

ARTICLE

Novel mutations in the *PEX12* gene of patients with a peroxisome biogenesis disorder

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The peroxisome biogenesis disorders (PBDs) form a genetically and clinically heterogeneous group of disorders due to defects in at least 11 distinct genes. The prototype of this group of disorders is Zellweger syndrome (ZS), with neonatal adrenoleukodystrophy (NALD) and infantile Refsum disease (IRD) as milder variants. Liver disease, variable neurodevelopmental delay, retinopathy and perceptive deafness are common to PBDs. PBD patients belonging to complementation group 3 (CG3) have mutations in the *PEX12* gene, which codes for a protein (PEX12) that contains two transmembrane domains, and a zinc-binding domain considered to be important for its interaction with other proteins of the peroxisomal protein import machinery. We report on the identification of five PBD patients belonging to CG3. Sequence analysis of their *PEX12* genes revealed five different mutations, four of which have not been reported before. Four of the patients have mutations that disrupt the translation frame and/or create an early termination codon in the *PEX12* open reading frame predicted to result in truncated protein products, lacking at least the COOH-terminal zinc-binding domain. All these patients display the more severe phenotypes (ZS or NALD). The fifth patient expresses two *PEX12* alleles capable of encoding a protein that does contain the zinc-binding domain and displayed a milder phenotype (IRD). The three biochemical markers measured in fibroblasts (DHAPAT activity, C26:0 β -oxidation and pristanic acid β -oxidation) also correlated with the genotypes. Thus, the genotypes of our CG3 patients show a good correlation with the biochemical and clinical phenotype of the patients.

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Introduction

The peroxisome biogenesis disorders (PBDs; MIM: 601539), which include Zellweger syndrome (ZS; MIM: 214100), neonatal adrenoleukodystrophy (NALD; MIM: 202370) and infantile Refsum disease (IRD; MIM: 266510), represent a spectrum of disease severity with ZS being the

most, and IRD the least severe disorders. Liver disease, variable neurodevelopmental delay, retinopathy and perceptive deafness are common to all the three PBDs.¹ Patients with ZS are severely hypotonic from birth and die before 1 year of age. Patients with NALD experience neonatal onset of hypotonia and seizures, and suffer from progressive white matter disease, dying usually in late infancy.² Patients with IRD may survive beyond infancy and some may even reach adulthood.³ Clinical differentiation between these disease states is not very well defined and patients can have overlapping symptoms.⁴

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The absence of functional peroxisomes in patients with a PBD leads to a number of biochemical abnormalities: (i) PBD patients have an impaired synthesis of plasmalogens, due to a deficiency of the two enzymes dihydroxyacetonephosphate acyltransferase (DHAPAT) and alkyl-dihydroxyacetonephosphate synthase (alkyl-DHAP-synthase).^{5,6} (ii) Peroxisomal fatty acid β -oxidation is defective, leading to the accumulation of very-long-chain fatty acids (VLCFAs), notably C26:0, the branched-chain fatty acid pristanic acid and the bile acid intermediates di- and trihydroxycholestanic acid (DHCA and THCA).¹ (iii) Phytanic acid α -oxidation and L-pipecolic acid oxidation are impaired.¹ While some peroxisomal enzymes are deficient, others show normal activity, including catalase, D-amino-acid oxidase, L- α -hydroxy-acid oxidase A and alanine:glyoxylate aminotransferase, although subcellular fractionation studies have shown that these enzymes are mislocalized to the cytoplasm.¹

The PBDs are caused by genetic defects in *PEX* genes encoding proteins called peroxins, which are required for the biogenesis of peroxisomes and function in the assembly of the peroxisomal membrane or in the import of enzymes into the peroxisome.⁷ After synthesis on free polyribosomes, peroxisomal matrix proteins carrying either a carboxy-terminal peroxisomal targeting sequence 1 (PTS1) or a cleavable amino-terminal PTS2 signal are translocated across the peroxisomal membrane.⁷ A defect in one of the peroxins of the peroxisomal import machinery leads to failure of protein import via the PTS1- and/or PTS2-dependent import pathway and, consequently, to functional peroxisome deficiency. Cell fusion complementation studies using patient fibroblasts revealed the existence of at least 11 distinct genetic groups, of which currently all the corresponding *PEX* genes have been identified. Most complementation groups are associated with more than one clinical phenotype.⁷

PBD patients belonging to complementation group 3 (CG3) have mutations in the *PEX12* gene (MIM: 601758).⁸ *PEX12* was first identified in the yeast *Pichia pastoris*,⁹ and more recent studies have led to the identification of the human homologue of this gene.^{8,10,11,12} *HsPEX12* encodes a 359 amino-acid protein (PEX12), with a molecular weight of ~41 kDa. PEX12 is an integral peroxisomal membrane protein with a zinc-binding motif at its COOH terminus.^{9,10} It spans the peroxisomal membrane twice and exposes its NH₂ and COOH termini to the cytoplasm. The protein interacts with PEX5 and PEX10 via its COOH-terminal zinc-binding domain, and is most likely involved in the actual process of translocation of peroxisomal matrix proteins across the peroxisomal membrane.¹³

In this study, we report the identification of novel mutations in the *PEX12* gene in five PBD patients, which, using cell fusion complementation analysis, were shown to belong to CG3. The correlations between genotypes and phenotypes are discussed.

Patients and methods

Patient samples

All patients analyzed showed the clinical characteristics of PBDs. Based on their clinical characteristics, patients have been diagnosed with ZS, NALD or IRD. Samples were collected from patients and sent to our laboratory for biochemical and molecular diagnosis.

Biochemical analysis

The biochemical diagnosis of a PBD was substantiated by detailed studies in primary skin fibroblasts, including the measurement of DHAPAT activity¹⁴ and C26:0 and pristanic acid β -oxidation,¹⁵ and immunofluorescence using antibodies against catalase, D-bifunctional protein and the PTS1 signal peptide SKL (Zymed laboratories, San Francisco, CA, USA).¹⁶

Complementation analysis

To identify the defective *PEX* gene in the patients, cell fusion complementation studies were performed.¹⁷ Fibroblasts from the patients were fused with index fibroblasts from known complementation groups. The resulting heterokaryons were assayed for complementation by catalase immunofluorescence as previously described.¹⁶

Mutation analysis

PEX12 mutation analysis in the patients was performed at the genomic DNA level. Genomic DNA was isolated from primary skin fibroblasts using the Wizard genomic DNA purification kit (Promega, Madison, WI, USA). The entire exons plus flanking intron sequences from the *PEX12* gene were amplified by PCR using the primer sets shown in Table 1. All forward and reverse primers used for mutation analysis were tagged with a -21M13 (5'-TGAAAAC-GACGGCCAGT-3') sequence and M13rev (5'-CAGGAAA-CAGCTATGACC-3') sequence, respectively. PCR fragments were sequenced in two directions using '-21M13' and 'M13rev' fluorescent primers on an Applied Biosystems 277A automated DNA sequencer, following the manufacturer's protocol (Perkin Elmer, Wellesley, MA, USA).

Quantitative real-time RT-PCR analysis

Total RNA was isolated from primary 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). Quantitative real-time PCR analysis of *PEX12* and β -2-microglobulin RNA was performed using the LightCycler FastStart DNA Master SYBR green I kit (Roche, Mannheim, Germany). The *PEX12* primers used were: PEX12-LC-F, 5'-CAGCCAGGAGTGTAGTGAG-3'; and PEX12-LC-R, 5'-GGTTTTACGACACAGTGGGC-3'. The β -2-microglobulin primers used were: b2M-FW, 5'-TGAA-TTGCTATGTGTCTGGG-3'; and b2M-REV, 5'-CATGTCTC-GATCCCACTAAC-3'. The PCR program comprised a

Table 1 Primer sets used for mutation analysis of the *PEX12* gene at chromosome 17q12

Amplicon	5' primer (forward)	3' primer (reverse)
Exon 1	[−21M13]-TGAGCACCCATCTGATACTC	[M13rev]-CGCTAGGCTACCAAATAAGC
Exon 2	[−21M13]-TGTGTCATGGAATGAATTTAC	[M13rev]-GGGATACGATTTTCGAATTTAC
Exon 3	[−21M13]-GGAGATAGTACCAGTCTACC	[M13rev]-TACCATGCTGAAACCAGCTC

Table 2 *PEX12* mutations and biochemical markers in 5 patients with a PBD

ID	Pheno type	Survival	Mutation in genomic DNA	Consequence	DHAPAT activity ^a	C26:0 β -oxidation ^b	Pris. acid β -oxidation ^b	Catalase IF
PEX12-01	ZS	9 months	887–888delTC (homo)	L296fs→307X	0.8	48	1	—
PEX12-02	IRD	2.5 years	273A>T (homo)	Arg91Ser	10.2	384	67	—
PEX12-03	ZS	4.5 months	625C>T	Gln209X	0.5	100	0	—
PEX12-04	NALD	5 months	887–888delTC	L296fs→307X	0.6	236	3	—
PEX12-05	ZS	2.5 months	604C>T (homo)	Arg202X	0.5	107	5	—
Control values					5.8–12.3	1200–1500	675–1100	+

^anmol/2 h mg protein.

^bpmol/h mg protein.

10 min initial denaturation step at 95°C to activate the hot start polymerase, followed by 40 cycles of 95°C for 10 s, 58°C for 2 s and 72°C for 11 s (9 s for β -2-microglobulin). Fluorescence was measured at 82°C for *PEX12* and 80°C for β -2-microglobulin. Melt curve analysis to show the generation of a single product for each reaction was carried out following the PCR program. Amplification of a single product of the correct size was also confirmed by agarose gel electrophoresis. Duplicate analysis was performed for all samples. Data were analyzed using LightCycler Software, version 3.5 (Roche, Mannheim, Germany). To adjust for variations in the amount of input RNA, the values for the *PEX12* gene are normalized against the values for the housekeeping gene β -2-microglobulin, and the patient ratios are presented as a percentage of the mean of two control fibroblast cell lines.

Results

In this study, we analyzed five patients affected by a PBD, as concluded from the finding of typical abnormalities in plasma (elevated levels of VLCFA, bile acid intermediates, pristanic and phytanic acid) and primary skin fibroblasts (deficient DHAPAT activity, C26:0 β -oxidation and pristanic acid β -oxidation and absence of catalase-positive particles visualized by immunofluorescence (Table 2)). In patient PEX12-2, normal levels of DHAPAT activity and a relatively high rate of C26:0 β -oxidation were found, but no peroxisomal localization of catalase. This prompted us to study the localization of other peroxisomal matrix proteins in these fibroblasts. D-bifunctional protein immunofluorescence revealed a particle-bound localization

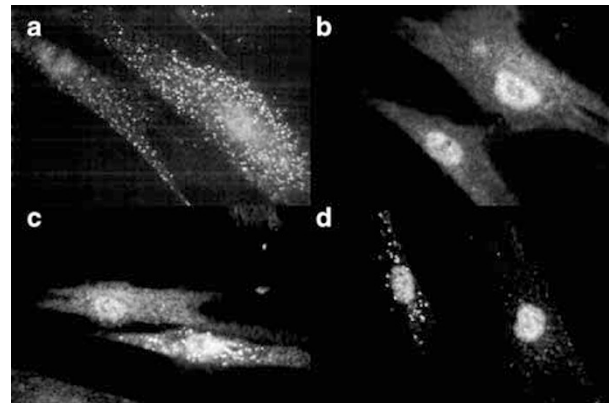


Figure 1 Immunofluorescent staining of fibroblasts from control (a) and patient PEX12-02 (b–d). Cells were stained with antibodies against catalase (a,b), D-bifunctional protein (c) and the PTS1 signal peptide SKL (d).

in approximately 40% of the cells, and immunofluorescence with antibodies against the PTS1 signal peptide SKL showed a particle-bound localization in approximately 50% of the cells (Figure 1). In the positive cells, peroxisomes were larger and less abundant. These results indicate that although catalase is almost exclusively localized in the cytosol, other peroxisomal matrix proteins display a mosaic distribution. This may account for the mild biochemical abnormalities found in these cells.

Cell fusion complementation studies revealed that the five patients belong to CG3, with *PEX12* as the causative gene. Sequence analysis of the *PEX12* gene of these patients revealed five different mutations, four of which

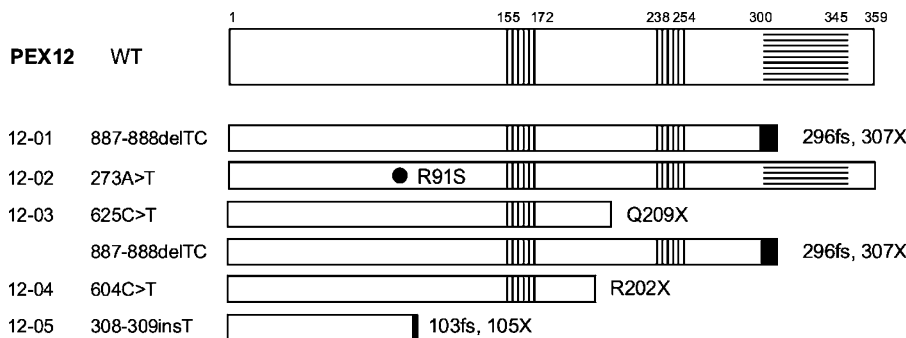


Figure 2 Deduced *PEX12* products of five PBD patients. The diagram shows the predicted protein product of each *PEX12* allele. The zinc-binding domain is indicated by a horizontally striped box and each of the transmembrane domains is indicated by a vertically striped box. The black boxes indicate the length of additional amino acids that are appended as a result of frame-shifting mutations.

have not been reported before. The mutations involve one deletion, one insertion, one missense and two nonsense mutations (Table 2). Patient PEX12-01 was homozygous for a 2-bp deletion (887–888delTC) that was previously described in a patient, who was compound heterozygous for this mutation.¹¹ This mutation results in a frameshift and premature termination of the protein before the COOH-terminal zinc-binding domain (Figure 2). Patient PEX12-02 was homozygous for a missense mutation (R91S) in the N-terminal part of the protein. Patient PEX12-03 was heterozygous for a nonsense mutation (Q209X) that truncates *PEX12* before the second transmembrane domain, and a 2-bp deletion that was also found in patient PEX12-01. Patient PEX12-04 was homozygous for a nonsense mutation (R202X) that truncates *PEX12* before the second transmembrane domain, and patient PEX12-05 was homozygous for a 1-bp insertion (308–309insT) that results in a frameshift and premature termination of the protein before the first transmembrane domain. Thus, four of the five mutations disrupt the translation frame and/or create an earlier termination codon in the *PEX12* open reading frame, and are predicted to result in a truncated protein product (Figure 2) or in no product at all.

In eucaryotic cells, the introduction of a nonsense codon into mRNA can also lead to nonsense-mediated decay of the mRNA and subsequent reduction in protein production, a process common in human genetic disease.^{18,19} To test for this latter possibility as a primary cause of *PEX12* dysfunction in these patients, RNA from the patient cell lines was analyzed by real-time RT-PCR to quantify *PEX12* mRNA. These analyses showed that the levels of *PEX12* mRNA in patient PEX12-02, carrying the missense mutation, were relatively normal (Figure 3). From the four patients with frameshift and/or nonsense mutations, patient PEX12-01, homozygous for the 887–888delTC mutation, displayed relatively normal *PEX12* transcript levels (>80%). The *PEX12* transcript level in patient PEX12-03, compound heterozygous for the mutation in

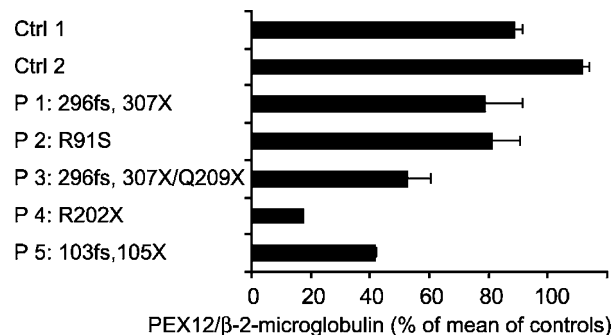


Figure 3 Quantitative real-time RT-PCR analysis of *PEX12*. The *PEX12*/β-2-microglobulin ratios expressed as percentages of the mean of controls 1 and 2 are given.

patient PEX12-01 and the nonsense mutation Q209X, was shown to be 50% of controls, whereas the level in patient PEX12-04 with the nonsense mutation R202X was markedly reduced to 20%. Patient PEX12-05, homozygous for the 308–309insT mutation, showed *PEX12* transcript levels of 40%. Except for patient PEX12-01, these results indicate that *PEX12* transcripts containing a nonsense codon are actively removed by nonsense-mediated decay.

Discussion

Mutations in *PEX12* are known to underlie the disease in patients with a peroxisome biogenesis disorder belonging to CG3.^{8,10} Previous studies have shown a relatively straightforward relationship between genotype and phenotype in seven patients of this group.¹¹ In this study, we determined the *PEX12* genotypes of five additional patients, diagnosed in our laboratory. After having assigned the patients to CG3 by cell fusion complementation studies, we found mutations in the *PEX12* gene of all the five patients, confirming that a defective *PEX12* is indeed responsible for the disease in these patients. Four patients

were apparent homozygotes for a mutation and one patient a heterozygote for two mutations. No parental DNA was available for confirmation of the zygosity. The mutations found involve one deletion, one insertion, one missense and two nonsense mutations. Four of the mutations have not been described previously.

Except for patient PEX12-02, all patients displayed the more severe phenotypes (ZS or NALD) and survived for less than 9 months. Patient PEX12-02 was diagnosed with IRD and survived for 2.5 years. All severely affected patients in our cohort lacked the COOH-terminal zinc-binding domain that is important for PEX12 function and interacts with PEX5 and PEX10.^{13,20} In cells of three of these four patients, reduced *PEX12* mRNA levels were found, which will contribute to a reduced PEX12 function, but cannot explain this entirely. Unfortunately, no antibodies raised against full-length PEX12 are available to study the protein stability of the truncated PEX12 products. The milder affected patient (patient PEX12-02) contained a missense mutation (R91S) in the N-terminus of the protein and, consequently, is predicted to produce full-length PEX12. In 1998, Chang and Gould described a patient with a 2-bp deletion at the N-terminal part of the protein that theoretically would produce an eight amino-acid protein.¹¹ This patient had the IRD phenotype and *in vitro* translation showed that translation was reinitiated at a downstream AUG codon, at position 94. This shows that the first part of the protein is not obligatory for import/function of PEX12. Extrapolation of this result to our own data suggests that the R91S mutation does not have a major deleterious effect on PEX12 function.

We found that, in our cohort, severe defects in PEX12 activity were associated with mutations that truncated PEX12 upstream of the COOH-terminal zinc-binding domain. Mutations in another zinc-binding domain-containing PEX10 have also been reported. In *PEX10*, one mutation leads to truncated PEX10 lacking the zinc-binding domain.^{21,22} All patients homozygous for this mutation were diagnosed with the severe ZS phenotype; so regarding the zinc-binding domain, the genotype-phenotype correlation for *PEX12* seems to be similar to *PEX10*.

Recent studies in fibroblasts have shown that DHAPAT activity, C26:0 β -oxidation and, to a lesser extent, pristanic acid β -oxidation correlate best with patients' survival.²³ The mutations in our cohort correlate rather good with the biochemical markers. All patients with truncated PEX12 proteins have a severely deficient DHAPAT activity and C26:0 and pristanic acid β -oxidation, whereas the IRD patient with the missense mutation has a normal DHAPAT activity, a mildly defective C26:0 and pristanic acid β -oxidation, and a mosaic distribution of peroxisomal matrix proteins, as demonstrated by immunofluorescence with antibodies against D-bifunctional protein and the PTS1 signal peptide SKL. Thus, the genotypes of our CG3

patients show a good correlation with the biochemical and clinical phenotype of the patients.

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