A de novo large deletion of 2.8 kB produced in ABCD1 gene causing Adrenoleukodystrophy disease

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A de novo large deletion of 2.8 kB produced in *ABCD1* gene causing Adrenoleukodystrophy disease

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Running title: De novo large deletion in *ABCD1* gene.

27 **Abstract**

28 X-linked adrenoleukodystrophy (X-ALD) is a neurodegenerative disorder caused by
 29 mutations in the *ABCD1* gene that encodes an ATP-binding cassette transporter protein,
 30 ALDP. The disease is characterized by increased concentrations of very long-chain fatty acids
 31 (VLCFAs) in plasma, adrenal, testicular and nervous tissues. In the present study, our
 32 objective is to conduct a clinical, molecular and genetic study of a Tunisian patient with X-
 33 linked adrenoleukodystrophy. The clinical diagnosis was based on clinical symptoms,
 34 biochemical levels, typical scanner pattern and molecular biology. Whereas, the molecular
 35 analysis was based on PCR, long range PCR and sequencing. The molecular analysis by long
 36 range PCR and direct sequencing of the *ABCD1* gene showed the presence of a *de novo* 2794
 37 bp deletion covering the whole of exon 2. Using bioinformatics tools, we demonstrate that the
 38 large deletion is located in a region rich of Alu sequences. Furthermore, we suggest that the
 39 AluJb sequence can be the cause of the large deletion of intron 1, exon 2 and intron 2 and
 40 creation of premature stop codon within exon 3. The present report, however, is the first
 41 report in which we demonstrate the breakpoints and the size of a large deletion in an X-ALD
 42 Tunisian patient.

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46 **Key words:** *ABCD1* gene; AluJb sequence; Large deletion; SINE sequences; X-
 47 Adrenoleucodystrophy.

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50 INTRODUCTION

51 X-linked adrenoleucodystrophy (X-ALD; OMIM 300100) is the most frequently
52 inherited peroxisomal neurodegenerative disorder affecting cerebral white matter, peripheral
53 nerves, adrenal cortex and testis (Moser et al. 2001). The incidence of X-ALD in males is
54 estimated at between 1:21,000 and 1:50,000 (Kemp et al. 2001). X-ALD is mainly classified
55 into cerebral ALD, adrenomyeloneuropathy, Addison-only, olivopontocerebellar ALD and
56 asymptomatic ALD (Kemp et al. 2001). The Cerebral ALD includes childhood (ccALD, onset
57 age: 3–10 years old), adolescent (adoALD; onset age: 11–21 years old) and adult forms
58 (acALD, onset age: after 21 years old) (Kemp et al. 2001; Moser et al. 2007). The ccALD
59 form is the most devastating phenotype, which is associated with a rapidly progressive
60 cerebral inflammatory, demyelinating reaction, common central demyelinating disease. In X-
61 ALD disease, many modifier genes and/or environmental factors are involved in the
62 pathogenesis of these highly variable phenotypes (Moser et al. 2001; Heinzer et al. 2003;
63 Singh and Pujol 2010). The diagnosis of X-ALD is based on clinical suggestive features,
64 neuro-radiological findings and elevated levels of saturated unbranched very long chain fatty
65 acids (VLCFA) particularly tetracosanoic (C24:0) and hexacosanoic acid (C26:0) (Moser et
66 al. 2001). The X-ALD disease is caused by a defect in the gene for the adenosine triphosphate
67 (ATP)-binding cassette protein, subfamily D, member 1 located on Xq28 (*ABCD1*) (Moser et
68 al. 2001). The *ABCD1* gene contains 10 exons, spanning 20 kb of genomic DNA and encodes
69 a half transporter protein (ALDP) of 745 amino acids. The protein ALDP is a structural
70 protein related to the transport of VLCFA across peroxisome membranes (Kemp and Wanders
71 2010), consists of two important domains: the transmembrane domain and the ATP binding
72 domain (Rees et al. 2009). The main biochemical abnormality associated with X-ALD is the
73 accumulation of unbranched saturated VLCFA in plasma and tissues, due to impaired β -
74 oxidation in peroxisomes. The increase in VLCFA levels provides a reliable diagnostic tool

for prenatal and postnatal identification of affected males (Moser et al. 2001). Since its identification, 1641 mutations have been reported in the *ABCD1* gene of which 46 cases of large deletion of one or more exons (<http://www.x-ald.nl/>). In this study, we identify a 2794 bp large deletion in a rich region of Alu sequence in *ABCD1* gene in a Tunisian X-ALD patient.

MATERIALS AND METHODS

Patient

Our study consists on an affected boy with X-ALD belonging to a Tunisian family was born to unrelated parents. The healthy parents and brother were also recruited. Informed consent was obtained from patient and control individuals in accordance with the ethics committee of La Rabta Hospital (Tunis, Tunisia). The diagnosis of X-ALD was based on the clinical manifestation, the cerebral scanner and the molecular analysis.

Methods

DNA extraction

Blood samples were collected from the patient and his parents. Genomic DNA was extracted from the whole blood following a standard phenol-chloroform method (Lewin and Stewart 1992).

***ABCD1* gene Amplification and sequencing**

The 10 exons and the flanking intronic regions of the *ABCD1* gene were tested for mutations in the X-ALD family by sequence analysis. PCR amplification of the 10 *ABCD1* fragments was performed using the primer sets (Table 1) as previously published (Boehm et al. 1999). All fragments were amplified in a thermal cycler (Applied Biosystem 2720) in a final volume of 50 µl containing 100 ng of genomic DNA, 0.2µM of each primer, 1x GoTaq®Buffer (Promega), 1.2 mM MgCl₂, 0.2 mM each dNTP, and 1,25U GoTaq®DNA Polymerase (Promega). The polymerase chain reaction conditions were as follows: initial

denaturation at 95°C for 5 minutes followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 60-73°C (depending upon the primers sets used) for 30 seconds and extension at 72°C for 45 seconds, and final extension at 72°C for 10 minutes. Each PCR product was then purified by enzyme reaction (Exonuclease I; 20 units/ μ l; 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.

Long Range PCR

During the amplification of the 10 exons of *ABCD1* gene, we noticed the absence of amplification of exon 2 of the patient. It was thought to amplify the fragment surrounding the exon 2 using the primer sense of exon 1c and the primer antisense of exon 3/4. The long range PCR was performed using primers exon 1c sense (5' CCACGCCTACCGCCTCTACTT3') and exon 3/4 antisense (5' GCAGCAGGTCAGCACCTGCA3') in the patient, his father and his mother to verify the deletion in the patient. The amplified fragment includes exons 1c, 2, 3 and 4 (10808bp). To limit the breakpoints deletion, an internal long range PCR amplification (3047 bp) of the region surrounding the exon 2 was performed using the primers sense 5'CTGGGACCTTGTCTCACTGG3' (intron1) and antisense 5'ATCACCTGACCAGGAGTTTCG3' (in intron 2).

The long range amplification was performed in a thermal cycler (Applied Biosystem 2720) in a final volume of 50 μ l containing 100 ng of genomic DNA, 0.2 μ M of each primer, 1x Long PCR Buffer with MgCl₂ (Fermentas), 0.2 mM each dNTP, and 1.25U Long PCR Enzyme Mix (Fermentas). The long range PCR conditions were as follows: initial denaturation at 93°C for 3 minutes followed by 10 cycles of denaturation at 93°C for 30 seconds, annealing at 58°C for 30 seconds and extension at 68°C for 12 minutes, 25 cycles of

denaturation at 93°C for 30 seconds, annealing at 58°C for 30 seconds and extension at 68°C for 12 minutes with a delay of 5 seconds per cycle and a final extension at 68°C for 11 minutes.

RESULTS

Clinical analysis of the X-ALD patient

The patient is a 16-year-old male, and was born normal and at full term. The parents are Tunisians by origin and not related. No positive neurological disease was present in the family history. The patient was normal up to the age of 8, after which he had skin pigmentation. At 11 years of age, the patient suffered from: a decline in scholar performance, reduced concentration, impaired memory and behavior, folding on itself, psychomotor retardation, hearing loss, anorexia, headache rebels, gumline, hyperpigmentation, atrophy of the hand and the foot. At 12 years of age, the patient complained of: lost his sight, agitation and behavioral disorders. After 3 years, the son becomes blind and bedridden. The patient was categorized into ccALD phenotype and was dead at the age of 16 years.

Radiological findings

A cerebral scanner for the patient at the age of 12 years was performed (Figure 1). The cerebral scanner of patient was suggestive of X-ALD.

Biochemical findings

The essential criteria in X-ALD are the high plasma VLCFA and the adrenocorticotrophic hormone (ACTH) levels. Analysis of the plasma VLCFA levels is based on tetracosanoic acid (C24:0), hexacosanoic acid (C26:0) and their ratios to docosanoic acid (C22:0). The ratios of C26:0/C22:0 was 0,003 (normal ratio= 0,002-0,018) and C24:0/C22:0 was 0, 86 (normal ratio = 0,5-0,98). The plasma VLCFA levels of our patient are normal. Biochemical analysis revealed a high ACTH level at 1890 ng/L (N< 48 ng/L).

Mutation analysis

Genomic DNAs from the patient, his parents, and his brother were amplified using specific human *ABCD1* gene primers and the PCR products were sequenced. During the PCR amplification, it was noticed the absence of a band corresponding to the fragment of exon 2 in the patient suggesting that this exon is deleted in a hemizygous state.

To map the deletion breakpoints, we designed a pair of primers in the genomic region between exon 1c and exon 4 of the *ABCD1* gene. The fragment, amplified by long-range PCR, covers the exon 1c, intron 1, exon 2, intron 2, exon 3, intron 3 and exon 4. The Long range PCR yielded a product of 10808 bp in the father, the mother and the brother and a product of approximately 8014 bp with a deletion of 2794 bp in the patient (Figure 2a). To map the deletion breakpoints, we designed a series of internal primers in the genomic region between exon 1c and exon 3/4. A pair of primers was selected to amplify a 3047 bp covering the exon 2. The parents and brother showed a band of 3047 bp of size, the patient showed a band of 253 bp (Figure 2b). The sequencing of PCR products from the deletion and wild-type alleles showed that a 2794 bp deletion eliminates exon 2 and flanking intronic sequences (Figure 3).

Bio-informatic analysis of the repeat sequences

Positions and subfamilies of Alu elements in the *ABCD1* gene were identified using RepeatMasker, a program that screens DNA sequences for interspersed repeats (<http://repeatmasker.org>). A total of 23 repeat sequences, including 10 SINE sequences (8 Alu -type and 2 MIR type), 10 LINE sequences (7 L1-type, 3 L2- type) and 3 LTR sequences were identified, and their positions on the gene defined. Of these, 8 SINE sequences (7 Alu -type and 1 MIR type), 8 LINE sequence (7 L1-type and 1 L2) were located in the genomic region type between exons 1c and exon 3. The majority of SINE and LINE sequences are located around exon 2 between intron 1 and intron 3 of *ABCD1* gene (Table 2). Sequence

analysis of the 253 bp product PCR of patient revealed a deletion of 2794 bp (152,992,724–152,995,517 in the H. sapiens chromosome X). The 5' part of the deletion point is located in intron 1 without an Alu element in intron 1. The 3' part of the deletion point is located in an AluJb element in intron 2 (Figure 4).

DISCUSSION

In this study, we diagnosed a Tunisian patient with ccALD. Elevated level of very long-chain fatty acids (VLCFA) in plasma is still the best initial biomarker for X-ALD in males. Our patient has normal values of plasma VLCFA: tetracosanoic acid (C24:0), hexacosanoic acid (C26:0) and their ratios to docosanoic acid (C22:0). In obligate carriers, VLCFA levels can be normal in around 20% of cases (Moser et al. 2001). Although the normal values of VLCFA, the patient presents a severe phenotype and he died at the age of 16. The X-ALD is caused by mutations in the *ABCD1* gene, and more than 1641 different mutations of the *ABCD1* gene have been reported up to date. Most of these mutations are missense, more than 46 mutations are deletion of one or more exons. In this study, we identified, in our patient, a *de novo* hemizygous large deletion encompassing exon 2 of the *ABCD1* gene. This deletion is absent in his mother and his brother. In a considerable number of cases, X-ALD is not inherited but the respective mutations occur spontaneously. While the majority of patients typically inherit the defective *ABCD1* allele from one parent, between 4.1% and 19% of X-ALD cases have been reported to carry mutations acquired *de novo* (Wiesinger et al. 2015). The next-generation sequencing technology applied in the detection and quantification of mutations can be a standard tool for the accurate estimation of recurrence risks in families for which a genetic disease is caused by an apparently *de novo* disease gene mutation event (Veltman et al. 2012). Sequencing of the PCR amplicon from the patient's genomic DNA shows the complete characterization of the deletion breakpoint (Figure 3). The deletion spans 2794 bp as follows: 1963 bp at the 3' of intron 1, along the exon

2 (181bp) and 650 bp at the 5' of intron 2. The *ABCD1* mRNA lacking exon 2 is predicted to have a premature termination four codons after codon 300 (the last codon of exon 1), compared with 745 amino acid residues of the mature ALDP protein. Alternatively, the formation of premature stop codons leading to the production of truncated forms, probably removed by the nonsense-mediated decay pathway (Zizza et al. 2015).

Large genomic rearrangements in the *ABCD1* gene have been identified in 4% to 7% of unrelated patients with various phenotypes including ccALD, acALD and AMN. (Mosser et al. 1993; Kok et al. 1995; Koike et al. 1995). In previous studies, many large deletions have been found in patients with ccALD phenotype (Kok et al. 1995; Krasemann et al. 1996), our study is in agreement with these finding. The exon 2 has been found deleted alone in two cases, with other exons in five cases (<http://www.x-ald.nl/>). Takano et al identified in his study on 29 patients three large rearrangements of 0.5, 7 and 10 kb in three acALD patients (Takano et al. 1999). In other study on 69 Japanese families, Shimosawa et al described two exon-skip mutations and three large deletions in acALD and ccALD patients (Shimozawa et al. 2010).

No large deletions quoted in X-ALD in previous studies are detailed in size, origin or mechanisms responsible. One of the mechanisms causing major gene rearrangements in humans involves Alu elements (Miki 1998; Deininger and Batzer 1999). Alu sequences are the most abundant repeats in the human genome, which contain more than 1 million copies of Alu sequences distributed throughout the genome with an average spacing of 4 kb. Alu-mediated rearrangement events have long been recognized as a common cause of local deletion and duplication events associated with human genetic diseases (Batzer and Deininger 2002; Sen et al. 2006). The present report is the first report on an X-ALD patient in which an Alu-mediated recombination is involved. Unequal homologous recombination may occurs between two Alu sequences oriented in the same direction, resulting in duplications, deletions

and translocations. In fact, around 0.3% of all human genomic diseases have been reported to likely result from Alu-mediated unequal homologous recombination (Lobachev et al. 2000). Usually the recombination event presumably occurred between highly similar regions of two sequences of the two members of distant subfamilies of Alu elements. However, in our patient there is one Alu sequence (Figure 4). This is a rare case where the deletion is surrounded by only one Alu sequence, the mechanism responsible for the deletion is not clear. Recent studies explain others mechanisms responsible to leading genomic rearrangements as the non-allelic homologous recombination (NAHR) mechanism (Gu et al. 2008; Stankiewicz and Lupski 2002). In addition, Non-homologous end joining (NHEJ) is a candidate recombination mechanism to explain some non-recurrent rearrangements (Gu et al. 2008; Lieber 2008). Although, the FoSTeS (Fork Stalling and Template Switching) and/or MMBIR (Micro homology-Mediated Break-Induced Replication) mechanisms can contribute to non-recurrent rearrangements causing other genomic disorders, structural variations and genome or single gene evolution (Zhang et al. 2009). Furthermore, 35% of the chromosomal events happen on the X chromosome. This phenomenon is due to the hemizyosity of X-linked genes in males and preferential retro-transposition on the X chromosome (Kazazian and Moran 1998).

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Conflict of Interest and sources of finding

The authors declare no conflict of interest.

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Table legends:

Table 1: Primers used in the amplification of the gene *ABCD1*.

Exon	Primers	T _m (°C)	Size (bp)
Exon 1a	F : 5' ACAACAGGCCCCAGGGTCAGA3'	62	422
	R : 5' AGGAAGGTGCGGCTCACCA 3'		
Exon 1b	F : 5' AACC GG GTATTCTGCAGCG 3'	60	385
	R : 5' ACTGGTCAGGGTTGCGAAGC 3'		
Exon 1c	F : 5' CCACGCCTACCGCCTCTACTT 3'	60	484
	R : 5' AGACTGTCCCCACCGCTC 3'		
Exon 2	F : 5' CCAGGAAGCCTCTCTGTGTC 3'	60	251
	R : 5' CCTGGCCTGGATCCTCTTGG 3'		
Exon 3/4	F : 5' GCAGAAGAGCCTCGCCTTTC 3'	64	570
	R : 5' GCAGCAGGTCAGCACCTGCA 3'		
Exon 5	F : 5' CTGCCAGGGATGGGAATGAG 3'	60	337
	R : 5' TCTCACCTTGACCTTGGCCC 3'		
Exon 6	F : 5' GCCATAGGGTACGGGAAGGG 3'	60	276
	R : 5' GCCTCTGCAGGAAGCCATGT 3'		
Exon 7	F : 5' CGATCCACTGCCCTGTTTGG 3'	60	491
	R : 5' CTTCCCTAGAGCACCTGG 3'		
Exon 8/9	F : 5' CTGAGCCAAGACCATTGCCCCCG3'	60	471
	R : 5' TGCTGCTGCCGGGCCCCGC 3'		
Exon 10	F : 5'GAGGGGAGGAGGTGGCCTGGC3'	73	427
	R : 5'GCGGGGTGCGTGCATGGGTGG3'		

Table 2. Repetitive sequences in the region around exon 2 of the *ABCD1* gene according to the Repeat Masker program.

<i>Position in query</i>				<i>Strand</i>	<i>Matching repeat</i>	
<i>score</i>	begin	end	(left)		repeat	class/family
12	117	156	(9788)	+	G-rich	Low_complexity
424	1771	1943	(8001)	+	MIRb	SINE/MIR
1781	2393	2675	(7269)	C	AluJr	SINE/Alu
2086	3747	4055	(5889)	C	AluJb	SINE/Alu
256	4060	4145	(5799)	C	L1MEd	LINE/L1
2446	4146	4454	(5490)	+	AluYk3	SINE/Alu
256	4455	4514	(5430)	C	L1MEd	LINE/L1
286	4598	4721	(5223)	C	L1MEd	LINE/L1
1534	4803	4840	(5104)	C	L1MEd	LINE/L1
822	4842	4857	(5087)	C	AluJo	SINE/Alu
26	4858	4898	(5046)	+	(TTTG)n	Simple_repeat
822	4899	5013	(4931)	C	AluJo	SINE/Alu
1534	5014	5886	(4058)	C	L1MEd	LINE/L1

Figure Legends:

Figure 1: Cerebral scanner of X-ALD patient. (A) Axial scanner section without injection of contrast reveals a diffuse decrease in density throughout the cerebral white matter which evokes demyelinating lesions. (B) Axial scanner section showed a discrete zone of contrast enhancement adjacent to the low density areas.

Figure 2: (A) Family pedigree of the patient. (B) Long range PCR showing a large de novo deletion in the patient. (C) PCR products using a pair of internal primers.

MW, Marker Weight; 1, father; 2, mother; 3, X-ALD patient; 4, brother; 5, healthy individual; C, control without DNA.

Figure 3: DNA sequence chromatograms: (A) Recombination sequence of *ABCD1* gene in X-ALD patient revealed the deletion of part of intron 1, exon 2 and part of intron 2. (B) The normal 5'upstream sequence of intron 1 in a healthy individual. (C) The normal 3'downstream sequence of intron 2 in a healthy individual.

Figure 4: Schematic representation of the deletion and the SINE and LINE sequences involved in the recombination.

Figure 1: Cerebral scanner of X-ALD patient. (A) Axial scanner section without injection of contrast reveals a diffuse decrease in density throughout the cerebral white matter which evokes demyelinating lesions. (B) Axial scanner section showed a discrete zone of contrast enhancement adjacent to the low density areas.
230x150mm (120 x 120 DPI)

Figure 2: (A) Family pedigree of the patient. (B) Long range PCR showing a large de novo deletion in the patient. (C) PCR products using a pair of internal primers.
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200x289mm (96 x 96 DPI)

Figure 3: DNA sequence chromatograms: (A) Recombination sequence of ABCD1 gene in X-ALD patient revealed the deletion of part of intron 1, exon 2 and part of intron 2. (B) The normal 5'upstream sequence of intron 1 in a healthy individual. (C) The normal 3'downstream sequence of intron 2 in a healthy individual. 443x144mm (96 x 96 DPI)

Figure 4: Schematic representation of the deletion and the SINE and LINE sequences involved in the recombination.
288x85mm (96 x 96 DPI)