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Identification of novel variants in hereditary spherocytosis patients by whole-exome sequencing

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ABSTRACT

Defects in erythrocyte membrane proteins can cause the most common type of inherited hemolytic anemia, so called hereditary spherocytosis (HS). It is characterized by the appearance of spherocytes in peripheral blood, hemolytic anemia, splenomegaly, jaundice and gallstones. Due to difficulty of diagnosis solely based on aforementioned parameters, the addition of genetic testing seems to be effective and most acknowledged. Up to date, pathogenic variations in five genes encoding membrane proteins (*ANK1*, *SPTA1*, *SPTB*, *SLC4A1*, *EPB42*) are identified to cause HS. Here, we have studied the genetic spectrum in forty-one patients with clinically suspected HS and their families, as well as their genotype-phenotype correlations. Pathogenic mutations in *ANK1*, *SPTB*, *SLC4A1* and *SPTA1* were found in 17 (41.5 %), 12 (29.3 %), 7 (17.1 %) and 5 (12.2 %) patients, respectively. Deleterious variants include 12 missense, 15 nonsense, 12 frameshift, and 4 splicing variants. Among these variations 32 were novel. In our genotype-phenotype analysis, platelet levels in *SPTB* ($p = 0.021$) and *SLC4A1* ($p = 0.02$) patients were found to be significantly lower than *ANK1* patients. In addition, LDH levels in *SPTB* patients were remarkably lower than patients with *ANK1* mutations ($p = 0.025$).

1. Introduction

From late 1660 s the first description of red blood cells (RBCs) features been made by two Dutch biologists and microscopists, striking improvements were achieved in understanding the morphology, structural component, biological and pathological function of RBC membrane and the proteins resided [1]. A major unique feature of erythrocyte is the high degree of membrane elastic properties, which play a crucial role when passing through small blood vessels and capillaries such as splenic sinus [2]. The capability of large reversible deformations of erythrocytes during peripheral blood circulation while maintaining structural integrity is largely accredited to the structure organization of red cell cytoskeleton. Defects in red cell membrane proteins thus may alter membrane function, compromise cell deformability that enable red blood cells be trapped and destroyed by spleen, and eventually cause congenital and hereditary hemolytic anemia (HA) such as hereditary spherocytosis (HS). The red cell membrane is composed of ~ 20 major proteins and more than 850 minor proteins that with differential functions and diverse abundance, ranging from a few hundred up to a million copies per erythrocyte [1,3,4]. One of the most abundant integral proteins in red cell membrane is called band 3, an anion-exchange channel which is positioned as the center of the macromolecular complexes of integral membrane proteins [5,6]. Band 3 is encoded by *SLC4A1* gene and constitute about 15–20 % of total membrane protein. The two-dimensional membrane skeleton appears as a pseudohexagonal meshwork that consists of α- and β-spectrin, ankyrin, protein 4.1R, actin and its associated proteins [4,7]. Therefore, the decrease in membrane cohesion caused by the reduction in the number of "vertical"

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connections between the bilayer and membrane skeleton can explain the underlying mechanism of membrane loss in HS [8]. Alterations in genes encoding five pivotal membrane proteins: band 3 (*SLC4A1*), ankyrin-1 (*ANK1*), α-spectrin (*SPTA1*), β-spectrin (*SPTB*), and protein 4.2 (*EPB42*) are well recognized as pathogenic mechanism in development of HS [9].

HS is the most prevalent membranopathy of erythrocyte and a common inherited hemolytic anemia, affecting approximately 1:2000 northern European ancestry and 1:100000 Chinese populations [10,11]. Inheritance pattern of HS patient can be autosomal dominant (75%), recessive or *de novo* [10]. Its characteristic is the presence of spherical red blood cells in peripheral blood with reduced surface area. While some patients are asymptomatic and may be miss-diagnosed if not tested thoroughly, this disease can cause life-threatening HA that requires erythrocyte transfusion and thus needs to be carefully treated. Diagnosis of HS is mainly based on positive family history, physical examinations such as jaundice, splenomegaly and gallstones, laboratory test results such as hemolytic anemia, osmotically fragility, as well as negative antiglobulin tests [12,13]. Common complications of HS include cholelithiasis, hemolytic episodes and aplastic crises [10]. With rapid development of next-generation gene sequencing (NGS) the utilization of molecular diagnosis in combination with clinical features become a more convenient and definitive tool in disease diagnosis as well as differential diagnosis since other genetic disorders such as enzymopathies and Gilbert syndrome can cause a similar phenotype.

In this study, we were able to analyze a cohort of 41 patients with clinical diagnosed HS and their families. Pathogenic variations were determined by using whole-exome sequencing (WES). The genetic associations with clinical features and laboratory findings were performed to analyze the genotype-phenotype correlations in these patients.

2. Materials and methods

2.1. Patients

Forty-one unrelated HS patients were enrolled in this study retrospectively on the basis of the NGS analyses performed at our laboratory from May 2022 to October 2023. This study was approved by the Ethics Committee of Institute of Hematology and Blood Disease Hospital. Appropriate informed consent was obtained from all the patients directly, or from the parents of pediatric patients. At the time of enrollment, the medical records of the patients were reviewed retrospectively, including clinical manifestations and family history, hematological parameters, erythrocyte morphology and other laboratory findings.

2.2. Genetic analysis

As low as 1–2 ml of peripheral blood was drawn from the patients and/or their parents, and their genomic DNAs were extracted by using Lab-Aid 896 genomic DNA kit (Zeesan, China) according to the manufacturer's protocol. Afterwards, DNAs were sheared into 200–300 bp fragments using Covaris ultrasonic instrument, and whole-exome sequencing (WES) was performed [14]. Briefly, library preparation was conducted using Twist EF Library Pre Kit (Twist Bioscience, CA, USA) and genomic DNAs were captured by Twist Exome 2.0 kit (Twist Bioscience, CA, USA). Then samples were sequenced on Illumina Novaseq 6000 apparatus according to manufacturer's protocol and differentiated by distinct barcode. Raw data was analyzed by Illumina built-in software DRAGEN. UCSC hg19 human reference genome was used for sequence alignment, and variants information was annotated by using databases including NCBI dbSNP (https://www.ncbi.nlm.nih.gov/snp/), 1000 Genomes (https://browser.1000genomes.org), Exome Aggregation Consortium (ExAC, https://exac.broadinstitute.org), Exome Sequencing Project Database (ESP6500, https://evs.gs.washington.edu/EVS/), and Genome Aggregation Database (GnomAD, https://gnomad.broadinstitute.org). Potential functional impacts of the variants were assessed by using different in silico softwares such as SIFT (https://sift.jcvi.org), PolyPhen-2 (https://genetics.bwh.harvard.edu/pph2), MutationTaster (https://www.mutationtaster.org/), and CADD (https://cadd.gs.washington.edu/). The pathogenicity of suspicious variants were assessed according to the guidelines of the American College of Medical Genetics and Genomics [15], and reported variants were verified from online databases like ClinVar (https://www.ncbi.nlm.nih.gov/clinvar) and The Human Gene Mutation Database (HGMD, https://www.hgmd.cf.ac.uk), or from literatures. Finally, Sanger sequencing was performed to verify the causing/likely causing variants of the patients and/or their parents.

2.3. Statistical analysis

Patients were divided into several groups according to different aspects: (1) Patients were classified as mild, moderate, and severe disease severity according to their hemoglobin (Hb) level [16]; (2) Patients were classified as *ANK1*, *SPTB*, *SLC4A1*, and *SPTA1* groups based on their mutated genes; (3) Patients were divided into missense, nonsense, frameshift, and splicing groups based on their mutational patterns. Afterwards, genotype-phenotype analysis was conducted to analyze and compare the baseline clinical features among different group of patients. Continuous variables were described as median (range). The intergroup association was analyzed by using Fisher's exact test or Kruskal-Wallis test. Pairwise comparison with Bonferroni adjustment was conducted if statistically significant by Kruskal-Wallis. The statistical analyses were performed by using SPSS software. All tests were two-tailed, and p value cutoff of < 0.05 was considered statistically significant.

3. Results

3.1. Clinical features of HS patients

A total of forty-one patients and their families were examined in this study. Among these patients, twenty-one (51.2%) were male. Most of the patients exhibited their clinical symptoms during childhood or neonatal period (Supplemental Table 1). Jaundice and splenomegaly were observed in majority of the patients (97.4% each), and splenectomy was carried out in three out of seventeen (17.6%) patients. Cholecystitis and/or cholecystolithiasis were identified in 71.9% of patients, and cholecystectomy was carried out in eight out of thirty-two (25%) patients. The median WBC count was 6.42×10^9/L, platelet count was 188×10^9/L, and median RBC count was 2.87×10^{12}/L. Subsequently, the median hemoglobin (Hb) level and F-Hb level was 92 g/L and 96.05 g/L, respectively. Moreover, the median MCV, MCH and MCHC level was 91.1 fL, 31 pg, and 339 f/L, respectively. The median hematocrit (HCT) was 27.1%, and the median reticulocyte (Ret) was 12.95%. Furthermore, the median red blood cell volume distribution width (RDW)-CV and RDW-SD were 20.1% and 65.5 fL, respectively. The median RBC lifespan was 16.5 days. Total bilirubin (T-Bil) and indirect bilirubin (I-Bil) levels were 79.4 μmol/L and 68 μmol/L, respectively, and the median LDH level was 246.75 U/L. In addition, as shown in Table 1 and Supplemental Table 1, 97.4% of the acid glycerol hemolysis tests (AGLT), 80.5% of the eosin maleimide (EMA) tests, as well as 82.5% of the Osmotic fragility tests (OFT) were found to be positive. All of the enzymes (*PK*, *G6PD*, *P5N* and *GPI*) tested were normal. All of the Coomb's tests were negative. All of the eleven karyotypes tested were normal except for one complex karyotype.

Based on the Hb level at the time of diagnosis patients were divided into normal, mild, moderate, and severe anemia group in order to investigate the impact on clinical parameters. In summary, as shown in Table 1, WBC, RBC, Hb, HCT, MCH and MCHC were found to be significantly different. In details, WBC count in moderate anemia group was significantly lower than in normal group ($p = 0.032$). HCT was markedly lower in moderate ($p < 0.001$ and $p = 0.006$, respectively) and

Table 1
Patients' clinical characteristics grouped by hemoglobin levels.

	Total (n = 41)	Normal (n = 6)	Mild (n = 17)	Moderate (n = 15)	Severe (n = 3)	p-value[a]
Age (yrs), median (range)	33 (3–69)	30.5 (17–69)	30 (3–69)	34 (4–65)	60 (58–65)	0.133
Gender, male (%)	21/41 (51.2 %)	4/6 (66.7 %)	8/17 (47.1 %)	8/15 (53.3 %)	1/3 (33.3 %)	0.84[b]
Jaundice (%)	37/38 (97.4 %)	5/5 (100 %)	15/15 (100 %)	15/15 (100 %)	2/3 (66.7 %)	0.079[b]
Splenomegaly (%)	38/39 (97.4 %)	6/6 (100 %)	14/15 (93.3 %)	15/15 (100 %)	3/3 (100 %)	1.0[b]
Splenectomy (%)	3/17 (17.6 %)	0/1 (0)	1/6 (16.7 %)	2/9 (22.2 %)	0/1 (0)	1.0[b]
Cholecystitis/cholecystolithiasis (%)	23/32 (71.9 %)	3/5 (60 %)	9/12 (75 %)	9/13 (69.2 %)	2/2 (100 %)	0.937[b]
Cholecystectomy (%)	8/32 (25 %)	1/5 (20 %)	4/13 (30.8 %)	3/12 (25 %)	0/2 (0)	1.0b
F-Hb (mg/L), median (range)	96.05 (11.7–346.5)	157.9 (75–165.4)	104.7 (42.8–346.5)	73.9 (15–206.4)	48.3 (11.7–173.2)	0.246
WBC ($\times10^9$/L), median (range)	6.42 (2.65–16.71)	7.755 (6.78–11.26)	6.72 (3.97–16.57)	5.33 (2.65–16.71)	5.57 (3.97–5.69)	**0.009**
RBC ($\times10^{12}$/L), median (range)	2.87 (1.61–5.24)	3.895 (3.76–5.24)	3.03 (2.4–3.63)	2.63 (2.23–3.25)	1.77 (1.61–1.91)	**<0.001**
Hb (g/dL), median (range)	92 (51–161)	134 (120–161)	99 (91–109)	78 (60–89)	56 (51–58)	**<0.001**
PLT ($\times10^9$/L), median (range)	188 (27–1673)	208.5 (167–250)	196 (26.7–502)	170 (70–397)	151 (106–1673)	0.267
HCT (%), median (range)	27.1 (16.2–44.3)	36.7 (33.2–44.3)	28.7 (25.3–33.2)	23.5 (19.3–28.8)	17 (16.2–17.4)	**<0.001**
MCV (fL), median (range)	91.1 (77.1–110.4)	89.95 (84.5–99.7)	93 (77.1–110.4)	88.6 (77.9–108.3)	96 (91.1–100.6)	0.413
MCH (pg), median (range)	31 (24.9–37.9)	32 (30.4–35)	32.5 (28.7–37.9)	29.3 (24.9–34.7)	31.6 (30.4–31.7)	**0.031**
MCHC (g/L), median (range)	339 (306–383)	362 (322–383)	352 (316–376)	333 (306–357)	329 (315–333)	**0.002**
Ret (%), median (range)	12.95 (2.89–30.76)	11.26 (5.04–15.08)	12.95 (2.89–30.76)	13.81 (5.34–21.71)	17.07 (7.63–23.59)	0.435
Ret# ($\times10^{12}$/L), median (range)	0.4013 (0.1049–0.9105)	0.45445 (0.2311–0.5851)	0.4386 (0.1049–0.9105)	0.3532 (0.1613–0.5254)	0.3021 (0.1457–0.3892)	0.33
RDW-CV (%), median (range)	20.1 (13.5–29)	19.55 (13.5–20.2)	19.6 (15.9–29)	21.8 (18.8–27.9)	22.6 (18.2–27.6)	0.061
RDW-SD (fL) , median (range)	65.5 (41.9–97.2)	58.3 (41.9–71.6)	65.4 (45.2–95.7)	66.6 (55.7–96.8)	66.7 (60.1–97.2)	0.191
T-Bil (μmol/L), median (range)	79.4 (27.7–285.6)	86 (45.9–123)	77.35 (33.6–285.6)	93.05 (39–170.8)	46.4 (27.7–184.3)	0.821
D-Bil (μmol/L), median (range)	11.6 (6.7–26.7)	12.85 (8–23.4)	11.2 (7–19.7)	12 (7.8–26.5)	12.9 (6.7–26.7)	0.763
I-Bil (μmol/L), median (range)	68 (21–268.5)	72.1 (37.9–108.8)	66.05 (26.3–268.5)	76.1 (30.1–152.8)	33.5 (21–157.6)	0.812
LDH (U/L), median (range)	246.75 (163.5–593.1)	269.05 (183.5–322.1)	217 (163.5–345.2)	266.85 (180.9–593.1)	354.9 (304.1–452)	0.057
AGLT50, positive (%)	38/39 (97.4 %)	4/5 (80 %)	16/16 (100 %)	15/15 (100 %)	3/3 (100 %)	0.205[b]
EMA, positive (%)	33/41 (80.5 %)	6/6 (100 %)	14/17 (82.4 %)	11/15 (73.3 %)	2/3 (66.7 %)	0.474[b]
OFT, positive (%)	33/40 (82.5 %)	3/5 (60 %)	16/17 (94.1 %)	10/15 (66.7 %)	3/3 (100 %)	0.094[b]

F-Hb: free hemoglobin; WBC: white blood cell; RBC: red blood cell; Hb: hemoglobin; PLT: platelet; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; Ret: reticulocyte; RDW: red blood cell distribution width; T-Bil: total bilirubin; D-Bil: direct bilirubin; I-Bil: indirect bilirubin; LDH: lactate dehydrogenase; AGLT50: acidified glycerol lysis test; EMA: eosin maleimide test; OFT: osmotic fragility test.
a: Kruskal-Wallis test.
b: Fisher's exact test.

severe anemia group ($p < 0.001$ and $p = 0.008$, respectively) than in normal and mild group. Moreover, MCH level in moderate anemia group was lower than in mild group ($p = 0.044$). Similarly, moderate group showed to have a significantly lower level of MCHC than normal ($p = 0.013$) and mild ($p = 0.037$) groups.

3.2. Mutational spectrum of HS patients

Through whole-exome sequencing we have investigated deleterious variants in all HS patients (see Supplemental Table 2 for quality information of sequencing). As shown in Table 2, we have identified a total of 52 variants in 41 patients. Among them, 5 variants were from genes not related to hereditery spherocytosis. For example, in patient #3, we have found a nonsense variant in *ABCG5* gene that is related to an autosomal recessive disorder sitosterolemia 2. In patient #8, we have identified a frameshift variant in *CDAN1* gene that may cause an autosomal recessive disorder congenital dyserythropoietic anemia (type Ia). In patient #20, we have identified a pathogenic variant in plasma membrane copper-transport protein *ATP7B* that accounts for wilson disease. Furthermore, in patient #25, a pathogenic deletion variant in *MMACHC* gene was found, which can cause methylmalonic aciduria and homocystinuria (type cblC). Additionally, in patient #36, a likely pathogenic variant in *G6PD* gene was identified that may lead to G6PD deficiency and relate to hemolytic anemia. However, all these variants detected were heterozygous, which bears a disconcordant inheritance with related congenital disorder.

In total, we have identified forty-six distinct variants in *ANK1*, *SPTA1*, *SPTB*, *SLC4A1* and *EPB42* genes (see in Table 2 and Fig. 1). Among them, 35 were novel. Patients #15 and #33 also carried a variant in *EPB42* gene, and patients #20 and #36 were also accompanied with a *SPTA1* alteration. All four variants were found to be unknown significance except for *SPTA1*-p.I75T which was also found in patient #38 with a compound heterozygous inheritance. Therefore, a total of 43 variants were identified to be pathogenic, including 17 *ANK1* (17 patients), 12 *SPTB* (12 patients), 7 *SLC4A1* (7 patients) and 7 *SPTA1* (5 patients) variants. Among them, 32 were novel. Deleterious variants consist of 12 missense, 15 nonsense, 12 frameshift, and 4 splicing variants. Loss of function (nonsense, frameshift, canonical splicing) variants remains to be the predominant disease-causing variants in *ANK1* and *SPTB* gene, accounting for 13/17 (76.5%) and 12/12 (100%) of cases, respectively. However, missense variants accounted for 4/7 (57.1%) cases in both *SCL4A1* and *SPTA1* gene, suggesting its predominant role than LOF variant. The diagram that depicts causal variants of HS patients was shown in Fig. 2. Variants detected in *ANK1*, *SPTB*, *SLC4A1* and *SPTA1* were wide-spread through the whole gene. The missense variants were undergoing structural analysis by HOPE (Supplemental Table 3) [17,18]. For example, as shown in Supplemental Figure 1, the ANK1 (p.A312E) variant introduces a charge in a buried residue which can lead to defective protein folding. Also, glutamic acid will cause loss of hydrophobic interactions in the core of the protein. For SLC4A1 (p.E522K) variant, the charge of the buried wild-type glutamic acid is reversed by lysine residue, this may cause repulsion between residues in the protein core, and thus would disturb the ionic interaction. Next, patterns of inheritance were available for analysis in 15 families (shown in Table 2 and Fig. 3). The chromatograms were shown in Supplemental Figure 2. Six patients (all caused by *ANK1* variation) were found to be *de novo*. Four patients (1 *ANK1*, 2 *SPTB* and 1 *SPTA1*) were found to be inherited from paternal allele and three patients (1 *ANK1* and 2 *SLC4A1*) were inherited from maternal allele. Two compound heterozygous variants in *SPTA1* gene were also found, with each allele inherited from either father or mother, respectively.

Table 2
Variants identified in HS patients.

ID #	Status	Gene	Location	cDNA change	Protein change	Inheritance	1000G/ gnomAD	SIFT	PolyPhen2	LRT	MutationTaster	dbscSNV	CADD	HGMD/ PMID	ACMG
1	Het	ANK1	Exon26	c.2768G > A	p.G923D	—	0/0	D	D	D	D	—	28.2	—	4-LP (PM1,PM2,PP3,PP4, BP1)
2	Het	ANK1	intron27	c.3116-2A > G	—	—	0/0	—	—	—	D	1	35	—	5-P (PVS1,PM2,PP4)
3	Het	ANK1	Exon19	c.2146_2183del	p.L716Cfs*64	de novo	0/0	—	—	—	—	—	—	—	5-P (PVS1,PS2,PM2,PP4)
	Het	ABCG5	Exon6	c.751C > T	p.Q251*	—	0/0	—	—	D	A	—	44	28521186; 30,985,648	5-P (PVS1,PM2,PP5)
4	Het	ANK1	Exon17	c.1867delC	p.Q623Sfs*14	—	0/0	—	—	—	—	—	—	—	5-P (PVS1,PM2,PP4)
5	Het	ANK1	Intron38	c.5096 + 1G > A	—	—	0/0	—	—	—	D	1	34	—	5-P (PVS1,PM2,PP4)
6	Het	ANK1	Exon37	c.4515_4516del	p. L1506Vfs*37	Inherited (F)	0/0	—	—	—	—	—	—	—	5-P (PVS1,PM2,PP4)
7	Het	ANK1	Exon9	c.841C > T	p.R281*	—	0/0	—	—	D	A	—	36	31,016,877	5-P (PVS1,PM2,PP4,PP5)
8	Het	ANK1	Exon20	c.2230C > T	p.Q744*	—	0/0	—	—	D	A	—	39	—	5-P (PVS1,PM2,PP4)
	Het	CDAN1	Exon19	c.2576delC	p.P859Rfs*8	—	0/0	—	—	—	—	—	—	—	5-P (PVS1,PM2,PP4)
9	Het	ANK1	Exon10	c.935C > G	p.A312G	de novo	0/0	D	P	D	D	—	29.5	—	4-LP (PVS1,PM2,PP4)
10	Het	ANK1	Intron21	c.2388 + 1G > A	—	—	0/0	—	—	—	D	0.9999	34	—	5-P (PVS1,PM2,PP4)
11	Het	ANK1	Exon10	c.935C > A	p.A312E	—	0/0	D	D	D	D	—	28.4	—	4-LP (PM1,PM2,PP3,PP4, BP1)
12	Het	ANK1	Exon17	c.1930dupC	p. Q644Pfs*149	de novo	0/0	—	—	—	—	—	—	—	5-P (PVS1,PS2,PM2,PP4)
13	Het	ANK1	Exon29	c.3387C > A	p.S1129R	de novo	0/0	D	D	D	D	—	24.9	—	5-P (PS2,PM1,PM2,PP3, PP4,BP1)
14	Het	ANK1	Exon4	c.327 + 1G > C	—	de novo	0/0	—	—	—	D	1	34	—	5-P (PVS1,PS2,PM2,PP4)
15	Het	ANK1	Exon14	c.1585C > T	p.Q529*	—	0/0	—	—	N	A	—	45	—	5-P (PVS1,PM2,PP4)
	Het	EPB42	Exon9	c.1280G > A	p.R427H	—	2e-04/ 2.985e-04	T	P	D	D	—	25		3-VUS (PM1,BS2)
16	Het	ANK1	Exon14	c.1519delC	p.L507Cfs*26	de novo	0/0	—	—	—	—	—	26.1	—	5-P (PVS1,PS2,PM2,PP4)
17	Het	ANK1	Exon1	c.24delC	p.E9Kfs*9	Inherited (M)	0/0	—	—	—	—	—	—	—	5-P (PVS1,PM2,PP4)
18	Het	SPTB	Exon16	c.3121delG	p. E1041Sfs*87	—	0/0	—	—	—	—	—	—	—	5-P (PVS1,PM2,PP4)
19	Het	SPTB	Exon20	c.4262dupT	p. K1422Efs*69	—	0/0	—	—	—	—	—	—	—	5-P (PVS1,PM2,PP4)
20	Het	SPTB	Exon2	c.48C > A	p.Y16*	Inherited (F)	0/0	—	—	D	A	—	38	—	5-P (PVS1,PM2,PP4)
	Het	ATP7B	Exon12	c.2755C > G	p.R919G	Inherited (M)	0/1e-04	D	P	D	D	—	28.5	CM980175; 23,159,873	4-LP (PM1,PM2,PP3,PP5)
	Het	SPTA1	Exon8	c.1088A > G	p.Y363C	—	4e-04/ 1.687e-04	T	P	N	D	—	22.2	—	3-VUS (PM1,BS2)
21	Het	SPTB	Exon14	c.2135dupC	p.Q713Afs*31	—	0/0	—	—	—	—	—	—	—	5-P (PVS1,PM2,PP4)
22	Het	SPTB	Exon3	c.188G > A	p.W63*	—	0/0	—	—	D	A	—	41	—	5-P (PVS1,PM2,PP4)
23	Het	SPTB	Exon23	c.4759C > T	p.Q1587*	—	0/0	—	—	D	A	—	52	31602632; 38,069,343	5-P (PVS1,PM2,PP4)
24	Het	SPTB	Exon4	c.376C > T	p.Q126*	—	0/0	—	—	D	A	—	43	33620149; 31,122,244	5-P (PVS1,PM2,PP4)
25	Het	SPTB	Exon16	c.2863C > T	p.R955*	—	0/0	—	—	D	A	—	37	31602632; 27,292,444	5-P (PVS1,PM2,PP4,PP5)
	Het	MMACHC	Exon4	c.658_660del	p.K220del	—	0/ 4.411e-05	—	—	—	—	—	21.4	16311595; 23,757,202	4-LP (PM1,PM2,PM4, PP5)
26	Het	SPTB	Exon23	c.4735C > T	p.R1579*	—	0/0	—	—	D	A	—	52	31602632; 29,572,776	5-P (PVS1,PM2,PP4,PP5)
27	Het	SPTB	Exon10	c.1080dupG	p.N361Efs*31	—	0/0	—	—	—	—	—	—	37,280,519	5-P (PVS1,PM2,PP4)

(*continued on next page*)

Table 2 (*continued*)

ID #	Status	Gene	Location	cDNA change	Protein change	Inheritance	1000G/ gnomAD	SIFT	PolyPhen2	LRT	MutationTaster	dbscSNV	CADD	HGMD/ PMID	ACMG
28	Het	SPTB	Exon26	c.5230C > T	p.Q1744*	—	0/0	—	—	D	A	—	49	—	5-P (PVS1,PM2,PP4)
29	Het	SPTB	Exon26	c.5266C > T	p.R1756*	Inherited (F)	0/0	—	—	N	A	—	44	CM094379; 26,830,532	5-P (PVS1,PM2,PP4,PP5)
30	Het	SLC4A1	Exon17	c.2279G > A	p.R760Q	—	0/0	D	D	D	A	—	29.2	CM951169; 34,093,240	4-LP (PM1,PM2,PP3,PP4, PP5)
31	Het	SLC4A1	Exon14	c.1665C > A	p.Y555*	—	0/0	—	—	U	A	—	33	—	5-P (PVS1,PM2,PP4)
32	Het	SLC4A1	Exon11	c.1210delC	p.Q404Rfs*38	—	0/0	—	—	—	—	—	—	—	5-P (PVS1,PM2,PP4)
33	Het	SLC4A1	Exon13	c.1564G > A	p.E522K	—	0/0	D	D	D	D	—	29.7	CM095580	4-LP (PM1,PM2,PP3,PP4, PP5)
	Het	EPB42	Exon8	c.1041G > T	p.Q347H	—	2e-04/ 3.978e-05	D	D	D	D	—	24.5	—	3-VUS (PM1,PP3,BS2)
34	Het	SLC4A1	Exon19	c.2608C > T	p.R870W	Inherited (M)	0/0	D	D	D	A	—	28.4	CM951173; 11,380,459	4-LP (PM1,PM2,PP3,PP4, PP5)
35	Homo	SLC4A1	Exon2	c.7G > T	p.E3*	—	0/0	—	—	N	A	—	34	—	5-P (PVS1,PM2,PP4)
36	Het	SLC4A1	Exon17	c.2173A > G	p.S725G	Inherited (M)	0/0	D	P	D	D	—	25	—	3-VUS (PM1,PM2,PP3, PP4)
	Het	SPTA1	Exon2	c.224 T > C	p.I75T	Inherited (F)	6e-04/ 3.487e-04	T	B	N	D	—	11.59	—	3-VUS (PM1,BS2)
	Het	G6PD	Exon7	c.766A > G	p.I256V	Inherited (M)	0/0	D	P	D	D	—	24.1	—	4-LP (PM1,PM2,PP3,PP4)
37	Het	SPTA1	Exon23	c.3190 T > C	p.Y1064H	Inherited (F)	2e-04/ 1.446e-04	D	D	N	D	0.8655	26.4	—	4-LP (PM1,PM2,PP3,PP4, BS2)
	Het	SPTA1	Exon7	c.833A > G	p.Q278R	Inherited (M)	0/ 8.032e-06	T	B	N	N	—	13.08	—	3-VUS (PM1,PM2,PP4, BP4)
38	Het	SPTA1	Exon33	c.4690 T > A	p.S1564T	Inherited (M)	4e-04/ 1.928e-04	T	B	N	D	—	14.65	—	3-VUS (PM1,PP4,BS2)
	Het	SPTA1	Exon2	c.224 T > C	p.I75T	Inherited (F)	6e-04/ 3.487e-04	T	B	N	D	—	11.59	—	3-VUS (PM1,BS2)
39	Het	SPTA1	Exon33	c.4698dupT	p.E1567*	Inherited (F)	0/0	—	—	—	—	—	—	—	5-P (PVS1,PM2,PP4)
40	Het	SPTA1	Exon41	c.5764_5776del	p. L1922Tfs*15	—	0/0	—	—	—	—	—	—	—	5-P (PVS1,PM2,PP4)
41	Het	SPTA1	Exon19	c.2671C > T	p.R891*	—	0/ 1.203e-05	—	—	N	A	—	35	CM148356; 32,266,426	5-P (PVS1,PM2,PP4,PP5)

Transcript: ANK1 (NM_000037); SPTB (NM_001355436); SLC4A1 (NM_000342); SPTA1 (NM_003126); EPB42 (NM_001114134); ABCG5 (NM_022436); CDAN1 (NM_138477); ATP7B (NM_000053); MMACHC (NM_015506); G6PD (NM_001360016).

Het: heterzygous; Homo: homozygous; Inherited (M): inherited from maternal allele; Inherited (F): inherited from paternal allele; P: pathogenic; LP: likely pathogenic; VUS: variant of unknown significance.

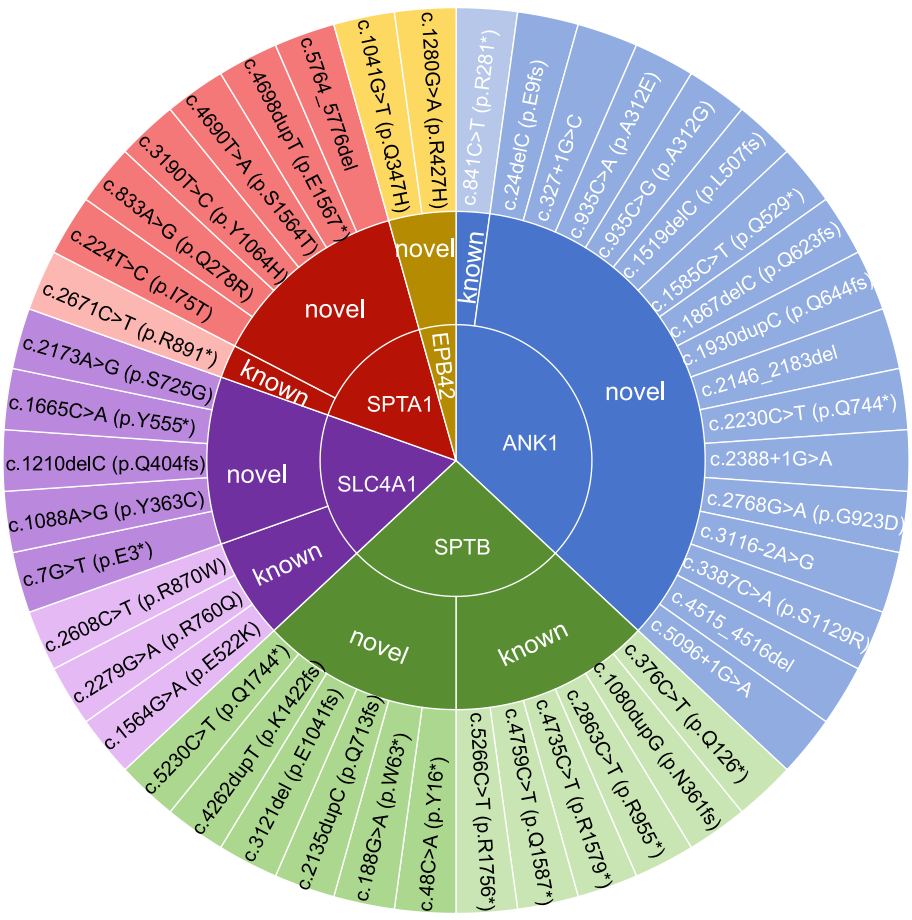

Fig. 1. Pie diagram overview of variations identified in *ANK1*, *SPTB*, *SLC4A1*, *SPTA1* and *EPB42* genes (novel as well as known).

3.3. Genotype-phenotype analysis of HS patients

In order to explore the underlying complication factors of clinical manifestations in HS patients, we next performed genotype-phenotype analysis to study the influence of mutated genes as well as mutational patterns on patients' clinical parameters. As shown in Table 3, four groups of patients (with *ANK1*, *SPTB*, *SLC4A1* and *SPTA1* alterations, respectively) exhibited no significant difference in most of the parameters including age, gender, percentage of splenomegaly, and hemtological values like F-Hb level, WBC count, Hb, MCV, MCH, MCHC, Ret count, levels of bilirubin, and positive results of laboratory examinations such as EMA and OFT *etc*. However, we did find a signicant difference in PLT level, LDH level and Ret percentage ($p = 0.004$, 0.015 and 0.043, respectively). Specifically, by pairwise comparison patients with *ANK1* variants showed a remarkably higher level of platelet count than that with *SPTB* and *SLC4A1* variants ($p = 0.021$ and 0.02, respectively). Moreover, patients with *SPTB* variants exhibited substantially lower level of LDH compared with *ANK1* mutated patients ($p = 0.025$). However, Ret percentage showed no significant difference in pairwise comparison (data not shown).

Next, we were intriguing to decipher whether different patterns of variants may shed light on influence in clinical presentations. To answer this question we divided the patients into four groups based on their mutational patterns (missense, nonsense, frameshift and splicing group, respectively). All patients were included for analysis except for patients with *SPTA1* variants in order to remove interference of compound heterozygous variants. As shown in Supplemental Table 4, all above baseline clinical features and laboratory parameters did not differ except for WBC count. Specifically, pairwise comparison showed that patients with splicing variants had a notably higher level of WBC count than patients with frameshift variants ($p = 0.01$), but not between any other groups. Our genotype-phenotype analysis suggested that patients with different gene alterations or different mutational types may exhibit subtle changes in some of hematological or biochemical parameters like WBC, PLT and LDH levels, which may provide some prediction values for clinical use.

4. Discussion

In this study, we have deciphered disease-causing variants in forty-one HS patients and depicted their mutational spectrum. In summary, we have found 17 patients caused by *ANK1* alteration, 12 patients by *SPTB*, 7 patients by *SLC4A1* and 5 patients by *SPTA1* alteration. Among 43 pathogenic variants detected, 32 were identified as novel variants. The finding of pathogenic/likely pathogenic variants in other genes except for *ANK1*, *SPTB*, *SLC4A1* and *SPTA1* suggested an underlying possibility of disease occurrence in their offspring and was not surprising. Four out of forty-one patients were found to be accompanied with additional variants other than pathogenic HS variants, and thus whether or not the concomitant variants in any of the five genes participate in disease causing is not known and merits further investigation. Moreover, we have also found that the majority of disease-causing variants in *ANK1* and *SPTB* gene were LOF variants, whereas more than half of *SLC4A1* and *SPTA1* variants were missense variants, indicating the distinct role of different mutational types in specific genes. Interestingly, among eight families with pathogenic *ANK1* alteration, six cases were *de novo*, compared with 0/2 and 0/2 cases for patients with *SPTB* and *SLC4A1* mutations, suggesting the possibility that *de novo* mutation may prone to take place in *ANK1* gene than any other genes.

In five HS patients with *SPTA1* variants, two were found to have

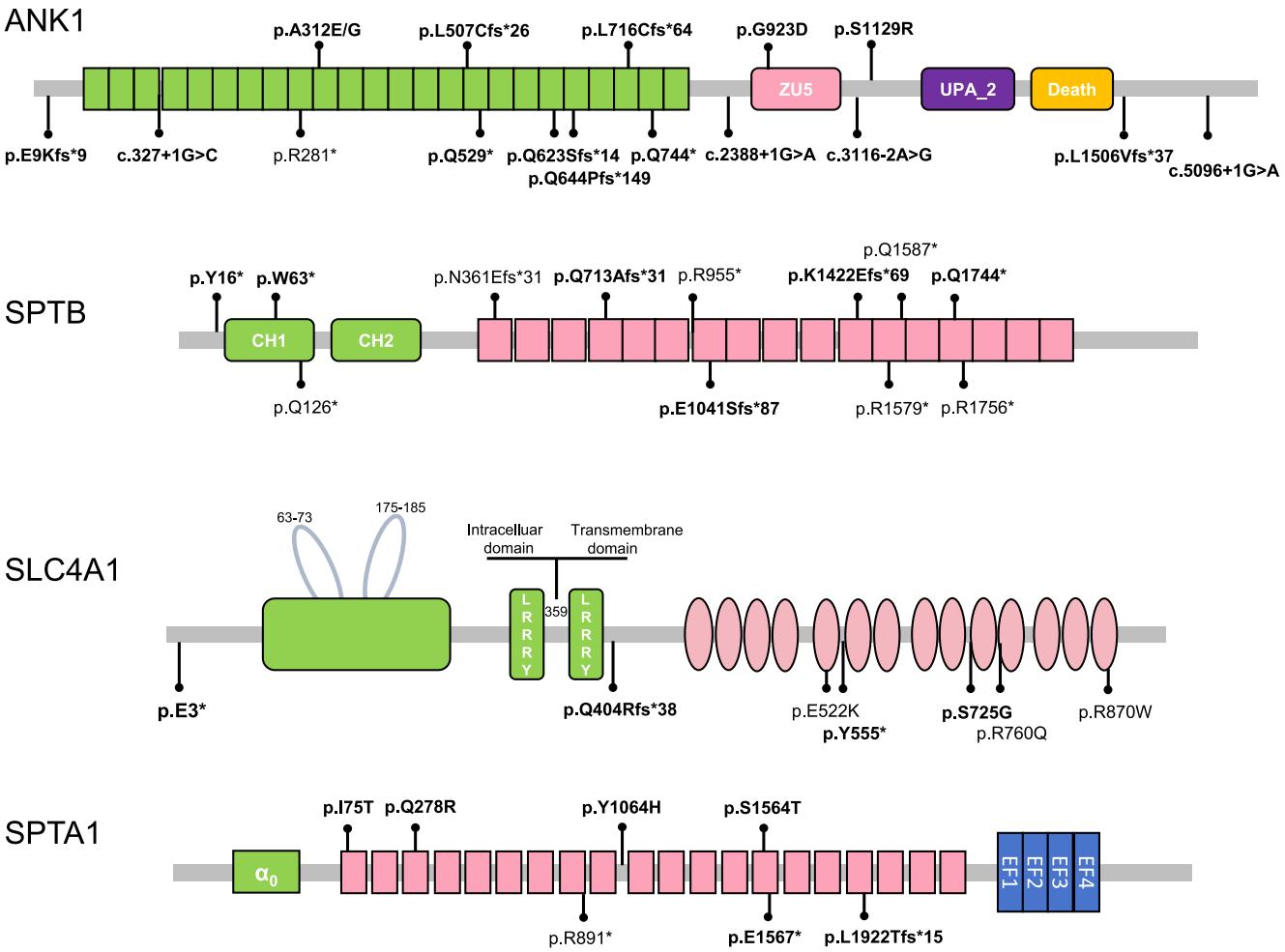

Fig. 2. Schematic representation of pathogenic variants identified in HS patients. Novel variants were depicted in bold. *ANK1* encodes erythroid ankyrin protein that consists of 24 ankyrin repeats (green box), a spectrin-binding domain ZU5 (pink box), a UPA_2 domain (violet box) and a C-terminal death domain (orange box). Repeats 7–24 contribute to band 3 binding. *SPTB* encodes erythroid β-spectrin protein that is comprised of 2 CH domains (green box) and 17 spectrin repeats (pink box). The CH domain is responsible for actin and protein 4.1R binding. The first 2 repeats contribute to dimerization while the last repeat contributes to tetramerization, and repeats 14 and 15 mediate ankyrin binding. *SLC4A1* encode band 3 and contains an intracellular domain and a transmembrane (TM) domain, the latter of which forms the anion exchange channel (consists of 14 TM segments; pink oval). *SPTA1* encodes α-spectrin protein that comprises 20 spectrin repeats (pink box) and specialized domains for self-association, protein 4.2 binding (blue box) and other proteins binding.

compound heterozygous variant, which was consistent with recessive inheritance. However, alteration in only one *SPTA1* allele were found in remaining three patients, which contradicted with the inheritance pattern and increased the difficulty for clinical diagnosis. One explanation for this is due to the lack of capability to identify the presence of low-expression alleles such as α^{LELY} which carries alterations simultaneously in exon 40 (c.5572C > G) and intron 45 (c.6531-12C > T) and leads to exon 46 (18 bp) skipping in half of the α^{LELY} transcripts [19–21]. The corresponding missing of 6 amino-acids in α-chains hinders the initiation of dimerization process [22]. Although the exon 40 variation and intron 45 variation were found to be heterozygous in all three patients (data not shown), whether the two variants coincided in the same allele (in cis) were not known. A study from Brizil demonstrated that the low expression α^{LELY} allele in trans with a null variant appeared to aggravate the phenotype even within a single family [23]. In contrary, Delaunay *et al* argued that the low expression α^{LELY} allele in trans to a null *SPTA1* allele did not cause HS because of sufficient supply of spectrin to meet the needs of the membrane [24]. Thus the conclusive role of α^{LELY} allele in trans to a null *SPTA1* variant in the severity of HS still merits further studies. Another low-expression polymorphism called α^{LEPRA} which carries a C to T transition in intron 30 (IVS30-99C > T) was not found in any three patients [25]. Consequently, whether participation of any low expression allele in disease severity or not is not known in this study. Interestingly, Wang *et al* reported two HS patients with one heterozygous *SPTA1* variant without either α^{LEPRA} or α^{LELY} allele, suggesting a likelihood of autosomal dominant inheritance [26]. Moreover, a group from Germany suggested a digenic cause of *SPTA1* due to the finding of only one *SPTA1* allele in concomitant with a missense variant in other genes like *EPB42* or *PIEZO1* in two patients [27]. Therefore, whether or not dominant or digenic transmission occurs in *SPTA1* is inconclusive and merits further study.

In our cohort, four patients (#6, #20, #21 and # 31) were complicated with Gilbert syndrome (GS) which was characterized by increased unconjugated bilirubin without hepatocellular disease or hemolysis. Although Gilbert syndrome is a benign condition present in 5–10% of the population, when in combination with other prevailing conditions such as thalassemia, spherocytosis, G6PD deficiency, or cystic fibrosis it can exacerbate severe hyperbilirubinemia and/or cholelithiasis [28,29]. The most common polymorphic variant in Caucasian and African GS populations is a homozygous TA-insertion in the TATA box A(TA)7TAA (also known as *UGT1A1**28) in *UGT1A1* gene [30,31]. Whereas in East Asian populations (Chinese, Japanese, and Korean), homozygous alteration of another variant *UGT1A1**6 (c.211G > A/p.G71R) maybe the main cause of GS [32,33]. However, in these four patients, except for

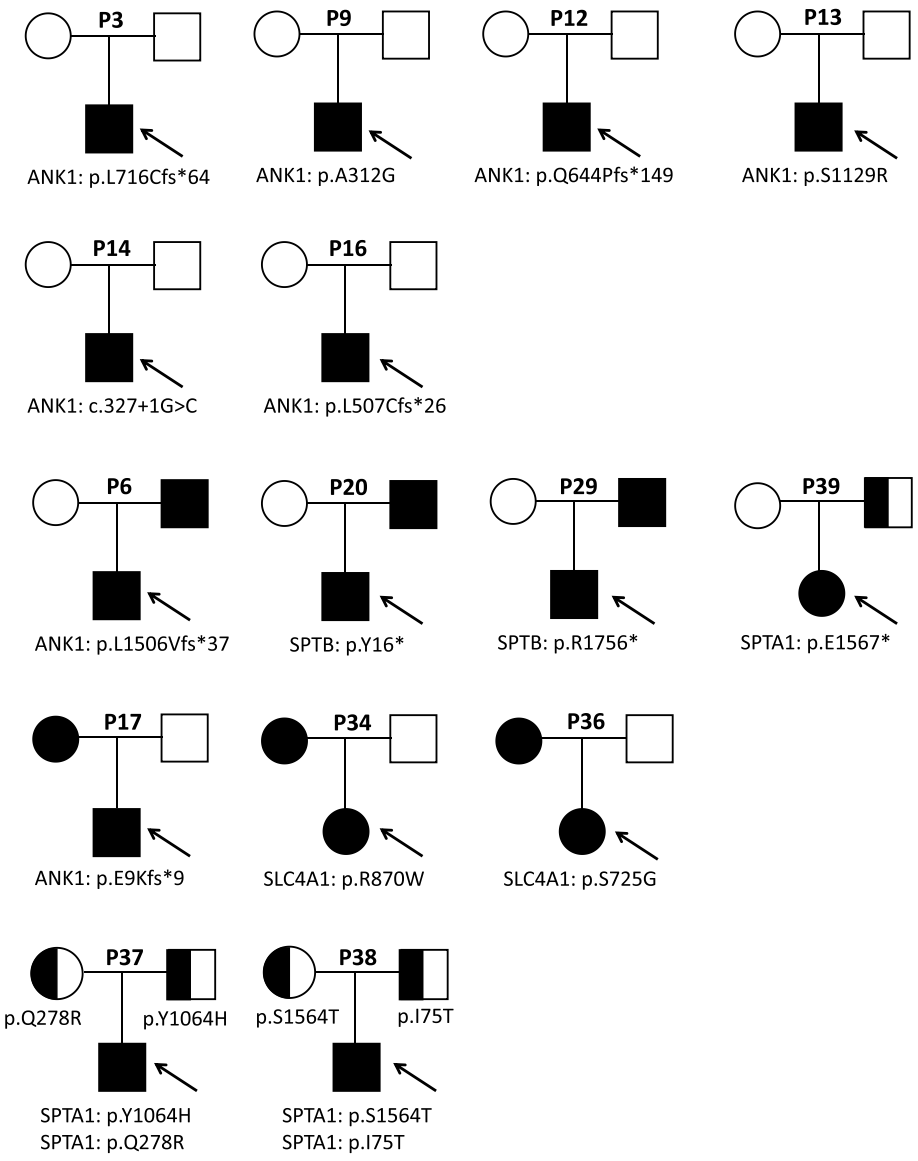

Fig. 3. Pedigree analysis of 15 families.

patient #21 in which we found a heterozygous *UGT1A1*6* variant (data not shown), we were unable to find any polymorphisms of *UGT1A1* including *UGT1A1*28* and any other known variants in the coding region. Therefore, we speculate that GS maybe miss-diagnosed in these patients due to the complication of HS condition. Another explanation for this is that GS may be caused by uncommon or unreported mechanism such as alterations in deep introns or gene rearrangement that we were unable to detect by WES, although this speculation is less likely to happen.

In this study, the sensitivity of EMA test and OFT test was 80.5% and 82.5%, respectively (see Supplemental Figures 3 and 4 for EMA graphs). The EMA binding assay utilizes flow cytometry to measure the fluorescence level from individual red blood cells (reflecting EMA-binding transmembrane proteins), and reports show that it has comparable specificity and sensitivity to AGLT and was superior to OFT [34–36]. According to other studies, a negative result of OFT test does not exclude the possibility of diagnosing HS and it may occur in 10–20% of cases, which was comparable to our results [37,38]. Although AGLT test is reported to have higher detection rate than OFT, the drawback of these two tests is that they apparently lack specificity in certain situations [37]. In addition to family history and clinical parameters such as blood count, hemolysis parameters, and blood smear, the guidelines suggest conducting HS specific testing to confirm diagnosis, which can be combined with either RBC fragility test (AGLT, OFT) and the EMA or, alternatively, ektacytometry can be chosen [39,40]. Osmotic gradient ektacytometry, another reference test for diagnosis of RBC membrane disorders, has not been widely employed in clinical laboratories and thus was not in regular use in this study. In our study, the combination of EMA with either OFT or AGLT showed a good sensitivity (95 % and 100 %, respectively).

In summary, our research detected pathogenic variations in 41 Chinese HS patients and found 35 novel variants of *ANK1*, *SPTB*, *SLC4A1*, and *SPTA1*. Our data revealed that *ANK1* and *SPTB* are the most prevalent genes mutated in Chinese population and that variations were widespread through the whole gene. Truncated variatns were predominant in *ANK1* and *SPTB* gene, whereas missense variations were most common in *SLC4A1* and *SPTA1* gene. Among 15 families available for pedigree analysis, 6 were found to have *de novo* alterations. All *de novo* variations were detected in *ANK1*, indicating a better chance of *de novo* inheritance in *ANK1* than others. Besides, patients with *ANK1* variants showed significant higher levels of platelet and LDH. We found no other correlation between the genotype and phenotype. HS patients

Table 3
Genotype-phenotype analysis according to genes mutated.

	ANK1 (n = 17)	SPTB (n = 12)	SLC4A1 (n = 7)	SPTA1 (n = 5)	p value[a]
Age (yrs), median (range)	23 (3–65)	33.5 (4–69)	42 (25–69)	35 (6–65)	0.288
Gender, male (%)	11/17 (64.7 %)	7/12 (58.3 %)	1/7 (14.3 %)	2/5 (40 %)	0.152[b]
Jaundice (%)	16/16 (100 %)	11/12 (91.7 %)	6/6 (100 %)	4/4 (100 %)	0.579[b]
Splenomegaly (%)	16/17 (94.1 %)	12/12 (100 %)	6/6 (100 %)	4/4 (100 %)	1.0[b]
Splenectomy (%)	3/5 (60 %)	0/6 (0)	0/6 (0)	–	0.015[b]
Cholecystitis/cholecystolithiasis (%)	9/12 (75 %)	8/11 (72.7 %)	5/6 (83.3 %)	1/3 (33.3 %)	0.539[b]
Cholecystectomy (%)	3/12 (25 %)	3/8 (37.5 %)	2/6 (33.3 %)	0/3 (0)	0.777[b]
F-Hb (mg/L), median (range)	120 (40.1–346.5)	96.05 (11.7–179)	75 (18.9–224.7)	82 (43.7–190.5)	0.679
WBC ($\times10^9$/L), median (range)	6.74 (3.57–16.71)	6.86 (3.18–14.51)	6.42 (2.65–8.21)	5.79 (3.97–6.09)	0.305
RBC ($\times10^{12}$/L), median (range)	2.85 (1.61–5.24)	2.75 (1.91–5.14)	3.02 (2.73–3.91)	3.02 (2.58–3.62)	0.671
Hb (g/dL), median (range)	92 (51–161)	89.5 (58–156)	95 (84–137)	89 (68–107)	0.646
PLT ($\times10^9$/L), median (range)	230 (120–1673)	168.5 (26.7–273)	146 (70–196)	199 (170–213)	**0.004**
HCT (%), median (range)	27.9 (16.2–44.3)	25.5 (17.4–43.7)	27.6 (25.3–35.8)	26.8 (20.1–30.6)	0.804
MCV (fL), median (range)	93 (81.5–110.4)	91.3 (80.1–99.7)	90.3 (88.1–101.1)	83.4 (77.1–101.5)	0.533
MCH (pg), median (range)	31.7 (27.4–37.9)	30.4 (24.9–34.7)	31.8 (29.5–35)	28.7 (26.4–35.3)	0.462
MCHC (g/L), median (range)	337 (306–367)	337.5 (309–376)	352 (324–383)	338 (332–373)	0.701
Ret (%), median (range)	17.07 (5.04–30.76)	11.035 (2.89–22.79)	12.95 (5.34–18.61)	8.77 (3.02–19.6)	**0.043**
Ret# ($\times10^{12}$/L), median (range)	0.458 (0.2641–0.9105)	0.3597 (0.1049–0.7202)	0.4013 (0.1613–0.5043)	0.2263 (0.1093–0.5174)	0.147
RDW-CV (%), median (range)	21.8 (13.5–29)	20.15 (18.2–24)	19.3 (16.9–22.5)	18.8 (15.9–24.3)	0.207
RDW-SD (fL), median (range)	68.6 (41.9–97.2)	65.3 (55.2–78.1)	63.3 (53.8–71.2)	60.8 (45.2–73.6)	0.209
T-Bil (μmol/L), median (range)	89.95 (33.6–285.6)	79.4 (27.7–170.8)	77.15 (45.9–118.6)	62.2 (53.4–79.4)	0.732
D-Bil (μmol/L), median (range)	13.1 (7–26.7)	11.65 (6.7–23.4)	11.4 (7.8–14)	10.4 (10.1–11.4)	0.593
I-Bil (μmol/L), median (range)	78.05 (26.3–268.5)	67.75 (21–152.8)	65.5 (37.9–106.6)	52.1 (43–68)	0.768
LDH (U/L), median (range)	333.65 (211.7–452)	215 (163.5–304.1)	216.9 (180.9–367.3)	255.9 (217.2–593.1)	**0.015**
AGLT50, positive (%)	16/16 (100 %)	12/12 (100 %)	5/6 (83.3 %)	5/5 (100 %)	0.282[b]
EMA, positive (%)	12/17 (70.6 %)	11/12 (91.7 %)	7/7 (100 %)	3/5 (60 %)	0.178[b]
OFT, positive (%)	14/16 (87.5 %)	10/12 (83.3 %)	5/7 (71.4 %)	3/5 (60 %)	0.484[b]

F-Hb: free hemoglobin; WBC: white blood cell; RBC: red blood cell; Hb: hemoglobin; PLT: platelet; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration; Ret: reticulocyte; RDW: red blood cell distribution width; T-Bil: total bilirubin; D-Bil: direct bilirubin; I-Bil: indirect bilirubin; LDH: lactate dehydrogenase; AGLT50: acidified glycerol lysis test; EMA: eosin maleimide test; OFT: osmotic fragility test.
a: Kruskal-Wallis test.
b: Fisher's exact test.

may be underestimated in the past, but with the advent and development of high-throughput NGS technology, more patients will be discovered and distinguished. Thus molecular testing can provide a definitive diagnosis, while pathogenic detection can provide further reference for genetic counseling and future research directions.

CRediT authorship contribution statement

Li Qin: Writing – original draft, Methodology, Formal analysis, Conceptualization. **Yujiao Jia:** Supervision, Methodology. **Haoxu Wang:** Visualization. **Yuan Feng:** Validation. **Junyan Zou:** Validation. **Jianfeng Zhou:** Methodology. **Changshun Yu:** Software, Data curation. **Bingqing Huang:** Data curation. **Ruixue Zhang:** Methodology, Data curation. **Lihui Shi:** Methodology. **Jigang Xiao:** Methodology. **Yuping Zhao:** Methodology. **Qi Sun:** Supervision. **Zhijian Xiao:** Supervision, Funding acquisition. **Huijun Wang:** Supervision, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cca.2024.119989.

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