

Exome-Based Trio Analysis for Diagnosis of the Cause of Congenital Severe Hemolytic Anemia in a Child

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Keywords

Whole exome sequencing · Variant detection · Spherocytosis · Hereditary · Hemolytic anemia

Abstract

Inborn hemolytic anemia requiring frequent blood transfusions can be a life-threatening disease. Treatment, besides blood transfusion, includes iron chelation for prevention of iron accumulation due to frequent blood transfusions. We present the results of a clinical investigation where the proband was diagnosed with severe hemolytic anemia of unknown origin soon after birth. Transfusion was required every 4–6 weeks. After whole exome sequencing of the proband and his parents as well as a healthy sibling, we established that the proband had a compound heterozygous state carrying two rare variants in the erythrocytic spectrin gene, *SPTA1*. The maternal allele was a stop mutation (rs755630903) and the paternal allele was a missense mutation (rs375506528). The healthy sibling had the paternal variant but not the maternal variant. These rare variants of *SPTA1* most likely account for the hemolytic anemia. A severely reduced osmotic resistance in the erythrocytes from the proband was demonstrated. Splenectomy considerably improved the hemolytic anemia and obviated the need for blood transfusion despite the severe clinical presentation.

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Introduction

Inborn hemolytic anemia can be due to hereditary spherocytosis, hereditary elliptocytosis, or pyropoikilocytosis. It has been estimated that hereditary spherocytosis and hereditary elliptocytosis are the most common red blood cell (RBC) membrane disorders in the world, with a prevalence of about 1:2,000 [1, 2]. The frequency in Denmark is increasing [3].

Other inborn variants in enzymes like RBC pyruvate kinase and glucose-6-phosphate dehydrogenase deficiency can also cause hemolysis of RBCs [2, 4, 5].

Some of the genes frequently causally involved in hemolytic anemia due to RBC cytoskeletal aberrations are ankyrin 1, *SLC4A1*, *SPTB*, *SPTA1*, and *EPB42* [6]. These genes are involved in maintaining the unique RBC biconcave membrane flexible structure and are involved in immobilizing the *SLC4A1* (the band 3 protein) in the RBC membrane. Spectrin alpha/beta tetramers associate with *EPB41* (erythrocyte membrane protein band 4.1) and actin to form the cytoskeletal superstructure of the erythrocyte plasma membrane [6].

Exome analysis is well suited for finding candidate genes for rare, heritable diseases in a single family with a single affected individual [7].

We present the results of a clinical investigation where the proband was diagnosed with severe hemolytic anemia of unknown origin soon after birth. Despite prior thor-

Table 1. Primers used for amplification of the paternal allele and NGS sequencing

Oligo name	Sequence (5'→3')
<i>SPTA1</i> up	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTCCGATCT GAGGCTCTCAGCAAAATGTT C
<i>SPTA1</i> low	CAAGCAGAAGACGGCATAACGAGATCGTGATGTGACTGGAGTTCAGACGTGTGCTCTCCGATCT CTGGGCTCTTTCCTTTTC CT

Bases in bold are gene specific.

ough investigations with genetic analyses for enzyme deficiencies as well as bone marrow biopsies and peripheral blood smears, no specific diagnosis as to the cause of the hemolytic anemia was established, only poikilocytosis was noted. We decided to perform an exome analysis of the proband, the parents, and a healthy sibling aiming to secure a molecular diagnosis of the hemolysis.

Materials and Methods

Proband

The proband was male and was given phototherapy shortly after birth and had a rapid bilirubin increase, developed anemia, and received the first blood transfusion day 6 after birth due to a hemoglobin of 3.8 mM. A blood transfusion was also given on day 12, and day 30, and since then, every 3–4 weeks until splenectomy at age 5 years. Due to reticulocytopenia and uncertain diagnosis, a bone marrow analysis was performed at age 4 months on the suspicion of DBA (Diamond van Black Anemia). The result was inconclusive, despite a repeated bone marrow biopsy. Many other diagnostic procedures were undertaken with no conclusive result. The anemia was macrocytic with a low B-12 level and reticulocytopenia, but from age 6 months the anemia changed and reticulocytosis was noted and the anemia was consequently classified as a transfusion-dependent hemolytic anemia of unknown etiology. To establish a molecular etiology, an exome study was initiated.

Other Family Members

Besides the proband, also the mother, father, and a healthy male sibling were included in the study.

Blood Samples

EDTA-anticoagulated blood was collected from each family member: father, mother, proband, and a healthy sibling. The sample from the proband was taken just before a transfusion was given. Both parents were healthy. Buffy coat mononuclear cells were purified on Lymphoprep (Thermo Fisher Scientific, Roskilde, Denmark), and DNA was purified from the mononuclear cells using an automated procedure on a Symphony robot (Qiagen, Copenhagen, Denmark). Genomic DNA concentration was measured on a Qubit fluorometer (Fisher Scientific, Roskilde, Denmark).

Blood Group Determination

Blood group determination for all family members except for the proband was performed according to the standard serological methods in the blood bank, at Copenhagen University Hospital. The blood groups of the proband only were determined using the ID CORE XT kit (BLOODchip; Progenika, Bilbao, Spain) because of the frequent transfusions. The following blood groups used for sample identity control were determined serologically or predicted

genetically: ABO, RhD, RhCE, Kell, Kidd, Duffy, and MSs. The independent serological and BLOODchip-based determination of the blood groups was compared to the blood groups deduced from the exomes and used as identity controls during exome analysis.

Library Production

Exome libraries were prepared with Illumina's TruSeq adaptors and sequenced on Illumina HiSeq 2500 (Illumina, San Diego, CA, USA). In brief, 1 µg of genomic DNA was fragmented to 150–200 bp by sonication using a Covaris S2 (Covaris, Woburn, MA, USA). End repair and adaptor ligation was performed on the SprITE (Beckman Coulter) using Illumina's adaptors (TruSeq DNA LT Sample Prep Kit) and SPRIworks Fragment Library Cartridges I with a size selection between 200 and 400 bp. The exome was captured using the SureSelect Human All Exon V5 (Agilent, Santa Clara, CA, USA) covering the coding exons without 5' and 3' UTR according to the manufacturer's instructions. The captured DNA was sequenced on the Illumina HiSeq 2500 platform as paired-end sequencing 2 × 101 bases resulting in approximately 100 million sequences in pairs from each sample with an average coverage of 92. The data were mapped using CLC workbench version 7.5 (Qiagen) after removal of duplicate reads (approximately 3%) and trimming of the 3' base of each read. Variants were called with a minimum coverage of 10x resulting in about 100,000 variants identified in each exome. Reads were aligned to *hg19* (February 2009 [GRCh37/hg19]) and variants scored using CLC software.

Analysis of Variants

The variants were analyzed as trio analysis assuming recessive, compound heterozygote or dominant inheritance mode, respectively, as well as analyzed for *de novo* mutations in the affected child using the QIAGEN Ingenuity Variant Analysis Software. SIFT 6.2.1 [8], Mutation taster (20 December 2020) [9] and PolyPhen function [10] predictions were also made. Frequencies of variants were checked in the genome aggregation database (gnomeAD). The sequences flanking the paternal variant rs375506528 of exon 31 of *SPTA1* were PCR amplified with an annealing temperature of 60°C and 35 PCR cycles using appended primers (Table 1) and NGS sequenced to ascertain heterozygosity of the paternal allele.

Osmotic Resistance Assay

For standardization, all samples were examined 24 h after they were drawn, and all procedures were carried out at room temperature. Before flow cytometry, 0.5 mL of EDTA blood was washed with 2 mL of phosphate-buffered saline (PBS, 17.3–17.8 mS/cm) and centrifuged at 2,200 g for 5 min. After the supernatant was discarded, 2 µL of RBCs were resuspended in 1 mL of PBS adjusted at various dilutions (100%, 60%, 50%, 40%, and 30%) with distilled water and incubated for exactly 3 min before analysis. 10,000 events were collected using a Canto flow cytometer (BD Biosciences, San Francisco, CA, USA) and the percentage of intact RBCs

Table 2. Variants found after analysis with ingenuity variant analysis software

Chromosome	Position*	Gene region	Gene symbol	Type of variant	Rs number	Protein variant	Translation	Frequency (European) [#]	Genotype of individual [§]	Read depth	SIFT score	Mutation taster score	PolyPhen function prediction
1	158613207	Exonic	<i>SPTA1</i>	c.4347G>T	375506528	p.K1449N	Missense	0.0001911	F, het M P, het S, het	F, 12 [‡] M, 0 P, 17 S, 34	0.0, damaging	94, disease causing	Probably damaging
1	158627401	Exonic	<i>SPTA1</i>	c.2671C>T	755630903	p.R891*	Stop gain	0.00001470	F M, het P, het S	F, 0 M, 63 P, 80 S, 0	–	Disease causing	–
3	10094159	Exonic	<i>FANCD2</i>	c.1634A>G	145222204	p.N545S	Missense	0.007395	F M P, het S	F, 0 M, 0 P, 12 S, 0	0.71, tolerated	Polymorphism	Benign
17	16068383	Exonic	<i>NCOR1</i>	c.528C>T; c.201C>T	199892481	p.L176L, p.L67L	Synonymous	–	F M P, het S	F, 0 M, 0 P, 42 S, 0	na	na	Na
17	16068396	Exonic	<i>NCOR1</i>	c.188C>T; c.515C>T	150910818	p.S172I, p.S63L	Missense	–	F M, het P, het S, het	F, 0 M, 65 P, 48 S, 60	0.49, tolerated	Disease causing	Benign
17	37855714	Promoter	<i>ERBB2</i>	c.-778delA; c.-18+4280delA	–	na	na	–	F M P, het S	F, 0 M, 0 P, 86 S, 0	na	na	na
21	36422282	Promoter	<i>RUNX1</i>	c.-878_-877insGT	71324342	na	na	0.3027	F M P, het S	F, 0 M, 0 P, 20 S, 0	na	na	na

na, not applicable. * Position according to GRCh37/hg19. [#] Frequency from gnomAD ver 3.1.2. [‡] F, father; M, mother; P, proband; S, sibling; het, heterozygous. [‡] Of which, only 2 reads were high quality.

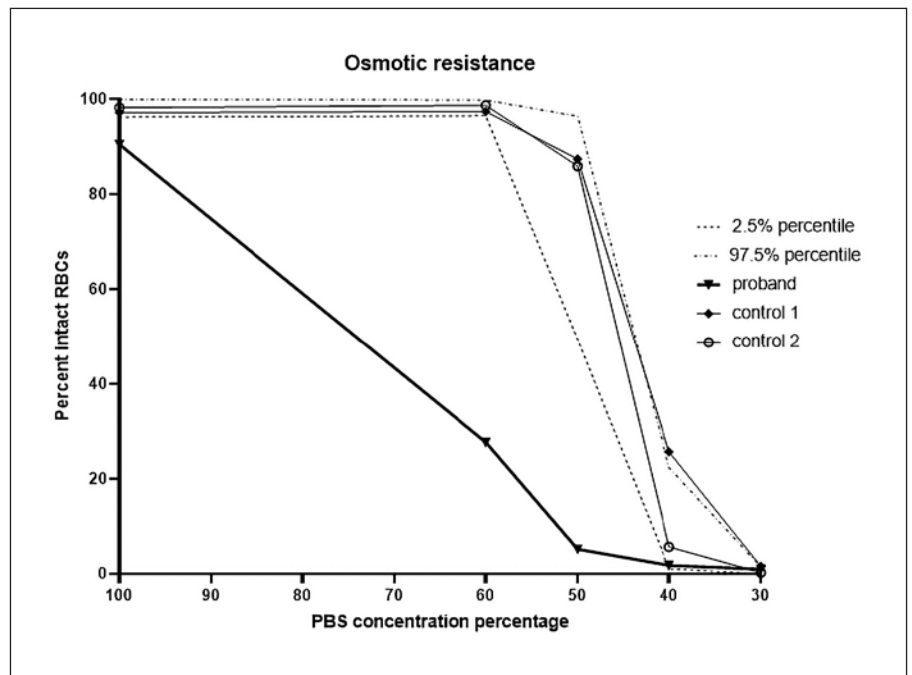


Fig. 1. Percent intact RBCs plotted as a function of PBS concentration in percent. Squares and the full line represent values obtained with erythrocytes from the proband. The upper dotted line represents the 97.5 percentile and the lower dotted line represents the 2.5 percentile.

was calculated using an FSC/SSC scatterplot. FACSDiva analyzing software was from BD Biosciences. Blood samples from 2 donors were measured in parallel (control 1 and 2 in Fig. 1). Blood samples from 129 Danish blood donors were used to establish the reference interval between the 2.5 percentile and the 97.5 percentile.

Results

Overall results for the exomes are summarized in Table 2. The average coverage of the exomes was 92x. Based on the individual family member's serological and genomic blood group determinations, all family members could be discerned and the correct identity of the family members was assured.

Sorting of variants with the ingenuity variant software was performed according to different types of heritability. After searching for possible causal variants, a short list of candidate variants in 5 different genes for a possible causative explanation of the hemolytic anemia resulted (Table 2). *SPTA1* variants are known to cause hemolytic anemia; the two *SPTA1* variants, both found via the Ingenuity software, are extremely rare and predicted to be deleterious and none of the other variants that were found were plausible causes of the hemolytic anemia, as judged by experienced clinical immunologists. It was concluded that the hemolytic anemia of the proband was most likely caused by compound heterozygosity of two rare variants of *SPTA1* with only two high quality reads of the paternal allele. PCR amplification of rs375506528 of the father's DNA was sequenced by NGS and showed two alleles in equal proportion as expected.

The paternal allele NM_003126.4:c.4546C>A, p.Lys1449Asn, rs375506528 [4, 11] was found in gnomAD v.3.1.2 with a frequency of 0.0001911 among Europeans (non-Finnish). The maternal allele was a stop gain NM_003126.4:c.2671C>T, p.Arg891*, rs755630903. This allele was found in the gnomAD v.3.1.2 with a frequency of 0.00001470 among Europeans (non-Finnish) but was not described at the time of this exome analysis and must be considered extremely rare [1, 12].

Neither of the variant alleles had at the time of the exome investigation been described in connection with pyropoikilocytosis, elliptocytosis, or spherocytosis, but are now mentioned in relation to this causality [11, 12]. The paternal and the maternal variants are both localized to exons outside the dimerization and tetramerization domain of alpha-spectrin. Both identified candidate variants were Sanger sequenced (online suppl. Fig. 1; see www.karger.com/doi/10.1159/000523706 for all online suppl. material), confirming the result from the NGS.

For the paternal allele, the SIFT score was 0.00, predicting a deleterious effect, and the MutationTaster gave a score of 94, predicting a disease-causing variant, PolyPhen function prediction gave a "probably damaging" estimation. A multiple sequence alignment with a selection of other species showed that the wild-type lysine is highly conserved (results not shown).

Proband RBCs osmotic resistance measurements demonstrated severely reduced osmotic resistance of the proband's RBCs (Fig. 1). The extent of RBC hemolysis from the proband was significantly greater than healthy controls. Hemolysis of the RBCs of the proband was also evident after centrifugation of a blood sample (Fig. 2).

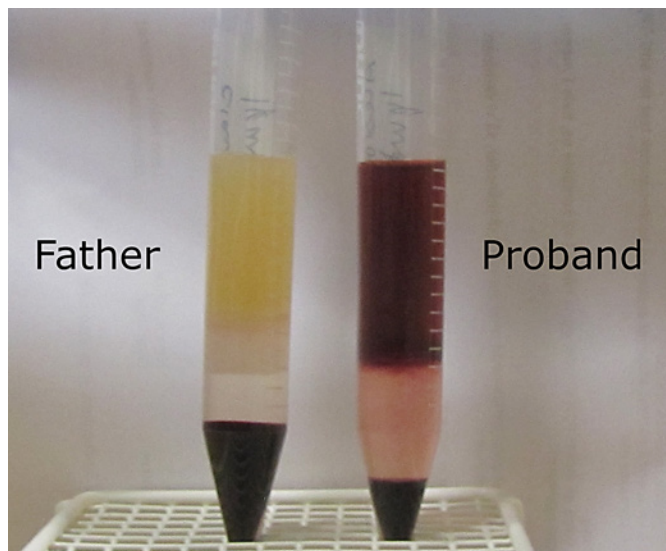


Fig. 2. The difference between a peripheral blood sample drawn from the father of the proband. In the sample from the proband, a striking degree of hemolysis and small amount of pelleted RBC is clearly visible. Samples were taken just prior to transfusion.

Discussion

The technology of NGS analysis of exomes and genomes has significantly propelled the capability for rapid diagnosis of genetic diseases. Several novel genetic causes for various diseases have been determined in recent years [7].

A trio exome study was undertaken to identify the potential molecular basis of hemolytic anemia in a child. In this investigation, we identified a compound heterozygote composed of two rare variants in the RBC spectrin gene, *SPTA1*, causing severe anemia. The osmotic fragility of RBCs further corroborated this conclusion.

We analyzed the exomes of the father, mother, and proband in a trio supplemented with a healthy sibling to establish the cause of a severe hemolytic anemia of unknown origin. The exomes were analyzed assuming various modes of inheritance as well as de novo mutations. The reasons for believing that the identified *SPTA1* variants are causal of the hemolytic anemia were the known involvement of *SPTA1* in maintenance of the structure of the RBC and other known variants of *SPTA1* giving rise to anemia, the absence of other plausible variants, the rarity and effect of the variants in *SPTA1* as tested by the SIFT and MutationTaster software and neither of the other family members had both variants. The other variants resulting from the ingenuity analysis were unlikely to be causally involved in the hemolytic anemia of the proband. We felt that all these elements, taken together, were very compelling. Furthermore, these two variants have now been described in similar hemolytic diseases by others [11, 12]. No damaging mutations were found in

the proband in any other of the genes known to be responsible for upholding the biconcave structure of the RBC.

The primers designed for validation of the paternal allele by NGS can be used for noninvasive prenatal diagnostics to determine if the fetus has inherited the paternal variant in subsequent pregnancies (Table 1) [13, 14]. This is done by PCR amplification of cfDNA purified from maternal plasma after GA 10 using the primers in Table 1 and deep sequencing by NGS with counting of the number of times the paternal allele is detected. If the paternal variant allele has been inherited, however, a CVS biopsy is needed to ascertain if the fetus has also inherited the maternal variant allele for compound heterozygosity of the two described *SPTA1* alleles to be present.

The maternal variant, with a stop codon close to the SH3 domain at a position that would truncate the protein to about half the normal size, is very likely damaging and may target the mRNA produced for nonsense mediated decay. The paternal allele with an asparagine (neutral) substituted for the normal allele of a lysine (positively charged) scored damaging by the SIFT algorithm and by the MutationTaster software. The lysine is highly conserved.

Based on the analysis and clinical information, it is highly likely that we have identified the causal mutations underlying the severe hemolytic anemia of the proband. The proband was later splenectomized and the need for regular transfusions ceased and the proband's hemoglobin is now stable.

In conclusion, by trio analysis supplemented with a healthy sibling, we identified that most likely the basis of the hemolytic anemia disease was compound heterozygosity of the *SPTA1* gene with two rare variants. The variants identified are consistent with known causes of deformation fragility of the affected RBCs. Reduced osmotic resistance measurements are further consistent with the interpretation that the identified variants are the genetic cause of the severe hemolytic anemia. The identification of the variants has enabled noninvasive prenatal screening in 50% of subsequent pregnancies for the paternal variant in this family and invasive screening for the maternal variant.

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Statement of Ethics

Informed consent was obtained from the parents including consent to publication. This work was part of a clinical diagnostic procedure and exempted from the Ethical Committee permission. The parents have given their written informed consent for publication of this case report.

Conflict of Interest Statement

Lars Jønson is now employed by a private company but at the time of the investigation was employed at a public hospital as were the rest of the authors. Otherwise, the authors have no conflicts of interest to declare.

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References

- 1 Bogardus H, Schulz VP, Maksimova Y, Miller BA, Li P, Forget BG, et al. Severe nondominant hereditary spherocytosis due to uniparental isodisomy at the *SPTA1* locus. *Haematologica*. 2014;99(9):e168–70.
- 2 Da Costa L, Galimand J, Fenneteau O, Mohandas N. Hereditary spherocytosis, elliptocytosis, and other red cell membrane disorders. *Blood Rev*. 2013;27(4):167–78.
- 3 Hansen DL, Glenthøj A, Møller S, Biemond BJ, Andersen K, Gaist D, et al. Prevalence of congenital hemolytic disorders in Denmark, 2000–2016. *Clin Epidemiol*. 2020;12:485–95.
- 4 Petkova-Kirova P, Hertz L, Danielczok J, Huisjes R, Makhro A, Bogdanova A, et al. Red blood cell membrane conductance in hereditary haemolytic anaemias. *Front Physiol*. 2019;10:386.
- 5 Grace RF, Barcellini W. Management of pyruvate kinase deficiency in children and adults. *Blood*. 2020;136(11):1241–9.
- 6 Delaunay J, Alloisio N, Morle L, Baklouti F, Dalla Venezia N, Mailliet P, et al. Molecular genetics of hereditary elliptocytosis and hereditary spherocytosis. *Ann Genet*. 1996;39(4):209–21.
- 7 Bergant G, Maver A, Lovrecic L, Čuturilo G, Hodzic A, Peterlin B. Comprehensive use of extended exome analysis improves diagnostic yield in rare disease: a retrospective survey in 1,059 cases. *Genet Med*. 2018;20(3):303–12.
- 8 Vaser R, Adusumalli S, Leng SN, Sikic M, Ng PC. SIFT missense predictions for genomes. *Nat Protoc*. 2016;11:1–9.
- 9 Schwarz JM, Cooper DN, Schuelke M, Seelov D. MutationTaster2: mutation prediction for the deep-sequencing age. *Nat Methods*. 2014;11:361–2.
- 10 Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, et al. A method and server for predicting damaging missense mutations. *Nat Methods*. 2010 Apr;7(4):248–9.
- 11 van Vuren A, van der Zwaag B, Huisjes R, Lak N, Bierings M, Gerritsen E, et al. The complexity of genotype-phenotype correlations in hereditary spherocytosis: a cohort of 95 patients – genotype-phenotype correlation in hereditary spherocytosis. *Hemasphere*. 2019;3(4):e276.
- 12 Chonat S, Risinger M, Sakthivel H, Niss O, Rothman JA, Hsieh L, et al. The spectrum of *SPTA1*-associated hereditary spherocytosis. *Front Physiol*. 2019;10:815.
- 13 Rieneck K, Bak M, Jonson L, Clausen FB, Krog GR, Tommerup N, et al. Next-generation sequencing: proof of concept for antenatal prediction of the fetal kell blood group phenotype from cell-free fetal DNA in maternal plasma. *Transfusion*. 2013;53(11 Suppl 2):2892–8.
- 14 Rieneck K, Egeberg Hother C, Clausen FB, Jakobsen MA, Bergholt T, Hellmuth E, et al. Next generation sequencing-based fetal ABO blood group prediction by analysis of cell-free DNA from maternal plasma. *Transfus Med Hemother*. 2020;47(1):45–53.

Author Contributions

K.R. suggested the experimental approach, did some of the bioinformatic analysis, and wrote the paper; B.L. was responsible for the patient and all laboratory investigations and arriving at a clinical diagnosis; F.B.C. substantially contributed to the writing of the manuscript and interpretation of some data; L.J. performed the exome sequencing and some of the analysis; A.T.H. did the osmotic fragility analysis and data interpretation; M.H.D. contributed to data analysis of the osmotic fragility analysis and supervised the work; all authors contributed to the manuscript, revised it critically for important intellectual content, approved the final version of the manuscript to be published, and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Data Availability Statement

Sequencing data are not publicly available on legal and ethical grounds. Further inquiries can be directed to the corresponding author.