but has failed to improve responses.[28-31] The combination of antithymocyte globulin and cyclosporine A is, therefore, still regarded as the standard IST regimen. Another possibility is that we have reached a ceiling in the percentage of patients with the capacity to respond to IST.[18] In patients refractory to IST, the pathophysiology of the disease may be different from that in patients responsive to IST, which is thought to involve autoimmune processes, although there are no good markers to routinely or reliably distinguish non-responders from responders.[13,32-34] Further studies are needed to identify patients refractory to IST, because these patients might benefit from prompt alternative donor SCT.

Importantly, all patients in the current analyses were treated with horse antithymocyte globulin (Lymphoglobulin), which has recently been withdrawn from Asian and European markets and replaced by rabbit antithymocyte globulin. To date, there are only limited studies using rabbit antithymocyte globulin as first-line IST for pediatric aplastic anemia, and thus, the effectiveness of this form of antithymocyte globulin for pediatric patients remains controversial.[35-38] The change of product might result in different outcomes in response to IST for children with SAA.

Survival after BMT from an MFD in children with SAA has exceeded 90% for the past two decades, and this has remained unchanged when compared with our previous observation in the 1980s. In this study, the major causes of treatment failure were primary and secondary graft failure, but notably, most patients with secondary graft failure were rescued by second transplantation or careful observation. In addition to short-term complications, long-term sequelae, such as chronic GVHD and late malignancy, should be taken into consideration to make optimal treatment decisions, especially in children. Our results showed that acute and chronic GVHD were relatively uncommon

in the setting of BMT from an MFD for pediatric SAA, which is consistent with recently reported results from the EBMT SAAWP, with 11% of grade II to IV acute GVHD and 4% of extensive chronic GVHD after BMT from an MFD for SAA in all age groups.[39] Regarding late malignancy, Kikuchi *et al.* recently published data from 329 Japanese children with SAA from the nationwide registry, confirming a low incidence of late malignancy after BMT from an MFD; the cumulative incidence of late malignancy was 0.8% at 10 years and 2.5% at 20 years, respectively, which was much lower than the cumulative incidences in reports from western countries.[40] In the present series, only one patient developed a late malignancy (myelodysplastic syndrome), and was saved by second BMT. These observations suggest that this approach has been already established as first-line treatment for children with SAA.

In conclusion, our updated data clearly demonstrate that children receiving BMT from an MFD as first-line treatment have a significant advantage over children managed with first-line IST, given the dramatically better failure-free survival and the lower incidence of associated long-term sequelae in the BMT group, which supports the current algorithm for treatment decisions that recommends BMT for pediatric SAA when an MFD is available. On the other hand, IST using the combination of antithymocyte globulin and cyclosporine A is the treatment of choice for children with SAA without an MFD considering the comparable overall survival with BMT from an MFD, which could possibly be ascribed to recent improvements in outcomes after SCT from an alternative donor. In other words, patients have an excellent chance of survival, even after failed first-line IST, when they undergo second-line SCT from an alternative donor.

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References

1. Kojima S, Horibe K, Inaba J, Yoshimi A, Takahashi Y, Kudo K, et al. Long-term outcome of acquired aplastic anaemia in children: comparison between immunosuppressive therapy and bone marrow transplantation. Br J Haematol. 2000;111(1):321-8.
2. Mathe G, Amiel JL, Schwarzenberg L, Choay J, Trolard P, Schneider M, et al. Bone marrow graft in man after conditioning by antilymphocytic serum. Transplant Proc. 1971;3(1):325-32.
3. Young NS. Acquired aplastic anemia. JAMA. 1999;282(3):271-8.
4. Bacigalupo A, Brand R, Oneto R, Bruno B, Socie G, Passweg J, et al. Treatment of acquired severe aplastic anemia: bone marrow transplantation compared with immunosuppressive therapy--The European Group for Blood and Marrow Transplantation experience. Semin Hematol. 2000;37(1):69-80.
5. Doney K, Leisenring W, Storb R, Appelbaum FR. Primary treatment of acquired aplastic anemia: outcomes with bone marrow transplantation and immunosuppressive therapy. Seattle Bone Marrow Transplant Team. Ann Intern Med. 1997;126(2):107-15.
6. Locasciulli A, van't Veer L, Bacigalupo A, Hows J, Van Lint MT, Gluckman E, et al. Treatment with marrow transplantation or immunosuppression of childhood acquired severe aplastic anemia: a report from the EBMT SAA Working Party. Bone Marrow Transplant. 1990;6(3):211-7.
7. Bacigalupo A, Broccia G, Corda G, Arcese W, Carotenuto M, Gallamini A, et al. Antilymphocyte globulin, cyclosporin, and granulocyte colony-stimulating factor in patients with acquired severe aplastic anemia (SAA): a pilot study of the EBMT SAA Working Party. Blood. 1995;85(5):1348-53.
8. Fuhrer M, Rampf U, Baumann I, Faldum A, Niemeyer C, Janka-Schaub G, et al. Immunosuppressive therapy for aplastic anemia in children: a more severe disease predicts better survival. Blood. 2005;106(6):2102-4.
9. Kojima S, Hibi S, Kosaka Y, Yamamoto M, Tsuchida M, Mugishima H, et al. Immunosuppressive therapy using antithymocyte globulin, cyclosporine, and danazol with or without human granulocyte colony-stimulating factor in children with acquired aplastic anemia. Blood. 2000;96(6):2049-54.
10. Locasciulli A, Oneto R, Bacigalupo A, Socie G, Korthof E, Bekassy A, et al. Outcome of patients with acquired aplastic anemia given first line bone marrow transplantation or immunosuppressive treatment in the last decade: a report from the European Group for Blood and Marrow Transplantation (EBMT). Haematologica. 2007;92(1):11-8.
11. Peinemann F, Bartel C, Grouven U. First-line allogeneic hematopoietic stem cell transplantation of HLA-matched sibling donors compared with first-line ciclosporin and/or antithymocyte or antilymphocyte globulin for acquired severe aplastic anemia. Cochrane Database Syst Rev. 2013;7:CD006407.
12. Kosaka Y, Yagasaki H, Sano K, Kobayashi R, Ayukawa H, Kaneko T, et al. Prospective multicenter trial comparing repeated immunosuppressive therapy with stem-cell transplantation from an alternative donor as second-line treatment for children with severe and very severe aplastic anemia. Blood. 2008;111(3):1054-9.
13. Yoshida N, Yagasaki H, Hama A, Takahashi Y, Kosaka Y, Kobayashi R, et al. Predicting response to immunosuppressive therapy in childhood aplastic anemia. Haematologica. 2011;96(5):771-4.
14. Deeg HJ. Prophylaxis and treatment of acute graft-versus-host disease: current state, implications of new immunopharmacologic compounds and future strategies to prevent and treat acute GVHD in high-risk patients. Bone Marrow Transplant. 1994;14 (Suppl 4):S56-60.
15. Glucksberg H, Storb R, Fefer A, Buckner CD, Neiman PE, Clift RA, et al. Clinical manifestations of graft-versus-host disease in human recipients of marrow from HL-A-matched sibling donors. Transplantation. 1974;18(4):295-304.
16. Shulman HM, Sullivan KM, Weiden PL, McDonald GB, Striker GE, Sale GE, et al. Chronic graft-versus-host syndrome in man. A long-term clinicopathologic study of 20 Seattle patients. Am J Med. 1980;69(2):204-17.
17. Marsh JC, Ball SE, Cavenagh J, Darbyshire P, Dokal I, Gordon-Smith EC, et al. Guidelines for the diagnosis and management of aplastic anaemia. Br J Haematol. 2009;147(1):43-70.
18. Passweg JR, Marsh JC. Aplastic anemia: first-line treatment by immunosuppression and sibling marrow transplantation. Hematology Am Soc Hematol Educ Program. 2010:36-42.
19. Scheinberg P. Aplastic anemia: therapeutic updates in immunosuppression and transplantation. Hematology Am Soc Hematol Educ Program. 2012:292-300.
20. Bayever E, Champlin R, Ho W, Lenarsky C, Storch S, Ladisch S, et al. Comparison between bone marrow transplantation and antithymocyte globulin in treatment of young patients with severe aplastic anemia. J Pediatr. 1984;105(6):920-5.
21. Bacigalupo A, Hows J, Gluckman E, Nissen C, Marsh J, Van Lint MT, et al. Bone marrow transplantation (BMT) versus immunosuppression for the treatment of severe aplastic anaemia (SAA): a report of the EBMT SAA Working Party. Br J Haematol. 1988;70(2):177-82.
22. Tichelli A, Schrezenmeier H, Socie G, Marsh J, Bacigalupo A, Duhrsen U, et al. A randomized controlled study in patients with newly diagnosed severe aplastic anemia receiving antithymocyte globulin (ATG), cyclosporine, with or without G-CSF: a study of the SAA Working Party of the European Group for Blood and Marrow Transplantation. Blood. 2011;117(17):4434-41.
23. Nair V, Sondhi V, Sharma A, Das S, Sharma

S. Survival after immunosuppressive therapy in children with aplastic anemia. Indian Pediatr. 2012;49(5):371-6.
24. Valdez JM, Scheinberg P, Nunez O, Wu CO, Young NS, Walsh TJ. Decreased infection-related mortality and improved survival in severe aplastic anemia in the past two decades. Clin Infect Dis. 2011;52(6):726-35.
25. Bacigalupo A, Socie G, Lanino E, Prete A, Locatelli F, Locasciulli A, et al. Fludarabine, cyclophosphamide, antithymocyte globulin, with or without low dose total body irradiation, for alternative donor transplants, in acquired severe aplastic anemia: a retrospective study from the EBMT-SAA Working Party. Haematologica. 2010;95(6):976-82.
26. Kennedy-Nasser AA, Leung KS, Mahajan A, Weiss HL, Arce JA, Gottschalk S, et al. Comparable outcomes of matched-related and alternative donor stem cell transplantation for pediatric severe aplastic anemia. Biol Blood Marrow Transplant. 2006;12(12):1277-84.
27. Yagasaki H, Takahashi Y, Hama A, Kudo K, Nishio N, Muramatsu H, et al. Comparison of matched-sibling donor BMT and unrelated donor BMT in children and adolescent with acquired severe aplastic anemia. Bone Marrow Transplant. 2010;45(10):1508-13.
28. Alsultan A, Goldenberg NA, Kaiser N, Graham DK, Hays T. Tacrolimus as an alternative to cyclosporine in the maintenance phase of immunosuppressive therapy for severe aplastic anemia in children. Pediatr Blood Cancer. 2009;52(5):626-30.
29. Scheinberg P, Nunez O, Weinstein B, Wu CO, Young NS. Activity of alemtuzumab monotherapy in treatment-naive, relapsed, and refractory severe acquired aplastic anemia. Blood. 2012;119(2):345-54.
30. Scheinberg P, Nunez O, Wu C, Young NS. Treatment of severe aplastic anaemia with combined immunosuppression: anti-thymocyte globulin, ciclosporin and mycophenolate mofetil. Br J Haematol. 2006;133(6):606-11.
31. Scheinberg P, Wu CO, Nunez O, Boss C, Sloand EM, Young NS. Treatment of severe aplastic anemia with a combination of horse antithymocyte globulin and cyclosporine, with or without sirolimus: a prospective randomized study. Haematologica. 2009;94(3):348-54.
32. Chang MH, Kim KH, Kim HS, Jun HJ, Kim DH, Jang JH, et al. Predictors of response to immunosuppressive therapy with antithymocyte globulin and cyclosporine and prognostic factors for survival in patients with severe aplastic anemia. Eur J Haematol. 2009;84(2):154-9.
33. Scheinberg P, Wu CO, Nunez O, Young NS. Long-term outcome of pediatric patients with severe aplastic anemia treated with antithymocyte globulin and cyclosporine. J Pediatr. 2008;153(6):814-9.
34. Yoshida N, Yagasaki H, Takahashi Y, Yamamoto T, Liang J, Wang Y, et al. Clinical impact of HLA-DR15, a minor population of paroxysmal nocturnal haemoglobinuria-type cells, and an aplastic anaemia-associated autoantibody in children with acquired aplastic anaemia. Br J Haematol. 2008;142(3):427-35.
35. Scheinberg P, Nunez O, Weinstein B, Biancotto A, Wu CO, Young NS. Horse versus rabbit antithymocyte globulin in acquired aplastic anemia. N Engl J Med. 365(5):430-8.
36. Marsh JC, Bacigalupo A, Schrezenmeier H, Tichelli A, Risitano AM, Passweg JR, et al. Prospective study of rabbit antithymocyte globulin and cyclosporine for aplastic anemia from the EBMT Severe Aplastic Anaemia Working Party. Blood. 2012;119(23):5391-6.
37. Afable MG 2nd, Shaik M, Sugimoto Y, Elson P, Clemente M, Makishima H, et al. Efficacy of rabbit anti-thymocyte globulin in severe aplastic anemia. Haematologica. 2011;96(9):1269-75.
38. Jeong D-C, Chung NG, Lee JW, Jang P-S, Cho B, Kim H-K. Long-term outcome of immunosuppressive therapy with rabbit antithymocyte globulin (rATG) for childhood severe aplastic anemia for 15 years. ASH Annual Meeting Abstracts. 2011;118(21):1346.
39. Bacigalupo A, Socie G, Schrezenmeier H, Tichelli A, Locasciulli A, Fuehrer M, et al. Bone marrow versus peripheral blood as the stem cell source for sibling transplants in acquired aplastic anemia: survival advantage for bone marrow in all age groups. Haematologica. 2012;97(8):1142-8.
40. Kikuchi A, Yabe H, Kato K, Koh K, Inagaki J, Sasahara Y, et al. Long-term outcome of childhood aplastic anemia patients who underwent allogeneic hematopoietic SCT from an HLA-matched sibling donor in Japan. Bone Marrow Transplant. 2012;48(5):657-60.