A novel bile acid biosynthesis defect due to a deficiency of peroxisomal ABCD3

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ABCD3 is one of three ATP-binding cassette (ABC) transporters present in the peroxisomal membrane catalyzing ATP-dependent transport of substrates for metabolic pathways localized in peroxisomes. So far, the precise function of ABCD3 is not known. Here, we report the identification of the first patient with a defect of ABCD3. The patient presented with hepatosplenomegaly and severe liver disease and showed a striking accumulation of peroxisomal C27-bile acid intermediates in plasma. Investigation of peroxisomal parameters in skin fibroblasts revealed a reduced number of enlarged import-competent peroxisomes. Peroxisomal beta-oxidation of C26:0 was normal, but beta-oxidation of pristanic acid was reduced. Genetic analysis revealed a homozygous deletion at the DNA level of 1758bp, predicted to result in a truncated ABCD3 protein lacking the C-terminal 24 amino acids (p.Y635NfsX1). Liver disease progressed and the patient required liver transplantation at 4 years of age but expired shortly after transplantation. To corroborate our findings in the patient, we studied a previously generated Abcd3 knockout mouse model. Abcd3−/− mice accumulated the branched chain fatty acid phytanic acid after phytol loading. In addition, analysis of bile acids revealed a reduction of C24 bile acids, whereas C27-bile acid intermediates were significantly increased in liver, bile and intestine of Abcd3−/− mice. Thus, both in the patient and in Abcd3−/− mice, there was evidence of a bile acid biosynthesis defect. In conclusion, our studies show that ABCD3 is involved in transport of branched-chain fatty acids and C27 bile acids into the peroxisome and that this is a crucial step in bile acid biosynthesis.

INTRODUCTION

Peroxisomes are single membrane-bound organelles present in nearly all eukaryotic cells. They are essential for a number of important metabolic pathways such as fatty acid alpha- and beta-oxidation and the biosynthesis of bile acids and ether phospholipids (1). For these pathways to function properly, the various substrates, co-factors and products must be transported across the peroxisomal membrane (2). A channel-forming protein, PXMP2, present in the peroxisomal membrane allows transmembrane passage of small solute molecules with a molecular mass of <400 Da (3). However, for bulky solute molecules (>400 Da), the membrane is an impermeable barrier and transporters are required (4). The mammalian peroxisomal

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membrane contains three ATP-binding cassette (ABC) transporters, which are thought to be involved in the transport of metabolites required for the metabolic processes inside the peroxisome (5,6). ABC transporters comprise a superfamily of membrane-bound proteins with a highly conserved structure and catalyze the ATP-dependent transmembrane transport of a wide variety of substrates. The three peroxisomal ABC transporters, classified into subfamily D, have a hydrophobic transmembrane domain with multiple membrane-spanning segments and a hydrophilic region with a nucleotide-binding domain defined by Walker A and B motifs and a conserved ABC signature (6). They are half-transporters that must dimerize, either as homodimers or as hetero-dimers, to form functional units. The three known peroxisomal ABC transporters are ABCD1 (adrenoleukodystrophy protein, ALDP) (7), ABCD2 (adrenoleukodystrophy-related protein, ALDR) (8,9) and ABCD3 (70-kDa peroxisomal membrane protein, PMP70) (10).

X-linked adrenoleukodystrophy (MIM300100), the most common peroxisomal disorder, is a neurodegenerative disorder caused by mutations in the ABCD1 gene (11–13). The ABCD1 defect causes impaired peroxisomal beta-oxidation of very-long-chain fatty acids (VLCFAs, ≥C22:0) and consequently, accumulation of VLCFAs, especially in brain and adrenal glands. There are different clinical phenotypes ranging from isolated adrenocortical insufficiency (Addison only), adrenomyeloneuropathy with progressive myelopathy and peripheral neuropathy, to the most severe form childhood cerebral adrenoleukodystrophy characterized by progressive cerebral demyelination with an inflammatory response in the white matter (11–13). Recent studies both in yeast and human cells have shown that very-long-chain acyl-CoA esters are the natural substrates for ABCD1 (14,15). Interestingly, in yeast and plants, it was shown that the acyl-CoAs are hydrolyzed during transport by the ABCD transporter and re-esterified in the peroxisomal interior by an acyl-CoA synthetase (16,17), whereas in man, very-long-chain acyl-CoAs are transported by ABCD1 into the peroxisome independent of an acyl-CoA synthetase (15).

Although the substrate specificity of ABCD2 partially overlaps with that of ABCD1, it is believed to play a specific role in the beta-oxidation of polyunsaturated fatty acids. Docosahexaenoic acid, C22:6ω3, is formed via peroxisomal beta-oxidation of C24:6ω3 (18). Beta-oxidation activity of C24:6ω3 in the brain of Abcd2 knockout mice was reduced compared with wild-type animals, and Abcd2 knockout mice have reduced levels of C22:6ω3 in primary neurons (19). Recent studies in yeast have confirmed this role of ABCD2 in docosahexaenoic acid production. Indeed, expression of human ABCD2 in baker’s yeast lacking the peroxisomal ABC transporters Pxa 1 and Pxa 2 resulted in the increased capacity of peroxisomes to beta-oxidize C24:6ω3 (20). To date no disease has been associated with a defect of ABCD2.

Originally, ABCD3 was thought to play a role in peroxisome biogenesis because mutations were identified in the ABCD3 gene in patients with Zellweger syndrome (21). Later studies, however, showed unambiguously that a PEX1 defect was the underlying cause of the defect in peroxisome biogenesis in these patients (22,23). ABCD3 is one of the most abundant peroxisomal membrane proteins, at least in hepatocytes (24), and it has been reported to be involved in the beta-oxidation of various substrates. Overexpression of ABCD3 in Chinese hamster ovary cells was found to stimulate palmitate beta-oxidation, whereas expression of ABCD3 with a mutation in the Walker A motif reduced palmitate beta-oxidation to a similar extent (25). RNAi-mediated silencing of ABCD3 expression in rat C6 glial cells reduced the rate of beta-oxidation of both palmitate and lignoceric acid (26). Recently, the residual C26:0-CoA beta-oxidation activity in fibroblasts of X-ALD patients was shown to be mediated by ABCD3 (15). Besides its role in the transport of straight- and very-long-chain acyl-CoAs, ABCD3 is believed to play an important role in the transport of 2-methyl branched-chain acyl-CoAs including pristanoyl-CoA and the bile acid intermediates di- and tri-hydroxycholestanoyl-CoA (D/TH-CoA). This conclusion was based on unpublished observations made in Abcd3 knockout mice presented at various international meetings (27,28).

Here, we report the identification of the first patient with a defect of ABCD3. The patient presented with hepatosplenomegaly and severe liver disease and had a striking accumulation of the peroxisomal bile acid intermediates DHCA and THCA in plasma. Our observations support the postulated role of ABCD3 in the transport of DHC-CoA and THC-CoA into the peroxisome, which is an important step in the biosynthesis of bile acids. Analysis of the Abcd3 knockout mice revealed similar abnormalities in bile acid biosynthesis.

RESULTS

Case description

The patient, a girl, was the second child of healthy consanguineous parents of Turkish descent. She had an elder sister who was well at the age of 9 years. The patient was born with normal spontaneous delivery weighing 3200 g. There were no neonatal problems except mild jaundice that was not treated. She was exclusively breastfed until the age of 5 months and continued to be breastfed until the age of 1.5 years with the addition of supplements. Developmental milestones were normal; she sat at the age of 7 months and walked at the age of 1 year. Social and language development was also normal.

At the age of 6 months, she had jaundice during 1 week for which the family did not seek medical evaluation. At the age of 1 year, abdominal distension was noticed. She was initially seen by the local health department, but no pathology was reported. At the age of 1.5 years, she had an episode of fever and gastroenteritis for which she was treated at a hospital. The clinical diagnosis was rotavirus infection, but the physician noticed paleness and hepatosplenomegaly. She had severe anemia for which she was referred to the emergency department. Physical examination revealed mild jaundice, palmar erythema and hepatosplenomegaly. The liver was palpable 5–6 cm and spleen 7–8 cm below the costal margins. Laboratory evaluation showed severe anemia (Hb: 4.7 g/dl, Hct: 16.4%, WBC: 14 300/µl, MCV: 57 fl, PLT: 96 000/µl) and coagulopathy (PT: 23.1, APTT: 62.5, INR: 1.9) unresponsive to 3 mg of parenteral vitamin K administration. Low erythrocyte MCV (57 fl), serum iron (25 µg/dl) and ferritin (5.8 ng/ml) and elevated total iron binding capacity (684 µg/dl) were in favor of anemia caused by iron deficiency. She received packed red blood cell...
transfusion also showed normoblastic hyperactivity in accordance with severe iron deficiency anemia, but no indication for malignancies and storage disorders was found. Liver enzymes (AST: 202 U/l; ALT: 57 U/l) were elevated. Additional workup for hepatosplenomegaly and mild hyperbilirubinemia (total bilirubin: 3.96 mg/dl, direct bilirubin: 1.3 mg/dl) yielded normal results for protein electrophoresis, virological tests and alpha-1-antitrypsin. Routine blood biochemistry was unremarkable; glucose: 76 mg/dl, BUN: 10 mg/dl, urea: 22 mg/dl, uric acid: 5.1 mg/dl, sodium: 138 mmol/l, potassium: 3.9 mmol/l, chloride: 103 mmol/l, calcium: 10.4 mg/dl, phosphorus: 4.4 mg/dl, alkaline phosphatase: 526 U/l, GGT: 27 U/l, reducing substance in urine was 2+, and monosaccharide thin-layer chromatography showed galactosuria. Initial treatment with a lactose restricted diet did not lead to any clinical improvement and was stopped after the diagnosis of galactosemia was ruled out by enzymatic analysis.

A liver core biopsy was taken at the age of 1.5 years, and microscopic examination of the biopsy revealed scant monocellular cell infiltration in the portal areas with minimal interface activity. Interlobular bile ducts were normal without any ductular reaction. Indication for hepatocellular regeneration such as crowding of hepatocellular trabeculae, rosette formation and double nuclei was detected. There was minimal intranuclear and intracytoplasmic cholestasis. No necrosis and steatosis were seen. Portal–portal and portal–central fibrosis as well as pericellular fibrosis throughout the liver parenchyma were detected. She was started on ursodeoxycholic acid treatment (10 mg/kg/day) at the age of 2 years.

During follow-up, liver and spleen were further enlarged to fill the abdomen, but she showed normal growth and development. She had several admissions for febrile infections and episplenia in the following 2 years. Pancycopenia caused by hypersplenism and bleeding caused by hepatic dysfunction were the main clinical features. After 3 years of age, she developed clinical symptoms of severe portal hypertension with umbilical collaterals, esophageal varices and progressive fatigue owing to hepatopulmonary syndrome. She also had mild jaundice owing to cholestasis. Bile acids in plasma were all increased (see Table1), and in particular, there was a marked accumulation of the peroxisomal C27-bile acid intermediates DHCA and THCA. Also the C29-dicarboxylic acid (3α,7α,12α-trihydroxy-27-carboxymethyl-5β-cholestan-26-oic acid), which is formed by chain-elongation of THCA was detected. VLCFA analysis revealed repeatedly increased C26:C22 and C24:C22 ratios owing to low levels of C22:0. Phytanic and pristanic acid levels were normal. Erythrocyte plasmalogen levels were reduced but not deficient. Based on these results, detailed functional and morphological studies of peroxisomes in the patient’s fibroblasts were initiated.

At the age of 4 years before receiving a final metabolic diagnosis, she deteriorated rapidly and decompensated liver cirrhosis associated with hepatopulmonary syndrome requiring oxygen supplementation necessitated liver transplantation. Total hepatectomy specimen measuring 19 × 9, 5 × 5 cm displayed micronodular cirrhotic features. She died 5 days after liver transplantation because of respiratory complications triggered by worsening hypoxemia at the intensive care unit.

**Peroxisomal parameters in skin fibroblasts**

The dramatic accumulation in plasma of the C27-bile acid intermediates DHCA and THCA and that of the C29-dicarboxylic acid in combination with abnormal ratios of VLCFA strongly suggested a peroxisomal disorder in the patient. To investigate this possibility, the presence and morphology of peroxisomes in cultured skin fibroblasts were studied by catalase immunofluorescence microscopy analyses. These analyses showed that the peroxisomal matrix enzyme catalase was localized within the peroxisome but that the number of peroxisomes was reduced and the size of the peroxisomes was enlarged (see Fig. 1A). Immunofluorescence microscopy analyses using antibodies against different peroxisomal membrane proteins revealed the normal presence of ABCD1 and PEX14, but the absence of ABCD3 in the peroxisomal membrane (Fig. 1A).

Subsequent immunoblot analysis with a homogenate of the patient’s fibroblasts confirmed the absence of the 70-kDa band corresponding to ABCD3 (Fig. 1B). Immunoblot analysis with antibodies against peroxisomal acyl-CoA oxidase 1 and classical 3-ketoacyl-CoA thiolase revealed normal presence and processing of these proteins (not shown). VLCFA (C26:0, C24:0, C22:0) levels in fibroblasts were marginally decreased (Table 1). Peroxisomal beta-oxidation studies in fibroblasts revealed normal beta-oxidation of C26:0 but reduced beta-oxidation of pristanic acid (59% of the mean activity in control fibroblasts, Table 1).

**Genetic analysis and functional complementation studies**

We sequenced ABCD3 cDNA synthesized from fibroblast mRNA in four overlapping fragments. The sequence of the first three fragments comprising exons 1 through 20 was normal; however, we were not able to amplify the last fragment comprising exons 21 t/m 24, despite the use of different sets of forward and reverse primers, suggesting the possibility of a large intragenic deletion. To investigate this in more detail, we performed 3' RACE-PCR and sequencing, which demonstrated a deletion of exon 24 and part of the 3' UTR (c.1903-573_∗1246). Subsequent analysis of genomic DNA confirmed a homozygous deletion at the DNA level of 1758bp: c.1903-573_∗1108, see Figure 1C. This deletion is predicted to result in a truncated ABCD3 protein lacking the C-terminal 24 amino acids, p.Y635NfsX1. Homozygosity was confirmed by testing parental DNA. Both parents were carriers for this deletion. Because the antibodies against ABCD3 used for the immunofluorescence microscopy studies and immunoblot analysis recognize a C-terminal epitope (last 15 amino acids), we repeated the immunoblot analysis with another antiserum that did show the presence of ABCD3 in fibroblasts of the patient (not shown) confirming that ABCD3 is truncated in the patient.

To determine whether the ABCD3 deletion was the underlying cause of the observed cellular phenotype characterized by a
Table 1. Biochemical parameters in blood and fibroblasts

<table>
<thead>
<tr>
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<th>Patient</th>
<th>Reference range</th>
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<tbody>
<tr>
<td><strong>Blood</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bile acids (μmol/l)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDCA</td>
<td>12.6</td>
<td>0.7–10</td>
</tr>
<tr>
<td>CA</td>
<td>15.7</td>
<td>0.1–4.7</td>
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<tr>
<td>UDCA</td>
<td>54.3</td>
<td>0–16</td>
</tr>
<tr>
<td>DHCA</td>
<td>6.3</td>
<td>0–0.02</td>
</tr>
<tr>
<td>THCA</td>
<td>5.3</td>
<td>0–0.08</td>
</tr>
<tr>
<td>C29DCA</td>
<td>1.4</td>
<td>0–0.001</td>
</tr>
<tr>
<td>VLCFA (μmol/l)</td>
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<td></td>
</tr>
<tr>
<td>C22:0</td>
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<tr>
<td>C24:0</td>
<td>13.8</td>
<td>33–84</td>
</tr>
<tr>
<td>C26:0</td>
<td>1.12</td>
<td>0.45–1.32</td>
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<td>C24/C22</td>
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<td>0.57–0.92</td>
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<tr>
<td>C26/C22</td>
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<td>0–0.02</td>
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<td>Branched-chain fatty acids (μmol/l)</td>
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<tr>
<td>Phytanic acid</td>
<td>5.8</td>
<td>0.49–9.88</td>
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<tr>
<td>Pristanic acid</td>
<td>1.6</td>
<td>0.12–2.98</td>
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<tr>
<td>Plasmalogens (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C18DMA</td>
<td>9.8</td>
<td>10.6–24.9</td>
</tr>
<tr>
<td>C16DMA</td>
<td>3.8</td>
<td>6.8–11.9</td>
</tr>
<tr>
<td>Fatty acid oxidation [pmol/(min mg)]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C26:0 oxidation</td>
<td>1247 b</td>
<td>1273–1431</td>
</tr>
<tr>
<td>Pristanic acid oxidation</td>
<td>553 a</td>
<td>748–975</td>
</tr>
<tr>
<td>VLCFA (μmol/g)</td>
<td></td>
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<tr>
<td>C22:0</td>
<td>0.17 b</td>
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<td>C24:0</td>
<td>6.11 b</td>
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<td>C22:0</td>
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<td>C26/C22</td>
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<tr>
<td>C24/C22</td>
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<tr>
<td>Plasmalogens (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C18DMA</td>
<td>7.0</td>
<td>7.2–11.5</td>
</tr>
<tr>
<td>C16DMA</td>
<td>8.9</td>
<td>8.6–13.0</td>
</tr>
<tr>
<td>Peroxosomal enzymes</td>
<td></td>
<td></td>
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<tr>
<td>DHAPAT [nmol/(2 h mg)]</td>
<td>7.9</td>
<td>5.4–10.5</td>
</tr>
<tr>
<td>ACOX1 [pmol/(min mg)]</td>
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<tr>
<td>DBP hydratase [pmol/(min mg)]</td>
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<td>115–600</td>
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<tr>
<td>DBP dehydrogenase [pmol/(min mg)]</td>
<td>285</td>
<td>25–300</td>
</tr>
<tr>
<td>SCPx [pmol/(min mg)]</td>
<td>103</td>
<td>10–39</td>
</tr>
</tbody>
</table>

CDCA, chenodeoxycholic acid; CA, cholic acid; UDCA, ursodeoxycholic acid; DHCA, dihydroxycholate acid; THCA, trihydroxycholate acid; C29DCA, C29-dicarboxylic acid; VLCFA, very-long-chain fatty acid; DMA, dimethylacetal; DHAPAT, dihydroxyacetonephosphate acyltransferase; ACOXI, acyl-CoA oxidase 1; DBP, D-bifunctional protein; SCPx, sterol carrier protein X.

Units are indicated between parentheses.

*Mean values of two independent experiments.

*Mean values of three independent experiments. All other values represent single measurements.

Reduced number of enlarged peroxisomes, we overexpressed wild-type ABCD3 in the patient’s fibroblasts followed by immunofluorescence microscopy using specific antibodies against ABCD3 and catalase. We found that ~20% of the cells were transfected and that all transfected cells that showed restoration of ABCD3 expression also showed normalization of peroxisomal number and size (Fig. 1D).

**Abcd3 knockout mice**

To corroborate our findings in the patient, we studied a previously generated Abcd3 knockout mouse model with a targeted disruption of the Abcd3 gene. The genotypes of offspring of Abcd+/− matings fitted Mendelian ratios (20 Abcd3+/+, 43 Abcd3+/− and 22 Abcd3−/− in a total of 85 pups from 10 litters). Newborn Abcd3−/− pups displayed normal activity and no obvious malformations. In routine necropsy studies done to survey Abcd3−/− animals for phenotypic abnormalities, we noted that the size of their liver was increased ~2-fold as compared with controls. Histologic examination of the liver showed no evidence of increased hepatic fat or fluid that might account for the difference in liver weight. No other obvious phenotype was observed in the Abcd3−/− mice. Analysis of mRNA and protein isolated from the liver showed no detectable mRNA or Abcd3 protein in the Abcd3−/− animals (Supplementary Material, Fig. S1C and D). Catalase immunofluorescence microscopy analyses in cultured skin fibroblasts of Abcd3−/− mice revealed that, just like in fibroblasts of the patient, peroxisomes were import-competent, but reduced in number and enlarged in size (Supplementary Material, Fig. S1E and F).

**Bile acid analysis in Abcd3−/− mice**

The accumulation of C27-bile acid intermediates in plasma of the patient strongly suggested an important role for ABCD3 in bile acid biosynthesis. We confirmed these findings by measuring bile acids by negative ion mass spectrometry in liver, bile, intestine and plasma from Abcd3−/− mice.

Similar to the patient, Abcd3−/− mice showed clear bile acid abnormalities. In all materials, there was a significant reduction of mature C24 bile acids. Total C24 bile acids in liver from Abcd3+/+ animals were 306 ± 113 nmol/g wet weight (mean ± SD) compared with 174 ± 111 nmol/g in liver from Abcd3−/− mice. This reduction is the result of a reduction in both taurine-conjugated cholic acid (taurine-CA) and especially taurine-conjugated muri-cholic acid (tauro-mCA) (see Fig. 2).

In bile, the concentration of total C24 bile acids was 98 ± 36 nmol/l in wild-type animals and 41 ± 28 nmol/l in Abcd3−/− mice. In contrast to Abcd3+/+ mice where only 5% of the liver C24 bile acids were unconjugated, 33% of the liver C24 bile acids (mainly cholic acid) in the Abcd3−/− mice were unconjugated. In bile, there was also an increase in the percentage of unconjugated C24 bile acids from 2% in wild-type animals to 22% in Abcd3−/− mice. In addition to the reduction in C24 bile acids, there was a significant increase in C27-bile acid intermediates in liver, bile and intestine from Abcd3−/− mice. Levels of taurine-conjugated and unconjugated DHCA, THCA and OH-THCA were all increased.

Total C27-bile acid level in liver from Abcd3−/− mice was 14 ± 10 nmol/g (wet weight) as compared with 0.4 ± 0.2 nmol/g in wild-type mice. In bile of Abcd3−/− mice, the concentration of total C27 bile acids was 3.1 ± 1.9 mmol/l compared with 0.03 ± 0.03 mmol/l in bile of wild-type mice. Thus, there was a marked bile acid biosynthesis defect in Abcd3−/− animals.

**Phytol loading studies in Abcd3−/− mice**

The finding of an impaired pristanic acid beta-oxidation observed in fibroblasts from the patient suggested that ABCD3 is involved in the transport of branched-chain fatty acids into
the peroxisome. To test this hypothesis, we supplemented the diet of Abcd3−/− mice and Abcd3+/+ littermates with phytol (0.25% w/w), the precursor of phytanic acid, and subsequently measured plasma levels of phytanic and pristanic acids. By Day 8, the plasma phytanic acid concentration had increased in the Abcd3−/− mice from 1.9 to 322 μM and
from 0.6 to 32 μM in wild-type mice (Fig. 3). Similarly, plasma levels of pristanic acid increased during this challenge from 0.7 to 14.9 μM in Abcd3−/− mice and from 0.3 to 0.7 μM in wild-type mice. Plasma levels of palmitate in both the Abcd3−/− and Abcd3+/+ animals remained unchanged over the 8-day phytol load (not shown). Thus, on a phytol diet, there was a
marked accumulation of phytanic and pristanic acid in plasma of Abcd3−/− animals.

DISCUSSION

We report for the first time the identification of a patient with a defect of the peroxisomal ABC half transporter ABCD3. The patient presented with severe hepatosplenomegaly, cholestasis and liver dysfunction and died shortly after liver transplantation at the age of 4 years owing to respiratory complications. Screening of peroxisomal parameters in blood revealed marked accumulation of the peroxisomal C27-bile acid intermediates. Subsequent studies of peroxisomal parameters in cultured skin fibroblasts showed the absence of ABCD3 protein in the peroxisomal membrane by immunofluorescence microscopy analyses whereas other peroxisomal membrane proteins like ABCD1 and PEX14 were normally present. The peroxisomes in fibroblasts were reduced in number and increased in size, which is also commonly observed in the peroxisomal fatty oxidation disorders ACOX1 deficiency and DBP deficiency. The underlying cause is unknown, but this change could be caused by the accumulation and/or shortage of a metabolite influencing peroxisome number and morphology. Genetic analysis identified a deletion of exon 24 and flanking sequence that results in truncated ABCD3 protein lacking the C-terminal 24 amino acids. Although characterization of ABCD3’s function has been subject of several studies in the past [reviewed in (5,6,29)], its function has never been established unambiguously. Identification of a patient with an ABCD3 defect finally makes this possible.

The beta-oxidation studies in cultured skin fibroblasts from the patient showed a reduced activity with pristanic acid as substrate strongly suggested that ABCD3 is involved in the transport of this branched-chain fatty acid into the peroxisome. Yet, pristanic acid and pristanic acid levels in plasma of Abcd3−/− mice were normal. These fatty acids are exclusively derived from the diet; however, this is possible in young patients. Indeed, this is often observed in patients with Zellweger syndrome, who lack functional peroxisomes (30). To further study the role of ABCD3 in peroxisomal degradation of the branched-chain fatty acids, we supplemented the diet of wild-type and Abcd3−/− mice with 0.25% (w/w) phytol, a precursor of pristanic and pristanic acid. Within 8 days, there was a marked increase in pristanic acid and pristanic acid levels in plasma from Abcd3−/− mice as compared with the levels in the wild-type mice fed the phytol-loaded diet, demonstrating that ABCD3 is involved in peroxisomal metabolism of these branched-chain fatty acids.

The marked accumulation of the peroxisomal C27-bile acid intermediates DHCA and THCA in blood from the patient strongly suggested that ABCD3 is also involved in the transport of the C27-bile acid intermediates across the peroxisomal membrane. Bile acids are synthesized in the liver from cholesterol, and after a series of reactions involving different enzymes localized in several different subcellular compartments, the peroxisomal C27-bile acid intermediates DHCA and THCA are formed (31). They are activated to CoA esters at the cytosolic side of the ER and then need to be transported into the peroxisome for shortening of the side chain by peroxisomal beta-oxidation to C24-bile acyl-CoAs. Finally, the mature C24-bile acyl-CoAs are conjugated to taurine or glycine by bile acyl-CoA: amino acid N-acyltransferase (BAAT) (31,32). As in the patient, in Abcd3−/− mice, there was a clear accumulation of C27-bile acid intermediates. C27-bile acid intermediates were increased in liver, bile and intestine from Abcd3−/− mice compared with wild-type animals, and at the same time, there was a significant reduction of C24 bile acids. Measurement of the enzymatic activities of the peroxisomal beta-oxidation enzymes involved in the beta-oxidation of pristanoyl-CoA and DHC-/THC-CoA [i.e. alpha-methylacyl-CoA racemase (AMACR), branched-chain acyl-CoA oxidase (ACOX2), DBP and Sterol Carrier Protein X (SCPx)] revealed that these enzymes were normally active in liver from Abcd3−/− mice (not shown) showing that the accumulation is not due to a deficiency of one of these peroxisomal enzymes. After formation of the mature C24-bile acyl-CoAs, they are conjugated in the peroxisome by BAAT with taurine or glycine. Abcd3−/− mice showed a clear increase in unconjugated C24 bile acids compared with wild-type animals, although BAAT activity was normal in liver homogenates from Abcd3−/− mice (not shown). These results suggest that at least part of the mature C24 bile acids in Abcd3−/− are formed via the
alternative microsomal route for bile acid synthesis because of a hampered transport into the peroxisome where shortening of the side chain followed by conjugation takes place. Taken together, these data show that ABCD3 transports branched-chain acyl-CoAs, including the peroxisomal bile acid intermediates, into the peroxisome where they are metabolized.

ABCD3 deficiency as reported in this paper represents a novel bile acid biosynthesis defect with a very severe clinical course, which can be detected by performing plasma bile acid analysis. Patients with Zellweger syndrome, who lack functional peroxisomes, and patients with a defect of one of the peroxisomal enzymes involved in bile acid biosynthesis, i.e. AMACR-, DBP- and SCPx-deficient patients, all accumulate C27-bile acid intermediates (30,33,34). These C27-bile acid intermediates have altered physical properties when compared with mature C24 bile acids. They are excreted in bile, but studies in DBP- and AMACR-deficient knockout mice have shown that they are not excreted as efficiently as C24 bile acids (35,36). It has been shown that the C27-bile acid intermediates are especially toxic, more toxic than their C24 products, especially when they are unconjugated (37). Accumulation of C27-bile acid intermediates contributes to the liver disease associated with peroxisomal disorders. Patients with Zellweger syndrome have cholestasis, hepatomegaly, elevated serum liver enzymes, excessive hepatocyte iron stores and steatorrhea. Lesions of cholangioles, inflammation and bile duct proliferation have also been reported (38,39). In two siblings with AMACR deficiency, who had cholestasis and a deficiency of fat-soluble vitamins, histological examination of the liver revealed giant cell transformation, the prevalence of necrotic hepatocytes and perportal hemosiderosis (40). Identification of more patients with an ABCD3 defect and further studies in the Abcd3−/− mice will provide more insight into the exact pathological mechanisms involved in this disorder.

MATERIALS AND METHODS

Metabolic, biochemical and microscopical analyses

Concentrations of very-long-chain fatty acids, phytanic and pristane acid, bile acids and plasmalogens were determined as described previously (41–43). Dihydroxyacetone phosphate acyltransferase (44), acyl-CoA oxidase 1 (ACOX1) (45), DBP (46) and SCPx (47) activities in cultured fibroblasts were measured as previously described. Beta-oxidation of C26:0 and pristane acid intermediates contributes to the liver disease associated with peroxisomal disorders. Patients with Zellweger syndrome have cholestasis, hepatomegaly, elevated serum liver enzymes, excessive hepatocyte iron stores and steatorrhea. Lesions of cholangioles, inflammation and bile duct proliferation have also been reported (38,39). In two siblings with AMACR deficiency, who had cholestasis and a deficiency of fat-soluble vitamins, histological examination of the liver revealed giant cell transformation, the prevalence of necrotic hepatocytes and perportal hemosiderosis (40). Identification of more patients with an ABCD3 defect and further studies in the Abcd3−/− mice will provide more insight into the exact pathological mechanisms involved in this disorder.

Genetic analysis

Mutation analysis was performed by sequencing cDNA prepared from total fibroblast mRNA. Total RNA was isolated from skin fibroblasts using Trizol (Invitrogen, Carlsbad, CA, USA) extraction, after which cDNA was prepared using a first-strand cDNA synthesis kit for RT-PCR (Roche, Mannheim, Germany). Genomic DNA was isolated from skin fibroblasts using the NucleoSpin Tissue genomic DNA purification kit (Macherey-Nagel, Germany, Düren). To determine the 3′ deletion in the ABCD3 cDNA, we used the 3′ rapid amplification of cDNA ends’ method [3′ RACE; (51)]. To this end, we first prepared cDNAs from total RNA isolated from the patient’s fibroblasts using as oligo-dT-adaptor primers AGTGGATATCAACGCA-GAGTACT20A/G/C. PCR was then used to amplify the 3′ end of ABCD3 cDNA using an ABCD3-specific primer (available upon request) and primer GTACTCTGCGTTGATACCT, which is complementary to the adaptor sequence. The resulting PCR fragment is then sequenced as described later. All forward and reverse primers used for sequencing (available upon request) were tagged with a -21M13 (5′ TGTTAAAACGACGGCCAGT 3′) sequence or M13rev (5′ CAGGAAACAGCTATGACC 3′) sequence, respectively. PCR fragments were sequenced in two directions using ‘-21M13’ and ‘M13rev’ primers by means of BigDye Terminator v1.1 Cycle Sequencing Kits (Applied Biosystems, Foster City, CA, USA) and analyzed on an Applied Biosystems 3130 × 1 or 3730 × 1 DNA analyser, following the manufacturer’s protocol (Applied Biosystems). ABCD3 sequence data were compared with the reference ABCD3 sequence (GenBank accession No. NM_002858.3) with nucleotide numbering starting at the first adenine of the translation initiation codon ATG.

Functional complementation

The coding regions of wild-type ABCD3 were amplified by PCR from reverse-transcribed RNA isolated from control fibroblasts and sub-cloned into a eukaryotic expression vector (pcDNA3; Invitrogen). The patient’s fibroblasts were transfected with the pcDNA3 expression plasmid containing the ABCD3 cDNA using the AMAXA nucleofector technology (Amaxa, Cologne, Germany). The fibroblasts were examined by catalase and ABCD3 immunofluorescence in a double-labeling experiment 4 days after transfection. FITC-conjugated polyclonal goat anti-mouse immunoglobulins (DAKO) and Alexa Fluor 555 goat anti-rabbit immunoglobulins (Molecular probes) were used for visualization.
Animal experiments

Targeted disruption of the murine Abcd3 gene, embryo manipulation and mice breeding is described in detail in Supplementary Material.

For the bile acid measurements, five adult wild-type and Abcd3−/− mice fed a normal chow diet were sacrificed and organs were harvested and stored at −80 °C until analysis. Bile acids in mouse liver, intestine, bile and plasma were analyzed by HPLC-negative ion electrospray tandem mass spectrometry as described (35). For the phytol feeding experiment, adult wild-type and Abcd3−/− mice were fed pelleted mouse chow without supplements (control) or supplemented with 0.25% (w/w) phytol (Sigma–Aldrich). Each group consisted of three animals and the animals received the diet for 8 days. At the end of the experiment, animals were sacrificed and phytic and pristanic acids levels in plasma were determined as described.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG online.

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Conflict of Interest statement. None declared.

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