Abnormalities of dystrophin, the sarcoglycans, and laminin α2 in the muscular dystrophies

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Abstract

Abnormalities of dystrophin, the sarcoglycans, and laminin α2 are responsible for a subset of the muscular dystrophies. In this study we aim to characterise the nature and frequency of abnormalities of these proteins in an Australian population and to formulate an investigative algorithm to aid in approaching the diagnosis of the muscular dystrophies. To reduce ascertainment bias, biopsies with dystrophic (n=131) and non-dystrophic myopathic (n=71) changes were studied with antibodies to dystrophin, α, β, and γ sarcoglycan, β dystroglycan, and laminin α2, and results were correlated with clinical phenotype. Abnormalities of dystrophin, the sarcoglycans, or laminin α2 were present in 61/131 (47%) dystrophic biopsies and in 0/71 myopathic biopsies, suggesting that immunocytochemical study of dystrophin, the sarcoglycans, and laminin α2 may, in general, be restricted to patients with dystrophic biopsies. Two patients with mutations identified in γ sarcoglycan had abnormal dystrophin (by immunocytochemistry and immunoblot), showing that abnormalities of dystrophin may be a secondary phenomenon. Therefore, biopsies should not be excluded from sarcoglycan analysis on the basis of abnormal dystrophin alone. The diagnostic yield was highest in those with severe, rapidly progressive limb-girdle weakness (92%). Laminin α2 deficiency was identified in 5/131 (4%) patients; 2/5 patients presented after infancy, indicating that abnormalities of laminin α2 are not limited to the congenital muscular dystrophy phenotype. Overall patterns of immunocytochemistry and immunoblotting provided a guide to mutation analysis and, on the basis of this study, we have formulated a diagnostic algorithm to guide the investigation of patients with muscular dystrophy.

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Keywords: muscular dystrophy; dystrophin; sarcoglycan; laminin α2

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The muscular dystrophies are a clinically and genetically heterogeneous group of disorders unified by the presence of "dystrophic" changes on muscle biopsy, defined as increased fibre size variability, increased connective tissue, and the presence of degenerating and regenerating fibres. They are broadly divided on the basis of age of onset and pattern of weakness into those with onset of weakness at birth (congenital muscular dystrophy) and those with later onset limb-girdle weakness, the X linked dystrophinopathies (Duchenne and Becker muscular dystrophy), and the limb-girdle muscular dystrophies (LGMD). The congenital muscular dystrophies (CMD) are traditionally further classified by the presence or absence of central nervous system involvement. The clinical course of those with limb-girdle weakness ranges from the severe phenotype typical of Duchenne muscular dystrophy (DMD), with onset in the first decade and rapid progression to loss of ambulation in the second decade, to milder forms with later onset and slower progression, such as in Becker muscular dystrophy (BMD).[1]

The cloning of the DMD/BMD gene at Xp21 and identification of its protein product, dystrophin, led to the isolation of a complex of associated proteins, the sarcolemmal "dystrophin associated proteins" (sarcoglycans, dystroglycans, and syntrophins) and laminin α2 in the extracellular matrix (fig 1).[2 3] Mutations in the genes encoding α, β, γ, and δ sarcoglycan have recently been described in autosomal recessive limb-girdle muscular dystrophy.[4 6] Mutations in the LAMA2 gene encoding the laminin α2 chain of merosin have been identified in a subset of "pure" congenital muscular dystrophy without clinical central nervous system involvement, but with characteristic changes in the white matter on cerebral MRI.[7–9] There has, to date, been no disease identified resulting from primary abnormalities of the dystroglycan or syntrophin complexes, although there is a recent report of a patient with muscular dystrophy and isolated β dystroglycan deficiency.[10] The classification of the muscular dystrophies has recently been restructured according to the primary gene defect.[11]

Initial studies to determine the frequency of sarcoglycan and laminin α2 abnormalities and the associated phenotypes have been limited by patient selection bias, either at the clinical or histopathological level. For example, the initial search for mutations in components of the sarcoglycan complex was restricted to patients with a severe LGMD phenotype; however, subsequent reports confirm considerable inter- and intrafamilial heterogeneity.[12 13] Similarly, the search for patients with laminin α2 deficiency focused on patients with "pure" CMD, and although patients with later onset disease resulting from laminin α2 deficiency have been identified, there have been no systematic studies to provide an estimate of frequency.[9 14 17]

The most comprehensive study to date is that of Duggan *et al*,[18] in which myopathic biopsies from 556 patients (Italian and American) were studied. Patients were excluded if

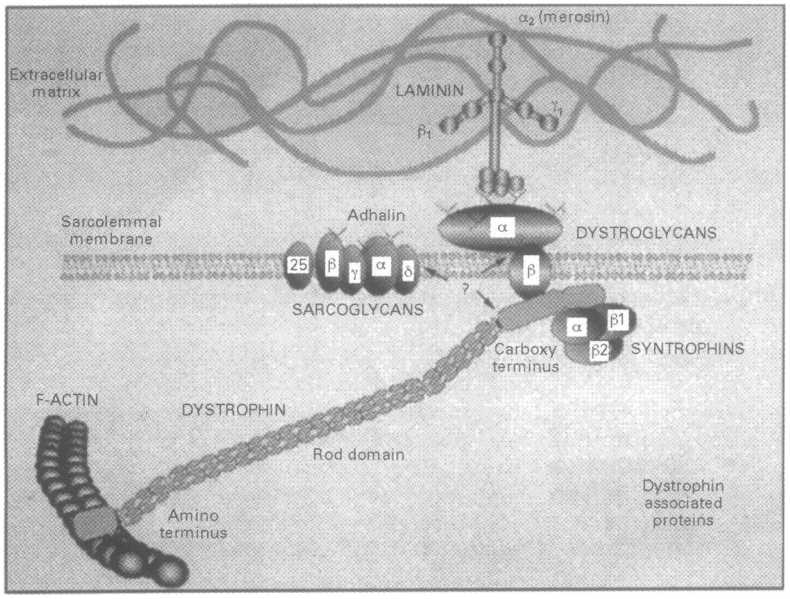

Figure 1 Proposed model of the dystrophin associated proteins. The dystrophin associated protein (DAP) complex can be divided into two main subcomplexes: the dystroglycans and the sarcoglycans. The sarcoglycan complex is composed of at least five transmembrane proteins: α (adhalin), β, γ, and δ sarcoglycan, and a 25 kDa protein. The dystroglycan (DG) complex is composed of α-DG and β-DG; β-DG is a transmembrane protein which forms the link between the cysteine rich and carboxy terminal domains of dystrophin within the cell, and α-DG is extracellular and binds to laminin α2 (merosin) in the basal lamina. Disruption of one component of the complex leads to loss of the integrity of the complex as a whole. It has been suggested that defects in this complex disrupt the binding of the extracellular matrix to the cytoskeleton, rendering the muscle fibre membrane unstable and resulting in cell damage and necrosis.[3] (Adapted from diagram by Alan Beggs (Beggs AH, De Girolami U. Skeletal muscle. In: Silverberg SG, De Lellis R, Frable WJ, eds. Principles and practice of surgical and cytopathology. 3rd ed. New York: Churchill Livingstone, in press. Fig 24-7B.)

dystrophin was abnormal (on the basis of immunocytochemistry and immunoblot), and laminin α2 was not studied. Ten percent (54/556) had abnormal immunocytochemical staining with antibodies to α sarcoglycan and, of these, 29/50 (58%) had detectable mutations of the sarcoglycan genes (α-SG (17), β-SG (8), γ-SG(4)). Vainzof *et al*[19] and Stec *et al*[20] estimated that among sporadic male patients with severe LGMD, 8-12% and 11.8% respectively carry autosomal rather than X chromosome mutations. Hayashi *et al*[21] found 5/208 (2%) Japanese muscular dystrophy patients had deficiency of α sarcoglycan with normal dystrophin. The reported frequency of laminin α2 deficiency among patients with "pure" congenital muscular dystrophy varies; American[22] and Japanese[23] studies found 1/12 (4.2%) and 2/34 (5.9%) patients respectively were laminin α2 deficient, whereas in the French[7] and British[24] experience, 13/20 (65%) and 11/24 (46%) respectively were laminin α2 negative.

In this study, we aim to characterise the nature and frequency of abnormalities of dystrophin, the dystrophin associated proteins, and laminin α2 in an Australian population. To address some of the limitations of previous studies, particularly related to ascertainment bias, we studied all available dystrophic muscle biopsies, as well as those with non-dystrophic myopathic changes. We have not limited study of the sarcoglycans to those biopsies with normal dystrophin. In studying patients with a wide range of biopsy findings and clinical phenotypes, we aim to formulate an investigative algorithm to aid in approaching the pathological diagnosis of abnormalities of dystrophin and associated proteins, and to determine the indications for immunocytochemical studies, immunoblotting, and gene mutation analysis.

Patients and methods

PATIENTS

Patients were ascertained retrospectively and prospectively through archived muscle biopsy material (1979-1996) at the University of Sydney, Sydney Children's Hospital, and Royal Alexandra Hospital for Children, major reference laboratories in the state of New South Wales for muscle disease. There were no limitations with respect to the age of the patient at biosy. Biopsies were divided into two groups: (1) "dystrophic" biopsies (as defined above) and (2) biopsies with non-dystrophic myopathic changes. Biopsies were excluded if there was an alternative clinical diagnosis (for example, myotonic dystrophy or facioscapulohumeral dystrophy), or the muscle pathology was strongly suggestive of an alternative diagnosis (for example, neuropathy, necrotising or mitochondrial myopathy, or specific ultrastructural changes such as central cores). Clinical data were collected from medical records, supplemented by clinical assessment where possible.

ANTIBODIES

The following antibodies were used in this study: monoclonal dystrophin (NCL-DYS1 [1:1], NCL-DYS2[1:2], NCL-DYS3[1:1], Novocastra) for immunocytochemistry, polyclonal antibody to the rod domain of dystrophin (DYS6-10[1:1000] provided by C Bonneman and L Kunkel) for immunoblotting, monoclonal α sarcoglycan (NCL-50DAG[1:50] Novocastra), affinity purified β and γ sarcoglycan (β-SG[1:50], γ-SG[1:1000]), β-DG[1:100] (provided by C Bonneman and L Kunkel), and laminin α2 (merosin) antibodies (MAB1922 [1:2000] Chemicon).

IMMUNOCYTOCHEMISTRY

All muscle biopsies were frozen in isopentane, pre-chilled with liquid nitrogen. Indirect immunofluorescence staining was applied to cryostat sections of 8 μm. Sections were incubated with primary antibodies in phosphate buffered saline (PBS) with 10% fetal calf serum for two hours at room temperature and washed with PBS. Secondary antibodies, FITC conjugated or Cy3 conjugated (Jackson ImmunoResearch Laboratories Inc, Westgrove, PA), were applied and incubated for 30 minutes at room temperature followed by three washes with PBS. Sections were mounted and sealed with Immumount (Shandon, Pittsburgh, PA) and examined and photographed with a Leica DMRBE fluorescence microscope. Staining was graded as negative (-), patchy (+ - ++), decreased but continuous (↓), or normal (N). Photographs were taken with Ektachrome P1600 film under identical conditions with the same exposure time and developed at 800 ASA.

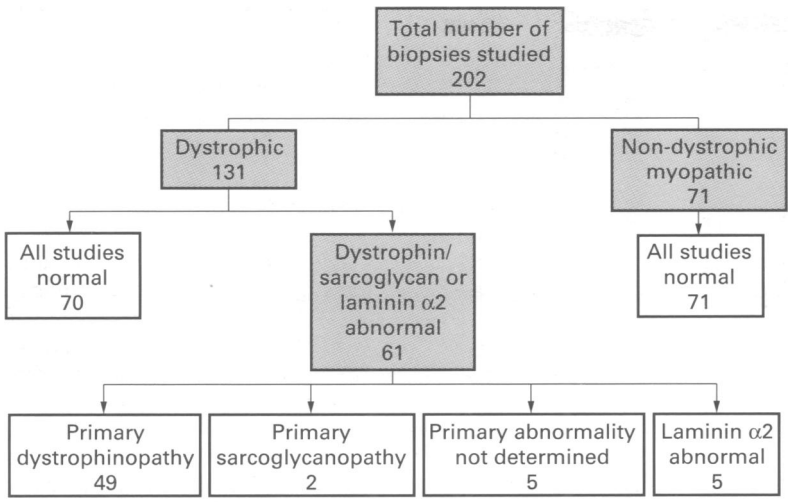

Figure 2 Results: overview.

IMMUNOBLOTTING

Western blots of biopsied skeletal muscle were prepared as described[25] to quantitate dystrophin and laminin α2. The following modifications were made to the published protocol: cryosections (10 × 4 μm) from biopsied skeletal muscle were collected in pre-chilled eppendorf tubes. Samples were vortexed in 100 μl of loading buffer (0.125 mol/l Tris-PO_4, pH 6.8, 5% sodium dodecyl sulphate (SDS), saturated bromophenol blue, 20% glycerol, 0.1 mol/l DTT, 0.02 mol/l EDTA, 3 mg/ml aprotinin, 2.5 mg/ml leupeptin, 1 mg/ml pepstatin, and 40 mg/ml PMSF), heated for five minutes, and stored at −20°C; 7.5% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) gels were used for dystrophin and laminin α2 assay. Twenty-five micrograms of total protein were loaded in each lane. Protein concentrations of muscle lysates were estimated using a colorimetric assay for staining by amido black[26] and equal loading was confirmed by staining of the gel with Coomassie blue. After transfer to the nitrocellulose, the blots were incubated with primary antibody for two hours. Primary antibodies were detected using the ECL chemiluminescence western blotting kit (Amersham, Bucks, UK), followed by exposure to Kodak XAR film for periods of 30 seconds to 15 minutes. Dystrophin and laminin α2 assays were designated normal, decreased in size/ amount, or negative.

Results

We studied a total of 202 muscle biopsies, 131 with dystrophic changes and 71 with non-dystrophic, non-specific myopathic changes (fig 2). The average age at biopsy of the patients was 13.7 years (range 0-73 years) with a median age of 5 years. To control for artefact in the immunocytochemical studies, we concurrently studied 30 biopsies in which there were no ultrastructural changes identified by light or electron microscopy, and all stains were normal.

LABORATORY DATA

Abnormalities of dystrophin, the sarcoglycans, or laminin α2 were present in 61/131 (47%) dystrophic biopsies and in 0/71 non-dystrophic myopathic biopsies. Primary dystrophinopathy accounted for 49/61 (80%); patients with abnormal dystrophin on immunocytochemical staining were considered to have a primary dystrophinopathy if they had (1) detectable deletion of the dystrophin gene, (2) clear pedigree evidence of X linked inheritance, (3) a mother with raised CK, or (4) complete absence or altered size of dystrophin on immunoblot. The sarcoglycans were secondarily abnormal, with patchy (discontinuous) staining in 87% of patients with a primary dystrophinopathy. In 7/61 (11%) patients, staining with antibodies to dystrophin or the sarcoglycans or both was abnormal or patchy, but did not satisfy the above criteria for a primary dystrophinopathy (table 1). Three of these patients had abnormalities suggestive of a primary sarcoglycanopathy (patients 1-3) with marked reduction or absence of all components of the sarcoglycan complex (fig 3). Dystrophin staining was markedly abnormal in 2/3 patients (patients 1 and 2) by immunocytochemistry, and of normal size but reduced in quantity on immunoblot. In both cases, mutations in γ sarcoglycan have been identified (C Bonnemann, personal communication). Patient 1 is heterozygous for a four base pair deletion in the splice donor site of intron 2 of the γ sarcoglycan gene and has a large scale deletion of the other allele. Patient 2 is homozygous for the same intronic splice site mutation of the γ sarcoglycan gene. No mutation has yet been identified in patient 3. In the remaining cases (patients 4-7) there was patchy

Table 1 Immunocytochemical results: abnormal dystrophin and associated proteins (excluding confirmed primary dystrophinopathy)

			Immunofluorescence									
	Patient's current age (sex)	*Pattern of weakness/clinical course*	*Dystrophin*			*Sarcoglycans*				*Laminin α2*	*Western blot*	
Patient No			*Dys 1*	*Dys 2*	*Dys 3*	*α-SG*	*β-SG*	*γ-SG*	*β-DG*		*Dystrophin*	*Diagnosis*
1(CS)	12 (F)	Limb-girdle (S)	++	↓	++	++	−−+	−	N	N	↓ amount, N size	γ-SG (mutation detected)
2 (MD)	9 (F)	Limb-girdle (S)	++	+	++	−−+	−−+	−	++	N	—	γ-SG (mutation detected)
3 (EA)	14 (F)	Limb-girdle (S)	N	N	↓	+	−	++	N	N	N	Probably primary sarcoglycanopathy
4 (SC)	33 (M)	Limb-girdle (M)	↓	N	↓↓	↓	↓	N	N	N	↓ amount, N size	Probably dystrophinopathy
5 (MG)	21(M)	Limb-girdle (M)	N	↓	−	↓	N	N	N	N	↓ amount, N size	Probably dystrophinopathy
6 (ME)	9 (M)	Limb-girdle (S)	−	+	+	++	+	++	N	N	↓ amount, N size	Probably dystrophinopathy/ ?sarcoglycanopathy
7 (MD)	19 (F)	Limb-girdle (S)	++	N	N	N	N	N	N	N	↓ amount, N size	?Dystrophinopathy/ ?sarcoglycanopathy

N = normal, - = negative, + and ++ = patchy (discontinuous) staining, ↓ = decreased staining, ↓ amount = decreased amount, N size = normal size, SG = sarcoglycan, β-DG = β-dystroglycan, CMD = congenital muscular dystrophy, (S) = severe limb-girdle weakness with rapid progression, (M) = milder limb-girdle weakness with slower progression.

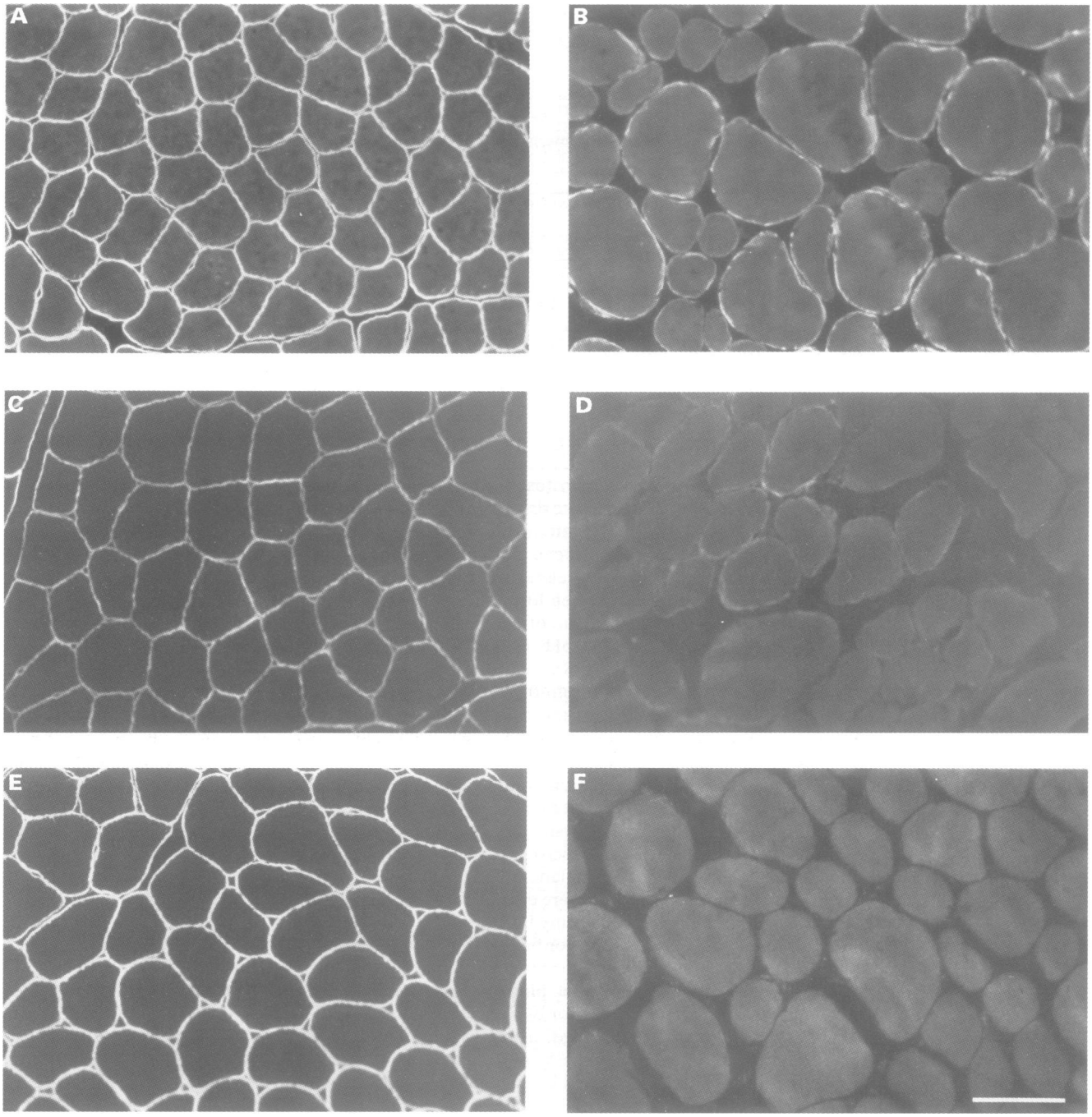

Figure 3 Immunocytochemistry of biopsied skeletal muscle from patient 1 (B, D, F), a 12 year old girl with primary γ sarcoglycanopathy and severe Duchenne-like muscular dystrophy, compared with a normal control (A, C, E). Sections of the biopsy were stained with antibodies to α sarcoglycan (A, B), β sarcoglycan (C, D), and γ sarcoglycan (E, F). γ sarcoglycan staining was completely absent and α and β sarcoglycan staining was markedly reduced and patchy. (Images examined under fluorescein microscopy at 20 × magnification; scale bar represents 100 μm.)

or discontinuous staining in dystrophin or sarcoglycans or both. In the absence of a family history suggesting X linked inheritance or detection of the primary gene mutation we were unable to classify these patients definitively.

Laminin α2 was abnormal in 5/131 (4%) dystrophic biopsies. In 3/5 patients (patients 8, 10, 11), laminin α2 was negative on immunoblot consistent with primary laminin α2 deficiency, and two patients (9, 12) had partial deficiency of laminin α2 (table 2). Patient 10, with absence of laminin α2, also had abnormal staining with dystrophin and α sarcoglycan. Laminin α2 staining was normal in all other patients with abnormal dystrophin or sarcoglycan staining.

CLINICAL DATA

Clinical data were available on 110/131 patients with dystrophic muscle biopsies (summarised in fig 4). Progressive limb-girdle weakness was present in 83/110 (75%) patients with dystrophic biopsies; of these, 52/83 (63%) had a severe phenotype (with onset of weakness in the first decade and rapid progression to loss of ambulation before 16 years), and in 31/83 (37%) the phenotype was milder with later onset and slower rate of progression. In 48/52 (92%) patients with the severe phenotype, there were abnormalities of dystrophin or sarcoglycans or both; 44/52 (85%) patients had confirmed primary dystrophinopathy (DMD), 3/52 had likely sarcoglycanopathy, 1/52 had likely dystrophinopathy, in 1/52 we were unable

Table 2 Immunocytochemical and clinical results: patients with abnormal laminin α2

Patient No	Current age (sex)	Immunofluorescence: Laminin α2	Dystrophin: Dys 1	Dystrophin: Dys 2	Sarcoglycans: Dys 3	Sarcoglycans: α-SG	Sarcoglycans: β-SG	Sarcoglycans: γ-SG	Western blot: Laminin α2	Clinical course	Cerebral MRI
8 (JD)	6 (F)	—	N	N	N	N	N	N	Negative	CMD	Typical white matter changes
9 (SM)	3 (F)	↓	N	N	N	N	N	N	↓ amount, N size	CMD, DD ?secondary to ICH	Typical white matter changes
10 (B-MD)	3 (F)	++	N	++	N	+−+ +	N	N	Negative	Non-progressive weakness	Typical white matter changes
11 (BD)	6 (M)	—	N	N	N	N	N	N	Negative	Non-progressive weakness	Typical white matter changes
12 (LR)	5 (F)	↓	N	ND	ND	N	N	N	↓ amount, N size	CMD, DD, seizures	Patchy white matter changes

N = normal, - = negative, + and ++ = patchy (discontinuous) staining, ↓ = decreased staining, ND = not done, ↓ amount = decreased amount, N size = normal size, SG = sarcoglycan, β-DG = β-dystroglycan, CMD = congenital muscular dystrophy, DD = developmental delay, ICH = intracranial haemorrhage.

to predict the primary abnormality, and in 3/52 patients all stains were normal. In contrast, only 8/31 (26%) of patients with the milder phenotype had abnormalities of dystrophin or sarcoglycans or both, five of these with definite primary dystrophinopathy (BMD) and two with likely primary dystrophinopathy.

Clinical data of the patients with abnormal sarcoglycans or dystrophin or both (excluding those with confirmed primary dystrophinopathy) are outlined in table 3. The two patients with confirmed primary γ sarcoglycan deficiency (patients 1 and 2) and patient 3 with probable primary sarcoglycanopathy had similar clinical presentations with a severe phenotype. All are female, white Australian, born to unrelated parents, presented between 6 and 9 years of age with progressive limb-girdle weakness, and remain ambulant over short distances only at 12, 9, and 14 years, respectively. CK was raised more than 25 times the upper limit of normal. Intellect, cardiac (ECG and echocardiogram), and respiratory function are normal to date, and cerebral MRI (performed in patient 1) was normal.

Nineteen of 110 (17%) patients with a dystrophic biopsy (for whom clinical data were available) fulfilled the diagnostic criteria for congenital muscular dystrophy, with presentation of hypotonia, weakness, or contractures within the first 6 months of life, and 1/19 had structural central nervous system involvement.[9 27] Laminin α2 deficiency was present in 3/19 (16%) patients with CMD (patients 8, 9, 12). Patients 8 and 9 had structurally normal brains on cranial MRI with characteristic white matter changes. Patient 9 has seizures and severe intellectual impairment; however, we believe this was on the basis of intracranial haemorrhage in the neonatal period. A mutation has been identified in one copy of the LAMA2 gene in this patient (P Guicheney, personal communication). Patient 12 has severe intellectual impairment, without eye abnormalities, and on cranial MRI there were patchy white matter changes and irregular

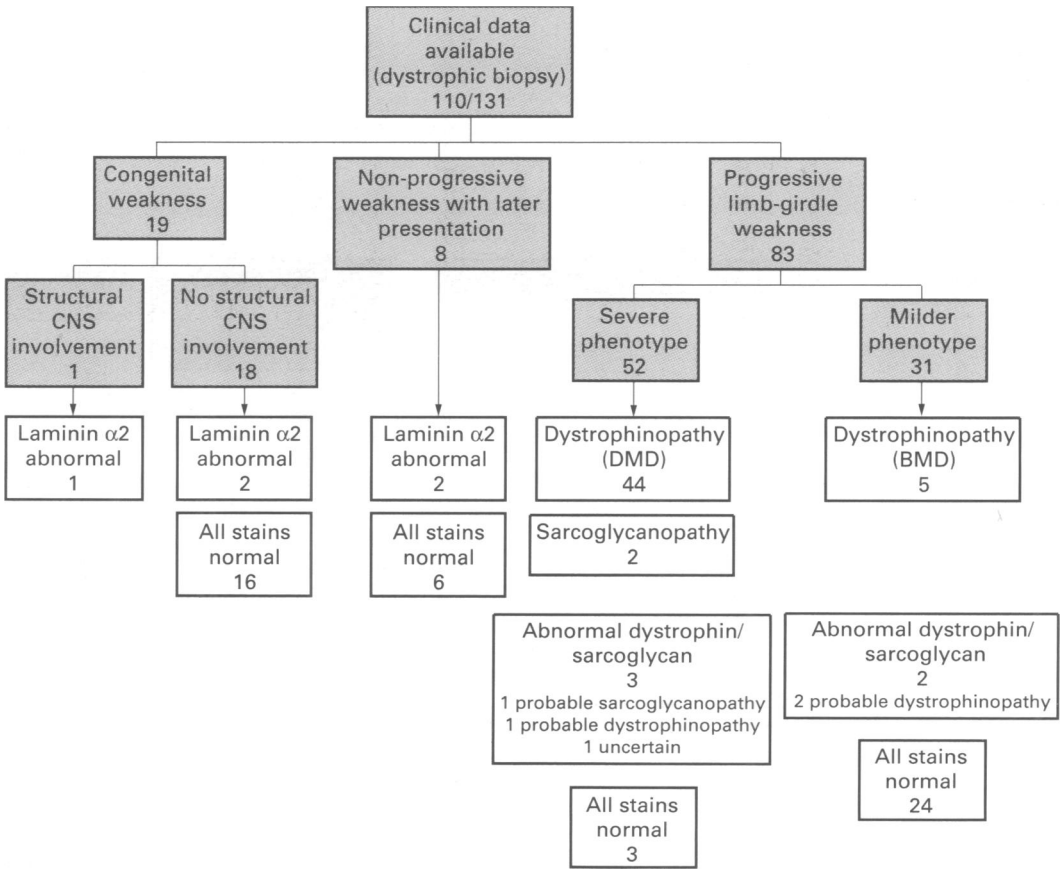

Figure 4 Clinical data: dystrophic biopsies. CNS=central nervous system.

Table 3 Clinical results: abnormal dystrophin and associated proteins (excluding primary dystrophinopathy)

Patient No	Current age (sex)	Pattern of weakness	Clinical course	Age at loss of ambulation	Highest serum CK (× normal upper limit) (age in y)	Calf hypertrophy	Cardiac involvement	Intellect	Other
1 (CS)	12 (F)	Limb-girdle	Rapid progression	Still ambulant	45× (12)	+	−	Normal	Short stature, scoliosis
2 (MD)	9 (F)	Limb-girdle	Rapid progression	Still ambulant	42× (8)	−	−	Normal	Asymmetrical weakness
3 (EA)	14 (F)	Limb-girdle	Rapid progression	Still ambulant	25× (10)	+	−	Normal	
4 (SC)	33 (M)	Limb-girdle	Slow deterioration	Still ambulant	15× (30)	−	−	Normal	Acromegaly
5 (MG)	21 (M)	Limb-girdle	Slow deterioration	Still ambulant	29× (17)	−	−	Normal	
6 (ME)	9 (M)	Limb-girdle	Rapid progression	Still ambulant	162× (9)	+	−	Normal	
7 (MD)	19 (F)	Limb-girdle	Rapid progression	13 years	140× (7)	+	−	Mild learning difficulties	

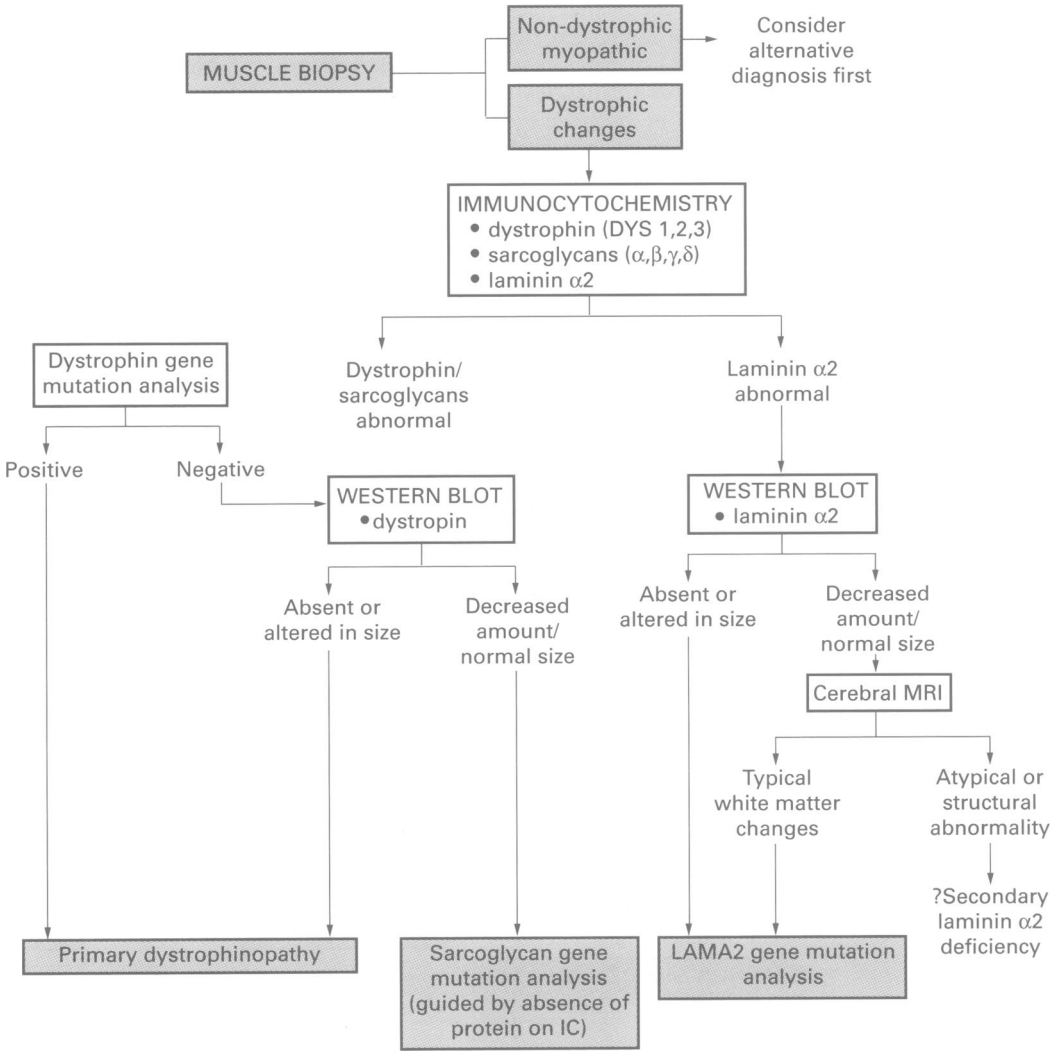

Figure 5 Investigative algorithm. The first line investigation for a male with limb-girdle weakness and raised CK is dystrophin gene mutation analysis, as this may immediately confirm the diagnosis of primary dystrophinopathy. Muscle biopsy should be performed if this is negative or to gain further prognostic information.

areas of pachygyria and polymicrogyria. Laminin α2 was present but reduced in quantity on immunocytochemistry and immunoblot, raising the possibility of secondary laminin α2 abnormality. These findings are consistent with Fukuyama congenital muscular dystrophy; the distribution of white matter changes does not correlate with those described as "occipital agyria" by van der Knaap *et al.*[28] CK was more markedly raised in the CMD patients with abnormal laminin α2 (range 2009-7121 U/l, normal <200 U/l, average 3750 U/l) than in the laminin α2 positive CMD patients (range 22-1710 U/l, average 324 U/l).

A subset of patients (8/110) presented after 6 months of age with non-progressive proximal or generalised weakness, with no abnormality noted in infancy. Two of these patients (patients 10 and 11) had abnormal laminin α2. Both patients have normal intellect with typical white matter changes on cerebral MRI. They presented at 2 and 4 years respectively with delayed motor milestones, lordotic, waddling gait, and use of a Gowers manoeuvre to rise from the floor. There has been no progression in weakness, and in patient 11 there has been some functional improvement; he is now able to rise from the floor without a Gowers

manoeuvre, his gait is almost normal, but his run remains slow and awkward.

Discussion

Abnormalities of dystrophin, the sarcoglycans, or laminin α2 were present in 61/202 (30%) myopathic muscle biopsies; however, abnormalities were only found in those with dystrophic changes. The diagnostic yield was therefore considerably increased when only dystrophic biopsies were considered (61/131, 47%). When further subdivided on the basis of clinical findings, abnormalities of either dystrophin, the sarcoglycans, or laminin α2 were present in 48/52 (92%) patients with severe limb-girdle weakness, compared with 8/31 (26%) patients with a milder phenotype, 3/19 (16%) with congenital muscular dystrophy, and 2/7 (29%) with non-progressive weakness. Our data suggest that sarcoglycanopathy in patients with myopathic biopsies is considerably less common in the Australian population (1.5%, 3/202) than in the American and Italian populations (10%).[18] However, we cannot directly compare the frequency of dystrophin and sarcoglycan abnormalities in our study with those of Duggan *et al*,[18] as although patients with a wide range of clinical phenotypes and muscle histology were included in their study, patients with dystrophic biopsies were not analysed separately, and patients with abnormal dystrophin were excluded from further sarcoglycan analysis.

Our results do not support the assumption that the diagnosis of a primary dystrophinopathy can be based exclusively on decreased or patchy staining with dystrophin antibodies and abnormalities on immunoblot. Secondary abnormalities of dystrophin staining were observed in three patients with sarcoglycanopathy (patients 1-3) and one patient with laminin α2 deficiency (patient 10), a finding previously noted by Vainzof *et al.*[12] In those patients with proven γ sarcoglycanopathy, dystrophin was abnormal on immunocytochemistry and immunoblot; staining was patchy and discontinuous, and the protein was of normal molecular weight but reduced in quantity in patient 1 and absent in patient 2. In contrast, patient 3 had absent staining with β sarcoglycan, patchy staining with α and γ sarcoglycan, and relatively normal dystrophin staining, a pattern suggesting a primary abnormality of another component of the sarcoglycan complex. The pattern of dystrophin abnormalities found in some primary sarcoglycanopathies is similar to that seen in the milder primary dystrophinopathies (BMD). Duggan *et al*[18] commented that "because biopsy specimens from patients with dystrophin gene abnormalities reveal secondary deficiencies of the sarcoglycans, testing for sarcoglycan proteins is informative only if dystrophin is normal", and therefore restricted sarcoglycan analysis to those patients with normal dystrophin staining. This may underestimate the incidence of primary sarcoglycanopathy, as the dystrophin abnormalities may be a secondary finding, and may have considerable genetic counselling implications.

All three patients with proven or probable sarcoglycanopathy had absent or markedly diminished staining with all components of the sarcoglycan complex. The complete absence of a protein on immunocytochemical staining may be used as a guide for determining the primary abnormality by mutation analysis, as suggested by Vainzof *et al.*[12] In patients 1 and 2 with primary γ sarcoglycanopathy, γ sarcoglycan staining was completely absent on immunocytochemistry and α and β sarcoglycan staining was markedly reduced and patchy. Our numbers of patients with proven primary sarcoglycanopathy are small, and it is difficult to make conclusions about genotype-phenotype correlation. However, both of our patients with γ sarcoglycan (patients 1 and 2) had normal intellect and normal cardiac and respiratory function in accordance with the clinical features described by Sewry *et al.*[29]

The frequency of laminin α2 abnormality in "pure" congenital muscular dystrophy without structural central nervous system involvement was 11% (2/18), higher than in the American (4.2%) and Japanese (5.9%) populations, but not approaching that seen in European studies (46-65%).[7 22-24] Two of five patients with abnormal laminin α2 presented after the first year of life with non-progressive generalised weakness; however, these patients may have had mild weakness from birth which was not noted. White matter changes were present on MRI, with a structurally normal brain. In both patients (10 and 11) there was no correlation between the severity of the phenotype and level of reduction of protein.

Conclusion

Immunocytochemical study of dystrophin, the sarcoglycans, and laminin α2 can, in general, be restricted to patients with dystrophic biopsies. However, there has been one patient with a primary sarcoglycanopathy in which the muscle pathology was myopathic rather than dystrophic, showing that there are always exceptions (C Bonnemann, personal communication). The diagnostic yield is highest in patients with a severe, rapidly progressive phenotype. Abnormalities of dystrophin should not be used to exclude patients from sarcoglycan analysis without confirmation of primary dystrophinopathy, as dystrophin abnormalities may be secondary. Immunocytochemistry and immunoblotting patterns can be used to guide mutation analysis, particularly in the sarcoglycanopathies. At this early stage of defining the characteristics of the sarcoglycanopathies, it is recommended that all available sarcoglycan antibodies are used in screening (α and β sarcoglycan, which are currently commercially available, γ and δ sarcoglycan). On the basis of this study, we have formulated an investigative algorithm to aid in approaching the diagnosis of abnormalities of dystrophin, the sarcoglycans, and laminin α2, particularly to determine the indications for immunocytochemical studies, immunoblotting, and gene mutation analysis (fig 5).

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