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Identification of Novel Mutations and Sequence Variation in the Zellweger Syndrome Spectrum of Peroxisome Biogenesis Disorders

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Abstract

Peroxisome biogenesis disorders (PBD) are a heterogeneous group of autosomal recessive neurodegenerative disorders that affect multiple organ systems. Approximately 80% of PBD patients are classified in the Zellweger syndrome spectrum (PBD-ZSS). Mutations in the *PEX1*, *PEX6*, *PEX10*, *PEX12*, or *PEX26* genes are found in approximately 90% of PBD-ZSS patients. Here, we sequenced the coding regions and splice junctions of these five genes in 58 PBD-ZSS cases previously subjected to targeted sequencing of a limited number of *PEX* gene exons. In our cohort, 71 unique sequence variants were identified, including 18 novel mutations predicted to disrupt protein function and 2 novel silent variants. We identified 4 patients who had two deleterious mutations in one *PEX* gene and a third deleterious mutation in a second *PEX* gene. For two such patients, we conducted cell fusion complementation analyses to identify the defective gene responsible for aberrant peroxisome assembly. Overall, we provide empirical data to estimate the relative fraction of disease-causing alleles that occur in the coding and splice junction sequences of these five *PEX* genes and the frequency of cases where mutations occur in multiple *PEX* genes. This information is beneficial for efforts aimed at establishing rapid and sensitive clinical diagnostics for PBD-ZSS patients and interpreting the results from these genetic tests.

Keywords

peroxisome biogenesis disorders; Zellweger syndrome; PBD-ZSS; neonatal adrenoleukodystrophy; infantile Refsum disease; *PEX1*; *PEX6*; *PEX10*; *PEX12*

INTRODUCTION

Peroxisomes are organelles present in almost all eukaryotic cells (Purdue and Lazarow, 2001; Schluter, et al., 2007; Wanders and Waterham, 2006). In humans, they are essential for the metabolism of branched chain and very long chain fatty acids, ether lipids, polyamines, amino acids, and glyoxylate (Schluter, et al., 2007; Steinberg, et al., 2006; Wanders and

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Waterham, 2006). During some of these metabolic processes, they generate and subsequently inactivate reactive oxygen species (Schrader and Fahimi, 2006).

Based on genetic, bioinformatic, and proteomic analyses, it has been estimated that at least eighty-five proteins are associated with peroxisome structure and function in humans (Schluter, et al., 2007). Peroxisome matrix proteins are synthesized on free ribosomes in the cytosol prior to import into the peroxisome. Peroxins, encoded by a family of sixteen human *PEX* genes (Steinberg, et al., 2004), are involved in peroxisome biogenesis with functions ranging from membrane synthesis and matrix protein import to organelle division.

Peroxisomal biogenesis disorders (PBD; MIM# 601539) are inherited in an autosomal recessive manner and are characterized by impaired peroxisome assembly (Wanders and Waterham, 2005; Weller, et al., 2003). Due to their heterogeneity, PBDs had been divided into four groups: Zellweger syndrome (ZS; MIM# 214100), neonatal adrenoleukodystrophy (NALD; MIM# 202370), infantile Refsum disease (IRD; MIM# 266510), and rhizomelic chondrodysplasia punctata (RCDP; MIM# 215100) Type 1. ZS, NALD, and IRD comprise the Zellweger syndrome spectrum (PBD-ZSS). Approximately 80% of PBD patients fall within the Zellweger syndrome spectrum, which has an overall incidence of 1:50,000 births in the United States and 1:500,000 births in Japan (Krause, et al., 2006; Shimozawa, et al., 2003; Steinberg, et al., 2004). PBD-ZSS patients with severe disorders generally die within the first year of life, while those with milder disorders can survive into young adulthood, but typically exhibit multiple organ dysfunction, sensorineural hearing loss, pigmentary retinal degeneration, and psychomotor impairments. This reflects the crucial roles peroxisomes play in neuronal migration, proliferation, differentiation and survival (Faust, et al., 2005; Krysko, et al., 2007; Powers and Moser, 1998).

It has been estimated that over 90% of PBD-ZSS patients have mutations in the *PEX1* (MIM# 602136), *PEX6* (MIM# 601498), *PEX10* (MIM# 602859), *PEX12* (MIM# 601758) or *PEX26* genes (MIM# 608666) (Steinberg, et al., 2004). *PEX1* and *PEX6* mutations account for ~70% and ~10% of PBD-ZSS patients, respectively (Steinberg, et al., 2004). PEX1 and PEX6 belong to the AAA (ATPases Associated with diverse cellular Activities) ATPase family of proteins and form heterodimers associated with the cytoplasmic side of peroxisome membranes. These heterodimers are involved in the recycling of *PEX5*, the receptor for proteins with peroxisomal targeting signals (PTS) (Tamura, et al., 2006; Thoms and Erdmann, 2006). In contrast, *PEX10*, *PEX12*, and *PEX26* mutations are less frequently observed in PBD-ZSS patients (Steinberg, et al., 2004). PEX26 is a peroxisomal membrane protein that assists in the localization of PEX1-PEX6 complexes to the peroxisomal membrane (Furuki, et al., 2006; Tamura, et al., 2006). PEX10 and PEX12 comprise part of a RING-finger complex of peroxisomal membrane proteins that function downstream of the initial association of cargo-laden PEX5 with its peroxisomal docking complex (Chang, et al., 1999; Okumoto, et al., 2000).

Here, we surveyed the mutational spectrum of the coding regions and splice junctions of the *PEX1*, *PEX6*, *PEX10*, *PEX12*, and *PEX26* genes in a cohort of fifty-eight PBD-ZSS patients. Overall, we identified eighteen novel sequence variants predicted to disrupt protein function and two novel sequence variants predicted to be neutral. Based on these results, we estimate the relative fraction of point mutations in the coding and splice junction sequences of these five *PEX* genes in PBD-ZSS patients. This information is valuable for predicting the sensitivity and specificity of existing and future genetic tests for the diagnosis of PBD-ZSS.

MATERIALS AND METHODS

Preparation of genomic DNA

Genomic DNAs extracted from cultured PBD-ZSS patient fibroblasts were obtained from the Kennedy Krieger Institute Peroxisomal Diseases Laboratory. Whole genome amplification of these samples was conducted using the Repli-G Mini Kit (Qiagen) and the manufacturer's recommended protocols.

Amplification

Primers flanking *PEX* gene coding exons of interest were designed using the Primer3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) (Supp. Table S1). The GenBank reference numbers for each *PEX* gene were as follows: *PEX1* (NM_000466.2), *PEX6* (NM_000287.2), *PEX10* (NM_002617.3), *PEX12* (NM_000286.1), and *PEX26* (AB089678.1). To simplify sequencing procedures, we appended T3 (5′-ATTAACCCTCACTAAAGGGA-3′) and T7 (5′-TAATACGACTCACTATAGGGA-3′) promoter sequences to the 5′-end of forward and reverse primers, respectively. PCR reactions were set up in 25 μL containing 60 ng of template DNA, 20 μM of each primer, 2.5 μL of 10× PCR buffer, 2.5 o μL of 25 mM $MgCl_2$, 1.25 μL of 10 mM dNTPs and 2 units of AmpliTaq Gold polymerase (Applied Biosystems). Thirty-five cycles (94 °C for 30 seconds, 60–68 °C for 30 seconds, and 72 °C for 90 seconds) of PCR were performed followed by a final extension at 72 °C for 10 minutes. DNA-free controls were run in parallel to monitor for template contamination. All template-free control reactions and randomly selected reactions with templates were analyzed on agarose gels to verify the presence of appropriately sized products. When no product was observed, 2% or 4% of DMSO was added to replicate reactions to increase product yields.

Sequencing

All amplicons were treated with 10 units of exonuclease (Amersham) and 1 unit of shrimp alkaline phosphatase (Amersham) according to manufacturer's recommended protocols in order to remove excess primer and dNTPs. Treated PCR products were sequenced using forward T3 (5′-ATTAACCCTCACTAAAGGGA-3′) and reverse T7 (5′-TAATACGACTCACTATAGGGA-3′) primers and the DNA sequencing kit with BigDye Terminator V3.0 from Applied Biosystems, according to manufacturer's recommendations. However, the BigDye Terminator was diluted 1:4 with halfBD (Genetix Ltd).

Sequencing reactions were evaluated on the ABI3700 DNA Sequencer (Applied Biosystems, Inc.) at the DNA Core Facility of the University of Southern California Norris Comprehensive Cancer Center. Sequence variants were detected using Sequencher software V4.6 (Gene Codes Corp.). In cases where only a single deleterious *PEX* allele was detected, the sequencing chromatograms of the *PEX* gene with the single deleterious allele was inspected by eye to maximize the possibility of detecting the second allele. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines (www.hgvs.org/mutnomen). The initiation codon is codon 1.

In instances where insertions and deletions were detected in the coding regions or flanking splice junctions of *PEX* exons and the nature of the mutation could not be unambiguously ascertained by analyzing the sequence traces, the appropriate amplicons were subcloned using the TA TOPOII Cloning Kit (Invitrogen) and individual subclones were sequenced to confirm the identity of mutations. In all cases, the recommended mutation nomenclature guidelines provided on the Human Genome Variation Society (HGVS) website (http://www.hgvs.org/mutnomen) were followed.

PCR amplification using the *PEX6* exon 10a primer set yielded two amplicons that could be readily resolved from one another via agarose gel electrophoresis in 9/60 (15%) samples. In order to avoid flanking intronic sequences not amenable to primer design, the forward and reverse exon 10a primers were designed to bind within exons 9 and exon 11 of *PEX6*, respectively. We sequenced the lower molecular weight band produced from the above mentioned nine samples and found that while *PEX6* exon 9, 10, and 11 sequences were present, the intronic sequences flanking *PEX6* exon 10 were fully deleted. Therefore, we hypothesized that this lower molecular weight amplicon was generated via priming off of the remnants of a *PEX6* pseudogene located elsewhere in the genomes of these nine individuals. However, PCR analyses using additional primer sets designed to interrogate for the presence of a *PEX6* pseudogene in these samples did not support this hypothesis (data not shown). Therefore, we designed a novel primer set (designated *PEX6* exon 10b) designed to partially overlap the intronic regions flanking *PEX6* exon 10 and found that it generated a single amplicon in the PCR analysis these same nine samples. These PCR products were sequenced and results of these analyses were included in our larger data set.

Complementation Assays

Cell fusion complementation analyses were conducted as previously described (Moser, et al., 1995). Primary fibroblasts from PBD704, PBD604, *PEX1* null, *PEX6* null, *PEX12* null and *PEX26* null patients were cultured in rich media (DMEM supplemented with vitamins, essential and nonessential amino acids, antibiotic and 10% fetal bovine serum) at 37 °C in 5% CO_2. Upon reaching confluence, co-cultures of admixture of equal numbers of cells for two different lines for crosses were generated and incubated overnight at 40 °C. Afterwards, cells were washed with HBSS to remove trace culture media. Whole cell fusions were initiated by adding consecutively: (i) Solution 1 (4 mL 72% (w/v) polyethylene glycol 4000 (PEG) solution in 15% (w/v) DMSO and MEM) for 60 seconds followed by the addition of 4 mL 72% (w/v) PEG 4000 solution in HBSS. After 90 seconds, the total solution was diluted with 16 mL HBSS and incubated for two minutes prior to washing with HBSS. Afterwards, the cells were cultured in DMEM with 10% FBS and incubated overnight at 40 °C. The genotypes of mutated cell lines used in our complementation analyses were as follows: *PEX1* null (c.2098insT/c.2916delA), *PEX6* null (c.1314_1320del/c.2362−2A>G), *PEX12* null (c.887_888delTC homozygote), and *PEX26* null (c.37_38delAG/c.667+2T>C).

Immunofluorescence Analysis

Fused or primary dermal fibroblasts were seeded onto glass coverslips and grown in six well plates for 72 hours at 40 °C. Cells were fixed for 20 min in 3% formaldehyde/1 × Dulbecco's PBS solution (DPBS). After fixation, cells were washed 3 times in 1 × DPBS and permeabilized in 1% Triton X-100/1 × DPBS for 5 minutes. Subsequently, the cells were washed three times in 1 × DPBS, followed by a 30 minute blocking with 0.1% BSA in 1 × DPBS. Afterwards, the cells were incubated with primary antibodies for 60 minutes, washed seven times in 1 × DPBS, and then incubated with the appropriate secondary antibodies for 60 minutes. Rabbit polyclonal antiserum to rat PMP70 and sheep antiserum to human catalase were obtained from Sigma Aldrich and The Binding Site, respectively. Secondary antibodies were obtained from Jackson ImmunoResearch. Micrographs were captured on a Zeiss LSM510 confocal multi-photon microscope.

RESULTS AND DISCUSSION

Previously, a cohort of 91 PBD-ZSS patients was subject to the *PEX* Gene Screen, an algorithm for the sequence analysis of select *PEX1*, *PEX2*, *PEX5*, *PEX6*, *PEX10*, *PEX12*, and *PEX26* gene exons (Steinberg, et al., 2004). In these studies, two putative disease-causing alleles were

found in fifty-five individuals and one putative disease-causing allele in seventeen individuals. No *PEX* gene mutations were found in the remaining nineteen individuals.

Here, we sequenced the complete coding regions and splice junctions of *PEX1*, *PEX6*, *PEX10*, *PEX12* and *PEX26* in fifty-eight of these ninety-one PBD-ZSS patients, irrespective of prior genotypes. Our cohort was comprised of twenty-four patients where two putative disease-causing alleles were known, seventeen patients where one putative disease-causing allele was known, and seventeen patients where no disease-causing alleles were previously identified. This cohort was selected for the purposes of: (i) determining the sensitivity of our sequencing analysis based on known sequence variants, (ii) identifying deleterious alleles missed by the *PEX* Gene Screen, and (iii) identifying common sequence variants in these five *PEX* genes.

All analyses were successful with the exception of *PEX10* exon 3, which was removed from consideration due to the inability to consistently produce robust specific PCR products despite multiple primer designs. Overall, 71 unique sequence variations were found scattered throughout these five genes (Tables 1–2). We placed these variants into three specific categories: (i) deleterious mutations predicted to inactivate gene function via nonsense-mediated decay, premature translation termination, frameshift, splicing error, or alteration of a conserved amino acid (i. e. same in human, rhesus monkey, mouse, and rat orthologs), (ii) single nucleotide polymorphisms (SNPs), and (iii) rare neutral variants. We screened the scientific literature and dbpex (www.dbpex.org) for prior reports of each of the deleterious mutations identified. The information found in dbpex is publicly available and kindly provided by Drs. Steven Steinberg (Kennedy Krieger Institute/Johns Hopkins University School of Medicine) and Nancy Braverman (Johns Hopkins University School of Medicine/McGill University). Mutations cited as (www.dbpex.org) were identified by Drs. Steinberg and Braverman, but not reported in the scientific literature.

We categorize SNPs as being reported in the National Center for Biotechnology Information (NCBI) Single Nucleotide Polymorphism database (dbSNP: http://www.ncbi.nlm.nih.gov/projects/SNP/) in more than one individual or the scientific literature. Previously unreported silent changes were classified as rare neutral variants, even though we recognize that such variants can be deleterious by introducing cryptic splice junctions or interfering with exonic splicing enhancer (ESE) motifs (Boichard, et al., 2008). We did not find any examples of missense alleles of ambiguous functional significance that either (i) was not found in multiple individuals in dbSNP or (ii) involved a poorly conserved amino acid in the orthologous mouse and rat proteins.

Sensitivity of *PEX* Gene Exon Sequencing

Previously, 59 sequence variants (28 unique) were identified in our patient cohort through *PEX* Gene Screen analyses (Steinberg, et al., 2004) (Tables 1–3). Based on our blinded sequencing analyses, we uncovered all 59 (100%) of these known variants. This indicates that our sequencing analyses display excellent sensitivity and can reproducibly detect mutations in these *PEX* genes. Below, we discuss all 71 unique sequence variants uncovered in our current study (Table 1).

Deleterious *PEX1* Alleles

We identified twenty-nine patients with two deleterious *PEX1* alleles and none with a single deleterious *PEX1* allele (Table 1). The alleles c.2098insT and c.2528G>A (G843D) are strongly represented here, because these are known common alleles that provide the basis of the *PEX* Gene Screen to identify *PEX1* deficient patients (Steinberg, et al, 2004). A total of twenty-six deleterious *PEX1* alleles were uncovered. These included twelve novel deleterious

mutations (7 frameshift: c.270delA p.Q91fs, c.643_647delACCAA p.T215fs, c. 782_783delAA p.Q261fs, c.1714_1715delCA p.H572fs, c.1840delA p.E615fs, c. 3693_3696delGTCA p.Q1231fs, c.3732_3736dupCATTA p.S1246fs; one nonsense: c. 547C>T p.R183X; four splice junction: c.473−1G>A, c.1240+1G>T, c.1900+2T>C, c.3438 +2T>C) and eight previously reported deleterious mutations (two nonsense: c.2614C>T p.R872X (Fukuda, et al., 1996), c.2992C>T p.R998X (FitzPatrick and Valle, 1996); three frameshift:: c.2097_2098insT p.1700fs p.S701fs (Collins and Gould, 1999;Maxwell, et al., 1999;Walter, et al., 2001), c.2537_2545del9insTCATGGT (Steinberg, et al., 2004), and c. 2916delA p.G973fs (Maxwell, et al., 2002); one in-frame duplication: c. 1952_1960dupCAGTGTGGA (Preuss, et al., 2002); one in-frame deletion: c. 3022_3024delCCT p.P1008del (www.dbpex.org); one splice junction: c.2926+1G>A (Crane, et al., 2005;Steinberg, et al., 2004)) that result in a truncated PEX1 protein lacking the AAA1 and AAA2 functional domains.

In addition, we identified three novel deleterious missense mutations (c.1769T>G p.L590R, c. 2843G>A p.R948Q, and c.3710C>A p.A1237E) affecting residues conserved in rhesus monkeys, mouse, and rat. However, the functional significance of the *PEX1* p.R948Q allele in patient 604 is subject to interpretation since this individual has two deleterious *PEX26* missense alleles, both p.R98W. Indeed, cell fusion complementation analyses with *PEX1* and *PEX26* null fibroblasts (Fig. 1A–E) indicate that cells derived from patient 604 have at least one functional *PEX1* allele (Fig. 1D), but lack *PEX26* function (Fig. 1E). Thus, we provide evidence that the p.R98W allele negatively impacts PEX26 function. However, we cannot rule out the possibility that PBD604 carries three inactivating *PEX* alleles (i.e. two *PEX26* p.R98W alleles and *PEX1* p.R948Q). R948 is localized in a region that is conserved in yeast, although this residue itself is only semi-conserved.

One previously identified missense mutation c.1777G>A p.G593R (Rosewich, et al., 2005; Walter, et al., 2001) associated with low PEX1 protein levels (Walter, et al., 2001) and two previously identified temperature-sensitive mutations, c.2528G>A p.G843D (Collins and Gould, 1999; Maxwell, et al., 2002; Maxwell, et al., 1999; Walter, et al., 2001) and c.2392C>G p.R798G (Crane, et al., 2005; FitzPatrick and Valle, 1996)) were detected. The c.2088A>G p.I696M allele present in two patients was reported to be either a rare variant or polymorphism in other studies (Crane, et al., 2005; Steinberg, et al., 2004) and is thus not included in Table 1.

PBD672 carries the deleterious p.G843D and p.R872X *PEX1* alleles and the *PEX6* p.R601Q allele. Although we do not know the phase of these *PEX1* mutations, most likely they are on different chromosomes and responsible for the NALD/IRD patient diagnosis. As discussed below, we uncovered functional evidence that the *PEX6* p.R601Q allele is deleterious in patient PBD704 (Fig. 1). Thus, we propose that PBD672 carries three inactivating *PEX* alleles. The significance of this complex genotype is uncertain since the clinical and biochemical phenotype of PBD672 is similar to *PEX1* deficient patients who are compound heterozygotes for p.G843D and a nonsense or frameshift mutation.

Deleterious *PEX6* Alleles

We identified four patients with two deleterious *PEX6* alleles, one patient with a single deleterious *PEX6* allele, and one patient with three deleterious *PEX6* alleles (Table 1). One was a novel frameshift (c.914delA p.D305fs) mutation that results in a truncated PEX6 protein lacking an intact AAA cassette domain. In addition, two previously reported splice junction mutations (c.882+1G>A (Steinberg, et al., 2004) and c.1962−1G>A (Steinberg, et al., 2004;Yahraus, et al., 1996)) were identified.

Two novel [c.1646C>T p.A549V and c.2546A>C p.N849T] and four previously reported deleterious missense mutations (c.821C>T p.P274L (Steinberg, et al., 2004), c.1802G>A p.R601Q (www.dbpex.org), c.2578C>T p.R860W (www.dbpex.org), and c.2579G>A p.R860Q (www.dbpex.org)) also were identified. All of the missense mutations affect amino acid residues conserved in rhesus macaque, mouse, rat, zebrafish and chicken orthologs. Nevertheless, one patient carried two protein-abolishing mutations (c.914delA p.D305fs and c.1962−1G>A) in addition to the p.A549V variant. Thus, the medical significance of the p.A549 allele is questionable, even though it is counted as a novel deleterious allele in Table 1 according to our nomenclature.

The functional significance of the c.2579G>A p.R860Q/c.1802G>A p.R601Q mutations in PBD704 were of particular interest given that this individual also carries the deleterious c.681−2A>C *PEX12* allele. As such, we conducted cell fusion complementation analyses with *PEX6* and *PEX12* null fibroblasts (Fig. 1F–J) and demonstrated that PBD704 fibroblasts lack *PEX6* function (Fig. 1I), but have at least one functional *PEX12* allele (Fig. 1J). Given the nature of the *PEX12* c.681−2A>C mutation, we propose that PBD704 carries three inactivating *PEX* alleles; it remains a tantalizing question as to whether this is of any biological or medical significance. However, we do not have any clinical information on PBD704 to make any genotype-phenotype assessment.

The previously reported *PEX6* c.235G>C p.A79P missense allele (Steinberg, et al., 2004) was found in one patient (627) with two deleterious *PEX1* alleles (both p.A1237E) and one patient (607) with two deleterious *PEX12* alleles (both p.L296fs). In addition, it is conserved in rhesus macaques and mouse, but not rat. Thus, this is highly likely a neutral variant not causative of disease.

Deleterious *PEX10* Alleles

We identified two patients with two deleterious *PEX10* alleles and none with a single deleterious *PEX10* allele (Table 1). We uncovered four previously reported deleterious *PEX10* mutations (c.4delG p.A2fs (Steinberg, et al., 2004), c.704_705insA p.L236fs (Warren, et al., 2000), c.730C>T p.R244X (Krause, et al., 2006) and c.835G>A p.E279X (Steinberg, et al., 2004)). All of those mutations result in a truncated PEX10 protein lacking the C-terminal RING finger domain (AA273-311) critical for its function (Krause, et al., 2006). No *PEX10* missense alleles were found in this study.

Deleterious *PEX12* Mutations

We identified five patients with two deleterious *PEX12* alleles and one patient with a single deleterious *PEX12* allele (Table 1). Overall, we uncovered nine previously reported deleterious *PEX12* mutations. Of those, four were frame-shift mutations: c.541_542insT p.Y181fs (Steinberg, et al., 2004), c.730_733dupGCCT p.L245fs (Chang and Gould, 1998), c.887delT p.L296fs (Steinberg, et al., 2004) and c.887_888delTC p.L296fs (Gootjes, et al., 2004b). Two in-frame deletions (c.531_533del p.Q178del and c.1047_1049del p.Q349del) were detected that affect residues conserved in rhesus, mouse and rat. The latter mutation lies in the C-terminal zinc-binding RING domain encompassing amino acids 260–359 (Chang, et al., 1999;Krause, et al., 2006;Okumoto and Fujiki, 1997). A previously reported splice site mutation (c.681−2A>C (Steinberg, et al., 2004)) was detected in PBD704, which we previously discussed as also carrying the *PEX6* p.R601Q and p.R860Q alleles. PBD673 was homozygous for the c.959C>T p.S320F missense allele, which was previously reported to reduce PEX12 binding affinity to PEX5 and PEX10 (Chang, et al., 1999). Interesting, in another study, this same allele also was found in the homozygous state in a patient with a relatively mild PBD (Gootjes, et al., 2004a).

In PBD649, we detected the c.102A>T p.R34S *PEX12* missense allele affecting an amino acid residue conserved in rhesus macaques, mouse, and rat, which was previously reported as a mild, temperature sensitive mutation in two probands (Zeharia, et al., 2007). We noted that this individual also carries two deleterious *PEX1* alleles (pI700fs and p.H572fs), although we did not test if they reside on separate chromosomes. PBD649 had a severe early onset presentation consistent with Zellweger syndrome, thus being compatible with having two null alleles rather than a mild missense allele in conjunction with a null allele. Although PEX12 p.R34S may be associated with mild disease in isolation (hence its inclusion in Table 1), triallelic inheritance is unlikely to be an important contributor to the biochemical and clinical phenotype of PBD649.

Deleterious *PEX26* mutations

We identified five patients with two deleterious *PEX26* alleles and no patients with a single deleterious *PEX26* allele (Table 1). Of the five unique deleterious *PEX26* mutations detected, four (c.37_38delAG p.R13fs, c.192_216del p.S64fs, c.296G>A p.W99X, and c.574C>T p.R192X) were previously identified as protein-truncating mutations (Steinberg, et al., 2004). One (c.667+2T>C) represents a previously reported splice junction mutation (Steinberg, et al., 2004). We also identified two reported missense mutations (c.292C>T p.R98W (Matsumoto, et al., 2003) and c.353C>G p.P118R (Steinberg, et al., 2004)). Both could disrupt a PEX6 binding domain present in residues 29–163 of PEX26 protein (Weller, et al., 2003). The c. 292C>T p.R98W *PEX26* missense allele has been associated with a temperature-sensitive peroxisomal matrix protein import defect (Furuki, et al., 2006). Furthermore, homozygosity for PEX26 p.R98W has been reported in association with neonatal adrenoleukodystrophy (Matsumoto, et al., 2003;Weller, et al., 2005) and PBD604 has the same clinical diagnosis.

Single Nucleotide Polymorphisms and Unreported Neutral Variants in *PEX* genes

We detected a total of sixteen unique SNPs previously reported in dbSNP and/or the scientific literature and two unique neutral variants in the *PEX1*, *PEX6*, *PEX10*, *PEX12*, and *PEX26* genes (Table 2). Twelve variants of this general type were found multiple times in our cohort. In contrast, six such variants (*PEX1* c.3762T>C p.A1254A; *PEX6* c.2770G>T p.A924S; *PEX6* c.210G>A p.G70G; *PEX6* c.271G>T p.P271A; *PEX12* c.733T>A p.L245I and *PEX12* c.867C>T p.D289D) were found only once in our cohort.

Combined Analyses with the *PEX* Gene Screen

Here, we identified at least one deleterious *PEX* allele in eleven of the seventeen individuals where the *PEX* Gene Screen uncovered no *PEX* mutation. This led us to combine the results from our current analyses with that of the *PEX* Gene Screen. Based on these combined analyses, we demonstrate that the disease-causing gene could be assigned in 91.2% of the ninety-one PBD-ZSS cases (Table 3). In keeping with reports from multiple laboratories, the bulk of the cases could be explained by *PEX1* mutations (58.2%). The remaining 33.0% of cases where at least one deleterious *PEX* allele was identified were distributed amongst *PEX6*, *PEX10*, *PEX12*, *PEX26*, *PEX2*, and *PEX5* mutations (each gene ranging from 1.1 – 9.9% of cases explained).

There are several possible explanations for our inability to uncover all predicted *PEX* gene defects. Most likely, mutations may lie outside of the exon and splice junction sequenced, either elsewhere in these five *PEX* genes or, alternatively, in other loci. In addition, sequence variation could be missed due to the sensitivity of our sequencing analysis. As in any PCR-based method, the loss of primer binding sites could prevent the amplification of mutant alleles. In a similar manner, genomic deletions that eliminate large segments of any of these five genes would not be detected using our non-quantitative PCR-based methodology.

Overall, this study supports the design of the *PEX* Gene Screen. Although a limited number of coding exons in the *PEX26* (two of five), *PEX12* (two of three), and *PEX10* (three of six) were sequenced in the *PEX* Gene Screen analysis of 91 patients (Steinberg, et al., 2004), we uncovered no deleterious mutations in the untested exons of *PEX* Gene Screen mutation-negative patients. Although it is likely that a select number of PBD patients have mutations localized solely to these exons untested by the *PEX* Gene Screen, our observations suggest that adding these exons to the *PEX* Gene Screen would not benefit test sensitivity significantly.

Implications of patients with predicted deleterious mutations in multiple *PEX* genes

The identification of four patients (PBD649, PBD704, PBD604, and PBD672) with two predicted deleterious mutations in one *PEX* gene and a single deleterious mutation in a second *PEX* gene highlights potential difficulties in the interpretation of genetic tests. It is highly likely (but not certain) that the gene containing two deleterious alleles is responsible for disease. However, it is possible that a third allele on a second *PEX* gene further reduces the efficiency of peroxisome assembly and thus contributes to the clinical phenotype in some patients. Thus far, we have not identified a case consistent with the triallelic inheritance reported in other disorders, such as Bardet-Biedl syndrome (Badano, et al., 2006). As a corollary, it is not formally correct to assign the disease gene in the two patients (PBD650 and PBD720) for which one deleterious allele was found. Nevertheless, given the observed frequency of PBD-ZSS patients with mutations in multiple *PEX* genes, we anticipate that the genes in these two patients for which we found only one mutation by sequencing are responsible for disease.

The complex mutational spectrum in some PBD-ZSS patients makes this an important testing ground for newly developed resequencing technologies. These including oligonucleotide microarrays (Greiner, et al., 2006; Hacia, 1999; Hacia, et al., 1996; Hacia, et al., 2000; Karaman, et al., 2005) and next generation sequencing methods (Albert, et al., 2007; Dahl, et al., 2007; Hodges, et al., 2007; Porreca, et al., 2007). The wealth of functional biochemical and genetic assays for peroxisome activity in cell culture model systems as well as blood lipid profiling (Gootjes, et al., 2002; Steinberg, et al., 2008) will play a major role in deciphering the results of future large-scale resequencing analyses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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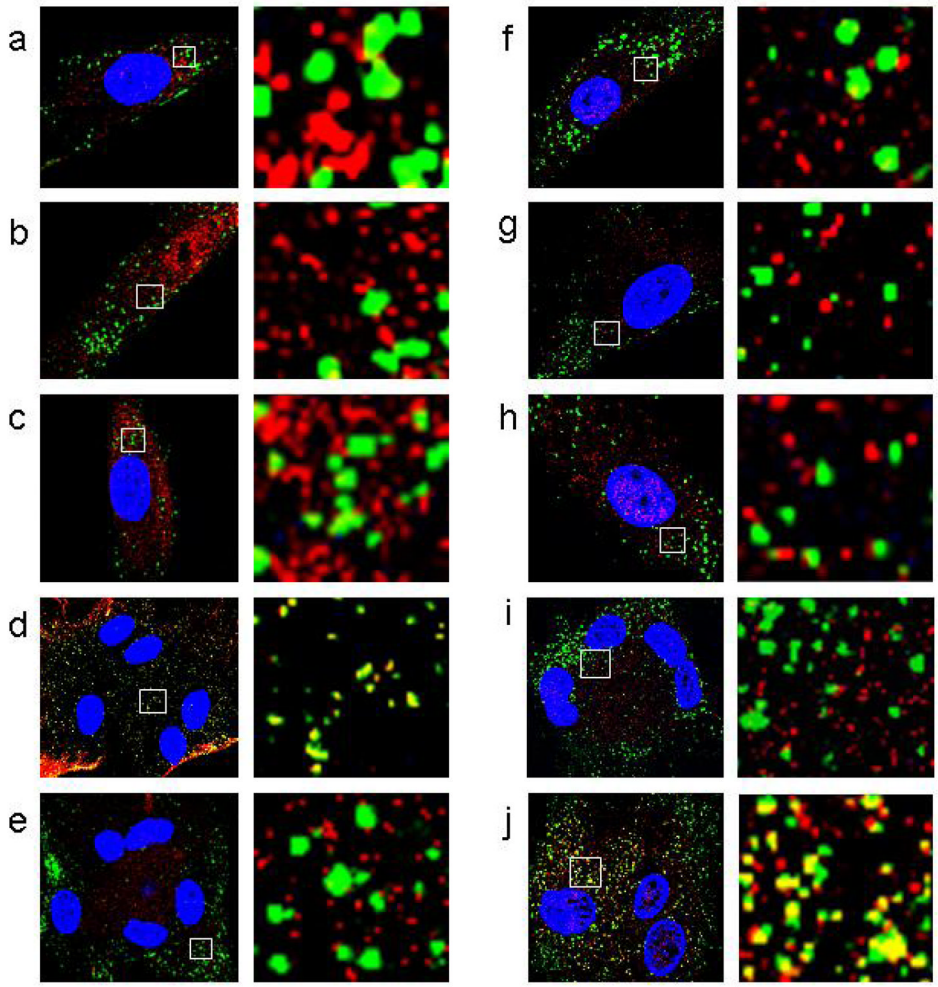

Figure 1. Complementation analyses of cells with deleterious alleles in multiple *PEX* genes

A representative immunofluorescence (IF) image of the indicated fibroblast culture along with a magnification of a boxed region is provided in each panel. Panels **A**–**E** depict the analysis of PBD604 fibroblasts with a single *PEX1* p. R948Q missense allele and two *PEX26* p. R98W alleles. **A:** PBD604 cells alone, **B:** *PEX1* null cells alone, **C:** *PEX26* null cells alone, **D:** fused PBD604 and *PEX1* null cells, and **E:** fused PBD604 and *PEX26* null cells. Panels **F**–**J** depict the analysis of PBD704 fibroblasts with two *PEX6* missense alleles (p.R601Q and p.R860Q) and a single *PEX12* c.681−2A>C allele. **F:** PBD704 cells alone, **G:** *PEX6* null cells alone, **H:** *PEX12* null cells alone, **I:** fused PBD704 and *PEX6* null cells, and **J:** fused PBD704 and *PEX12* null cells. Signals were generated using anti-PMP70 (green) and anti-catalase (red) antibodies, as markers of peroxisomal membrane and matrix proteins. Co-localization of PMP70 and catalase, as indicated by yellow signals, indicate proper peroxisome assembly. Nuclei were stained with DAPI (blue). The genotypes of the *PEX1*, *PEX6*, *PEX12*, and *PEX26* null cells are provided in Methods.

Table 1

Summary of Predicted Deleterious Mutations Identified in Cohort of ZSS-PBD Patients

		1st mutation[b]			2nd mutation			Other mutations[c]			
Diagnosis[a]	**Patient ID**	**Exon**	**Coding**	**Protein**	**Exon**	**Coding**	**Protein**	**Gene**	**Exon**	**Coding**	**Protein**
PEX1	674	Intron 4	c.473−1G>A		Intron 4	c.473−1G>A					
	701	Intron 5	c.1240+1G>T		10	c.1714_1715delCA	p.H572fs				
	719	5	c.547C>T	p.R183X	23	c.3732_3736dupCATTA	p.S1246fs				
	715	10	c.1769T>G	p.L590R	10	c.1769T>G	p.L590R				
	627	23	c.3710C>A	p.A1237E	23	c.3710C>A	p.A1237E				
	707	2	c.270delA	p.Q91fs	15	c.2528G>A	p.G843D				
	651	5	c.547C>T	p.R183X	15	c.2537_2545del9insTCATGGT	p.H846fs				
	662	5	c.643_647delACCAA	p.T215fs	13	c.2097_2098insT	p.I700fs				
	611	5	c.782_783delAA	p.Q261fs	13	c.2097_2098insT	p.I700fs				
	640	5	c.782_783delAA	p.Q261fs	15	c.2528G>A	p.G843D				
	649	10	c.1714_1715delCA	p.H572fs	13	c.2097_2098insT	p.I700fs	*PEX12*	1	c.102A>T	p.R34S
	638	10	c.1714_1715delCA	p.H572fs	15	c.2528G>A	p.G843D				
	716	11	c.1840delA	p.E615fs	15	c.2528G>A	p.G843D				
	610	Intron 11	c.1900+2T>C		13	c.2097_2098insT	p.I700fs				
	601	13	c.2097_2098insT	p.I700fs	Intron 21	c.3438+2T>C					
	660	13	c.2097_2098insT	p.I700fs	23	c.3693_3696delGTCA	p.Q1231fs				
	717	10	c.1777G>A	p.G593R	12	c.1952_1960dupCAGTGTGGA	p.W653_M654insTVW				
	712	13	c.2097_2098insT	p.I700fs	15	c.2528G>A	p.G843D				
	635	14	c.2392C>G	p.R798G	Intron 18	c.2926+1G>A					
	672	15	c.2528G>A	p.G843D	16	c.2614C>T	p.R872X	*PEX6*	8	c.1802G>A	p.R601Q
	606	15	c.2528G>A	p.G843D	19	c. 2992C>T	p.R998X				
	602	13	c.2097_2098insT	p.I700fs	13	c.2097_2098insT	p.I700fs				
	608	13	c.2097_2098insT	p.I700fs	15	c.2528G>A	p.G843D				
	646	13	c.2097_2098insT	p.I700fs	15	c.2528G>A	p.G843D				
	671	13	c.2097_2098insT	p.I700fs	18	c.2916delA	p.G973fs				
	702	13	c.2097_2098insT	p.I700fs	18	c.2916delA	p.G973fs				
	605	15	c.2528G>A	p.G843D	15	c.2528G>A	p.G843D				
	708	15	c.2528G>A	p.G843D	18	c.2916delA	p.G973fs				
	616	15	c.2528G>A	p.G843D	19	c.3022_3024delCCT	p.P1008del				

		1st mutation[b]			2nd mutation			Other mutations[c]			
Diagnosis[a]	**Patient ID**	**Exon**	**Coding**	**Protein**	**Exon**	**Coding**	**Protein**	**Gene**	**Exon**	**Coding**	**Protein**
PEX6	710	2	c.914delA	p.D305fs	Intron 9	c.1962−1G>A		*PEX6*	7	c.1646C>T	p.A549V
	609	8	c.1802G>A	p.R601Q	14	c.2546A>C	p.N849T				
	704[d]	8	c.1802G>A	p.R601Q	14	c.2579G>A	p.R860Q	*PEX12*	Intron 2	c.681−2A>C	
	713	8	c.1802G>A	p.R601Q	14	c.2579G>A	p.R860Q				
	636	Intron 1	c.882+1G>A		1	c.821C>T	p.P274L				
	720	14	c.2578C>T	p.R860W	---	---	---				
PEX10	603	1	c.4delG	p.A2fs	5	c.835G>T	p.E279X				
	652	4	c.704_705insA	p.L236fs	4	c.730C>T	p.R244X				
PEX12	669	2	c.531_533delACA	p.Q178del	2	c.531_533delACA	p.Q178del				
	633	2	c.541_542insT	p.Y181fs	3	c.730_733dupGCCT	p.L245fs				
	607	3	c.887_888delTC	p.L296fs	3	c.887_888delTC	p.L296fs				
	673	3	c.959C>T	p.S320F	3	c.959C>T	p.S320F				
	644	3	c.1047_1049delACA	p.Q349del	3	c.887delT	p.L296fs				
	650	3	c.887_888delTC	p.L296fs							
PEX26	604[e]	3	c.292C>T	p.R98W	3	c.292C>T	p.R98W	*PEX1*	18	c.2843G>A	p.R948Q
	714	2	c.192_216del	p.S64fs	3	c.353C>G	p.P118R				
	632	2	c.37_38delAG	p.R13fs	Intron 3	c.667+2T>C					
	634	3	c.292C>T	p.R98W	4	c.574C>T	p.R192X				
	676	3	c.296G>A	p.W99X	3	c.296G>A	p.W99X				

[a] In cases where only one deleterious *PEX* gene allele was found, we assigned this gene as disease-causing even though it is formally possible that another gene is causative of disease. The GenBank reference numbers for each *PEX* gene were as follows: *PEX1* (NM_000466.2), *PEX6* (NM_000287.2), *PEX10* (NM_002617.3), *PEX12* (NM_000286.1), and *PEX26* (AB089678.1).

[b] Novel unreported variants are shown in red. Variants previously not found in these samples, but reported in other datasets are shown in black. Variants previously found in these samples via the *PEX* Gene Screen are shown in blue. Samples are ordered based on the number of novel deleterious variants uncovered and sub-grouped based on the number of novel alleles discovered in our study. Nucleotide numbering reflects cDNA numbering with +1 corresponding to the A of the ATG translation initiation codon in the reference sequence, according to journal guidelines. The initiation codon is codon 1.

[c] All missense changes occur in amino acids conserved in human, rhesus macaque, mouse, and rat orthologs.

[d] Individual listed has defective PEX6 function via genetic complementation analysis.

[e] Individual listed has defective PEX26 function via genetic complementation analysis.

Table 2

SNPs and neutral variants observed

Gene	Allele[a]		Frequency[b]
PEX1	c.2088A>G	p.I696M	0.03
	c.2331C>A	p.G777G	0.84
	c.3762T>C	p.A1254A	0.01[c]
PEX6	c.210G>A	p.G70G	0.01[c]
	c.235G>C	p.A79P	0.02
	c.399G>T	p.V133V	0.37
	c.813G>T	p.A271A	0.01[c]
	c.1802G>A	p.R601Q	0.03
	c.2364G>A	p.V788V	0.04
	c.2426C>T	p.A809V	0.03
	c.2644G>A	p.V882I	0.03
	c.2770G>T	p.A924S	0.01[c]
	c.2814G>A	p.E938E	0.43
	c.2816C>A	p.P939Q	0.35
PEX10	c.820A>G	p.T274A	0.03
PEX12	c.733T>A	p.L245I	0.01[c]
	c.867C>T	p.D289D	0.01[c]
PEX26	c.457C>G	p.L153V	0.03

[a] Newly discovered silent variants in red

[b] Based on the analysis of 116 chromosomes

[c] Allele found only once in cohort

Table 3

PEX Gene Screen (PGS) Results: 91 patients total

Gene	Patients with one mutation (PGS)	Patients with two mutations (PGS)	Total (at least one mutation PGS)	Newly identified cases by current study	% cases (at least one mutation by PGS and/or sequencing)[a]
PEX1	16	32	48	6	58.2
PEX6	0	4	4	5	9.9
PEX10	0	3	3	0	3.3
PEX12	1[b]	6	7	1	7.7
PEX26	0	5	5	0	5.5
PEX2	0	5	5	0[c]	5.5
PEX5	0	1	1	0[c]	1.1
Total	17	56	73	12	91.2

[a] Cases with deleterious alleles detected in multiple *PEX* genes are counted a single time based on the assigned diagnosis provided in Table 1

[b] The current study identified a *PEX6* defect by sequencing and complementation analyses

[c] The current study does not screen for *PEX2* or *PEX5* mutations