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Absence of Mutations Raises Doubts about the Role of the 70-kD Peroxisomal Membrane Protein in Zellweger Syndrome

To the Editor:

The 70-kD peroxisomal membrane protein (PMP70) was first purified from rat liver tissue, and analysis of its sequence showed that it belonged to a family of ATP-binding proteins, the ABC transporters (Kamijo et al. 1990). In rat liver, PMP70 is induced by peroxisomal proliferators, and it was proposed that the protein may be part of the import machinery for peroxisomal proteins (Kamijo et al. 1990). Import of proteins into the peroxisome is defective in patients with a range of clinical phenotypes, including those with Zellweger syndrome, neonatal adrenoleukodystrophy, and infantile Refsum disease. Complementation analysis has indicated that this group of disorders can be caused by defects in a number of different genes (Shimozawa et al. 1993; Moser et al. 1995) and that all of the aforementioned phenotypes can occur within the one complementation group. In 1992, Gärtner et al. (1992*a*) proposed that defects in the gene for PMP70 are the cause of the peroxisomal assembly defect in patients belonging to complementation group 1 (Kennedy-Krieger Institute nomenclature). This hypothesis was based on the identification of both a donor splice-site mutation in a single patient and a missense mutation in two affected sibs, all belonging to this complementation group. However, mutations were not identified on the other PMP70 allele from these individuals, nor on alleles from a further 19 complementation group 1 patients (Gärtner et al. 1992*a*). Further support for the involvement of PMP70 in peroxisomal biogenesis, but not necessarily in complementation group 1, has come from transfection studies using a peroxisome-deficient Chinese hamster ovary (CHO) cell mutant carrying a point mutation in another peroxisomal membrane protein, PMP35 (Gärtner et al. 1994). Overexpression of normal PMP70 cDNA was able to overcome the peroxisomal biogenesis defect caused by the defect in PMP35. However, overexpression of a PMP70 mutant cDNA, carrying the predicted 23-bp insertion resulting from the donor splice-site mutation found in one of the patients, did not complement the PMP35 defect (Gärtner et al. 1994).

To gain further information on the possible role of the PMP70 gene in patients with peroxisomal biogenesis disorders belonging to complementation group 1, we have investigated, with the approval of The Women's and Children's Hospital Research Ethics Committee, 12 Australian patients for the presence of mutations in the PMP70 gene. The seven female and five male patients investigated in this study all had a clinical phenotype indicative of a peroxisomal biogenesis disorder. Eight of the patients showed the more severe phenotype of Zellweger syndrome, and the remaining four patients had the milder, infantile Refsum disease phenotype. In each case the clinical diagnosis was confirmed biochemically by methods described elsewhere (Paton et al. 1996). There was no known consanguinity in any of the families. The patients studied here were assigned to complementation group 1 by N. Shimozawa, Y. Suzuki, and T. Orii, all at The Gifu University School of Medicine, using their catalase immunofluorescence method (Yajima et al. 1992), and this was confirmed for three of the patients by use of pristanic acid oxidation to assess peroxisomal integrity (Paton et al. 1996). Four of the patients had both affected and nonaffected sibs, a pattern consistent with autosomal recessive inheritance, given that their parents were not affected.

To exclude the possibility of a rearrangement of the DNA in the gene for PMP70, genomic DNA was extracted from the fibroblasts of all 12 patients, as well as from controls, by use of DNAzol^{TM} reagent (GIBCO BRL Life Technologies). The DNA was digested separately with *Eco*RI and *Hin*dIII and hybridized with a mixed probe after Southern blotting. The probe was prepared from overlapping PCR products for PMP70 (corresponding to fragments A+B, C+D, E+F, G+H, and I+J; table 1) prepared from control cDNA. The PCR products were randomly labeled with [α-^{32}P]CTP by use of an Amersham Multiprime DNA labeling kit. When compared with controls, no differences were observed in the restriction-enzyme digest patterns for any of the 12 patients studied.

RNA of patients was extracted from fibroblast pellets by use of the method of Chomczynski and Sacchi (1987), and cDNA was prepared by use of reverse transcriptase. Ten overlapping PCR fragments (A–J; table 1) of the PMP70 cDNA sequence (Gärtner et al. 1992*a*) were then generated from the cDNA of the 12 patients, as well as from that of controls. In every case, PCR products of the correct size were generated, ruling out the possibility of all but very small insertions or deletions in the coding region of the expressed alleles. Although over- or underexpression of the PMP70 gene could be deleterious, there was no suggestion that this was the case in any of the patients, with the amount of PCR product generated from the patients' samples being similar to that in controls.

To maximize detection of mutations, SSCP analysis (Orita et al. 1989) of the PCR products (40–200 ng of DNA) was conducted under two conditions, either with gels containing 7.6% acrylamide, 0.4% bisacrylamide, 10% (v/v) glycerol, and 1 × Tris-borate EDTA, which were run at ambient temperature for 20 h at 21 W, or with 9.8% acrylamide, 0.2% bisacrylamide, 4%(v/v) glycerol, and 1 × Tris-borate EDTA gels, which were run at 4°C for 6 h at 75 W. Prior to electrophoresis, the samples were mixed with 6 vol of loading dye (96% formamide, 20 mM EDTA, 0.5 mg bromophenol blue/ml, 0.5 mg xylene cyanol/ml, and 10 mM NaOH), then denatured by boiling for 3 min, and rapidly cooled on ice. After electrophoresis, the gels were silver stained by use of the method of Budowle et al. (1991). In addition, PMP70 products A–D and F–I were digested with the indicated restriction enzyme (table 1), to yield products close to the optimal size for detection of conformational polymorphisms (Sheffield et al. 1993), and then also were analyzed on gels. Mobility differences under both SSCP conditions for fragment A, either before or after restriction-enzyme digestion, indicated the presence of a polymorphism in this region of the cDNA sequence. The PCR products were sequenced by use of the dideoxy chain-termination method (Sanger et al. 1977), with 75 pmol of PCR product and 20 ng of [γ-^{32}P] end-labeled primer (table 1) per reaction and 25 amplification cycles (95°C for 20 s, 60°C for 30 s, and 72°C for 1 min). This confirmed that the polymorphism corresponded to a known synonymous (K54K) polymorphism (Gärtner et al. 1992*b*). Allele frequencies for the polymorphism were consistent with those published by Gärtner et al. (1992*b*), and heterozygosity at nucleotide 162 in five of the patients provided evidence that both alleles were transcribed in these individuals.

Apart from the aforementioned polymorphism, no clear differences were observed on SSCP gels. Nevertheless, some other minor variations were noted, and these were investigated further by sequencing the relevant regions, but no nucleotide differences were found. For all 10 samples (6 patients and 4 controls) sequenced at nucleotide 33 (numbered from the initiation codon), the base differed from that published by Gärtner et al. (1992*a*) but agreed with the alternative human PMP70 sequence published by Kamijo et al. (1992). The amount of PMP70 cDNA sequenced for nine of the patients is indicated in table 2. For three patients, almost the entire cDNA for PMP70 was sequenced, and no mutations were identified. The possibility of an mRNA-negative allele was excluded in these individuals, since they were heterozygous for the polymorphism at nucleotide 162.

Under the conditions used, we believe that SSCP analysis should have detected most mutations in the PMP70 cDNA sequence. In addition to the polymorphism at nucleotide 162 of the PMP70 cDNA sequence, SSCP

Table 1

Oligonucleotide Primers Used to Generate PCR Products from PMP70 cDNA

Product	Oligonucleotide Sequence (5′→3′)	Location	Product Size (bp)	Restriction Enzyme	Single-Stranded Fragment Sizes (Nucleotides)
A	GGCGGCCTTCAGCAAGTACTTGAC ATGTTCGAGACACCAGCATAACAG	3–26 292–269	290	*Bsu*RI	99, 99 191, 191
B	AGGTGTTTTTCTCAAGGCTCATAC AGTGAGCCTTACTCGGAAGCACAG	182–204 477–454	296	*Taq*I	104, 106 190, 192
C	TCAACTTCATCGCTGCCATGCCTC CCAAGTAGGCCATCATGCTCGCTG	380–403 712–689	333	*Hin*fI	149, 152 181, 184
D	ACCAGCTGCTTACACAAGATGTAG TCGGAAGACTGAGTGGACTGTCTG	557–580 891–868	335	*Hin*cII	106, 106 229, 229
E	CGACTTCGAAGACCCATTGGTAAG ACAACTAGGTAACCAACAACAGTG	736–759 998–957	263		
F	TTGTTTCGGTTTTCAATGGGCTTC GACCATTGTGCGCTCATATTTGCC	922–945 1233–1210	312	*Xba*I	138, 142 170, 174
G	ATAGTTTTGGCTGGGCGTGAAATG GCCATTTGGACCACAAATTAGAAC	1120–1143 1428–1405	309	*Hin*PI	102, 104 205, 207
H	TGATCATGTTCCTTTAGCAACGCC TTCAAGGATATGACCCAACTGGAC	1326–1349 1668–1645	343	*Bsu*RI	145, 145 198, 198
I	GGACGAGAAGATCAGAAAAGGAAG AGACACAGTGAAGAGAGTGATGCC	1582–1605 1872–1849	291	*Hin*cII	228, 228 63, 63
J	GTTAGTGTCGACGTGGAAGGCTAC TTCACTGAAGCAGGTATAGTTCTC	1801–1824 2013–1990	213		

analysis has readily detected the presence of mutations in homologous proteins such as those coded by the ALDP gene (Kok et al. 1995) and the CFTR gene (Chillón et al. 1994; Ravnik-Glavac et al. 1994). Also, the polymorphism at nucleotide position 162 makes it unlikely that all of the patients' alleles share a common mutation that we have failed to detect. Indeed, given that our 12 patients were all unrelated, a number of different mutations (as many as 24) might be expected.

It has been suggested that some PMP70 mutations (e.g., the splice-site mutation leading to a 23-bp insertion [Gärtner et al. 1992*a*]) could have a dominant-negative effect, in which case an individual with a single mutant PMP70 allele would manifest symptoms of the disorder, and fewer mutant alleles would be expected/found among the relevant patients. Some support for this hypothesis comes from the knowledge that PMP70 contains only half the necessary domains to form a transporter and that therefore, in order to function, must form a dimer with a similar ABC transporter. Members of the ABC transporter family with half the complement of domains are known to form either homo- or heterodimers (Higgins 1992). If a mutant PMP70 allele produces a stable polypeptide product, then it is possible that its incorporation into dimers might have a dominant-negative effect on transporter function. However, as indicated earlier, a dominant inheritance pattern is unlikely in four of our families, unless a gonadal mosaic involving a mutant allele with a dominant-negative effect were involved. If PMP70 forms a heterodimer, insufficient functional transporter might also be formed if the individual is heterozygous for mutations in both components of the transporter; and, again, fewer mutant PMP70 alleles would be expected in the patients. Interestingly, two genes associated with peroxisome-deficient *Hansenula polymorpha* mutants have recently been shown to involve both dominant-negative and recessive mutant alleles (Tan et al. 1995). In addition, two *Saccharomyces cerevisiae* genes have recently been described that code for ABC transporters. Mutants of either gene are unable to oxidize oleic acid, an entirely per-

Table 2

Sequencing of Patients' cDNA for PMP70 (1,980 Nucleotides, Including Stop Condon)

	No. (%) of Nucleotides Sequenced	
Patient	In Both Directions	In at Least One Direction
1	355 (17.9)	556 (28.1)
2	1,580 (79.8)	1,936 (97.8)
3	354 (17.9)	467 (23.6)
4	1,746 (88.2)	1,945 (98.2)
5	1,475 (74.5)	1,934 (97.7)
6	597 (30.2)	708 (35.8)
7	911 (46.0)	1,102 (55.7)
8	454 (22.9)	707 (35.7)
9	149 (7.5)	267 (13.5)

oxisomal function in yeast, and it has been suggested that the two gene products may be subunits of the same transporter (Shani et al. 1995). Thus, it is possible that the human disorder might be caused by heterozygosity for mutations in two interacting components of the peroxisomal biogenesis machinery. To our knowledge, none of the parents of our patients were consanguineous, so the results of this study are compatible with the aforementioned possibility. However, if patients from complementation group 1, in which the parents are consanguineous, exist elsewhere, it would be more probable that only one gene, rather than two, is involved in the disorder in individual patients.

Although we cannot exclude the possibility that defects in PMP70 are involved in a subset of patients with peroxisomal biogenesis disorders from complementation group 1, our failure to identify any mutations in our group of patients, along with the sparsity of mutations detected in the patients studied by Gärtner et al. (1992*a*), makes it unlikely that defects in the PMP70 gene are a major cause of the peroxisomal biogenesis defect in patients from complementation group 1. Our findings are also consistent with the recent report, from Shimozawa et al. (1996), that transfection of fibroblasts from complementation group 1 with human cDNA encoding PMP70 failed to correct the peroxisomal biogenesis defect. To date, there has been no convincing evidence of functional loss of PMP70 in patients. In particular, to our knowledge, expression of the reported missense mutation (Gärtner et al. 1992*a*) has not been attempted, and, although Gärtner et al. (1994) expressed the deletion resulting from the donor splice-site mutation in PMP35-deficient CHO cells, no information was supplied on the proportion of aberrantly spliced message generated from this allele in the patient (Gärtner et al. 1992*a*). If defects in PMP70 are involved only in some patients, then complementation group 1 must be genetically heterogeneous, and an explanation is required for the failure of cells with genetically different disorders to complement each other. To our knowledge, no complementation group 1 patient has been shown to have two defective alleles for PMP70, and, although more complicated inheritance patterns may be involved (see above), until more is known about the function of PMP70 and whether it acts as a homo- or heterodimer, or both, these possibilities remain speculative. We therefore advise caution in ascribing the cause for complementation group 1 of the peroxisomal biogenesis disorders to mutations in the gene for PMP70.

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Disease Relevance of the So-Called Secondary Leber Hereditary Optic Neuropathy Mutations

To the Editor:

In the August issue of the *Journal,* Mackey et al. (1996) addressed the open question of the pathogenic role of the mtDNA mutation at nucleotide position (np) 15257 in Leber hereditary optic neuropathy (LHON). This was done by investigating a total of 159 LHON families from Australia/New Zealand and from several European countries. As a major result, the 15257 mutation was found in 6 (4%) of 159 families, and in every one of these instances it was associated with one of the established primary LHON mutations, at np 11778 (in 4 [5%] of 78 families), at np 3460 (in 1 [7%] of 14 families), and at np 14484 (in 1 [4%] of 23 families). Because the 15257 mutation did not occur in isolation of an established primary LHON mutation, Mackey et al. questioned whether there is a primary pathogenic role for this mutation in the cytochrome b gene. However, they also discussed possible doubts about this conclusion. As one of the points, they cited a report by our group (Obermaier-Kusser et al. 1994) on a German LHON family that carries the 15257 mutation but lacks any of the three established primary mutations. We would like to comment on this point. In the cited paper, we described 17 families and sporadic cases in which the mutation pattern included at least one primary or intermediate mutation. The 15257 mutation was found in three index patients; in two cases in association with the 11778 or 14484 mutation and in one case without any of the three primary mutations. Maternal inheritance and male predominance in combination with the clinical features confirm the classification of LHON in this latter family. Of the eight families tested in that paper, the singleton 15257 family showed the highest penetrance (56%, all males). Meanwhile, we identified 38 further LHON index cases. Our total LHON collective ($n = 55$) now consists of 24 carriers of 11778, 5 carriers of 3460, and 21 carriers of 14484; the 15257 mutation is found three times associated with np 11778, six times associated with np 14484, and five times without any of the established primary mutations. The percentage of LHON patients carrying the 15257 mutation (25%) by far exceeds the finding by Mackey et al. (1996). In the five cases without primary mutation, the 15257 mutation behaves like a primary LHON mutation; however, we cannot exclude the possibility that another, still-unknown primary mutation is present. Two other groups also reported singleton 15257 LHON cases (Howell et al. 1993; Brown et al. 1995), and, in these studies, other pathogenic mtDNA mutations could be excluded by screening most of the mtDNA coding regions.

The pathogenicity of the 15257 mutation is also discussed with respect to its haplogroup specificity. This mutation is nearly always associated with the mutations at nps 4216 and 13708 (Johns and Berman 1991; Brown et al. 1992). Mackey et al. (1996, p. 484) concluded that "the increased frequency of the 15257 mutation among LHON patients can be explained solely in terms of population history and genetics, rather than as a reflection of its pathogenic role." In order to look for an association of certain haplogroups with distinct neurodegenerative disorders, we currently are investigating