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Short Report

Further genotype–phenotype correlation emerging from two families with *PLP1* exon 4 skipping

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Proteolipid protein 1 (*PLP1*) gene-related disorders due to mutations in the *PLP1* include a wide spectrum of X-linked disorders ranging from severe connatal Pelizaeus–Merzbacher disease (PMD) to spastic paraplegia 2 (SPG2). Duplications, deletions or point mutations in coding and noncoding regions of the *PLP1* gene may occur. We report the clinical, neuroradiologic and molecular findings in six patients from two unrelated families. The affected males showed severe mental retardation, spastic tetraparesis, inability of walking and pes cavus at onset in early infancy. Brain magnetic resonance imaging (MRI) showed hypomyelination and brain atrophy. Nystagmus was never observed. The affected females showed adult-onset progressive spastic paraparesis leading to wheel-chair dependency and subtle white matter changes on brain MRI. Molecular studies in the two families identified two different intronic mutations, the novel c.622+2T>C and the known c.622+1G>A, leading to the skipping of *PLP1*-exon 4. The clinical presentation of the affected males did not consistently fit in any of the *PLP1*-related disorder subtypes (i.e., connatal or classic PMD, SPG2 and '*PLP1* null syndrome'), and in addition, the carrier females were symptomatic despite the severe clinical picture of their respective probands. This study provides new insight into the genotype–phenotype correlations of patients with *PLP1* splice-site mutations.

Conflict of interest

The authors declare that they have no conflict of interest.

Roberta Biancheri[a], Serena Grossi[b,†], Stefano Regis[b,†], Andrea Rossi[c], Fabio Corsolini[b], Daniela Paola Rossi[a], Pietro Cavalli[d], Mariasavina Severino[c] and Mirella Filocamo[b]

[a]Child Neurology and Psychiatry Unit, [b]UOSD Centro di diagnostica genetica e biochimica delle malattie metaboliche, [c]Pediatric Neuroradiology Unit, Istituto G. Gaslini, Genova, Italy, and [d]Servizio di Genetica, Istituti Ospedalieri di Cremona, Cremona, Italy

[†]These authors equally contributed.

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Corresponding authors: Roberta Biancheri, MD, PhD, Child Neurology and Psychiatry Unit, Istituto G. Gaslini, Largo G. Gaslini 5, 16147 Genova, Italy.
Tel.: +39 010 5636432;
fax: +39 010 381303;
e-mail: roberta@biancheri.com
and
Mirella Filocamo, BSc, PhD, UOSD, Centro di diagnostica genetica e biochimica delle malattie metaboliche, Istituto G. Gaslini, Largo G. Gaslini 5, 16147 Genova, Italy.
Tel.: +39 010 5636792;
fax: +39 010 383983;
e-mail: mirellafilocamo@ospedale-gaslini.ge.it

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Proteolipid protein 1 (*PLP1*) gene-related disorders include a wide spectrum of X-linked disorders due to mutations in the proteolipid protein gene (*PLP1*; MIM# 300401) ranging from severe connatal Pelizaeus–Merzbacher disease (PMD) to spastic paraplegia 2 (SPG2) (1–3).

The gene *PLP1* containing seven exons encodes both the 277 amino acid PLP and its 242 amino

acid isoform, DM20, which is derived from the use of a developmentally regulated internal splice donor site within *PLP1* exon 3 (4). Duplications, deletions or point mutations in coding and noncoding regions of the *PLP1* gene may occur. The classic PMD form is typically related to *PLP1* duplications and is characterized by nystagmus, hypotonia progressing to spasticity, ataxia, and cognitive impairment at onset in infancy or early childhood. Intragenic lesions may cause both severe and mild phenotypes (3). Different brain MRI features correlate with the different clinical and genetic forms. Myelination may be completely absent in the severe connatal form, whereas it is variably deficient in the classic PMD. A milder diffuse or patchy hypomyelination may occur in the *PLP1* null syndrome and in SPG2 (3).

Here we report the clinical and neuroradiologic findings associated with two point mutations leading to the skipping of *PLP1*-exon 4 in hemizygous and heterozygous affected subjects from two unrelated Italian families.

Materials and methods

Patients

The patients are three males from two unrelated Italian families (Fig. 1), who underwent clinical examination, laboratory, neurophysiological and neuroradiological investigations. Their respective symptomatic carrier females were evaluated according to the same protocol.

Molecular studies

Genomic DNA and total RNA was extracted from cultured fibroblasts using standard methods. *PLP1* gene exons and exon–intron boundaries were polymerase chain reaction (PCR) amplified and directly sequenced, as previously described (5).

Putative c.622+1G>A and c.622+2T>C mutations were confirmed by sequencing duplicate PCR products as previously described (5). To detect both transcripts, *PLP* being rarer than *DM20*, full-length PCR amplification, performed using primers placed within the 5′ and 3′ untranslated regions, respectively, was followed by two nested PCR amplifications, with primers in the *PLP*-transcript specific region (exon 3B).

To exclude the presence of potential transcripts containing exon 4, a specific PCR was performed using primer lying in exons 2 and 4, respectively.

Results

Family #1

The propositus is a 33-year-old man born at term after uneventful pregnancy and delivery. During the first six months of life, failure to thrive, developmental delay, muscular hypotonia were noticed. On the basis of available clinical records, we were able to review the neurological findings and disease course. In the first years of life, severe mental retardation and progressive spasticity became evident with inability of walking even with support. Over the years, the clinical picture showed a slowly progressive worsening. A percutaneous gastrostomy was performed due to severe dysphagia at 27 years. Nystagmus was never noticed. He never suffered from seizures. At 28 years, the patient was referred to us. Neurological examination showed severe spastic tetraparesis with bilateral Babinski sign, dystonia, poor tendon reflexes, pes cavus, scoliosis, and severe dysarthria. Funduscopy showed optic atrophy. Brain MRI showed white matter signal abnormalities consistent with diffuse hypomyelination associated with mild cerebral and cerebellar atrophy (Fig. 1A). Nerve conduction velocity (NCV) studies were not performed. At last follow-up the clinical picture was unchanged.

His mother is a 55-year-old woman who presented with neurogenic bladder at 38 years followed by spastic paraparesis leading to be wheelchair dependent at age 54. The last neurological examination showed spasticity prevailingly at the lower limbs, pes cavus, poor tendon reflexes and bilateral Babinski sign. Brain MRI showed normal signal intensity of the white matter except for a minimal indistinct hyperintensity in the parieto-occipital regions (Fig. 1A). Lower limb motor and sensory NCV studies showed conduction velocity slowing (<40 m/s) and reduced amplitude of the compound action potential consistent with axonal/demyelinating neuropathy.

The maternal grandmother is a 70-year-old woman presenting with neurogenic bladder at 28 years followed by progressively worsening gait difficulties leading to wheelchair-dependency since 40 years. Brain MRI performed when she was 61 years showed aspecific loss of white matter bulk and minimal periventricular white matter hyperintensities (Fig. 1A). NCV studies were not performed.

Family #2

The proband, a 32-year-old male and his 42-year-old brother were born at term after uneventful pregnancy and normal delivery. Both patients were initially diagnosed as having cerebral palsy. They showed a similar clinical picture characterized by developmental delay and muscular hypotonia at onset during the first months of life. They never achieved the ability of walking. Over the years they showed a slowly progressive neurological impairment. Nystagmus was never noticed in both of the patients. They never suffered from seizures. We were able to clinically assess the proband at 22 years. Neurological examination showed severe spastic tetraparesis with bilateral Babinski sign, poor tendon reflexes, pes cavus, severe mental retardation, and cerebellar signs. Funduscopy showed optic atrophy. Brain MRI showed diffuse white matter signal abnormalities consistent with hypomyelination associated with mild cerebral atrophy (Fig. 1B). NCV studies were not performed. Neurological examination of the older brother at 32 years showed similar findings to those described in his sibling. In the last years, we were

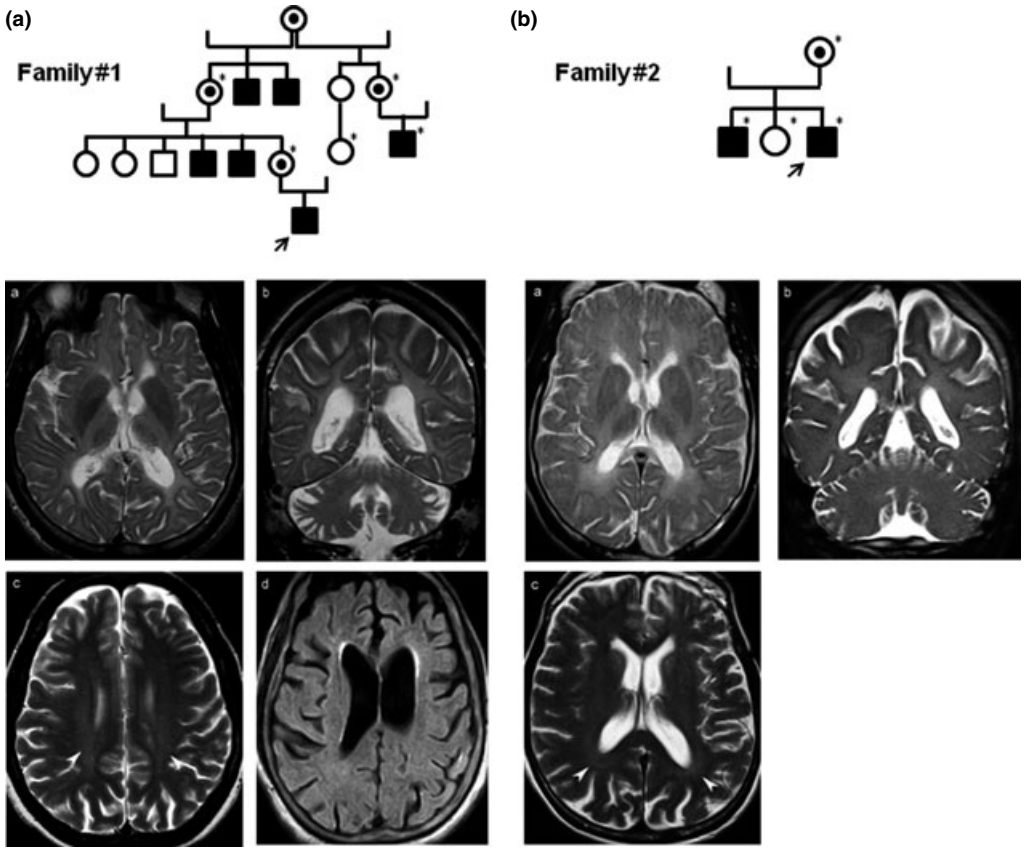

Fig. 1. (**A**) Pedigrees and brain MRI examinations of Family 1. Axial (a) and coronal (b) T2-weighted images of the propositus at 28-years old show a pattern of diffuse and severe hypomyelination associated with mild brain atrophy; (c) axial T2-weighted image of the mother at 45-years reveals normal signal intensity of the white matter except for a minimal indistinct hyperintensity in the parieto-occipital regions (arrowheads); (d) axial FLAIR image of the grandmother at 61 years depicts aspecific loss of white matter bulk and minimal periventricular white matter hyperintensities. (**B**) Pedigrees and brain MRI examinations of Family 2. Axial (a) and coronal (b) T2-weighted images of one sibling at 22-years old show complete absence of supra- and infratentorial myelin associated with mild brain atrophy; (c) axial T2-weighted image of the mother reveals near-normal signal intensity of the white matter with subtle hyperintensities in the parieto-occipital regions (arrowheads). Pedigree key: closed square and filled circle denote affected individuals; the arrow indicates the proband; open square and open circle indicate healthy individuals; asterisk marks individuals confirmed by molecular analysis.

not able to further evaluate the patients whose clinical picture was referred as progressively worsened.

Their mother is a 64-year-old woman affected by spastic paraparesis at onset at around 42 years. She was wheelchair-dependent since 60 years. Brain MRI showed near-normal signal intensity of the white matter with subtle hyperintensities in the parieto-occipital regions (Fig. 1B). NCV studies were not performed.

Molecular studies

The molecular testing identified a novel intronic mutations c.622+2T>C (Family #1) and c.622+1G>A (Family #2), both occurring at the invariant donor 'GT' dinucleotide splice site of intron 4 (Fig. 1).

Analysis of the RT_PCR products confirmed the skipping of the exon 4 resulting from both the two genomic mutations, c.622+2T>C and c.622+1G>A, by demonstrating the direct junction exon 3B-exon 5 in the PLP transcripts and exon 3A-exon 5 in the DM20 transcripts (Fig. 2c).

The specific exon 4 PCR confirmed the absence of exon 4-containing transcripts in the patients (Fig. S2, supporting information).

Discussion

PLP1 gene mutations cause a range of different phenotypes termed *PLP1*-related disorders. While the classic PMD phenotype due to gene duplication has been extensively described, there are only limited case series and reports of patients harbouring *PLP1* intragenic mutations (3). The clinical presentation of the present male patients did not consistently fit in any of the *PLP1*-related disorder subtypes. The connatal and classic PMD were excluded due to the absence of nystagmus. The milder SPG2 and the so-called '*PLP1* null syndrome' could not be considered due to the severe cognitive and motor impairment with inability of walking. On the other hand, brain MRI of affected male patients revealed diffuse hypomyelination associated with mild cerebral and cerebellar atrophy consistent with PMD diagnosis.

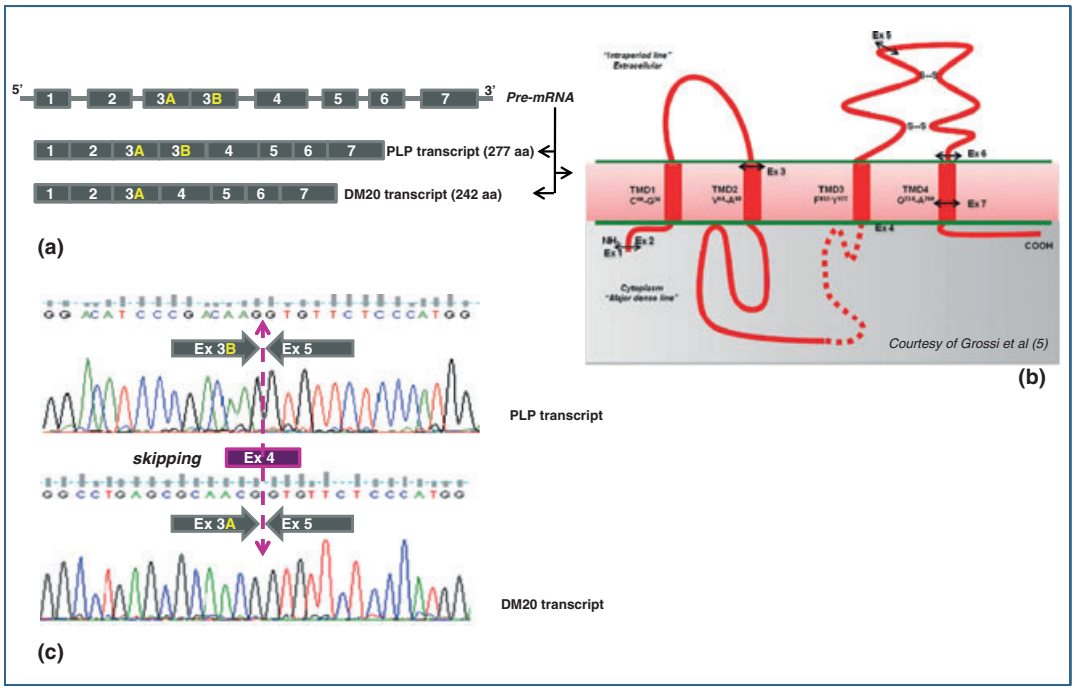

Fig. 2. (**a**) Graphical representation of *PLP1* gene transcription. The gene containing seven exons (grey boxes) encodes the proteolipid protein (PLP) and its isoform, DM20 by the alternative use of a developmentally regulated internal splice donor site within *PLP1* exon 3 (4). Hence, PLP (277 amino acids, aa) and DM20 (242 aa) are identical except for the presence of 35 aa. (**b**) Model of the tetraspan proteolipid proteins, PLP and DM20. The four predicted transmembrane domains (TMD1-4) are depicted, the first and last amino acids being indicated for each TMD. The PLP1-specific region, exon 3B (which is absent from DM20) is denoted by a dotted line. (**c**) Sequence analysis of the RT-PCR products from the patients. The results of the analysis confirmed the skipping of the exon (Ex) 4 resulting from both the genomic mutations c.622+2T>C (family #1) and c.622+1G>A (family #2), by demonstrating the direct junction Ex 3B–Ex 5 in the PLP transcripts and Ex 3A–Ex 5 in the DM20 transcripts. The predicted abnormally truncated PLP (p.F152VfsX28) and DM20 (p.F117VfsX28) proteins lack two transmembrane domains (TMD3 and TMD4) of the canonical four present in the normal PLP and DM20 proteins. Note that only the involved exonic regions are graphically shown.

Molecular analysis of the affected individuals of the two independent families identified two distinct intronic mutations affecting the 5′ donor splice site of intron 4: c.622+2T>C in family #1 and c.622+1G>A in family #2. The functional relevance of these mutations, addressed by RT-PCR analysis on the male patients' mRNA, revealed the presence of two out-of-frame PLP and DM20 transcripts lacking the entire coding sequence of exon 4 (Figs 2a–c and S1a,b). Generally, these types of mutations, known as 'null alleles', are associated with mild phenotypes. The phenotype of our patients instead was quite severe. One possible explanation is that the identified premature mutant transcripts, escaping the nonsense mediated decay (NMD) pathway (6), produced abnormally truncated PLP (p.F152VfsX28) and DM20 (p.F117VfsX28) proteins, lacking therefore two of the four transmembrane domains of the normal PLP and DM20 proteins (Fig. 2b). It has been shown that such severely misfolded proteins, resulting from the alteration of the tetraspan structure, are retained in the endoplasmic reticulum (7). As a consequence, the unfolded protein response (UPR) might be activated therefore impairing oligodendrocyte function (8, 9).

The c.622+1G>A mutation identified in family #2 of the present study has also been previously reported in two brothers showing a mild clinical phenotype with nystagmus and peripheral neuropathy (10). RT-PCR analyses, performed in this latter study on a sural nerve biopsy from one patient, showed the simultaneous presence of both transcripts lacking exon 4 and entire transcripts with the retention of additional 10 nucleotides of intron 4. In view of these findings, we experimentally excluded the simultaneous presence of additional transcripts containing exon 4 in our patients (Fig. S2).

We can suppose that a relation might exist between the different phenotypes of these patients carrying the same mutation but showing a different pattern of PLP1 gene transcription although it remains to be formally demonstrated that the different profile of gene transcription simply reflect tissue-related specificities (fibroblasts *vs* sural nerve).

Other splicing defects affecting PLP1 intron 6 have been reported associated with different phenotypes (11). Mild or severe phenotype has been also observed in mice carrying the rumpshaker mutation in response to different genetic backgrounds (C3H or C57BL/6) (12). According to this study, the only remarkable difference observed between the two strains was the higher expression of the endoplasmic reticulum (ER) stress apoptotic factor CHOP in C57BL/6 mice compared to C3H mice (13). In connection with this, one suggestion could be that the different phenotype features of our

patients, compared to the previously reported ones (10), might reflect a different response to misfolded proteins in the ER.

The carrier females of the present study are symptomatic despite the severe clinical picture of the probands. Indeed, they showed spastic paraparesis and mild myelin involvement on brain MRI. Interestingly, a similar pattern of brain involvement has been described in the mother of two young boys with 4-bp deletion of the *PLP1* gene, in whom the nearly normal-appearing white matter presented reduction in fractional anisotropy and elevation in trace and radial diffusivity on diffusion tensor imaging (DTI), consistent with subtle changes in the microstructure of the white matter due to abnormal myelin (14). It is known that *PLP1* heterozygous females are generally non-affected. However, they may occasionally show neurological symptoms with an inverse relationship between the severity of manifestations in males and the likelihood of heterozygous females to be symptomatic. (3, 15). In particular, carriers of nonsense mutations are more likely to develop symptoms than for other genetic lesions (15). The occurrence of disease symptoms in females carrying disease-causing mutations in the PLP1 gene has been explained through a mechanism accounting for the cellular toxicity effects and X-inactivation pattern in heterozygotes (15). In carrier females, the oligodendrocytes expressing mutant *PLP1* alleles on active X chromosome and undergoing apoptosis are replaced over time by oligodendrocytes expressing normal allele on the active X chromosome (16). Hence, females carrying severe *PLP1* are usually asymptomatic and show skewed inactivation of the X chromosome (3). Both the mothers of the present study, tested according to the human androgen receptor assay (HUMARA), showed a random pattern of X-inactivation (data not shown). Although a skewed X-chromosome inactivation, occurring only in the brain as result of secondary selection upon the myelination, should be formally excluded, our data suggest that oligodendrocytes containing the PLP1 mutations in these two families survive into adulthood, thus potentially leading to the severity of the phenotype in carrier mothers.

In conclusion, this study provides new insight into the genotype-phenotype correlations of patients with *PLP1* splice-site mutations. These rare intragenic variants may cause either similar phenotypes related to different genomic mutations, as demonstrated by our patients, or different clinical phenotypes caused by the same mutation. Further studies are needed to better characterize the clinical and neuroradiologic spectrum of specific intronic *PLP1* mutations.

Supporting Information

The following Supporting information is available for this article:

Fig S1. RT-PCR analysis in patients' fibroblasts. (**a**) Full-length cDNA was amplified with a set of primers encompassing exons 1–7. The analysis revealed a shorter DM20 product of 745-bp in size, corresponding to an anomalous isoform with the entire skipping of exon 4 in the two probands (lanes 2 and 3) compared to the two normal, nonaffected, controls (lanes 1 and 4) showing the expected product (914-bp). Note that the PLP product (1019-bp) is not visible in any of the samples likely due to the low amount of PLP transcribed in fibroblasts. (**b**) To selectively identify the PLP product, two consecutive nested PCRs were performed on full-length cDNA product: (i) the first nested PCR, performed by a set of primers lying on exons 1 and 3B, amplified the expected PLP product (558-bp) in both the patients (lanes 2 and 3) and the controls (lanes 1 and 4); (ii) the second nested PCR, performed with a set of primers lying on exons 3B and 7, revealed the expected product (565-bp) in normal controls (lanes 1 and 4), while a shorter product of 396-bp, corresponding to the entire skipping of exon 4, in the patients (lanes 2 and 3). Note that the primers lying on exon 3B are PLP sequence specific (details in Fig. 2). M: ΦX DNA HaeIII digested molecular weight marker; C: no template control.
Fig S2. *PLP1* exon 4-specific RT-PCR. The analysis, performed on RNA samples (extracted from fibroblasts), PCR amplified by a set of primers, lying on exons 2 and 4, showed the expected DM20 isoform (348 bp) in the normal, nonaffected, controls (lanes 3 and 4), while no product was present in the two probands (lanes 1 and 2). Note that the other expected product of 453 bp in size (PLP isoform) is not visible in any of the samples likely due to the low amount of PLP isoform transcribed in the fibroblasts. M: ΦX DNA HaeIII digested molecular weight marker; C: no template control.

Additional Supporting information may be found in the online version of this article.

Acknowledgments

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Ethics approval

Following ethical guidelines, all cell and nucleic acid samples were obtained for analysis and storage with the patients' (and/or a family member's) written informed consent. The consent was sought using a form approved by the local Ethics Committee.

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