資料１

平成２４年度
厚生労働科研費補助金（難治性疾患克服研究事業）
「先天性大脳白質形成不全症の診断と治療目指した研究」

第１回班会議

平成２５年２月１０日（日）
１５－１７時
東京女子医科大学
心臓血圧研究所　会議室
〒１６２－００５４
東京都新宿区河田町８－１

研究代表者：　井上　健
研究分担者：　小坂　仁
黒澤健司
高梨潤一（欠席）
出口貴美子
山本俊至
研究協力者：　中村祥子

【演題名】先天性大脳白質形成不全症研究班の推進について

【演者】氏名：井上　健
所属：国立精神・神経医療研究センター　神経研究所

【目的】先天性大脳白質形成不全症の研究班は、昨年度で3年間の研究を終了した。本年度初めの継続申請が採択されなかったことから、研究の推進は大幅にペーズダウンせざるを得ない状況に陥ったが、幸い追加募集に採択され、平成24年10月より、正式に第2期研究班が発足した。本発表では、研究班で重点的に実施する研究課題について、臨床研究、基礎研究それぞれについての概要をまとめ、今後の方針について検討する。
【方法】本研究班では、MRI データベースの構築と画像臨床研究の推進、新規疾患原因遺伝子の同定を含む遺伝子解析研究、治療候補薬や食事療法を用いた治療研究の推進、患者登録制度の確立と登録の推進、市民公開講座の継続実施と患者を取巻くネットワークの確立をおこなう。基礎研究面では、幹細胞移植を用いた治療法開発の基礎研究、既存薬ライブラリーを用いた治療薬候補の検索などを実施する。また、本疾患群の代表的疾患である Pelizaeus-Merzbacher 病（PMD）の細胞分子病態の解明を行う。
【結果】MRI データベースの構築に関しては、37 症例について MRI 画像および臨床データを登録することができた。遺伝子解析については、PLP1 をはじめとする既知の遺伝子に関する解析は神奈川県立こども医療センターを中心に継続して実施した。また、少数例の診断未確定例について、エクソーム解析を開始し、新規疾患遺伝子を探索している。基礎研究で見出された治療薬候補の臨床応用研究については、クルクミンを提供する会社との臨床治験の実施を計画中である。食事療法などと合わせ、今後臨床表現型の変化の評価スケールの作成が必要であるので、これを行う。患者登録制度は、来年度の実施に向け、システムを他疾患で実施されているものを参考に今後構築していく。第4回市民公開講座を実施し、27 家族の参加を得た。来年度も実施予定である。基礎研究面では、モデルマウスを用いた幹細胞移植に関する基盤技術の確立した。既存薬ライブラリーを用いた新規治療薬候補の検索は、ライブラリーの調達に成功し、来年度より開始する予定である。PMD の細胞分子病態として、PLP1 点変異が小胞体ストレスを誘導する際に、小胞体シャペロンの細胞内動態に変化を来すこと、およびゴルジ体の形態変化を来すことなどを見出した。
【結論】臨床研究、および基礎研究の2つを軸に本疾患群に関する研究を推進した。今後は特に、新規の疾患遺伝子の同定や治療法の開発などのインパクトの大きな成果を目指しつつ、地道な試料の収集や共同研究のための環境整備を進めていきたい。

【演題名】先天性大脳白質形成不全症の診断と治療
【演者】小坂仁[1]、新保裕子[1]、永井淳一[2]、黒澤健司[3]

所属：地方独立行政法人神奈川県立病院機構神奈川県立こども医療センター
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【抄録】
1, 研究方法；2009 年 4 月から 2012 年 12 月に当院に PLP1 解析依頼のあった症例のうち、臨床所見より、PMD が除外できる患者および女子例をのぞいた 33 例を対象とした。PMD の診断は、参考文献により、診断基準を満たさない患者は、今回の症例からは除外している。
2, 研究結果；33 例の内、25 例で PLP1 異常を認めた。量的異常としては、重複が 9 例、3 倍が 1 例、エクソン 2-7 の欠失が 1 例であった。点突然変異は 13 例で認め、ATG ＞ AGG (initiation codon)(exon1), Phe32Val(exon2), Ala39Val(exon2), Ileu176Asn (exon4), Asp203His(exon4), Arg205Lys(exon4), Ala214Asp(exon5), Gly217Asp(exon5) Ala247Val(exon6), Ala248Glu(exon6) IVS2;-1G＞A (intron1), IVS4;-2A＞C (intron3) はそれぞれ 1 例に、Phe240Leu (exon6) は血縁関係のない 2 例に認めた。
3, 考察；PMD 患者における PLP1 異常の占める割合は、報告例では 30%～70%と非常に幅が広いが、臨床所見と画像所見を加味した診断基準を適応した今回の解析では 24/33 例(72%)と高率に変異を見出すことが可能であった。診断基準を適応することにより、大多数の患者においては PLP1 異常を見出すことが可能であると考えられる。

【演題名】転座切断点重複欠失を伴う t(X;6)転座による Pelizaeus-Merzbacher 病女児例の切断点解析

黒澤健司[1]　田中学[2]　井田一美[1]　成戸卓也[1]　永井淳一[3]　小坂仁[4]
1. 神奈川県立こども医療センター遺伝科
2. 埼玉県立小児医療センター神経科
3. 神奈川県立こども医療センター検査科
4. 神奈川県立こども医療センター神経内科

X 連鎖劣性遺伝形式をとる Pelizaeus-Merzbacher 病では、まれに女児例の報告がある(Fonseca et al., 2013; Carrozzo et al., 1997; Yiu et al., 2009; Ida et al., 2003)。多くの場合、構造異常と不活化パターンの偏りが原因となっている。今回我々は、46,X,t(X;6)(q22;p21)と一見均衡型を呈しながら、その転座切断点に欠失（6p21.2）、重複（Xq22.1-q22.2）を認め、Xq22 の量的効果により Pelizaeus-Merzbacher 病を発症した女児の切断点解析を試みたのでまとめた。【対象と方法】症例の臨床経過は既に報告されている（Tanaka et al., 2008)。切断点解析を目的にアレイ解析（CGH SurePrint G3 1M アレイ）を行い、6p21 に 339kb の欠失、Xq22 に 862kb の重複を確認した。アレイのオリゴプローブの位置に加え、STS を手掛かりとして、複数の PCR プライマーの組み合わせを検討し、切断点を詰めた。【結果と考察】der(6)t(X;6)(q22.1;p21.2)の転座切断点はPCR産物を得ることができ、明らかにすることができた(chrX:102,496,490, chr6:39,391,450)。しかし、der(X)t(X;6)(q22.2;p21.2)の切断点は、予想される切断点をはさむように設計し、方向性も考慮し、可能性のある複数のプライマーを組み合わせても PCR 産物を得ることができなかった。この事実は、これまで報告されてきた重複断片の Xqter（distal side）の複雑なゲノム構造を支持するものでもある。同様症例との発症機序を比較し、まとめた。

【演題名】第４回先天性大脳白質形成不全症市民公開セミナーの開催

【演者】氏名：出口貴美子[1]、井上　健[2]
所属：１　出口小児科医院、慶應義塾大学　解剖学
２　国立精神・神経医療研究センター　神経研究所

【抄録】先天性大脳白質形成不全症市民公開セミナーは、平成２１年７月に第１回会合を神奈川県立こども医療センターで開催して以来、本年まで毎年１回、継続して開催され、本年で第４回会合を迎えることができた。日程は、例年通り７月の「海の日」週末であった。今年は、場所を台場の日本科学未来館の隣にある産業科学総合研究所に移してセミナーを行った。今年も、昨年同様に多数の参加者に恵まれた。昨年度までは、厚労科研費難治性疾患克服研究事業（奨励研究）の一環として、研究班の事業としてこの公開セミナーを行ってきたが、本年度は開催時が本補助金追加採択前であったため、公的な研究費によるサポートがほとんどない状況で実施せざるを得なかった。しかしながら、ボランティア参加でいただいた方々、運営にも関与していただいた親の会の皆さん、そして班員とその施設のスタッフのおかげで、これまでにも増して、充実したセミナーを盛会のうちに終了することができた。このセミナーは、疾患に関する情報提供の場、専門家と直接相談が出来る場としての役割のみならず、疾患が稀少であるために、普段顔をあわせて話をすることが出来ない家族同士のコミュニケーションを育てる有意義な機会となっている。また、本疾患の家族会は、このセミナーを基盤に立ち上がっており、昨年度からはその年次総会の場としても機能している。Ｈ２５年度も、継続してこのセミナーを開催する予定である。

【演題名】先天性大脳白質形成不全症のゲノム診断

【演者】氏名：○山本俊至

所属：東京女子医科大学統合医科学研究所

研究目的：先天性大脳白質形成不全症においては、主な責任遺伝子である *PLP1* 以外にも関連遺伝子が複数関わっており、診断を進めていくにあたって戦略的な対応が必要となる。我々はこれまでに診断の進め方に関するフローチャートを作成してきたが、それに則り新規例の解析を行った。
研究方法：臨床的に先天性大脳白質形成不全症を疑わせる臨床症状や頭部 MRI 所見を示す女性例を対象とした。既知の遺伝子を *PLP1*>*GJC2* の順に解析した。最終的には発症メカニズムを明らかにするためにマイクロアレイによる解析を行った。
研究結果：患者においては *PLP1* の重複や塩基置換は認められなかったため、*GJC2* の全翻訳領域を解析したところ、新規ミスセンス変異がホモ接合で認められた。母親はこの変異のヘテロ接合であり保因者と考えられたが、父親はすでに他界しており、解析できなかった。*GJC2* の位置する 1 番染色体領域のヘテロ欠失や loss-of-heterozygosity (LOH)の可能性を疑い、SNP タイピングを搭載した comparative genomic hybridization (CGH)解析を行ったところ、*GJC2* 領域の欠失はないものの、LOH を示していた。さらに 1 番染色体のハプロタイプは全て母親由来であることから、母親由来の片親性ダイソミー(uniparental disomy; UPD)により、LOH を生じたものと考えられた。
結論：今回女児例において、非常に稀な *GJC2* ホモ接合変異を認め、発症機構として UPD が関わっていることを明らかにした。常染色体劣性遺伝性によって発症する遺伝子のホモ接合が認められた場合には、CGH+SNP 解析を行うことが有用であることが示唆された。

資料２

第４回市民公開セミナーのお知らせ

先天性大脳白質形成不全症の克服へ向けて

～リハビリテーションから研究の進歩まで～

第４回市民公開セミナー

先天性大脳白質形成不全症は、ペリツェウス・メルツバッハ病などの稀ながら重度の障害を伴う小児難治性神経疾患です。４回目のセミナーとなる今回は、特別講師として栗原まな先生をお迎えし、こどものリハビリテーションに関するお話を伺います。ほかに本疾患に関する医学研究の歩みに関する話題を取り上げ、患者さんご家族など一般の方々を対象に、わかりやすく解説をします。

日時；平成２４年７月１５日（日）　１３時～１６時
場所；産業技術総合研究所　臨海副都心センター別館　１１階　会議室
（日本科学未来館のとなりです）

【第一部　教育講演】

１．先天性大脳白質形成不全症の臨床研究

神奈川県立こども医療センター　神経内科　小坂　仁

２．先天性大脳白質形成不全症の治療法開発の歩み

国立精神・神経医療研究センター　神経研究所　井上　健

３．災害時への備え：宮城県で震災から学んだこと

国立精神・神経医療研究センター　神経研究所　沼田有里佳

４．【特別講演】こどものリハビリテーションの進歩

神奈川県総合リハビリテーションセンター　小児科　栗原まな

【第二部　家族会連絡懇親会】

お茶とお菓子を用意します。気軽な相談や、ご家族同士のご歓談に！

主催　先天性大脳白質形成不全症リサーチ・ネットワーク
代表研究者　井上　健

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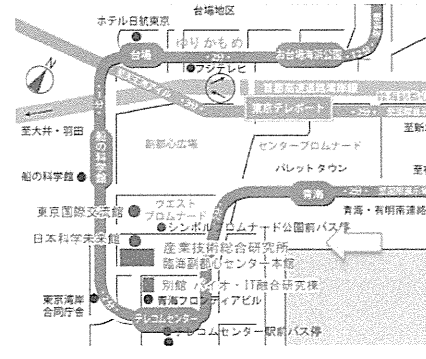
アクセスの詳細は産業技術総合研究所ホームページ
（http://unit.aist.go.jp/waterfront/）をご参照ください。



研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
井上　健、小坂　仁	先天性大脳白質形成不全症	遠藤文夫	新領域別症候群シリーズNo.20「先天代謝異常症候群（下）—病因・病態研究，診断・治療の進歩—」	日本臨牀社	大阪	2012	p897-901.
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雑誌

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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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Keywords: Pelizaeus–Merzbacher disease (PMD); proteolipid protein 1 gene (*PLP1*); induced pluripotent stem (iPS) cells; partial duplication; dysmyelination

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.[1–3] 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*.[1–3]

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.[4,5] 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.[6] In 2007, Takahashi *et al.* established iPS cells from human skin fibroblasts.[7] 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.[8] 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.[8] 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.[9] 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.[8] 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.[8] 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.[8]

PCR and direct sequencing of all seven exons of *PLP1* was performed by standard methods using the primers reported by Hobson *et al.*[4] 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^{-1} recombinant basic fibroblast growth factor (# RCHEMD001; Repro CELL, Yokohama, Japan) and passaged as described previously.[7,10]

Generation of iPS cells

Disease-specific iPS cells were generated from patients' skin fibroblasts as previously described.[7] 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^{-1} recombinant basic fibroblast growth factor (# RCHEMD001; 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,[10] 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.[10] 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.[10] 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),[7] 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, Clonetech, 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 *Sac*I and *Sac*II 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 [α-^{32}P] dCTP (PerkinElmer, Waltham, MA, USA) and used as probes for northern blotting.

Hybridization was performed as described previously.[11] Briefly, 30 μg 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, iPS 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, Clonetech). Subsequent analysis included the 12 iPS 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).[9] Patient 2 had a duplication of all 7 exons of *PLP1*, and subsequent aCGH analysis

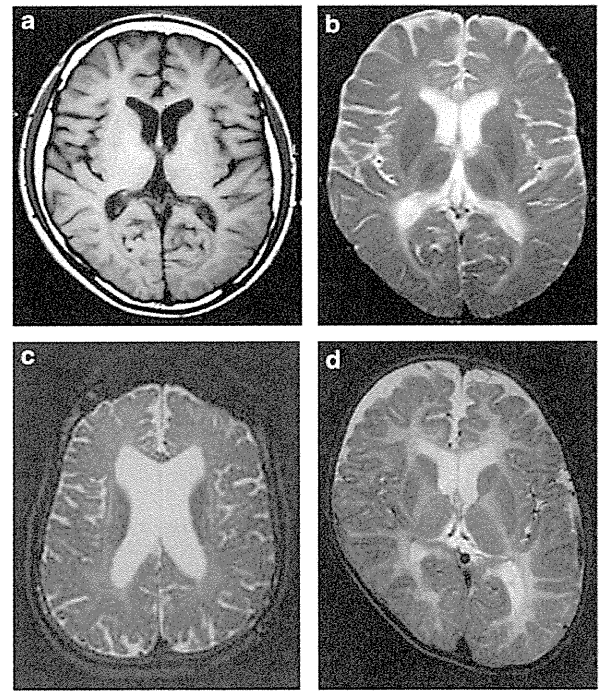

Figure 1 Brain MRI findings of the patients T1- (**a**) and T2 (**b**) weighted images of Patient 1 show mild and diffuse volume loss of the brain and high-intensity signals of the deep white matter in T2 indicating mild dysmyelination. T2-weighted image of Patient 2 (**c**) shows diffuse volume loss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

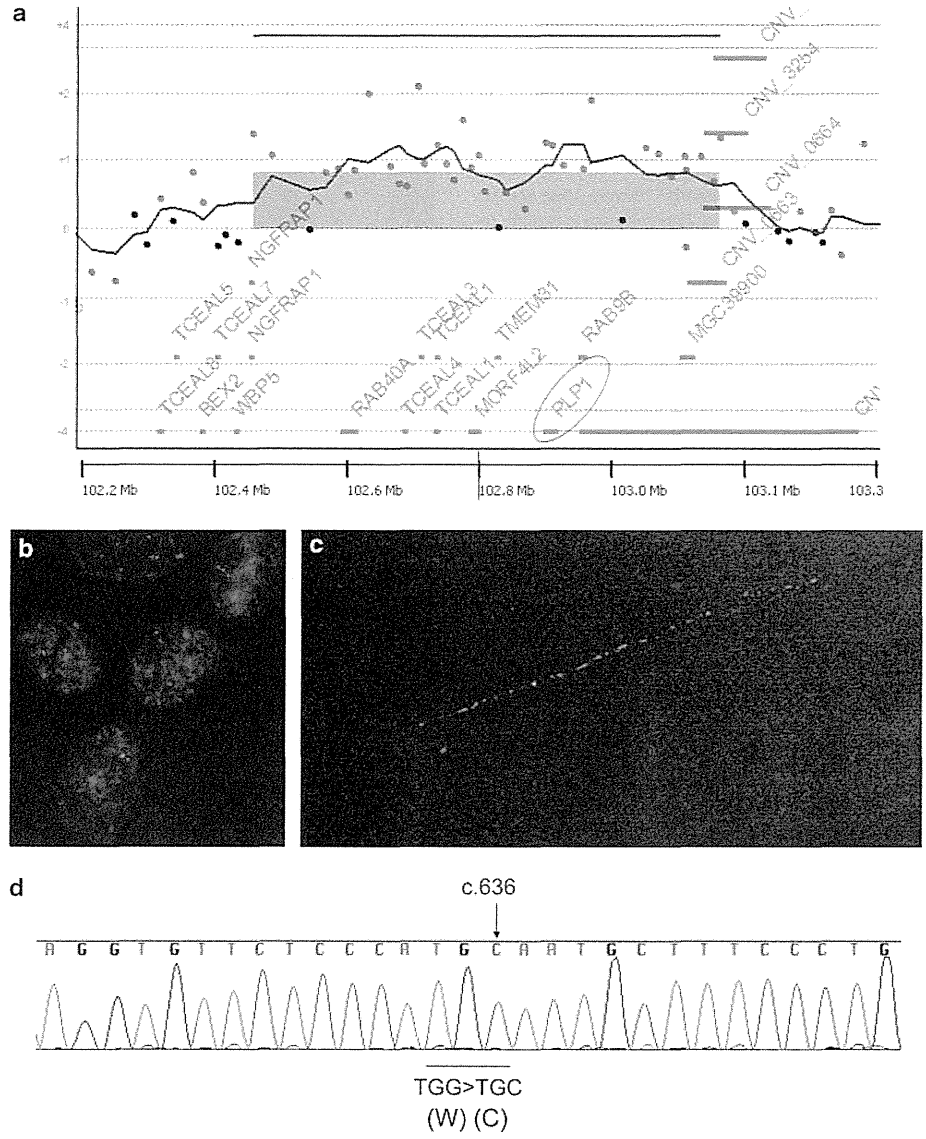

Figure 2 Genotyping and cytogenetic analyses for Patients 2 and 3. (**a**) A microchromosomal duplication including *PLP1* is shown in GeneView of Agilent Genomic Workbench (Agilent Technologies). The location of *PLP1* is highlighted by a red circle. (**b**) Interphase fluorescence *in-situ* hybridization 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