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Splicing defects in *ABCD1* gene leading to both exon skipping and partial intron retention in X-linked adrenoleukodystrophy Tunisian patient

Fakhri Kallabi[a,*], Ikhlass Hadj Salem[a], Amel Ben Chehida[b], Ghada Ben Salah[a], Hadhami Ben Turkia[b], Neji Tebib[b], Leila Keskes[a], Hassen Kamoun[a,c]

[a] *Laboratoire de Génétique Moléculaire Humaine, Faculté de Médecine de Sfax, Université de Sfax, Tunisia*
[b] *Service de Pédiatrie, Hôpital La Rabta de Tunis, Tunisia*
[c] *Service de Génétique Médicale, Hôpital Hédi Chaker Sfax, Tunisia*

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ABSTRACT

X-linked adrenoleukodystrophy (X-ALD) affects the nervous system white matter and adrenal cortex secondary to mutations in the *ABCD1* gene that encodes a peroxisomal membrane protein: the adrenoleukodystrophy protein. The disease is characterized by high concentrations of very long-chain fatty acids in plasma, adrenal, testicular and nervous tissues. Various types of mutations have been identified in the *ABCD1* gene: point mutations, insertions, and deletions. To date, more than 40 point mutations have been reported at the splice junctions of the *ABCD1* gene; only few functional studies have been performed to explore these types of mutations. In this study, we have identified de novo splice site mutation c.1780 + 2T>G in *ABCD1* gene in an X-ALD Tunisian patient. Sequencing analysis of cDNA showed a minor transcript lacking exon 7 and a major transcript with a partial intron 7 retention due to activation of a new intronic cryptic splice site. Both outcomes lead to frameshifts with premature stop codon generation in exon 8 and intron 7 respectively. To the best of our knowledge, the current study demonstrates that a single splicing mutation affects the *ABCD1* transcripts and the ALDP protein function.

1. Introduction

X-linked adrenoleukodystrophy (X-ALD; OMIM # 300100) is one of the most frequent monogenic inherited peroxisomal neurodegenerative disorders. It affects the cerebral white matter, peripheral nerves, adrenal cortex and testis (Moser et al., 2002). It is a serious and progressive genetic disorder characterized by abnormal accumulation of saturated very long chain fatty acids (VLCFA) in body fluids and affected tissues, most notably in the brain and adrenal cortex due to an impaired β-oxidation in peroxisomes (Moser et al., 2002; Bezman et al., 2001; Ferrer et al., 2010; Valianpour et al., 2003). Affected individuals may present with seven different clinical forms that are classified according to phenotypic expression and age at initial symptoms. Most frequent clinical phenotypes, accounting for 70–80% of the patients, include severe progressive, inflammatory, demyelinating childhood cerebral form (ccALD) and slowly progressive, noninflammatory, adult adrenomyeloneuropathy (AMN) affecting mainly peripheral nerves and spinal cord (Van Geel et al., 1997; Jardim et al., 2010; Kemp et al., 2001). Other less frequently occurring phenotypes include adolescent cerebral (AdolCALD), adult cerebral (acALD), olivopontocerebellar, addison-only and asymptomatic patients (Kemp et al., 2001). The biochemical diagnosis of X-ALD patients and carriers is based on the elevated levels of C24:0 and C26:0 in plasma. However, in 0.1% of affected males, the plasma C26:0 level is at borderline of the healthy subjects and 15% of female heterozygotes have normal levels of VLCFA (Valianpour et al., 2003; Moser et al., 1999; Igarashi et al., 1976). The diagnosis of X-ALD is based on the clinical manifestation, the cerebral magnetic resonance imaging (MRI), the elevated levels of: C24:0 and C26:0 and the ratios: C24:0/C22:0 and C26:0/C22:0 but the molecular analysis is the only effective and reliable method to unambiguous determination of the genetic status of X-ALD patients (Moser et al., 2007).

X-ALD occurs due to alterations in the ATP-binding cassette, subfamily D, member 1 (*ABCD1*) gene localized at Xq28 encoding the adrenoleukodystrophy protein (ALDP) (Boehm et al., 1999). If

* Corresponding author at: Laboratoire de Génétique Moléculaire Humaine, Faculté de Médecine de Sfax, Avenue Magida Boulila, 3029 Sfax, Tunisia.
Tel.: +216 74 24 18 88x159; fax: +216 74 46 14 03.
E-mail address: fakhrikallabi@yahoo.fr (F. Kallabi).

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the ALDP protein function is impaired, there is no peroxisomal β-oxidation of VLCFAs, hence their accumulation in body fluids and tissues leading either to neuroinflammation and demyelination in the brain characterizing the ccALD form or to axonal degeneration in spinal cord in AMN form (Migeon et al., 1981; Mosser et al., 1993).

Currently, all over the world, 1585 distinct mutations have so far been reported in *ABCD1* gene as the cause of a wide spectrum of clinical severity (http://www.x-ald.nl/). Many *ABCD1* intronic variants have been identified during diagnostic screening: they account for about 3% of all variants listed at X-ALD database. For the majority of intronic variations, the consequences on mRNA splicing have been only inferred by in-silico analysis, whereas experimental demonstration of their pathogenicity has been obtained by mRNA studies for only few of them (Chiu et al., 2006; Shi et al., 2003; Guimarães et al., 2001, 2002).

In this study, we performed a molecular genetic analysis of the *ABCD1* gene in X-ALD Tunisian patient. The availability of RNA splicing analysis from blood samples was exploited to demonstrate the effect of the c.1780 + 2T>G splicing mutation in *ABCD1* gene on mRNA splicing.

2. Materials and methods

The patient is a 16-year old boy belonging to a Tunisian family. He was born from unrelated healthy parents. One additional healthy sibling and the parents were also recruited. Informed consent was obtained from patients and control individuals in accordance with the ethics committee of La Rabta Hospital (Tunis, Tunisia). The diagnosis of X-ALD was made on the basis of clinical manifestation; cerebral magnetic resonance imaging (MRI) and accumulation of very long chain fatty acid (VLCFA). Blood samples were collected from four family members and healthy individuals. Genomic DNA was extracted from the whole blood following a standard phenol-chloroform method (Lewin and Stewart-Haynes, 1992).

2.1. Mutation analysis of ABCD1 gene

The 10 exons and flanking intron region of the *ABCD1* gene were tested for mutation in the X-ALD patient by sequence analysis. PCR amplification of all 10 *ABCD1* fragments was performed using the primer sets as previously published (Boehm et al., 1999). All exons were amplified in a thermal cycler (Applied Biosystem 2720) in a final volume of 50 μl containing 100 ng of genomic DNA, 0.2 μM of each primer, 1x PCR buffer (Promega), 1.2 mM $MgCl_2$, 0.2 mM each dNTP, and 1 U Taq DNA polymerase (Promega). The polymerase chain reaction conditions were as follows: initial denaturation at 95 °C for 5 min followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 63–71 °C (depending on the melting temperatures of the primer pairs used) for 30 s and extension at 72 °C for 45 s, and final extension at 72 °C for 10 min. Each PCR product was then purified by enzyme reaction (Exonuclease I; 20 units/μl; Fermentas), and directly sequenced using a Big-Dye di-deoxy-terminator cycle sequencing kit and an ABI-PRISM 3100 automated sequencer (Applied Biosystems). The BLAST homology searches were performed using the programs available at the NCBI (National Center for Biotechnology Information) website and compared the human *ABCD1* gene sequence with the wild-type sequence.

2.2. Bioinformatics prediction of splice consensus score

To evaluate the strength of the altered splice-site of c.1780 + 2T>G mutation, splice site scores were predicted by the Human Splicing Finder software (HSF V2.4 at http://www.umd.be/HSF) (Desmet et al., 2009).

2.3. RNA extraction and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis

The functional effect of the c. 1780 + 2T>G mutation was assessed by RT-PCR analysis of nuclear lymphocytes RNAs obtained from affected and control individuals. Total RNA was isolated from 10 ml of blood samples using PureLink™ Micro-to-Midi Total RNA Purification System (Invitrogen, Karlsruhe, Germany). Nucleic acids were quantified using the Nano Drop ND-1000 UV–Vis spectrophotometer. RT-PCR, covering the coding sequence of exons 6–10, was performed for the patient carrier of splicing mutation c.1780 + 2T>G, using the following primers: 5′ACGTACGGTGGTGTGCTCTA3′ (forward primer 69 pb downstream of exon 6) and 5′ CATCGAACTGTAGCAAGTGT3′ (reverse primer 12 pb upstream of exon 10), according to the manufacturer's recommendations of SuperScript Tm One-Step RT-PCR with platinum® Taq kit (Invitrogen). The expected RT-PCR products of 448 bp were separated and visualized under UV light by electrophoresis on 2% agarose gel stained with ethidium bromide. Direct sequencing of RT-PCR products was performed by standard conditions in both directions.

2.4. Estimation of expression levels of transcripts

To estimate the transcript expression levels of *ABCD1* gene, separate bands on the agarose gel were quantified using the Quantity 1D analysis software. Band intensity is expressed in arbitrary units (intensity × area) calculated by the software.

3. Results

In this current study, we identified a Tunisian family with one X-ALD affected male. The healthy parents were unrelated and originated from South Tunisia. Clinical and biological data indicated that the initial presentation was adrenal insufficiency followed by neurological manifestations.

3.1. Clinical analysis of X-ALD patient

The 16-year old male patient was asymptomatic up to the age of 5 after which he presented clinical data suggesting melanodermia and generalized hypotonia. He presented difficulties in understanding spoken language, and hearing deficit. Plasma VLCFAs levels of the patient showed C26:0 to be 3.78 μmol/L (normal level <1.31 μmol/L), C24:0 to be 46.44 μmol/L (normal level: 11–39 μmol/L) and C22:0 to be 36 μmol/L (normal level = 22–43 μmol/L).

The C26:0/C22:0 ratio was 0.105 (normal ratio = 0.002–0.018) and C24:0/C22:0 was 1.29 (normal ratio = 0.5–0.98). Hormonal dosages showed high plasmatic ACTH levels (1890 ng/L at 11 years old; normal level <48 ng/L), therefore glucocorticoid replacement therapy was initiated for adrenal insufficiency. His neurological abilities have worsened with progressive impairment of cognition and behavior. A brain magnetic resonance imaging (MRI) exam revealed a signal abnormality in the bilateral cerebral white matter, predominantly in the parieto-occipital region: low signal intensity in T1 (Fig. 1a) and high signal intensity in T2 (Fig. 1b). The patient was categorized into ccALD phenotype.

3.2. De novo c.1780 + 2T>G mutation in the ABCD1 gene

The sequencing analysis of coding regions and intron–exon boundaries of *ABCD1* gene of the patient revealed de novo splicing site mutation T>G at the second nucleotide of intron 7 (c.1780 + 2T>G) already described (Fig. 2). Using direct sequencing, the mutation was absent in the mother and the brother.

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