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Protein *O*-mannosyltransferase activities in lymphoblasts from patients with α-dystroglycanopathies

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Abstract

Defects in *O*-mannosylation of α-dystroglycan cause some forms of congenital muscular dystrophy (CMD), the so-called α-dystroglycanopathies. Six genes are responsible for these diseases with overlapping phenotypes.

We investigated the usefulness of a biochemical approach for the diagnosis and investigation of the α-dystroglycanopathies using immortalized lymphoblasts prepared from genetically diagnosed and undiagnosed CMD patients and from control subjects. We measured the activities of protein *O*-mannose β1,2-*N*-acetylglucosaminyltransferase 1 (POMGnT1) and protein *O*-mannosyltransferase (POMT). Lymphoblasts from patients harbouring known mutations in either *POMGNT1* or *POMT1* showed a marked decrease in POMGnT1 or POMT activity, respectively, compared to controls. Furthermore, we identified pathogenic mutations in *POMGNT1*, *POMT1* or *POMT2* in six previously genetically uncharacterised patients who had very low enzyme activity. In conclusion, the lymphoblast-based enzymatic assay is a sensitive and useful method (i) to select patients harbouring *POMGNT1*, *POMT1* or *POMT2* mutations; (ii) to assess the pathogenicity of new or already described mutations.

Keywords: POMT1; POMT2; POMGnT1; Congenital muscular dystrophy; Enzyme activity

1. Introduction

α-Dystroglycan (α-DG) is a heavy glycosylated membrane protein, and its glycan groups have been shown

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to play a major role in binding to extracellular matrix proteins such as laminin, neurexin, and agrin [1,2]. In humans, defects in the *O*-glycosylation of α-DG cause a large spectrum of autosomal recessive congenital muscular dystrophies (CMD), from mild forms without central nervous system (CNS) involvement to more severe forms with structural brain and eye abnormalities such as muscle–eye–brain disease (MEB: OMIM 253280) and Walker–Warburg syndrome (WWS: OMIM 236670). These entities are now collectively classified as the α-dystroglycanopathies [3].

Mutations in *POMGNT1* were first identified in patients with MEB [4,5] and soon after mutations in *POMT1* [6–9] and *POMT2* [10] were reported in children affected with WWS. In fact, the clinical spectrum associated with these three genes is broad and extends from patients with severe mental retardation and limited life expectancy to being able to walk and survive into the second decade of life.

In addition to MEB and WWS, other muscular dystrophies have been reported to be associated with an abnormal glycosylation of α-DG: Fukuyama-type congenital muscular dystrophy (FCMD: OMIM 253800), CMD type 1C (MDC1C: OMIM 606612), limb–girdle muscular dystrophy type 2I (LGMD2I: OMIM 607155), and CMD type 1D (MDC1D: OMIM 608840). FCMD, due to mutations in *fukutin*, has been reported almost exclusively in patients of Japanese origin who present with a CMD associated with mental retardation, brain malformations with or without eye involvement [11]. Regarding MDC1D, two cases with *LARGE* mutations have been reported in the literature, in a mentally retarded girl with brain abnormalities [12] and a family with WWS [13]. In contrast, *FKRP* mutations are responsible for the most common form of α-dystroglycanopathy in Western countries, a relatively mild disease that presents with limb–girdle weakness in childhood or adulthood and normal intelligence (LGMD2I). Less commonly, mutations in *FKRP* have been reported in patients with mental retardation, transitory brain white matter abnormalities and brain stem and *posterior fossa* malformations (MDC1C) [14–17] and even in patients with MEB and WWS, showing the overlap in phenotypes that is possible among the α-dystroglycanopathies [18]. As a further example of this overlap, *POMGNT1*, *POMT1* and *POMT2* mutations have been recently identified in patients with much milder phenotypes than the initial descriptions of a typical MEB or WWS [17,19–23]. Since multiple genes are known to cause α-dystroglycanopathies, with an extremely broad clinical spectrum and relatively poor phenotype–genotype correlation, at present molecular diagnosis of α-dystroglycanopathy patients is difficult and requires searching for mutations gene by gene.

With the aim of improving the diagnosis and investigation of patients with an α-dystroglycanopathy, we investigated the utility of a biochemical approach. At present, of the six known α-dystroglycanopathy genes, the function of the protein product is clear only for POMT1 and POMT2, which are responsible for the catalysis of the first step in *O*-mannosyl glycan synthesis [24] and POMGnT1 which forms a GlcNAcβ1–2 Man linkage of *O*-mannosyl glycans on α-DG [4]. An assay for POMGnT1 activity in lymphoblasts has previously been described [25]. In previous studies, we demonstrated by a specific enzymatic activity assay that mutations in *POMGNT1* and *POMT1* lead to defects in POMGnT1 and POMT activity, respectively, using mutant constructs transfected into cell lines [24,26]. In order to screen patients with confirmed or suspected forms of α-dystroglycanopathy, we developed an assay for lymphoblast POMT activity and measured the activities of both POMT and POMGnT1 in lymphoblasts from a series of patients. We observed reductions in POMGnT1 or POMT activity in several uncharacterised patients, in whom secondary targeted sequencing led to the identification of mutations in *POMT1*, *POMT2* or *POMGNT1*.

2. Experimental procedures

2.1. Patients

Blood from seven healthy subjects and 24 CMD patients with high serum creatine kinase levels, and with or without mental retardation was collected for B lymphoblast immortalization and DNA extraction after informed consent. Eleven patients had already been genetically characterised. Seven patients had mutations in *FKRP*, two in *POMGNT1* [4] and two in *POMT1* [21,22] (Table 1). Thirteen other patients were genetically uncharacterised (GUC1–GUC13) before the enzymatic studies. These 13 patients were all CMD patients with or without mental retardation, brain abnormality and ocular involvement, and in five of them, marked hypoglycosylation of α-DG was observed by immunohistochemistry or Western blot analysis in muscle biopsies. The α-DG status in the eight other cases could not be evaluated, because no muscle was available. *FKRP* had been sequenced and excluded in all of these 13 cases prior to the enzymatic studies. Afterwards *LARGE* and *fukutin*, and then *POMT1*, *POMT2* and *POMGNT1* were sequenced in the patients for whom no decrease in the enzymatic activities was found.

2.2. Cell culture and extract preparation

B lymphoblastoid cell lines were obtained after immortalization by Epstein–Barr virus and cultured in RPMI 1640 (Invitrogen, Carlsbad, CA) according to standard protocols to obtain 100×10^6 cells. After centrifugation at 800*g* for 5 min, the pellets were rinsed twice with 50 ml then with 12 ml of phosphate-buffered saline (PBS). The final pellets were frozen at −80 °C. The cells ($\sim 7.5 \times 10^6$ cells) were homogenized in 10 mM Tris–HCl, pH 7.4, 1 mM EDTA, 250 mM sucrose, 1 mM dithiothreitol, with a protease inhibitor cocktail (3 μg/ml pepstatin A, 1 μg/ml leupeptin, 1 mM benzamidine–HCl, and 1 mM PMSF). After centrifugation at 900*g* for 10 min, the supernatant was subjected to ultra centrifugation at

Table 1
POMGNT1, *POMT1* and *POMT2* mutations evidenced in patients with low POMGnT1 or POMT activities

Patient	Gene	Allele 1	Allele 2	References
GC8	*POMT1*	p.Gly65Arg	p.Trp582Cys	[21]
GC9	*POMT1*	p.Ala200Pro	p.Ala200Pro	[20]
GC10	*POMGNT1*	c.1539+1 G>A	c.1539+1 G>A	[4]
GC11	*POMGNT1*	p.Arg442His	p.Arg442His	Unpublished
GUC1	*POMGNT1*	p.Ser153X	p.Cys269del	Unpublished
GUC2	*POMT1*	p.Ala669Thr	p.Gly722fs>730x	Unpublished
GUC3	*POMT2*	p.Tyr96Cys	p.Tyr96Cys	Unpublished
GUC4	*POMT2*	p.Tyr666Cys	p.Tyr666Cys	[22]
GUC5	*POMT2*	p.Tyr666Cys	Splicing abnormality	Unpublished
GUC6	*POMT2*	p.Tyr666Cys	p.Trp748Arg	[22]

(GC: genetically characterised patient, GUC: genetically uncharacterised patient).

100,000*g* for 1 h. The precipitate was used as the microsomal membrane fraction. Protein concentrations were determined by the BCA assay (PIERCE, Rockford, IL). About 40 μg of protein was obtained in the microsomal membrane fraction prepared from 1×10^6 cells.

2.3. Assay for POMGnT1 activity

POMGnT1 activity was obtained from the amount of [^{3}H]GlcNAc transferred to a mannosylpeptide [4,25]. The reaction buffer containing 140 mM MES buffer (pH 7.0), 1 mM UDP-[^{3}H]GlcNAc (225,000 dpm/nmol) (Perkin-Elmer, Inc., Waltham, MA), 1 mM mannosyl nanopeptide (Ac-Ala-Ala-Pro-Thr(Man)-Pro-Val-Ala-Ala-Pro-NH_2), 10 mM $MnCl_2$, 2% Triton X-100, 5 mM AMP, 200 mM 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.

2.4. Assay for 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 previously [27]. Briefly, the reaction mixture contained 20 mM Tris–HCl (pH 8.0), 100 nM of [^{3}H]-mannosylphosphoryldolichol (Dol-P-Man, 125,000 dpm/pmol) (American Radiolabeled Chemical, Inc., St. Louis, MO), 2 mM 2-mercaptoethanol, 10 mM EDTA, 0.5% *n*-octyl-β-D-thioglucoside, 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, USA), rotated at 4 °C for 1 h, and washed three times with 20 mM Tris–HCl (pH 7.4) containing 0.5% Triton X-100. The radioactivity adsorbed to the beads was measured using a liquid scintillation counter.

2.5. Assay for GnT1 activity

The GnT1 (UDP–GlcNAc: α-3-D-mannoside β1,2-*N*-acetylglucosaminyltransferase 1, EC 2.4.1.101) activity was performed in a total volume of 20 μl reaction mixture containing 100 mM MES buffer, 10 μM pyridylaminated $Man_5GlcNAc_2$ (M5-PA, Takara Bio Inc., Otsu, Japan), 2 mM UDP–GlcNAc, 5 mM AMP, 0.5% Triton X-100, 0.2% BSA, 20 mM $MnCl_2$ 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 mM, 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 nm and 400 nm, respectively.

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 (Perkin-Elmer Applied Biosystems, Wellesley, MA). Sequences were analyzed on an ABI PRISM 31130 capillary sequencer (Applera, CA). One hundred unrelated healthy individuals served as control subjects to test the presence of the missense mutations p.Tyr96Cys and p.Tyr666Cys (*POMT2*), and p.Ala669Thr (*POMT1*) by sequencing.

3. Results

3.1. GnT1 activity

GnT1 present in the Golgi apparatus is the entry point for the conversion of oligomannose to hybrid and complex *N*-glycans. Since GnT1 is not involved in *O*-mannosylglycan biosynthesis, it is not affected in α-dystroglycanopathies and represents a suitable control to normalise samples for baseline microsomal activity. The high consistent levels of cellular GnT1 activity as shown in Fig. 1, gives an indication of the quality of the cells we used for enzyme analysis. The GnT1 activity mean (±standard deviation) of all samples was 0.53 (±0.06) nmol/h/mg total proteins.

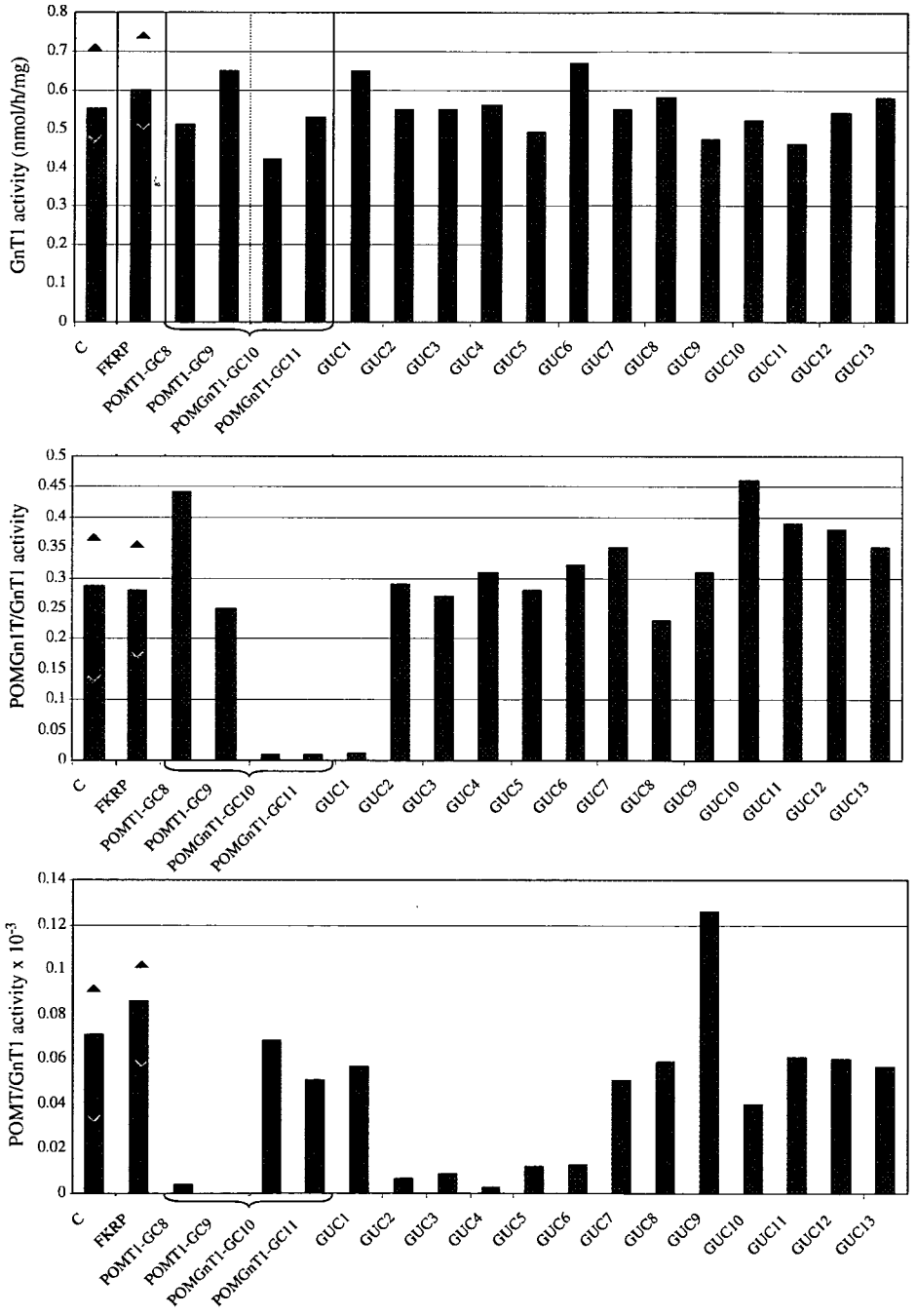

Fig. 1. Lymphoblast GnT1, POMGnT1/GnT1 and POMT/GnT1 activities from controls and patients with FKRP mutations, and patients with congenital muscular dystrophy: genetically characterised patients (GC8–GC11) and uncharacterised patients (GUC1–GUC13). For Controls (C) and FKRP-patients (FKRP) mean [minor–major value].

3.2. POMGnT1 activity

The average POMGnT1 activity measured in lymphoblasts of control subjects was 0.163 (±0.042) nmol/h/mg total proteins. When we assessed the POMGnT1 activity of patient lymphoblasts, we observed a decrease for three of them. Two had previously been genetically confirmed with mutations in *POMGNT1* (Table 1). Patient GC10 carried the common MEB mutation, c.1539 + 1G > A in the homozygous state, and patient GC11 harboured the mutation p.Arg442His, also in homozygous state. POMGnT1 activity in these lymphoblasts was much lower (0.005 and 0.006 nmol/h/mg total protein, respectively.) than in the control subjects and the other patients.

Among all the other patients, only one patient, GUC1, showed low POMGnT1 activity (0.008 nmol/h/mg), similar to GC10 and GC11, 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), which probably leads to a loss of function, and a deletion of three base pairs c.805–807delTGC, which is expected to delete one amino acid, cysteine at position 269 (p.Cys269del). A missense mutation, p.Cys269Tyr, affecting the same amino acid has already been reported [28], and *in vitro* enzymatic activity was undetectable in the mutant protein carrying this substitution [24]. Thus, the 269 cysteine deletion is very likely to be pathogenic. DNA study confirmed the heterozygous carrier status of both parents.

The ratios of POMGnT1 activity to GnT1 activity were on average 0.01 in patients with POMGNT1 mutations ($n = 3$), and 0.29 in controls ($n = 7$) (Fig. 1). The presence of different FKRP mutations did not affect lymphoblast POMGnT1 activity in the seven patients, as shown by POMGnT1/GnT1 activity ratio, which was similar to that of controls (0.28).

3.3. POMT activity

Using the same controls as for POMGnT1, we assayed the POMT activity for the first time in lymphoblasts of control subjects and found the average value was 0.041 (±0.013) pmol/h/mg proteins. It is notable that the POMT activity in these cells was much lower than the POMGnT1 activity.

When we assessed POMT activity in the 17 patient lymphoblasts without *FKRP* mutations, we observed markedly reduced activity in seven of them (GC8 and 9, GUC2, 3, 4, 5 and 6) (Fig. 1). Two had been previously genetically confirmed with mutations in the *POMT1*. Patient GC9 was homozygous for the missense mutation p.Ala200Pro [21], and patient GC8 was a compound heterozygous carrier of two missense mutations, p.Gly65Arg and p.Trp582Cys [22]. The enzyme activity in these patient lymphoblasts was extremely low: undetectable in patient GC9 and 0.002 pmol/h/mg proteins in patient GC8. These last results strongly support the pathogenicity of the previously identified mutations.

The five other patients were secondarily screened for *POMT1* and *POMT2* (Table 1). We found two heterozygous mutations, in *POMT1* for patient GUC2: p.Ala669Thr (c.2005G > A), associated with c.2167insG which leads to a premature stop codon in amino acid 730. These mutations were already described [6,20]. POMT activity of this patient was 0.004 pmol/h/mg proteins. Targeted sequencing of patients GUC3, GUC4, GUC5 and GUC6, whose POMT activity was 0.005, 0.001, 0.006 and 0.009 pmol/h/mg proteins, respectively, showed *POMT2* mutations (Table 1). Patients GUC3 and GUC4 harboured missense mutations in the homozygous state, respectively, p.Tyr96Cys (c.287A > G) and p.Tyr666Cys (c.1997A > G). These missense mutations are situated in highly conserved regions. The p.Tyr666Cys recently described as a founder mutation [23], was found in the heterozygous state in patients GUC5 (maternal allele) and GUC6. In patient GUC6, the second mutation was a missense mutation, p.Trp748Arg (c.2242T > C). In addition, patient GUC5 had on the other (paternal) allele a four base pair deletion in exon 1, 46 base pairs before the initiating ATG (c.1–46_49del CAGA). This change was also found in 4.2% of control DNA samples, and therefore is very unlikely to be pathogenic. Further analysis of patient cDNA showed a *POMT2* transcript missing exons 3–5, which is predicted to lead to a premature stop codon (data not shown). This transcript was also found in cDNA generated from paternal lymphoblasts. The corresponding pathogenic mutation remains to be identified at the genomic level. Patient GUC3 carrying a homozygous *POMT2* missense mutation p.Tyr96Cys had a classical severe WWS phenotype and died at age 2.5 years. In contrast, patients carrying the p.Tyr666Cys mutation in the homozygous or heterozygous state were less severely affected, having CMD with mental retardation, but without severe brain abnormalities on MRI. The p.Tyr96Cys (c.287A > G) *POMT2* mutation has not been previously described. The screening of 200 alleles from healthy unrelated individuals did not reveal any of these mutations.

The ratios of POMT/GnT1 activity were on average 0.005×10^{-3} in patients with *POMT1* or *POMT2* mutations, and 0.071 $(\pm 0.019) \times 10^{-3}$ in controls, respectively (Fig. 1). As for POMGnT1 activity, the seven patients with *FKRP* mutations presented similar lymphoblast POMT activity, compared to control values, as confirmed by the POMT/GnT1 activity ($0.086 \pm 0.019 \times 10^{-3}$).

4. Discussion

The recent identification of six genes associated with the α-dystroglycanopathies has made it possible to establish a precise molecular diagnosis in many families, but since there is such an overlap in clinical features between genes, successive genes must be sequenced to identify the causative mutations in sporadic cases. These methods are expensive and time-consuming. To direct genetic analysis, firstly a POMGnT1 enzymatic assay was reported based

on extracts of muscle biopsies [29], and more recently fibroblast- and lymphoblast-based assays have been developed [25]. The average residual POMGnT1 activity in fibroblasts and lymphoblasts from MEB patients was about 13% and 15% of normal controls, and this fibroblast- and lymphoblast-based assay was proposed as a rapid and relatively simple diagnostic test for MEB patients [25].

In a similar manner, we have developed an assay to determine POMT activity in patient lymphoblasts. We had previously measured *in vitro* POMT activity in Sf9 cells transfected with various *POMT1* mutants [26]. We now show that POMT activity can be measured in control and patient lymphoblasts. As the previous descriptions [25,29], measuring GnT1 activity in parallel allows validation of the cell preparation and the POMT and POMGnT1 activity measurement quality. When testing cells from patients with known *POMT1* mutations, activity levels were almost undetectable. A marked reduction in POMT activity was also found in five genetically uncharacterised patients, which led us to sequence *POMT1* and *POMT2*. We identified mutations in all of these patients, and most of them are either previously reported or novel missense mutations that were absent from control chromosomes. As for POMGnT1 activity, the lymphoblast-based enzymatic POMT assay is inexpensive and precise, and may bypass the need for invasive muscle biopsies when clinical findings are highly suggestive of an α-dystroglycanopathy. These tests can also be used to assess the pathogenicity of the new mutations, as well as those already described, to ascertain their pathogenicity.

Patients GC9 and GC8 carrying *POMT1* missense mutations showed relatively mild phenotypes compared to typical WWS. The Turkish patient (GC9), homozygote for the p.Ala200Pro mutation, had LGMD2I with mild mental retardation and microcephaly [21], while the phenotype of the Italian patient (GC8), who is compound heterozygous for the p.Gly65Arg and p.Trp582Cys mutations, had CMD with calf muscle pseudohypertrophy, microcephaly and severe mental retardation, but no eye abnormalities, and was still living at the age of 19 years [22]. These phenotypic features had led us to expect that these mutations would not completely abolish POMT activity. However, these patients have very low or undetectable enzyme activities. At this stage, the discrepancy between clinical severity and the level of POMT activity of these patients remains unexplained.

To date, *POMT2* mutations have been reported in CMD patients with both a severe phenotype, WWS, and a milder phenotype with mental retardation [22,23,30]. The new homozygous missense *POMT2* mutation, p.Tyr96Cys, caused a typical WWS phenotype. In contrast the other three patients carrying p.Tyr666Cys were affected less severely [23].

In seven CMD patients, no decrease in POMT or POMGnT1 activity was observed. Two of these patients were confirmed to have marked hypoglycosylation of α-DG on muscle immunohistochemistry. To further investigate the basis of the CMD, we sequenced the coding exons and flanking intronic regions of all six genes known to cause α-dystroglycanopathy, without finding mutations. These results suggest that new genes involved in α-DG metabolism may be responsible for the disease in these patients.

Patients with *FKRP* mutations did not show reduced activity for POMT and POMGnT1 suggesting that FKRP is not associated with POMT1 or POMT2, or with POMGnT1. However, it has been recently reported that fukutin is associated with POMGnT1 in the Golgi compartment. Although fukutin has no proven glycosyltransferase activity, transgenic knock-in mouse carrying the retrotransposon insertion in the *fukutin* gene showed a 30% reduction of POMGnT1 activity [31], suggesting that fukutin modulates POMGnT1 activity in muscle. It will be interesting to test POMGnT1 activity in muscle, lymphoblasts and fibroblasts from FCMD patients to determine whether mutations in *fukutin* could modulate POMGnT1 activity.

Initially, muscle biopsies were used to test enzyme activity since α-DG, one of the only *O*-mannosylated glycoproteins identified up to now, is expressed in this tissue in addition to brain. This study shows that not only POMGnT1 but POMT1 and POMT2 are expressed in lymphoblasts also. Their precise role in lymphoblasts is not known, but α-DG was recently identified at both the mRNA and protein level in murine T and B lymphocytes [32]. It is also possible that additional proteins may also be *O*-mannosylated in human blood cells. In conclusion, our investigations show that the lymphoblast-based enzymatic assays are accurate and useful methods to select patients harbouring *POMT1*, *POMT2* and *POMGNT1* mutations among those with a suspected or confirmed α-dystroglycanopathy. In addition, they are robust functional tests that can be used to assess the pathogenicity of mutations.

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