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Review

First functional analysis of a novel splicing mutation in the *B3GALTL* gene by an *ex vivo* approach in Tunisian patients with typical Peters plus syndrome

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ABSTRACT

Peters plus syndrome is a rare recessive autosomal disorder comprising ocular anterior segment dysgenesis, short stature, hand abnormalities and distinctive facial features. It was related only to mutations in the *B3GALTL* gene in the 13q12.3 region. In this study, we undertook the first functional analysis of a novel c.597-2 A > G splicing mutation within the *B3GALTL* gene using an *ex-vivo* approach. The results showed a complete skipping of exon 8 in the *B3GALTL* cDNA, which altered the open reading frame of the mutant transcript and generated a PTC within exon 9. This finding potentially elicits the nonsense mRNA to degradation by NMD (nonsense-mediated mRNA decay). The theoretical consequences of splice site mutations, predicted with the bioinformatics tool Human Splice Finder, were investigated and evaluated in relation to *ex-vivo* results. The findings confirmed the key role played by the *B3GALTL* gene in typical Peters-plus syndromes and the utility of mRNA analysis to understand the primary impacts of this mutation and the phenotype of the disease.
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Abbreviations: B3GALTL, beta-1,3-glucosyltransferase; cDNA, DNA complementary to RNA; PTC, premature translation stop codon; mRNA, messenger ribonucleic acid; NMD, nonsense-mediated mRNA decay; PPS, Peters plus syndrome; DNA, deoxyribonucleic acid; B3GTL, beta3-glycosyltransferase-like; βGal, β-galactosidase; aa, amino acid(s); G418, Geneticin; ss, splice site; Ins, insertion; PCR, polymerase chain reaction; bp, base pair(s); F, forward; R, reverse; DNase, deoxyribonuclease; dNTP, deoxyribonucleoside triphosphate; WT, wild type; RPMI, Roswell Park Memorial Institute; MMLV, Moloney Murine Leukemia Virus; HSF, human splicing finder; ESE, exonic splicing enhancer; RT-PCR, reverse transcription polymerase chain reaction; CV, consensus value; TMR, transmembrane region; SR, stem region; CD, catalytic domain; PolyA, polyadenylation.

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1. Introduction

Peters plus syndrome (PPS) is a rare recessive autosomal disorder, with less than 70 cases reported throughout the world. It is characterized by anterior chamber-eye anomalies, disproportionate short stature, developmental/intellectual disruptions, dysmorphic facial features, and cleft lip/palate (Maillette and Hennekam, 2002). This condition is related to mutations in the *B3GALTL* gene, originally identified by Heinonen and collaborators (Heinonen et al., 2003) and later found to encode a glycosyltransferase (Kozma et al., 2006; Sato et al., 2006). The latter gene contains 15 coding exons and spans 132 kb of genomic DNA. It is transcribed in a wide range of human tissues in the form of three transcripts resulting in three different alternative polyA sites, all in exon 15 (Heinonen et al., 2003). The B3GTL protein spans 498 amino acids and contains a short N-terminal tail (aa 1–4), a transmembrane region (aa 5–28), a so-called stem region (aa 29–260), and a C-terminal catalytic domain (aa 261–498) (Heinonen et al., 2003). This protein is a glycosyltransferase: the β-1,3-glycosyltransferase involved in the synthesis of the disaccharide Glc-β1,3-Fuc-O- that occurs on thrombospondin type 1 repeats (TSRs) of many biologically important proteins (Hess et al., 2008).

Up to now, only seven mutations in the *B3GALTL* gene were identified in patients with PPS. The most frequent mutation occurring in 75% of cases is the c.660 + 1G > A located at the donor splice site (5′ss) of exon 8 and harbored in several populations (Oberstein et al., 2006). Other mutations were also reported including the c.230insT mutation in exon 4, the c.347 + 5 G > A variation in exon 5, the c.459 + 1G > A transition in exon 6, the c.1178G > A mutation in exon 13 (Dassie-Ajdid et al., 2009), and a p.Tyr366X substitution in exon 13 (Aliferis et al., 2010). More recently, two Tunisian patients were found to have a c.597-2A > G mutation (Siala et al., 2012), whose effect in the acceptor splice site of exon 8 of the *B3GALTL* gene on the mRNA splicing process using an *ex-vivo* approach was studied in the present study.

2. Materials and methods

The study concerned two Tunisian patients belonging to two unrelated non-consanguineous families suspected to be affected by PPS. Both patients were underweight at birth, with disproportionate short stature and microcephaly. They also showed facial dysmorphism, including dolichocephaly, round face, and bilateral corneal opaqueness. The progress of the latter was monitored by the determination of visual acuteness, followed by cornea transplantations in both patients.

2.1. DNA extraction

Total DNA extraction from blood leukocytes was performed according to a previously described protocol (Kawasaki, 1990).

2.2. PCR amplification

The PCR amplification of exon 8 (64 bp) and its intron boundaries was performed using the following primers F: 5′GATAAGGGGTCACC AAAGCTTATGACTTTTTTCC3′ containing a mismatch to create a *Bste*II restriction site and reverse primer R: 5′CCCATTCAGCTAGCTTAAAAGTA AAGAATCATG3′ containing a mismatch to create a *Nhe*I endonuclease restriction site (restriction sites are underlined). These primers led to a 615 bp PCR product containing 296 bp of intron 7 and 255 bp of intron 8. Amplification was performed in a thermal cycler (Perkin Elmer Gene A PCR System 9700) in a total volume of 25 μl containing 0.05 μg of genomic DNA, 10 mM dNTP, 25 mM MgCl2, 20 pmol of each primer, 5 μl of 5× buffer, and 0.5 unit of Go Taq DNA polymerase (Go Taq, Promega). The PCR conditions were as follows : 2 min at 95 °C followed by 35 cycles, each consisting of 45 s at 95 °C, 60 s at 57 °C, 40 s at 72 °C, and final elongation at 72 °C for 7 min.

2.3. Cloning of wild type and mutant mini-genes

A splicing cassette (p(13,17)/cytomegalovirus [CMV]) was designed to contain the 2 adjacent constitutive exons 13 and 17 of human 4.1 gene, with their downstream and upstream flanking intron sequences, respectively (Deguillien et al., 2001). Final PCR products were digested and ligated into the *BstE*II/*Nhe*I sites of the cassette as 615 bp generated from the genomic fragment. After the subcloning of the mutant and wild type vectors, transformation was performed with TOP 10 competent cells (Invitrogen). The cells were grown to an optical density value of 0.8 at 37 °C. The cassette, as well as the WT and mutant inserts were fully sequenced to ensure the absence of any additional mismatch resulting from PCR or cloning errors. The size of recombinant cassette was 1200 bp, a feature that was used to distinguish the recombinant from the native plasmids.

2.4. Ex-vivo splicing assays: cell culture and transfection

Hela cells were cultured in RPMI (Roswell Park Memorial Institute) with glutamine in flask disk plates T-25. The cells were transfected with 2.5 μg of wild type or mutated minigene constructs using Fugen 6 Transfection reagent (ROCHE) according to the protocol provided by the manufacturer. Transfected cells were selected for 6–8 days in the same medium containing 600–800 μg G418/ml (Geneticin invitrogen).

2.5. RNA extraction and RT-PCR

Total RNA was extracted from the cells using a Trizol reagent (invitrogen) and was then treated with recombinant DNase I/RNase-free (Takara). Briefly, 10^7 cells were lysed using 1 ml of Trizol reagent. 200 μl of chloroform were added to the tubes and centrifuged at 12,000 tr for 25 min at 4 °C. The aqueous phase was precipitated with 1 vol 70% ethanol. The RNA was pelleted by centrifugation at 10,000 tr for 1 min with Rnase-free water, and the RNA preparation was then stored at −80 °C. RNA content was measured at 260 nm using a NanoDrop ND1000 spectrophotometer (NanoDrop Technologies). It was treated with MMLV reverse transcriptase (Fermentas) in a 10 μl of reaction in accordance with the manufacturer's instructions. 10 μl of the cDNA was added to a final volume of 50 μl PCR reaction mixture containing 125 μM of dNTP, 25 μM of forward and reverse primers and 1 unit of Go Taq DNA polymerase (promega). The forward primer (5′ CGCCTAGATGCCTCTGCTAA3′) and the reverse primer (5′AAGCGCTTG TCCCACTCGCT3′) were located at each extremity of the splicing cassette. The splicing products were extracted from the agarose gel and sequenced to confirm the identity of each product.

2.6. Bioinformatics prediction

The effect of the c.597-2A > G mutation on the splicing process of the *B3GALTL* gene was predicted by a new bioinformatics tool, the human splicing finder (HSF) software that is freely available online (http://www.umd.be/HSF/). This program includes new algorithms to evaluate the strength of 5′ss, 3′ss, and branch points (Beroud et al.,

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