

RESEARCH ARTICLE

Molecular Diagnosis in LGMD2A: Mutation Analysis or Protein Testing?

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Communicated by Jean-Claude Kaplan

Limb girdle muscular dystrophy (LGMD) type 2A (LGMD2A) is caused by mutations in the CAPN3 gene encoding for calpain-3, a muscle specific protease. While a large number of CAPN3 gene mutations have already been described in calpainopathy patients, the diagnosis has recently shifted from molecular genetics towards biochemical assay of defective protein. However, an estimate of sensitivity and specificity of protein analysis remains to be established. Thus, we first correlated protein and molecular data in our large LGMD2A patient population. By a preliminary immunoblot screening for calpain-3 protein of 548 unclassified patients with various phenotypes (LGMD, myopathy, or elevated levels of serum creatine kinase [hyperCKemia]), we selected 208 cases for CAPN3 gene mutation analysis: 69 had protein deficiency and 139 had normal expression. Mutation search was conducted using SSCP, denaturing high performance liquid chromatography (DHPLC), amplification refractory mutation system (ARMS-PCR), and direct sequencing methods. We identified 58 LGMD2A mutant patients: 46 (80%) had a variable degree of protein deficiency and 12 (20%) had normal amount of calpain-3. We calculated that the probability of having LGMD2A is very high (84%) when patients show a complete calpain-3 deficiency and progressively decreases with the amount of protein; this new data offers an important tool for genetic counseling when only protein data are available. A total of 37 different CAPN3 gene mutations were detected, 10 of which are novel. In our population, 87% of mutant alleles were concentrated in seven exons (exons 1, 4, 5, 8, 10, 11, and 21) and 61% correspond to only eight mutations, indicating the regions where future molecular analysis could be restricted. This study reports the largest collection of LGMD2A patients so far in which both protein and gene mutations were obtained to draw genotype–protein–phenotype correlations and provide insights into a critical protein domain. Hum Mutat 24:52–62, 2004. © 2004 Wiley-Liss, Inc.

KEY WORDS: limb-girdle muscular dystrophy; LGMD2A; calpain-3; protein deficiency; CAPN3 mutation detection

DATABASES:

CAPN3 – OMIM: 114240, 253600 (LGMD2A); GenBank: AF209502.1
www.dmd.nl. (Leiden Muscular Dystrophy Pages Database)

INTRODUCTION

Autosomal recessive limb girdle muscular dystrophies (LGMD type 2) are a clinically and genetically heterogeneous group of disorders, characterized by progressive involvement of proximal limb girdle muscles. LGMD2A (MIM# 253600), whose locus has been mapped to chromosome 15q15.1 [Beckmann et al., 1991], is considered to be the most frequent form of recessive LGMD. LGMD2A is caused by single or small nucleotide changes widespread along a 40-kb gene, named CAPN3 (MIM# 114240), which encodes for the calpain-3 protein [Richard and Beckmann, 1995]. More than 130 different CAPN3 gene mutations have been described, mostly of missense type [Fardeau et al.,

1996; Richard et al., 1997, 1999; Anderson et al., 1998; Chou et al., 1999; Minami et al., 1999; Chae et al., 2001; Pollitt et al., 2001].

Received 13 October 2003; accepted revised manuscript 19 March 2004.

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Grant sponsor: Telethon-Italy; Grant numbers: GUP030516; GTF02009; C61; P10; Grant sponsor: Italian Ministry of University and Scientific Research and Technology (MURST); Grant number: 2001068328; Grant sponsor: EuroBioBank Network; Grant number: QLRT-2001-027769.

DOI 10.1002/humu.20058

Published online in Wiley InterScience (www.interscience.wiley.com).

LGMD2A is caused by defects in a protein with an enzymatic function, calpain-3, the muscle specific member of a family of Ca^{++} activated neutral proteases. Calpain-3 is a multidomain protein, with three exclusive sequence inserts (NS, IS1, and IS2): domain I has regulatory role, domain II is the proteolytic module, domain III has a C2-like domain, and domain IV binds Ca^{++} ions.

Though the main calpain-3 biochemical activities are known [Sorimachi et al., 1989, 1993; Ono et al., 1998], i.e., autocatalytic regulation, protein hydrolysis (e.g. α -fodrin), and binding to titin, the physiological role of calpain-3 is still under investigation. The protein has been localized in the N2 and M-line regions of titin in the myofibrils [Sorimachi et al., 1993; Keira et al., 2003], and this binding is thought to stabilize calpain-3 to prevent its autolysis. The dissociation from titin would lead to local activation of calpain-3 in myofibrils, where the proteolysis of many cytoskeletal or myofibrillar proteins occurs [Poussard et al., 1996; Huang and Fosberg, 1998; Chae et al., 2001]. On the other hand, the search for specific substrates of calpain-3 has been so far unsuccessful. Under particular conditions, calpains can be relocalized from the cytosol to the nucleus [Ma et al., 2001], where they can proteolyze nuclear proteins and transcription factors, such as those controlling survival genes and apoptosis [Baghdiguian et al., 1999; Richard et al., 2000].

The cascade of pathogenetic events that follows CAPN3 gene mutations, leading to calpain-3 deficiency, and finally to muscular dystrophy, is still poorly understood. While it is expected that patients homozygous for null mutations (inactivating) have absent protein and severe phenotype, the effect of missense mutations is largely unpredictable, as they are associated with variable disease severity and protein amount. Functional studies by site-directed mutagenesis [Ono et al., 1998] have demonstrated that the loss of calpain-3 protease activity (rather than the loss of its autocatalytic function) underlies the molecular mechanism of LGMD2A. While it is unlikely that calpain-3 is implicated in myoblast fusion, it may possibly be involved in the disassembly of myofibrils during early stages of turnover [Huang et al., 1998] or cause a postfusion defect in muscle maturation and differentiation [Spencer et al., 2002; Yajima and Kawashima, 2002].

Molecular diagnosis of LGMD2A is now possible thanks to calpain-3 antibodies, used for protein diagnosis, and progress in gene mutation detection. Nevertheless, a sizeable number of LGMD2A cases currently remain undiagnosed, due to either incomplete sensitivity of the methods used for protein and gene analysis or the restrictive criteria used to select candidate patients.

Genetic corroboration of the results obtained from protein analysis is essential to the molecular characterization of the disease, but it is also fundamental for determining the proportion of missed diagnoses, for learning from genotype-phenotype correlations, and for providing insights into critical protein domains.

METHODS

Patient Selection Criteria

The muscle biopsy bank at the Neuromuscular Centre at the University of Padova, which contains more than 5,300 specimens, was surveyed for patients affected by an unidentified muscular dystrophy or myopathy that fulfilled the following criteria: normal muscle protein expression of dystrophin, α -sarcoglycan, dysferlin, caveolin-3, merosin, and emerin; muscle biopsy histopathology consistent with a dystrophic or myopathic process; and increased creatine kinase (CK) level (>500 U/L). Patients affected with congenital dystrophy, distal myopathy, scapulo peroneal dystrophy, dominant or other genetic myopathies (myotonic dystrophy, facio-scapulo-humeral dystrophy) were a priori excluded from this study. A total of 548 patients matched our selection criteria and were selected for calpain-3 protein screening: 199 had received the diagnosis of LGMD, 219 of myopathy, and 130 were asymptomatic, with only high CK levels.

Clinical Examination and Muscle Biopsy

Most patients underwent periodical clinical examinations, the first at the time of diagnosis and then during follow-ups as the disease progressed.

Onset. The age at onset of muscle weakness (or at first report of hyperCKemia) and clinical phenotype were recorded. For the purpose of this study we used the following clinical classification: 1) "early onset LGMD," patients with onset of muscle weakness occurring in the pelvic girdle before the age of 12 years; 2) "LGMD," patients with onset of weakness in the pelvic-femoral girdle (the classical Leyden-Mobius type) between the age of 13 and 29 years; 3) "late onset LGMD," patients in whom the onset of weakness occurred in the pelvic girdle at more than 30 years of age; and 4) "Erb dystrophy," patients with the scapular-humeral phenotype, with onset of weakness predominantly in the shoulder girdle.

Severity and progression. During periodical clinical examination, muscle atrophy and hypertrophy, gait and posture, presence of joint contractures, scoliosis, scapular winging, individual muscle weakness, difficulty in climbing stairs or in performing Gowers' maneuver, and age at loss of independent ambulation were recorded. The clinical severity of muscle disease at last examination was graded according to the following functional scale: grade 0 = hyperCKemia, all activities normal; grade 1 = normal gait, unable to run freely; grade 2 = tip-toe walking, waddling gait, initial Gowers' sign; grade 3 = overt muscle weakness, climbing stairs with banister, difficulty rising from a chair; grade 4 = unable to rise from the floor; grade 5 = unable to rise from a chair or to climb stairs; grade 6 = unable to walk unassisted; grade 7 = unable to eat, drink or sit without assistance. In our practice, the patients were subdivided in the following categories, according to the degree of muscle weakness: 1) asymptomatic, only elevated CK (functional grades 0-1); 2) mild, with objective muscle

weakness in lower and/or upper girdle (functional grades 2–3); 3) moderate, unable to rise from floor or a chair or climbing stairs (functional grades 4–5); and 4) severe, loss of ambulation or difficulty in daily activities (functional grades 6–7).

Muscle biopsy. At the time of diagnosis, an open biopsy of quadriceps femoris or biceps brachii muscle was obtained after written informed consent. Immediately after surgery, muscle specimens were frozen in isopentane chilled in liquid nitrogen and then stored at -80°C until processed.

Calpain-3 Immunoblot Analysis and Autocatalytic Assay

Calpain-3 immunoblot analysis was performed as previously described [Fanin et al., 2001]. The muscle biopsies of those patients showing CAPN3 gene mutations and normal protein expression by calpain-3 immunoblot were subjected to biochemical assay to test calpain-3 autocatalytic activity [Fanin et al., 2003].

Molecular Studies of CAPN3 Gene

Following the preliminary calpain-3 protein screening by immunoblot on 548 muscle biopsies, a subset of 208 patients was selected for CAPN3 gene mutation analysis: 69 cases were chosen on the basis of the detection of calpain-3 protein deficiency in their muscle, and they were analyzed using denaturing high performance liquid chromatography (DHPLC), amplification refractory mutation system (ARMS-PCR), or SSCP; 139 patients were chosen on the basis of phenotype/histopathology pattern compatible with LGMD2A, although calpain protein was normally expressed, and they were analyzed using only allele-specific tests to limit the effort required. The genomic DNA from all 208 patients was extracted from blood leukocytes or muscle tissue (after written informed consent), using a DNA extraction kit (Sigma Chem, <http://pubs.acs.org/pin/sigma/sigma.html>). Each mutation found by SSCP, DHPLC, or direct sequence was confirmed using an allele-specific test on patients' samples; mutations found by ARMS-PCR test were confirmed by direct sequencing.

ARMS-PCR analysis. A total of 15 different mutations were selected for screening purposes and studied using a classic ARMS-PCR test (c.550delA, p.G222R, p.R448H, p.R489W, p.R572Q, p.R572W, and p.R748Q) or tetra-primer ARMS-PCR (p.D77N, p.T184M, p.R448C, p.R489Q, p.R490W, p.R490Q, p.G496R, and p.S606L) [Fanin et al., 2003]. Additional new missense mutations found in patients' samples by other methods were checked by ARMS-PCR on 100 normal chromosomes to exclude the possibility they could be neutral polymorphisms.

SSCP analysis. Both the coding sequence (except for exons 9, 12, 14, 18, 23, and 24, which are less often involved in mutations) and the promoter region were amplified by PCR using the conditions and genomic primers published elsewhere [Richard et al., 1995] with

minor changes. PCR products were mixed with denaturing loading buffer, denatured at 95°C for 5 minutes, and immediately placed on ice. Gel electrophoresis was conducted at 3 W for 5–6 hr at 4°C using 10% acrylamide gel with and without 5% glycerol, in Tris-borate-EDTA buffer. The gels were silver stained. The DNA sequences containing nucleotide changes (distinguishable from aberrant migration bands) were directly sequenced.

DHPLC analysis. PCR products were prepared in a buffer containing 20 mM Tris-Hepes (pH 8), 10 mM KCl, 10 mM ammonium sulfate, 2 mM MgCl_2 , and 2.5% glycerol, using Amplitaq Gold polymerase (Applied Biosystems, www.appliedbiosystems.com). PCR products are directly set into the autosampler of the Wave Transgenomics apparatus (at the CRIBI Biotechnology Center, University of Padova), and processed using a DNA-separation column and tri-ethyl-amine acetate buffer. The initial conditions of analysis were suggested by the Navigator software and then modified according to the peak shape at different temperatures. The optimal temperature required in order to partially melt the DNA was chosen. The concentration of acetonitrile solvent was increased by 2% per minute, so that DNA was gently extracted from the column. The DNA sequences containing aberrant peak shape were directly sequenced.

DNA sequencing. PCR products were purified using the Microcon Amicon device (Millipore, www.millipore.com), and directly sequenced using the Big Dye di-deoxy-terminator cycle sequencing kit (Applied Biosystems). Extension products were purified, and the samples run on an ABI-PRISM 3700 automated sequencer (Applied Biosystems) at the CRIBI Biotechnology Centre, University of Padova. Sequence analysis was obtained using Chromas software, www.techne.lysium.com.au/chromas.html with the human CAPN3 gene sequence as reference (GenBank accession #AF209502.1). The DNA mutation numbering was based on the cDNA sequence using the nomenclature "c." symbol before the number, and using the A of the ATG start codon as +1.

Genotype-Protein-Phenotype Correlations

Any correlation between the genotype and either the protein or the clinical phenotype was obtained only from patients in whom both mutant alleles have been identified.

Statistical Analysis

We used the rank Spearman test to correlate the age at last examination with the functional severity score, and we used the χ^2 test in a 2×2 contingency table to determine whether the observed difference in sex distribution among mutant patients was significant. Comparison, between male and female patient groups, of the mean age at onset of muscle weakness and loss of ambulation was carried out using Student's *t*-test. Statistical significance was $P < 0.05$.

RESULTS

Calpain-3 Protein Deficiency and Corresponding Gene Mutation Identification

Of the 548 muscle biopsies with an unclassified dystrophy/myopathy that underwent calpain-3 immunoblot screening, 79 showed protein deficiency (14%): calpain-3 was absent or in traces in 43 cases (54%) and was reduced in amount (5–80% of control) in 36 cases (46%) (Fig. 1).

Gene mutation screening was conducted on 69 out of 79 cases with calpain-3 protein deficiency (in 10 cases the DNA sample was not available or insufficient).

Of the 208 patients who underwent gene mutation analysis, 28% of cases received molecular diagnosis of LGMD2A, but this is almost certainly an underestimate of the real situation, as screening was incomplete.

We identified a total of 58 patients with primary LGMD2A (Table 1): 80% had calpain-3 deficiency and 20% had normal protein. CAPN3 gene mutations were detected in 46 out of 69 patients with variable protein defect (67%) and in 12 out of 139 patients with normal protein (9%).

Based on our molecular results, we attempted to predict the probability of having primary LGMD2A only through protein testing. The a priori probability of having LGMD2A is 67% when patients show calpain-3 protein defect; it is extremely high (84%) when there is a total defect of calpain-3, it is 69% when there is a severely defective protein (<10% of norm), and it falls with an increase in the amount of the protein (from 20% with 20–80% protein, to 9% with 100% protein) (Fig. 2).

Clinical Phenotype of Patients

We studied 58 LGMD2A patients from 56 families: one pair of siblings and one pair of cousins were included. Most of our patients are isolated cases, 17 have positive family history, and four have consanguineous parents.

The whole series of mutant patients included 38 males and 20 females (sex ratio 1.9:1); a gender difference was also observed in the clinical subgroups of patients with hyperCKemia (male–female ratio 3:1), early onset LGMD (male–female ratio: 1.7:1), LGMD (male–female ratio: 2.2:1), late onset LGMD (male–female ratio: 2:1), whereas the ratio of male to female patients showing Erb or late onset Erb phenotype was balanced. This different

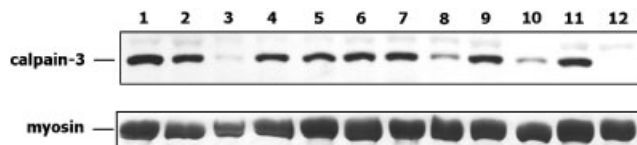


FIGURE 1. Calpain-3 Western blot analysis. The calpain-3 bands correspond to a 94-kDa molecular weight protein (upper panel). Myosin content in the posttransfer Coomassie blue stained gel (lower panel) is used to normalize the calpain-3 amount in each lane. Muscle samples numbered 3, 8, 10, and 12 correspond to patients showing a variable reduction in calpain-3 protein level. In the remaining samples, the protein is fully expressed, as compared to control (number 1).

distribution was not statistically significant; it rather reflects the same skewed ratio between the sexes that was observed in the 208 selected cases (male–female ratio 1.6:1). Moreover, no sex difference was observed in age at onset of weakness (male: 19.0, female: 20.6 years) or at loss of ambulation (male: 39.4, female: 39.3 years).

When the whole series of LGMD2A patients was considered, our population appeared rather variable, both when we analyzed age and phenotype at onset, and progression of the disease (Table 2). At onset, eight patients (14%) were asymptomatic with only high CK, six cases (10%) had muscle weakness first evident in the upper girdle (Erb phenotype), and 44 (76%) had lower girdle LGMD phenotype. The mean age at onset of muscle weakness was 19.6 years. At the time of the study, 13 patients had lost ambulation, on average at 39.4 years (Table 2). However, most patients in the LGMD and in the Erb group are still walking.

When we correlated age at last examination with their functional severity score, our patients were clearly distributed into two independent clusters (Fig. 3): cases with “early onset LGMD” corresponded to a rather homogeneous group, in which progress of the disease was fairly linear ($r = 0.81$), whereas patients with either adult and late-onset LGMD or Erb dystrophy were a more heterogeneous group ($r = 0.24$), in which progress of the disease was variable, even though it was significantly slower than in the early onset LGMD group.

Phenotype–Protein–Genotype Correlations

We obtained correlations only from the series of 33 patients in whom both mutant alleles have been identified (Table 1), and we discuss the results accordingly. We subdivided our patients on the basis of the age and phenotype at onset of weakness (see Methods section).

Patients in the “early onset LGMD” group (46%) frequently had ankle contracture and were often tip-toe walkers; their muscle weakness progressed from the pelvic to the shoulder girdle. Patients in this group are clearly the most severely affected, and 5 out of 15 (33%) had already lost ambulation, despite the fact that their current mean age is 26 years. Calpain-3 protein was absent in most patients (13 out of 15); six patients had two null mutant alleles (four homozygous, two compound heterozygous), four had two missense mutant alleles (two homozygous, two compound heterozygous), and the remaining five patients were compound heterozygous for one null and one missense mutant allele.

Patients with the “LGMD” phenotype at onset had some degree of upper girdle weakness, atrophic thighs, and no calf hypertrophy. These patients can be considered to be affected by typical, moderately severe LGMD, although 2 of the 11 patients involved had already lost ambulation. Calpain-3 protein was absent or severely reduced in most cases (9 out of 11); seven patients had two missense mutant alleles (six homozygous, one compound heterozygous), one patient had two null mutant alleles (homozygous), and the remaining

TABLE 1. Clinical and Molecular Data in 58 LGMD2A Patients

Pt. no.	Sex	Fam. hist	At onset			Severity (grade)	Age	Calpain on WB(%)	Nucleotide change (referred to cDNA)	Aminoacid change	Exon	Zigosity	Effect of mutation
			Age	Phenotype	At last examination								
4 ^a	M	+	11	e.o. LGMD	Moderate (4)	26	100	c.1469G>A	p.R490Q	11	Homo	Missense	
6	M	Cons.	11	e.o. LGMD	Moderate (5)	35	0	c.533T>C	p.I178T	4	Homo	Missense	
8	M	-	17	LGMD	Moderate (5)	46	100	c.1469G>A	p.R490Q	11	Homo	Missense	
11	M	+	10	e.o. LGMD	Severe (6)	35	0	c.550delA	-	4	Homo	Null	
12	F	-	10	e.o. LGMD	Moderate (5)	25	0	c.550delA	-	4	Homo	Null	
15	M	Cons.	21	LGMD	Severe (6)	30	0	c.1342C>T	p.R448C	10	Homo	Missense	
20	M	-	16	LGMD	Mild (3)	27	10	c.1061T>G	p.V354G	8	Homo	Missense	
24	F	+	28	ERB	Moderate (5)	36	100	c.1469G>A	p.R490Q	11	Homo	Missense	
25	M	+	15	LGMD	Severe (6)	24	0	c.550delA	-	4	Homo	Null	
26	F	Cons.	27	LGMD	Mild (2)	27	0	c.848T>C	p.M283T	6	Homo	Missense	
27 ^a	M	+	37	I.o. ERB	Mild (3)	49	100	c.1469G>A	p.R490Q	11	Homo	Missense	
42	F	-	10	e.o. LGMD	Mild (2)	15	0	c.550delA	-	4	Homo	Null	
43	M	-	45	I.o. LGMD	Mild (3)	50	5	c.550delA	-	4	Homo	Null	
46	F	+	15	LGMD	Moderate (4)	25	<5	c.1345A>C	p.N449H	10	Homo	Missense	
49	M	Cons.	21	LGMD	Moderate (5)	39	0	c.2243G>A	p.R748Q	21	Homo	Missense	
53	M	-	10	e.o. LGMD	Moderate (5)	21	0	c.2230_2231insAACA	-	21	Homo	Null	
9	M	-	11	high CK	Asymptomatic (1)	18	100	c.1469G>A	p.R490Q	11	Hetero	Missense	
14	F	-	8	e.o. LGMD	Moderate (5)	31	0	c.1466G>A	p.R489Q	11	Hetero	Missense	
17	M	-	16	ERB	Mild (3)	33	0	c.1992+1G>T	-	Intr.17	Hetero	Splicing, null	
21 ^b	M	+	6	e.o. LGMD	Severe (6)	26	0	c.1061T>G	p.V354G	8	Hetero	Missense	
23	M	-	4	e.o. LGMD	Severe (6)	40	0	c.1291G>A	p.V431M	10	Hetero	Missense	
28	F	+	12	e.o. LGMD	Severe (6)	35	0	c.1343C>T	p.R448C	10	Hetero	Missense	
29	M	-	18	LGMD	Mild (2)	26	100	c.550delA	-	4	Hetero	Null	
32	M	-	10	e.o. LGMD	Mild (2)	11	0	c.229G>A	p.D77N	1	Hetero	Missense	
34 ^b	F	+	21	LGMD	Moderate (4)	29	0	c.245C>T	p.P82L	1	Hetero	Missense	
2	M	+	4	e.o. LGMD	Severe (6)	25	<5	c.697 G>C	p.G233R	5	Hetero	Missense	
5	F	-	7	e.o. LGMD	Moderate (5)	24	0	c.1319 G>A	p.R440Q	10	Hetero	Missense	
36	M	-	7	e.o. LGMD	Moderate (4)	14	0	c.1343G>A	p.R448H	10	Hetero	Missense	
40	F	-	24	LGMD	Moderate (4)	43	0	c.550delA	p.R490Q	11	Hetero	Missense	
41	M	-	11	e.o. LGMD	Moderate (4)	25	100	c.550delA	-	4	Hetero	Null	
48	M	-	24	ERB	Mild (2)	26	100	c.1992+1G>T	p.R490W	Intr.17	Hetero	Splicing, null	
								c.1468G>T	-	11	Hetero	Missense	
								c.551 C>T	p.T184M	4	Hetero	Null	
								c.259_260insI	-	1	Hetero	Missense	

55	M	-	14	LGMD	Mild (2)	16	0	c.1621C>T	p.R541W	13	Hetero.	Missense
57	M	-	48	I.o. LGMD	Severe (6)	74	100	c.598_612del	p.F200_L204del.	4	Hetero.	In-frame Null
1	M	-	67	I.o. LGMD	Mild (3)	71	0	c.550delA	-	4	Hetero.	Null
3	M	-	8	e.o. LGMD	Moderate (5)	23	0	c.1309 C>T	p.R437C	10	Hetero.	Missense
7	F	-	12	e.o. LGMD	Severe (6)	35	0	c.1303G>A	p.E435K	10	Hetero.	Missense
10	F	-	8	high CK	Asymptomatic (1)	10	0	c.550delA	-	4	Hetero.	Null
13	F	+	49	I.o. ERB	Mild (2)	50	5	c.1992+1G>T	-	4	Hetero.	Null
16	F	+	26	LGMD	mild (3)	58	10	c.229G>A	p.D77N	1	Hetero.	Splicing, null
18	M	-	10	high CK	Asymptomatic (0)	15	20	c.229G>A	p.D77N	1	Hetero.	Missense
19	M	-	4	e.o. LGMD	mild (2)	12	0	c.245C>T	p.P82L	1	Hetero.	Missense
22	M	-	16	high CK	Mild (3)	28	100	c.J792_I79SdelAAAA	-	15	Hetero.	Null
30	F	+	49	I.o. LGMD	Mild (3)	59	<5	c.229G>A	p.R490W	11	Hetero.	Missense
31	M	-	24	high CK	Asymptomatic (0)	24	10	c.479C>G	p.D77N	1	Hetero.	Missense
33	F	-	34	I.o. LGMD	Moderate (5)	47	10	c.1061T>G	p.A160G	3	Hetero.	Missense
35	F	-	28	ERB	Severe (6)	48	80	c.1621C>T	p.V354G	8	Hetero.	Missense
37	M	+	27	LGMD	Mild (3)	37	5	c.2242C>T	p.R541W	13	Hetero.	Missense
38	M	-	18	LGMD	Mild (3)	22	0	c.2242C>T	p.R748X	21	Hetero.	Null
39	M	-	48	high CK	Asymptomatic (1)	48	50	c.755T>G	p.R748X	21	Hetero.	Null
44	F	-	8	e.o. LGMD	Mild (2)	13	0	c.100delG	P.M252R	5	Hetero.	Missense
45	M	-	26	LGMD	Severe (6)	55	10	c.2288A>G	-	1	Hetero.	Null
47	M	+	11	high CK	Asymptomatic (1)	11	10	c.2242C>T	p.Y763C	22	Hetero.	Missense
50	M	+	12	e.o. LGMD	Severe (6)	52	10	c.I714C>T	p.R748X	21	Hetero.	Null
51	F	-	6	high CK	Asymptomatic (0)	7	5	c.550delA	p.R572W	13	Hetero.	Missense
52	F	-	3	e.o. LGMD	Moderate (4)	8	0	c.2257G>A	-	4	Hetero.	Null
54	M	-	18	LGMD	Moderate (5)	40	0	c.1333C>A	p.D753N	21	Hetero.	Missense
56	M	-	3	e.o. LGMD	Severe (6)	33	100	c.1486G>A	p.G445R	10	Hetero.	Missense
58	M	+	50	I.o. LGMD	Mild (2)	56	100	c.1343G>A	p.G496R	11	Hetero.	Missense
									p.R448H	10	Hetero.	Missense

*Patients are subdivided according to the presence of homozygous mutation, compound heterozygous and only one mutant allele identified. GenBank accession #AF209502.1. The DNA mutation numbering was based on cDNA sequence using the nomenclature "c." symbol before the number, using the A of ATG start codon as + 1.

^{a,b}Pairs of relatives.

Cons., consanguineous parents.

three patients were compound heterozygous for one null and one missense mutant allele.

The progress of the disease in patients in the “late onset LGMD” group was slow and they showed a mild to moderate functional score (still ambulant) at ages 50 and 73 years.

Onset in patients showing the “Erb phenotype” invariably occurred only in adulthood (from 16 to 37 years of age) and the disease had a relatively mild course, as patients showed mild to moderate functional scores at 26, 33, 36, and 49 years of age. Calpain-3 protein amount was variable but mostly at normal levels (though functionally aberrant); three patients had two missense mutant alleles (two homozygous, one compound heterozygous) and one patient was compound heterozygous for one null and one missense mutant allele.

CAPN3 Gene Mutations

A total of 37 different mutations have been detected (Table 1, Fig. 4): 27 of missense type (73%), two of nonsense type (5%), two splice-site mutations (5%), one

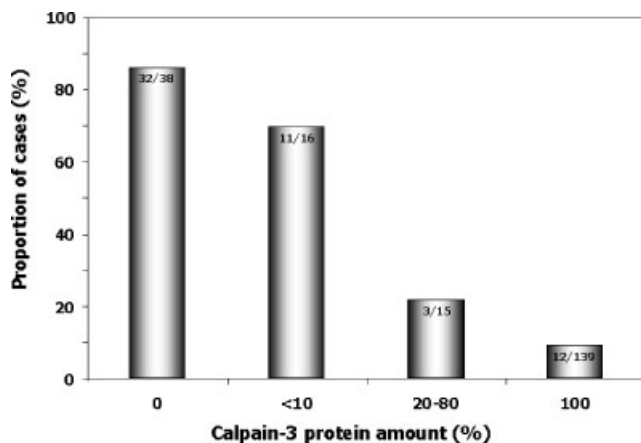


FIGURE 2. Proportion of cases with CAPN3 gene mutations in relation to the amount of calpain-3 protein in muscle biopsy. The likelihood of obtaining a molecular diagnosis of LGMD2A is high when patients show totally or severely defective calpain-3 protein, and the probability progressively decreases with the amount of protein.

in-frame deletion (3%), and five small deletions or insertions with consequent frame shifting (14%).

Of the mutations found, 10 (27%) are new (according to the Leiden Muscular Dystrophy database): six missense, and four frame-shifting insertions or deletions. To ensure that the six new missense mutations were not polymorphisms, 100 normal chromosomes were checked; furthermore, these nucleotide changes led to the substitution of an amino acid highly conserved in mammalian calpains, with an amino acid of different type (e.g., polar uncharged to charged).

In 57% of the patients, both mutant alleles were identified (28% homozygous, 72% compound heterozygous), and in 25 cases one mutant allele was found. Thus, we were able to identify 78% of mutant alleles in 58 patients, though the entire coding region was not screened. During this study, we identified several polymorphic variants (e.g., the substitution c.706G>A causing a p.A236T amino acid change in exon 5, the c.2380+12delA in intron 22) and synonymous nucleotide changes (e.g., the substitution c.96T>C producing a

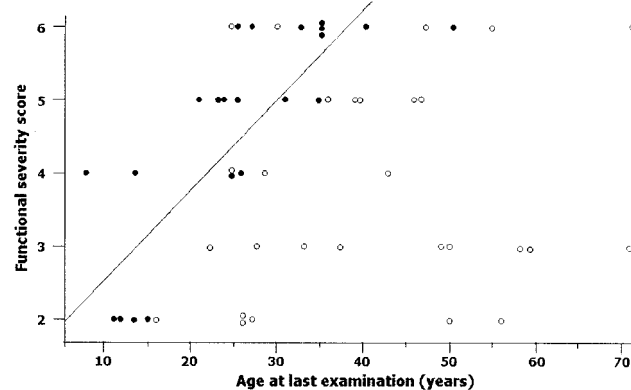


FIGURE 3. Correlation between age at last examination and functional severity score. Our patients are clearly distributed into two independent clusters: patients with early onset LGMD (filled circles) which are the most severely affected, are a rather homogeneous group, whose disease progression is fairly linear (see best interpolating line); patients with adult and late-onset LGMD or Erb dystrophy (empty circles) include a heterogeneous group of patients, whose rate of disease progression is variable, but nevertheless significantly slower than in the early onset LGMD group.

TABLE 2. Clinical Phenotype and Disease Progression in LGMD2A Patients

Phenotype at onset of weakness	Age at onset			Age at wheel chair bound		
	No. of cases	Mean age (yrs)	Age range (yrs)	No. of cases	Mean age (yrs)	Age range (yrs)
LGMD						
Early onset LGMD	22	8.2	3–11	8	35.1	25–52
LGMD	16	20.2	14–27	3	36.4	24–55
Late onset LGMD	6	48.8	34–67	1	74.0	
Erb						
Erb	4	24.0	16–28	1	48.0	–
Late onset Erb	2	43.0	37–49	–	–	
Total cases	50	19.6	3–67	13	39.4	24–74

A considerable number of patients, who in the past had been diagnosed only on a clinical basis, underwent molecular diagnosis of LGMD2A following the systematic protein and gene mutation results from this study. The strategies adopted in earlier studies for this purpose had missed a proportion of LGMD2A cases for a variety of reasons: one is that gene mutation analysis was carried out in patients selected only on the basis of their LGMD phenotype. Our study has demonstrated that some mutant patients might present clinical phenotypes that are different from LGMD or that match with the preclinical stage of the disease (e.g., mild myopathy or only hyperCKemia). Such screening, which involves a wide spectrum of phenotypes, may make it possible to obtain an earlier diagnosis (which is important for genetic counseling) to prevent and treat the complications of the disease (e.g., joint contractures, scoliosis, respiratory insufficiency), and, consequently, to improve patients' quality of life.

Calpain-3 Protein Analysis

In most of the literature, the results of CAPN3 gene mutation analysis in LGMD2A patients has been reported even though little or nothing was known of the corresponding calpain-3 protein expression; in some studies, protein data has been provided to support gene mutation findings. In our study, we adopted a different procedure; wide protein screening was used to select patients for gene mutation analysis: this approach made it possible to identify a high number of primary defects. Currently, most diagnostic laboratories tend to restrict the workup of calpainopathies to protein testing, thus only patients who have shown a biochemical deficiency undergo gene mutation analysis. However, there are many more pitfalls in calpain-3 protein testing than there are in using the same method for structural muscle proteins; we have demonstrated that the former is not completely specific or sensitive. A number of patients with calpain-3 protein deficiency showed no CAPN3 gene mutations, suggesting that false-positive results may be due to mutation in other genes (as reported for dysferlin and titin) or to poorly conserved tissue samples [Anderson et al., 1998]. We have also shown that some mutations do not result in protein deficiency despite primary gene defect, suggesting that protein testing may also give false-negative results [Talim et al., 2001], and that these false-negatives are more frequent than previously thought: 20% of all mutant LGMD2A patients in our series had normal calpain-3 protein expression. Our study has demonstrated that at least 67% of the patients with a variable calpain-3 protein defect are indeed primary LGMD2A, but this rises to 84% when absent protein is considered. Although in the minority of cases that show partially reduced calpain-3 protein levels where the precise quantitation of calpain-3 bands on Western blot is often difficult, this issue is not relevant for the significance of the results (Fig. 2).

CAPN3 Gene Mutations

This study provides important clues to aid future CAPN3 gene mutation screening studies, offering possible answers to the question of which methods should be used for molecular diagnosis. In our population, eight mutations alone accounted for about 61% of the mutant alleles, indicating that allele-specific methods could be useful for preliminary, rapid, and cheap mutation screening. Furthermore, the fact that there are "private" mutations found in particular geographical regions of northern Italy, suggests that these methods should be used in patients from these areas.

As about 27% of gene mutations identified in this study have been detected for the first time, we recommend using proper methods for the detection of unknown point mutation (e.g., SSCP, DHPLC) in future screening. Since these techniques are relatively laborious and expensive, they could perhaps be used to analyze only those exon sequences that are most often involved in mutations (i.e., exons 1, 4, 5, 8, 10, 11, and 21): 87% of the mutant alleles in our series were localized in these seven exons.

We showed that using a combination of methods yielded a higher detection rate of gene mutations: we detected 78% of the mutant alleles in 58 affected patients, even though the entire coding sequence was not screened (the smallest exons were not analyzed), and the SSCP and DHPLC methods are not completely sensitive. Furthermore, the pathogenetic role of many nucleotide variants which are assumed to be "neutral" (e.g., synonymous and intronic nucleotide changes) could not be demonstrated in this study, but they should be looked at carefully as they are potential gene mutations [Richard and Beckmann, 1995].

Unlike the expected effect of homozygous null mutations on the abolition of protein synthesis, most of our cases with at least one missense allele (homozygous, compound heterozygous for two different missense alleles, compound heterozygous for one missense and one null allele) had no calpain-3 protein, though their genetic condition is theoretically compatible with the synthesis of some protein. One possible explanation is that such missense mutations might have severely affected the protein function, possibly causing more rapid calpain-3 autodegradation [Ono et al., 1998].

Genotype-Protein-Phenotype Correlations

The amount of calpain-3 protein appears to have a relative prognostic value: patients with early onset LGMD usually had no calpain-3 protein, but absent or markedly reduced protein levels were also found in patients with late or adult onset LGMD. On the contrary, almost all patients with normal levels of calpain-3 protein (but with frequent loss of normal autocatalytic function) had late or adult onset muscular dystrophy.

A strong correlation exists between the genotype and its effect on protein and on the phenotype, especially when two null mutant alleles are detected (either

homozygote or compound heterozygote); eight such patients in this study showed no calpain-3 protein, and most of them (6 out of 8) had early onset LGMD. No definite correlations can be drawn from two missense mutant alleles (either homozygote or compound heterozygote): these patients may present a variable clinical phenotype at onset and also a variable level of calpain-3 protein (from 0–100%). Patients with Erb phenotype at onset frequently carried two missense mutations and had variable calpain-3 protein expression (mostly at normal level). A high degree of variation, both in the age and phenotype at onset, and in the severity of the disease, was observed among the four patients who are homozygous for the same p.R490Q missense mutation. This suggests that epigenetic factors play a role in modulating phenotype expression.

Patients with early onset LGMD are the most severe cases, showing loss of ambulation in the third to fifth decade of life. Adult or late onset LGMD patients usually have a mild and slowly progressive form of the disease, as do patients with Erb phenotype at onset. Comparing our LGMD2A population with other large series in the literature [Fardeau et al., 1996; Richard et al., 1997, 1999], we observed a similar mean age at onset but a significantly later mean age at loss of ambulation in our series (39.4 years vs. 17.3 years [Richard et al., 1999], 18.7 years [Fardeau et al., 1996], and 29.2 years [Richard et al., 1997]). This wide variability in age at loss of ambulation could be attributed to a different methodology of patient ascertainment: while the LGMD2A patients described in the literature were selected on the basis of overt LGMD phenotype, our diagnostic screening was carried out on a more general myopathic population (including patients with only high CK or mild LGMD in adolescence or adulthood). Our approach might therefore have identified a number of patients in an early stage of the disease, who are therefore expected to lose ambulation at a later than average age.

The apparent greater proportion of affected males than females we observed in our study is not statistically significant and it exactly matches the proportion observed in the total screened population.

Moreover, we observed no sex difference in age at onset of weakness or at loss of ambulation, suggesting that the disease similarly affects patients of both genders.

In conclusion, this work describes a study carried out on the largest collection of LGMD2A patients so far in which both protein and gene mutation analysis were obtained, thus enabling us to draw more reliable conclusions about the prevalence of primary calpainopathy. In our population, at least 28% of the cases of previously unclassified muscular dystrophy in patients analyzed for the CAPN3 gene were due to primary LGMD2A.

We examined for the first time the probability of obtaining LGMD2A molecular diagnosis only from protein testing. This could be an important tool for genetic counseling and has practical relevance in all neuromuscular diagnostic centers at which CAPN3 gene

mutation analyses are not feasible, and whenever patients' DNA samples are not available.

The probability of identifying primary calpainopathies among patients showing partial or complete protein deficiency or showing normal protein expression depends mainly on the different effects of gene mutation at both protein level and function level.

ACKNOWLEDGMENTS

We acknowledge all the clinicians who examined the patients at diagnosis, and we thank the patients and families involved in the study for their active participation and support. Supported by grants from Telethon-Italy (GUP030516 to M.F., GTF02009 to C.A., C61 to L.P., and P10 to V.N.), the Italian Ministry of University and Scientific Research and Technology MURST (2001068328), and the EuroBioBank network (QLRT-2001-027769 to C.A. and L.P.).

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