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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; $\geqslant$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

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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, neuropathological, 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 Qiaquick PCR Purification Kit (Qiagen). Cycle sequencing was performed with a rhodamine didesoxy 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′-deoxycytidine 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 *Hinf*I 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 *Hinf*I (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

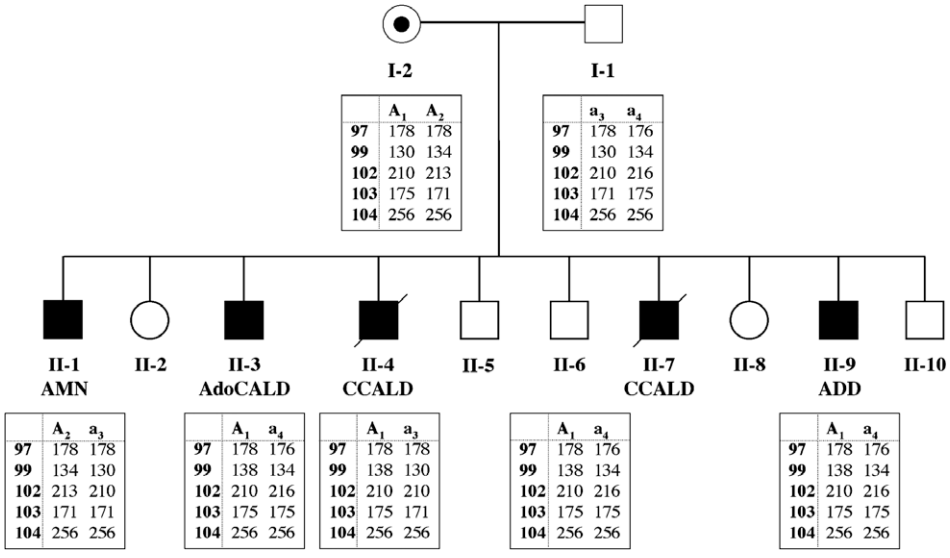

Fig. 1. Pedigree of a large X-ALD family and segregation analysis of the *ABCD2* gene. In all affected brothers and the female carrier, the same mutation (P484R) and no other mutation has been identified in the *ABCD1* gene. Two brothers with CCALD died at the age of thirteen and nine years, respectively (diagonal slash). The markers close to the *ABCD2* gene are listened 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.

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