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Short Report

PLP1 gene analysis in 88 patients with leukodystrophy

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Pelizaeus–Merzbacher disease (PMD) is caused in most cases by either duplications or point mutations in the *PLP1* gene. This disease, a dysmyelinating disorder affecting mainly the central nervous system, has a wide clinical spectrum and its causing mutations act through different molecular mechanisms. Eighty-eight male patients with leukodystrophy were studied. *PLP1* gene analysis was performed by the Multiplex Ligation-dependent Probe Amplification technique and DNA sequencing, and, in duplicated cases of *PLP1*, gene dosage was completed by using array-CGH. We have identified 21 patients with mutations in the *PLP1* gene, including duplications, short and large deletions and several point mutations in our cohort. A customized array-CGH at the Xq22.2 area identified several complex rearrangements within the *PLP1* gene region. Mutations found in the *PLP1* gene are the cause of PMD in around 20% of the patients in this series.

Conflict of interest

The authors declare that they have no conflict of interest.

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The leukodystrophies are a group of rare genetic disorders that affect the central nervous system (CNS) and less frequently peripheral nerves. They produce an important and progressive neurological disability. The name leukodystrophy refers to the deterioration of the white matter of the brain. Most leukodystrophies develop early during childhood or adolescence, and have a progressive course, leading to premature death. Pelizaeus–Merzbacher disease (PMD) is a leukodystrophy due to CNS hypomyelination caused by mutations in the *PLP1* gene located in the long arm of chromosome X. The *PLP1* gene encodes two proteins expressed mainly in oligodendrocytes, the proteolipid protein (PLP) and its differently spliced isoform DM20. PLP is a transmembrane protein that plays a major role in myelin sheath formation by promoting sheath compaction. An internal splice donor site within exon 3 creates an alternative transcript that encodes a 20-kDa

protein known as DM20. These two proteins are identical except for 35 amino acids that are present in PLP and absent in DM20. Both proteins, PLP and DM20, are the major protein components of myelin in the CNS.

More than 90 different mutations have been reported in the *PLP1* gene, the duplication of the gene accounting for around 60% of the total (1, 2). Male patients present clinical forms ranking from the more severe connatal to the mild spastic paraplegia 2. Connatal forms are associated with missense mutations that cause misfolding of PLP and DM20, leading to their retention in the endoplasmic reticulum. The accumulation of PLP in the endoplasmic reticulum produces finally apoptosis of the oligodendrocytes and the number of these cells is severely reduced. The classic PMD, with a later onset, is usually due to duplication of the *PLP1* gene suggesting that overdose can be a cause of PMD (3, 4). Other genes next to *PLP1* gene were also duplicated in these patients as it has been reported previously in different publications (5, 6).

In this work, we performed genetic analysis of the *PLP1* gene in patients with leukodystrophy using Multiplex Ligation-dependent Probe Amplification (MLPA) technique, Sanger sequencing and array-CGH techniques. Range of analysis have been included (10 Mb; chrX:98039492..108039491).

Materials and methods

Patients

Eighty-eight non-related males (Spanish origin 77 and Greek origin 11) with congenital hypomyelination and clinical diagnosis of leukodystrophy based on magnetic resonance imaging were included in this study. Neurophysiologic investigations were performed following standard protocols established at the respective institutions. For all patients informed consent was obtained. Patients were referred to our laboratory from the Neurology Services of several Spanish hospitals and from the Attiko and Aghia Sofia Hospitals of Athens, Greece. In the majority of patients, other causes of leukodystrophy had been ruled out. This study was approved by the ethics committee of the Hospital Universitario La Paz and complies with the principles of the Declaration of Helsinki. Essentially, we classified patients into five forms based on their best motor function according to the criteria proposed by Garbern and Hobson (7): severe connatal, classic PMD, PLP null syndrome, complicated spastic paraplegia and uncomplicated spastic paraplegia.

Methods

Genomic DNA was extracted from peripheral blood leucocytes by standardized procedures (kit Purogene, Qiagen GmbH, Hilden, Germany). All DNA samples were screened for *PLP1* gene duplications using the MLPA technique (SALSA P022, MRC-Holland, Amsterdam, the Netherlands). *PLP1* gene sequencing analysis was performed on patients who were negative for the duplication. Intronic primers were designed to amplify the seven exons and flanking intronic sequences of the *PLP1* gene. Amplified DNA products were sequenced by the dideoxy termination method (BigDye Sequencing Kit, Applied Biosystems, Foster City, CA). Molecular karyotyping was performed using a *PLP1* custom oligonucleotide array-CGH (Agilent-based 8x15K, Agilent Technologies, Santa Clara, CA). Statistically significant aberrations were determined using the ADM-2 (Aberration Detection Method 2) algorithm in CGH Analytics version 3.5 (Agilent Technologies). This targeted oligonucleotide chip includes *PLP1* gene and neighbor genes within 10 Mb. Its average resolution is around 600 bp. The features were selected from Agilent's eArray (Agilent Technologies, https://earray.chem.agilent.com/earray) probe library in a custom high-resolution format of 8x15k.

Results

Molecular analysis of *PLP1* gene in our cohort revealed gene alterations in 21 of 88 patients with leukodystrophy including nine *PLP1* duplications or triplication, eight point mutations, two deletions of 3 and 5 coding nucleotides, respectively, one deletion of 8 nucleotides in conserved regions of intron 1 and one deletion of exons 1–4 extending to the 5′ end of the *PLP1* gene (Tables 1 and 2).

PLP1 gene dosage studies by MLPA assay found *PLP1* duplications in nine males, and their correspondent mothers (with the exception of patient PM-057, where duplication appears to be *de novo*), and one large deletion. To address a possible correlation between *PLP1* genotypes and PMD phenotypes, an array-CGH was performed in duplicated samples. The array-CGH analysis for eight of these patients established a ranging size of duplications between 0.181 and 5.1 Mb and showed complex rearrangements in five of eight patients (PM-4, PM-25, PM-53, PM-67, and PM-68) (Fig. 1; Table 1).

Point mutations found were missense mutations in eight cases and one splicing mutation affecting the guanine in position 5 of the splice donor site of the junction exon/intron 6 (Table 2). Interestingly, five of these point mutations had not been reported before as a cause of PMD (p.Leu31Arg, p.Tyr207His, p.Leu224Phe, p.Asn264Ile and c.762+5G>A). Indeed, mutated residues Leu31, Tyr207, Leu224 and Asn264 are highly conserved throughout evolution, thus providing a strong argument to consider their change as disease-causing mutations. In this sense, three more mutations found in this work had also not been reported before: a deletion of five nucleotides (c.364_368delAGGGG; p.Arg124Phefs*78) that would give rise to a truncated PLP; a large deletion affecting the 5′UTR, and exons 1–4 of *PLP1* gene; a third mutation, found in a Greek patient, is a eight-nucleotide deletion in intron 1 (c.4+71delGGGGTTCG) that is not present in 100 healthy Greek controls. Finally, we also

Table 1. Phenotype of Pelizaeus–Merzbacher (PM) patients compared to the size of duplication

Patient	Onset/ present age	Complex rearrangement	Molecular karyotype (kb). NCBI37. hg19	PLP1 gene dose	Phenotype	Mother status
PM-4	8 months/deceased at 3 years	Yes	arr (102716771–102760337)x2, (102882791–103174234)x3 and (103174264–103356460)x2, 43 kb duplication, 291 kb triplication and 182 kb duplication	X3	Severe connatal PMD	Carrier
PM-25	3 months/4 years	Yes	arr (102768567–102809422)x2, (102809482–102825521)x0 and (102825581–103329714)x2, 40 kb duplication, 16 kb deletion and 504 kb duplication	X2	Classic PMD	Carrier
PM-53	2 months/7 years	Yes	arr (101855870–102556292)x2, (102556322–102638357)x3, (102638387–103057500)x2, (103175720–103243137)x2 and (103261627–103329714)x2, 700 kb duplication, 82 kb triplication, 419 kb duplication, 67 kb duplication and 68 kb duplication	X2	Classic PMD	Carrier
PM-57	8 months/5 years	No	arr (107209818–102039646)x2, 5170 kb duplication	X2	Classic PMD	Non-carrier
PM-67	2 months/1 years	Yes	arr (102475034–102679203)x2 and (102728403–103305096)x2, 204 kb duplication and 576 kb duplication	X2	Classic PMD	Carrier
PM-68	3 months/9 years	Yes	arr (102696732–103122533)x2 and (103337497–103643310)x2, 425 kb duplication and 306 kb duplication	X2	Classic PMD	Carrier
PM-101	2 months/5 years	No	arr (102159731–103353105)x2, 1193 kb duplication	X2	Severe connatal PMD	Carrier
PM-154	11 months/3 years	No	arr (102895728–103077302)x2, 182 kb duplication	X2	Classic PMD	Carrier

PMD, Pelizaeus–Merzbacher disease.

Table 2. Mutations in the *PLP1* gene

Patient	Onset age/ present age	Mother	Mutation	Protein domain	Reference	Phenotype
PM-28	At birth/4 years	Carrier	p.Leu224Phe	Extracellular 2	This study	Severe connatal PMD
PM-39	8 months/6 years	Non-carrier	p.Phe79del	Transmembrane 2	Shimojima et al. 11	Complicated spastic paraplegia
PM-43	4–6 months/16 years	Carrier	5′UTR-exon 4 deletion		This study	Classic PMD
PM-71	4 months/	Carrier	p.Cys33Tyr	Transmembrane 1	Hübner et al. (8)	Classic PMD
PM-83	2 months/deceased at 12 years	nd	p.Asn264Ile	Transmembrane 4	This study	Severe connatal PMD
PM-103	1 month/3 years	Carrier	c.762+5G>A	Intron 6	This study	PLP null syndrome
PM-106	8 months/4 years	Carrier	p.Leu31Arg	Transmembrane 1	This study	Severe connatal PMD
PM-121	6 months/8 years	nd	c.364_368del	Intracellular 2	This study	PLP null syndrome
PM-133	At birth/11 months	Carrier	p.Gly73Arg	Transmembrane 2	Doll et al.	Severe connatal PMD
PM-159	At birth/7 months	Carrier	p.Thr43Ile	Extracellular 1	Pratt et al. (9)	Severe connatal PMD
PM-81	1 year/deceased at 13 years	nd	c.4+71delGGGGTTCG	Intron 1	This study	Complicated spastic paraplegia
PM-119	3 months/28 years	Carrier	p.Tyr207His	Extracellular 2	This study	Classic PMD

PMD, Pelizaeus–Merzbacher disease.

found four mutations in the *PLP1* gene that had been previously reported elsewhere [p.Cys33Tyr, p.Thr43Ile, p.Gly73Arg and p.Phe79del (8–11)] (Table 2).

Discussion

This study is a survey of mutations found in the *PLP1* gene in patients with leukodystrophy. In our series, 21.6% (21 of 88) of patients with leukodystrophy carried mutations in the *PLP1* gene. Nine of 21 (42.8%) patients carried a duplication or triplication within chromosomal region Xq22 that includes *PLP1* gene. In the series by Mimault et al. (1), duplications of *PLP1* gene account for 60% of mutations in PMD patients. Our yields are closer to a previous study by Shimojima et al. (11) that reported 50% of *PLP1* gene duplications.

Twelve patients showed other kind of mutations different from the frequently observed duplication. We found missense mutations in eight cases (38%). In many cases, missense mutations in the *PLP1* gene caused the most severe PMD connatal syndrome (2). Patient PM-28 carried mutation Leu224Phe that is located in the second external loop of PLP. Substitutions in this loop caused severe PMD because this region is crucial to maintain the intraperiod line in compact myelin.

Fig. 1. Customized array-CGH of the Xq22.2 region from Pelizaeus–Merzbacher disease (PMD) patients with duplication or triplication of *PLP1* gene. Statistically significant aberrations on Xq22.2 were determined using the ADM-2 (Aberration Detection Method 2) algorithm in CGH Analytics version 3.5 (Agilent Technologies). The vertical axis indicates physical position of chromosome X and the horizontal axis indicates the signal log2 ratio. Positive and negative numbers indicate gain (red) and loss (green) of genomic copy numbers, respectively. Known copy number variations (CNVs) are represented as blue bars. Black arrows indicate the location of *PLP1* gene.

Accordingly, Leu224Phe is a missense mutation that caused a severe form of PMD (Table 2). Mutations of amino acid residue Leu224 are recurrent as mutations Leu224Ile and Leu224Pro have been reported previously.

Patient with mutation Asn264Ile in our series (PM-83) suffered from connatal PMD (Table 2). This mutation may interfere with correct folding of the polypeptide and misfolding and retention may induce oligodendrocyte apoptosis and cause a severe phenotype (12). Patient PM-106 in this cohort carried mutation Leu31Arg and he presented a very severe clinical form of PMD (Table 2).

As previously reported (13), PLP null syndrome presented with peripheral neuropathy and so was in the two cases in our series. Mutations in the 5′ splice donor site of exon 6 have been shown by Hobson et al. (14) to produce the skipping of exon 6 of *PLP1* gene. In our series, patient PM-103 carried a novel splice mutation, c.762+5G>A and he presented a PLP null syndrome. Two brothers studied by Hobson et al. (14) carried the splice mutation IVS6+3G>T that, as shown by these authors, causes skipping of exon 6 of *PLP1* gene.

We found a small deletion in patient PM-121 (mutation c.364_368delAGGGG; p.Arg124Phefs*78) that would give rise to a truncated PLP polypeptide. These kinds of mutations, affecting the expression of PLP1 but conserving the expression of the isoform DM20, are usually associated to a mild syndrome (15, 16). In fact, patient PM-121 corresponds to a PLP1 null syndrome following the criteria given by Garbern and Hobson (7).

The large deletion affecting the 5′UTR and exons 1–4 of *PLP1* gene would lead to the absence of PLP protein. Patient studied here suffers from classic PMD. While many cases of PMD are due to duplications of the *PLP1* gene, large deletions have seldom been described up to now (17, 18).

Mutation c.4+71delGGGGTTCG localized in a conserved region of intron 1 related to enhancer sequences (19) has not been proven yet to affect the expression of *PLP1*.

The array-CGH assay showed that the minimal duplication found in this series in the Xq22.2 region was 0.181 Mb and the larger one expands to 5.1 Mb (Fig. 1). The smaller duplication (0.181 Mb) includes, in addition to *PLP1*, five genes, *MORF4L2*, *LOC340544*, *GLRA4*, *TMEM31* and *RAB9B*, and a pseudogene, *LOC100130176*. The larger duplication (5.1 Mb) is over 150 times the size of the *PLP1* locus and includes at least 70 genes. Other genes affected by the duplication as well as the positions of the breakpoints, which might disrupt other genes, could contribute to the overall phenotype. The results of array-CGH in this series indicate that there is no direct correlation between extension of the duplication and the severity of the illness, as it was previously suggested by other studies (6, 8). However, there is one patient carrying triplication in the *PLP1* gene (PM-004) presenting a more severe clinical evolution (Table 1), a fact that has been reported previously by Wolf et al. (20). According to Lee et al. (5), 65% of patients with *PLP1* duplications have complex rearrangements at nucleotide sequence level. We detected herein, five of eight (62.5%) cases, a similar percentage. This fact may support the idea suggested by others (6) that no genotype/phenotype correlation is possible as a consequence of the existence of these complex rearrangements within *PLP1* gene region.

Our results from array-CGH on duplications of *PLP1* gene agree with previous results in the literature and, on the other hand, the sequencing analysis adds new data to the spectrum of mutations causing PMD.

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