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Identification of Novel Mutations in Children with Hereditary Spherocytosis by Targeted Exome Sequencing: A Single Center Experience

Hedeflenmiş Ekzom Dizilimi ile Kalıtsal Sferositozlu Çocuklarda Yeni Mutasyonların Belirlenmesi: Tek Merkez Deneyimi

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ABSTRACT

Aim: Hereditary spherocytosis (HS) is a prevalent cause of congenital hemolytic anemia in Northern Europeans. It is characterized by spherocytes resulting from defects in the erythrocyte structural membrane proteins spectrin and ankyrin. To date, more than five candidate genes, including ANK1, SPTB, SPTA1, SLC4A1, and EPB42 have been linked to HS. Here, we aim to investigate the presence of novel as well as known mutations in eight Turkish children with clinically suspected HS.

Material and Methods: We presented the clinical features of the patients and identified the causative gene variants using targeted exome sequencing. Eight children who were clinically suspected of having HS enrolled in this study. A family and medical history, clinical examination, relevant laboratory test results, osmotic fragility test (OFT), and genetic results were evaluated.

Results: Six causative variants, including three ANK1 variants, two SPTB variants and one SLC4A1 variant were detected. All these mutations were novel variants. ANK1 and SPTB are the most common mutant genes in children with HS.

Conclusion: This study expanded the mutation spectrum of ANK1, SPTB and SLC4A1. This is the first study to determine the genetic and clinical characteristics of children with HS in Turkey.

Keywords: ANK1, Hereditary spherocytosis, Pathogenic, SPTB, Targeted exome sequencing

ÖZ

Amaç: Kalıtsal sferositoz (HS), Kuzey Avrupalılarda konjenital hemolitik aneminin yaygın bir nedenidir. Eritrosit yapısal membran proteinleri spektrin ve ankirin'deki kusurlardan kaynaklanan sferositlerle karakterizedir. Bugüne kadar, ANK1, SPTB, SPTA1, SLC4A1 ve EPB42 dahil olmak üzere beşten fazla aday gen, HS ile ilişkilendirilmiştir. Burada, klinik olarak HS şüphesi olan sekiz Türk çocuğunda bilinen ve yeni mutasyonların varlığını araştırmayı amaçladık.

Gereç ve Yöntemler: Hastaların klinik özelliklerini sunduk ve hedeflenen ekzom dizilimi kullanarak nedensel gen varyantlarını belirledik. Klinik olarak HS olduğundan şüphelenilen sekiz çocuk bu çalışmaya alındı. Aile ve tıbbi öykü, klinik muayene, ilgili laboratuvar test sonuçları, ozmotik frajilite testi ve genetik sonuçlar değerlendirildi.

Bulgular: Üç ANK1 varyantı, iki SPTB varyantı ve bir SLC4A1 varyantı dahil olmak üzere 6 nedensel varyant tespit edildi. Bütün bu mutasyonlar yeni varyantlardı. ANK1 ve SPTB, HS'li çocuklarda en sık görülen mutant genlerdir.

Sonuç: Bu çalışma, ANK1, SPTB ve SLC4A1 genlerinin mutasyon spektrumunu genişletti. Bu, Türkiye'de HS'li çocukların genetik ve klinik özelliklerini belirleyen ilk çalışmadır.

Anahtar Sözcükler: ANK1, Kalıtsal sferositoz, Patojenik, SPTB, Hedeflenmiş ekzom dizilimi

INTRODUCTION

Hereditary spherocytosis (HS) is a common cause of congenital hemolytic anemia in Europeans. The clinical characteristics of HS include anemia, jaundice, reticulocytosis, cholelithiasis, splenomegaly and increased osmotically fragile in spherocytes. At present, the diagnosis of HS mainly relies on family history, clinical features, peripheral blood smear results, and osmotic fragility tests (OFT) (1). In the blood smears of HS patients, reticulocytes and spherocytes may be present as spherical-shaped cells. HS is widespread throughout the world, with a high prevalence in Northern Europe and Northern America (2). There is no data on the prevalence of HS in Turkish population.

HS is a genetic disorder in which both heredity and molecular characteristics are heterogeneous. Genetic transmission is autosomal dominant (AD) trait in 75% of cases with a de novo mutation (3). The pathogenic mutations ANK1 (ankyrin 1), SPTA1 (spectrin alpha chain), SPTB (spectrin ß chain), SLC4A1 (solute carrier family 4, anion exchanger, member 1) and EPB42 (protein 4.2) genes are the major causes of HS (4-6).The ANK1, SPTB, and SLC4A1 genes are associated with AD traits, whereas SPTA1 and EPB42 genes are related to autosomal recessive traits (Table 1) (5). The ANK1 gene variations are reportedly the most common (~50%), followed by those in the SPTB (~20%) , SLC4A1 (15-20%), and SPTA1 (~5-10%) (7).

However, the mutation profile of HS children has not been well defined in the Turkish population to date. In this study, we identified the causative gene variants in a group of Turkish children with hereditary spherocytosis.

MATERIAL and METHODS

In this retrospective study, eight patients with hereditary spherocytosis were evaluated. All were under age 18 at diagnosis and were followed between October 2020 to September 2022 by the Pediatric Hematology Department of Eskisehir City Hospital (Turkey). Patient data were analyzed retrospectively from patient files and the computer information system. Data gathered included clinical manifestation of patients at admission, sex and gender, family history, hematologic and biochemical data, ultrosonography findings and complications.

Table 1: Main features of the genes encoding the red cell membrane proteins.

Gene	Protein	Location	Inheritance
SPTA1	alpha spectrin	1q22-q23	Recessive
SPTB	beta spectrin	14q23-q24.1	Dominant
ANK1	ankyrin	8p11.2	Dominant
SLC4A1	band 3	17q21-q22	Dominant
EPB42	protein 4.2	15q15-q21	Recessive

ANK1: ankyrin 1, **SPTA1:** spectrin alpha chain, **SPTB:** spectrin ß chain, **SLC4A1:** solute carrier family 4, anion exchanger, member 1 and EPB42: protein 4.2

HS was diagnosed on the basis of clinical history, physical examination, and laboratory test results, including complete blood count, blood smear, reticulocyte count, bilirubin concentration, positive osmotic fragility test, and abdominal ultrasonography. All children were diagnosed as HS; four patients were male and four were female. Informed consent was obtained from the parents of all patients. The study was approved by the ethics committee of Eskişehir Osmangazi Medical Faculty (Protocol number: 2022-227) in accordance with the Declaration of Helsinki. All procedures were performed according to approved guidelines.

Genomic DNA purified from the patients' peripheral blood was performed using the QIAamp DNA Blood Midi Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The genomic DNA was sequenced on the Illumina NovaSeq Platform using the Agilent SureSelect V5 kit (Agilent, Santa Clara, CA, USA) at a mean depth of 100×. Raw data were analyzed with the "QIAGEN Clinical Insight (QCI) Interpret", aligning to the GRCh37/hg19 human genome.

Variant frequencies were subjected to dbSNP (www.ncbi.nlm.nih.gov/snp), 1000 Genomes (http://browser.1000genomes.org) and the Aggregation Consortium (ExAC, http://exac.broadinstitute.org). Polyphen-2, SIFT and MutationTaster software programs were used for pathogenicity prediction. Moreover, the identified variants were evaluated in Human Gene Mutation Database and Clinvar (http://www.ncbi.nlm.nih.gov/ clinvar). In addition, the pathogenicity of all variants was classified according to the American College of Medical Genetics and Genomics (ACMG) guidelines (8).

Used SPSS 15.0 for statistical analysis. Descriptive statistics (frequency, percentage distribution, mean, median etc.) were used for statistical analysis.

RESULTS

Demographic, clinical, and laboratory characteristics of the patient with hereditary spherocytosis are shown in Table 2. The median age at diagnosis was 7.6 (4.80) years old, and four out of eight (50%) patients were male. Only one patient had a positive family history (1/8; 12,5%). There was jaundice in seven patients and anemia in all patients. Only one patient had splenomegaly on abdominal ultrasound.

The median red blood cell (RBC) count was 2.23 × 1012/L and the median hemoglobin level was 8.57 g/dL. The median mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), and mean corpuscular hemoglobin concentration (MCHC) were 81.57 fL (normal range: 80-95), 29.65 pg (normal range: 27-31), and 36.76 g/L (normal range: 32-36), respectively. The median reticulocyte count

was 8.73% (normal range: 0.5%–2.5%) and the total bilirubin concentration was 5.35 mg/dL (normal value: 0.2-0.8). The mean lactate dehydrogenase (LDH) value was 485 units/L (normal range: 140-280). Peripheral blood smears demonstrated spherocytes and anisocytosis in all patients except one patient (P3). An increase in erythrocyte osmotic fragility was observed in five of the patients (5/8; 67.5%).

The genetic result was not detected in two patients (2/8; 25%). In total, six different heterozygous new variants were identified including ANK1 (three variants), SPTB (two variants) and SLC4A1 (one variant) genes (Table 3). Two novel variants result in a premature terminator codon in the coding sequence of ANK1 (c.3022G>T and c.4022C>A) and they have been suggested as deleterious. The other vari-

Table 2: The clinical and laboratory features of the Turkish children with HS.

Patients	P1	P2	P3	P4	P5	P6	P7	P8
Gender	F	M	M	M	M	F	F	F
Age	6y	7y	15y	4y	3y	12y	12y	2y
Family history	-	-	-	-	-	-	-	+
Jaundice	+	+	+	+	+	+	-	+
Anemia	+	+	+	+	+	+	+	+
Splenomegaly	-	-	+	-	-	-	-	-
LAB TESTS								
Rbc ($\times 10^{12}$/L)	1.96	1.92	2.48	1.8	2.16	3.2	2.4	1.94
Hemoglobin (g/dl)	7.6	8.4	9.5	8.2	7.3	10.4	7.8	9.4
MCV (fL)	72.8	80.2	82.5	70.6	89.4	94.4	91.5	71.2
MCH (pg)	23.5	30.6	29.4	28.7	34.0	32.7	27.8	30.5
MCHC (g/L)	35.5	37.4	37.0	36.3	35.8	36.6	38.2	37.3
Reticulocytes (%)	4.6	3.85	8.63	5.76	8.15	12.42	14.65	11.82
Total bilirubin (mg/dL)	5.8	4.0	3.9	4.2	4.8	9.8	5.6	4.7
LDH (U/L)	361	472	346	355	1400	256	308	389
Spherocytes on the peripheral blood smears	+	+	-	+	+	+	+	+
Increased Osmotic Fragility	-	+	+	NA	+	+	+	-

M: Male, **F:** Female, **HS:** Hereditary spherocytosis, **LDH:** Lactate dehydrogenase, **MCH:** Mean corpuscular hemoglobin, **MCHC:** Mean corpuscular hemoglobin concentration, **MCV:** Mean corpuscular volume, **RBC:** red blood cell, **NA:** Not available, **y:** Year(s), **+:** Present, **–:** Absent.

Table 3: Mutations analyzed by targeted exome sequencing in the children with HS.

Patients	Gene	Exon	cDNA	Protein	Mutation Type	Novelty	SIFT	Polyphen2	Mutationtaster	ACMG
P1	ANK1	27	c.3022G>T	p.Glu1008Ter	nonsense	Novel	damaging	-	disease causing	Likely pathogenic
P2	ANK1	33	c.4022C> A	p.Ser1341Ter	nonsense	Novel	damaging	-	disease causing	Likely pathogenic
P3	SPTB	22	c.4550T>G	p.Met1517Arg	missense	Novel	damaging	probably damaging	disease causing	Uncertain Significance
P4	SPTB	13	c.2386C>T	p.Arg796Cys	missense	Novel	damaging	probably damaging	disease causing	Likely pathogenic
P5	-	-	-	-	-	-	-	-	-	-
P6	-	-	-	-	-	-	-	-	-	-
P7	SLC4A 1	13	c.1486T>C	p.Trp496Arg	missense	Novel	damaging	probably damaging	disease causing	Likely pathogenic
P8	ANK1	17	c.1912C>T	p.Pro605Ser	missense	Novel	damaging	probably damaging	disease causing	Likely pathogenic

ACMG: American College of Medical Genetics and Genomics, **SIFT:** Sorting Intolerant From Tolerant.

ants in SPTB, SLC4A1 and ANK1 were missense mutations that resulted in an amino acid change (Table 3). According to the ACMG, all five detected novel variants were classified as pathogenic, and one variant was rated as a variant of unknown significance (VUS) (SPTB: c.4550T>G). None of the detected variants have been previously reported in any human gene mutation database. The details of the identified variants is given in Table 3. The mutation images of P2 (ANK1:c.4022C> A) and P3 (SPTB: c.4550T>G) patients are given in Figure 1.

DISCUSSION

Hereditary spherocytosis (HS) is a hereditary hemolytic disease that is caused by encoding-gene mutations in ANK1, SPTB, SLC4A1, EPB42, and SPTA1 genes (9). It occurs with a higher incidence in the population of Northern Europe and Northern America (approximately 1:2000) (10,11). Approximately 75% of HS cases can be inherited as an autosomal dominant trait (12). HS caused by mutations in ANK1, SPTB, and SLC4A1 is inherited in an autosomal dominant manner, while the mutations in SPTA1 and EPB42 is inherited in an autosomal recessive manner (9). Genetic testing is critical both to confirm the diagnosis and to provide genetic counseling. The phenotype of HS is heterogeneous, but typical symptoms include hemolysis, anemia, jaundice, and splenomegaly. The diagnosis of HS is based on having a family history, typical clinical features, evaluation of biochemical markers of hemolysis, peripheral red blood cell morphology examination and a positive erythrocyte osmotic fragility test (13). The advancement of genetic molecular methods has recently allowed for the definitive diagnosis of HS patients.

The application of next-generation sequencing (NGS) has led to impressive progress in the diagnosis of genetic diseases (14,15). NGS is an extremely important test in the molecular diagnosis of HS in clinical practice, including targeted exome sequencing (TES) and whole exome sequencing (WES) (16). Previous studies have demonstrated the usefulness of the targeted NGS methods in investigating the causal gene variants in patients with HS (17,18). In the present study, we found six novel gene mutations in children with HS. Targeted exome sequencing has identified 3 patients with an ANK1 gene mutation, two patients with a SPTB gene mutation, and one patient with a SLC4A1 gene mutation. This study indicated that the most frequently affected genes in Turkish children with HS were ANK1and SPTB. This result is similar to previous results found in American, European and Chinese populations (19-21). All mutations are novel and unique, having not previously been reported in a different population. We did not find any mutations in the EPB42 and SPTA1 genes, which were thought to be related to the sample size. Genetic studies on HS in Turkey are very limited, and there are only descriptive studies including clinical pathological phenotypes (22, 23). To our knowledge, this is the first report to describe the mutational spectrum in Turkish children affected by HS.

The ANK1 gene encodes a protein called ankyrin-1, which is found in the cytoplasmic membrane of red blood cells. This protein interacts with transmembrane proteins to form the membrane skeleton of cells (6,13). To date, more than 200 ANK1 gene mutations have been reported in HS patients. The reported mutations included nonsense, missense, and splicing mutations, small or gross deletions; and insertions

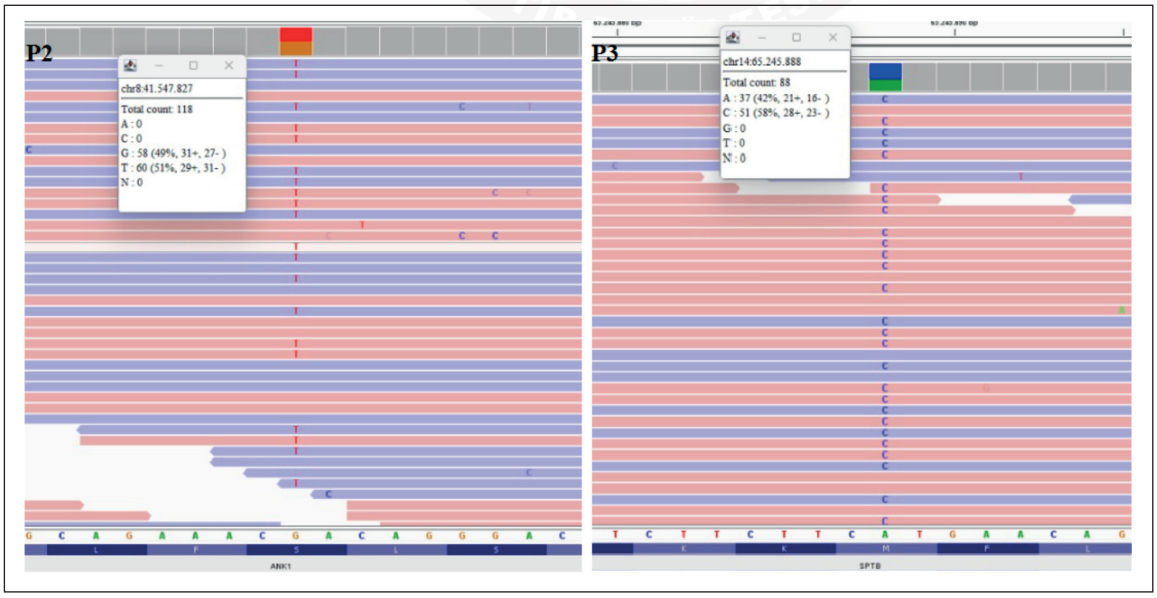

Figure 1. The mutation images of P2 (ANK1:c.4022C> A) and P3 (SPTB: c.4550T>G) patients.

(24).In this study, three novel mutations were identified in ANK1, including two nonsense mutations (c.3022G>T and c.4022C>A) and one missense (c.1912C>T) mutation. They were predicted as likely pathogenic by in silico protein programs. Based on the ACMG criteria, they were also categorized as likely pathogenic (Table 3).

The SPTB gene, which encodes α- and β spectrin, is localized on 14q23.3 and consists of 35 exons. These spectrin proteins play a crucial role in the formation and stability of the erythrocyte membrane (25). There are more than 40 reported mutations in the SPTB gene associated with HS (26). The types of mutations include nonsense and missense mutations, splicing mutations, and small or gross deletions, insertions or indels (27). In the present study, we found two novel missense SPTB mutations (c.4550T>G and c.2386C>T) associated with HS. According to in silico prediction tools, these novel variants were predicted as likely pathogenic.

The SLC4A1 (solute carrier family 4, anion exchanger, member 1) gene contains 20 exons and encodes both erythroid and kidney isoforms of anion exchanger 1. The N-terminal 40kDa domain is located in the cytoplasm and functions as a binding site for the red cytoskeleton through its interactions with transmembrane proteins (28). The C-terminal domain contains the binding sites for carbonic anhydrase II and is responsible for anion exchange. Mutations in the SLC4A1 gene have been linked to HS, ovalocytosis, and renal tubular acidosis (29). Currently, more than 70 pathogenic SLC4A1 gene mutations have been identified in HS (30). Here, we identified a novel missense (SLC4A1: c.1486T>C; p. Trp496Arg) mutation in a child with HS. This variant is absent in the public databases.

Herediter spheroctosis is genetically and phenotypically highly heterogeneous. In a study from the Netherlands, SPTA1, ANK1, and SPTB, EPB42 ranked as the top four genes with identified variants in patients with HS, respectively (31). However, SPTA1 and EPB42 gene variants were not found in our cohort group.

Anemia with varying degrees of increased reticulocyte count was detected in all of our HS cases. The fact that 75% of our patients had MCHC> 36.0 g/l indicates that this parameter is an effective factor in the diagnosis of HS in our population. Spherocytes were detected in peripheral blood smears of 87.5% of the patients. In this study, the osmotic fragility test was significant in 5 of 8 patients (67.5%). A parental history of HS was reported in only one of the patients with HS (12.5%). Among the 8 subjects, only one had splenomegaly (12.5%, Table 2).

The observed severity of clinical and laboratory findings was similar to the extent that they could not be classified according to gene variants. Therefore, we could not establish a significant genotype-phenotype correlation. It was not possible to find a clear correlation, probably due to the small sample size in this study.

This study reported on eight Turkish children with suspected clinical features of HS and identified six novel gene variants (3 novel in ANK1, 2 novel in SPTB, and 1 novel in SLC4A1) by targeted exome sequencing. Our study is the first to use the TES approach for the genetic diagnosis of Turkish children with HS. We clarified the mutational spectrum in patients with HS: ANK1 and SPTB genes are the most common causes of HS in Turkish children. Our study also broadens the spectrum of ANK1 and SPTB mutations and provides valuable information about the genotyping of HS in Turkish children. Future studies in larger study groups are needed to detect the mutational diversity in Turkish children with HS.

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None.

Author Contributions

The idea of presenting the study to the literature and collecting the data of the study: **Ayça Kocaağa.** Analysis of patient's data, writing of article and preparation of images: **Ayça Kocaağa, Hatice Mine Cakmak.**

Conflicts of Interest

Each author confirms that they do not have any conflict of interest.

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Ethical Approval

The study was approved by the ethics committee of Eskişehir Osmangazi Medical Faculty in accordance with the Declaration of Helsinki (Protocol number: 2022-227).

Review Process

After the blind peer-review process, it was found suitable for publication and accepted.

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