Figure legends

Fig. 1. Immunoblots of sarcospan (a), β-spectrin (b) and dystrophin (c) expressions in normal human quadriceps femoris muscles (lane 1 of a, b and c, respectively), in muscles of children with Fukuyama congenital muscular dystrophy (FCMD) (lane 2 of a, b and c, respectively) and in muscles of boys with Duchenne muscular dystrophy (DMD) (lane 3 of a, b and c, respectively). Electrophoresis and blotting were done as described in Materials and Methods. In normal human muscle extracts, bands of sarcospan, β-spectrin and dystrophin were at 25kDa, 270kDa and 427kDa, respectively (lane 1 of a, b and c, respectively). The reaction for sarcospan both in FCMD and DMD muscle extracts markedly decreased compared with that of normal muscle extracts (a); while the reaction for β-spectrin in FCMD and DMD muscle extracts showed almost normal intensity to a slight decrease compared with normal control muscle extracts (b). Reactions for dystrophin in extracts of normal and FCMD muscles had almost the same intensity, whereas the reaction for dystrophin in extracts of DMD muscles showed no band (c). Numbers to the left of each figure are the molecular masses of standards.

Fig. 2. Immunohistochemical staining of serial muscle sections of FCMD muscles with anti-sarcospan (a), anti-β-spectrin (b) and anti-neonatal myosin (c) antibodies. Sporadic large diameter FCMD myofibers

(arrows in a) express the sarcospan discontinuously at their myofiber surface, although large diameter FCMD myofibers (asterisks in a) do not necessarily express the sarcospan molecule. Most small diameter FCMD myofibers do not express sarcospan at their surface membrane (a). In b, both small and large diameter FCMD myofibers, including large diameter fibers with arrows and asterisks, express β-spectrin more or less at their myofiber surface. In c, small diameter FCMD myofibers contain immature myosin with neonatal myosin positive immunoreactivity, while large diameter FCMD myofibers, including those with arrows and asterisks in a and b, show negative immunoreactivity with anti-neonatal myosin antibody. (a, b, c ×300)

Fig. 3. Immunohistochemical staining of normal control muscle with anti-sarcospan (a) and anti-β-spectrin (b) antibodies, that of DMD muscle with anti-sarcospan (c), anti-β-spectrin (d) and anti-dystrophin (e) antibodies and that of myotonic dystrophy muscle (f) and facioscapulohumeral muscular dystrophy (FSHD) muscle (g) with anti-sarcospan antibody. Immunoreactivity is noted continuously at the normal control myofiber surface with anti-sarcospan (a) and anti-β-spectrin (b) antibodies. In DMD muscle, immunoreactivity with anti-sarcospan antibody (c) is markedly reduced compared with that of anti-β-spectrin antibody (d) and anti-dystrophin antibody immunoreactivity is negative (e). Anti-sarcospan antibody

immunoreactivity is nearly normal in myotonic dystrophy (f) and FSHD (g) muscles. (a-g ×300)

Fig. 4. Immunohistochemical staining of FCMD and normal control muscles with anti-glycosylated α-dystroglycan (IIH6C4) (a, b), anti-β-dystroglycan (c, d), anti-merosin (e, f), anti-α-sarcoglycan (g, h), anti-β-sarcoglycan (i, j), anti-γ-sarcoglycan (k, l), anti-δ-sarcoglycan (m, n) and anti-dystrophin (o, p) antibodies. Glycosylated α-dystroglycan expression is absent, while expression of β-dystroglycan, α-, β-, γ-, δ-sarcoglycan is more or less reduced, and that of merosin and dystrophin is nearly normal compared with that of normal control muscles immunostained with antibodies raised against the respective molecule. (a-p ×300)

Fig. 5. Co-labeling immunohistochemistry with anti-sarcoglycan and sarcospan antibodies using serial FCMD muscle sections. Immunostainings with antibodies against α-sarcoglycan (a), β-sarcoglycan (c), γ-sarcoglycan (e), δ-sarcoglycan (g) and sarcospan (b, d, f, h) are shown. Sarcoglycan immunostaining preparations show the presence of continuously immunopositive, partially immunopositive and immunonegative myofibers, while sarcospan immunostaining specimens showed generally reduced expression of sarcospan at the myofiber surface. Continuously immunopositive myofibers with

anti-sarcoglycan antibodies (asterisks in a, c, e, g) also expressed more or less sarcospan molecule (asterisks in b, d, f, h). Myofibers partially expressing sarcoglycan (arrows in a, c, e, g) tended to show negative immunostaining with anti-sarcospan antibody (arrows in b, d, f, h).

(a-h ×300)

Fig. 6 Immunohistochemical stainings of serial FCMD muscle sections with anti-α-dystroglycan core protein (a), sarcospan (b), α-dystroglycan glycosylated sugar (IIH6C4) (c) and neonatal myosin heavy chain (d) antibodies. Immunostaining with antibody against α-dystroglycan core protein (a) shows the presence of immunopositive FCMD myofibers that tend to be large diameter myofibers (a), while immunostaining specimen with anti-sarcospan antibody reveals reduced expression of sarcospan (b). Immunohistochemical preparation with anti-glycosylated α-dystroglycan antibody (IIH6C4) (c) shows almost total deficiency of glycosylated α-dystroglycan expression. Anti-neonatal myosin antibody immunoreactivity (d) reveals that immunopositive myofibers (arrows in d) tend to be small diameter FCMD myofibers. (a-d ×600)

Table 1 Primary antibodies

Antigen	Antibody	Concentration of dilution for IHC	Reference or source
α-Dystroglycan, rabbit skeletal muscle membrane preparation, Clone IIH6C4	Mouse monoclonal (IgM)	1:50	Upstate Temecula CA, USA
α-Dystroglycan core protein, mixture of recombinant α-dystroglycan N- and C-terminal domains	Goat polyclonal (IgG)	5μg of IgG/ml	Kanagawa et al. (unpublished data)
β-Dystroglycan, amino acid residues 880-894 (C-terminus), synthetic peptide	Sheep polyclonal	5μg of IgG/ml	Ibraghimov-Beskrovnaya et al. (1992) Wakayama et al. (1995)
Dystrophin (Dys3), amino terminal domain (between amino acids 321-494) of human dystrophin	Mouse monoclonal (IgG)	1:20	Novocastra Laboratories Ltd., Newcastle upon Tyne, UK
Merosin, purified human merosin	Mouse monoclonal (IgG)	1:30	Chemicon International Inc., Temecula CA, USA
Neonatal myosin, rabbit neonatal myosin heavy chain	Mouse monoclonal (IgG)	1:50	Vector Laboratories Inc., CA, USA
α-Sarcoglycan, amino acid residues 217-289 of the rabbit α-sarcoglycan sequence, fusion protein	Mouse monoclonal (IgG)	1:100	Ylem, Rome, Italy
β-Sarcoglycan, fusion protein RBSG-NT of the human β-sarcoglycan sequence, fusion protein	Mouse monoclonal (IgG)	1:50	Novocastra Laboratories Ltd., Newcastle upon Tyne, UK
γ-Sarcoglycan, amino acid residues 167-178 of the rabbit γ-sarcoglycan sequence, synthetic peptide	Mouse monoclonal (IgG)	1:100	Novocastra Laboratories Ltd., Newcastle upon Tyne, UK
δ-Sarcoglycan, amino acid residues 1-19 at the N-terminus of the human δ-sarcoglycan sequence, synthetic peptide	Mouse monoclonal (IgG)	1:50	Novocastra Laboratories Ltd., Newcastle upon Tyne, UK
Sarcospan CAADRQPRGQQRQGDAAGPD N-terminal domain of human sarcospan, synthetic peptide	Rabbit polyclonal	5μg of IgG/ml	Crosbie et al. (1997) Hayashi et al. (2006)
β-Spectrin (C-terminus of 270- kDa muscle isoform), synthetic peptide	Rabbit polyclonal	1:200	Porter et al. (1992) Wakayama et al. (1995)

IHC: Immunohistochemistry

Table 2 Immunoreaction for β-spectrin and sarcospan in dystrophic muscles

cases	anti-β-spectrin immunoreactivity*		
	(1)	(2)	(3)
5 FCMD	46.2 ± 10.8	38.7 ± 6.0	15.1 ± 5.1
5 DMD	45.6 ± 18.8	43.4 ± 6.7	11.0 ± 2.2
5 DC	88.3 ± 2.6	11.7 ± 2.6	0
5 NC	95.2 ± 1.1	4.8 ± 1.1	0
cases	anti-sarcospan immunoreactivity*		
	(1)	(2)	(3)
5 FCMD	2.0 ± 0.9	25.3 ± 3.6	72.7 ± 4.3
5 DMD	0.9 ± 0.5	21.5 ± 3.4	77.6 ± 3.6
5 DC	88.4 ± 3.1	11.6 ± 3.1	0
5 NC	94.6 ± 1.2	5.4 ± 1.2	0

* group mean percentage of myofibers ± standard error of the mean

(1)=group 1: continuously positive immunostaining pattern with more than 90% myofiber surface immunolabeling and normal immunoreactivity

(2)=group 2: partially positive immunostaining pattern with 10 to 90% myofiber surface immunolabeling and normal immunoreactivity

(3)=group 3: negative immunostaining pattern with less than 10% myofiber surface immunolabeling

DMD: Duchenne muscular dystrophy
FCMD: Fukuyama congenital muscular dystrophy
DC: disease control (myotonic dystrophy + facioscapulohumeral dystrophy)
NC: normal control

Table 3 Immunonegative fiber ratio (group 3)

	anti-β-spectrin antibody immunonegative fiber ratio	anti-sarcospan antibody immunonegative fiber ratio
	P<0.0001	
5 FCMD	15.1 ± 5.1%*	72.7 ± 4.3%*
	P<0.0001	
5 DMD	11.0 ± 2.2%#	77.6 ± 3.6%#
5 disease control (3 myotonic dystrophy + 2 facioscapulohumeral dystrophy)	0%	0%
5 normal control	0%	0%

* P<0.0001 by two-tailed t test

# P<0.0001 by two-tailed t test

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Original article

POMT2 intragenic deletions and splicing abnormalities causing congenital muscular dystrophy with mental retardation

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ABSTRACT

Background: Alpha-dystroglycanopathies are a group of congenital muscular dystrophies (CMDs) with autosomal recessive inheritance characterized by abnormal glycosylation of alpha-dystroglycan. Although six genetic causes have been identified (*FKTN, POMGNT1, POMT1, POMT2, FKRP,* and *LARGE*) many alpha-dystroglycanopathy patients remain without a genetic diagnosis after standard exon sequencing. To date *POMT2* mutations have been identified in CMD cases with a wide range of clinical severities from Walker–Warburg syndrome to limb girdle muscular dystrophy without structural brain or ocular involvement.

Methods: We analyzed *POMT2* in six CMD patients, who had severe diffuse muscle weakness, generalized joint contractures, microcephaly, severe mental retardation and elevated CK levels. Eye involvement was absent or limited to myopia or strabismus. We sequenced the coding regions of POMT2 using genomic DNA and cDNA generated from blood lymphocytes or B lymphoblastoid cell lines. Quantitative PCR analysis of genomic DNA was used to identify and determine the breakpoints of large deletions.

Results: We report five novel mutations in *POMT2*, four of which were outside of coding exons, two large genomic deletions and two intronic single base substitutions that induced aberrant mRNA splicing.

Conclusions: Large scale DNA rearrangements (such as large deletions) and cryptic splice mutations, that can be missed on standard sequencing of genomic DNA, may be relatively common in *POMT2*. Additional techniques, such as sequencing of cDNA are needed to identify all mutations. These results also confirm that *POMT2* mutations are an important cause of the less severe alpha-dystroglycanopathy phenotypes.

1. Introduction

Congenital muscular dystrophies (CMDs) are a group of autosomal recessive inherited myopathies, characterized by early onset hypotonia, weakness and delayed motor development. Some CMDs are associated with central nervous system abnormalities and variable eye involvement, in addition to muscular dystrophy: Fukuyama type congenital muscular disorder (FCMD: OMIM253800), muscle–eye–brain disease (MEB: OMIM 253280),

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Walker–Warburg syndrome (WWS: OMIM 236670), and congenital muscular dystrophies MDC1C (OMIM 606612) and MDC1D (OMIM 608840) [19]. These forms of CMD were recently found to share a common pathogenic mechanism linked to abnormal glycosylation of alpha-dystroglycan (α-DG). Mutations in the genes encoding six putative enzymes have been identified as causes of the so-called alpha-dystroglycanopathies: fukutin (*FKTN*), protein *O*-mannose beta-1,2-*N*-acetylglucosaminyltransferase (*POMGNT1*), protein *O*-mannosyltransferase 1 (*POMT1*), protein *O*-mannosyltransferase 2 (*POMT2*), fukutin-related protein (*FKRP*), and acetylglucosaminyltransferase-like protein (*LARGE*) [11,5,29,2,13,23,24,25]. The functions of only POMT1, POMT2 and POMGnT1 have been elucidated. POMT1 and POMT2 are responsible for the catalysis of the first step in *O*-mannosyl glycan synthesis in endoplasmic reticulum [16] and POMGnT1 catalyzes the transfer of *N*-acetylglucosaminyl residue from UDP-GlcNAc to *O*-mannosyl glycoproteins [29,30] in the Golgi apparatus.

Mutations in *POMT1* or *POMT2* [2,10,7,24] were first identified as causes of WWS, but can also result in milder phenotypes, including limb girdle muscular dystrophy with mental retardation or MEB-like phenotypes [1,8,26]. In this study, we identified five novel mutations in *POMT2*, four of which were outside coding regions in addition to the previously described common p.Tyr666Cys mutation [27].

2. Materials and methods

Blood samples were obtained from six patients (five unrelated families) presenting with congenital muscular dystrophy and mental retardation. Informed consent for genetic analysis was obtained according to the ethics committee of our institutional review board. Although muscle for study of alpha-dystroglycan expression was only available in two of the patients, the presence of typical features of alpha-dystroglycanopathies in the remaining children (increased CK levels and muscle pseudohypertrophy) led to a high suspicion of α-dystroglycanopathy. Mutations in *FKRP*, *POMGNT1*, and *POMT1* had been previously excluded in all the patients by direct sequencing before *POMT2* study was performed. A previous study of lymphoblast POMT activity showed a drastic reduction of POMT activity for Patient 1 (previously described GUC5 patient) [15]. The clinical features of two children (Patients 5 and 6) from an Argentinean family with 4 affected siblings have been previously described (cases 1 and 2) [21].

2.1. Genetic analysis of POMT2 from genomic DNA

Genomic DNA (gDNA) was extracted using standard methods from peripheral blood lymphocytes. For mutation screening, primer pairs were designed to amplify the 21 coding exons of *POMT2* and flanking intronic sequences. Primer sequences and PCR conditions are given in Supplementary online Table 1. The amplification products were purified and directly sequenced with the BigDye® Terminator v3.1 Cycle Sequencing Kit (Perkin Elmer Applied Biosystems, Wellesley, MA). Sequences were analyzed on an ABI PRISM 3130 capillary sequencer (Applera, CA).

2.2. Genetic analysis of POMT2 from cDNA

Total RNA from patient B lymphoblastoid cell lines obtained after immortalization by Epstein–Barr virus was extracted using TRIzol® Reagent (Invitrogen, La Jolla, CA). The first-strand cDNA synthesis reaction was catalyzed by SuperScript™ II Reverse Transcriptase (Invitrogen). Two series of PCR primers were used to amplify and sequence cDNA corresponding to the full coding sequence *POMT2* (NM_013382.4) in 5 and 9 overlapping amplicons respectively (Supplementary online Tables 2 and 2bis). The PCR products were separated by electrophoresis on 1.5% agarose gels and normal and aberrant bands were purified and directly sequenced using the above methods. Primer pairs Ex1-1F/Ex6R (series 1) and 3s/3r (series 2) were the best at detecting the deletions of exons 3–5 and 10–12 on cDNA from Patients 1 and 2.

2.3. Quantitative fluorescent PCR (qPCR) of POMT2

We used quantitative PCR (qPCR) to confirm the presence of large heterozygous deletions of *POMT2* and to estimate the size of the deletions. We designed a series of qPCR primer pairs, that amplified sequences within and flanking exons that appeared absent on cDNA. Primer sequences, their location, and amplicon sizes are provided in Supplementary online Table 3. To normalize qPCR results for standard diploid DNA we amplified *TEX2*, a gene located on chromosome 17 and used gDNA samples from a healthy individual as a control. Results of relative quantification were expressed as a normalized ratio comparison between control and patients: a ratio around 1.0 means that two *POMT2* alleles have been amplified, ratio of 0.5 corresponds to the amplification of only one *POMT2* allele. LightCycler 480SYBR Green Master (Roche Diagnostic, Basel, Switzerland and relative quantification analysis with the LighCycler 480 (Roche Diagnostics).

2.4. Genetic analysis of long-range PCR amplification on POMT2 gDNA

Long-range PCR amplification was performed to determine the precise breakpoints of the genomic deletions. Intronic primer pairs were designed up- and downstream of the regions identified as deleted by qPCR. The PCR products were obtained with the Platinum® *Pfx* DNA Polymerase kit (10 cycles at 60 °C and 30 cycles at 50 °C) and directly sequenced using the above methods.

3. Results

3.1. Patients

Clinical features and laboratory data of the six patients are summarized in Table 1. Patients 5 and 6, two siblings, have been previously described in detail [21]. These two patients and Patient 1 suffered a fatal cardiorespiratory arrest at 15 and 16 years, respectively, probably as the consequence of their progressive respiratory insufficiency rather than to a cardiac event, since there were no prior signs or symptoms of heart involvement. The three remaining patients are still alive. The six patients had many common clinical features. At birth, all presented with congenital hypotonia and psychomotor development was delayed from early infancy. They were mentally retarded and had microcephaly, which was present at birth in all the patients for whom the information was available (Table 1). On examination, all had clinical features typical of alpha-dystroglycanopathy patients [20], including progressive muscle weakness of the face, trunk and girdle muscles, tongue and/or calf muscle enlargement and, in the advanced stages of the disease multiple joint contractures and severe respiratory insufficiency. All had markedly elevated serum CK levels (more than 10 times normal levels) and electromyograms were myopathic. In all the cases, muscle biopsies revealed dystrophic changes with variability of fibre size, replacement of myofibres by fat and connective tissue, type 1 fibre atrophy, and necrotic or regenerative fibres. α-DG immunohistochemistry or immunoblotting analyses were performed in two biopsies (Table 1) and revealed a marked reduction of α-DG. Brain MRI abnormalities varied between patients. Five patients had cerebellar vermis

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Table 1
Clinical features of the six CMD patients with *POMT2* mutations.

Patient		1	2	3	4	5 and 6
Current age		Died at 16 y	15 y	4 y	5 y	Both died at 15 y
Gender		Female	Female	Male	Male	Male/Female
Origin		French	French	French	Portuguese	Argentinean
Mental retardation		+++	++	+++	+++	+++
Microcephaly		30.5 cm (−3 SD) at birth, 50 cm (−3 SD) at 14 y	32 cm (−2 SD) at birth, 46.5 cm (−3 SD) at 8 y	Yes	Yes	48.5 cm at 5 y (−2 SD)/48.5 cm at 8 y (−2.5 SD)
Cerebral dysplasia		Pachygyria (parieto-frontal)	Normal	Normal	Polymicrogyria, heterotopia	Yes focal (opercular, peri-rolandic and parietal)
White matter abnormalities		No	No	Parietal–occipital	Discrete	Minute periventricular
Cerebellar abnormalities		Vermis hypoplasia	Vermis hypoplasia, cerebellar cysts	No	Vermis hypoplasia	Vermis hypoplasia, cerebellar cysts
Brainstem involvement		No	No	No	No	Hypoplasia
Ocular involvement		Moderate myopia	Strabismus	No	No	No
Congenital hypotonia		Yes	Yes	Yes	Yes	Yes
Maximum motor function		Standing at 4 years, no walking	Sitting unsupported	Sitting unsupported	Sitting unsupported	Sitting unsupported
Muscle pseudo-hypertrophy		Legs	Legs, tongue	Legs	Tongue	Legs
CK levels		5100 (×50 times) at 1 y	2100 (×10 times) at 4 y	Increased	Increased	1300 (×10 times) at 8 y/1900 (×14 times) at 7 y
Muscle biopsy		Dystrophic	Dystrophic	Dystrophic	Dystrophic	Dystrophic
Merosin immunostaining		Normal	Normal	Slightly reduced	Normal	Normal
α-DG immunostaining		Not performed	Not performed	Reduction	Reduction	Not performed
Others			Lip cleft			Febrile seizures
POMT2 mutations						
Allele 1	Nucleotide change	c.1997A>G	c.1997A>G	c.1997A>G	c.1333−14G>A	c.1333−14G>A
	Transcript	r. 1997A>G	r. 1997A>G	r. 1997A>G	r.1332_1333ins1333-12_1333-1	r.1332_1333ins1333-12_1333-1
	Protein	p.Tyr666Cys	p.Tyr666Cys	p.Tyr666Cys	p.Ile444_Asn445ins-LLWQ	p.Ile444_Asn445insLLWQ
Allele 2	Nucleotide change	c.334-2172_657-763del6602insCCTG	c.1117-817_1333-379del 6187;1117-827delT	c.248+5G>C	c.1333−14G>A	c.1445G>T
	Transcript	r.334_654del	r.117_1332del	r.248_249ins-248+1_248+72	r.1332_1333ins1333-12_1333-1	r.1445G>T
	Protein	p.Met112AlafsX16	p.Val373_Ile444del72	p.Cys83TrpfsX2	p.Ile444_Asn445ins-LLWQ	p.Gly482Val

hypoplasia (Table 1 and [21]), and in three of them (Patients 2, 5 and 6) cerebellar cysts were observed. Supratentorial migration defects were detected in four patients (Patient 1, pachygyria; Patient 4, polymicrogyria and heterotopia; Patients 5 and 6, focal cortical dysplasia and bilateral opercular simplification). Finally, four patients showed focal or minor brain white matter abnormalities (Patients 3–6). Eyes appeared normal in the patients, except for moderate myopia and/or strabismus (Patients 1 and 2). It is noteworthy that Patients 5 and 6 had febrile seizures, and Patient 2 had a lip cleft.

3.2. Mutation detection

Sequencing *POMT2* from gDNA revealed a previously described heterozygous mutation [27] in Patients 1, 2, and 3 in exon 19 (c.1997A>G), which leads to the replacement of tyrosine 666 by a cysteine residue (p.Tyr666Cys) (Table 1). For Patients 1 and 2, a second mutation was not found on gDNA (Supplementary online Fig. 1). However, PCR amplification of *POMT2* cDNA in both patients revealed abnormally small PCR products due to the loss of several exons. In Patient 1, we found a cDNA transcript with deletion of exons 3–5 (c.334_654del), that is predicted to result in a frame shift and a premature stop codon (p.Met112AlafsX16). In Patient 2, a cDNA transcript was identified lacking exons 10–12 (r.1117_1332del), which is predicted to remove 72 amino acids from POMT2 (p.Val373_Ile444del72) (Supplementary online Fig. 1). In Patient 3, in addition to p.Tyr666Cys, a second heterozygous nucleotide change was detected on gDNA, at position +5 in intron 1 (c.248+5G>C) that induced abnormal gene splicing. Analysis of cDNA showed a transcript containing an extra 72 base pairs from intron 1 inserted between exons 1 and 2 (r.248_249ins248+1_248+72), which is predicted to substitute tryptophan for cysteine 83 and then encode a premature stop codon (p.Cys83TrpfsX2) (Supplementary online Fig. 1). In Patient 4, a homozygous nucleotide change was detected in intron 12

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(c.1333−14G>A). This mutation creates a new acceptor splice site and was associated with a 12-bp insertion between exon 12 and exon 13 in *POMT2* cDNA (r.1332_1333ins1333-12_1333-1), which is predicted to insert 4 amino acids (Leu-Leu-Trp-Gln) between p.Ile444 and p.Asn445 in the POMT2 protein (p.Ile444_Asn445insLLWQ). We also identified the same mutation in a heterozygous state in Patients 5 and 6 together with a novel missense mutation p.Gly482Val (c.1445G>T). This mutation affects a region in the third MIR domain of POMT2 that is conserved in mammals, chicken and zebrafish. The three novel point mutations were not found in 112 control subjects.

3.3. Quantitative fluorescent PCR (qPCR) and long-range PCR of gDNA

To determine whether the deletions detected at the transcript level were due to genomic deletions and to localize the breakpoints, 'walking qPCR' was performed on genomic DNA from Patients 1 and 2, and their family members. As shown in Fig. 1B-1, the normalized qPCR ratios for Patient 1 and her father were around 0.5 for distal intron 2 (Int2-2) to proximal intron 5 (Int5-1), while they normalized to 1.0 upstream of the middle region of intron 2 (Int2-1) and downstream of the distal part of intron 5 (Int5-2). Normal qPCR results were obtained from gDNA from the mother. In Patient 2, the normalized qPCR ratios were 0.5 between distal intron 9 (Int9-2) and distal intron 11 (Int11) while they normalized to around 1.0 for the middle region of intron 9 (Int9-1) and the distal part of intron 12 (Int12) (Fig. 1B-2). Genomic DNA from Patient 2's father gave normal qPCR results.

To confirm the presence of large genomic heterozygous deletions in Patients 1 and 2 and to determine the breakpoints, we designed primer pairs for long-range PCR (Fig. 1A). As seen in Fig. 1C-1, using primers specific for introns 2 and 5, PCR products of around 1100 bp were detected for Patient 1 and her father. No amplification was seen for a control subject and Patient 1's mother, likely because the PCR product expected from normal gDNA (7687 bp) was too large for amplification. In the same manner, PCR products of around 1500 bp were found for Patient 2 and her mother using primers specific for introns 9 and 12, but not from

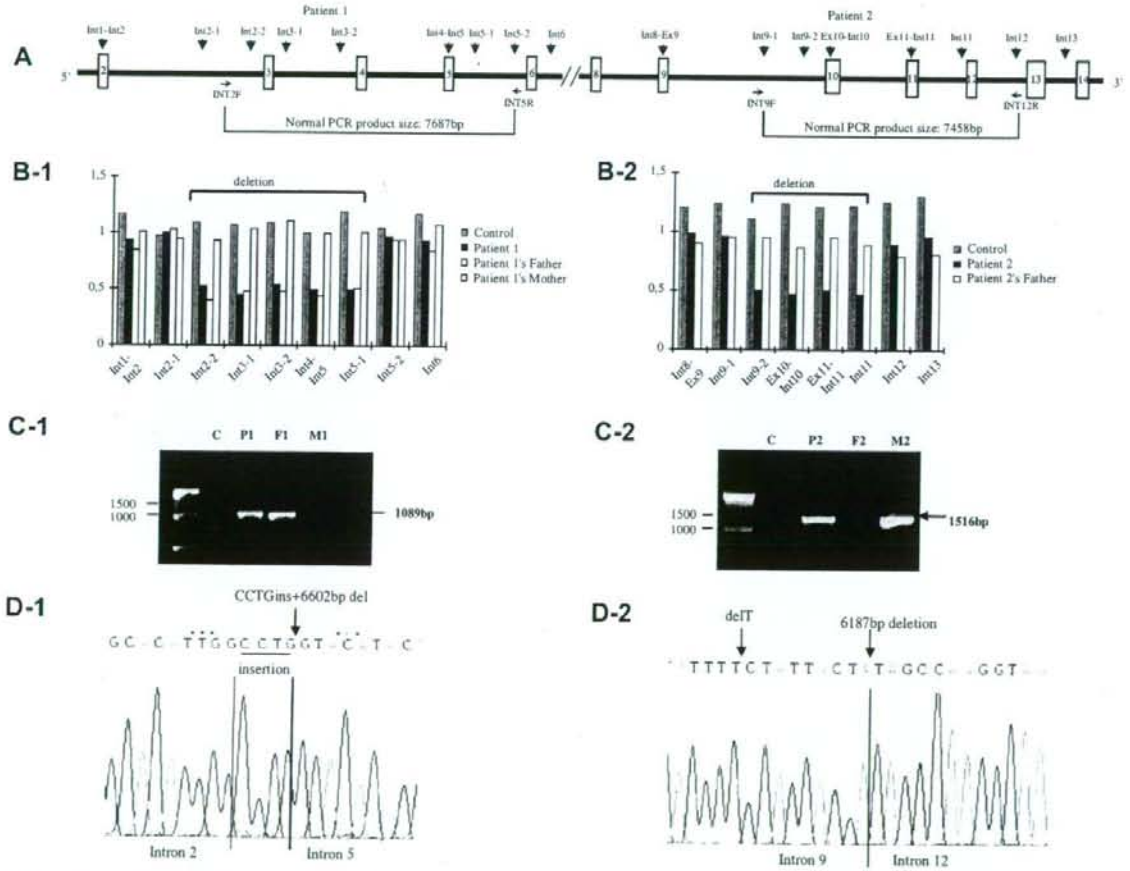

Fig. 1. Analysis of the large intragenic deletions of Patients 1 and 2 (**A**): location of the *POMT2* primer pairs used for relative quantification analysis (upper side) and long-range PCR (lower side). (**B**) Relative quantification analysis performed on gDNA from Patient 1 and her parents (**B-1**) and for Patient 2 and her father using LightCycler® 480 (**B-2**). (**C**) Electropherogram of *POMT2* gDNA long-range PCR amplification products on 2% agarose gel. (**C-1**) A 1089-bp PCR fragment, amplified by the INT2F-INT5R primer pair, was detected in Patient 1 (P1) and her father (F1). (**C-2**) A 1516-bp PCR fragment, amplified by the INT9F-INT12R primer pair, was observed for Patient 2 (P2) and her mother (M2). No PCR product was detected for the control subject in line C with the two primer pairs. (**D**) Sequence electrophoregrams of *POMT2* long-range PCR products on gDNA from Patient 1 (**D-1**) and Patient 2 (**D-2**).

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control gDNA or from Patient 2's father (Fig. 1C-2). Sequencing the two short PCR products (Fig. 1D) revealed the breakpoints of the two *POMT2* deletions. Patient 1 had a heterozygous 4-bp (CCTG) insertion and 6602-bp deletion beginning in intron 2 and ending in exon 5 (c.334-2172_657-763del6602insCCTG) (Fig. 1D-1). Patient 2 had a heterozygous 1-bp deletion at the position −827 of exon 10, and a 6187-bp deletion that began in intron 9 and extended to intron 12 (from −817 of exon 10 to −379 of exon 13; c.1117-817_1333-379del6187) (Fig. 1D-2).

4. Discussion

CMDs associated with abnormal O-glycosylation of α-DG have a broad clinical spectrum that ranges from the most severe forms of WWS with major structural brain and ocular involvement and early death, to LGMD forms with a benign course [19]. In spite of the variable severity, patients with alpha-dystroglycanopathy usually show particular common features including increased CK levels, muscle enlargement and facial and proximal muscle weakness, with progressive and severe impairment of motor and respiratory function [20]. There is a large degree of overlap in the spectrum of clinical severity associated with each of the six known genes (*POMT1*, *POMT2*, *POMGNT1*, *FKTN*, *FKRP* and *LARGE*) which complicates genetic testing [9]. The first *POMT2* mutations were published in three cases of WWS [24,3,15] and other studies have confirmed this association [4,28,15]. More recently, *POMT2* mutations have been identified in a number of CMD patients with less severe phenotypes including MEB/FCMD-like CMD [17,9], CMD associated with mental retardation with or without cerebellar abnormalities [9,27,18] and LGMD with or without mental retardation [9]. We previously described a founder missense mutation c.1997A>G, p.Tyr666Cys and confirmed its pathogenicity by enzymatic testing of POMT [27]. Here, we report the c.1997A>G mutation in three patients in association with two large intragenic deletions and a 5′ splice site mutation leading to a premature stop codon, and an intronic mutation in two unrelated families resulting in an elongated protein.

The phenotypes of the six patients here reported are within the spectrum of CMDs with severe mental retardation and structural brain abnormalities with no major eye abnormalities and confirms that *POMT2* is an important cause of this phenotype [17,9,18]. Compared to the clinical findings reported in similar cases due to other alpha-dystroglycanopathy gene defects, the most interesting and particular feature of the series is the presence in all of microcephaly, confirmed at birth in most of them. This is also observed in cases due to *POMT1* mutations [17,9] but has not been reported in patients with *POMGNT1* or *FKTN* mutations [9]. Patients with *FKRP* mutations may show acquired microcephaly [14]. Our results continue a previously reported trend for such patients to have at least one missense mutation [9,18]. The most likely explanation is that missense mutations are often associated with a residual amount of POMT2 enzyme activity that ameliorates the phenotype. In contrast, patients with WWS generally have two 'null' mutations that are predicted to completely abolish protein function [24,4,22].

We report the first large intragenic deletions in *POMT2* gene. These two heterozygous deletions were found by cDNA analysis and were confirmed with quantitative and long-range PCR. Interestingly, we first analyzed the cDNA of *POMT2* by amplifying relatively short PCR fragments that spanned two or three exons but results appeared normal due to amplification of the non-deleted allele that carried the c.1997A>G missense mutation. We only saw evidence of deletions when we amplified fragments that spanned many exons. This illustrates that the detection of deletions of several exons by PCR is highly dependent on the choice of the primer pairs. Other screening techniques, such as multiplex ligation-dependent probe amplification (MLPA) do not have this limitation. Concerning the mechanisms of these deletions, neither of the two large deletions exhibited any significant sequence similarity between the ±500 bp flanking their 5′ and 3′ breakpoints. This suggests that non-homologous end joining rather than homologous recombination was the mutational mechanism underlying both cases [12].

We also identified two new splicing mutations. Patient 3 carried a heterozygous mutation at position +5 of intron 1, where guanine is the most frequent nucleotide in human genes [6]. In-silico analysis of this mutation predicts reduced efficiency of the donor site (http://www.genet.sickkids.on.ca/~ali/splicesitefinder.html). Therefore it is consistent that a change of this nucleotide might affect the splicing efficiency. cDNA analysis showed abnormal splicing due to the preferential utilization of a downstream donor site, causing the insertion of the first 72 bp of intron 1 into the mRNA transcript and the creation of a premature stop codon. In Patients 4, 5 and 6, we identified the c.1333-14G>A variant in intron 12. Due to its intronic position, this single base change could have been mistaken for a harmless polymorphism. Analysis of cDNA clearly showed this mutation creates a new splice acceptor site leading to 12-bp insertion at the beginning of exon 13 and resulting in a 4 amino acid insertion.

Most of *POMT2* mutations so far reported affect coding regions located in exons. In our study, we identified two large intragenic deletions, and two intronic mutations that alter splicing, leading to insertion of intronic material into the *POMT2* mRNA transcripts. These atypical mutations required detection or confirmation in *POMT2* cDNA. These results raise the possibility that intragenic *POMT2* deletion that may not be identified by standard sequencing because of heterozygous status may cause alpha-dystroglycanopathy in a proportion of patients who currently lack genetic diagnosis.

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Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.ejmg.2008.12.004.

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Caveolin-3 regulates myostatin signaling. Mini-review

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Caveolins, components of the uncoated invaginations of plasma membrane, regulate signal transduction and vesicular trafficking. Loss of caveolin-3, resulting from dominant negative mutations of caveolin-3 causes autosomal dominant limb-girdle muscular dystrophy (LGMD) 1C and autosomal dominant rippling muscle disease (AD-RMD). Myostatin, a member of the muscle-specific transforming growth factor (TGF)-β superfamily, negatively regulates skeletal muscle volume. Herein we review caveolin-3 suppressing of activation of type I myostatin receptor, thereby inhibiting subsequent intracellular signaling. In addition, a mouse model of LGMD1C has shown atrophic myopathy with enhanced myostatin signaling. Myostatin inhibition ameliorates muscular phenotype in the model mouse, accompanied by normalized myostatin signaling. Enhanced myostatin signaling by caveolin-3 mutation in human may contribute to the pathogenesis of LGMD1C. Therefore, myostatin inhibition therapy may be a promising treatment for patients with LGMD1C. More recent studies concerning regulation of TGF-β superfamily signaling by caveolins have provided new insights into the pathogenesis of several human diseases.

Key words: caveolin-3, limb-girdle muscular dystrophy 1C (LGMD1C), autosomal dominant rippling muscle disease (AD-RMD), myostatin, transforming growth factor-β (TGF-β)

Caveolins are primary components of caveolae

Caveolae, uncoated invaginations of the plasma membrane, are an abundant feature of many terminally differentiated cells, such as adipocytes, endothelial cells, and muscle cells. Caveolin family proteins, 21-24 kDa integral membrane proteins, are the principle components of caveolae, designated as caveolin-1, -2, and -3 (1, 2). Caveolin-1 and caveolin-2 are coexpressed and form heterooligomers in nonmuscle cells, whereas caveolin-3 is muscle specific and forms homooligomers in muscle cells (3, 4). De novo synthesized caveolins assemble to about 350 kDa oligomers in the endoplasmic reticulum, subsequently target to the plasma membrane via the trans-Golgi network, and play a crucial role in the formation of caveolae. These caveolin family proteins have been implicated in numerous cellular events including vesicular trafficking, lipid metabolism, and signal transduction (1-6). They directly bind to and regulate specific lipid and lipid-modified proteins including cholesterol, G-protein, G-protein coupled receptors, Src family kinase, Ha-Ras, and nitric oxide synthases (5-7). The interaction between caveolins and these molecules is mediated by a caveolin-binding motif on the target protein and a scaffolding domain in caveolin (7). The number of *in vitro* studies linking caveolins to signal transduction pathways has grown exponentially. To date, however, only a few studies have been concluded the exact roles of caveolins to signal transduction *in vivo* (3).

Dominant-negative mutations of caveolin-3 gene causes LGMD1C/AR-RMD

Many mutations in caveolin-3 gene have been detected in autosomal dominant limb-girdle muscular dystrophy (LGMD) 1C and autosomal dominant rippling muscle disease (AD-RMD) (8, 9). Mutations of the caveolin-3 gene cause a significant reduction in the cell surface level of caveolin-3 protein in a dominant-negative fashion and, to a lesser extent, mistargeting of the mutant caveolin-3 protein to the Golgi complex (8-10).

The loss of caveolin-3 by mutations of the caveolin-3 gene in LGMD1C/AD-RMD patients has resulted in subsequent abnormalities of caveolin-3-binding molecules. The

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enzymatic activity of neuronal nitric oxide synthase, which is strongly suppressed by caveolin-3, increases in the skeletal muscles from a transgenic mouse model of LGMD1C and LGMD1C/AD-RMD patients (11, 12). Consistently, cytokine-induced NO production increases in C2C12 myoblast cells transfected with LGMD1C/AD-RMD-type mutant caveolin-3 compared to ones transfected with wild-type caveolin-3 (9). Src tyrosine kinase, a membrane tyrosine kinase whose activation regulates the balance between cell survival and cell death, is extremely activated and accumulates not in the plasma membrane but in the perinuclear region in cells transfected in LGMD1C/AD-RMD mutant caveolin-3 (13). Muscle-specific phosphofruktokinase, an enzyme of central importance in the regulation of glycolytic metabolism is also significantly reduced in cells transfected with LDMD1C/AD-RMD mutant caveolin-3 probably through ubiquitin-proteasomal degradation (14). Noteworthy also is the finding that dysferlin, a membrane-repair molecule deficient in LGMD2B/Miyoshi myopathy, mistargets to the cytoplasm from sarcolemma in skeletal muscle from LGMD1C/AD-RMD patients, probably due to the caveolin-3's delivery function to the correct targeting of plasma membrane (15-18).

Despite these findings, the underlying molecular mechanism leading to LGMD1C/AD-RMD in caveolin-3-deficient muscle remains to be elucidated.

Myostatin, a muscle-specific TGF-β superfamily member, is a therapeutic target of muscular dystrophy

Myostatin is a muscle-specific transforming growth factor (TGF)-β superfamily member and negatively regulates skeletal muscle growth and skeletal muscle volume (19). Overexpression of myostatin causes severe muscle atrophy, whereas targeted disruption of myostatin increases skeletal muscle mass in mice (19, 20). Like most members of the TGF-β superfamily, myostatin is synthesized as a precursor protein and undergoes proteolytic processing to generate an N-terminal prodomain and a biologically active, C-terminal disulfide-linked dimer (21). In the inactive state, the prodomain strongly inhibits the biological activity of the C-terminal dimer (22, 23), as do follistatin, and the follistatin-related gene (FLRG); which are collectively called natural inhibitors for myostatin (24). The circulating active form of myostatin directly binds to and phosphorylates the type II serine/threonine kinase receptor, namely activin receptor IIB (ActRIIB) (Fig. 1) (25). This, in turn, phosphorylates the type I serine/threonine kinase receptors, namely activin receptor-like kinase 4/5 (ALK4/5) at the plasma membrane (25-27). The activation of a heteromeric receptor complex consisting of phosphorylated type II and type I serine/threonine kinase receptors induces the phosphorylation of intracellular effectors, receptor-regulated Smads (R-Smads), namely Smad2/3 (26, 27). Phosphorylated R-Smads translocate to the nucleus from the cytoplasm, where it regulate the transcription of specific target genes inducing skeletal muscle atrophy (26-28).

Notably, administration of a blocking antibody against myostatin, myostatin vaccine, and myostatin prodomain, or genetic introduction of a follistatin-derivative ameliorates the pathophysiology of dystrophin-deficient *mdx* mice (29-32). In addition, a blocking antibody against myostatin improves the condition of young model mice with δ-sarcoglycan-deficient LGMD2F (33). An adeno-associated virus (AAV)-mediated myostatin prodomain has ameliorates the pathology of calpain-3-deficient LGMD2A model mice (34). Therefore, myostatin inhibition through different strategies has recently come to be considered for a therapeutic option for muscular dystrophies. However, the precise molecular mechanism by which myostatin inhibition improves the above dystrophic skeletal muscle is not fully understood; i.e. the molecular interaction of myostatin and the dystrophin-glycoprotein complex is unknown.

Caveolin-1 regulates TGF-β superfamily signaling *in vitro*

Recently, caveolin-1 has drawn attention as a regulator of TGF-β superfamily signaling. Caveolin-1 binds to and suppresses activation of the type I receptor of TGF-β1, which induces growth arrest in nonmuscle cells (35). Consistently, the binding affinity of caveolin-1 with type I TGF-β1 receptor decreases after stimulation with TGF-β1. In addition, caveolin-1 associates with the type II receptor of TGF-β1 (36-38). Caveolin-1 also facilitates ligand–bound TGF-β1 receptors internalization and degradation via the formation of endocytic vesicles with ubiquitin-ligase (39, 40). In addition, caveolin-1 interacts with type II and type I receptors of bone morphogenic proteins (BMPs) *in vivo* (41). These findings indicate that caveolin-1 regulates TGF-β superfamily signaling, including TGF-β1 and BMPs, at its receptor level.

Caveolin-3 suppresses myostatin signaling through its type I receptor *in vitro*

Upon consideration of molecular analogy and tissue distribution, we hypothesized that caveolin-3 inhibits myostatin signaling in a similar manner to that of inhibition

of caveolin-1 to multiple TGF-β superfamily signaling in nonmuscle cells. We found several caveolin-3 binding motifs (7); ϕXϕXXXXϕXXϕX, where ϕ indicates aromatic or aromatic-like amino acids in the cytoplasmic kinase domain of type I serine/threonine myostatin receptors, ALK4/5 (42). Therefore, we cotransfected caveolin-3 and these type I myostatin receptors in COS-7 monkey kidney cells and found that caveolin-3 colocalized with type I myostatin receptor. Immunoprecipitation and subsequent immunoblot analysis revealed that caveolin-3 associates with the type I myostatin receptor. In addition, phosphorylation level of the type I myostatin receptor decreased with the addition of caveolin-3 in cells cotransfected with constitutively active type I receptor and caveolin-3. Moreover, caveolin-3 eventually suppressed subsequent intracellular myostatin signaling; the phosphorylation level of an R-Smad of myostatin, Smad2 as well as the transcription level of the Smad-sensitive $(CAGA)_{12}$-reporter gene. Therefore, caveolin-3 suppresses the myostatin signal at its type I receptor level, in a similar manner to caveolin-1 for TGF-β1 signaling *in vitro*.

Caveolin-3 deficient muscles exhibit enhanced intracellular myostatin signaling

We previously generated transgenic (Tg) mice overexpressing mutant caveolin-3 ($CAV\text{-}3^{P104L}$) to develop a mouse model of LGMD1C/AD-RMD (11). The skeletal muscle phenotype of the transgenic mice showed severe myopathy with loss of caveolin-3. To determine whether caveolin-3 regulates myostatin signaling *in vivo*, we generated and characterized the double-transgenic mice showing myostatin deficiency and myostatin inhibition. Heterozygous mating of mutant caveolin-3 Tg mice with other Tg mice overexpressing myostatin prodomain ($MSTN^{Pro}$) (43), a potent inhibitor of myostatin signaling, gave rise to mice with four distinct phenotypes: wild-type, mutant caveolin-3 Tg, mutant MSTN Tg, and double-mutant Tg ($CAV\text{-}3^{P104L}/MSTN^{Pro}$). Growth curves revealed that the double-mutant Tg mice were significantly larger than the mutant caveolin-3 Tg mice and similar in size to the wild-type mice beginning at 6 weeks until 16 weeks of age (42). The muscle atrophy seen in the mutant caveolin-3 Tg was reversed in the double-mutant Tg with increased myofiber size and myofiber number. Thus, myostatin inhibition reverses caveolin-3-deficient muscular atrophy *in vivo*.

Caveolin-3-deficient muscle from mutant caveolin-3 Tg mice showed hyperphosphorylation of an R-Smad of myostatin, Smad2 and significant upregulation of a myostatin target gene, p21. These *in vivo* findings were consistent with our *in vitro* study in which caveolin-3 suppresses myostatin signaling. In the double-mutant Tg mouse, the levels of phospho-Smad2 and p21 gene expression were significantly reduced compared to those in the mutant caveolin-3 Tg mice and were similar to those in the wild-type mice. Thus, myostatin inhibition by genetic introduction of myostatin inhibitor normalized enhanced myostatin signaling and also reversed muscular phenotype in the caveolin-3 deficient mouse.

Myostatin inhibition therapy reversed muscular atrophy in caveolin-3 deficiency

We injected a soluble form of the extracellular domain of type II myostatin receptor, ActRIIB, which can inhibit myostatin-its type II receptor binding (25, 44), into the mutant caveolin-3 Tg mice to develop myostatin inhibition through its type II receptor as a therapeutic strategy for patients with LGMD1C. Intraperitoneal injection of soluble ActRIIB four times significantly increased skeletal muscle mass and reversed myofiber hypotrophy accompanied with suppression of Smad2 phosphorylation and downregulation of p21. This finding, therefore, suggests that myostatin inhibition therapy may be a reasonable and promising therapy for caveolin-3-deficient muscular dystrophy associated with enhanced myostatin signaling.

Conclusions and prospective for future research

Caveolin-3 has been considered to regulate numerous signal pathways for maintaining the normal integrity of skeletal muscles, but the *in vivo* significance of signal alterations by loss of caveolin-3 in the pathogenesis of LGMD1C/AD-RMD has not been well delineated. As reviewed herein, caveolin-3 regulates myostatin signaling *in vitro*, and thus disrupted interaction between caveolin-3 and myostatin could contribute to the pathogenesis of caveolin-3-deficient muscular dystrophy (Fig. 1).

We could not conclude that activated intracellular signaling molecules, hyperphosphorylation of an R-Smad, Smad2, and upregulation of p21 in the caeveolin-3 deficient skeletal muscle result simply from enhanced myostatin signaling by loss of caveolin-3, because the myostatin prodomain or the soluble myostatin receptor suppresses not only myostatin, but also other TGF-β ligands including growth and differentiation factor 11 (GDF11) (22, 25, 44, 45). In fact, evidence of an unknown TGF-β ligand exists in the form of a similar negative regulator of muscle mass like myostatin (45, 46). Thus TGF-β ligands other than myostatin also could be

involved in the pathogenesis of caveolin-3 deficieny via the Smad2-p21-mediated pathway. Crossing of mutant caveolin-3 mice with myostatin-null mice is a prospective project for obtaining straightforward evidence that hyperphosphorylation of Smad2 and upregulation of p21 in caveolin-3-deficient muscles is the simple result of enhanced myostatin signaling.

More recent studies have shown to be caveolins as an exact negative regulator of TGF-β superfamily signaling because the loss of caveolins has play important roles in the pathogenesis of human disorders. Mutations of the caveolin-1 gene or downregulation of caveolin-1 protein have been detected in some sporadic breast cancers (47) and epithelial cells derived from caveolin-1 null mice have shown hyperphosphorylation of Smad2 and epithelial mesenchymal transition, corresponding to premalignant status (48). In addition, loss of caveolin-1 has been strongly associates with idiopathic pulmonary fibrosis (49, 50). Caveolin-1 protein has been found to be reduced in the lung tissue from patients with idiopathic pulmonary fibrosis. TGF-β1-induced extarcellular matrix production, which is indicative of fibrosis, significantly increases in

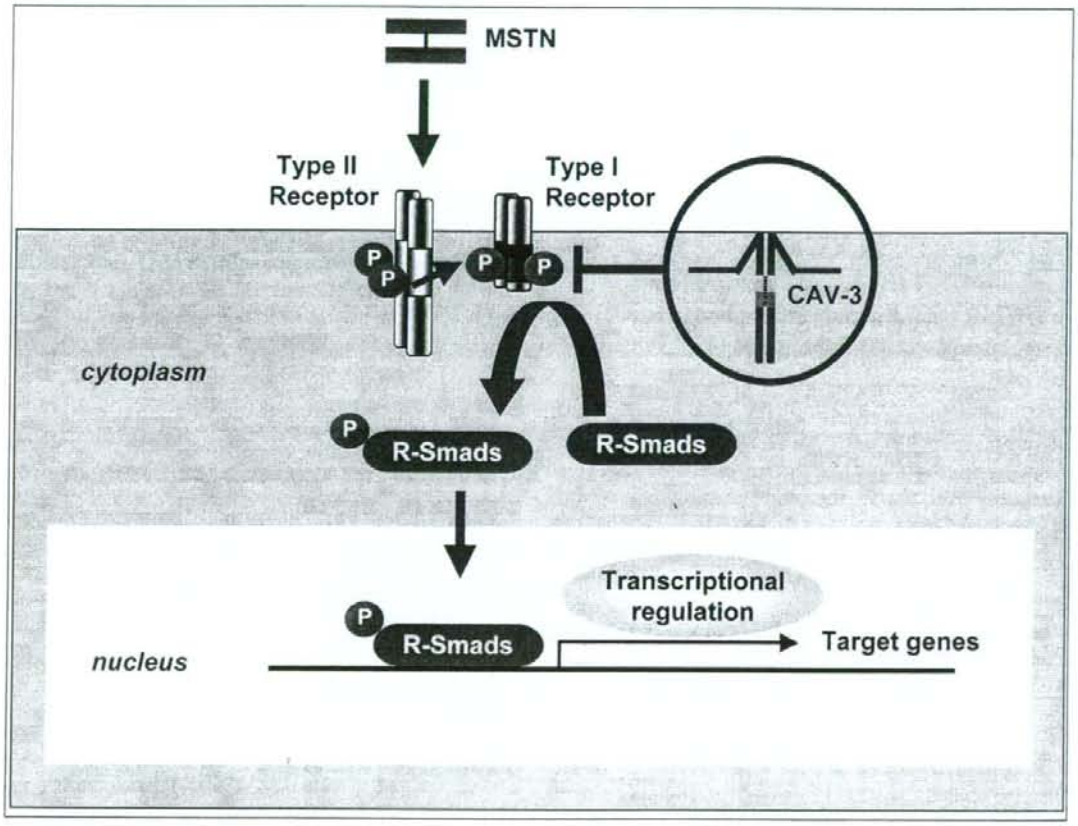

Figure 1. Putative scheme of the regulation of myostatin signaling by caveolin-3. Myostatin (MSTN) signaling is propagated through the myostatin receptor, a heteromeric complex consisting with transmembrane receptor serine/threonine kinases. Myostatin binds to and phosphorylates its type II serine/threonine kinase receptor (Type II Receptor). Subsequently, its type I serine/threonine kinase receptor (Type I Receptor) is phosphorylated by Type II Receptor and is recruited into the heteromeric complex, which in turn phosphorylates receptor-regulated Smads (R-Smads), a family of transcription factor controlling the expression of specific target genes. Caveolin-3 (CAV-3) binds to and suppresses activation of the Type I Receptor of MSTN at the plasma membrane and suppresses intracellular myostatin signaling, including phosphorylation of R-Smads and transcription of specific target genes. Loss of caveolin-3 resulting from dominant negative mutations of the caveolin-3 genes in patients with LGMD1C could enhance intracellular myostatin signaling, and thereby result in muscle mass reduction. Type II Receptor, ActRIIB; Type I Receptor, ALK4/5; R-Smads, Smad2/3. P indicates phosphorylation.

primary fibroblasts isolated from patients with idiopathic pulmonary fibrosis. Moreover, retroviral introduction of caveolin-1 ameliorates bleomycin-induced lung fibrosis in mice. Together with this review, it may be concluded that aberrant TGF-β superfamily signaling by loss of caveolins participate in the pathogenesis of some human diseases, including LGMD1C/AD-RMD, breast cancer, and idiopathic pulmonary fibrosis.

Myostatin inhibition therapy is effective, to some extent, with mouse models of several types of muscular dystrophies (29-34). Further investigation is needed to determine which types of myostatin inhibition therapy could be applied and to clarify the molecular mechanism by which myostatin-inhibition improves muscular dystrophy for prospective treatment of patients with muscular dystrophy. As reviewed herein, myostatin inhibition may be a potent therapy for caveolin-3-deficient muscular dystrophy with enhanced myostatin signaling.

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Recombinant Adeno-Associated Virus Type 8-Mediated Extensive Therapeutic Gene Delivery into Skeletal Muscle of α-Sarcoglycan-Deficient Mice

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Abstract

Autosomal recessive limb-girdle muscular dystrophy type 2D (LGMD 2D) is caused by mutations in the α-sarcoglycan gene (α-SG). The absence of α-SG results in the loss of the SG complex at the sarcolemma and compromises the integrity of the sarcolemma. To establish a method for recombinant adeno-associated virus (rAAV)-mediated α-SG gene therapy into α-SG-deficient muscle, we constructed rAAV serotypes 2 and 8 expressing the human α-SG gene under the control of the ubiquitous cytomegalovirus promoter (rAAV2-α-SG and rAAV8-α-SG). We compared the transduction profiles and evaluated the therapeutic effects of a single intramuscular injection of rAAVs into α-SG-deficient ($Sgca^{-/-}$) mice. Four weeks after rAAV2 injection into the tibialis anterior (TA) muscle of 10-day-old $Sgca^{-/-}$ mice, transduction of the α-SG gene was localized to a limited area of the TA muscle. On the other hand, rAAV8-mediated α-SG expression was widely distributed in the hind limb muscle, and persisted for 7 months without inducing cytotoxic and immunological reactions, with a reversal of the muscle pathology and improvement in the contractile force of the $Sgca^{-/-}$ muscle. This extensive rAAV8-mediated α-SG transduction in LGMD 2D model animals paves the way for future clinical application.

Introduction

LIMB-GIRDLE MUSCULAR DYSTROPHY TYPE 2D (LGMD 2D) is caused by mutations in the α-sarcoglycan (α-SG) gene, and is the most frequent cause of the autosomal recessive LGMD. LGMD 2D patients have the clinical characteristics of progressive muscle necrosis in the proximal limb muscles (Eymard *et al.*, 1997). Sarcoglycans (SGs) are essential constituents of the dystrophin-associated protein (DAP) complex, which consists of several membrane-spanning and cytoplasmic proteins, including dystroglycans (α and β), SGs (α, β, γ, and δ), sarcospan, syntrophins (α_1, β_1, and β_2), and dystrobrevins that directly or indirectly associate with dystrophin (Ervasti *et al.*, 1990; Yoshida and Ozawa, 1990; Iwata *et al.*, 1993). A defect in any one of the four SGs can disrupt the entire SG complex. Mutations in four genes encoding α-, β-, γ-, and δ-SG are responsible for autosomal recessive LGMD 2D, 2E, 2C and 2F, respectively (Ervasti *et al.*, 1990; Bonnemann *et al.*, 1995; Noguchi *et al.*, 1995; Nigro *et al.*, 1996; Eymard *et al.*, 1997; Fanin *et al.*, 1997).

Many *in vivo* studies have demonstrated that recombinant adeno-associated virus (rAAV) packaged in various serotypes of AAV capsids exhibits serotype-specific tissue or cell tropism with different transduction efficiencies (Fisher *et al.*, 1997; Greelish *et al.*, 1999; Gao *et al.*, 2002, 2004; Wang *et al.*, 2005). rAAV has been shown to mediate long-term transgene expression in many tissues without evoking severe immune reactions. Some rAAVs efficiently transduce skeletal muscle (Kessler *et al.*, 1996; Xiao *et al.*, 1996; Fisher *et al.*, 1997). rAAV serotype 2 (rAAV2)-mediated muscle gene therapy is a promising approach, but it is effective only locally. In contrast, rAAV serotype 8 (rAAV8)-mediated gene transfer is capable of crossing capillary blood vessels to achieve systemic gene delivery, and effectively transduces genes into cardiac and skeletal muscle (Wang *et al.*, 2005). Therefore, rAAV8 is a good candidate for a therapeutic tool.

To assess the efficacy and therapeutic potential of rAAV8 for LGMD 2D, we directly injected rAAV2-α-SG and rAAV8-α-SG into the tibialis anterior (TA) muscles of 10-day-old α-SG-deficient mice (neonatal $Sgca^{-/-}$ mice). Our data suggested not

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