Targeted next-generation sequencing identifies a novel nonsense mutation in SPTB for hereditary spherocytosis

A case report of a Korean family

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Abstract
Rationale: Hereditary spherocytosis (HS) is an inherited disorder characterized by the presence of spherical-shaped red blood cells (RBCs) on the peripheral blood (PB) smear. To date, a number of mutations in 5 genes have been identified and the mutations in SPTB (erythrocytic beta spectrin) gene account for about 20% of all affected individuals with HS. A novel nonsense mutation in SPTB for hereditary spherocytosis was identified by targeted next-generation sequencing (NGS). The nonsense mutation, c.1956G>A (p.Trp652*), in exon 13 was confirmed by Sanger sequencing and thus the proband was diagnosed with HS.

Patient concerns: A 65-year-old female had been diagnosed as hemolytic anemia 30 years ago, based on a history of persistent anemia and hyperbilirubinemia for several years. She received RBC transfusion several times and a cholecystectomy roughly 20 years ago before. Round, densely staining spherical-shaped erythrocytes (spherocytes) were frequently found on the PB smear. Numerous spherocytes were frequently found in the PB smears of symptomatic family members, her 3rd son and his 2 grandchildren.

Diagnosis: One heterozygous mutation of SPTB was identified by targeted NGS. The nonsense mutation, c.1956G>A (p.Trp652*), in exon 13 was confirmed by Sanger sequencing and thus the proband was diagnosed with HS. The proband underwent a splenectomy due to transfusion-refractory anemia and splenomegaly.

Interventions: After the splenectomy, her hemoglobin level improved to normal range (14.1 g/dL) and her bilirubin levels decreased dramatically (total bilirubin 1.9 mg/dL; direct bilirubin 0.6 mg/dL).

Lessons: We suggest that NGS of causative genes could be a useful diagnostic tool for the genetically heterogeneous RBC membrane disorders, especially in cases with a mild or atypical clinical manifestation.

Abbreviations: AD = autosomal dominant, HS = hereditary spherocytosis, NGS = next-generation sequencing, PB = peripheral blood, RBC = red blood cell, WES = whole exome sequencing.

Keywords: hereditary spherocytosis, nonsense mutation, SPTB gene, targeted next-generation sequencing

1. Introduction

Hereditary spherocytosis (HS) is a common inherited red cell membrane disorders characterized by nonautoimmune hemolytic anemia, jaundice, splenomegaly, and gallstone.[1] HS has a heterogeneous spectrum of clinical severity, and its prevalence is 1.39 cases per 100,000 people of Chinese population.[2] The main lesion in HS is loss of red blood cell (RBC) membrane surface, leading to reduced deformability due to defects in the RBC membrane proteins such as ankyrin, spectrin, band 3, or protein 4.2.[3]

Erythrocytic beta spectrin (SPTB) plays a role in RBC membrane organization and stability, along with ankyrin. The protein encoded by this locus functions in stability of RBC membranes, and mutations in this gene have been associated with HS type 2, hereditary elliptocytosis, and neonatal hemolytic anemia.[4] Beta spectrins are typically composed of 4 structural domains: actin binding domain, dimerization domain, spectrin repeats, and ankyrin binding domain. Spectrin and ankyrin interact to tether the spectrin cytoskeleton to the RBC membrane as major components of the RBC membrane skeleton. The structure of the spectrin binding domain of ankyrin and the ankyrin binding domain of spectrin have been solved to elucidate the structural basis for ankyrin–spectrin recognition.[5] The structure of spectrin repeats shows that these repeats are similar to all other spectrin repeats. SPTB mutations have been detected in about 20% of all affected individuals with HS who usually exhibit an autosomal dominant (AD) inheritance. Clinical manifestation ranges from mild to severe depending on the degree of the RBC membrane defect.[6] Recent advances in next-generation sequencing (NGS) technology have led to a paradigm shift, leading the laboratory field of genetic testing away from Sanger sequencing.[7] Cost-effective, high-throughput NGS has led to the clinical implementation of

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targeted NGS or whole exome sequencing (WES). WES has contributed greatly to the discovery of novel mutations responsible for Mendelian diseases. It is widely employed as a diagnostic method, as it allows researchers to screen the entire coding regions of genes. In this report, we identified a novel SPTB mutation responsible for HS in a Korean family using targeted NGS. This demonstrates that targeted NGS is an effective method both for identifying novel causal mutations and for diagnosing additional disease cases.

2. Case presentation

A 65-year-old female (Fig. 1A, individual I-2) was referred to the Department of Internal Medicine, Daejeon St. Mary’s Hospital (Daejeon, Republic of Korea) for a further evaluation. She had been diagnosed as hemolytic anemia 30 years ago, based on a medical history of persistent anemia and hyperbilirubinemia for several years. She received RBC transfusion several times and a cholecystectomy roughly 20 years ago before without knowing the reason for the operations. The laboratory findings at the time of admission to our hospital were as follows: hemoglobin, 6.6 g/dL; red cell distribution width, 23.9%; reticulocytes, 22.5%; haptoglobin, <20 mg/dL; erythropoietin, 4080 mIU/mL; total bilirubin, 8.7 mg/dL; direct bilirubin, 1.9 mg/dL; and lactate dehydrogenase, 250 IU/L. Autoimmune hemolytic anemia was ruled out because irregular antibody screening, Coombs test, and cold agglutinin test were negative, even though increased osmotic fragility. On the peripheral blood (PB) smear, round, densely staining spherical-shaped RBCs (spherocytes) were frequently found about 10 to 20 cells per high power field (Wright–Giemsa stain, ×1000 magnification).

Figures 1. Pedigree analysis and peripheral blood smears of the proband. (A) Pedigree analysis of a Korean hereditary spherocytosis with a novel nonsense mutation in SPTB. Proband (indicated by the arrow) revealed the c.1956G>A; p.Trp652* in the heterozygous state. Gray symbols indicate clinically affected individuals not tested for the mutation. (B) Peripheral blood smears demonstrate moderate spherocytosis, about 10 to 20 cells per high power field (Wright–Giemsa stain, ×1000 magnification).

Table 1

| Characteristics                  | Proband (I-2) | Son (II-3) | Grand daughter (III-1) | Grandson (III-2) |
|----------------------------------|---------------|------------|------------------------|------------------|
| Sex/age, y                       | F/65          | M/37       | F/6                    | M/1              |
| RBC, ×10^12/L (4.2–5.1)          | 1.84          | 3.96       | 4.39                   | 4.18             |
| Hemoglobin, g/dL (12–16)         | 6.6           | 12.1       | 13.3                   | 14.1             |
| MCV, fL (85–100)                 | 105.4         | 82.6       | 82.7                   | 93.3             |
| MCHC, % (32–36)                  | 35.9          | 30.6       | 30.3                   | 33.7             |
| RDW, % (11.5–14.5)               | 23.9          | 18.3       | 16.2                   | 17.9             |
| Reticulocytes, % (0.5–2)         | 22.5          | 5.6        | Not done               | 2.2              |
| Haptoglobin, mg/dL (30–200)      | <20           | <20        | Not done               | Not done         |
| Erythropoietin, mIU/mL (4.3–29)  | 4080          | 37.7       | Not done               | Not done         |
| LDH, IU/L (155–250)              | 250           | 193        | Not done               | 271              |
| Total bilirubin, mg/dL (0.2–1.2) | 8.7           | 4.5        | 1.6                    | 1.9              |
| Direct bilirubin, mg/dL (0.0–3)  | 1.9           | 0.5        | Not done               | 0.5              |
| Irregular Ab                     | Negative      | Not done   | Not done               | Not done         |
| Coombs (direct/indirect)         | Negative/negative | Negative/negative | Negative/negative | Negative/negative |
| Splenomegaly                     | Positive      | Positive   | Negative               | Negative         |
| Neutrophil jaundice              | Not available | Not available | Negative            | Negative         |
| Treatment                        | Transfusion   | Done       | Not done               | Not done         |
|                                 | Splenectomy   | Done       | Not done               | Not done         |
|                                 | Cholecystectomy | Done     | Not done               | Not done         |

LDH = lactate dehydrogenase, MCH = mean corpuscular hemoglobin, MCHC = mean corpuscular hemoglobin concentration, MCV = mean corpuscular volume, RBC = red blood cell, RDW = red cell distribution width.
OMIM 612653), and EPB42 (HS type 5, OMIM 612690). All subjects provided written informed consent for clinical and molecular analyses, and the study protocol was approved by the Institute Review Board of the Catholic University of Korea. Brieﬂy, genomic DNA was extracted from the PB. Capture of the target regions was performed with reagents from a custom design HaloPlex Target Enrichment kit (Agilent Technologies, Inc., Santa Clara, CA). Massively parallel sequencing was performed on the Illumina HiSeq 2000 platform (Illumina, Inc., San Diego, CA). Average coverage of depth of the entire panel was 101×, and 98.3% of targeted bases were covered by 10× sequence reads. Sequence reads were aligned to hg19 with Burrow–Wheeler Aligner (version 0.7.12, MEM algorithm). Duplicate reads were removed by using Picard-tools1.96. Local realignment and base quality recalibration were done by the Genome Analysis Toolkit (GATK ver 3.5). Variant calling was performed by GATK HaplotypeCaller. Variants were annotated by SnpEff ver 4.2. As a result, 1 heterozygous nonsense mutation, c.1956G>A; p.Trp652∗ (reference sequence: NM_001024858.2), was identiﬁed in the exon 13 of SPTB gene (Fig. 2). This novel mutation was not found in public population sequence databases such as 1000 Genomes Project Database, ESP6500, and ExAC, as well as in Korean population sequence databases (KRGDB, http://152.99.75.168/KRGDB/menuPages/intro.jsp). Any (likely) pathogenic variants were not identiﬁed in other 4 HS-associated genes according to the American College of Medicine and Genetics guidelines for the interpretation of sequence variation. Thus, this nonsense mutation of the SPTB was conﬁrmed to cause HS in the proband and may affect symptomatic family members who could not be tested for the mutation.

3. Discussion
Here, we describe a Korean family with 4 members affected clinically by HS. To the best of our knowledge, this is the ﬁrst report describing HS with the novel SPTB mutation detected by targeted NGS in a Korean family. Among them, novel nonsense mutation (p.Trp652∗) of SPTB was identiﬁed by targeted NGS and conﬁrmed by Sanger sequencing in the proband. Unfortunately, genetic testing was not available in symptomatic family members. The proband’s son received a cholecystectomy and her grandchildren experienced neonatal hyperbilirubinemia, indicating a high possibility of spherocytosis, in spite of his incomplete medical history. On the other hand, abnormal spherocytes are trapped and destroyed in the spleen and this is the main cause of hemolysis in HS. Common complications are hemolytic episodes, cholelithiasis, and aplastic crises. Splenectomy is curative but
should be undertaken only after careful assessment of the risks and benefits.\textsuperscript{11} In our proband, spleen removal was performed as an effective therapeutic option, thus, eliminated anemia, hyperbilirubinemia, and lowers the high reticulocyte count to nearly normal levels, effectively.

Confirmation of hereditary RBC membrane disorders at a molecular level is important for not only clinical management of the patient but also genetic counseling. Understanding of the genotype-phenotype correlation is valuable to prepare genetic-based practice in HS. Ankyrin gene (up to 50\%) is most commonly responsible for HS, followed by mutations in spectrin gene (SPTB, up to 20\%; SPTA1, up to 5\%), SLCA41 (up to 15\%), and EPB42 (up to 10\%).\textsuperscript{12} In the Korean population, ANK1 and SPTB are the major HS which corresponded to AD inheritance.\textsuperscript{13} In other ethnic population, ANK1 or SPTB mutations were frequently detected: ANK1 mutations in 31\% (15/49) Japanese HS,\textsuperscript{14} SPTB mutations in 25\% (10/40) the United States and Europe HS.\textsuperscript{15}

To date, 16 missense/nonsense mutations in SPTB have been reported among patients with HS.\textsuperscript{13} Like the novel p.Trp652 mutant mutation reported in this report, 7 have been nonsense mutations sparsely located in the parts of beta spectrin repeats. Deficient beta spectrin protein levels due to nonsense mutations sited on beta spectrin repeats have been described as a cause of HS.\textsuperscript{11} Beta spectrin proteins containing spectrin di-repeat structures may serve a controlled flexibility to membrane-associated scaffolds, as well as an intrinsic mechanosensing switch designed to control the disposition of ligands and signal-transducing molecules in response to cellular deformation or stretch.\textsuperscript{16} Our proband shows increased osmotic fragility, which is well-known pathological feature of HS type 2 caused by an osmotic fragility test.

RBC membrane disorders can be readily screened by various laboratory approaches such as PB smear, osmotic fragility test, and sodium dodecyl sulfate polyacrylamide gel electrophoresis.\textsuperscript{16} However, the confirmatory diagnosis of HS is based on mutational analysis of gene encoding RBC membrane proteins.\textsuperscript{13} An attempt to diagnose the process was described using Sanger sequencing of multiple genes in a sequential manner, which is, however, labor-intensive and expensive. Because RBC membrane disorders are clinically and genetically heterogeneous diseases with broadly overlapping clinical symptoms\textsuperscript{20} Detects in various membrane proteins involved in linking the lipid bilayer to the membrane skeleton result in loss in membrane cohesion leading to surface area loss.\textsuperscript{20} Recently, the advent NGS is a time and cost-effective method of detecting causal mutations associate to various conditions and a large number of candidate genes are reported among patients with HS.\textsuperscript{13} Like the novel p.Trp652 mutation reported in this report, 7 have been nonsense mutations

## 4. Conclusion

In summary, a novel nonsense mutation (p.Trp652*) in SPTB was identified using NGS in a Korean family affected by HS. Five known causative genes involving in RBC cytoskeleton formation are considered in HS. Moreover, more than 20 genes are associated with hyperbilirubinemia and bilirubin metabolism.\textsuperscript{22} Thus, to exactly discover the mutation causing the patient’s clinical manifestations, both pedigree analysis and genetic testing are required simultaneously. We suggest that NGS of causative genes could be a useful diagnostic tool for the genetically heterogeneous RBC membrane disorders, especially in cases with a mild or atypical clinical manifestation.

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