Clinical and genetic diagnosis of thirteen Japanese patients with hereditary spherocytosis

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INTRODUCTION

Hereditary spherocytosis (HS) is the most frequent cause of hereditary hemolytic anemia1. Generally, patients with HS show hemolytic anemia in association with jaundice, reticulocytosis, osmotically fragile red blood cells, gallstones, and splenomegaly2. Cholelithiasis and aplastic crises are also common complications3. The clinical severity of hemolytic anemia in patients with HS varies widely, ranging from asymptomatic to severe life-threatening hemolytic anemia. Thus, an accurate diagnosis is important to support decision-making pertaining to subsequent treatment strategies, including splenectomy. According to the Online Mendelian Inheritance in Man database (OMIM: https://www.omim.org/), HS is classified into five subtypes associated with five different genes responsible for the deficiency or dysfunction of red blood cell membrane proteins, including ankyrin 1 (ANK1; MIM #182000 [SPH1]), β-spectrin (SPTB; MIM #616649 [SPH2]), α-spectrin (SPTA1; MIM #270970 [SPH3]), band 3 protein (SLC4A1; MIM #612653 [SPH4]), and protein 4.2 (EPB42; MIM #612690 [SPH5]) (Supplemental Table S1). SPH1, SPH2, and SPH4 are associated with the autosomal dominant (AD) trait, whereas SPH3 and SPH5 are associated with autosomal recessive (AR) traits. Therefore, it is important to obtain an accurate diagnosis for proper genetic counseling. For this purpose, it is important to not only evaluate family history, the clinical course, and physical findings but to also perform laboratory examinations3. With the recent development of molecular analysis methods, it has become necessary to detect causative gene variants to obtain a final diagnosis for HS patients.

Recently, we developed an originally designed target capture sequencing (TCS) panel for the precise diagnosis of hemolytic anemia. Here, some of the results obtained with this panel are summarized to clarify the clinical and genetic features of patients with HS in association with the five well-established subtypes, SPH1-5.

MATERIALS AND METHODS

This study aimed to elucidate the molecular basis of HS in Japanese patients. For this purpose, we enrolled patients with hemolytic anemia, including HS, in accordance with the Declaration of Helsinki, followed by the approval of the Ethics Committee of our institution. After obtaining written informed consent, blood samples were collected from patients. From the attending doctors, we also obtained detailed clinical information, including family histories, clinical courses, and physical findings. Between 2016 and 2018, 51 patients showed clinical histories of hemolytic anemia associated with/without morphological abnormalities in red blood cells according to routine laboratory examinations and were enrolled as the subjects of this study.

In most of the patients, when possible, we first performed additional chemical tests, including the acidified glycerol hemolysis time test, the flow-cytometric osmotic fragility (FCM-OF) test, and the eosin-5′-maleimide (EMA) binding test with a negative direct antiglobulin test as per previously reported methods4,5. Genomic DNA was extracted from the patients’ peripheral blood using a QiAamp DNA extraction kit according to the manufacturer’s instructions (QIAGEN, Hilden, Germany). The Haloplex HS target enrichment system (Agilent Technologies, Santa Clara, CA, USA) was used as the target panel. The target panel was designed using SureDesign (https://earray.chem.agilent.com/suredesign/home.htm) to include all coding exons and intron-exon boundaries of 74 possible candidate genes (Supplemental Table S2). Massive parallel sequencing was performed using the Illumina MiSeq.

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platform (Illumina Inc., San Diego, CA, USA). Raw data were aligned to the GRCh37/hg19 human genome. The generated FASTQ files were imported into SureCall ver3.5 (Agilent Technologies) for variant calling.

The obtained variants were filtered according to the following strategy: (1) variant frequencies were below 1% in 1000G, EAS and ALL (1000 Genomes), HGDV, and dpSNP; (2) synonymous variants were excluded (nonsynonymous variants, variants associated with a frameshift, insertion/deletion variants, and variants in splicing donor/acceptor sites were included); (3) variants with allele frequencies of less than 30% within the total read depth were excluded; and (4) the CADD_phred value was higher than 20 if obtained. Variant information obtained through wANNOVAR (http://wannovar.wglab.org/) was used for curation. The Integrative Genomics Viewer (IGV) (https://software.broadinstitute.org/software/igv/) was used for visual evaluation. The final conclusion was reached following the American College of Medical Genetics and Genomics (ACMG) guidelines.

The effect of the splicing site variants was evaluated with in silico software using the Berkeley Drosophila Genome Project (https://www.fruitfly.org/seq_tools/splice.html) and DTU Bioinformatics (http://www.cbs.dtu.dk/services/NetGene2/) databases.

RESULTS
The 13 variants identified in the genes related to SPH1-5 (ANK1, SPTB, SPTA1, SLC4A1, and EPB42), together with the clinical data and laboratory test results, are summarized in Table 1. Among the 51 subjects, eight patients (patients 1, 3, 4, 7, 8, 9, 10, and 12) were primarily and clinically suspected of having HS and showed HS-related variants, indicating a 100% detection ratio. On the other hand, HS was primarily not suspected in five other patients who showed HS-related variants.

Although two variants identified in patients 6 and 10 were evaluated as “variants of uncertain significance (VUS)” in accordance with ACMG guidelines, the prediction scores for the variant in patient 6 suggested “damage”, and the variant in patient 10 was quite unique. Thus, we considered these variants to likely be related to disease occurrence. Seven variants were novel and was quite unique. Thus, we considered these variants to likely not have been reported previously but was found to be similar to the underlying abnormal splicing.

Thus, we concluded that this variant was the likely cause of patient 11. Also, the parents of patient 11 are first cousins, it is suspected that both parents are heterozygous carriers. Patient 11 also showed a homozygous SPTB variant (c.426 + 4.426 + 7del) has not been reported previously but was found to be similar to the Ankyrin Shiga variant (c.426 + 4.426 + 4insA). Through in silico analyses using two different websites, this insertion was predicted to cause the loss of the donor site (Supplementary Figs. S1, S2). Thus, we concluded that this variant was the likely cause underlying abnormal splicing.

Patient 13 harbored a homozygous EPB42 variant. Since the parents of patient 13 are first cousins, it is suspected that both parents are heterozygous carriers. Patient 11 also showed a homozygous splicing variant in SPTA1; however, consanguinity was not found in this patient’s family history.

Among the 38 patients who showed no pathogenic variants in the five genes, 20 patients were analyzed with the FCM-OF test, and only 5 patients showed low values (data not shown).

DISCUSSION
Inoue et al. analyzed the genetic backgrounds of Japanese HS patients using sodium dodecyl sulfate–polyacrylamide gel electrophoresis and reported band 3 deficiency (SPH4) in 32% of the patients, spectrin deficiency (corresponding to SPH2 and SPH3) in 15%, protein 4.2 deficiency (SPH5) in 6%, ankyrin deficiency (SPH1) in 2%, combined deficiency in 36%, and no abnormality in 9%.

In contrast, Yawata et al. analyzed 60 Japanese patients with HS using a similar method and detected protein 4.2 deficiency (SPH5) in 45% of the patients, band 3 deficiency (SPH4) in 20%, and ankyrin deficiency (SPH1) in 7%, while 28% of the patients had an unknown etiology.

Genetic variants in ANK1 were analyzed by Nakanishi et al., who identified 16 variants in 49 patients with HS, suggesting that ANK1 variants (SPH1) are not rare in the Japanese population.

Remarkable progress has been made in genomic analyses in the last decade. Next-generation sequencing (NGS) is being extensively used in this field. This has helped to expand our understanding of the genetic heterogeneity associated with HS.

Some studies have demonstrated the usefulness of the targeted NGS approach for the investigation of specific subtypes of patients with hemolytic anemia. Exome sequencing has also been applied to hereditary hemolytic anemia.

To confirm ethnic differences in HS-related variants, Choi et al. reviewed the available reports regarding HS-related mutations in comparison with the results of the present study. In reports from the United States, SPTA1 mutation (SPH3) was found to be the most common. A study in the Netherlands revealed that SPTA1, ANK1, and SPTB (SPH3, SPH1, and SPH2) were ranked as the top three genes with identified variants. In Korea, SPTB mutation (SPH2) was found to be the most common, followed by ANK1 mutation (SPH1). Another study in Korea reported that 25 patients with HS carried mutations in ANK1 (SPH1; n = 13) or SPTB (SPH2; n = 12). A study from China reported that among 23 patients, 13 mutations were observed in ANK1 (SPH1), while 10 mutations were observed in SPTB (SPH2). Other studies have reported similar observations. The distribution of the variants is summarized in Supplementary Table S3.

In this study, ANK1 variants (SPH1) were found to be the most common, being found in 46% (6/13) of the patients. SPTB variants (SPH2) were identified in 31% (4/13) of the patients. Thus, the distribution of the variants was similar to those observed in other Asian countries but was different from those observed in non-Asian countries. Previously, EPB42 (SPH5) was considered the most common subtype. However, the distribution of HS-related variants observed in this study was different from that identified in a previous study on Japanese patients with HS. The reason for this difference is unknown; however, the total number of samples examined in the present study is too small to be compared with this previous study. Thus, the analysis of more samples is necessary to better understand the genetic basis of HS in Japanese patients.

As mentioned above, ANK1 (SPH1), SPTB (SPH2), and SLC4A1 (SPH4) are related to AD traits, whereas SPTA1 (SPH3) and EPB42 (SPH5) are related to AR traits. In this study, variants in AD-related genes were found in 11 patients (85%). As six patients (46%) showed a positive family history, the identified variants were considered to be inherited from the affected ancestors. In comparison, five patients with variants in AD-related genes (45%) had no family history, and it remains unknown whether the identified variants occurred de novo or if they were inherited from nonsymptomatic parents. This is a limitation of this study, as parental analysis was not conducted.

We did not find any genotype-phenotype correlation. The observed severities of the clinical and laboratory findings were variable, even within the subgroups classified in accordance with the gene variants. Similar findings have been reported previously. Phenotypic variabilities have been reported in a pair of twins with de novo ANK1 missense variants. Thus, even though we found no clear genotype-phenotype correlation, our results are not contradictory to those reported previously.

Regarding laboratory testing, it is difficult to detect HS using only one method because its clinical phenotypes are widely variable. Therefore, more than one test is generally recommended. Previously, the osmotic fragility test was considered to be the gold standard for HS diagnosis. In this study, we found that only the results obtained from FCM-OF matched the results of
Table 1. Results of this study.

| Patient 1 | Patient 2 | Patient 3 | Patient 4 | Patient 5 | Patient 6 | Patient 7 | Patient 8 | Patient 9 | Patient 10 | Patient 11 | Patient 12 | Patient 13 |
|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|-----------|
| Subtype   | SPH1      | SPH1      | SPH1      | SPH1      | SPH1      | SPH2      | SPH2      | SPH2      | SPH2      | SPH3      | SPH4      | SPH5      |
| Gender    | Female    | Female    | Male      | Male      | Male      | Male      | Female    | Male      | Female    | Male      | Male      | Male      |
| Age       | 37 y      | 71 y      | 6 y       | 1 y       | 6 m      | 2 m      | 15 y      | 14 y      | 16 y      | 13 y      | 27 y      | 62 y      | 45 y      |
| Family history | –        | +         | –         | –         | +        | –        | –         | +        | –         | +         | –         | +         |
| Clinical histories |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Splenomegaly |     | +        | +         | +         | –        | +        | +         | –         | +         | –         | +         | +         |
| Splenectomy |     | –        | –         | –         | –        | –        | –         | +         | –         | –         | –         | –         |
| Gallstone  |     | +        | –         | –         | –        | –        | –         | +         | –         | +         | –         | –         |
| Blood transfusion | NA     | +        | +         | +         | –        | –        | –         | +        | –         | +         | –         | –         |
| Laboratory testing |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Hb (g/dL) | 11.7      | 7.1      | 13.5      | 7.9       | 8.2      | 12.6     | 15.6      | 11.2      | 11        | 12.9      | 5.9       | 15.6      | 51        |
| [13 < : male, 12 < : female] |     |     |     |     |     |     |     |     |     |     |     |     |     |
| MCV (fL)  | 86.8      | 106.1    | 80.2      | 85.2      | 87.6     | 95       | 86.5      | 84.9      | 82        | 88.9      | 106.9     | 96        | 113.2     |
| [31–33%]  | 34.6      | 36.2     | 37        | 31.1      | 34       | 34.8     | 35.3      | 33        | 35.5      | 33.5      | 32.1      | 35.5      | 31.6      |
| Reticulocytes (%) | 92      | 55       | 186       | 248       | 26       | 44       | 165       | 69        | 17.9      | 30        | 34        | NA        | 195       |
| [0.2—2.7%] | 162      | 187      | 200       | 535       | 281      | 272      | 246       | 222       | 246       | 240       | 156       | 157       | 286       |
| LDH (U/L) | 114       | 187      | 100       | 335       | 281      | 272      | 246       | 222       | 246       | 240       | 156       | 157       | 286       |
| Total Bilirubin (mg/dL) | 3.7 | 9.9 | 0.4 | 1.8 | 2.2 | 3.2 | 1 | 6.5 | 3.8 | 1.5 | 3.4 | 30 | 18 |
| [0.2—1.2 mg/dL] | 0.6 | 5.1 | 0.1 | 0.8 | 2.1 | 27 | 0.6 | 6 | 2.6 | 0.8 | 3.1 | NA | 1 |
| Indirect Bilirubin (mg/dL) | 0.2—1.0 mg/dL | 3.7 | 9.9 | 0.4 | 1.8 | 2.2 | 3.2 | 1 | 6.5 | 3.8 | 1.5 | 3.4 | 30 | 18 |
| RBC morphology |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Anisocytosis, spherocytosis, stomatocytosis |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Elliptocytosis, spherocytosis, stomatocytosis |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Codocyte, spherocyte |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Anisocytosis, spherocytosis, stomatocytosis |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Anisocytosis, codocyte, stomatocytosis |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Specific examination |     |     |     |     |     |     |     |     |     |     |     |     |     |
| AGT | 150 s | 30 min | NA | NA | NA | NA | 30 min | NA | NA | NA | NA | 30 min | NA | 45 sec |
| EMA (% of control) | 77.7 | 93 | 35.8 | 70.1 | 67.7 | 97.3 | 34.8 | 32 | 15.3 | NA | 106.3 | 15.8 | 902 |
| FCM-OF (460–689 nm) | 23.8 | NA | 102 | 7.2 | NA | NA | NA | 14.9 | 172 | 4.2 | NA | 18 | NA |
| Other findings |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Spina bifida |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Epilepsy, developmental delay, hearing difficulty |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Variant information |     |     |     |     |     |     |     |     |     |     |     |     |     |
| Chromosome | Chr8 | Chr8 | Chr8 | Chr8 | Chr8 | Chr8 | Chr14 | Chr14 | Chr14 | Chr14 | Chr14 | Chr14 | Chr15 |
| Gene | ANK1 | ANK1 | ANK1 | ANK1 | ANK1 | ANK1 | SPTB | SPTB | SPTB | SPTB | SPTA1 | SLC4A1 | EBFB2 |
| Genomic coordinate (GRCh37/hg19) | Start | 41655025 | 41584867 | 41518408 | 41587671 | 41573626 | 65259974 | 65249125 | 65264577 | 65239994 | 158632491 | 42334875 | 43507389 |
| End | 41655025 | 41584867 | 41518408 | 41587671 | 41573626 | 65259974 | 65249125 | 65264577 | 65239994 | 158632491 | 42334875 | 43507389 |
| HGVS (Coding) | NM_000037.4 | NM_001142446.2 | NM_000037.4 | NM_000037.4 | NM_000037.4 | NM_000037.4 | NM_000037.4 | NM_000037.4 | NM_000037.4 | NM_000037.4 | NM_000037.4 | NM_000037.4 | NM_000037.4 | NM_000037.4 |
| Location | Intron 1 | Intron 4 | Intron 5 | Intron 5 | Intron 5 | Exon 9 | Exon 9 | Exon 13 | Exon 19 | Exon 20 | Exon 25 | Intron 17 | Exon 30 |
| cDNA change | c.27—5G>C | c.427—7G>T | c.382—386del | c.426—4del | c.841C>T | c.1204G>A | c.2407del | c.1204G>A | c.4194dup | c.4393G>A | c.5456_5457del | c.2464—1G>A | c.1469G>A | c.4240A |
| Protein change | p.Lys128Phefs*7 | p.Arg281* | p.Glu402Lys | p.Glu803Serfs*17 | p.Arg1384Alafs*7 | p.Glu1447* | p.Glu1447* | p.Glu1819Val | p.Arg490His | p.Ala142Thr |
| Type          | Splicing Frameshift | Splicing Nonsense Missense Frameshift Frameshift Nonsense Missense Splicing Missense Missense |
|--------------|---------------------|----------------------------------|
| rs1350849760 | –                   | –                                 |
| rs778901641 | –                   | –                                 |
| rs774632615 | –                   | –                                 |

**Clinvar**
- Not reported

**SIFT (score)**
- NA
- NA
- NA
- NA
- 0.028
- NA
- NA
- NA
- NA
- NA
- 1
- 1
- 0
- 0

**Polyphen2**
- NA
- NA
- NA
- NA
- 1
- NA
- NA
- NA
- NA
- NA
- 1
- 1
- 0
- 0

**CADD_phred**
- NA
- 27.5
- NA
- NA
- 37
- 34
- NA
- NA
- 38
- NA
- 25
- 29.6
- 29.5

**ACMG criteria**
- PVS1, PM2, PM4, PM2, PM4, PM2, PM4, PM2, PM4, PM2, PM4
- PS1, PM2, PM4, PS1, PM2, PM4, PS1, PM2, PM4, PS1, PM2, PM4

**Interpretation**
- Pathogenic
- Likely pathogenic
- Novel

**References**
1. Zamora E. A., Schaefer C. A. Hereditary Spherocytosis. In: StatPearls. Treasure Island (Fl): StatPearls Publishing. Copyright © 2021, StatPearls Publishing LLC.; 2021.
2. Ciepielka, O. Old and new insights into the diagnosis of hereditary spherocytosis. *Ann. Transl. Med.* 6, 339 (2018).
3. Perrotta, S., Gallagher, P. G. & Mohandas, N. Hereditary spherocytosis. *Lancet* 372, 1411–1426 (2008).
4. Won, D. I. & Suh, J. S. Flow cytometric detection of erythrocyte osmotic fragility. *Cytoimmunology & Cytokine Research* 29, 135–141 (2009).
5. Ciepielka, O., Adamowicz-Salach, A., Zgodzińska, A., Łazowska, M. & Kotula, I. Flow cytometric osmotic fragility test: Increased assay sensitivity for clinical application in pediatric hematology. *Cytoimmunology & Cytokine Research* 29, 189–195 (2018).
6. Zanella, A. et al. Acidified glycerol lysis test: a screening test for spherocytosis. *Br. J. Haematol.* 45, 481–486 (1980).
7. King, M. J. et al. ICSH guidelines for the laboratory diagnosis of nonimmune hereditary red cell membrane disorders. *Int. J. Lab. Hematol.* 37, 304–325 (2015).
8. King, M. J. et al. Using the eosin-5-maleimide binding test in the differential diagnosis of hereditary spherocytosis and hereditary pyropoikilocytosis. *Cytoimmunology & Cytokine Research* 24, 244–250 (2008).
9. Richards, S. et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genet. Med.* 17, 405–424 (2015).
10. Dhermy, D. et al. Heterogenous band 3 deficiency in hereditary spherocytosis related to different band 3 gene defects. *Br. J. Haematol.* 98, 32–40 (1997).
11. Bouhassira, E. E. et al. An alanine-to-threonine substitution in protein 4.2 cDNA is associated with a Japanese form of hereditary hemolytic anemia (protein 4.2NIPPON). *Blood* 79, 1846–1854 (1992).
12. Nakanishi, H., Kanzaki, A., Yawata, A., Yamada, O. & Yawata, Y. Ankyrin gene related to different band 3 gene defects. *Int. J. Lab. Hematol.* 71, 54–63 (2001).
13. van Vuren, A. et al. The complexity of genotype-phenotype correlations in hereditary spherocytosis: a cohort of 95 patients. *Int. J. Lab. Hematol.* 73, 372–376 (1994).
14. Inoue, T. et al. Uniquely higher incidence of isolated or combined deficiency of band 3 and/or band 4.2 as the pathogenesis of autosomal dominantly inherited hereditary spherocytosis in the Japanese population. *Int. J. Hematol.* 60, 227–238 (1994).
15. Yawata, Y. et al. Characteristic features of the genotype and phenotype of hereditary spherocytosis in the Japanese population. *Int. J. Hematol.* 71, 116–135 (2000).
16. Yawata, Y., Kanzaki, A., Yawata, A., Nakanishi, H. & Kaku, M. Hereditary red cell membrane disorders in Japan: their genotypic and phenotypic features in 1014 cases studied. *Hematology* 6, 399–422 (2001).
17. Andolfi, I., Russo, R., Gambale, A. & Iolascon, A. New insights on hereditary erythrocyte membrane defects. *Hematologica* 101, 1284–1294 (2016).
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COMPETING INTERESTS
The authors declare no competing interests.

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