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Stefano Regis, Serena Grossi, Fabio Corsolini, Roberta Biancheri, Mirella Filocamo. PLP1 gene duplication causes overexpression and alteration of the PLP/DM20 splicing balance in fibroblasts from Pelizaeus-Merzbacher disease patients. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, 2009, 1792 (6), pp.548. 10.1016/j.bbadis.2009.04.002 . hal-00488810

HAL Id: hal-00488810

<https://hal.science/hal-00488810>

Submitted on 3 Jun 2010

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Accepted Manuscript

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PII: S0925-4439(09)00091-X
DOI: doi:[10.1016/j.bbadis.2009.04.002](https://doi.org/10.1016/j.bbadis.2009.04.002)
Reference: BBADIS 62952

To appear in: *BBA - Molecular Basis of Disease*

Received date: 2 December 2008
Revised date: 1 April 2009
Accepted date: 2 April 2009



Please cite this article as: Stefano Regis, Serena Grossi, Fabio Corsolini, Roberta Biancheri, Mirella Filocamo, PLP1 gene duplication causes overexpression and alteration of the PLP/DM20 splicing balance in fibroblasts from Pelizaeus-Merzbacher disease patients, *BBA - Molecular Basis of Disease* (2009), doi:[10.1016/j.bbadis.2009.04.002](https://doi.org/10.1016/j.bbadis.2009.04.002)

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PLP1 gene duplication causes overexpression and alteration of the PLP/DM20 splicing balance in fibroblasts from Pelizaeus-Merzbacher disease patients

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Keywords: gene duplication; gene expression; PLP1; PMD; fibroblasts; alternative splicing

Abstract

The PLP1 gene encodes two protein isoforms (PLP and DM20) which represent the predominant protein portion in myelin of the central nervous system. The two products are generated from the same primary transcript by alternative splicing. Defects of the PLP1 gene cause Pelizaeus-Merzbacher disease (PMD) or X-linked spastic paraplegia type 2 (SPG2). Duplication of the PLP1 gene is the most frequent gene defect, usually responsible for the classic form of PMD. To investigate the effects of PLP1 gene over dosage on gene expression, we analysed the PLP/DM20 expression profile in fibroblasts from three PMD patients with a PLP1 gene duplication. Gene expression was evaluated by real-time PCR using two different PLP1 amplicons and two different reference genes (GAPDH and GUSB). Fibroblasts from the three patients showed a 4-5 fold increase of PLP1 gene expression compared to fibroblasts from three normal controls. The contribution of the two alternatively spliced transcript isoforms (PLP and DM20) to the whole PLP1 gene expression was investigated using a DM20-specific amplicon. The three patients showed a decrease of the DM20/(DM20+PLP) ratio in comparison to the three normal controls, suggesting a prominent contribution of the PLP transcript to the PLP1 gene overexpression detected in the patients. Therefore, PLP1 gene duplication seems to result both in overexpression and in a shift of the PLP/DM20 splicing balance in direction of the PLP isoform.

Introduction

The proteolipid protein 1 gene (PLP1; GeneID: 5354) is a myelin-related gene encoding two highly hydrophobic transmembrane proteins, PLP and DM20, which are the major protein components in myelin of the central nervous system [1,2]. The two products are generated from the same primary transcript by two competing 5' donor sites in exon 3 to the same 3' acceptor site in exon 4 [3]. As a result of the alternative splicing, DM20 lacks 35 amino acids in comparison to PLP which is 276 amino acids long.

Defect of the PLP1 gene can cause a broad spectrum of clinical phenotypes ranging from the congenital form of Pelizaeus-Merzbacher disease (PMD) to the pure form of X-linked spastic paraplegia type 2 (SPG2). Symptoms vary from a severe picture evident at birth, with spasticity, stridor and nystagmus, to a relatively mild condition of paraparesis with no cognitive impairment. PMD/SPG2 is inherited as an X-linked trait, the PLP1 gene lying in Xq22.

The role of the PLP/DM20 proteins has not been fully elucidated. In knock-out mice lacking the *Plp1* gene myelination occurs, although myelin shows a reduced physical stability [4]. Moreover, axonal degeneration and impaired axonal transport have been observed [5]. Therefore, PLP/DM20 seem to have a function in the stabilization of the myelin sheaths, a neuroprotective function of CNS myelin [6] and a role in axonal transport [7].

Point mutations of the PLP1 gene are responsible for 20% of the PMD/SPG2 cases causing a wide range of clinical phenotypes. Rare total deletions of the PLP1 gene have been reported to cause a mild form of PMD/SPG2, with moderate spastic paraplegia and mild cognitive delay. Duplication of a genomic fragment containing the PLP1 gene is the most frequent gene defect, accounting for 60-70% of PMD cases [2]. Patients carrying the PLP1 gene duplication usually show the classic form of PMD, with onset in the first year of life, characterized by nystagmus, mental retardation, spasticity. The presence of more than two PLP1 gene copies has been reported in a small number of patients showing a severe form of PMD [8]. Among patients carrying a PLP1 gene duplication, although usually displaying the classic form of the disease,

the degree of clinical severity can vary. This variability does not correlate to the variability of the PLP1 gene duplication extension [9].

Increased PLP1 gene dosage is presumed to cause overexpression. However, this has not been experimentally confirmed in human patients [1]. Conversely, studies on transgenic mice with additional copies of the Plp1 gene have shown that the severity of the disease is proportional to gene dosage and overexpression [10-12].

Expression of the PLP1 gene is mainly detected in oligodendrocytes, which are the myelinating cells in the central nervous system. DM20 is the predominant isoform at embryonic stages, but adult myelin oligodendrocytes prevalently express PLP rather than DM20 transcripts [13,14]. PLP/DM20 expression in Schwann cells, the myelinating cell in the peripheral nervous system, is much lower than that of oligodendrocytes. These cells predominantly express the DM20 isoform. PLP/DM20 expression has been detected in several non-myelinating cells. In these cells a prevalence of the DM20 form has been observed [1]. Cultured fibroblasts express at low levels the PLP1 gene, producing only a small amount of PLP transcript [15]. We used cultured fibroblasts obtained from skin biopsies of three PMD patients carrying a PLP1 gene duplication to analyse their PLP/DM20 expression profile in comparison to that of fibroblasts from normal controls grown under the same culture conditions. As expected, an evident overexpression of the PLP1 gene was detected in patients with duplication of the PLP1 gene. Besides overexpression, a shift of the PLP/DM20 balance towards the PLP isoform was observed in patients with duplication compared to normal controls.

Materials and Methods

Fibroblast cell lines

Fibroblasts from three PMD male patients and from three male normal controls were obtained from the “Cell Line and DNA Bank from Patients affected by Genetic Diseases” collection (<http://www.gaslini.org/labppm.htm>). Patients were classified as PMD form 3, according to the PMD score based on the level of the best motor performance [16].

DNA, RNA extraction and first-strand cDNA synthesis

Genomic DNA was extracted using the QIAamp DNA Blood Mini kit (Qiagen, Valencia, CA, USA). Total RNA was extracted using the RNeasy Mini Kit (Qiagen). First-strand cDNA synthesis was performed using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocols. Priming was performed using simultaneously oligodT (1.25 μ M) and random hexamers (1.25 ng for each reaction). Each first-strand cDNA was obtained using 1 μ g of total RNA in a volume of 20 μ l.

Real-time PCR procedure

Amplifications by real-time PCR were performed from genomic DNA and from first strand cDNA of patients and controls to determine the PLP1 gene dosage and to analyze the PLP/DM20 expression profile, respectively. Amplicons composed of two primers and a TaqMan probe were designed for the real-time PCR runs using the Primer Express 1.5 software (Applied Biosystems, Foster City, CA, USA) according to the previously reported requirements [17]. The real-time PCR experiments were performed using the Applied Biosystems 7500 Real-Time PCR System and the TaqMan chemistry as previously described [17]. The standard curve method with amplification of the target and reference sequences in separate tubes was used (User Bulletin #2. Relative quantitation of gene expression. www.appliedbiosystems.com/). Samples were always run in quadruplicate. Standards were run in duplicate. All the used TaqMan probes were labelled with FAM as the reporter fluorophore at their 5'-end and with TAMRA as the quencher at their 3'-end. Primers and probes were purchased by TIB Molbiol (Genoa, Italy).

PLP1 gene dosage determination

DNA samples of the patients were tested by real-time PCR for PLP1 gene dosage using amplicon PLP1gen, located in exon 3 of the PLP1 gene, and amplicon GAPgen, located in exon 7 of the glyceraldehyde-3-phosphate dehydrogenase gene (GAPDH), used as reference [17]. To exclude incomplete PLP1 gene duplications, the copy number of two flanking amplicons was also tested: amplicon 830gen, located in intron 1 of the PLP1 gene, and amplicon 459gen, located in the untranslated portion of exon 7 (Table 1).

PLP1 gene expression analysis

Patients' and controls' cDNAs were used for PLP1 gene expression analysis performed by real-time PCR. Combined PLP/DM20 and selective DM20 expression were evaluated using specifically designed amplicons. For the combined amplification of the PLP and DM20 cDNAs, amplicons 151 and PL20, lying in a region common to the two isoform, were designed. Amplicon D2 was designed to selectively amplify the DM20 cDNA, avoiding the simultaneous amplification of the PLP cDNA. This selectivity was obtained thanks to the reverse primer of the amplicon, lying across the splicing boundaries of the DM20 isoform (Fig. 1). Amplicons GAD and SB (Table 1) were used for the amplification of the GAPDH gene and the beta-glucuronidase gene (GUSB) cDNAs, respectively. Both GAPDH and GUSB genes were used as reference genes. Sequence of primers and probe of the GAD amplicon was obtained by User Bulletin #2 by Applied Biosystems.

Total PLP1 gene expression was at first determined after normalization to the GAPDH gene expression using amplicon 151 as target and amplicon GAD as reference. Subsequently, gene expression was determined after normalization to the GUSB gene expression using amplicons PL20 as target and amplicon SB as reference.

Expression of the DM20 transcript normalized to the GUSB gene expression was evaluated using amplicon D2 as target and amplicon SB as reference.

Expression of the DM20 transcript normalized to the total PLP1 gene expression was determined using amplicon D2 as target and amplicon PL20 as reference.

In all the above mentioned experiments, standard wells contained the equivalent of the cDNA obtained from 50, 20, 10, 5, 2, 1, 0.5 ng of total RNA extracted from a normal male. Each patient and control well contained the equivalent of the cDNA obtained from 10 ng of total RNA.

Expression of DM20 transcript normalized to the total PLP1 gene expression was also evaluated, again with amplicons D2 and PL20, by using as standard a plasmid containing the DM20 cDNA. This experimental profile allowed the determination of the DM20/(DM20+PLP) ratio, that is the fraction of DM20 on the total PLP+DM20 amount, in patients and controls. Standard wells contained 100×10^{-8} , 50×10^{-8} , 25×10^{-8} , 10×10^{-8} , 5×10^{-8} , 2.5×10^{-8} , 1×10^{-8} ng/ μ l of the DM20 cDNA plasmid. This experimental system was at first validated using three kinds of samples, containing respectively the DM20 cDNA plasmid, a PLP cDNA plasmid and a mix of the two plasmids. Each sample well contained 10×10^{-8} ng/ μ l of plasmid DNA. Subsequently, patient and control samples were analysed, using in each well the cDNA obtained from 10 ng of total RNA.

To avoid any bias due to between-experiment variation, each experiment included simultaneously all the analysed patients and controls.

Preparation of the PLP and DM20 cDNA containing plasmids

In order to amplify the entire open reading frame of the PLP1 gene, an endpoint RT-PCR using primers PLPF: 5'-AGACCGAAGAAGGAGGCTGGAGAGACCAGG-3' and PLP1R: 5'-GAGTTAAGATGGGAGACGCAGCATTGTAGG-3' was performed using first strand cDNA obtained from a normal control. RT-PCR product was cloned in pCR2.1 (Invitrogen). Plasmids containing the PLP and DM20 cDNAs were selected and subsequently sequenced to exclude the presence of undesired mutations.

Analysis of selectivity of the PLP/DM20-related amplicons

To test the selective amplification of the PLP and/or DM20 cDNAs by the PLP1-related amplicons 151, PL20 and D2, end-point PCR amplifications using forward and reverse primers from each amplicon were performed using as template plasmids containing the PLP cDNA, the DM20 cDNA, or both.

Results*PLP1 gene dosage determination*

DNA samples from PMD male patients, of whom a fibroblast cell line was available, were tested for the presence of the PLP1 gene duplication according to the real-time PCR-based procedure by Regis et al. [17], in which the copy number of a portion of PLP1 gene exon 3, covered by amplicon PLP1gen, is determined. As reported in table 2, three patients (P1, P2 and P3) were found to carry two PLP1 gene copies showing ratios proximal to the expected value 2 and to the ratio obtained for the positive control carrying the duplication (DC). To exclude a partial gene duplication, the copy number of two flanking regions was also tested with amplicon 830, located in intron 1 of the PLP1 gene, and amplicon 459, located in the untranslated portion of exon 7 (Table 1). Experimental findings confirmed a good uniformity of the data across the entire gene in each of the three patients (Table 2).

Selectivity of the PLP/DM20 amplicons

Three PLP/DM20-related amplicons, each composed of a forward, a reverse primer and a TaqMan probe, were designed. Amplicons 151 and PL20 matched both the PLP and DM20 cDNAs. Amplicon D2 was designed to amplify selectively the DM20 cDNA and not the PLP cDNA (Fig. 1). To verify the selectivity of each PLP/DM20-related amplicon, primers were tested by endpoint-PCR using as template plasmids containing the PLP or DM20 cDNAs, or a mixture of the two plasmids. As expected, a PCR product was obtained in the presence of either

PLP or DM20 cDNA using primers of amplicons 151 and PL20, a PCR product was obtained only in the presence of the DM20 cDNA using primers of amplicon D2 (Fig. 2).

Combined PLP/DM20 expression normalized to the GAPDH and GUSB expression

Once the selectivity of each PLP1-related amplicon was established, two amplicons from two different genes chosen as reference were designed: amplicon GAD, from the GAPDH gene, and amplicon SB, from the GUSB gene (Table 1). We used amplicon 151 as target and amplicon GAD as reference to quantify the whole PLP1 gene expression normalized to the GAPDH gene expression in fibroblasts from patients and normal controls. The three repeated experiments revealed a certain variability of the PLP1 gene expression among the normal controls as well as among the patients. However, PLP1 gene expression in patients largely exceeded that in normal controls. Considering the mean values, patients showed more than a 4-fold increase in gene expression compared to normal controls (Fig. 3A).

To verify these results, excluding undesired error possibly introduced by the used reference gene or amplicons, we quantified the whole PLP1 gene expression normalized to the GUSB gene expression using PL20 and SB as target and reference amplicon, respectively. Results of the triplicate replicas were in line with previous data. Variability among controls and patients was confirmed. Considering again the mean values, patients had more than a 5-fold increase in gene expression compared to normal controls (Fig. 3B).

Selective DM20 expression normalized to the GUSB expression

After confirming the PLP1 gene overexpression of the patients with duplication, we focused on the contribution of PLP and DM20 transcripts to the observed overexpression. Hence, we used amplicon D2 as target and amplicon SB as reference in order to evaluate the DM20 expression normalized to the GUSB expression in fibroblasts from patients and normal controls. Mean values of the triplicate replicas showed that the patients had a 2-fold increase in DM20 expression compared to normal controls (Fig. 4).

Selective DM20 expression normalized to the whole PLP/DM20 expression

To confirm the indication of a limited contribution of the DM20 transcript to the PLP1 gene overexpression, we evaluated the DM20 expression normalized to the whole PLP1 gene expression using amplicons D2 as target and PL20 as reference. We found that fibroblasts from patients showed a decrease of the DM20/(DM20+PLP) ratio compared to normal controls, indicating a shift in the balance between the PLP and DM20 transcripts (Fig. 5).

Determination of the DM20/(DM20+PLP) ratio in patients and controls

To quantify the fraction of DM20 present in normal controls and in patients with duplication, we used the same D2 and PL20 amplicons previously employed, but the standard curves were generated using a plasmid containing the DM20 cDNA instead of a cDNA from a normal control. Using this experimental profile the expected ratio directly corresponded to the DM20/(DM20+PLP) ratio. In detail, expected ratios of 1, 0.5, 0 corresponded respectively to a 100%, 50%, 0% content of DM20 in the total DM20+PLP amount. To validate this experimental profile we performed three real-time PCR runs using three kind of samples, containing the DM20 cDNA plasmid, an equal amount of the DM20 and PLP cDNAs plasmids, or the PLP cDNA plasmid, respectively. As shown in table 3, the concordance between expected and obtained results was evident. Subsequently, the same experimental profile was used to test cDNAs from patients and normal controls. Patients showed a mean DM20/(DM20+PLP) ratio of 0.7, corresponding to a DM20 content in the total DM20+PLP amount of about the 70%, while normal controls showed a mean DM20/(DM20+PLP) ratio of 1.0, corresponding to a DM20 content near to the 100% (Fig. 6).

To confirm data obtained using the DM20-specific amplicon, we evaluated the PLP expression normalized to the whole PLP1 gene expression using a PLP-specific amplicon named P2 as target and the PL20 amplicon as reference. We found that fibroblasts from patients showed an increase of the PLP/(DM20+PLP) ratio compared to normal controls (data not shown), thus

confirming the decrease of the DM20/(DM20+PLP) ratio detected using the DM20-specific amplicon.

Discussion

Duplication of the PLP1 gene is the most frequent gene defect responsible for PMD. Although it is likely that the increased gene dosage causes overexpression of the gene, up to now, however, it has not been experimentally confirmed in patients with duplication [1]. Moreover, no data exists concerning the relative frequency of the PLP and DM20 transcripts in patients carrying the duplication compared to normal controls.

To investigate on these issues we used fibroblast cells. These cells are easily obtained from patients and exhibit an acceptable level of PLP1 gene expression to perform quantitative determinations. Recently, fibroblasts have been used to study PLP1 splicing abnormalities [18]. However, it must be underlined that fibroblasts do not have a role in the pathogenesis of PMD. Their PLP1 expression is considerably lower than that of oligodendrocytes. Moreover, unlike oligodendrocytes, fibroblasts show a predominant expression of the DM20 isoform. Nevertheless, fibroblasts can be considered a simple cellular model in which the consequences of an altered PLP1 gene dosage can be ascribed to basic mechanisms of regulation of PLP1 gene expression.

Preliminarily to the expression analysis, copy number of the PLP1 gene in the selected patients was determined in three different portions of the gene to exclude incomplete gene duplications. We used real-time PCR for the PLP1 gene expression study, due to the sensitivity of this technique in performing quantitative determinations of rare transcripts.

Whole PLP1 gene expression was analysed using two different portions of the PLP/DM20 cDNA as target (Fig. 1) and two different reference genes. While GAPDH is a widely used reference gene, the GUSB gene has a lower expression level than GAPDH, more similar to the levels of the PLP1-related transcripts.

Results of the two parallel real-time PCR determinations were sufficiently comparable to confirm the robustness of the experimental design and the reliability of the two reference genes used. The obtained data show a certain degree of variability, which might reflect different levels of expression among patients and controls, or be due to cell culturing, or be intrinsic to the used experimental profile. Despite this variability, the 4-5 fold increase of the PLP1 gene expression in patients versus controls is evident. It is not clear why a more than two-fold overexpression was detected. A sort of synergistic effect between the two gene copies may be hypothesized. Or again, the wide overexpression detected may be in part due to the cell culturing or to the used experimental system.

To evaluate the contribution of the PLP and DM20 isoforms to PLP1 gene expression we directly quantified the expression of DM20, which is the prevalent transcript isoform in fibroblasts, and indirectly we deduced the expression of PLP.

DM20 expression levels showed an only two-fold increase in patients compared to controls. These findings were suggestive of a possible more significant contribution of PLP than of DM20 to overexpression.

Quantification of the DM20 expression level normalized to the total PLP1 expression level confirmed this indication showing a decrease of the $DM20/(DM20+PLP)$ ratio in patients compared to controls.

To assign values to the $DM20/(DM20+PLP)$ ratio in patients and controls, the DM20 expression normalized to the total PLP1 expression was estimated using a plasmid containing the DM20 cDNA as standard. Mean $DM20/(DM20+PLP)$ ratios resulted 0.7 in PMD patients and 1.0 in controls, thus confirming that fibroblasts from patients show a shifting of the splicing ratio in direction of the PLP isoform, besides the expected increased expression of the PLP1 gene.

Alterations of the PLP/DM20 splicing ratio have been reported, in each case due to specific mutations lying in exon3b (the portion of exon 3 spliced in DM20 and not in PLP) or in intron 3 [15,19-21]. By studying these mutations an intronic splicing enhancer (ISE) [19] and two exonic splicing enhancers (ESE) [20,21] involved in the PLP/DM20 splicing have been described.

Overexpression of ASF/SF2 [20] and reduction in the hnRNPH/F expression [21] have been reported as correlating with PLP 5' splice selection in oligodendrocytes. These RNA-binding factors and enhancers seem to be part of a complex splicing machinery regulating the PLP/DM20 splicing pattern. We can speculate that the PLP1 gene expression level is one of the factors able to influence this splicing machinery in a dose-dependent manner. In view of this, the PLP1 gene overexpression observed in fibroblasts from duplicated patients could be the cause of the PLP/DM20 balance alteration observed respect to normal controls. Broadly speaking, the level of PLP1 gene expression could drive the PLP/DM20 splice selection being responsible, at least in part, for the different PLP/DM20 ratios observed in different cell types and in different developmental stages.

ACKNOWLEDGEMENTS

The samples were obtained from the "Cell Line and DNA Biobank from Patients Affected by Genetic Diseases" (G. Gaslini Institute) - Telethon Genetic Biobank Network (project no. GTB07001A).

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Figure legends

Fig. 1. PLP/DM20-related amplicons used for the gene expression analysis. Amplicons PL20 and 151 (on the right side of the diagram) amplified simultaneously PLP and DM20 cDNAs. Amplicon D2 (on the left side of the diagram) amplified selectively DM20 cDNA but not PLP cDNA. The portions of the amplicons corresponding to primers and probes are underlined. DM20 and PLP cDNAs are schematically represented in the middle part of the diagram. Green bar (PLP cDNA) represents the specific genomic region of 105 nucleotides of the PLP isoform. Blue and red bars (PLP and DM20 cDNAs) represent the cDNA regions shared by the two isoforms. Sequences at the blue-red boundary (complementary to the reverse primer of amplicon D2) and blue-green /green-red boundaries (not-complementary to the reverse primer of amplicon D2) are reported. Red arrows indicate the location of forward and reverse primers of amplicon PL20 (located at cDNA exon 4 and 5, respectively); blue arrows indicate the location of forward and reverse primers of amplicon 151 (located at cDNA exon 5 and exons 6-7, respectively).

Fig. 2. Endpoint PCR reactions performed using forward and reverse primers of amplicons 151, D2 and PL20. Plasmids containing the PLP cDNA (P), the DM20 cDNA (D), or a mixture of them (B) were used as templates. C= amplification without DNA. M= molecular weight marker Φ x DNA Hae III digested.

Fig. 3. Combined PLP/DM20 expression normalized to the GAPDH and GUSB genes expression. Histogram data (top-half of the figure) derived from subset data of the related tables (bottom-half of the figure) report the results of the real-time PCR experiments performed using amplicon 151 as target (Tamp) and amplicon GAD as reference (Ramp) (Fig. 3A) or amplicon PL20 as target and amplicon SB as reference (Fig. 3B). The repeated experiments (Exp 1, 2, 3) showed that the PLP1 gene expression in patients (P1, P2, P3) largely exceeded that in normal controls (C1, C2, C3).

Fig. 4. Selective DM20 expression normalized to the GUSB gene expression. Histogram data derived from subset data of the related table report the results of the real-time PCR experiments performed using amplicon D2 as target (Tamp) and amplicon SB as reference (Ramp). Mean values of the triplicate replicas showed that the patients (P1, P2, P3) had a 2-fold increase in DM20 expression compared to normal controls (C1, C2, C3).

Fig. 5. Selective DM20 expression normalized to the whole (PLP+DM20) expression. Histogram and the related tabular data show results of the real-time PCR experiments performed using amplicon D2 as target (Tamp) and amplicon PL20 (Ramp) as reference. Mean values of the triplicate replicas from patients (P1, P2, P3) showed a decrease of the DM20/(DM20+PLP) ratio compared to normal controls (C1, C2, C3).

Fig. 6. Determination of the DM20/(DM20+PLP) ratio in patients and controls. Experiments were carried out using amplicons D2 and PL20. The asterisk (D2/PL20*) marks that, differently from previous experiments, the standard curves were generated using a plasmid containing the DM20 cDNA, instead of a cDNA from a normal control. Histogram and related tabular data show a mean DM20/(DM20+PLP) ratio of 0.7 and 1.0 for patients and controls, respectively.

Table 1

Amplicons used for PLP1 gene dosage determination and expression analysis

GENE DOSAGE DETERMINATION		
Denomination	Gene	Sequence
Amplicon PLP1gen	PLP1	<u>AGTCAGGCAGATCTTTGGCGACTACAAGACCACCATCTGCGGCAAGGGCCTGAGCGCAACGGTAAACAGG</u> <u>GGGCCAGAAGGGGAGGGGTTCCAGAGGCCAACATCAAGTCATTCTTTGGAGCGGGTGTGTC</u>
Amplicon 830gen	PLP1	<u>GAGAGCCAAGGAACCTTCCCTAGAAATCTCTCCAGCCTTCTTGAATCAAACCTCACACCCTTCAACCA</u> <u>GGAGGGGTTCTGAAATGGAGAGCTTTTGGCT</u>
Amplicon 459gen	PLP1	<u>TCACATAACAGAATGCACCAGTCATCAGCTATTCAGTTGGTAAGCTTCCAGGAAAAAGGACAGGCAGAA</u> <u>AGAGTTTGAGACCTGAATAGCTCCAGATTC</u>
Amplicon GAPgen	GAPDH	<u>GGGCTCTCCAGAACATCATCCCTGCCTCTACTGGCGCTGCCAAGGCTGTGGGCAAGGTCATCCCTGAGCT</u> <u>GAACGGGAAGCTCACTGGCATGGCCTTCCGTGTCCCACTGCCAACGTGTCAGTGGTGGAC</u>
GENE EXPRESSION ANALYSIS		
Denomination	Gene	Sequence
Amplicon GAD	GAPDH	<u>GAAGGTGAAGGTCGGAGTCAACGGATTTGGTCGTATTGGGCGCCTGGTCACCAGGGCTGCTTTTAACTCT</u> <u>GGTAAAGTGGATATTGTTGCCATCAATGACCCCTTCATTGACCTCAACTACATGGTTTACATGTTCCAATA</u> <u>TGATTCCACCCATGGCAAATTCCATGGCACCGTCAAGGCTGAGAACGGGAAGCTTGTTCATCAATGGAAA</u> <u>TCCATCACCATCTC</u>
Amplicon SB	GUSB	<u>TAAGGTGCCAGGTGTCAGCCTCTGGTGGCCGTACCTGATGCACGAACGCCCTGCCTATCTGTATTCATTG</u> <u>GAGGT GCAGCTGACTGCACAGACGTCCTGG</u>

Amplicon portions corresponding to primers and probes are underlined. Amplicons GAD and SB were used as reference for the PLP/DM20 expression studies; PLP/DM20-related amplicons are reported in figure 1.

Table 2
 PLP1 gene dosage determination

Amplicon (genomic localization)	P1	P2	P3	DC	MC
830gen (intron 1)	2.0	2.0	2.0	2.1	0.9
PLP1gen (exon 3)	1.9	2.0	2.1	2.1	1.0
459gen (exon 7 UTR)	2.2	2.0	2.2	2.0	0.9

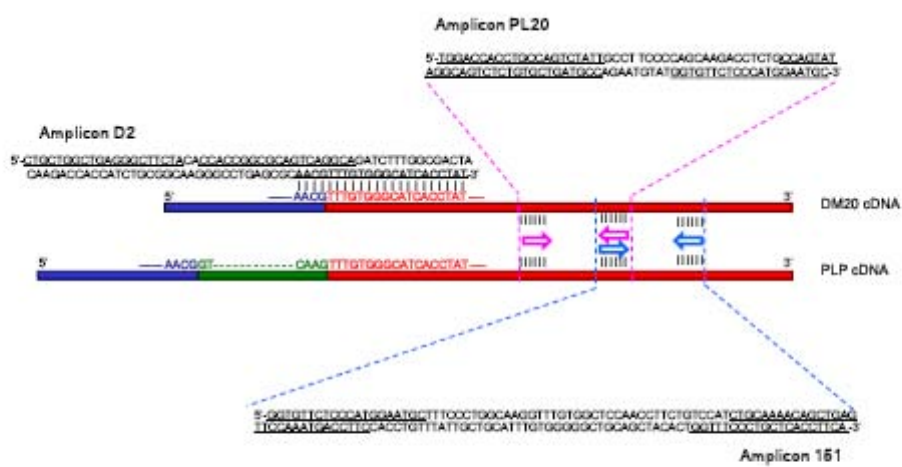
P1, P2, P3: the three PMD patients; DC: male patient carrying the PLP1 gene duplication; MC: normal male.

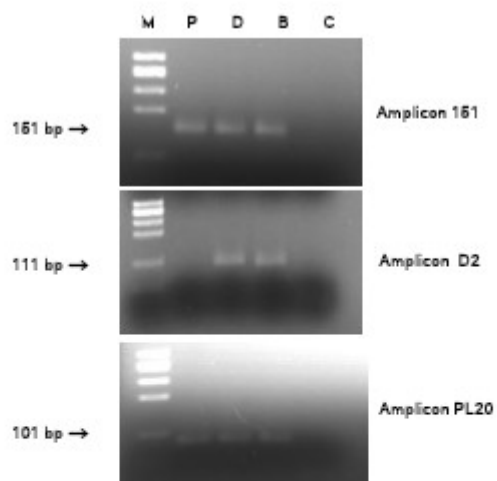
Table 3

Verification of the real-time PCR profile for the estimation of the DM20/(DM20+PLP) ratio

Tamp/Ramp D2/PL20*	Plasmids containing specific isoform		
	DM20 cDNA	DM20 cDNA+PLP cDNA	PLP cDNA
Exp 1	1.0	0.5	0
Exp 2	0.9	0.5	0
Exp 3	0.8	0.4	0
Mean	0.9	0.5	0
Expected values	1.0	0.5	0

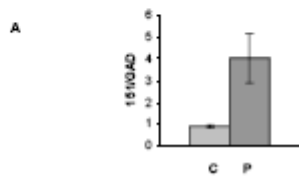
Tamp= target amplicon; Ramp= reference amplicon; * this symbol marks the use of a specific plasmid DNA as standard (in place of the previously used cDNA from a normal male). Means of the three replicas and the expected values are in bold.



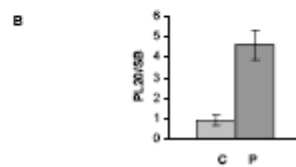


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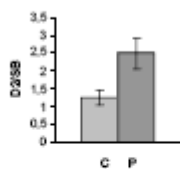
Temp/Ramp	Controls (C)			Patients (P)		
	C1	C2	C3	P1	P2	P3
151/GAD						
Exp 1	0.7	0.7	1.0	4.9	4.6	2.9
Exp 2	1.0	1.1	1.0	5.4	3.7	2.4
Exp 3	0.9	0.8	1.1	4.6	4.4	3.2
Mean	0.9	0.9	1.0	5.0	4.2	2.8
St. Dev.	0.2	0.2	0.1	0.4	0.5	0.4



Temp/Ramp	Controls (C)			Patients (P)		
	C1	C2	C3	P1	P2	P3
PL20/5B						
Exp 1	0.6	1.0	0.6	5.7	5.0	3.7
Exp 2	0.5	1.1	1.2	4.7	4.6	4.3
Exp 3	0.6	1.1	0.8	5.3	4.8	3.4
Mean	0.6	1.1	1.0	5.2	4.8	3.8
St. Dev.	0.1	0.1	0.3	0.5	0.2	0.5

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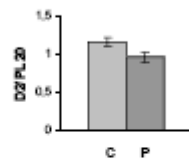
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Temp/Ramp	Controls (C)			Patients (P)		
	C1	C2	C3	P1	P2	P3
D2/SB						
Exp 1	1.0	1.1	1.4	3.1	2.1	2.1
Exp 2	1.2	1.1	1.5	3.2	2.6	2.4
Exp 3	1.5	1.0	1.5	2.8	2.3	2.0
Mean	1.2	1.1	1.5	3.0	2.3	2.2
St. Dev.	0.3	0.1	0.1	0.2	0.3	0.2

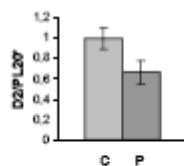
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Temp/Exp	Controls (C)			Patients (P)		
	C1	C2	C3	P1	P2	P3
Exp 1	1.1	1.3	1.2	1.0	0.8	1.1
Exp 2	1.3	1.2	1.3	1.1	1.0	1.0
Exp 3	1.2	0.9	1.2	1.0	0.8	0.9
Mean	1.2	1.1	1.2	1.0	0.9	1.0
St. Dev.	0.1	0.2	0.1	0.1	0.1	0.1

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Temp/Ramp	Controls (C)			Patients (P)		
D2/P1 L20*	C1	C2	C3	P1	P2	P3
Exp 1	1.0	0.9	1.1	0.8	0.7	0.7
Exp 2	1.0	0.9	1.0	0.7	0.6	0.6
Exp 3	0.9	0.9	1.1	0.8	0.6	0.6
Mean	1.0	0.9	1.1	0.8	0.6	0.6
St. Dev.	0.1	0.0	0.1	0.1	0.1	0.1