HMG Advance Access published July 28, 2015

A novel type of rhizomelic chondrodysplasia punctata, RCDP5, is caused by loss of the PEX5 long isoform

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Abstract

Import of peroxisomal matrix proteins, crucial for peroxisome biogenesis, is mediated by the cytosolic receptors PEX5 and PEX7 that recognize proteins carrying peroxisomal targeting signals 1 or 2 (PTS1 or PTS2), respectively. Mutations in *PEX5* or twelve other *PEX* genes cause peroxisome biogenesis disorders, collectively named the Zellweger spectrum disorders (ZSD), whereas mutations in *PEX7* cause rhizomelic chondrodysplasia punctata type 1 (RCDP1). Three additional RCDP types, RCDP2-3-4, are caused respectively by mutations in *GNPAT, AGPS* and *FAR1*, encoding enzymes involved in plasmalogen biosynthesis. Here we report a fifth type of RCDP (RCDP5) caused by a novel mutation in *PEX5*. In four patients with RCDP from two independent families, we identified a homozygous frame shift mutation c.722dupA (p.Val242Glyfs*33) in *PEX5* (GenBank: NM_001131023.1). *PEX5* encodes two isoforms, PEX5L and PEX5S, and we show that the c.722dupA mutation, located in the PEX5L specific exon 9, results in loss of PEX5L only. Both *PEX5* isoforms recognize PTS1-tagged proteins, but PEX5L is also a co-receptor for PTS2-tagged proteins. Previous patients with *PEX5* mutations had ZSD, mainly due to deficient import of PTS1-tagged proteins. Similarly to mutations in *PEX7*, loss of PEX5L results in deficient import of PTS2-tagged proteins only, thus causing RCDP instead of ZSD. We demonstrate that PEX5L expression restores the import of PTS2-tagged proteins in patient fibroblasts. Due to the biochemical overlap between RCDP1 and RCDP5, sequencing of *PEX7* and exon 9 in *PEX5* should be performed in patients with a selective defect in the import of PTS2-tagged proteins.

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Introduction

Peroxisomes are subcellular organelles that perform several metabolic functions, e.g. fatty acid α- and β-oxidation and biosynthesis of plasmalogens, a class of membrane glycerophospoholipids (1). Peroxisomal import of matrix proteins, crucial for peroxisome biogenesis, is initiated when proteins carrying peroxisomal targeting signals 1 or 2 (PTS1 or PTS2) are recognized by their cytosolic receptors, encoded by *PEX5* (peroxisome biogenesis factor 5) or *PEX7* (peroxisome biogenesis factor 7) (2).

Mutations in *PEX5* and 12 other *PEX* genes result in peroxisome biogenesis disorder (PBD) group A (1), the Zellweger spectrum disorders (ZSD), including Zellweger syndrome (ZS; MIM 214100) and infantile Refsum disease (IRD; MIM 202370), while mutations in *PEX7* cause PBD group B (1), rhizomelic chondrodysplasia punctata type 1 (RCDP1; MIM 215100) (3-5). Two additional RCDP types, RCDP2 (MIM 222765) and RCDP3 (MIM 600121), caused by mutations in *GNPAT* (glyceronephosphate O-acyltransferase) (6) and *AGPS* (alkylglycerone phosphate synthase) (7), are also disorders of peroxisome function, specifically plasmalogen biosynthesis (1). *FAR1* encodes fatty acyl-CoA reductase 1, producing fatty alcohols used in plasmalogen biosynthesis (8). Mutations in *FAR1* were recently shown to cause peroxisomal fatty acyl-CoA reductase 1 disorder (PFCRD; MIM 616154) (9), also referred to as RCDP4 (10).

The most severe form of the ZSDs is characterized by muscular hypotonia, poor feeding, distinctive facial dysmorphisms, global developmental delay and seizures, hepatic cysts and dysfunction, and usually death in the first year of life (11,12), whereas patients with the milder forms of ZSD may have developmental delays, hearing loss, vision impairment, liver dysfunction and episodes of hemorrhage, with prolonged survival possibly into adulthood (13). Similarly, the severe form of RCDP is characterized by rhizomelia, chondrodysplasia punctata, congenital cataracts, profound growth retardation and intellectual disability, seizures,

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and death within the first decade of life (14-16), while some individuals lack rhizomelia, show less profound growth and developmental delays, and have longer survival (14-16).

Here we describe four patients from two independent families carrying the same novel homozygous frame shift mutation c.722dupA (p.Val242Glyfs*33) in *PEX5* (GenBank: NM_001131023.1). We show that the c.722dupA, located in the PEX5 long (PEX5L) isoform specific exon 9, affects the synthesis of PEX5L only, resulting in a peroxisomal dysfunction due to selective defect in the import of PTS2-tagged proteins, and causing RCDP instead of ZSD. Mutations in *PEX5* affecting PEX5L only are a novel genetic cause of RCDP, suggested by us to be designated RCDP type 5 (RCDP5).

Results

Clinical description

Family A has three affected siblings of first cousin parents from Jammu and Kashmir in Pakistan (Figure 1A). The oldest affected female (FA.II-1; Figure 1A-D) was born at term with normal growth parameters. She had congenital cataracts, and was operated at 5 months. After the age of 18-24 months she showed growth delay and microcephaly. Her cognitive development, assessed at 7, 16 and 27 years of age, was consistent with severe intellectual disability (IQ 20-34, Supplementary Material, Table S1). Hearing assessed at age 7 was normal. At the age of 17 years she developed seizures, and EEG revealed epileptiform activity. Radiological examination at age 27 showed mild shortening of the humeri (3.6 cm < the 10th centile) (17), short femoral necks, narrow ilii and coxa vara**,** in addition to slight thoracic lordosis and scoliosis and short pedicles. At the last clinical examination at 27 years, her height was 10 cm below the 3rd centile, weight for height was at the 10th centile, and her occipitofrontal circumference (OFC) was at the 2.5th centile. She was ambulatory with a broad-based gait. There were signs of peripheral neuropathy with areflexia, generalized

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muscle atrophy in the extremities, contractures of the large joints, swan neck deformity of the hands, pes cavus and pes equinus. Neurophysiological examination suggested pronounced, demyelinating motor and sensory neuropathy with nerve conduction velocities between 12 and 20 m/s (normal > 50 m/s). She had mild palmoplantar keratodermia, but no ichthyosis. Cardiac ultrasound was normal, but a 24 hours EKG investigation registered episodes of sinus tachycardia. Abdominal ultrasound did not reveal organ pathology. She wore aphakic spectacles (right eye +18 and left eye +16). Examination of the fundi did not indicate retinitis pigmentosa. She had a pleasant personality, and performed simple tasks at a work centre.

The second affected female (FA.II-3; Figure 1A) was born with normal growth parameters. Congenital cataracts were operated at 4 months, followed by several operations due to secondary cataracts. Her vision remained severely impaired. After the age of 18 months she showed growth delay and microcephaly, with height and OFC below the 3rd centiles. She had severe bronchial asthma from the first year of life. A gastric feeding tube was placed at 4 years. At the same age she had a vocabulary of three single words and could not sit unaided. Her psychomotor development was regarded as profoundly delayed. Hearing was not formally assessed, but she reacted to sounds and voices. At 8 years peripheral neuropathy was suspected due to muscular atrophy, contractures and hyporeflexia. Walking was limited to short distances with support. She had vertical and horizontal nystagmus. Radiological examination at 5 years revealed punctate calcifications around the hips, mild shortening of the humeri (< 10th centile) (17) irregular metaphyses, small and irregular femoral capital epiphyses**,** narrow ilii and coxa vara, and at 8 years slight s-shaped scoliosis and compression fractures. At age 8 she developed epilepsy, and two EEG examinations showed epileptiform activity. From 9 years she had recurrent respiratory infections, and she died in association with non-convulsive status epilepticus and pneumonia at age 13.

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The youngest child, an affected male (FA.II-6; Figure 1A, E-H), was born at term with normal birth parameters. He had congenital cataracts, operated at 2 months. After 2 years he showed growth delay and microcephaly. Similar to FA.II-3 he developed bronchial asthma, complicated by frequent airway infections. At 3 years he had a vocabulary of about 20 words and walked a few steps independently. His cognitive level, assessed at 6.5 and 10.5 years of age, was consistent with severe intellectual disability (IQ 20-34, Table S1). He mostly spoke single worlds, but could utter some short sentences. Cerebral MRI examination at 7 years was normal. Radiological examinations showed normal length of long bones at age 8 months, but mild shortening of the humeri (< 10^{th} centile) (17) and metaphyseal cupping at 6 years. Radiographs and additional description from skeletal surveys are provided in Figure 2A-D. At the last clinical visit at 10.5 years his height was 19 cm below the 3^{rd} centile, weight for length was at the 50^{th} centile and his OFC was 1.5 cm below the 2.5^{th} centile. He had independent ambulation. Peripheral neuropathy was suspected due to hyporeflexia, muscular weakness and muscular atrophy. Neurophysiological examination was incomplete due to lack of cooperation, but indicated delay of F-responses. He recently developed seizures, and an EEG showed epileptiform activity. Dermatological examination at age 10 years was normal. Abdominal ultrasound at the same age did not reveal organ pathology. He wore aphakic spectacles (right eye +16 and left eye +14.5). A recent electroretinography examination did not indicate retinitis pigmentosa. He had a positive and pleasant behaviour and was attending a special education class, where he had friends and cooperated well.

Family B has a single affected female (FB.II-1; Figure 1I) born to first cousin parents from Punjab in Pakistan. She was born after an uncomplicated pregnancy with normal weight and OFC. She had congenital cataracts, which were operated at 6 months of age with replacement of intraocular lenses. She showed postnatal growth delay and microcephaly. At 3 years her developmental age corresponded to 12-18 months, and she walked independently from 4

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years. Cerebral MRI examination at age 3 was normal. At the last clinical examination at 6.5 years, her height and OFC were both below the 0.4th centile, and weight for height was at the 25th centile. She was ambulant with no contractures, but had raised tone at the ankles. Reflexes were normal and she did not have abnormalities of foot or hand posture to indicate a peripheral neuropathy. At clinical assessment, her impressive and expressive language skills were judged as corresponding to about 12 months, and her gross and fine motor skills to about 18 months. Radiological examination of the spine and left radius and ulna at the age of 7 years was normal. She required help to meet all her care needs.

Identification of a novel variant in the PEX5L specific exon 9 in *PEX5*

Whole-exome sequencing of DNA from individuals FA.I-1, FA.II-1 and FA.II-6 was performed (sequencing statistics are provided in Supplementary Material, Table S2). The genetic variants were filtered using FILTUS (http://folk.uio.no/magnusv/filtus.html) assuming recessive inheritance due to multiple affected siblings. Variants were removed when they occurred with a frequency higher than 0.01 in the 1000 Genomes Project (http://browser.1000genomes.org), the NHLBI Exome Sequencing Project Exome Variant Server (http://evs.gs.washington.edu/EVS), or in our in-house database of 268 exomes, or were predicted to have low impact according to snpEff (18), SIFT (19) or PolyPhen-2 (20). This resulted in homozygous variants in five candidate genes (Supplementary Material, Table S3). Among these, the homozygous c.722dupA (p.Val242Glyfs*33) in *PEX5* (GenBank: NM_001131023.1) was considered to be likely disease-causing because of the established role of *PEX5* in peroxisome biogenesis. *PEX5* encodes a long (PEX5L) and a short (PEX5S) isoform, distinguished by the 111 bp exon 9 (coding exon 7) present in PEX5L only (21,22). The novel c.722dupA variant in *PEX5* is an insertion of one nucleotide after genomic position chr12:7354871, hg19. It is located in exon 9, thus resulting in a frame shift affecting PEX5L

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only, starting with p.Val242Gly and introducing a stop codon 32 codons downstream. The variant was not reported in the Exome Aggregation Consortium Browser (http://exac.broadinstitute.org/) containing more than 60 000 exomes (accessed April 28th 2015). Sanger sequencing confirmed that the affected siblings FA.II-1, FA.II-3 and FA.II-6 were homozygous for the c.722dupA variant in *PEX5*, and both parents were heterozygous (Figure 1A and J, Supplementary Material, Table S4). The c.722dupA (p.Val242Glyfs*33) variant in *PEX5* was submitted to the ClinVar database (http://www.ncbi.nlm.nih.gov/clinvar), accession number SCV000222895.

FB.II-1 was independently referred for metabolic analyses due to suspicion of a peroxisomal disorder. The results of the studies of peroxisomal parameters in cultured fibroblasts indicated a PTS2 protein import defect and thus *PEX7* sequencing was performed, followed by *PEX5* sequencing. This revealed no pathogenic mutations in *PEX7*, but identified the same homozygous c.722dupA (p.Val242Glyfs*33) variant in *PEX5* (Figure 1I and J).

The novel variant in *PEX5* results in selective loss of PEX5L

We assessed the predicted loss of PEX5L in fibroblasts from the patients. Quantitative real time PCR (qRT-PCR) showed that the mean PEX5L transcript level in fibroblasts from FA.II-3 and FA.II-6 was 25% of the level in controls (Figure 3A), indicating it was subjected to nonsense-mediated decay. Furthermore, immunoblot analysis using an antibody which recognizes both *PEX5* isoforms detected two bands, corresponding to PEX5L and PEX5S in control fibroblasts, whereas only the single band corresponding to PEX5S was detected in fibroblasts from FA.II-1, FA.II-3 and FB.II-1 (Figure 3B). No bands corresponding to the two *PEX5* isoforms were present in the *PEX5* deficient human fibroblast cell line, PEX5.11 (Figure 3B). This confirmed that the novel c.722dupA variant in *PEX5* leads to a selective loss of the PEX5L isoform.

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Loss of PEX5L results in a selective peroxisomal import defect of PTS2-tagged proteins

In order to characterize the peroxisomal dysfunction resulting from the selective loss of PEX5L, biochemical parameters relevant for import of PTS1- and PTS2-tagged proteins were measured in blood from the three living patients (FA.II-1, FA.II-6 and FB.II-1), and in fibroblasts from all four patients (Table 1 and 2). Most peroxisomal matrix proteins are targeted to the organelle by a PTS1 (2), including several enzymes involved in peroxisomal fatty acid β-oxidation (23). Accumulation of very long chain fatty acids (VLCFAs) is the biochemical hallmark in ZSDs (24,25), whereas levels are normal in RCDP (26). PTS1-mediated peroxisomal protein import was evaluated by measuring the amount of VLCFAs in plasma, and the peroxisomal β-oxidation capacity in fibroblasts using C26:0 and pristanic acid as substrates. The results were within reference ranges (Table 1), except for a mildly elevated C26/C22 ratio in individual FB.II-1 in plasma (Table 1), but not in fibroblasts (0.05, ref. 0.03-0.07), confirming normal import of the PTS1-tagged enzymes involved in peroxisomal β-oxidation. PTS1-targeted protein import was further evaluated by immunoblot analysis using an antibody against the first enzyme of the peroxisomal β-oxidation system, acyl-CoA oxidase (ACOX1). This revealed normal intra-peroxisomal processing of ACOX1 in fibroblasts from all four patients similar to the control fibroblasts, indicating normal import of ACOX1 in these cell lines (Figure 4A). In contrast, a ZSD human fibroblast cell line only showed unprocessed ACOX1 (Figure 4A). Furthermore, immunofluorescence microscopy analysis performed using an antibody against PTS1-tagged catalase, a commonly used marker for PTS1 protein import (27). The results showed peroxisomal staining in control cells (Figure 4B) as well as in fibroblasts from all patients (Table 1, Figure 4C), indicating normal peroxisomal import of PTS1-tagged catalase.

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Three PTS2-tagged peroxisomal matrix proteins are known: AGPS, involved in plasmalogen biosynthesis (7), phytanoyl-CoA 2-hydroxylase (PHYH) (28), involved in phytanic acid α-oxidation, and acetyl-CoA acyltransferase 1 (ACAA1) (29), which has a redundant function in fatty acid β-oxidation (30). All RCDP types are characterized by low levels of plasmalogens (31-33), while patients with RCDP1 in addition show accumulation of phytanic acid and reduced intra-peroxisomal processing of the precursor form of ACCA1 (26,34). Peroxisomal import of PTS2-tagged proteins was evaluated by measuring the levels of plasmalogens in erythrocytes or fibroblasts, and the activity of the PTS1-targeted GNPAT in fibroblasts. Reduced activity of GNPAT is typically observed in cells with deficient import of the PTS2-targeted AGPS (35,36). The plasmalogen levels were mildly reduced in all four patients, as was the GNPAT activity measured in FA.II-1, FA.II-3 and FA.II-6 (Table 2), indicating deficient import of AGPS. Import of PTS2-tagged PHYH was assessed by measuring the level of phytanic acid in plasma from the patients, and the phytanic acid α-oxidation in fibroblasts. The levels of phytanic acid were highly elevated (Table 2), and phytanic acid α-oxidation markedly reduced (Table 2), indicating deficient import of PHYH. Immunoblot analysis using an antibody against PTS2-tagged ACAA1, showed peroxisomal processing in the control cells, while only the unprocessed ACAA1 was detected in all four patients, as well as in a peroxisome deficient ZSD cell line (Table 2, Figure 4D), indicating deficient import of ACAA1 in these cell lines. Finally, immunofluorescence microscopy analysis using the same ACAA1 antibody showed peroxisomal staining in control cells (Figure 4E), while the patient cells showed cytoplasmic staining (Table 2, Figure 4F), further confirming the deficient peroxisomal import of ACAA1.

Together, the results confirm that the selective loss of PEX5L in the patients results in a peroxisomal dysfunction due to deficient import of PTS2-tagged proteins only.

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Expression of PEX5L in patient fibroblasts restores the peroxisomal import of PTS2-tagged proteins

In order to confirm that the isolated defect in import of PTS2-tagged proteins was indeed caused by loss of PEX5L, we ectopically expressed either PEX5L or PEX5S in fibroblasts from FA.II-3 and the *PEX5* deficient ZSD human fibroblast cell line PEX5.11, aiming to restore the peroxisomal import of PTS2-tagged proteins. The cells were co-transfected either with PTS2-tagged green fluorescent protein (PTS2-GFP) and PEX5S, or with PTS2-GFP and PEX5L. Both the ZSD cell line and the patient fibroblasts transfected with PEX5S showed a cytoplasmic fluorescence pattern (Figure 5A and B), indicating that PEX5S could not restore peroxisomal import of PTS2-tagged proteins in either cell line. In contrast, both cell lines transfected with PEX5L showed a peroxisomal fluorescence pattern consistent with restored import of PTS2-tagged proteins (Figure 5C and D). These results confirm that loss of PEX5L is responsible for the peroxisomal dysfunction due to the isolated defect in import of PTS2-tagged proteins in our patients.

DISCUSSION

Previously reported patients with mutations in *PEX5* had ZSD (21,37-39), primarily due to deficient import of PTS1-tagged proteins, but often accompanied by impaired import of PTS2-tagged proteins (37). The patients with the novel c.722dupA (p.Val242Glyfs*33) mutation in *PEX5* lacked several features typically associated with ZSD, such as hepatic dysfunction and sensorineural hearing loss (11-13). In contrast, some of their key features such as congenital cataracts and chondrodysplasia punctata are usually associated with RCDP (14-16). Altogether, the phenotype in our patients, including congenital cataracts, postnatal growth delay, microcephaly and severe intellectual disability in all four patients, mild

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shortening of humeri in three patients, and chondrodysplasia punctata in two patients, was consistent with RCDP (14-16).

Both PEX5L and PEX5S recognize PTS1-tagged proteins (40), but PEX5L is also a co-receptor critical for PEX7-mediated import of PTS2-tagged proteins (21,41,42). This suggested that the novel c.722dupA mutation in *PEX5* demonstrated to result in a selective loss of PEX5L, might cause RCDP due to an isolated defect in import of PTS2-tagged proteins. Indeed, the normal import of PTS1-tagged proteins in the patients excluded that they have ZSD, and their isolated defect in the peroxisomal import of PTS2-tagged proteins is similar to the peroxisomal defect in RCDP1, thus explaining their RCDP. Ectopic expression of PEX5L restored the peroxisomal import of PTS2-tagged proteins in cultured patient fibroblasts, thus we prove that the homozygous c.722dupA mutation in *PEX5* causes selective loss of PEX5L, resulting in an isolated defect in the peroxisomal import of PTS2-tagged proteins, and thereby give rise to RCDP5.

Plasmalogen deficiency is regarded as the primary cause of RCDP (31-33). Plasmalogens are components of cellular and subcellular membranes, as well as specialized membranes, such as myelin, and secreted membranes, such as synaptic vesicles (31). The plasmalogen level is relatively low in the brain of newborns, but increases during development to reach its peak around 30 years of age (31,43). Plasmalogens provide structural support to membranes, functions as a reservoir for lipid second messengers, and protect against oxidative damage (31,44). However, the pathomechanism of plasmalogen deficiency in RCDP is unknown (31). Our patients with RCDP5 had a less pronounced reduction in tissue plasmalogen level compared to patients with severe RCDP, and their clinical phenotype is analogous to other RCDP patients with milder biochemical defects, who often have less pronounced skeletal abnormalities, later onset of epilepsy, and less profound growth delay and intellectual disability (14-16,36). Similar to other RCDP patients with partial plasmalogen deficiency,

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patients with RCDP5 reached more developmental milestones, such as self-feeding, independent ambulation and limited impressive and expressive language skills, than most RCDP patients with severely reduced plasmalogen levels (14-16,36), but it is still apparent that even partial plasmalogen deficiency has significant consequences. Bilateral cataract does not seem to correlate with the severity of the plasmalogen deficiency, as it is a consistent finding in RCDP patients and knock out mouse models with both severe and partial plasmalogen deficiencies (14-16,36,45-47). PEX5L is regarded as critical for PEX7-mediated peroxisomal import of PTS2-tagged proteins (21,41,42), but loss of PEX5L did not result in the most severe clinical and biochemical RCDP phenotypes in the RCDP5 patient. This suggests that PEX7 sustains low level import of the PTS2-targeted AGPS in the absence of the co-receptor PEX5L.

Similarly to patients with RCDP1 (15,16), the patients in this study had accumulation of phytanic acid in plasma, due to deficient import of PTS2-tagged PHYH. Accumulation of phytanic acid due to mutations in *PHYH*, results in adult Refsum disease (ARD; MIM 266500) (28), characterized by onset from adolescence to adulthood with anosmia, early-onset retinitis pigmentosa and variable combinations of neuropathy, deafness, ataxia, and ichthyosis (48-51). ARD patients are also at risk of developing cardiac arrhythmias (48,49). Clinical and neurophysiological studies in the three affected siblings in family A indicated they had demyelinating peripheral neuropathy, which to the best of our knowledge has not been reported in RCDP, but is an important sign of ARD (48,49). However, additional signs of ARD were not recognized in our patients (anosmia was not assessed). Phytanic acid accumulates with age and in a diet dependent manner in patients with deficient phytanic acid α-oxidation (52). Due to the considerably elevated levels of phytanic acid, a diet restricted in phytanic acid was recently initiated in the patients, aiming to prevent toxic effects of accumulation.

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To conclude, we report a novel c.722dupA mutation in *PEX5*, the first to exclusively affect PEX5L expression, and that unlike previous mutations in *PEX5*, results in RCDP instead of ZSD. The peroxisomal dysfunction in the patients was due to an isolated defect in import of PTS2-tagged proteins, previously described from mutations in *PEX7* only. Mutations in *PEX5* resulting in a selective loss of PEX5L represents a fifth type of RCDP. Due to the clinical and biochemical overlap between RCDP1 and RCDP5, sequencing of *PEX7* and the PEX5L specific exon 9 in *PEX5* should be considered in patients with an isolated defect in peroxisomal import of PTS2-tagged proteins

Materials and Methods

Consent

This study was approved by the Norwegian South-Eastern Regional Ethics Committee. Informed written consent was obtained from the parents in both families investigated.

Whole-exome sequencing

Genomic DNA from FA.I-1, FA.II-1 and FA.II-6 was used to capture exomes with the Illumina TruSeq Exome Enrichment kit and sequenced on an Illumina HiSeq2000 with 100 bp paired-end reads. Alignment against the GRCh37 human reference genome was performed with a Burrows-Wheeler Aligner (BWA, v.0.5.9) (53) PCR-duplicates marking and removal with Picard (v.1.104), and indel realignment, base quality recalibration, and joint variant calling with the Genome Analysis Toolkit (GATK, v.2.5) (54). Finally, functional annotation was performed with snpEff (18) and Variant Effect Predictor (VEP) (55) using Ensembl 71. Variants were analyzed with FILTUS (v.99-9) (http://folk.uio.no/magnusv/filtus.html), assuming recessive inheritance due to parental consanguinity. Variants with a high probability

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of being technical artifacts, as computed by the GATKs "variant quality score recalibration" procedure, were removed in the filtering process.

Sanger sequencing

Sanger sequencing was performed with Big Dye™ Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems) on an ABI 3730 sequencer (Applied Biosystems), using primers for *PEX5* (37) and *PEX7* (56) in individual FB.II-1 as described, and primers for validation and segregation analysis for the novel variant in *PEX5* in family A provided in Supplementary Material, Table S4.

qRT-PCR analysis

RNA from fibroblasts was converted to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qRT-PCR amplifications were performed using the SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich) on a QuantStudio™ 12K Flex Real-Time PCR System (Applied Biosystems). Samples were run in triplicates, and amplification levels were calculated according to the 2-ΔΔCt method (57). Two PEX5L specific primer sets and two primer sets amplifying *PMM1* and *GADPH* transcripts as endogenous references were used and provided in Supplementary Material, Table S5. Dissociation curve analysis revealed a single product for each primer pair. Negative template controls and genomic DNA controls (cDNA synthesis reactions without RT enzyme) did not show amplification. Statistical significance was evaluated using a two tailed student's t-test.

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Cell culture

Skin biopsies were obtained and cultured according to standard procedures. Fibroblasts were transfected as described (37). A cell line deficient of both *PEX5* isoforms, PEX5.11 (37), was used as control in several experiments.

Biochemical analysis

Concentrations of VLCFAs and phytanic acid in plasma (58), and peroxisomal phytanic acid α-oxidation (59), peroxisomal β-oxidation using C26:0 and pristanic acid as substrates (60), and the activity of GNPAT (61) were measured in cultured fibroblasts as described.

Immunoblot analysis

Immunoblot analysis was performed on protein extracts from cultured fibroblasts using an antibody against ACOX1 (in house generation), ACAA1 (Atlas antibodies, article number HPA007244) and PEX5 (Proteintech Europe, 12545-1-AP) as described (62).

Immunofluorescence microscopy analysis

Immunofluorescence microscopy analysis was performed on cultured fibroblasts using an antibody against catalase (in house generation) and ACAA1 (Atlas antibodies, HPA007244) as described (27).

Supplementary Material

Supplementary Material is available on HGM online.

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Acknowledgements

We are grateful to the families who participated in this study. We thank Madeleine Fannemel, Kristin Ørstavik, Eli Ormerod and Heidi-Karin Lundlie from Oslo University Hospital, Oslo, for genetic counselling, electrophysiological studies, taking skin biopsies and collecting skeletal images, respectively. We thank Petra Mooyer and Conny Bakker-Dekker from the Laboratory Genetic Metabolic Diseases, Academic Medical Center, Amsterdam, for the study of peroxisomal parameters in fibroblasts. This study was supported by the Norwegian Southeastern Regional Health Authorities [2011071]; and "Legatet til Henrik Homans Minde" to TB; and "Anders Jahres fond til vitenskapens fremme" to DM. The sequencing service was provided by the Norwegian High-Throughput Sequencing Centre, a national technology platform supported by the 'Functional Genomics' and 'Infrastructure' programs of the Research Council of Norway and the Southeastern Regional Health Authorities.

Conflict of Interest statement.

None declared.

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Legends to Figures

Figure 1. Pedigrees and pictures of affected individuals. (A) Pedigree of family A (FA), (B-D) FA.II-1 at 27 years of age, (B) note bushy eyebrows and thick spectacle prisms after removal of cataracts, (C) swan neck deformity of the hands, (D) and distal muscle wasting with pes equinus deformity, compatible with chronic demyelinating neuropathy. (E-H) FA.II-6 at 9.5 years of age (E, F and H) and 10.5 years of age (G), (E) note similar bushy eyebrows and thick spectacle prisms after removal of cataracts as in his elder sister, (F) similar signs of neuropathy with swan neck deformity of the hands, (G) and muscle wasting in the lower extremities, but without foot deformity, (H) and shortening of the extremities and multiple joint contractures (elbow, hip, knee), thoracic kyphosis and scoliosis. (I) Pedigree of family B (FB), (J) chromatogram of the Sanger sequence showing the heterozygous c.722dupA mutation in *PEX5* in FA.I-1 and FA.I-2 (top), and the homozygous c.722dupA mutation in FA-II-1, FA.II-3, FA.II-6 and FB.II-1 (bottom). fs, frame shift.

Figure 2. Radiographs of patient FA.II-6. (A) Radiograph of the knees at 8 months showing extensive punctate calcification (indicated by arrow). (B) Pelvis at 6 years showing narrow ilii, small and fragmented capital femoral epiphyses (indicated by arrow) with subtle calcifications. (C and D) Frontal and lateral view of the spine at 11 years showing slight s-shaped scoliosis, osteopenia and anisospondyly.

Figure 3. Loss of PEX5L in patient fibroblasts. **(**A) Relative quantification of the PEX5L transcript level in fibroblasts from patients FA.II-3 and FA.II-6 using two independent primer sets amplifying PEX5L only (PEX5L-1 and PEX5L-2) showed a significant reduction in the mean level compared to five controls ($p < 0.005$). Data from a representative experiment is

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presented as the mean PEX5L level in patients (n=2) and controls (n=5). *p < 0.005. Error bars represents the 95% confidence intervals. (B) Immunoblot analysis using an antibody binding to both PEX5L and PEX5S isoforms showing the band corresponding to PEX5L in HEK293T cells transfected with PEX5L (PEX5L) and the band corresponding to PEX5S in HEK293T cells transfected with PEX5S (PEX5S). Both isoforms are detected in fibroblasts from controls (C1 and C2), while only the band corresponding to PEX5S was detected in the three patients FA.II-3, FA.II-1 and FB.II-1. The *PEX5* deficient fibroblast cell line PEX5.11 lacked bands for both PEX5 isoforms. Two different exposure times are shown. Equal loading was confirmed by Ponceau-S staining.

Figure 4. Selective defect in import of PTS2-tagged proteins in patient fibroblasts. (A) Immunoblot analysis using an antibody against PTS1-tagged ACOX1 in fibroblasts showed bands corresponding both to the unprocessed 72 kDa ACOX1 and the processed 50 kDa ACOX1 in control (C1), and in all four patients (FA.II-6, FA.II-1, FA.II-3, FB.II-1). Only unprocessed 72 kDa ACOX1 was detected in a peroxisome deficient ZSD cell line (ZSD). These results indicated normal import of ACOX1, which is cleaved from the 72 kDa precursor to the 50 kDa ACOX1 protein after peroxisomal entry. (B) Immunofluorescence microscopy analysis using an antibody against PTS1-tagged catalase in fibroblasts showed peroxisomal staining in control (B) as well as in all four patients; shown is cells from FA.II-3 (C). (D) Immunoblot analysis using an antibody against ACAA1 in fibroblasts showed a band corresponding to the processed 41 kDa ACAA1 in the control (C1), while only a band corresponding to the unprocessed 44 kDa ACAA1 was detected in the four patients (FB.II-1, FA.II-3, FA.II-1 and FA.II-6), as well as in cells from a peroxisome deficient ZSD cell line (ZSD), indicating abnormal peroxisomal processing of ACAA1 due to deficient import of PTS2-tagged ACAA1 in these cell lines. (E) Immunofluorescence microscopy analysis using the same antibody against ACAA1 showed peroxisomal staining in control cells (E), whereas

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the staining was cytosolic in cells from the four patients, indicating deficient peroxisomal import of PTS2-tagged ACAA1; shown are cells from FA.II-3 (F).

Figure 5. PEX5L expression restored import of PTS2-tagged proteins in patient fibroblasts. (A and B) Fibroblasts from FA.II-3 and a *PEX5* deficient cell line, PEX5.11 were co-transfected with PEX5S and PTS2-GFP (C and D) or PEX5L and GFP-PTS2. A cytoplasmic fluorescence pattern compatible with defective import of PTS2-tagged proteins was observed in both cell lines transfected with PEX5S (A and B), whereas both cell lines transfected with PEX5L showed a peroxisomal fluorescence pattern compatible with restored import of PTS2-tagged proteins.

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Tables

Table 1. Normal peroxisomal import of PTS1-tagged proteins in patients with the *PEX5* c.722dupA

Reference values are from Laboratory Genetic Metabolic Diseases, Amsterdam, The Netherlands. [a]Reference values from Laboratory Medicine, Sheffield Children's Hospital, Sheffield, UK: C26:0, 0.33-1.5 µmol/l; C26/C22, 0.005-0.030. The two measurements were performed one month apart at age 5.5 years. [b]The values for RCDP1-3 are the same as for the reference. FBRO, fibroblasts; IB, immunoblot; IF, immunofluorescence; ND, not done; peroxisomal processing, cleavage of the 72 kDa precursor to the 50 kDa protein, PLA, plasma; VLCFAs, very long chain fatty acids; ZS, Zellweger syndrome.

	VLCFAs (PLA) µmol/l		β-oxidation (FBRO) pmol/(hr x mg) protein		IB ACOX1 (FBRO) peroxisomal processing	IF catalase (FBRO) peroxisomal staining
	C26	C26:0/C22:0	C26:0	Pristanic acid		
FA.II-1	0.92	0.02	983	1010	+	+
FA.II-3	ND	ND	830	1627	+	+
FA.II-6	0.57	0.01	815	1098	+	+
FB.II-1	1.3, 1.45[a]	0.037, 0.045[a]	ND	ND	+	+
Reference[b]	0.45-1.32	0.00-0.02	800-2040	790-1690	+	+
ZS	1.8-8.1	0.069-0.453	50-350	0-30	-	-

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Table 2. Deficient peroxisomal import of PTS2-tagged proteins in patients with the *PEX5* c.722dupA

Reference values are from Laboratory Genetic Metabolic Diseases, Amsterdam, The Netherlands. [(a)]Measurement in fibroblasts using two controls showing values for C16:0 Dimethylacetal (DMA)/C16:0 of 12.0% and 12.9% and for C18:0 DMA/C18:0 of 8.9% and 9.9%. [(b)]Reference values from Laboratory Medicine, Sheffield Children's Hospital, Sheffield, UK: PLA phytanic acid, <19.3 µmol/l. The two measurements were performed one month apart at age 5.5 years. [(c)]Can be normal, increases with age. ERY, erythrocytes; FBRO, fibroblasts; IB, immunoblot; IF, immunofluorescence; ND, not done; PLA, plasma; peroxisomal processing, peroxisomal cleavage of 44 kDa ACAA1 to 41 kDa; ZS, Zellweger syndrome.

	Plasmalogens (ERY or FBRO) %		GNPAT activity (FBRO) nmol/(2hr x mg protein)	Phytanic acid (PLA) µmol/l	α-oxidation (FBRO) pmol/(hr x mg protein) phytanic acid	ACAA1 (FBRO)	
	C16 DMA/C16:0	C18:0 DMA/C18:0				IB peroxisomal processing	IF peroxisomal staining
FA.II-1	2.2	7.2	3.8	427	7	-	-
FA.II-3	ND	ND	4.3	ND	11	-	-
FA.II-6	2.5	6.7	4.3	343	8	-	-
FB.II-1	3.8[(a)]	2.7[(a)]	4.6, 4.8	360, 397[(b)]	ND	-	-
Reference	6.8-11.9	10.6-24.9	5.4-10.6	0.5-9.9	28-95	+	+
RCDP1	<6.8	<10.6	1.0-4.2	>9.9[(c)]	≤22	-	-
RCDP2	<6.8	<10.6	<0.1	0.5-9.9	28-95	+	+
RCDP3	<6.8	<10.6	1.0-4.2	0.5-9.9	28-95	+	+
ZS	<6.8	<10.6	0.1-0.9	1.6-115.7	0-10	-	-

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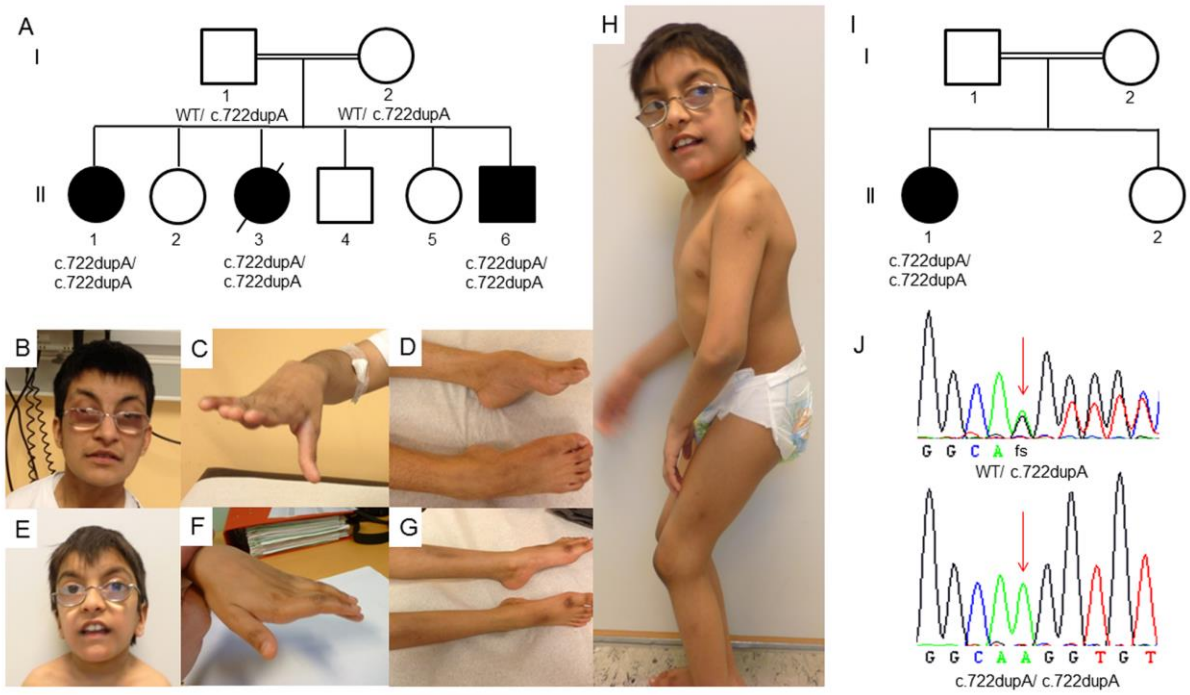

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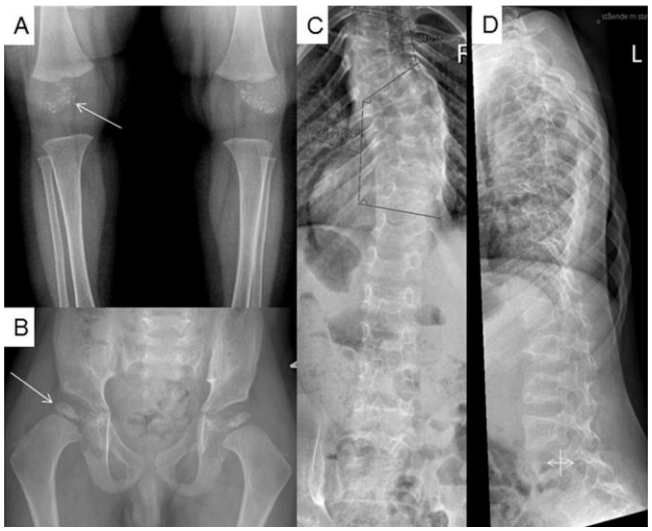

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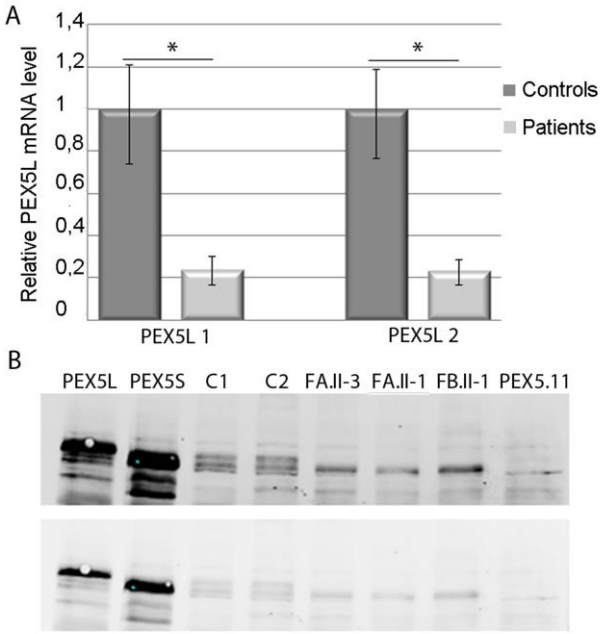

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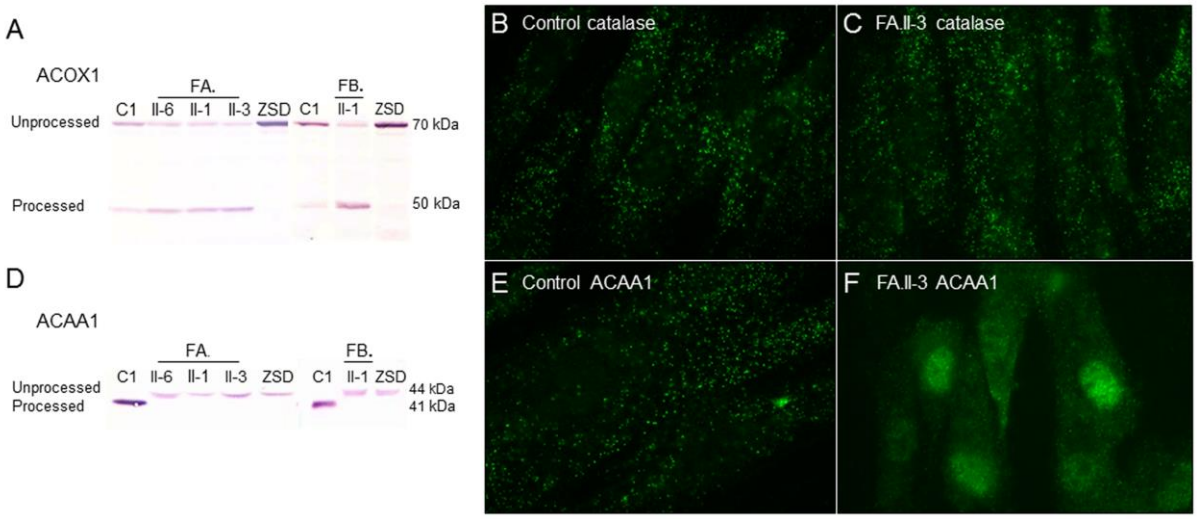

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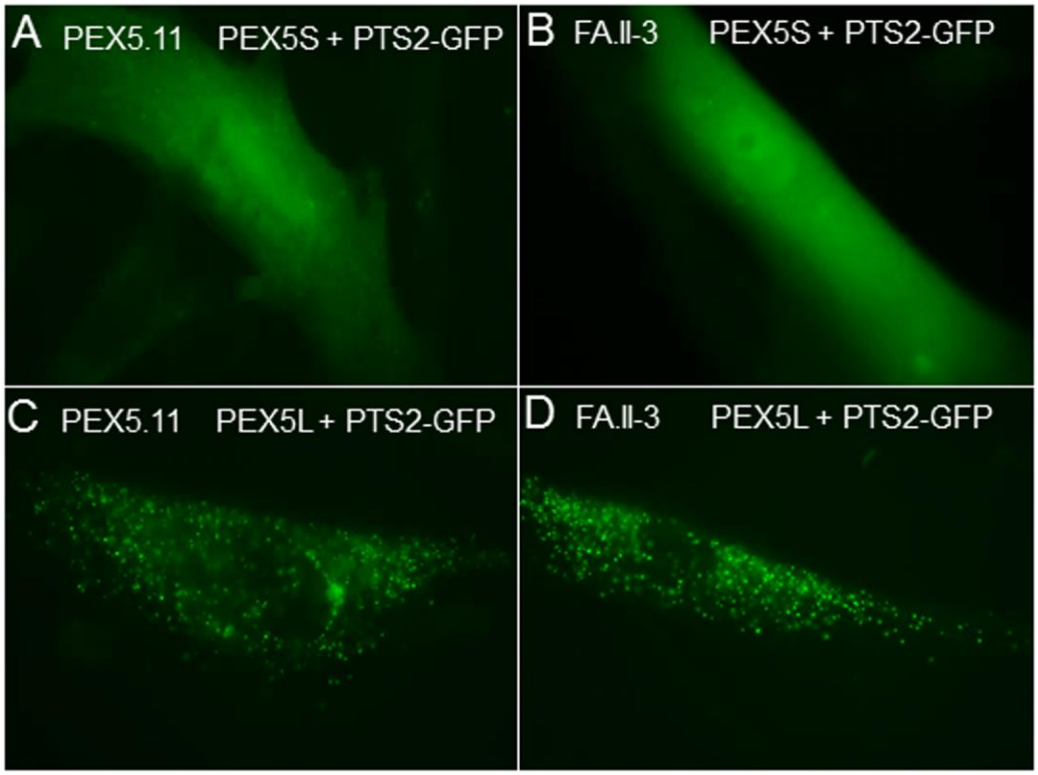

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