Molecular Investigation of Iranian Patients Suspected to Hereditary Spherocytosis

Shahab-Movahed Z, Majd A, Torbati ES and Zeinali S*1

1Department of Cellular and Molecular Biology, Faculty of Biological Sciences, Islamic Azad University, North Tehran Branch, Tehran, Iran
2Department of Genetic, Faculty of Science, North Tehran Branch of Islamic Azad University, Tehran, Iran
3Department of Medical Molecular, Biotechnology Research Center, Pasteur Institute of Tehran, Iran
4Kowsar Human Genetic Research Center, Tehran, Iran

*Corresponding author: Sirous Zeinali, Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran

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Abstract

Introduction: Hereditary spherocytosis is a heterogeneous disorder with mild to moderate anemia. The aim of this study was to evaluate the inherited spherocytosis gene mutations in patients with RBC cytoplasmic disorders in Iranian population.

Materials and Methods: In this study, Whole Exome Sequencing (WES) was performed for patients suspected to hereditary spherocytosis and their relatives.

Results: Sequence analysis of the probands and their parents identified variations in ANK1 gene (NM_001142446.1: c.127-2A>G), SPTB (c. 14delC, p.Thr5LysfsTer41), SPTA1 (c.466C>T), SLC4A1 (c.2494C>T) and SLC25A38 gene (c.683G>T, NP_060345.2:p.Gly228Val) that could be related to the patients clinical manifestation.

Conclusion: Findings are in line with the appropriate diagnostic yield of WES in determining the causative variant especially in those disorders that many genes are involved like anemia. This is the first report of a cohort of Iranian patients with anemia suspected to that were investigated using WES technology. Further studies are needed to investigate the distribution of gene mutations in patients with RBC membrane disorders in Iran

Keywords: Hereditary spherocytosis; Whole-exome sequencing; Hemolytic anemia; Erythrocyte membrane protein; ANK1

Introduction

Red cell membrane disorders that affect its membrane stability results in irregular red cell shape and disorders including Hereditary Spherocytosis (HS), Hereditary Distal Renal Tubular Acidosis; AE1: Anion Exchanger 1; WES: Whole Exome Sequencing; ACMG: American College of Medical Genetics; ANK1: Ankyrin 1; EPB42: Erythrocyte MEMBRANE PROTEIN PROTEIN BAND 4.2; HS: Hereditary Spherocytosis; MCH: Mean Corpuscular Hemoglobin; MCHC: Mean Corpuscular Hemoglobin Concentration; MCV: Mean Corpuscular Volume; PCR: Polymerase Chain Reaction; RBCs: Red Blood Cells; SLC4A1: Solute Carrier Family 4 Member 1; SPTA1: Spectrin α Erythrocytic 1; SPTB: Spectrin β; Variants of Unknown Significance (VUS)

Abbreviations

HS: Hereditary Spherocytosis; DRTA: Hereditary Distal Renal Tubular Acidosis; AE1: Anion Exchanger 1; WES: Whole Exome Sequencing; ACMG: American College of Medical Genetics; ANK1: Ankyrin 1; EPB42: Erythrocyte MEMBRANE PROTEIN PROTEIN BAND 4.2; HS: Hereditary Spherocytosis; MCH: Mean Corpuscular Hemoglobin; MCHC: Mean Corpuscular Hemoglobin Concentration; MCV: Mean Corpuscular Volume; PCR: Polymerase Chain Reaction; RBCs: Red Blood Cells; SLC4A1: Solute Carrier Family 4 Member 1; SPTA1: Spectrin α Erythrocytic 1; SPTB: Spectrin β; Variants of Unknown Significance (VUS)

Materials and Methods

Patients and study design

Patients with anemia and primary clinical diagnosis of Hereditary Spherocytosis (HS) were referred from the Blood disorder clinic of Ali-Asghar Children’s Hospital, Iran University of Medical Sciences, Tehran, Iran to Dr. Zeinali’s Medical Genetics Lab, from September 2017 to March 2018 for genetic counseling and genetic diagnosis. Each patient was reviewed to determine clinical features, laboratory data, disease history (age at diagnosis, jaundice, transfusion history and age at first transfusion and family history). Families with clinical evidence of congenital hemolytic anemia suspected to HS were recruited to the study. After genetic counseling, drawing family pedigree, recording the clinical characteristic and obtaining informed consent, peripheral blood sample (10 ml) were taken from patients and other family
members on tubes containing EDTA. Hematologic tests including Complete Blood Count and Analysis (CBC), reticulocyte count, NaCl osmotic fragility test (on fresh and incubated blood), red blood cell distribution width (RDW), peripheral blood smear examination, Direct Antiglobulin Test (DAT) and hemoglobin electrophoresis were performed at Ali-Asgar Children’s Hospital laboratory. Genomic DNA was extracted using salting out Proteinase K method (Kawser Biotech Co., Tehran, Iran) following the manufacturer’s instruction. Quantity and quality of isolated DNA were measured by Nanodrop spectrophotometer (ThermoScientific®, Waltham, USA) and gel electrophoresed on 1% agarose, respectively. This study was approved by the ethics committee of Kawasaki Human Genetics Research Center (KHGRC).

Whole genome sequencing was performed using Illumina’s HiSeq X-ten machines (deCODE Genetics, Reykjavik, Iceland) (Supple S1). More than 95% of the targeted sequences were covered adequately for high-confidence variant calling (>30 X coverage). Rare variants with minor allele frequency less than 1% in the population were included in final analysis. We considered coding and splicing variants with high impact (stop, frameshift, and splice site) and moderate impact (missense, in frame and splice region) on protein function and focused on Single-Nucleotide Polymorphisms (SNPs) and small indels (< 20 base pairs) and prioritized the analysis on mutations in phenotypically relevant genes, i.e. genes with a known link to Mendelian disorders according to Online Mendelian Inheritance in Men (OMIM) (http://www.omim.org) that fit the phenotypes of the index and the inspected inheritance model (especially those relevant to HS including ANK1 (HS type 1, OMIM #182900), SPTB (HS type 2, OMIM #616649), SPTA1 (HS type 3, OMIM #270970), SLC4A1 (HS type 4, OMIM #612653), and EPB42 (HS type 5, OMIM #612690) genes). Variants in candidate genes that were suspected of the index and the inspected inheritance model (especially those relevant to HS including ANK1 (HS type 1, OMIM #182900), SPTB (HS type 2, OMIM #616649), SPTA1 (HS type 3, OMIM #270970), SLC4A1 (HS type 4, OMIM #612653), and EPB42 (HS type 5, OMIM #612690) genes). Variants in candidate genes that were suspected of being involved in the disease pathogenesis (Variants of unknown significance (VUS), Likely Pathogenic and Pathogenic) were selected for Sanger sequencing in the proband, her/his parents and other family members, if present. Specific forward and reverse primers according to the identified variants were designed with NCBI Primer-Blast Tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast) and synthesized commercially by Metabion Co., Germany. A list of the designed primers and amplification reaction were provided in Supple S2 and Table S1.

### Results

Twenty-five samples (9 affected cases and 16 healthy family members from 5 consecutive families (consanguineous marriage in 2 families) were enrolled to the study by anemia and primary clinical diagnosis of Hereditary Spherocytosis (HS). Clinical characteristics and laboratory data in probands at the time of first visit are summarized in Table S1. Following variant filtering of WES results in the probands and their parents, two pathogenic/likely pathogenic variants in ANK1 and SPTB genes were identified in two separate families and confirmed by segregation analysis within the family by direct Sanger sequencing (Table 1, Figure 1 and Figure 2). Furthermore, we identified some variants that were selectively present in other families that underwent further segregation within the family to confirm their presence and pathogenicity, as follows.

In family 1 (family 363821), the proband was a 13 YO girl with neonatal presentation of jaundice and pallor. Clinical feature and the pedigree were shown in Table S1 and Figure 1 respectively. Similar presentation and diagnosis were performed for her mother who underwent splenectomy and cholecystectomy. Sequencing the affected child, her affected mother and healthy father, maternal aunt and grandmother revealed a heterozygote pathogenic splicing variant in ANK1 gene (NM_001142446.1:c.127-2A>G) in the affected cases

### Table 1: Assessing the pathogenicity of mutations identified in 5 Iranian families, using bioinformatics tools and ACMG Standards.

| Family ID | Gene | Position (hg38) HGVSc/HGVSp | Variant type (Exon No/ Total exon No) | ACMG classification/Inheritance |
|-----------|------|-----------------------------|-------------------------------------|---------------------------------|
| 363821    | ANKI | chr8: 41758139 NM_001144246.1c: 127-2A>G | Hetero, splice_acceptor_variant, Intron 1/42 | Pathogenic variant, AD          |
| 364002    | SPTB | Chr14: 64823080 NM_001024658.2:c.14 delC | Hetero, Frameshift_variant Exon 2/36 | Pathogenic variant, de novo     |
| 3364803   | SLC4A1 | chr17:44251320 NM_000343.2:c.2484C>T NP_000333.1:p.Arg832Cys | Homo, Missense variant Exon19/20 | VUS, AR                        |
|           | SPTA1 | chr1:156681592 NM_003128.2:c.466C>T NP_003117.2:p.Arg56Trp | Hetero, Missense variant, Exon4/52 | VUS, AD                        |
| 366364    | HBB  | Chr11: 5226930 NM_00051 804:c.92G>C NP_000509.1:p.Arg3Thr | missence variant 1/3 | Likely pathogenic AD           |
|           | EPB41 | NM_001166005.1:c.1228G>T NP_001159477.1:p.Asp410Tyr | Het (affected father) missnce_vairiant, 9/21 | vus               |
|           | PIEZO1 | NM_001142864.2:c.2857C>T NP_001136336.2:p. Arg636Cys | Het (affected father) missence_vairiant 21/51 | vus               |
|           | KIF22 | chr16:29803561 NM_007317.2:c.1563-1564delAG NP_015556.1:p.Ala523LysTer3I | Het (affected son frameshift_variant HIGH impact 10/14 | Likely pathogenic AD           |
|           | NOTCH3 | NM_000435.2:c.2647C>T NP_000426.2:p.Arg883Ter | Het (affected son) stop_gained_vairiant, HIGH impact 17/33 | Likely pathogenic AD           |
| 318635    | SLC25A38 | Chr3:39394467 NM_017767.2:c.683G>T NP_060345.2:p.Gly228Val | Homo, missence_variant, (6/7) | Likely pathogenic, AR          |

Het: Heterozygous; AD: Autosomal Dominant; VUS: Variant of Uncertain Significance.
(mother and daughter) that were confirmed by Sanger sequencing (Table 2). The variant is located in a conserved position with GERP score = 4.37 and \textit{in silico} analysis with Human Splicing Finder (HSF) predicted it as most probably affecting splicing.

In family 2 (family 364002), the proband was an 18 YO boy with neonatal presentation of jaundice, pallor and hyperbilirubinemia. Clinical feature and the pedigree were shown in Table S1 and Figure 2 respectively. No history of hemolytic anemia was noted in his parents. He underwent splenectomy and cholecystectomy due to severe anemia at age 6 YO and clinical and laboratory presentation of the anemia was ameliorated after surgery. Sequencing the affected child, her healthy parents and, sibling and grandmothers revealed a heterozygote pathogenic frameshift de novo variant in SPTB gene (c. 14delC, p.Thr5LysfsTer41) in the affected case that was absent in the other healthy family member (confirmed by Sanger sequencing) (Figure 2). The variant is located in a conserved position with GERP score = 5.05.

In family 3 (family 3364803, (the proband was a 5 YO girl with early neonatal onset of severe jaundice, pallor, anemia and hemolysis who received blood transfusion at one month of age due to clinical diagnosis of HS disease. Anemia was reported in her half-sibling and paternal cousins, suspected to thalassemia minor (samples were not available to test). Recurrent urinary tract infection was reported in the affected case from 1 month. DMSA renal scan showed left poles damage with no signs of nephrolithiasis and hydronephrosis in renal ultrasound study. In addition, proband did not present with auditory deficits or cognitive impairment. Her parents were consanguineous and had no splenomegaly, renal failure or bone disease in the time study. There was some hematologic presentation of HS including peripheral blood spherocytes in PBS and increased osmotic fragility test without any frank presentation of anemia in her mother. Clinical feature of the proband and the pedigree were shown in Table S1 and
Two different Variants of Uncertain Significance (VUS) in SPTA1 and SLC4A1 genes were shown in the proband (Table 1). Segregation analysis in the family (Sanger sequencing of the proband and her parents) showed that the homozygote variant in SLC4A1 gene (c.2494C>T) in the affected case, was inherited from heterozygote carrier parents. The heterozygote variant in SPTA1 gene (c.466C>T) was inherited from her heterozygote mother (confirmed by Sanger sequencing in the proband and her mother (Figure 3). The presence of SLC4A1 homozygote mutation in the proband beside a history of failure to thrive and urinary infection in neonatal age was in line with the diagnosis of incomplete distal renal tubular acidosis with AR inheritance that could be more severe presented due to the co-presence of the variant in the SPTA1 gene.

In family 4 (family 366364), the proband was a 8 YO boy with neonatal presentation of jaundice and pallor from 2 months old that was initially has been diagnosed as thalassemia minor (C30;
AGG>ACG) that was inherited from his father with anemia, icterus, and reticulocytosis. Clinical feature and the pedigree were shown in Table S1 and Figure 4 respectively. As the clinical presentation couldn’t be explained with thalassemia minor, whole exome analysis was made for the affected case, his parents and uncle (Trio-plus). Two heterozygous Variants of Uncertain Significance (VUS), in KIF22 (c.1563_1564delAG) and NOTCH3 genes (c.2647C>T) have been detected in the proband that were absent in the proband’s parents (confirmed by Sanger sequencing). We also detected two different heterozygote variants of uncertain significance (VUS), in EPB41 (c.1228G>T) and PIEZO1 genes (c.2857C>T) in the proband’s father that were absent in the proband and his mother (confirmed by Sanger sequencing) (Table 1).

In family 5 (family 318635), the proband was a 5 YO girl with microcytic hypochromic anemia who receive monthly blood transfusion from 3 months old with clinical diagnosis of thalassemia major. There was a family history of thalassemia major in her maternal cousin (IVSI-5 homozygote). Her mother was known carrier of thalassemia minor (IVSI-5 heterozygote, known mutation) and her brother also receives blood transfusion with similar manifestation. Clinical feature and the pedigree were shown in Table S1 and Figure 5 respectively. Molecular studies revealed mutation in HBB gene (IVSI-5 heterozygote) and no other HBB gene mutation in this sibling. No deletion and duplication in HBB gene was found in the proband’s brother investigation using MLPA technique. As the primary clinical diagnosis of thalassemia major, hadn’t been confirmed, whole exome sequencing was made for the proband and her healthy parents to investigate other genes with related to the other genetic cause of anemia like hereditary spherocytosis. We detected a previously reported homozygote missense variant (c.683G>T, NP_060345.2:p.Gly228Val) in SLC25A38 gene that was present in both affected children (homozygote) and their parents (heterozygote). This variant has been reported previously in HGMD database (CM114292) (PMID: 21393332), and likely pathogenic variant in the SLC25A38 gene was reported in sideroblastic anemia-2, pyridoxine-refractory: SIDBA2 (PMID: 21393332 and 19412178) (OMIM # 205950, AR).

**Discussion**

Hereditary Spherocytosis (HS) is a heterogeneous inherited disorder with wide range of clinical presentation and different mode of inheritance. HS originates from the mutations in the many genes coding various components of RBC membrane and molecular study of the related genes and detection of the causative variations is the mainstream of its diagnosis. Different genes are correlated to the pathogenicity of HS so far including ANK1, SPTB, SPTA1, SLC4A1, and EPB41. However some previous studies, were reported cell membrane disorders and hemolytic anemia based on clinical and laboratory characteristics in Iran [11-14], to our knowledge this is the first report of molecular study of hereditary spherocytosis using Next Generation Sequencing (NGS) method in Iran. In the present study, we detected the pathogenic variation in ANK1 and SPTB in two unrelated families. In two other families, we found VUS, candidate variant that their correlation and pathogenicity hadn’t been confirmed through familial segregation study. In one case the final molecular diagnosis was sideroblastic anemia.

In family 1, the causative pathogenic variant was found in the ANK1 gene that encodes a 206-kD protein (Ankyrin protein) and is the most common cause of HS (Approximately half of all HS patients) following by SLC4A1 and SPTB genes [7]. The major function of ankyrin is to stabilize the membrane structure by interacting with beta-spectrin, protein 4.2, and band 3 protein and the deficiency of ankyrin protein leads to a decrease in spectrin assembly on the membrane skeleton thus, causes Loss of membrane [15]. Different kind of variation have been determined along the gene, including missense, nonsense, frameshift and splicing mutations (more than 60) with autosomal dominant or autosomal recessive pattern [16,17]. Ankyrin is combined of three major domains, including a multiple ankyrin repeats N-terminal domain, a spectrin-binding center region, and a regulatory C-terminal domain [18]. A de novo pathogenic variant in the SPTB gene was detected in family 2 that encodes β-spectrin protein. It plays a significant role in the strength of the erythrocyte membrane [16] and mutation in the gene may change the RBC membrane structure and surface area, and thus inducing
families for this research project. I’d also like to thank Dr. Shahla Ansari Damavandi (Ali-Asghar Children’s Hospital) for guidance.

Healthy child. Study of HS take advantage of assessing the risk of HS for the other recognize hemolytic effect and type of inherited anemia. Molecular especially hereditary spherocytosis is more complicated mainly variants of uncertain significant and also genes that has not correlated further familial study and population data to classify the yielded the causative pathogenic variant in many cases. However, we need further familial study and population data to classify the variants. Sequencing the proband in family 5 showed a likely pathogenic variant in SLC25A38 gene that is related to a form correlation study within the family and also further population data to classify the variants. Sequencing the proband in family 5 showed a likely pathogenic variant in SLC25A38 gene that is related to a form of anemia in which the bone marrow produces ringed sideoblasts rather than healthy peripheral erythrocytes.

In conclusion, sequencing and analysis of the multiple genes in a single test using high-throughput Next-Generation Sequencing (NGS) test, seems to act as a powerful genetic diagnostic tool and increase diagnostic efficiency [16] for congenital hemolytic anemia especially if done as parents-offspring trio test. It enables us to find the causative pathogenic variant in many cases. However, we need further familial study and population data to classify the yielded variants of uncertain significant and also genes that has not correlated to the phenotype yet. Genetic diagnosis of congenital anemia and especially hereditary spherocytosis is more complicated mainly because of the genetic heterogeneity and the large size of the genes related to the phenotype [2]. Therefore, next-generation sequencing could be a cost-effective for the molecular diagnosis of spherocytic hemolytic anaemia, especially when family history is uninformative, physical examination or when routine laboratory findings cannot recognize hemolytic effect and type of inherited anemia. Molecular study of HS take advantage of assessing the risk of HS for the other relatives and also help them with their future pregnancies to have a healthy child.

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Highlights

- Red cell membrane disorders that affect its membrane stability results in irregular red cell shape.
- Hereditary spherocytosis is a heterogeneous inherited disorder due to red blood cell structural proteins with a wide range of clinical presentation and different mode of inheritance.
- Next-generation sequencing could be cost-effective for the molecular diagnosis of spherocytic hemolytic anemia.

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