X-linked adrenoleukodystrophy phenotype is independent of ABCD2 genotype

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ABSTRACT

Strikingly variable clinical phenotypes can be found in X-linked adrenoleukodystrophy (X-ALD) even with the same ABCD1 mutation. ABCD2 is the closest homolog to ABCD1. Since ABCD2 overexpression complements the loss of ABCD1 in vivo and in vitro, we have investigated the possible role of the ABCD2 gene locus as determinant of X-ALD phenotypes. Sequence and segregation analysis of the ABCD2 gene, in a large X-ALD family with different phenotypes disclosed that the identical ABCD2 alleles were inherited in brothers affected by mild (noncerebral) versus severe (childhood cerebral) X-ALD phenotypes. Moreover, two independent association studies of ABCD2 polymorphisms and clinical phenotypes showed an even allele distribution in different X-ALD phenotypes and controls. Based on these findings ABCD2 can be excluded as a major modifier locus for clinical diversity in X-ALD. These findings are of particular importance for the attempt of pharmacological induction of ABCD2 as a possible therapeutic approach in X-ALD.

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X-linked adrenoleukodystrophy (X-ALD; MIM #300100) is a neurodegenerative metabolic disease characterized by the elevation of saturated, unbranched very long chain fatty acids (VLCFAs; ≥C22:0) in tissue and plasma likely due to a combination of increased elongation and impaired β-oxidation of VLCFA [1,2]. A wide spectrum of clinical phenotypes ranging from very mild late onset adrenomyeloneuropathy to childhood rapidly progressive demyelinating disease can be observed even within a single family. It has been speculated that in addition to the obligate defect of the ABCD1 gene (coding for the adrenoleukodystrophy protein ALDP) both, environmental factors and genetic modifiers, may contribute to this phenomenon [3,4]. ABCD2 (coding for the adrenoleukodystrophy-related protein; ALDRP) is the closest homologue of ABCD1 [5,6]. The genomic structure of human [7] and mouse [8] ABCD2 revealed a striking similarity to ABCD1, suggesting a recent duplication event of a common ancestor. The gene products ALDP and ALDRP belong to the family of peroxisomal ATP-binding cassette (ABC) half-transporters. Homo- as well as heterodimerization has been reported to occur between ALDP and ALDRP [9]. The expression pattern of mouse and human ABCD2 was found to be distinct from that of ABCD1 [5]. Cell lines or tissues expressing high levels of ABCD1 expressed no or low levels of ABCD2 and vice versa [10–12]. This finding was interpreted as an indication that ALDP and ALDRP are not obligatory partners but might rather fulfill similar metabolic functions in different tissues. It was indeed shown, that the impaired VLCFA β-oxidation in ALDP-defective fibroblasts could be corrected by expression of transfected ABCD2 [13]. Moreover, transgenic overexpression of Abcd2 in Abcd1-deficient mice prevents both VLCFAs accumulation and the neurodegenerative features, whereas double mutants for ABCD1 and ABCD2 exhibit an earlier onset and more severe disease when compared with Abcd1 single mutants [14]. These results provide direct evidence
for functional redundancy or overlap between both transporters in vivo and in vitro. These observations have substantiated the concept of a new therapeutic strategy in X-ALD through drug-induced transcriptional upregulation of the “surrogate” gene ABCD2 [15]. Furthermore, due to the functional similarity of ALDP and ALDRP, ABCD2 appeared to be a good candidate for being a modifier gene in X-ALD, which may account for the heterogeneity of clinical phenotypes. To test this hypothesis, we performed sequencing and segregation analyses of ABCD2 within a large X-ALD family, affected by different X-ALD phenotypes but carrying the identical mutated ABCD1 allele (P484R) [16]. Additionally, we have used an ABCD2 polymorphisms for association studies of mild versus severe X-ALD phenotypes.

Patients, material and methods

Patients. The pedigree of the family is shown in Fig. 1. In all hemizygote brothers and the female carrier, the same (P484R) and no other mutation has been identified in the ABCD1 gene [16]. Two boys (II-4 and II-7), who suffered from childhood cerebral X-ALD, died at the age of thirteen and nine years, respectively. Three male patients (II-1, adrenomyeloneuropathy; II-3, adolescent onset cerebral X-ALD; and II-9, Addison only at the age of 20) and the female carrier (I-2) showed increased VLCFA levels in fibroblasts, plasma, and leukocytes as compared with controls. Normal values were detected in all other female (II-2) and male (I-1, II-5, II-6 and II-10) members of the family [16]. Clinical, pathological, ultrastructural, and neurochemical findings in this family had been described previously [17,18].

Two independent association studies (rs11172566 and rs1172661) were performed enrolling 45 unrelated X-ALD patients (17 childhood cerebral X-ALD; 28 noncerebral X-ALD at any age) and 147 controls in the first study and 72 X-ALD patients and 200 controls in the second. In the latter study, the analysis was focused on two extreme phenotypes: childhood cerebral X-ALD with an onset before the age of 12 (n=44) and “pure” AMN (n=28) where MRI has been performed after the age of 45 years proving the absence of inflammatory demyelination.

Sequence analysis of the coding and promoter region of the human ABCD2 gene. Analyses of patients were performed from genomic DNA isolated from fibroblasts, blood leukocytes, or skeletal muscle. Single exons of the ABCD2 gene or fragments of the putative promoter region (2903 bp) were PCR-amplified with Taq DNA polymerase (Roche). The PCR products were column-purified using a Qiagroup PCR Purification Kit (Qiagen). Cycle sequencing was performed with a rhodamine dideoxy dye terminator kit (ABI/Perkin-Elmer) using amplification primers and internal primers as given in Table 1. Samples were then separated on an ABI 377 sequencer.

Segregation analysis. Genomic DNA was amplified with marker-specific primers (Table 2) using Taq DNA polymerase (Roche). The fragments were labeled with rhodamine[R110] 2’-deoxyctydine 5’-triphosphate (ABI/Perkin-Elmer), which was added at a final concentration of 1 μM to the PCR-reaction. Fragment-lengths were determined with the GeneScan 672 Software using a GeneScan-350 [ROX] standard (ABI/Perkin-Elmer).

Association studies of ABCD2 and clinical forms of X-ALD. The SNP rs11172566 is located in the 3'-untranslated region of the ABCD2 mRNA at position 3165 (Ref. sequence NM005164). The A to G polymorphism creates an HinfI restriction site. Using PCR primers Oli283 and Oli284, an 880 bp fragment of the 3'-untranslated region of ABCD2 (Table 1) is amplified. Genomic DNA was used as template, and PCR fragments were subsequently digested with HinfI (Roche) restriction endonuclease leading to 5 fragments of 32, 37, 60, 317, and 434 bp in case of c.2614A, and to 4 fragments of 32, 37, 60, and 751 bp in case of c.3165G, respectively, (Fig. 2).

In a separate set of patients the SNP rs11172661, a A to G transition, located in the intron 9 of the ABCD2 gene (position 2110440 of the Ref. contic sequence NT029419) has been investigated using allele specific sense primers Oli1125 (specific for A) or Oli1126 (specific for G), and antisense primer Oli1127. A 193 bp fragment was amplified in separate reactions. Each set of allele-specific primers was mixed with a pair of primers Oli11 and Oli12 amplifying a 774-bp fragment from the ASA gene (Arylsulfatase A) as a PCR control (Fig. 3).

Results

Comparative analyses of ABCD2 DNA sequence in brothers affected by different X-ALD phenotypes

To elucidate the hypothesized role of ABCD2 in X-ALD, we took advantage of a large X-ALD kindred: Five affected brothers and four unaffected siblings had been described previously [17,18]. The markers close to the ABCD2 gene are listed in Table 3. A1, A2: maternal alleles; a3, a4: paternal alleles. AMN: adrenomyeloneuropathy; AdoCALD: adolescent onset cerebral ALD; CCALD: childhood cerebral ALD; ADD: Addison only.
presenting with four different X-ALD phenotypes, ranging from severe cerebral demyelination (II-3, II-4, II-7) to mild noncerebral forms (II-1, II-9) of X-ALD (Fig. 1). To study the influence of the \(ABCD2\) gene on the phenotypic variability within this family, we performed \(ABCD2\)-mutation analysis. By sequencing all coding exons and exon-flanking regions of patients II-1, II-3, II-4, and II-9, we could not reveal any difference to the wild type sequence of \(ABCD2\).

### Table 1
**ABCD2 PCR and sequencing primers**

<table>
<thead>
<tr>
<th>Exon</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ia AGACTATGGGAGCCTGATGGAC</td>
<td>lb TCTGGCACTGCGACTGGCAAATTG</td>
<td>1111</td>
</tr>
<tr>
<td>2</td>
<td>Ila AGATGCAATGATAATGCCATAC</td>
<td>lbb CATAAACTCACTAGCAACTCTC</td>
<td>346</td>
</tr>
<tr>
<td>3</td>
<td>IIIa AAAAAACTGATTGTGTTTGT</td>
<td>IIVb GTAATTACGTGATGATTAAGTG</td>
<td>234</td>
</tr>
<tr>
<td>4</td>
<td>IVa AGTCGACAGGCTTCTGAATACTG</td>
<td>IVb GTGACTTACTAAAGACTCTTC</td>
<td>345</td>
</tr>
<tr>
<td>5</td>
<td>Va TTAATCGAGATTTGTAGTTTGT</td>
<td>Vb GTATTGTCGTGATGCAACACTC</td>
<td>196</td>
</tr>
<tr>
<td>6</td>
<td>Via2 TGCCCAAACCTAGTTTGTATG</td>
<td>Viiib AAGTCTTATGCTTCTGAGAG</td>
<td>456</td>
</tr>
<tr>
<td>7</td>
<td>Viii TGTGCTGAGTTGATGATACTG</td>
<td>Vllib TTTGCTTAAACAACTGGCAC</td>
<td>286</td>
</tr>
<tr>
<td>8</td>
<td>IXa TGGATCTAAGTACCACTACTATG</td>
<td>IXb GTGACCATAAAGAAATATG</td>
<td>345</td>
</tr>
<tr>
<td>9</td>
<td>Xa GGATGCAATGATAATGCCATAC</td>
<td>37r CTTAGCTTAACATCTTACG</td>
<td>391</td>
</tr>
</tbody>
</table>

### Table 2
**Markers close to the \(ABCD2\) gene**

<table>
<thead>
<tr>
<th>No.</th>
<th>Marker</th>
<th>Alias</th>
<th>Forward primer (5' → 3')</th>
<th>Reverse primer (5' → 3')</th>
<th>Repeat type</th>
</tr>
</thead>
<tbody>
<tr>
<td>97</td>
<td>D12S331</td>
<td>AFM092wd11</td>
<td>TAAACTTTATTCATCTCCATAGA</td>
<td>ACATGTAAGAGAGACTCTCAGA</td>
<td>CA</td>
</tr>
<tr>
<td>99</td>
<td>D12S1029</td>
<td>CHLC.GATA47G02</td>
<td>ATACTCACTAGTTGAAAGACTGAGA</td>
<td>ATTTACCAAAGAAACTCCCTTG</td>
<td>Tetratetraoid</td>
</tr>
<tr>
<td>102</td>
<td>D12S1048</td>
<td>CHLC.ATA29H01</td>
<td>GGTCTGCTAGGTCCCTTTT</td>
<td>AAGGAACACTCCCAATCTG</td>
<td>Trinucleotide</td>
</tr>
<tr>
<td>103</td>
<td>AFMA106VD5</td>
<td>CAAATCTGACCTGAACTGACG</td>
<td>TTATCTTTTATTGATATCCTGAG</td>
<td>CA</td>
<td></td>
</tr>
<tr>
<td>104</td>
<td>D12S315</td>
<td>AFM210WF6</td>
<td>AAATGCAATGATGATGATGATG</td>
<td>TGGACGAAGGAGAGGAGGCCAG</td>
<td>CA</td>
</tr>
</tbody>
</table>

![Fig. 2](image-url) **Fig. 2.** Restriction endonuclease assay of polymorphism rs11172566 (A → G) within the 3'-UTR of the \(ABCD2\) gene. 1, 3: undigested PCR product (880 bp); 2: Hinfl digestion; The A-allele leads to products of 32, 37, 60, 434 and 317 bp; The G-allele leads to products of 32, 37, 60 and 751 bp; 2: Heterozygosity for A/G; 4: Homozygosity for the A allele. St, nucleic acid standard.

![Fig. 3](image-url) **Fig. 3.** Allele specific PCR assay of polymorphism rs11172661 in intron 9. Separate PCR reactions are performed using A (left side) or G (right side) specific primers respectively. The genotype of the numbers 1–3 represent three patients with the genotype indicated at the bottom of the gel. The size of the 774 bp ASA control fragment and the 193 bp \(ABCD2\) allelespecific fragment are indicated at the right margin.
boundaries of the ABCD2 gene, we sequenced a 2903 bp region upstream the translational start codon representing the putative promoter region in a severe cerebral phenotype (II-4) and a noncerebral mild phenotype (II-9). This region includes a 1.3 kb segment that has been reported to exhibit functional promoter activity and which is sufficient to stimulate transcription of a reporter gene by 9-cis-retinoic acid and forskolin [19]. Moreover, a functional regulatory element has been identified within this region consisting of a sterol regulatory element (SRE), which overlaps with a direct repeat separated by 4 nucleotides (DR-4). The DR-4 serves as binding motif for nuclear receptors suggesting cross-talk between different transcription factors [20–22]. No sequence difference was observed, neither in the regulatory element, nor in any other position within the 2903 bp region of the promoter investigated when compared to the control ABCD2 sequence.

**ABCD2 segregation analysis in a X-ALD family with differently affected children**

Sequencing the ABCD2 coding region and the putative promoter region did not reveal any difference in brothers affected by different clinical phenotypes. Since we could not exclude the possibility of cryptic mutations (e.g., mutations in intronic regions), segregation analysis was performed. We used the microsatellite markers D12S331, D12S1029, D12S1048, AFMA106VD5 and D12S315 (Table 2) comprising a genomic region of about 3133 kb (NCBI Map Viewer; www.ncbi.nlm.nih.gov/mapview) close to ABCD2. Segregation analysis disclosed that the patient presenting the (mild) Addison-only-phenotype at age of 20 (II-9, Fig. 1) had identical ABCD2 alleles (A1a4) compared to his brother who had died from cerebral X-ALD at the age of 13 years (II-3, Fig. 1). Thus, no correlation between a specific X-ALD phenotype and a characteristic maternal A1 allele as compared to the A1 alleles of all brothers form cerebral X-ALD at the age of 13 years (II-3,Fig. 1). Thus, no sequence difference was observed, neither in the regulatory element, nor in any other position within the 2903 bp region of the promoter investigated when compared to the control ABCD2 sequence.

**Association study of ABCD2 SNPs and clinical phenotypes in X-ALD**

In two independent studies the SNPs rs11172566 and rs11172661 were investigated in unrelated X-ALD patients and controls (Table 3). Both analyses revealed no evidence of the polymorphisms to be associated with X-ALD in general, nor with a severe (cerebral) or mild (noncerebral) phenotype, not even in the second study analyzing well characterized “extreme” phenotypes.

**Discussion**

The unpredictable variablility of clinical courses of X-ALD has been a matter of speculation for a long time. There is no general correlation between the type of ABCD1 gene mutation and the clinical phenotype. Although it cannot be excluded that residual ALDP transport activities (over the peroxisomal membrane) in individual ALDP–mutations, might prevent the development of the inflammatory cerebral form in X-ALD this would only explain the clinical manifestation in a small set of patients. Strikingly different clinical phenotypes can occur (i) in one nuclear family; (ii) in patients affected by mutations that lead to a complete absence of ALDP (e.g., large deletions); (iii) in unrelated families with the identical ABCD1 mutation (e.g. 1801delAG); (iv) in monzygotic twins [3]. These findings in addition to genome wide association studies predict genetic and environmental factors to influence phenotypic heterogeneity in X-ALD.

Several candidate genes have been suggested as potential modifiers: peroxisomal ABC-half transporters related to ABCD1 (ABCD2, ABCD3); Very long chain fatty acid elongation (ELOVL1-6 [23]) and activation system (ACSVL1 and BGI [24,25]); genes related to initiation or maintenance of inflammation (CD1A-E, HLA-locus, TNFz, MOG [26–30]) and cystathionine beta-synthase involved in the methionine metabolism [31,32]. It is tempting to speculate that more than one genetic variation might modulate the X-ALD phenotype.

We had hypothesized that the ABCD2 gene could be such a modifier. There was good evidence since ABCD2 is able to compensate for the loss of ABCD1 in vivo and in vitro. However, our combined data of sequence analysis, segregation analysis, and polymorphism association studies may be regarded as evidence against the hypothesis that ABCD2 is a modifier gene contributing to the clinical heterogeneity in X-ALD. Nevertheless, a genetically distant locus that influences ABCD2 expression and thus contributes to clinical variations was not excluded by our methodology. Regulation of ABCD2 transcription is recognized to be exceptionally complex and can be modulated by a variety of nuclear receptors. However, in a recent study it was demonstrated that the expression levels of ABCD2 in the normal–appearing white matter of brains derived from patients displaying different X-ALD phenotypes were similar and did not differ significantly from the levels observed in age-matched controls [25]. Therefore, the existence of a distant locus that influences ABCD2 expression is unlikely in this sample. Likewise, epigenetic factors, such as methylation might influence the expression of ABCD2 in X-ALD patients and cannot be excluded by our methodology. The promoter of the ABCD2 gene, however, does not contain a high content of GC-sites (40%). Together with the observation of similar expression levels of ABCD2 in brain tissue of X-ALD patients and controls, this renders differences in methylation to occur unlikely.

As ABCD2 can compensate ABCD1 deficiency, major attempts have been undertaken to get insights on the complex regulation of ABCD2 gene expression with the final aim to pharmacologically induce the ABCD2 gene expression in cell types where the loss of ABCD1 contributes to the progression of the disease in order to ameliorate the severity of the disease. The reported data may not be regarded as evidence for ABCD2 to be a modifier gene in X-ALD, but since a functional ABCD2 gene is prerequisite for therapeutic interventions, the finding of an intact ABCD2 gene in all phenotypes of X-ALD sets an additional basis toward the aim of pharmacological surrogate gene therapy in X-ALD.

**Table 3**

<table>
<thead>
<tr>
<th>rs11172566</th>
<th>Patients (n)</th>
<th>AA (%)</th>
<th>AG (%)</th>
<th>GG (%)</th>
<th>Alleles (n)</th>
<th>A (%)</th>
<th>G (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ccALD</td>
<td>17</td>
<td>15 (88)</td>
<td>2 (12)</td>
<td>0</td>
<td>34</td>
<td>32 (94)</td>
<td>2 (6)</td>
</tr>
<tr>
<td>X-ALD non cerebral</td>
<td>28</td>
<td>25 (89)</td>
<td>3 (12)</td>
<td>0</td>
<td>56</td>
<td>53 (95)</td>
<td>3 (5)</td>
</tr>
<tr>
<td>Normal population</td>
<td>147</td>
<td>131 (89)</td>
<td>15 (10)</td>
<td>1 (&lt;1)</td>
<td>294</td>
<td>277 (94)</td>
<td>17 (6)</td>
</tr>
<tr>
<td>rs11172661</td>
<td>Patients (n)</td>
<td>GG (%)</td>
<td>AG (%)</td>
<td>AA (%)</td>
<td>Alleles (n)</td>
<td>G (%)</td>
<td>A (%)</td>
</tr>
<tr>
<td>ccALD</td>
<td>44</td>
<td>26 (59)</td>
<td>15 (34)</td>
<td>3 (7)</td>
<td>88</td>
<td>67 (76)</td>
<td>21 (24)</td>
</tr>
<tr>
<td>“pure” AMN</td>
<td>28</td>
<td>21 (75)</td>
<td>6 (21)</td>
<td>1 (4)</td>
<td>56</td>
<td>48 (86)</td>
<td>8 (14)</td>
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<tr>
<td>Normal population</td>
<td>200</td>
<td>131 (66)</td>
<td>62 (31)</td>
<td>7 (4)</td>
<td>400</td>
<td>324 (81)</td>
<td>76 (19)</td>
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</tbody>
</table>
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References


