表 1　Complex IV 欠損症関連の核遺伝子のまとめ

遺伝子	正式名称	局在	OMIM	code しているタンパク・働き	遺伝形式	報告されている疾患
サブユニット異常						
COX6B1	cytochrome c oxidase subunit VIb polypeptide 1	19q13.1	124089	サブユニット 6B	A.R.	乳児ミトコンドリア病
COX4I2	cytochrome c oxidase subunit IV isoform 2	20q11.21	607976	サブユニット 4 isoform 2	A.R.	膵外分泌不全，赤血球異型性貧血（Dyserythropoeitic Anemia），頭頂部の頭蓋骨骨化過剰の合併症例
COX7B	cytochrome c oxidase subunit VIIb	Xq21.1	300887	サブユニット VIIb	A.R.	先天性皮膚形成不全症（APLCC）
NDUFA4	NADH dehydrogenase（ubiquinone）1 alpha subcomplex, 4, 9kDa	7p21.3	603833	additional COX subunit	A.R.	Leigh 脳症
アセンブリータンパク・呼吸鎖生合成（biogenesis）の異常						
SURFI	surfeit 1	19q13.12	185620	アセンブリー因子	A.R.	Leigh 脳症や重篤な神経疾患
SCO2	SCO cytochrome oxidase deficient homolog 2	22q13.33	604272	copper transport	A.R.	乳児ミトコンドリア病（心筋症，脳筋症）
SCO1	SCO cytochrome oxidase deficient homolog 1	17p13.1	603644	copper transport	A.R.	乳児ミトコンドリア病（心筋症，脳症，肝症）
COX10	COX10 homolog, cytochrome c oxidase assembly protein, heme A: farnesyltransferase	17p12	602125	Heme A farnesyltransferase	A.R.	尿細管障害を伴う脳症，Leigh 脳症，乳児ミトコンドリア病（難聴，心筋症）
COX15	COX15 homolog, cytochrome c oxidase assembly protein	10q24	603646	Heme A synthesis	A.R.	Leigh 脳症（長期生存例あり）や乳児ミトコンドリア病（脳症，心筋症，小頭症）
COX20	COX20 cytochrome C oxidase assembly factor	1q44	614698	アセンブリー因子	A.R.	小児の成長障害，筋緊張低下，小脳失調
ETHE1	ethylmalonic encephalopathy 1	19q13.31	608451	sulfur dioxygenase ; Ethylmalonic acid metabolism	A.R.	エチルマロン酸脳症
FASTKD2	FAST kinase domains 2	2q33.3	612322	アポトーシスに関与？	A.R.	乳児ミトコンドリア病（脳症）
COA（*C2orf64*）	cytochrome c oxidase assembly factor 5	2q11.2	613920	アセンブリー因子	A.R.	新生児期のミトコンドリア心筋症
ミトコンドリア DNA の発現に関わる異常						
LRPPRC	leucine-rich pentatricopeptide repeat containing	2p21	607544	アセンブリー因子	A.R.	Leigh脳症（French-Canadian Leigh disease）

る．はじめにサブユニット異常についてである．

COX6B1 遺伝子異常については，2008 年にこの遺伝子異常により重篤な乳児の脳筋症をきたした症例が報告された[4]．数少ないサブユニットを code した核の遺伝子である．

COX4I2 遺伝子異常は，脂肪吸収障害を伴う膵外分泌不全(cystic fibrosis や Shwachman-Diamond 症候群や Pearson 症候群などで見られる)や，赤血球異型性貧血(dyserythropoeitic Anemia)や，頭頂部の頭蓋骨骨化過剰きたす症例で見つかっている[5]．

次はアセンブリータンパクの異常である．

Surf1 は病因遺伝子としては最も有名であり，complex IV 欠損によって生じる Leigh 脳症の主要因となる遺伝子である．また，その他の重篤な神経疾患や多臓器障害もきたす．特に新生児期〜乳児期に発症することが多い[6]．

SCO1 および *SCO2* はミトコンドリア内の銅の成熟化とサブユニット II の合成に関わる．前者は肝症＋脳症，心筋症＋脳症を引き起こした報告がある[7][8]．

COX10 は Heme A farnesyltransferase をコードしており complex IV の機能発現と Complex IV 内のヘム A に関わる成熟機構に関係する．この遺伝子の phenotype は，表 1 に示すように Leigh 脳症を含めさまざまである．Leigh 脳症の症例や乳児ミトコンドリア病の症例は輸血が必要な重度の貧血をきたしている[9]．初回報告例は尿細管障害を伴う脳症としての報告であるが，本遺伝子異常に特異的ではないだろう．

COX15 は HemeA 合成に関わる遺伝子であり，complex IV の生合成に関わるタンパクである．Leigh 脳症の長期生存例の報告がある一方，新生児早期の致死性心筋症でも報告がある[10]．

ETHE1 はエチルマロン酸血症(脳症)として知られ，ミトコンドリアマトリックス内にある硫化

図1　哺乳類の complex IV アセンブリーモデル
(Fornuskova D, et al: Novel insights into the assembly and function of human nuclear-encoded cytochrome c oxidase subunits 4, 5a, 6a, 7a and 7b. Biochem J 428: 363-374.2010 より引用)

物の異化に関わる sulfur dioxygenase をコードしている遺伝子である．硫化物は強力な complex IV 阻害物質であるため，何らかの硫黄物による complex IV 活性の間接的な低下が機序として示唆されている．エチルマロン酸血症は，タンデムマス(アシルカルニチン分析，C4-6 上昇)および尿中有機酸分析にて化学診断が可能である．

FASTKD2 はこれまで新生児のミトコンドリア病(脳症)の報告がある[11]．*FASTKD2* はミトコンドリア内部分画のタンパクを code しており，アポトーシスへの関与が考えられている．

C2orf6 は Complex IV のアセンブリー過程の早期に関与する因子と考えられており，新生児の肥大型心筋症として報告されている[12]．

LRPPRC はケベック州(Saguenay-Lac-Saint-Jean 地域)のフランス系カナダ人に好発する Leigh 脳症を起こす病因遺伝子として知られている[13]．この遺伝子はサブユニットやアセンブリータンパクではなく，mtDNA の発現に関わることがわかってきた．

NDUFA4 は complex I のサブユニットをコードしている遺伝子と思われたが，実際には Leigh 脳症の患者で complex IV のサブユニットであることが示唆された[14]．

診断と鑑別診断

本症の診断は，臨床症状や CT，MRI などの画像所見，生化学所見などから呼吸鎖異常症を疑い，皮膚線維芽細胞，または障害臓器(肝臓，筋肉，心筋)などを用いて呼吸鎖酵素活性を測定することである．Complex III のアッセイと同様にこの酵素アッセイは非常に速いため，手際よく測定しないと偽性 complex IV 欠損症をつくってしまう．また，COX 染色は病理検査としても行われており，合わせて評価することは有意義である．

治療と予後

本症の根治的治療法はなく，一般的に高脂肪食およびミトコンドリアカクテル等を使用していくことになる．

Complex IV 欠損症に関して，ベザフィブレート(Bezafibrate: BZF)を使用することにより，complex IV の活性が上昇し ATP 産生が増すことが *COX10* や他のアセンブリー因子欠損マウスで示されており[15]，さらに complex IV 欠損症症例の培養細胞でも complex I, III, IV 活性の上昇効果が示されている．

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e) Complex V（ミトコンドリア呼吸鎖複合体V）欠損症
Complex V deficiency

疾患の要点

- Complex V が欠損ないし活性が低下することにより，エネルギー産生低下が起こり臓器障害を引き起こす
- ミトコンドリア DNA および核DNA の両方とも原因になりうる
- Complex V 欠損症の頻度は，酵素診断されているうち約4% が complex V 欠損症である mtDNA の異常は酵素診断されることは少なく，それを含めると相当数になると思われる
- mtDNA の異常では，Leigh 脳症，MELAS，（運動不耐性）ミオパチー，LHON，急性脳症，心筋症，前立腺癌など臨床病型は様々であり，重症度は症例によって異なる．核DNA では新生児ミトコンドリア病（先天性高乳酸血症）が多く，生化学的に 3-メチルグルタコン酸尿症を呈することが多い
- 診断は Complex V の低下・欠損を酵素学的に証明することである（*in vitro* 酵素活性，BN-PAGE 解析など）

■欠損酵素：ATP 合成酵素（ATP sythetase），EC 3.6.1.14
■遺伝情報／遺伝形式／OMIM：表 1 中に記載

ミトコンドリア呼吸鎖複合体 V(complex V)は ATP 合成酵素(ATP sythetase)であり，呼吸鎖の最終段階に位置する．電子伝達によって complex I, III, IV は H^+低濃度のマトリックスから H^+高濃度の膜間部(サイトゾルにつながっている)にプロトンを汲み出す．こうして生じる電気化学勾配(プロトン駆動力)に従ってプロトンが膜間腔からマトリックス内に流入するときに，内膜に結合した ATP 合成酵素が ADP と無機リン酸から ATP を合成する(図 1)[1].

この complex V ＝ ATP 合成酵素の欠損ないし活性が低下によってエネルギー産生が低下して臓器障害を引き起こすものが，ミトコンドリア呼吸鎖複合体 V 欠損症(complex V 欠損症)である．Complex V 欠損症の臨床診断としては Leigh 脳症，新生児・乳児ミトコンドリア病，心筋症などさまざまである．

疫　学

Complex V 欠損症の頻度は，はっきりと記載されたものは少ない．Honzik らはオーストリアとチェコでの 20 年間で新生児に発症した 129 人の MRCD 患者のうち，31 人(24%)が complex V 欠損であると報告しており，欧州(特に東欧地域)における新生児発症 MRCD においては大きな割合を占めている[2]．その多くは *TMEM70* 遺伝子異常である．また，Rodenberg の論文を読み解くと，酵素診断されているうち約 4% が complex V 欠損症(ちなみに complex I 欠損が 31%，複数の呼吸鎖欠損が 27%，complex IV 欠損が 19%，complex III 欠損が 12%，complex II 欠損が 7%)である[1]．しかし complex V 欠損症の多くの症例は，新生児のミトコンドリア病であり，高アンモニア血症を認めたり，尿中有機酸分析にて 3-メチルグルタコン酸が検出されたりすることが多く，診断の十分な手がかりとなる．筆者らは全国の新生児高乳酸血症や高アンモニア血症の症例に対して，尿中有機酸分析を含む代謝スクリーニング検査を行う機会が多いが，3-メチルグルタコン酸はほとんど検出されておらず，わが国での発症は欧州ほど多くことが予想される．

mtDNA 由来では a，A6L サブユニットをコードしている，*MT-ATP6*，*MT-ATP8* 遺伝子が病因遺伝子として挙げられる．特に *MT-ATP6* 遺伝子異常による Leigh 脳症をはじめとしたミトコンドリア病の報告は多い．

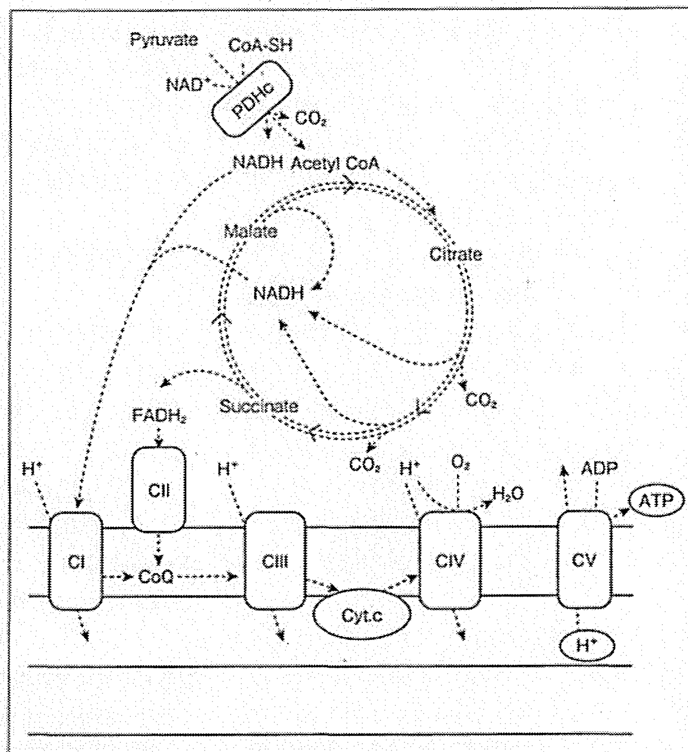

図１　TCA回路およびミトコンドリア呼吸鎖の模式図
（Rodenburg RJ: Biochemical diagnosis of mitochondrial disorders. J Inherit Metab Dis 34: 283-292. 2011より抜粋，改変）

病因・病態

現在病因遺伝子として報告されているものは，本酵素のサブユニット異常およびアセンブリー因子を含む呼吸鎖生合成(biogenesis)の異常である．これらの異常に伴いcomplex Ⅴの酵素活性が低下し，症状が出現する．本症は，新生児期に症状を呈することが多い．

以下，既知の病因遺伝子について述べる．

mtDNA由来の病因遺伝子としては，サブユニットaをcodeしている*MT-ATP6*とサブユニットA6Lをcodeしている*MT-ATP8*である．Complex Ⅴ欠損症で最初に発見された遺伝子異常は，*MT-ATP6*である[3]．これまで*MT-ATP6*遺伝子内に20を超えるの点変異の報告がなされている(http://www.mitomap.org/MITOMAP)．特に m.8993T>G/Cおよびm.9176T>G/C変異は，孤発性運動失調，NARP (Neuropathy, Ataxia, and Retinitis Pigmentosa)，両側線条体壊死，Leigh脳症(MILS; Maternally Inherited Leigh Syndromeともいう)といった症状を呈することが多い[4]．その他多くの変異が報告されているが，Leigh脳症，MELAS，(運動不耐性)ミオパチー，LHON，急性脳症，心筋症，前立腺癌など臨床病型はさまざまであり，重症度は症例によって異なる．*MT-ATP8*遺伝子異常によるMRCDの報告は2008年から，脳症，心筋症などが報告されている[5][6]．

次に核DNA由来の既知の変異について述べる(表1)．サブユニット異常に関して，現在報告されている遺伝子は*ATP5E*(サブユニットε)と*ATP5A1*(サブユニットα)でありかつ，それぞれ1例ずつの報告である．*ATP5E*遺伝子は22歳女性の報告で，新生児期に発症し，乳酸アシドーシスと3-メチルグルタコン酸高値を認め，軽度の精神遅滞，末梢神経障害を呈しているという報告である[7]．ATP5A1遺伝子は新生児の進行性脳症の原因として報告されている[8]．

最も多い核由来の異常は*TMEM70*(Transmem-

brane protein 70)である．*TMEM70*はミトコンドリア内膜に関連しているタンパクで，complex Vの生合成に重要である(ancillary factor)ことがわかっている[5]．臨床的には新生児ミトコンドリア病を呈する．すなわち高乳酸血症，脳症，心筋症，奇形，新生児白内障，消化管の機能不全，先天性の筋緊張亢進など多彩な症状を呈しうる[9)10)]．Honzikは25症例の*TMEM70*異常症例をまとめている[10]．それによると早産やIUGRも7割近く認めている．無呼吸発作，筋緊張低下がともに92%，肥大型心筋症が76%，停留精巣が67%，尿道下裂が54%，高乳酸血症(5～36mmol/L)を92%，高アンモニア血症(100～520μmol/L)を86%に認めている．40%が6週以内に死亡しており，予後はよくない．この遺伝子異常はロマニー族(Romanies)に集積していることが明らかになっている．本症は尿中有機酸分析で3-メチルグルタコン酸高値が必発である．*ATPAF2*遺伝子は*ATP12*遺伝子とも言われ，complex Vのアセンブリータンパクである．*TMEM70*と同様に重症な新生児ミトコンドリア病を呈し，脳症，奇形等を呈した報告がある[11]．

診断と鑑別診断

本症は大きな特徴は，新生児期に発症することが多いことである．したがって新生児高乳酸血症や新生児心筋症などの中に含まれていることになる．Complex Vの酵素活性での診断は困難なことが多い．一部の新生児ミトコンドリア病は尿中3-メチルグルタコン酸の有無である程度鑑別できる．また，組織や培養細胞を用いたBN-PAGE解析は，他の呼吸鎖欠損と同様に診断の一助になる．

表2　ATP合成酵素の組成

バクテリア	哺乳類	酵母菌
α (55.2 kDa)	α (55.1 kDa)	α (55.3)
β (50.1)	β (51.6)	β (52.5)
γ (32.4)	γ (30.2)	γ (30.6)
δ (19.3)	OSCP	OSCP (20.9)
ε (14.9)	δ (15.1)	δ (14.5)
–	ε (5.7)	ε (6.6)
–	Inhibitor protein	
a	a	Subunit 8 (5.87)
b	b	Subunit 6 (27.9)
c	c	Subunit 9 (7.79)
–	d	d (19.66)
–	e	
–	f	b (P25)
–	g	
–	A6L	
–	F6	

治療と予後

本症の根治的治療法はなく，一般的に高脂肪食およびミトコンドリアカクテルなどを使用していくことになる．Complex V欠損症に特化した治療法は今のところ出てきていない．また，新生児ミトコンドリア病は多くは致死性の経過をとることが多く，予後は非常に厳しい．エクソーム解析やiPS細胞の活用など新しい技術によって，新たな治療展開が生まれることを期待したい．

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表 1　Complex V 欠損症関連の核遺伝子のまとめ

遺伝子	正式名称	局在	OMIM	コードしているタンパク・働き	遺伝形式	報告されている疾患
サブユニット異常						
ATP5E	ATP synthase, H^+ transporting, mitochondrial F1 complex, epsilon subunit	20q13.32	606153	subunit ε	A.R.	新生児ミトコンドリア病(22歳時に報告)
ATP5A1	ATP synthase, H^+ transporting, mitochondrial F1 complex, alpha subunit 1	18q21	164360, 615228	subunit α	A.R.	新生児ミトコンドリア病(進行性脳症)
呼吸鎖生合成（biogenesis）の異常						
ATPAF2 (*ATP12*)	ATP synthase mitochondrial F1 complex assembly factor 2	17p11.2	608918	F_1 のアセンブリー因子	A.R.	新生児ミトコンドリア病（脳筋症）
TMEM70	transmembrane protein 70	8q21.11	612418	Complex V の生合成に関わる，アセンブリー因子？	A.R.	新生児ミトコンドリア病（心筋症，脳筋症），3-メチルグルタコン酸尿症（ほぼ必発）

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ORIGINAL INVESTIGATION

A hemizygous *GYG2* mutation and Leigh syndrome: a possible link?

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Abstract Leigh syndrome (LS) is an early-onset progressive neurodegenerative disorder characterized by unique, bilateral neuropathological findings in brainstem, basal ganglia, cerebellum and spinal cord. LS is genetically heterogeneous, with the majority of the causative genes affecting mitochondrial malfunction, and many cases still remain unsolved. Here, we report male sibs affected with LS showing ketonemia, but no marked elevation of lactate and pyruvate. To identify their genetic cause, we performed whole exome sequencing. Candidate variants were narrowed down based on autosomal recessive and X-linked recessive models. Only one hemizygous missense mutation (c.665G>C, p.W222S) in glycogenin-2 (*GYG2*) (isoform a: NM_001079855) in both affected sibs and a heterozygous change in their mother were identified, being consistent with the X-linked recessive trait. *GYG2* encodes glycogenin-2 (GYG2) protein, which plays an important role in the initiation of glycogen synthesis. Based on the structural modeling, the mutation can destabilize the structure and result in protein malfunctioning. Furthermore, in vitro experiments showed mutant GYG2 was unable to undergo the self-glucosylation, which is observed in wild-type GYG2. This is the first report of *GYG2* mutation in human, implying a possible link between GYG2 abnormality and LS.

Introduction

Glycogen is a large branched polysaccharide containing linear chains of glucose residues. Glycogen deposits in skeletal muscle and liver serve as shorter-term energy storage in mammals, while fat provides long-term storage. Glycogen biosynthesis begins with self-glucosylation of glycogenins by covalent binding of UDP-glucose to tyrosine residues of the glycogenins and the subsequent extension of approximately ten glucose residues (Pitcher et al. 1988; Smythe et al. 1988). Glycogen particles are formed by the continued addition of UDP-glucose to the growing

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glycogen chain by glycogen synthase, and introduction of branches every 10–14 residues by the glycogen branching enzyme (Krisman and Barengo 1975; Larner 1953). To date, two glycogenin paralogues have been identified in human, glycogenin-1 (GYG1) and glycogenin-2 (GYG2). These proteins have been shown to form homodimers, heterodimers and larger oligomers (Gibbons et al. 2002). GYG1 (muscle form) is expressed predominantly in muscle while GYG2 (liver form) is expressed mainly in liver, heart and pancreas (Barbetti et al. 1996; Mu et al. 1997). Biallelic GYG1 abnormality is known to cause muscle weakness and cardiac arrhythmia in humans through GYG1 autoglucosylation failure (Moslemi et al. 2010). However, human disease due to GYG2 abnormality has never been reported.

Leigh syndrome (LS; MIM #256000) was first described as a subacute necrotizing encephalomyelopathy by Dr. Denis Leigh in 1951 (Leigh 1951). LS is a progressive neurodegenerative disorder with an estimated incidence of 1:40,000 live births (Rahman et al. 1996). Onset is usually in early childhood (typically before age 2) (Naess et al. 2009; Ostergaard et al. 2007). Clinical manifestations of LS are observed in the central nervous system (CNS) (developmental delay, hypotonia, ataxia, convulsion, nystagmus, respiratory failure and dysphagia), peripheral nervous system (polyneuropathy and myopathy) and extraneural organs (deafness, diabetes, cardiomyopathy, kidney malfunction and others) (Finsterer 2008). The neurological features depend on the affected regions and degree of severity. The presence of bilateral, symmetrical, focal hyperintense T2-weighted MRI signals in basal ganglia (mainly putamen), thalamus, substantia nigra, substantia ruber, brainstem, cerebellum, cerebral white matter or spinal cord is diagnostic of LS (Farina et al. 2002; Medina et al. 1990). Neuropathological studies revealed that these lesions reflect neuronal necrosis, gliosis and vascular proliferation (Brown and Squier 1996; Leigh 1951). In the majority of LS cases, lactate, pyruvate or the lactate/pyruvate ratio is increased in blood and cerebrospinal fluid (Finsterer 2008). To the best of our knowledge, 37 nuclear genes are known to be mutated in LS, in addition to some mitochondrial genes (Antonicka et al. 2010; Debray et al. 2011; Finsterer 2008; Lopez et al. 2006; Martin et al. 2005; Quinonez et al. 2013). Thus, inheritance patterns of LS include mitochondrial, autosomal recessive and X-linked recessive modes (Benke et al. 1982; van Erven et al. 1987).

We encountered a Japanese family with affected brothers showing atypical LS without marked elevation of lactic or pyruvic acid and unknown etiology. A unique genetic variant was identified by whole exome sequencing (WES), which may be associated with atypical LS phenotype in this family.

Materials and methods

Subjects

Peripheral blood samples of affected brothers diagnosed with LS and their parents were collected after obtaining written informed consent. DNA was extracted from peripheral blood leukocytes using QuickGene-610L (Fujifilm, Tokyo, Japan) according to the manufacturer's instructions. Lymphoblastoid cell lines derived from all family members were established. The Institutional Review Boards of Yokohama City University School of Medicine approved this study.

Causative gene identification

Whole exome sequencing was performed in two affected individuals (II-2 and II-3 in Fig. 1a) as described in the Supplementary methods. All candidate variants based on autosomal and X-linked recessive models were checked by Sanger sequencing in the parents and affected siblings. PCR products amplified with genomic DNA as a template were sequenced on an ABI3500xl autosequencer (Applied Biosystems, Foster City, CA) and analyzed using Sequencher 5.0 (Gene Codes Corporation, Ann Arbor, MI). As the pedigree tree might also indicate mitochondrial inheritance of this disease and LS is known to be caused by mitochondrial genome mutations, we screened the entire mitochondrial genome by the algorithm reported previously (Picardi and Pesole 2012), using exome data (detailed in Supplementary methods).

Structure modeling

To evaluate the effect of the GYG2 missense mutation (c.665G>C, p.W222S in isoform a: NM_001079855) on its function at the molecular structural level, the mutated molecular structure was constructed, and the free energy change caused by the mutation was calculated using the FoldX software (version 3.0) (Guerois et al. 2002; Khan and Vihinen 2010). As crystal structure of human GYG2 is unavailable, that of human GYG1 (Protein Data Bank code; 3T7O) was used as a structural model. The mutation was introduced into one subunit of the GYG1 homodimer. The ligands included in the crystal structure of GYG1 were ignored in the calculation, because the FoldX energy function could not deal with the ligands. The calculation was repeated three times, and the resultant data were presented as an average value with standard deviations.

Preparation for mammalian expression vectors

Human glycogenin-2 isoform a cDNA clone (IMAGE Clone ID: 100008747) integrated in pENTR221 was purchased from Kazusa DNA Research Institute (Chiba, Japan). The

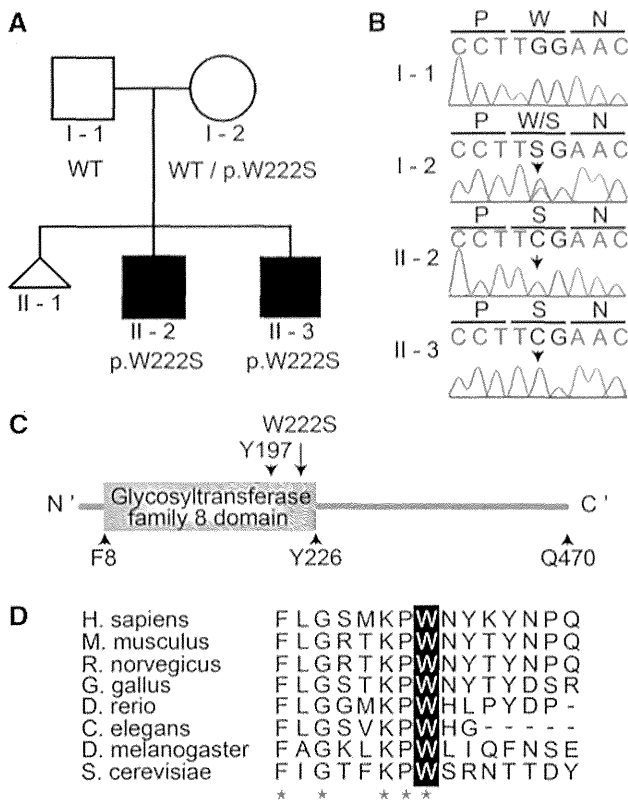

Fig. 1 Mutation Analysis of *GYG2*. **a** Pedigree of the family with a unique type of LS and a *GYG2* mutation (c.665G>C, p.W222S). *Square*, *circle* and *triangle* denote male, female and spontaneous abortion, respectively. *White* and *black symbols* indicate unaffected and affected individuals, respectively, while the affection status of the spontaneous abortion is unknown. **b** Electropherograms of a *GYG2* mutation. **c** The functional domain of human GYG2 (isoform a). The substitution of p.W222S is located within the glycosyltransferase family 8 domain (*yellow square*). **d** The evolutionary conservation of the W222 in GYG2. *Red stars* indicate identical amino acids from *S. cerevisiae* to *H. sapiens*. Sequences were aligned using CLUSTALW (http://www.genome.jp/tools/clustalw/)

missense mutation (c.665G>C, p.W222S) was introduced by Site-directed mutagenesis using the QuikChange II XL site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). Wild-type and mutant C' V5/6xHis tagged GYG2 constructs were created using pcDNA-DEST40 (Invitrogen, Carlsbad, CA) by LR recombination in Gateway system (Invitrogen). To create the untagged construct, the last codon was altered to a stop codon by mutagenesis.

Self-glucosylation analysis

Glucosyltransferase activity of GYG2 was measured as previously described (Lomako et al. 1988), with slight modifications. In brief, COS-1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, Schnelldorf, Germany) containing 10 % heat-inactivated fetal bovine serum (FBS) (Gibco-BRL, Grand Island, NY), 2 mM L-glutamine (Sigma-Aldrich) and 1 % penicillin–streptomycin (Sigma-Aldrich). As previously described (Mu and Roach 1998), the ~80 % confluent COS-1 cells (~1×10^7) were transiently transfected by X-treamGENE9 DNA transfection reagent (Roche Applied Science, Foster City, CA) with 5 μg of either a wild-type Human GYG2 (isoform a) expressing plasmid or the same plasmid into which the W222S encoding mutation had been introduced. After 24 h, the cells were collected and lysed in 300 μl of buffer consisting of 50 mM HEPES, 0.5 % Triton X-100, 1 × EDTA-free protease Inhibitor Cocktail tablets (Roche Applied Science), 1 × phosphatase inhibitor cocktail (Nacalai Tesque Inc., Kyoto, Japan) and 0.5 mM β-mercaptoethanol (Mu et al. 1997). After centrifugation at 14,000 rpm for 15 min, 10 μl of the soluble fractions were mixed with 10 μl of 2 × reaction buffer containing 100 mM HEPES (pH7.5), 10 mM MgCl, 4 mM dithiothreitol (DTT) and 40 μM UDP-[^{14}C]-glucose (250 mCi/mmol; PerkinElmer, Waltham, MA) (Cao et al. 1993). After incubation at 30 °C for 30 min, the reaction was stopped by addition of 20 μl of 2 × Laemmli sample buffer (Sigma-Aldrich) (Viskupic et al. 1992). 15 μl of each sample was subjected to SDS-polyacrylamide gel electrophoresis. After treatment with Gel drying solution (Bio-Rad Laboratories, Hercules, CA) for 30 min, gels were dried. Dried gels were then exposed on X-ray film for 2 weeks to detect the incorporation of UDP-[^{14}C]-glucose into GYG2. In addition, the ^{14}C-signal intensities were evaluated using an imaging analyzer, BAS2500 (Fujifilm). Three independent experiments were performed.

Western blot analysis

For the detection of GYG2 protein, rabbit polyclonal anti-GYG2 antibodies (1:500 dilution; Abcam Inc., Cat.#HPA005495, Cambridge, MA) and horse-radish peroxidase (HRP)-conjugated anti-rabbit IgG (1:10,000 dilution; Jackson ImmunoResearch, Cat.#111-035-003, West Grove, PA) were used. Immunoblot chemiluminescence was performed using SuperSignal West Dura as substrate (Thermo Fisher Scientific, Waltham, MA). The chemiluminescence signal images were captured by FluorChem 8900 (Alpha Innotech, San Leandro, CA). Signal intensities were measured by AlphaEase FC (Alpha Innotech). Three independent experiments were performed.

Results

Clinical finding

Patient II-2 (Fig. 1a; Table 1) is a 26-year-old male born to non-consanguineous parents. His mother previously had a

Table 1 Clinical features of the presenting patients affected with LS

	II-2	II-3
Sex	M	M
Age (years)	26	19
Common clinical phenotype		
Psychomotor retardation	+	+
Failure to thrive	+	+
Swallowing difficulties	−	−
Spasticity	+	+
Rigidity	+	+
Pathological reflexes	+	+
Ataxia	+	+
Athetoid movements	+	+
Convulsions	+	+
Ophthalmoplegia	+	+
Strabismus	+	+
Gastrointestinal problems	+	+
Renal agenesis	NA	+
Pes equinovarus	+	+
Uncommon clinical phenotype		
Increase of ketone body	+	+

NA not assessed

spontaneous abortion. He was born at 39 weeks gestation without asphyxia after an uneventful pregnancy. His body weight was 3,680 g (+1.6 SD), his height was 50.0 cm (−0.5 SD), and his head circumference (HC) was 34.0 cm (−0.5 SD). His early developmental milestones were normal with head control and reach to toys at 4 months, roll at 6 months and grasp with two fingers at 7 months. At 10 months, he was referred to our hospital because of an inability to sit. His body weight was 9,120 g (±0.0 SD), his height was 76.0 cm (+1.3 SD), and his HC was 48.0 cm (+1.4 SD). He could smile and swallow well. Bilateral strabismus was noted. No minor anomalies were noticed. Muscle tone was normal. Deep tendon reflexes were normal with negative Babinski sign. He showed athetoid movements of trunk and extremities. He showed pes equinovarus at traction response. Levels of lactate and pyruvate were normal with 12.2 and 0.89 mg/dl (L/P ratio = 13.7), respectively. Other laboratory examinations, including blood gas, blood sugar, ammonia, AST, ALT, BUN, Creatine, TSH, T3, T4, amino acids, and urine organic acid analyses were all normal. Electroencephalogram (EEG) showed no abnormalities. He was suspected to have dyskinetic cerebral palsy and referred to the division of rehabilitation. He could crawl at the age of 2. At 6 years, he experienced a loss of consciousness followed by generalized tonic–clonic convulsion with fever and was admitted to another hospital. He was diagnosed with bilateral infarction of the basal ganglia. Although EEG showed no abnormalities, clonazepam was started with the suspicion of symptomatic epilepsy. At the age of 9, he was referred to us again. His weight was 19.1 kg (−4.5 SD), his height was 115.0 cm (−2.8 SD). He lost the ability to speak several words and switched handedness from right to left. He also showed other signs of regression: including spasticity with elevated deep tendon reflexes and positive Babinski sign. In addition, he suffered bilateral hip joint dislocations and the foot deformity became worse. Contractures were noted in all extremities. Brain magnetic resonance imaging (MRI) revealed a bilateral necrotic lesion of the globus pallidus (Fig. 2a, b). EEG and motor conduction velocities were normal. Laboratory examinations, including lactate and pyruvate, were all normal. At the age of 12, he was admitted with acute bronchitis, at that time he showed an increase of blood ketone bodies: acetoacetic acid, 720 μmol/l; 3OHBA, 974 μmol/l and urine ketone (+++). Blood levels of ammonia (18 μmol/l), sugar (125 mg/dl) and lactate/pyruvate (5.1/0.29 mg/dl) were all within normal range. The values of blood ketone bodies returned to normal level with the cease of fever. Deficiencies of 3-ketothiolase and succinyl-CoA:3-oxoacid CoA transferase were ruled out by enzyme analysis using fibroblasts. His clinical symptoms and repeated MRI show the non-progressive course of his disease. Currently he is unable to sit or speak any words. Despite the addition of carbamazepine and lamotrigine, he still exhibits generalized tonic–clonic convulsion a few times a year. He also takes medicine for hypertonicity including dantrolene sodium, diazepam, baclofen and levodopa.

Patient II-3 (Fig. 1a; Table 1), the younger brother of II-2, was born uneventfully. He was born at 37 week's gestation without asphyxia after an uneventful pregnancy. His body weight was 3,668 g (+1.5 SD), his height was 50.0 cm (+0.5 SD), and his HC was 36.0 cm (−0.5 SD). He suffered from bacterial meningitis of unknown origin at 1 month of age. He became unconscious followed by convulsion and gastroenteritis at 1 year and 11 months. Brain MRI showed marked swelling of the basal ganglia (Fig. 2c, d). He was diagnosed with bilateral infarction of the basal ganglia. After this event, he became left handed. When he was 2 years old, surgery was performed to correct bilateral inner strabismus. He was referred to our hospital at the age of 4 for evaluation. His body weight was 11.0 kg (−2.2 SD), his height was 92.5 cm (−1.2 SD), and his HC was 49.5 cm (−1.3 SD). He could respond with a smile to his mother's voice. Motor milestones were delayed with no head control. No minor anomalies were noticed. Muscle tone was hypotonic. Deep tendon reflexes were exaggerated with positive Babinski sign and ankle clonus. He showed pes equinovarus. He showed a significant increase of blood acetoacetic acid of 1,270 μmol/l and 3-OHBA of 3,270 μmol/l. Levels of blood lactate and pyruvate were normal (6.2 and 0.48 mg/dl, respectively, L/P ratio = 12.9).

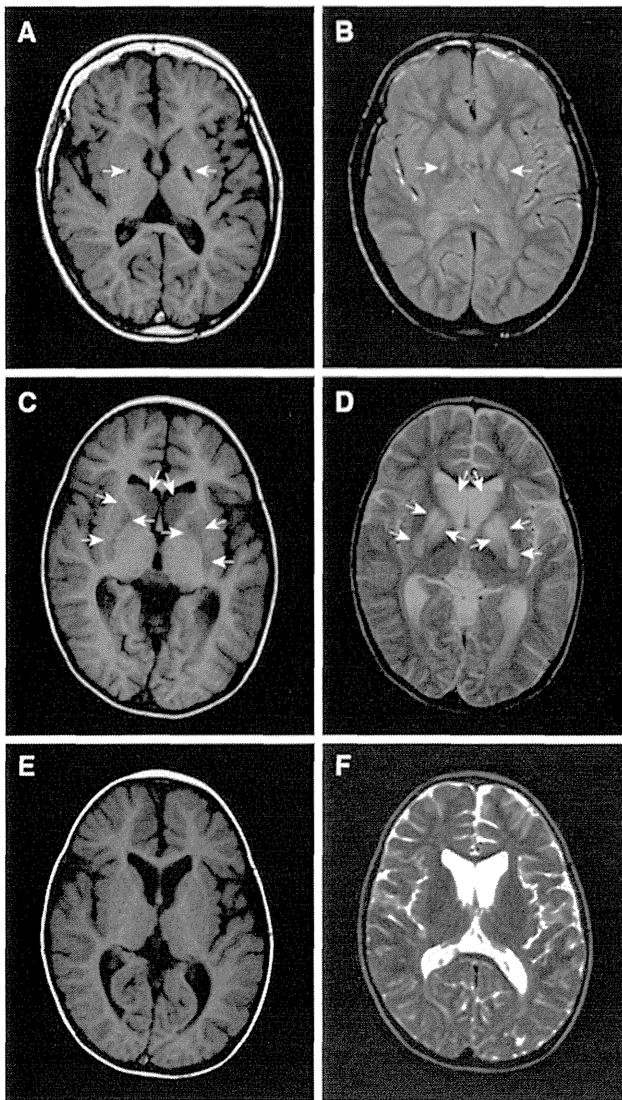

Fig. 2 Brain MRI of affected patients with a *GYG2* mutation. **a**, **b** (Patient II-2): T1 (**a**) and T2 (**b**) weighted brain magnetic resonance imaging (MRI) show necrotic lesion of bilateral globus pallidus (*arrows*). T2 elongation is observed at deep white matter at 1 year. **c–f** (Patient II-3): MRI at 1 year and 11 months shows swellings of caudate nuclei, globus pallidus, and putamen with the decreased T1 intensity (**c**) and increased T2 signals (**d**). *Arrows* indicate swollen lesions in basal ganglia. At 4 years (**e**, **f**), swelling of basal ganglia disappeared with continued mild high intensity in T2 weighted image (**f**)

Lactate and pyruvate levels of cerebrospinal fluid were slightly elevated with 11.3 and 1.11 mg/dl, respectively. Other laboratory examinations, including blood gas, blood sugar, ammonia, AST, ALT, BUN, Creatine, TSH, T3, T4, amino acids, and lysosomal enzymes were all normal. Urine organic acid analyses showed an increase of acetoacetic acid, 3-OHBA, and 3-OH-isovaleric acid. EEG showed no paroxysmal discharges. Muscle biopsy showed no specific abnormalities and no ragged red fibers. Staining for cytochrome c oxidase was normal (data not shown).

Brain MRI disclosed T2 elongation in the basal ganglia and cerebral deep white matter (Fig. 2e, f). At the age of 5, he showed lethargy with fever. At 6 years, he again showed lethargy. Biochemical analysis disclosed a significant increase of blood ketone bodies: acetoacetic acid, 1,337 μmol/l; 3-OHBA, 4,845 μmol/l and urine ketone (+++). Blood levels of ammonia (28 μmol/l), sugar (78 mg/dl), lactate (5.1 mg/dl) and pyruvate (0.43 mg/dl) were all within normal range. Blood gas analysis revealed metabolic ketoacidosis with an increase of anion gap; 22.4 mEq/l (normal range 12 ± 2). His consciousness and biochemical measurements returned to normal within a few days with intravenous fluid infusion. Similar ketoacidosis attacks were repeatedly observed and agenesis of the left kidney and neurogenic bladder were recognized at the age of 8. He started intermittent urinary catheterization, and suffered from repeated urinary tract infections, resulted in chronic renal failure. Repeated brain MRI shows the progression of cerebral and cerebellar atrophy. He is now 19 years old and shows no gain of motor or intellectual abilities from the age of 4. He takes dantrolene sodium and diazepam for hypertonicity, and spherical charcoal, allopurinol for renal failure.

Identification of a *GYG2* variant by exome sequencing

A total of 2,433,011,483 bps (II-2) and 7,926,169,749 bps (II-3) were mapped to RefSeq coding DNA sequence (CDS). 83.3 and 96.0 % of CDS were covered by ten reads and more. We used only NGS data of II-3 for selecting candidate variants as the lower-quality NGS data of II-2 may lead to erroneous conclusion. Based on the hypothesis that this syndrome is inherited in an autosomal recessive or an X-linked recessive fashion, we focused on homozygous or compound heterozygous variants on autosomes and hemizygous variants on the X chromosome. While nine variants in four candidate genes were selected by in silico flow, only one hemizygous missense mutation in *GYG2* gene agreed with the familial segregation pattern (autosomal recessive or X-linked recessive) (Table S1, S2). The c.665G>C (p.W222S) in *GYG2* (isoform a: NM_001079855) was hemizygous in affected sibs and heterozygous in their mother, consistent with the X-linked recessive model, and was confirmed by Sanger sequence (Fig. 1b). The variant was absent in our in-house Japanese exome data ($n = 418$), the 1,000 Genomes database and ESP6500. Furthermore, no pathological variants in mtDNA were detected by exome sequence (Supplementary Results, Figure S1). In addition, a total of 21 LS patients (12 males and 9 females) were screened, but no pathological changes were found in *GYG2*.

GYG2 encodes GYG2 proteins with at least five isoforms: isoform a (NM_001079855), isoform b (NM_003918),

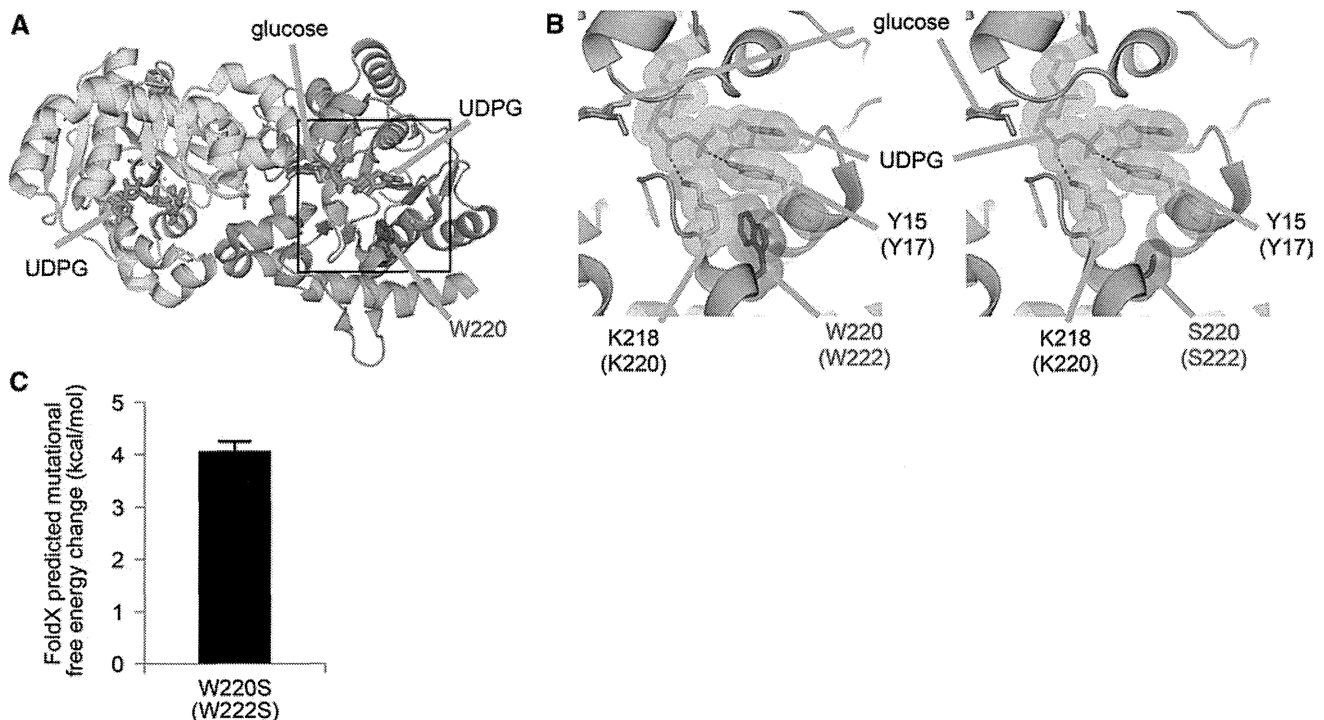

Fig. 3 Molecular structural consideration of the W222S mutation of GYG2. **a** Crystal structure of human GYG1 (Protein Data Bank code; 3T7O) (Chaikuad et al. 2011). Each monomer is colored *yellow* and *cyan*. α-helices, β-sheet and loops are drawn as *ribbons*, *arrows* and *threads*, respectively. The side chain of W222, glucose and UDP-glucose (UDPG) are shown as *sticks* in *red*, *orange* and *green*, respectively. Amino acid numbering shown is for human GYG1 with that for human GYG2 in *parenthesis*. The *squared area* corresponds to the close-up views in (**b**). **b** Detailed views of structures of the wild-type (*left*) and mutated GYG2 (p.W222S) (*right*). Amino acid residues at positions of 15, 218 and 220 and UDPG are shown as sticks with van der Waals representation and annotations. Hydrogen bonds are depicted as *dotted lines*. **c** Calculated free energy change upon the p.W222S mutation of GYG2 using FoldX software. All the molecular structures were drawn using PyMOL (www.pymol.org)

isoform c (NM_001184702), isoform d (NM_001184703), and isoform e (NM_001184704). At least two GYG2 isoforms (isoform a and b) are expressed preferentially in liver, heart and pancreas (Mu et al. 1997), while the detailed expression and function of other isoforms are undetermined. GYG2 has a glycosyltransferase family 8 domain and initiates glucose addition on its Tyrosine residue (Y197 in isoform a) via *O*-glycosylation (self-glucosylation) and can also attach an additional 7–10 residues of UDP-glucose to itself (Bollen et al. 1998; Lomako et al. 2004; Zhai et al. 2001). The W222 within the glycosyltransferase family 8 domain is evolutionarily highly conserved from *S. cerevisiae* to *H. sapiens* (Fig. 1c, d). In addition, all isoforms contain this residue. Thus, it is thought that this mutation may impair its biological function.

Structural consideration of the p.W222S mutation in human GYG2

The amino acid residue W222 of GYG2 (isoform a) was mapped to the crystal structure of human GYG1 (Chaikuad et al. 2011), since no experimental structure of GYG2 was available. W222 is involved in a hydrophobic core near the UDP-glucose (UDPG) binding site along with Y17 and K220 (Fig. 3a, b). The side chains of Y17 and K220 are hydrogen-bonded to UDPG, and the former also makes van der Waals contacts with the uridine ring of UDPG in a stacking mode. Therefore, the formation of the hydrophobic core appears to be a prerequisite for UDPG binding. To estimate the impact of the W222S mutation on the protein stability, we modeled the mutant structure and calculated the free energy change upon the mutation using the FoldX software. As a result, the mutation was predicted to destabilize the protein structure with about 4 kcal/mol increase in free energy (Fig. 3c). This suggests that the W222S mutation would impair UDPG binding (Fig. 3b).

Self-glucosylation analysis

To see the functional effects of the *GYG2* mutation in vitro, glucosyltransferase activity monitoring by self-glucosylation was measured using wild-type (WT) and W222S mutant (Mut) GYG2 (isoform a) transiently

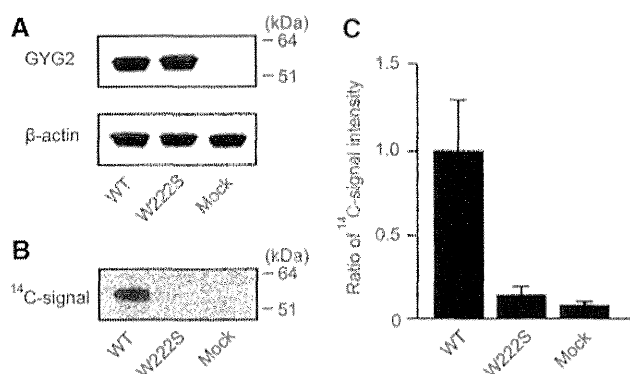

Fig. 4 Enzyme activity of GYG2. **a** Western blot analysis of recombinant GYG2. Wild-type (WT) and mutant (p.W222S) GYG2 was detected at the expected size (52 kDa). β-actin (42 kDa) was used as an internal control. **b** Autoradiography images presenting ^{14}C glucosylation toward GYG2. The signal was detected in WT, but undetected in mutant, with similar levels to Mock. **c** Graphic presentation of autoglucosylation of GYG2. The activity detected in Mock might be due to the endogenous glycogenin. *Error bars* represent the standard error of the mean

overexpressed in COS-1 cells. By immunoblotting, the expected 52 kDa bands of recombinant WT and Mut GYG2 were detected with similar expression levels (Fig. 4a). While WT GYG2 showed reasonable glucosyltransferase activity, Mut GYG2 almost completely lost the enzyme activity and was similar to the Mock level (Fig. 4b, c).

Expression analysis of GYG1 and GYG2

To observe tissue distribution of the human *GYG1* and *GYG2*, expression analysis was performed using multiple tissue cDNA panels. *GYG1* was expressed preferentially in skeletal muscle and heart from fetus to adult stages as previous reports (Barbetti et al. 1996). *GYG2* is dominantly expressed in liver from fetus through adult stages and moderately expressed in brain, heart, pancreas and kidney (Supplementary Results, Figure S2). To be marked, *GYG1* is not expressed in liver and brain where *GYG2* is highly expressed.

Discussion

In this study, we analyzed unique brothers affected with LS who were born to non-consanguineous healthy parents after uneventful pregnancies. Patient II-2 and II-3 developed LS accompanied by delayed developmental milestones at 10 months and 13 months of age, respectively. Their age of onset, clinical features and brain imaging were compatible with the diagnosis of LS. Interestingly, CNS abnormalities were observed (developmental delay, convulsion, athetoid movements, nystagmus, hypotonia, spasticity, increased deep tendon reflex and abnormal reflection), but involvement of peripheral nerve and extraneural organs was obscure. Based on the facts including (1) male (X-linked recessive), (2) normal lactate/pyruvate, (3) ketonemia/ketonuria, and (4) CNS predominant symptoms, the hemizygous *GYG2* mutation was highlighted a primary culprit.

In this study, we first identified a human *GYG2* mutation in affected brothers with LS with ketonemia/ketonuria but normal blood lactate/pyruvate. We can hypothesize a pathomechanism of the *GYG2* impairment in this family based on the canonical pathway of glycogen metabolism (Fig. 5). As glycogen storage in liver might be decreased because of the GYG2 malfunction, glucose is easily depleted. To keep appropriate blood glucose concentrations, the metabolism would be shifted toward gluconeogenesis and beta-oxidation to create glucose and energy sources like Acetyl-CoA (Garber et al. 1974; Laffel 1999; Randle et al. 1964). Excess beta-oxidation would result in overproduction of ketone bodies, consistent with the observation of ketonemia and ketonuria. However, pyruvate and lactate could be normally metabolized in gluconeogenesis and/or TCA cycle and would not accumulate in the body as seen in the majority of LS patients. Interestingly, both patients showed normal blood glucose level while showing LS manifestations which might be due to tissue energy depletion. In GYG2-deficient patients, the CNS was dominantly affected, while the effect of this abnormal metabolism was thought to extend to the entire body. This predominance could be explained by high glucose consumption as the primary energy source in brain (Amaral 2012; Magistretti and Pellerin 1999) and glycogen depletion in brain tissue level, while the blood sugar level was maintained by the other compensatory mechanism. This is similar to the muscle specific phenotypes (muscle weakness and arrhythmia) observed in patients with deficiencies of "muscle form" GYG1 in the absence of hypoglycemia (Moslemi et al. 2010). Remarkably, glycogen was less in the muscle tissue of GYG1 depleted patient (Moslemi et al. 2010). These evidences might indicate that it is not always linked between glucose level in the peripheral blood and glycogen/energy supply in tissue level while we could not show the loss of glycogen in liver or brain tissues because the materials were not available. In addition, deficiencies in two paralogous enzymes, GYG1 and GYG2, result in different human diseases suggesting they are unable to compensate each other in specific organs.

The *GYG2* mutation is probably causative for LS in this family. However, it is possible that the mutation is just coincidence because we just showed genetic evidences (due

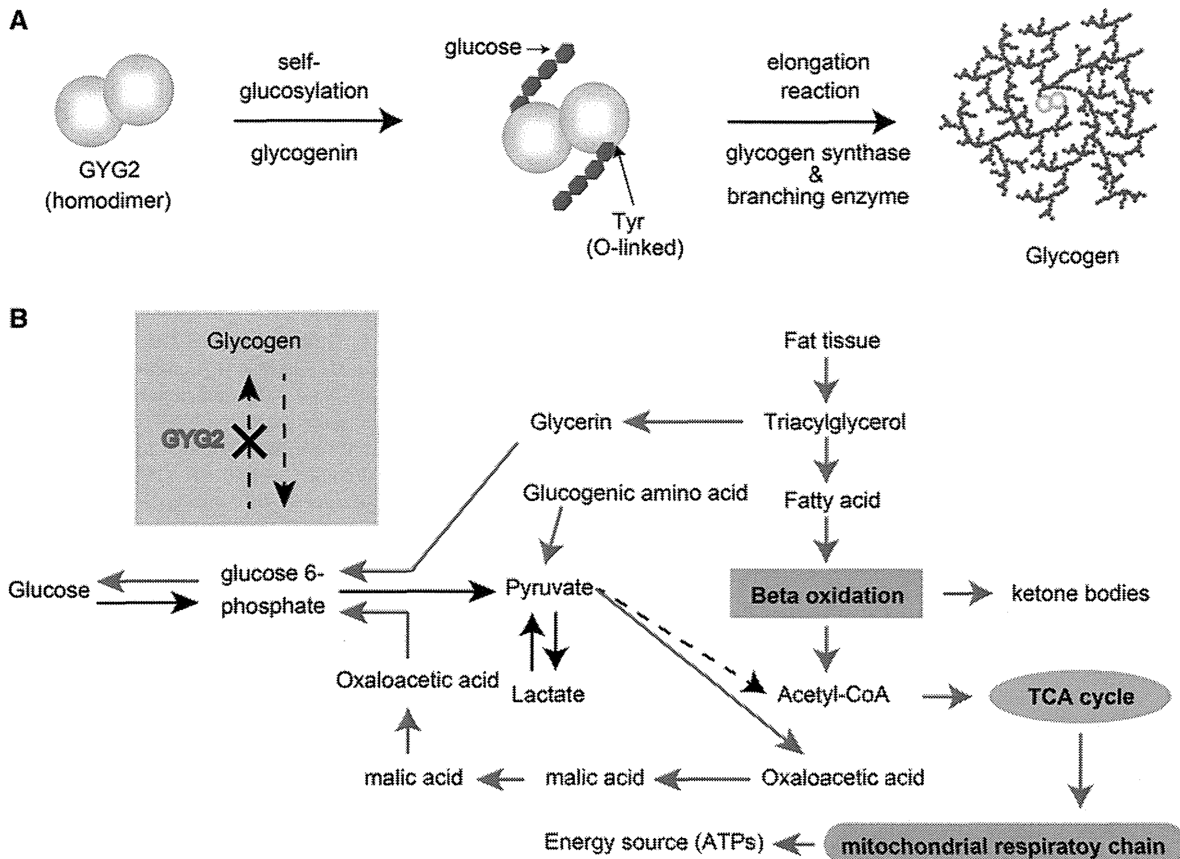

Fig. 5 Biochemical metabolisms in glycogen storage and glycolysis pathways. **a** Schematic presentation of glycogen biosynthesis. GYG2 has a catalytic capability for *O*-linked self-glucosylation at Tyrosine (Y197 in isoform a) and adds approximately 10 glucose molecules. By the subsequent elongating reactions by glycogen synthase and branching enzyme, giant molecule "glycogen" is formed. **b** Modeled biochemical pathway in GYG2 impairment. As the GYG2 impairment results in the absence of glycogen storage, glycogen is easy to be depleted and gluconeogenesis is induced from fat tissues and glucogenic amino acids. The reactions in mitochondria are shown in *yellow shadow*. While increased acetyl-CoA inhibits the pyruvate dehydrogenase complex which irreversibly converts pyruvate to acetyl-CoA (as shown as *dotted line*), it accelerates gluconeogenesis through pyruvate–oxaloacetic acid–malic acid–oxaloacetic acid. Triacylglycerol was metabolized into glycerin and fatty acid. Fatty acid was used for beta-oxidation and ketone production. The *arrows* indicate the directions of normal metabolism. *Red arrows* indicate the predicted predominant pathways in GYG2-deficient patients

to its rarity and familial co-segregation) and GYG2 loss of function by in vitro study without showing any sufficient data on how the *GYG2* mutation causes LS.

In conclusion, we describe the first human variant of *GYG2* which may be associated with the atypical LS phenotype in this family. Further studies are absolutely needed to conclude whether *GYG2* abnormality leads to atypical LS observed in this family.

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Conflict of interest The authors declare that they have no conflict of interest.

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CASE REPORT

A Japanese Adult Case of Guanidinoacetate Methyltransferase Deficiency

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Abstract Guanidinoacetate methyltransferase (GAMT) deficiency is a rare disorder of creatine synthesis resulting in cerebral creatine depletion. We present a 38-year-old patient, the first Japanese case of GAMT deficiency. Developmental delay started after a few months of age with a marked delay in language, which resulted in severe intellectual deficit. She showed hyperactivity and trichotillomania from childhood. Epileptic seizures appeared at 18 months and she had multiple types of seizures including epileptic spasms, brief tonic seizures, atypical absences, complex partial seizures with secondary generalization, and "drop" seizures. They have been refractory to multiple antiepileptic drugs. Although there have been no involuntary movements, magnetic resonance imaging revealed T2 hyperintense lesions in bilateral globus pallidi. Motor regression started around 30 years of age and the patient is now able to walk for only short periods. Very low serum creatinine levels measured by enzymatic method raised a suspicion of GAMT deficiency, which was confirmed by proton magnetic resonance spectroscopy and urinary guanidinoacetate assay. *GAMT* gene analysis revealed that the patient is a compound heterozygote of c.578A>G, p.Gln193Arg and splice site mutation, c.391G>C, p.Gly131Arg, neither of which have been reported in the literature. We also identified two aberrant splice products from the patient's cDNA analysis. The patient was recently started on supplementation of high-dose creatine and ornithine, the effects of which are currently under evaluation. Although rare, patients with developmental delay, epilepsy, behavioral problems, and movement disorders should be vigorously screened for GAMT deficiency, as it is a treatable disorder.

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Introduction

Guanidinoacetate methyltransferase (GAMT; OMIM 601240) deficiency is a rare autosomal recessive disorder of creatine synthesis resulting in cerebral creatine depletion (Stöckler et al. 1994, 1996b). Guanidinoacetate (GAA) accumulates in body fluids. Symptoms of GAMT deficiency usually emerge after a few months of life, such as intellectual disability, speech delay, autistic behaviors, epileptic seizures, and involuntary movements (Mercimek-Mahmutoglu et al. 2006). Making a diagnosis of GAMT deficiency is challenging; nonetheless, early diagnosis is crucial because this disorder is treatable (Stöckler et al. 1996a). Only approximately 80 cases have been reported to date, mostly from Europe and the Middle East. Here we report on the first Japanese patient with GAMT deficiency with two novel gene mutations.

Case Report

The patient, a 38-year-old female with intractable epilepsy and severe mental retardation, was born at full term with a birth weight of 3,260 g. There were no pre- or perinatal complications. She is the third of four children of Japanese non-consanguineous healthy parents. The first child, a boy, started having epileptic seizures after 1 year of age with unknown cause and died at 28 years of age at an institution for the mentally handicapped. The other two children have been healthy.

Although the patient showed a social smile by 3 months and head control by 4 months of age, her development has been delayed since then. She sat alone at 14 months, walked alone at around 20 months, and became able to take the stairs one step at a time with support around 5 years of age. She has spoken no meaningful words and gained little language comprehension. Her medical chart at 7 years of age described her as speechless, unable to follow verbal commands, but able to run and walk up the stairs one step at a time without support. She showed no involuntary movements. She was hyperactive and had trichotillomania. Neuropsychological assessment at 7 years 7 months by analytic test for development in infancy and childhood (Enjoji and Yanai 1961) demonstrated her developmental quotient was 14. Around 30 years of age, she was unable to walk for a long time but was able to take the stairs with support. At 32 years of age, she was no longer able to run. Currently, at 38 years of age, the patient has severe intellectual deficit with no speech or language comprehension. She still has trichotillomania. Her transport is mostly by wheelchair, although she is able to walk slowly for short periods. Her muscle tone is normal and there are no involuntary movements.

The onset of epilepsy was at around 18 months of age, characterized by epileptic spasms and brief tonic seizures. At 2 years of age, atypical absences appeared. Despite therapy with multiple antiepileptic drugs, the patient continued to have these seizures until 15 years of age, when her seizures were suppressed by valproic acid and clonazepam. When they recurred at 20 years of age, her seizures were characterized by consciousness impairment with head and body version to left followed by generalized tonic-clonic convulsions lasting up to 1 minute, suggesting complex partial seizures with secondary generalization. At around 23 years, brief "drop" seizures occurring in clusters started. She has continued to have these seizures since then, although she has been tried on multiple antiepileptic drugs including phenobarbital, valproic acid, clonazepam, phenytoin, clobazam, topiramate, lamotrigine, and levetiracetam.

Electroencephalograms (EEGs) at 2–12 years of age showed a slow background activity, generalized 1.5–2.5 Hz slow spike-wave bursts and some multifocal spikes, consistent with Lennox-Gastaut syndrome. EEGs after adolescence showed multifocal spike-waves with anterior head predominance and intermittent generalized slow spike-waves. The most recent EEG at 38 years of age demonstrated background slowing and no spikes during wakefulness but intermittent focal polyspikes and polyspike-waves over bilateral anterior and left posterior head regions during sleep.

Laboratory blood tests demonstrated low levels of serum creatinine (5–7 μmol/L by enzymatic method; normal range 40–71 μmol/L). Subsequent tests using enzymatic methods demonstrated serum creatine levels were below detection limit (normal range 23–92 μmol/L). Proton magnetic resonance spectroscopy (^{1}H-MRS) demonstrated absent creatine peak (Fig. 1a). Brain magnetic resonance imaging (MRI) demonstrated T2 high-intensity lesions in globus pallidi (Fig. 1b). Analysis of urinary creatine metabolites by weak-acid ion chromatography (Wada et al. 2012) demonstrated elevated GAA (548.53, 782.52 mmol/mol creatinine; normal 3–78 mmol/mol creatine (Almeida et al. 2004)) and creatine below detection limit. These findings suggested GAMT deficiency.

Genomic DNA analysis of the *GAMT* gene (Suppl. Table 1) showed a compound heterozygosity for two novel point mutations, an exonic splicing mutation c.391G>C located at the last nucleotide of exon 3 and a missense mutation c.578A>G, p.Gln193Arg in exon 6 (Fig. 2a). Analysis of cDNA revealed two aberrantly spliced transcription products at the allele of splicing mutation (Fig. 2b, c). One transcript had the complete exon 3 (64-bp) deletion by exon skipping and the other transcript was aberrantly spliced at exon 2 involving intron 2 insertion (44-bp) followed by exon 3 skipping, resulting in a 20-bp deletion. Both transcripts are expected to result in frame shift and premature termination of p.Val110Glyfs*30 and p.Ile111Profs*73, respectively. A novel A to G transition on exon 6 (c.578A>G) results in the replacement of arginine by glutamine at position 193 (p.Gln193Arg). This missense variation was not found in 100 control alleles. Glutamine193 is highly conserved in evolution (Fig. 2d), suggesting this mutation represents a pathogenic mutation.

This patient was recently started on supplementation of high-dose creatine and ornithine, and its effects are currently under evaluation.

Discussion

We reported on the first Japanese case of an adult patient with GAMT deficiency. Cases have been reported mostly from Europe and the Middle East (Mercimek-Mahmutoglu et al. 2006).

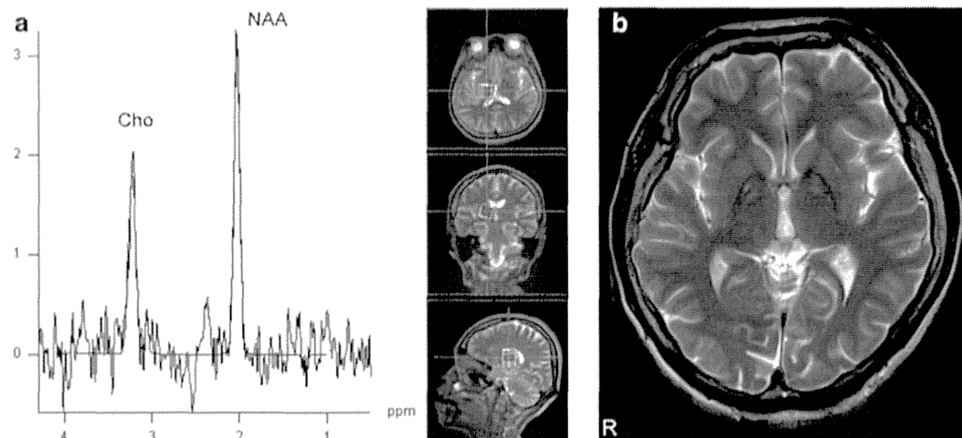

Fig. 1 MR spectroscopy and MRI from the patient with GAMT deficiency. (**a**) ^{1}H-MRS at the right basal ganglia demonstrates absence of creatine peak. (**b**) T2-weighted brain MRI shows high-intensity lesions in bilateral globus pallidi. *Cho* choline; *NAA* N-acetylaspartate

Compared with cases in the literature, our patient showed similar MRI findings and clinical course, with severe intellectual deficit, intractable epilepsy, behavioral problems, but she lacked involuntary movements. Although no definite progression of symptoms was seen during adolescence and young adulthood, motor regression slowly started around 30 years of age. This suggests GAMT deficiency can be slowly progressive if untreated.

Onset of symptoms in GAMT deficiency is from a few months to young childhood (Longo et al. 2011). Intellectual disability is seen in all cases and is severe (IQ < 35) in the majority, especially with profound speech disturbance (Mercimek-Mahmutoglu et al. 2006). Epilepsy is the second most frequent symptom, intractable in most cases, and partially responsive to antiepileptic drugs in two thirds (Leuzzi et al. 2013). Various types of seizures, such as generalized tonic-clonic seizures, absences, myoclonic seizures, myoclonic-astatic seizures, and partial seizures with secondary generalization, have been reported (Leuzzi et al. 2013). Involuntary movements, behavioral problems, and abnormal MRI signals in globus pallidi are seen in some cases. Adult cases that help to understand the natural history of GAMT deficiency are scarce (Schulze et al. 2003; Caldeira Araújo et al. 2005). Progression of neurological deficits, such as paraparesis, hypertonia, and rigidity, has been reported in some cases (Caldeira Araújo et al. 2005).

GAMT gene analysis revealed a compound heterozygosity of two novel mutations: c.391G>C splice donor site of exon 3 and c.578A>G, p.Gln193Arg in exon 6. The former led to two abnormal transcripts lacking exon 3, resulting in a premature stop codon. Reverse transcription polymerase chain reaction detected a higher expression level of the allele with the c.578A>G mutation, which implies the degradation of mRNA from the allele with the splice site mutation by nonsense-mediated mRNA decay (Fig. 2b). Gln193Arg substitution by the latter mutation is presumed to destabilize the tertiary structure of GAMT (Komoto et al. 2002) by increasing the bulkiness and changing the neutral to a positively charged residue, as Gln193 is situated in the middle of α-helix and protrudes into this enzyme.

Making a diagnosis of GAMT deficiency is challenging, because of its nonspecific symptoms and limited access or capacity of ^{1}H-MRS. GAA assay may not be readily available. While not as specific as GAA, measurement of creatinine is helpful, as creatinine can be low in GAMT deficiency (Verhoeven et al. 2000). It should be warned that creatinine may also be low in patients with decreased muscle volume. Another caveat is that creatinine measurement by Jaffé method is not as sensitive in detecting GAMT deficiency as the enzymatic method or high-performance liquid chromatography (Verhoeven et al. 2000). Our patient showed low levels of serum creatinine as determined by enzymatic method, which directed us to the diagnosis of GAMT deficiency. The assay of creatine and creatinine is also important to detect creatine transporter 1 deficiency, another type of cerebral creatine deficiency, as the urinary creatine/creatinine ratio is elevated in this disorder (Salomons et al. 2003; Verhoeven et al. 2005). GAA is a more sensitive marker than creatine and creatinine in GAMT deficiency and arginine: glycine amidinotransferase deficiency, the other type of cerebral creatine deficiency (Verhoeven et al. 2005). Therefore, blood and urine tests of creatinine, creatine and GAA should be a part of the workup for developmental delay and/or epilepsy with unknown cause, if creatine and GAA measurements are available.

Early diagnosis is crucial to achieve a favorable outcome in GAMT deficiency. Ideally, treatment should be initiated as early as possible before the creatine pool supplied from maternal body during gestation becomes