Genetic and Clinical Characteristics of Patients With Hereditary Spherocytosis in Hubei Province of China

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Hereditary spherocytosis (HS) is an inherited disorder characterized by anemia, splenomegaly, and spherical-shaped erythrocytes, caused by mutations in erythrocyte membrane Protein Genes (ANK1, SPTB, SLC4A1, SPTA1, and EPB42). We investigated molecular spectrum and genotype-phenotype correlation in HS patients in Hubei province, central China. Twenty-three patients with HS were included. A next-generation sequencing (NGS) panel targeting ANK1, SPTB, SLC4A1, SPTA1, and EPB42 genes was used to screen potential variants. Sanger sequencing was applied to validate variants. Of the twenty-three patients, thirteen patients carried ANK1 variants, and ten patients harbored SPTB variants, including ten non-sense, six indel, four splice site, one start-loss, and one missense variant. Four out of twenty-two variants in our study were known, and eighteen variants were novel. Most ANK1 and SPTB variants were indel (5/12) or non-sense (7/10), respectively. Family member analysis in thirteen families showed that six variants were de novo. Variable expressivities were observed in a pair of twins with ANK1 c.341C > T variant, and two unrelated patients both carried ANK1 c.2T > A variant. Genotype-phenotype analysis found no significant difference between ANK1 and SPTB regarding the levels of Hb, RBC, MCV, MCH, and MCHC. However, variants in the ANK1 death domain were associated with lower levels of MCV and MCH compared to other ANK1 domains. In conclusion, NGS is a fast way to provide a molecular HS diagnosis. We also identified unique genetic and clinical characteristics of patients with HS in Hubei Province, China. However, a large sample size is needed to further investigate the genotype-phenotype correlation.

Keywords: hereditary spherocytosis, ANK1, SPTB, variable expressivity, mutation

INTRODUCTION

Hereditary spherocytosis (HS) is an inherited disorder characterized by anemia, splenomegaly, spherical-shaped erythrocytes on blood smear, osmotically fragile spherocytes, and jaundice, and it can present with or without cholelithiasis. Its clinical manifestation varies from asymptomatic to severe and life-threatening anemia. HS is caused by mutations in various erythrocyte membrane protein genes, including ANK1 (ankyrin), SPTB (β-spectrin), SLC4A1 (Band 3), SPTA1 (α-spectrin), and EPB42 (protein 4.2) with significant heterogeneity in the molecular deficiency...
(He et al., 2018). Autosomal dominant (AD) and autosomal recessive (AR) patterns of inheritance account for 75% and 25% of all the HS cases, respectively. HS prevalence varies among different racial and ethnic regions, affecting approximately 1 in 2000 individuals in northern Europe, North America, and Japan, but it is less common in African-American and southeast Asian people (Perrotta et al., 2008). The estimated prevalence is 1:100,000 in the Chinese population (Wang et al., 2015).

HS is usually diagnosed based on a positive family history, spherocytosis, jaundice, and splenomegaly. The HS phenotype may also be modified by co-occurrence with other disorders like glucose-6-phosphate dehydrogenase (G6PD) and Gilbert syndrome (Aggarwal et al., 2019; Zou et al., 2020). Application of next-generation sequencing (NGS) has led to impressive progress in genetic disorder diagnostics, and it has provided unprecedented benefits for both personalized laboratory medicine and patients with rare genetic disorders (Di Resta and Ferrari, 2018; Prodan Zitnik et al., 2018). NGS has promoted HS molecular diagnosis compared to clinical practice, including both targeted panel and whole exome sequencing (WES) (Xue et al., 2019; Qin L. et al., 2020).

Genetic and clinical features of Chinese HS cases have been frequently identified. Systematic investigation of the genotype-phenotype correlation in Chinese HS patients has been seldom reported (Qin L. et al., 2020). Here, we systematically studied the genetic and clinical characteristics of patients with HS in Hubei province, China, and we investigated the genotype-phenotype correlation in those patients.

MATERIALS AND METHODS

Subjects
Twenty-three unrelated Chinese patients with suspected HS in Hubei province from Dec 2016 to Dec 2019 were included in this study. Patients were diagnosed according to the guidelines from the British Society for Hematology (Bolton-Maggs et al., 2012). This study was approved by Ethics Committee of Tongji Hospital, and written informed consent was obtained from patients or their legal guardians if under 18 years of age.

Next-Generation Sequencing
Targeted NGS was performed as previously described (Wang X. et al., 2018). Genomic DNA was extracted from peripheral blood with the PANA9600 Automated Nucleic Acid Extraction System (Tianlong, Xi’an, China). DNA libraries were built using the AmpIseq Library Preparation Kit (Thermo Fisher, San Diego, United States) targeting the mentioned genes (ANK1, SPTB, SLC4A1, SPTA1, and EPB42). PCR products purified using AMPure XP beads (Beckman Coulter, Brea, CA, United States) were pooled together and amplified using the Ion PGM Hi-Q OT2 Kit on One-Touch Two (OT2) system, and they were further enriched on the ES machine and semiconductor sequencing was conducted with the Hi-Q Sequencing Kit using the Ion 316 or Ion 318 chip on the Ion Torrent Personal Genome Machine (PGM).

Raw data were aligned to the human hg19 reference genome sequence. Variant annotation was performed according to the Human Genome Variation Society (HGVS) recommendations. Annotated variants were filtered if the MAF > 0.01 in any of the following databases, including gnomAD1, ExAC2, and 1000G3. Filtered variants were further queried from the HGMD database4. Functional prediction of missense variants was performed using VarCards5 (Li et al., 2018). Splice site variants were predicted using the GENIE6, NetGene27, and HSF3.18 (Brunak et al., 1991; Hebsgaard et al., 1996; Desmet et al., 2009) programs. Identified variants were further confirmed using Sanger sequencing on an ABI 3500 Dx Genetic Analyzer (Thermo Fisher, San Diego, United States).

Statistical Analysis
Patients were grouped into ANK1 and SPTB groups based on mutated genes. Genotype-phenotype analysis was performed by comparing hemoglobin (Hb), red blood cells (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) between different groups. Statistical analyses were performed using the GraphPad prism 5 software (GraphPad Software, Inc., San Diego, CA, United States). All data were analyzed using the Mann-Whitney U-test, and a p-value of < 0.05 was considered statistically significant.

RESULTS

Clinical Features of Hereditary Spherocytosis Patients
The clinical features of these index patients were listed in Table 1. In summary, more than half of the patients (13/23) were less than 14 years old, and 14 out of 23 (60.9%) patients were female. Seven patients received splenectomy before their Hb and RBC levels recovered to normal, and the remaining sixteen patients were classified as moderate (Hb > 8.0 g/dL) to moderately severe (Hb: 6.0–8.0 g/dL). Eight of the moderate to moderately severe cases were aged ≤ 6 years old. The MCV, MCH, and MCHC levels were in the normal range for most patients. The Hb levels in patients before splenectomy were not available.

Spectrum of Variants in Hereditary Spherocytosis Patients
Analysis of the targeted NGS panel data yielded causal variants in all 23 patients. Thirteen patients carried ANK1 (NM_0000373.3) variants, accounting for 57%. Ten patients had SPTB (NM_001024858.2) variants, accounting for 43% (Table 2). These variants included ten non-sense (45%), six

1http://gnomad.broadinstitute.org/
2http://exac.broadinstitute.org/
3http://asia.ensembl.org/index.html
4http://www.hgmd.cf.ac.uk/ac/index.php
5http://varcards.biols.ac.cn/
6http://rulai.cshl.edu/new_alt_exon_db2/HTML/score.html
7http://www.cbs.dtu.dk/services/NetGene2/
8http://www.umd.be/HSF3/
showed a deleterious effect (Supplementary Table S1) by VarCards showed that 16 out of 19 algorithms non-sense (7/10), respectively. The ANK1 

In silico analysis of the missense variant (Supplementary Table S1) and was highly conserved among species (Supplementary Table S2). This missense variant was found in a pair of twins, and it was de novo. 

In silico analysis of the four splice site variants (ANK1 c.1800 + 1G > A, ANK1 c.2559-2A > C, SPTB c.2805-1G > T, and SPTB c.5799-2A > G) was performed using GENIE, NetGene2, and HSF 3.1. The splice score was decreased or the splice site was lost based on all three algorithms (Supplementary Table S3). We sequenced the reverse transcribed cDNA from peripheral blood in families carrying ANK1 c.1800 + 1G > A and SPTB c.5799-2A > G, respectively. However, only normally spliced mRNA sequence was detected. Real time PCR and RNA sequencing may help to evaluate the expression and abnormally spliced mRNA.

Genotype-Phenotype Correlation in Hereditary Spherocytosis Patients

Genotype-phenotype correlation was analyzed by comparing the indices including Hb, RBC, MCV, MCH, and MCHC in different groups based on mutated genes. We found no significant difference for any indices between patients carrying ANK1 or SPTB variants (Supplementary Table S4). However, ANK1 death domain variants tended to be associated with lower levels of MCV and MCH compared to other ANK1 domains (Figure 2).

Variable expressivities were observed. A pair of twins (number 17) carried the de novo ANK1 missense ANK1 c.341C > T (p.Pro114Leu) variant. The proband was admitted for cholelithiasis, anemia, and splenomegaly, but her twin sister did

### Table 1: Clinical and laboratory features of these included hereditary spherocytosis patients.

| ID | Gender | Age | Hb  | RBC  | MCV  | MCH  | MCHC | Osmotic fragility | Spherocytosis | Splenomegaly | Splenectomy |
|----|--------|-----|-----|------|------|------|------|------------------|--------------|-------------|-------------|
| 1  | Female | 28  | 12.0| 4.08 | 88.7 | 29.4 | 33.1 | –                | –            | Yes         | Yes         |
| 2  | Male   | 31  | 12.3| 3.82 | 90.3 | 32.2 | 35.7 | ↑                | Yes          | Yes         | Yes         |
| 3  | Female | 11  | 15.8| 5.53 | 80.8 (↓)| 28.6 | 35.3 | –                | Yes          | Yes         | Yes         |
| 4  | Female | 1   | –   | –    | –    | –    | –    | –                | –            | Yes         | No          |
| 5  | Male   | 4   | –   | –    | –    | –    | –    | –                | –            | Yes         | No          |
| 6  | Male   | 7   | 11.9| 4.33 | 80.6 (↓)| 27.5 | 34.1 | ↑                | Yes          | Yes         | Yes         |
| 7  | Male   | 24  | 12.3| 3.82 | 90.3 | 32.2 | 35.7 | ↑                | Yes          | Yes         | Yes         |
| 8  | Male   | 14  | 15.9| 5.35 | 84.9 | 29.7 | 35.0 | ↑                | Yes          | Yes         | Yes         |
| 9  | Female | 23  | 8.4  | 2.7  | 84.8 | 31.1 | 36.7 | –                | Yes          | No          | No          |
| 10 | Female | 12  | 8.6  | 2.89 | 90.3 | 29.8 | 33.0 | ↑                | Yes          | No          | No          |
| 11 | Female | 2   | 7.4  | 2.87 | 78 (↓)| 25.8 (↓)| 33.0 | ↑                | Yes          | No          | No          |
| 12 | Female | 40  | 8.7  | 2.83 | 98.1 | 30.7 | 27.8 (↓)| ↑                | Yes          | No          | No          |
| 13 | Female | 15  | 7.8  | 2.63 | 90.9 | 29.7 | 32.6 | ↑                | Yes          | No          | No          |
| 14 | Male   | 4   | –   | –    | –    | –    | –    | –                | –            | Yes         | No          |
| 15 | Female | 30  | 8.3  | 2.51 | 98.4 | 33.1 | 33.6 | ↑                | Yes          | No          | No          |
| 16 | Female | 28  | 12.6 | 4.29 | 89.7 | 29.4 | 32.7 | ↑                | Yes          | Yes         | Yes         |
| 17 | Female | 19  | 8.3  | 2.62 | 87.8 | 31.7 | 36.1 | ↑                | Yes          | Yes         | Yes         |
| 18 | Female | 13  | 8.6  | 2.79 | 90   | 30.8 | 34.3 | ↑                | Yes          | Yes         | Yes         |
| 19 | Male   | 6   | 9.2  | 3.4  | 80.6 (↓)| 27.1 | 33.6 | –                | –            | Yes         | No          |
| 20 | Female | 12  | 8.1  | 2.59 | 90.3 | 31.3 | 34.6 | –                | –            | Yes         | No          |
| 21 | Male   | 3   | 8.8  | 2.99 | 86   | 29.4 | 34.2 | ↑                | Yes          | Yes         | Yes         |
| 22 | Male   | 6   | 8.2  | 3.3  | 74.8 (↓)| 24.8 (↓)| 33.2 | –                | Yes          | Yes         | No          |
| 23 | Female | 2   | 8.5  | 3.25 | 80.6 (↓)| 26.2 (↓)| 32.4 | –                | –            | Yes         | No          |

Hb, hemoglobin; RBC, red blood cell; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration. Hb (g/dL) ref: 11.5-15.0; RBC (10¹²/L) ref: 3.8-5.1; MCV (fL) ref: 82.0-100.0; MCH (pg) ref: 27.0-34.0; MCHC (g/dL) ref: 31.6-35.4. Osmotic fragility ref (g/L): start NaCl concentration, 2.8-3.2, complete NaCl concentration, 4.2-4.6. The Hb levels before splenectomy of patients received splenectomy were not available.
not suffer cholelithiasis. Moreover, patient 8 showed lower MCV and higher MCHC compared to patient 12, although they both harbored the heterozygous ANK1 c.2T > A (p.Met1Lys) variant. In other analyzed families, affected family members showed similar phenotypes.

### DISCUSSION

HS is the most common non-immune inherited hemolytic anemia, with variable expressivity (Iolascon et al., 2019), and the majority of HS was inherited in an AD manner. HS shows wide phenotypic and genotypic heterogeneity. The prevalence varies in different racial and ethnic regions, and the molecular spectrum differed in different regions. For example, the ANK1 variant accounts for 40–65% in United States and Europe but 5–10% in Japan (Perrotta et al., 2008). Here, ANK1 variants accounted for 57%, different to that observed in the Japanese population but similar to that in the Korean population (Park et al., 2016) in a cohort of 95 HS patients from the Netherlands, SPTAI, ANK1, and SPTB ranked as the top three genes with identified variants (van Vuren et al., 2019).

Here, Hb and RBC were both decreased in all patients without splenectomy. The MCV, MCH, and MCHC levels were all in the normal range, similar to a recent Indian study (Aggarwal et al., 2019). Moreover, 89% of the patients (17/19) showed MCHC < 35.9 g/dL, different from the findings reported in a study by Michaels et al., demonstrating that most HS cases had MCHC > 35.9 g/dL (Michaels et al., 1997). Our study further confirms that MCHC is less effective for determining HS.

NGS has promoted molecular HS diagnosis, including both targeted panel and WES (Roy et al., 2016; Russo et al., 2018; Xue et al., 2019; Qin L. et al., 2020). Here, the causal variants could be found in 100% of the cases using targeted NGS. In a recent retrospective study of children with HS by Tole et al. (2020), 21 disease-causing variants had been identified in 160/166 (97%) children with HS. WES may help find the phenotype modifying genes (Aggarwal et al., 2019). Here, all variants were from ANK1 and SPTB genes. Most ANK1 and SPTB variants were indel (5/12) and non-sense (7/10), respectively. In the studies reported by Qin L et al. and Wang R et al., which included 35 Chinese patients from Tianjin of China and 38 patients from Beijing China, respectively, it was shown that ANK1 and SPTB genes were the most frequently mutated genes (Wang R. et al., 2018; Qin L. et al., 2020). Qin L et al. found that six out of eight families showed a de novo variant, while we found that six out of thirteen families showed a lower rate of carrying de novo variants. Moreover, 21 out of 34 variants in the study by Qin et al. study were novel (Qin L. et al., 2020), and 18 out of 22 variants in our study were novel. These data indicate unique genetic characteristics of patients with HS in Hubei province, China.
FIGURE 1 | Spectrum of variants in HS patients. (A), Locations of ankyrin. Ankyrin contained an 89 kDa domain, two ZU5 domains, a UPA domain, and a 55 kDa regulatory domain. The 89 kDa domain included 23 ANK repeats ranging from exon 1–21, which formed a spiral around a large central cavity involved in the binding of ion transporters. The two ZU5 and UPA domains, ranging from exon 26–33, forms a structural supramodule required for ankyrin’s function, and mediated interaction with $\beta$-spectrin. The 55-kDa domain, ranging from exon 34–42, included a death domain and was involved in the regulation of $\beta$-spectrin binding with protein band 3. The variants in $\textit{ANK1}$ gene were distributed in initial codon, ANK Repeats, UPA domain, and death domain. (B), Location of $\beta$-spectrin. $\beta$-spectrin contains an actin-binding region (exon 1–7), 17 Spectrin repeats (exon 8–30), and a C-terminal. Variants in $\textit{SPTB}$ gene were mainly distributed in spectrin repeats, and two variants were in actin-binding region.

Moreover, the laboratory indices showed unique clinical characteristics in Hubei province compared to Tianjin, China (Qin L. et al., 2020). We found no significant difference for any indices between patients carrying $\textit{ANK1}$ or $\textit{SPTB}$ variants. Our data were similar to the those reported in a study by Aggarwal et al. (2019) and Tole et al. (2020), where they found that the indices in patients with $\textit{ANK1}$ variants were similar to the $\textit{SPTB}$ group. Moreover, Tole et al. (2020) found that the variant type or location within each gene did not predict the disease severity. Here, $\textit{ANK1}$ death domain variants were associated with lower levels of MCV and MCH compared to other $\textit{ANK1}$ domains, but the number of patients was limited. MCV and MCH were much higher in the $\textit{ANK1}$ group than the $\textit{SPTB}$ group in the study by Qin L. et al. (2020). In a cohort of 25 Korean HS patients, anemia was most severe in the $\textit{ANK1}$ spectrin-binding domain (Park et al., 2016). Similarly, van Vuren et al. found that variants affecting spectrin-binding of $\textit{SPTA1}$, $\textit{ANK1}$, and $\textit{SPTB}$ led to more severe phenotypes. They also found that red blood cell deformability measurements were associated with HS severity (van Vuren et al., 2019). Variable expressivity was observed in a
In summary, we discovered 18 novel variants in the ANK1 and SPTB genes from 23 Chinese patients with HS from Hubei province, central China. We found that NGS was an effective tool for rapid molecular diagnosis of HS. This is the first study to determine the genetic and clinical characteristics of patients with HS in Hubei Province, China.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the SRA database with the following link: https://www.ncbi.nlm.nih.gov/sra/?term=PRJNA649395.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of Tongji Hospital. Written informed consent to participate in this study was provided by the participants’ legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

YL and LMC: project leads and designing the project. MH and LC: data collection. XW and YL: analysis. AZ and QH: data collection. XW: manuscript writing. All authors contributed to the article and approved the submitted version.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fgene.2020.00953/full#supplementary-material

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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