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Mutations in the human *LARGE* gene cause MDC1D, a novel form of congenital muscular dystrophy with severe mental retardation and abnormal glycosylation of α-dystroglycan

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The congenital muscular dystrophies (CMD) are a heterogeneous group of autosomal recessive disorders. A new pathomechanism has recently been identified in a group of these disorders in which known or putative glycosyltransferases are defective. Common to all these conditions is the hypoglycosylation of α-dystroglycan. Fukuyama CMD, muscle–eye–brain disease and Walker–Warburg syndrome, each associated with eye abnormalities and neuronal migration defects, result from mutations in *fukutin*, *POMGnT1* and *POMT1*, respectively, while mutations in the *fukutin-related protein* (*FKRP*) gene cause congenital muscular dystrophy 1C, typically lacking brain involvement. Another putative glycosyltransferase, *Large*, is mutated in the myodystrophy mouse. The human homologue of this gene is therefore a strong candidate for involvement in novel forms of muscular dystrophy. We studied 36 patients with muscular dystrophy and either mental retardation, structural brain changes or abnormal α-dystroglycan immunolabelling, unlinked to any reported CMD loci. Linkage analysis in seven informative families excluded involvement of *LARGE* but sequencing of this gene in the remaining 29 families identified one patient with a G1525A (Glu509Lys) missense mutation and a 1 bp insertion, 1999insT. This 17-year-old girl presented with congenital muscular dystrophy, profound mental retardation, white matter changes and subtle structural abnormalities on brain MRI. Her skeletal muscle biopsy showed reduced immunolabelling of α-dystroglycan. Immunoblotting with an antibody to a glycosylated epitope demonstrated a reduced molecular weight form of α-dystroglycan that retained some laminin binding activity. This is the first description of mutations in the human *LARGE* gene and we propose to name this new disorder MDC1D.

INTRODUCTION

The congenital muscular dystrophies (CMD) are a heterogeneous group of autosomal recessive disorders which present within the first 6 months of life with hypotonia, muscle weakness and contractures, associated with dystrophic changes on skeletal muscle biopsy (1). Recently, a new pathomechanism has been identified in a group of these conditions in which the peripheral membrane protein α-dystroglycan is hypoglycosylated (2,3). The defective genes in these disorders encode known or putative glycosyltransferases. There are three severe CMD forms with eye abnormalities and neuronal migration

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defects: Fukuyama congenital muscular dystrophy caused by mutations in *fukutin* [FCMD (OMIM 253800)] (4), muscle–eye–brain disease due to mutations in *POMGnT1* [MEB (OMIM 253280)] (5) and Walker–Warburg syndrome [WWS (OMIM 236670)] (6), in which *POMT1* is mutated in around 20% of cases. Mutations within the *fukutin-related protein* (*FKRP*) gene result in both a severe form of congenital muscular dystrophy [MDC1C (OMIM 606612)] (7) and a milder form of limb girdle muscular dystrophy [LGMD2I (OMIM 607115)] (8). Neither disorder is associated with brain involvement, although it is now evident that rare and more severe *FKRP* mutations can result in structural cerebellar changes (9), or more extensive structural brain and eye involvement similar to that seen in MEB and WWS (unpublished data). An identical pathomechanism is also responsible for a spontaneous form of muscular dystrophy in the myodystrophy mouse (*myd*; now renamed Large^{myd}), in which the *Large* gene is mutated, again encoding a putative glycosyltransferase (10). In addition to these genetically characterized forms, a number of conditions with similar biochemical features, unlinked to any known CMD loci, have been reported (11).

Dystroglycan is a crucial component of the dystrophin–glycoprotein complex (12). The *DAG1* gene encodes a precursor protein that is post-translationally cleaved into α and β subunits (13). α-Dystroglycan is a peripheral membrane glycoprotein that associates with transmembranous β-dystroglycan at the sarcolemma. One of its functions is to bind components of the extracellular matrix, thereby linking it, via β-dystroglycan and dystrophin, to the actin associated cytoskeleton (14). Binding of some extracellular matrix molecules, including laminin α2 and perlecan, is mediated by α-dystroglycan's carbohydrate moieties, while others, such as biglycan, bind to the core protein (15).

α-Dystroglycan contains predominantly *O*-linked glycans (16), including rare *O*-linked mannosyl structures that appear to be essential for laminin binding (17). Both *POMT1* and *POMGnT1*, encode enzymes potentially involved in the biosynthesis of these glycans. The biochemical activities of fukutin, FKRP and Large are unknown. In all these disorders the hypoglycosylation of α-dystroglycan is accompanied by a secondary reduction in the laminin α2 chain of merosin in skeletal muscle (7,18–20). Recent immunoblot analysis of α-dystroglycan with an antibody that recognizes an unknown glycosylated epitope failed to detect any expression in skeletal muscle in patients with FCMD, MEB or the myodystrophy mouse, while an antibody specific for a peptide epitope identified a form of α-dystroglycan whose molecular weight was reduced by more than 50 kDa (21). This lower molecular weight form of α-dystroglycan failed to bind laminin, agrin or neurexin on overlay assays. These results strongly suggest that α-dystroglycan is hypoglycosylated in these disorders and the resultant disruption of its interactions with extracellular matrix molecules plays a crucial role in the pathology of these conditions (2,3,21).

Myodystrophy mice have a relatively severe phenotype, characterized by small size, abnormal posturing of hind limbs when suspended by the tail, and abnormal shuffling gait. They develop progressive thoracic kyphosis and have shortened lifespan (22). Other features include cardiomyopathy, abnormal brainstem evoked potential and elecroretinogram and a neuronal migration defect affecting the cerebrum and cerebellum (21,23,24). Serum creatine kinase is elevated almost 10-fold and muscle histology shows diffuse, progressive myopathic changes and discontinuous basal lamina (22,23).

The spontaneously arising myodystrophy mutation is an autosomal recessive 100 kb genomic deletion that removes exons 5–7 of *Large*, resulting in a frameshift and premature termination of translation (10). The Large protein is unusual in that it is predicted to contain two putative catalytic domains. The first has 22% amino acid identity to the bacterial WaaIJ family of putative α-glycosyltransferases, which are involved in synthesis of bacterial outer membrane lipopolysaccaride (25). The second has 28% amino acid identity with β-1,3-*N*-acetylglucosaminyltransferase (iGnT), an enzyme responsible in part for the synthesis of the poly-*N*-acetyllactosamine backbone of the erythrocyte i antigen (26). The human *LARGE* gene maps to chromosome 22q12.3–13.1. It is the fifth largest human gene, covering over 660 kB and is ubiquitously expressed, with highest levels in heart, brain and skeletal muscle (27). The predicted protein product shows 98% amino acid identity with its murine homologue (27).

LARGE is a strong candidate for involvement in human muscular dystrophies, although no patients with defects in this gene have yet been reported. We collected a cohort of 36 patients with muscular dystrophy and either mental retardation, structural brain changes or abnormal immunolabelling of α-dystroglycan, unlinked to any known CMD loci. We undertook linkage analysis in seven informative families in the cohort and excluded linkage to the *LARGE* locus. We subsequently undertook direct sequencing of the *LARGE* gene in the remaining 29 families and in one patient with a distinct clinical phenotype we identified compound heterozygous mutations. This is the first description of mutations in the human *LARGE* gene. In keeping with the currently adopted classification we propose to name this novel form of congenital muscular dystrophy MDC1D.

RESULTS

Clinical details

The patient is the 17-year-old daughter of healthy, non-consanguineous, British parents. She was born after a pregnancy complicated only by hyperemesis. No resuscitation was required and she had no feeding difficulties. Presentation was at 5 months with global developmental delay, hypotonia and mirror movements of the upper limbs. She sat unsupported aged 2.5 years, walked with hands held aged 3 years and walked independently at 4.5 years. Maximal motor function was achieved aged 9 years when she was able to walk 200 yards and to jump. Since then, there has been a gradual deterioration in her mobility and currently she is able to walk only a few steps independently.

On examination at age 17 years, her height was 151 cm (2nd centile), weight was 69 kg (90th centile) and head circumference was 56.5 cm (75th centile). She had profound mental retardation and only had 10 single words, used without meaning, and was able to understand simple one-step commands. Mirror movements were present in the upper limbs

and the fingers were held in a flexed position, with thumbs adducted. She had moderate quadriceps, calf and arm muscle hypertrophy but no macroglossia or muscle atrophy. There was mild facial weakness and extraocular movements were full, but she had horizontal nystagmus on lateral gaze. On observation she had a combination of motor difficulties related both to her muscle weakness and spasticity. She was unable to rise from the floor independently. Weakness affected proximal muscles predominantly and lower limbs were more severely affected than upper limbs. In the upper limbs, tone and deep tendon reflexes were normal. In the lower limbs tone was increased, reflexes exaggerated and plantar responses extensor, indicating pyramidal tract involvement. There were only mild bilateral tendoachilles and elbow contractures. Fundoscopy was normal. Hearing and vision appeared normal.

Serum creatine kinase was elevated, ranging between 467 and 4500 IU/l. ECG and echocardiogram were normal. Peripheral motor and sensory nerve conduction studies were normal: amplitudes were 7 μV in both sural and tibial nerves, and conduction velocities were 40 and 42 m/s, respectively, at age 3.5 years. EMG showed short duration, small amplitude motor unit potentials, in keeping with a myopathy. Auditory evoked responses were normal.

The flash elecroretinogram was recorded using skin electrodes. Amplitude of the flash ERG response was measured from the peak of the a-wave to the peak of the b-wave. Results showed a significant reduction in the amplitude of the evoked response, mainly due to a reduction in amplitude of the b-wave. The b-wave did not show the expected increase in amplitude after dark adaptation.

CT scans at 4 years of age showed low attenuation throughout the white matter of the cerebral hemispheres, but no other abnormalities were visualized. Brain MRI at age 14 years was abnormal: the most striking abnormality was extensive and symmetrical white matter changes affecting the periventricular region to the arcuate fibres, more evident anteriorly and in the temporal regions (Fig. 1A and B). The white matter in the internal capsule, corpus callosum, optic radiations and infratentorial structures was normal. Additional features also suggested abnormal neuronal migration, similar to that described in the cobblestone complex (28). In particular, there was a hypoplastic brainstem due to mild kinking at the isthmus and a mildly flat pons (Fig. 1C). There was mild pachygyria with moderately thick and dysplastic cortex in the frontal lobes and mildly simplified gyri with shallow sulci in the posterior frontal, temporal and parietal regions (Fig. 1D).

Genetic analysis

We studied 36 patients with muscular dystrophy and either mental retardation, structural brain changes or abnormal immunolabelling of α-dystroglycan. Linkage analysis using dinucleotide repeat markers spanning the *LARGE* locus excluded the involvement of this gene in seven informative families. Direct sequencing of *LARGE* in the remaining 29 patients revealed one patient with compound heterozygous mutations. This patient had a missense G1525A mutation in exon 13, which changes a glutamic acid residue to lysine at amino acid position 509 (Fig. 2B). Glu509 is conserved between mouse and human and resides within the putative second catalytic domain of LARGE. Her mother was heterozygous for the mutation. This change introduces a *Sty*I restriction site into exon 13 (see Methods) and RFLP analysis showed the mutation to be absent in 200 British control chromosomes. Direct sequencing also revealed a heterozygous 1base pair insertion, 1999insT, in exon 15 that leads to a frameshift and premature termination of translation at codon 693 (Fig. 2B). This insertion is also within the second putative catalytic domain of LARGE. Her father was heterozygous for the insertion, and an unaffected brother carried neither mutation (Fig. 2A).

Immunohistochemical analysis

α-Dystroglycan expression was analysed using three antibodies: IIH6, directed against the glycosylated laminin α2 binding site; VIA4-1 recognizing an as yet unidentified glycosylated epitope; and a sheep polyclonal that recognizes the core protein. IIH6 and VIA4-1 antibodies showed a significant overall reduction in the level of immunolabelling, which was more marked with VIA4-1 than with IIH6 (Fig. 3E–H). The extent of this reduction varied, with some fibres almost negative and others showing some residual labelling which was discontinuous around the basement membrane. A small proportion of fibres were brightly labelled and corresponded to the occasional fibres that displayed weak labelling with antibody to the core dystroglycan protein. Except for these isolated fibres, labelling of dystroglycan using the core antibody was normal (Fig. 3C and D). β-Dystroglycan expression was normal (Fig. 3A and B). Expression of the laminin α2 chain was assessed using an antibody against the 300 kDa fragment and an antibody against its C-terminal 80 kDa fragment. In both cases the labelling was comparable to control samples (Fig. 3I–L).

Western blotting and overlay analysis

Western blot analysis of skeletal muscle α-dystroglycan showed a small but significant shift in its average molecular weight relative to a control using an antibody against an unidentified glycosylated epitope of α-dystroglycan (VIA4-1; Fig. 4A). By contrast β-dystroglycan was normal. The 80 kDa fragment of laminin α2 chain was also reduced on western blot and was resolved as two bands of closely related molecular weight (Fig. 4A). A laminin overlay assay demonstrated that residual α-dystroglycan retained to ability to bind laminin (Fig. 4B).

DISCUSSION

We have identified compound heterozygous mutations in the human *LARGE* gene in a patient with congenital muscular dystrophy and hypoglycosylation of α-dystroglycan. She has severe mental retardation and brain MRI shows extensive white matter abnormalities and subtle structural changes indicative of a neuronal migration defect. Although similar to the myodystrophy mouse, her phenotype is in many respects considerably milder.

Whilst it is difficult to compare the severity of muscle weakness between humans and mice, our patient has milder

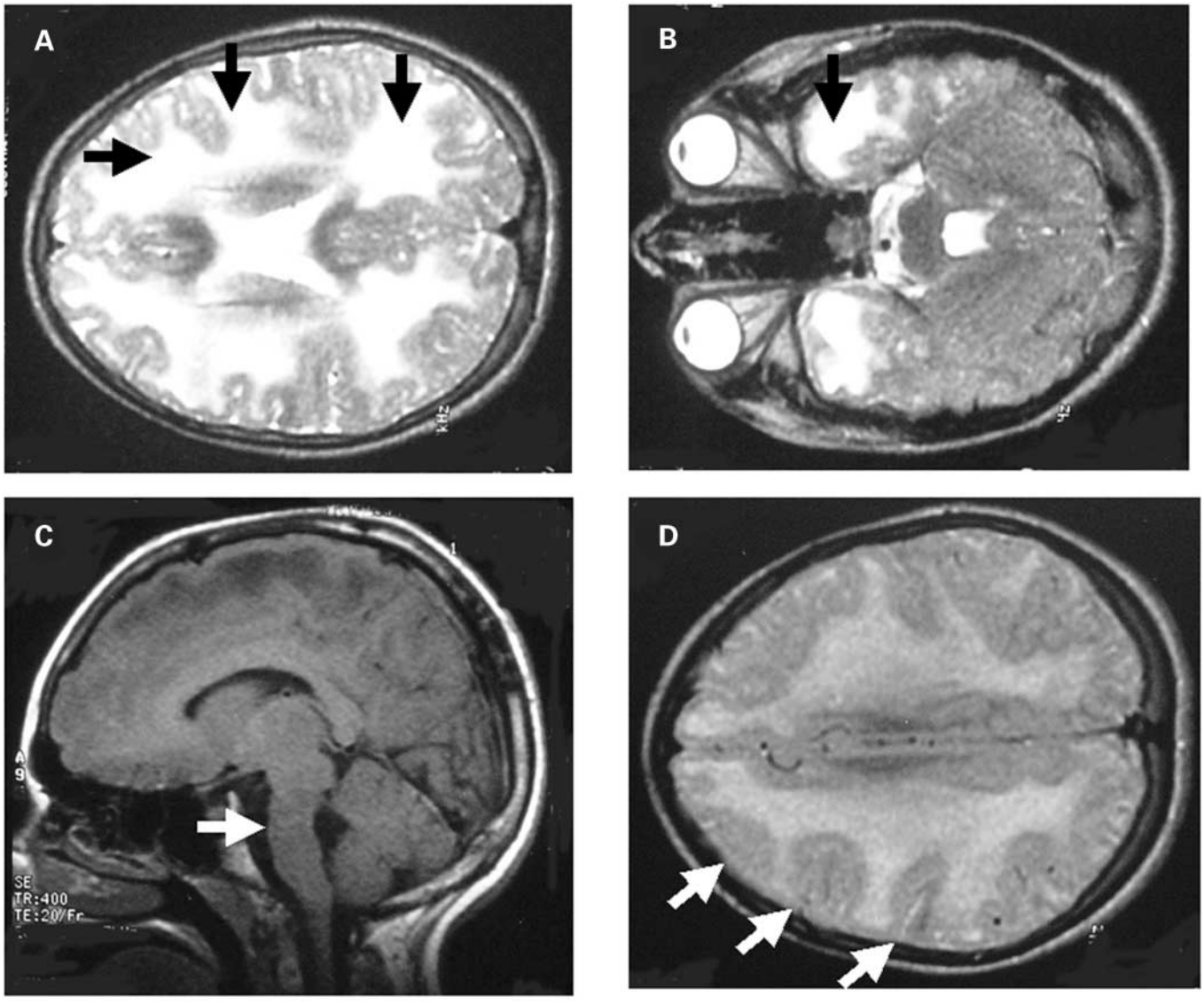


Figure 1. Magnetic resonance imaging of brain, age 14 years. (**A**) T_2-weighted image, transverse plane. There is bilateral, symmetrical, increased signal intensity in white matter (arrows). (**B**) T_2-weighted image, transverse plane. Increased signal intensity of white matter preferentially affects temporal regions (arrow). (**C**) T_1-weighted image, saggital plane. There is a mildly hypoplastic brainstem. The pons is slightly flattened (arrow). (**D**) T_1-weighted image, transverse plane. There is mild pachygyria with moderately thickened cortex in the frontal lobes, and mildly simplified gyri with shallow sulci in the posterior frontal, temporal and parietal regions (arrows).

histological abnormalities. Myodystrophy mice show almost complete loss of α-dystroglycan immunolabelling with antibodies directed against glycosylated epitopes on immunohistochemistry (21,24), whereas in our patient, although severely reduced, some labelling was still present. Immunoblotting in the myodystrophy mouse again shows a virtual absence of immunolabelling with antibodies against glycosylated epitopes (21,24), while our patient showed a small shift towards a lower molecular weight form. The core peptide of α-dystroglycan shows normal immunolabelling in skeletal muscle in myodystrophy mice but reduced molecular weight on immunoblotting, consistent with its hypoglycosylation (21). Our patient also shows normal immunolabelling with the core antibody, although suitable core antibodies for use in immunoblotting were not available. In keeping with her milder phenotype, the residual α-dystroglycan present in the skeletal muscle biopsy retained laminin-binding on an overlay assay, whereas this was lost in the myodystrophy mouse (21,24). Laminin immunolabelling in skeletal muscle of myodystrophy mice has been reported to be both normal (21) and reduced (24) in two conflicting studies. In our patient laminin α2 immunolabelling was normal using antibodies recognizing both a full length protein and the 80 kDa fragment. In contrast, immunoblotting demonstrated a slight reduction in the level of laminin α2 expression.

The structural brain involvement in our patient is probably less pronounced than the changes seen in the myodystrophy mouse. These mice have disturbed cortical lamination

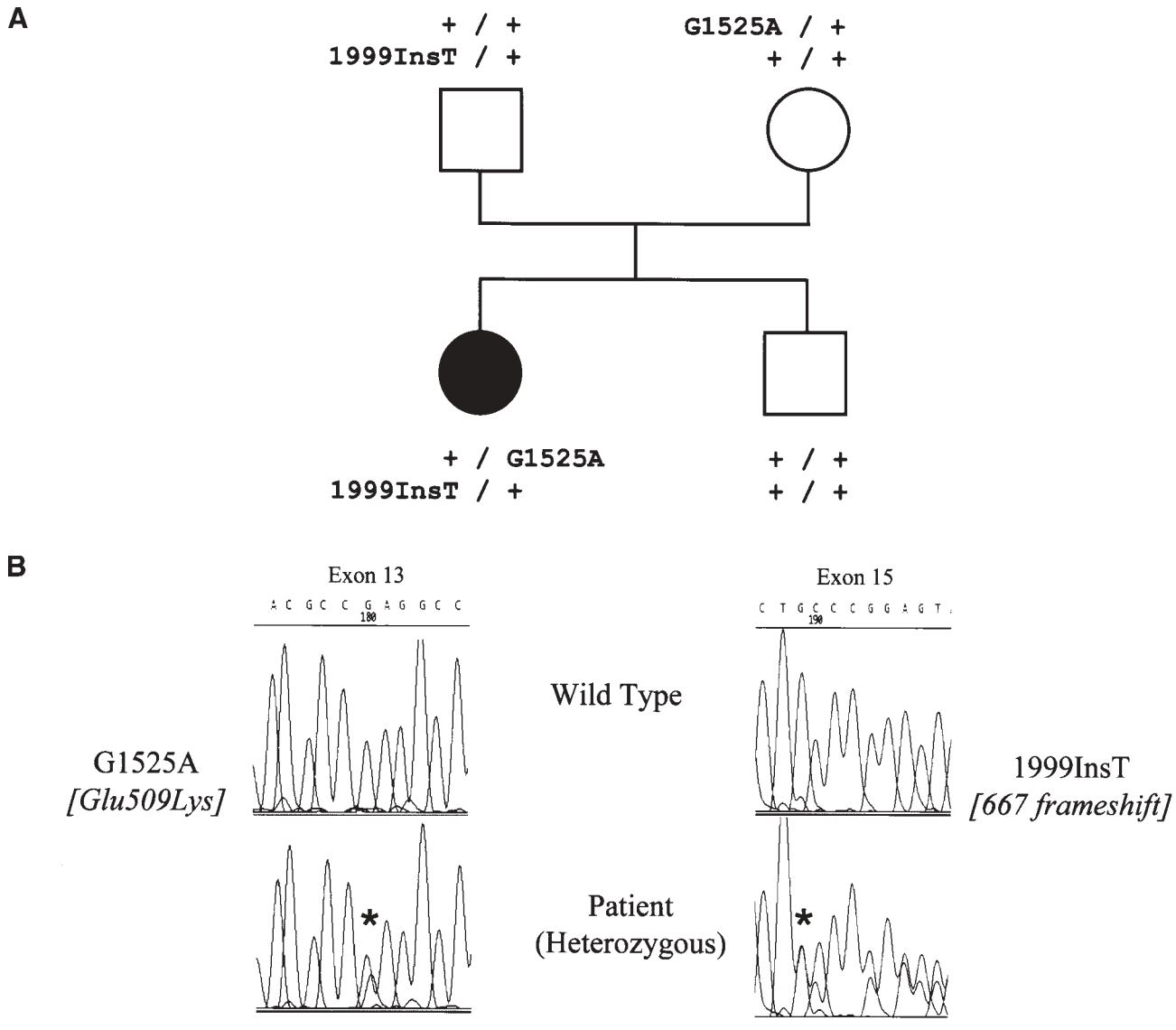
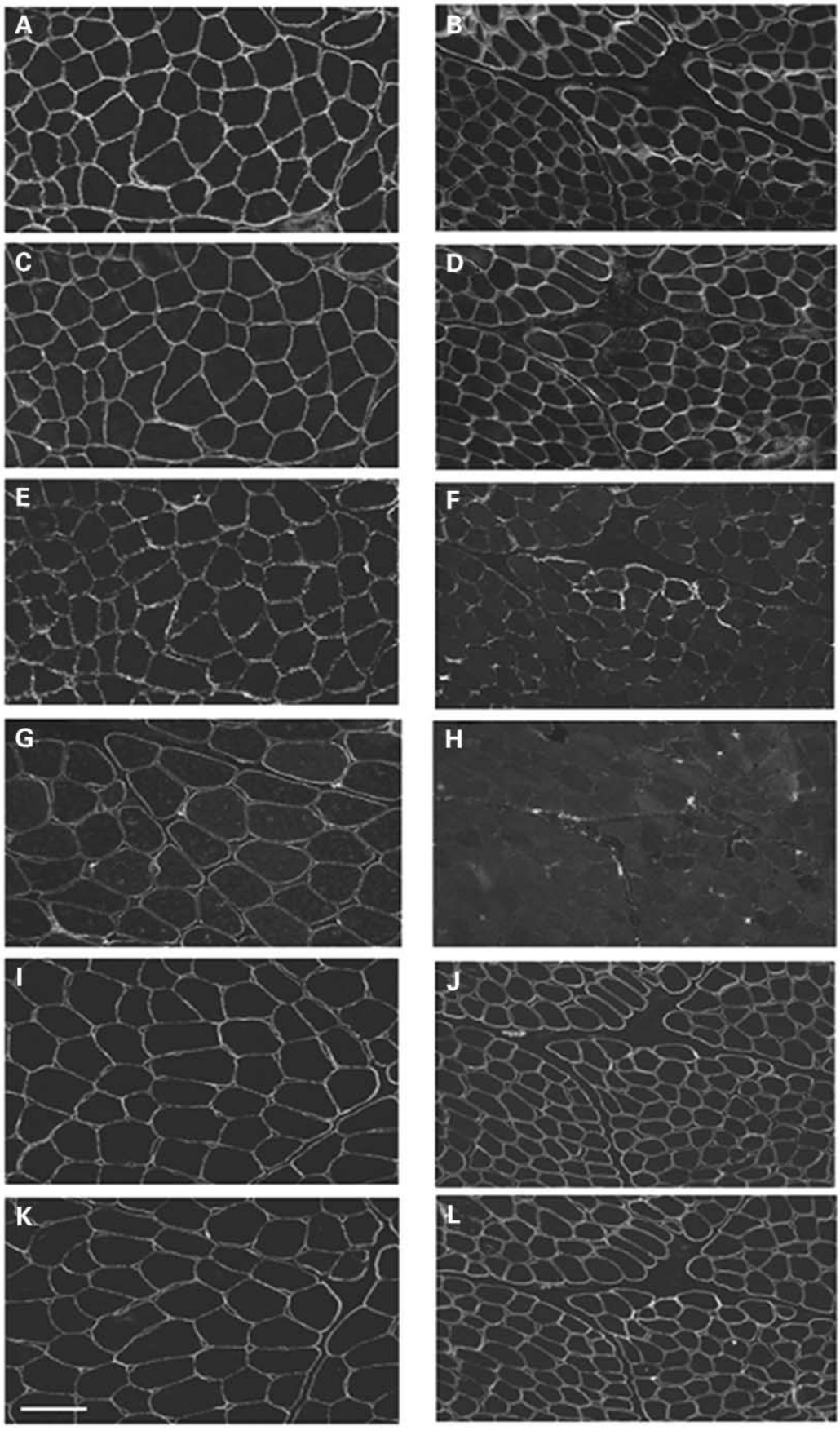


Figure 2. Mutation analysis. (**A**) Family pedigree. Females are represented by open circles, males are represented by open squares. The solid symbol represents the affected patient. The carrier status for the two mutations is indicated above each individual; (+) wild type. (**B**) Sequencing chromatograms of *LARGE* in affected patient, and healthy control. The patient shows a heterozygous missense mutation G1525A (Glu509Lys) in exon 13 (left), and a heterozygous 1 bp insertion, 1999insT, in exon 15 (right).

throughout the cerebrum and cerebellum with neuronal heterotopia throughout the outer, molecular layer of the cortex (24). There is abnormal migration of granule cells in the cerebellum and hippocampus and clusters of heterotopic neurones are present in the cerebellar white matter (21,24). Severe focal disruptions are also seen in the glia limitans of the cerebellum (21). This pattern of neuronal migration defect (cobblestone lissencephaly) is also observed FCMD, WWS and MEB (29). Compared to these conditions, our patient had similar but milder structural defects, most closely resembling those seen in MEB. However in comparison with MEB, the white matter involvement in our patient was much more extensive.

The mechanism underlying the neuronal migration defect in CMD is not entirely understood, but a key factor appears to be the integrity of the glia limitans, the outer limiting membrane of the brain (29,30,31). Disruption of this membrane results in the migration of neurones and glial cells through the pia into the subarachnoid space, giving rise to a 'cobblestone' appearance of the brain surface (21). Other findings commonly associated with this cortical neuronal migration defect include a flattened brainstem, cerebellar vermis hypoplasia, ventricular dilatation and white matter abnormalities, referred to as the 'cobblestone complex' (28). α-Dystroglycan appears to play a major role in neuronal migration. Targeted deletion of the *Dag1* gene in the mouse brain results in a neuronal migration defect similar to cobblestone lissencephaly (32). In addition, α-dystroglycan from the brain of the myodystrophy mouse shows almost complete loss of binding to its ligands laminin, neurexin and agrin on an overlay assay (21).



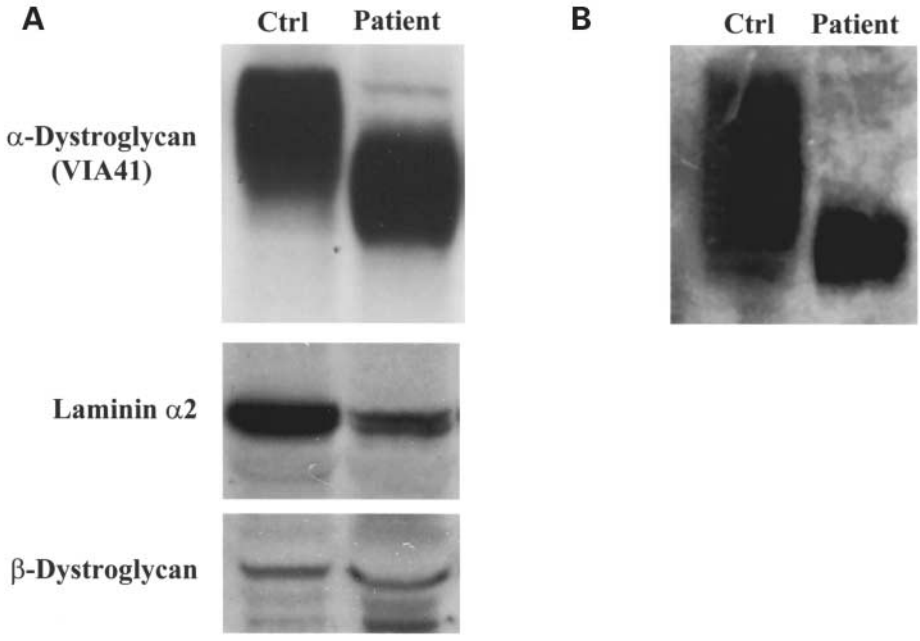


Figure 4. (**A**) Skeletal muscle immunoblot analysis. The molecular weight of α-dystroglycan in the patient using the V1A4I antibody shows a shift towards lower molecular weight forms relative to a control. The laminin α2 80 kDa fragment was slightly reduced and resolved as a doublet. β-Dystroglycan expression was similar to a control and demonstrates equal loading between samples. (**B**) Skeletal muscle laminin overlay assay. The patient's residual α-dystroglycan retains some ability to bind laminin. Ctrl = control muscle.

Abnormal electroretinographic (ERG) studies have been described in myodystrophy mice and fukutin-deficient chimaeric mice, comprising a reduction in implicit time and amplitude of the b-wave of evoked potentials, more marked after dark adaptation (24,33). The b-wave is generated by the Muller glia, in response to depolarization of bipolar cells, which process the initial neural signal from photoreceptors (34). The normal a-wave in the myodystrophy mouse implies normal photoreceptor function and, as retinal morphology is normal, the b-wave abnormality reflects abnormal processing of the visual signal. It is interesting that a similar pattern of reduction in amplitude of the evoked potentials, affecting predominantly the b-wave, was detected in our patient. Dystroglycan is strongly expressed in the retina, within the basal lamina in the inner limiting membrane and blood vessels, and in Muller glia and photoreceptors (35). The abnormal ERG in our patient and the myodystrophy mouse suggests that abnormalities in α-dystroglycan affect processing of the visual signal, presumably by a similar pathomechanism as seen in skeletal muscle and brain.

Other abnormalities described in the myodystrophy mouse include cardiomyopathy and abnormal auditory evoked responses (23,24). Both features were absent in our patient.

The enzymatic activity of LARGE has yet to be defined and its role in α-dystroglycan glycosylation is unclear. Its two putative catalytic domains have homology to classes of glycosyltransferases whose proposed biochemical activities result in modifications not found on α-dystroglycan. However the characterization of α-dystroglycan carbohydrates is far from complete and structures compatible with the proposed activities of LARGE may remain undetected. Alternatively, LARGE may act indirectly on α-dystroglycan processing via interaction with other proteins. The milder phenotype of our patient, in comparison with the myodystrophy mouse, may reflect differing levels of LARGE activity. The myodystrophy mouse is expected to have a virtual absence of enzyme activity (10). Both mutations identified in our patient are located within the putative second catalytic domain of LARGE and may permit some residual enzyme activity. One mutation is a 1 bp insertion after nt1999 resulting in a 693 amino acid truncated protein lacking the last 63 amino acids. The second mutation is a missense mutation G1525A (Glu509Lys).

Our results indicate that *LARGE* mutations are not common among patients with a CMD phenotype, although the possibility of large scale rearrangements within the gene exists. However, mutations in *LARGE* could cause a wide spectrum of clinical phenotypes, as demonstrated in patients with *FKRP* mutations (7–9). LARGE therefore remains a good candidate for involvement in the whole range of muscular dystrophies.

In conclusion, we have described a novel form of congenital muscular dystrophy with hypoglycosylation of α-dystroglycan, due to mutations in *LARGE*. The patient is ambulant at age 17 years and has profound mental retardation, white matter brain

Figure 3. Immunohistochemical analysis of control (**A**, **C**, **E**, **G**, **I**, **K**) and patient (**B**, **D**, **F**, **H**, **J**, **L**) muscle sections using antibodies to β-dystroglycan (A and B), α-dystroglycan (C and D, core antibody; E and F, IIH6 antibody; G and H, VIA4-1 antibody) and laminin α2 chain (I and J, MAB1922 antibody; K and L, 4H8 antibody). Scale bar = 50 μm. The serial sections of the patient show a profound reduction of labelling with IIH6 (F) and more severe reduction with V1A41 (H), while the core protein is normally expressed (D).

abnormalities and mild structural brain involvement within the spectrum of the cobblestone complex. We propose to name this novel form MDC1D.

MATERIALS AND METHODS

Linkage analysis

DNA was extracted from whole blood using standard procedures after obtaining informed consent. Markers *D22S280*, *D22S1162* and *D22S1158*, spanning the *LARGE* locus, were used in linkage analysis. Primers amplifying these markers were purchased from Invitrogen, with the forward primer modified at the 5′ end by the addition of either a FAM or NED fluorescent label. PCR products were separated on a 5% denaturing gel (Amresco) in a ABI 377A automated DNA sequencer (Applied Biosystems) and analysed using Genescan version 3.01 and Genotyper version 2.01 software (Applied Biosystems).

Sequencing analysis

The 14 coding exons of *LARGE* were amplified by PCR using AmpliTaq Gold (Applied Biosystems) and a PE9600 Thermalcycler (Applied Biosystems). PCR products were purified using a Qiagen PCR purification kit. All exons were sequenced in both directions using a BigDye Cycle sequencing kit (Applied Biosystems). Sequencing products were separated on a 5% denaturing gel (Amresco) on a ABI 377A automated DNA sequencer (Applied Biosystems) and analysed using Sequence Analysis software version 2.01 (Applied Biosystems).

RFLP analysis

The G1525A mutation was investigated in controls using RFLP analysis. Exon13 was amplified using primers Large 13F and 13R. The presence of the mutation created a *Sty*I restriction site that cleaved the 581 bp product into two fragments of 201 and 380 bp. Ten microliters of PCR product were incubated at 37°C for 2 h with 20U of *Sty*I (Promega). Digestion products were separated on a 1% agarose gel and visualized with ethidium bromide staining.

Immunohistochemistry

Frozen muscle sections were incubated with antibodies to α-dystroglycan; sheep polyclonal raised against a 20 amino acid peptide from the chick α-dystroglycan sequence that recognizes the core protein of α-dystroglycan (1 : 500; kind gift of Dr Stephan Kröger) and monoclonal antibodies IIH6 and VIA4-1 (1 : 200 and 1 : 50 respectively; both antibodies were a kind gift of Dr K. Campbell).

Laminin α2 chain expression was analysed using the monoclonal antibody MAB1922 (Chemicon, 1 : 4000) and the polyclonal antibody 4H8 (Alexis 1 : 200). All primary antibodies were applied for 1 h and revealed with an appropriate biotinylated secondary antibody for 30 min, followed by streptavidin conjugated to Alexa 594 (Molecular Probes) for 15 min. All dilutions and washings were made in phosphate buffered saline. Sections were mounted in aqueous mountant and viewed with epifluorescence using a Leica DMR microscope linked to Metamorph (Universal Imaging). Control sections were labelled without primary antibodies, and all sections were compared with control samples from other patients with neuromuscular disorders and with normal muscle.

Immunoblotting

Muscle proteins were extracted in sample buffer consisting of 75 mM Tris–HCl, 1% SDS, 2-mercaptoethanol, plus a cocktail of protease inhibitors (antipain, aprotinin and leupeptin). Soluble proteins were resolved using a NuPage Pre-cast gel (4–12% Bis–Tris; Invitrogen) and then transferred electrophoretically to nitrocellulose membrane (Hybond-ECL, Amersham). Nitrocellulose strips were blocked in 3% BSA (IgG- and protease-free, Jackson) in Tris-buffered saline buffer, probed with antibodies to α-dystroglycan (VIA4-1 and IIH6, Upstate) and β-dystroglycan (Novocastra). After washing they were incubated with HRP-anti-mouse IgG (Jackson). For IIH6 biotinylated IgM (Dako) and HRP streptavidin (Dako) were used. Membranes were visualized using chemiluminescence (ECL + Plus, Amersham).

Overlay assay

Nitrocellulose membranes were blocked for 1 hour in laminin binding buffer (LBB: 10 mM triethanolamine, 140 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, pH 7.6) containing 5% non-fat dry milk (laminin) followed by incubation of mouse Engelbreth-Holm-Swarm laminin (Gibco) overnight at 4°C in LBB. Membranes were washed and incubated with anti rabbit laminin (Sigma) followed by HRP-anti rabbit IgG (Jackson). Blots were visualized using chemiluminescence (ECL + Plus, Amersham).

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