Genotype and Protein Expression After Bone Marrow Transplantation for Adrenoleukodystrophy

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Background: X-linked adrenoleukodystrophy (X-ALD) is the most common inherited peroxisomal disorder. It is caused by impaired function of ALD protein that results in accumulation of very long-chain fatty acids in tissues and body fluids. So far, hematopoietic stem cell transplantation (HSCT) constitutes the only curative approach able to prevent the progression of cerebral X-ALD. However, biological mechanisms of this beneficial approach are still unknown.

Objective: To describe the effect of HSCT in a family with various X-ALD disease forms by ALD mutation and protein expression analysis.

Design, Setting, and Patients: In a family with various X-ALD forms, an ALD mutation screening was performed. Two boys had cerebral X-ALD and underwent HSCT. One of them is alive and well without any further neurological deterioration, whereas his cousin died of transplantation-related complications at day 76. The postmortem specimens were analyzed by genotyping and immunohistochemical assays.

Results: All of the affected family members carry an as-yet-undescribed large ALD gene deletion (NC_000023:g.152512130-152520645del) resulting in a complete lack of ALD protein expression on immunofluorescence analysis. After engraftment, both patients who underwent HSCT showed complete chimerism in blood. Postmortem studies in 1 patient revealed both mutant and wild-type ALD sequences in each of 23 analyzed tissues, indicating mixed chimerism. Furthermore, immunohistochemical staining for ALD protein revealed no differences between patient and control tissues including blood cells, bone marrow, and glial cells as well as neurons.

Conclusion: To our knowledge, our analysis provides the first evidence for the stable development of a wild-type X-ALD genotype and peroxisomal ALD protein expression in a great variety of human tissues following HSCT.

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is located at chromosome Xq28 and contains 10 exons spanning approximately 21 kilobases (kb). It encodes for ALD protein (ALDP) (also named ABCD1), which is a member of the adenosine triphosphate–binding cassette transport protein family (ABC transporters). Adrenoleukodystrophy protein is most likely involved in the transport of fatty acyl coenzyme A substrates or their cofactors into peroxisomes. If ALDP function is impaired, VLCFAs accumulate in body fluids and tissues. The consequence of increased VLCFAs on cerebral and adrenal dysfunction is still poorly understood.

In more than 250 affected families, various ALD gene mutations have been identified, involving all functional ALDP domains with a clustering around the nucleotide-binding fold. Approximately 75% of these mutations result in complete loss of ALDP expression. Notably, there is no apparent correlation between genotype and clinical phenotype even within the same family. Therefore, knowing the genotype cannot predict the outcome of individual patients. However, it is helpful to identify female carriers who have normal VLCFA plasma concentrations.

To date, allogeneic hematopoietic stem cell transplantation (HSCT) constitutes the only curative therapeutic approach. Standard sources of hematopoietic stem cells are bone marrow as well as peripheral blood after mobilization of hematopoietic stem cells with granulocyte colony-stimulating factor. As an alternative stem cell source, cord blood is used more frequently, showing a comparable outcome of HSCT. Nevertheless, biological mechanisms of HSCT at the cellular level have yet to be elucidated. Owing to considerable toxicity, HSCT is restricted to patients with cerebral X-ALD at early disease stages. In an international report on 126 patients with cerebral X-ALD treated with HSCT, the 5-year survival rate was 56%. Notably, a significant difference in the 5-year survival rate was noticed between patients who underwent transplantation at an early stage of disease (92%) and those who underwent transplantation later (45%). In our study, we describe the clinical and genetic data of a family with several affected individuals and different clinical forms of X-ALD. The family has a previously undescribed large interstitial ALD gene deletion resulting in loss of ALDP expression. One family member with cerebral X-ALD died after HSCT owing to transplantation-related complications. We were able to do a postmortem analysis of gene and protein expression in various tissues to study the therapeutic impact of HSCT at the cellular level.

**METHODS**

**PATIENTS AND MATERIAL**

The study includes a family with X-ALD with 6 female carriers and 5 affected males (Figure 1). Molecular analysis of the ALD gene was able to be done in 6 family members (patients II:4, III:3, III:4, III:5, III:6, and IV-1) (Figure 1 and Table). Their clinical phenotypes are described in the Table. At the time of clinical manifestation, cerebral MRI of patient III:4 revealed a disease-specific extensive demyelination pattern restricted to occipital white matter with gadolinium enhancement. Owing to progressive and pronounced loss of neurological functions, a 6/6 HLA antigen–matched unrelated HSCT was performed. At day 49 after HSCT, a complete chimerism of bone marrow and peripheral blood was demonstrated, indicating that all of the bone marrow cells and blood leukocytes were derived from the donor. Unfortunately, patient III:4 developed severe acute graft-vs-host disease complicated by hemolytic uremic syndrome, and he died at day 76. At postmortem examination, 12 central nervous system (CNS) and 11 extraneural paraffin-embedded tissue samples were collected for mutation and protein expression analysis. Tissues from the CNS included the frontal lobe, right and left occipital lobe, central region, Ammon horn, mesencephalon, pons, basal ganglia, cerebellum, medulla oblongata, and thoracic and lumbar spinal cord. Extraneural tissues included bone marrow, adrenal gland, kidney, liver, spleen, large and small bowel, heart, lung, testis, and skin.
In contrast to patient III:4, his cousin (patient III:6) underwent unrelated HSCT soon after presenting the first neurological symptoms (Table). At this time, cerebral MRI showed mild symmetric demyelination in occipital lobes. Now after 4 years of follow-up, the patient does not show further progression of neurological symptoms or demyelination on cerebral MRI. He has complete chimerism in bone marrow and peripheral blood. For mutation analysis, blood samples before and after HSCT were available. In addition, his fibroblasts were able to be cultivated for protein expression analysis.

GENOTYPE ANALYSIS

The DNA from formalin-fixed tissue samples was extracted from 20- to 30-µm paraffin sections with the DNeasy Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Isolation of genomic DNA from blood leukocytes, amplification, and mutation screening were performed according to previously described protocols. The oligonucleotide primers, their annealing temperatures, and the length of amplification products are available from us (S.S.).

EXPRESSION ANALYSIS IN PARAFFIN-EMBEDDED TISSUE

Immunohistochemical detection of peroxisomal ALDP was performed on 3- to 5-µm tissue sections mounted onto coated microscope slides (Superfrost Plus; Menzel-Glaser, Braunschweig, Germany). Two different mouse monoclonal ALDP antibodies (Chemicon Corp, Temecula, Calif) were used. The antibody MAB2162 binds to the ALDP nucleotide-binding fold and MAB2164 to its N-terminal. Both antibodies were applied in a 1:20 000 dilution using the DAKO antibody diluent (DAKO Corp, Carpinteria, Calif) to reduce nonspecific background staining. In addition, 2 different primary antibodies were used as controls, namely synaptophysin (DAKO Corp, Carpinteria, Calif) and peroxisomal catalase (Europa Bioproducts Ltd, Cambridge, England). The Catalyzed Signal Amplification System (DAKO Corp) was selected as a very sensitive staining method. Slide preparation, incubation, and immunohistochemical detection were performed according to the manufacturer’s instructions.

EXPRESSION ANALYSIS IN FIBROBLASTS

Human fibroblasts were cultured using standard protocols. Immunofluorescence assays were done as previously described. Peroxisome-specific antibodies against ALDP or catalase were used in a dilution of 1:250 and a secondary antibody (goat-anti-mouse IgG or goat-anti-rabbit IgG; Dianova, Hamburg, Germany) in a dilution of 1:50.

Molecular analysis of the ALD gene within this family revealed that the C-terminal part of exon 1 (exon 1B + 1C) and exon 2 could not be amplified, indicating the presence of an internal deletion. Using oligonucleotide primers on both sides of the assumed deletion, a 2.8-kb fragment instead of the expected 11.3-kb wild-type fragment was amplified. Sequencing revealed the large internal deletion NC_000023:g.152512130-152520645del (Figure 2A), corresponding to amino acids 316 to 489 of ALDP. As a result, this family carries an as-yet-undescribed ALD gene deletion including part of exon 1, the complete intron 1 (>3 kb), exon 2, and a large portion of intron 2 (Figure 2B).

Immunofluorescent staining of cultured skin fibroblasts of patient III:6 showed a regular pattern of the peroxisomal marker enzyme catalase (Figure 2C) but absent signals for ALDP using 2 different ALDP antibodies, one against the deleted protein part (MAB2164, amino acids 279-482) and the other against a C-terminal non-deleted protein region (MAB2162, amino acids 495-648) (Figure 2D). Thus, the identified ALD gene deletion results in a complete loss of ALDP.

For genotype analysis of other family members as well as various tissue samples, we designed primers inside and outside the identified ALD gene deletion (Figure 2B). Polymerase chain reaction amplification with these primer pairs resulted in specific amplification of a 159-base pair (bp) fragment in the case of an intact ALD gene on at least 1 allele. In contrast, a 68-bp fragment rather than an 8.6-kb fragment was amplified if the described deletion was present. The results of these genotype analyses within the family are included in Figure 1. In the female patient II:4, both fragments could be amplified, indicating carrier status. In contrast, carrier status of her daughter (patient III:3) was excluded by the detection of a sole 159-bp fragment. In the male patients III:4, III:5, III:6, and IV:1, only the

Table. Clinical Characteristics of the Recruited Family With X-linked Adrenoleukodystrophy

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Sex</th>
<th>Present Age, y, mo</th>
<th>Age at Onset, y</th>
<th>Age at HSCT, y, mo</th>
<th>Clinical Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>II:4</td>
<td>F</td>
<td>49, 7</td>
<td>NA</td>
<td>NA</td>
<td>Asymptomatic</td>
</tr>
<tr>
<td>II:8</td>
<td>M</td>
<td>39, 0</td>
<td>Unknown</td>
<td>NA</td>
<td>Asymptomatic</td>
</tr>
<tr>
<td>III:3</td>
<td>F</td>
<td>29, 6</td>
<td>NA</td>
<td>NA</td>
<td>Asymptomatic</td>
</tr>
<tr>
<td>III:4</td>
<td>M</td>
<td>15, 4</td>
<td>13</td>
<td>15, 1</td>
<td>Asymptomatic</td>
</tr>
<tr>
<td>III:5</td>
<td>M</td>
<td>12, 3</td>
<td>6</td>
<td>NA</td>
<td>Addison disease, no neurological dysfunctions</td>
</tr>
<tr>
<td>III:6</td>
<td>M</td>
<td>8, 8</td>
<td>4</td>
<td>4, 2</td>
<td>Before HSCT: mild disturbance of auditory and visual perception; unrelated HSCT at early stage of disease, no further disease progression</td>
</tr>
<tr>
<td>IV:1</td>
<td>M</td>
<td>5, 6</td>
<td>NA</td>
<td>NA</td>
<td>Asymptomatic</td>
</tr>
</tbody>
</table>

Abbreviations: HSCT, hematopoietic stem cell transplantation; NA, not applicable; X-ALD, X-linked adrenoleukodystrophy.
68-bp fragment could be amplified, indicating the presence of the ALD gene deletion. After HSCT, blood samples from patient III:6 had the 159-bp fragment of the intact ALD gene amplified exclusively.

In contrast, genotype analysis of the 12 CNS and 11 extraneural postmortem tissues collected after HSCT resulted in amplification of both the 68-bp and 159-bp fragments, indicating mixed chimerism with the presence of a wild-type allele and a mutated allele (Figure 3). To confirm that HSCT also restores protein expression, we performed immunohistochemical analysis of paraffin-embedded tissues of patient III:4 with antibodies against the nondeleted (MAB2162) and deleted (MAB2164) regions of ALD. Because the subcellular localization of ALDP as a peroxisomal integral membrane protein may affect its accessibility to antibodies, antigen retrieval and a catalyzed signal amplification staining technique were applied. Notably, there was no difference between patient and control tissues regarding intensity and specificity of the staining pattern. In bone marrow, both antibodies showed a coarse-soiled cytoplasmatic staining of granulopoietic cells and megakaryocytes. The antibody MAB2164 as a peroxisomal integral membrane protein may indicate the presence of peroxisomal catalase staining (C) but absent adrenoleukodystrophy protein signals with antibody MAB2162 (D). Thus, the identified ALD gene deletion results in the loss of adrenoleukodystrophy protein.

**Figure 2.** Genotype and protein expression analyses. Comparison of the wild-type ALD sequence (ALD exon 1 and intron 2) with the mutant sequence of patient III:4 and his mother, patient II:4. The ALD gene is deleted at base pair (bp) 948 of the complementary DNA (NM_000033.2) in exon 1 and continues in intron 2 at the chromosome X position 152520646 (NC_000023) (A), revealing a large internal 8516-bp deletion (B). Immunofluorescent staining of cultured skin fibroblasts of patient III:6 indicates regular peroxisomal catalase staining (C) but absent adrenoleukodystrophy protein signals with antibody MAB2162 (D). Thus, the identified ALD gene deletion results in the loss of adrenoleukodystrophy protein.

**Figure 3.** Mutation analysis of paraffin-embedded tissues of patient III:4. In control tissues from healthy donors, only the wild-type ALD complementary DNA sequence could be amplified. In contrast, polymerase chain reaction amplification of bone marrow and adrenal gland tissue from patient III:4 yielded both a wild-type (68-base pair [bp]) and a mutant (159-bp) ALD complementary DNA sequence.
rons was confirmed by staining serial brain sections with synaptophysin as a neuronal marker (data not shown). The adrenal gland showed a distinct difference between adrenal medulla and cortex with a preferential staining of the cortex, especially the zona glomerulosa. In kidneys, punctuate staining of tubular epithelial tissue was detectable. These observations suggest that HSCT results in a widespread presence of intact, peroxisomally located donor ALDP.

**COMMENT**

Our genotype analysis in a family affected with X-ALD revealed a previously undescribed large internal deletion of the ALD gene in affected males and female carriers. Our observation supports the hypothesis of a profound genetic heterogeneity of X-ALD that is incompatible with a deduction from a founder family. Thus, almost every affected family bears its private mutation.6 Deletion of 1 or several exons are rare events that account for approximately 4% of ALD gene mutations and result mainly in a total loss of ALDP expression (X-linked Adrenoleukodystrophy Database, http://www.x-ald.nl).6

Despite the profound cellular impact of this deletion on ALDP expression, 3 different clinical phenotypes were observed in the affected family (Table). These results are in accordance with previous studies1 and underscore that there is no correlation between an ALD gene deletion, ALDP expression, and the clinical phenotype. In fact, our findings substantiate the current hypothesis of disease pathogenesis that additional genetic or epigenetic factors play a pivotal role in determining the clinical phenotype in the presence of a mutated or deleted ALD gene. Moreover, a combined genotype of methionine metabolism polymorphisms that is associated with demyelination in patients treated with methotrexate might be involved in developing cerebral changes in X-ALD.13 Besides these polymorphisms, mutations and polymorphisms in genes encoding proteins that are especially involved in protein-protein interaction with ALDP may also play a role in determining X-ALD disease forms.

In the described family, 2 affected boys (patients III:4 and III:6) developed a childhood cerebral form and
underwent unrelated HSCT. Whereas patient III:6 showed only mild neurological symptoms at the time of HSCT, patient III:4 had more pronounced symptoms. Following HSCT, no clinical progression of the disease occurred with either patient. For the last 4 years after HSCT, patient III:6 had neither clinical nor radiological signs of disease progression and VLCFA levels were within the reference range, thus supporting previous studies of HSCT in X-ALD.10 Our data support current recommendations to perform HSCT at a very early stage of cerebral X-ALD. In this context, regular clinical, neuropsychological, and neuroradiological follow-up of asymptomatic boys with X-ALD is highly recommended.10,14

Little is known about the pattern of physiological ALDP expression in peripheral tissues and in the CNS. However, the observation of an accumulation of VLFCAs preferably in the white matter, the adrenal cortex, and the testis in patients with X-ALD indicates the essential role of ALDP expression in these organs.15 Previous studies with conventional immunohistochemical techniques show that in the brain, ALDP expression is restricted to white matter and endothelial cells with astrocytes and microglia expressing ALDP more strongly than oligodendrocytes.16 Notably, our immunohistochemical analysis with the highly sensitive Catalyzed Signal Amplification System assay reveals that ALDP expression in the brain is not restricted to endothelial and glial cells but can also be detected in neurons. This finding is in accordance with a study on the occurrence of catalase messenger RNA and protein in adult rats in which catalase could be detected in neurons with a punctuate staining pattern of the cytoplasm.17 The colocalization of ALDP with the peroxisomal marker protein catalase in our analysis strongly indicates its peroxisomal localization. While a potential cross reaction of the applied antibodies related to alterations in protein structure during formalin fixation cannot be excluded completely, the specificity of the detected staining pattern is conversely supported by control experiments on cultured fibroblasts. In conclusion, our immunohistochemical analysis provides good evidence that HSCT restores ALDP expression in various tissues, including the CNS. Thus, restoration of ALDP expression and function is most likely the major curative effect of allogeneic HSCT and more important than a potential effect of immunosuppressive therapy on cerebral inflammatory processes.18

At the cellular level, the therapeutic effect is mediated by transplantation of genetically normal donor stem cells that correct metabolic abnormalities, including VLFCFA metabolism. The effect results in the regeneration of normal membrane structure and function, allowing for remyelination and prevention of progressive demyelination.19 Despite this, the cellular mechanisms that contribute to the beneficial effects of HSCT are still controversial. First, bone marrow–derived donor cells may be able to migrate into the brain tissue and transdifferentiate into organ-specific cells such as glial cells. However, the detection of ALDP in neurons as soon as 76 days after HSCT argues against this hypothesis. Second, normal donor stem cells may fuse with organ-specific cells.10 This hypothesis is supported by studies in a murine model of type I tyrosinemia. In this model, hematopoietic donor cells migrate into the liver and merge with metabolically inactive hepatocytes, thus transferring the genome of stem cells to the organ-specific cells, resulting in recovery of normal liver function. Third, wild-type ALDP might be secreted from genetically normal hematopoietic donor cells and transferred into the peroxisome membrane of neurons by endocytosis. Recent HSCT studies in ALDP-deficient mice reveal that the metabolic impairment is not corrected by cell fusion but rather by direct cell-cell contact.20 Thus, the most likely cellular mechanism in humans preventing disease progression in patients with X-ALD after HSCT is that healthy donor cells assist affected cells in metabolic function by cell-cell contact and, in particular, deprive X-ALD cells of accumulated VLFCFAs.

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**Correction**

Misspellings and Incorrect First Name. In the article titled “Two Novel Epilepsy-Linked Mutations Leading to a Loss of Function in LGI1” by Chabrol et al, published in the February issue of the ARCHIVES (2007;64: 217-222) INSERM was misspelled in the “Author Affiliations” and “Correspondence” sections on pages 217 and 222, respectively. Also in the “Author Affiliations” and “Correspondence” sections, the parenthetical information should have read as follows: “(Institut National de la Santé et de la Recherche Médicale, Unité Mixte de Recherche 679).” The beginning of the second sentence of the “Acknowledgment” section on page 222 should have read as follows: “We would like to thank Merle Ruberg, PhD, for critical reading of the manuscript...”