at −80°C. The embryos were rehydrated in PBS, and hybridization of a DIG-labeled RNA probe was carried out in a hybridization buffer containing 50% formamide, 25% 20×SSC, 1% Tween20, 9 mM citric acid and 1 mg/ml heparin at 65°C for 16 h. Embryos hybridized with a DIG-labeled RNA were incubated with anti-DIG antibody conjugated to alkaline phosphatase (Roche Diagnostics) (1:2000) at 4°C for 12 h, stained with BM purple (Roche Diagnostics) at room temperature for 2 h and fixed with 4% PFA-PBS. The embryos were observed using ECLIPSE E600 (Nikon Corp., Tokyo, Japan).

Localization of 3′UTRs in zPOMT1 and zPOMT2 genes

The *EGFP* gene was cloned into pSP72 vector (Promega, Madison, WI). The3′UTRs of *zPOMT1* and *zPOMT2* were fused separately downstream of *EGFP* gene. The 3′UTR of zebrafish *NUDT2* was used as a control. Capped mRNAs of *EGFP-zPOMTs-3′UTR* were synthesized using mMESSAGE mMACHINE High Yield Capped RNA Transcription Kit (SP6, Ambion Inc., Austin, TX). Capped mRNAs at a concentration of 0.05 μg/μL were injected into fertilized eggs from one to two cells. The embryos were observed until 24 hpf using ECLIPSE E600 and a mercury lamp (Nikon).

Knockdown analysis of zPOMT1 and zPOMT2

Antisense MOs targeted to interfere with *zPOMT1* and *zPOMT2* translation were purchased from Gene Tools LLC (Philomath, OR). The antisense sequences of *zPOMT1* and *zPOMT2* genes were designed using the 50 sequence around the putative start of translation of *zPOMT1* and *zPOMT2* mRNA (accession nos. AB281275 and AB281276). The morpholino sequences were *zPOMT1*-MO: 5′-gacgggcagtttaacacactgcatg-3′ and *zPOMT2*-MO: 5′-gtccattcttgaagatgaagaggac-3′. The sequence of control MO was 5′-gtacgtcacacaatttgacgggcag-3′. MOs at a concentration of 0.25, 0.5 or 1.0 mM were injected into embryos at the one- to two-cell stage.

Immunohistochemistry

For immunohistochemistry, embryos were fixed overnight in 4% paraformaldehyde solution, embedded in paraffin and sectioned at 10 μm and mounted on slides. Sections were left to dry for 2 h. After being dewaxed and rehydrated, some sections were stained with hematoxylin and eosin, while others were subjected to immunohistostaining as described in (Mulero et al. 2007). Anti-glycosylated α-DG IIH6 (Upstate, Millipore, Billerica, MA) was used at a dilution of 1:100 with PBS. Slides were washed 3× 10 min with PBS containing 0.1% Tween (PBSTw) and incubated with secondary antibody for 1 h. The secondary antibody used was Alexa Fluor 488 Goat Anti-Mouse IgM (Molecular Probes Invitrogen Life Technologoies Corp., Tokyo, Japan) at a dilution of 1:500 with PBS. Embryos were fixed in 4% PFA-PBS and transferred into 100% methanol. The embryos were rehydrated in PBS containing 0.1% Tween-20 (PBT) and incubated in PBT containing anti-glycosylated α-DG antibody (IIH6) overnight at 4°C followed by several washes with PBT and incubation with secondary antibody (goat anti-mouse IgM AlexaFluor-488,). The embryos were observed using ECLIPSE E600 and a mercury lamp (Nikon).

Assay for protein O-mannosyltransferase activity

Expression plasmids of zPOMT1 and zPOMT2 were constructed using pcDNA3.1 Hygro (+) vector (Life Technologoies Corp., Tokyo, Japan) and confirmed by the sequencing. The expression plasmids were transfected into HEK293T cells, and the cells were cultured for 3 days in complete medium, harvested, and homogenized. Protein *O*-mannosyltransferase activity was based on the amount of [^{3}H]-mannose transferred from [^{3}H]-mannosylphosphoryldolichol to a glutathione-S-transferase fusion α-DG (GST-α-DG) as described previously (Manya et al. 2004). Approximately 80 μg of microsomal membrane fractions were collected from HEK293T cells coexpressing combinations of *POMT1* and/or *POMT2* genes from either human or zebrafish, suspended in a 20-μL reaction buffer containing 10 μg of GST-α-DG. The reaction mixture was incubated at 22°C for 1 h, and GST-α-DG was purified using glutathione-Sepharose 4B beads (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). The radioactivity adsorbed to the beads was measured by using liquid scintillation counter.

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The nucleotide sequences in this paper have been submitted to the Genbank/EMBL/DDBJ Nucleotide Sequence Database. The accession numbers AB281275 and AB281276 have been assigned to the cDNA sequences of zebrafish POMT1 and POMT2, respectively.

Abbreviations

α-DG, α-dystroglycan; DGC, dystrophin–glycoprotein complex; DIG, Digoxigenin; *EGFP, enhanced green fluorescent protein*; FKRP, fukutin-related protein; HEK293T cells, human embryonic kidney 293T cells; MO, morpholino oligonucleotide; ORF, open reading frame; PBS phosphate-buffered saline; PBT PBS containing 0.1% Tween-20; PFA paraformaldehyde; POMGnT1, protein *O*-mannose β1,2-*N*-acetylglucosaminyltransferase1; POMT, PMT, protein *O*-mannosyltransferase; RT-PCR, reverse transcriptase-polymease chain reaction; SD, standard deviation; WWS, Walker–Warburg syndrome.

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CHAPTER NINETEEN

POMGnT1, POMT1, and POMT2 Mutations in Congenital Muscular Dystrophies

Tamao Endo,* Hiroshi Manya,* Nathalie Seta,† *and* Pascale Guicheney‡

Contents

Abstract

α-Dystroglycanopathies are a group of rare inherited neuromuscular disorders characterized by reduced glycosylation of α-dystroglycan (α-DG). Mutations in six genes (*POMT1*, *POMT2*, *POMGNT1*, *FKTN*, *FKRP*, and *LARGE*) have been identified in patients with α-dystroglycanopathies. Due to an extremely broad clinical spectrum and relatively poor phenotype–genotype correlation, diagnosis of α-dystroglycanopathies is difficult and requires searching for mutations gene by gene. At present, of the six proteins involved on α-dystroglycanopathies, the function of the gene products is only known for POMT1, POMT2, and POMGnT1, all responsible for the *O*-mannosylglycan biosynthesis. This chapter describes the assay protocols to diagnose patients with α-dystroglycanopathy by measuring glycosyltransferase activity.

* Molecular Glycobiology, Tokyo Metropolitan Institute of Gerontology, Itabashi-ku, Tokyo, Japan
† Laboratoire de Biochimie Métabolique et Cellulaire, AP-HP, Hopital Bichat, Paris, France
‡ Génétique, Pharmacologie et Physiopathologie des Maladies Cardiovasculaires, Groupe Hospitalier Pitié-Salpêtrière, Paris, France

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1. Overview

Recent studies indicate that *O*-mannosylation of α-dystroglycan (α-DG), a highly glycosylated surface membrane protein, plays an important role in muscle and brain development. Defects in glycosylation of α-DG cause several forms of autosomal recessive muscular dystrophy, also called α-dystroglycanopathies, that share common features such as high serum creatine kinase and muscle hypertrophy. Six genes (*POMT1*, *POMT2*, *POMGNT1*, *FKTN*, *FKRP*, and *LARGE*) are responsible for these diseases with overlapping phenotypes (Michele and Campbell, 2003; Muntoni *et al.*, 2008).

Muscle–eye–brain disease (MEB) is an autosomal recessive disorder characterized by congenital muscular dystrophy (CMD), ocular abnormalities, and brain malformation (type II or cobblestone lissencephaly). Mutations in the *POMGNT1* gene were first identified in patients with MEB (Yoshida *et al.*, 2001). A selective deficiency of glycosylated α-DG in MEB patient muscle biopsies was found, suggesting that hypoglycosylation of α-DG may be the pathomechanism of MEB. Walker–Warburg syndrome (WWS) is the most severe form characterized by CMD, major structural brain defects and eye malformations. The first mutations in *POMT1* and *POMT2* were reported in patients with WWS (Beltran-Valero De Bernabe *et al.*, 2002; van Reeuwijk *et al.*, 2005). The gene *POMGNT1* encodes the protein *O*-linked mannose β1,2-*N*-acetylglucosaminyltransferase 1 (POMGnT1) which forms GlcNAcβ1-2Man linkage of *O*-mannosyl glycans (Yoshida *et al.*, 2001), and the protein products of *POMT1* and *POMT2*, protein *O*-mannosyltransferase 1 and 2, are responsible for the catalysis of the first step in *O*-mannosyl glycan synthesis on α-DG (Manya *et al.*, 2004). *POMT1* and *POMT2* are both required for protein *O*-mannosyltransferase activity (Akasaka-Manya *et al.*, 2006; Manya *et al.*, 2004). In WWS patients, as in MEB patients, the highly glycosylated α-DG was selectively deficient in skeletal muscle and brain. WWS and MEB are similar disorders, but the clinical spectrum associated with both diseases is broad (Biancheri *et al.*, 2006; D'Amico *et al.*, 2006).

Other forms of muscular dystrophies have been suggested to be caused by abnormal glycosylation of α-DG, for example, Fukuyama-type congenital muscular dystrophy (FCMD), CMD type 1C (MDC1C), limb-girdle muscular dystrophy 2I (LGMD2I), and CMD type 1D (MDC1D), since highly glycosylated α-DG was also found to be selectively deficient in the skeletal muscle of these patients. These gene products are thus thought to have glycosyltransferase activity or be involved in glycan stability.

FCMD is the second most common form of muscular dystrophy in Japan, and characterized by central nervous system involvement. Severe mental retardation and epilepsy are characteristic clinical features of FCMD, with brain showing polymicrogyria/pachygyria caused by altered neuronal migration. Kobayashi *et al.* (1998) identified the gene responsible for FCMD, which

encodes a protein named fukutin. Sequence analysis of fukutin predicts it to be an enzyme that could modify glycoconjugates. In addition, mutations in a homologue of fukutin, the fukutin-related protein (*FKRP*), were found in MDC1C patients (Brockington *et al.*, 2001a). MDC1C is characterized by a rapidly progressive muscle disease leading to a complete loss of muscle function and lethal respiratory insufficiency during the second decade (Quijano-Roy *et al.*, 2002). Mental retardation and cerebellar cysts have been observed in some patients. In contrast, allelic mutations in the *FKRP* gene cause a milder and more common form of myopathy, named LGMD2I, with a variable onset ranging from adolescence to adulthood (Brockington *et al.*, 2001b). Patients with *FKRP* mutations have reduced expression of glycosylated α-DG, broadly correlating with disease severity (Brown *et al.*, 2004). Finally, the gene *LARGE* encodes a putative glycosyltransferase (Grewal *et al.*, 2001). However, its biochemical activity has not yet been confirmed. Mutations in the *LARGE* gene cause MDC1D, a novel form of CMD also with a variable degree of mental retardation and brain abnormalities (Longman *et al.*, 2003; van Reeuwijk *et al.*, 2007).

Since multiple genes are known to cause α-dystroglycanopathies, with an extremely broad clinical spectrum and relatively poor phenotype–genotype correlation (Mercuri *et al.*, 2009), at present molecular diagnosis of α-dystroglycanopathy patients is difficult and often requires the analysis of several genes, which is expensive and time consuming. At present, of the six known α-dystroglycanopathy genes, the functions of the protein products are clear only for POMT1, POMT2, and POMGnT1. To assess the pathogenicity of several mutations, we demonstrated by a specific enzymatic assay that mutations in *POMGNT1* and *POMT1* lead to defects in respective enzymatic activities using mutant constructs transfected into cell lines (Akasaka-Manya *et al.*, 2004; Manya *et al.*, 2003). Another group reported POMGnT1 enzymatic assay in lymphoblasts and muscle biopsies (Vajsar *et al.*, 2006; Zhang *et al.*, 2003). Recent established mouse models for α-dystroglycanopathy will help our understanding between glycosylation and pathophysiology of these diseases (Kanagawa *et al.*, 2009; Liu *et al.*, 2006; Miyagoe-Suzuki *et al.*, 2009).

This chapter describes the assay protocols to diagnose patients with α-dystroglycanopathy by measuring glycosyltransferase activity in lymphoblast microsomal preparations (Manya *et al.*, 2008).

2. Methods

2.1. Cell Culture and Preparation of Microsomal Membrane Fraction

Blood from five healthy subjects and seven patients with CMD, with mental retardation, or hypoglycosylation of α-DG, or both, was collected for B lymphoblasts immortalization and DNA extraction after informed consent

Table 19.1 Summary of patients examined in the present study

Patient	Clinical diagnosis	Molecular diagnosis	
1	MEB	*POMGNT1*	IVS17+1G>A homozygous
2	MEB	*POMGNT1*	p.Arg442His homozygous
3	CMD-MR	*POMT1*	p.Gly65Arg + Trp582Cys heterozygous
4	LGMD-MR	*POMT1*	p.Ala200Pro homozygous
5	MEB	Uncharacterized	
6	CMD-MR	Uncharacterized	
7	MEB	Uncharacterized	

CMD, congenital muscular dystrophy; LGMD, limb-girdle muscular dystrophy; MR, mental retardation.

from the parents. Four patients had already been genetically characterized (patients 1 and 2 for *POMGNT1* and patients 3 and 4 for *POMT1*; Table 19.1). Three other patients (patients 5–7) were genetically uncharacterized.

B lymphoblasts were obtained after immortalization by Epstein–Barr virus and cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) according to standard protocols to obtain 100 $\times$ 10^6 cells. After centrifugation at 800$\times g$ for 5 min, the pellets were rinsed twice with 50 ml then with 12 ml of PBS buffer. The final pellets were frozen at –80 °C. The cells ($\sim$7.5 $\times$ 10^6 cells) were homogenized in 10 m*M* Tris–HCl, pH 7.4, 1 m*M* EDTA, 250 m*M* sucrose, 1 m*M* dithiothreitol, with a protease inhibitor cocktail (3 μg/ml pepstatin A, 1 μg/ml leupeptin, 1 m*M* benzamidine–HCl, and 1 m*M* PMSF). After centrifugation at 900$\times g$ for 10 min, the supernatant was subjected to ultracentrifugation at 100,000$\times g$ for 1 h. The precipitate was used as the microsomal membrane fraction (enzyme source). Protein concentration was determined by BCA assay (PIERCE, Rockford, IL). About 40 μg proteins of microsomal membranes were obtained from 1 $\times$ 10^6 cells.

2.2. Assay for glycosyltransferase activity

Since GnT1 (UDP-GlcNAc: α-3-D-mannoside β1,2-*N*-acetylglucosaminyltransferase 1, EC 2.4.1.101) is not involved in *O*-mannosylglycan biosynthesis, it is not affected in α-dystroglycanopathies and represents a suitable control to normalize samples for baseline microsomal activity.

2.3. GnT1 activity

The GnT1 activity was performed in a total volume of 20 μl reaction mixture containing 100 m*M* MES buffer, 10 μ*M* pyridylaminated $Man_5GlcNAc_2$ (M5-PA, Takara Bio, Inc., Otsu, Japan), 2 m*M* UDP-GlcNAc, 5 m*M*

AMP, 0.5% Triton X-100, 0.2% BSA, 20 m*M* $MnCl_2$, and enzyme source (100 μg of microsomal membrane fraction) at 37 °C for 2 h. The samples were then analyzed by reversed phase HPLC with a COSMOSIL 5C18-AR-II column (4.6 × 250 mm, Nacalai Tesque, Kyoto, Japan). The solvent used was a 100 m*M*, pH 6.0, ammonium acetate buffer containing 0.15% 1-butanol, and the substrate and the product were isocratically separated. Fluorescence was detected with a fluorescence detector (RF-10AXL, Shimadzu Corp., Kyoto, Japan) at excitation and emission wavelengths of 320 and 400 nm, respectively. The GnT1 activity mean (±standard deviation) of all samples was 0.53 (±0.06) nmol/h/mg total proteins with high constancy.

2.4. POMGnT1 activity

The POMGnT1 activity was based on the amount of [^{3}H]GlcNAc transferred to a mannosylpeptide (Ac-Ala-Ala-Pro-Thr(Man)-Pro-Val-Ala-Ala-Pro-NH_2) as described in a previous chapter of this series (Endo and Manya, 2006). The mannosylpeptide is not commercially available but it is possible to use Benzyl-Man, which is commercially available, as a substitute as described previously (Endo and Manya, 2006). Therefore, the procedures are described briefly here. The reaction buffer containing 140 m*M* MES buffer (pH 7.0), 1 m*M* UDP-[^{3}H]GlcNAc (225,000 dpm/nmol) (PerkinElmer, Inc., Waltham, MA), 1 m*M* mannosyl nanopeptide, 10 m*M* $MnCl_2$, 2% Triton X-100, 5 m*M* AMP, 200 m*M* GlcNAc, 10% glycerol, and enzyme source (100 μg of microsomal membrane fraction) in 20 μl total volume was incubated at 37 °C for 4 h. After boiling for 3 min, the mixture was analyzed by reversed phase HPLC with a Wakopak 5C18-200 column (4.6 × 250 mm, Wako Pure Chemical Industries, Ltd., Osaka, Japan). Solvent A was 0.1% trifluoroacetic acid in distilled water and solvent B was 0.1% trifluoroacetic acid in acetonitrile. The peptide was eluted at a flow rate of 1 ml/min using a linear gradient of 1–25% solvent B. The peptide separation was monitored continuously at 214 nm, and the radioactivity of each fraction was measured using a liquid scintillation counter. The average POMGnT1 activity measured in lymphoblasts of control patients was 0.163 (±0.042) nmol/h/mg total proteins.

2.5. POMT activity

The POMT activity was based on the amount of [^{3}H]-mannose transferred to a glutathione-*S*-transferase fusion α-DG (GST-αDG) as described also in a previous chapter of this series (Endo and Manya, 2006). Therefore, the procedures are described briefly here. The reaction mixture contained 20 m*M* Tris–HCl (pH 8.0), 100 n*M* of [^{3}H]-mannosylphosphoryldolichol (Dol-P-Man, 125,000 dpm/pmol) (American Radiolabeled Chemical, Inc., St. Louis, MO), 2 m*M* 2-mercaptoethanol, 10 m*M* EDTA, 0.5% *n*-octyl-β-D-thioglucoside (Dojindo Laboratories, Kumamoto, Japan), 10 μg

GST-α-DG, and enzyme source (80 μg of microsomal membrane fraction) in 20 μl total volume. After 1 h incubation at 22 °C, the reaction was stopped by adding 150 μl PBS containing 1% Triton X-100, and the reaction mixture was centrifuged at 10,000×*g* for 10 min. The supernatant was removed, mixed with 400 μl of PBS containing 1% Triton X-100 and 10 μl of Glutathione Sepharose 4B beads (GE Healthcare Bio-Sciences Corp., NJ), rotated at 4 °C for 1 h, and washed three times with 20 m*M* Tris–HCl (pH 7.4) containing 0.5% Triton X-100. The radioactivity adsorbed to the beads was measured using a liquid scintillation counter. The average POMT activity in lymphoblasts of control subjects was 0.041 (±0.013) pmol/h/mg proteins.

2.6. Mutation analysis

Genomic DNA was extracted from lymphoblasts using standard methods. Primer pairs were designed to amplify all coding exons and flanking intronic sequences of *POMT1* (9q34.1), *POMT2* (14q24), and *POMGNT1* (1p34.1). The primer sequences and PCR conditions are available upon request. The generated amplicons were purified and directly sequenced with the BigDye terminator kit (PerkinElmer Applied Biosystems, Wellesley, MA). Sequences were analyzed on an ABI PRISM 31130 capillary sequencer (Applera, CA). For patient 7, to find the second mutation, total RNA extracts from lymphoblasts were reversed transcribed and *POMT2* cDNA was amplified by nested PCR as previously reported (Yanagisawa *et al.*, 2009).

3. Procedures for Enzymatic Activity and Mutation Search

When we assessed the POMGnT1 activity in lymphoblasts from patients 1 and 2, enzymatic activity in these lymphoblasts was much lower than in the control subjects (Fig. 19.1). Those had previously been genetically confirmed with mutations in the *POMGNT1* gene (Table 19.1). Patient 1 carried the mutation c.1539+1 G>A in the homozygous state, and patient 2 harbored the mutation p.Arg442His, also in homozygous state.

When we assessed POMT activity in lymphoblasts from the patients who were been previously genetically confirmed with mutations in the *POMT1* gene (Table 19.1). Patient 3 was a compound heterozygous carrier of two missense mutations, p.Gly65Arg and p.Trp582Cys (van Reeuwijk *et al.*, 2006). Patient 4 was homozygous for the missense mutation p.Ala200Pro (Balci *et al.*, 2005). The enzyme activity in these patient lymphoblasts was extremely low.

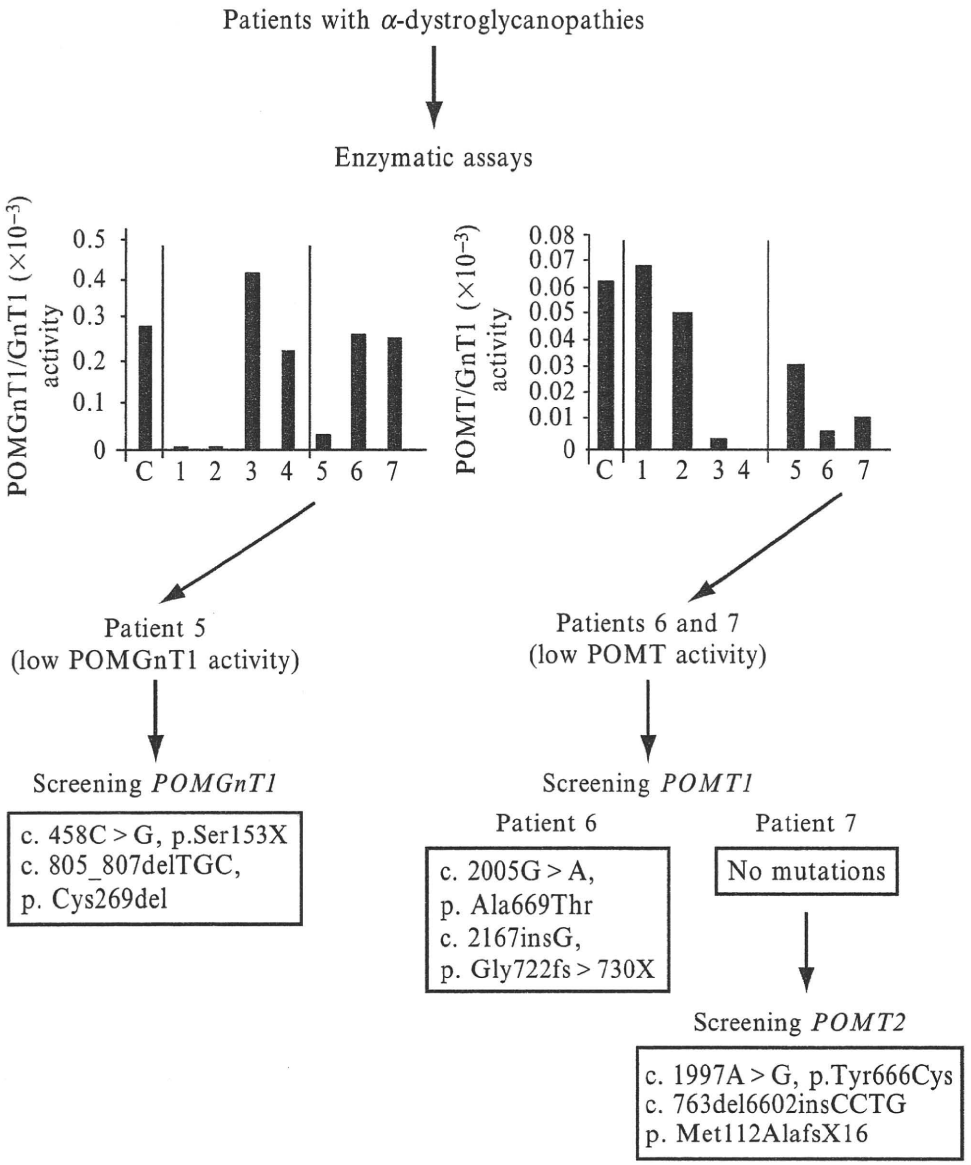

Figure 19.1 Schematic illustration of procedures for enzymatic activity and mutation search. Enzymatic activities in lymphoblasts from uncharacterized patients with α-dystroglycanopathies were measured. If a patient showed low enzymatic activity, the potential responsible gene was screened. Patient 5 showed low POMGnT1 activity, and *POMGNT1* was thus screened. Patients 6 and 7 showed low POMT activity, and thus patients 6 and 7 were screened for *POMT1* at first. However, since no mutations were found in the *POMT1* gene of patient 7, then the *POMT2* gene was further studied.

Among the uncharacterized patients, patient 5 showed low POMGnT1 activity and was thus secondarily screened for *POMGNT1*. The DNA study of this patient revealed two heterozygous mutations: a nonsense mutation, p.Ser153X (c.458C>G), and a deletion of three nucleotides c.805-807delTGC, which is expected to delete one amino acid, cysteine at position 269 (p.Cys269del), localized in the stem domain of the protein (Leu59-Leu300) (Manya *et al.*, 2008).

When we assessed POMT activity in the uncharacterized patients, we observed a markedly reduced activity in patient 6 and patient 7 (Fig. 19.1). Then patient 6 and patient 7 were secondarily screened for *POMT1* at first. We found two heterozygous mutations, in *POMT1* for patient 6: p.Ala669Thr (c.2005G>A), associated with c.2167insG which leads to a premature stop codon in amino acid 730 (Manya *et al.*, 2008). However, no mutation was found in the *POMT1* gene of patient 7. Then we screened *POMT2* for mutations and finally found two heterozygous mutations: a missense mutation, p.Tyr666Cys, and a large deletion 763del6602insCCTG leading to a premature stop codon (Yanagisawa *et al.*, 2009).

In conclusion, the lymphoblast-based enzymatic assay is an accurate and extremely useful method to select the patients harboring *POMT1*, *POMT2*, and *POMGNT1* mutations among those with suspected α-dystroglycanopathies. In other words, the enzymatic assay can be used as a first screening tool for narrowing the responsible gene in α-dystroglycanopathies. Interestingly, the same POMT assay was successfully used in skin fibroblasts from patients (Lommel *et al.*, 2010). The combinatory study of enzyme activity and gene mutation screening will help surveying patients with α-dystroglycanopathies and better understanding the clinical spectrum of theses pathologies.

ACKNOWLEDGMENT

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ARTICLE

Congenital muscular dystrophy type 1D (MDC1D) due to a large intragenic insertion/deletion, involving intron 10 of the *LARGE* gene

Nigel F Clarke[1,2,11], Svetlana Maugenre[1,2,11], Aurélie Vandebrouck[1,2], J Andoni Urtizberea[3], Tobias Willer[4], Rachel A Peat[1,2], Françoise Gray[5], Céline Bouchet[6], Hiroshi Manya[7], Sandrine Vuillaumier-Barrot[6], Tamao Endo[7], Eliane Chouery[8], Kevin P Campbell[4], André Mégarbané[8,9,10] and Pascale Guicheney*[,1,2]

Mutation of the *LARGE* gene is the rarest of the six known genetic causes of α-dystroglycanopathy. We report further a family with MDC1D due to a complex genomic rearrangement that was not apparent on standard sequencing of *LARGE*. Two sisters in a consanguineous family had moderate mental retardation and cerebellar malformations, together with dystrophic changes and markedly reduced α-dystroglycan glycosylation staining on muscle biopsy. There was homozygous linkage to the *LARGE* locus but sequencing of *LARGE* coding regions was normal. Analysis of *LARGE* cDNA showed an abnormal sequence inserted between exons 10 and 11, in most of the transcripts, predicted to introduce a premature stop codon. The abnormal sequence mapped to a spliced EST (DA935254) of unknown function, normally located at 100 kb centromeric of *LARGE* on chromosome 22q12.3. Quantitative PCR analysis of the EST and adjacent regions showed twice the normal copy number in patients' genomic DNA samples, consistent with a large intra-chromosomal duplication inserted into intron 10 of *LARGE* in a homozygous state. This insertion was associated with deletion of a central region of intron 10, but the exact break points of the deletion/duplication were not found, suggesting that an even more complex rearrangement may have occurred. The exact function of LARGE, a golgi protein, remains uncertain. POMT and POMGnT enzyme activities were normal in patients' lymphoblast cells, suggesting that defects in LARGE do not affect the initiation of *O*-mannosyl glycans.
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Keywords: congenital muscular dystrophy 1D; alpha-dystroglycan; DNA duplication; muscle-eye-brain disease

INTRODUCTION

Alpha-dystroglycan (αDG) is a highly glycosylated cell surface protein that has important roles in neuronal cell migration and cell-to-extracellular matrix interactions in muscle. Abnormality in the glycosylation of αDG is the hallmark histological abnormality and the likely pathogenic mechanism for a group of congenital muscular dystrophies (CMD), collectively called the 'α-dystroglycanopathies'.[1] At present, six genes have been linked to this common clinicopathological presentation (*POMT1, POMT2, POMGNT1, FKTN, FKRP* and *LARGE*). All of these encode confirmed or putative enzymes that are thought to function in the O-mannosyl glycosylation pathway. Abnormal αDG glycosylation correlates with an increasingly severe pattern of tissue effects that extend from an adult-onset limb-girdle muscular dystrophy with normal intelligence (LGMD type 2I) to severe congenital brain and eye malformations (Walker–Warburg syndrome, WWS).[1] The *LARGE* gene became a candidate for human CMD after the discovery of the myodystrophic (myd) mouse that has a spontaneous null mutation in *Large* because of a genomic deletion involving exons 5 to 7.[2–4] Another spontaneous murine mutant, named veils (vls) also results from an intragenic deletion leading to loss of exons 3 to 5 and premature protein termination.[5] The two strains share typical cerebral, ocular and muscular changes of an α-dystroglycanopathy. MDC1D due to mutations in *LARGE* is the rarest of the α-dystroglycanopathies identified to date, with only three confirmed families previously described.[6–8] Affected children have had typical neurological and muscle abnormalities associated with the α-dystroglycanopathies, but with very different severities; one family had mild muscle-eye-brain disease (MEB) and the other two had typical WWS. Another WWS patient has been reported with a single heterozygous nonsense mutation in *LARGE*.[9] Confirmation that this patient has CMD due to *LARGE* awaits the discovery of a second mutation. In this paper, we describe the fourth confirmed family with MDC1D due to a homozygous *LARGE* mutation, in which two sisters from consanguineous parents have moderate mental retardation, cerebellar and

[1]Inserm, U956, Faculté de Médecine Pierre et Marie Curie, Paris, France; [2]UPMC Univ Paris 06, Paris, France; [3]AP-HP, Hôpital Marin, Hendaye, France; [4]Howard Hughes Medical Institute, Departments of Molecular Physiology and Biophysics, Neurology and Internal Medicine, University of Iowa Roy J and Lucille A Carver College of Medicine, Iowa City, IA, USA; [5]AP-HP, Hôpital Lariboisière, Service Central d'Anatomie et de Cytologie Pathologiques, Paris, France; [6]AP-HP, Hôpital Bichat-Claude Bernard, Biochimie Métabolique, Paris, France; [7]Tokyo Metropolitan Institute of Gerontology, Glycobiology Research Group, Foundation for Research on Aging and Promotion of Human Welfare, Itabashi-ku, Tokyo, Japan; [8]Université Saint Joseph, Faculté de Médecine Unité de Génétique Médicale, Beirut, Lebanon; [9]Institut Jérôme Lejeune, Paris, France; [10]Inserm, UMR910, Génétique Médicale et Génomique Fonctionnelle, Faculté de Médecine de la Timone, Marseille, France
*Correspondence: Dr P Guicheney, Unité UMR_S956, Faculté de Médecine Pierre et Marie Curie, site Pitié-Salpêtrière, 91 boulevard de l'Hôpital, 75634 Paris Cedex 13, France. Tel: +33 1 40 77 9805; Fax: +33 1 40 77 9645; E-mail: pascale.guicheney@upmc.fr
[11]These authors are co-first authors.
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pontine hypoplasia, and persistent white matter abnormalities on brain magnetic resonance imaging (MRI), features consistent with mild MEB. We identified an unusual genetic mechanism for *LARGE* gene disruption, namely the splicing of an abnormal sequence into the *LARGE* mRNA transcript likely due to a duplication/deletion event involving intron 10. This report extends our knowledge of the clinical phenotype associated with *LARGE*, and is the second family in which disease results from a large-scale gene rearrangement.

The *POMT1*, *POMT2* and *POMGNT1* genes encode enzymes that together mediate the first two steps in the *O*-mannosyl glycosylation pathway. A recent study suggests that *LARGE* participates in post-phosphoryl glycosylation of a novel phosphorylated *O*-mannosyl glycan on the mucin-like domain of recombinant αDG, which is required for laminin binding.[10] Evidence from cell culture and a *fukutin* transgenic mouse suggests that fukutin physically interacts with POMGnT1 and can cause reduced POMGnT1 activity when abnormal.[11] To investigate whether LARGE may also function by influencing the first steps of the *O*-mannosyl pathway, we assessed the activity levels of POMT and POMGnT1 in cultured lymphoblasts from one of our patients.

MATERIALS AND METHODS

Case reports

The two affected girls are the only children of first-cousin parents from Lebanon. There is no other family history of neuromuscular disease. Both children were born full-term after uneventful pregnancies with normal foetal movements and deliveries. Birth weights, lengths and head circumferences were within normal limits.

Patient 1: The older sibling first presented to medical attention at an age of 2.5 months with hypotonia and regurgitation. Acquisition of all motor skills was delayed. She sat at an age of 9 months, stood unsupported at an age of 2.5 years and at an age of 8 years, she could climb stairs with difficulty but could not run. Her full-scale IQ was 61. On examination at an age of 8 years, she weighed 24 kg (50th centile), her height was 122.5 cm (35th centile) and the occipitofrontal circumference (OFC) was 53 cm (75th centile). Her speech was restricted to dysarthric simple words, and she followed simple commands. She had a myopathic face with an open-mouth posture and mild tongue hypertrophy. There was generalised muscle hypertrophy, most prominent in the calves, thighs and the shoulder girdle. This contrasted with moderate proximal lower limb weakness that manifested as a waddling gait and a positive Gowers' manoeuvre. Upper limb strength was within normal limits. There were no joint contractures, except for mild tightness of the Achilles' tendons, associated with toe walking. The only abnormalities found on eye assessment were mild myopia and strabismus. There was no history of seizures. Cardiac and respiratory examinations were normal. Brain MRI performed at ages 3 ½ and 6 years showed marked cerebellar atrophy (particularly affecting the vermis) and cerebellar cysts (Figure 1). There was mild generalised cerebral and pontine atrophy, dilatation of all ventricular spaces and pachygyria of the frontal lobes (Figure 1). Diffuse symmetrical high signal abnormalities were seen within the periventricular and deep white matter of both cerebral hemispheres, with sparing of the subcortical U fibres. These hyperintensities were unchanged at the age of 6 years. A generalised skeletal X-ray survey, abdominal ultrasound, echocardiogram, electroencephalogram and auditory-evoked potentials with brainstem responses were all normal. Creatine kinase (CK) levels were 30 times higher than the upper limit of normal.

Patient 2: The younger sister followed a clinical course similar to her sister, but was less delayed in her motor and cognitive skills. She stood unsupported at an age of 16 months and walked independently at an age of 2 years. On examination at an age of 4 ½ years, her weight was 15 kg (25th centile), height was 106 cm (75th centile) and OFC was 51 cm (50th centile). There was generalised hypotonia, firm hypertrophic calves and a mild equinovarus foot position. Mild myopia was the only abnormality observed on eye examination. Myopathic features were noted on EMG analysis, whereas nerve conduction studies were normal. Brain MRI performed at 2 years showed diffuse high signal white matter abnormalities, mainly in the frontal and parieto–occipital regions, dilated ventricular spaces and marked cerebellar hypoplasia. CK levels were 60 times above the upper limit of normal.

Muscle biopsy findings

Muscle and skin biopsy samples were taken from Patient 1 from the right gastrocnemius muscle. Standard histochemical analysis revealed a dystrophic pattern characterised by fibre size variation, markedly increased connective tissue, internalised nuclei and scattered necrotic fibres. Immunohistochemistry (IHC) for αDG showed markedly reduced staining using VIA4-1 (Millipore, Billerica, MA, USA), an antibody that recognises a glycosylated epitope. Laminin-α2 expression was mildly reduced by IHC using antibodies that recognize both the 80 kDa (clone 5H2; Millipore) and the 300 kDa (NCL-merosin; Novocastra Labs, Newcastle, UK) fragments. Staining for β-dystroglycan (βDG), dystrophin, collagen VI and sarcoglycans (α and γ) was normal.

WESTERN BLOT ANALYSIS

Western blotting was used to investigate the degree of abnormal αDG glycosylation using a skin fibroblast cell line obtained from Patient 1, as frozen muscle was not available. Fibroblasts were maintained at 37°C and 5% CO_2 in DMEM medium plus 20% foetal bovine serum and 0.5% penicillin-streptomycin (Gibco, Invitrogen Corp., Carlsbad, CA, USA). Cultured cells were lysed, and glycoproteins from cell homogenates were enriched with WGA lectin as previously described.[11] We performed immunoblots (for αDG and βDG) and laminin ligand overlay assays using polyvinylidene difluoride membranes, and blots were developed by horseradish peroxidase-enhanced chemiluminescence (Pierce, Thermo Fisher Scientific, Rockford, IL, USA).[12] The monoclonal antibodies IIH-6 and VIA4-1, which are specific for functionally glycosylated αDG,[13] showed no detectable binding affinity to

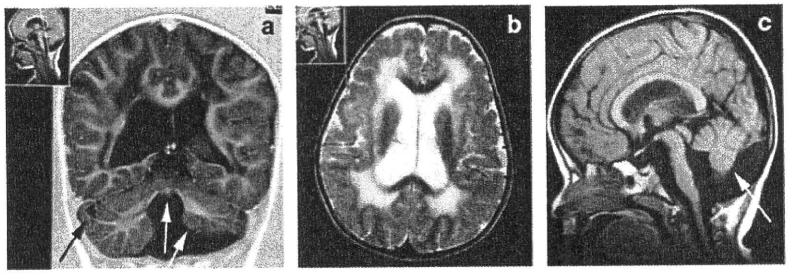

Figure 1 Brain MRI images obtained from Patient 1 performed at an age of 3.5 years. (**a**) Coronal T1-weighted image showing cerebellar and vermis hypoplasia (white arrows), cerebellar cysts (black arrow), generalised white matter atrophy and increased CSF spaces. (**b**) Transverse T2-weighted image showing abnormal high white matter signal and dilated lateral ventricles. (**c**) Sagittal T1-weighted image showing cerebellar (white arrow) and pontine hypoplasia.

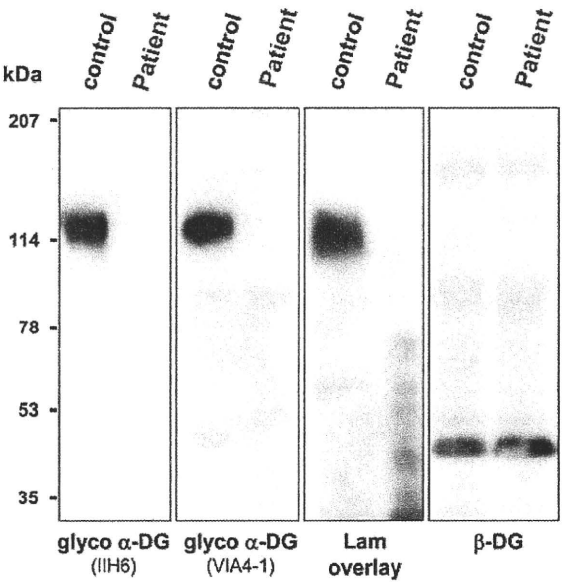

Figure 2 Western blot analysis of α-dystroglycan. Immunoblot analysis of WGA-enriched homogenates from fibroblasts obtained from Patient 1 and a healthy individual (control). Absent staining of antibodies IIH6 and VIA4 that bind to glycosyl epitopes on α-dystroglycan (α-DG) and a laminin-1 (Lam) overlay assay show abnormalities in αDG glycosylation in patient fibroblasts that is associated with loss of laminin-1 protein binding. Beta-dystroglycan (β-DG) is used as a loading control.

αDG obtained from patient fibroblast cells, even though βDG (monoclonal antibody 7D11) was normally present, consistent with a glycosylation defect in αDG (Figure 2). This defect in αDG glycosylation also resulted in loss of laminin-1 ligand binding (Figure 2).

GENETIC ANALYSIS

We performed linkage analysis using microsatellite markers to the five α-dystroglycanopathy loci known at the time (*POMGNT1*, *FKRP*, *POMT1*, *POMT2* and *FKTN*) and the *DAG1* gene that encodes DG, but we identified no homozygous linkage. A genome-wide linkage study using 400 microsatellite markers showed potential linkage to the newly identified *LARGE* locus and analysis of further markers supported homozygous linkage (Figure 3). We sequenced the coding regions of *LARGE* bi-directionally by standard techniques. Oligonucleotide primers, at least 80 base pairs (bp) outside the exons, were chosen for PCR amplification and sequencing on ABI 3730 capillary electrophoresis system (Applied Biosystems, Life Technologies Corp., Carlsbad, CA, USA), but no abnormalities were found. Subsequently, mRNA was extracted from cultured EBV-transformed lymphoblasts obtained from Patient 1 (Trizol method), cDNA was generated using oligodT Superscript II First-Strand Synthesis (Invitrogen, Carlsbad, CA, USA) and the *LARGE* gene transcript was sequenced using 14 overlapping primer pairs. Two abnormally large PCR products, together with a faint product of the expected size, were obtained for primers that spanned between exons 9 and 12 (Figure 4a). Sequencing revealed that most mRNA transcripts contained an abnormal 197 bp sequence inserted between exons 10 and 11. Low levels of a 171 bp insertion (identical to the 197 bp sequence except lacking 26 bases from the 3′ end) and a normally spliced transcript were also obtained. Identical splicing abnormalities were also seen in mRNA from cultured patient fibroblast cells, except a greater proportion of transcripts showed normal splicing

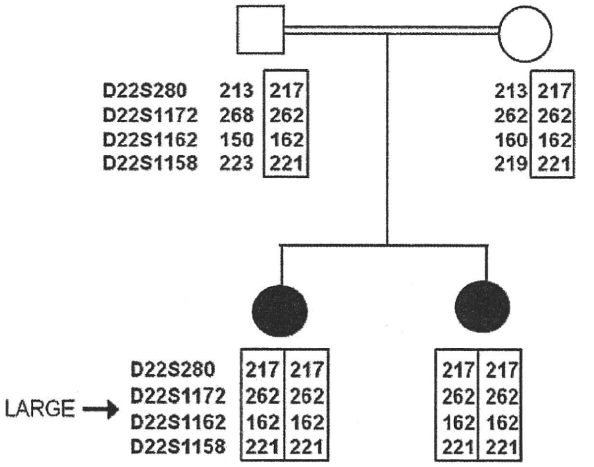

Figure 3 Genotyping for the *LARGE* locus. Family tree showing analysis of microsatellite markers in close proximity to the *LARGE* locus (22q12.3). Both of the affected children are homozygous for four contiguous markers, consistent with homozygosity by descent for this chromosomal region.

(Figure 4a). The abnormal sequences mapped to a part of a spliced EST (GenBank accession no: DA935254) of unknown function, originally identified in a human splenic cDNA library,[14] normally located at 100 kb centromeric to the *LARGE* gene on chromosome 22q12.3 (Figure 5). The presence of the two most abundant abnormally inserted sequences are predicted to immediately introduce a premature stop codon in the *LARGE* mRNA transcript, resulting in truncation of the LARGE protein mid-way through translation (Figure 4b). Analysis of the inserted EST sequence identified potential splice acceptor and donor sites for both the larger and smaller insertions that likely mediate the abnormal splicing (Supplementary E, Figure 1). Archived frozen muscle was no longer available to verify the abnormalities in *LARGE* splicing in muscle.

To investigate whether the abnormal *LARGE* splicing arose because of a chromosomal duplication, we performed quantitative PCR (qPCR) analysis of the EST sequence obtained from patients' genomic DNA (gDNA) using the LightCycler 480 (Roche Diagnostics, Basel, Switzerland) and the SYBR Green I detection system (Roche Applied Science, Indianapolis, IN, USA). We normalised gDNA concentrations between individual samples using PCR primers for a distant gene (*PTPLA* on chromosome 10). Supplementary E, Table 1 lists the key primer pairs used. The EST (DA935254) was present in double the expected copy number in both the patients compared with two healthy unrelated individuals (Figure 4c), consistent with a homozygous duplication of the EST sequence in both patients (ie, they have four copies instead of two). To investigate the size of the putative duplication, we repeated qPCR analysis for genomic regions at variable distances up- and downstream of the EST sequence (Supplementary E, Table 1). These studies indicate that between 40825 and 42937 bp of gDNA, surrounding the EST sequence, is present at twice the normal copy number in gDNA in both patients (Figure 4c). Both parents had 1.5 times the normal copy number, consistent with each having one copy of the chromosome-22 duplication and a normal allele. The break point at the 3′-end is likely located in a 1313 bp region located between regions 3b and 3c (Chr 22:33548218–33549530; GRCh37/hg19 assembly) and the 5′-break point in a 799 bp region located between regions 5b and 5c (Chr 22:33590356–33591154).

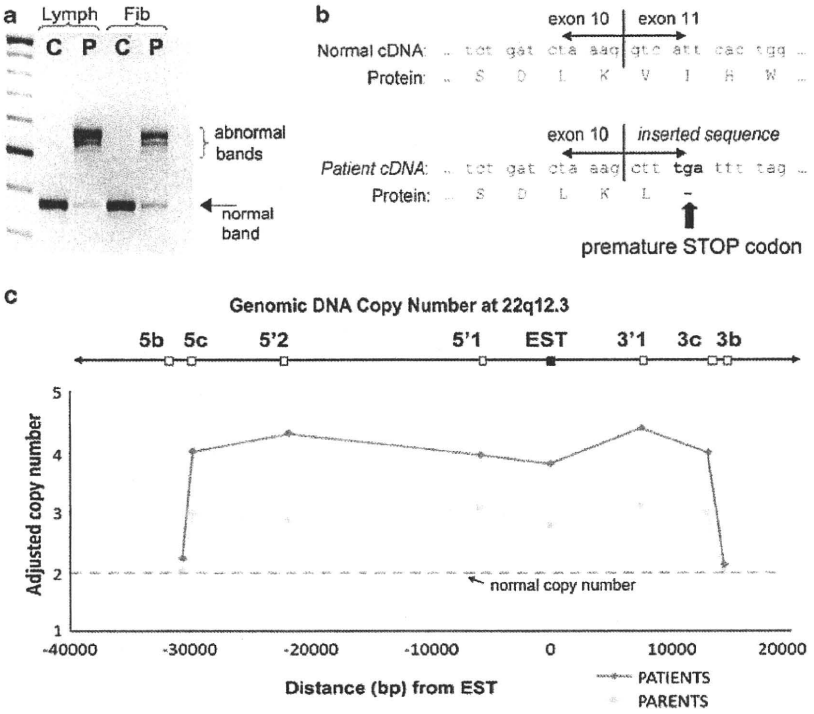

Figure 4 Analysis of the *LARGE* gene insertion in genomic and cDNA. (**a**) Agarose gel showing the PCR products from patient (P) and control (C) cDNA generated from cultured lymphoblasts (Lymph) and fibroblasts (Fib) using primers that span from exons 9 to 12 of *LARGE* (Supplementary E, Table 1). In patient cDNA sample, two abnormally high bands predominate that correspond to insertions of abnormal DNA sequences between exons 10 and 11. A faint band of the normal size is also seen (arrow). (**b**) Diagram showing the amino acid sequence that is coded by normal and patient cDNA around the exon 10/11 boundary. The abnormal insertion found in patient cDNA results in the introduction of a premature STOP codon, predicted to cause protein termination mid-way through the translation of the LARGE protein. (**c**) Results from qPCR analysis of genomic DNA (gDNA) for EST DA935254 and the surrounding chromosomal region. gDNA concentrations between samples were adjusted using qPCR results from a distant gene (*PTPLA*) and results were scaled so that control samples had a mean copy number of 2 at each point (data not shown). In patients' gDNA sample, qPCR analysis of the EST sequence and for five regions, located at variable distances upstream and downstream, shows twice the expected copy number consistent with homozygous duplication of this region. Genomic copy number returns to normal for markers 5b and 3b in the patient gDNA samples, and therefore these markers define the maximum size of the duplication. Analysis of gDNA from both parents shows that both had three copies of the duplicated region, consistent with each having the duplication in a heterozygous state. All results are shown relative to the reverse gDNA strand.

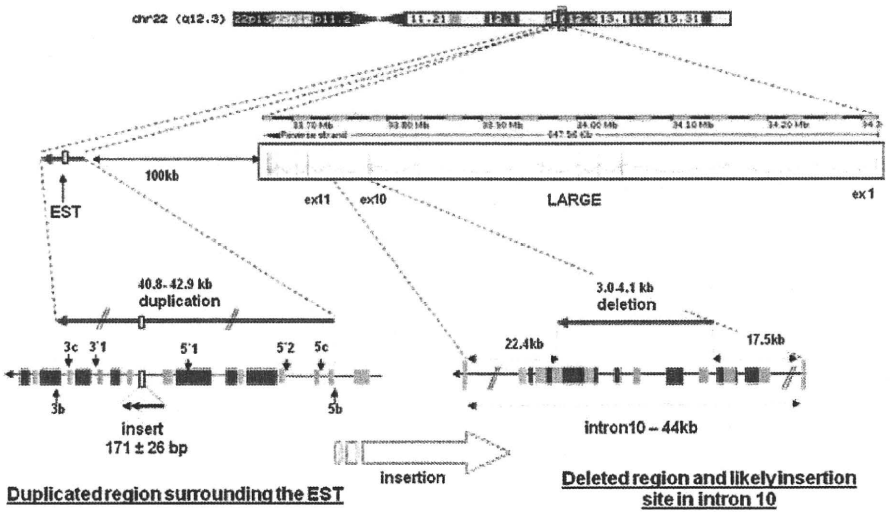

Figure 5 Diagram of the insertion/deletion mutation involving intron 10 of the *LARGE* gene. The EST DA935254, normally located at 100 kb centromeric of *LARGE*, is duplicated together with ~40 kb of flanking DNA and likely inserted into intron 10 of *LARGE*. Numerous repetitive elements, which may have contributed to the large intra-chromosomal rearrangement, are present in both intron 10 and flanking the EST, including SINEs (green), LINEs (red) and LTRs (blue). bp=base pairs.

Together, these results indicate that the patients are homozygous for a duplication of gDNA around EST (DA935254), and the most likely site of insertion for the duplicated DNA is intron 10 of *LARGE*, leading to the abnormal splicing of the EST into the *LARGE* mRNA transcript between exons 10 and 11.

To identify the site of the insertion in intron 10 of LARGE, we designed twelve pairs of overlapping primers (Supplementary E, Figure 2) to cover the whole length of the intron (44117 bp). With two primer pairs (5aF-5aR and 6aF-6aR), we amplified the expected bands in controls and parents but not in the two patients, indicating that the insertion should be in this region (Supplementary E, Figure 2). We repeated the same strategy with several other primer pairs chosen inside this region and in the bordering regions to finally determine a region from 3012 to 4175 bp that could never be amplified in the patients, which likely corresponds to a deleted region associated with the insertion. By qPCR analysis using primer pairs L5F-L6R and L6F-L7R (Supplementary E, Table 1), both parents had reduced amplification compared with controls, consistent with them being heterozygous for a deletion in this region. We were not able to amplify any product with primers chosen on the borders of the insertion and on the non-deleted sides of the intron, and thus did not identify the exact break points for the insertion/deletion rearrangement.

As repetitive sequences are implicated in the pathogenesis of recurrent chromosomal deletion or duplication syndromes (eg, CMT1A),[15] we analysed intron 10 and the duplicated region around EST DA935254 for homologous sequences. Both regions shared multiple SINE, LINE and LTR repeats that may have had roles in the genesis of the mutation, but no extended regions of homology were found.

POMT AND POMGNT1 ACTIVITY IN PATIENTS' LYMPHOBLAST CELLS

The POMT and POMGnT1 enzymes mediate the first and second steps, respectively, of the *O*-mannosyl glycosylation of αDG. To investigate whether *LARGE* mutations may disrupt the glycosylation of αDG by influencing the activity of either POMTs or POMGnT1, we assessed the enzymatic activity of these enzymes in cultured patients' lymphoblast cells using previously validated assays.[16] The POMT activity was based on the amount of [^{3}H]mannose transferred from [^{3}H]mannosylphosphoryldolichol to a glutathione-*S*-transferase fusion αDG, and the POMGnT1 activity was based on the amount of [^{3}H]GlcNAc transferred from UDP-[^{3}H]GlcNAc to a mannosyl-peptide. In brief, we incubated microsomal membrane fractions prepared from patients' and control EBV-immortalised B lymphocytes, with an acceptor substrate and radiolabelled sugar donor substrate. We then assessed the incorporation of radioactivity into each acceptor substrate. We normalised control and patient samples using the activity level of GnT1, a golgi enzyme involved in the *N*-glycosylation pathway, which functions independently of *O*-glycosylation. Results showed no difference between the patient and eight control samples for POMT/GnT1 activity (patient 0.283 *vs* control 0.287 ± 0.067 SD) or for POMGnT1/GnT1 activity (patient 0.068 *vs* control 0.071 ± 0.019 SD).

DISCUSSION

In this paper, we report the fourth confirmed family with MDC1D due to mutations in *LARGE*. The clinical abnormalities are typical for the α-dystroglycanopathy group of disorders. Both sisters have typical features of an α-dystroglycanopathy, such as raised CK levels, dystrophic changes on muscle histology associated with reduced glycosylated α-dystroglycan staining, intellectual disability and brain malformations. In terms of severity of neurological involvement, the two sisters are best classified as having mild MEB disease, even though there were only minor ocular abnormalities. The findings were similar to the first family identified with mutations in *LARGE* with mild/moderate developmental abnormalities of the cerebellum, pons, brain stem and cerebrum, although our patients are the first ones to be reported with cerebellar cysts.[6]

Even though standard sequencing of the coding regions of *LARGE* was normal in the proband of this family, we persisted with analysis of *LARGE* because there was homozygous linkage to the gene locus in the context of consanguinity. Analysis of cDNA, generated from mRNA derived from patients' lymphoblast cells, showed an abnormally large product for a PCR reaction that spanned from exons 9 to 12. Sequencing of this product showed that an abnormal sequence of either 197 or 171 bases was spliced between exons 10 and 11 in almost all transcripts. The abnormal sequence is normally situated on chromosome 22 centromeric to the 3′ end of *LARGE*. In this position, it would not usually be incorporated into the *LARGE* transcript, even though the sequence contains the necessary splice donor and acceptor sites. To explain its incorporation into the *LARGE* mRNA, we hypothesised that the EST sequence was translocated into intron 10 of *LARGE* in our family, possibly through duplication of this genomic region. We assessed the copy number of the EST sequence by qPCR relative to other genes in patients' gDNA samples and showed twice the expected levels, consistent with a genomic duplication in a homozygous state. Both parents had a 1.5-fold increase in copy number (an increase from two to three copies of the sequence) that was consistent with both of them being asymptomatic heterozygous carriers of the duplication. To map the approximate size of the duplication, we repeated qPCR analysis up- and downstream of the EST sequence and showed the duplication spans between 40 and 43 kb of gDNA. Even though we identified the likely site of insertion in the central part of intron 10 and delimited a large intronic region that cannot be amplified in the patients, mapping the precise break points of the proposed duplication/insertion was not feasible, possibly because of the presence of a more complex rearrangement than anticipated.

Analysis of *LARGE* mRNA transcripts from patients' lymphoblast and fibroblast cells suggest that most *LARGE* mRNA transcripts contain an abnormal sequence. Both versions of the abnormal insertion are predicted to immediately introduce a premature stop codon mid-way through protein translation, just before the second catalytic domain, and result in a non-functional protein (Figure 4b). Small amounts of normally spliced transcript were also seen and the levels appeared slightly higher in fibroblasts than in lymphoblasts, suggesting this may vary in different tissues. Unfortunately, it was not possible to assess levels of normally spliced *LARGE* mRNA or protein function either in muscle or in brain, the two main tissues that show pathology in the α-dystroglycanopathies. We observed no functionally glycosylated αDG by western blot analysis in patient fibroblast cells, but it is likely that low levels of functional αDG are present in patient tissues at levels below the threshold of detection by western blot for several reasons. Residual glycosylated αDG staining was present in the muscle biopsy, and both sisters have less severe cognitive deficits and brain malformations than a patient with homozygous null mutations in *LARGE* who had a WWS phenotype.[7] We also detected low levels of a normally spliced *LARGE* gene mRNA transcript in both lymphoblasts and fibroblasts, which should allow normal LARGE protein to be produced at low levels.

We also investigated whether LARGE may exert some of its effects on αDG glycosylation through interactions with either the POMT or the POMGnT1 enzymes. Both POMT and POMGnT1 activities were normal in patients' lymphoblast cells, strongly suggesting that the two initial steps of αDG glycosylation occur independently of LARGE.

At present, it appears that *LARGE* is a rare causative of CMD, as the coding regions of the gene have been screened in large cohorts of patients with α-dystroglycanopathy of unknown cause.[6,8] Although experience is very limited at present, it is notable that two of the four confirmed families with CMD, caused by *LARGE* mutation, have had large gene rearrangements, either a deletion involving several exons[7] or a likely intragenic insertion/deletion (our family). In another family, a heterozygous nonsense mutation (p.Trp516X) was identified on standard gene sequencing, leaving open the possibility that a large deletion/duplication or rearrangement on the other allele was overlooked.[9] In addition, consanguineous families are described that link to the *LARGE* locus but no mutation has been found by exon sequencing.[7,17] LARGE was so named for the enormous genomic region it occupies on chromosome 22q12.3 (647 kb) because of the presence of very large introns enriched in low-copy repeats (LCRs). The structure of the *LARGE* gene may predispose to mutations that involve duplication or deletion of large regions of DNA because of non-allelic homologous recombination between LCRs, the proposed mechanism for many genomic disorders.[18] Sequencing of coding regions from gDNA will not identify many large gene rearrangements. Therefore, sequencing LARGE from cDNA and the application of CGH, qPCR, and other methods for detecting rearrangement mutations should be considered routinely.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Follistatin-derived peptide expression in muscle decreases adipose tissue mass and prevents hepatic steatosis

Masashi Nakatani,[1] Masahiro Kokubo,[2] Yutaka Ohsawa,[3] Yoshihide Sunada,[3] and Kunihiro Tsuchida[1]

[1]*Division for Therapies against Intractable Diseases, Institute for Comprehensive Medical Science (ICMS), and* [2]*Joint Research Laboratories, Fujita Health University, Toyoake; and* [3]*Division of Neurology, Department of Internal Medicine, Kawasaki Medical School, Kurashiki, Japan*

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Nakatani M, Kokubo M, Ohsawa Y, Sunada Y, Tsuchida K. Follistatin-derived peptide expression in muscle decreases adipose tissue mass and prevents hepatic steatosis. *Am J Physiol Endocrinol Metab* 300: E543–E553, 2011. First published January 4, 2011; doi:10.1152/ajpendo.00430.2010.—Myostatin, a member of the transforming growth factor (TGF)-β superfamily, plays a potent inhibitory role in regulating skeletal muscle mass. Inhibition of myostatin by gene disruption, transgenic (Tg) expression of myostatin propeptide, or injection of propeptide or myostatin antibodies causes a widespread increase in skeletal muscle mass. Several peptides, in addition to myostatin propeptide and myostatin antibodies, can bind directly to and neutralize the activity of myostatin. These include follistatin and follistatin-related gene. Overexpression of follistatin or follistatin-related gene in mice increased the muscle mass as in myostatin knockout mice. Follistatin binds to myostatin but also binds to and inhibits other members of the TGF-β superfamily, notably activins. Therefore, follistatin regulates both myostatin and activins in vivo. We previously reported the development and characterization of several follistatin-derived peptides, including FS I-I (Nakatani M, Takehara Y, Sugino H, Matsumoto M, Hashimoto O, Hasegawa Y, Murakami T, Uezumi A, Takeda S, Noji S, Sunada Y, Tsuchida K. *FASEB J* 22: 477–487, 2008). FS I-I retained myostatin-inhibitory activity without affecting the bioactivity of activins. Here, we found that inhibition of myostatin increases skeletal muscle mass and decreases fat accumulation in FS I-I Tg mice. FS I-I Tg mice also showed decreased fat accumulation even on a control diet. Interestingly, the adipocytes in FS I-I Tg mice were much smaller than those of wild-type mice. Furthermore, FS I-I Tg mice were resistant to high-fat diet-induced obesity and hepatic steatosis and had lower hepatic fatty acid levels and altered fatty acid composition compared with control mice. FS I-I Tg mice have improved glucose tolerance when placed on a high-fat diet. These data indicate that inhibiting myostatin with a follistatin-derived peptide provides a novel therapeutic option to decrease adipocyte size, prevent obesity and hepatic steatosis, and improve glucose tolerance.

myostatin; adipocyte; fatty liver; glucose tolerance

THE TRANSFORMING GROWTH FACTOR (TGF)-β superfamily is one of the largest families of secreted growth and differentiation factors and plays important roles in regulating tissue development and homeostasis (37). Myostatin, a member of the TGF-β superfamily, acts as a negative regulator of muscle growth (19, 22). Mutations in the myostatin gene in cattle, sheep, dogs, and humans cause an increase in skeletal muscle mass, indicating conservation of its function in mammals (5, 8, 23, 26, 32, 33). Myostatin is expressed predominantly in skeletal muscle and at significantly lower levels in adipose tissue (22). Inhibition of myostatin causes an increase in skeletal muscle mass and ameliorates several models of muscular dystrophies. Therefore, myostatin inhibitors are a promising therapeutic target to treat muscular atrophy and muscular dystrophy (19, 29, 37).

The loss of myostatin by gene disruption prevents an age-related increase in adipose tissue mass and partially attenuates the obese and diabetic phenotypes (14, 24). The serum leptin concentration and adipose tissue leptin mRNA expression were lower in myostatin null mice than in wild-type mice (24). Inhibition of myostatin by transgenic (Tg) expression of myostatin propeptide was also reported to prevent diet-induced obesity (41, 42). Even when fed a high-fat diet (HFD), these mice exhibited normal insulin sensitivity, unlike wild-type mice (42). Furthermore, ΔACVR2B, a soluble extracellular form of the activin type IIB receptor, effectively decreased the adipose tissue mass (1). These results suggest that inhibition of myostatin signaling could be useful to prevent and/or treat obesity and diabetes.

There are several strategies to block the functions of myostatin, including myostatin propeptide, follistatin, follistatin-related gene (FLRG), follistatin domain-containing growth and differentiation factor-associated serum protein-1 (GASP-1), the potent myostatin inhibitor ΔACVR2B, neutralizing antibodies, and small chemical compounds that block receptor serine kinases (2, 15, 37, 38).

Follistatin was shown to bind to myostatin and inhibit its activity. However, follistatin inhibits other members of the TGF-β superfamily, including GDF11 and activin (11, 37, 38). Although GDF11 and myostatin show a high degree of sequence similarity at the amino acid level, GDF11 is unlikely to regulate skeletal muscle mass, because GDF11 controls skeleton and kidney development rather than regulating muscle mass (21). Like myostatin, activin regulates skeletal muscle mass (12). However, unlike myostatin, activin has many pleiotropic roles including ovarian and neuronal functions (30, 36). In our previous study, we reported the development and characterization of a myostatin inhibitor derived from follistatin, designated FS I-I. FS I-I is unable to neutralize activin but still binds to and inhibits myostatin (27). Tg expression of FS I-I using a skeletal muscle-specific promoter caused a widespread increase in skeletal muscle mass and ameliorated muscular dystrophy. In addition, muscle strength was recovered when the FS I-I Tg mice were crossed with *mdx* mice (27). In this study, we explored whether FS I-I Tg mice are resistant to diet-induced obesity and hepatic steatosis. We found that FS I-I Tg mice exhibited reduced fat accumulation even when fed a normal diet (NFD). Adipocytes were also much smaller than those of wild-type littermates. Furthermore, the FS I-I Tg mice

Address for reprint requests and other correspondence: K. Tsuchida, Division for Therapies against Intractable Diseases, Institute for Comprehensive Medical Science (ICMS), Fujita Health Univ., Toyoake, Aichi 470-1192, Japan (e-mail: tsuchida@fujita-hu.ac.jp).