Diagnosis and etiology of congenital muscular dystrophy

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ABSTRACT

Objective: We aimed to determine the frequency of all known forms of congenital muscular dystrophy (CMD) in a large Australasian cohort.

Methods: We screened 101 patients with CMD with a combination of immunofluorescence, Western blotting, and DNA sequencing to identify disease-associated abnormalities in glycosylated α-dystroglycan, collagen VI, laminin α2, α7-integrin, and selenoprotein.

Results: A total of 45% of the CMD cohort were assigned to an immunofluorescent subgroup based on their abnormal staining pattern. Abnormal staining for glycosylated α-dystroglycan was present in 25% of patients, and approximately half of these had reduced glycosylated α-dystroglycan by Western blot. Sequencing of the *FKRP*, *fukutin*, *POMGnT1*, and *POMT1* genes in all patients with abnormal α-dystroglycan immunofluorescence identified mutations in one patient for each of these genes and two patients had mutations in *POMT2*. Twelve percent of patients had abnormalities in collagen VI immunofluorescence, and we identified disease-causing *COL6* mutations in eight of nine patients in whom the genes were sequenced. Laminin α2 deficiency accounted for only 8% of CMD. α7-Integrin staining was absent in 12 of 45 patients studied, and *ITGA7* gene mutations were excluded in all of these patients.

Conclusions: We define the distribution of different forms of congenital muscular dystrophy in a large cohort of mixed ethnicity and demonstrate the utility and limitations of current diagnostic techniques. ***Neurology*® 2008;71:312–321**

GLOSSARY

CK = creatine kinase; **CMD** = congenital muscular dystrophy; **EMG** = electromyogram; **FKRP** = fukutin related protein; **LGMD2I** = limb-girdle muscular dystrophy type 2I; **MDC1C** = congenital muscular dystrophy type 1C; **MEB** = muscle-eye-brain disease; **PVDF** = polyvinylidene fluoride; **SSCP** = single strand conformational polymorphism; **UCMD** = Ullrich congenital muscular dystrophy; **WWS** = Walker-Warburg syndrome.

The congenital muscular dystrophies (CMDs) are a heterogeneous group of inherited muscle disorders, defined by a combination of early onset hypotonia and weakness, contractures, variable progression, normal or elevated serum creatine kinase (CK), and myopathic changes on electromyogram (EMG), usually associated with a dystrophic muscle biopsy.[1] This group of conditions is thought to be among the most common of autosomal recessive neuromuscular disorders,[2] with reported estimates of the incidence ranging between 4.7 per 100,000 live births in the north of Italy[3] and 6.3 per 100,000 live births in western Sweden.[4]

There are currently 12 genetically defined forms of CMD[5,6] that fall into three groups on the basis of the classes of proteins that are affected.[7,8] Several forms of CMD are caused by mutations in genes encoding structural proteins of the basement membrane or extracellular matrix

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of skeletal muscle fibers: collagen VI associated with Ullrich CMD, MIM 254090 (*COL6A1*, MIM 120220; *COL6A2*, MIM 120240; *COL6A3*, MIM 120250), laminin α2 associated with MDC1A, MIM 607855 (*LAMA2*, MIM 156225) and α7-integrin, *ITGA7*, MIM 600536. Mutations in genes encoding putative or proven glycosyltransferase enzymes (*POMT1*, MIM 607423; *POMT2*, MIM 607439; *POMGnT1*, MIM 606822; *fukutin*, MIM 607440; *FKRP*, MIM 606596; and *LARGE*, MIM 603590) involved in the glycosylation of α-dystroglycan result in a number of forms of CMD, often associated with neuronal migration defects (FCMD, MIM 253800; muscle-eye-brain disease [MEB], MIM 253280; Walker-Warburg syndrome [WWS], MIM 236670; congenital muscular dystrophy type 1C [MDC1C], MIM 606612; and MDC1D, MIM 608840). Mutations in the *SEPN1* gene, MIM 606210, which encodes an endoplasmic reticulum protein of unknown function, are associated with CMD with rigid-spine syndrome (RSMD1, MIM 602771).

Accurate description of the clinical phenotype, coupled with comprehensive protein and genetic analysis, are necessary to definitively assign a diagnosis of a specific form of CMD. For example, overlapping CMD phenotypes associated with abnormal expression of glycosylated α-dystroglycan can be caused by mutations in any of the genes encoding putative glycosyltransferases (*POMT1*, *POMT2*, *POMGnT1*, *fukutin*, *FKRP*, and *LARGE*). Conversely, mutations in the gene encoding fukutin-related protein (*FKRP*) can cause multiple different phenotypes: MDC1C as well as limb-girdle muscular dystrophy type 2I (LGMD2I, MIM 607155),[9,10] MEB, WWS, and atypical forms of CMD.[11-13]

The frequencies of the different forms of CMD are not known. It was initially suggested that primary deficiency in laminin α2 was the cause of 50% of all CMDs,[7,14] although estimates from the last 5 years are closer to 30 to 40% in European patients, with regional variations.[8] Ullrich congenital muscular dystrophy (UCMD) is emerging as the second most common form of CMD, and CMDs associated with abnormal glycosylation of α-dystroglycan expression are being identified more frequently.[7,8,15] Primary deficiency of α7-integrin appears to be a very rare form of CMD with only three patients identified to date.[8,16,17]

The aim of this study was to determine the frequencies of the CMD subtypes in a large cohort of Australasian patients of mixed ethnic origin and with undefined etiology, using a combination of protein and molecular diagnosis to maximize the diagnostic yield and to distinguish between primary and secondary immunofluorescent abnormalities. In this study, we subclassified 45% of our cohort into distinct immunofluorescent groups, and reached a definitive diagnosis in 24% of patients. This is a comprehensive report of the relative frequencies of the known subtypes of CMD in a large cohort.

METHODS

Patients. Patients were ascertained retrospectively and prospectively through archived muscle biopsy material (1979 to 1996) at the University of Sydney and patient biopsies referred for CMD analysis at The Children's Hospital at Westmead until June 2006. Muscle biopsies were reported histopathologically as dystrophic, myopathic, non-specific, or normal.

All patients referred for analysis of CMD with evidence of onset of muscle weakness within the first 2 years of life and dystrophic changes on muscle biopsy were included. If the muscle biopsy was not dystrophic, then patients were included if they or a sibling had clinical features consistent with the diagnosis of CMD such as muscle weakness or hypotonia at <2 years of age, delayed gross motor milestones, congenital/early contractures or scoliosis, abnormal brain or evidence of white matter changes, disorders of migration, cerebellar or brainstem abnormalities, or a raised CK. We used these broad inclusion criteria since patients with CMD can have muscle histopathology varying from normal to dystrophic.[18] All fetuses or neonates referred for CMD analysis were included. Patients were excluded when no frozen muscle biopsy was available to study or when muscle pathology suggested a non CMD diagnosis, for example rods, cores, or inclusions that suggest nemaline or multi-minicore myopathy, or when an alternative diagnosis was subsequently made such as Prader-Willi syndrome and rickets. Muscle biopsies were available on 101 patients and clinical data on 97 patients. We included only the proband in our analysis when there were multiple affected individuals in a family. Clinical data were collected from the referring doctor, medical records, and where possible by patient assessment. This study was approved by the Ethics Committees of The Children's Hospital at Westmead, The University of Sydney, and The Royal Children's Hospital, Melbourne.

Antibodies. These antibodies were used at the following dilutions in the study: anti-dystrophin, 1:2,000 (clone DYS6-10, provided by Prof. Louis Kunkel, Boston, MA); anti-collagen VI, 1:30,000 (clone 3C4, MAB1944, Chemicon International, CA);

anti-collagen VI, 1:5,000 (clone 70-XR95, Fitzgerald Industries International Inc., MA); anti-perlecan, 1:5,000 (clone A7L6, MAB1948, Chemicon International, CA) used in combination with the 70-XR95 anti-collagen VI antibody; anti-α-dystroglycan, specific for the glycosylated form of α-dystroglycan, 1:200 for immunofluorescence (clone VIA4-1, Upstate, MA) and 1:100 for Western blot (gift from Professor Kevin Campbell, Howard Hughes Medical Institute, University of Iowa); anti-laminin α2 to the 80 kDa antigen was used at 1:20,000 for immunofluorescence and 1:500 for Western blot (clone 5H2, MAB1922, Chemicon International, CA); anti-laminin α2 to the 300 kDa antigen was used at 1:400 for immunofluorescence (clone 4H8-2, Alexis Biochemicals, Switzerland); anti-β-dystroglycan was used at 1:50 for immunofluorescence and 1:250 for Western blot (clone 43DAG1/8D5, Novocastra Laboratories Ltd, UK); anti-spectrin, 1:200 (clone RBC2/3D5, NCL-SPEC1, Novocastra Laboratories Ltd, UK); antibodies to the cytoplasmic domain of the α7-integrin chain [A2(346) and B2(347)] were used as previously described.[19,20] Fluorescent secondary antibodies used for immunofluorescence were Cy™-3 conjugated AffiniPure goat anti-rabbit antibody (Jackson ImmunoResearch Laboratories, PA) used at 1:250, and Alexa-Fluor® 488 conjugated goat anti-mouse antibody (Molecular Probes, OR) used at 1:200. For Western blotting, HRP-conjugated sheep anti-mouse secondary antibody (GE Healthcare, UK) was used at 1:1,000.

Immunofluorescence. Immunofluorescence was performed as previously described[21] with the following changes. Sections to be stained for glycosylated α-dystroglycan were fixed in ice-cold 17.47 N glacial acetic acid and ethanol in a 1:1 ratio for 1 minute, and then rinsed in 1× PBS; for detection of α7-integrin, sections were fixed in ice cold methanol for 1 minute and rinsed in 1× PBS, the standard method was then followed. Images were taken on an Olympus BX50F4 microscope (Japan) at 40× magnification. Staining was graded as absent, severely reduced (very patchy and reduced in intensity), moderately reduced (patchy and reduced), mildly reduced (fibers are not patchy, but all show a reduction in staining intensity), or normal. For controls, we concurrently studied age-matched biopsies with no ultrastructural changes identifiable by light or electron microscopy and previously shown to be normal for all routine histologic and immunofluorescent stains.

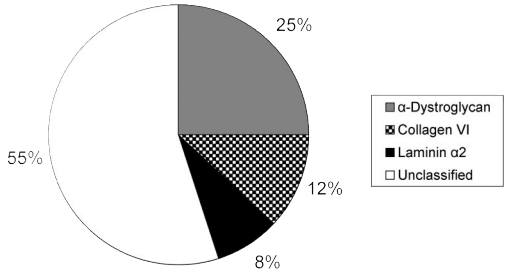
Western blotting. Western blotting for the glycosylated form of α-dystroglycan and laminin α2 was performed on frozen skeletal muscle biopsies as previously described.[22] Equal sample loading was evaluated using myosin band intensity on mini-gels. Samples adjusted for myosin loading were separated on 3 to 8% NuPAGE gels (Invitrogen, CA), transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica MA), probed with indicated antibodies, and developed with ECL chemiluminescent reagents (GE Healthcare, UK). Glycosylated α-dystroglycan and laminin α2 were noted as present, absent, reduced in amount, or reduced in size (molecular weight). Loading was controlled for by probing with the antibody raised against β-dystroglycan and staining of the PVDF membranes with Coomassie Brilliant Blue (Sigma Aldrich) to visualize the amount of myosin loaded on the gel.

Mutation analysis. Fukutin related protein (*FKRP*): PCR primers were designed to amplify the coding and flanking intronic sequence (exon 4) of *FKRP* (accession number AJ314847) as follows: primer pair 1 FKRP3F 5′-TGACAATCAGCTGCTGCCT-3′ and FKRP6R 5′-AGTGGTTCGGCTGCAAC-3′ and primer pair 2 FKRP5F 5′-AGCTGCTGGACTTGACCTT-3′ and FKRP4R 5′-AGGAGAGCATGAGAGAAGGCT-3′ (Invitrogen, Australia, and SigmaGenoSys, Australia).

PCRs were performed using Platinum *Pfx* enzyme (Invitrogen, CA) and the following cycling conditions: 95 °C for 5 minutes, 40 cycles of 94 °C for 30 s, 62 °C for 30 s, 68 °C for 1 min, and one cycle of 68 °C for 7 minutes. The PCR products were agarose-gel purified and sequenced.

Mutational analysis of the three collagen VI genes (*COL6A*) by RT-PCR, genomic PCR, and DNA sequencing[23] and mutational analysis of α7-integrin (*ITGA7*) by single strand conformational polymorphism (SSCP) analysis of cDNA[17] were performed as previously described. Mutational analysis of laminin α2 (*LAMA2*)[24] and selenoprotein N1 (*SEPN1*) was performed as previously described.[25]

Figure 1 **Classification of patients with congenital muscular dystrophy (CMD)**



Classification of patients with CMD by abnormal immunofluorescent staining, n = 101.

RESULTS Histopathologic information was obtained and immunofluorescence performed on 101 muscle biopsies (52 female and 49 male patients). The ethnic origin was known for approximately half of the probands' parents. Of those, approximately one-third were of Caucasian decent and the remainder were of multiple ethnicities reflecting the ethnic diversity of the Australian population (Afghani, Australian Aboriginal, Bangladeshi, Chinese, Egyptian, Filipino, Greek, Indian, Iraqi, Italian, Lebanese, Mauritian, Pakistani, Palestinian, Salvadorian, Sri Lankan, and Turkish).

All biopsies from the 101 patients were analyzed together for immunofluorescence. The age at biopsy ranged from 19 weeks gestation to 29 years. Sixty-eight biopsies (67%) had dystrophic histopathology, 16 were myopathic, 9 were nonspecific, 7 were normal, and histopathology was not available for 1 biopsy. Biopsies were stained with antibodies to spectrin, β-dystroglycan, the glycosylated form of α-dystroglycan, laminin α2, dystrophin, collagen VI, and perlecan. All patients had normal β-dystroglycan and dystrophin staining. Forty-five percent of patient biopsies were classified into an immunofluorescent subgroup based on a moderate to severe reduction in antibody staining (figure 1

Table 1 Clinical information according to abnormal immunofluorescent subgroups

	Abnormal immunofluorescent subgroups			
	α-Dystroglycan	Collagen VI	Laminin α2	Unclassified
No. of patients	23	12	8	43
Male/female	9/14	7/5	3/5	23/20
Distribution of weakness	5 generalized, 14 proximal	6 generalized, 5 proximal	4 generalized, 4 proximal	17 generalized, 16 proximal
Facial weakness	11 (18)	5 (8)	5 (7)	18 (30)
Distal laxity	2 (8)	10 (10)	1 (2)	9 (12)
Delayed motor milestones	16 (17)	9 (10)	8 (8)	22 (29)
Impaired intellect	8 (18)	0 (11)	2 (7)	11 (26)
Abnormal neuroimaging	7 (17)	2 (4)	6 (8)	11 (23)
Ocular anomalies	4	1	3	12
Cardiac anomalies*	5 (18)	2 (9)	0 (6)	6 (26)
Respiratory anomalies	9 (18)	2 (8)	4 (7)	16 (28)
Feeding difficulties	5 (17)	2 (8)	4 (8)	21 (30)
Contracture(s)	13 (17)	11 (11)	6 (8)	17 (27)
Scoliosis	7 (15)	2 (6)	1 (8)	13 (30)
Elevated creatine kinase	11 (18)	8 (10)	8 (8)	15 (33)
Abnormal EMG	6 (8)	5 (7)	5 (5)	15 (23)
Abnormal nerve conduction	3 (10)	2 (7)	0 (5)	3 (25)

Clinical information was not available on all patients for all variables. Hence the results shown represent the number of patients documented to have a particular variable, with the number of patients for whom information regarding that variable was available in parentheses.
*Cardiac involvement details are provided in table e-2.

and table e-1 on the *Neurology*® Web site at www.neurology.org).

Detailed clinical data were available for 86 of 90 patients with CMD (not including the fetuses and neonates). Nine of these patients have died. Eight patients had affected siblings, all parents of these children were without overt muscle weakness, and 12 of 60 patients had consanguineous parents. This is consistent with most types of CMD being inherited in an autosomal recessive manner. The clinical features associated with the immunofluorescent classification are shown in table 1 and table e-2.

Eleven of 101 patients were fetuses or neonates who died within the first 2 weeks of life and their results are shown in table 2. We report clinical data from the fetuses and neonates separately because of the limited clinical information available.

Glycosylated α-dystroglycan abnormalities. α-Dystroglycan is a highly glycosylated protein that is expressed abundantly at the membrane of skeletal muscle fibers. The *DAG1* gene encodes both α- and β-dystroglycan,[26] and no disease-causing mutations have yet been identified in this gene. Glycosylated α-dystroglycan staining is abnormal in patients with mutations in the putative glycosyltransferase genes *FKRP*,[9,10,27] *Fukutin*,[28] *POMT1*,[29] *POMT2*,[6] *POMGnT1*,[30,31] and *LARGE*.[32] Abnormal glycosylation of α-dystroglycan can result in a loss of localization to the sarcolemmal membrane and a secondary partial reduction in laminin α2, its binding partner in the extracellular matrix.[10] Twenty-five of 101 (25%) patients had absent to moderately reduced staining of glycosylated α-dystroglycan compared to age-matched controls (figure 2A). Sufficient muscle was available for Western blot on 19 patients with abnormal glycosylated α-dystroglycan staining. Glycosylated α-dystroglycan was absent or reduced in amount by Western blot in 11 patients. Ten of these patients also had decreased levels of laminin α2 by Western blot despite having normal laminin α2 immunofluorescence. All patients had normal immunofluorescence staining for β-dystroglycan.

We sequenced *FKRP* in all 25 patients with abnormal immunofluorescence for glycosylated α-dystroglycan. We identified disease-causing mutations in only one patient, who had two previously reported mutations, one on each allele, [g.162_165 dupGGAG] + [g.826 C>A]. This patient (Patient 63) had severely reduced glycosylated α-dystroglycan immunofluorescence (figure 2B), and no detection of glycosylated α-dystroglycan and reduced amounts of laminin α2 by Western blot (figure 2C).

Table 2 Clinical and histopathologic information for fetuses and neonates referred for congenital muscular dystrophy analysis

Patient	Histopathology	IF abnormal subgroup	Affected siblings	Consanguineous	Gestation (wk)	Age at death	CNS	Contractures
14	N	Unclassified	—	Yes	26	Day 1	Lissencephaly	No
20	N	Unclassified	—	Yes	32	Day 13	Microcephaly, dilated ventricles, probable polymicrogyria, calcification of periventricular region	—
23	Dystrophic	Unclassified	Yes	No	21	N/A	Normal brain and spinal cord	Yes
25	Dystrophic	Unclassified	Yes	No	39	Day 3	Normal brain and spinal cord	Yes*
33	Dystrophic	Unclassified	—	Yes	34	Day 2	Normal brain	Yes
46	Myopathic	Unclassified	Yes	—	Term	Day 1	Normal brain and spinal cord	Yes*
71	Dystrophic	Unclassified	—	Yes	20	N/A	Normal brain	Yes*
74	N	Unclassified	—	—	19	N/A	Multiple brain abnormalities, thought to be the result of an ischemic event	No
87	Nonspecific	Unclassified	Yes	No	19	N/A	Normal brain	Yes
92	Myopathic	Unclassified	—	No	33	Day 1	Normal brain and spinal cord	Yes*
100	N	α-DG	Yes	No	29	Day 1	Normal brain	Yes

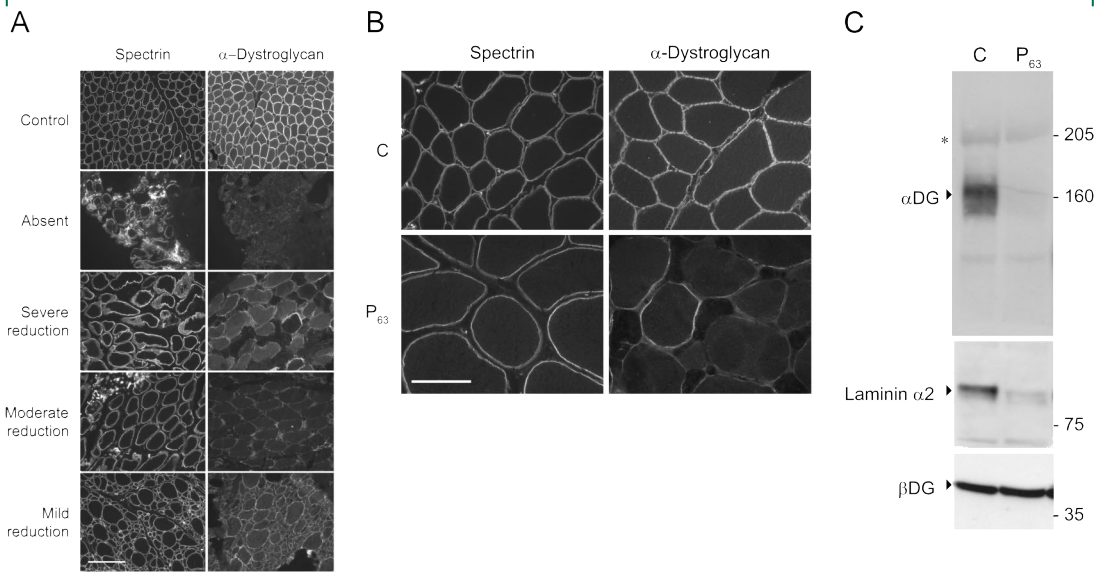
IF = immunofluorescence; — = data not available; N/A = not applicable; Yes* = yes arthrogryposis; N = normal; α-DG = α-dystroglycan.

DNA from 24 patients with abnormal glycosylated α-dystroglycan immunofluorescence has been referred to Prof. Francesco Muntoni for screening of the other known glycosyltransferase genes *LARGE, POMT1, POMT2, Fukutin,* and *POMGnT1*. Mutations have been identified to date in 5 of 24 (21%) patients in whom the coding regions of all genes have been sequenced.[32a] All patients in whom mutations have been identified and had sufficient muscle available for Western blot had reduced glycosylated α-dystroglycan and laminin α2 expression.

Eleven patients had abnormal Western blotting for glycosylated α-dystroglycan, in addition to abnormal immunofluorescence, and hence are likely to have primary defects in the glycosylation of α-dystroglycan. Ten patients had proximal muscle weakness and one generalized weakness; only three patients were noted to have prominent calves. Five had impaired intellect and two of eight patients in whom neuroimaging was performed had abnormalities detected (one had white matter changes and normal brain structure and the other had cerebellar cortical cysts, a dysplastic superior vermis, hypoplastic pons, large irregular ventricles, polymicrogyria of frontal lobes, and frontal white matter changes). One patient is known to have cataracts, one thin atrophic retinas, and one ophthalmoplegia. Three of eight patients in whom cardiac evaluation was performed had cardiac conduction abnormalities with normal echocardiograms; only one of nine patients to date requires nocturnal ventilation. Nine patients have elevated CK levels. The one patient (Patient 63) with mutations in the *FKRP* gene has progressive weakness with loss of ambulation and development of a severe scoliosis in the teenage years. Intellect is normal. CK was markedly elevated (4,100 U/L, normal <200 U/L). At 35 years of age, she has myopathic facies, dysarthria, and tongue and calf hypertrophy. No neuroimaging has been performed.

Collagen VI abnormalities. Collagen VI is a ubiquitous extracellular matrix protein found adjacent to the sarcolemmal membrane and in the interstitial tissue between the muscle fibers and fascia of skeletal muscle.[33] Abnormalities in collagen VI expression are associated with UCMD and Bethlem myopathy (MIM 158810). Twelve patients (12%) had severe to moderately abnormal collagen VI staining (figure 3), including one patient who was also abnormal for glycosylated α-dystroglycan. Collagen VI mutational analysis was able to be performed in 9 of the 12 patients, and pathogenic collagen VI mutations were identified in 8 patients. One patient had a homozygous single base deletion in *COL6A1* c.1660delG that led to the introduction of a premature stop codon, and one patient had compound heterozygous mutations in *COL6A2,* c.1855_1860 del6 (p.V619_I620del2) and c.1771-1 G>T that led to skipping of exon 24, a frame shift and the introduction of a premature stop codon. In both cases the parents were heterozygous carriers of the mutations. Six patients had heterozygous dominant mutations within the triple helical domain, *COL6A1* c.887 G>T (p.G296V), *COL6A2* c.785 G>A (p.G262D), *COL6A3* c.6284 G>T (p.G2095V), *COL6A3* c.6210 + 1 G>A led to skipping of exon 16,[23] *COL6A2* c.801 + 2 T>C caused skipping of exon 5, and *COL6A2* c.954 G>T led to skipping of exon 9. Collagen VI mutational analysis was not performed in the patient

Figure 2 **Immunofluorescent staining and Western blotting**



(A) Immunofluorescent staining of muscle biopsies from four patients and control with antibodies to spectrin and glycosylated α-dystroglycan demonstrating variation in the glycosylated α-dystroglycan staining patterns observed. Control muscle demonstrates normal glycosylated α-dystroglycan localization and intensity at the muscle fiber membrane. Absent glycosylated α-dystroglycan and normal spectrin straining is demonstrated in Patient 30 (refer to patient details in table e-1) with very dystrophic muscle. Patients were classified with a severe reduction in glycosylated α-dystroglycan when the staining was very patchy and reduced in intensity (Patient 101). Patchy and reduced glycosylated α-dystroglycan staining (Patient 67) was classified as a moderate reduction. The last panel demonstrates a mild reduction in glycosylated α-dystroglycan (Patient 36), where fibers are not patchy, but all show a reduction in staining intensity and a more diffuse distribution at the membrane. Scale bar is 100 μm. (B) Immunofluorescent staining of muscle biopsies from control (C) and Patient 63 (P_{63}) in whom mutations in *FKRP* were identified. Spectrin staining in the patient is similar to control staining, whereas glycosylated α-dystroglycan is patchy and reduced compared to control. Scale bar is 100 μm. (C) Western blotting of glycosylated α-dystroglycan and laminin α2 from Patient 63 compared with control. Patient 63 has primary mutations in the *FKRP* gene with absent glycosylated α-dystroglycan and a secondary reduction of laminin α2. β-Dystroglycan shows equal loading of total muscle protein. *Cross-reactivity with myosin by primary antibody.

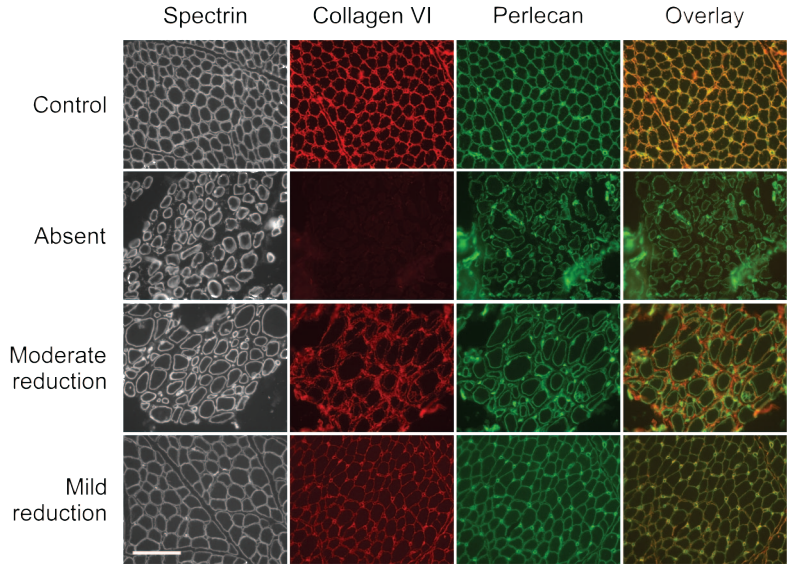
with abnormalities in glycosylated α-dystroglycan and collagen VI due to insufficient specimen, and the patient was lost to follow-up, preventing the attainment of a further specimen for analysis.

Eleven of the 12 patients with a severe reduction in collagen VI had dystrophic histopathology and one patient had a myopathic biopsy. Eight out of 10 patients in whom CK was measured had a mildly increased CK level (1.5—4× upper limit of normal). Ten patients presented in the neonatal period with developmental dysplasia of the hip, contractures, arthrogryposis, and decreased muscle bulk. Eight patients had a papular ("sandpaper") rash, prominent heels, or prominent ears. Eleven of the patients had a clinical phenotype consistent with a clinical diagnosis of UCMD.[2]

Laminin α2 abnormalities. Laminin α2 (also known as merosin) is an essential component of the structural scaffolding of the basement membrane in normal skeletal muscle.[34] Abnormalities of laminin α2 are associated with congenital muscular dystrophy, either as the result of primary recessive mutations in *LAMA2* (MDC1A) or secondary to CMD with abnormally glycosylated α-dystroglycan (see above). Eight of 101 patients (8%) had absent or severely reduced immunofluorescence for laminin α2, and absent or markedly reduced laminin α2 band on Western blot (table e-1),[35] consistent with primary abnormalities of laminin α2. To date, the *LAMA2* gene has been sequenced and primary mutations identified in four of these eight patients. There were 12 patients with normal laminin α2 staining, but abnormal laminin α2 by Western blot and often abnormal staining of the glycosylated form of α-dystroglycan. These 12 patients also had immunofluorescence performed with an antibody recognizing the 300 kDa epitope of laminin α2. All patients had normal staining, indicating that the laminin α2 abnormality seen on Western blot is likely to be secondary.

All patients had an abnormal EMG, an elevated CK level, and dystrophic muscle biopsy. Two patients had impaired intellect. Six of eight patients have had cranial MRI; four had white matter abnormalities, one patient had occipital pachygyria, and the remaining patient had possible polymicrogyria.

Figure 3 **Immunofluorescent staining of muscle biopsies from three patients with Ullrich congenital muscular dystrophy demonstrating the range of variation in collagen VI abnormalities compared to control**



Spectrin is used as a marker of membrane integrity and shows the dystrophic pattern of the muscle fibers. Collagen VI (red) is normally expressed adjacent to the muscle fiber membrane and in the interstitial tissue as part of the extracellular matrix. To determine collagen VI localization and intensity, the image is merged with a perlecan stain (green). Perlecan is a heparan sulfate proteoglycan and is expressed at the muscle membrane and also in the blood vessels. Absent collagen VI expression which is readily apparent with the single collagen VI stain and the overlay is seen in Patient 11. A moderate reduction of collagen VI is noted when collagen VI is present in the interstitium, but absent at the muscle membrane, this is best visualized with the overlay and is shown in Patient 98. A mild reduction in collagen VI is seen in the muscle of Patient 21, where collagen VI is localized correctly to the membrane and the interstitium, but generally reduced in intensity overall. Scale bar is 100 μm.

Unclassified patients and α7-integrin abnormalities. α7-Integrin is a transmembrane laminin receptor originally identified as a marker for skeletal muscle myogenesis.[36] We screened 45 patients for abnormalities in α7-integrin by immunofluorescence, and 31/45 patients exhibited altered α7-integrin expression; 12 patients had absent α7-integrin staining. SSCP analysis and sequencing of cDNA from the 12 patients with absent α7-integrin staining revealed no pathogenic changes in the coding region of the α7-integrin gene (*ITGA7*). Therefore, loss of α7-integrin is a common secondary manifestation in CMD so these patients were included in the unclassified group. We did not screen the remainder of the cohort for abnormalities in α7-integrin.

Patients who displayed mild generalized reductions in the intensity of staining for glycosylated α-dystroglycan or collagen VI by immunofluorescence (figures 2A and 3) were also included in the unclassified group. α-Dystroglycan was normally glycosylated by Western blot for all patients who had a mild reduction in glycosylated α-dystroglycan by immunofluorescence. All three collagen VI cDNAs were sequenced in 5 of 10 patients with mildly reduced collagen VI, and no mutations were identified. Thus we conclude that staining of mildly reduced intensity for these proteins is likely to be secondary.

Selenoproteinopathies. Mutations in *SEPN1* are associated with a distinct clinical phenotype—early and severe scoliosis and respiratory muscle involvement with relative preservation of limb strength.[37,38] Mutations in *SEPN1* have been reported in four histopathologic phenotypes—congenital muscular dystrophy with spinal rigidity (RSMD1, MIM 602771), multi-minicore disease (MIM 602771.0007 and 0.0008), desmin related myopathy with Mallory body-like inclusions (MIM 602771.0009), and congenital fiber-type disproportion.[25] We have previously identified eight patients with mutations in the selenoprotein N gene (*SEPN1*), one of whom satisfied inclusion criteria for this cohort.[25] We performed immunofluorescence on this patient (Patient 81) and staining with all antibodies was normal (table e-1). Based on clinical findings consistent with a *SEPN1* phenotype, we sequenced the selenoprotein N gene in nine additional patients in our cohort. We identified one other patient (Patient 27) who was homozygous for a previously reported *SEPN1* mutation (c.1397 G>A)[39] and who also had reduced staining for α7-integrin.

Consistent with our ascertainment based on clinical findings, both patients developed a severe scoliosis requiring surgical intervention and require nocturnal respiratory support. Both presented with hypotonia in the neonatal period, had normal early motor milestones, but had progressive weakness with loss of ambulation by the age of 10 years. One patient (Patient 27) had a dystrophic muscle biopsy and impaired intellect. The other patient (Patient 81) had nonspecific myopathic features on muscle biopsy and normal intellect.

DISCUSSION We have characterized a cohort of 101 patients using immunofluorescent staining, Western blotting, and DNA sequencing. Based on the immunofluorescent screen we were able to assign 45% of patients to an immunofluorescent classification. To date we have identified the primary genetic cause in 20 patients; an additional four patients are likely to have primary laminin α2 deficiency; thus we have reached a definitive diagnosis in 24/101 (24%). Once patients are classified according to abnormal antibody staining, we were able to reach a definitive diagnosis in 22/45 (49%), demonstrating the diagnostic utility of immunofluorescence. Our findings also suggest that mutation analysis of the glycosyltransferases could be further restricted to patients in whom glycosylated α-dystroglycan is abnormal by Western blot—although this needs to be confirmed in a larger series. Our ability to identify the primary genetic cause will improve as new forms of CMD are

identified, and immunofluorescent classification is used to guide future molecular analysis. A total of 38/68 (56%) of dystrophic biopsies were able to be assigned to a subgroup based on abnormal immunofluorescence findings, and 7/33 (21%) of all nondystrophic biopsies. Thus we confirm that screening for CMD should not only be restricted to patients with dystrophic pathology. Our data demonstrate the approximate distribution of the CMDs in the Australasian population of mixed ethnicity.

Patients with CMD with abnormal glycosylated α-dystroglycan immunofluorescence were the most common in our cohort (25%). Eleven of 19 patients tested (58%) had abnormally hypoglycosylated α-dystroglycan by Western blotting. The more severe the reduction in glycosylated α-dystroglycan staining, the more likely a patient was to have an abnormal Western blot. Our data suggest that immunofluorescence is more sensitive at detecting glycosylated α-dystroglycan abnormalities but that Western blotting may be more specific. Further studies with larger patient numbers are required to clarify this. Eleven patients in the unclassified group had mildly abnormal glycosylated α-dystroglycan; 10 of these patients on whom Western blotting was performed were normal for glycosylated α-dystroglycan. The mild alterations in glycosylated α-dystroglycan immunofluorescence may be nonspecific and due to the dystrophic process or secondary to mutations in as yet unknown genes encoding proteins that interact with α-dystroglycan in the dystrophin-associated glycoprotein complex. All patients with abnormal glycosylated α-dystroglycan staining were screened for mutations in the *FKRP* gene, and disease-causing mutations were identified in only one patient. Mutations in the *FKRP* gene are thus a rare cause of CMD in the Australasian population. Interestingly, our group has also shown that LGMD2I (due to mutations in *FKRP*) are not a common cause of limb girdle muscular dystrophy in Australia (2.5% compared to <30% in European populations).[39a]

In agreement with current literature,[8,23,40] we detected a number of collagen VI abnormalities by immunofluorescence, with 12/101 (12%) patients having severely to moderately reduced collagen VI. These patients presented with a strikingly similar UCMD phenotype. Nine of these patients were screened for collagen VI mutations and pathogenic changes were found in all but one. This demonstrates a high degree of correlation between typical UCMD clinical presentation, greatly reduced collagen VI in the muscle biopsy, and pathogenic mutations. Only two of the eight patients had recessive mutations, while six had heterozygous dominant mutations, consistent with reports suggesting that dominant mutations are common in UCMD.[23,41] Seven patients in the unclassified subgroup had mildly reduced collagen VI. Four of these patients were screened for collagen VI mutations, and no mutations were detected, suggesting that these changes are secondary to the dystrophic process or primary alterations in other interacting proteins.

Only 8% of the cohort demonstrated a severe reduction in laminin α2 staining, consistent with a primary laminin α2 abnormality. Early studies suggested that laminin α2 abnormalities accounted for 40% of all CMD cases in France and the United Kingdom,[42] but the frequency is now known to have regional variability.[43] Even so, abnormal laminin α2 is still often cited to be the most common single form of CMD.[7,8,18]

Previous studies have shown altered α7-integrin expression in patients with CMD.[44] A screen of 113 Japanese patients identified three patients lacking α7-integrin and who had mutations in the *ITGA7* gene.[16] To date these are still the only patients identified with definite primary integrin deficiency. A study of 210 Italian patients with undefined muscular dystrophies identified 17% of patients with reduced α7-integrin by immunofluorescence. One patient had a mutation in one allele of the α7-integrin gene; no mutations were detected in the other allele and no further mutations were identified in this cohort.[17] In our study, absent α7-integrin was seen in 12 patients; however, no *ITGA7* mutations were identified in the coding region of this gene. Together these studies suggest that *ITGA7* mutations in patients with undefined CMD are relatively rare but secondary deficiencies in α7-integrin expression are common; routine screening for α7-integrin abnormalities in patients with CMD is thus of limited utility.

We have defined the frequencies of different forms of CMD in a large cohort of diverse ethnicity and demonstrate the utility of immunofluorescent sorting of patients into categories to guide further protein and molecular analysis. Molecular studies for these disorders have major implications for genetic counseling, and highlight the importance of further studies into understanding the genetic basis and phenotypic variability of the CMDs.

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