Panneerselvam K, Freeze HH (1996) Mannose enters mammalian cells using a specific transporter that is insensitive to glucose. J Biol Chem 271:9417–9421
Pelletier VA, Galeano N, Brochu P, Morin CL, Weber AM, Roy CC (1986) Secretory diarrhea with protein-losing enteropathy, enterocolitis cystica superficialis, intestinal lymphangiectasia, and congenital hepatic fibrosis: a new syndrome. J Pediatr 108:61–65
Pirard M, Collet JF, Matthijs G, Van Schaftingen E (1997) Comparison of PMM1 with the phosphomannomutases expressed in rat liver and in human cells. FEBS Lett 411: 251–254
Powell LD, Paneerselvam K, Vij R, Diaz S, Manzi A, Buist N, Freeze H, et al (1994) Carbohydrate-deficient glycoprotein syndrome: not an N- linked oligosaccharide processing defect, but an abnormality in lipid-linked oligosaccharide biosynthesis? J Clin Invest 94:1901–1909
Proudfoot AE, Turcatti G, Wells TN, Payton MA, Smith DJ (1994) Purification, cDNA cloning and heterologous expression of human phosphomannose isomerase. Eur J Biochem 219:415–423
Reyes A, Cardenas ML (1984) All hexokinase isoenzymes coexist in hepatocytes. Biochem J 221:303–309
Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2d ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
Sols A, Crane RK (1954) Substrate specificity of brain hexokinase. J Biol Chem 210:581–595
Stibler H, Jaeken J (1990) Carbohydrate deficient serum transferrin in a new systemic hereditary syndrome. Arch Dis Child 65:107–111
Thorens B, Sarkar HK, Kaback HR, Lodish HF (1988) Cloning and functional expression in bacteria of a novel glucose transporter present in liver, intestine, kidney and β-pancreatic islet cells. Cell 55:281–290
Van Schaftingen E, Jaeken J (1995) Phosphomannomutase deficiency is a cause of carbohydrate-deficient glycoprotein syndrome type I. FEBS Letters 377:318–320
Weinhouse S (1976) Regulation of glucokinase in liver. Curr Top Cell Regul 11:1–50

Address for correspondence and reprints: Dr. J. Jaeken, University Hospital Gasthuisberg, Herestraat 49, B-3000 Leuven, Belgium. E-mail: Jo.Vencken@uz.kuleuven.ac.be
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Temperature-Sensitive Phenotypes of Peroxisome-Assembly Processes Represent the Milder Forms of Human Peroxisome-Biogenesis Disorders

To the Editor:

Peroxisome-biogenesis disorders (PBDs) are lethal hereditary diseases caused by abnormalities in the assembly processes of peroxisomes (Moser et al. 1995). The peroxisome is a ubiquitous organelle involved in vital metabolic functions, such as oxidative processes involving H_2O_2, β-oxidation of fatty acids, and biosynthesis of plasmalogens (Van den Bosch et al. 1992). PBDs are characterized by multiple defects in these functions, as well as by the lack of morphologically normal peroxisomes. They are genetically classified into complementation groups (CGs), the number of which is $\geqslant 11$ (Shimozawa et al. 1993; Moser et al. 1995; Poulos et al. 1995). Each CG contains significantly different clinical phenotypes—for example, Zellweger syndrome (ZS), neonatal adrenoleukodystrophy (NALD), and infantile Refsum disease (IRD). ZS patients have severe neurological abnormalities, dysmorphic features, hepatomegaly, and multiple renal cysts, and most die at age <6 mo. NALD patients have similar symptoms, but they survive considerably longer, dying during early childhood. In contrast, IRD patients do not exhibit significant abnormalities in the CNS, and they have the longest average life span among patients with PBDs (Lazarow and Moser 1995; Moser et al. 1995). Although the causal genes (*PEX*s) for several CGs have been cloned and the mutations have been identified at the molecular level (Shimozawa et al. 1992 ; Dodt et al. 1995; Wiemer et al. 1995; Fukuda et al. 1996; Yahraus et al. 1996; Chang et al. 1997; Okumoto and Fujiki 1997; Portsteffen et al. 1997; Reuber et al. 1997), it is unknown why such diverse clinical phenotypes occur in the same CGs although, in all CGs, the phenotypes are very similar. We report that milder forms of PBDs are characterized by temperature-sensitive (TS) phenotypes of peroxisome-assembly processes in the fibroblasts of patients.

In spite of the variations in the clinical features, the fibroblasts from patients of all three PBD phenotypes generally lack peroxisomes. Although the occurrence of a reduced number of peroxisomes occasionally has been noted in several PBD cell lines (Arias et al. 1985; Wiemer et al. 1991; Slawecki et al. 1995), no correlation with clinical features has been apparent. We assumed that limited types of leaky mutations in the *PEX* genes could be the causes of the milder forms of PBDs. As a possible parameter representing such leakiness, we examined temperature sensitivity. Fibroblasts from PBD patients with different CGs were incubated at 30°C and at 37°C and were subjected to immunofluorescence staining with anti-catalase antibody. After 72 h incubation at 30°C, punctate staining of catalase typical of peroxisomes was detected in the fibroblasts of all six patients with IRD and in three of five of those with NALD, belonging to four different CGs (fig. 1*b* and table 1), whereas no peroxisomes appeared in the same cells after incubation at 37°C (fig. 1*a*). Catalase and the 70-kD peroxisomal membrane protein (PMP70) were colocalized in these

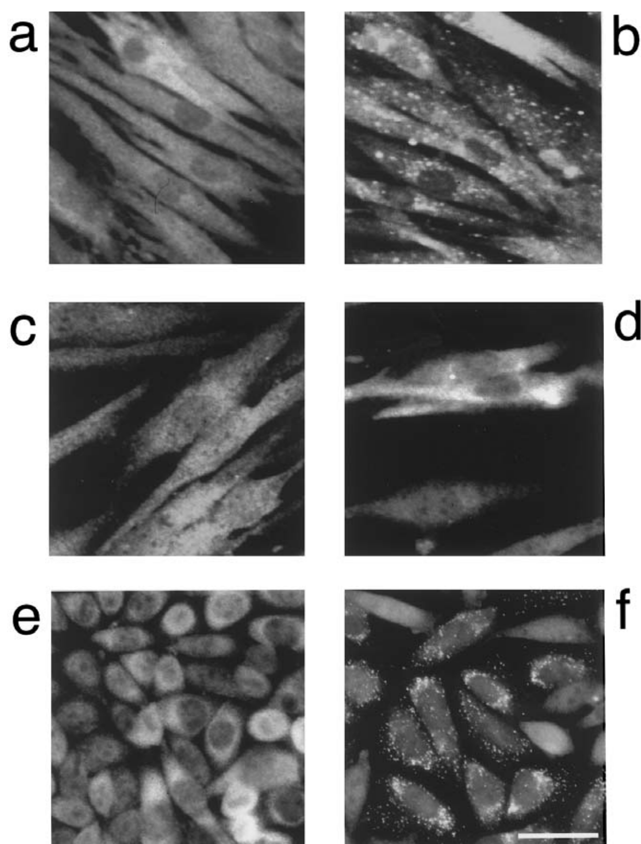

Figure 1 Immunofluorescence staining of peroxisomes in patients' fibroblasts and in Z65 mutant Chinese-hamster-ovary cells. Cells were cultured for 72 h at either 37°C (*a, c,* and *e*) or 30°C (*b, d,* and *f*) and were stained with either anti–human catalase antibody (*a–d*) or anti–rat catalase rabbit antibody (*e* and *f*). *a* and *b*, Fibroblasts of an IRD patient (F-05). *c* and *d*, Fibroblasts of a ZS patient (F-01). *e* and *f*, Z65 transformant with $Pex2^{E55K}$. (Scale bar = 50 μm)

cells after incubation for 72 h at 30°C (data not shown), thereby confirming the identity of these catalase-positive granules as peroxisomes. In the fibroblasts of ZS patients with any CGs tested, no peroxisomes were found at either 30°C or 37°C incubation (fig. 1*c* and *d* and table 1). Fibroblasts of normal controls had punctate patterns of catalase at both 30°C and 37°C incubation (data not shown).

We confirmed the formation of functional peroxisomes in the TS cells at 30°C, on the basis of three biochemical criteria: the peroxisomal β-oxidation activity of very-long-chain fatty acids (Suzuki et al. 1991), the activity of peroxisomal dihydroxyacetonephosphate acyltransferase (Shimozawa et al. 1988) involved in the plasmalogen biosynthesis, and the proteolytic processing of peroxisomal acyl-CoA oxidase (Tsukamoto et al. 1995), which occurs within the peroxisomes. These activities were deficient in the ZS cells (F-01), at both 30°C and 37°C incubation, whereas in the TS cells (F-05), these functions were markedly improved by incubation at 30°C but not by incubation at 37°C (data not shown).

To identify the mutation responsible for the TS phenotype, the *PEX2* gene (GenBank), the causal gene for CG-F (Shimozawa et al. 1992), was amplified by PCR from the genomic DNA of TS cells F-05. PCR reaction was performed by use of *PEX2* gene–specific antisense (complementary to the *PEX2* cDNA sequence of positions 967–991, 5′-ATA CTT AGG ATG ACT AAT ATT AAG-3′) and sense (an intron sequence of the *PEX2* gene, starting 48 bp upstream of the 5′ end of the coding exon, 5′-CAA GAT TGC AAC TCT TTG CTA ATG-3′) oligonucleotides. PCR conditions were as follows: initial heating for 1 min at 94°C, followed by 40 cycles of denaturation for 1 min at 94°C, annealing for 2 min at

Table 1

Temperature Sensitivity of Peroxisome Biogenesis in Fibroblasts of PBD Patients

CG and Patient[a]	Phenotype	Peroxisome-Positive Cells Incubated at[b] (%) 37°C	30°C	Age at Death or Last Follow-up[c]
A (8):				
A-06	ZS	0	10	4 mo
A-05	NALD	0	90	
A-08	NALD	0	80	3 years 1 mo
A-04[d]	IRD	0	60	
C (4):				
C-03[e]	ZS	0	0	8 mo
C-08	ZS	0	0	4 mo
E (1):				
E-14	ZS	0	0	4 mo
E-01[f]	NALD	5	5	2 years 9 mo
E-13	NALD	1	1	1 year 8 mo
E-05[d]	IRD	0	90	
E-24	IRD	0	60	1 year 7 mo[g]
E-25	IRD	0	60	10 years 7 mo[g]
E-26	IRD	0	50	6 years 1 mo[g]
F (10):				
F-01[h]	ZS	0	0	8 mo
F-04[i]	ZS	0	0	3 mo
F-05	IRD	0	70	
…[j] (6):				
6-01	NALD	5	80	4 years 6 mo

[a] The letter designation is that provided by Gifu University (Japan), and the number designation (in parentheses) is that provided by the Kennedy Krieger Institute.

[b] Data are averages of several view fields, at ×200.

[c] Data are for traceable cases only.

[d] Purchased from Coriell Cell Repositories (Camden, NJ); the cell line designations are ———GM08771 (A-04) and GM08770 (E-05).

[e] Source: Fukuda et al. (1996).

[f] Source: Maeda et al. (1990).

[g] Parents were alive after the age at the last follow-up (i.e., age shown).

[h] Source: Shimozawa et al. (1992).

[i] Source: Shimozawa et al. (1998).

[j] No designation of CG was available from Gifu University.

55°C, and extension for 3 min at 72°C. Nucleotide-sequence comparison with the normal *PEX2* gene revealed that this patient was heterozygous for two point mutations. One was a G→A substitution at nucleotide position 163,, relative to the A residue of the initiation codon, causing an amino acid alteration (E55K). The other was a C→T substitution at nucleotide position 355, resulting in the change of the codon 119 to a stop codon, TGA (R119 Stop). These two mutations were also found in the *PEX2* cDNA obtained by reverse transcriptase–PCR of the mRNA from F-05. The latter nonsense mutation is identical to that reported by Shimozawa et al. in both ZS patient F-01 (Shimozawa et al. 1992) and another CG-F patient (Shimozawa et al. 1993) (both cases were homozygous for the mutation), and it previously had been established that this mutation is nonfunctional. Accordingly, we investigated the relationship between the E55K mutation and the TS phenotype, by gene transfection. The $PEX2^{E55K}$ gene sequence subcloned in the expression vector pUcD2SRαMCS (Tsukamoto et al. 1995) was transfected to a *PEX2*-deficient Chinese-hamster-ovary cell mutant (Z65) (Tsukamoto et al. 1991, 1994), and stable transformants were produced. The transformants revealed a punctate distribution of catalase after 72 h incubation at 30°C, whereas no catalase-positive granules were observed for incubation at 37°C (fig. 1*e* and *f*); Z65 transfected with wild-type *PEX2* had catalase-positive granules at both 30°C and 37°C; and the cells transfected with the empty vector exhibited no peroxisomal staining at either 30°C or 37°C (data not shown). Thus, the TS phenotype of peroxisome biogenesis of the IRD fibroblasts (F-05) is caused by the E55K mutation of the *PEX2* gene.

The present results indicate that the peroxisome-assembly process is TS in the fibroblasts of patients with the mildest form of PBD (i.e., IRD), irrespective of CGs, and that this phenotype is directly linked to the specific genotype of the responsible *PEX* gene, at least in F-05. Such a TS phenotype was not observed for the most severe form of PBD (i.e., ZS), whereas only a subset of the cell lines were TS for NALD, the intermediate form of PBD. In this regard, it is interesting to note that the NALD patients with the TS phenotype (patients A-08 and 6-01) had longer life spans than did those with the non-TS phenotype (patients E-01 and E-13), even though the latter two patients exhibited slight leakiness at both temperatures. Thus, the TS phenotypes of peroxisome assembly in the cultured fibroblasts represent the mildness of the clinical symptoms of PBD. Patients with the TS phenotypes may be mosaic for peroxisome occurrence from cell to cell in the body at normal body temperatures. A mosaicism of peroxisomes was indeed reported in the liver of a PBD patient who had a relatively long life span (Giros et al. 1996). It is also possible that TS patients have partially functional peroxisomes. In any case, the TS patients probably have higher gross peroxisomal activities than do the patients with non-TS leaky phenotypes (patients E-01 and E-13).

Temperature- or cold-sensitive phenotypes have been noted in a few genetic diseases. In epidermolysis bullosa simplex, the disturbance of the skin becomes worse at higher temperatures, whereas, in paramyotonia congenita, exposure to lower temperatures causes myotonia. In these instances, the symptoms are understood to be direct effects of the temperature- and cold-sensitive phenotypes of the corresponding gene products, keratin (Morley et al. 1995) and Na^+-channel protein (McClatchey et al. 1992), respectively. In maple syrup–urine disease, symptoms sometimes worsenwhen there is a high fever (Chuang and Shih 1995); however, the responsible mutation has not been identified. Thus, TS phenotypes directly linked to specific genotypes possibly occur in various genetic diseases. Among these, PBD cases are unique in that a complex cellular process, peroxisome assembly, becomes TS because of a single gene mutation, causing distinct clinical features.

Our present results would raise several clinical implications. First, the severity of prognosis could be diagnosed, by examination of the temperature sensitivity of peroxisome assembly in the fibroblasts of newborn PBD patients. Second, precaution against fever may be necessary in the treatment TS PBD patients. Third, hypothermal therapy might be applicable to TS PBD patients. Such therapy might increase the frequency of cells having functional peroxisomes, thereby improving clinical symptoms.

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Atsushi Imamura,[1] Toshiro Tsukamoto, [1]
Nobuyuki Shimozawa,[2] Yasuyuki Suzuki,[2]
Zhonghi Zhang,[2] Tsuneo Imanaka,[4] Yukio Fujiki,[5]
Tadao Orii,[3] Naomi Kondo,[2] and Takashi Osumi[1]
[1]*Department of Life Science, Himeji Institute of Technology, Kamigori, Hyogo, Japan;* [2]*Department of Pediatrics, Gifu University School of Medicine, and* [3]*Faculty of Human Welfare, Chubu Gakuin University, Seki, Gifu, Japan;* [4]*Department of Microbiology and Molecular Pathology, Faculty of Pharmaceutical Sciences, Teikyo University, Sagamiko, Kanagawa, Japan; and* [5]*Department of Biology, Faculty of Science, Kyushu University, Fukuoka, Japan*

Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

GenBank, http://www.ncbi.nih.gov/Web/Search/index/html (for human peroxisome-assembly factor-1 [*hsPEX2;* accession number M86852])

References

Arias JA, Moser AB, Goldfischer SL (1985) Ultrastructural and cytochemical demonstration of peroxisomes in cultured fibroblasts from patients with peroxisomal deficiency disorders. J Cell Biol 100:1789–1792

Chang CC, Lee WH, Moser H, Valle D, Gould SJ (1997) Isolation of the human PEX12 gene, mutated in group 3 of the peroxisome biogenesis disorders. Nat Genet 15:385–388

Chuang DT, Shih VE (1995) Disorders of branched chain amino acid and keto acid metabolism. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) The metabolic and molecular bases of inherited disease. McGraw-Hill, New York, pp 1239–1278

Dodt G, Braverman N, Wong C, Moser A, Moser HW, Watkins P, Valle D, et al (1995) Mutations in the PTS1 receptor gene, PXR1, define complementation group 2 of the peroxisome biogenesis disorders. Nat Genet 9:115–125

Fukuda S, Shimozawa N, Suzuki Y, Zhang Z, Tomatsu S, Tsukamoto T, Hashiguchi N, et al (1996) Human peroxisome assembly factor-2 (PAF-2): a gene responsible for group C peroxisome biogenesis disorder in humans. Am J Hum Genet 59:1210–1220

Giros M, Roels F, Prats J, Ruiz M, Ribes A, Espeel M, Wanders RJ, et al (1996) Long survival in a case of peroxisomal biogenesis disorder with peroxisome mosaicism in the liver. Ann N Y Acad Sci 804:747–749

Lazarow PB, Moser HW (1995) Disorders of peroxisome biogenesis. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) The metabolic and molecular bases of inherited disease. McGraw-Hill, New York, pp 2287–2324

Maeda K, Terada A, Murakami Y, Terabe K, Oki S, Hattori M, Matsumoto N, et al (1990) Clinical and biochemical analysis of infant with neonatal adrenoleukodystrophy (in Japanese). Acta Paediatr Jpn 94:1869–1876

McClatchey AI, van den Bergh P, Pericak VM, Raskind W, Verellen C, McKenna YD, Rao K, et al (1992) Temperature-sensitive mutations in the III-IV cytoplasmic loop region of the skeletal muscle sodium channel gene in paramyotonia congenita. Cell 68:769–774

Morley SM, Dundas SR, James JL, Gupta T, Brown RA, Sexton CJ, Navsaria HA, et al (1995) Temperature sensitivity of the keratin cytoskeleton and delayed spreading of keratinocyte lines derived from EBS patients. J Cell Sci 108: 3463–3471

Moser AB, Rasmussen M, Naidu S, Watkins PA, McGuinness M, Hajra AK, Chen G, et al (1995) Phenotype of patients with peroxisomal disorders subdivided into sixteen complementation groups. J Pediatr 127:13–22

Okumoto K, Fujiki Y (1997) *Pex12* encodes an integral membrane protein of peroxisomes. Nat Genet 17:265–266

Portsteffen H, Beyer A, Becker E, Epplen C, Pawlak A, Kunau WH, Dodt G (1997) Human PEX1 is mutated in complementation group 1 of the peroxisome biogenesis disorders. Nat Genet 17: 449–452

Poulos A, Christodoulou J, Chow CW, Goldblatt J, Paton BC, Orii T, Suzuki Y, et al (1995) Peroxisomal assembly defects: clinical, pathologic, and biochemical findings in two patients in a newly identified complementation group. J Pediatr 127: 596–599

Reuber BE, Germain LE, Collins CS, Morrell JC, Ameritunga R, Moser HW, Valle D, et al (1997) Mutations in PEX1 are the most common cause of peroxisome biogenesis disorders. Nat Genet 17:445–448

Shimozawa N, Suzuki Y, Orii T, Moser A, Moser HW, Wanders RJ (1993) Standardization of complementation grouping of peroxisome-deficient disorders and the second Zellweger patient with peroxisomal assembly factor-1 (PAF-1) defect. Am J Hum Genet 52:843–844

Shimozawa N, Suzuki Y, Orii T, Yokota S, Hashimoto T (1988) Biochemical and morphologic aspects of peroxisomes in the human rectal mucosa: diagnosis of Zellweger syndrome simplified by rectal biopsy. Pediatr Res 24:723–727

Shimozawa N, Suzuki Y, Tomatsu S, Nakamura H, Kono T, Takada H, Tsukamoto T, et al (1998) A novel mutation, R125X in peroxisome assembly factor-1 responsible for Zellweger syndrome. Hum Mutat Suppl 1:S134–S136

Shimozawa N, Tsukamoto T, Suzuki Y, Orii T, Shirayoshi Y, Mori T, Fujiki Y (1992) A human gene responsible for Zellweger syndrome that affects peroxisome assembly. Science 255:1132–1134

Slawecki ML, Dodt G, Steinberg S, Moser AB, Moser HW, Gould SJ (1995) Identification of three distinct peroxisomal protein import defects in patients with peroxisome biogenesis disorders. J Cell Sci 108:1817–1829

Suzuki Y, Shimozawa N, Yajima S, Yamaguchi S, Orii T, Hashimoto T (1991) Effects of sodium 2-[5-(4-chlorophenyl)pentyl]-oxirane-2-carboxylate (POCA) on fatty acid oxidation in fibroblasts from patients with peroxisomal diseases. Biochem Pharmacol 41:453–456

Tsukamoto T, Miura S, Fujiki Y (1991) Restoration by a 35K membrane protein of peroxisome assembly in a peroxisome-deficient mammalian cell mutant. Nature 350:77–81

Tsukamoto T, Miura S, Nakai T, Yokota S, Shimozawa N, Suzuki Y, Orii T, et al (1995) Peroxisome assembly factor-2, a putative ATPase cloned by functional complementation on a peroxisome-deficient mammalian cell mutant. Nat Genet 11:395–401

Tsukamoto T, Shimozawa N, Fujiki Y (1994) Peroxisome assembly factor 1: nonsense mutation in a peroxisome-deficient Chinese hamster ovary cell mutant and deletion analysis. Mol Cell Biol 14:5458–5465

Van den Bosch H, Schutgens RBH, Wanders RJA, Tager JM (1992) Biochemistry of peroxisomes. Annu Rev Biochem 61: 157–197

Wiemer EA, Nuttley WM, Bertolaet BL, Li X, Francke U, Wheelock MJ, Anne UK, et al (1995) Human peroxisomal targeting signal-1 receptor restores peroxisomal protein im-

port in cells from patients with fatal peroxisomal disorders. J Cell Biol 130:51–65

Wiemer EA, Out M, Schelen A, Wanders RJ, Schutgens RB, van den Bosch H, Tager JM (1991) Phenotypic heterogeneity in cultured skin fibroblasts from patients with disorders of peroxisome biogenesis belonging to the same complementation group. Biochim Biophys Acta 1097:232–237

Yahraus T, Braverman N, Dodt G, Kalish JE, Morrell JC, Moser HW, Valle D, et al (1996) The peroxisome biogenesis disorder group 4 gene, PXAAA1, encodes a cytoplasmic ATPase required for stability of the PTS1 receptor. EMBO J 15:2914–2923

Address for correspondence and reprints: Dr. Takashi Osumi, Department of Life Science, Himeji Institute of Technology, Kamigori, Hyogo 678-1297, Japan. E-mail: osumi@sci.himeji-tech.ac.jp

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A Breast Cancer Patient of Scottish Descent with Germ-Line Mutations in *BRCA1* and *BRCA2*

To the Editor:

Ramus et al. (1997) previously described an Ashkenazi Jewish patient found to have germ-line mutations in both breast and ovarian cancer–susceptibility genes, *BRCA1* and *BRCA2*. We report the first such example for the non-Jewish Caucasian population. The patient, who is indicated by an arrow in pedigree 232 (fig. 1), was of Scottish origin. She was diagnosed with breast cancer (grade 2 adenocarcinoma) at age 35 years. Simultaneous screening by protein truncation test of both *BRCA1* (exon 11) and *BRCA2* (exon 11) detected truncating

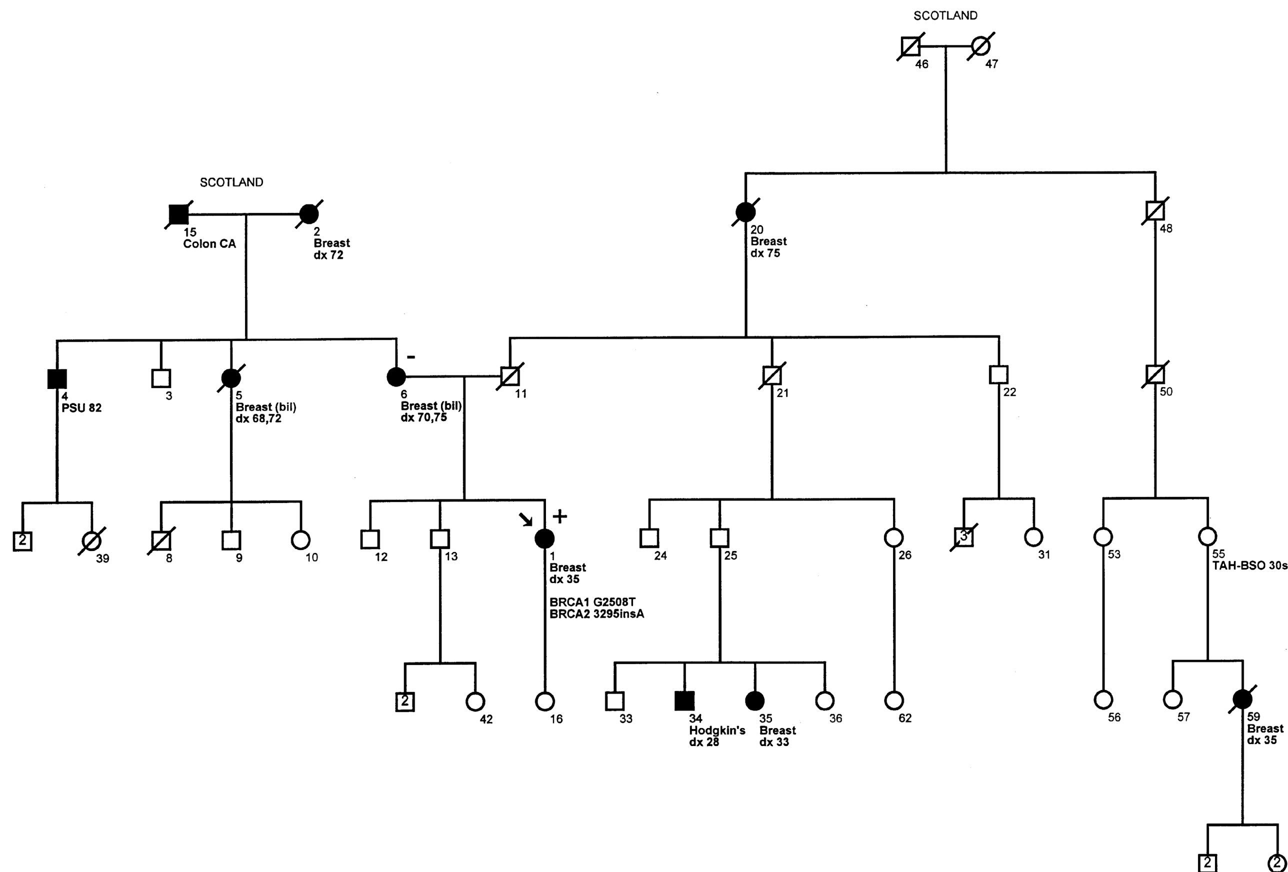

Figure 1 Pedigree of family 232. Blackened circles indicate affected women; blackened squares indicate affected men; and a diagonal slash indicates that the patient is deceased. Individual identification numbers appear directly below the symbols. Breast cancer is indicated with age at diagnosis ("dx"); bilateral breast cancer is indicated ("bil") with ages at diagnoses; "PSU" indicates primary site not known; and "TAH-BSO" indicates a complete abdominal hysterectomy, including bilateral oophorectomy. The plus sign (+) indicates the presence of the *BRCA1* G2508T mutation and the *BRCA2* 3295insA mutation, in the proband tested. The minus sign (−) indicates the absence of these two mutations in *BRCA1* and *BRCA2*.