CASE REPORT

Adrenoleukodystrophy – a new mutation identified

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Abstract

Introduction: **X-linked adrenoleukodystrophy from the group of peroxisomal disorders presents with an extensive spectrum of phenotypes. The mutation affects the ABCD1 gene encoding a peroxisomal membrane protein. So far, its detailed function has not been clarified. However, it plays an essential role in the ethiopathogenesis of X-linked adrenoleukodystrophy. Its defect causes accumulation of the very long chain fatty acids in the tissues of the central and peripheral nervous system, adrenal glands and in the body fluids.**
Purpose: **To review the clinical presentations and diagnostic issues in X-adrenoleukodystrophy diagnosed in the one affected family.**
Methods: **A case report. Measurement of very long chain fatty acids. Molecular analysis of the adrenoleukodystrophy gene.**
Results: **A new "unique" mutation in the initiation codon in the first exon of ABCD1 gene was identified. We present a phenotype description of a patient with this mutation.**
Conclusions: **X-linked adrenoleukodystrophy is a disease with the incidence rate approximately 1: 16 800. Detection of new mutations contributes to better understanding of this rare disease and makes the diagnostic more available and precise. The importance of an adequate diagnosis is justified not only by a different therapeutic approach, but also by the need of prenatal diagnostics and the need of genetic counselling in the affected families. As demonstrated in our case, it is necessary to consider this diagnosis also in the adult age, e.g. within the differential diagnosis of spastic paraparesis *(Tab. 1, Fig. 4, Ref. 23)*. Full Text (Free, PDF) *www.bmj.sk*.**
Key words: adrenoleukodystrophy, peroxisomal disorders, adrenoleukodystrophy protein, very long chain fatty acids, adrenomyeloneuropathy.

Abbreviations: ABCD1 – ATP Binding Cassette transporter subfamily D member 1, ACTH – adrenocorticotrophin, ALDP – adrenoleukodystrophy protein, ALDR – adrenoleukodystrophy related protein, AMN – adrenomyeloneuropathy, CT – computed tomography, EMG – electromyography, HSCT – hematopoetic stem cell transplantation, Kb – kilobase, MEP – motor evoked potential, MRI – magnetic resonance imaging, PMP 70 – 70kDa peroxisomal membrane protein, P70R – PMP70-related protein, VLCFA – very long chain fatty acid, X-ALD – adrenoleukodystrophy linked to X-chromosome

Adrenoleukodystrophy (X-ALD) is a disorder of the peroxisomal compartment. Its heritability is recessive, linked to X-chromosome. It is the most common disease of peroxisomes and the most frequent monogenetic inherited demyelinating disorder. Because of its heredity linked to X-chromosome, it is fully manifested only in males, even though to some extent it may affect females, too. However, these phenotypes represent milder variants. The women, heterozygous for X-ALD, develop an adrenomyeloneuropathy-like syndrome in middle or later age (1). The estimated incidence of X-ALD (hemizygotes plus heterozygotes) is 1:16 800 (2).

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The current clinical classification includes a wide range of phenotypic manifestations and criteria such as age of onset, organs involved, and the rate of progression of neurological symptoms (3, 4). There are at least six distinct types of disorder's manifestation (in a decreasing severity):
– childhood cerebral,
– adolescent and adult cerebral,
– adrenomyeloneuropathy,
– Addison-only,
– asymptomatic,
– phenotypes in female carriers.

The principal inherited defect in X-ALD is the mutation of *ABCD1* gene (ATP Binding Cassette transporters subfamily D member 1). Gene encodes a 745 amino acid peroxisomal transmembrane protein with a general structure of an ATP-Binding Cassette transporter. Up to date, almost 500 different mutations in ABCD1 have been identified (www.x-ald.nl). The mutations are distributed equally among the entire coding region of the gene that covers approximately 19-kb, contains 10 exons and 9 introns (5). It is obvious, that there is no disease-associated mutation within the first 88 N-terminal amino acids and the last 45 C-terminal amino acids (6). There is no general correlation between the type of *ABCD1* gene mutation and the clinical phenotype (6). Mutations that are known to cause a complete loss of adrenoleukodystrophy protein (ALDP) can be associated with all different clinical phenotypes, even with the very late onset adrenomyeloneuropathy (AMN) (7).

Methods of biochemical and molecular diagnostics

Gas chromatography-mass spectrometry (GC-MS) analysis of VLCFA

The Folch extraction method was used to harvest VLCFA from plasma/serum (8). All lipid fractions were derivatised (methylated) for GC-MS (9).

GC-MS analysis was performed with a GCQ-Gas Chromatograph Finnigan MAT equipped with the capillary column DB-XLB 122-1232, connected to the GCQ – Mass Spectrometer Finnigan MAT. Helium was used as a carrier gas. The flow rate was 40 cm/s and the oven temperature was 60 °C for 1 min, increasing to 200 °C at 20 °C/min and then to 280 °C at 5 °C/min, then held for 11 min. The temperatures were 250 °C, 250 °C and 200 °C for the injection port, transfer line and ion source respectively. The metylesters of fatty acids in hexane (1l) were manually injected in the splitless mode. The fatty acids were identified by their retention time and mass spectrum. These values were compared with commercial standards (Sigma).

Mutation analysis of ABCD1 gene

*Genomic DNA (gDNA) was prepar*ed from the whole blood in the presence of anticoagulant (EDTA) using a NucleoSpinr Blood QuickPure kit (Macherey-Nagel) according to the manufacturer's protocol.

The gDNA served as a template for PCR product, which contained the part of the first exon of the *ABCD1* gene. PCR was performed using PhusionTM high fidelity PCR kit (Finnzymes). PCR primer pairs S1 (CCAGGGCACTGACAGGACAGGAGA) -A1 (CCACCAGACGGCTGCGGAACGACA) was used.

The PCR mixture (total volume 50 µl) contained: (final concentration in brackets) 5x Phusion buffer GC – for GC rich templates (1x), dNTPs (200 µM each), primers (0.5 µM each), DMSO (5 %), Phusion DNA polymerase (0.02 U/µl), cDNA (0.1 µg) and water (to 50 µl).

The PCR cycling conditions were: Initial denaturation at 98 °C for 30 s (1 cycle); denaturation at 98 °C for 10 s, annealing at 68 °C for 10 s and extension at 72 °C for 30 s (35 cycles); final extension at 72 °C for 5 min (1 cycle); held at 4 °C.

The PCR products were purified using QIAquickr PCR purification (Qiagene, Germany), quantified by the agarose gel electrophoresis and sequenced using the ABI Genetic Analyser 310 (ABI 310) with Big Dye Terminator Cycle v3.0 sequencing Ready reaction kit (Applied Biosystems) according to the manufacturer's protocol. The complete coding region (both sense and antisense chains) was sequenced.

The mutation found in proband and family members was confirmed by PCR/RFLP from genomic DNA. Since mutation c.3G>C (M1I) creates a restriction site for enzyme *FokI*, screening and segregation analysis was performed by digestion of PCR products for 16 hours at 37 °C with restriction enzyme according to the manufacturer's protocol (New England Biolabs).

Case report

We report a case of a 28 years old male with a 6 months history of progressive lower limbs weakness, gait ataxia and postural instability. Sensitive symptoms included feelings of cold in dis-

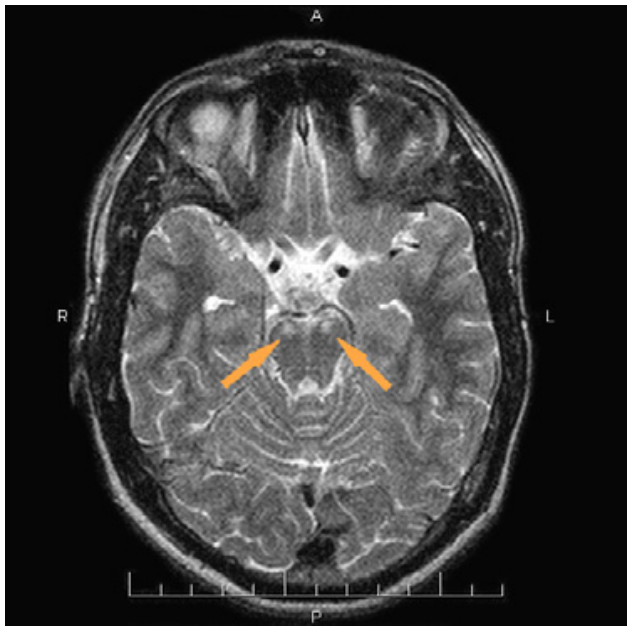

Fig. 1. Two areas of signal hyperintensity in T2 weighted MRI scan longitudinally shaped 4–6x9 mm in ventral part of mesencephalon bilateral.

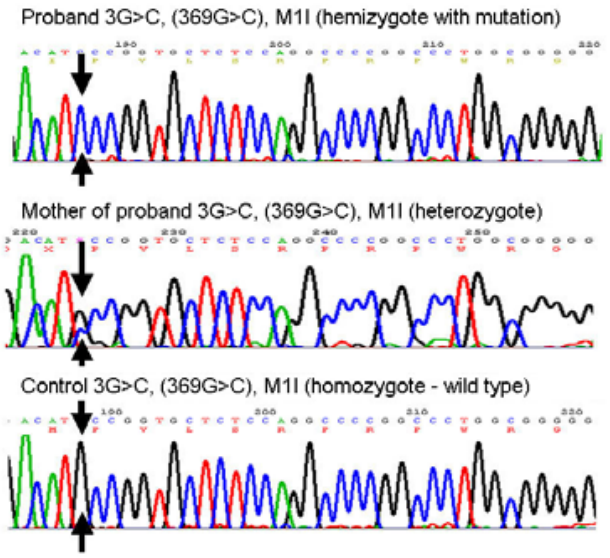

Fig. 2. Molecular analysis of the first exon of ABCD1. Partial sequence determination of the novel c.3G>C (M11) mutation in ABCD1 (the arrow indicates the involved nucleotide). A – hemizygous proband, B – heterozygous proband's mother, C – control sample – homozygous wild type.

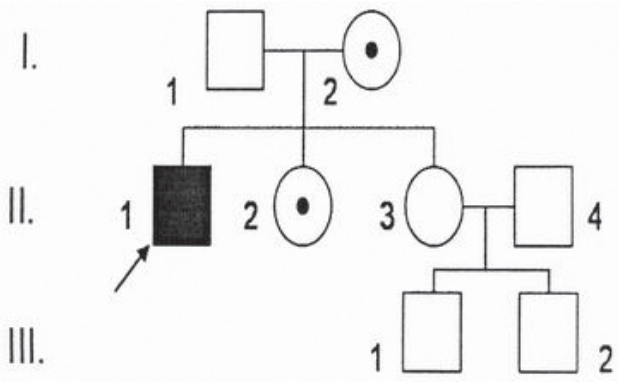

Fig. 3. Genealogical chart of family members. The proband is indicated by the arrow in the pedigree.

tal legs. Urinary dysfunction was also present. The patient hasn't noticed his difficulties much. He has found physician's help after a mild head and back injury during a "friendly" battle shortly before he entered the hospital. In the physical examination, tetraparesis with dominance in the lower limbs, limb ataxia, spasticity and dissociated sensory loss and no signs of trauma was found. Serum biochemistry, blood count and hemocoagulatory parameters showed no signs of pathology. In the cerebrospinal fluid, only a subtle hyperproteinorhachy (496 mmol/l) was present, other parameters were within normal range. There were no signs of blood-brain-barier disruption, no oligoclonal band or intrathecal immunoglobulin production. Eye fundus examination was normal. Neuropsychological testing revealed no cognitive decline. Spinal neuroimaging studies (contrast-enhanced computer

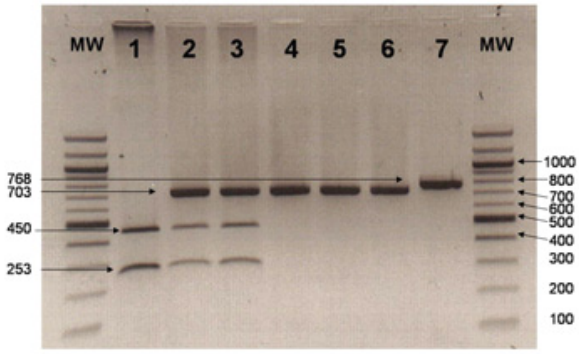

Fig. 4. PCR/RFLP – polymerase chain reaction/analysis of restriction fragments lenght polymorphism. RFLP analysis after digestion of the 768 bp amplified PCR product with *FokI*. The mutant allele gives rise to three bands of 450, 253 and 65 bp, while the normal allele presents only two fragments 703 and 60 bp. MW: 100 bp DNA ladder; 1st proband II.1; 2nd proband's sister II.2 – carrier of mutation; 3rd proband's mother I.2 – carrier of mutation; 4st proband's sister II.3 – healthy; 5st proband's nephew III.1 – healthy; 6st proband's nephew III.2 – healthy; 7st undigested PCR product.

tomography) did not show any spinal pathology, which could be responsible for the clinical symptoms. Magnetic resonance imaging of the brain depicted lesions of atypical signal intensity longitudinally shaped 4-6x9 mm in the area of pons and mesencephalon (Fig. 1). Electroencephalogram was normal. Nerve conduction studies did not prove any neuropathic changes (latency of F waves ulnar nerve right 29.8 ms, left 31 ms, peroneal nerve right 57.2 ms, left 54.6 ms); the central conduction time latencies for all the extremities were delayed (ulnar nerve right 3.6 ms, left 3.4 ms, peroneal nerve right 4.2 ms, left 5.8 ms). There were no signs of adrenal insufficiency. Because of MRI finding, analysis of VLCFAs by gas chromatography/mass spectrometry (GC/MS) was performed. A typical pattern, characterised by an increase of VLCFAs in the serum, was observed. The value of serum cerotic acid exceeded markedly beyond the reference values (Tab. 1). Index values (C24:0/C22:0 and C26:/C22:0) were elevated as well. The sequence analysis of the ABCD 1 gene revealed mutation in the position 3 of the first exon with substitution of G nucleotide for C (Fig. 2). This mutation affects the initial codon ATG so the target protein most probably will not be translated. When we examined the patient's family, we found that the patient had inherited the mutation from his mother, clinically healthy. In his mother, the values of C26:0 reached 0.957ug/ml; (Tab. 1). The

Tab. 1. Values of VLCFA and values of the index parameters.

Case	I.2	II.1	II.2	Reference values µg/ml
C26:0	0.97	0.79	0.55	<0.39
C26:0/C22:0 ratio	0.051	0.048	0.026	<0.019
C24:0/C22:0 ratio	1.65	1.46	1.23	<1.05

mother simultaneously transmitted the mutation to the half of the female progeny (Figs 3 and 4).

Discussion

We present a new mutation, which, at the time of diagnosis, was not recorded in the World Register of mutations related to gene for the adrenoleukodystrophy protein (www.x-ald.nl). Phenotype, clinical manifestation, age of onset and sex, were consistent with the presentation of adrenomyeloneuropathy. It might be possible that the atypical cerebral lesions are the first presentation of cerebral involvement with the disease progression and a "cerebral AMN" could develop. Considering the mutation in the initial codon, we suppose a complete translation failure to the target product. Despite, the onset of the disease is relatively late and phenotype rather mild. This supports the previous observations that the severity of the mutation doesn't correlate well with the disease phenotype. The proband was the only affected member of the family. He inherited the mutation from his mother who was an asymptomatic carrier. Asymptomatic carrier was also the sister of the patient, single, without any progeny at present. Second sister, married, with two sons, is fortunately healthy; she isn't the carrier of pathologic gene. Uniformly, her sons are clinically and genetically healthy. Due to a negative family history regarding neurological disorder, or early demise in family from the mother side, we suppose a possible formation of a new mutation in mother's genotype.

It's possible that residual ALDP activity might prevent the development of the inflammatory cerebral form in X-ALD patients, leading to a milder phenotype (9). Most likely, our proband hasn't any product of pathologic gene. Therefore we would expect a cerebral involvement in a more severe stage, but the MRI finding presents only "incipient" involvement of brain. In addition to the functional ABCD1 gene, on Xq28 several autosomal non-processed pseudogenes are present on several different chromosomes (9). ALDP structurally represents a half-ABC transporter, with one hydrophobic transmembrane domain and one hydrophilic nucleotide-binding domain, where dimerisation is necessary in order to become a functional unit (10). Three other mammalian half-ABC transporters, structurally similar to ALDP, have been identified: the ALD related protein (ALDR) (11, 12), the 70kDa peroxisomal membrane protein (PMP70) (13, 14) and the PMP70-related protein (P70R) (15, 16). Today, it isn't obvious, whether ALDP dimerises as a homodimer, or forms a heterodimer with one specific heterodimerisation partner, or forms different heterodimers in different tissue-specific cell types in accordance with the availability of individual peroxisomal-half ABC transporters. The four peroxisomal ABC transporters show remarkably distinct expression patterns among different cell types. Natural substrate is established for none of the four peroxisomal ABC transporters. But there is an assumption that ALDP and PMP70 transport substrates only in one direction from the cytoplasm to the peroxisomal lumen using the energy from ATP hydrolysis. Accumulation of saturated, unbranched, very long chain fatty acids (VLCFA – fatty acids with the number of carbons more than 22) in all tissues (peripheral nervous system, central nervous system, adrenal glands) and body fluids is the only biochemical alteration known to be present in all clinical variants of X-ALD, including presymptomatic individuals (8). The link between the loss of the ability to transport a substrate from the cytoplasm to the peroxisomal lumen, and the accumulation of VLCFA isn't clarified enough. But a "direct" (old) and also an "indirect" (new) hypothesis exists for the molecular mechanism of disease. The "direct" hypothesis generally assumed that the primary cause of elevated VLCFA is a decreased peroxisomal ß-oxidation associated with a reduced activity of the peroxisomal enzyme, very long chain acyl-CoA synthetase, which converts VLCFA to their CoA thioesters (17, 18). Though the link between the function of VLCFA-synthetase and ALDP is not established, it is hypothesised that ALDP transported either VLCFA, VLCFA-CoA, or even VLCFA-synthetase into the matrix of peroxisomes, or is needed to stabilise the VLCFA-synthetase (6). The "indirect" hypothesis assumes that tissue levels of VLCFA are not directly correlated with the rate of peroxisomal VLCFA ß-oxidation and suggests that ABCD1 may not participate directly in the degradation of VLCFA. An enhanced, unrestricted fatty acyl-chain elongation in fibroblasts of peroxisome biogenesis disorders has been observed (19, 20). It is obvious that VLCFA accumulation is necessary but not sufficient for a manifestation of different clinical phenotypes X-ALD (21).

One of the broad hypotheses for the pathogenesis of central nervous system lesions is that the VLCFA lead to myelin instability, followed by an inflammatory or immune mediated process that destroys myelin (22). The VLCFA fraction in the brain cholesterolesters and the adrenocortical cells increases from early 5 % to 20–60 %. By this, the ganglioside structure is being changed and is distinguished by the immune system as unfamiliar, therefore the immune response is pointed towards them with the start of demyelinisation's process. Myelin is supposed to be able to activate complement with the presence of antibody, and activated complement can cause lysis of other areas of myelin (22). An adrenal insufficiency is explained in a way that adrenal cells membranes loose their earlier solubility in their new composition. As a result, the answer of sensory receptors to ACTH stimulation is suppressed and the adrenocortical hormones become deficit. No signs of hormonal pathology of Addison disease type were present in the affected family.

Nowadays, X-ALD still belongs to the group of disorders which are therapeutically not well manageable. Therapeutic approaches can be divided into dietetic, pharmacotherapeutic, immunosuppressive, cell transplantations and gene therapies.

The best effect was reported after the transplantation of bone marrow and haematopoietic stem cells (HSCT), although they are accompanied by quite serious side effects and possible complications of the therapy. It's commonly recommended, that HSCT should be reserved for X-ALD patient with early but definite evidence of cerebral disease by brain MRI (23). Unlike HSCT, for most neurometabolic diseases in which replacement of the deficient enzyme is the cornerstone of the therapeutic approach,

the attainment of a new immune system post-HSCT appears to be the key feature in the success of HSCT in X-ALD (22).

Conclusions

According to the estimated frequency of X-ALD in the population and the real number of patients diagnosed with X-ALD, we assume that this is a rare disease in Slovakia and therefore often misdiagnosed. We present the phenotype of a new mutation in ABCD1 gene. The importance of adequate diagnostics is justified not only by a different therapeutic approach, but also with the need of prenatal diagnostics and the need of genetic counselling in the affected families. As demonstrated in our study, it is necessary to consider this diagnosis also in the adult age, e.g. within the differential diagnostics of spastic paraparesis.

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