

Original article

Clinical and molecular spectrum of inherited hemolytic anemia associated with recurrent *SPTB* variants and *KLF1* mutations in northeastern Thailand

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Abstract:

Background: Inherited hemolytic anemia (IHA) caused by red cell membrane and erythroid transcription factor defects remains underrecognized in Northeastern Thailand. We aimed to describe the clinical spectrum, molecular findings and transfusion outcomes of pediatric IHA at a regional tertiary care center. **Methods:** We conducted a prospective cohort study at Maharat Nakhon Ratchasima Hospital from 2023 to 2025. Twenty-nine children with clinically suspected inherited hemolytic anemia were enrolled. Clinical features, hematologic parameters, transfusion history and family studies were reviewed. Common *SPTB* variants, including Providence c.6055T>C, Buffalo c.6074T>G and Chiang Mai c.6224A>G, were tested using multiplex ARMS-PCR with high-resolution melting analysis. *KLF1* testing was performed in selected cases. **Results:** *SPTB* variants were identified in 22 of 29 patients. Hereditary pyropoikilocytosis (HPP) was the most common phenotype and was associated with homozygous or compound heterozygous *SPTB* variants, particularly Buffalo-containing genotypes. Most patients with HPP had severe neonatal-onset anemia, transfusion dependency and frequent perinatal complications, including hydrops fetalis. Patients with hereditary elliptocytosis and heterozygous *SPTB* variants had mild, non-transfusion-dependent disease. Two patients had compound *KLF1* variants and transfusion-dependent anemia, one of whom had confirmed neonatal-onset disease. Most affected families were from Nakhon Ratchasima Province. **Conclusion:** Recurrent *SPTB* variants, particularly the Buffalo variant, were frequently identified among children with suspected IHA referred to our center and were associated with severe HPP phenotypes. Early molecular diagnosis may facilitate accurate classification, counseling and timely management.

Keywords : ● Inherited hemolytic anemia ● Hereditary pyropoikilocytosis ● Hereditary elliptocytosis ● *SPTB* ● *KLF1*

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นิพนธ์ต้นฉบับ

ลักษณะทางคลินิกและพันธุกรรมของภาวะซีดจากเม็ดเลือดแดงแตกทางกรรมพันธุ์ที่สัมพันธ์กับการกลายพันธุ์ของ *SPTB* และ *KLF1* ในภาคตะวันออกเฉียงเหนือของประเทศไทย

ปฐณวิช คงพิทักษ์สกุล

หน่วยโลหิตวิทยาและมะเร็ง กลุ่มงานกุมารเวชกรรม โรงพยาบาลมหาราชนครราชสีมา

บทคัดย่อ

บทนำ ภาวะซีดจากเม็ดเลือดแดงแตกทางกรรมพันธุ์ ที่เกิดจากความผิดปกติของเยื่อหุ้มเม็ดเลือดแดง และความผิดปกติของ transcription factor ที่เกี่ยวข้องกับการสร้างเม็ดเลือดแดง ยังอาจได้รับการวินิจฉัยไม่เพียงพอในภาคตะวันออกเฉียงเหนือของประเทศไทย การศึกษานี้มีวัตถุประสงค์เพื่ออธิบายลักษณะทางคลินิก ผลการตรวจทางพันธุกรรมระดับโมเลกุล และผลลัพธ์ด้านการให้เลือดในผู้ป่วยเด็กที่มีภาวะซีดจากเม็ดเลือดแดงแตกทางกรรมพันธุ์ ณ โรงพยาบาลตติยภูมิระดับภูมิภาค **วิธีการศึกษา** การศึกษานี้เป็นการศึกษาแบบ prospective cohort ณ โรงพยาบาลมหาราชนครราชสีมา ระหว่างปี พ.ศ. 2566-2568 มีผู้ป่วยเด็กที่สงสัยภาวะซีดจากเม็ดเลือดแดงแตกทางกรรมพันธุ์จำนวน 29 รายเข้าร่วมการศึกษา โดยทบทวนข้อมูลลักษณะทางคลินิก ผลตรวจทางโลหิตวิทยา ประวัติการได้รับเลือด และการศึกษาครอบครัว ตรวจหาการกลายพันธุ์ของยีน *SPTB* ที่พบบ่อย ได้แก่ Providence c.6055T>C, Buffalo c.6074T>G และ Chiang Mai c.6224A>G ด้วยวิธี multiplex ARMS-PCR ร่วมกับ high-resolution melting analysis และตรวจยีน *KLF1* ในผู้ป่วยบางราย **ผลการศึกษา** พบการกลายพันธุ์ของยีน *SPTB* ในผู้ป่วย 22 จาก 29 ราย พีโนไทป์ที่พบบ่อยที่สุดคือ hereditary pyropoikilocytosis ซึ่งสัมพันธ์กับการกลายพันธุ์ของ *SPTB* แบบ homozygous หรือ compound heterozygous โดยเฉพาะจีโนไทป์ที่มี *SPTB* Buffalo variant ร่วมด้วย ผู้ป่วย HPP ส่วนใหญ่มีภาวะซีดรุนแรงตั้งแต่ระยะแรกเกิด ต้องได้รับเลือดต่อเนื่อง และพบภาวะแทรกซ้อนปริกำเนิดบ่อย รวมถึง hydrops fetalis ผู้ป่วยที่มี hereditary elliptocytosis ร่วมกับการกลายพันธุ์ของ *SPTB* แบบ heterozygous มีอาการไม่รุนแรงและไม่ต้องได้รับเลือดต่อเนื่อง ผู้ป่วย 2 รายมีการกลายพันธุ์ของยีน *KLF1* แบบ compound และมีภาวะซีดที่ต้องได้รับเลือดต่อเนื่อง โดยยืนยันการเริ่มแสดงอาการในระยะแรกเกิดได้ 1 ราย ครอบครัวของผู้ป่วยส่วนใหญ่มาจากจังหวัดนครราชสีมา **สรุป** พบการกลายพันธุ์ของยีน *SPTB* ที่เกิดซ้ำ โดยเฉพาะ Buffalo variant บ่อยในกลุ่มผู้ป่วยเด็กที่ได้รับการส่งต่อด้วยภาวะซีดจากเม็ดเลือดแดงแตกทางกรรมพันธุ์ในการศึกษานี้ และสัมพันธ์กับพีโนไทป์ HPP ที่รุนแรง การวินิจฉัยทางพันธุกรรมระดับโมเลกุลตั้งแต่ระยะแรกอาจช่วยในการจำแนกโรค การให้คำปรึกษาทางพันธุกรรม และการดูแลรักษาอย่างเหมาะสมและทันที่

คำสำคัญ : ● ภาวะซีดจากเม็ดเลือดแดงแตกทางกรรมพันธุ์ ● เฮเรดิทารีไพโรพอยคิโลไซโทซิส ● เฮเรดิทารีเอลลิปโตไซโทซิส ● ยีน *SPTB* ● ยีน *KLF1*

วารสารโลหิตวิทยาและเวชศาสตร์บริการโลหิต. 2569;36:273-82.

Background

Inherited hemolytic anemia (IHA) comprises a heterogeneous group of red blood cell disorders caused by membrane defects,¹⁻³ enzymopathies or hemoglobinopathies. In clinical practice, a subset of pediatric patients presents with hemolytic anemia without identifiable thalassemia mutations, with disease severity ranging from mild anemia to severe neonatal disease,⁴ including hydrops fetalis⁵ and transfusion dependency.

Recent studies in Thailand⁶⁻⁸ have identified recurrent *SPTB* mutations—particularly *SPTB* Providence^{6,9-11} (c.6055T>C; p.Ser2019Pro), Buffalo^{6,9,12} (c.6074T>G; p.Leu2025Arg), and Chiang Mai^{6,13} (c.6224A>G; p.Glu2075Gly)—as major molecular causes of hereditary elliptocytosis (HE) and hereditary pyropoikilocytosis (HPP).¹⁴ These mutations account for a substantial proportion of inherited red blood cell membrane disorders in Thai populations, with emerging evidence suggesting possible regional clustering.

However, clinical and molecular data from Northeastern Thailand remain limited,¹⁵ particularly regarding genotype–phenotype correlations and early-life disease severity. This study aimed to characterize the clinical presentation, molecular spectrum and transfusion outcomes of pediatric patients with IHA at a tertiary care center in this region.

Material and methods

Study objectives

This study aimed to identify pediatric patients with IHA at Maharat Nakhon Ratchasima Hospital and to describe their clinical characteristics, hematologic findings, molecular spectrum, genotype–phenotype correlations and transfusion outcomes.

Study design and setting

This prospective observational cohort study enrolled patients from January 2023 to December 2025. Clinical data obtained before enrollment were additionally retrieved from medical records at the Division of Pediatric Hematology–Oncology, Maharat Nakhon Ratchasima

Hospital, a tertiary referral center in Health Region 9 of Northeastern Thailand.

Participants

Children younger than 18 years with clinically suspected IHA, including HPP, HE, hereditary spherocytosis (HS), Southeast Asian ovalocytosis (SAO) or unexplained chronic hemolytic anemia, were enrolled as index cases. Parents and siblings were invited to participate in family studies and molecular testing when available. Hemoglobin typing and/or molecular testing for common thalassemia variants were reviewed when available. Co-inheritance was determined only among patients with interpretable test results documented in the medical records. Patients with severe thalassemia syndromes, including Bart's hydrops fetalis, β -thalassemia major and β -thalassemia/HbE disease, were excluded. Patients with anemia primarily caused by acute blood loss or acquired hemolysis were also excluded.

Bone marrow disorders affecting erythropoiesis, including inherited bone marrow failure syndromes, aplastic anemia, congenital dyserythropoietic anemia,¹⁶ and myelodysplastic syndrome, were excluded based on clinical history, complete blood count patterns, reticulocyte response, peripheral blood smear morphology, the absence of persistent pancytopenia or dysplastic leukocyte or platelet features and clinical follow-up. Bone marrow examination was performed when clinically indicated.

Clinical and laboratory data collection

Demographic data, age at presentation, birth history, perinatal complications, transfusion history, physical findings, complete blood count, reticulocyte count, bilirubin profile, lactate dehydrogenase level, direct antiglobulin test results and peripheral blood smear findings were reviewed. Available hemoglobin typing and thalassemia testing results were reviewed to assess the co-inheritance of thalassemia or hemoglobin variants. Co-inherited thalassemia traits or hemoglobin variants, when present, were recorded and interpreted in conjunction with red cell morphology and molecular findings.

Molecular testing

Genomic DNA was extracted from EDTA-anticoagulated whole blood. Common recurrent *SPTB* variants, including Providence c.6055T>C, Buffalo c.6074T>G and Chiang Mai c.6224A>G, were detected using multiplex amplification refractory mutation system-polymerase chain reaction combined with high-resolution melting analysis, as previously described.¹⁷ This assay was designed to detect only these three recurrent *SPTB* variants. Samples with atypical high-resolution melting profiles were retested or confirmed by Sanger sequencing when available.

KLF1 mutation analysis was performed in selected patients with severe hemolytic anemia without a fully explanatory *SPTB* genotype or with clinical features suggestive of an erythroid transcription factor disorder. *KLF1* variants were reported using Human Genome Variation Society (HGVS) nomenclature at the coding DNA and protein levels when available. For patients tested through the Thailand Genomic Medicine Program, candidate variants related to red cell membrane disorders, erythroid transcription factors and congenital hemolytic anemia were reviewed in relation to the clinical phenotype and inheritance pattern. Variants outside the targeted *SPTB* loci, including *KLF1* mutations or other rare variants, required broader molecular testing and were confirmed by Sanger sequencing when available.

Disease severity definitions

Disease severity was classified according to transfusion requirements and the perinatal clinical course. Severe disease was defined as transfusion-dependent anemia, the need for intrauterine transfusion or exchange transfusion or a severe neonatal presentation such as hydrops fetalis, severe neonatal hemolytic anemia or persistent pulmonary hypertension of the newborn (PPHN). Mild disease was defined as the absence of chronic transfusion requirement and stable clinical status during follow-up after the neonatal period. Variable severity was used when patients within the same diagnostic or genotype group demonstrated heterogeneous transfusion requirements.

Data analysis

Data analysis was performed using descriptive and analytical statistical methods. Data were analyzed using Stata version 18 (StataCorp LLC, College Station, TX, USA). Continuous variables were presented as mean $\pm$ standard deviation (SD) or median (interquartile range [IQR]), as appropriate. Categorical variables were summarized as frequencies and percentages. Comparisons of hematologic parameters among the four diagnostic groups (HPP, HE, HS and *KLF1*-related disease) were performed using the Kruskal-Wallis test. Effect size (η^2) was calculated using an approximation based on the Kruskal-Wallis H statistic: $(H - k + 1) / (N - k)$, where H is the Kruskal-Wallis test statistic, k is the number of groups, and N is the total sample size. A two-sided p -value of < 0.05 was considered statistically significant. All data were de-identified and stored securely.

Ethical considerations

This study was approved by the Institutional Review Board of Maharat Nakhon Ratchasima Hospital (IRB No. SUB66187). Written informed consent was obtained from parents or legal guardians before enrollment.

Results

A total of 29 pediatric patients with IHA were enrolled between 2023 and 2025. The distribution of clinical diagnoses and the demographic and perinatal characteristics of each diagnostic group are summarized in Table 1. Molecular findings, transfusion status and disease severity are presented in Table 2. HPP was the most common phenotype, followed by HE, HS and *KLF1*-related erythroid disorder. The median age at the time of the complete blood count (CBC) selected for hematologic analysis was 1 day (IQR, 1-55 days), and 20 of 29 patients (69.0%) had the selected CBC obtained during the neonatal period. There was a female predominance, with 17 females and 12 males (female-to-male ratio, 1.42:1). Most patients (24/29, 82.8%) originated from Nakhon Ratchasima Province.

Table 1 Demographic, clinical and perinatal characteristics by diagnostic group

Variable	HPP (n = 16)	HE (n = 6)	HS (n = 5)	KLF1 (n = 2)
A. Demographic and birth characteristics				
Male, n (%)	6 (37.5%)	2 (33.3%)	3 (60.0%)	1 (50.0%)
Age at first clinical presentation, median (IQR), days	1 (1-1)	42 (12.5-700)	185 (2-395)	1 (n = 1)
Neonatal onset, n/N (%)	14/16 (87.5%)	3/6 (50%)	3/5 (60%)	1/2 (50%)
Gestational age, median (IQR), weeks	36 (30-39), n = 15	38 (37-39), n = 5	37.5 (33-40), n = 4	39 (38-40), n = 2
Preterm birth (< 37 weeks), n/N (%)	9/15 (60%)	0/5 (0%)	2/4 (50%)	0/2 (0%)
Birth weight, median (IQR), g	1,971 (1,560-2,200) (n = 14)	3,175 (2,842.5-3,700) (n = 4)	2,315 (1,922.5-2,850) (n = 4)	2,720 (n=1)
Low birth weight (< 2,500 g), n/N (%)	12/14 (85.7%)	0/4 (0%)	2/4 (50.0%)	0/1 (0%)
Small for gestational age, n/N (%)	8/13 (61.5%)	0/3 (0%)	1/3 (33.3%)	0/1 (0%)
Apgar score < 7 at 1 minute, n/N (%)	5/13 (38.5%)	0/3 (0%)	0/3 (0%)	0/1 (0%)
Inborn, n/N (%)	10/16 (62.5%)	3/6 (50%)	2/5 (40%)	1/2 (50%)
Nakhon Ratchasima residence, n (%)	12 (75.0%)	6 (100%)	5 (100%)	1 (50%)
B. Fetal compromise				
Cesarean delivery, n/N (%)	10/13 (76.9%)	4/5 (80%)	1/3 (33.3%)	2/2 (100%)
Fetal indication for cesarean delivery, n/N (%)	8/11 (72.7%)	1/4 (25%)	1/2 (50%)	2/2 (100%)
Hydrops fetalis, n/N (%)	8/16 (50%)	0/6 (0%)	1/4 (25%)	0/2 (0%)
Fetal distress, n/N (%)	6/11 (54.5%)	0/4 (0%)	1/2 (50%)	1/2 (50%)
Fetal anemia, n/N (%)	2/14 (14.3%)	0/6 (0%)	0/5 (0%)	1/2 (50%)
Intrauterine transfusion, n/N (%)	2/14 (14.3%)	0/6 (0%)	0/5 (0%)	0/2 (0%)
Fetal cardiomegaly, n/N (%)	2/9 (22.2%)	0/4 (0%)	0/4 (0%)	1/2 (50%)
Oligohydramnios, n/N (%)	4/11 (36.3%)	0/4 (0%)	0/4 (0%)	0/2 (0%)

- Neonatal onset was defined as anemia or jaundice requiring medical attention within 28 days of life.
- Mode-of-delivery data were available in 13 patients with HPP.
- The exact age at first clinical presentation was unavailable for one patient with a *KLF1*-related erythroid disorder; therefore, the summary was based on one patient with a documented presentation date.

Table 2 Genotype-phenotype correlation (n = 29)

Mutation Type	n	%	Phenotype	Transfusion-dependent	Severity
Homozygous <i>SPTB</i> Buffalo	7	24.1	HPP	Yes	Severe
Compound heterozygous <i>SPTB</i> Providence/Bufalo	5	17.2	HPP	Yes	Severe
Compound heterozygous <i>SPTB</i> Buffalo/Chiang Mai	2	6.9	HPP	Yes	Severe
Heterozygous <i>SPTB</i> Providence	2	6.9	HPP	Yes	Severe
Heterozygous <i>SPTB</i> Providence	5	17.2	HE	No	Mild
Heterozygous <i>SPTB</i> Buffalo	1	3.4	HE	No	Mild
<i>KLF1</i> -related erythroid disorder	2	6.9	<i>KLF1</i>	Yes	Severe
<i>SPTB</i> -negative	5	17.2	HS	Variable	Mild-severe

- Both patients with compound *KLF1* variants developed transfusion-dependent anemia; neonatal onset was confirmed in one patient. The other patient developed symptoms during early infancy, although the exact age at onset was unavailable.
- Two cases were transfusion-dependent, one was transfusion-dependent only during the first year, and two were non-transfusion-dependent.

Hemoglobin typing and/or molecular testing for common thalassemia variants were available in 17 of 29 patients (58.6%). Co-inherited hemoglobin variants or α -thalassemia deletions were identified in 8 of the 17 tested patients (47.1%), including five patients with HPP, one with HE and two with HS. No patient was confirmed to have Hb H disease. Detailed variant-level information regarding co-inherited hemoglobin variants and α -thalassemia was not consistently available because testing was incomplete and nonuniform. Therefore, their specific effects on hematologic parameters and clinical severity could not be evaluated.

Perinatal characteristics by diagnostic group are summarized in Table 1. Perinatal manifestations were most severe in the HPP group, including preterm birth, low birth weight, small-for-gestational-age status, fetal distress, hydrops fetalis, intrauterine transfusion, exchange transfusion and severe PPHN. Notably, two patients with homozygous *SPTB* Buffalo variants died during the neonatal period because of severe hydrops fetalis complicated by persistent pulmonary hypertension, likely resulting from intrauterine hypoxia secondary to profound hemolytic anemia.

Overall, these exploratory findings suggest that HPP was associated with more severe anemia and greater erythropoietic stress than HE and HS within this cohort, supporting the clinical classification of HPP as a more severe phenotype within the IHA spectrum.⁷

SPTB variants were identified in 22 of 29 patients. All patients with HPP carried *SPTB* variants, predominantly homozygous *SPTB* Buffalo, compound heterozygous *SPTB* Providence/Buffalo and compound heterozygous *SPTB* Buffalo/Chiang Mai. Fourteen of 16 patients with HPP had confirmed neonatal-onset disease, and all were classified as having severe, transfusion-dependent anemia. In contrast, patients with HE carried heterozygous *SPTB* Providence or heterozygous *SPTB* Buffalo variants and had mild disease without a chronic transfusion requirement.

Two patients were classified as having *KLF1*-related erythroid disorder. The first patient carried compound

heterozygous *KLF1* variants, c.892G>C (p.Ala298Pro) and c.649C>T (p.Gln217Ter), and presented with severe neonatal-onset transfusion-dependent anemia. The second patient carried *KLF1* c.892G>C (p.Ala298Pro) together with c.525_526insCGGCGCC (p.Gly176Argfs*179).

The second patient presented with severe anemia requiring transfusion from approximately one month of age and was referred to our center at five months of age with severe anemia and hepatosplenomegaly. The patient subsequently remained transfusion-dependent. The CBC selected for hematologic analysis was obtained after transfusion at five months of age.

A representative family with homozygous *SPTB* Buffalo-associated HPP is shown in Figure 1. The index patient was a term male neonate born at 38 weeks of gestation by cesarean delivery, with a birth weight of 3,100 g and Apgar scores of 5, 8 and 8 at 1, 5 and 10 minutes, respectively. He presented at birth with non-immune hydrops fetalis, profound hemolytic anemia, hepatosplenomegaly, thrombocytopenia and severe PPHN. The pretransfusion CBC showed a hemoglobin (Hb) level of 3.8 g/dL, hematocrit (Hct) of 13.9%, mean corpuscular volume (MCV) of 105.9 fL, mean corpuscular hemoglobin concentration (MCHC) of 27.1 g/dL, red cell distribution width (RDW) of 36.4%, platelet count of $73 \times 10^9/L$ and 260 nucleated red blood cells (NRBC) per 100 white blood cells (WBC). Marked red cell fragmentation, elliptocytosis and anisopoikilocytosis reflected severe red cell membrane instability and supported the diagnosis of HPP rather than isolated HS. He subsequently underwent total exchange transfusion and required inhaled nitric oxide, inotropic support and pulmonary vasodilators for severe pulmonary hypertension. Despite intensive cardiopulmonary support, he died on day 4 of life from complications of severe persistent pulmonary hypertension.

Hematologic parameters from the CBC selected for analysis are shown in Table 3. One CBC record was selected for each patient. Whenever possible, pretransfusion values were used. Hb levels, Hct, RBC counts, NRBC counts, corrected WBC counts and platelet counts

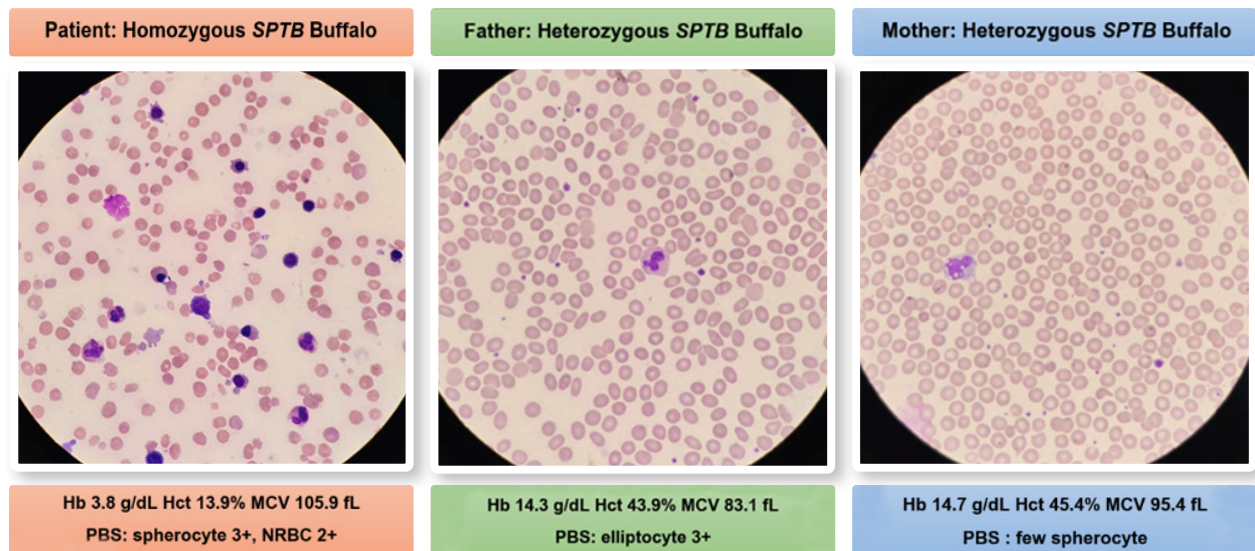


Figure 1 Representative family with homozygous *SPTB* Buffalo-associated hereditary pyropoikilocytosis. The index patient was a term male neonate presenting at birth with non-immune hydrops fetalis, profound neonatal hemolytic anemia, hepatosplenomegaly, thrombocytopenia and severe persistent pulmonary hypertension of the newborn. The pretransfusion peripheral blood smear demonstrated marked anisopoikilocytosis with elliptocytes, elongated cells, red blood cell fragments, numerous nucleated red blood cells and occasional spherocytes. These findings reflected severe red cell membrane instability and supported the diagnosis of HPP rather than isolated HS. Both parents were heterozygous carriers of the *SPTB* Buffalo variant. Wright-Giemsa stain, 100× objective.

differed significantly among diagnostic groups. Patients with HPP and *KLF1*-related erythroid disorder exhibited more severe anemia and greater erythropoietic stress than patients with HE or HS. Hematologic parameters were obtained at different ages, and post-transfusion values were used for four patients with HPP and one patient with a *KLF1*-related erythroid disorder because pretransfusion results were unavailable. Therefore, between-group comparisons should be interpreted cautiously because hematologic parameters were obtained at different ages and post-transfusion values were used for five patients.

These analyses were exploratory and intended to describe between-group differences within this cohort rather than establish age-independent diagnostic differences. Markers of erythropoietic stress were also significantly elevated in HPP and *KLF1*-related erythroid disorders. NRBC counts differed significantly among groups ($p = 0.008$; $\eta^2 = 0.337$), with the highest values observed in the *KLF1* group, followed by HPP. Corrected WBC counts were significantly higher in HPP and *KLF1*-related erythroid disorders than in HE and HS ($p = 0.045$; η^2

$= 0.137$). Platelet counts significantly differed among diagnostic groups ($p = 0.015$; $\eta^2 = 0.297$), although this finding should be interpreted cautiously because of the small sample size and differences in age at testing.

In contrast, MCV, MCH, RDW and MCHC/MCV ratio did not show statistically significant differences among diagnostic categories and had small effect sizes, suggesting limited discriminatory value for these indices in differentiating disease severity in this cohort. Arora, et al.¹⁸ previously proposed an MCHC cutoff of 35 g/dL for HS. Christensen, et al.¹⁹ previously proposed the MCHC/MCV ratio as a screening index for neonatal HS, with a cutoff of > 0.36 ; however, this index did not clearly discriminate among diagnostic groups in the present cohort.

Eosin-5'-maleimide (EMA) flow cytometry was performed in 10 of 29 patients. Reduced EMA fluorescence was observed in all tested HPP cases and in selected patients with HE, HS and *KLF1*-related erythroid disorder, suggesting overlapping membrane instability across different molecular subtypes.

Table 3 Hematologic parameters from the CBC selected for analysis, stratified by diagnosis

Parameter	HPP (n = 16)	HE (n = 6)	HS (n = 5)	KLF1 (n = 2)	p-value [†]	η ²
Age at CBC, median (IQR), days	1 (1-1)	485 (35.5-1,801.5)	185 (2-395)	87.5 (44.5-130.8)	-	-
Pretransfusion CBC	12/16 (75%)	6/6 (100%)	5/5 (100%)	1/2 (50%)	-	-
Neonatal CBC	16/16 (100%)	1/6 (16.7%)	2/5 (40%)	1/2 (50%)	-	-
Hemoglobin (g/dL)	5.4 (4.05-8.8)	12.85 (10.4-15.7)	11.8 (10.9-15.8)	5.15 (4.4-5.9)	0.007	0.370
Hematocrit (%)	17.6 (14.8-27.5)	36.15 (33.6-47.3)	37.5 (32.5-46)	19.3 (15.8-22.8)	0.013	0.310
RBC (×10 ¹² /L)	2.285 (1.39-3.28)	5.175 (4.88-5.24)	4.73 (4.35-5.39)	2.095 (1.96-2.23)	0.006	0.374
MCV (fL)	92.65 (79.4-102.1)	71.65 (65.7-88.1)	72.1 (69.6-114.9)	91.5 (80.7-102.3)	0.200	0.066
MCH (pg)	28.45 (25.55-31.3)	25.45 (20.3-29.5)	24.7 (22.9-37.9)	24.3 (22.3-26.3)	0.435	0.000
MCHC (g/dL)	32.5 (28.5-33.05)	33.35 (30.8-34.8)	33 (31.4-34.3)	26.7 (25.8-27.6)	0.079	0.151
MCHC/MCV	0.346 (0.305-0.421)	0.457 (0.380-0.469)	0.451 (0.287-0.476)	0.297 (0.252-0.342)	0.137	0.101
RDW (%)	35.5 (20.65-41.2)	24.35 (15.2-35.4)	20.5 (16.3-23.6)	38.8 (38.4-39.2)	0.363	0.008
NRBC (per 100 WBC)	150.5 (15-329)	0 (0-0)	3 (0-11)	352.5 (36-669)	0.008	0.337
Corrected WBC (×10 ⁹ /L)	22.2 (13.5-26.8)	14.05 (9.3-15.2)	11.7 (9.7-23.2)	49.85 (46.4-53.3)	0.045	0.137
Platelet (×10 ⁹ /L)	224.5 (108-368.5)	561 (452-654)	401 (348-539)	141.5 (61-222)	0.015	0.297

[†]Kruskal-Wallis test

- MCV varies with age. The HS group included two neonates, one young infant and two older children, which likely contributed to the wider IQR. In the *KLF1* group, laboratory values for one patient were obtained after transfusion because of clinical urgency.
- Group comparisons were performed using the Kruskal-Wallis test across four diagnostic categories (HPP, HE, HS and *KLF1*). Effect size (η²) was estimated from the Kruskal-Wallis H statistic using the formula: $\eta^2 = (H - k + 1) / (N - k)$, where k represents the number of groups (k = 4) and N represents the total sample size (N = 29).
- Hemoglobin typing and/or molecular testing for common thalassemia variants was available in 17 of 29 patients. Percentages for co-inherited variants were calculated using the number of patients with available test results as the denominator. The analyzed CBC was defined as the CBC selected for hematologic comparison. Whenever possible, pretransfusion values were used; however, only post-transfusion values were available among four patients with HPP and one patient with a *KLF1*-related erythroid disorder.
- Age at selected CBC, pretransfusion CBC status and neonatal CBC status were presented descriptively and were not subjected to formal between-group statistical testing.

Discussion

This study demonstrates a distinct genotype–phenotype severity gradient among pediatric patients with IHA in Northeastern Thailand. *SPTB* Buffalo-containing genotypes were strongly associated with HPP, severe neonatal hemolytic anemia, transfusion dependency and

perinatal complications. In contrast, heterozygous *SPTB* variants were associated with HE and a milder, non-transfusion-dependent phenotype. Co-inherited hemoglobin variants or α -thalassemia deletions may have modified red cell indices and disease severity; however, their effects could not be conclusively determined because

testing was incomplete and nonuniform, and the number of patients within each subgroup was small.

The predominance of *SPTB* Buffalo in this cohort suggests possible regional enrichment in Nakhon Ratchasima and surrounding areas. Previous Thai studies^{6-8,17} have shown that recurrent *SPTB* variants are important causes of hereditary red cell membrane disorders, and our findings extend these observations by highlighting the severe perinatal phenotype associated with Buffalo-containing genotypes in Northeastern Thailand.

The clinical distinction between HPP and HE was supported by transfusion requirements, perinatal severity and family studies. Homozygous or compound heterozygous *SPTB* variants were associated with severe disease, whereas heterozygous *SPTB* variants among parents or mildly affected children were associated with milder red cell morphology changes and preserved Hb levels. These findings support the value of family-based molecular testing for clarifying inheritance pattern and providing genetic counseling.

KLF1-related erythroid disorder²¹ represented a small but clinically important subgroup. Compound *KLF1* mutations²¹ may mimic severe IHA or HS-like disease and should be considered in patients with transfusion-dependent anemia without a fully explanatory *SPTB* genotype. Reporting *KLF1* variants using standardized HGVS nomenclature is important for comparison across studies and future variant interpretation.

EMA flow cytometry²⁰ was useful as a screening test for red cell membrane instability; however, reduced EMA fluorescence was not limited to HS in this cohort. Reduced EMA fluorescence in HPP, HE or *KLF1*-related erythroid disorder may reflect overlapping membrane instability and should be interpreted together with morphology, family study and molecular results.

This study had several limitations. The sample size was small, and referral bias may have enriched the cohort for severe neonatal and transfusion-dependent cases. Comprehensive molecular testing was unavailable for all patients, and the targeted ARMS-PCR/HRM assay

detected only three recurrent *SPTB* variants. Therefore, rare *SPTB* variants, defects in other red cell membrane genes or modifier variants may have been missed. Hemoglobin typing and molecular testing for common thalassemia variants were not uniformly available, with interpretable results documented in only 17 of 29 patients. Therefore, the frequency and phenotypic contribution of co-inherited hemoglobin variants could not be ultimately determined. Testing was performed according to clinical indications and test availability rather than systematically in all enrolled patients.

Geographic variation in allele frequency may contribute to the observed clustering of *SPTB* Buffalo mutations in this cohort. Previous studies in Thailand have identified recurrent *SPTB* mutations as major causes of inherited red cell membrane disorders, accounting for a substantial proportion of HE and pyropoikilocytosis cases⁷⁻⁸. A recent case series from Northeastern Thailand¹⁵ also demonstrated a predominance of homozygous and compound heterozygous *SPTB* Buffalo-containing genotypes, supporting a possible regional founder effect.

Based on previously reported allele frequencies and assuming Hardy-Weinberg equilibrium, the expected frequency of disease associated with biallelic *SPTB* Providence, Buffalo or Chiang Mai variants was theoretically estimated to be approximately 1 in 100,000 births¹⁷. The high proportion of severe, transfusion-dependent cases in the present study likely reflects referral enrichment and should not be interpreted as population prevalence, even in regions where these variants are relatively prevalent. These findings support early genetic screening among neonates with unexplained hemolytic anemia in regions where these recurrent variants are relatively prevalent.

Conclusion

In conclusion, recurrent *SPTB* variants, particularly the Buffalo variant, were frequently identified in this referral cohort of children with IHA in Northeastern Thailand. Early recognition of neonatal hemolytic anemia, hydrops fetalis or transfusion dependency should prompt

molecular evaluation for recurrent *SPTB* variants in this region. *KLF1*-related erythroid disorder should also be considered in severe *SPTB*-negative or atypical cases.

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