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ORIGINAL ARTICLE

Limb-Girdle Muscular Dystrophy in the United States

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Abstract

Limb-girdle muscular dystrophy (LGMD) has been linked to 15 chromosomal loci, 7 autosomal-dominant (LGMD1A to E) and 10 autosomal-recessive (LGMD2A to J). To determine the distribution of subtypes among patients in the United States, 6 medical centers evaluated patients with a referral diagnosis of LGMD. Muscle biopsies provided histopathology and immunodiagnostic testing, and their protein abnormalities along with clinical parameters directed mutation screening. The diagnosis in 23 patients was a disorder other than LGMD. Of the remaining 289 unrelated patients, 266 had muscle biopsies sufficient for complete microscopic evaluation; 121 also underwent Western blotting. From this combined evaluation, the distribution of immunophenotypes is 12% calpainopathy, 18% dysferlinopathy, 15% sarcoglycanopathy, 15% dystroglycanopathy, and 1.5% caveolinopathy. Genotypes distributed among 2 dominant and 7 recessive subtypes have been determined for 83 patients. This study of a large racially and ethnically diverse population of patients with LGMD indicates that establishing a putative subtype is possible more than half the time using available diagnostic testing. An efficient approach to genotypic diagnosis is muscle biopsy immunophenotyping followed by directed mutational analysis. The most common LGMDs in the United States are calpainopathies, dysferlinopathies, sarcoglycanopathies, and dystroglycanopathies.

Key Words: Calpain-3, Dysferlin, Dystroglycan, FKRP, Lamin A/C, Limb-girdle muscular dystrophy, Sarcoglycan.

INTRODUCTION

Among the hereditary disorders of muscle, Walton and Nattrass established limb-girdle muscular dystrophy (LGMD) as an entity distinct from other muscular dystrophies in 1954 (1). Clinically, the age of onset, rate of progression, and degree of muscle weakness vary across a broad range from adult onset with mild clinical impairment and indolent course to childhood onset with severe disability, sometimes early respiratory failure, and death. In 1995, the European Neuromuscular Centre Workshop established more precise criteria for diagnosis and classification of LGMD, grouping the different subtypes according to their genetic characteristics (2, 3). The designation for autosomal-dominant limb-girdle dystrophies is now LGMD1, whereas autosomal-recessive forms are LGMD2. Each distinct gene locus has a unique letter. Since 1995, the discovery of new genetic information has brought us to the current state of 15 LGMD loci for which 13 genes are delineated (Table 1 adapted from *Neuromuscular Disorders* (2006;16:64–65) and published online at http://www.musclegenetable.org.).

Numerous reviews and individual reports summarize the clinical and pathologic features of each specific LGMD subtype. Many reflect the experience at regional medical centers (1, 4–19). The publication of many studies was before our current knowledge of the molecular defects underlying the different subtypes of LGMD (1, 4–6), whereas others focus only on certain LGMD subtypes (20–23). Collectively, this published work suggests that autosomal recessive is much more common than autosomal-dominant LGMD. Among patients with LGMD2, types 2A (calpain-3), 2B (dysferlin), and 2I (fukutin-related protein) appear to be the more common subtypes. As a group, the

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TABLE 1. Classification of Limb-Girdle Muscular Dystrophy (LGMD)*

Designation	Locus	Gene	Protein	Key References
Autosomal dominant				
LGMD 1A	5q31	*TTID*	Myotilin	70
LGMD 1B	1q11–q21	*LMNA*	Lamin A/C	71
LGMD 1C	3p25	*CAV3*	Caveolin-3	72, 73
LGMD 1D	6q23			74
LGMD 1E	7q			75
Autosomal recessive				
LGMD 2A	15q15.1	*CAPN3*	Calpain-3	76
LGMD 2B	2p13	*DYSF*	Dysferlin	77, 78
LGMD 2C	13q12	*SGCG*	γ-sarcoglycan	79
LGMD 2D	17q12–q21.33	*SGCA*	α-sarcoglycan	80
LGMD 2E	4q12	*SGCB*	β-sarcoglycan	81, 82
LGMD 2F	5q33	*SGCD*	δ-sarcoglycan	83
LGMD 2G	17q12	*TCAP*	Telethonin	84
LGMD 2H	9q31–q34	*TRIM32*	E3-ubiquitin ligase	85
LGMD 2I	19q13	*FKRP*	Fukutin-related protein	46
LGMD 2J	2q31	*TTN*	Titin	86

*, Adapted from *Neuromuscul Disord* 2006;16:64–65; also periodically updated and published online at: http://www.musclegenetable.org.
TCAP is telethonin.

sarcoglycanopathies (LGMDs 2C, 2D, 2E, and 2F) are nearly as frequent.

Because most of these studies evaluated patients outside the United States and many represent populations with a high degree of consanguinity (1, 4–8, 21), we set forth to better understand LGMD as it exists in the United States. Accomplishing this end result requires a collaborative multicenter effort to enroll and study patients carrying the clinical diagnosis of LGMD. Although the evaluation of this patient population is ongoing, we report here the current immunophenotype and genotype data (as of May 31, 2006) for the 370 patients enrolled. The information provided derives from a broad geographic referral area and is important for a number of reasons. The U.S. healthcare system does not direct patients to centers of excellence that allow population-based analysis of disease epidemiology. Therefore, the data presented here is likely to provide the best current estimate of the hierarchy of distribution of LGMD subtypes and their genotypes in the United States. This information should enhance the ability of investigators representing academia, industry, and private foundations to plan clinical trials for LGMD, which, in fact, represents the driving force behind this effort originally directed by the Muscular Dystrophy Association. The LGMD population in the United States is highly relevant to the potential allocation of resources in view of the MD-CARE Act (Public Law 107-84) approved by Congress and signed into law by the President of the United States in 2001 pertaining to federally sponsored centers of excellence for muscular dystrophy research.

MATERIALS AND METHODS

Patient Population

Six university medical centers (Columbus Children's and The Ohio State University Hospitals, The University of Iowa, University of Rochester, Vanderbilt University, Washington University in St. Louis, and University of Pennsylvania) evaluated patients with LGMD referred from all parts of the United States. These patients were 2 years of age or older with onset of clinical disease after the neonatal period (defined as the first 4 weeks of life). Patients excluded were those with known dystrophinopathy (Duchenne and Becker muscular dystrophy) or a confirmed diagnosis of other neuromuscular diseases (e.g. myotonic disorder, facioscapulohumeral dystrophy [FSHD], congenital muscular dystrophy, congenital myopathy, inflammatory myopathy, glycogen or lipid storage myopathies, or mitochondrial myopathy). In some cases, patients with LGMD referred for evaluation showed prominent scapular winging or other features suggesting a diagnosis of FSHD, although they did not have facial weakness (24, 25). We enrolled these patients and performed DNA analysis for deletions at 4q35.

Clinical Evaluation

Each patient underwent a standardized history, medical record review, and clinical examination. Patients were evaluated for the following historical features: age, gender, race, ethnicity, marital status, age at onset, age at diagnosis, initial site of symptoms, parental country of origin, consanguinity, concurrent medical diseases, and present medications. Physical evaluation included height, weight, vital signs, general medical examination for involvement of other organ systems, manual muscle testing of 34 muscle groups (graded by a modified MRC scale) (26), and assessment for joint contractures. Functional tests of walking speed, stair climbing, arising from a chair, drinking, and pulmonary functions (forced vital capacity) were assessed along with global measures of upper and lower extremity function as previously described (26, 27). Serum creatine kinase and an electrocardiogram were obtained on each patient.

Muscle Biopsy Evaluation

A muscle biopsy was evaluated on every patient unless an affected relative's muscle was available or unless DNA testing confirmed the suspicion of FSHD. A total of 312 biopsies were evaluated. Frozen sections were evaluated centrally (Steve Moore at The University of Iowa). The analysis included standard hematoxylin and eosin (H&E) and immunostaining for dystrophin (rod domain and carboxy terminus), spectrin, utrophin, sarcoglycans (α-, β- , γ-, and δ-), dystroglycans (α- and β-), laminin-α2 (merosin), caveolin-3, emerin, dysferlin, and collagen VI (Table 2). Immunostains were analyzed by standard fluorescence microscopy, and the intensity of staining with each antibody was graded from zero (absent) to 3+ (normal expression). Control human skeletal muscle was included with patient material on each glass slide immunostained in the study. If the H&E histopathology suggested diagnoses other than LGMD, appropriate enzyme histochemistry or immunostaining was carried out.

In a subset of 121 patients with normal immunostaining for dystrophin, sarcoglycans, and emerin, frozen muscle was prepared for Western blotting to further evaluate dysferlin and calpain-3. Some of these samples were separately frozen at the time of biopsy, whereas others were residual muscle from the frozen block used for histology. This procedure involved homogenizing frozen muscle samples in 125 mM Tris-HCl, 4% SDS, 4 M urea, 5% β-mercaptoethanol, 10% glycerol, 0.005% bromophenol blue sample buffer at pH 6.8, which was then heated in a hot water bath and spun down at 3100 rpm for 5 minutes.

Samples were electrophoresed for 16 hours through 1.5-mm-thick SDS-polyacrylamide multiple biphasic gradient gels using Protean-II gel system (BioRad, Hercules, CA) as previously described (28). Migrated protein bands were transferred to 0.45-μm nitrocellulose membranes overnight at a constant 200 V maintained at 13.5°C. Post-transfer polyacrylamide gels were fixed and stained for total residual protein. Nitrocellulose membranes were blocked and incubated with primary (Table 2) and secondary antibodies in heat-sealed bags at room temperature for 1 hour.

Band quantification on scanned images of dried gels and membranes was performed using Phoretix 1D Advanced software from Nonlinear Dynamics, Ltd. (Newcastle, U.K.). Band intensity was normalized for total protein load established by residual myosin heavy chain on post-transferred gels. Standard curves were developed using Minitab Statistical software to identify statistical significant ($p < 0.005$) decreases in protein quantity using parallel 5-point dilution series of control samples with correlation coefficients greater than 0.95 between controls. Antibodies against β-dystroglycan were used as an internal control for each sample.

TABLE 2. Antibodies Used for Immunofluorescence and Immunoblotting

Antibody	Vendor*	Cat. Number	Clone	Immunofluorescence Dilution	Immunoblot Dilution
Dystrophin (rod domain)	Novocastra	NCL-DYS1	Dy4/6D3	Neat	1:50
Dystrophin (carboxy term)	Novocastra	NCL-DYS2	Dy8/6C5	NA†	1:50
Dystrophin (carboxy term)	BioGenex	AM2445m	Dy8/6C5	Neat	NA
Dystrophin (amino term)	Novocastra	NCL-DYS3	Dy10/12B2	NA	1:50
Utrophin	Novocastra	NCL-DRP1	DRP1/12B6	1:10	NA
Spectrin	Novocastra	NCL-SPEC1	RBC2/3D5	1:200	NA
α-dystroglycan‡			IIH6	1:200	NA
α-dystroglycan	Upstate	05-298	VIA4-1	1:25	NA
β-dystroglycan	Novocastra	NCL-b-DG	43DAG1/8D5	1:25	1:200
α-sarcoglycan (adhalin)§	Upstate	05-262	IVD3-1	1:200	NA
α-sarcoglycan (adhalin)§	Novocastra	NCL-a-sarc	Adl/20A6	1:200	NA
β-sarcoglycan	Novocastra	NCL-b-sarc	b-sarc/5B1	1:50	NA
γ-sarcoglycan	Novocastra	NCL-g-sarc	35DAG/21B5	1:50	NA
δ-sarcoglycan	Novocastra	NCL-d-sarc	d-sarc3/12C1	1:50	NA
Merosin (α2-laminin)§	Gibco BRL	12076-014		1:2000	NA
Merosin (α2-laminin)§	Novocastra	NCL-MEROSIN	Mer3/22B2	1:2000	NA
Caveolin-3	Becton Dickinson	610420	26	1:500	NA
Eemerin	Novocastra	NCL-EMERIN	4G5	1:100	NA
Dysferlin	Novocastra	NCL-HAMLET	Ham1/7B6	1:10	1:300
Dysferlin	Novocastra	NCL-HAMLET2	Ham3/17B2	NA	1:300
Calpain-3	Novocastra	NCL-CALP-2C4	Calp3d/2C4	NA	1:10
Calpain-3	Novocastra	NCL-CALP-12A2	Calp3d/12A2	NA	1:20
Collagen VI	RDI	RDI-600-401-108		1:50	NA

*, Vendor locations: Novocastra, distributed by Vision BioSystems Inc., Norwell, MA; BioGenex, San Ramon, CA; Upstate, Charlottesville, VA; Gibco BRL, Gaithersburg, MD; Becton Dickinson, San Jose, CA; RDI (Research Diagnostics, Inc.), Concord, MA.

†, NA, not applicable; the antibody was not used for either immunofluorescence staining or Western blotting.

‡, The IIH6 antibody was provided by Kevin P. Campbell.

§, Vendor changes were necessary in the midst of these studies as a result of availability of antibodies. Initial studies used the first listed source of this antibody.

Mutation Analysis

The analysis of peripheral blood or skeletal muscle genomic DNA for mutations in specific genes was primarily directed by the results of muscle biopsy protein findings in combination with clinical parameters such as family history or the presence of specific signs or symptoms. For example, a search for sarcoglycan gene mutations was undertaken in subjects whose biopsies demonstrated reduced sarcoglycan immunofluorescence staining. For disorders without a demonstrable skeletal muscle protein deficiency such as LGMD1B, *LMNA* gene mutations were sought in patients exhibiting unique phenotypic features such as cardiac disease, especially in combination with limb contractures. Atrial, atrioventricular, and ventricular dysrhythmias and dilated cardiomyopathy even without contractures were an indication for considering *LMNA* gene defects (29).

RESULTS

Population Demographics

Three hundred seventy patients have been enrolled representing 337 unrelated families. The patients' average age at enrollment was 36.4 years (range, 2–80 years; median age, 35.8 years) and females comprised 43.8% of the group. A total of 79 patients (23%) were 18 years or younger. Our overall population was 87% white, 5.0% black, 2.3% Asian-American, and 2.3% other racial groups. Three percent considered themselves Hispanic, but we did not collect data on the race of these individuals. However, according to U.S. Census data (http://www.census.gov/population/pop-profile/dynamic/RACEHO.pdf), Hispanics are 92% white, 4% black, and smaller percentages of other racial groups. Our patients were the product of consanguineous marriages in 3.7% of the cases.

Nonlimb-Girdle Muscular Dystrophy Diagnoses

Twenty-three patients had diagnoses other than LGMD despite their referral diagnosis of LGMD. Six patients had reduced dystrophin expression indicative of Becker muscular dystrophy. Of 13 patients with features suggestive of facial-sparing FSHD and tested for 4q35 deletions, 11 were confirmed to have FSHD. Absence of emerin by immunofluorescence led to DNA testing in 2 patients who were subsequently confirmed by genetic testing to have X-linked Emery-Dreifuss muscular dystrophy (EDMD). Two patients with selective merosin deficiency by muscle immunofluorescence likely have congenital muscular dystrophy (MDC1A) (30). One patient had inclusion bodies with appropriate staining qualities on H&E, modified Gomori trichrome, and desmin immunoperoxidase to make the diagnosis of myofibrillary myopathy. Hereditary inclusion body myopathy resulting from compound heterozygous mutations of UDP-*N*-acetylglucosamine 2-epimerase/*N*-acetylmannosamine kinase (GNE) was diagnosed in one patient because of typical clinical and histopathologic features (31). In none of the biopsies was collagen VI staining of muscle fiber basal laminae judged to be reduced.

Immunophenotypes—Limb-Girdle Muscular Dystrophy Diagnoses Based on Protein Expression

Biopsies from 312 patients were available and evaluated centrally (by Steve Moore). Of the 289 patients without a specific, non-LGMD diagnosis (see previous paragraph), 266 had muscle biopsies adequate to perform histopathologic and immunofluorescence evaluation. Biopsies were regarded insufficient for evaluation because of end-stage disease (6 cases), being entirely adipose tissue (5 cases), or being too small (12 cases). To date, 121 biopsies have also undergone Western blotting for calpain-3 and dysferlin (by Chris Shilling). Combining this data, we assigned immunophenotypes to patients based on protein deficiencies. Each diagnosis is based on the protein with greatest reduction on immunofluorescence staining of frozen sections, Western blot analysis, or both. Patients with calpainopathy have reduced to absent calpain-3 on Western blot; patients with dysferlinopathy have reduced to absent dysferlin on immunostaining or Western blot; patients with sarcoglycanopathy have reduced to absent immunostaining for one or more of the 4 sarcoglycans; and patients with dystroglycanopathy have reduced to absent immunostaining using glycosylation-dependent antibodies for α-dystroglycan. The distribution of immunophenotypes is 12% (n = 31) calpainopathy, 18% (n = 47) dysferlinopathy, 15% (n = 40) sarcoglycanopathy, 15% (n = 40) dystroglycanopathy, and 1.5% (n = 4) caveolinopathy (Table 3). Some patients (9% [n = 24]) have reduced expression of more than one protein.

Genotypes—Limb-Girdle Muscular Dystrophy Diagnoses Based on Mutational Analysis

Mutation analysis has established the genotype for 83 unrelated patients with LGMD (Table 4). Genotypes are distributed according to the following percentages: <1% myotilin (LGMD1A), 4% lamin A/C (LGMD1B), 6% calpain-3 (LGMD2A), 2% dysferlin (LGMD2B), 2% γ-sarcoglycan (LGMD2C), 4% α-sarcoglycan (LGMD2D), 4% β-sarcoglycan (LGMD2E), <1% δ-sarcoglycan (LGMD2F), and 4% fukutin-related protein (FKRP) (LGMD2I). Total sarcoglycanopathies constitute 11% of the total patients with LGMD.

Histopathology

Although these biopsies had a broad variety of histopathologic features, the majority was myopathic; 52% had active, necrotizing myopathic features, whereas another 36% were myopathic without ongoing myonecrosis or regeneration. Several biopsies had mixed neurogenic and myopathic features (8%) and 4% were considered to have no diagnostic histopathologic abnormalities. Among the more common immunophenotypes, active myonecrosis was present in 40% of the calpainopathies, 75% of the dysferlinopathies, 80% of the sarcoglycanopathies, and 70% of the α-dystroglycanopathies. Forty-three biopsies contained rimmed vacuoles, and 12 of these cases were classified as dysferlinopathies. Rimmed vacuoles were also seen in occasional biopsies from patients with calpainopathy, sarcoglycanopathy,

TABLE 3. Distribution of Immunophenotypes

Diagnosis*	Presumptive LGMD Subtype	Number of LGMD cases	Percent†	Concurrent Abnormal Protein Expression‡
Caveolinopathy	1C	4	1.5%	Calpain-3, dysferlin, α-dystroglycan
Calpainopathy§	2A	31§	12%§	Caveolin-3, dysferlin, α-dystroglycan
Dysferlinopathy‖	2B	47	18%	Calpain-3, caveolin-3, α-dystroglycan
Sarcoglycanopathy	2C, D, E, and F	40	15%	
Dystroglycanopathy	2I	40	15%	Calpain-3, caveolin-3, dysferlin, merosin

*, Diagnosis is based on the protein with greatest reduction on immunofluorescence staining of frozen sections, Western blot analysis, or both.
†, The denominator for calculating percentage is the total number of biopsies from unrelated patients with sufficient muscle to undergo immunofluorescence and/or Western blot analysis. This number is 266.
‡, Nine percent (n = 24) of the biopsies evaluated by immunofluorescence or Western blot had partial reduction in the expression of other proteins.
§, To date, 121 of these 266 biopsies have also undergone Western blot analysis. Because calpain-3 can only be evaluated on Western blotting, the number of calpainopathies identified so far in our study population is almost certainly underrepresented.
‖, Patients classified as dysferlinopathy have sarcolemmal immunostaining for dysferlin scored as zero or 1+ with or without cytoplasmic immunostaining.
LGMD, limb-girdle muscular dystrophy.

and dystroglycanopathy. Ragged-red fibers were noted in 15 biopsies, and 7 of these were dysferlinopathies.

Immunophenotype–Genotype Correlations

Protein deficiency was highly predictive of genotype in many of the patients studied to date. The best correlation between protein deficiency and genotype is among the sarcoglycanopathies, in part as a result of the much more complete status of the mutational analysis. Extensive molecular evaluation is complete in 37 of the 40 families with sarcoglycan deficiency detected in a biopsy by immunofluorescence. Thirty-four of these 37 families (92%) have had sarcoglycan mutations identified. Furthermore, the pattern of sarcoglycan expression is predictive of the sarcoglycan mutation in many cases. For instance, selectively greater reduction of γ-sarcoglycan in the biopsy was predictive of a γ-sarcoglycan mutation in 7 of 7 cases. In fact, no γ-sarcoglycan was detected by immunofluorescence in any of these cases, whereas the remaining sarcoglycans were reduced to varying degrees. A relatively greater reduction of α-sarcoglycan predicted mutations in the corresponding gene in 8 of 12 cases. Near absence (scored 1+) or absence (scored zero) of all 4 sarcoglycans was seen in 2 of 12 patients with α-sarcoglycan mutations and 8 of 13 patients with β-sarcoglycan mutations.

The predictive value of immunostaining and/or immunoblotting for caveolin-3 (LGMD1C) remains to be determined. We have no caveolin-3 mutational data. However, this protein is reduced in the biopsies of 8 of our patients; 5 of the 8 have additional partial protein deficiencies (including calpain-3, dysferlin, or merosin) or abnormal staining (α-dystroglycan).

Of the 31 patients with a calpain-3 deficiency discovered by Western blot, 22 have undergone *CAPN3* mutation analysis and 17 have mutations (77%). One additional patient is heterozygous for a *CAPN3* missense mutation but has normal protein expression by Western blot.

The dysferlinopathies present a more complex situation, because abnormalities in dysferlin immunostaining are very common in myopathic disease (32). For purposes of this study, we classified patient biopsies as dysferlinopathy only if sarcolemmal staining was zero or 1+ with or without cytoplasmic staining. Milder reductions in sarcolemmal dysferlin accompanied by cytoplasmic, immunoreactive dysferlin were considered less likely to predict *DYSF* mutations, because this pattern of dysferlin staining has been seen in Duchenne and Becker muscular dystrophy, several types of LGMD, and inflammatory myopathies (32). In our small sample of 6 patients with LGMD2B with both biopsy and DNA data, all six had zero to 1+ sarcolemmal immunofluorescence and only one had cytoplasmic staining. Dysferlin Western blotting has been completed on 4 of these same patients; 2 had a complete deficiency, whereas 2 appeared normal. In one of these latter cases, a missense

TABLE 4. Distribution of Genotypes

LGMD Subtype	Number of Cases	Percent*
LGMD1A†	1	<1%
LGMD1B‡	12	4%
LGMD2A§	17	6%
LGMD2B‖	6	2%
LGMD2C¶	7	2%
LGMD2D¶	12	4%
LGMD2E¶	13	4%
LGMD2F¶	2	<1%
LGMD2I**	13	4%

*, The denominator for calculating the percentage of each mutation-proven LGMD subtype is the total number of unrelated patients with LGMD enrolled in the study. This number is 297 (289 with biopsies + 8 without biopsies). To date, only 179 of these 297 patients have undergone DNA analysis; thus, the number (and percentage) of most LGMD subtypes identified so far in our study population is almost certainly underrepresented. The exceptions to this underrepresentation are the sarcoglycanopathies. By virtue of the high predictive value of immunofluorescence and the availability of DNA testing, 37 of the 40 unrelated patients with biopsy-proven sarcoglycanopathy have undergone complete DNA analysis. Thirty-four of the 37 were found to have mutations. Thus, the percent distribution of LGMD 2C, 2D, 2E, and 2F is likely to be accurate. Mutation-proven sarcoglycanopathies collectively represent 11% of the patients with LGMD in our population.
†, Patient belongs to family reported in *Hum Mol Genet* 2000;9:2141–47 (70).
‡, *LMNA* mutational analysis was carried out by C. A. Brown.
§, *CAPN3* mutational analysis was carried out by C. Serrano and D. Darvish.
‖, *DYSF* mutational analysis was carried out by M. Rosario dos Santos, Instituo de Genética Médica Jacinto Magalhães, Portugal.
¶, Sarcoglycan mutational analysis was carried out by C. Stolle.
**, *FKRP* mutational analysis was carried out by D. E. Michele, A. Stence, and T. Winder.

mutation was present on only one allele (the second mutation not found). We presume that this patient was unable to localize dysferlin to the sarcolemma. The other patient with a normal Western blot harbored a compound heterozygous missense dysferlin mutation but nevertheless was able to localize some of the mutant protein to the membrane (1+ staining).

To date, 35 of the 40 patients with reduced α-dystroglycan staining using glycosylation-dependent antibodies (IIH6 and VIA4-1) have undergone FKRP gene sequencing. Only 13 of these 35 patients (37%) have confirmed FKRP mutations. Thus, additional genotypes may be represented among this population of patients with LGMD with hypoglycosylated α-dystroglycan.

DISCUSSION

This report summarizes immunophenotypic information gleaned from a racially and ethnically diverse population of U.S. patients presenting with LGMD. Previous publications have determined the prevalence of LGMD to vary widely: at least 8.1 per million in a nationwide study in The Netherlands (5), 48 per million in the highly inbred Reunion Island population (21), 69 per million in the geographically isolated Basque Country of Spain (7), and 20 to 40 per million in a worldwide survey (33). Nigro suggests there is a collective 65 per million prevalence of LGMD2 (16). Because our study is not a complete nationwide ascertainment of patients with LGMD, we were unable to generate prevalence data. However, our data do provide information about the relative proportion of LGMD subtypes in the United States. Among our 266 unrelated patients with LGMD with biopsy data, dysferlinopathies represented the largest subgroup (approximately 18%) followed closely by dystroglycanopathies (approximately 15%), sarcoglycanopathies (approximately 15%), and calpainopathies (approximately 12%). Those with lamin A/C (*LMNA*) mutations were the next most common confirmed subtype (approximately 4% of all unrelated patients).

The relative distribution of LGMD subtypes has been estimated for several other LGMD patient populations. Among Brazilian patients with LGMD, dysferlinopathies account for approximately 14% (24 of 166 patients) and sarcoglycanopathies for 20% (23 of 115 patients) (34–36). The immunophenotypic distribution reported among Italian patients is quite varied. In a recent review, Angelini reports that approximately 30% of northern Italy patients with LGMD are calpainopathies, 20% dysferlinopathies, and 15% sarcoglycanopathies (17). Protein screening data of 191 patients with LGMD from this same group showed 53 (28%) with reduced to absent calpain-3 but only 2 (1%) with reduced or absent dysferlin (13). These 191 patients with LGMD were known to have normal dystrophin, merosin, and sarcoglycan expression. For another Italian cohort, Nigro provides no hard data but suggests a distribution of approximately 35% calpainopathy, <10% dysferlinopathy, 10% sarcoglycanopathy, and 10% dystroglycanopathy (16). Of 38 Turkish families, 18% were calpainopathy, 8% dysferlinopathy, and 39% sarcoglycanopathy (12); and a more recent report from this same group indicates that 23% of 93 LGMD2 families have *CAPN3* mutations (37). In a study of 99 patients with LGMD2 from Denmark, 38% were dystroglycanopathy, 23% sarcoglycanopathy, and only 2% dysferlinopathy (38). A publication of Dutch patients with LGMD reported a similar percentage were sarcoglycanopathy patients (8 of 40 unrelated patients [20%]) but did not comment on the diagnosis of the remaining patients (39).

In addition to these comparisons to worldwide geographic LGMD distributions, some attention should be paid to the racial distribution of our patients. The cohort reported here is approximately 90% white, 5% black, and 2.3% Asian-American as compared to U.S. Census data from 2000 that indicates the U.S. population as a whole is 75% white, 12% black, and 3.6% Asian (http://www.census.gov/population/www/pop-profile/profile.html). Hispanic or Latino origin is considered separately from race by the U.S. federal government. The most recent U.S. Census data indicates that Hispanics represent 14% of the U.S. population and that 92% of these individuals are white and 4% black. Compared with these U.S. population figures, our patient cohort is skewed toward more whites, fewer black, and fewer Hispanics.

Previously published work suggests that pediatric patients with LGMD are more likely to have calpain- or sarcoglycanopathies (12, 34, 39, 40). In our own study, the age range of patients on entry was 2 to 80 years. Among the 45 patients 18 years or younger with a defined diagnosis, 13% (n = 6) are laminopathy (LGMD1B), 13% (n = 6) are calpainopathy, 4% (n = 2) are dysferlinopathy, 49% (n = 22) are sarcoglycanopathy, and 18% (n = 8) are dystroglycanopathy. Thus, nearly 70% of pediatric patients with LGMD in our study have primary abnormalities in the dystrophin–glycoprotein complex. As a percentage of the total patients in each LGMD subtype, these pediatric patients make up 50% of the laminopathies, 19% of the calpainopathies, 4% of the dysferlinopathies, 55% of the sarcoglycanopathies, and 20% of the dystroglycanopathies.

The LGMDs represent a heterogeneous group of disorders resulting from mutations in 15 or more genes (19). These gene defects affect various sites throughout the muscle fiber, including the nuclear envelope, sarcomere, sarcoplasm, and sarcolemma. Clinical phenotype does not allow accurate prediction of genotype (9, 15, 19). For example, specific mutations in dysferlin may present as LGMD, Miyoshi myopathy, or equally represented proximal and distal weakness (41, 42); sarcoglycan gene mutations may produce childhood onset muscular dystrophy with rapid progression or a slowly progressive, mild affliction with onset later in life (20, 43); and FKRP can lead to either LGMD or CMD phenotypes, the allelic disorders LGMD2I and MDC1C, respectively (44–46).

Despite clinical overlap in phenotypes among and within subtypes of LGMD, distinct muscle biopsy features help focus the application of definitive mutational analysis. Although no histopathologic features (e.g. myonecrosis, myocyte regeneration, lobulated fibers, rimmed vacuoles, endomysial fibrosis, or fatty replacement) are predictive, protein expression analysis quite often suggests the appropriate gene (or small group of genes) for mutational analysis.

Perhaps the best example is in sarcoglycan-deficient patients (LGMD subtypes 2C, 2D, 2E, and 2F). Immunostaining alone is sufficient to identify patients with sarcoglycanopathy, and furthermore, can frequently predict which sarcoglycan gene is responsible. In our study, sarcoglycan deficiency was highly predictive of sarcoglycan mutations (34 of 37 unrelated patients) and the pattern of sarcoglycan deficiency helped target mutational analysis. Seven of 7 patients with LGMD2C had relatively selective loss of γ-sarcoglycan, whereas 8 of 12 patients with LGMD2D had relatively greater loss of α-sarcoglycan. Furthermore, 8 of 13 patients with LGMD2E had lost all 4 sarcoglycans. These data are similar to the experience of other groups (39, 40, 47).

Detection of dystroglycanopathies can also be accomplished by immunostaining alone, although our experience indicates that the use of glycosylation-dependent antibodies identifies more than just patients with FKRP mutations. Of 35 patients with abnormal α-dystroglycan immunostaining who have undergone FKRP sequencing, only 13 (37%) have FKRP mutations. Several groups have indicated that FKRP mutations are found in substantial numbers of patients with LGMD in Europe (38, 45, 48–50). However, our finding of large numbers of patients with dystroglycanopathy with LGMD clinical phenotypes, but no FKRP mutations may be unique. Sveen et al report that all 38 of their patients with LGMD with FKRP mutations had reduced muscle biopsy immunostaining for α-dystroglycan, but these authors do not specify whether any additional patient biopsies in the study had abnormalities of α-dystroglycan expression (38). Candidate genes are known for patients with dystroglycanopathy LGMD without FKRP mutations, primarily additional genes known to be involved in posttranslational glycosylation of α-dystroglycan in various types of patients with CMD (POMT1, POMT2, POMGnT1, LARGE, and fukutin [51]), but these genes have not yet been evaluated in our patients.

For the dysferlinopathies, immunostaining alone is known to be predictive of *DYSF* mutations when dysferlin is greatly reduced or absent. In 3 published studies, a critical inclusion criterion for patients with LGMD was absence (or near absence) of dysferlin on immunostaining and/or immunoblotting. Among patients with this biopsy immunophenotype, all harbored *DYSF* mutations, a total of 32 Italian patients from 2 different studies (52, 53) and 34 French patients (54). Mutations were found in only 60% of similar patients in a Japanese study (55). These authors report that 37 of 53 LGMD biopsies had abnormal dysferlin immunostaining; however, only the 10 patients with dysferlin deficiency on Western blot (defined as <30% of normal) underwent *DYSF* mutational analysis. It remains to be determined how often other patterns of abnormal dysferlin immunostaining or more moderate degrees of dysferlin deficiency are seen in patients with LGMD2B. We found *DYSF* mutations in all 6 patients evaluated to date by immunostaining, immunoblotting, and mutational analysis. However, 2 of these patients with LGMD2B had greatly reduced to absent dysferlin by immunostaining yet essentially normal dysferlin on Western blot. This suggests that the immunophenotypic spectrum of LGMD2B may have more breadth than currently appreciated.

In contrast to the sarcoglycans, α-dystroglycan, and dysferlin, immunoblotting plays a central role in the evaluation of calpain-3 (28, 56, 57). In our 17 patients with proven LGMD2A, 16 had significantly reduced calpain-3 in their biopsies. Much larger studies show that protein quantitation by Western blotting is extremely variable across the entire population of LGMD2A patients (57). Work by Fanin et al (56, 58, 59) suggests that as many as 20% of patients with LGMD2A may have normal amounts of calpain-3 but mutations that alter the proteolytic activity of protein.

A putative identification of patients with LGMD1B is possible by a combination of characteristic clinical features (e.g. autosomal-dominant inheritance, contractures, cardiac conduction defects) and normal expression of proteins such as calpain-3, dysferlin, sarcoglycans, and α-dystroglycan in a muscle biopsy (60). In our study, we screened *LMNA* in 85 patients without a specific protein deficiency who manifested any of the following features: autosomal-dominant inheritance, contractures in the extremity or neck, or cardiac conduction defects. Twelve of these patients harbored *LMNA* mutations. One patient had autosomal-dominant inheritance and an LGMD phenotype but without contractures or cardiac disease. All others had contractures that affected elbows plus either neck or Achilles tendon. If only elbows were affected, *LMNA* mutations were seen in those also manifesting cardiac conduction defects.

Patients with diagnoses other than LGMD may present with LGMD-like phenotypes. Ten patients referred to clinicians in our study with a limb-girdle pattern of weakness were shown to harbor 4q35 deletions diagnostic of FSHD. Normal facial muscle strength was present in these patients, but the distinctive reversed axillary folds with pectoral and scapular stabilizer muscle weakness were otherwise typical of FSHD (24, 25, 61). Likewise, occasional patients with a dystrophinopathy and Duchenne or Becker phenotypes presented with normal genetic screening for dystrophin gene mutations and reports of normal dystrophin immunostaining on an outside biopsy. Subsequent reanalysis of the biopsy tissue revealed subtle deficits in dystrophin staining confirmed by abnormalities on Western blot. Confirmation of dystrophinopathies should become more certain with enhanced efforts at identifying small gene mutations by the substantially more sensitive DOVAM genetic testing (62) or rapid methods of gene sequencing (63).

At the current time, no effective treatment is available to patients with LGMD. However, an array of potential therapies on the horizon emphasizes the importance of accurate immunophenotypic and genotypic classification of patients with LGMD. These therapies include gene transfer (64–66), exon skipping (67), stop codon suppression (68), and myostatin inhibition (69). Advancement of these and other therapeutic interventions for LGMD, and muscular dystrophies in general, will depend on the availability of well-characterized patient populations.

In summary, this study documents the distribution of LGMD subtypes in the United States based primarily on immunophenotypic characterization of muscle biopsies. This report serves to emphasize the extensive evaluation required to reach a specific diagnosis in patients with LGMD phenotypes.

This is not a trivial issue considering that combined tissue and molecular testing are required. Further analysis of this large LGMD patient population and further accrual of patients will better refine each subtype's relative prevalence, whereas longitudinal assessment over years will generate valuable natural history data.

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