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Case Report

Case Report

Severe Congenital Hypothyroidism Due to a Novel Deep Intronic Mutation in the TSH Receptor Gene Causing Intron Retention

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Abstract

In 3 Somalian siblings with severe nongoitrous congenital hypothyroidism, exome sequencing identified a variant in *TSHR* predicted to be benign in isoform 3 but leading to an intronic mutation in isoform 1 (NM_00369:c.692 + 130C>A), which is the isoform expressed in the thyroid. This mutation creates a pseudoexon that results in a protein that, if transcribed, would lack the transmembrane domain, thereby hampering its expression at the cell surface. Our findings illustrate that the interpretation of exome analysis requires knowledge of the relevant isoform expression and of the biology of the disease. This is the first description of a deep intronic mutation creating a pseudoexon and inactivating the thyroid stimulating hormone (TSH) receptor.

Key Words: nongoitrous congenital hypothyroidism, whole exome sequencing, diagnosis, birth defects, TSHR inactivation

Congenital hypothyroidism (CH) with orthotopic thyroid hypoplasia may be caused by inactivating mutations in various transcription factors or in *TSHR* [1]. Specifically, biallelic loss-of-function mutations in *TSHR* lead to a wide spectrum of phenotypes, ranging from isolated hyperthyrotropinemia to severe CH with apparent athyreosis [2].

In 3 Somalian siblings with such severe phenotype, whole exome sequencing (WES) was performed. While WES pointed to the gene likely responsible for the phenotype, knowledge of the expression of this gene and of the biology of the disease was required to design additional studies to prove that the variant was indeed disease-causing.

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Patients' Reports

The clinical presentation of the 3 siblings was published in 2016 [3]. Briefly, in a family without known consanguinity who had emigrated from Somalia to the United States, 3/9 siblings were found at ages 0.5, 7.7, and 14.8 years old to have severe CH and orthotopic thyroid hypoplasia (Fig. 1A). The parents and the other siblings were clinically euthyroid.

Methods

Patients and Sample Collection

Written consent for genetic testing was obtained from all subjects. Deoxyribonucleic acid (DNA) was extracted from leucocytes using phenol-chloroform-isoamyl alcohol and ribonucleic acid (RNA) from whole blood using the Tempus Spin

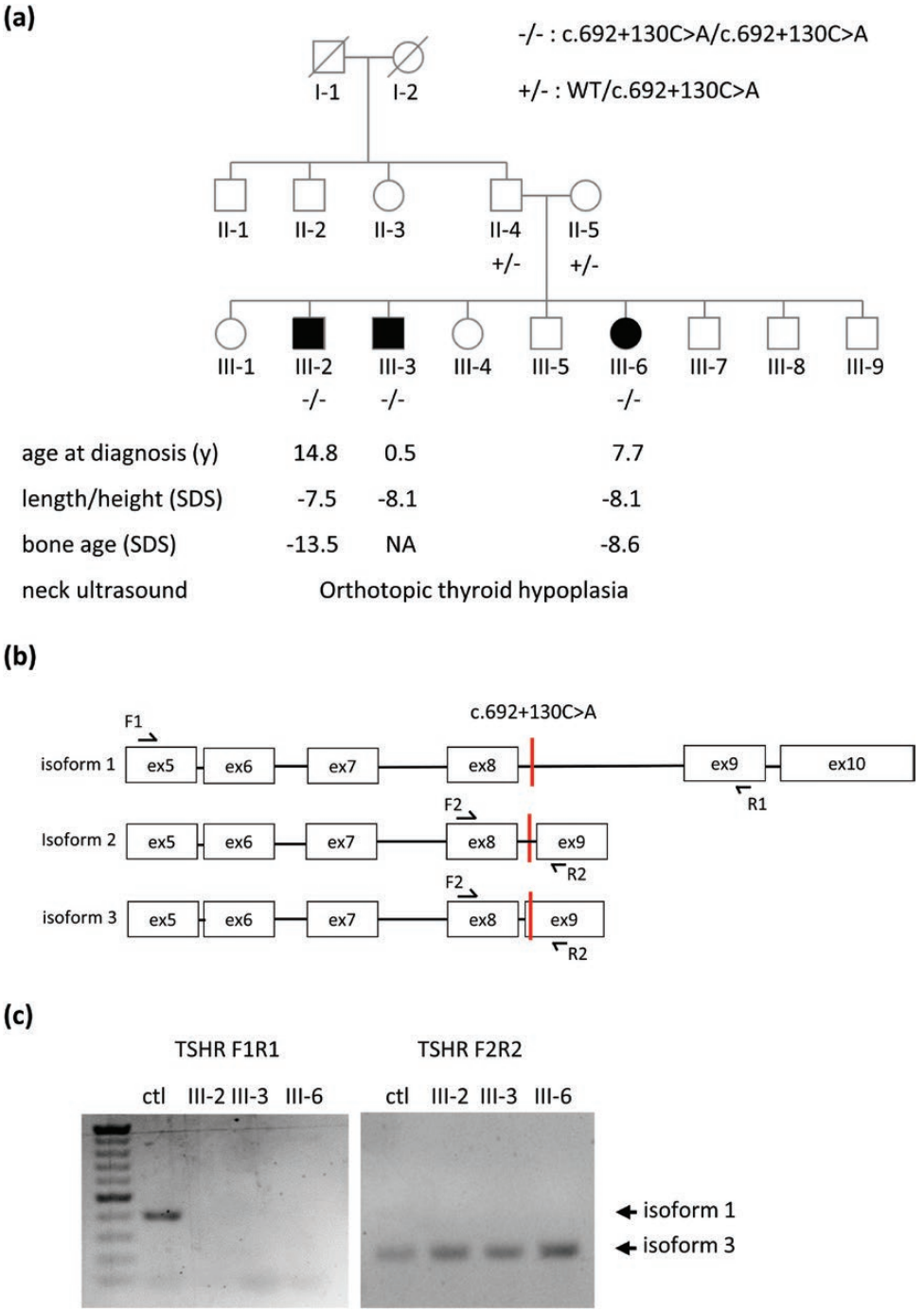

Figure 1. **A:** Family pedigree. Patients, in black, are homozygous for an intronic mutation, while the parents are heterozygous. **B:** Schematic representation of a portion of *TSHR* isoform 1, 2, and 3 with the variant's position. The first 8 exons are identical, while exon 9 is different. Only isoform 1 includes exon 10, which encodes the transmembrane domain and intracellular loop. Specific primers were designed for isoform 1 and for isoforms 2 and 3. **C:** RT-PCR revealed the absence of isoform 1 in patients' RNA, while isoform 3 is present in all samples. Abbreviations: ctl: control; NA, not reported; RNA, ribonucleic acid; RT-PCR, reverse transcription polymerase chain reaction.

RNA isolation kit (Applied Biosystems, California, Foster City). Complementary DNA was reverse transcribed from RNA using RevertAid H minus reverse transcriptase, and polymerase chain reaction (PCR) was performed using Phusion High-Fidelity DNA polymerase (Life Technologies, California, Carlsbad).

Whole Exome Sequencing

Whole exome sequencing and Sanger sequencing were performed at McGill University and Genome Quebec Innovation Centre. Whole exome sequencing was performed using the Agilent SureSelect exon capture library, followed by Illumina base pair (2 × 100 bp) HiSeq 2000 sequencing technology. Raw data were aligned to the reference genome (GRCh37) using the Burrow-Wheeler Alignment tool and the variants were called using the Genome Analysis Toolkit haplotype caller. Finally, the variant dataset was annotated by ANNOVAR (http://annovar.openbioinformatics.org/en/latest/). Only rare variants (minor allele frequency [MAF] < 0.01 in the Exact Database and 1000 Genomes Project) and protein altering variants (nonsynonymous, frameshift, stop-gain, stop-loss, exonic, or exonic splicing) were kept. An autosomal recessive model was used to analyze WES data.

In Silico Analysis

In silico analysis for exonic variants was performed using SIFT (https://sift.bii.a-star.edu.sg/) and Polyphen2 via the Ensembl Variant Effect Predictor (https://grch37.ensembl.org/Homo_sapiens/Tools/VEP) and Mutation Taster webtool (http://www.mutationtaster.org/). The potential effect of intronic variants on splicing was assessed with the Human Splicing Finder (http://www.umd.be/HSF/).

Minigene Assay

A minigene assay was carried out using the exon trapping vector pSPL3b [4]. Exon 8 of *TSHR* flanked by 128 bp of intron 7 and 195 bp of intron 8, with or without the identified variant GrCh37: chr14: 81574926C>A (see below), was PCR-amplified using the following primers: forward 5'-GAGCGGCCGCTGCA*GGATCCTATCTTCTAAATTCTTGAAATCAGTCA*-3' and reverse 5'-GTACGGGATCACCA*GATATCTCTCAAAGGACAAGGACTTTCT*-3'. Restriction sites included in primers for standard cloning are underlined and the sequences in italic are complementary to *TSHR* DNA. Deoxyribonucleic acid from a control subject and from patient III-2 were used as template. The PCR products were cloned in pSPL3b. The wild type or mutant vector was transfected in HEK293 cells using 10 µl of lipofectamine 2000 (Life Technologies) and 5 µg of vector. Two days after transfection, RNA was extracted from cells using Trizol Reagent (Life Technologies), according to the manufacturer's protocol. Reverse transcription PCR (RT-PCR) on extracted RNA using the following pSPL3b specific primers: SD6, 5'- TCTGAGTCACCTGGACAACC-3' and SA2, 5'- ATCTCAGTGGTATTTGTGAGC-3' followed by Sanger sequencing of RT-PCR fragments was performed to evaluate the effect of the variant on splicing.

Results

Whole exome sequencing, performed on DNA from 2 affected siblings and from the parents, identified a novel homozygous variant not reported in public databases in isoform 3 of *TSHR* (GrCh37: chr14: 81574926C>A; NM_001142626:c.C726A; p.F242L). This variant was the only rare, protein-altering variant homozygous in both siblings and heterozygous in the parents. *TSHR* has 3 isoforms that share the first 8 exons. Exon 9 is different for each isoform and isoform 1 is the only one containing exon 10, which encodes the transmembrane domain and the intracellular loop. Isoform 1 is therefore the only full-length functional isoform (Fig. 1B).

Sanger sequencing was used to confirm WES results in all 3 siblings and the parents. The exonic variant mapping to exon 9 in isoform 3 was classified as benign by Polyphen2, SIFT, and Mutation Taster. However, the phenotype of the patients was consistent with complete *TSHR* inactivation. In isoform 1, the functional isoform, the variant is intronic and maps to intron 8 (NM_00369:c.692 + 130C>A). While some heterozygous variants in other genes related to CH were found, the *TSHR* variant is the only one that is present in the homozygous state in the affected siblings and in the heterozygous state in both parents, thus segregating perfectly with the family pedigree. Reverse transcription PCR on all 3 patients' RNA showed absence of isoform 1 and presence of isoform 3 (Fig. 1C). Unfortunately, RNA from the parents or an unaffected sibling was not available.

In silico analysis to evaluate the potential effect of the variant on splicing suggested that it introduced a new enhancer splicing element or eliminated a silencer splicing element. To investigate whether this variant affected splicing of isoform 1, a minigene assay was performed. Exon 8 and part of intron 7 and 8 of wild type and mutant *TSHR* were cloned in pSPL3b vector (Fig. 2A). Constructions were transfected in HEK293 cells followed by RNA extraction and RT-PCR. The generated fragments were sequenced by Sanger and resulted in a fragment of 335 bp for the wild type vector and 455 bp for the mutant (Fig. 2B), confirming that the variant induced insertion of

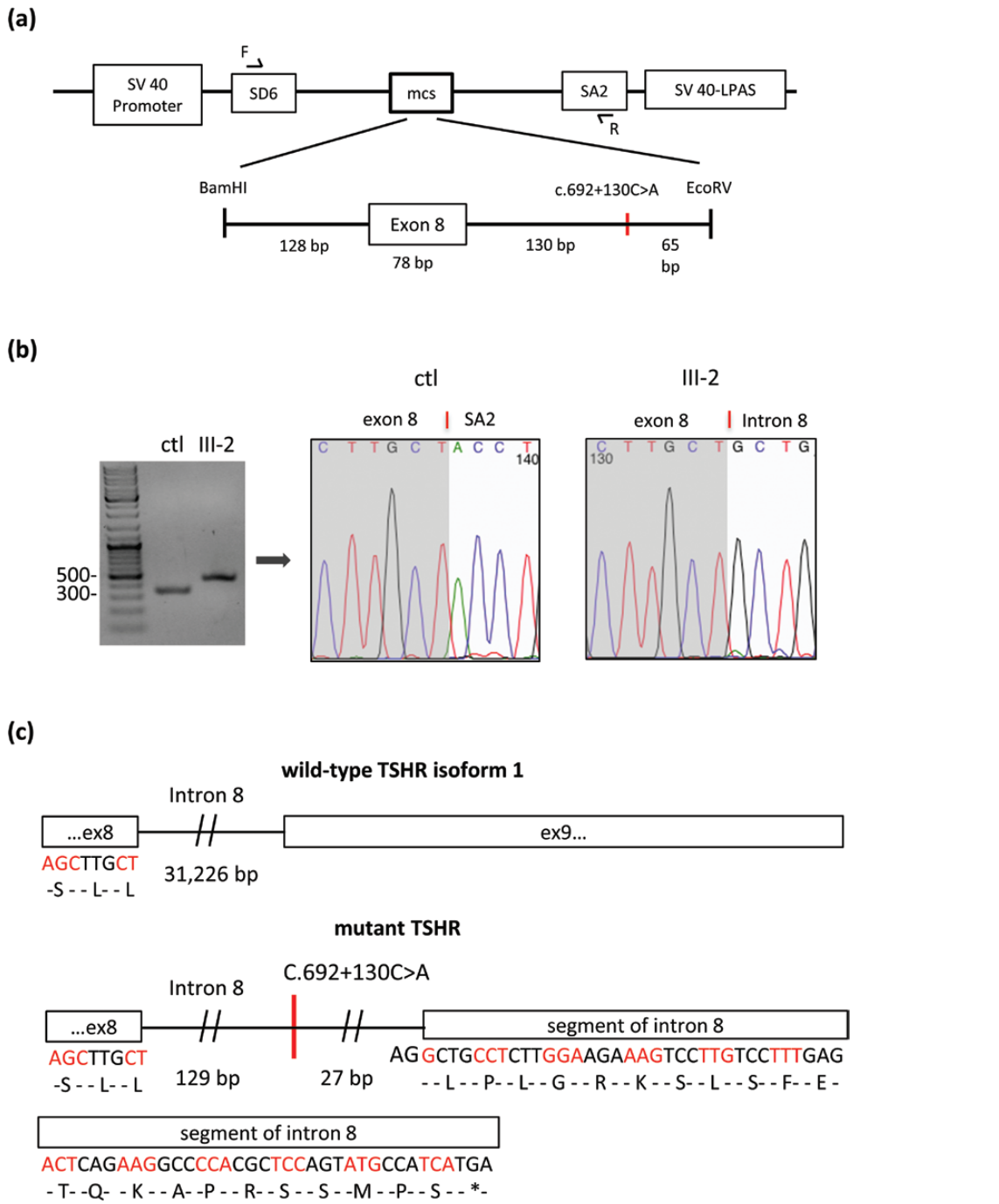

Figure 2. **A:** Minigene assay. Exon 8 flanked by 128 bp of intron 7 and 195 bp of intron 8 was cloned in pSPL3b vector. DNA from a control subject and from patient III-2 was used to amplify the region. **B:** RT-PCR on RNA using SD6 and SA2 primers show a higher fragment for the mutant vector compared with the wild type, suggesting that the variant induced retention of part of intron 8. Sanger sequencing revealed insertion of a segment of intron 8. **C:** Schematic representation of the putative truncated protein. The insertion likely introduced a stop codon 24 amino acids after exon 8. Abbreviations: ctl, control; DNA, deoxyribonucleic acid; MCS, multiple cloning site; RNA, ribonucleic acid; RT-PCR, reverse transcription polymerase chain reaction.

a segment of intron 8 starting 30 nucleotides downstream of the variant (position GrCh37: chr14: 81574956), thus creating a pseudoexon. This pseudoexon follows a cryptic AG acceptor splice site. If transcribed, this would lead to a shorter protein containing only exon 1 to 8, thus a nonfunctional protein.

Discussion

The objective of our study was to identify the gene mutation causing severe nongoitrous CH in 3 siblings. Whole exome sequencing analysis identified a novel homozygous variant in exon 9 of isoform 3 of *TSHR* (NM_001142626:c.C726A; p.F242L), predicted to be

benign by 3 predictive tools. Thus, while WES analysis pointed to the right gene, the variant was first annotated to a nonfunctional isoform. However, the patients' phenotype was consistent with complete *TSHR* inactivation and the variant is 130 bp after exon 8 in the functional isoform 1 (NM_00369:c.692 + 130C>A). *In silico* analysis suggested this variant could affect regulatory splicing elements, which may lead to partial intron retention [5]. More importantly, this variant is the only one to segregate with the CH phenotype in the homozygous state.

We showed that isoform 1 messenger RNA was absent in all 3 patients, while it was present in a control subject (Fig. 1C). Moreover, the minigene assay and Sanger sequencing revealed that the variant induced the insertion of a segment of intron 8, allowing the spliceosome machinery to recognize a cryptic donor splice site 28 bp downstream of the variant. This pseudoexon likely introduces an in-frame stop codon after exon 8, resulting in a shorter nonfunctional protein, lacking the transmembrane domain and intracellular loop (Fig. 2C). Moreover, the absence of isoform 1 may be explained by an in-frame stop codon, since it probably leads to nonsense-mediated messenger RNA decay. Pseudoexon inclusion has been reported with deep intronic variants in large introns and has been shown to cause diseases such as cystic fibrosis and androgen insensitivity [5]. To our knowledge, we present the first evidence for this mechanism of *TSHR* inactivation.

Conclusion

Whole exome sequencing is a valuable tool to identify the cause of familial CH. Our findings illustrate that the interpretation of exome analysis requires knowledge of the expression of the various isoforms of the relevant genes and of the biology of the disease.

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Disclosure Summary: The authors have nothing to disclose.

Data Availability: Some or all datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on request.

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