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Report

Identification of *PEX7* as the Second Gene Involved in Refsum Disease

Daan M. van den Brink,[1] Pedro Brites,[2] Janet Haasjes,[2] Anthony S. Wierzbicki,[3,4] John Mitchell,[5] Michelle Lambert-Hamill,[3] Jacqueline de Belleroche,[5] Gerbert A. Jansen,[1] Hans R. Waterham,[2] and Ronald J. A. Wanders[1,2]

Departments of [1]Clinical Chemistry and [2]Pediatrics, Emma Children's Hospital, Academic Medical Center, University of Amsterdam, Amsterdam; [3]Department of Chemical Pathology, King's College London, St. Thomas' Hospital Campus, [4]Refsum's Disease Clinic, Chelsea & Westminster Hospital, and [5]Department of Neuromuscular Disease, Faculty of Medicine, Imperial College Medical School, Charing Cross Hospital Campus, London

Patients affected with Refsum disease (RD) have elevated levels of phytanic acid due to a deficiency of the peroxisomal enzyme phytanoyl-CoA hydroxylase (PhyH). In most patients with RD, disease-causing mutations in the *PHYH* gene have been identified, but, in a subset, no mutations could be found, indicating that the condition is genetically heterogeneous. Linkage analysis of a few patients diagnosed with RD, but without mutations in *PHYH*, suggested a second locus on chromosome 6q22-24. This region includes the *PEX7* gene, which codes for the peroxin 7 receptor protein required for peroxisomal import of proteins containing a peroxisomal targeting signal type 2. Mutations in *PEX7* normally cause rhizomelic chondrodysplasia punctata type 1, a severe peroxisomal disorder. Biochemical analyses of the patients with RD revealed defects not only in phytanic acid α-oxidation but also in plasmalogen synthesis and peroxisomal thiolase. Furthermore, we identified mutations in the *PEX7* gene. Our data show that mutations in the *PEX7* gene may result in a broad clinical spectrum ranging from severe rhizomelic chondrodysplasia punctata to relatively mild RD and that clinical diagnosis of conditions involving retinitis pigmentosa, ataxia, and polyneuropathy may require a full screen of peroxisomal functions.

Refsum disease (RD [MIM 266500]) is a peroxisomal disorder of branched-chain lipid metabolism, characterized by progressive adult retinitis pigmentosa, peripheral neuropathy, anosmia, and cerebellar ataxia. Additional symptoms include nerve deafness, skeletal dysplasia, ichthyosis, cataracts, and cardiac arrhythmias. The adolescent onset of clinical symptoms is due to a gradual accumulation of phytanic acid (3,7,11,15-tetramethylhexadecanoic acid), the only pathognomic biochemical marker of RD (Wanders et al. 2001*a,* 2001*b;* Wierzbicki et al. 2002). Because of the presence of a 3-methyl group, phytanic acid cannot be degraded directly by β-oxidation but instead undergoes one round of α-oxidation, during which the terminal carboxyl group is removed to yield pristanic acid. The peroxisomal enzyme phytanoyl-CoA hydroxylase (PhyH) catalyzes the first step of α-oxidation, and mutations in the *PHYH* gene have been identified in many patients with RD (Jansen et al. 1997, 2000; Mihalik et al. 1997; Mukherji et al. 2001). Recent observations, however, pointed to genetic heterogeneity among patients with RD. First, in some patients with RD, genetic analysis failed to identify mutations in the *PHYH* gene, despite a marked deficiency in phytanic acid α-oxidation (our unpublished data). Second, genetic linkage studies revealed that not all patients with RD could be linked to the *PHYH* locus at 10p13 (Wierzbicki et al. 2000).

We conducted biochemical studies in fibroblasts from two probands clinically diagnosed with RD but in whom no mutations could be identified in the *PHYH* gene. Proband 1 comes from a family of eight with three affected sibs. He was born following an uneventful, full-term pregnancy, became ataxic at age 12 years, and, on neurological examination at age 19, had established retinitis pigmentosa, with limited ocular fields, absence of night blindness, and a normal electroretinogram (ERG). Other

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Address for correspondence and reprints: Dr. Ronald J. A. Wanders, Laboratory for Genetic Metabolic Diseases (F0-224), Department of Pediatrics, Emma Children's Hospital, Academic Medical Center, University of Amsterdam, P.O. Box 22700, 1100 DE Amsterdam, The Netherlands. E-mail: R.J.Wanders@amc.uva.nl

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Table 1

Results of Biochemical Analyses

Subject	Phytanic Acid α-Oxidation (pmol/hr/mg)[a]	PhyH Activity (nmol/hr/mg)[b]	Presence (+) or Absence (−) of Peroxisomes[c]	Pristanic Acid β-Oxidation (pmol/hr/mg)[d]	Plasmalogen Synthesis (ratio Peroxisomal: Microsomal)[e]
Control Individual	44 – 82	.10 – 1.00	+	675 – 1121	40 – 330
Patient with Classic RD	2	0	+	810	125.0
Patient with Zellweger	11	0	−	5	6.0
Patient with RCDP type 1	5	0	+	634	.9
Proband 1	5	.03	+	926	2.4
Proband 2	4	.04	+	ND[f]	3.0

[a] Phytanic acid α-oxidation was performed in intact cultured skin fibroblasts, as described elsewhere (Wanders and Van Roermund 1993).

[b] Phytanoyl-CoA activity was measured in skin fibroblast homogenates incubated for 30 min at 37°C in a reaction mixture containing 25 mM Tris-HCl, 0.25 mM dithiothreitol, 3 mM ATP, 1.5 mM magnesium chloride, 0.2 mM coenzyme A (CoA), 0.25 mM ammonium iron (II) sulphate, 1 mM 2-oxoglutarate, 2 mM ascorbate, 10 μM phytanoyl-CoA, final pH 7.5. After termination of the reaction by addition of HCl, the product, 2-hydroxyphytanoyl-CoA, was hydrolyzed to form 2-hydroxyphytanic acid, which was measured by GC-MS, essentially as described elsewhere (ten Brink et al. 1992).

[c] Presence of peroxisomes was ascertained by catalase immunofluorescence in cultured skin fibroblasts (Heikoop et al. 1991).

[d] Pristanic acid β-oxidation in intact cultured skin fibroblasts was performed as described elsewhere (Wanders et al. 1995).

[e] De novo plasmalogen synthesis was measured in intact cultured skin fibroblasts as described elsewhere (Schrakamp et al. 1988).

[f] Not determined.

features included anosmia, a short fifth metacarpal and palmar ichthyosis, pes cavus, muscle weakness, and nerve hypertrophy, which was confirmed by sural nerve biopsy. His sister presented at age 20 years with postpartum ataxia and numbness in her fingers and toes. She had profound retinitis pigmentosa, an abnormal ERG, anosmia, sensory and motor dysfunction with nerve hypertrophy, and mild pes cavus. The third affected sib, a brother, had mild retinitis pigmentosa and anosmia but no other signs, on screening at age 24 years. Initial plasma phytanic acid levels in the three individuals were 400 μM, 1,950 μM, and 372 μM (normal <30 μM), respectively.

Proband 2 originates from a family comprising eight sibs with two living affected members. Although born with bilateral cataracts, she presented to a neurology clinic, at age 20 years, with polyneuritis and onset of ataxia at age 19 years. She had bilateral short fifth metacarpals and metatarsals, "arthritis" since age 25 years, and mild retinitis pigmentosa. Her brother presented at age 34 years with mild ataxia and mild retinitis pigmentosa but no night blindness, obvious anosmia, or deafness. Neither patient had any episodes of ichthyosis or signs of deafness. One other male sib died of supposed "poliomyelitis" with symptoms of weakness and ataxia at age 12 years. Their initial plasma phytanic acid levels were 198 μM and 142 μM, respectively.

Patients were reviewed by specialized neurological units and had full ophthalmological (Claridge et al. 1992), smell test, audiological, balance, and electrophysiological investigations performed. Skeletal changes were assessed by a radiological survey (Plant et al. 1990). Dietary phytanic acid intake was assessed by questionnaire (Brown et al. 1993). Phytanic acid levels were measured in plasma and adipose tissue biopsy specimens by use of standard gas chromatographic techniques (Moser and Moser 1991). Sural nerve biopsy was performed for gross nerve hypertrophy.

A deficiency of phytanic acid α-oxidation and of PhyH activity was found in cultured skin fibroblasts of both probands (table 1), which is in agreement with the clinical diagnosis of RD. To identify the defective gene in these patients, we performed multipoint linkage analysis in the two families and found linkage to a region on chromosome 6, which extended from D6S292 to D6S441, with a peak LOD score of 1.92 between D6S314 and D6S308, close to the loci for PEX7 and fatty acid–binding protein 7 (fig. 1) (Wierzbicki et al. 2000). Previous studies have shown that mutations in the *PEX7* gene cause rhizomelic chondrodysplasia punctata type 1 (RCDP type 1 [MIM 215100]) (Braverman et al. 1997; Motley et al. 1997, 2002; Purdue et al. 1997; Braverman et al. 2000). The *PEX7* gene codes for the peroxin 7 protein, which has a function as a mobile receptor required for import into the peroxisome of proteins containing a peroxisomal targeting signal type 2 (PTS2). In RCDP type 1, a number of peroxisomal functions are deficient, including PhyH activity, but peroxisomal biogenesis is not affected (Purdue et al. 1999). Hence, we extended our biochemical analysis in the probands' fibroblasts. A normal pristanic acid β-oxidation (table 1) and a normal punctate pattern of catalase immunofluorescence was found (results not shown), but plasmalogen synthesis was deficient (table 1). The latter finding has never been observed in RD but is characteristic of patients who have RCDP type 1 (Hoefler et al. 1988),

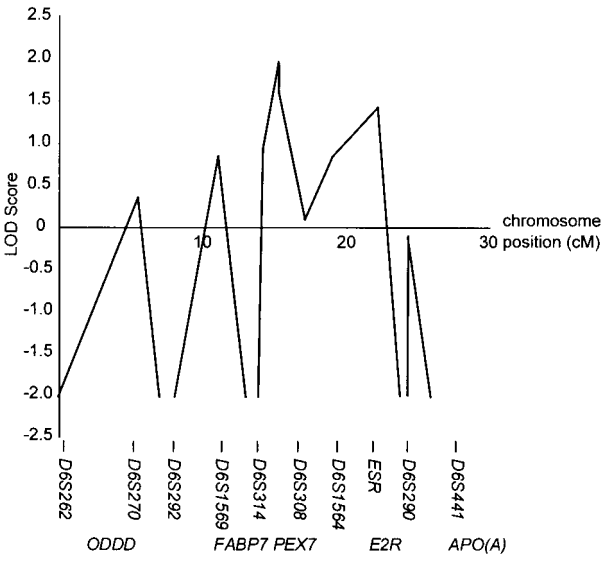

Figure 1 Multipoint linkage analysis of markers on chromosome 6, with approximate locations of known loci. With approval from the ethics committee of each institution, 23 individuals, including 4 living patients with a clinical RD phenotype, from the two families were recruited through a specialized clinic, using diagnostic criteria specified elsewhere (Wierzbicki et al. 2000). A panel of 11 fluorescein-labeled dinucleotide repeat markers (Perkin Elmer) spanning 6q14-6qter (GenLink home page) was amplified from extracted patient lymphocyte DNA (Puregene), by PCR, and typed on an ABI 310 analyzer with GeneAmp software (Perkin Elmer). The data were analyzed using the GENEHUNTER linkage analysis program (Kruglyak et al. 1996), assuming complete penetrance for a recessive disorder and a mutation rate of 10^{-5}. The allele frequency for the RD trait was assumed to be 0.001 on the basis of the prevalence of RD of 10^{-6} in the United Kingdom. Distances are in centimorgans from D6S242, which was taken arbitrarily as zero. Apolipoprotein (a) = APO(A); estrogen receptor = E2R; peroxin 7/RCDP type 1 = PEX7; fatty acid binding protein 7 = FABP7; oculodentodigital dysplasia = ODDD (MIM 164200).

and it prompted us to further investigate possible aberrations in PTS2 protein import.

All of the currently known human PTS2 proteins undergo processing upon import into the peroxisome. Peroxin 7 deficiency, however, leads to mislocalization and cytosolic accumulation of unprocessed PhyH, alkyldihydroxyacetonephosphate synthase (ADHAPS), and peroxisomal 3-oxoacyl-CoA thiolase 1 (thiolase). The difference in molecular weight between the unprocessed precursor and mature proteins can be readily visualized by a mobility shift on immunoblot (Heikoop et al. 1990; Swinkels et al. 1991). As expected, ADHAPS was found in its mature 67-kDa form in fibroblast homogenates from both a normal control individual and a patient with classic RD with mutations in the *PHYH* gene (fig. 2*a*). In fibroblast homogenates from a patient with RCDP type 1, the unprocessed 73-kDa form was found, which is in agreement with the defective import of this protein in patients with RCDP type 1 (Purdue et al. 1999). In the two probands, both forms of the protein were detected, suggesting a (partial) defect in PTS2 protein import. Similar results were obtained when we performed immunoblot analysis for peroxisomal thiolase (fig. 2*b*). PhyH protein levels appeared to be much lower or absent in the probands (fig. 2*c*), although, in fibroblast homogenates of proband 2, some protein could be detected on longer exposures (not shown). The precursor of PhyH could not be detected either in probands or in material from patients with RCDP, which might indicate that it is degraded more rapidly than precursors of ADHAPS and peroxisomal thiolase.

When the results shown in figure 2 are examined closely, it is clear that levels of processed thiolase and PhyH are somewhat higher in proband 1 than in proband 2, whereas the opposite is found for ADHAPS. The underlying basis of this finding is not clear at present but may well have to do with the nature of the two distinct (*or* different) mutations found in the probands, each affecting PTS2-protein import differently (see below).

Since the data described above suggested a deficiency of peroxin 7, we performed mutation analysis of the *PEX7* gene, as described elsewhere (Motley et al. 2002), and found that both patients were compound heterozygotes for mutations in *PEX7* (table 2). The Y40X nonsense mutation, found in both probands, introduces a premature stop codon in the N-terminal region of the protein. This mutation has also been found in patients

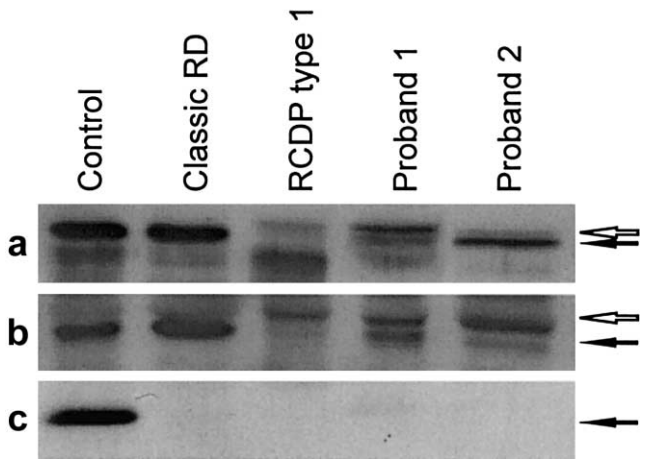

Figure 2 Processing of PTS2 proteins into peroxisomes in control, classic RD, RCDP type 1, and probands 1 and 2. Lysates of fibroblasts were subjected to western blotting, using antibodies ADHAPS (*a*), peroxisomal 3-oxoacyl-CoA thiolase (*b*), and PhyH (*c*). Solid and open arrows indicate the position of the unprocessed and mature forms, respectively. Immunoblot analyses were performed, as described, for peroxisomal thiolase (Heikoop et al. 1990), ADHAPS (de Vet et al. 1997), and PhyH with affinity-purified antibodies (Jansen et al. 2000).

Table 2

Mutations in *PEX7* in the Probands

Subject	Nucleotide Change	Amino Acid Change
Proband 1	120 C→G	Y40X
	12-18dupGTGCGGT	Frameshift
Proband 2	120 C→G	Y40X
	40 A→C	T14P

with classic RCDP type 1 with a severe clinical presentation (Motley et al. 2002), which makes it unlikely to be responsible for the mild clinical presentation observed in the probands. In proband 1, a 7-nt duplication (12-18dupGTGCGGT; table 2) was found on the second allele, predicted to cause a frameshift leading to a premature stop codon at amino acid position 57. However, the duplication occurs between two in-frame initiation codons, which would suggest that use of the second ATG may produce a protein that lacks the first 10 amino acids but retains partial peroxin 7 transport function. The second mutation in proband 2 was a T14P amino acid substitution. Recent data from Braverman et al (2002) would suggest that the T14P mutation lies in a stretch of amino acids just before the first β strand. An amino acid substitution in a β strand of peroxin 7 is thought either to interfere with folding of the protein or to reduce the affinity for its binding partners (Braverman et al. 2002). It remains to be established how the T14P mutation interferes with the function of peroxin 7.

To investigate the effect of the mutations on the function of peroxin 7, we expressed the three patient *PEX7* alleles, together with a PTS2-tagged GFP construct in cultured skin fibroblast derived from a patient with RCDP type 1 (fig. 3*A*). Expression of control *PEX7* resulted in a punctate fluorescence, indicating that PTS2-protein import into the peroxisomes was restored. As shown elsewhere (Motley et al. 2002), expression of the Y40X allele does not restore PTS2-protein import, as concluded from the diffuse, cytosolic fluorescence of PTS2-GFP (fig. 3*B*). Expression of the 7-nt duplication allele resulted in a punctate fluorescence, suggesting that this allele, when overexpressed, can restore PTS2-protein import (fig. 3*C*). Similar results with a relatively mild *PEX7* mutation have been shown elsewhere (Motley et al. 2002). Restoration of import, however, could not be observed upon expression of the T14P allele, with all cells showing cytosolic fluorescence (fig. 3*D*).

To study the function of peroxin 7 endogenously, we used immunofluorescence with peroxisomal thiolase antibodies to investigate the subcellular localization of peroxisomal thiolase in cultured fibroblasts. Control fibroblasts showed a peroxisomal fluorescence, which was absent in fibroblasts derived from a patient with RCDP type 1, in whom a cytosolic labeling was observed (fig. 4). Likewise, in fibroblasts from proband 1, fluorescence could be observed only in the cytosol, indicative of defective PTS2-protein import into the peroxisomes in these cells. The normal presence of peroxisomes and the import of PTS1 proteins were established in all cell lines, using immunofluorescence with catalase antibodies (fig. 4). These experiments could not be carried out in cells from proband 2, since no cultured fibroblasts were available for study.

From these results, it appears that the 7-nt duplication allele can restore PTS2-protein import when overexpressed, although, in an endogenous setting, peroxisomal thiolase and other PTS2-proteins are predominantly present in the cytosol in fibroblasts from proband 1, as observed by immunofluorescence and western blotting. In this context, it is surprising that the T14P allele does not restore PTS2-protein import when overexpressed, even though immunoblot results showed a mild import defect. Further study is needed to explain this apparent inconsistency.

As mentioned above, *PEX7* is the gene involved in RCDP type 1. It is interesting that the usual clinical

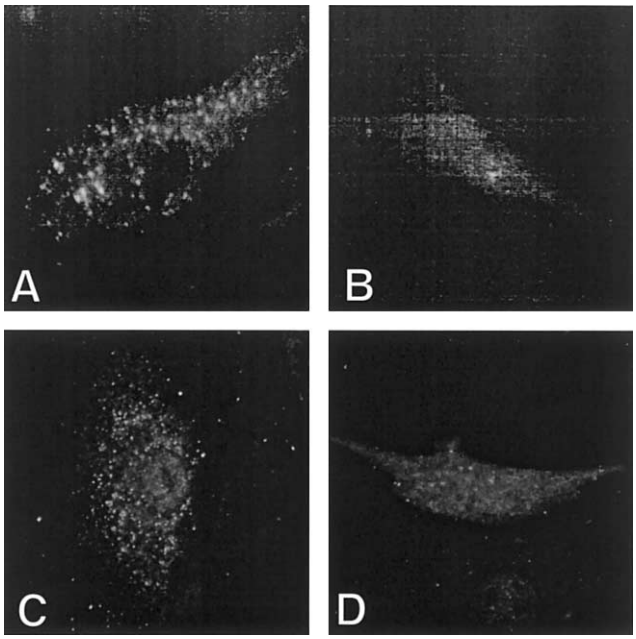

Figure 3 Functional complementation of PTS2-mediated peroxisomal protein import by *PEX7* alleles. The three mutant *PEX7* alleles identified in the two probands were coexpressed with PTS2-tagged GFP in cultured fibroblast of a patient with RCDP type 1, as described elsewhere (Motley et al. 1997, 2002), to test their ability to restore PTS2-mediated peroxisomal protein import. Expression of control *PEX7* resulted in punctate peroxisomal fluorescence (*A*), whereas the Y40X allele resulted in cytosolic fluorescence (*B*). Expression of the 7-nt duplication (*C*) and the T14P allele (*D*) resulted in peroxisomal and cytosolic fluorescence, respectively.

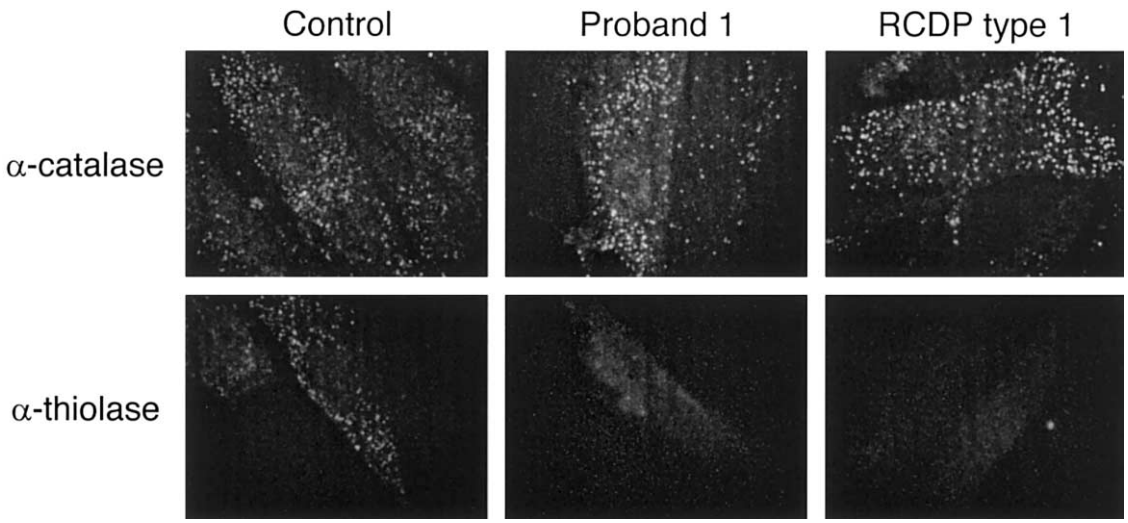

Figure 4 Localization of peroxisomal thiolase in fibroblasts by immunofluorescence. Cultured fibroblasts derived from a control individual, a patient with RCDP type 1, and proband 1 were stained, as described elsewhere, for catalase and peroxisomal thiolase (Heikoop et al. 1991). No peroxisomal thiolase could be detected in the peroxisomes of proband 1, giving a cytosolic staining similar to that observed in the patient with RCDP type 1.

presentation of patients affected with RCDP type 1 is severe and quite different from RD, with patients showing growth retardation, profound developmental delay, cataracts, rhizomelia, dysostoses, and ichthyosis, with death occurring within the first years of life. In addition, a number of patients with a milder phenotypic variant of RCDP type 1 have been described, but all followed a classical presentation, with differences in degree of cognitive impairment, longer life expectancy, and lack of rhizomelia or chondrodysplasia (Poll-The et al. 1991; Smeitink et al. 1992; Barth et al. 1996). These patients all have been diagnosed with RCDP type 1 early in life, whereas the patients we describe here were diagnosed with classical RD in adulthood.

In conclusion, we have identified mutations in the *PEX7* gene in a subgroup of patients clinically diagnosed with RD. In each patient, we found one mutation predicted to have a mild effect, which correlates with some residual functional activity of peroxin 7 and, consequently, also of the PTS2 proteins imported by peroxin 7, leading to a clinical phenotype normally associated with RCDP instead of RD. This finding suggests that mutations in the *PEX7* gene can give rise to an overlapping clinical presentation ranging from RD to RCDP similar to that of classical peroxisomal biogenesis disorders, whose presentation ranges from severe Zellweger syndrome to infantile Refsum disease (Gould et al. 2001). Thus, investigation of patients with retinitis pigmentosa, ataxia, and neuropathy may require a full screen of peroxisomal functions to identify the underlying genetic cause of the disorder.

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Electronic-Database Information

Accession numbers and URLs for data presented herein are as follows:

GenLink, http://www.genlink.wustl.edu/

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for RD [MIM 266500], RCDP type 1 [MIM215100], and Zellweger syndrome [MIM 214100])

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