Molecular biology and pharmacogenetics of x-linked adrenoleukodystrophy
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Chapter 2

Identification of a two base pair deletion in five unrelated families with X-linked adrenoleukodystrophy: A possible hot spot for mutations

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Chapter 2

Abstract

The gene for X-linked adrenoleukodystrophy (X-ALD) was recently identified. Intragenic deletions of several kilobases were found in about 7% of patients. Point mutations, expected to be very heterogeneous, were identified so far in only two patients. We report the identification of a two base pair deletion at position 1415-1416 of the ALD cDNA, located within the fifth exon of the ALD gene, which precedes the two consensus motives for ATP-binding. This micro-deletion was found in five out of 40 (12.5%) unrelated X-ALD kindreds, indicating that this position is a hot spot for mutations. The mutation was observed both in patients with childhood cerebral ALD (CCALD) and in patients with adrenomyeloneuropathy (AMN).

Introduction

X-linked adrenoleukodystrophy (X-ALD) is a peroxisomal disorder, characterized by demyelination of the nervous system and adrenocortical insufficiency. X-ALD has a highly variable phenotypic expression. The most frequent phenotypes are childhood cerebral ALD (CCALD) and adrenomyeloneuropathy (AMN). Both CCALD and AMN may co-occur within the same kindred as different manifestations of the same genetic defect. Biochemically, X-ALD is characterized by accumulation of saturated very long-chain fatty acids (VLCFA) in white matter, adrenal cortex, fibroblasts and plasma, caused by the inability to β-oxidize these VLCFA in peroxisomes.

In 1993, a putative ALD gene was identified. The deduced amino acid sequence of the X-ALD protein (ALDP) showed significant homology to the peroxisomal membrane protein PMP70, belonging to the ‘ATP-binding cassette’ superfamily of transporter proteins. Using immunoelectron microscopy with monoclonal antibodies raised against ALDP, Mosser et al. demonstrated that the ALDP is associated with the peroxisomal membrane. The molecular weight of the ALDP was determined to be 75 kDa. The localization of ALDP in the peroxisomal membrane and the presence of the ATP-binding sites in ALDP may indicate that X-ALD is caused by an impaired peroxisomal transport process rather than by a deficiency of β-oxidation of VLCFA directly.

So far, six intragenic deletions ranging from 1.6 to 19 kb were reported, accounting for about 7% of X-ALD patients analyzed. At the cDNA level only two point mutations, a missense mutation at position 871 (Glu291Lys), and one
nonsense mutation at position 1768 (Gln590Stop) were reported. We investigated the part of the ALD cDNA containing the consensus motives for ATP-binding, and we identified a small deletion of two base pairs within the fifth exon of the ALD gene.

Patients, Materials and Methods

Patients: ALD#3 is a Vietnamese family in which a boy died of CCALD. ALD#4 is a Dutch family with two asymptomatic carriers and one male AMN patient. ALD#16 is a Dutch family with one carrier and two AMN patients. ALD#25 (family B, reference 8) is a German family with one male CCALD patient and a young symptomatic heterozygote. ALD#31 is a Dutch family with two AMN patients.

Oligonucleotide primers: Primers were selected from the human ALD cDNA sequence (EMBL database N° Z21876, where ATG = +1) using the Primer computer program (S.E. Lincoln, M.J. Daly and E.S. Lander, MIT Center for Genome Research and Whitehead Institute, Cambridge, USA). For nomenclature we used the following abbreviations: ALD, followed by the starting nucleotide position, followed by F or R for forward or reverse, respectively. The following primers were used: ALD914F 5'-TGC TAC AGC GCT CCT ACC AG-3'; ALD1295F 5'-GTC ACT TCA AGA GGC CCA GG-3'; ALD1395F 5'-CCA GGT GGT GGA TGT GGA A-3'; ALD1475R 5'-CTG GCC ACC ACC ACC TCT-3'; ALD1589R 5'-ACA CCA CCG TAC GTG GGC-3' and ALD1630R 5'-GCG GGA TGT AGA ACA TGC G-3'. Primers were synthesized on a Cyclone Plus DNA synthesizer (Millipore).

RT-PCR and sequence analysis: Fibroblasts were grown (37°C, humidified air, 5% CO₂) in DMEM with 10% fetal calf serum, 2 mM glutamine, 50 U/ml penicillin and 50 U/ml streptomycin. A part of the fibroblasts was stored as a dry pellet at −20°C for total DNA isolation. Total mRNA from fibroblast cell lines was extracted with guanidinium isothiocyanate, DNA was removed with acidic phenol, RNA was precipitated and dissolved in water, and concentrations were measured spectrophotometrically. RNA was converted in cDNA by random primed cDNA synthesis using M-MLV reverse transcriptase (Gibco BRL). The cDNA was amplified using primers ALD914F and ALD1630R. Subsequent PCR reactions contained total cDNA derived from approximately 250 ng of total RNA in 50 µl of 2 mM MgCl₂, 50 mM KCl, 10 mM Tris.Cl (pH 8.3), 5% DMSO, 200 µM of each dNTP, 0.8 U of Taq polymerase (Gibco BRL) and 50 ng of each primer. PCR was performed in a multiwell thermocycler (MJ Research, Waltham, MA) for 35 cycles (each cycle was 1 min at 93°C, 1.5 min at 57°C and 2 min at 72°C). Free primers and nucleotides were removed using QIAquick-spin PCR purification kit (Qiagen). Sequencing was performed with primers ALD1295F and ALD1589R, using the SequiTherm Cycle Sequencing Kit (Epicycle technologies, Madison, USA) according to the protocol with [γ-32P]ATP (Amersham Life Sciences, UK) labeled primers. Some changes were made, we used 200 ng of template DNA, reactions were carried out in a multiwell thermocycler and PCR cycles consisted of 25 rounds of 45 s at 92°C, 55°C and 72°C. Sequence reactions were loaded on a 7% and a 5% polyacrylamide/8 M urea sequence gel and after electrophoresis at 65 W for 2 h and 3 h, respectively, the gels were exposed on a Phospho Imager™ (Molecular Dynamics, Sunnyvale, CA). Sequences were analyzed using the Bioimage packet (Millipore, USA).
**Deletion verification:** Deletion of the nucleotides 1415 and 1416 of the ALDP gene in 5 patients was verified using chromosomal DNA of these patients together with DNA obtained from additional family members. Primers used for amplification of exon 5 were ALD1395F and ALD1475R. The PCR reaction contained: 50 ng of total chromosomal DNA in 10 µl of 2 mM MgCl₂, 50 mM KCl, 10 mM Tris.Cl, 5% DMSO, 70 µM of dCTP, dTTP and dGTP, 7 µM of dATP, 0.4 U of Taq polymerase, 35 ng of each primer and 1 µCi of [α-32P]dATP. After 25 rounds of PCR, consisting of 1 min at 93°C, 45 s at 55°C and 45 s at 72°C, PCR product were denatured and separated on a 6% polyacrylamide/8 M urea sequence gel. After electrophoresis for 2 h at 65 W gels were exposed to X-ray film for 6-12 h at -70°C with an intensifying screen.

**DNA isolation:** DNA isolation from blood samples anticoagulated with EDTA was performed according to standard techniques. Fibroblasts were used to obtain chromosomal DNA from deceased X-ALD patients. Fibroblasts were left at room temperature overnight in a lysis solution containing: 1x SSC/20 mM EDTA/1% SDS and Proteinase K (10 µg/ml), after phenol extraction and precipitation the DNA was dissolved in 10 mM Tris/0.5 mM EDTA.

**Haplotype analysis:** Probes used for haplotype analysis can be found in the Genome Data Base under the following accession numbers: DXS15/Ba, (microsatellite: GDB Id: G00-207-346), DXS707/p2-55 (GDB Id: G00-180-336), DXS605/2-19 (GDB Id: G00-178-856), F8C/IVS13 (GDB Id: G00-185-865), DXS1108 (GDB Id: G00-191-645).

**Figure 1.** Partial DNA sequence of the normal and mutant ALD cDNA. Two nucleotides (AG, underlined) at cDNA position 1415-1416 were deleted, resulting in a frame shift at amino acid residue Glu471 (Fs E471) and a prematurely terminated ALDP at amino acid position 554.

**Figure 2.** 6% sequence gel showing radiolabeled PCR amplified genomic DNA fragments, internally. DNA samples were derived from two healthy individuals (H), two carriers (C) and one X-ALD patient (P). The normal allele is represented by a PCR fragment of 81 bp and the mutant allele, carrying a two base pair deletion, by a fragment of 79 bp.

**Results**

Fibroblasts from X-ALD patients were used isolate total mRNA. With RT-PCR and internal primers derived from the ALD cDNA sequence published by Mosser et al. we generated PCR fragments for sequencing. Without the addition of dimethyl...
sulfoxide (DMSO) only fragments up to 150-300 bp could be amplified, which is probably due to the high G/C content of the ALD cDNA (65%). Larger PCR fragments, up to 1 kb, could be generated by addition of 5% DMSO to the PCR reaction.

We focussed our attention on the region containing and surrounding the consensus sequences for ATP-binding, being Walker A at the amino acid residues 507-520 (nt 1519-1560) and Walker B at the amino acid residues 605-630 (nt 1813-1891). A small deletion of two base pairs (nt AG) at ALD cDNA position 1415-1416, which precedes the Walker A motif, was found in four out of 30 unrelated X-ALD kindreds,10 kindreds ALD#3, ALD#4, ALD#16 and ALD#25 (Figure 1).

To verify that the microdeletion 1415delAG was not a PCR artifact, a PCR on total genomic DNA with primers ALD1395F and ALD1475R was performed in the presence of [α-32P]dATP. The full-length PCR fragments were separated on a 6% sequence gel. In case of a normal allele an 81 bp PCR fragment is found, while a two base pair deletion mutant allele is represented by an 79 bp PCR fragment (Figure 2). Other family members were investigated for the presence of the microdeletion: all investigated carriers were heterozygous for the microdeletion, all male patients carried the 79 bp allele, while all non-affected males and females only had the wild type allele (data not shown). Ten additional X-ALD kindreds of whom no fibroblast material was available were also tested for the presence of this mutation on genomic DNA. We identified one additional family (ALD#31) with an identical two base pair deletion.

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Table 1: Haplotype analysis in Xq28

Haplotype analyses of five X-ALD patients carrying an identical mutation in the ALD gene. Markers are located in Xq28.

We identified two silent intragenic nucleotide polymorphisms at ALD cDNA positions 1548 and 2246. At position 1548 kindred ALD#4 carried an adenosine residue where the other four kindreds contained a guanine as in the ALD cDNA
sequence published by Mosser et al. At position 2246, kindred ALD#16 had a guanine substituting the cytosine residue (Table 1). The adenosine residue at ALD cDNA position 1548 was found in 5 out of 34 X-chromosomes investigated (15%) and the cytosine at ALD cDNA position 2246 in 8 out of 33 X-chromosomes (24%). To further exclude linkage between the five kindreds we performed haplotype analysis in Xq28 at loci listed in Table 1. These results showed that all five kindreds are unrelated.

Discussion

We identified a two base pair deletion upstream of the ATP-binding domain in ALDP in five out of 40 unrelated X-ALD kindreds. This microdeletion is located in exon 5 at cDNA nucleotide positions 1415 and 1416. The mutation results in a frameshift at amino acid residue Glu471 (Fs E471) and a premature stop codon at amino acid position 554. The predicted ALDP protein derived from the mutant allele would not contain the consensus nucleotide-binding domain for ATP and will therefore, most likely, be inactive.

Haplotype analysis was carried out to determine whether the deletion originated independently in the five kindreds. Polymorphisms in the region of Xq28, containing the ALD gene, showed differences at four or more markers between kindreds ALD#3, ALD#4, ALD#16 and ALD#25 (Table 1). In kindred ALD#25 only fibroblasts from a carrier were available and therefore the allele segregating with the disease could not be determined. The differences between the alleles strongly suggested that these five kindreds are unrelated. Even silent polymorphisms in the ALD gene were present in kindreds ALD#4 (1548G>A) and ALD#16 (2246C>G). Furthermore, kindred ALD#3 was of different ethnic origin.

Ketterling and Sommer have proposed a possible mechanism for the generation of microdeletions. In their model a quasipalindromic structural intermediate is formed, of which the non-matching nucleotides are deleted followed by re-ligation. Another possible mechanism for such mutations is slippage of DNA polymerase involving a short tandem repeat sequence. We were not able to identify neither a quasipalindromic sequence, nor a tandem repeat surrounding position 1415-1416 and therefore a different mechanism could be involved in the generation of the two base pair deletion of the ALD gene.

The majority of severe X-linked disorders are caused by a wide variety of mutations, as can be seen for example in Duchenne muscular dystrophy and Lesch-Nyhan syndrome. An exception is Haemophilia A, where a large inversion in
the Factor VIII gene is often found. We identified an identical mutation in five out of 40 unrelated X-ALD kindreds, which indicates that this position is a hot spot for mutations in the ALD gene. This will facilitate carrier detection and prenatal diagnosis in the families concerned by a relatively simple and rapid method as shown in Figure 2.

It is remarkable that a deletion of two nucleotides, resulting in a truncated and non-functional ALDP protein, results in both CCALD and AMN. In two of the investigated kindreds cases of CCALD were found (ALD#3 and ALD#25), while in the other three kindreds (ALD#4, ALD#16 and ALD#31) this mutation caused AMN. These results strongly support the hypothesis that a modifier gene, and/or environmental factors are involved in modulating the clinical expression of X-ALD.

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