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New *POMT2* mutations causing congenital muscular dystrophy

Identification of a founder mutation



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ABSTRACT

Background: Dystroglycanopathies are a group of congenital muscular dystrophies (CMDs) with autosomal recessive inheritance, often associated with CNS and ocular involvement. They are characterized by the abnormal glycosylation of alpha-dystroglycan, and caused by mutations in at least six genes encoding enzymes: *FKTN*, *POMGNT1*, *POMT1*, *POMT2*, *FKRP*, and *LARGE*. *POMT2* mutations have recently been identified in Walker-Warburg syndrome and in a milder muscle-eye-brain disease-like form.

Methods: We studied mentally retarded patients with CMD, analyzed *POMT2* by sequencing the coding regions, and also performed a haplotype analysis in all patients and their family members carrying the new *POMT2* mutation.

Results: We report three novel *POMT2* mutations. One of these, p.Tyr666Cys, was homozygous in two unrelated patients and in a compound heterozygous state in others. All patients showed severe diffuse muscle weakness, microcephaly, severe mental retardation, and marked lordoscoliosis with hyperextended head. Elevated CK levels, cerebral cortical atrophy, and cerebellar vermis hypoplasia were constant findings. Mild cardiac abnormalities, focal white matter abnormalities, or partial corpus callosum hypoplasia were detected in single cases. Eye involvement was absent or mild. By genotype analysis, we defined a distinct 170kb haplotype encompassing *POMT2* and shared by all the subjects harboring the mutation p.Tyr666Cys.

Conclusions: Our results broaden the clinical spectrum associated with *POMT2* mutations, which should be considered in patients with CMD associated with microcephaly, and severe mental retardation with or without ocular involvement. **Neurology**® 2007;69:1254-1260

Congenital muscular dystrophies (CMDs) are a heterogeneous group of inherited myopathies, most of them with autosomal recessive transmission. They are characterized clinically by early onset hypotonia, weakness, and delayed motor development, and morphologically by the finding of dystrophic changes on the muscle biopsy.^{1,2} Spinal deformities and joint contractures are frequently observed in the course of the disease.

There exist a number of entities which associate CNS abnormalities and variable eye involvement, in addition to the muscular dystrophy: Fukuyama congenital muscular dystrophy (FCMD: OMIM 253800), muscle-eye-brain disease (MEB: OMIM 253280), Walker-Warburg syndrome (WWS: OMIM 236670), and congenital muscular dystrophies MDC1C (OMIM 606612) and MDC1D (OMIM 608840). Intense research in the last few years has revealed that these forms share a common pathogenic mechanism linked to the abnormal glycosylation of alpha-dystroglycan (α -DG), and are thus called secondary alpha-dystroglycanopathies. They are caused by mutations in at least six genes

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encoding enzymes: 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*).³⁻⁸ The functions of these proteins are being elucidated. *POMT1* and *POMT2* are responsible for the catalysis of the first step in *O*-mannosyl glycan synthesis in endoplasmic reticulum,⁹ and *POMGNT1* catalyzes the transfer of *N*-acetylglucosamine from UDP-GlcNAc to *O*-mannosyl glycoproteins in the Golgi apparatus.⁴

Recessive mutations in either *POMT1* or *POMT2* cause WWS.¹⁰⁻¹⁴ *POMT1* mutations may also result in phenotypes milder than severe WWS. These include limb-girdle muscular dystrophy with early onset mental retardation, with or without minor brain abnormalities.¹⁴⁻¹⁷ While the first three homozygous *POMT2* mutations were described in typical WWS patients,¹⁸ two compound heterozygous missense mutations were recently reported in a girl with a MEB-like phenotype.¹⁹

In the present study, we identified new *POMT2* mutations in CMD patients with mental retardation.

METHODS Blood samples were obtained from CMD patients and family members. Informed consent for genetic analysis was obtained according to the ethics committee of our institutional review board. Specimens for light microscopy were immediately frozen and processed using standard histologic and histochemical techniques according to previously described procedures.²⁰

Immunohistochemistry analysis. Unfixed frozen 8 μ m sections were incubated with monoclonal antibodies to dystrophin (NCL-DYS1-3), sarcoglycan (NCL- α - γ SARC), merosin (80kD, Chemicon; 300 kD, NCL-merosin), β -DG (NCL- β -DG), and glycosylated α -DG (VIA4-1, Upstate, USA). They were then revealed with a biotinylated secondary antibody followed by streptavidin conjugated to molecular probes, and were visualized with epifluorescence microscopy.

Immunoblot analysis. Muscle proteins were extracted in sample buffer, and transferred to nitrocellulose membrane by electrophoresis. The membranes were blocked, and probed with antibodies to α -DG. They were then washed, incubated with monoclonal antibody II H6 (1:500, provided by Dr. Kevin Campbell), and visualized with chemiluminescence.⁷

Genetic analysis. Genomic DNA was extracted using standard methods from peripheral blood lymphocytes. Mutations in *FKRP*, *POMGNT1*, and *POMT1* were excluded by direct sequencing. For *POMT2*, primers pairs were designed to amplify the 21 coding exons and flanking intronic sequences. Except for exons 1, 17, 18, 19, and 20, we used previously described primers.¹⁸ The optimal working conditions were determined by running a gradient PCR. The primer sequences and PCR conditions are available on request. The amplification products were purified and directly sequenced with the BigDye Terminator v3.1 Cycle Sequencing Kit (Perkin Elmer Applied Biosystems). Sequences were analyzed on an ABI PRISM 3730 capillary sequencer (Applied Biosystems, CA). The presence of the mutation c.1997A>G (p.Tyr666Cys) was confirmed by restriction enzyme analysis with *Bts1* (New England Biolabs) on the PCR products with the following primers: ACAGCAAGGAAGGGGCGAG and TGCTCTGTCTCCCAAGTCCAG and by single strand conformation analysis (SSCP). This method was used to screen the absence of the mutation in 100 unrelated European control individuals.

Haplotype analysis. Haplotype analysis was performed in all patients carrying the c.1997A>G *POMT2* mutation and their family members. All of them were typed for six extragenic microsatellites markers, and five intragenic single nucleotide polymorphisms (SNPs). As shown in figure E-1 (on the *Neurology*[®] Web site at www.neurology.org), the *POMT2* gene is found in a telomeric to centromeric orientation, and the extragenic microsatellite markers were located close to *POMT2* as follows: cen-D14S279-D14S983-CA1_POMT2 (GT repeats)-*POMT2*-CA3_POMT2 (GT repeats)-CA4_POMT2 (CA repeats)-D14S59-tel. Based on the annotated genomic assembly (<http://www.ensembl.org/index.html>), D14S279 and D14S983 are at a distance of 310 and 220 kb from *POMT2*, and D14S59 is 290 kb away. We identified and analyzed new informative microsatellite markers closer to *POMT2*: CA1_POMT2 located 80 kb centromeric (downstream to the transcriptional orientation of *POMT2*); CA3_POMT2 (40 kb telomeric); and CA4_POMT2 (50 kb telomeric). Primer sequences of CA1_POMT2, CA3_POMT2, and CA4_POMT2 were as follows: CA1_POMT2 (forward) 5'-ACACCCAGAG-AGGAGGAGTG-3', (reverse) 5'-AGGTCACATTCCTGCTTCC-3', CA3_POMT2 (forward) 5'-GGGCG-ACAGAGCAAGACTC-3', (reverse) 5'-CCTGGG-TGATGGAGTGAGG-3', and CA4_POMT2 (forward) 5'-CCTGCATCAGCCTCTCAAGT-3', (reverse) 5'-TCCT-CCCATGTCAGTCCCTA. The PCR products were analyzed on the ABI PRISM 310 (Perkin Elmer).

RESULTS Patients. Clinical features and laboratory data of the four patients are summarized in the table. Patient 1 died at age 24 years due to respiratory failure; the three other patients remain alive. The four patients had a number of major clinical features in common. At birth, each presented with congenital hypotonia and microcephaly, and psychomotor development was delayed in the early stages. All of them were severely mentally retarded. Upon examination, all had microcephaly; severe diffuse weakness including the

Table	Clinical features of patients with congenital muscular dystrophies			
	Patient 1	Patient 2	Patient 3	Patient 4
Current age, y	Died at 24	27	14	6
Gender	Female	Female	Male	Male
Origin	Moroccan	French	Sicilian/French	French
Consanguinity	Unknown	No	No	No
Congenital hypotonia	Yes	Yes	Yes	Yes
Maximum motor function	Sits unaided	Ambulant between ages 2.5–10 y	Sits unaided	Sits unaided
Mental retardation	Yes	Yes	Yes	Yes
Microcephaly	Yes	Yes	Yes	Yes
Ocular involvement	No	Retinal peripheral abnormal pigmentations	Mild myopia	Mild strabismus
Brain MRI				
Cerebral cortex atrophy	Mild, predominantly in the parietal area	Discrete and diffuse	Mild	Moderate, predominantly in the frontal-temporal area
Cerebellar vermis hypoplasia	Yes	Mild	Mild	Moderate
Brainstem involvement	No	No	No	No

face, trunk, and girdle muscles; tongue and calf muscle hypertrophy; diffuse joint contractures; and diminished or absent deep tendon reflexes. In addition, except for Patient 4 (who was only 6 years old at examination), all showed severe spinal deformity with marked fixed hyperextension of cervical, dorsal, and lumbar regions (figure 1).

There are, however, some variations in the presence and severity of clinical features among the four patients. Only one patient (Patient 2) gained the ability to sit unaided (at age 1 year); she also was able to walk without support at age 2.5 years, but lost ambulation at age 10 years. Although all patients could understand simple orders, only one patient (Patient 3) could speak about 20 words (although he was not able to make sentences). Patients 1 and 4 had bilateral hip dislocation, and Patient 4 underwent surgery at age 5 years for correction. Only one patient (Patient 2) had significant ophthalmologic findings, consisting of retinal peripheral abnormal pigmentations; the other three patients had no or mild ocular involvement, without ocular malformation or retinal dysplasia. Only one patient (Patient 1) had cardiac involvement, consisting of left ventricular hypertrophy as shown by echocardiogram at age 3.5 years, but it was not progressive.

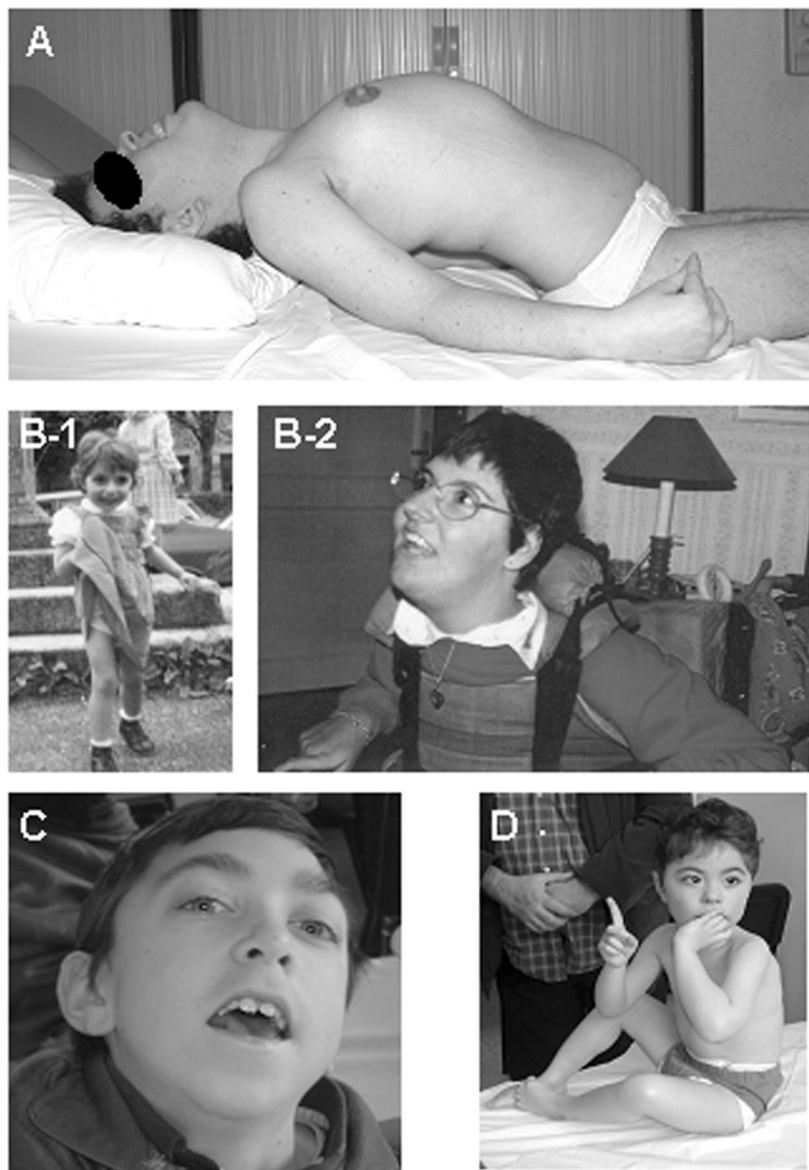
Brain MRI documented moderate or mild cerebral cortex atrophy in four patients, consisting of moderate enlargement of the lateral ventricles (Patient 1), hypoplasia of the median and posterior regions of corpus callosum (Patient 2), and localized T2 hypersignal lesions in the occipital periventricular white matter (Patient 3). All four

patients showed moderate or mild cerebellar vermis hypoplasia without any brainstem involvement (figure 2). Serum CK levels were markedly elevated, from 2.5 to 100 times the upper limit of normal. Electromyogram (EMG) showed myopathic changes. Muscle CT, performed in Patient 1 at age 22 years, showed severe diffuse wasting and low density of the axial and limb muscles with relative preservation of the anterior tibial muscles. In the four cases, muscle biopsies revealed dystrophic changes with variability of fiber size, replacement of myofibers by fat and connective tissue, type-1 fiber atrophy, and necrotic or regenerative fibers.

Immunohistochemistry. Immunohistochemistry analysis of muscle biopsies showed some irregularity in the laminin $\alpha 2$ chain labeling, upregulation of the laminin $\alpha 5$ chain in two patients (Patients 2 and 4), and a drastic and diffuse reduction of the glycosylated α -DG labeling on almost all the muscle fibers in three patients (Patients 1, 2, and 4) (figure 3). Immunolabeling of dystrophin, sarcoglycan, merosin, dysferlin, caveolin-3, collagen VI, lamin A/C, emerin, and β -DG was normal. In Patient 3 the expression of laminin $\alpha 2$ chain was normal, but insufficient muscle was available to assess α -DG status.

Immunoblot analysis. Immunoblot analysis of muscle specimens showed that the pattern of glycosylated forms of α -DG (a dispersed, dense band of 156 kDa) was drastically reduced in Patients 1, 2, and 4, compared to control samples, suggesting defective glycosylation (data not shown). Immu-

Figure 1 Patients



(A) Patient 1 at age 22 years. (B) Patient 2 at age 2.5 years (B-1) and 20 years (B-2). (C) Patient 3 at age 10 years. (D) Patient 4 at age 6 years. Note the lower limb (particularly calf) muscle hypertrophy, facial muscle weakness, and the open mouth in all patients. Patients 1, 2, and 3 have cervical-dorsal lordosis, with stiff hyperextended necks. In Patient 1, this is associated with lumbar lordosis. Patient 4 has slight strabismus.

noblot analysis was not performed on Patient 3.

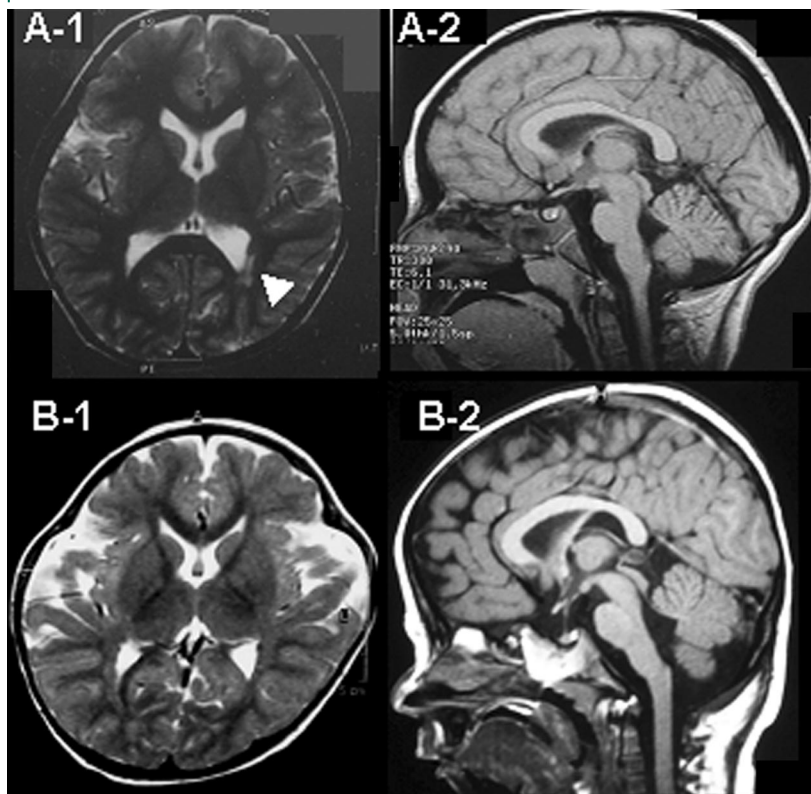
Mutation detection. The same homozygous mutation, c.1997A>G, in exon 19 of *POMT2* was identified in Patients 1 and 2 (figure E-2A). This mutation leads to the substitution of tyrosine at position 666 to cysteine (p.Tyr666Cys), and generates a new *BtsI* restriction site. The parents of Patient 2 harbored the mutation in the heterozygous state (figure E-2B). Amino acid alignment shows that the tyrosine 666 is conserved in vertebrate *POMT2* and *POMT1* orthologues, and even in more distantly related orthologues such as

Drosophila melanogaster (figure E-2C). The same mutation was also found in the heterozygous state in Patients 3 and 4, and a novel mutation was also detected in each. Patient 3 had a novel nonsense mutation (c.1941G>A, p.Trp647X) in exon 19. Patient 4 had a novel missense mutation (c.2242T>C, p.Trp748Arg) in exon 21. One disease allele was carried by each parent of Patients 3 and 4. The c.1997A>G was also identified in the heterozygous state in four additional CMD patients from France or Spain, for whom we did not find a second mutation (data not shown), but not found in 200 alleles from healthy unrelated individuals, as well as the two other mutations.

Haplotype analysis. Six microsatellite markers and five intragenic SNPs were genotyped in families carrying at least one heterozygous c.1997A>G *POMT2* mutation to determine whether it is inherited on the same genetic background (figure E-1). As seen in figure E-3, the four families shared the same haplotype, limited by CA1_POMT2 and D14S59, on the allele harboring the c.1997A>G mutation. Different backgrounds are associated with the two other mutations as shown by the haplotypes either transmitted to Patient 3 by his father (who carries the c.1941G>A mutation) or to Patient 4 by his mother (who carries the c.2242T>C mutation). The four other families in which we identified c.1997A>G as the sole disease allele shared most of the common haplotype, with the exception of a recombination at CA4_POMT2 in three families. Thus the disease-associated haplotype as defined by the minimum combination of three microsatellites and five SNPs is (CA1_POMT2) 231-C-G-G-C-T-249-205 (CA4_POMT2). This common haplotype was not found in any parental chromosome without the c.1997A>G mutation.

DISCUSSION Defects in O-glycosylation of α -DG are associated with a broad spectrum of autosomal recessive CMDs, from forms without CNS involvement and with a benign course to forms with structural brain and eye abnormalities, absent motor development, and early death.^{1,2} The most severe form is the Walker-Warburg syndrome (WWS) in which CMD is associated with CNS abnormalities including type II cobblestone lissencephaly, hydrocephalus, brainstem and cerebellar malformations, and eye anomalies. Most children with WWS die before the age of 3 years.²¹ At present, up to 20% of WWS cases have identifiable defects in *POMT1* or *POMT2*, and only rare cases have been associated with mutations in *FKTN* and *FKRP*; the ge-

Figure 2 Brain MR images



T2-weighted axial (A-1 and B-1) and T1-weighted sagittal (A-2 and B-2) brain MR images of Patient 3 at 5 years old (A) and Patient 4 at 6 years old (B). The MRI of Patient 3 shows hypersignal lesions at the occipital periventricular white matter (A-1, arrow), mild hypoplasia of the cerebellar vermis (especially the inferior part) without brainstem abnormalities (A-2), and mild atrophy of the frontal and temporal cerebral cortex (A-1). Patient 4 demonstrates moderate cortical atrophy, predominantly in the frontal and temporal cortex (B-1 and B-2).

netic cause in the remainder of WWS cases is unknown.^{10,11,18,22-24} In *POMT2*, only three homozygous mutations have been reported to date in WWS patients (two nonsense mutations and one splice site mutation),¹⁸ along with two compound heterozygous missense mutations in a MEB-like patient.¹⁹

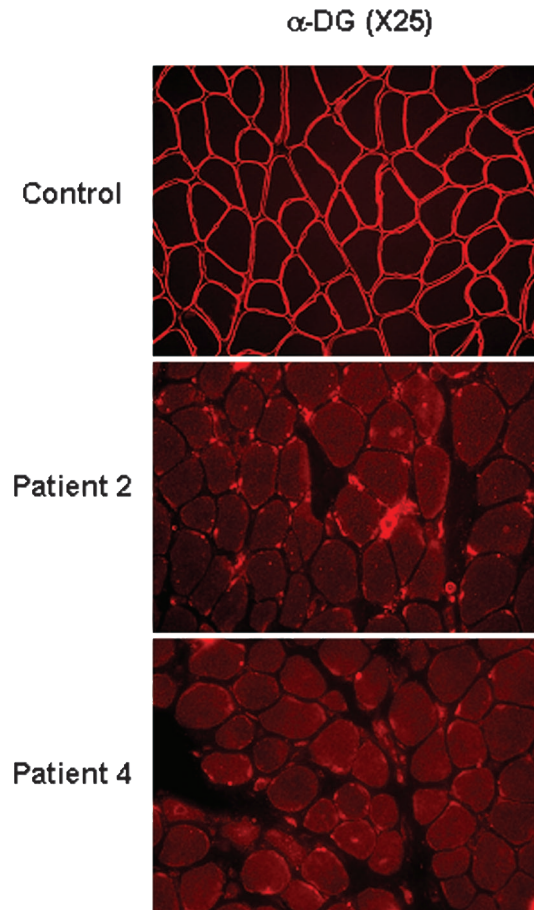
Here, we identified the common missense mutation in *POMT2* (c.1997A>G, p.Tyr666Cys) in the homozygous state in two unrelated patients. The same heterozygous mutation was found in two other patients, associated with two new *POMT2* mutations (c.1941G>A, p.Trp647X and c.2242T>C, p.Trp748Arg). All four patients who were either homozygous or heterozygous for the p.Tyr666Cys mutation presented with CMD associated with microcephaly and severe mental retardation, but they had only mild structural CNS abnormalities revealed by the brain MRI (cerebral atrophy and cerebellar vermis hypoplasia), and only three of them had minor ocular abnormalities even at late ages (abnormal pigmentation of the peripheral retina, myopia, or strabismus), in contrast with WWS and MEB.

Other than the neurologic findings, the patients showed several clinical features often observed in patients with secondary alpha-dystroglycanopathies, especially at late ages. All patients had very high CK levels and facial and proximal limb predominant muscle weakness. The three patients older than 10 years showed a progressive motor and respiratory insufficiency, and diffuse joint contractures. In spite of having the same gene defect, both patients carrying the homozygous mutation had marked variability in the severity of motor dysfunction, suggesting additional influencing factors. While one patient never walked and died in young adulthood due to respiratory insufficiency, another was able to walk without support at age from 2 to 10 years and at 22 years had a respiratory insufficiency but did not require mechanical ventilation. Interestingly, in all patients older than 10 years, axial muscle weakness led to a very similar spinal lordotic deformity characterized by a marked fixed hyperextension of the cervical, dorsal, and lumbar spine. This type of deformity is partly due to the occurrence of neck and paravertebral extensor muscle contractures in the absence of strong neck flexor muscles. In our experience, it is not infrequent in the course of early onset muscular dystrophies, especially those due to mutations in *LAMA2*, *SEPN1*, and *LMNA*. However, this sort of deformity has not been previously described in patients with dystroglycanopathy, even at very late stages of the disease. This could be due to the early death of most patients, to differences in orthopaedic treatment among patients, or to incomplete data regarding this specific feature in previous publications. Further clinical reports will be therefore required to elucidate if this is a distinct feature of *POMT2* mutations.

Compared with previously reported patients with congenital dystroglycanopathies, the phenotype observed in our first patient is different from the MEB phenotype because our patient had brain atrophy and posterior fossa malformation but no eye involvement. Her clinical and neuroradiologic picture was close to that described in certain *FKRP* mutated patients with mental retardation and CNS malformation,²⁵⁻²⁷ although no brainstem hypoplasia or cerebellar cysts were observed in our patient compared to the *FKRP* patients. Our remaining three patients had mild eye involvement and minor CNS abnormalities, and therefore can be considered as a mild “MEB-like phenotype,” very similar to that described in a series of four Italian patients with CMD, mental retardation, microcephaly, and calf and thigh en-

Both demonstrate absence of staining for the glycosylated epitope of α -DG, compared with normal staining of control.

Figure 3 Immunofluorescence analysis of muscle biopsies for Patients 2 and 4



largement.²⁸ Subsequently, four different *POMT1* missense or nonsense mutations were identified in three of them.¹⁴ Recently, two *POMT2* missense mutations (p.Gly353Ser and p.Gly726Glu) have been identified in the fourth patient, who presented with microcephaly, severe mental retardation, muscle hypertrophy, diffuse muscle weakness with marked facial and proximal limb involvement, and mild myopia. Neuroradiologically, cortical-subcortical brain atrophy, pons and cerebellar vermis hypoplasia, and abnormal signal of the periventricular white matter were observed.¹⁹ It is not surprising that patients with *POMT1* and *POMT2* mutations could share the same phenotype because *POMT1* and *POMT2* form a heterodimeric complex responsible for the catalysis of the first step in *O*-mannosyl glycan synthesis.⁹

In the present study, although two patients harbored homozygous copies of the same mutation, the patients were unrelated, and none of the families was known to be consanguineous. In this setting, by analysis of the chromosomal background upon which a mutation occurs, one can

infer whether a specific disease-causing mutation is due to a founder effect. Genotype data defined a distinct 170 kb haplotype encompassing *POMT2* and shared by all the subjects harboring the p.Tyr666Cys mutation. These results suggest that this mutation is a founder mutation present in the European population. Further studies will be needed to determine its precise geographic origin and frequency. This study provides confirmation that, as reported for other forms of alpha-dystroglycanopathies, *POMT2* mutations are associated with a wide clinical and neuroradiologic heterogeneity. In addition to the previous reported phenotypes including WWS and MEB-like, our results demonstrate that *POMT2* mutations may be responsible for a CMD associated with mental retardation and posterior fossa hypoplasia but no ocular involvement, similar to the phenotype previously described in patients with *FKRP* mutations and CNS involvement. Variable phenotypic severity was observed in patients with the same genotype, suggesting the influence of additional pathogenic factors.

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