

## ORIGINAL ARTICLE

# Reduced *PLP1* expression in induced pluripotent stem cells derived from a Pelizaeus–Merzbacher disease patient with a partial *PLP1* duplication

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Pelizaeus–Merzbacher disease (PMD) is an X-linked recessive disorder characterized by dysmyelination of the central nervous system (CNS). We identified a rare partial duplication of the proteolipid protein 1 gene (*PLP1*) in a patient with PMD. To assess the underlying effect of this duplication, we examined *PLP1* expression in induced pluripotent stem (iPS) cells generated from the patient's fibroblasts. Disease-specific iPS cells were generated from skin fibroblasts obtained from the indicated PMD patient and two other PMD patients having a 637-kb chromosomal duplication including entire *PLP1* and a novel missense mutation (W212C) of *PLP1*, by transfections of *OCT3/4*, *C-MYC*, *KLF4* and *SOX2* using retro-virus vectors. *PLP1* expressions in the generated iPS cells were examined by northern blot analysis. Although *PLP1* expression was confirmed in iPS cells generated from two patients with the entire *PLP1* duplication and the missense mutation of *PLP1*, iPS cells generated from the patient with the partial *PLP1* duplication manifesting a milder form of PMD showed null expression. This indicated that the underlying effect of the partial *PLP1* duplication identified in this study was different from other *PLP1* alterations including a typical duplication and a missense mutation.

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## INTRODUCTION

Pelizaeus–Merzbacher disease (PMD; MIM #312080) is an X-linked recessive neurodegenerative disorder characterized by dysmyelination of the central nervous system (CNS). Patients with PMD often present with nystagmus as the initial symptom, and psychomotor developmental delay associated with spasticity and ataxia is seen later in development.<sup>1–3</sup> The proteolipid protein 1 gene (*PLP1*; MIM #300401), located on chromosome Xq22.2, is the gene responsible for PMD. It encodes 2 isoforms, PLP1 and DM20, as a consequence of differential splicing of exon 3. The genetic basis of PMD is unique because two-thirds of *PLP1* abnormalities identified in PMD patients are duplications of small chromosomal segments that include *PLP1*. The remaining one-third of *PLP1* abnormalities are nucleotide alterations in the *PLP1* coding sequence. The nucleotide alterations in *PLP1* are varied and are scattered along the entire coding region of *PLP1*.<sup>1–3</sup>

Because *PLP1* is mainly expressed in oligodendrocytes in the CNS and cultured skin fibroblasts express low levels of *PLP1*, gene

expression in the fibroblasts has been analyzed by comparative reverse-transcription (RT)-PCR analysis.<sup>4,5</sup> The use of technology to establish induced pluripotent stem (iPS) cells has now made it possible to examine gene expression and function in greater detail.<sup>6</sup> In 2007, Takahashi *et al.* established iPS cells from human skin fibroblasts.<sup>7</sup> This revolutionary technology has stimulated and accelerated research in embryogenesis and genetics. In this study, we established iPS cells from skin fibroblasts of patients with PMD and examined *PLP1* expression. This is the first report analyzing *PLP1* expression in PMD disease-carrying iPS cells.

## MATERIALS AND METHODS

### Subjects

For our ongoing study identifying genomic mutations in *PLP1*, three new patients with dysmyelination were referred to us for genetic diagnosis based on the clinical diagnosis of PMD.<sup>8</sup> Clinical information and radiographic findings by MRI for the patients were obtained from attending doctors. Based on

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approval by the ethical committees at the institutions, written informed consent was obtained from each patient and/or their family. Peripheral blood samples were collected from the patients and genotyping was performed as described.<sup>8</sup> After genetic diagnosis of PMD was made, another written informed consent for the iPS cell study was obtained from each patient and/or their family. Skin fibroblasts were collected from three patients and a healthy male control.

### Genotyping of the patients

Genomic DNAs were extracted from peripheral blood samples from patients and others by using standard methods. Initial screening for *PLP1* duplication was performed by multiplex ligation-dependent probe amplification analysis by using the PLP1 Kit (P022; MRC-Holland, Amsterdam, The Netherlands) according to the manufacturer's instruction.<sup>9</sup> In case of *PLP1* duplication, the aberration region was confirmed by microarray-based comparative genomic hybridization (aCGH) using the Agilent Human 105A CGH Kit (Agilent Technologies, Santa Clara, CA, USA) as described previously.<sup>8</sup> To detect the small duplication in Patient 1, a custom array was designed using e-array, a web-based software (<https://earray.chem.agilent.com/earray/>), and 29 918 probes in chrX:98 000 000–104 500 000, around *PLP1*, were selected. The average interval of the probes was 217 bp in this region.

*PLP1* duplication was confirmed by two-color fluorescence *in-situ* hybridization as described previously.<sup>8</sup> Two bacterial artificial chromosome clones, RP11–75D20 (located at Xp22.13) and RP11–832L2 (located at Xq22.2), were selected from the UCSC Human Genome Browser (<http://genome.ucsc.edu/>) and used as probes. The fixed metaphase and interphase spreads of the specimens were derived from patients' peripheral blood samples and generated iPS cells. The direction of the duplicated segment identified in Patient 2 was analyzed by fiber-fluorescence *in-situ* hybridization analysis as described previously.<sup>8</sup>

PCR and direct sequencing of all seven exons of *PLP1* was performed by standard methods using the primers reported by Hobson *et al.*<sup>4</sup> The designs of the primers for all exons and the breakpoint searches of the duplicated segments in Patient 1 are listed in Supplementary Table 1.

### Cell culture

Human fibroblasts, the Plat-E Retroviral Packaging Cell Line (Cell Biolabs, San Diego, CA, USA), 293FT cells (Life Technologies, Foster City, CA, USA) and mouse fibroblast STO cell line (SNL) feeder cells (ECACC, Salisbury, UK) were grown in Dulbecco's modified Eagle's medium (DMEM 14247-15; Nacalai Tesque, Japan) containing 10% fetal bovine serum and 0.5% penicillin and streptomycin (Life Technologies). Human iPS cells were maintained on SNL feeder cells treated with mitomycin C in Primate ES Cell Culture Medium supplemented with 4 ng ml<sup>-1</sup> recombinant basic fibroblast growth factor (# RCHMD001; Repro CELL, Yokohama, Japan) and passaged as described previously.<sup>7,10</sup>

### Generation of iPS cells

Disease-specific iPS cells were generated from patients' skin fibroblasts as previously described.<sup>7</sup> Briefly, recombinant lentivirus produced from 293FT cells, in which pLenti6/Ubc/mSlc7a1 (AddGene, Cambridge, MA, USA) was transfected by use of Virapower Lentiviral Expression System (Life Technologies), was infected into cultured fibroblasts for 24 h. Then, four retroviruses produced with Plat-E Packaging Cells (Cell Biolabs), in which pMXs-hOCT3/4, pMXs-hSOX2, pMXs-hKLF4 and pMXs-hc-MYC (AddGene) were transferred independently, were infected into mSlc7a1-expressing human fibroblasts. Six days after retroviral infection, the fibroblasts were placed onto mouse fibroblast SNL feeder cells (ECACC, Salisbury, UK) at the appropriate concentration. The following day, DMEM 14247-15 (Nacalai Tesque, Japan) was replaced with Primate ES Cell Culture Medium supplemented with 4 ng ml<sup>-1</sup> recombinant basic fibroblast growth factor (# RCHMD001; Repro CELL, Yokohama, Japan). Thirty days after transduction, each embryonic stem (ES) cell-like colony was individually placed onto SNL feeder cells. Each colony was tested to determine whether they had indeed acquired pluripotency. After validation,<sup>10</sup> three independent iPS cell clones were selected from the candidates generated from each patient's skin fibroblasts.

### Validation of the pluripotency of iPS cells

Initially, alkaline phosphatase staining was performed for validation of iPS cells. Leukocyte Alkaline Phosphatase (AP) kit 86R (Sigma-Aldrich, St Louis, MO, USA) was used for this purpose.

Reactivation of endogenous pluripotency genes and the silencing of artificially induced retroviral transgenes indicated successful reprogramming of putative iPS cell clones. To confirm this, RT-PCR analysis and real-time PCR were performed as described below.

Total RNAs were extracted from iPS cells using ISOGEN (Nippon Gene, Tokyo, Japan) and contaminating genomic DNAs were removed by DNase (Takara, Ohtsu, Japan) according to the manufacturer's instructions. Subsequently, total RNAs were reverse transcribed into complementary DNAs by using the Superscript VILO cDNA Synthesis Kit (Life Technologies) according to the manufacturer's instructions.

Quantitative real-time PCR was performed for *OCT3/4*, *SOX2*, *KLF4*, *C-MYC*, *NANOG*, *REX1*, *GAPDH* and actin beta using the Power SYBR Green PCR Master Mix (Life Technologies) and analyzed with the 7300 Real-Time PCR System (Life Technologies). Primer sequences are shown in Supplementary Table 1.

Immunocytochemistry was also performed for all putative iPS cells. For this purpose, the following primary antibodies were used: anti-SSEA4 (1:200, MAB1435, R&D systems, Minneapolis, MN, USA), anti-OCT3/4 (1:200, AF1759, R&D systems), anti-TRA-1-60 (1:200, MAB4360, Millipore, Billerica, MA, USA), and Anti-TRA-1-81 (1:200, MAB4381, Millipore). Secondary antibodies included Alexa488-conjugated donkey anti-mouse IgG, Alexa488-conjugated goat anti-mouse IgM, and Alexa594-conjugated donkey anti-mouse IgG (1:1000, Life Technologies). Nuclei were stained with Hoechst 33342 (1:1000, Life Technologies).

### Validation of the differentiation ability of iPS cells

Determination of the differentiation ability of established iPS cells is important for the selection of putative iPS cell clones. To confirm their pluripotency to differentiate into three embryonic germ layers, we used floating cultivation to form embryoid bodies as described previously.<sup>10</sup> iPS cells were grown as floating cultures for 8 days. After embryoid body formation, the cells were cultured on gelatin-coated dishes for an additional 8 days.

Immunocytochemistry was performed to confirm expression of the three germ layers as described elsewhere.<sup>10</sup> In this case, three primary antibodies were used; anti-βIII tubulin (1:1000, MRB435P, Covance, Princeton, NJ, USA) as the ectoderm marker, anti-α smooth muscle actin (1:200, A2547, Sigma-Aldrich) for mesoderm, and anti-αAFP (1:100, A8452, Sigma-Aldrich) for endoderm. Donkey anti-mouse IgG labeled with Alexa Fluor 594 and donkey anti-rabbit IgG labeled with Alexa Fluor 488 (1:1000, Life Technologies) were used as secondary antibodies. Nuclei were stained with Hoechst 33342 (1:1000, Life Technologies) for nuclear staining.

### Validation of the karyotypes of iPS cells

To check the artificial chromosomal rearrangements, conventional G-banding by trypsin treatment stained with Giemsa and aCGH analyses using the same methods described above were performed for the generated iPS cell clones. iPS cell lines that acquired chromosomal rearrangements were eliminated from this study.

### Database analysis

Preliminary gene expression analysis was performed using online data sets. Two microarray data sets, GSM242095 for adult human dermal fibroblasts and GSM241846 for iPS cells (clone 201B7),<sup>7</sup> were retrieved from NCBI Gene Expression Omnibus (GEO) and analyzed using GeneSpring GX10 (Agilent Technologies).

### Northern blotting

The full-length mRNA of *PLP1* (920 bp) and a partial sequence of actin beta (*ACTNB*; MIM #102630) mRNA (91 bp) were amplified by RT-PCR by using Human Brain Total RNA (#636530, Clontech, Mountain View, CA, USA) as a template. Primer sequences are listed in Supplementary Table 1. The PCR product was subcloned into pGEM-T Vector System (Promega, Madison, WI,

USA) and grown in LB Broth overnight. Plasmid DNAs were extracted by an automated DNA isolation system, PI-80X (Kurabo, Osaka, Japan). DNA inserts were digested with *SacI* and *SacII* restriction enzymes. Following agarose gel electrophoresis, product bands were excised and extracted using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). The DNA fragments were then labeled using [ $\alpha$ - $^{32}$ P] dCTP (PerkinElmer, Waltham, MA, USA) and used as probes for northern blotting.

Hybridization was performed as described previously.<sup>11</sup> Briefly, 30  $\mu$ g of total RNA was extracted using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions, separated on a 1% agarose/0.6 M formaldehyde gel, visualized using Radiant Red RNA Stain (Bio-Rad, Hercules, CA, USA), transferred to a nylon membrane and subsequently hybridized for 24 h with either *PLP1* or *ACTNB* probes. Images were captured using the FLA-5100 scanner (Fujifilm, Tokyo, Japan).

Initial analysis included seven samples: mitomycin-treated and -untreated SNL feeder cells, Epstein-Barr virus-infected immortalized lymphocytes derived from a normal human control, human skin fibroblasts derived from the normal control, iPSC cells generated from the normal human control and two brain samples purchased from a provider (Human Fetal Brain Total RNA #636526 and Human Brain Total RNA #636530, Clontech). Subsequent analysis included the 12 iPSC cell lines generated in this study.

## RESULTS

### Clinical features

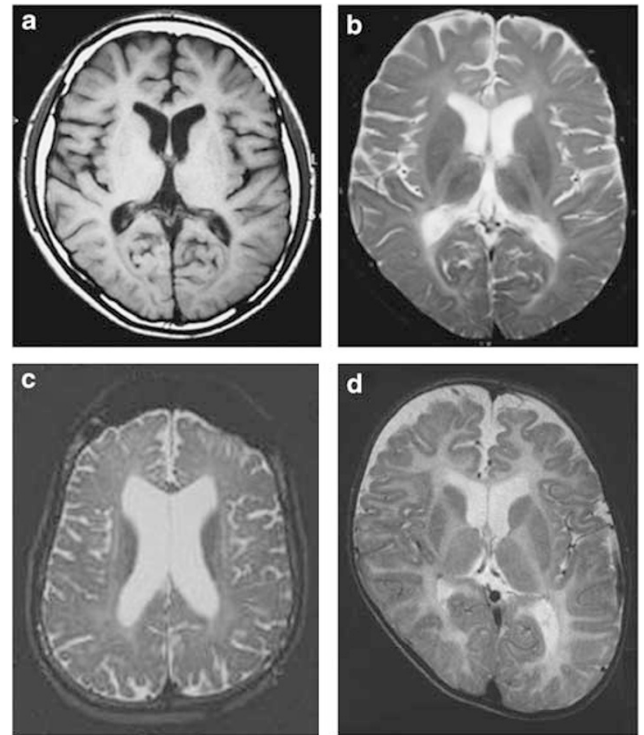
Patient 1 was a 16-year-old male, born by spontaneous delivery at 40 weeks gestation, with a weight of 3054 g. Soon after birth he showed nystagmus. At 4 months, he exhibited poor neck control and was diagnosed with spastic paraplegia. Psychomotor development was moderately delayed with walking alone at his age of 2 years and his intelligence quotient was estimated below 50. At 15 years, he was prescribed medication for depression. At that time, his fine motor ability allowed the use of chopsticks but he needed a wheel chair to move. His speech was dysarthric. One month later, he had an epileptic attack and was admitted to the hospital. An electroencephalogram revealed occipital spikes. Although auditory brain response was normal, brain magnetic resonance imaging (MRI) revealed a pattern of mild dysmyelination (Figures 1a and b).

Patient 2 was a 46-year-old male with two healthy female siblings. As he lacked neck control at 1 year of age, he was diagnosed with spastic cerebral palsy. Then, at 4 years, he could turn over but could not sit unaided. He lacked the ability to speak effectively, being limited to two-word sentences. At 15 years, he could use a wheel chair by himself. Subsequently, the quality of his daily life declined gradually. At 39 years, MRI revealed atrophic white matter displaying dysmyelination (Figure 1c). At present, he can move only his upper body very slowly and is bedridden. He is able to comprehend what his siblings say, but he is severely dysarthric and is able to speak only a few words very slowly.

Patient 3 was a 32-month-old boy with a birth weight of 3869 g delivered at 39 weeks gestation. He has a healthy brother. Owing to respiratory problems since birth, he was intubated and tracheostomy was performed at 58 days. He also required tube feeding. He is currently bedridden and has continuous nystagmus. Auditory-brain-response audiometry showed no waves after the first wave. A brain MRI revealed high-intensity lesions of the white matter in a T2-weighted image, indicating severe hypomyelination (Figure 1d).

### Molecular analyses

Initial multiplex ligation-dependent probe amplification analysis using a PLP1 Kit (P022; MRC-Holland) identified duplications of *PLP1* in Patient 1 and 2 (data not shown).<sup>9</sup> Patient 2 had a duplication of all 7 exons of *PLP1*, and subsequent aCGH analysis

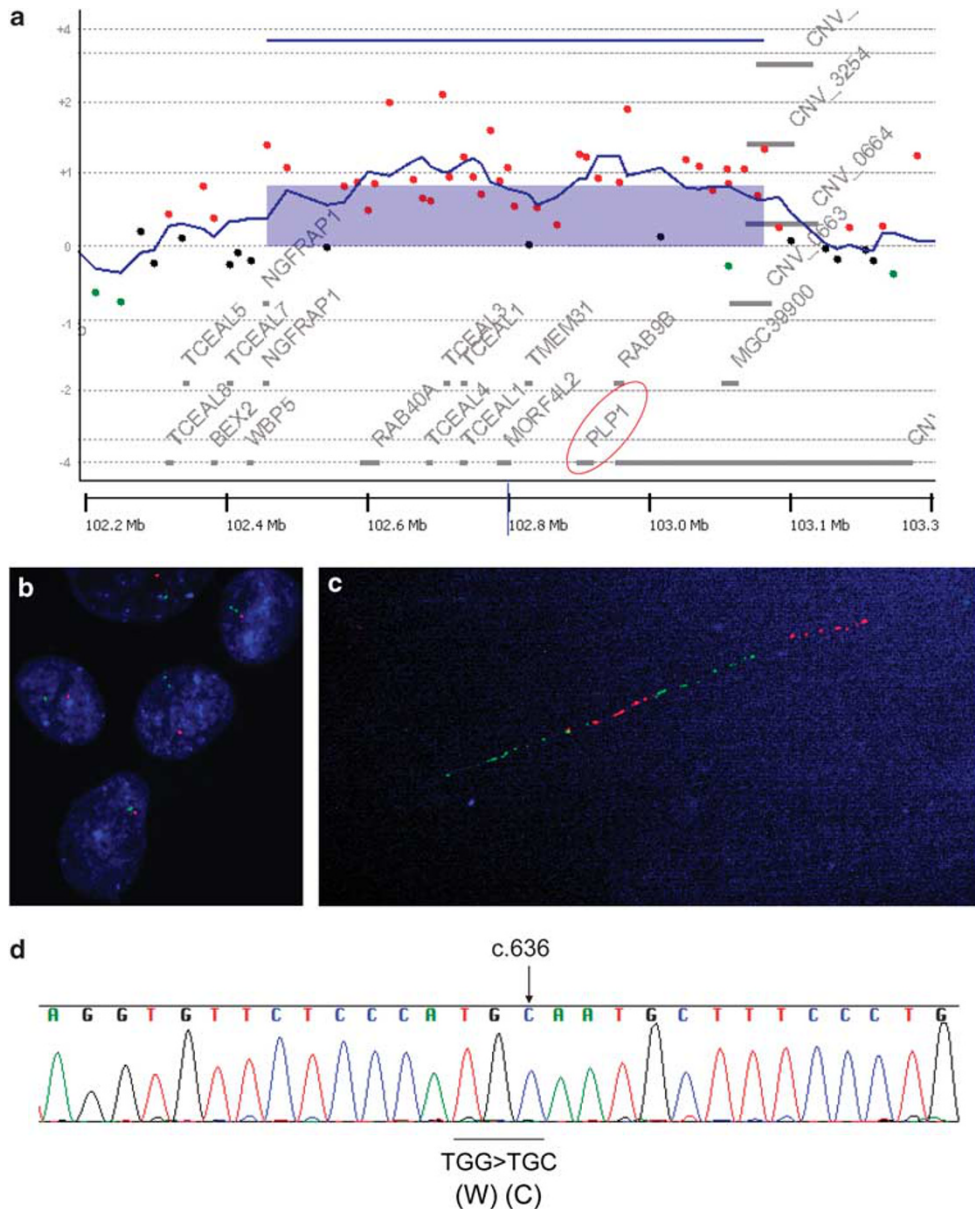


**Figure 1** Brain MRI findings of the patients T1- (a) and T2 (b) weighted images of Patient 1 show mild and diffuse volume loss of the brain and high-intensity signals of the deep white matter in T2 indicating mild dysmyelination. T2-weighted image of Patient 2 (c) shows diffuse volume loss resulting in the dilatation of the ventricles and dysmyelination in the white matter. T2-weighted image of Patient 3 (d) shows extremely hypomyelinated pattern with high intensity in all white matter.

by using the Human Genome CGH Microarray 105 K (Agilent Technologies) revealed that the duplicated region was chrX:102 519 000–103 155 851 (636 851 bp) with an average  $\log_2$  ratio of +0.83, which is a typical duplication region seen in PMD patients with *PLP1* duplications (Figure 2a). The duplication was confirmed by fluorescence *in-situ* hybridization (Figure 2b), and the direction of the duplicated segment, including *PLP1*, was shown to be in a tandem configuration by fiber-fluorescence *in-situ* hybridization analysis (Figure 2c).

The duplication identified in Patient 1 was unique because only the first 3 exons (exons 1–3) of *PLP1* were included in the duplicated region. To confirm this partial duplication, we designed a custom aCGH chip and used it to detect the precise duplication region. As shown in Figure 3a, the duplicated region was chrX:102 912 361–102 928 360 (15 999 bp) with an average  $\log_2$  ratio of +0.72. To determine the location of the duplicated segment, we sought to detect the breakpoint by PCR direct sequencing, using primers A and B (Supplementary Table 1). A 775-bp band was obtained and re-sequenced (Figures 3b and c). Ultimately, an extremely small duplication of 16 208 bp, which has never been previously reported, was identified. The sample from Patient 1's mother was also analyzed and she was found to be a carrier of this duplication (Figures 3b and c).

In Patient 3, a novel missense mutation, c.636G>C (W212C), was identified in exon 5 of *PLP1* (Figure 2d). The *PLP1* sequence is completely conserved among species and this novel mutation was not identified in 100 normal control samples (50 males and 50 females). This patient's mother declined to have her genotype analyzed.



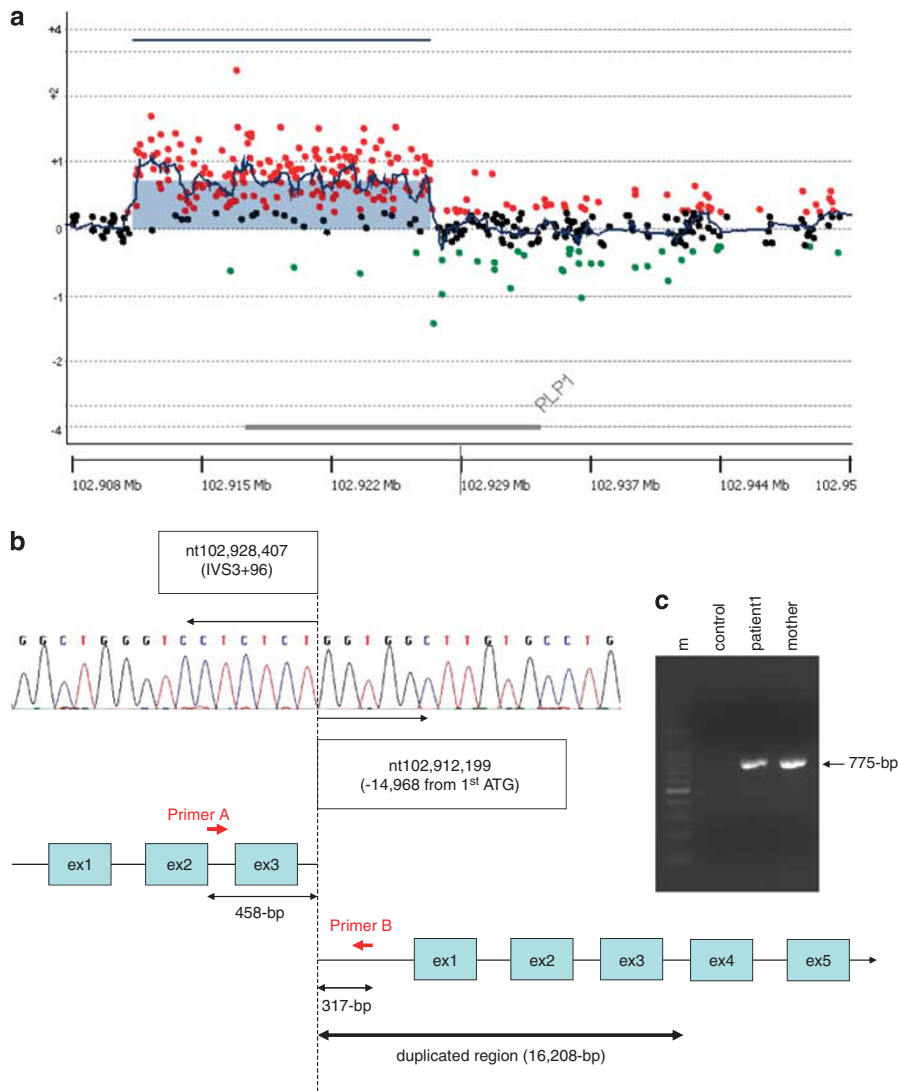
**Figure 2** Genotyping and cytogenetic analyses for Patients 2 and 3. **(a)** A microchromosomal duplication including *PLP1* is shown in GeneView of Agilent Genomic Workbench (Agilent Technologies). The location of *PLP1* is highlighted by a red circle. **(b)** Interphase fluorescence *in-situ* hybridization analysis shows two green signals labeled on RP11-832L2 (located at Xq22.2) in the nucleus. Red signals labeled on RP11-75D20 (located at Xp22.13) are the marker for X chromosome. **(c)** Fiber-fluorescence *in-situ* hybridization analysis indicates tandem configuration of the duplicated segments labeled with green and red probes. **(d)** Electropherogram shows a novel missense mutation c.636G>C (W212C) in Patient 3.

### Generation of iPS cells

We successfully generated iPS cells from three patients with PMD and a normal male control (Supplementary Figure 1). At least three independent clones were validated using the following three categories: (1) silencing of four transfected genes (*OCT3/4*, *C-MYC*, *KLF4* and *SOX2*; Supplementary Figure 2); (2) expression of endogenous pluripotency genes (*OCT3/4*, *SOX2*, *KLF4*, *C-MYC*, *NANOG* and *REX1*; Supplementary Figures 3 and 5); and (3) confirmation of the differentiation potency by immunocytochemistry (Supplementary Figure 4). Karyotype and aCGH analyses for the resulting iPS cells showed no artificial chromosomal rearrangements.

### PLP1 expression

Preliminary *PLP1* expression levels were compared between two online data sets for human skin fibroblasts and iPS cells. The results showed that *PLP1* expression levels were  $\times 40.70$  ( $\log_2 = 6.38$ ) higher in iPS cells than in skin fibroblasts (Supplementary Figure 6). Subsequently, our initial experiments for *PLP1* expression in several samples were performed by northern blot analysis, which revealed predominant *PLP1* expression in the brain (fetal brain had weaker expression than adult brain). Although the other samples showed no *PLP1* expression, we could detect the *PLP1* band in iPS cells (Figure 4); the differentiation between two isoforms for *PLP1* and *DM20* could not be detected owing to small size differences as same as



**Figure 3** Molecular analyses of the *PLP1* duplication identified in Patient 1. (a) The result of a custom-designed aCGH shows partial duplication of *PLP1*. (b) A schematic representation of the breakpoint analyzed by PCR and subsequent sequencing. (c) A 775 bp PCR product including a breakpoint is amplified by Primer A and B and is shown in electrophoresis. m; molecular marker OneSTEP Ladder 50 (Nippon Gene).

the previous study.<sup>12</sup> Finally, total RNA samples extracted from the 12 iPSC clones generated in this study were analyzed. Although we could detect the *PLP1* band in iPSCs from normal individual and Patients 2 and 3, we could not detect the *PLP1* band in the iPSCs generated from Patient 1, indicating null expression of *PLP1* caused by the partial duplication of *PLP1* (Figure 5). In Patients 2 and 3, *PLP1* signals appeared to be somewhat stronger than in controls, but because of the large variation in signal intensity among different cell lines, it was inconclusive in our limited experiments.

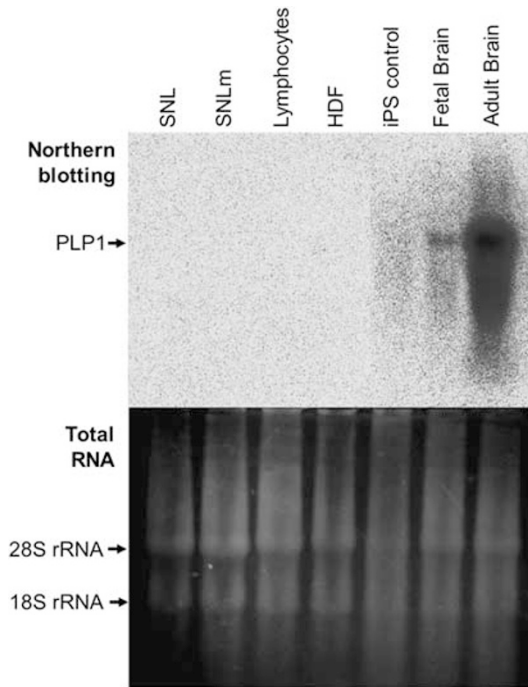
## DISCUSSION

In this study, we identified different *PLP1* abnormalities in three patients with PMD. Patient 3 showed a novel missense mutation, c.636G>C (Tyr212Cys), which is in the extramembrane region of the PLP1 protein.<sup>2</sup> A missense substitution in the same codon, but resulting in a change into a different amino acid, c.634T>C (Tyr212Arg), has been reported to be a pathogenic mutation by others.<sup>13</sup> Frequently, a cysteine residue changes the three-dimensional protein conformation drastically owing to disulfide bond formation

with other cysteines.<sup>14</sup> Thus, the amino-acid substitution to cysteine in our patient is likely a pathogenic mutation, causing PMD. Previous genotype-phenotype correlation study showed that the phenotype of patients with *PLP1* missense mutations was more severe than those with *PLP1* duplications.<sup>1-3</sup> Indeed, Patient 3 showed severely delayed psychomotor development complicated by respiratory and feeding difficulties. His condition can be classified as form 0 according to the classification proposed by Cailloux *et al.*,<sup>13</sup> as form 0 is the most severe form of PMD. Dysmyelination in this patient was particularly severe.

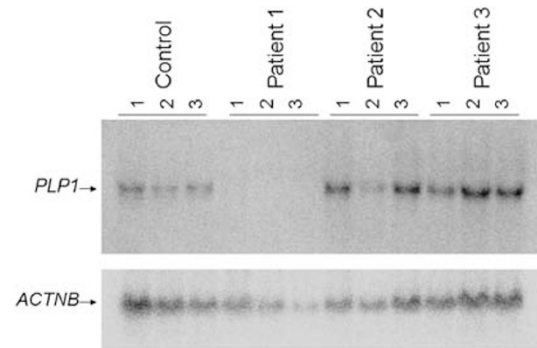
Patient 2 had a 0.6-Mb duplication including *PLP1*. This size is typical for PMD patients with *PLP1* duplications.<sup>8,15,16</sup> This patient is now 43 years old and does not show any deterioration of neurological abilities. Despite being bedridden, he can verbally communicate with several words. His clinical condition can be classified as form 2, because his maximum motor ability was sitting. His dysmyelination is milder than that of Patient 3.

The most intriguing result in this study is the partial duplication of *PLP1* identified in Patient 1. Although there have been reports



**Figure 4** *PLP1* expression analysis by the northern blotting for various samples. (Upper) Predominant expression of *PLP1* is shown in the brain samples. Although control iPS cells show weak expression, there are no expressions of *PLP1* in the other samples. (Bottom) Agarose gel staining for the total RNAs before subsequent northern blotting indicates the same amounts of total RNAs loaded in each lane. SNLm, SNL-feeder cells treated with mitomycin; HDF, human dermal fibroblasts

of partial deletions in *PLP1* identified by multiplex ligation-dependent probe amplification analysis,<sup>9</sup> this is the first report of a partial duplication of *PLP1*. The duplicated segment included the promoter region and the first three exons. Therefore, we hypothesized that a very short mRNA or long fusion mRNA might be expressed by this duplication together with the normal mRNA. To confirm this hypothesis, we analyzed *PLP1* expression by using northern blot analysis, the only way to detect the length and the quantity of mRNAs. As expression levels of *PLP1* in skin fibroblasts were too low to be examined by northern blot analysis, we generated iPS cells from the patients. Contrary to expectation, northern blot analysis showed no *PLP1* bands in the iPS cell generated from the fibroblasts of Patient 1. Although there may be a limitation to detect short-unstable mRNAs in our method, this possibly indicated that the expression of *PLP1* mRNA was disturbed by the *PLP1* partial duplication identified in Patient 1. Regarding the clinical severities of the patients, Patient 1 showed milder phenotype than Patient 2, and his condition can be classified as form 3. Previous genotype-phenotype correlation study have shown that patients with *PLP1* missense mutations show severe manifestation associated with severe hypomyelination, which is recognized as the consequence of accumulated mutant protein in the endoplasmic reticulum as a gain-of-toxic function of the mutant protein.<sup>2</sup> Excessive *PLP1* protein resulting from genomic duplications may accumulate in late endosomes/lysosomes, promoting its incorporation into other myelin components.<sup>1</sup> In contrast, patients with *PLP1* null mutations escape severe impairments because of the absence of any gain-of-toxic function.<sup>2,13,17</sup> Indeed, knockout mice with a functionally null *Plp1* gene do not develop classical signs of *Plp1*-related disease; their



**Figure 5** *PLP1* expression analyses using northern blotting. Three iPS cells of Patient 1 show no *PLP1* band, whereas the other iPS cells from normal control, Patient 2 and Patient 3 show expressions of *PLP1*. *ACTNB* (actin beta) is used for internal control.

oligodendrocytes develop normally and synthesize compact myelin sheaths.<sup>3,18</sup> However, the mice show ultrastructural abnormalities, including swelling of the small-diameter axons and late-onset axonal degeneration.<sup>3,19</sup> Consequently, a loss-of-function mutation of *PLP1* does not induce oligodendrocyte cell death, possibly serving as a mechanism underlying the milder phenotypic consequences observed in patients with null *PLP1* mutations. Although length-dependent axonal degeneration has been described in *PLP1* null mutations,<sup>20</sup> there is no information about peripheral neuropathy in Patient 1. Thus, it was unclear whether the clinical condition of Patient 1 is compatible with that of *PLP1* null mutations. However, the lack of *PLP1* expression in iPS cells derived from Patient 1 clearly demonstrated that the underlying mechanism of PMD in Patient 1, with a partial *PLP1* duplication, is different from the other two patients in this study.

Immortalized lymphocytes and skin fibroblasts derived from patients are often used for expression studies or biological analyses, as these cells are easy to be obtained and handled. However, many tissue-specific genes are not sufficiently expressed by these cells; *PLP1* being one of them. Although there are reports examining *PLP1* expression by RT-PCR, using mRNA extracted from skin fibroblasts,<sup>4,5,21</sup> the expression of *PLP1* mRNA in skin fibroblasts is too low to be examined by northern blotting as shown here. In this study, our microarray database search showed over 40 times higher *PLP1* expression in iPS cells than that in skin fibroblasts. Our initial northern blot analysis confirmed faint but detectable *PLP1* expression in iPS cells, whereas no expression was observed in skin fibroblasts, lymphocytes or SNL feeder cells. This study also confirmed a lack of *PLP1* expression in SNL feeder cells. Therefore, this study demonstrates that iPS cells express endogenous *PLP1*, and that the possibility of contamination from SNL feeder cells or original skin fibroblasts can be excluded. Although being detectable by northern blotting, *PLP1* expression in iPS cells appeared to be much lower than that in mature oligodendrocytes and may be simply cryptic rather than functional. If so, this allows us to evaluate the native transcriptional level of each mutant (and wild-type) *PLP1* allele, which is the primary focus of our study. Meanwhile, terminal differentiation of iPS cells into the oligodendrocyte lineage would result in an enhanced *PLP1* expression with functional consequence. However, this requires technological breakthrough in the induction of terminal differentiation into oligodendrocyte lineage, which is currently unavailable.

In conclusion, we identified the first PMD patient having a partial *PLP1* duplication. The absence of *PLP1* expression in iPS cells, generated from the patient's skin fibroblasts, proved the underlying effects of the partial *PLP1* duplication for the PMD development.

### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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WEB SITE: NCBI GEO; <http://www.ncbi.nlm.nih.gov/guide/genes-expression/OMIM>; <http://omim.org/>

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