Summary: We identified a child with KLF1-E325K congenital dyserythropoietic anemia type IV who experienced a severe clinical course, fetal anemia, hydrops fetalis, and postnatal transfusion dependence only partially responsive to splenectomy. The child also had complete sex reversal, the cause which remains undetermined. To gain insights into our patient’s severe hematologic phenotype, detailed analyses were performed. Erythrocytes from the patient and parents demonstrated functional abnormalities of the erythrocyte membrane, attributed to variants in the α-spectrin gene. Hypomorphic alleles in SEC23B and YARS2 were also identified. We hypothesize that coinheritance of variants in relevant erythrocyte genes contribute to the clinical course in our patient and other E325K-linked congenital dyserythropoietic anemia IV patients with severe clinical phenotypes.

Key Words: congenital dyserythropoietic anemia, KLF1, hydrops fetalis, sex reversal, CD44, CD71, DRAQ5, alpha spectrin, HPFH

Received for publication June 16, 2017; accepted September 18, 2017.

From the Departments of Pediatrics and Biochemistry, Wayne State University School of Medicine, Children’s Hospital of Michigan/Karmanos Cancer Institute, Detroit, MI, and Departments of Pediatrics, Pathology and Genetics, Yale University School of Medicine, New Haven, CT.

Y.R.: designed diagnostic strategies and wrote the manuscript; G.G., M.G., and S.B.: executed and interpreted laboratory studies; P.G.G.: performed genetic analyses and assisted in writing the manuscript; R.M.J.: supervised laboratory testing, assisted in genetic analyses, and assisted in writing the manuscript.

Presented in part in abstract form at the American Society of Hematology annual meeting [ASH Annual Meeting Abstracts. 2011;118(21):2101].

Supported by the Georgia Ginopolis Chair Award (Y.R.); the Melissa Ann Krinsky Research Fund, the David Carr Memorial Fund and NIDDK RO1DK104046.

The authors declare no conflict of interest.

Reprints: Yaddanapudi Ravindranath, MBBS,* Robert M. Johnson, PhD,* Gerard Goyette, BS,* Steven Buck, MS,* Manisha Gadgeel, MD,* and Patrick G. Gallagher, MD‡

‡Address correspondence to this author. E-mail: ravig@med.wayne.edu.

Supplemental Digital Content is available for this article. Direct URL citations appear in the printed text and are provided in the HTML and PDF versions of this article on the journal’s website, www.jpho-online.com.

Copyright © 2017 The Author(s). Published by Wolters Kluwer Health, Inc. This is an open-access article distributed under the terms of the Creative Commons Attribution-Non Commercial-No Derivatives License 4.0 (CCBY-NC-ND), where it is permissible to download and share the work provided it is properly cited. The work cannot be changed in any way or used commercially without permission from the journal.
TABLE 1. Hematologic Findings Before and After Splenectomy

|                        | Before Splenectomy | After Splenectomy | Current Values |
|------------------------|--------------------|-------------------|---------------|
|                        | Presplenectomy*    | 1.5              | 11            |
| Age at sample          |                    |                  | 10 y          |
| Hb (g/dL)              | 7.4-9.3            | 11.3             | 8.1           |
| Hct (%)                | 22-28              | 37.5             | 27            |
| RBC (mL/L)             | 2.6-3.07           | 3.77             | 2.45          |
| MCH (pg)               | 28.1-29.6          | 30.9             | 30            |
| MCHC (g/dL)            | 30-33              | 30.1             | 30            |
| RDW                    | 15.8-23.1          | 21.2             | 25.9          |
| Reticulocytes (%)      | 10-14              | 14.9             | 10.7          |
| nRBC (10³/μL)          | 1.3-9.8            | 40.6             | 134.11        |
| WBC (10³/μL)           | 9.9-14.3           | 13.6             | 18.14         |
| Platelets (10³/μL)     | 220                | 872              | 1040          |
| Hb A/A2/F (%)          | 63.6/1.8           | 63.6/1.8         | 63.6/1.8      |

*The presplenectomy and 1.5 months after splenectomy values reflect admixture of transfused red blood cells within the previous 2 to 3 months.

Hb indicates hemoglobin; Hct, hematocrit; MCH, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; nRBC, nucleated red blood cell; RDW, red cell distribution width; WBC, white blood cells.

expression on red cells was used as a screening tool and additional confirmatory tests were performed. CD44 expression (CD71+ population) was absent on mature erythrocytes from the proband (Fig. 2B) compared with its expression on mature erythrocytes from the parents and a control. CD44 was also reduced or absent in CD71+ cells from the proband (reticulocytes and nucleated red blood cells [CD45−, DRAQ5+, CD71+ cells) in contrast to its presence on reticulocytes and nRBC from a thalassemia major patient with elevated numbers of circulating nRBC and on cells in a normal control bone marrow (Fig. 2C). CD44 expression was present in patient’s lymphocytes but was decreased compared with control values (not shown). Sanger sequencing of the KLF1 gene, performed as described,7 revealed the proband was heterozygous for the E325K KLF1 variant associated with CDA type IV (NM_006563:c.G973A:p.E325K) (Fig. 1E). Neither parent carried the E325K variant (Fig. 1F; data on father not shown), suggesting this occurred de novo or 1 parent is mosaic.

Identification of the KLF1 E325K mutation prompted detailed hemoglobin analyses based on previous cases. Isoelectric focusing of erythrocyte hemoglobin yielded a pattern identical to that published by Arnaud and colleagues, indicating the presence of embryonic hemoglobins (Fig. 1F). Presence of ζ-globin was confirmed by proteomic analysis of low-molecular-weight bands on the sodium dodecyl sulfate polyacrylamide gel electrophoresis of red cell membranes (not shown). By flow cytometry the distribution of Hbf was heterocellular (Fig. 2A).

Additional genetic variants contributing to the patient’s clinical course were sought. With parental consent; patient and parental samples were subjected to whole exome sequencing (WES) to identify other coinherit mutated in red cell genes. Data were processed using the SeattleSeq annotation platform (http://www.tumor.sanger.ac.uk/SeattleSeqAnnotation137) to identify the pathologic variants. Erythrocyte membrane protein genes were initially analyzed due to the abnormal erythrocyte EMA binding and ektacytometry results in the proband and her parents. Numerous nonsynonymous coding sequence variants in the ζ-spectrin gene (SPTA1) were found in the proband (Supplemental Table 1, Supplemental Digital Content 1, http://links.lww.com/JPHO/A231).
The proband inherited several variant SPTA1 alleles from the father and mother (Supplemental Table 1, Supplemental Digital Content 1, http://links.lww.com/JPHO/A231) including the αLELY allele (NM_003126:c.5572C>G:p.L1858V), which may result in reduced α-spectrin membrane content.8,9 Another variant allele was the αBughill allele (NM_003126:c.2909C>A:p.A970D), inherited from the mother. This allele is associated with recessive hereditary spherocytosis, is associated with a mild hereditary spherocytosis "carrier state,"10 and is frequently inherited with the αLEPRA mutation. The αLEPRA allele was not detected in either parent or the proband. No functional studies of the αBughill allele have been performed to inform its contribution to spectrin function, but αBughill homozygous patients without the αLEPRA mutation exhibit mild spectrin deficiency and the hereditary spherocytosis carrier state.10 No predicted pathogenic mutations were found in the coding regions or intron/exon junctions of the β-spectrin, ankyrin-1, band 3, protein 4.2 or other erythrocyte membrane genes. These studies do not exclude deep intronic or regulatory element mutations or small genic deletions.

Other red cell disease genes were examined for potential variants contributing to the proband’s phenotype. Hypomorphic variants with significantly elevated CADD scores were noted in SEC23B (NM_001172745:c.1467C>G:p.H489Q), the CDA type II gene, and in YARS2 (NM_001040436:c.572G>T:p.G191V0), a pathogenic variant in a gene linked to a type of sideroblastic anemia.

**DISCUSSION**

Phenotypic features and the clinical course of 5 cases of KLF1 E325K-associated CDA type IV have been reported (Table 2). Review reveals 2 distinctive clinical courses, one characterized by a mild course, with childhood anemia or transfusion dependence in infancy that resolves spontaneously, and the other characterized by a more severe course. Severely affected patients present with fetal anemia and hydrops fetalis followed by postnatal transfusion dependence; splenectomy is palliative but not curative. To gain insight into our patient’s severe hematologic phenotype and the sex reversal phenotype, detailed laboratory and genetic analyses were performed.

Abnormal EMA binding and ektacytometry of erythrocytes from the patient and parents identified functional abnormalities of the erythrocyte membrane. Analyses of WES data, while finding numerous membrane protein gene variants, did not provide a specific genetic diagnosis for the mild HS/HS carrier phenotype in either parent. The mother’s mild clinical picture could be attributed to the αBughill allele as previously described10,15 or other as yet unidentified variants. It is possible that the numerous membrane protein gene variants, for example in SPTA1, in cis or trans may lead to a cumulative effect on the protein of interest, with variants interacting to produce a clinical phenotype as described in other proteins.16

Other variant alleles in 2 other red blood cell disease-associated genes, SEC23B and YARS2, that may have contributed to the severity of our patient’s clinical course were also identified. Hypomorphic mutations in SEC23B have been associated with mild phenotypes in CDA type...
II. Similarly, a wide spectrum of phenotypic and genotypic variability have been described in the YARS2 mitochondrial myopathy, lactic acidosis, and sideroblastic anemia syndrome, including isolated anemia presenting in adulthood.18

Taken together, we hypothesize that coinheritance of variants in relevant erythrocyte genes contribute to the clinical course in our patient and other E325K-linked CDA IV patients with severe clinical phenotypes.

In 2 of the cases with severe phenotypic features, the patients were phenotypic males with urogenital anomalies.2 One patient had microopen with hypospadias and had growth retardation unresponsive to human growth hormone while our case had sex reversal. In both cases, the etiology of growth retardation unresponsive to human growth hormone and thyroid supplementation.2

...
have normalized soon thereafter. In our case, ferritin values normalized 9 months after splenectomy without chelation and remain normal to date. In cases with growth and endocrine changes, imaging studies with magnetic resonance imaging (T2* or superconducting quantum interference device) may be necessary to exclude tissue iron accumulation. Although our patient can maintain a hemoglobin of 9 to 10 g/dL and shows partial skipping of exon 46.

**Summary**

A comparison of the first 5 cases with KLF1 E325K mutation suggests that the spectrum of clinical effects of this mutation can vary from moderate to severe, transfusion-dependent anemia. The presence of modifier alleles in non-KLF1 genes associated with congenital anemia may lead to the wide variability in clinical phenotypes observed. The concurrent presence of urogenital anomalies in 2 of the karyotypic males remains unexplained.

**ACKNOWLEDGMENTS**

Dr Senthil Sundaram provided invaluable help to the primary author in getting started with exome sequencing studies and the use of various annotation Websites. A special thanks to Dr Laurence Boxer (University of Michigan) for his encouragement and sharing in the care of this child. The authors acknowledge the contributions of James Hoyer (Mayo Laboratories for Isoelectric focusing studies of hemoglobin), James Fais (Rochester, NY for in vitro erythroid colony studies), Douglas Whitten (Michigan State University, Lansing Michigan for proteomic studies), and Adele Kruger for assistance with WES analyses. The authors also acknowledge the contribution of the child and her parents to these investigative studies.

**REFERENCES**

1. Wickramasinghe SN, Illum N, Wimberley PD. Congenital dyserythropoietic anemia with novel intra-erythroblastic and intra-erythrocytic inclusions. Br J Haematol. 1991;79:322–330.
2. Arnaud L, Saison C, Helias V, et al. A dominant mutation in the gene encoding the erythrotranscription factor KLF1 causes a congenital dyserythropoietic anemia. Am J Hum Genet. 2010;87:721–727.
3. Jaffray JA, Mitchell WB, Gnanapragasam MN, Jaffray JA, et al. Case Report of Erythroid Transcription Factor EKLF Mutation Causing a Rare Form of Congenital Dyserythropoietic Anemia in a Patient of Taiwanese Origin. Am J Hematol. 1997;54:233–241.
4. Taras W, Cai SP, Eng B, et al. Expression of embryonic zeta-globin and epsilon-globin chains in a 10-year-old girl with congenital anemia. Blood. 1993;81:1636–1640.
5. Parsons SF, Jones J, Ansee DJ, et al. A novel form of congenital dyserythropoietic anemia associated with deficiency of erythroid CD44 and a unique blood group phenotype [In(a-b+), Co(a-b-)]. Blood. 1994;83:860–868.
6. Agre P, Smith BL, Baumgarten R, et al. Human red cell Aquaporin CHIP. II. Expression during normal fetal development and in a novel form of congenital dyserythropoietic anemia. J Clin Invest. 1994;94:1050–1059.
7. Russo R, Langella C, Esposito MR, et al. Hypomorphic mutations of SEC23B gene account for mild phenotypes of congenital dyserythropoietic anemia type II. Blood Cells Mol Dis. 2013;51:17–21.
8. Riley LG, Menezes MJ, Rudinger-Thirion J, et al. Phenotypic variability and identification of novel YARS2 mutations in YARS2 mitochondrial myopathy, lactic acidosis and sideroblastic anemia. Orphanet J Rare Dis. 2013;8:193.
9. Magor GW, Tallack MR, Gillinder KR, et al. KLF1-null neonates display hydrops fetalis and a deranged erythroid transcriptome. Blood. 2015;125:2405–2417.
10. Gillinder KR, Isley MD, Nebor D, et al. Promiscuous DNA-binding of a mutant zinc finger protein corrects the transcriptome and diminishes cell viability. Nucleic Acids Res. 2017;45:1130–1143.
11. Planutis A, Xue L, Trainer CD, et al. Neomorphic effects of the neonatal anemia (Nan-Eklf) mutation contribute to deficits throughout development. Development. 2017;144:430–440.
12. Perkins A, Xu X, Higgs DR, et al. Kruppeling erythropoiesis: an unexpected broad spectrum of human red blood cell disorders due to KLF1 variants. Blood. 2016;127:1856–1862.
13. Chen K, Liu J, Heck S, et al. Resolving the distinct stages in erythroid differentiation based on dynamic changes in membrane protein expression during erythropoiesis. Proc Natl Acad Sci USA. 2009;106:17413–17418.
14. Liu J, Zhang J, Ginzburg Y, et al. Quantitative analysis of murine terminal erythroid differentiation in vivo: novel method to study normal and disordered erythropoiesis. Blood. 2013;121:e43–e49.
15. Zhou HS, Carter BZ, Andreeff M. Bone marrow niche-mediated survival of leukemia stem cells in acute myeloid leukemia: Yin and Yang. Cancer Biol Med. 2016;13:248–259.
16. Zoller M, CD44, hyaluronan, the hematopoietic stem cell, and leukemia-initiating cells. Front Immunol. 2015;6:235.
17. Biason-Lauber A. Control of sex development. Best Pract Res Clin Endocrinol Metab. 2010;24:163–186.