

DATA REPORT

A novel mutation in the β -spectrin gene causes the activation of a cryptic 5'-splice site and the creation of a *de novo* 3'-splice site

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The analysis of genes involved in hereditary spherocytosis, by next-generation sequencing in two patients with clinical diagnosis of the disease, showed the presence of the c.1795+1G>A mutation in the *SPTB* gene. cDNA amplification then revealed the occurrence of a consequent aberrant mRNA isoform produced from the activation of a cryptic 5'-splice site and the creation of a newly 3'-splice site. The mechanisms by which these two splice sites are used as a result of the same mutation should be analyzed in depth in further studies.

Human Genome Variation (2015) 2, 15029; doi:10.1038/hgv.2015.29; published online 27 August 2015

Hereditary spherocytosis (HS) is a heterogeneous hemolytic anemia characterized by a defect in erythrocyte membrane proteins, particularly ankyrin, α - and β -spectrin, band 3 or protein 4.2.¹ As result, abnormal erythrocytes are trapped and destroyed in the spleen, which is the main cause of hemolysis in this disorder.²

The clinical manifestations of HS vary widely. Typical manifestations of the disease are hemolytic anemia, jaundice, reticulocytosis, gallstones and splenomegaly.³ HS is inherited in an autosomal dominant manner in approximately two thirds of patients, and mutations in the ankyrin (*ANK1*), β -spectrin (*SPTB*) or band 3 (*SLC4A1*) genes are associated with HS. In the remaining patients, inheritance is non-dominant due to a *de novo* mutation or autosomal recessive inheritance associated with mutations of either the α -spectrin (*SPTA1*) or protein 4.2 (*EPB42*) genes.^{4,5}

Defects in β -spectrin account for ~15–30% of cases of HS in northern European populations. Patients with β -spectrin deficiency typically have mild to moderately severe disease.^{4,6} Here we describe a novel *SPTB* mutation identified in a Bolivian family by next-generation sequencing. This mutation affects splicing and is likely to result in a shortened protein.

The proband was a 1-month-old female baby who presented moderately severe hemolytic anemia with hyperbilirubinemia and spherocytes in the peripheral blood smear. In particular, her hemoglobin level was 7.8 g/dl, with a reticulocyte count of 15% and indirect bilirubin of 3.2 mg/dl. The proband's mother was clinically diagnosed with HS in Bolivia, but there were no data from molecular studies. Splenectomy had been performed years before. Informed consent was obtained for genetic testing in both.

DNA was extracted from 100 μ l of EDTA-anticoagulated whole blood using MagNA Pure (Roche Diagnostics, West Sussex, UK). The targeted resequencing library was performed using the TruSight One (Illumina, San Diego, CA, USA) kit, which allows the enrichment and final analysis of a panel of ~5,000 genes, including all genes known to be related to HS (*ANK1*, *SPTB*, *SLC4A1*, *SPTA1* and *EPB42*). Quantification and validation of the genomic library was performed using a Qubit 2.0 Fluorometer system (Life Technologies, Carlsbad, CA, USA) and 2100 Bioanalyzer Instruments (Agilent Technologies, Santa Clara, CA, USA).

Paired-end sequencing was performed using the MiSeq sequencer (Illumina). The variants of the genes mentioned above were generated by alignment with a reference genome (UCSC hg19) using the MiSeq Reporter software (Illumina), which employs the Burrows–Wheeler Aligner and the Genome Analysis Toolkit variant caller. The variants reported in the VCF file were also evaluated and visualized via integrative genome viewer (IGV).

Exon 12, the flanking intronic region in intron 11 and the entire intron 12 of the *SPTB* gene were amplified by PCR using Promega Go Taq Hot Start Colorless Master Mixes (Promega, Madison, WI, USA) with the following primers: sense primer 5'-AGAGA CATGCCCTTGCAGG-3' (coordinate number 65261404–65261423, GRCh37.p13/hg19) and antisense primer 5'-CCATGTTGCTCAG CTCCTCA-3' (coordinate number 65260505–65260524, GRCh37.p13/hg19). Detailed conditions are available upon request. The resulting PCR amplicon was purified by Millipore plates (Darmstadt, Germany) and sequenced in both directions using the BigDye Direct Cycle Sequencing Kit (Life Technologies) and an ABI Prism 3130 DNA Analyzer (Applied Biosystems, Foster City, CA, USA), according to the manufacturer's instructions.

Total cellular RNA was prepared from peripheral blood using the Illustra RNAspin mini RNA isolation kit (GE Healthcare, Princeton, NJ, USA). RNA was reverse transcribed into cDNA using the RT-PCR (AMV) kit (Roche Diagnostics, West Sussex, UK). The cDNA from the proband was amplified with primers encompassing the coding sequence from exon 12 to 14 (sense primer 5'-CCGAGTTTGGGAAGCACTTG-3' (coordinate number 65261300–65261319, GRCh37.p13/hg19) and antisense primer 5'-GGTTACACCATCAATCTGAGTC-3' (coordinate number 65258516–65258538, GRCh37.p13/hg19). Detailed methods are available upon request. The cDNA amplification products were analyzed by 2% agarose gel electrophoresis and further sequenced.

Analysis of the genes involved in HS in the proband showed several polymorphisms and the presence of a G>A transition in a heterozygous state, at position +1 of the intron 12 donor splice site (c.1795+1G>A) of the shortest isoform of the *SPTB* gene (NM_000347.5; Figure 1a). Exon 12 is conserved in the other

isoform (NM_001024858) of the gene, as reported in the Refseq gene database. This substitution, also found in her mother, is a novel variant not previously reported in the public databases (dbSNP, HGMD, LOVD and NHLBI ESP). It was further confirmed using the Sanger method (Figure 1b). No additional mutations were found in intron 12 of the gene.

As this splice site mutation could affect mRNA splicing, we further examined the cDNA of *SPTB*. Amplification by primers localized in exons 12 and 14 resulted in two different PCR products in the patient, one of the expected size of 1.065 bp and a shortened one of 576 bp, suggesting that this extra band of lower molecular weight arose from aberrant mRNA splicing (Figure 2a). Sequencing of these PCR products revealed a *SPTB* mRNA isoform

lacking the last 16 nucleotides of exon 12 and the first 473 nucleotides of exon 13 (Figures 2b and c). The total number of nucleotides deleted was a multiple of three, and the reading frame was not altered. This deletion results from the activation of a cryptic 5'-splice site (5'-ss) at position -16 and, at the same time, from the creation of a *de novo* 3'-splice site (3'-ss) within exon 13.

Here we present a novel mutation in the *SPTB* gene that abolishes the natural donor sequence and is predicted to produce aberrant splicing and lead to a shortened protein. The *SPTB* gene encodes the β I subunit of spectrin, which contains 106 contiguous amino-acid sequence motifs called 'spectrin repeats'.⁷ As a result of the presence of the c.1795+1G>A mutation, the final eight amino acids of spectrin 3, the entirety of spectrin 4 and the first 49

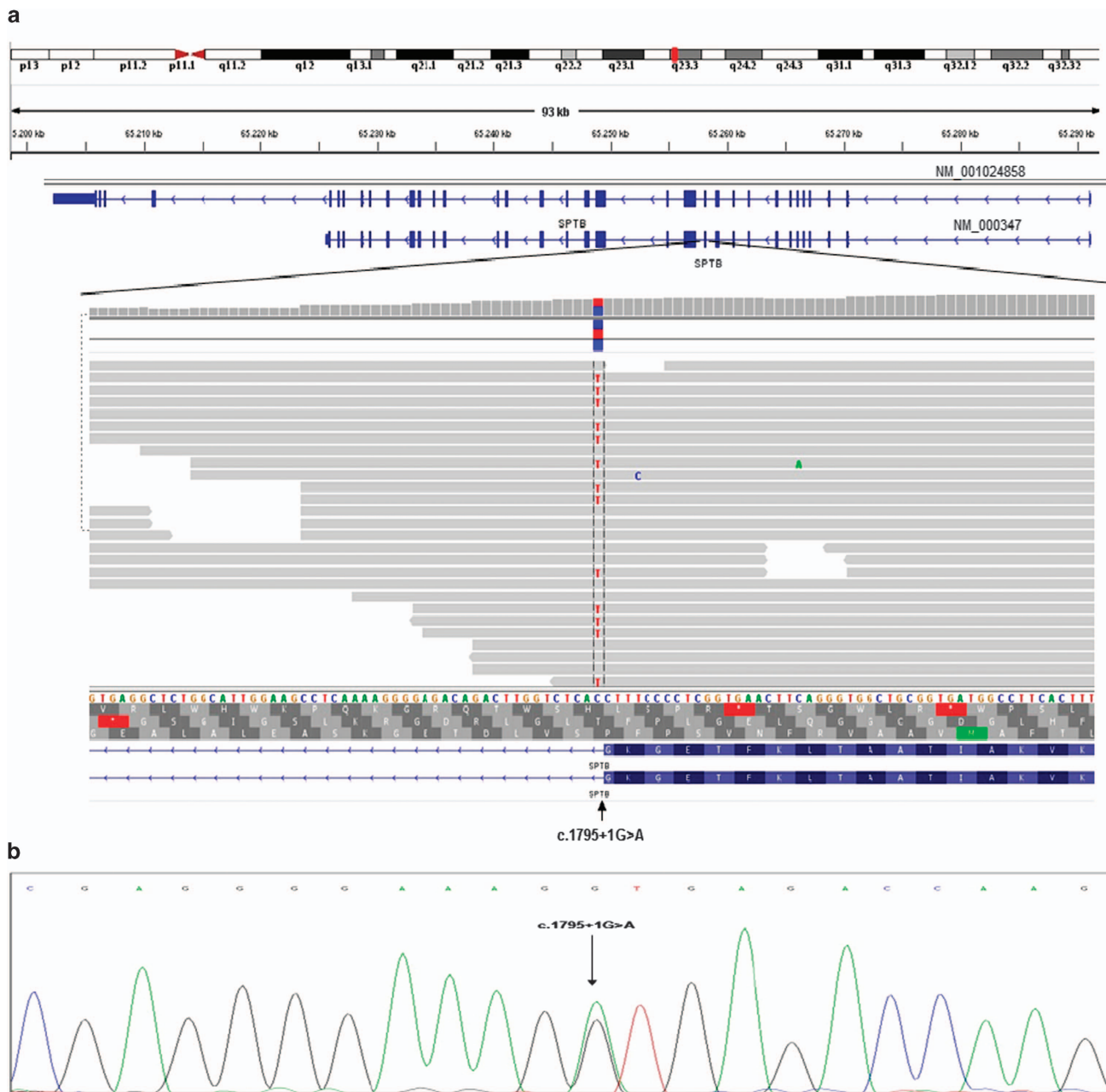


Figure 1. Molecular characterization of the mutation c.1795+1G>A. (a) Visualization of the boundary of exon 12 of *SPTB* gene using IGV (the mutation is shown as a C \rightarrow T change because IGV always displays the forward strand, and in the *SPTB* gene, the coding strand is the reverse one). (b) Sanger sequencing of PCR-amplified genomic DNA of exon 12 of the *SPTB* gene confirms that the nucleotide +1 in intron 12 was changed from G to A. IGV, integrative genome viewer.

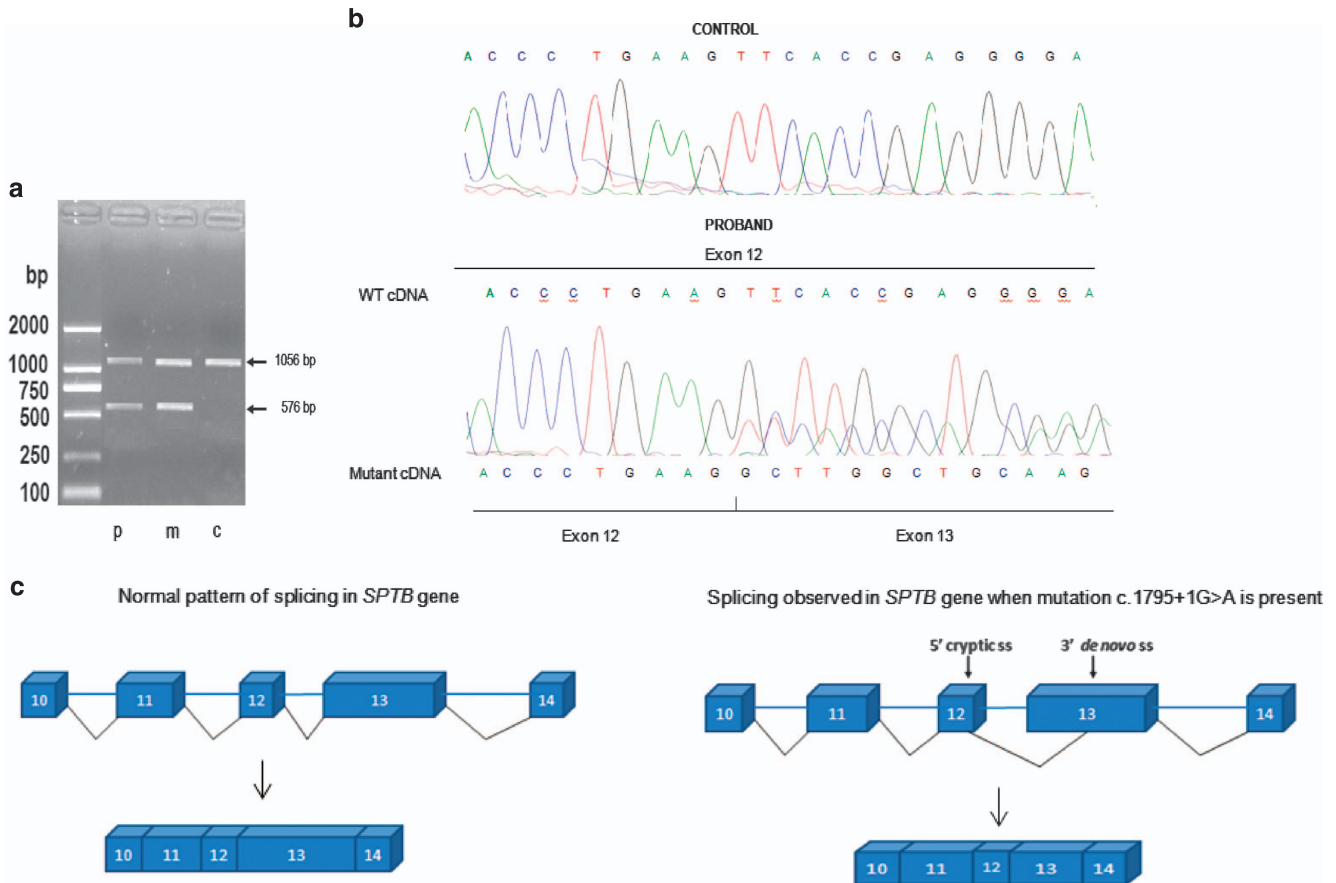


Figure 2. cDNA analysis. **(a)** After RT-PCR with primers localized in exons 12 and 14, two bands of 1,065 and 576 bp were observed in the sample of the propositus (p) and her mother (m). The abnormal band was absent in the control (c). **(b)** cDNA sequencing shows that the final 16 nucleotides of exon 12 and the first 473 nucleotides of exon 13 were spliced out. As a result, the proband and her mother present a shortened mutant cDNA and a WT cDNA. **(c)** Schematic diagram showing the effect of the c.1795+1G>A mutation on *SPTB* mRNA. WT, wild type.

amino acids of spectrin 5 are predicted to be lost. Nevertheless, aberrant spliced mRNAs are subject to instability, and it is possible that no protein was produced.

Mutations that inactivate natural donor sites usually cause skipping of their associated exon.⁸ However, in a considerable number of cases, three additional events can also take place: full intron inclusion, cryptic splice site activation and pseudoexon inclusion.⁹

Cryptic splice sites are splice sites that are only used by the cell when a mutation disrupts the use of authentic splice sites. In contrast, the term *de novo* refers to all aberrant splice sites that are induced by mutations elsewhere than in the splice site consensus.¹⁰ In some cases, when an authentic acceptor site is disrupted, the use of a created 5'-ss may happen in conjunction with the activation of a 3'-cryptic ss^{11,12} leading to intron retention and defining a new exon. Nevertheless, to our knowledge, a mutation at a donor splice site leading to the creation of a *de novo* 3'-ss within an exon in addition to the activation of a cryptic 5'-ss, which occurs in this case for c.1795+1G>A, has not been previously reported.

We do not know the exact mechanisms by which this exonic 3'-ss is recognized. Most *de novo* 3'-ss are intronic, due to AG creation in the polypyrimidine tract (PPT). Exonic *de novo* 3'-ss are much less common than intronic ones and are more often induced by distant mutations.¹³ Because of the size of intron 12 (599 bp), the c.1795+1G>A mutation is far away from the authentic 3'-ss. Královicová *J et al.*¹³ have suggested that PPT, branchpoint sequences or distant auxiliary splicing signals are

important for the activation of exonic 3'-ss. The c.1795+1G>A mutation did not alter the PPT or branchpoint sequence, and therefore unidentified splicing signals may be activated in this case.

The creation of a *de novo* 3'-ss within exon 13 allows the maintenance of the reading frame and leads to a mild phenotype. If splicing occurred in the cryptic 5'-ss at position -16 and in the authentic 3'-ss in intron 12, the mutation would cause a frameshift and a premature stop codon in exon 13, with 72% of the protein lost and a more severe phenotype. Nevertheless, it is possible that this frameshifted mRNA is really produced but is then degraded by the nonsense-mediated mRNA decay pathway.

In conclusion, we report a novel splicing mutation in the *SPTB* gene that produces an aberrant mRNA by activating a cryptic 5'-ss and creating a *de novo* 3'-ss at the same time, an event not previously published to date. Further studies are needed to clarify the mechanism of the splicing mutation.

HGV DATABASE

The relevant data from this Data Report are hosted at the Human Genome Variation Database at <http://dx.doi.org/10.6084/m9.figshare.hgv.670>.

COMPETING INTEREST

The authors declare no conflict of interest.

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