MUTATION IN BRIEF

Seventeen Novel *PLP1* Mutations in Patients with Pelizaeus-Merzbacher Disease

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Pelizaeus-Merzbacher disease (PMD) is a rare X-chromosomal neurodegenerative disorder that affects primarily the white matter of the central nervous system and is caused by mutations of the *PLP1* (proteolipid protein 1) gene. We performed mutation analysis of 133 male patients with suspected PMD. Following SSCP analysis of all coding exons of *PLP1*, we found most likely pathogenic mutations (single base substitutions and small rearrangements) including 17 novel sequence variants in 21 (15.8%) patients. Most patients with missense mutations had a severe phenotype. Twelve patients (9.0%) carried a duplication of the entire gene, as demonstrated by quantitative real-time PCR, and presented with a variable clinical phenotype including mild, classical, and severe courses of disease. Two patients had large deletions, spanning approximately 115 kb, that included the *PLP1* gene. In total, we identified pathogenic mutations involving *PLP1* in 35 (26.3%) of the 133 patients analyzed.

KEY WORDS: Pelizaeus-Merzbacher disease; *PLP1*; duplication; deletion; quantitative PCR

INTRODUCTION

Pelizaeus-Merzbacher disease (PMD; MIM# 312080) belongs to inherited leukodystrophies, diseases affecting primarily the white matter of the central nervous system (CNS). Within the heterogeneous group of dysmyelinating disorders, PMD accounts for 6.6 % of all cases (Heim et al., 1997). PMD is caused by mutations of the *PLP1* gene (MIM# 300401) on Xq22. The gene encodes two proteins expressed abundantly in oligodendrocytes, the proteolipid protein (PLP) and its differently spliced isoform DM20. The two proteins are identical except for 35 amino acids that are present in PLP but absent in DM20 and account for more than half of the total protein mass of myelin in the CNS. PLP is thought to play a major role in myelin sheath formation by promoting sheath compaction (Boison et al., 1995). PLP dosage seems to be tightly regulated since duplication of *PLP1* is a frequent cause of PMD. X-linked spastic paraplegia type 2 (SPG2; MIM# 312920) is the second major phenotype associated with mutations of *PLP1*. Here we report 17 novel *PLP1* mutations in patients with PMD.

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PATIENTS AND METHODS

Patients

We analyzed DNA samples of 133 unrelated male patients with clinical suspicion of PMD. For all patients informed consent was obtained.

For an arbitrary classification of the patients' phenotypes several clinical signs and symptoms were considered. 'Mild' PMD was distinguished by the absence of nystagmus and the presence of mild spastic quadriparesis that mostly affected the legs, with ataxia and mild multifocal demyelinating peripheral neuropathy. Patients with 'classic' PMD manifested first symptoms such as nystagmus, hypotonia, and cognitive impairment in infancy or early childhood, nystagmus later often disappeared and ataxia, spasticity, involuntary movements as well as optic atrophy and microcephaly developed. Ambulation was often achieved in classic PMD. The term 'severe' PMD was used for patients with onset of symptoms within the first year of life with rapid progression, some with yet unknown outcome.

PLP1 mutation analysis

Genomic DNA was isolated from peripheral blood lymphocytes by standard methods. Each of the 7 coding exons of the *PLP1* gene was PCR-amplified using intronic primers (Doll et al., 1992; Strautnieks et al., 1992). SSCP analysis (Gu et al., 1996) was followed by direct sequencing of selected samples. Sequence variants resulting in a change of the restriction map of the respective amplicon were confirmed by restriction digestion and agarose gel electrophoresis. Fifty unrelated women without family history for PMD were genotyped for sequence variants of unknown relevance for the disease. The *PLP1* cDNA sequence (GenBank ID number NM_000533.3) was used to describe the variants identified, the A of the ATG initiation codon being +1.

In two samples, none of the *PLP1* exons could be amplified suggesting the presence of a large deletion. To identify the breakpoints, sequence tagged sites (STS) were amplified at every 5,000 to 10,000 bp centromeric and telomeric to the *PLP1* gene (primers available on request). Finally, a mutation-specific junction fragment was amplified and sequenced.

To estimate the copy number of *PLP1*, exon 4 of *PLP1* and exon 4 of *CFTR* were amplified simultaneously (Mimault et al., 1999; Woodward and Malcolm, 2001) in a quantitative PCR using the SYBR-Green PCR Master Mix (Applied Biosystems) and the Rotor-Gene real time cycler (Corbett Research, Australia). Each sample was amplified in duplicates and gave consistent results in two independent experiments. *PLP1* amplification efficiency was normalized to that of *CFTR*. For *PLP1* dosage estimation a *PLP1* exon 4 amplicon of an unaffected female individual was used as a calibrator and set to value 1.0 (Fig. 1). Validation of the assay was performed by analysis of DNA samples of 20 unaffected female and male individuals. Normalized mean values were 0.47 and 1.04 for male and female controls, respectively. Normalized values within 0.35 - 0.59 (mean ± 2 STD) and 0.86 - 1.18 (mean ± 2 STD) were considered to correspond to one and two copies of *PLP1*, respectively.

RESULTS AND DISCUSSION

Clinical diagnosis of PMD relies on the typical neurological presentation, diffusely abnormal myelin on magnetic resonance imaging (MRI), and, in familial cases, X-linked inheritance. Depending on the clinical criteria used for selecting the patients to be included in mutation analysis, up to 80% may carry a *PLP1* mutation (Mimault et al., 1999). Our cohort of 133 patients was selected less stringently and included patients without cranial MRI evaluation and those with unclassified leukodystrophy, taking into account the difficulty of distinguishing different myelin disorders by clinical presentation. In total, we identified pathogenic *PLP1* mutations in 35 (26.3%) of the 133 patients. These included duplications or deletions of the complete gene and point mutations or small rearrangements of *PLP1*.

Duplications

PLP1 duplications were reported to be the most frequent cause of PMD with a phenotype at the milder end of the spectrum (Inoue et al., 1999). In a group of 82 critically selected sporadic cases, 39 (47.5%) patients were found to carry a duplication of *PLP1* and 24 (29.3%) patients had various 'short length' mutations (Mimault et al., 1999). Though insertions of the duplicated *PLP1* at a greater distance have also been observed, the duplication is

usually tandem in nature, occurring in a 800 kb region of the X chromosome which includes the *PLP1* gene, and arises from an intrachromosomal recombination or sister chromatid exchange predominantly during male meiosis (Inoue et al., 1999). Various techniques have been proposed to detect *PLP1* duplications, including Southern blot (Ellis and Malcolm, 1994; Sistermans et al., 1998), multiplex PCR (Sistermans et al., 1998; Woodward et al., 1998), and FISH (Woodward et al., 1998), but in our hands all were found to be less reliable and more labor-intensive than the quantitative real-time PCR used here.

Of the 133 patients studied by us, 12 (9%) carried a duplication of *PLP1*, a frequency much lower than reported by others (Mimault et al., 1999). Clearly, the low detection rate in our cohort may be in part due to the less stringent inclusion criteria of patients, the higher frequency of patients with severe phenotype (patients with a very mild disease such as spastic paraplegia are probably underrepresented in our cohort), or different ethnical backgrounds.

Although the long-term outcome is still undefined for most of the 12 patients in our cohort with *PLP1* duplications, we found strikingly variable clinical phenotypes ranging from severe (5 cases; onset either connatal or within the first months of life) to classic (3 cases) and mild (4 cases) courses of disease. In mild cases, no nystagmus was noted and ambulation was achieved. Clearly, the different clinical pictures could be explained by the known variation of size of duplicated region as well as by a particular location of the breakpoints.

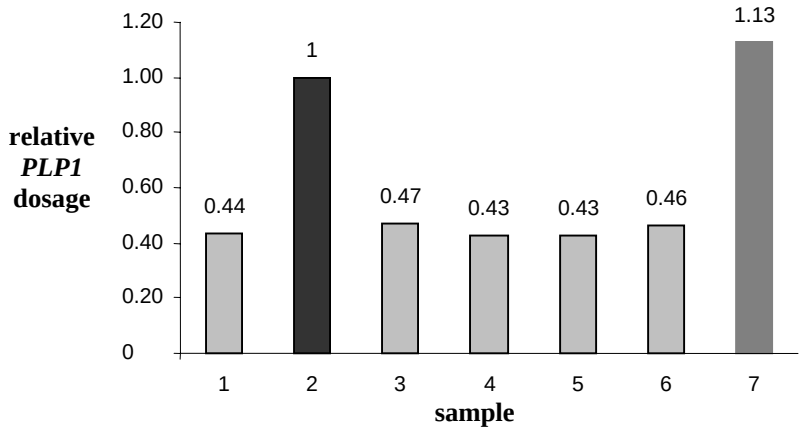

Figure 1. Representative results of a quantitative PCR run with 6 male DNA samples after normalization to *CFTR* and an unaffected female control (*PLP1* calibrator; sample 2). One patient (sample 7) carried a duplication of *PLP1*.

Deletions

In two samples, none of the *PLP1* exons could be amplified suggesting the presence of a large deletion. Only three families with gross *PLP1* deletions have been described so far (Raskind et al., 1991; Inoue et al., 2002). In those families, the authors claimed that the deletions included at least two further genes. First, *RAB9L*, that is a member of the RAB family of small G proteins regulating intracellular vesicle trafficking, including exocytosis, endocytosis, and recycling with yet unknown function. Second, *TMSNB*, that encodes a protein belonging to the thymosin ß family and is thought to bind to actin monomers thus preventing actin polymerization (Huff et al., 2001). In the first patient ascertained by us, the deletion spanned approximately 115 kb and included *PLP1* and *RAB9B*. Sequencing of a deletion-specific junction fragment revealed the exact boundaries of the deletion (available on request). By amplifying the junction fragment we could show that the deletion was absent in the peripheral lymphocyte DNA of the patient's mother suggesting a *de novo* event. The clinical phenotype of this patient fits classic PMD. In the second family, we identified a deletion of approximately the same size, including again *PLP1* and *RAB9B*. As a deletion-specific junction fragment could not be amplified, the exact boundaries are yet undetermined. The two affected brothers were severely handicapped and had a disease onset shortly after birth.

Point Mutations

Point mutations and small rearrangements of *PLP1* account for approximately 15 to 20% of PMD alleles (Sistermans et al., 1998; Garbern et al., 1999; Mimault et al., 1999), and are distributed throughout the gene. We identified a gene defect in 15.8% (21 of 133 samples) of our patients. Thus the rate of point mutations in our patient group was within the expected range, which argues against a major sampling bias due to less stringent inclusion criteria compared to other studies. Four mutations (p.A39T, p.L46P, p.L239P, and c.696+2T>G) have already been reported by others. All 17 novel sequence variants identified in this study are most likely pathogenic for they either result in truncation of the protein, substitution of an evolutionarily conserved amino acid, or affect splice consensus sequences (Table 1). Seven of the missense mutations (p.C33Y, p.C35R, p.A76P, p.V162E, p.S170P, p.P173S, and p.G246A) lie within the hydrophobic transmembrane spans (Fig. 2) and may interfere with correct folding of the polypeptide. In general, missense mutations are thought to lead to retention of the mutant PLP/DM20 protein in the endoplasmic reticulum. Misfolding and retention may induce oligodendrocyte apoptosis and thus cause a severe phenotype (Gow et al., 1994; Gow and Lazzarini, 1996; Thomson et al., 1997). In the patients studied here, mutations p.C33Y, p.C35R, p.V162E, p.P173S, p.Y50C, and p.A76P were associated with severe PMD, p.Y50C and p.A76P presenting as connatal manifestations, whereas p.S170P and p.G246A were associated with a mild clinical course.

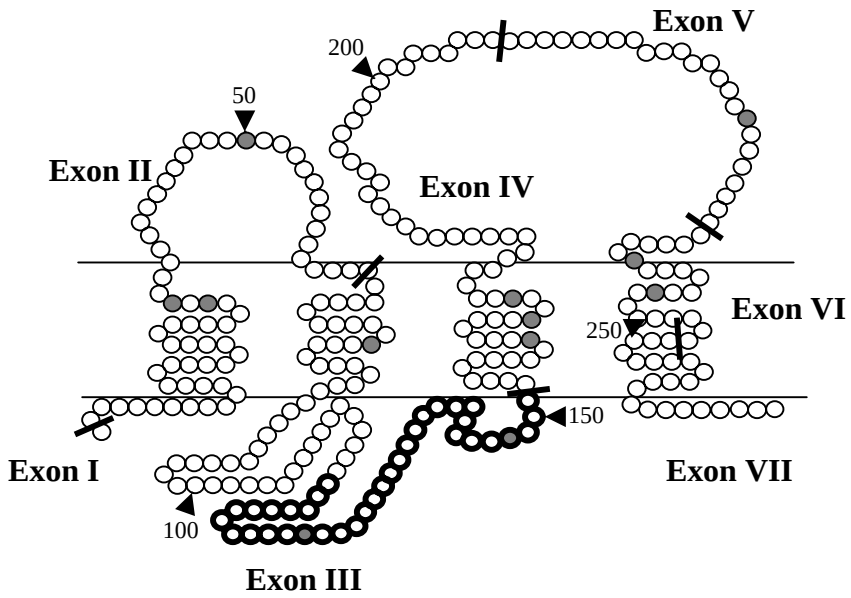

Figure 2. Cartoon of the structure of PLP. Amino acids absent in DM20 are shown in bold. Novel amino acid substitutions identified in this study are shown in gray.

We detected three novel donor splice site sequence variants (c.191+3A>T, c.762G>T, and c.453+7A>G). Splice sites are sequences that determine the boundaries of exons. The donor splice site is characterized by the consensus RNA sequence exon…AG/guragu…intron, where r denotes a pyrimidine. There are online software programs that predict splice sites by a classification approach (a set of consensuses) based on the assumption that high frequency of some nucleotides in definite positions reflects the functional importance of a given nucleotide at this position. Nucleotides at different site positions are considered to be mutually dependent, thus forming the structure recognized by components of the splicing machinery (Rogozin and Milanesi, 1997). Indeed, analysis of the wild-type allele c.191+3A with the online software Spliceview (www.itba.mi.cnr.it/webgene) revealed a score of 0.81 for the donor site whereas the program did not recognize a splice site on the mutated c.191+3T allele. c.762G is the last nucleotide of exon 6, and a G is found at this position in about 80% of cases compared to a T in 8% (Zhang, 1998). The isocoding variant c.762G>T resulted in a complete loss of the donor site, as predicted by Spliceview.

As this software does not consider the 7th intronic nucleotide, the online software of the Berkeley Drosophila genome project (http://www.fruitfly.org/seq_tools/splice.html) (Reese et al., 1997) was used for analyzing the c.453+7A>G transition in IVS3. Indeed, the score of the donor site was decreased from 0.69 to 0.54 by c.453+7A>G. Nevertheless, the precise molecular consequences of the sequence variants on the *PLP1* mRNA splicing can only be determined by functional assays. The two patients with IVS2 and IVS3 mutations identified in this study had a classic manifestation with onset of PMD during early childhood. This is in line with the observation of two other patients with classical PMD phenotype and mutations involving the splice site of intron 3 (Hobson et al., 2000).

Table 1. Sequence Variants of *PLP1* Identified in the Present Study

Exon/Intron	Nucleotide Change	Amino Acid Change / Consequence	Phenotype	Onset
Novel Mutations				
2	c.98G>A	p.C33Y	severe	?
2	c.103T>C	p.C35R	severe	first year
2	c.149A>G	p.Y50C	severe	connatal
3	c.226G>C	p.A76P	severe	connatal
3	c.385C>T	p.Q129X	classic	?
3	c.442C>T	p.H148Y	?	?
4	c.481_482dupGT	p.V162LfsX37	severe	first year
4	c.485T>A	p.V162E	severe	first year
4	c.508T>C	p.S170P	mild	first year
4	c.517C>T	p.P173S	severe	first year
5	c.630delC	p.P211HfsX25	severe	?
5	c.674T>C	p.L225P	classic	first year
6	c.716T>C	p.L239P	?	?
6	c.737G>C	p.G246A	mild	first year
6	c.762G>T	splicing?	mild	second year
IVS2	c.191+3A>T	splicing?	classic	?
IVS3	c.453+7A>G	splicing?	classic	first year
Recurrent Mutations				
2	c.115G>A	p.A39T (A38T in Cailloux et al., 2000)	?	?
2	c.137T>C	p.L46P (L45P in Hodes et al., 1999)	classic	first year
5	c.716T>C	p.L239P (L223P in Strautnieks et al., 1992)	?	?
IVS5	c.696+2T>G	splicing (Aoyagi et al., 1999)	classic	first year
Unknown Significance				
Promoter	c.-210_-220del	?	n.a.	n.a.
IVS4	c.622+28C>G	?	n.a.	n.a.
Polymorphisms				
Promoter	c.-102C>T	not pathogenic	n.a.	n.a.

The *PLP1* cDNA sequence (GenBank ID number NM_000533.3) was used to describe the variants identified, with the A of the ATG initiation codon being +1. n.a.: not applicable

The significance of c.-210_-220del and c.622+28C>G variants in the pathogenesis of the patients' disease is unknown for the time being. The sequence variant c.-102C>T detected in the *PLP1* promoter region was also present in an unaffected brother of the index patient and thus represents most likely a non-pathogenic variant.

Of the 133 male patients suspected to suffer from PMD, 35 (26.3%) had a disease-relevant mutation of the *PLP1* gene. In view of this high proportion of patients carrying a *PLP1* mutation in our cohort, we feel that screening unselected patients with leukodystrophy for *PLP1* mutations may be considered an option in reaching diagnosis in this very heterogeneous group of disorders.

REFERENCES

Aoyagi Y, Kobayashi H, Tanaka K, Ozawa T, Nitta H, Tsuji S. 1999. A de novo splice donor site mutation causes in-frame deletion of 14 amino acids in the proteolipid protein in Pelizaeus-Merzbacher disease. Ann Neurol 46:112-115.

Boison D, Bussow H, D'Urso D, Muller HW, Stoffel W. 1995. Adhesive properties of proteolipid protein are responsible for the compaction of CNS myelin sheaths. J Neurosci 15:5502-5513.

Cailloux F, Gauthier-Barichard F, Mimault C, Isabelle V, Courtois V, Giraud G, Dastugue B, Boespflug-Tanguy O. 2000. Genotype-phenotype correlation in inherited brain myelination defects due to proteolipid protein gene mutations. Clinical European Network on Brain Dysmyelinating Disease. Eur J Hum Genet 8:837-845.

Doll R, Natowicz MR, Schiffmann R, Smith FI. 1992. Molecular diagnostics for myelin proteolipid protein gene mutations in Pelizaeus-Merzbacher disease. Am J Hum Genet 51:161-169.

Ellis D, Malcolm S. 1994. Proteolipid protein gene dosage effect in Pelizaeus-Merzbacher disease. Nat Genet 6:333-334.

Garbern JY, Cambi F, Lewis R, Shy M, Sima A, Kraft G, Vallat JM, Bosch EP, Hodes ME, Dlouhy S, Raskind W, Bird T, Macklin W, Kamholz J. 1999. Peripheral neuropathy caused by proteolipid protein gene mutations. Ann N Y Acad Sci 883:351-365.

Gow A, Friedrich VL, Jr., Lazzarini RA. 1994. Many naturally occurring mutations of myelin proteolipid protein impair its intracellular transport. J Neurosci Res 37:574-583.

Gow A, Lazzarini RA. 1996. A cellular mechanism governing the severity of Pelizaeus-Merzbacher disease. Nat Genet 13:422-428.

Gu SM, Orth U, Veske A, Enders H, Klunder K, Schlosser M, Engel W, Schwinger E, Gal A. 1996. Five novel mutations in the L1CAM gene in families with X linked hydrocephalus. J Med Genet 33:103-106.

Heim P, Claussen M, Hoffmann B, Conzelmann E, Gartner J, Harzer K, Hunneman DH, Kohler W, Kurlemann G, Kohlschutter A. 1997. Leukodystrophy incidence in Germany. Am J Med Genet 71:475-478.

Hobson GM, Davis AP, Stowell NC, Kolodny EH, Sistermans EA, de Coo IF, Funanage VL, Marks HG. 2000. Mutations in noncoding regions of the proteolipid protein gene in Pelizaeus-Merzbacher disease. Neurology 55:1089-1096.

Hodes ME, Zimmerman AW, Aydanian A, Naidu S, Miller NR, Garcia Oller JL, Barker B, Aleck KA, Hurley TD, Dlouhy SR. 1999. Different mutations in the same codon of the proteolipid protein gene, PLP, may help in correlating genotype with phenotype in Pelizaeus-Merzbacher disease/X-linked spastic paraplegia (PMD/SPG2). Am J Med Genet 82:132-139.

Huff T, Muller CS, Otto AM, Netzker R, Hannappel E. 2001. beta-Thymosins, small acidic peptides with multiple functions. Int J Biochem Cell Biol 33:205-220.

Inoue K, Osaka H, Imaizumi K, Nezu A, Takanashi J, Arii J, Murayama K, Ono J, Kikawa Y, Mito T, Shaffer LG, Lupski JR. 1999. Proteolipid protein gene duplications causing Pelizaeus-Merzbacher disease: molecular mechanism and phenotypic manifestations. Ann Neurol 45:624-632.

Mimault C, Giraud G, Courtois V, Cailloux F, Boire JY, Dastugue B, Boespflug-Tanguy O. 1999. Proteolipoprotein gene analysis in 82 patients with sporadic Pelizaeus-Merzbacher Disease: duplications, the major cause of the disease, originate more frequently in male germ cells, but point mutations do not. The Clinical European Network on Brain Dysmyelinating Disease. Am J Hum Genet 65:360-369.

Reese MG, Eeckman FH, Kulp D, Haussler D. 1997. Improved splice site detection in Genie. J Comput Biol 4:311-323.

Rogozin IB, Milanesi L. 1997. Analysis of donor splice sites in different eukaryotic organisms. J Mol Evol 45:50-59.

Sistermans EA, de Coo RF, De Wijs IJ, Van Oost BA. 1998. Duplication of the proteolipid protein gene is the major cause of Pelizaeus-Merzbacher disease. Neurology 50:1749-1754.

Strautnieks S, Rutland P, Winter RM, Baraitser M, Malcolm S. 1992. Pelizaeus-Merzbacher disease: detection of mutations Thr181Pro and Leu223Pro in the proteolipid protein gene, and prenatal diagnosis. Am J Hum Genet 51:871-878.

Thomson CE, Montague P, Jung M, Nave KA, Griffiths IR. 1997. Phenotypic severity of murine Plp mutants reflects in vivo and in vitro variations in transport of PLP isoproteins. Glia 20:322-332.

Woodward K, Kendall E, Vetrie D, Malcolm S. 1998. Pelizaeus-Merzbacher disease: identification of Xq22 proteolipid-protein duplications and characterization of breakpoints by interphase FISH. Am J Hum Genet 63:207-217.

Woodward K, Malcolm S. 2001. CNS myelination and PLP gene dosage. Pharmacogenomics 2:263-272.

Zhang MQ. 1998. Statistical features of human exons and their flanking regions. Hum Mol Genet 7:919-932.