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Refining genotype–phenotype correlations in muscular dystrophies with defective glycosylation of dystroglycan

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Muscular dystrophies with reduced glycosylation of α-dystroglycan (α-DG), commonly referred to as dystroglycanopathies, are a heterogeneous group of autosomal recessive conditions which include a wide spectrum of clinical severity. Reported phenotypes range from severe congenital onset Walker–Warburg syndrome (WWS) with severe structural brain and eye involvement, to relatively mild adult onset limb girdle muscular dystrophy (LGMD). Specific clinical syndromes were originally described in association with mutations in any one of six demonstrated or putative glycosyltransferases. Work performed on patients with mutations in the *FKRP* gene has identified that the spectrum of phenotypes due to mutations in this gene is much wider than originally assumed. To further define the mutation frequency and phenotypes associated with mutations in the other five genes, we studied a large cohort of patients with evidence of a dystroglycanopathy. Exclusion of mutations in *FKRP* was a prerequisite for participation in this study. Ninety-two probands were screened for mutations in *POMT1*, *POMT2*, *POMGnT1*, *fukutin* and *LARGE*. Homozygous and compound heterozygous mutations were detected in a total of 31 probands (34 individuals from 31 families); 37 different mutations were identified, of which 32 were novel. Mutations in *POMT2* were the most prevalent in our cohort with nine cases, followed by *POMT1* with eight cases, *POMGnT1* with seven cases, *fukutin* with six cases and *LARGE* with only a single case. All patients with *POMT1* and *POMT2* mutations had evidence of either structural or functional central nervous system involvement including four patients with mental retardation and a LGMD phenotype. In contrast mutations in *fukutin* and *POMGnT1* were detected in four patients with LGMD and no evidence of brain involvement. The majority of patients (six out of nine) with mutations in *POMT2* had a Muscle–Eye–Brain (MEB)-like condition. In addition we identified a mutation in the gene *LARGE* in a patient with WWS.
Our data expands the clinical phenotypes associated with *POMT1*, *POMT2*, *POMGnT1*, *fukutin* and *LARGE* mutations. Mutations in these five glycosyltransferase genes were detected in 34% of patients indicating that, after the exclusion of *FKRP*, the majority of patients with a dystroglycanopathy harbour mutations in novel genes.

Keywords: congenital muscular dystrophy; limb girdle muscular dystrophy; alpha dystroglycan; glycosylation; glycosyltransferase

Abbreviations: CMD = congenital muscular dystrophy, LGMD = limb girdle muscular dystrophy, α-DG = alpha-dystroglycan

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Introduction

Muscular dystrophies with reduced glycosylation of α-dystroglycan (α-DG) are a clinically and genetically heterogeneous group of autosomal recessive muscular dystrophies with variable neurological and ophthalmic involvement. Pathologically these disorders share the common feature of a hypoglycosylated form of α-dystroglycan (α-DG) on skeletal muscle biopsy (Muntoni *et al.*, 2002) which led to the term dystroglycanopathy (Toda *et al.*, 2003; Brockington and Muntoni, 2005; Mercuri *et al.*, 2006). Alpha and beta dystroglycan are derived from the same precursor peptide and are major components of the dystrophin-associated glycoprotein complex (DGC) that forms a link between the actin associated cytoskeleton and the extracellular matrix. α-DG is a highly glycosylated peripheral membrane protein that binds many of its extracellular matrix partners through its carbohydrate modifications. In the dystroglycanopathies, these modifications are either absent or reduced resulting in the decreased binding of its ligands, such as laminin-2, agrin and perlecan in skeletal muscle and neurexin in the brain (Barresi and Campbell, 2006). Primary mutations in the gene encoding dystroglycan (*DAG1*) have never been reported and a dystroglycan knockout mouse is embryonically lethal (Williamson *et al.*, 1997).

To date, mutations in six known or putative glycosyltransferase genes have been identified in these disorders: *Protein-O-mannosyl transferase* 1 (*POMT1*; OMIM 607423), *Protein-O-mannosyl transferase 2* (*POMT2*; OMIM 607439), *Protein-O-mannose 1,2-N-acetylglucosaminyltransferase 1* (*POMGnT1*; OMIM 606822), *fukutin* (OMIM 607440), *Fukutin-related protein* (*FKRP*; OMIM 606596) and *LARGE* (OMIM 603590) (Kobayashi *et al.*, 1998; Brockington *et al.*, 2001*a*; Yoshida *et al.*, 2001; Beltran-Valero de Bernabe *et al.*, 2002; Longman *et al.*, 2003; van Reeuwijk *et al.*, 2005*b*). These genes are thought to be involved in the addition of carbohydrate residues onto the α-DG backbone either via the process of *O*-mannosylation (*POMT1, POMT2, POMGnT1*) (Yoshida *et al.*, 2001; Manya *et al.*, 2003; Akasaka-Manya *et al.*, 2004) or via other not fully characterized mechanisms (*fukutin, FKRP and LARGE*) (de Paula *et al.*, 2003; Brown *et al.*, 2004; Brockington *et al.*, 2005; Xiong *et al.*, 2006).

The phenotypic severity of dystroglycanopathy patients is extremely variable. At the most severe end of the clinical spectrum are Walker–Warburg Syndrome (WWS), Muscle–Eye–Brain (MEB) disease and Fukuyama congenital muscular dystrophy (FCMD). These conditions are characterized by congenital muscular dystrophy (CMD) with severe structural brain and eye abnormalities, which in WWS results in early infantile death (van Reeuwijk *et al.*, 2005*a*). Conversely, individuals at the mildest end of the clinical spectrum may present, in adult life, with limb-girdle muscular dystrophy (LGMD) with no associated brain or eye involvement (Brockington *et al.*, 2001*b*). A number of intermediate phenotypes between these extremes have also been described including congenital muscular dystrophy type 1C (MDC1C), a CMD variant in which the brain can be entirely normal and LGMD2K, a variant with microcephaly and mental retardation but a relatively mild LGMD-like phenotype (Brockington *et al.*, 2001*a*; Balci *et al.*, 2005).

These syndromes were originally described in association with mutations in specific genes: WWS [OMIM 236670] was associated with mutations in *POMT1* and *POMT2* (Beltran-Valero de Bernabe *et al.*, 2002; Currier *et al.*, 2005; van Reeuwijk *et al.*, 2005*b*); these enzymes form a heterodimer and have been shown to catalyse the first step in *O*-mannosylation (Akasaka-Manya *et al.*, 2006). MEB [OMIM 253280] was originally described within the Finnish population in association with mutations in *POMGnT1*, an enzyme involved in the second step of *O*-mannosylation of α-DG by transferring *N*-acetylglucosamine to a protein *O*-linked mannose (Yoshida *et al.*, 2001). Recent molecular genetic studies have demonstrated that the high prevalence of MEB in the Finnish population is due to a founder splice site mutation (Diesen *et al.*, 2004). FCMD [OMIM 253800] was described within the Japanese population where it is the second most common form of muscular dystrophy after Duchenne muscular dystrophy (Kobayashi *et al.*, 1998). The high incidence of FCMD in Japan is related to a founder retrotransposal mutation in the 3′UTR of *fukutin*, which is found in the homozygous state in ~90% of all Japanese FCMD patients. MDC1C [OMIM 606612] and MDC1D [OMIM 608840] are two rare CMD syndromes, secondary to mutations in *FKRP and LARGE* respectively (Brockington *et al.*, 2001*a*; Longman *et al.*, 2003). The increased availability of mutation analysis in patients with a dystroglycanopathy has subsequently led to the widening of the clinical spectrum observed for several of these genes. This is best exemplified by the range of phenotypes resulting from mutations in *FKRP*. Following the initial description of its involvement in MDC1C (Brockington *et al.*, 2001*a*), it has subsequently been shown to cause a very common and relatively mild variant, LGMD2I [OMIM 607155] (Brockington *et al.*, 2001*b*), and more recently CMD variants with associated mild structural brain [MDC1C and cerebellar cysts (Topaloglu *et al.*, 2003; Mercuri *et al.*, 2006)] or severe brain and eye involvement (WWS and MEB-like disorders) (Beltran-Valero de Bernabe *et al.*, 2004; Mercuri *et al.*, 2006). It has recently been documented that several of these genes are involved in both milder and more severe phenotypes than originally reported. This includes the finding of *fukutin* mutations in two families with WWS (de Bernabe *et al.*, 2003) and in two families with a LGMD variant (Godfrey *et al.*, 2006) as well as the involvement of *POMT1* in patients with LGMD2K, with associated microcephaly and mental retardation (Balci *et al.*, 2005).

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All previous studies have been conducted on a small number of families or individuals. This causes inevitable difficulties in applying mutation frequencies to the general population. In addition such reports make it difficult to establish whether the described clinical spectrum is truly representative of the phenotypic variability as well as how common the originally described core phenotypes are for each of these genes. In order to address these points, we have systematically screened a large population of patients with a dystroglycanopathy phenotype for mutations in the associated genes. As the spectrum of phenotypes secondary to *FKRP* involvement has been previously reported by us and others, we studied 92 patients in whom involvement of this gene had been excluded before proceeding with analysis of the five remaining genes. Our large and unbiased study redefines the clinical spectrum associated with each of the glycosyltransferases genes studied, identifies the frequency of individual gene defects and suggests that, after the exclusion of *FKRP*, the majority of patients with a dystroglycanopathy do not harbour mutations in any of the known genes.

Patient and methods

Patients

The cohort consisted of 92 unrelated individuals. This included a large group of patients from Australia (27 patients) and Turkey (16 patients). The majority of the remaining patients were recruited via the Hammersmith Hospital National Commissioning Group (NCG) service and included DNA from individuals referred from across the UK and Europe with a few samples from further a field. Mutations in *FKRP* had previously been excluded in all cases (Brockington *et al.*, 2001*a*).

The inclusion criteria were specified as hypoglycosylation of α-DG at the sarcolemma by immunolabelling of skeletal muscle sections (Brown *et al.*, 2004; Dubowitz and Sewry, 2007). Eighty patients met this criteria whilst in the remaining 12 cases there was no muscle available to perform α-DG studies. This later group of patients were included due to their clinical phenotype being highly suggestive of a dystroglycanopathy and consisted of children with CMD, elevated serum CK and brain MRI evocative of a cobblestone lissencephaly. All the patients who had had a muscle biopsy, were studied by standard immunocytochemical and/or Western blotting analysis in order to rule out dystrophinopathy, LGMDs such as sarcoglycanopathies, calpainopathy and dysferlinopathy, merosin deficient CMD and collagen VI deficiency (Dubowitz and Sewry, 2007). Clinical data was collated and patients were divided into phenotypic categories. This study was approved by Hammersmith Hospital Ethics Committee REC 2000:/5802.

Molecular genetics

Genomic DNA was extracted in the referring centre's laboratory using standard protocols. All mutation scanning was performed in the DNA laboratory at Guy's Hospital. The complete coding regions, including intron/exon boundaries of *POMT1*, *POMT2*, *POMGnT1*, *fukutin* and *LARGE* were amplified by PCR (primers are available in supplementary information, Table 1). Single nucleotide polymorphisms (SNP) within the primer binding sites were avoided using the Diagnostic SNP Check software (www.ngrl.man.ac.uk/SNPCheck). Amplicons were screened for mutations using a combination of unidirectional sequencing (standard dideoxynucleotide methodology) and heteroduplex analysis as previously described (Godfrey *et al.*, 2006). Where available, parental DNA was studied once a sequence alteration was identified in the proband. In two families, further segregation analysis was carried out to investigate the potential pathogenicity of unclassified variants. In families where a *de novo* mutation was suspected, paternity was confirmed using 11 STR markers (data not shown). Mutation nomenclature based on the following GeneBank Accession numbers; *POMT1*; NM_007171.2, *POMT2*; NM_013382.3, *POMGnT1*; NM_017739.1, *fukutin*; NM_006731.1 and *LARGE*; NM_133642.2, with nucleotide number 1 corresponding to the first base of the translation initiation codon.

Results

Clinical findings

Patients were classified as having either a CMD or LGMD phenotype and further subdivided according to the degree of structural and functional brain involvement. CMD was defined as onset of weakness prenatally or within the first 6 months of life. LGMD was defined by later onset weakness, specifically after having acquired ambulation. The cohort consisted of a total of 64 patients with CMD and 25 patients with LGMD, a total of 59 patients had brain involvement. In three cases the clinical information available was insufficient to determine phenotypic classification. Patients were divided into 1 of 7 broad phenotypic categories described below;

(1) WWS (and WWS-like): Onset prenatally or at birth. Patients assigned to this category had severe structural brain abnormalities including complete agyria or severe lissencephaly with only rudimentary cortical folding, marked hydrocephalus, severe cerebellar involvement and complete or partial absence of the corpus callosum. Eye abnormalities including congenital cataracts, micropthalmia and buphthalmus were common. When MRI evidence was not available, death before 1 year of age was taken as suggestive of this category if other clinical findings were supportive (Cormand *et al.*, 2001). Motor development was typically absent in these patients. Five patients were assigned to this group.
(2) MEB/FCMD-like: These categories were merged due to the overlapping phenotypic features. Included in

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Table 1 Clinical characteristics of 33 individuals from 31 families in whom mutations were detected

Patient	ADG	Phenotype	Age at onset[a]	CK	Motor ability[b]	Contractures[c]	Hypertrophy[d]	Spine[e]	Eyes[f]	Weakness[g]	IQ[h]	Microcephaly[i]	MRI[j]	Other[k]
1	LOW	WWS	P	4000	NS	Y	Y	Sc, RS	Poor visual attention	LL>UL	L	Y	H, CHy, WM, Lis	Gastrostomy
2	LOW	MEB-FCMD	P	3500	N/A	Y	N/A	N/A	CG	N/A	L	Y	H, BS,WM, CC,CHy	N/A
3	LOW	LGMD-MR	I	2000	W	N/A	Y	U	N/A	N/A	L	Y	Normal	N/A
4	LOW	CMD-MR	I	7800	NW 2yr	N/A	N/A	U	U	N/A	L	N/A	WM	N/A
5	LOW	LGMD-MR	I	4000	W	N	Y	U	U	N/A	L	Y	Normal	N/A
6	LOW	LGMD-MR	3 Yr	8000	W		Y	N/A	U	N/A	L	Y	WM- minimal	N/A
7	LOW	CMD-MR	I	3600	St	N	Y	U	U	N/A	L	Y	Normal	N/A
8	LOW	CMD-MR	4 m	18000	W	N/A	Y	RS	N/A	N/A	L	Y	WM- minimal	Choreic Movement disorder
9	LOW	MEB-FCMD	N	5500	S	Y	Y	RS, Sc	N/A	N/A	L	Y	WM, BS	N/A
10	LOW	MEB-FCMD	4 Yr	5200	NW	N	Y	U		N/A	L	Y	Encephalocele	N/A
11	LOW	MEB-FCMD	7 m	N/A	NS	Y	N	U	Hm	N/A		N/A	H, WM,CC,	N/A
12	LOW	MEB-FCMD	N	3100	NS	Y	N/A	RS		UL>LL	L	Y	WM	N/A
13	N/A	MEB-FCMD	8 m		W	N/A	Y	U	My	UL>LL	L	N	WM, CDys, CC,PMG	N/A
14	LOW	MEB-FCMD	N	6000	S	Y	Y	Sc	CC	N/A	L	Y	BS,H,WM	SE, RIP age 11yr
15a	N/A	CMD-cerebellar	I	4700	W	Y	Y	N/A	N/A	UL>LL	L	Y	N/A	N/A
15b	N/A	CMD-cerebellar	I	5200	S	N/A	N/A	N/A	N/A	N/A	L	N/A	CHy	Micropenis and cryptorchidism
16	LOW	LGMD-MR	18 m	1900	W	N	Y	U	N/A	N/A	L	N/A	NO MRI	RBBB on ECHO
17	LOW	MEB-FCMD	N	2000	NS	Y	Y	N/A	My	UL,LL	L	Y	CHy, H	Macroglossia
18	LOW	MEB-FCMD	I	780	NW	N	N	N/A	CG	N/A	L	N/A	BS,CC,WM,H	N/A
19	LOW	MEB-FCMD	P	1000	W	Y	Y	U	OA, My	N/A	L	N	WM,CC	SE, feeding difficulties
20	LOW	LGMD-NOMR	12 Yr	12000	R	N	Y	U	My	LL>UL	N	N		N/A
21	LOW	MEB-FCMD	N	1200	NONE	N	N	U	RD	N/A	L	N/A	H,WM,CC	SE, feeding difficulties.

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22	LOW	MEB-FCMD	12 m	2800	R	N	N/A	U	Pt, RA	N/A	L	N/A	CHy, CC, WM,H	Dyspraxia, feeding difficulties, SE
23	LOW	MEB-FCMD	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	H,CC,WM	N/A
24	N/A	WWS	N	1300	NS	N/A	N/A	U	N/A	N/A	L	N/A	CC,CHy, WM, H,Lis	N/A
25	LOW	WWS	P	5700	NONE	Y			RDy		L		H,WM,CHy, Lis	Feeding difficulties. RIP 8 weeks
26	LOW	CMD-NOMR	3 Yr	3200	S	N	N	U	U	G	N	N/A	WM-MILD	hypothyroid
27	N/A	MEB-FCMD	I	4000	S	N	Y	U	U	N/A	L	N/A	CC,WM,H	
28	LOW	WWS	N	7000	N/A	Y	Y	U	RD,Mo	N/A		N/A	WM,CHy, BS, H	Dysmorphic
29a	LOW	LGMD-NOMR	4 m	10000	W	N	Y	N/A	N/A	UL>LL	N	N	N/A	Steroid responsive
29b	LOW	LGMD-NOMR	4 m	13000	W	N	Y	U	U	LL>UL	N	N	Normal	Steroid responsive
30	LOW	LGMD-NOMR	10 m	60000	W	N	Y	U	U	LL>UL	N	N	H-MILD	Steroid responsive
31a	LOW	LGMD-NOMR	4 yr	9000	R	Y	N/A	U	N/A	N/A	N	N/A	N/A	N/A
32b	LOW	LGMD-NOMR	9 m	5700	R	Y	Y	U	N/A	LL>UL	N	N/A	Normal	CDH. Increased weakness with fever.

WWS = Walker–Warburg Syndrome; MEB/FCMD = Muscle-Eye-Brain/Fukuyama Congenital Muscular Dystrophy Like; CMD-MR = Congenital Muscular Dystrophy with Mental Retardation; CMD-NOMR = Congenital Muscular Dystrophy with No Mental Retardation; CMD-Cerebellar = Congenital Muscular Dystrophy with cerebellar Involvement; LGMD-MR = Limb Girdle Muscular Dystrophy with Mental Retardation; LGMD-NOMR = Limb Girdle Muscular Dystrophy with No Mental Retardation.
[a]P = prenatal onset; N = neonatal onset; I = infant onset; Yr = years; m = months. [b]W = walk; S = sit; St = stand; R = run; Prefix N = never. [c]Y = yes; N = no. [d]Y = yes; N = no. [e]RS = rigid spine; Sc = scoliosis; U = unaffected [f]CG = congenital glaucoma; RD = retinal detachment; RA = Retinal Atrophy; CC = Congenital cataracts; OA = optic atrophy; My = myopia; Mo = micropthalmia; Pt = ptosis; U = unaffected; Hm = hypermetropia; RDy = retinal dysplasia [g]UL = Upper limbs; LL = lower limbs; G = generalised [h]N = Normal intelligence; L = low [i]Y = yes; N = no [j]H = Hydrocephalus; CC = cerebellar cysts; BS = brainstem involvement; WM = white matter abnormality; CHy = cerebellar hypoplasia; Lis = lissencephaly; CDys = cerebellar dysplasia [k]SE = seizures; CDH = congenital dislocation of hip; RBBB = Right bundle branch block.

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this group were CMD with brain abnormality less severe than that seen with WWS. MRI findings include pachygyria with preferential fronto-parietal involvement, polymicrogyria, cerebellar hypoplasia, cerebellar dysplasia and frequent flattening of the pons and brainstem. Eye abnormalities are often seen and include congenital glaucoma, progressive myopia, retinal atrophy and juvenile cataracts. Individuals may, rarely, acquire the ability to walk although this is delayed. Rarely patients manage to learn a few spoken words. Thirty patients were assigned to this group, including one in whom the clinical information was limited.

(3) CMD-CRB (CMD with cerebellar involvement): This category included CMD with mental retardation and cerebellar involvement on MRI scan as the only structural abnormality. Cerebellar abnormalities may include cysts, as described relatively frequently in individuals with *FKRP* gene defects (Mercuri *et al*., 2006), or cerebellar hypoplasia or dysplasia. Four patients were assigned to this group.

(4) CMD-MR (CMD with mental retardation): CMD with mental retardation and structurally normal brain. Patients with isolated microcephaly or minor white matter changes on MRI are included in this group. Fifteen patients were assigned to this group, including two with limited clinical information.

(5) CMD-no MR (CMD with no mental retardation): Several patients within this group have had no neuroimaging but had entirely normal intellectual function. Ten patients were assigned to this group, one with limited information.

(6) LGMD-MR (LGMD with mental retardation): LGMD with mental retardation and structurally normal brain. Patients with minor white matter abnormalities and microcephaly were included in this group. This category includes patients with a phenotype resembling LGMD-2K (Balci *et al*., 2005). Five patients were assigned to this group.

(7) LGMD-no MR (LGMD with no mental retardation): LGMD with no mental retardation. This category includes the LGMD phenotypes resembling LGMD2I and 2L (Godfrey *et al*., 2006). Twenty patients were assigned to this group, six with limited clinical information.

The division of phenotypes within the cohort is shown in Table 4. Detailed clinical information is contained in Table 1 for those patients in whom pathogenic mutations were detected.

Mutation analysis

Mutation screening of *POMT1*, *POMT2*, *POMGnT1*, *fukutin* and *LARGE* was performed on 92 probands in whom *FKRP* mutations had been previously excluded. Homozygous and compound heterozygous mutations were detected in a total of 31 probands (34 individuals from 31 families). Thirty-seven different mutations were identified, 32 of which had not been previously reported. Pathogenic mutations are summarized in Table 2 and the comparative mutation frequencies between genes are represented in Table 4.

Without further RNA studies and functional biochemical analysis it is difficult to determine the pathogenicity of unclassified variants within these genes, this is exacerbated by the abundance of missense variants. For the purposes of this study, nonsense mutations, insertions and deletions, splice-site mutations as well as previously reported mutations were classified as pathogenic. Both exonic and intronic sequence alterations were categorized as polymorphisms if they were present on The Single Nucleotide Polymorphism database (http://www.ncbi.nlm.nih.gov), the Leiden database (http://www.dmd.nl) or present as an additional change in a patient with two proven pathogenic mutations (polymorphisms are available in supplementary information, Table 2). Amino acid substitutions were classified as pathogenic if they were detected in conjunction with a clearly pathogenic mutation or if they have been shown to segregate with disease in a large pedigree. In addition, two patients with homozygous missense mutations and one patient with compound heterozygous missense mutations have been included in Table 2 as they are non-conservative amino acid changes that affected an evolutionary conserved amino acid residue (Patient 16, Patient 18 and Patient 27). Patients in whom only a single-sequence alteration was detected are summarized in Table 3. We have been unable to determine whether these are rare polymorphisms or pathogenic alterations in patients who harbor a second undetectable mutation. These six patients have not been included in the 34% of patients detected with mutations. Patient 25 has been included in Tables 1, 2 and 4 despite the absence of a second detectable mutation due to the presence of a nonsense mutation.

A variety of mutation types were identified; 37 missense mutations; 7 nonsense mutations; 9 frameshift mutations; 1 insertion/deletion mutation; 1 deletion and 6 splice-site mutations. No mutation hotspots were identified. From a total of 37 mutations, 8 were found to be recurrent within the cohort. The p.Ala200Pro mutation in *POMT1*, previously described as prevalent within the Turkish population (Balci *et al*., 2005), was detected in three patients, two of Turkish origin and one of Greek decent (Patient 8). The *POMGnT1* donor splice-site mutation c.1539 + 1G>A found to account for the enrichment of MEB within the Finnish population was detected in two patients (Diesen *et al*., 2004). Three further novel mutations were detected more than once, specifically the p.Tyr666Cys mutation which was found both in the homozygous and heterozygous state in four patients. Segregation of this novel missense mutation was studied in a large pedigree and was found to

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Table 2 A summary of pathogenic mutations detected in this study

Patient	Gene	Exon/intron	Nucleotide change	Predicted amino acid change	Mutation type	Reference
1	*POMT1*	20	c.2179.2180delTC	p.Ser727fs	Frameshift	Novel
	POMT1	20	c.2179.2180delTC	p.Ser727fs	Frameshift	Novel
2	*POMT1*	20	c.2179.2180delTC	p.Ser727fs	Frameshift	Novel
	POMT1	20	c.2179.2180delTC	p.Ser727fs	Frameshift	Novel
3	*POMT1*	7	c.598G>C	p.Ala200Pro	Missense	Leiden database
	POMT1	7	c.598G>C	p.Ala200Pro	Missense	Leiden database
4	*POMT1*	18	c.1847.1849delGGT	p.Trp616del	Deletion	Novel
	POMT1	3	c.193G>A	p.Gly65Arg	Missene	Leiden database
5	*POMT1*	11	c.1081C>T	p.Gln361X	Nonsense	Novel
	POMT1	19	c.2005G>A	p.Ala669Thr	Missene	Novel
6	*POMT1*	6	c.517.523delTTCTTCAinsG	p.Phe173.Asn175delinsAsp	Insertion/deletion	Novel
	POMT1	18	c.1868G>C	p.Arg623Thr	Missene	Novel
7	*POMT1*	7	c.598G>C	p.Ala200Pro	Missense	Leiden database
	POMT1	7	c.598G>C	p.Ala200Pro	Missense	Leiden database
8	*POMT1*	5	c.427G>T	p.Glu143X	Nonsense	Novel
	POMT1	7	c.598G>C	p.Ala200Pro	Missene	Leiden database
9	*POMT2*	21	c.2150T>C	p.Phe717Ser	Missense	Novel [a]
	POMT2	21	c.2177G>A	p.Gly726Glu	Missene	Leiden database
10	*POMT2*	19	c.1997A>G	p.Tyr666Cys	Missense	Novel
	POMT2	19	c.1997A>G	p.Tyr666Cys	Missense	Novel
11	*POMT2*	19	c.1997A>G	p.Tyr666Cys	Missense	Novel
	POMT2	11	c.1238G>C	p.Arg413Pro	Missene	Novel
12	*POMT2*	20	c.2047A>C	p.Thr683Pro	Missense	Novel
	POMT2	9	c.1051delG	p.Ala351fs	Framshift	Novel
13	*POMT2*	5	c.593T>A	p.Ile198Asn	Missense	Novel
	POMT2	19	c.1997A>G	p.Tyr666Cys	Missene	Novel
14	*POMT2*	10	c.1117G>T	p.Val373Phe	Missense	Novel
	POMT2	5	c.593T>A	p.Ile198Asn	Missense	Novel
15a, 15b [b]	*POMT2*	19	c.1997A>G	p.Tyr666Cys	Missense	Novel
	POMT2	19	c.1997A>G	p.Tyr666Cys	Missense	Novel
16	*POMT2*	5	c.551C>T	p.Thr184Met	Missense	Novel
	POMT2	21	c.2243G>C	p.Trp748Ser	Missene	Novel
17	*POMT2*	9	c.1057G>A	p.Gly353Ser	Missense	Novel [1]
	POMT2	21	c.2177G>A	p.Gly726Glu	Missene	Novel [1]
18	*POMGnT1*	6	c.526A>C	p.Thr176Pro	Missense	Novel
	POMGnT1	6	c.526A>C	p.Thr176Pro	Missense	Novel
19	*POMGnT1*	7	c.652+1G>A	Donor splice site	Splice site	Novel
	POMGnT1	17	c.1469G>A	p.Cys490Tyr	Missene	Leiden database
20 [b]	*POMGnT1*	20	c.1666G>A	p.Asp556Asn	Missense	Novel [2]
	POMGnT1	20	c.1666G>A	p.Asp556Asn	Missense	Novel [2]
21	*POMGnT1*	17	c.1539+1G>A	Donor splice site	Splice site	Leiden database
	POMGnT1	17	c.1539+1G>A	Donor splice site	splice site	Leiden database
22	*POMGnT1*	12	c.1100G>A	p.Arg367His	Missense	Novel
	POMGnT1	17	c.1539+1G>A	Donor splice site	Splice site	Leiden database
23	*POMGnT1*	20	c.1785+2T>G	Donor splice site	Splice site	Novel
	POMGnT1	20	c.1785+2T>G	Donor splice site	Splice site	Novel
24	*POMGnT1*	17	C1425G>A	p.Trp475X	Nonsense	Novel
	POMGnT1	17	C1425G>A	p.Trp475X	Nonsense	Novel
25	*LARGE*	13	c.1548C>G	p.Trp516X	Nonsense	Novel
26	*fukutin*	8	c.920G>A	p.Arg307Gln	Missense	Leiden database
	fukutin	8	c.920G>A	p.Arg307Gln	Missense	Leiden database
27	*fukutin*	8	c.915G>A	p.Trp305Cys	Missense	Novel
	fukutin	8	c.915G>A	p.Trp305Cys	Missense	Novel
28	*fukutin*	8	c.919C>T	p.Arg307X	Nonsense	Novel
	fukutin	8	c.919C>T	p.Arg307X	Nonsense	Novel
29a, 29b	*fukutin*	8	c.920G>A	p.Arg307Gln	Missense	Novel [3]

(continued)

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Table 2 Continued

Patient	Gene	Exon/intron	Nucleotide change	Predicted amino acid change	Mutation type	Reference
	fukutin	9	c.1167dupA	p.Phe390fs	Frameshift	Leiden database[3]
30	fukutin	9	c.1167dupA	p.Phe390fs	Frameshift	Leiden database[3]
	fukutin	10	c.1363delG	p.Asp455fs	Frameshit	Novel[3]
31a, 31b	fukutin	4	c.340G>A	p.Ala114Thr	Missense	Novel
	fukutin	7	c.859delA	p.Thr286fs	Frameshift	Novel

Probands are numbered, affected siblings are indicated with letters.
[a]*de novo* mutation. [b]Family studies carried out to investigate segregation of the variant through the pedigree.
The following patients were included in this cohort and have recently been reported individually; [1]Patients previously described in Mercuri *et al*., 2006. [2]Patient described individually in Clement *et al*., 2007, *Archives of Neurology* in press. [3]Patients previously described in Godfrey *et al*., 2006.

Table 3 Summary of unclassified variants

Patient	Gene	Exon/intron	Nucleotide change	Predicted amino acid change	Mutation type	Reference
32	*POMT1*	9	c.905T>G	p.Phe302Cys	missense	Novel
33	*POMT1*	19	c.1922C>T	p.Ala641Val	missense	Novel
34	*POMT1*	20	c.2203C>T	p.Arg735Cys	missense	Novel
35	*POMT1*	20	c.2244+5A>G	intronic	intronic	Novel
	POMT1	20	c.2244+5A>G	intronic	intronic	Novel
36	*POMT1*	20	c.2246G>A	synonymous	synonymous	Novel
37,38,39	*POMGnT1*	21	c.1867A>G	p.Met623Val	missense	Novel
39	*LARGE*	4	c.309C>A	synonymous	synonymous	Novel
40	*LARGE*	12	c.1431C>T	synonymous	synonymous	Novel
41	*LARGE*	13	c.1640G>A	p.Arg547His	missense	Novel
42,43	*LARGE*	14	c.1827A>T	synonymous	synonymous	Novel

Table 4 The phenotypic distribution of patients within the cohort, the frequency of mutations in each of the five glycosyltransferase genes analysed and the comparative mutation frequencies for individual clinical categories

	Number of patients							
	WWS	MEB FCMD	CMD CRB	CMD MR	CMD no MR	LGMD MR	LGMD no MR	Total
POMT1	1	1	–	3	–	3	–	8
POMT2	–	6	2	–	–	1	–	9
POMGnT1	–	6	–	–	–	–	1	7
fukutin	1	1	–	–	1	–	3	6
LARGE	1	–	–	–	–	–	–	1
Mutation detected	3 (60%)	14 (47%)	2 (50%)	3 (20%)	1 (10%)	4 (80%)	4 (20%)	31 (34%)
Total	5	30	4	15	10	5	20	92[a]

WWS = Walker–Warburg syndrome; MEB/FCMD = muscle eye brain syndrome/Fukuyama congenital muscular dystrophy; CMD CRB = congenital muscular dystrophy with cerebellar involvement; CMD-MR = congenital muscular dystrophy with mental retardation; CMD-no MR = congenital muscular dystrophy with no mental retardation; LGMD-MR = limb girdle muscular dystrophy with mental retardation; LGMD-no MR = limb girdle muscular dystrophy with no mental retardation.
[a]Includes three patients not assigned a clinical classification due to insufficient clinical information.

segregate with the disease (Patient 15). Parental samples were studied for 11 probands to ensure that compound heterozygous mutations were in *trans* and that apparent homozygous mutations in the proband were not masking undetected deletions. Where parental DNA was tested (22 families in total) a single paternal mutation was found to occur *de novo* (p.Phe117Ser, *POMT2*). A relatively similar frequency of patients with mutations were detected

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in *POMT1*, *POMT2*, *POMGnT1* and *fukutin* (Table 4). In contrast, only a single patient was found to have a pathogenic mutation in *LARGE* although we were unable to identify a second mutation (Patient 25).

Genotype–phenotype correlations

The spectrum of phenotypes associated with mutations in *POMT1* included WWS (one case), MEB-FCMD (one case), CMD-MR (three cases) and LGMD-MR (three cases). *POMT2* mutations were observed in patients with MEB-FCMD (six cases), CMD-CRB (two cases) and LGMD-MR. Six patients with *POMGnT1* mutations had WWS and a single case had LGMD-no MR. Phenotypes associated with mutations in *fukutin* were detected in patients with WWS (one case), MEB-FCMD (one case), CMD-no MR (one case) and LGMD-no MR (three cases). A mutation in *LARGE* was detected in a single patient with WWS.

Although α-DG immunostaining was not systematically quantified as part of this study, we noticed a broad correlation between the amount of depleted glycosylated epitope and phenotypic severity. For example the WWS patient found to have a mutation in *LARGE* had complete absence in immunostaining, while the previously reported milder case of MDC1D had only a reduction in the amount of immunofluorescence (Longman *et al*., 2003). Similarly the *POMGnT1* patient with LGMD-no MR (Patient 20) had only a subtle deficiency in immunofluorescence (Clement *et al*., 2007, *Archives of Neurology*, in press), in contrast to the virtually absent expression in patients with MEB-FCMD.

There was no clear difference in phenotype or pattern of dystroglycan expression between patients with and without mutations in any of these genes. The phenotypic spectrum of patients without identifiable mutations was similar to that of patients with mutations.

Discussion

Dystroglycanopathies are a recently defined, common group of muscular dystrophies encompassing an extremely wide spectrum of clinical severity and are caused by mutations in at least sex genes encoding putative or demonstrated glycosyltransferases. The comparatively small coding region of *FKRP* has facilitated the rapid correlation between genotype and phenotype, allowing the discovery of pathogenic mutations in patients with LGMD2I, MDC1C, MEB-like and WWS-like disorders. However, there is no information regarding the frequency of involvement or the genotype–phenotype relationships for the remaining five glycosyltransferase genes in a large and unbiased population.

In this study we have systematically screened for mutations in *POMT1*, *POMT2*, *POMGnT1*, *fukutin* and *LARGE* in patients in whom we had previously ruled out *FKRP* gene involvement. Mutations were detected in 34% of these patients.

Fukutin mutations

Mutations in *fukutin*, typically associated with FCMD in Japan were found in six patients, none of whom are of Japanese origin. Only two of these patients had structural brain involvement; one patient affected by WWS (Patient 28) and one by a MEB-FCMD phenotype (Patient 27). The remaining patients had no structural brain involvement; one case had CMD-no MR (Patient 26) and never acquired the ability to walk but has normal IQ and five individuals from three families have entirely normal intellect and a mild LGMD phenotype (LGMD2L) (Patients 29, 30 and 31). Interestingly in the latter two of these families, a dramatic response to steroid therapy was noted (Godfrey *et al*., 2006). In striking contrast to what has previously been reported in FCMD, none of these five patients have evidence of central nervous system involvement. Our findings together with the recent description of individuals with *fukutin* mutations presenting with a predominant cardiomyopathy (Murakami *et al*., 2006), suggest that the majority of mutations outside Japan give rise to conditions milder than FCMD and are not usually associated with structural brain involvement.

POMGnT1 mutations

Mutations in *POMGnT1* were also associated with a wider than reported spectrum of clinical severity, which include a relatively mild form of LGMD. The majority of patients (6) had an MEB like disorder with only a single patient possessing a LGMD phenotype, suggesting that *POMGnT1* mutations more frequently give rise to congenital disorders with associated structural brain involvement. The LGMD patient (Patient 20) has entirely normal intellectual function and disease onset in the second decade of life, dramatically expanding the phenotypes associated with mutations in *POMGnT1* (Clement *et al*., 2007, *Archives of Neurology*, in press).

POMT1 mutations

Mutations in *POMT1* have previously been reported in patients with WWS, CMD-MR and LGMD-MR (LGMD-2K). Within our cohort, all patients with mutations in *POMT1* had evidence of functional brain involvement either with no clear associated structural brain abnormalities (three patients with LGMD2K, and three patients with CMD-MR), or more severe conditions with structural brain defects (one patient with WWS, and one individual with a MEB-like phenotype). This suggests that the majority, if not all patients with *POMT1* mutations have either functional or structural central nervous system involvement, including those patients with relatively mild muscle weakness. This is in contrast to patients in the present study with mutations in *fukutin* and *POMGnT1* and to that previously reported for *FKRP* mutations.

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POMT2 mutations

Mutations in *POMT2* were confined to patients with evidence of brain involvement. Nine patients had pathogenic *POMT2* mutations; six with a MEB-FCMD phenotype, two with a CMD-cerebellar phenotype and a single patient with LGMD-MR. This latter patient has learning difficulties and remains ambulant at age 20 having presented, at 18 months of age, with developmental delay (Patient 16). These findings indicate that like *POMT1*, the majority or all patients with mutations in *POMT2* have evidence of central nervous system involvement. In addition, we have identified the mildest phenotype associated with mutations in *POMT2* reported to date in an individual with LGMD-MR.

LARGE mutations

We were only able to identify a single pathogenic *LARGE* mutation in a patient with typical WWS phenotype who died in the first few months of life (Patient 25). Absent immunofluorescence staining was demonstrated on this patient's skeletal muscle biopsy using antibodies which recognise the glycosylated epitope of α-DG. Unfortunately neither sufficient DNA nor frozen muscle from this patient was available to investigate the presence of a second, as yet undetected, mutation. However, it remains possible that this mutation contributed to the patient's phenotype.

Mutation frequencies

Mutations in *POMT2* were the most prevalent with nine cases, followed by *POMT1* with eight cases, *POMGnT1* with seven cases, *fukutin* with six cases and finally *LARGE* with only a single case.

We have previously identified *FKRP* mutations in 79 patients. Approximately 75% of these patients have a LGMD2I phenotype (Brockington *et al*., 2001*a*; Topaloglu *et al*., 2003; Beltran-Valero de Bernabe *et al*., 2004; Mercuri *et al*., 2006). The relative frequency of *FKRP* involvement needs to be considered with caution as it clearly reflects the genetic origin of patients studied in our unit. For example screening of 79 Australian LGMD patients detected only two *FKRP* mutations. However, when amalgamating these results, it remains clear that *FKRP* mutations are the most frequently found mutations in this group of conditions. Both ourselves and others have previously published extensively on the spectrum of these mutations (Brockington *et al*., 2001*a*, *b*; Mercuri *et al*., 2003; Topaloglu *et al*., 2003; Harel *et al*., 2004; Mercuri *et al*., 2006; Vieira *et al*., 2006; Lin *et al*., 2007).

Genotype–phenotype correlations

Pathogenic mutations were detected in 3 of 5 patients with WWS syndrome (60%), 14 of 30 patients with a MEB/FCMD phenotype (47%), 2 of 4 patients with CMD CRB (50%), 3 of 15 patients with CMD-MR (20%), 1 of 10 patients with CMD-no MR (10%), 4 of 5 patients with LGMD-MR (80%) and 4 of 20 patients with LGMD-no MR (20%) (Table 4).

Patients with associated structural brain defects belonging to the severe end of the clinical spectrum showed no apparent difference in their pattern of skeletal muscle weakness or central nervous system involvement in relation to the gene involved. However, the four LGMD patients with associated MR and microcephaly all had mutations in either *POMT1* or *POMT2*. No mutations were identified in the remaining patients. Conversely a number of patients with more severe muscle weakness and no brain involvement (CMD-no MR) were found to have mutations in *fukutin*, similar to that previously described in MDC1C (Brockington *et al*., 2001*a*). This suggests that there may be a hierarchical involvement of muscle and brain arising from individual gene mutations, with *POMT1* and *POMT2* being associated with significant central nervous system involvement even in patients with relatively mild weakness and who remain ambulant (LGMD2K). This does not appear to be a feature of *fukutin* or *FKRP*. These results suggest that in some individual subcategories, certain genes are more likely to be involved than others and this should be taken into account when undertaking mutation analysis in the dystroglycanopathies.

The results of this study demonstrate that the phenotypic spectrum of disorders associated with mutations in the six known glycosyltransferase genes is significantly wider than initially suspected, in part due to the high prevalence of founder mutations within specific populations (Kobayashi *et al*., 1998; Diesen *et al*., 2004). We have expanded the clinical phenotypes associated with mutations in *POMT1*, *POMT2*, *POMGnT1*, *fukutin* and *LARGE*, although we have not observed a full spectrum of phenotypes associated with each gene, in particular *POMT1*, *POMT2* and *LARGE*. A large number of patients with clinico-pathological features indistinguishable from the ones detailed in this manuscript were not found to have mutations in any of the genes studied. Finally, this work suggests that more, as yet undefined, genes are likely to be involved in the pathogenesis of the dystroglycanopathies. The identification of these genes may provide additional information on the pathway of glycosylation of α-dystroglycan.

Supplementary material

Supplementary material is available at *Brain* online.

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