same high dose DEX pulse regimen as our patient's [7,8], half of patients attained complete, sustained OMS remission and one fifth had no neurological sequelae, without serious adverse reactions despite treatments exceeding 1 year. Thus, DEX pulse therapy has several advantages over other treatment regimens for OMS: DEX is cheap, only minor side effects (e.g., dysphoria, weight gain, and reversible glucosuria) have been reported [7,8], and out-patient treatment is possible.

As with other OMS patients [3,6], the OMS in our patient was associated with low PB CD4-positive T-cell frequencies, low PB CD4/CD8 ratios, higher CSF B-cell frequency, and high CSF/PB B-cell ratio, and DEX pulse therapy gradually reversed these aberrant values and improved the neurological symptoms. The analyzed cell number in CSF for immunophenotyping in our case was more than thousand, which was a reasonable amount as in previous reports [4,11]. It is reported that glucocorticoid treatment restores the impaired suppressive function of regulatory T cells (both CD4 and CD25-positive cells) in patients with multiple sclerosis [12] that is autoimmune central nervous system (CNS) disease as well as OMS. In our patient immune dysregulation attributed to underlying reduced CD4-positive T-cell frequency might be ameliorated by the DEX pulse therapy. The evidences supporting an important role of CSF B-cell expansion in the pathogenesis of OMS have been accumulated, such as intrathecal autoantibody production, neurological severity correlating with the degree of CSF B-cell expansion and promising therapeutic effects of B-cell depletion with rituximab [11]. The expanding B lineage cells can survive in the inflammatory CNS environment for many years [13], which might be associated with remitting relapsing manner of OMS. Our patient who showed greater B-cell expansion than has been reported previously [11] may have had severe OMS. The alterations of lymphocyte subsets observed in our patient may be responsible for the development of OMS and these could serve as DEX pulse treatment markers.

He has a considerably good prognosis in the motor development; however, we need further careful follow-up for his verbal development to determine the efficacy of this promising treatment.

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連載

小児・思春期精神医学⑧

自閉症の原因診断と病態に基づいた治療の可能性について*

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Key Words : autism, genetic disorder, synaptic function, metabolic disorder, biomarker

はじめに

自閉症あるいは広い意味で自閉症スペクトラム障害(ASD)は社会性の障害，コミュニケーションの障害，興味や行動の範囲が限定的であるなどの症状を示す疾患であるが，その原因は多岐にわたっている．診断基準にあてはめて，自閉症と診断がつくことは単なるスタートラインに着いたにすぎない．なぜならば，さらに絞り込んで病因を突き止め，今後の治療に繋げることが重要な課題となるからである．自閉症の治療については，原因如何にかかわらず，療育方法，対応方法は非常に重要な部分を占めるのは言うまでもない．また，症状に合わせた薬物療法も必要である．しかし，病態に即した治療は，原因を突き止めることにより，はじめてその開発が可能となっていくものである．数は少ないものの原因療法の可能な自閉症も報告されてきている[1]．今回ここでは，自閉症の原因として注目されている疾患，特に現在原因療法がアプローチされている疾患について紹介する．

自閉症には，症候性(syndromic)，非症候性(nonsyndromic)と分類する方法がある．一般的に，症候性の場合は，顔貌や身体的特徴などを手掛かりとして診断が絞りこまれ，確定していくことが多い．特異的顔貌や先天性心疾患などの身体所見が明瞭な場合は，出生後早い時期に染色体検査ですでに特異的診断が確定し，それぞれの疾患特異的に必要な治療計画が立てられている．自閉症状を持つ頻度が高い疾患では，その症状出現を予測しての対応が治療計画に盛り込まれていくこととなる．臨床的身体所見が乏しく自閉症の診断が先になされ後に原因疾患が確定した場合は，自閉症は診断名としてではなくその疾患に併存する一つの症状として認識されていくこととなる．

ASDの原因疾患と遺伝子

自閉症の原因の多くは多因子遺伝と考えられている．すなわち，common genetic variants of weak allele(自閉症リスク遺伝子の変異の)蓄積で起こる．しかし，それだけではなくrare genetic variants of strong allele(自閉症遺伝子の機能異常)それ単独で自閉症をひき起こすことも知られている[2]．自閉症の原因は不明な部分が多いが，近年における分子生物学の進歩に伴い，おそらくその15～20％は特定できるとされる．自閉症の原因の中で，染色体レベルの異常を示す疾患群は5％以下，コピー数変異(CNV)として検出

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されるものが5～10%，単一遺伝子疾患の判明するものが5%未満とされている[3]．CNVの解釈にも課題は残っているものの，身体所見が明瞭でない自閉症でもCNVを含む染色体検査で異常の認められることがあり，今後積極的な検査がなされるとこの割合は増加していくものと推測される．そして，自閉症に限らず，多くの単一遺伝子疾患において，遺伝子異常の部位の多様性と症状にも広がりが認められ，遺伝子異常が認められても無症状であることも珍しくないことも判明してきている．このことからも，自閉性障害のみに限定して考えるよりは，スペクトラム，あるいは幅広い表現型(broad phenotype)を考慮していく必要性もうなずけるのである．

ASDに関与する遺伝子は100以上あり，さらに増え続けているとBetancurは紹介している[4]．症候性の疾患が圧倒的に多い．その一つ一つの疾患における自閉症状を示す割合が，決して多くないものも含まれている．その中で，①ASDがhallmarks(顕著な特徴)となる疾患，②ASDが一般によく認められる疾患，③ASDは頻繁ではないが繰り返し報告される疾患の3郡に分けている．その分け方を参考にして，それぞれの疾患における自閉症状の認められる割合，ASDと診断された症例におけるその疾患の占める割合，その疾患の発症頻度[5][6]を表1にまとめた．

ASDとシナプスの障害

ASDの原因の中で，頻度が多いとされる疾患は脆弱X症候群，結節性硬化症，Angelman症候群，SHANK3/22q deletion syndrome, Prader-Willi症候群があげられる．これらの遺伝子がコードするタンパクの機能に共通していることは，シナプスにおける機能にかかわっているということである．

脆弱X症候群は精神遅滞の原因として最も頻度の高い(2.6～8.7%[7]，日本人では0.8～2.4%[8])疾患として知られている．その半数以上で自閉症状〔自閉性障害30%，pervasive developmental disorder-not otherwise specified(PDD-NOS)30%〕を示し，ASDの1～3%に本症が認められる．欧米では男性4,000人に1人(女性8,000人)に1人とされるが，日本ではそれより少なく1万人に1人とされる[7][8]．身体所見として特徴的顔貌，巨大睾丸，関節過伸展があるが，小児期には典型的な特徴に乏しく，臨床兆候だけからは診断できない非特異的な症例についても，今後の調査が進めばこの頻度は増える可能性がある．この疾患は*FMR1*遺伝子のCGGのリピート数が200以上となると遺伝子の発現が抑制されることにより発症する．*FMR1*遺伝子にコードされるタンパク質のFMRPはmRNAの転写を調節しており，シナプスの構造や調節にかかわっている．脆弱X症候群ではシナプスにおける代謝型グルタミン酸受容体(mGluR1/5)の機能が促進されていることが示され[9]，この病態から脆弱X症候群の治療として，このmGluR1/5を拮抗する薬物が動物実験を経て，アメリカでは治験段階に入っている[1]．また，脆弱X症候群で増加しているタンパクの1つにmatrix metalloproteinase 9(MMP-9)がある．ミノマイシンにMMC-9の異常活性を抑制する作用があり，同様に治験されている[1]．

そのほかにシナプスの形成と機能にかかわっている遺伝子には***neuroligin 3/neuroligin 4, neurexin 1, CNTNAP2, PAK2/PAK3, MeCP2***などがあげられる．

染色体15q11-q13領域は欠失や重複が起こりやすい部位であり，Angelman症候群，Prader-Willi症候群を含んでいる．この領域に含まれるcytoplasmic FMR1-interacting protein 1(Cyfip1)はFMRPと結合してシナプスでmRNAの翻訳を調節している．

22q13欠失症候群は重度の表出言語の遅れ，筋緊張低下，自閉症様行動特性，過成長，軽度の顔貌異常を特徴とする．しかし，遺伝子解析スクリーニングで偶然診断されるケースもあり[10]，外表奇形に乏しく診断未定の例が存在する可能性がある．この領域に含まれる*SHANK3*遺伝子の異常が原因と考えられている．そして，この*SHANK3*遺伝子はシナプスのグルタミン酸受容体と細胞骨格の間をつなぐ足場タンパクをコードしている．

結節性硬化症は神経皮膚症候群の一疾患で知的障害，てんかん，白斑などに全身性の過誤腫，腫瘍性病変を特徴とする．発症にかかわる*TSC1*遺伝子と*TSC2*遺伝子はシナプスにおける情報伝

表1　ASDの原因遺伝疾患，他の身体症状を伴う疾患群(syndromic ASD)

	疾患	遺伝子	その疾患のASD症状頻度	ASDにおけるその疾患の頻度	疾患の頻度(10万人につき)
・ASDが主な臨床症状の一つとなる疾患					
単一遺伝子	脆弱X症候群	*FMR1*	男60%，女20%	1～3％	25人/男，12.5人/女，10人/日本人
単一遺伝子	レット症候群	*MECP2*		女子の1.3%	7～10人
単一遺伝子	結節性硬化症	*TSC1, TSC2*	16～60%	1.1～1.3%	10人
単一遺伝子	Timothy症候群	*CACNA1C*	60～80%		稀
単一遺伝子		*ADSL*	～50%		稀
単一遺伝子	Smith-Lemli-Opitz症候群	*DHCR7*	50～75%		2.5～5人，日本人稀
単一遺伝子		*CNTNAP2*			
単一遺伝子	SHANK3/22q欠失症候群	*SHANK3*	44%～しばしば	1.0～1.3%	
単一遺伝子	Angelman症候群	*UBE3A*	50～63%	1～3％	5～10人
・ASDの症状がよく認められる疾患					
単一遺伝子	脳クレアチン欠損症	*SLC6A8*ほか	しばしば		
単一遺伝子	Cornelia de Lange症候群	*NIPBL, SMC1A*	47～67%		10人
単一遺伝子	CHARGE症候群	*CHD7*	68%		12人
単一遺伝子	Cohen症候群	*VPS13B*	49%		1人
単一遺伝子	Joubert症候群と関連症候群	*AHI1*ほか	13～36%		
単一遺伝子	Myotonic dystrophy type1	*DMPK*	35～50%		
単一遺伝子	Potocki-Lupski症候群	*RAI1*	しばしば		
単一遺伝子	Smith-Magenis症候群	*RAI1*	しばしば90%		4～6人
単一遺伝子	X-linked female-limited epilepsy and ID	*PCDH19*	22～38%		
単一遺伝子	Xq28欠失症候群	*MECP2*			
隣接遺伝子	5pモノソミー(Cri du Chat syndrome)	*5p*欠失	39%		
隣接遺伝子	Williams症候群	*7q11.23*欠失	50%		
隣接遺伝子	7q11.23重複症候群	*7q11.23*重複	40%		
隣接遺伝子	8p23.1欠失	*8q23.1*欠失	57%		
隣接遺伝子	WAGR症候群	*11p13*欠失	52%		
隣接遺伝子	Prader-Willi症候群	*15q11-q13*	23%		4～6人
隣接遺伝子	16p11.2微小欠失と微小重複	*16p11.2*欠失・重複		1％	
隣接遺伝子	17q12欠失	*17q12*欠失	66%		
隣接遺伝子	22q11欠失症候群(velocardiofacial/DiGeorge syndrome)	*22q11*欠失	14～50%		
・ASDが高頻度ではないがよく報告されている疾患					
単一遺伝子	Duchenne型とBecker型筋ジストロフィー症	*DMD*	19%		
単一遺伝子	PTEN関連症候群	*PTEN*	15%	大頭症の4.7%	0.5人？
単一遺伝子	Cardio-facio-cutaneous症候群	*KARS, BRAF*ほか	23%		
単一遺伝子	Noonan症候群	*PTPN11*	8％		40～100人，10人(日本人)
隣接遺伝子	1q21.1微小重複症候群		～10%		発達遅滞+ID+先天奇形0.2%
隣接遺伝子	2q37欠失症候群		24%		
隣接遺伝子	3q29微小欠失		26%		
隣接遺伝子	9qサブテロメア欠失症候群				
隣接遺伝子	11q欠失症候群		13%		
隣接遺伝子	15q24微小欠失症候群		22%		
・ASDのリスクの認められる染色体異数体					
染色体異常	ダウン症候群	21トリソミー	5～15%		126人
染色体異常	ターナー症候群	45, X	女3.3%		女子で50～100人
染色体異常	クライネフェルター症候群	47, XXY	11～48%		男子で166人
染色体異常	XYY症候群	47, XYY	19%		
染色体異常	45, X/46, XYモザイク症	45, X/46, XY	7％		

達経路上にあるmammalian target of rapamycin (mTOR)の活性を調節している．

PTEN (the phosphatase and tensin homologue gene) hamartoma tumor syndromeのなかでもBannayan-Rikey-Ruvalcaba症候群(BRRS)は大頭症，脂肪腫，血管腫，陰茎色素沈着，消化管の過誤腫性ポリポーシスを主徴とし，ASDを伴うことが報告されている．大頭症を伴う自閉症の4.7%に*PTEN*遺伝子の異常が検出されており[10]，3SD≧の大頭症の自閉症ではスクリーニングの必要があるであろう．この*PTEN*遺伝子も*TSC1*や*TSC2*同様にmTORの活性調節にかかわっている．

それぞれの疾患が自閉症に占める割合は多くてもそれぞれ約1%程度しかないが，シグナルパスウェイから考えると共通した治療法が導き出される可能性がある．現実の治療への応用までにはまだまだ課題は多いと思われるが，このように遺伝子の異常が判明すると，その遺伝子の機能を手掛かりとして治療へと発展させていくことの可能性が出てくる．

ASDと代謝異常症

ASDの原因として代謝異常症は非常に稀である．しかし，よく知られているように，フェニルケトン尿症は，新生児期にスクリーニングされて早期治療が始まり，治療開始以前にみられた症状は，自閉症状を含めて治療開始後には改善し認められなくなっている．このように，治療が可能である疾患が含まれているため，代謝異常に起因する自閉症を発見し診断することは予後を考えると重要である．自閉症状を示す症例報告のあった代謝異常症とその参考症状，バイオマーカー，治療法を**表2**に示した．

これらの代謝異常の中で現在最も注目されているのは，脳クレアチン欠損症である(**表3**)．脳内クレアチンは脳内のATPの貯蔵と再生に重要な役割を担っている．脳クレアチン欠損症候群(cerebral creatine deficiency syndrome；CCDSs)には現在3つの遺伝子の異常が知られている．クレアチン合成障害として，①guanidinoacetate methyltransferase (GAMT)欠損症，②arginine：glycine aminotransferase deficiency (AGAT)欠損症の2種類，クレアチンのuptakeの障害である，③creatine transporter (SLC6A8)欠損症(X連鎖性のクレアチン症候群)である．症状には幅があり，非特異的知的障害，てんかん，錐体外路症状，自閉症などさまざまである．一般的にCCDSsの症状は，知的障害が主体であるが，GAMT欠損症では，てんかんが93%，行動異常(自閉行動や自傷)が80%，錐体路/錐体外路症状が45%に認められる．発症は3カ月から3歳である．AGAT欠損症の報告はまだ少なく5例のみであり行動異常はない[11]．SLC6A8欠損症は軽度から重度の知的障害や言語の遅れ，てんかん(<10%)と，自閉症様の行動異常が認められる．発症は2歳から66歳と幅が広い．女子のヘテロの約50%に学習や行動の問題がみられる．GAMT欠損症とAGAT欠損症では経口creatine monohydrateで脳内クレアチンを増加させる治療がなされている．さらに，GAMT欠損症では食事のアルギニン制限とオルニチンのサプリメント使用が併用される．SLC6A8欠損症でも経口creatine monohydrateが試みられているが現在のところ効果は認められていない．Lアルギニン補充療の効果の報告では効果なしと効果あるとで分かれている[12]．早期に治療を開始した場合の効果の違いについてはまだ不明であり，今後症例の蓄積が必要である．診断の手掛かりとなる尿所見については，GAA (guanidinoacetate)濃度はGAMT欠損症で高く，AGAT欠損症で低く，SLC6A8欠損症で正常である．クレアチン濃度はGAMT欠損症とAGAT欠損症で低く，SLC6A8欠損症で高い(ただし女子では正常あるいは高値)クレアチン/クレアチニンはGAMT欠損症とAGAT欠損症で正常，SLC6A8欠損症の男児で高い(女子では正常あるいは高値)である．血清のGAAはGAMT欠損症で正常の20～30倍，AGAT欠損症で低く，SLC6A8欠損症では正常である．血清クレアチンはGAMT欠損症で低い．確定診断ではGAMT酵素活性，AGAT酵素活性を測定する．SLC6A8欠損症ではクレアチン吸収試験と遺伝子診断を行う．頻度については，SLC6A8欠損症は非症候性知的障害の男児の2.1%[13]，IQ 70以下の全体的遅れのある男子の2.2%[14]との報告がある．男児の知的障害で高頻度であることを報告したLion-François[15]らは，スポット尿でクレアチン/クレアチニンとGAA/クレアチニンを測定し，異常値を示した症例には脳MRS (H-MRS)と*SLC6A8*

表2　ASDと代謝異常疾患

疾患	遺伝子	ASD症状割合	参考症状	バイオマーカー	治療
フェニールケトン尿症	*PAH*	治療前5.7% 治療後0%	小頭症，てんかん	血中フェニルアラニン高値，BH4代謝正常	治療ミルク，食事療法
スミス-Lemli-オピッツ症候群	*DHCR7*	50～86%	多発奇形，発育障害	低コレステロール血症	高コレステロール食，胆汁酸
脳クレアチン欠損症					
アルギニン：グリシンアミノトランスフェラーゼ欠損症	*GATM* (*AGAT*)	16%	てんかん高率，錐体外路症状，失調	尿中GAA高値，GAMT酵素活性	経口クレアチン水和物，アルギニン制限食，経口オルニチン
グアニジンアミノアセテートトランスフェラーゼ欠損症	*GAMT*		行動異常なし，まだ報告が少ない	尿中GAA低値，クレアチン低値，AGAT酵素活性	経口クレアチン水和物
クレアチン輸送担体欠損症	*SLC6A8*	しばしば	てんかん10%以下，行動異常	尿クレアチン/クレアチニン高値	Lアルギニン補充療法(効果はまだ不定)
アデニロコハク酸リアーゼ欠損症	*ADSL*	50%	筋緊張低下，てんかん	尿・髄液S-AdoとSAICArの検出	
コハク酸アルデヒド脱水素(SSADH)欠損症(4-ヒドロキシ酪酸尿症)	*ALDH5A1*	12%	筋緊張低下，けいれん，多動，小脳失調，睡眠障害	尿中4-hydroxybutyric acd増加，培養リンパ芽球SSADH活性低下	Vigavatorin(確証なし)
ビオチニダーゼ欠損症	*BDT*	症例報告	筋緊張低下，けいれん，皮膚炎	酵素活性	ビオチン投与
L2ヒドロキシグルタル酸尿症	*L2HGDH*		小脳・錐体外路症状，大頭症，基底核・歯状核を含む白質脳症	L-2-HGAの増加	脳腫瘍併発観察
オルニチントランスカルバミラーゼ欠損症	*OTC*		反復嘔吐，意識障害，男児重症，女児軽症	アンモニア高値，血漿シトルエイン低値など	高アンモニア血症対策
Sanfilippo病A型	*SGSH*		多動，多毛，粗毛，顔貌	尿中ヘパラン硫酸の排泄	酵素補充量の計画中
カルシナーゼ欠損症	*CNSN*		発達遅滞	尿中カルノシン増加	
シトリン欠損症	*SLC25A*		タンパク嗜癖，低身長，けいれん，遷延性黄疸，反復昏睡	高ガラクトース血症，複数アミノ酸高値	
ヒスチジン血症	*HAL*		けいれん，言語障害，無症状例がほとんど	高ヒスチジン血症	

BH4：tetrahydrobiopterin, GAMT：guanidinoacetate methyltransferase, AGAT：L-arginine：glycine amidinotransferase, *SLC6A8*：*SLC6A8*-related creatine transporter, GAA：guanidinoacetate, S-Abo：succinyladenosine, SAICAr：succinylaminoimidazole carboxamide ribotide, SSADH：succinic semialdehyde dehydrogenase, L-2-HGA：L-2-hydroxyglutaric acidia

か*GAMT*の遺伝子検査で確定診断を行っている．症例は軽度から中等度精神遅滞の118名(男114例，女74例)のうち男子のみの5例に異常が発見され，その5例の知的障害は重度で，5例中3例に自閉症状が認められている．したがって，頻度は全体の2.7%，男児の4.4%であった．家族性は2例で5.4%，非家族性は3例で1.9%という報告であった．

他の報告によると，原因不明とされていた精神遅滞の944例(3歳から76歳)中7例(0.74%)において尿検査で先天代謝異常が検出されたとの報告がある[16]．7例のうち自閉的行動を合併していた5例の内訳は，脳クレアチン欠損症3例，adenylosuccinate lyase欠損症1例，フェニールケトン尿症(スクリーニング以前に出生)1例であった．このように身体的所見が非特異的で乏しく，従来原因不明と考えられていた症例の中にも代謝異常症を念頭に調べてみると原因の見つかる症例もあり，特に脳クレアチン欠損症がその中でも頻度が多い可能性がある．

表3　脳クレアチン欠損症候群の症状と尿所見

欠損	ASDの頻度	症状			尿所見		
		知的障害程度	てんかん頻度	錐体外路症状	GAA濃度	クレアチン濃度	クレアチン/クレアチニン比
GAMT	16%	軽～重	93%	無～重	高	低	正
AGAT		軽～中	20%	無	低	低	正
SLC6A8	しばしば	軽～重	<10%	無	男 正	高	高
					女 正	正～高	正～高

GAMT：guanidinoacetate methyltransferase, AGAT：L-arginine : glycine amidinotransferase, SLC6A8：SLC6A8-related creatine transporter, GAA：guanidinoacetate

まとめ

自閉症スペクトラム障害(ASD)の原因検索の必要性を述べた．しかし，ASDの患者で，血液や尿検査をして異常の見つかることは非常に少ない．最も検出される検査は，染色体，アレイCGH, MLPA法などの臨床遺伝的検査であろう．しかし，検査依頼方法やコストのほかに，倫理的な面その後の対処法も含めてさまざまな配慮が必要であり，どこにおいても検査を行うわけにはいかないのが現状である．もう少し簡便にスクリーニングできる方法が開発されることを切望する．

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Case report

Muscle glycogen storage disease 0 presenting recurrent syncope with weakness and myalgia

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Abstract

Muscle glycogen storage disease 0 (GSD0) is caused by glycogen depletion in skeletal and cardiac muscles due to deficiency of glycogen synthase 1 (GYS1), which is encoded by the *GYS1* gene. Only two families with this disease have been identified. We report a new muscle GSD0 patient, a Japanese girl, who had been suffering from recurrent attacks of exertional syncope accompanied by muscle weakness and pain since age 5 years until she died of cardiac arrest at age 12. Muscle biopsy at age 11 years showed glycogen depletion in all muscle fibers. Her loss of consciousness was gradual and lasted for hours, suggesting that the syncope may not be simply caused by cardiac event but probably also contributed by metabolic distress.

Keywords: Glycogen storage disease; Glycogen synthase; Glycogen; Syncope; Sudden death

1. Introduction

Glycogen is a high molecular mass polysaccharide that serves as a repository of glucose for use in times of metabolic need. It is stored in liver, cardiac and skeletal muscles, and broken down to glucose to produce ATP as energy as needed. For the synthesis of glycogen, at least two proteins, glycogenin (GYG) and glycogen synthase (GYS), are known to be essential. GYG is involved in the initiation reactions of glycogen synthesis: the covalent attachment of a glucose residue to GYG is followed by elongation to form an oligosaccharide chain [1]. GYS catalyzes the addition of glucose monomers to the growing glycogen molecule through the formation of alpha-1,4-glycoside linkages [2].

Defect in either GYG or GYS can cause glycogen depletion. Recently, muscle glycogen deficiency due to a mutation in a gene encoding muscle GYG, *GYG1*, was reported [3] and named as glycogen storage disease type XV. In contrast, glycogen depletion caused by the *GYS* gene mutation is called glycogen storage disease type 0 (GSD0). GSD0 was first reported in 1990 in patients with type 2 diabetes who had a defect in glycogen synthesis in liver, which was caused by a defect in liver GYS, GYS2, and the disease was named as liver GSD0 (or also called GSD0a) [4,5].

The disease of muscle GYS, GYS1, was first described in 2007 in three siblings and named muscle GSD0, which is

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also called GSD0b [6]. One of the patients initially manifested exercise intolerance, epilepsy and long QT syndrome since the age of 4 years, then died of sudden cardiac arrest after exertion when he was 10.5-year-old. The other two siblings were then genetically confirmed as muscle GSD0 with mutations in *GYS1* and cardiac involvement was also found in both. The second muscle GSD0 family was reported in 2009 [7]. The 8-year-old boy had been healthy before collapsing during a bout of exercise, resulting in death. Post-mortem examinations and studies verified the diagnosis of muscle GSD0. He had a female sibling who died at 6 days of age of undetermined cause. Here we report the first muscle GSD0 patient in Asia with some distinct clinical manifestations from other reported cases.

2. Case report

An 11-year-old Japanese girl with repeated episodes of post-exercise loss of consciousness, weakness, and myalgia since age 5 years, was admitted to the hospital. She was the first child of unrelated healthy parents. She was born uneventfully and was normal in psychomotor development. At age 2 years, she developed the first episode of generalized tonic-clonic seizure while she was sleeping. At age 4 years, she had the second episode of generalized tonic-clonic seizure when she was under general anesthesia for tonsillectomy, whose cause was thought to be hypoglycemia due to prolonged fasting. In both episodes, seizure was followed by strong limb pain. At age 5 years, she suffered from the first episode of syncope while climbing up stairs. She recovered after a few hours. One year later, she had the second syncopal attack after running 50 m, which was accompanied by subsequent limb muscle weakness and myalgia. Since then, similar episodes were repeated several times a year. For each bout, she first developed leg muscle weakness immediately after exercise, making her squat down, and gradually lost the consciousness. She recovered her consciousness after a few hours but always experienced strong myalgia in legs which lasted for several hours. Blood glucose level was not decreased during these attacks.

On admission, general physical examination revealed no abnormal finding. On neurological examination, she had mild proximal dominant muscle weakness and mildly limited dorsiflexion of both ankle joints. T1-weighted images of skeletal muscle MRI showed high signal intensities in gluteal and flexor muscles of the thigh, which were assessed to be fatty degeneration (Fig. 1). Systemic investigations including electrocardiography, echocardiography, stress cardiac catheterization, stress myocardial scintigraphy, brain imaging, electroencephalography, and screening tests for metabolic diseases revealed no abnormality except for a mild ischemic finding on exercise electrocardiography. Ischemic and non-ischemic forearm exercise tests [8] showed the lack of lactate elevation, raising a possibility of glycogen storage disease. A few months later, resting electrocardiography, 24-h holter monitoring and resting echocardiography were re-evaluated and again revealed normal findings.

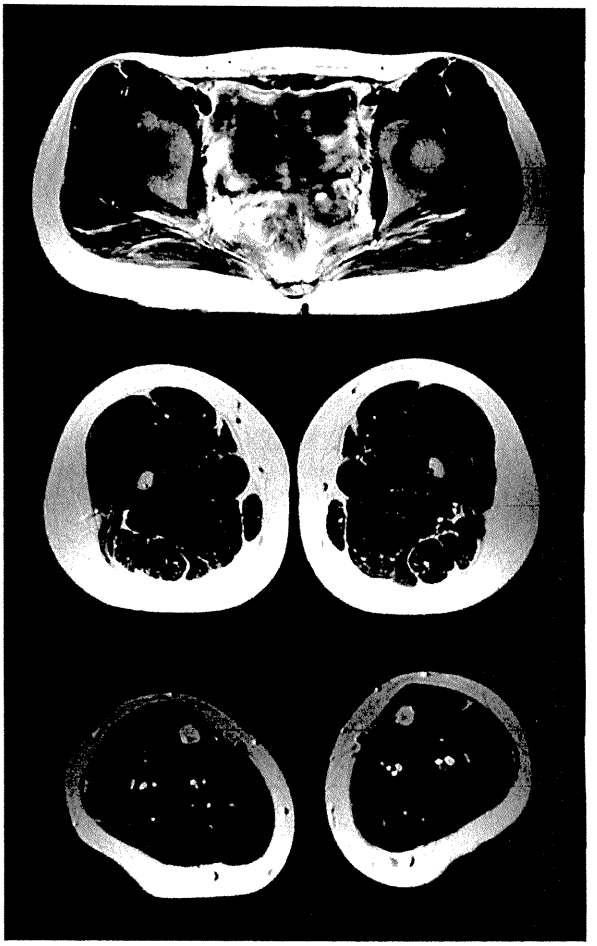

Fig. 1. Muscle MRI, T2WI, axial. It shows high intensity in gluteus maximus and biceps femoris muscles.

3. Histological analysis of skeletal muscle

Muscle biopsy was performed from biceps brachii. Serial frozen sections were stained with hematoxylin and eosin, modified Gomori trichrome, and a battery of histochemical methods. The most striking finding was depletion of glycogen in all muscle fibers but not in the interstitium on periodic acid-schiff (PAS) staining (Fig. 2A). Phosphorylase activity was also deficient in all fibers (Fig. 2B). Mitochondria especially at the periphery of muscle fibers were prominent on modified Gomori trichrome (Fig. 2D). ATPase staining revealed type 2 fiber atrophy. Electron microscopic analysis showed mitochondrial proliferation at the periphery of muscle fibers with no notable intramitochondrial inclusions (Fig. 2E).

4. Biochemical and molecular analysis

Both the activity of GYS1 and the amount of glycogen in the skeletal muscle were markedly reduced (Table 1). On western blotting, GYS1 in the patient's skeletal muscle was undetectable (Fig. 2F). The *GYS1* gene sequence analysis revealed compound heterozygous mutation of

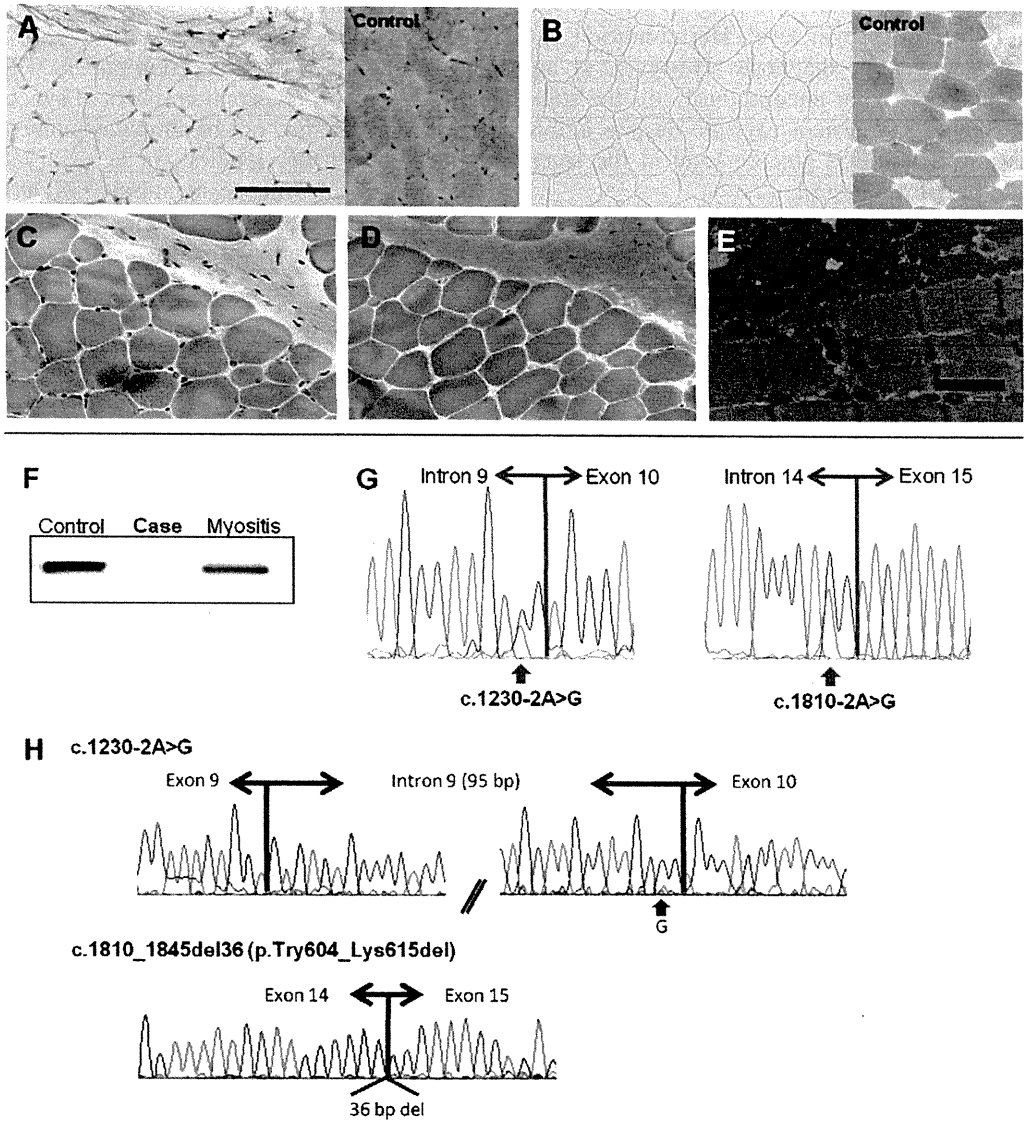

Fig. 2. Histological, genetic and protein analyses. Periodic acid-schiff (PAS) staining shows marked depletion of glycogen in muscle fibers but not in the interstitium (A). Phosphorylase activity is also deficient in all fibers (B). Hematoxylin and eosin staining shows mild fiber size variation (C). On modified Gomori trichrome, mitochondria are prominent especially at the margin of each muscle fiber (D). On electron microscopy (EM), mitochondria are increased in number at the periphery of muscle fibers (E). Bars represent 100 μm for histochemistry and 7 μm for EM. On western blotting using anti-GYS1 antibody (Abcam), GYS1 protein is absent in skeletal muscle from the patient (F). Sequence analysis for the *GYS1* gene reveals a compound heterozygous mutation of c.1230-2A > G and c.1810-2A > G (G). cDNA analysis showed insertion of intron 9 between exon 9 and 10 and 36-bp deletion from the beginning of exon 15 (H).

Table 1
Analyses of enzymatic activity and glycogen content. The activity of GYS and glycogen content in skeletal muscle were markedly reduced.

	Glycogen synthase (mol/min/mg)	UDPG-pyrophosphorylase (nmol/min/mg)	Glycogen contents (% of wet weight)
Patient	*0.9*	30.5	*0.03*
Control	42.0 ± 11.2	31.2 ± 3.5	0.94 ± 0.55

Italicized values: lower than control range.

c.1230-2A > G in intron 9 and c.1810-2A > G in intron 14 (Fig. 2G). cDNA analysis confirmed the insertion of the full-length intron 9 between exons 9 and 10 and a 36-bp deletion in the beginning of exon 15 (Fig. 2H).

5. Clinical course after diagnosis

Upon the diagnosis of GSD0, exercise was strictly limited to avoid syncope resulted from glucose depletion. In

addition, oral intake of cornstarch (2 g/kg, every 6 h) was started to maintain blood sugar level. Her condition had been stable for 1 year after diagnosis. However, at age 12 years, she was found lying unconsciously on the stairs at her school. She had persistent asystole despite ambulance resuscitation. The blood glucose level in the emergency room was above 100 mg/dl.

6. Discussion

We identified the first Asian patient with muscle GSD0, who manifested recurrent episodes of syncope with subsequent muscle weakness and myalgia, and eventually developed cardiac arrest.

Findings in our patient seem to be similar to previous reports, but some differences indicated the possibility of another pathogenesis of the disease. Our patient repeatedly suffered from episodes of syncope. In contrast to two earlier reports, those patients never had syncope, although the last attack led to sudden death [6,7]. In support of this notion, most muscle glycogen synthase knock-out mice died soon after birth due to impaired cardiac function [8]. However, the pattern of loss of consciousness in our patient cannot be explained by simple cardiac dysfunction, as she lost her consciousness gradually after exercise and took hours to regain, which is different from typical cardiac syncope, usually showing sudden loss of consciousness and rapid recovery. Alternatively, defective glycogen synthesis in brain may be related to syncope, as GYS1 is also expressed in brain, albeit not so much as in cardiac and skeletal muscles. Another possibility may be intermittent arrhythmia. However, electrocardiogram during the episode was never obtained. Further studies are necessary to answer this question.

On muscle pathology and electron microscopy, we found profound deficiency of glycogen in all muscle fibers accompanied by mitochondrial proliferation, which is similar to previous reports. The mitochondrial proliferation may reflect a compensatory mechanism for supplying ATP to glycogen-depleted muscles. Interestingly, phosphorylase activity on histochemistry seemed deficient. This is consistent with the fact that endogenous glycogen is used as a substrate of phosphorylase on histochemistry. Previous reports described the reduced number of type 2 fibers. In our patient, type 2 fiber atrophy, but not type 2 fiber deficiency, was seen. Although type 2 fiber atrophy is a nonspecific finding, this picture might also reflect the dysfunction of glycogen-dependent muscle fibers.

7. Conclusion

We identified the first Asian patient with muscle GSD0. In our patient, recurrent episodes of syncope and eventual sudden death may not be simply explained by cardiac dysfunction. Further studies are necessary to elucidate the mechanism of syncope in muscle GSD0 and to establish appropriate guideline of management for these patients to prevent sudden death.

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CONFIRMATION OF THE EFFICACY OF VITAMIN B_6 SUPPLEMENTATION FOR MCARDLE DISEASE BY FOLLOW-UP MUSCLE BIOPSY

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ABSTRACT: No effective treatment for McArdle disease exists. We report a Japanese patient with McArdle disease who was treated with vitamin B_6 supplementation (60–90 mg/day). After treatment, increased muscle phosphorylase activity was confirmed by follow-up muscle biopsy (3.8 times higher than pretreatment levels). Increased lactate levels were seen on the forearm exercise test, and regular work activities could be resumed. Vitamin B_6 supplementation can enhance residual phosphorylase activity and improve insufficient anaerobic glycolysis of skeletal muscle.

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McArdle disease is a rare metabolic myopathy caused by a deficiency in muscle phosphorylase, which has an important role in anaerobic glycolysis of skeletal muscle. Clinical features of McArdle disease include muscle cramps, myalgia, exercise intolerance, fatigue, and slowly progressive weakness, although the type and amount of exercise needed to precipitate these symptoms varies from patient to patient and from day to day. Muscle necrosis and myoglobinuria caused by an inadequate energy supply to skeletal muscle during exercise occur in about half of patients, and half of them develop acute renal failure.[1] A diagnosis of McArdle disease is suspected based on patient history and elevation of serum creatine kinase (CK) levels.[1]

The forearm exercise test, during which serum ammonia and lactate levels are measured, is a simple, sensitive, and specific test for disorders of muscle glycolysis. In McArdle disease, patients fail to produce lactate during this test.[2]

Several groups have reported the use of vitamin B_6 treatment in McArdle disease. However, a repeat muscle biopsy in the same patient after treatment has not been performed; thus, the efficacy of this treatment is not well documented. To date, there is no conclusive evidence of significant benefits from nutritional or pharmacological treatments in McArdle disease.[3]

We report an adult Japanese patient who was treated with oral vitamin B_6 supplements (60–90 mg/day) for >2 years. Efficacy of treatment was evaluated using manual muscle testing (MMT: −4 = paralysis; −3.5 = paralysis–severe weakness; −3 = severe weakness; −2.5 = severe–moderate weakness; −2 = moderate weakness; −1.5 = moderate–mild weakness; −1 = mild weakness; −0.5 = mild weakness–normal power; 0 = normal power), the forearm exercise test, and a follow-up muscle biopsy; we also measured serum CK levels.

CASE REPORT

In March 2008, a 41-year-old Japanese man was brought to our emergency room with severe myalgia and brown urine after being injured in a fight with his brother. He indicated that, since childhood, he had experienced muscle cramps and myalgia after exercising. His parents were consanguineous, and he had a history of hypertension and subarachnoid hemorrhage. Blood chemistry showed markedly elevated serum CK level (420,950 IU/L), but vitamin B_6 levels were within the normal range (pyridoxine <0.3 ng/ml, pyridoxamine <0.2 ng/ml, pyridoxal 6.5 ng/ml). He developed severe rhabdomyolysis and acute renal failure, but hemodialysis in the intensive care unit greatly improved his renal function.

In April 2008, his height and body weight were 167 cm and 54 kg, respectively, and neurological examination showed moderate weakness of proximal muscles in the upper and lower limbs (MMT = −2). The forearm exercise test revealed virtually no increase in serum lactate level, although increases in lactate levels after the forearm exercise test were five- or sixfold higher than baseline levels in healthy subjects (Fig. 1).[2] An electromyogram was normal. Computed tomography scans of all his extremities indicated slight atrophy of the proximal muscles. Muscle biopsy of his left biceps

Abbreviations: CK, creatine kinase; H&E, hematoxylin and eosin; MMT, manual muscle testing; PAS, periodic acid–Schiff; PLP, pyridoxal 5′-phosphate

Key words: anaerobic glycolysis; follow-up muscle biopsy; McArdle disease; muscle phosphorylase; vitamin B_6 supplementation

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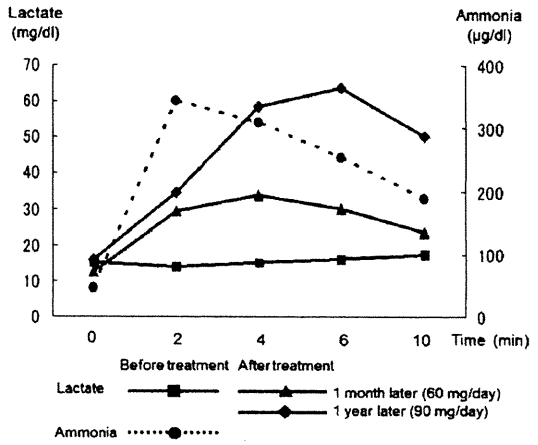

FIGURE 1. Forearm exercise test. For this test, rhythmic (1-Hz) handgrip exercise at maximal voluntary contraction was performed for 2 minutes. Results showed no increase in serum lactate levels before treatment with vitamin B_6 was started. An increase in serum lactate levels was seen 1 month after starting oral vitamin B_6 supplementation at 60 mg/day (1.1 mg/kg/day). Moreover, lactate markedly increased 1 year after treatment with 90 mg/day (1.6 mg/kg/day). Plasma ammonia concentrations were only measured before treatment with vitamin B_6. Time = 0: before exercise. Normal values (at rest): lactate 5.0–20.0 mg/dl; ammonia 9–45 μg/dl.

brachii, which was performed 40 days after the severe rhabdomyolysis, showed variation of muscle fiber size and frequent internal nuclei on hematoxylin and eosin (H&E) staining (Fig. 2A). When muscle phosphorylase activity is preserved, muscle fibers are stained brown or violet with phosphorylase because of their reaction to the iodine–potassium iodide solution used for phosphorylase staining. The higher the activity of phosphorylase, the deeper violet the muscle fibers are stained. However, his muscle fibers did not show phosphorylase staining (Fig. 2B). Periodic acid–Schiff (PAS) staining revealed many glycogen deposits under the sarcolemma of muscle fibers. Under histochemical staining for ATPase activity at pH 4.4, the proportion of type 1 and type 2 fibers was 44% and 56%, respectively. Muscle phosphorylase activity was 3.8 nmol/min/mg protein [control: 58.9 ± 17.5 nmol/min/mg protein (mean ± SD)]. He was found to be homozygous for a single-codon deletion at codon 708/709 in exon 17, which is the most common mutation of muscle phosphorylase among Japanese patients with McArdle disease.[4]

In August 2008, treatment with oral vitamin B_6 supplements (60 mg/day, 1.1 mg/kg/day) was started and, 1 month later, the forearm exercise test showed an increase in lactate levels (Fig. 1). Neurological examination revealed muscle strength improvement (MMT = −0.5). Serum CK levels were normal (146 IU/L) (Fig. 3). In November 2008, we increased the dosage of vitamin B_6 from 60 to 90 mg/day (1.6 mg/kg/day) because serum CK had increased due to more severe physical stress associated with his job. Serum CK levels normalized 1 month after administration of 90 mg/day of vitamin B_6. Subsequently, his muscle weakness gradually improved.

In July 2009, 1 year after starting treatment, the patient's lactate levels markedly increased on the forearm exercise test (Fig. 1). In October 2009, a follow-up muscle biopsy of his right biceps brachii was performed with his informed consent. The fiber size variation was minimized, and 70–80% of the muscle fibers were stained brown with phosphorylase (Fig. 2C and D). The proportions of type 1 and type 2 fibers in the posttreatment sample were 31% and 69%, respectively. Little accumulation of glycogen was observed in the muscle fibers by PAS staining. Muscle phosphorylase activity was 14.4 nmol/min/mg protein, which was 3.8 times higher than before treatment.

The patient's serum CK levels ranged from 120 to 2,093 IU/L (mean 576 IU/L), depending on his physical activities (Fig. 3). However, his clinical condition was stable regardless of the heavy labor he performed during his daily work as a fish dealer. Furthermore, there had been no adverse effects caused by vitamin B_6, including sensory neuropathy.[5–7]

DISCUSSION

Oral vitamin B_6 supplementation (60–90 mg/day) in this patient led to improvements in both muscle weakness and inadequate anaerobic glycolysis; a follow-up muscle biopsy confirmed the presence of increased muscle phosphorylase activity after treatment. Our patient has continued to be engaged in his work for 2 years and 2 months. Although the phosphorylase activity after vitamin B_6 treatment is not completely normal, it is sufficient for him to maintain his regular work activities.

McArdle disease is transmitted as an autosomal recessive trait. The gene for muscle phosphorylase is localized on chromosome 11q13. Deficiency of this enzyme results in inability to metabolize skeletal muscle glycogen during anaerobic metabolism, followed by clinical symptoms such as muscle weakness. There is almost no detectable muscle phosphorylase activity in the majority of affected individuals,[1,4] but some residual activity (i.e., up to 10% of normal values) has been observed in some cases.[1] Our patient had 6.5% of normal muscle phosphorylase activity before treatment, 40 days after the severe rhabdomyolysis in March 2008. The residual activity may have been caused by several regenerating muscle fibers expressing the fetal isoform of muscle phosphorylase.[8,9] In addition, the difference in the proportion of muscle fiber types before and after vitamin B_6 treatment might have been caused by regeneration of skeletal

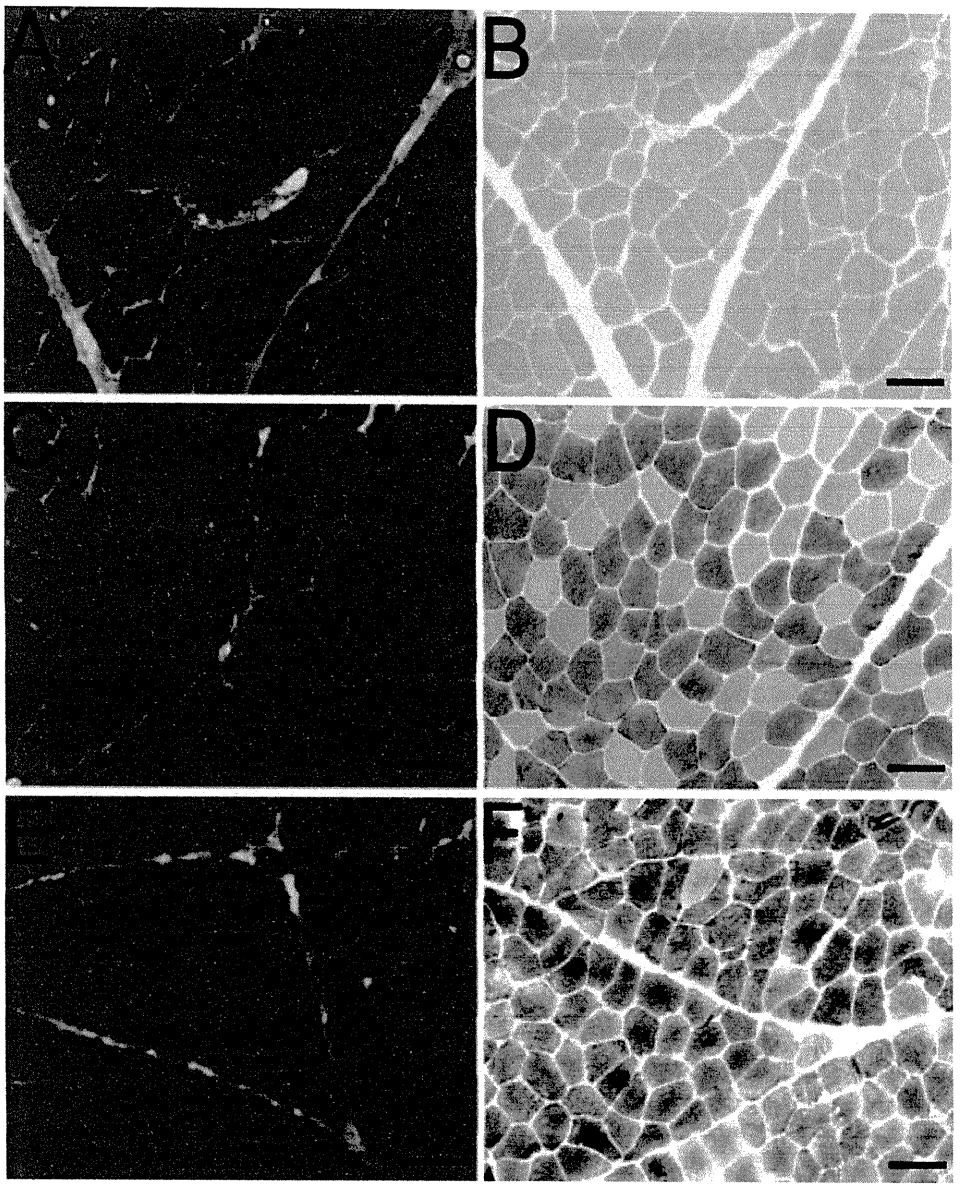

FIGURE 2. Muscle biopsy samples from the patient and a control subject. **(A, B)** Before treatment with vitamin B_6: **(A)** a sample stained with H&E; and **(B)** a sample stained with phosphorylase. Samples showed variation in muscle fiber size and frequent internal nuclei. No muscle fibers were stained with phosphorylase. **(C, D)** After treatment with vitamin B_6: **(C)** a sample stained with H&E; and **(D)** a sample stained with phosphorylase. The muscle fibers stained with phosphorylase increased markedly and muscle fiber size was almost uniform. **(E, F)** Control: **(E)** a sample stained with H&E; and **(F)** a sample stained with phosphorylase. Muscle biopsy samples before and after treatment were stained at the same time as the control sample. Bars = 100 μm.

muscle fibers, especially type 2C fibers. However, we believe that the increased muscle phosphorylase activity of the follow-up muscle biopsy at 1 year and 3 months after treatment was due to treatment with vitamin B_6 because enough time had passed since the episode of severe rhabdomyolysis, and there had been only mildly increased CK levels during treatment.

Lactate increased more dramatically on the forearm exercise test after treatment with higher doses of vitamin B_6 (90 mg/day, 1.6 mg/kg/day) than with lower doses (60 mg/day, 1.1 mg/kg/day). On the other hand, we decreased the dosage of vitamin B_6 from 90 to 60 mg/day in April 2009, because we believed that the toxicity of vitamin B_6 (90 mg/day) resulted in transient exacerbation of muscle weakness and a reduction in regular work activities in this period. However, we determined that the worsening was due to more severe physical work, and we returned the dosage to 90 mg/day in May 2009. Except for this episode, he has been in good condition under treatment with 90 mg/day of vitamin B_6. Thus, these results suggest that the effects of vitamin B_6 may depend on the dosage.

Many trial treatments other than oral vitamin B_6 supplementation have been used for McArdle

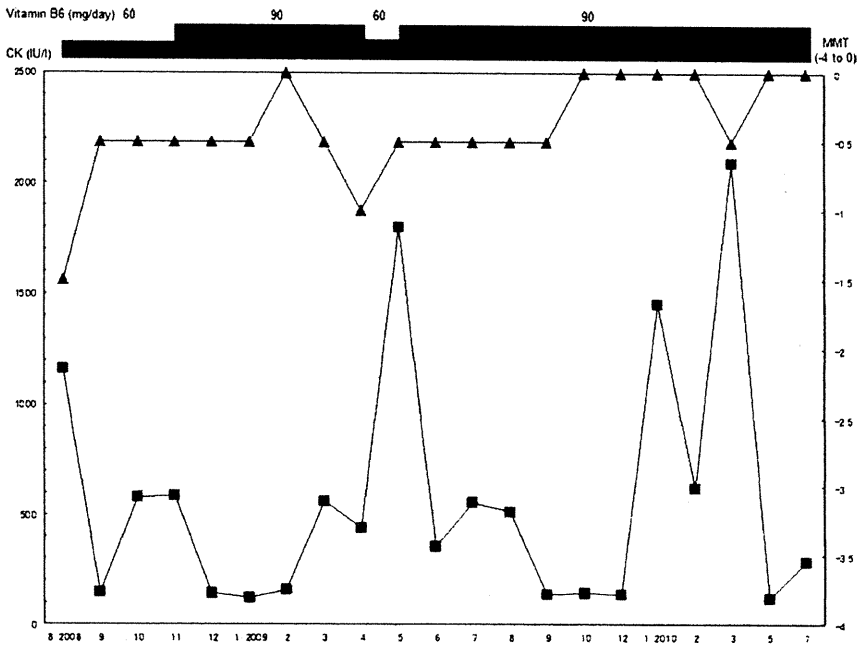

FIGURE 3. Clinical course of the patient. Muscle strength was evaluated by mean MMT of the neck flexors, deltoid muscles, and iliopsoas muscles. The dosage of vitamin B_6 was 60 mg/day from August 2008 to November 2008, 90 mg/day from November 2008 to April 2009, 60 mg/day from April 2009 to May 2009, and 90 mg/day from May 2009 onward. Squares: CK; triangles: MMT. Normal value of CK: 55–290 IU/L.

disease, such as high oral doses of ribose, a fat-rich diet, glucagon, verapamil, a high-protein diet, branched-chain amino acid supplementation, dantrolene sodium, low- or high-dose creatine, oral sucrose, intravenous gentamicin, a ketogenic diet, a high-carbohydrate diet, and ramipril. However, there has been no definitive evidence of any significant benefit from these treatments.[3] On the other hand, the withdrawal of vitamin B_6 supplementation from a patient after 2 years of daily administration resulted in decreased exercise tolerance and increased muscle cramps,[10] which suggested the efficacy of therapy with vitamin B_6 supplements. In addition, a Japanese patient with a very mild case of McArdle disease was treated with vitamin B_6 supplementation (90 mg/day) for 3 months, and the forearm exercise test showed improved glycogenolysis, as in our patient.[11]

In normal individuals, skeletal muscle contains at least 80% of the total body pool of vitamin B_6, bound as pyridoxal 5′-phosphate (PLP) to muscle phosphorylase. One molecule of PLP covalently bound to a lysine residue of each muscle phosphorylase subunit is essential for enzyme activity.[12,13] The decreased phosphorylase in McArdle disease substantially diminishes PLP in skeletal muscle.[12,13] The action of vitamin B_6 supplementation may require the presence of some residual muscle phosphorylase, as in our patient, and probably would not be seen in patients with null mutations, including the R50X mutation, which is most common among Caucasians.[1,14,15]

As noted earlier, most patients lack detectable muscle phosphorylase, as detected by sodium dodecylsulfate–polyacrylamide gel electrophoresis, immunoblot, and enzyme-linked immunosorbent assay.[1,4] This may result from rapid decay of unstable proteins. Thus, we hypothesize that vitamin B_6 supplementation can restore some stability to the mutant enzyme and enhance the residual phosphorylase activity in skeletal muscle of patients, followed by improvement in insufficient anaerobic glycolysis of skeletal muscle. However, other mechanisms are also possible.

Our study suggests that supplementation of vitamin B_6 may be an effective therapy for McArdle disease, especially for patients who have some residual muscle phosphorylase activity, although further studies, including a double-blind, placebo-controlled study, are necessary to draw firm conclusions about the effects of vitamin B_6 supplementation.

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RELAPSED ACUTE MYELOGENOUS LEUKEMIA OF BRACHIAL PLEXUS AFTER MARROW TRANSPLANT

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ABSTRACT: We present a detailed description of brachial plexus infiltration by acute myelogenous leukemia (AML) in the setting of a remission bone marrow biopsy, without evidence of leukemia by flow cytometric analysis. This case illustrates the possibility of dormant leukemic cells in the peripheral nervous system (PNS) in a patient in apparent clinical remission. In patients with an unexplained brachial plexopathy and a history of AML, leukemic infiltrate of the PNS must be considered.

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Metastatic tumors to the brachial plexus are a relatively rare disease entity. Involvement of the brachial plexus by metastatic tumors occurs in most instances via direct extension of the tumor or by means of lymphatic or hematogenous spread.[1] Primary tumors with reported metastases to this region of the peripheral nervous system most frequently include carcinomas of the breast and lung, lymphomas, and melanoma.[2] Although involvement of peripheral nerves by a leukemic infiltrate has been reported rarely, this is a detailed description of brachial plexus pathology by a leukemic infiltrate based on immunohistochemical studies. We describe a patient who had a peripheral nervous system (PNS) relapse of acute myelogenous leukemia (AML) manifested by brachial plexopathy. Of particular interest is that the patient had received a gender-mismatched bone marrow transplant 6 years earlier. The relapse occurred in the setting of a remission bone marrow biopsy with a normal female donor karyotype and with no evidence of leukemia by flow cytometric analysis. A normal complete blood count (CBC) had been present on multiple tests over 6 years.

Abbreviations: ALL, acute lymphoblastic leukemia; AML, acute myelogenous leukemia; CBC, complete blood count; CLL, chronic lymphocytic leukemia; CNS, central nervous system; CSF, cerebral spinal fluid; EMG,

Key words: brachial plexus, myelogenous leukemia, peripheral nerve metastasis, transplant

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CASE REPORT

History and Neurological Examination. A 33-year-old man was diagnosed with AML when he presented with a hemoglobin of 8.9 g/dl, hematocrit of 26%, leukocytosis [55,000 white blood cells (WBC)/μl], and thrombocytopenia (121,000 platelets/μl). Peripheral blood smear evaluation revealed an abnormal white cell differential with 91% blasts. A bone marrow aspirate and biopsy showed AML with maturation, based on the World Health Organization (WHO) classification.[3] Flow cytometric analysis of the blasts revealed immunophenotypic features indicative of myeloblasts (CD34, CD117, CD33, HLA-DR, CD15, and CD13 positive). Cytogenetic analysis of the bone marrow revealed a trisomy 8 karyotype.

The patient went into remission after chemotherapy, which consisted of daunorubicin and cytarabine (Ara-C), but 1 year later he had a relapse followed by leukemic meningitis. He received intrathecal Ara-C and high-dose intravenous Ara-C (2 g/m^2) and later underwent a gender-mismatched allogeneic bone marrow transplant. His chemotherapeutic regimen for the transplant consisted of ^{131}I monoclonal antibody and fludarabine in addition to low-dose total body radiation. He again went into remission, but his course was complicated by graft-versus-host disease

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ORIGINAL ARTICLE

Copy-number variations on the X chromosome in Japanese patients with mental retardation detected by array-based comparative genomic hybridization analysis

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X-linked mental retardation (XLMR) is a common, clinically complex and genetically heterogeneous disease arising from many mutations along the X chromosome. Although research during the past decade has identified >90 XLMR genes, many more remain uncharacterized. In this study, copy-number variations (CNVs) were screened in individuals with MR from 144 families by array-based comparative genomic hybridization (aCGH) using a bacterial artificial chromosome-based X-tiling array. Candidate pathogenic CNVs (pCNVs) were detected in 10 families (6.9%). Five of the families had pCNVs involving known XLMR genes, duplication of Xq28 containing *MECP2* in three families, duplication of Xp11.22-p11.23 containing *FTSJ1* and *PQBP1* in one family, and deletion of Xp11.22 bearing *SHROOM4* in one family. New candidate pCNVs were detected in five families as follows: identical complex pCNVs involved in dup(X)(p22.2) and dup(X)(p21.3) containing part of *REPS2*, *NHS* and *IL1RAPL1* in two unrelated families, duplication of Xp22.2 including part of *FRMPD4*, duplication of Xq21.1 including *HDX* and deletion of Xq24 noncoding region in one family, respectively. Both parents and only mother samples were available in six and three families, respectively, and pCNVs were inherited from each of their mothers in those families other than a family of the proband with deletion of *SHROOM4*. This study should help to identify the novel XLMR genes and mechanisms leading to MR and reveal the clinical conditions and genomic background of XLMR.
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Keywords: array CGH; *FRMPD4*; *HDX*; *MECP2*; pCNV; *PQBP1*; *SHROOM4*; XLMR

INTRODUCTION

Mental retardation (MR) is characterized by nonprogressive cognitive impairment and affects 1–3% of the general population. The predominance of males in the MR population has been attributed to genes located on the X chromosome. In fact, individual X-linked genes were recently estimated to contribute to 10–12% of all MR cases in males.[1] X-linked MR (XLMR) conditions have been divided into syndromic (MRXS representing approximately one-third of XLMR) and nonsyndromic (MRX representing approximately two-third of XLMR).[2] As MRX have no obvious and consistent phenotypes other than MR, XLMR conditions are clinically diverse and genetically heterogeneous disorders. In excess of 215 XLMR conditions have been recorded (http://xlmr.interfree.it/home.htm and http://www.ggc.org/xlmr.htm) and 90 XLMR genes have been identified.[3,4] Genes for 87 conditions have been mapped by linkage analysis and/or cytogenetic breakpoints, but for 38 conditions, genes have been neither identified nor mapped to candidate loci. In addition, more than 300 X-linked protein-coding genes are expressed in brain tissue, suggesting that many XLMR genes remain to be unidentified.[5]

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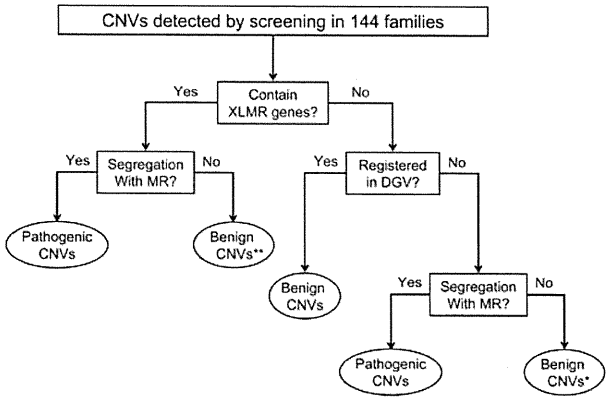

Figure 1 The flowchart of the screening of MR-associated pathogenic CNV. DGV means database of genomic variants. Asterisks indicate types of benign CNVs corresponding to asterisks in Supplementary Table S1.

Array-based comparative genomic hybridization (aCGH) has revealed copy-number variations (CNVs) to be the cause of MR.[6–8] Although Tarpey *et al.*[4] screened for mutations in the coding regions of 718 genes on the X chromosome in probands from 208 families by means of resequencing, only three XLMR-associated genes have been identified, suggesting structural variations other than point mutations, including CNVs or variants in regulatory regions, to contribute to unidentified XLMR conditions.

In this study, we examined CNVs in individuals with MR from 144 families with at least one affected male by aCGH using an in-house bacterial artificial chromosome (BAC)-based X-tiling array (MCG X-tiling array).[9] We detected 10 candidate pathogenic CNVs (pCNVs) according to a flowchart of our procedure (Figure 1), suggesting that pCNVs on the X chromosome could be found at a constant rate by the high-density aCGH in heterogeneous MR patients and our approach is useful to identify known as well as novel XLMR genes, resulting in a better understanding of the clinical conditions and genetic background of XLMR, although further study is needed to assess the significance of candidate XLMR-related genes.

MATERIALS AND METHODS

Patients

We selected 144 families with at least one male having MR. 'Familial type' MR, that is more than two members of the family affected, was identified in 76 families and 'sporadic type' MR, that is only one male affected, was found in 68 families. The male probands were subjected to an aCGH using the MCG X-tiling array.[9] In 131/144 cases, conventional karyotyping was performed, and an abnormal karyotype of 46,XY,der(18)t(5;18)(p13;p11.3)pat(20/20) was detected in one case.

Cell culture

Peripheral blood samples were obtained with informed consent approved by the Institutional Review Board, National Center of Neurology and Psychiatry, Japan. Epstein–Barr virus-transformed lymphoblast cell lines (LCLs) were established from peripheral blood cells. All LCLs were cultured in RPMI1640 medium supplemented with 10% fetal bovine serum and antibiotics.

aCGH using an in-house BAC array

aCGH hybridization using the MCG X-tiling array was performed as described previously with DNA extracted from sex-matched normal lymphocytes as a reference.[10] Acquired images from hybridized slides were analyzed with GenePix Pro 6.0 (Axon Instruments, Foster City, CA, USA). Fluorescence ratios were normalized so that the mean of the middle third of $\log_2$ ratio across the array was zero. The thresholds for copy-number gain and loss were set at log2 ratios of 0.4 and −0.4, respectively.

High-density oligonucleotide aCGH

A genome-wide oligonucleotide aCGH was performed using 244K (Agilent Technologies, Santa Clara, CA, USA) according to the directions provided by the manufacturer. The hybridized arrays were scanned using an Agilent scanner, and the CGH Analytics program version 3.4.40 (Agilent Technologies) was used to analyze copy-number alterations after data extraction, filtering and normalization by Feature Extraction software (Agilent Technologies).

FISH

Metaphase chromosomes were prepared from normal peripheral lymphocytes and from each of the LCLs in all family members using the standard method. Fluorescent *in situ* hybridization (FISH) analyses were performed as previously described,[10] using BAC clones located around the region of interest as probes.

Quantitative real-time reverse transcriptase-PCR

cDNAs were synthesized from total RNA extracted from LCLs established from the patients, their parents and six normal controls (three males and three females). Quantitative real-time reverse transcriptase PCR was performed with the ABI PRISM 7500 sequence detection System (Applied Biosystems, Foster City, CA, USA) using TaqMan Gene Expression Assays (Hs00202185_A1 FTSJ1, Hs00172868_A1 PQBP1, Applied Biosystems) according to the manufacturer's instructions. mRNA levels of the genes of interest were normalized against a housekeeping gene, *GAPDH* (Hs9999905_A1 GAPDH, Applied Biosystems), as an internal control to collect the relative expression data. Each assay was performed in triplicate for each sample.

The *androgen receptor* X-inactivation assay and late replication assay

The pattern of X-chromosome inactivation in the females was first evaluated using the *androgen receptor* X-inactivation assay[11] with minor modifications. Briefly, DNA was modified with sodium bisulfite and amplified with primers specific for a methylated or unmethylated DNA sequence at the human *androgen receptor* locus where methylation correlates with X-inactivation. Two different sized products, which were gained from the paternal and maternal alleles because of the polymorphism of the triplet repeat, were analyzed on a 3130 Genetic Analyzer (Applied Biosystems), and peak images of each PCR product were measured by GeneMapper Software v4.0 (Applied Biosystems). An imbalance of X-chromosome inactivation (skewing) was judged from the ratio between the amount of PCR product from paternal and maternal alleles. These ratios were corrected using a calculation previously described.[11]

A late replication assay was performed using a replication G-banding technique as previously reported[12] with minor modifications. Metaphase chromosomes were prepared with adjunction of 5-bromo-2-deoxiuridine in the last 6 h of cell culture after thymidine synchronization. The chromosome slides were stained with Hoechst 33258 (1 mg ml^{-1}) (Sigma, Saint Louis, MO, USA) for 5 min, and exposed to 254-nm ultraviolet light (Stratalinker UV Crosslinker 1800; Agilent Technologies) at a distance of 20 cm for 10 min after heating at 75 °C for 10 min. These chromosomes were used for FISH to estimate the ratio of inactivation of the affected X chromosome.

RESULTS

Classification of CNVs

We screened CNVs on the X chromosome in probands of 144 families with at least one affected male, by array CGH using the MCG X-tiling array to identify novel XLMR-related genes. We designed a flowchart for the classification of CNVs (Figure 1). If we detected a CNV containing known XLMR-related genes or of unknown biological or clinicopathological significance (National Center for Biotechnology

Information, http://www.ncbi.nlm.nih.gov/) and not registered in the Database of Genomic Variants (DGVs, http://projects.tcag.ca/variation/) in the male proband, we examined other family members using FISH. If the same CNV was segregated into cases of MR in the same family, it was considered a candidate for a pCNV, although CNVs observed in unaffected females in the same family or sporadic type were not excluded. Consistent with previous reports (5/108=4.6%[13] or 8/54=14.8%[14]), putative MR-associated pCNVs were detected in 10 families (6.9%, Table 1; Figure 2). The CNVs detected in five families contained known XLMR genes, whereas five candidate pCNVs seemed to be novel, although their pathogenic significance will need to be determined. The detection rates for the 'familial type' and 'sporadic type' were 7.9% (6/76) and 5.9% (4/68), respectively, suggesting that we cannot ignore the 'sporadic type' in the screening of candidate pCNVs. Each of pCNVs detected in 9 of 10 families was inherited from probands' mothers, respectively, suggesting that those CNVs were not altered through the establishment of each of the Epstein–Barr virus-transformed LCLs. In these 10 families, no abnormality was detected by conventional cytogenetics. In addition, no CNV possibly related to MR was detected in autosomes with the high-density oligonuculeotide array. Family trees of the 10 families are presented in Figure 3.

Frequent duplication at Xq28 including *MECP2*

Duplications at Xq28 including *MECP2* (OMIM 300005) were observed in 3 of 144 families (2.1%; MRYB6, MR1P3 and MR347 families in Table 1). These patients had several common phenotypes, such as severe MR, muscular hypotonia, absence of speech and recurrent respiratory infections as reported,[15–19] although the size of genes within the affected regions differed among the three families (Supplementary Figure S4a). The smallest region of overlap was ~437 kb and contained 13 genes including *L1CAM* (OMIM 08840) and *MECP2*. FISH using an *MECP2*-specific probe revealed that the mothers in all three families were carriers (Supplementary Figures S1a, S1b and S1c) and had a skewed X-inactivation pattern (Table 1) and dominant late replication pattern of the *MECP2*-duplicated allele (data not shown). In patients and the mother of family MR347, an ~182-kb deletion at Xp22.31, which contains no protein-coding gene (Supplementary Figure S4b), was detected simultaneously by aCGH analyses using an X-tilling array and Agilent oligonucleotide array, suggesting this CNV to be of unknown biological or clinical pathological significance. In addition, the CNVs at autosomal region not registered in the DGV were detected in each of the probands in three families. (Supplementary Table S2) According to ISCN 2009,[20] these CNVs are as follows: MRYB6 had arr 15q21.2 (50 711 956–50 777 075)×1; MR1P3 had arr 20p13(897 451–956 849)×1; MR347 had arr 4q13.1(61 867 547–61 924 356)×1 and arr 15q23(65 693 871–65 713 056)×1.

Aberrations at Xp11.22-p11.23 detected in two families contain known MR-related genes

We detected candidate pCNVs at Xp11.22-p11.23 in 2 of 144 families (1.4%; MR67H and MRF91 in Table 1), although the affected regions showed no overlap between these two families (Figure 4).

MRF91

The male proband (III-1) of family MRF91 showed moderate MR and speech delay. In this patient, an ~1.37-Mb duplication at Xp11.23 was detected (Figure 2). Information from the UCSC genome browser (http://genome.ucsc.edu/cgi-bin/hgGateway) revealed that the duplicated region is gene-dense, and includes three known MR-related genes, *FTSJ1* (OMIM 300499), *PQBP1* (OMIM 300463) and *SYP* (OMIM 313475). No mutation was detected in those genes in the proband (data not shown). FISH revealed the duplication in the proband (III-1), mother (II-2) and his affected younger sister (III-2) (Supplementary Figure S1d). The mRNA levels of *FTSJ1* and *PQBP1* in LCLs determined by quantitative real-time reverse transcriptase-PCR were highest in the proband (Supplementary Figure S2). *SYP* mRNA levels could not be evaluated due to low expression in LCL. The X-chromosome inactivation in LCL showed a skewed pattern in the unaffected mother and random pattern in the affected sister (Supplementary Figure S3a). In addition, dup(X)(p11.23) showed a late replicating pattern in 39/50 cells (78%) of the unaffected mother and 24/50 cells (48%) of the affected sister (Supplementary Figure S3b). The high-density oligonucleotide aCGH revealed that the duplication at Xp11.23 in family MRF91 was flanked distally by a segmental duplication containing a synovial sarcoma X breakpoint families (*SSX1*, *SSX9*, *SSX4*, *SSX3* and *SSX4B*) and proximally by an additional segmental duplication containing G-antigen (*GAGE*) families (Figure 4). The aberration is as follows: arr Xp11.23 (48 089 045–49 246 795)×2 mat.

MR67H

The male proband of family MR67H showed only moderate MR. In this patient (III-1), an ~2.86-Mb deletion at Xp11.22, which has never been reported, was detected (Figure 2). Information from the UCSC genome browser revealed that the deleted region contains *SHROOM4* (OMIM 300579), reported to be a causative gene for XLMR.[21] Sample of his mother (II-4) was not available. The high-density oligonucleotide aCGH revealed that the deletion at Xp11.22 in family MR67H was also flanked distally by a sequence gap and proximally by a complex repeat-rich locus containing *SSX* families (*SSX7* and *SSX2*), *melanoma antigen* (*MAGE*) families and *X-antigen* (*XAGE*) families (Figure 4). The aberration is as follows: arr Xp11.22 (50 040 995–52 710 691)×0.

Other novel candidate pCNVs in five families

Identical complex pCNVs detected in nonconsanguineous MR22T and MRK13 families. The proband (III-1) of MR22T was diagnosed with West syndrome from electroencephalogram and showed severe MR, epilepsy, absence of speech and atrophy of the hippocampus, whereas patients (II-1, II-2) of family MRK13 manifested moderate MR, speech delay and autistic feature. Although the MR22T and MRK13 families are not consanguineous, identical duplications at the same two loci were detected: dup(X)(p22.2) containing part of *NHS* (OMIM 300457) and part of *REPS2* (OMIM 300317), and dup(X)(p21.3) containing part of *IL1RAPL1* (OMIM 300206), which was identified as an XLMR-related gene (OMIM 300143) (Supplementary Figures S4c and d). The aberration is as follows: arr Xp22.2 (16 898 131–17 635 375)×2 mat and arr Xp21.3 (28 711 594–28 812 042)×2 mat. The X-chromosome inactivation of mothers in both families showed a skewed pattern (Table 1). FISH analysis revealed that the signal for a BAC RP11-438J7 at Xp21.3 appeared separately at Xp21.3 and Xp22.2 and the signal at Xp22.2 could be detected more strongly (Figure 5).

MR1WK

The male proband (II-2) of family MR1WK showed mild MR and autism. An ~0.57-Mb duplication at Xp22.2 including a part of *FRMPD4* was detected in the patient. The high-density oligonucleotide aCGH revealed that the duplication also includes *MSL3* distal to *FRMPD4* (Supplementary Figure S4e). The aberration is as