Muscular dystrophies due to defective glycosylation of dystroglycan

F. Muntoni1, M. Brockington1, C. Godfrey1, M. Ackroyd1, S. Robb1, A. Manzur1, M. Kinali1, E. Mercuri1, M. Kaluarachchi1, L. Feng1, C. Jimenez-Mallebrera1, E. Clement1, S. Torelli1, C.A. Sewry1,2, S.C. Brown1

1 Dubowitz Neuromuscular Centre, Department of Paediatrics, Imperial College Healthcare NHS Trust, Hammersmith Hospital, Du Cane Road, London, UK; 2 Wolfson Centre for Inherited Neuromuscular Diseases, Robert Jones & Agnes Hunt Orthopaedic Hospital, Oswestry, UK

Muscular dystrophies are a clinically and genetically heterogeneous group of disorders. Until recently most of the proteins associated with muscular dystrophies were believed to be proteins of the sarcolemma associated with reinforcing the plasma membrane or in facilitating its re-sealing following injury. In the last few years a novel and frequent pathogenic mechanism has been identified that involves the abnormal glycosylation of alpha-dystroglycan (ADG). This peripheral membrane protein undergoes complex and crucial glycosylation steps that enable it to interact with LG domain containing extracellular matrix proteins such as laminins, agrin and perlecan.

Mutations in six genes (POMT1, POMT2, POMGnT1, fukutin, FKRP and LARGE) have been identified in patients with reduced glycosylation of ADG. While initially a clear correlation between gene defect and phenotype was observed for each of these 6 genes (for example, Walker Warburg syndrome was associated with mutations in POMT1 and POMT2, Fukuyama congenital muscular dystrophy associated with fukutin mutations, and Muscle Eye Brain disease associated with POMGnT1 mutations), we have recently demonstrated that allelic mutations in each of these 6 genes can result in a much wider spectrum of clinical conditions. Thus, the crucial aspect in determining the phenotypic severity is not which gene is primarily mutated, but how severely the mutation affects the glycosylation of ADG.

Systematic mutation analysis of these 6 glycosyltransferases in patients with a dystroglycan glycosylation disorder identifies mutations in approximately 65% suggesting that more genes have yet to be identified.

Key words: Muscular dystrophy, glycosylation, alpha dystroglycan, neuronal migration, glycosyltransferases

Introduction

A significant number of muscular dystrophies (MD) are secondary to mutations in proteins located in the extracellular matrix, sarcolemma or nuclear envelope. Most of these proteins play a major structural role in muscle fibres although some have also been implicated in signaling or in chromatin binding and organization. However, recently it has become evident that a number of forms of congenital muscular dystrophy (CMD) and several variants of limb girdle muscular dystrophy (LGMD) are associated with mutations in a number of genes encoding for proteins that are either putative or demonstrated glycosyltransferases (1-5). These include four severe forms of CMD that are associated with severe structural brain involvement and variable associated eye abnormalities: Walker-Warburg syndrome (WWS), Muscle-Eye-Brain disease (MEB), Fukuyama congenital muscular dystrophy (FCMD) and congenital muscular dystrophy type 1D (MDC1D). The CMD variant MDC1C and a relatively mild form of limb girdle muscular dystrophy (LGMD2I) are not typically associated with brain involvement. A characteristic and diagnostic feature of these MD variants is their association with abnormalities in the glycosylation of α-dystroglycan (ADG), and this has led to the suggestion of the name “dystroglycanopathy” to identify them (6-10). The abnormal glycosylation of ADG was only described in 2001 but is now a recognized common pathogenetic mechanism responsible for several forms of muscular dystrophy. Mutations in 6 genes have been identified in patients with dystroglycanopathies, initially each associated with a specific clinical entity. However it is now clear that allelic mutations in each of these 6 genes are responsible for an extremely wide spectrum of clinical conditions; in addition thorough genetic analysis of these 6 genes in patients with a dystroglycanopathy only identifies mutations in ~ 65% of cases suggesting that further genetic heterogeneity exists.
Glycosylation defects and muscular dystrophies

There are two main forms of protein glycosylation: N-linked glycosylation in which the oligosaccharide is attached to the amide group of an asparagine residue and O-linked glycosylation where the oligosaccharide is attached to a hydroxyl group of a serine or threonine residue. O-mannosylation is a very rare form of glycosylation and in humans only ADG has so far been shown to contain these modified glycans (1, 11-16). ADG is a very heavily glycosylated glycoprotein: while its primary sequence predicts a molecular mass of 72 kDa, its molecular mass in mammalian skeletal and cardiac muscle is 156 kDa and 140 kDa respectively and in brain and peripheral nerve 120 kDa. Although O-mannosylation does not represent the only form of O-glycosylation on ADG, it is required for binding to a number of LG domain containing extracellular matrix proteins such as laminin, perlecans and agrin in muscle, and neurexin in the brain (17).

To date, mutations in 6 known or putative glycosyltransferase genes have been identified in dystroglycanopathies. Protein-O-mannosyl transferase 1 (POMT1; OMIM 607423), Protein-O-mannosyl transferase 2 (POMT2; OMIM 607439), Protein-O-mannose 1,2-N-acetylgalcosaminyltransferase 1 (POMGnT1; OMIM 606822), fukutin (OMIM 607440), Fukutin-related protein (FKRP; OMIM 606596) and LARGE (OMIM 603590) (18-23). Three of these genes are clearly involved in the process of O-mannosylation (POMT1, POMT2, POMGnT1) (20, 24, 25), while the function of the remaining 3 genes, fukutin, FKRP and LARGE is still not clear (26-29). Of these 6 genes, the most frequently mutated in the Caucasian population is FKRP. While this was the first gene to be associated with an extremely wide range of clinical severity, more recent data suggests that this is also a common theme for mutations in other genes.

The FKRP gene

Our group originally described mutations in the fukutin-related protein gene (FKRP) in patients with a form of CMD (MDC1C) characterized by onset at birth or in the first few months of life with profound weakness, markedly elevated serum CK and inability to achieve independent ambulation or standing (22). Intelligence was preserved and brain imaging normal. These patients had a significant reduction of the glycosylation of ADG both on immunocytochemistry and Western blot analysis (22). Shortly after, our group also identified involvement of the FKRP gene in a much milder variant of limb girdle muscular dystrophy, LGMD2I, which had already been mapped to chromosome 19q13 where the FKRP gene lies (30). In contrast with MDC1C, the onset of the condition in LGMD2I varied from childhood to late adult life; typical patients with LGMD2I have a hypertrophic phenotype and a proximal distribution of weakness, limited or no contractures, markedly elevated serum CK and frequent cardiac complications (30-32). Both intelligence and brain imaging are entirely normal.

Surprisingly, this form of LGMD was subsequently found to be the most common LGMD variant in the UK population, due to the high frequency of a C826A mutation, with an estimated heterozygote frequency of ~1:400 (32). This particular mutation was also found at high frequency in other Caucasian populations, such as in Germany (33) and Scandinavian countries (34), while it was less common in Italians, and even less common in LGMD patients from Brazil (27, 35) and Japan. The expression of glycosylated ADG was only moderately reduced in LGMD2I, in keeping with the less severe muscle involvement compared to children with MDC1C (28).

Subsequent studies clarified that the originally described MDC1C phenotype did not represent the most severe end of the clinical spectrum, as we then identified FKRP mutations in patients with a CMD variant resembling MDC1C but with additional features such as mental retardation and cerebellar dysplasia and cysts on brain MRI (36), followed by the identification of mutations in patients with severe supratentorial cortical dysplasia and structural eye involvement, mimicking classical Muscle-Eye Brain disease (MEB) and Walker Warburg syndrome (WWWS) (37). The severity of loss of ADG glycosylation in these patients was more severe than previously found in MDC1C, in keeping with their more severe clinical phenotype. None of the FKRP patients described was ever found to be homozygous for nonsense mutations and this led to the speculation that the homozygosity for FKRP null alleles might be incompatible with life. Despite the frequency of the involvement of this gene and the observation that ADG hypoglycosylation is associated with these forms of muscular dystrophy, there is no clear idea of the precise role of FKRP. Several studies have localized recombinant FKRP proteins to the Golgi apparatus of cultured cells (38-40), and more recent studies have noted an association of FKRP with the dystroglycan complex in skeletal muscle (41). While some authors have described mislocalisation of mutant proteins in transfected cells (39, 42), we have not confirmed these findings in our experiments (38, 43), suggesting that the pathogenesis of this condition is due to impaired function rather than altered localisation within the cell. In order to further evaluate this aspect our group has recently generated an animal model with reduced FKRP expression that recapitulates the severe brain and eye involvements observed in patients with MEB. These mice also have a very marked reduction of glycosylated ADG in their skeletal muscle. The detailed characterization of the phenotype of this animal model is currently being undertaken.
The **POMT1** and **POMT2** genes

Mutations in the *O-*mannosyltransferase 1 (*POMT1*) were originally described in a proportion (20%) of patients affected by the severe condition Walker Warburg syndrome (20). POMT1 catalyses in combination with POMT2 the first step in *O-*mannosyl glycan synthesis (44); as ADG is so far the only protein in which this type of glycosylation has been demonstrated, the finding of its abnormal processing in patients with *POMT1* mutations is not a surprise. A few years after the identification of *POMT1* mutations in WWS, mutations in *POMT2* were also identified in a subgroup of patients with WWS (19). Both conditions are characterized by a very severe depletion of ADG recognized by an antibody which identifies a glycosylated epitope, but also a marked reduction of the epitope recognized by an antibody raised to the core ADG originally produced in the laboratory of Kroger (45), though not by an anti-core ADG antibody produced in the laboratory of Campbell (46). These observations indicate that ADG may not be completely absent but rather abnormally glycosylated thus exposing different epitopes. Markedly reduced expression of glycosylated ADG in peripheral nerve has also been documented in WWS patients with a *POMT1* mutation (47).

More recent studies have indicated a wider spectrum of clinical and pathological features for mutations in both *POMT1* and *POMT2* genes than originally reported (48, 49). Allelic mutations in the *POMT1* gene have recently been described in ambulant patients with a phenotype resembling LGMD, but with associated microcephaly and mental retardation, despite apparently normal brain scan (LGMD2K) (50). Milder allelic *POMT2* mutations have also been recently reported in patients with milder features compared to WWS, ranging from MEB-like phenotypes to ambulant patients with microcephaly and mental retardation, with features indistinguishable from LGMD2K (51, 52).

POMT1/2 enzymatic activity can be measured in both fibroblasts and lymphocytes, and this has been exploited as a diagnostic test in patients with mutations in these 2 genes. It has also been proposed that the finding of reduced POMT enzymatic activity could be used as a more rapid form of patient screening (53).

Targeted inactivation of *Pomt1* has been achieved in mice but is embryonic lethal in the homozygous mice due to defects in the formation of Reichert’s membrane, the first basement membrane to form in the mouse embryo which requires normally glycosylated dystroglycan (54).

The **POMGnT1** gene

The *POMGnT1* gene encodes protein *O-*linked mannose B1,2-N-acetylglucosaminyltransferase 1, an enzyme involved in the transfer of an *N*-acetylglucosamine residue to an *O-*linked mannose (25). Mutations in *POMGnT1* were originally identified in patients affected by typical MEB, a condition characterized by CMD together with cortical dysplasia and ocular involvement (20). Following the original demonstration of *POMGnT1* mutations in the Finnish and Turkish MEB patient population, collaborative papers highlighted the spectrum of phenotypes in a world wide study, which ranged from typical MEB to more severe patients with structural brain involvement overlapping WWS. Mutations located at the 5’ end of the gene were on the whole associated with a more severe phenotype than those located at the 3’ end of the gene (55). *POMGnT1* enzymatic activity can also be measured from frozen muscle, lymphocytes and fibroblasts (56).

Regarding the spectrum of clinical severity in patients with *POMGnT1* mutations, our group very recently identified a patient with a form of LGMD with onset in the second decade of life, with entirely normal intelligence, who followed a relatively rapid progression of muscle weakness, becoming wheelchair dependant aged 19 following a fracture. Enzymatic studies on the patient’s fibroblasts showed an altered kinetic profile, however this was less marked than in patients with MEB, and in keeping with the patient’s relatively mild phenotype. This patient therefore is the mildest patient with *POMGnT1* mutations reported so far and her phenotype is that of a LGMD with proximal muscle weakness and markedly elevated serum CK (Clement et al., manuscript in press).

An animal model of MEB due to mutations in *POMGnT1* exists, following the introduction of null alleles in this gene. These mutant mice were viable with multiple developmental defects in muscle, eye, and brain, similar to the phenotypes observed in human MEB disease (57, 58).

The **Fukutin** gene

Fukuyama-type congenital muscular dystrophy (FCMD) is a condition confined to Japan. Similar to MEB and WWS, it is characterized by the combination of CMD and central nervous system involvement. On the whole the severity of brain involvement is milder than WWS and MEB, and the eyes are only occasionally severely affected. A high proportion (87%) of FCMD patients carry a retrotransposon insertion into the 3’ untranslated region of the *fukutin* gene which leads to a reduction in *fukutin* mRNA levels (21). This ancestral mutation explains the high prevalence of the disease in Japan where it represents the second most common form of muscular dystrophy after Duchenne. Patients homoygous for this mutation are relatively mild compared to those with compound heterozygosity between the ancestral mutation and a more severe loss-of-function mutation.

Inactivation of the *fukutin* gene in mice leads to lethality at embryonic day 6.5-7.5 (59), hence mice chimeric for normal and fukutin deficient cells have been generated (60). Those with a high proportion of fukutin
deficient cells show a typical muscular dystrophy with reduced survival and a marked disorganisation of the laminar structures of both the cerebral and cerebellar cortices with pathological features of a cobblestone lissencephaly, and eye abnormalities.

Several recent reports have significantly increased the spectrum of conditions due to fukutin mutations, mostly due to patients outside Japan, without the retrotransposonal insertion in the fukutin gene (61). Initially patients resembling WWS with homozygous null alleles of the fukutin gene were identified. These patients had a more profound depletion of ADG immunolabelling compared to typical FCMD patients. More recently the spectrum has been expanded towards milder patients: we recently described three children with CMD and no structural brain involvement, and in addition three families with a LGMD-like condition, with onset in the first few years of life, without any evidence of central nervous system involvement (62). In two of these families with LGMD the affected children where initially considered to have an inflammatory myopathy, and were administered corticosteroids which resulted in a significant clinical improvement. Even milder patients have recently been described carrying intragenic fukutin mutations: six patients were identified with dilated cardiomyopathy with the absence of, or minimal limb girdle muscle involvement and normal intelligence (63).

The LARGE gene

Mutations in the LARGE gene were originally identified in the myodystrophy mouse (myd; now renamed Large<sup>W</sup>), a spontaneous model of CMD (64). Several detailed studies of the skeletal and cardiac muscles, eyes and central nervous system involvement of these mice have been reported (65-68). These mice also show a profound deficiency of glycosylated ADG. We originally reported mutations in the LARGE gene in a single family, where the propositus was affected by congenital onset of weakness, profound mental retardation, white matter changes and subtle structural abnormalities on brain MRI; this novel condition was named MDC1D (23). A moderate reduction of glycosylated ADG was identified. More recently, allelic mutations in LARGE were identified in a patient with a severe form of CMD resembling WWS (69). This patient had an out of frame intragenic deletion, leading to a complete loss of LARGE function, and thereby explaining the milder phenotype of the previously described patient with missense mutations. We also recently identified a patient with a WWS-like phenotype, who carried a nonsense mutation at the heterozygous state. These results therefore identify LARGE as a gene rarely involved in CMD, with severity ranging from MDC1D to WWS.

### Table 1. The phenotypic distribution of patients within a cohort of 92 dystroglycanopathy patients. 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.

<table>
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<th>WWS</th>
<th>MEB</th>
<th>CMD</th>
<th>CMD</th>
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<th>LGMD</th>
<th>LGMD</th>
<th>Total</th>
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<td>3</td>
<td></td>
<td>3</td>
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<td>8</td>
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<td></td>
<td>1</td>
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<tr>
<td>fukutin</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td>3</td>
<td></td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>LARGE</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
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<td>2 (50%)</td>
<td>3 (20%)</td>
<td>1 (10%)</td>
<td>4 (80%)</td>
<td>4 (20%)</td>
<td>31 (34%)</td>
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<td>4</td>
<td>15</td>
<td>10</td>
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terion was the evidence of a dystroglycanopathy. Ninety two probands were screened for these 5 genes (FKRP had been previously excluded) irrespective of their age, ethnicity and clinical features, including the presence or absence of central nervous system involvement. We identified mutations in a total of 31 probands (34 individuals from 31 families); 37 different mutations were identified, of which 32 were novel. Concerning the frequency of involvement of individual gene defects in this cohort, mutations in POMT2 were the most prevalent with 9 cases, followed by POMT1 with 8 cases, POMGnT1 with 7 cases, fukutin with 6 cases and finally LARGE with only a single case (Table 1).

Regarding phenotype/genotype correlations for POMT1, POMT2, POMGnT1, fukutin and LARGE, we detected pathogenic mutations in 3 of 5 patients with WWS syndrome, 14 of 30 patients with a MEB/FCMD phenotype, in 2 of 4 patients with CMD and cerebellar involvement, 3 of 15 patients with CMD-mental retardation but otherwise structurally normal brain, 1 of 10 patients with CMD and absent mental retardation (resembling what has been previously described in MDC1C), 4 of 6 patients with LGMD and associated reduced IQ (resembling what has been previously described for LGMD2K), and 4 of 23 patients with LGMD and no mental retardation or other evidence of central nervous system involvement. In most instances there was no apparent difference in the pattern of skeletal muscle weakness or central nervous system involvement in patients with associated structural brain defects belonging to the severe end of the clinical spectrum such as WWS. However, regarding the LGMD subgroups with mental retardation and microcephaly (ie. LGMD2K and similar phenotypes), we found this specific phenotype only in patients with mutations either in POMT1 or POMT2 (70). On the other hand, we identified a number of patients with considerably more severe muscle weakness than LGMD2K, clinically resembling MDC1C (i.e. non ambulant children), with absent brain involvement, due to mutations in fukutin. This suggests that while involvement of any of these genes can give rise to a very wide spectrum of clinical syndromes with overlapping features, there might be at the same time subtle differences in the involvement of brain and muscle secondary to specific gene mutations. POMT1 and POMT2 are apparently associated with more severe central nervous system involvement even in patients with relatively mild weakness who remain ambulant (LGMD2K) whereas this phenotype has so far not been observed for POMGnT1, LARGE, fukutin or FKRP. These results may therefore allow the targeting of specific gene defects in individual subcategories of patients with dystroglycanopathies.

The results also suggest that the original descriptions of several “core phenotypes” associated with each of these genes is related to the high prevalence of founder mutations within specific populations, such as the “Finnish” POMGnT1 mutation in MEB disease, and the “Japanese” fukutin mutation responsible for FCMD, and not to the fact that mutations in these genes are not capable of inducing different conditions.

These observations therefore expand the clinical phenotypes associated with mutations in POMT1, POMT2, POMGnT1, fukutin and LARGE, and provide an indication of the relative frequency of their involvement in Caucasian patients with a dystroglycanopathy. Adding together the patients recently studied for mutations in POMT1, POMT2, POMGnT1, fukutin and LARGE, and those in whom we have previously identified FKRP mutations (77 cases, Muntoni et al, personal observation) we have been able to identify causative mutations in approximately 65% of patients with a dystroglycanopathy. This means that a significant number of patients did not have mutations in any of the genes we know are associated with this phenotype, suggesting that more, as yet undefined gene(s) are likely to be implicated in the pathogenesis of the dystroglycanopathies. The identification of these other genes may provide additional information on the pathway of glycosylation of α-dystroglycan.

Conclusions

All these forms of muscular dystrophies are characterized by the hypoglycosylation of ADG in both patients skeletal muscle biopsies and the skeletal muscle of equivalent animal models, suggesting the existence of a common pathogenetic pathway. Whilst POMT1, POMT2 and POMGnT1 encode enzymes involved in the biosynthesis of O-mannosylated glycans on dystroglycan, the function of fukutin, FKRP and LARGE have yet to be identified. In keeping with the hypothesis of a common pathogenetic pathway, allelic mutations of any of these genes results in conditions of variable severity broadly correlated with the degree of ADG hypoglycosylation. Molecular genetic analysis of patients with a dystroglycanopathy therefore should include all these 6 genes; however, approximately 35% of patients have no identifiable mutations, strongly pointing towards further genetic heterogeneity. Genetic analysis suggests that the possibility of a single major locus accounting for the remaining dystroglycanopathies is unlikely and we must be prepared to search for multiple genes associated with the glycosylation of ADG.

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