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
高梨潤一	小児神経疾患	日本磁気共鳴医学会 Proton MRS の臨床有用性検討会編	Proton MRS の臨床有用性コンセンサスガイド 2009 年度版	日本磁気共鳴医学会	東京	2009	32-42
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雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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研究成果の刊行物・別刷（抜粋）

Pelizaeus-Merzbacher 病
—Double,Double...and Trouble—

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要旨

Pelizaeus-Merzbacher 病（PMD）は中枢神経系の髄鞘形成不全を特徴とするまれなX連鎖性疾患である．原因遺伝子 *PLP1* の変異でもっとも頻度が高いのは，遺伝子全体を含むゲノム領域の重複である．本稿では，ゲノム重複の発見とその診断法の開発について解説する．*PLP1* の変異には，重複以外に点変異や欠失などがある．変異によって異なる分子病態を介してさまざまな重症度の表現型を引き起こすので，PMD の遺伝子診断は予後の推定や将来の治療法の選択に重要である．

Key words：ゲノム病，遺伝子重複，ミエリン，遺伝子診断，分子病態

はじめに

表記の副題は，われわれが Pelizaeus-Merzbacher 病（ペリツェウス・メルツバッハ病：PMD）の原因遺伝子変異として，それまで知られていた点変異とは別に，ゲノム重複（genomic duplication）が疾患の主要な原因であることを 1996 年に American Journal of Human Genetics に報告した時に[1]，その号の巻頭に当時 PMD 臨床研究の第一人者であった故 Hodes 博士が記した Invited Editorial のタイトルである[2]．PMD の原因遺伝子である proteolipid protein 1 遺伝子（*PLP1*）のコピー数の変化が PMD の原因となりうることは，X 染色体の可視的な（巨大な）部分重複をもつ患児が PMD 様の症状を呈することから，あるいはサザンブロット法で *PLP1* 遺伝子シグナルの増強を示す患者がいることから，ある程度予期されていた[3][4]．しかしながら，遺伝子の質的変化ではなく，重複や欠失（deletion）といったゲノム構造変化（structural change）による量的変化がヒトの遺伝性疾患の原因になるという genomic disorder（ゲノム病）という概念そのものがまだ一般的ではなかった[5]．

われわれのこの仕事は，蛋白コード領域の変異がない多くの典型的な PMD 患者において，*PLP1* 遺伝子ゲノム領域が重複することが，PMD の主要な遺伝的病因であることを示すことができたばかりでなく，ゲノム病の疾患概念の確立にわずかながら資することができたのではないか，と考えている．本稿では，その発見からその後の研究の展開を追いながら，PMD の遺伝子診断と分子病態について解説する．

Ⅰ Pelizaeus-Merzbacher 病の *PLP1* 遺伝子重複の発見

PMD は，中枢神経系の髄鞘形成不全（dysmyelination）を病理学的な本態とするX連鎖遺伝性疾患である[6]．すなわち，1 次的な髄鞘化の行程そのものを完結できないことが病態であり，他の多くの白質変性症のように，一度完成した髄鞘があとから破壊される（demyelination）のとは異なる．臨床的には，生後の発達発育の遅れに加え，多くは 1 カ月以内に気づかれる眼振が特徴的である．生後半年ぐらいまで

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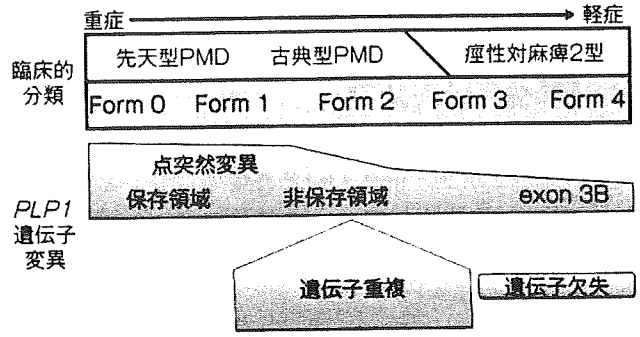

図1 PMDとSPG2の臨床分類と*PLP1*遺伝子変異の関連

上段は従来の分類とBoespflug-Tanguyらによる分類．左から右に行くに従い，臨床的には軽症となる．下段は対応する*PLP1*変異を示す．点変異は重症な先天型での頻度が高いが，古典型や痙性対麻痺でもみられる．重複はもっとも頻度が高く，古典型に多い．欠失は非常にまれで，ニューロパチーを合併する点が特徴的である．

の早い時期は筋緊張の低下，後には小脳失調やアテトーゼ様の異常肢位を伴う痙性四肢麻痺が主徴になる．最重症型の先天型（connatal form），もっとも頻度の高い古典型（classic form），そしてより軽症な痙性対麻痺2型と分類されるが，実際には重症度に応じた連続的なパラダイムを形成するので，より詳細な臨床的徴候に基づいて5段階に分類したBoespflug-Tanguyらの分類[7]が有用である（図1）．

PMDが中枢神経系の主要な髄鞘蛋白をコードする*PLP1*遺伝子の変異で起こることは，1989年に先のHodesら他により報告された[8][9]．これは，遺伝性神経疾患の原因遺伝子の同定としては，比較的早い部類に入る．同時期MRIの導入により，剖検脳の病理学的診断を待つことなく，画像検査で診断を行えるようになったことは，臨床現場では画期的な進歩であった．PMDのMRI所見は非常に特徴的で，T2強調画像で皮質下の白質に至るまで，大脳白質全体にびまん性の高信号を呈する（図2-a）．画像診断の進歩に伴い，生化学的な確定診断の必要性も高まり，PMD症例の*PLP1*遺伝子解析が行われた．しかし，期待とは裏腹に，*PLP1*遺伝子のコード領域に変異が見つかるのはわずかに2〜3割の症例のみで，臨床的には典型的と思われた症例に変異がない，つまり臨床的に一疾患単位として考えられていたPMDに変異のある症例群とない症例群が存在するという，非常に不可思議な状況が生じたのである．

冒頭で述べた通り，その当時いわゆるゲノム病の疾患概念はまだ存在しなかった．ゲノム病とは，疾患遺伝子を含む比較的大きなゲノム領域の欠失や重複，あるいは反転（inversion）といったゲノム構造の変化が原因で起こる遺伝性疾患を指し，現在PMDはゲノム病の代表的な疾患の一つとして数えられている．*PLP1*遺伝子のゲノム変異が主要なPMDの遺伝子異常として「再発見」される前に，いくつかそのプロローグとなるような知見が存在した．*PLP1*遺伝子を含む大きな染色体領域の重複をもつ症例で，PMD様の痙性麻痺や発達遅滞がみられたこと[3]，正常*PLP1*遺伝子を過剰発現するトランスジェニックマウスが髄鞘形成不全を呈すること[10][11]，そして染色体異常を伴わないPMD2家系のサザンブロット法において*PLP1*遺伝子のシグナルが増強しており，遺伝子重複が疑

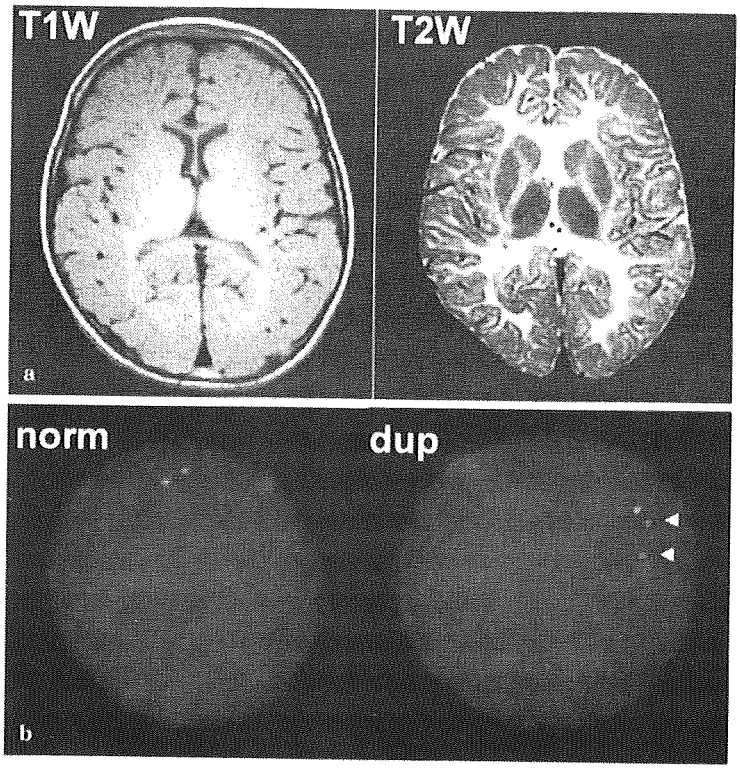

図2　特徴的なPMDのMRI画像とFISH法による*PLP1*重複の同定

a：典型的なPMD患者のMRI所見．T1強調画像（左）では明らかな異常を認めないが，T2強調画像（右）では基底核から内包，脳室周囲，皮質下の白質に至るまでびまん性に著明な高信号を認め，髄鞘形成不全を示す．

b：間期FISH法を用いた*PLP1*重複の遺伝子診断．正常（左）では1つの*PLP1*シグナル（赤）と同じX染色体上の対照シグナル（*BTK*；緑）1つのみ観察されるのに対し，PMD患者（右）では*PLP1*シグナル（赤）が2つ観察され（矢頭），*PLP1*遺伝子を含むゲノム領域が重複していることを示す．

われたことである[9]．

これらの知見をもとに，われわれは*PLP1*遺伝子コード領域に点変異がないPMD症例について，PCRによる*PLP1*遺伝子量の検定のための半定量解析法（現在の定量的PCRに比べるとはるかに原始的ではあるが，原理は同じである）を開発し，これを用いて*PLP1*の重複の有無を検証した．驚いたことに，点変異のないPMD5家系のうち4家系で*PLP1*遺伝子量が2倍に増加していたのである[11]．正常な遺伝子のコピー数が1つ増えることが，PMDの主要な遺伝子変異であることがこのとき明らかになったのである．

II　*PLP1*遺伝子重複の検出法

当時のPCR半定量解析法は，そのまま診断に用いることができるほど，簡便かつ信頼度の高いものではなかった．遺伝子重複の診断を積極的に行うためには，より信頼度の高い方法を

開発する必要があった．そこでわれわれは，患者のリンパ球の核を顕微鏡下に観察し，蛍光でラベルした*PLP1*遺伝子のコピー数を直接数える間期FISH（interphase fluorescence *in situ* hybridization）法を用いた[12]．FISH法を用いると，患者男児や保因者の女性は，通常1つしかない（女性は2つ）*PLP1*遺伝子のシグナルが2つ（保因者では3つ）観察され，*PLP1*遺伝子が重複していることを明瞭に観察することができる（図2-b）．筆者が米国でこの診断法を確立し，数カ月後には臨床細胞遺伝検査室での細胞遺伝学的検査として一般臨床に応用された．研究レベルでの成果を臨床に応用する迅速さは驚くべきものであった．FISH法を用いた多数症例の遺伝子診断により，PMD症例の半分以上はこの*PLP1*遺伝子の重複が原因であることが明らかになった[12]．

FISH法は遺伝子診断のみならず，*PLP1*遺伝子の重複の大きさや向きについてのゲノム解析に大きな力を発揮した．驚いたことに，*PLP1*の重複の大きさは症例により多様で，しかしながら重複の向きはすべての症例で直列繰り返し（タンデム配列）であることがわかった[12]．後述するが，これはゲノム病一般に知られているメカニズムからすると特異であり，どのようにしてこのゲノム重複変異が起こるのかは，いまだに不明である．

重複の領域の大きさに幅があるということは，もしある患者のゲノム重複の大きさがFISH法の検出限界（約50 Kb）よりも小さい場合は，見逃してしまう可能性があることを示唆する．実際に，そういった症例がまれながら存在することが明らかになるにつれ，他の解析方法が必要になってきた．そこで導入されたのがリアルタイムPCRである．これは，増幅されたDNA量を経時的にモニターすることにより，元の鋳型DNA量を正確に定量することができるシステムである．RT-PCRを用いることにより，少量のDNAのみで*PLP1*重複の診断ができるようになった．現在，本邦でPMDの遺伝子診断を診療の一部として行っている神奈川県立こども医療センターではこの方法を採用している（図3）．

さらに近年は，DNAチップを用いたアレイCGH（array comparative genomic hybridization）を用いて，PMDを含むゲノム病すべてに関与するゲノム量の変化を網羅的に検索できる方法も開発され，米国を中心に臨床応用されている．この方法を用いることにより，複数のゲノム病の検索が一度にできるのみならず，重複（あるいは欠失）がゲノム上のどの範囲にわたって起こっているのかなどの詳細なゲノム情報を，たった1回の解析で得ることができる[13]．

III *PLP1*遺伝子変異と臨床表現型との関連—PMDと遺伝性痙性対麻痺

*PLP1*遺伝子変異の種類と臨床表現型との間に，一定の関連性があることが明らかになっている（図1）[7]．まったく髄鞘化が起こらず，多くが10歳代で死亡する最重症型の先天型の症例は，*PLP1*コード領域のうち，進化的に保持されているアミノ酸（つまり変異が起こると種として維持できなくなる部位で，機能的に重要であることが示唆される）のミスセンス変異による症例が多い．もっとも頻度が高く，臨床的にもある程度の発達を獲得する古典型では遺伝子重複の頻度がもっとも高く，遺伝子診断にはまず重複の検索を行うべきである．それ以外の症例では，アミノ酸置換型の点変異が見出される．さらに，臨床的には痙性対麻痺に分類されるような症例も，*PLP1*変異が原因となることが明らかになった．

遺伝性痙性対麻痺は，常染色体優性，劣性，X連鎖性とさまざまな遺伝形式をとり，多くの疾患原因遺伝子が存在する遺伝的多様性を有する疾患群である．このうち，X連鎖性痙性対麻痺2型（SPG2）に白質病変を合併するものが存

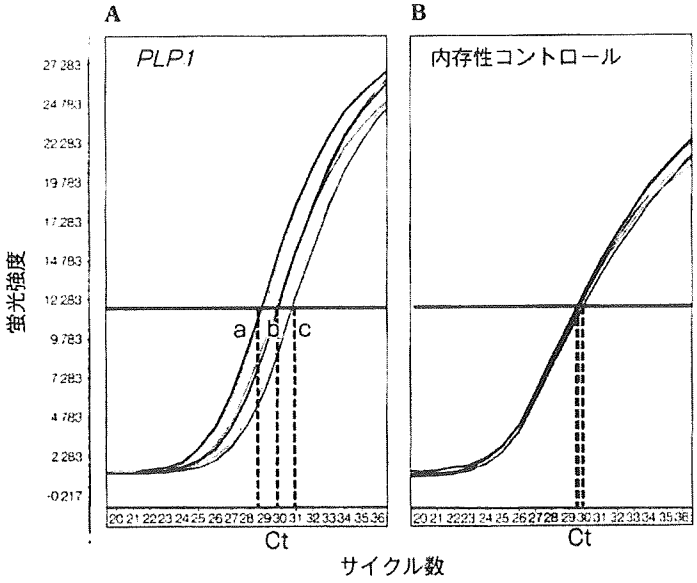

図3　RT-PCR（ΔΔCt 法）を用いた *PLP1* 重複の遺伝子診断の実例

ある一定の蛍光レベルに達したときのサイクル数（Ct）を内因性コントロール遺伝子の Ct で正規化（ΔCt）した後に検体間で比較する（ΔΔCt）ことにより，遺伝子のコピー数を検定する方法．

A：*PLP1* 遺伝子エクソン 7 を用いたリアルタイム PCR の検量線．a：*PLP1* 遺伝子重複の保因者女性，b：*PLP1* 遺伝子重複をもつ患者男性，c：正常男性を示す．

B：内因性コントロールに対するリアルタイム PCR の検量線．一定の蛍光強度（横線）に対応するサイクル数（Ct）を示す．ΔΔCt 法により *PLP1* 遺伝子のコピー数は，a：b：c＝3：2：1 と判定された．ここでは DNA 濃度をそろえているため，内因性コントロールに対する Ct にはほとんど差がみられない．n＝3．（提供：神奈川県立こども医療センター永井惇一専門技師）

症することが知られていたが，これらの家系に *PLP1* 遺伝子の変異が見出されたことから，PMD と SPG2 は異なる疾患ながら，同じ遺伝子の変異が原因で起こること，すなわち対立遺伝子疾患（allelic disorders）であることがわかった[14][15]．

SPG2 型の臨床経過をとる症例にみられる変異には，次の 3 種類が存在する．まず，2 つある *PLP1* の選択的スプライス産物（蛋白レベルでは，長い PLP1 とエクソン 3 の後半 35 残基が欠ける短い DM20）のうち，PLP1 に特異的な 35 残基の部分（エクソン 3B とよばれる部分）にアミノ酸置換が起こり，その結果，PLP1 蛋白のみが変異体となるが，DM20 には変化をきたさないもの，次に，進化的に保持されていない部分（すなわち，機能的に必須でないと予想される場所）にアミノ酸置換をきたすもの，そして，*PLP1* ゲノム領域の欠失や翻訳開始領域近くのナンセンスコドン変異など，PLP1 蛋白産生そのものが欠落するものである[16][17]．

興味深いのは，3 つ目の欠失例で，PLP1 蛋白が存在しなくとも臨床表現型は比較的軽症であること，すなわち生体にとって変異体 PLP1 蛋白や過剰量の野生型蛋白の存在は，PLP1 が存

在しないことよりもずっと有害であることを示唆する．また，PLP1は微量ながら末梢神経の髄鞘にも発現されているが，欠失の症例のみで軽度ニューロパチーを呈することが知られている．臨床的にはより重症な重複あるいは点変異の症例では，末梢神経症状は電気生理学的な検索をしても検出されない．この分子病理メカニズムは，まだ解明されていない．

IV 今後の研究の展望

今後，PMDを取り巻く研究の進歩は2つの方向性をもっていくと考えられる．まず第1に，ゲノム重複のメカニズムを明らかにすることである．なぜ，ヒトゲノムの特定の領域に重複あるいは欠失が繰り返し起こり，多くの同一の疾患が世界中で引き起こされるのであろうか．この疑問は，多くのゲノム病，例えばCharcot-Marie-Tooth病1A型やWilliams症候群などについては，ほぼ解明されている．詳細は他書に譲るが，一般的なゲノム病の発生メカニズムには，ヒトゲノムの構造的な特徴が大きく関与している．その中でも，疾患責任遺伝子を含むゲノム重複あるいは欠失領域を挟んで存在する巨大（数十から数百Kb）な繰り返し配列（low copy repeat：LCR）の間で非均等相同性組み換え（non-allelic homologous recombination）が起こることが，その分子機序としてほぼコンセンサスになっている[18]．したがってどの患者でも同じ場所で同じ大きさのゲノム変異が起こる．これに対して，PMDはそういった位置関係にあるLCRが存在せず，また重複あるいは欠失ゲノム領域の大きさが患者ごとに異なるというユニークな特徴をもっており，まだそのゲノム変異の機序が確立されていない[13]．しかし，最近この特異的なゲノム構造異常が，組み換えではなく複製のエラーであるという仮説が提唱されており，これが今後検証されると思われる[19]．

第2はPMDの治療法開発に向けた取り組みである．現在PMDに対する有効な根治療法はない．しかし，分子病態の解明や新たな遺伝子工学あるいは幹細胞技術などの進歩により，さまざまな治療の可能性が生まれてきている．薬剤による治療法開発のためには，変異によって異なる分子病態に沿った治療薬を見出す必要がある．点変異によるアミノ酸置換を伴う変異PLP1蛋白は，細胞内小胞体に蓄積して細胞毒性を発揮すると考えられている．であれば小胞体ストレスを軽減するような薬剤に治療効果が期待できる．また，*PLP1*重複は遺伝子産物の過剰が原因であるので，*PLP1*の発現を低下させるような薬剤を見出せば治療に用いることができるかもしれない．現在，こういった仮説に基づき，治療薬の検索が行われている．RNA干渉やアンチセンスオリゴなどを用いた*PLP1*遺伝子発現の抑制も，PMDの分子標的治療として期待される．さらに，iPSをはじめとする幹細胞を用いた細胞移植治療は，今後実用化に向けた技術革新が進むと期待されるが，PMDもその対象疾患として有力な候補である．

おわりに

発見から10余年を経ているにもかかわらず，なぜ*PLP1*遺伝子重複がPMDの原因になるのか，そしてこのもっとも頻度の高い変異をどうしたら克服できるのか，問題は未解決のまま，依然われわれの眼前に残されている．Double, Double…and Trouble, Left Unsolvedである．

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Case report

Mild phenotype in Pelizaeus-Merzbacher disease caused by a *PLP1*-specific mutation

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Abstract

We present the case of a 26 year-old man who developed normally until he began having difficulty walking at age 12. He subsequently became unable to stand at 15 years old and exhibited mental regression and generalized tonic convulsions by age 20. Magnetic resonance imaging revealed incomplete myelination of cerebral white matter, which resembled that of Pelizaeus-Merzbacher disease. By sequencing the proteolipid protein 1 (*PLP1*) gene, we found a novel mutation (c.352_353delAG (p.Gly130fs)) in the latter half of exon 3 (exon 3B) that is spliced out in the DM20 isoform. Exon 3B mutations are known to cause a mild phenotype since they do not disturb DM20 production. Mutations that truncate PLP1 correlate with a mild phenotype by activating the nonsense-mediated decay mechanism that specifically detects and degrades mRNAs containing a premature termination codon. This attenuates the production of toxic mutant PLP1. The very mild presentation in the present case seems to be derived from the unique nature of the mutation, which preserves DM20 production and decreases mutant PLP1.

Keywords: Pelizaeus-Merzbacher disease; Proteolipid protein 1; *PLP1*

1. Introduction

Proteolipid protein (PLP) 1 and its splice isoform DM20 are encoded by the *PLP1* gene. PLP1/DM20 proteins are major components of myelin expressed in oligodendrocytes in central nervous system (CNS) [1]. PLP1/DM20 translated in the endoplasmic reticulum (ER) is transported to the cell surface and integrated into plasma membrane presumably via four membrane-spanning domains with both amino- and carboxy-terminal ends on cytoplasmic side. Owing to its strong hydrophobicity, PLP1/DM20 can form a stable compact myelin sheath in cooperation with other myelin proteins [1]. Expression of PLP1 and DM20 are spatially and temporally regulated. DM20 expresses preferentially in embryonic stages in a variety of cell types, whereas PLP1 expresses postnatally in oligodendrocytes. Both PLP1 and DM20 constitute the predominant protein in myelin [2].

Pelizaeus-Merzbacher disease (PMD) is a severe X-linked recessive disorder caused by mutations of the *PLP1* gene [1]. A deficiency in PLP1/DM20 at the cell

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membrane by PLP1 mutation leads to the arrest of myelination. Moreover, mutant PLP1 elicits a response in the ER, which attempt to refold the misfolded mutant PLP1/DM 20 protein [3]. However, if the level of misfolded protein exceeds the controllable limit within the ER quality control system, apoptotic signals are transduced from ER [3]. This cellular process is reflected in developmental regression and atrophy of the CNS.

Exonic or intron/exon boundary mutations are found in 20–30% of PMD patients and phenotypes are severer than other mutations such as total deletion and duplication of *PLP1*. An exception has been observed in the latter half of exon 3 (exon 3B) of *PLP1* [4–8]. Mutations within this region are predicted not to disturb DM20 expression and function. Mutations that truncate PLP1 are related to a mild phenotype presumably by activating the nonsense-mediated decay (NMD) pathway, a mechanism that specifically detects and degrades mRNAs containing a premature termination codon [9]. This attenuates the production of mutant PLP1 levels and thus likely lessens the ER stress responses. In this report, we present a PMD patient with a very mild phenotype. We identified a novel *PLP1* gene mutation that is predicted to preserve DM20 production and results in a frame-shifted mutant PLP1 protein.

2. Materials and methods

2.1. Patient

This 26 year-old boy was born uneventfully at full term to Japanese parents. He was born with a body weight of 3660 g and an Apgar score of 9/9 at 1 and 5 min. No stridor or nystagmus was noted. He gained head control at 4 months, could sit without support at 8 months, and could walk without assistance at 4 years. He was pointed out spasticity of lower limbs and EEG abnormalities at 1 year. He was treated with carbamazepine for 14 years. No seizures occurred during that period. He could speak a few words at 2 years of age. He attended a special class in normal elementary and junior high school. He had no difficulties in daily conversation and writings. The patient began having difficulty walking at 12 years of age and became unable to stand at 15 years. He showed frequent urination and was diagnosed as neurogenic bladder at 15 years. MRI taken at that time revealed only mild ventricular enlargement. Myelination was not evaluated because of the motion artifact. At age 20, he showed signs of mental regression and began speaking fewer words. He exhibited generalized tonic convulsions and was treated with valproic acid at 24 years. He was subsequently referred to a hospital for evaluation. He was not small for his ages with a height of 167 cm and a weight of 54 kg. He could conversate with combining two words. He showed no nystagmus and exhibited alternating outer-nystagmus and oculomoter apraxia. He could walk with assistance. His muscle tone was hypertonic in the upper limbs. Clumsiness was observed with all extremities displaying exaggerated tendon reflexes and bilateral extensor plantar responses. Speech was slurred and dysmetry with terminal oscillation and dysdiadochokinesis were observed. Routine laboratory examinations revealed no biochemical abnormalities in the level of serum ammonia, lactate and pyruvate, very long chain fatty acids, or arylsulfatase A. Nerve conduction velocities and electromyographic studies were all normal. Measurement of auditory evoked brain responses revealed only wave I. MRI revealed a completion of myelination in the T1 signal. Myelination in the white matter was incomplete in the T2 signal (Fig. 1).

2.2. Genomic DNA sequencing

Genomic DNA from this patient was prepared from white blood cells using the Wizard Genomic DNA puri-

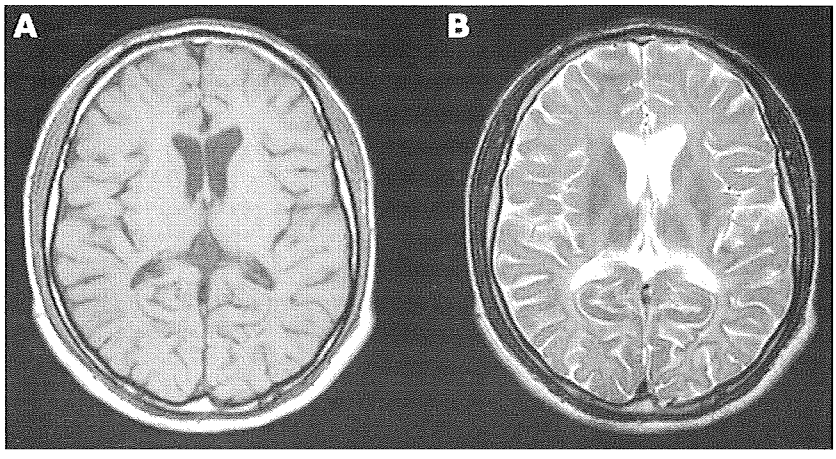

Fig. 1. Magnetic resonance imaging (MRI) at 26 year-old patient shows disappearance of contrast between cortex and white matter (A) on a T1-weighted image. T2-weighted image shows the incompletion of myelination in the white matter (B).

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fication kit (Promega, Madison, WI USA). PCR of seven exons and promoter regions of the *PLP1* gene was performed as previously described [10]. Subsequent sequencing analyses of the PCR fragments were performed by direct sequencing using the Big Dye Terminators v1.1 Cycle Sequencing kit (Applied Biosystems Foster City, CA). Duplication was screened by FISH as described [10].

3. Results

By direct sequencing of the patient's *PLP1* gene exons, exon/intron boundaries and a promoter region, we found a novel mutation in exon 3: c.352_353delAG (p.Gly130fs) (Fig. 2). No other sequence alterations were found and this mutation was not detected in more than 200 alleles. This two nucleotide deletion occurs in the latter half of exon 3 (exon 3B), which is not involved in *DM20* mRNA production (Fig. 3). FISH analysis showed normal copy numbers in this patient.

4. Discussion

Pelizaeus-Merzbacher disease belongs to leukodystrophies, one of a group of disorders that affect the white matter of the CNS. Genetic defects in PLP1/DM20, the most predominant myelin proteins, causes dual pathology: defects in CNS myelin formation (dysmyelination)

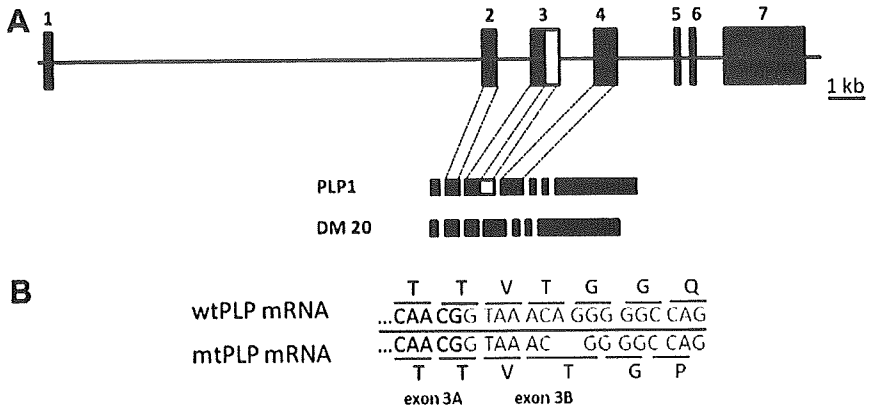

Fig. 2. Splicing the *PLP1* gene into *PLP1* mRNA and *DM20* mRNA. (A) Schematic presentation of *PLP1* gene structure. (upper panel) PLP1 gene is composed of seven exons. (lower panel) mRNA of PLP1/DM20 differs in only the latter half of exon 3 that is spliced out for the production of DM20 mRNA. (B) Two nucleotide deletion and subsequent frame shift in the Patient. Novel mutation in exon 3B, c.352_353delAG (p.Gly130fs), causes the frame shift in *PLP1* mRNA.

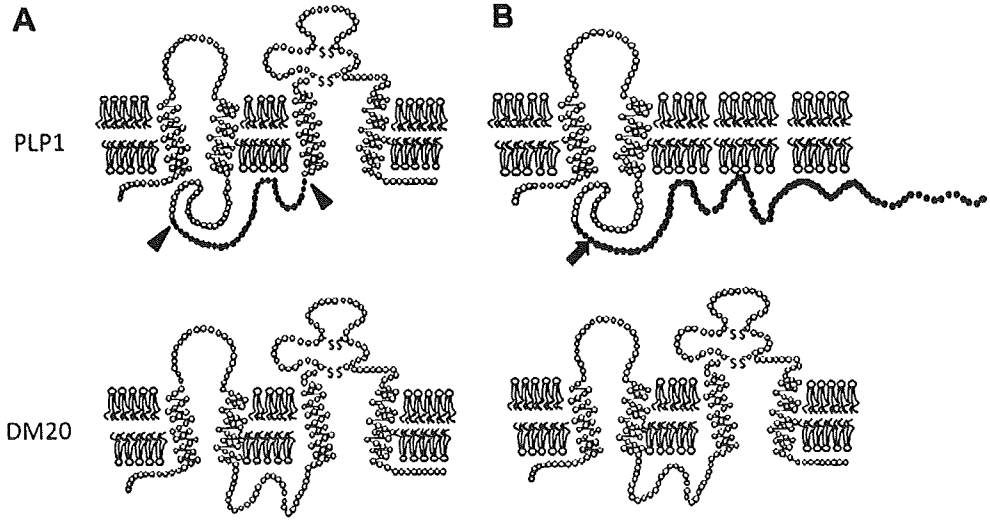

Fig. 3. Deduced PLP1 gene products; PLP1 and DM20. (A) Wild-type PLP1 (upper) and DM20 (lower) which are thought to include 4 membrane-spanning domains. Thirty-five intracellular amino acids (gray circle; between arrow head) are lacking in DM20. One circle corresponds to one amino acid. (B) PLP1 and DM20 of the patient. (upper) A two nucleotides deletion in exon 3B, c.352_353delAG (p.Gly130fs), causes a frame shift (arrow) and extension that are composed of 82 nonsense peptides. (lower) DM20 is identical to wild-type in this patient.

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and oligodendrocytes cell loss via apoptosis. PLP1 and DM20 are required for myelin compaction. Mutations in the *PLP1* gene, such as total deletion and truncation mutations, cause an inability to form normal myelin, which is easily revealed by diffuse high signals in all CNS white matter in T2-weighted MRI scans. Since PLP1/DM20 are constitute more than 50% of the protein in oligodendrocytes, mutant PLP1/DM20 cause the excessive ER stress responses and subsequent cell death that can be visualized by MRI/CT as brain atrophy.

Typically, patients with PMD show neonatal nystagmus and developmental delay that becomes apparent during infancy. Impairments of motor functions involve spastic paresis from the defect in the corticospinal tract, intention tremor from abnormalities in the cerebellar pathway, and choreoathetosis and rigidity due to basal ganglia dysfunction. Although all patients exhibit mental retardation, psycho-intellectual development is greater than motor development. Lesions are restricted in the myelinated portion in the CNS but disease severity varies considerably.

Cailloux et al. graded the clinical severity of PMD patients by their maximal motor achievements. Patients with Form 0 never gain head control ability, whereas patients with Form 1 can achieve head control. Form 2 includes the patients who are able to maintain a sitting position. Form 3 includes patients who can walk with support, while patients with Form 4 can walk autonomously. This last form overlaps the clinical phenotype of X-linked spastic paraplegia type 2, the allelic disease to PMD [8]. The patient described in the present case report belongs to Form 4, the mildest symptom group.

Amino acid substitutions, especially conserved amino acids in DM20/PLP1 within species, usually cause a severe phenotype [6]. Duplication of PLP1 causes a milder form, in which patients gain head control or sitting ability. Two types of mutations cause the mildest form of PMD. One type is total gene deletion, a truncation mutation that does not cause the mutant PLP1 that elicit the ER stress responses, and the second is the *PLP1*-specific exon 3B mutation.

Here, we described a patient with a mild form of PMD who could speak meaningful words and walk independently until 15 years of ages. He had two nucleotide deletions within exon 3B which are spliced out during *DM20* messenger RNA production. This mutation preserves the expression and function of DM20 protein. Moreover, this mutant protein is much shorter than wild-type PLP (277/241aa). It should easily be degraded via the activation of nonsense-mediated decay (NMD) pathway, a mechanism that specifically detects and degrades mRNAs containing a premature termination codon. The very mild phenotype observed is probably due to the dual effect of mutation: conservation of DM20 and the inability to elicit an ER stress response.

Thirteen different mutations have been reported in exon 3B (c.384C>G, 385C>T, 388C>T, 409C>G, 409C>T, 410delG, 418C>T, 430A>T, 434G>A, 441A>T, 442C>T, 446C>T). Twelve of them are one nucleotide changes and are predicted to preserve DM20 expressions. Clinical presentations are reported in 9 cases and 6 fit the criteria of Form 4, reinforcing the importance of DM20 function in addition to PLP1. Thus far, only one example of an exon 3B mutation that causes normal DM20 and truncated PLP1 has been reported (440delG; R137fsX8) [6]. This mutation caused two patients with Form 3 and one with Form 4. Our case is the second examples of an exon 3B mutation that produce normal DM20 and truncational PLP1. Our case, together with reports of other exon 3B mutations, supports the hypothesis that frame-shift mutations of PLP1 in exon 3B underlies the very mild phenotype in PMD.

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Original article

Comprehensive genetic analyses of *PLP1* in patients with Pelizaeus–Merzbacher disease applied by array-CGH and fiber-FISH analyses identified new mutations and variable sizes of duplications

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Abstract

Pelizaeus–Merzbacher disease (PMD; MIM#312080) is a rare X-linked recessive neurodegenerative disorder. The main cause of PMD is alterations in the proteolipid protein 1 gene (*PLP1*) on chromosome Xq22.2. Duplications and point mutations of *PLP1* have been found in 70% and 10–25% of all patients with PMD, respectively, with a wide clinical spectrum. Since the underlining genomic abnormalities are heterogeneous in patients with PMD, clarification of the genotype-phenotype correlation is the object of this study. Comprehensive genetic analyses using microarray-based comparative genomic hybridization (aCGH) analysis and genomic sequencing were applied to fifteen unrelated male patients with a clinical diagnosis of PMD. Duplicated regions were further analyzed by fiber-fluorescence *in situ* hybridization (FISH) analysis. Four novel and one known nucleotide alterations were identified in five patients. Five microduplications including *PLP1* were identified by aCGH analysis with the sizes ranging from

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374 to 951-kb. The directions of five *PLP1* duplications were further investigated by fiber-FISH analysis, and all showed tandem duplications. The common manifestations of the disease in patients with *PLP1* mutations or duplications in this study were nystagmus in early infancy, dysmyelination revealed by magnetic resonance imaging (MRI), and auditory brain response abnormalities. Although the grades of dysmyelination estimated by MRI findings were well correlated to the clinical phenotypes of the patients, there is no correlation between the size of the duplications and the phenotypic severity.

Keywords: Array-based comparative genomic hybridization (aCGH); Fiber-FISH; Fluorescence *in situ* hybridization (FISH); Pelizaeus–Merzbacher disease (PMD); Proteolipid protein 1 (*PLP1*)

1. Introduction

Pelizaeus–Merzbacher disease (PMD; MIM#312080) is a rare X-linked recessive neurodegenerative disorder characterized by early onset nystagmus and hypotonia later evolving into spastic tetraparesis, dystonia, ataxia, and developmental delay usually beginning in the first year [1–3]. The main cause of PMD is alterations in the proteolipid protein 1 gene (*PLP1*; MIM#300401) on chromosome Xq22.2 [4–6], which encodes 2 proteins, PLP1 and the splicing variant, DM20, both of which are abundantly expressed in oligodendrocytes [3]. PLP1 is thought to play a major role in myelin sheath formation by promoting sheath compaction [7]. Within the heterogeneous group of dysmyelinating disorders, PMD accounts for 6.5% of all cases [8].

It has been proposed that patients with *PLP1*-related inherited dysmyelinating disorders should be clinically divided into 3 subgroups in order of decreasing severity: connatal, classic, and X-linked spastic paraplegia type 2 (SPG2; MIM#312920) [9]. Duplications of *PLP1* can be found in up to 70% of all patients with PMD, indicating that increased *PLP1* dosage is deleterious for normal myelination [10,11]. Point mutations in *PLP1* have been found in 10–25% of PMD cases with the entire clinical spectrum [11], ranging from the most severe connatal form to the least severe SPG2 form, depending on the affected domain of the protein [9]. Although there are characteristic clinical and radiological features of PMD [1,12], molecular and/or cytogenetic analyses are necessary for final diagnosis because *PLP1* is only expressed in the central nervous system and there are no practical biochemical tests available. The first step in genetic testing should be a genomic dosage analysis of *PLP1* because the major genetic aberration is duplication of *PLP1*. For this purpose, various methods have been used, including southern blotting [6], quantitative polymerase chain reaction (PCR) [13], fluorescence *in situ* hybridization (FISH) [14], multiplex ligation-dependent probe amplification (MLPA) [15], and multiplex amplifiable probe hybridization (MAPH) [16]. Recently, microarray-based comparative genome hybridization (aCGH) has emerged as a novel technology that enables detection and determination of the size of the duplicated or deleted genomic intervals [17]. In case of normal dosage of *PLP1*, nucleotide sequences of *PLP1* should be examined [18]. Here, we report our recent studies to develop comprehensive molecular and cytogenetic analyses to diagnose patients with PMD and to understand the pathogenic mechanism of PMD and its correlation between clinical phenotypes.

2. Materials

Fifteen unrelated male patients (age span from 1 year to 20 years old) with congenital dysmyelination were referred to us for genetic diagnosis based on the clinical diagnosis as PMD. Clinical information and radiographic findings by magnetic resonance imaging (MRI) for the patients were obtained from attending doctors. Based on the approval by the ethics committee at the institution, informed consents were obtained from patient's families, and peripheral blood samples were obtained from all patients. Lymphoblast cell lines were established from lymphocytes extracted from peripheral blood samples by immortalization with Epstein-Barr virus. Three of the fifteen patients (P1, P3, P4) had been diagnosed as having *PLP1* duplications by previously performed comparative PCR amplification method (data not shown).

Genomic DNAs of the patients were extracted from peripheral blood samples using the QIAquick DNA Extraction Kit (QIAGEN, Hamburg, Germany). Metaphase or prometaphase chromosomes were prepared from phytohemagglutinin-stimulated peripheral blood lymphocytes or lymphocyte cell lines according to standard techniques.

One extra cell line (S1) that showed duplication of Xp22.31 including steryl-sulfatase precursor gene (*STS*) as determined by aCGH, was derived from a non-PMD mentally retarded patient and used for fiber-FISH analysis as a positive control of *STS* duplication.

Population-based control DNA samples were obtained from 100 healthy Japanese volunteers.

3. Methods

3.1. aCGH analysis

aCGH analysis, using the Human Genome CGH Microarray 105A chip (Agilent Technologies, Santa

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