ORIGINAL ARTICLE

Molecular Genetics & Genomic Medicine
Open Access WILEY

Clinical manifestation and phenotypic analysis of novel gene mutation in 28 Chinese children with hereditary spherocytosis

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Abstract

Purpose: Objective to summarize the clinical features and laboratory findings of 28 Chinese children with hereditary spherocytosis (HS), and analyze these mutations.

Method: Collected and analyzed the clinical data of all children and their parents, and completed the relevant laboratory examinations of all children. Analyzed the sequence of related genes by second-generation sequencing technology, and verified the suspected mutations by Sanger sequencing method. Analyzed all biological information using the Single Nucleotide Polymorphism database, the 1000 Human Genome Project, and the Exosome Aggregation Consortium.

Result: New mutations were detected in the HS coding region of 28 children. Among them, there were 13 cases (46.4%) with *ANK1* mutation, 10 cases (35.7%) with *SPTB* mutation, three cases (10.7%) with *SLC4A1* mutation, and two cases (7.2%) with *SPTA1* mutation. All mutations cause amino acid changes in the coding gene, as well as subsequent changes in protein structure or loss of function.

Conclusion: All the newly discovered gene coding region mutation sites detected are the suspected pathogenic causes of the 28 Chinese children. At the same time, the second-generation gene sequencing technology is an effective means to diagnose HS. Different mutation types and different mutation regions have no significant correlation with the severity of anemia. The novel gene mutation sites in 28 children studied in this paper have not yet been included in the human genome database, dbSNP (v138), or ExAC database. The new gene mutations found in HS children can provide a theoretical basis for further exploring the genetic causes of HS in Chinese children.

KEYWORDS
ANK1, children, gene mutation, hereditary spherocytosis, *SLC41*, *SPTA1*, *SPTB*

1 | INTRODUCTION

Hereditary spherocytosis (HS) is a kind of hemolytic anemia with jaundice, hemolysis, and splenomegaly (Butorac et al., 2018; He et al., 2018) as the main clinical features, caused by changes in the structure of red blood cell membrane proteins. This disease occurs all over the world, with different incidences in different regions. Studies have shown that the incidence of HS is about 1/2000 (Perrotta et al., 2008) in Northern Europe and 1/5000 (Jung, 2013) in South Korea. In China, the incidence of HS in adults is about 1/100,000 (Wang et al., 2015), and there has been no clear incidence reported in Chinese children. The clinical manifestations of the disease vary in children with different degrees. Mild children may have no clinical symptoms, while severe children may exhibit severe anemia, jaundice, splenomegaly, and gallstones (King et al., 2015). Some children with severe anemia often need repeated blood transfusions to maintain normal life and activities, which is a heavy burden for families and society (Hassoun & Palek, 1996). At present, splenectomy is still the main treatment for moderate to severe HS. According to the study, about 10% of children require total splenectomy (Pugi et al., 2018). Laparoscopic splenectomy can relieve clinical symptoms and improve blood indicators in children with HS.

The HS-related mutant genes are currently believed to be mainly *SPTA1* (1q21, OMIM 182860), *SPTB* (14q23.3, OMIM 182870), *ANK1* (8p11.21, OMIM 612641), *SLC4A1* (17q21.31, OMIM 109270), and *EPB 42* (15q15.2, OMIM 177070). *ANK1* and *SPTB* gene mutations are the most common (Park et al., 2016), which are also the main mutation genes in Chinese HS patients (Wang et al., 2018). In terms of heredity, HS is mainly autosomal dominant inheritance, but there are also some related reports about autosomal recessive inheritance (Iolascon et al., 2008).

In recent years, with the rapid development of post-gene sequencing technology and its application in medical testing and clinical practice, clinical gene sequencing technology has gradually shifted from first-generation sequencing to second-generation sequencing (Koboldt et al., 2013), and this technology has also been used in the detection of hereditary anemia. Since the phenotype of HS varies greatly among different patients, traditional laboratory examination methods have many factors affecting it, and it is impossible to screen all HS patients. Therefore, genetic testing technology provides a more accurate means for determining the cause of HS (Farias, 2017). This study used the second-generation gene sequencing technology to detect 271 anemia-related sites in children with clinically suspected congenital anemia. HS new mutation sites were detected in twenty-eight Chinese children and were verified using the first-generation sequencing technique. The results of gene testing were consistent with clinical symptoms and traditional laboratory tests, demonstrating the effectiveness, accuracy, and convenience of this gene testing method in HS diagnosis of children.

1.1 | Objectives and methods

This study was approved by the Ethics Committee of the First Affiliated Hospital of the Naval Military Medical University (CHEC2020-115). Written consent was obtained from all guardians of child patients prior to the clinical evaluation and blood sample collection.

1.2 | Patient information

A retrospective analysis of all inpatients in the Department of Pediatrics of the First Affiliated Hospital of Naval Military Medical University from May 2016 to March 2020 included 28 children diagnosed with HS. We analyzed the clinical manifestations, physical examination, family history, and relevant laboratory test results of the abovementioned children. All the children came from different families and had no blood relationship. All children completed blood routine (including red blood cell morphology microscopy and reticulocyte count), urine routine, blood biochemistry (including total bilirubin, direct, and indirect bilirubin), gallbladder, liver, and spleen ultrasound, AGLT50 test, infiltration Test, G-6-PD and PK enzyme examination, hemoglobin electrophoresis, and autoantibody examination. All children received gene sequencing. The diagnosis of HS was based on the literature standard (Bolton-Maggs et al., 2012).

2 | METHODS

2.1 | Second-generation gene sequencing

Took 2 ml of venous blood from the child, and used DNA extraction kit (product of Beijing Tiangen Biochemical Technology Co., Ltd.) and BloodGen Midi kit (CWBIO, China) to extract the patient's whole genome DNA. Established A DNA library according to the GenCap custom kit (Product of Beijing Mygeno Gene Co., LTD.), and screened out 271 congenital anemia-related genes (including five erythrocyte membrane protein coding genes related to the pathogenesis of HS) using gene capture technology. Use a biotin-labeled capture probe (80–120-mer) to cover all exons in non-repetitive regions. The average gene coverage of the target area was 94.86%, the average sequencing depth was 436.75×, the coverage of 90.14% of the target area was >30×, and the coverage of 60.29% of the target area was >200×. Sequence the enriched DNA library through the Illumina HiSeq sequencing platform. Combined the

TABLE 1 Clinical manifestations and routine laboratory findings of 28 children with newly diagnosed HS mutations in China

Clinical characteristics	Total (*n* = 28)	Mild (*n* = 6, 21%)	Moderate (*n* = 9, 32%)	Severe (*n* = 13, 47%)
Males, *n* (%)	15 (54)	5 (83)	3 (33)	7 (54)
Age (years); median (range)	3.5 (0.1–10.2)	7.4 (0.7–9.4)	1.3 (0.1–8.6)	3.1 (0.2–10.2)
Family history, n (%)	19 (68)	6 (100)	4 (44)	9 (69)
Hemoglobin (g/L), median (range)	61.5 (39–103)	95.5 (90–103)	71 (61–87)	53 (39–60)
MCV (fl), median (range)	84.8 (59–110.5)	81.8 (59–88)	86.5 (70–110.5)	84.9 (76.6–100.8)
MCHC (%), median (range)	327.5 (259–359)	354.5 (317–359)	329 (259–341)	323 (277–355)
Reticulocytes (%), median (range)	10.38 (0.23–25.07)	5.74 (0.23–19.63)	12.8 (7.23–20.78)	10.3 (1.6–25.07)
Total bilirubin (μmol/L), median (range)	44.3 (5.3–168.9)	40.5 (5.3–85.4)	38.8 (17.3–106.9)	47.3 (23.3–168.9)
Splenomegaly, *n* (%)	16 (57)	2 (33)	6 (67)	8 (62)
Neonatal jaundice, *n* (%)	18 (64)	3 (50)	5 (56)	10 (77)
Transfusion, *n* (%)	21 (75)	0 (0)	8 (89)	13 (100)

Abbreviations: MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume.

sequencing results with the single nucleotide polymorphism (SNP) database (http://www.ncbi.nlm.nih.gov/projects/SNP/), 1000 Human Genome Project (www.1000genomes.org), and Exome Aggregation Consortium database (http://exac.broadinstitute.org/) for comparison.

2.2 | Sanger sequencing

For the HS-related gene mutations detected by second-generation sequencing, the sites with frequencies lower than 0.01 in the 1000 Genomes and ExAC databases and whose functions are predicted to be harmful were confirmed by Sanger sequencing. Obtained the genomic DNA of the patient's parents for Sanger sequencing. Carried out specific PCR amplification of the sample DNA and purification of the PCR products and sequenced the products by ABI 3730XL sequencer using the terminator cycle sequencing method. Compared the DNA sequence with the corresponding GenBank (www.ncbi.nlm.nih.gov) reference sequence to identify the mutation site. The gene reference sequence transcripts were NM_001142446 (ANK1), NM_001024858 (SPTB), NM_003126 (SPTA1), NM_000342 (SLC4A1), and NM_000119 (EPB42).

2.3 | Pathogenicity prediction of mutation sites and analysis of protein function impact

Applied *Protein Variation Effect Analyzer* (http://provean.jcvi.org/index.php), *Sorting Intolerant from Tolerant* (http://sift.jcvi.org), *PolyPhen-2* (http://genetics.bwh.harvard.edu/pph2), and *Mutation Taster* (http://Mutationtaster.org) for predictive analysis of protein and structural hazards.

For the prediction of gene effect on protein structure, we use the following three-dimensional protein structure model to forecast (http://www.proteinmodelportal.org).

2.4 | Statistical methods

Used SPSS 22.0 for statistical analysis. Described non-normal measurement data as median (range) and count data as percentage. Used the Kruskal-Wallis H test for comparison between groups. Considered $p < .05$ to be statistically significant.

3 | RESULTS

3.1 | Clinical characteristics and laboratory findings of HS children in China

The clinical manifestations and laboratory results of 28 Chinese children with HS are shown in Table 1. Among all the children, there were 15 males (54%) and 13 females (46%), all of whom were unrelated. Twenty-three cases (23/28, 82%) were onset within 1-year-old. However, the median age of HS diagnosis was 3 years and 6 months. In routine laboratory examinations, the median percentage of reticulocytes in all children was 10.38% (one of the children with severe anemia had a reticulocyte ratio of 1.6%, and he had undergone blood transfusion before admission to the hospital, which is considered to be affected). The median total bilirubin level was 44.3 μmol/L. Except for one child with a recent blood transfusion, which was classified according to the lowest hemoglobin level in the past, the other 27 children who had no blood transfusion more than 30 days before admission were classified according to the

TABLE 2 Gene mutation spectrum of 28 children with newly diagnosed HS mutations in China

Patients	Sex	Age	Inheritance	Gene	Location	cDNA change	Protein change	Type
1	Male	5 years and 3 months	De novo	ANK1	Exon 4	c.399Thr>Gly	p.Tyr133X,1765	Nonsense
2	Female	2 months	Hereditary	ANK1	Exon 17	c.1914_1918delTTTGC	p.Pro638Profs*14	Frameshift
3	Male	8 months	De novo	ANK1	Exon 14	c.1564delC	p.Leu522Cysfs*44	Frameshift
4	Female	3 months	Hereditary	ANK1	Exon 37	c.4439dupA	p.Asn1480Kfs*20	Frameshift
5	Male	9 years and 2 months	Hereditary	ANK1	Exon 37	c.4510_4513del	p.Asn1504Trpfs*17	Frameshift
6	Female	7 years and 11 months	Hereditary	ANK1	Exon 27	c.2961delC	p.T988P fs*30	Frameshift
7	Female	8 years and 11 months	Hereditary	ANK1	Exon 18	c.2142dupT	p.Pro715Serfs*111	Frameshift
8	Male	8 years and 7 months	Hereditary	ANK1	Intron 26	c.2858+1G>C	—	Splicing error
9	Female	4 months	De novo	ANK1	Exon 28	c.3235delG	p.Glu1079fs	Frameshift
10	Male	4 years and 1 month	Hereditary	ANK1	Exon 39	c.4739A>G	p.Gln1580Arg	Missense
11	Female	7 years	De novo	ANK1	Exon 25	c.2638-2 A>G	—	Splicing error
12	Male	3 months	De novo	ANK1	Exon 27	c.2926C>T	p.Arg976Ter	Nonsense
13	Male	3 years and 1 month	Hereditary	ANK1	Exon 34	c.4153C>T	p.Arg1385X	Nonsense
14	Male	7 years and 5 months	Hereditary	SPTB	Exon 7	c.876_880del	p.Lys292Asnfs*2	Frameshift
15	Female	4 years	De novo	SPTB	Exon 18	c.3866G>A	p.Trp1289X,1040	Nonsense
16	Female	2 years and 3 months	Hereditary	SPTB	Exon 22	c.4885C>A	p.Gln1629Ter	Nonsense
17	Male	8 years	Hereditary	SPTB	Exon 15	c.3103C>T	p.Arg1035Trp	Missense
18	Male	2 years and 8 months	Hereditary	SPTB	Exon 15	c.3471G>A	p.Trp1157Ter	Nonsense
19	Male	1 year and 3 months	De novo	SPTB	Exon 3 het	c.376C>T	p.Gln126X	Nonsense
20	Female	1 month 18 days	Hereditary	SPTB	Exon 26 het	c.5530 G>T	p.Glu1844 X	Nonsense
21	Female	9 years and 6 months	Hereditary	SPTB	Exon 19	c.4105A>G	p.Lys1369Glu	Missense
22	Male	5 months	Hereditary	SPTB	Exon 11	c.1249C>T	p. Gln417X	Nonsense
23	Male	8 months	Hereditary	SPTB	Exon 29	c.5970G>C	p.Arg1900Ser	Missense
24	Male	10 years and 2 months	De novo	SLC4A1	Exon 16	c.2423G>A	p.Arg808His	Missense
25	Female	1 year and 1 month	Hereditary	SLC4A1	Exon 12	c.G1388G>A	p.Gly463Asp	Missense
26	Female	6 years and 11 months	De novo	SLC4A1	Exon 16	c.1979C>T	p.Pro660Leu	Missense
27	Male	4 years and 1 month	Hereditary	SPTA1	Exon 1	c.82C>A	p.Arg28Ser	Missense
28	Female	4 months	Hereditary	SPTA1	Exon 30	c.4418C>T	p.Thr1473Met	Missense

De novo, no family history with parents' genetic study.

hemoglobin level at the time of admission, and the children with mild, moderate, and severe anemia were 6/28 (21%), 9/28 (32%), and 13/28 (47%). Eighteen cases (18/28, 64%) had a history of neonatal jaundice, but this was not related to the severity of anemia in the future. Twenty-one cases (21/28, 75%) had a history of blood transfusion; 16 cases (16/28, 57%) had splenomegaly. All children had normal G6PD and PK test results, no abnormal hemoglobin electrophoresis, increased osmotic fragility, shortened AGLT50 test time, and negative Coombs test results.

3.2 | Gene mutation characteristics in Chinese children with HS

The characteristics of novel gene mutations in 28 Chinese children with HS are detailed in Table 2. Among the 28 children, 15 were males (54%) and 13 were females (46%). There was no significant difference in the gender of onset. Nineteen cases (68%) had a family genetic history, 11 cases (58%) had mutations from mothers, and eight cases (42%) were from fathers, and all the above mentioned parents had a history of anemia and HS-related clinical manifestations. The remaining nine cases (32%) were spontaneous mutations with no family genetic history. Among all mutation types, there were eight cases of frameshift mutation, two cases of splice site mutation, nine cases of nonsense mutation, and nine cases of missense mutation. None of these children's mutation sites have been included in the genome database, dbSNP (v138), or ExAC database, neither is there any relevant literature report.

3.3 | Comparison of clinical symptoms in children with different types of membrane protein gene mutations

The clinical symptoms of children with different types of membrane protein gene mutations are shown in Table 3. Among the 28 Chinese children with novel HS gene mutations, nonsense mutation and missense mutation were the most common, with nine cases (32%) and nine cases (32%), respectively, followed by a frameshift mutation in eight cases (29%) and splice site mutation in two cases (7%). In terms of the degree of anemia corresponding to different mutation types, the median hemoglobin concentration of missense mutations, nonsense mutations, frameshift mutations, and splice site mutations were all moderate, which may be related to the relatively small number of cases and patients in this study and the limitations of the patients, and need to be further explored in the follow-up study on Chinese children's HS.

3.4 | Distribution characteristics of novel mutation sites in 28 Chinese children with HS in *ANK1*, *SPTB*, *SLC41*, and *SPTA1* anchoring areas

The distribution of novel ANK1 mutation sites in the *ANK1* anchoring area in 13 Chinese children is shown in Figure 1. Of these, four cases of novel mutations were in the N-terminal region, five cases were in the central structure domain, and four were in the C-terminal regulatory domain. Among the 13

TABLE 3 Comparison of clinical symptoms in children with different membrane protein gene mutation types

Clinical characteristics	Total (*n* = 28)	Frameshift (*n* = 8, 29%)	Splicing error (*n* = 2, 7%)	Missense (*n* = 9, 32%)	Nonsense (*n* = 9, 32%)
Males, *n* (%)	15 (54)	3 (38)	1 (50)	5 (56)	6 (67)
Age (years); median (range)	3.5 (0.1–10.2)	4.1 (0.2–9.2)	8.0 (7.3–8.6)	4.1 (0.3–10.2)	2.1 (0.1–5.3)
Family history, *n* (%)	19 (68)	6 (75)	1 (50)	7 (78)	5 (56)
Hemoglobin (g/L), median (range)	61.5 (39–103)	67 (53–102)	64 (56–71)	60 (39–103)	56 (39–99)
MCV (fl), median (range)	84.8 (59–110.5)	84.2 (70–86.5)	91.6 (90.2–92.9)	88 (59–110.5)	81.2 (76.6–102.2)
MCHC (%), median (range)	327.5 (259–359)	335 (323–359)	324 (307–341)	317 (259–357)	324 (277–351)
Reticulocytes (%), median (range)	10.38 (0.23–25.07)	11.28 (7.23–19.36)	22.15 (19.23–25.07)	4.86 (0.23–15.9)	12.08 (5.39–20.78)
Total bilirubin (μmol/L), median (range)	44.3 (5.3–168.9)	53.1 (28.3–67.9)	46.5 (17.3–75.6)	35.9 (5.3–168.9)	74.6 (22.8–111.8)
Splenomegaly, *n* (%)	16 (57)	4 (50)	2 (100)	4 (44)	6 (67)
Neonatal jaundice, *n* (%)	18 (64)	3 (38)	2 (100)	5 (56)	8 (89)
Transfusion, *n* (%)	21 (75)	5 (63)	2 (100)	5 (56)	9 (100)

Abbreviations: MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume.

novel *ANK1* mutations, seven cases of frameshift mutations were the most common (54%), followed by three nonsense mutations cases (23%), two splicing site mutations (15%), and one missense mutation (8%). According to the degree of anemia, there were 4/4 (100%), 4/5 (80%), and 3/4 (75%) in children with moderate to severe anemia from the N-terminal domain, the central domain to the C-terminal regulatory region. In this study, there was no significant difference in the distribution of *ANK1* mutations and anemia severity in different regions ($p = .602$).

The distribution of *SPTB* mutation sites in the *SPTB* anchoring area in 10 Chinese children is shown in Figure 2. One of the novel mutations was in the actin-binding domain, two novel mutations were in the dimerization domain, and seven were in the spectrin repeats domain. Among these 10 mutations, there was one case, six cases, and three cases of frameshift mutation, nonsense mutation, and missense mutation. There were three children (30%) with severe anemia.

The gene loci distribution of all children with novel mutations in *SLC4A1* and *SPTA1* is shown in Figures 3 and 4. In the three children with novel *SLC4A1* mutations, all mutation sites were located in the HCO3 region, all of which were missense mutations. According to the degree of anemia, one case (33%) was severe anemia. In two children with novel *SPTA1* mutations, all mutation sites were in the spectrin repeat region, all of them were missense mutations, and two cases (100%) were severe anemia.

4 | DISCUSSION

Hereditary spherocytosis is hemolytic anemia characterized by changes in the structure of red blood cell membrane proteins. Its clinical manifestations mainly include jaundice, hemolysis, hepatosplenomegaly, and gallstones. Statistics

in related articles in China show that it accounts for 84% (Jinying et al., 2005) of red blood cell membrane diseases. Genetically, about 75% of HS patients are autosomal dominant, and about 25% are autosomal recessive or novel mutations (Miraglia del Giudice et al., 2001; Perrotta et al., 2008; Tse & Lux, 1999). From onset age, HS can appear in any age group, but they are most common in newborns or childhood. The median age of onset is about 5.6 years old, and there is no significant difference in gender (Konca & Soker, 2015; Oliveira et al., 2012). In this study, the median age of onset of all children is 3.5 years old, earlier than reported in the literature. Considering that most patients with mild anemia did not pay attention to see a doctor or outpatient examination, this study was for inpatients, while moderate to severe anemia accounted for 79%, which was easier to detect early. We have also found that if the child has severe clinical manifestations in the infancy, it will be more conducive for us to make a clear diagnosis of children's HS in the early stage.

Literature reports that children with HS have a positive family history (Perrotta et al., 2008). Among the 28 children in this study, 19 (68%) had parents with the same mutation gene mutation site, and the parents with these mutation sites had similar clinical manifestations of anemia with the children. Some parents underwent splenectomy after diagnosis, and their condition improved after surgery. The median age of children with splenomegaly is 6.9 years old, and there is no case of splenomegaly in infancy. There was no gallstone formation found in any children during the examination. This may be related to the long-term chronic process of splenomegaly and gallstone formation.

Studies have shown that the sites associated with HS gene mutations are mainly located in the following genes: *ANKl*, *SPTB*, *SPTA1*, *EPB42*, and *SLC4A1*. *ANK1* mutation accounts for about 50% (An & Mohandas, 2008; Gundel et al., 2011; Yang et al., 2011) of all HS gene mutations, followed by
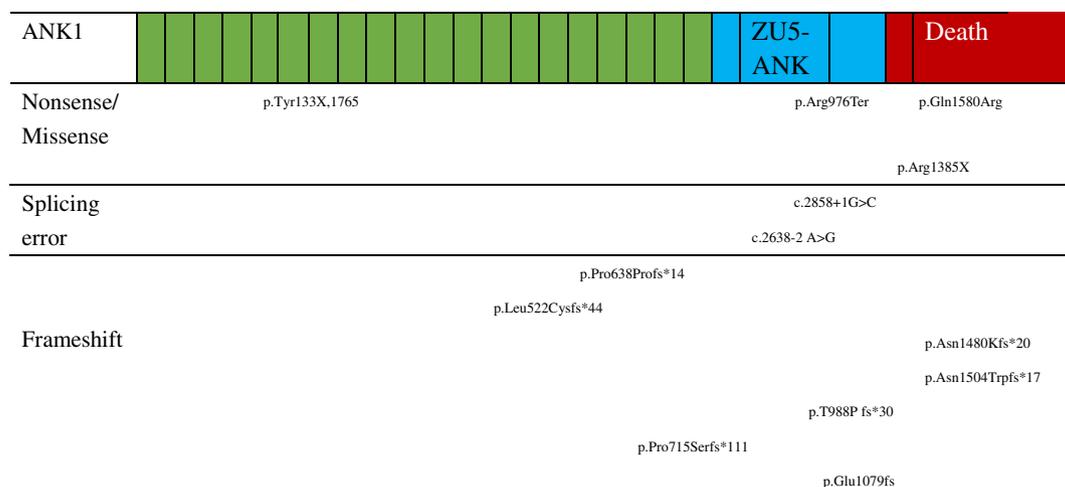
FIGURE 1 Schematic diagram of ANK1 anchoring domain. In the figure, the green region indicates the N-terminal domain, blue indicates the central domain, and red indicates the C-terminal regulatory region

SPTB	CH	CH	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Frameshift			p.Lys292Asn fs*2																
Nonsense			p.Gln126X		p. Gln417X						p.Trp1157Ter			p.Gln1629Ter				p.Glu1844 X	
											p.Trp1289X,1040								
Missense												Lys1369Glu						p.Arg1900Ser	
				p.Arg1035Trp															
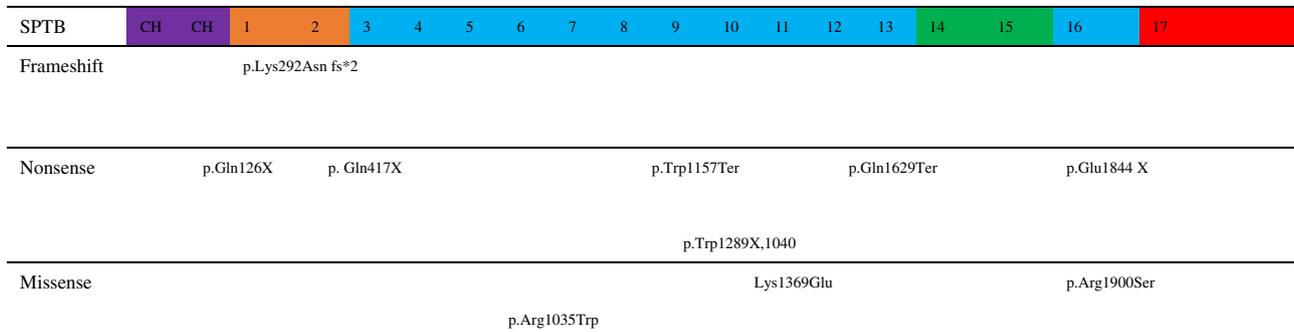
FIGURE 2 Schematic diagram of SPTB anchoring domain. In the figure, the purple region indicates the actin binding domain, orange indicates the dimerization domain, blue indicates the parts of spectrin repeats domain, green indicates the ankyrin binding domain, and red indicates the tetramerization domain

SLC4A1	AE	HCO3	AE
			p.Arg808His
Missense		p.Gly463Asp	
		p.Pro660Leu	
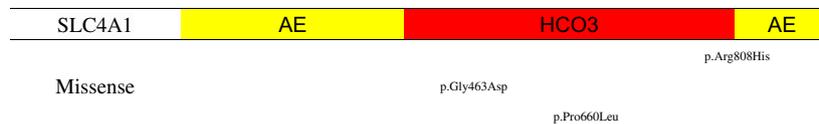
FIGURE 3 Schematic diagram of SLC4A1 anchoring domain. In the figure, the yellow region indicates the anion exchange protein domain, red indicates the HCO3-transporter famoly domain

SPTA1				
Missense	p.Arg28Ser		p.Thr1473Met	
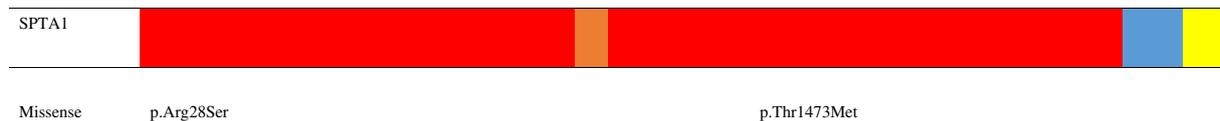
FIGURE 4 Schematic diagram of SPTA1 anchoring domain. In the figure, the red region indicates the spectrin repeats domain, orange indicates the src homology 3 domain, blue indicates the EF-hand domain, and yellow indicates the Ca^{2+} insensitive EF hand domain

SLC4A1 and *SPTB* gene mutations (Gre et al., 1982; Gallagher, 2005). The types of mutant genes in this study are *ANK1* (46%), *SPTB* (36%), *SLC4A1* (11%), and *SPTA1* (7%), which are consistent with previous reports. At present, there are few studies on children's HS mutant genes, most of them are case reports, and there is a lack of large sample studies (Min et al., 2016; Xiong et al., 2017; Xin et al., 2018). None of the 28 HS mutations found in this study have been included in thousands of human genome databases, dbSNP (v138), and ExAC databases. Further research is needed to explore the genetic causes and specificity of HS in Chinese children.

Structurally, the changes of red blood cell membrane proteins corresponding to different mutant genes are also different. The most common membrane proteins are ankyrin 1 (encoded by the *ANK1* gene) and spectrin β-1 (encoded by the *SPTB* gene), spectrinα-1 (encoded by the *SPTA1* gene), erythrocyte protein 4.2 (encoded by the EPB42 gene), and solute carrier family 4-1 (encoded by the *SLC4A1* gene) (Hughes et al., 2011; Iolascon et al., 2008).

The erythrocyte ankyrin encoded by the *ANK1* gene is expressed in the erythrocyte membrane by mainly connecting the anion channel, Rh complex, and contracted ovalbumin (Hughes et al., 2011). One end of ankyrin binds to the self-connection point of the contractile protein β chain tail, and the other end binds to the band 3 protein, which fixes the membrane skeleton in the lipid bilayer and plays an important role in stabilizing the red blood cell membrane (Ipsaro et al., 2009). The mutation of *ANK1* leads to changes in the structure of the ankyrin it encodes, which reduces the plasticity and stability of the red blood cell membrane, thus accelerating the dissolution and destruction of red blood cells (Barcellini et al., 2011). Of the 13 *ANK1* mutation cases in this article, the mutations in all children were harmful in the prediction of protein structure. The 3D model prediction indicated that there was a change in protein structure, and after the structure had changed, the stability of the red blood cell membrane structure decreased, and red blood cell destruction occurred.

Structurally, ankyrin usually consists of three domains: An N-terminal domain containing multiple ankyrin repeat sequences, a central region containing the blood chromatin-binding domain and a C-terminal regulatory domain. Studies have shown that patients with mutations in the central region have the most severe anemia (Park et al., 2016) compared with patients with mutations in other domains (patients with *ANK1* mutations in the spectrin-binding

domain have the most severe anemia). Among the 13 children with *ANK1* mutation reported in this paper, five cases of mutations are in the central region, of which four cases are moderate to severe anemia, which prove the severity of anemia after mutation in this region. However, in this study, children with moderate to severe anemia in the N-terminal domain and C-terminal regulatory domain accounted for 100% and 75% of all children with mutations, respectively. This result is different from the previous study and may be related to the ethnic specificity and regionality of Chinese children. It also needs to be confirmed by further expansion of the research sample.

There are currently more than 60 *ANK1* gene mutations associated with human HS (Wang & Quanfa, 2015). None of the 13 *ANK1* gene mutations reported in this paper has been reported. Combined with the related clinical manifestations of the children's parents with inherited mutations, the clinical manifestations and laboratory tests of all the children consistent with HS, and other causes excluded, the above novel mutation site can be considered as the pathogenic gene mutation site of these children. In this study, the proportion of children with moderate to severe anemia was higher than previously reported. This may be because most of what came to our hospital for treatment were children referred from other hospitals, and most of them suffered from severe disease early. There was no significant difference between the different types of mutations and the severity of anemia. Due to the limited number of cases, it is still necessary to collect cases in the future to further confirm whether this conclusion is true.

Previous reports have shown that *SPTB* gene mutations encoding β-spectrin account for about 15%-50% of all HS patients, and the main genetic pattern is autosomal dominant inheritance (Park et al., 2016; Perrotta et al., 2008). *SPTB* gene expresses a 246 kD spectrin subunit. Spectrin is composed of two similar α and β subunits. It consists of three domains: the CH structural calmodulin homologous domain, the anchoring protein repeat domain, and the C-terminal domain (Winkelmann et al., 1990). Mutations in the *SPTB* gene lead to failures in the formation of normal polymer structures, resulting in the loss of the support of skeleton proteins in the red blood cell membrane and inducing hemolytic anemia (Hughes et al., 2011). Previous studies have also shown that most *SPTB* gene mutations are frameshift mutations, nonsense mutations, or splicing mutations. There are only three cases of gene mutations in the *SPTB* gene with deletions of large fragments reported in the literature, which may be related to the inability of large fragment deletions to survive in the fetal period (Griswold et al., 2011; Hassoun et al., 1995; Lybaek et al., 2008). In the 10 cases of *SPTB* mutations in this study, there was no report of deletion of macromolecular fragments, which further verifies the fact that complete or partial deletion of macromolecular fragments is rare. In

this study, *SPTB* mutations account for 36% (10/28) of all HS mutations, and eight children (80%) with a family genetic history were in line with autosomal dominant inheritance characteristics. In terms of mutation types, the number of children with nonsense mutations, missense mutations, and frameshift mutations is six, three, and one, respectively. The prediction of protein structure and function in all the above mutation sites is harmful, and there is no report in the past.

The band 3 protein encoded by the *SLC4A1* gene, with a molecular weight of 102 KD, accounts for about 25% of membrane protein. It is the most numerous proteins on the erythrocyte membrane. The structure mainly contains the following three related domains: The hydrophilic N-terminal domain that serves as the anchor site for other membrane proteins, the hydrophobic cytoskeleton of 14 transmembrane fragments with anion exchange function, and the terminal domain (Ipsaro et al., 2009). Of the three children with novel *SLC4A1* mutations, all the mutation types were missense mutations and they were all located in HCO3 region. In the follow-up study of HS cases, we should further explore whether the distribution characteristics of mutation types and mutation regions have ethnic as well as racial characteristics.

For the *SPTA1* gene, missense mutations and splicing mutations are the most common types of mutations. The encoded protein is connected to the surface of the plasma membrane through band 3 protein and ankyrin, forming an important part of the red blood cell skeleton. Related reports suggest that *SPTA1* homozygous or compound heterozygous mutations may be the pathogenesis of recessive hemagglutinin-deficient HS. While in heterozygous individuals, sufficient α-hemagglutinin can still be produced to balance β-hemagglutinin and structurally maintain the stability of the red blood cell skeleton (Nussenzveig et al., 2014). Nevertheless, in HS case reports associated with the recessive form of inheritance caused by *SPTA1* mutations, the presence of two null allele mutations is considered fatal (Narla & Mohandas, 2017; Perrotta et al., 2008). Therefore, it is difficult for children with such mutations to survive in the fetal period. In the two novel *SPTA1* mutation cases studied in this article, all the mutation types are missense mutations, which are consistent with previous studies.

With the development and application of genetic testing technology in various genetic diseases in recent years, its higher sensitivity and accuracy in the detection of genetic diseases have also been proved to be more effective than common detection methods (Choi et al., 2019), and it has been gradually applied to the detection of hereditary red blood cell membrane diseases, including HS (He et al., 2017). At the same time, the second-generation gene sequencing technology provides an economical, efficient, rapid, and direct method for detecting complex and diverse genes, especially for patients (Lu et al., 2014) whose etiology cannot be determined by routine laboratory tests or whose blood

transfusions are frequent. And the high sequencing sensitivity of NGS up to 90% (Jun et al., 2019) also provides a more favorable guarantee for the definite diagnosis of diseases.

To sum up, in all 28 Chinese children with new HS gene mutations in this study, we can conclude that based on the synthesis of clinical manifestations, family genetic history, traditional laboratory tests, genetic testing, and protein harmfulness prediction results, the mutation sites in all these children are pathogenic mutation sites of theirs. Meanwhile, no mutation site of these children has been found in relevant gene banks or literature reports. We can think that these mutation sites are novel in these children with HS, and these sites have the characteristics of Chinese children with HS. The particularity also provides a theoretical basis for clinical practice such as the decision-making time of the follow-up corresponding to clinical treatment measures and genetic counseling for children with HS.

CONFLICT OF INTEREST
The authors declare that they have no competing interests.

AUTHOR CONTRIBUTIONS
FX, LL, and LZ performed the clinical examinations, and participated in the analysis and writing up of the manuscript. FX, BC, LG, YG, and XL did the clinical assessment and recruitment of the patients and their family members. LZ and JJ designed the study, analyzed the data, and wrote up the manuscript. All authors have been involved in revising the manuscript and have given approval of the final version.

DATA AVAILABILITY STATEMENT
The data generated and analyzed as part of the current study are not publicly available due to personal data information on the patients. Anonymized parts of the data can be retrieved from the corresponding author.

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