Molecular biology and pharmacogenetics of x-linked adrenoleukodystrophy
Kemp, S.

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Summary and conclusions
X-linked adrenoleukodystrophy (X-ALD) is the most common peroxisomal disorder. Biochemically it is characterized by increased plasma and tissue levels of saturated very long-chain fatty acids (VLCFA: \( C > 22 \)). The clinical manifestations of X-ALD are highly variable with respect to age of onset, the organs principally affected and rate of progression. The X-ALD phenotypes range from the rapidly progressive childhood cerebral (CCALD) form, which often leads to severe disability and death before the age of 10, to the milder and slowly progressive adrenomyeloneuropathy (AMN). Most frequently AMN manifests between 20 and 40 years of age and may be compatible with survival into the eighth decade. Some patients have adrenocortical insufficiency without demonstrable nervous system involvement ("Addison-only" phenotype). In many X-ALD kindreds these different phenotypes are represented and often they co-occur in the same nuclear family.

VLCFAs are degraded exclusively in peroxisomes through \( \beta \)-oxidation. Prior to their degradation they must be activated to their CoA thioesters, a process that is performed by the peroxisomal enzyme very long-chain fatty acyl-CoA synthetase (VLCS). As VLCS activity is strongly reduced in X-ALD this gene was the most likely candidate for X-ALD. The \( ALD \) gene was identified in 1993 using positional cloning strategies. Surprisingly, the gene product referred to as adrenoleukodystrophy protein (ALDP), appeared to be is a peroxisomal membrane protein and a member of the ATP-binding cassette (ABC) superfamily of transporter proteins. ALDP has the topology of a half-transporter protein and it is hypothesized that it has to form a dimer with itself or with another peroxisomal ABC half-transporter in order to form a functional ABC transporter. ALDP has no homology to VLCS.

The identification of the \( ALD \) gene opened the possibility for molecular biology to attempt to unravel the many questions surrounding X-ALD. The \( ALD \) gene is located on Xq28; it consists of 10 exons and encodes a protein of 745 amino acids with a molecular mass of 70 kDa.

In Chapter 1 the history, clinical manifestations and pathogenesis of X-ALD is described. Additionally, the X-ALD mouse model and past, present and future therapies are addressed.

In Chapter 2 a mutation (1415delAG) that is present in the fifth exon of the \( ALD \) gene is described. This mutation was found in five out of 40 (12%) Dutch X-ALD kindreds. Haplotype analysis excluded a founder effect; the kindreds were genetically unrelated. This mutation results in a frame shift and a premature stop codon upstream of the ATP-binding domain. The mutant \( ALD \) protein can not be
functional because the ATP-binding domain is absent. Interestingly, this mutation was found both in patients with CCALD and AMN. This was the first demonstration at the molecular level that identical mutations in the *ALD* gene result in different phenotypes.

In Chapter 3 the first systematic analysis of mutations in the *ALD* gene is reported. Mutations were detected in all 28 unrelated Dutch X-ALD kindreds investigated. These data confirmed that the gene identified as the *ALD* gene is the only gene responsible for X-ALD. *ALD* mRNA was detected in all patients, indicating that none of the patients had complete gene deletions. The systematic approach provided the opportunity to study the association between genotype and phenotype. A correlation between genotype and phenotype does not exist. In addition to the findings described in Chapter 2, CCALD appeared to be associated with ‘mild’ mutations involving a single amino acid change (missense mutation) as well as more severe mutations, like nonsense mutations and frame shifts.

The observations of Chapters 2 and 3 strongly support the hypothesis that a modifier gene is involved in modulating the clinical expression of X-ALD. Furthermore, the systematic approach used provided a strategy for efficient screening of mutations in the *ALD* gene. In particular regions of the *ALD* gene mutations were found more frequent than in other regions. For example, the region in exon 1 that encodes the putative transmembrane domains 2 to 4, exon 5, and the exons encoding the ATP-binding cassette.

The consequence of different mutations on the stability of ALDP was investigated in Chapter 4. ALDP expression in fibroblasts from 24 male X-ALD patients representing 17 unrelated Dutch X-ALD kindreds was studied. Two methods for detection of ALDP were used: immunofluorescence (IMF) and immunoblotting (Western blotting). IMF revealed a punctate peroxisomal staining pattern in only five (29%) kindreds. The majority (71%) had no detectable ALDP both by IMF and Western blot analysis. All mutations other than missense mutations resulted in the absence of detectable protein. This study showed that besides the absence of a genotype-phenotype correlation there is also no correlation between ALDP expression and the different phenotypes. The discovery that 70% of X-ALD kindreds have no detectable level of ALDP is also an important aid to diagnosis. VLCFA analysis provides a reliable diagnostic tool for prenatal and postnatal diagnosis of affected males. In contrast, approximately 15% of obligate carriers have normal VLCFA levels. If it is demonstrated that a mutation in a particular X-ALD kindred results in non-detectable ALDP, IMF analysis of fibroblasts may
facilitate the identification of female carriers in that kindred. Furthermore, early identification of male fetuses will be possible as IMF and protein blotting can be performed in chorionic villus biopsy material. These relatively easy and sensitive techniques can be used to diagnose X-ALD.

In Chapter 5 all known X-ALD mutations (over 200) are categorized and analyzed. In the X-ALD literature two different nucleotide numbering systems are used by different groups. One method uses the variant introduced by Mosser et al. (Nature 361:726-730, 1993) where nucleotide numbering starts at the first nucleotide of the ALD cDNA; the adenosine of the ATG (methionine) initiator codon is nucleotide number 387. The second, more common variant, starts counting at the start of the open-reading frame and designates the adenosine of the ATG as nucleotide number +1. In this chapter a table is included in which all mutations are categorized according to both numbering systems. Extensive analyses of these 211 X-ALD mutations revealed the absolute absence of genotype-phenotype correlation. Of all mutations, missense mutations are most frequent (52.6%), followed by frame shifts (24.2%), nonsense (8.5%), large deletions involving one or more exons (6.2%), in-frame amino acid insertion or deletions (5.7%) and mutations involving splice sites (2.8%). Most X-ALD kindreds (70%) have unique or ‘private’ mutations.

Missense mutations, which affect only a single amino acid, are most informative for the identification of functional domains within the protein. Ninety percent of the missense mutations are found in only three regions: 1) a region that might function in the homo- or heterodimerization of ALDP, the binding of ALDP to VLCS, or in substrate recognition by ALDP, 2) a region of unknown function that is evolutionary conserved among ABC transporters, even in prokaryotes, and 3) the ATP-binding domain.

Mutation analysis in X-ALD is hampered by the existence of autosomal pseudogenes (Chapter 3). Each of these pseudogenes contains approximately 10 kb of DNA that has a 92-96% nucleotide identity with the exons 7-10 of the ALD gene. Data presented in Chapter 5 showed that a genomic PCR with exon 7 specific primers yielded a single DNA fragment. Further analysis, however, revealed that this PCR amplicon was not pure, it contained DNA derived from several of the pseudogenes. If mutation analysis is to be performed on genomic DNA, ALD gene specific PCR primers for this region must be designed (Chapter 3). The autosomal pseudogenes have originated fairly recent in evolution; they do not exist in mouse and rhesus monkey; the first presence of pseudogenes is observed in Orangutan.
Besides ALDP three additional peroxisomal ABC half-transporters have been identified: ALDRP, PMP70 and PMP69 (P70R). By comparison to known ABC half-transporter proteins, it has been suggested that ALDP may function as a homodimer and/or as a heterodimer with one or more of these peroxisomal ABC half-transporters. In Chapter 5 it was demonstrated that, at least in vitro, ALDP forms homodimers and heterodimers with both PMP70 and ALDRP. PMP69 was not tested. It was also demonstrated that ALDP, PMP70 and ALDRP have related and/or overlapping functions. It had already been demonstrated that overexpression of ALD cDNA in fibroblasts derived from X-ALD patients corrected the biochemical defect. The functional redundancy of the peroxisomal half-transporters was demonstrated by restoration of the biochemical defect in X-ALD cell lines upon overexpression of either ALDR or PMP70 cDNA. While the precise role of ALDP in peroxisome metabolism is unknown, its identification as a member of the ABC transporter superfamily suggests that it might transport VLCFAs, activated VLCFAs, CoA, ATP or other required metabolites into the peroxisome. It may also be possible that ALDP functions as an anchor for VLCS in the peroxisomal membrane and that association and dissociation of ALDP as a homodimer or heterodimer in a cell type specific manner might reflect different or changing metabolic states.

Genetic segregation analyses support the hypothesis that at least one autosomal gene plays a role in the phenotypic variation observed in X-ALD. The peroxisomal half-transporters PMP70 and ALDRP are candidates for such modifier effects. A third potential modifier candidate was introduced in Chapter 5. Mouse fibroblasts have no detectable VLCS protein or VLCS mRNA, however, they actively degrade VLCFAs, a reaction that requires VLCS. Fibroblasts generated from the X-ALD knock out mouse have reduced VLCFA β-oxidation and increased VLCFA levels. These latter data indicate that a yet unidentified ALDP dependent VLCS exists. Surprisingly, overexpression of VLCS cDNA in mouse X-ALD fibroblasts corrected VLCFA β-oxidation to the same extent as overexpression of either ALD, ALDR, or PMP70 cDNA did. The VLCS activity appears to be ALDP independent and may contribute to the tissue variability of VLCFA β-oxidation and VLCFA levels.

In Chapter 6 a potential pharmacological gene therapy approach for X-ALD is described. Cell lines from X-ALD patients and X-ALD knockout mice were treated with 4-phenylbutyrate (4PBA). This resulted in decreased VLCFA levels and increased VLCFA β-oxidation. The mechanism of action does not involve ALDP but increased expression of the functionally related peroxisomal protein ALDRP. It
was also demonstrated, for the first time in human cells, that 4PBA induced peroxisome proliferation. X-ALD cells lacking ALDP have residual activity for β-oxidation; therefore, an increase in peroxisome numbers could also contribute to increased VLCFA metabolism. A significant decrease of VLCFAs in the brain and adrenal glands of X-ALD mice demonstrated the *in vivo* efficacy of dietary 4PBA. The demonstration that 4PBA lowers brain VLCFA levels suggests that this therapy may ameliorate or prevent the onset of cerebral inflammatory demyelination found in several of the X-ALD phenotypes.

Hopefully, this thesis will contribute to the unveiling of the function of ALDP, the identification of the modifier gene, and easier and more efficient screening of potentially affected persons. After all, X-ALD is a devastating disease, and new cases can be prevented.

A co-operation between the Kennedy Krieger Institute in Baltimore and the Academic Medical Center in Amsterdam has resulted in the initiation of an international database for X-ALD mutations. This database will be accessible on the internet.

Treatment with 4PBA has shown its potential in the X-ALD mouse and fibroblasts obtained from X-ALD patients. Already, a pilot study in a small group of affected male patients has been initiated, and the first results will become available soon. Nevertheless, further studies are warranted to determine whether 4PBA will be a future therapeutic option for X-ALD.