Candidate-gene testing for orphan limb-girdle muscular dystrophies

S. Aurino[1,2], G. Piluso[1], V. Saccone[2], M. Cacciottolo[2], F. D'Amico[1], M. Dionisi[2], A. Totaro[2], A. Belsito[2], U. Di Vicino[2], V. Nigro[1,2]

[1] *Telethon Institute of Genetics and Medicine (TIGEM), Naples;* [2]*Laboratorio di Genetica Medica, Dipartimento di Patologia Generale, Seconda Università di Napoli, Naples, Italy*

The term limb-girdle muscular dystrophies (LGMD) identify about two dozens of distinct genetic disorders. Additional genes must play a role, since there are LGMD families excluded from any known locus. The aim of our work is to test a number of candidate genes in unclassified LGMD patient and control DNA samples.

We selected the following 11 candidate genes: myozenin 1, 2 and 3), gamma-filamin, kinectin-1, enolase-3 beta, ZASP, TRIM 11 and TRIM 17, OZZ and zeta –sarcoglycan. These candidates were chosen for a combination of different reasons: chromosomal position, sequence homology, interaction properties or muscular dystrophy phenotypes in animal models.

The exon and flanking intron sequences were subjected to molecular testing by comparative mutation scanning by HT-DHPLC of LGMD patients versus control.

We identified a large number of variations in any of the genes in both patients and controls. Correlations with disease or possible modifying effects on the LGMD phenotype remain to be investigated.

Key words: limb-girdle muscular dystrophies

Introduction

The Limb-Girdle Muscular Dystrophies (LGMD) are an important subgroup of muscular dystrophy, grouped together on the basis of common clinical features: they all primarily and predominantly affect proximal muscles of the scapular and the pelvic girdles. The clinical course is characterized by great variability, ranging from severe forms with rapid onset and progression to very mild forms allowing affected people to have fairly normal life spans and activity levels (1).

In addition, clinical characteristics such as hypertrophy of the calves, selectivity of muscle involvement and late stage cardiac complications are associated more or less specifically with each of the different forms (2). The molecular basis of the diseases is also highly heterogeneous (3).

LGMDs are divided into autosomal dominant (LGMD1) and autosomal recessive (LGMD2) forms with a lettering system denoting the chronology of locus identification (A to G for dominant and A to O for recessive LGMDs). Only 3 out of 7 autosomal dominant gene have been identified (4-6) whereas all but one of the causative genes have been identified for the 15 LGMD2 (7-21).

Despite several comprehensive studies developed over the last few years, there are at least 25% of families who are not linked to any known locus and 40% of isolated cases with a severe or intermediate LGMD phenotype with no mutation in any known gene.

The presence of many patients, both sporadic and familiar, not associated with any of the known LGMD loci has led us to explore new potential candidate genes for yet unassigned form of muscular dystrophies.

We screened a large cohort of LGMD patients, with a clear pathogenesis but without molecular diagnosis, by extensive mutation scanning in several candidate genes.

Materials and methods

DNA samples

Genomic DNA was extracted by phenol/chloroform to be used for DHPLC analysis. DNA was quantified and diluted at 20ng/μL for the amplification by PCR (22). We selected 180 DNA samples belonging to unassigned LGMD for which mutations in calpain 3 (LGMD2A), sarcoglycans (LGMD2C-2F), Telethonin (LGMD2G), TRIM32 (LGMD2H), FKRP (LGMD2I), POMT1 (LGMD2K), lamin A/C (LGMD1B) and caveolin 3 (LGMD1C) were excluded. Dysferlin (LGMD2B) and titin (LGMD2I) genes were also excluded by fluorescent microsatellite analysis. DNA samples were spotted into two 96-well plate, together with three empty wells and one control, then amplified and analyzed by DHPLC.

Address for correspondence: Vincenzo Nigro, Telethon Institute of Genetics and Medicine (TIGEM), via Pietro Castellino 111, 80131 Naples, Italy - Tel/Fax. +39-081/5665704. E-mail: nigro@tigem.it,

Polymerase Chain Reaction

The sequences of all candidate genes were downloaded from web interface Genome Browser Santa Cruz (*http://genome.ucsc.edu/cgi-bin/hgGateway*). All coding exons and intron-flanking regions were amplified by PCR from genomic DNA using primers pairs available on request. Primers were compared with results of the web-based program Primer3 (PRIMER3; primer3_www. cgi, v 0.2; *http://frodo.wi.mit.edu*). Each oligonucleotide was also checked by Blastn against the NCBI data bank genome for specificity (BLAST, *www.ncbi.nlm.nih.gov/ BLAST*; NCBI, *www.ncbi.nlm.nih.gov*).

For PCR analysis, 60 ng of genomic DNA was amplified with a DNA Thermocycler System. An initial denaturation step at 95°C for 7 min was set, followed by 34 cycles (95°C for 30 s, 60–61°C for 1 min and 30 s, and 68°C for 1 min) followed by 95°C and a final extension at 68°C for 10 min.

DHPLC Analysis

We performed comparative mutation scanning to select amplicons for aberrant DHPLC profiles not shared by normal controls. Primers were longer than 25 nucleotides to reduce the allele preference determined by sequence differences located in the region of annealing. DHPLC was performed on a WAVE DNA fragment analysis system (Transgenomic Inc.) equipped with a DNASep column (3,500 High Throughput [HT]) employing a UV-C scanner to detect eluted DNA (23). Based on DHPLC requirements, special buffer formulations and primer design were used to improve sensitivity and specificity (22, 23).

Genomic DNA Sequence Analysis

Both strands were sequenced using BigDyes Terminator sequencing chemistry (Applied Biosystems). An ABI3130XL automatic DNA sequencer (Applied Biosystems) was used to analyze the product of the sequence reaction. We verified each nucleotide change by direct sequencing of a second amplified PCR product obtained with different primers. Mutations were numbered based on proteins and cDNA sequences in GenBank (Table 1). Nucleotides were numbered according to international recommendation (24).

Results

Selection of candidate genes

We have selected a pool of eleven candidate genes with different methodology: yeast-two hybrid and bioinformatics approach. It consists in selecting genes with a combination of interesting characteristics: muscle specific expression or localization (sarcomeric or sarcoplasmatic); function (known or hypothetic for muscle); structure (similarity with other LGMD proteins).

Myozenins (1, 2 and 3)

These three genes codify for three small Z-disk proteins which specifically binds calcineurin. Transgenic mice that overexpress the calcineurin develop a progressive cardiac hypertrophy, which causes stroke and death (25). Myozenin 1 is also known as FATZ for the interaction with three sarcomeric proteins: alpha-actinin, filamin C and telethonin. Gontier et al., in 2005 (26) observed the interaction of calsarcins with ZASP and myotilin, confirming the importance of the pathway in which they are involved.

Filamin C

The FLNC gene codify for the muscle-specific filamin isoform. It is involved in a form of autosomal dominant

Table 1. Genes and relative nucleotide and protein accession numbers.

MYOZ1	NM_021245	NP_067068
MYOZ2	NM_016599	NP_057683
MYOZ3	NM_133371	NP_588612
FLNC	NM_001458	NP_001449
KTN1	NM_004986	NP_004977
ENO3	NM_001976	NP_001967
ZASP –variant 3	NM_001080115	NP_001073584
TRIM11	NM_145214	NP_660215
TRIM17	NM_016102	NP_057186
OZZ	NM_080749	NP_542787
SGCZ	NM_139167	NP_631906

Table 2. Myozenins.

MYOZ1					
Exon	**Status**	**Variation**		**Patient alleles**	**Control alleles**
		n.	**p.**		
2	Het	147G>A	L48L	2	
3	Het	282G>T	V93V	1	
4	Het	516C>T	G171G	1	

MYOZ2					
Exon	**Status**	**Variation**		**Patient alleles**	**Control alleles**
		n.	**p.**		
2	Het	237A>G	A78A	1	
3	Het	245-18A>G		13	
4	Het	459A>G	E152E	2	

MYOZ3					
Exon	**Status**	**Variation**		**Patient alleles**	**Control alleles**
		n.	**p.**		
2	Het	61-46G>T		3	
5	Het	424-51G>A		9	
6	Het	587-56T>A		7	

myofibrillar myopathy (MFM) described by Vorgerd et al. in 2005 (27). Patients presented with slow progressive skeletal-muscle weakness, beginning in the lower extremities, which is compatible with the clinical signs of LGMD.

ZASP

Z-band alternatively spliced PDZ-motif containing protein is a sarcomeric protein expressed in human cardiac and skeletal muscle at the Z-disk (28). Several mutations in ZASP gene have been identified as responsible for different dominant disorders: MFM and dilated cardiomyopathy (29-31). The clinical phenotype in patients is heterogeneous, with variable age of onset, proximal or distal presentation and variable occurrence of cardiomyopathy.

Kinectin 1

It is a 160kDa transmembrane protein located on the cytoplasmic vesicles of the endoplasmic reticulum. This is probably present on the vesicles that operate the transport of proteins from the endoplasmic reticulum to the Golgi. It may mediate the binding between kinesin and vesicle membrane to be transported (32).

To date no disease has been linked to mutation in kinectin 1 gene.

Enolase 3-b

The enolase enzyme catalyze the conversion of 2-phosphoglycerate into 2-phosphoenolpyruvate, and the beta isoform is muscle specific.

In 2001, Comi et al. (33) described a patient with a metabolic myopathy showing myalgia, fatigue and stress-induced weakness. This patient resulted compound heterozygous for two missense mutations in ENO3 gene.

TRIM11 and TRIM17

These are two small cytosolic proteins belonging to tripartite motif containing protein family (TRIM) as the muscular dystrophy 2H gene TRIM32. The two genes map at chromosome 1 in the critical region for the congenital muscular dystrophy 1B (MDC1B, OMIM #604801). The disease is characterized by proximal muscle weakness with hypertrophy, respiratory failure and increased CK serum levels.

ζ-sarcoglycan

It is a well known gene whose protein product belongs to sarcoglycans protein family (34). It has been demonstrated its ability to form an alternative complex with α, β and δ in different tissues if γ-sarcoglycan is absent.

Table 3. Filamin C FLNC.

Exon	Status	Variation		Patient alleles	Control alleles
		n.	p.		
2	Het	87C>T	H29H	10	
2	Het	212A>C	N71T	1	0
7	Het	972C>T	V324V	13	
10	Het	1374C>T	R418R	1	
11	Het	1554C>T	Y518Y	9	
14	Het	2007+6G>A		1	
16	Het	2052C>G	L684L	1	
16	Het	2389+54G>T		2	
22	Het	3149 C>T	P1050L	1	0
22	Het	3661C>T	R1221C	1	0
23	Het	3772A>C	R1258R	1	
23	Het	3964+41G>A		13	
24	Het	3913C>T	L1305L	16	
24	Het	3996C>T	R1332R	9	
28	Het	4738+28G>A		6	
31	Het	5011G>A	D1671N	1	0
32	Het	5239+8G>A		1	
33	Het	5338+16T>C		14	
37	Het	5944+11G>A		3	
37	Het	5944+19G>A		2	
38	Het	6145+84C>T		4	
44	Het	7161C>T	D2387D	12	
48	Het	7817G>A	S2606N	15	7
48	Het	7849A>C	K2617Q	11	6

Table 4. ZASP.

Exon	Status	Variation		Patient alleles	Control alleles
		n.	p.		
1	Het	1-93T>C		10	
2	Het	245+41G>A		1	
4	Het	530C>T	A177V	1	0
4	Het	352G>A	V118M	1	0
4	Het	343G>A	G115S	1	0
5	Het	718+47G>C			
6	Het	719-34G>A			
7	Het	1074C>T	A258A	>5	
12	Het	2016C>T	C672C	1	

Four sarcoglycans gene are mutated in LGMDs (α, β, γ and δ) and ε- is the gene mutated in the myoclonic dystonic syndrome (DYT11, 35).

OZZ

It is a small muscle specific protein and is a member of SOCS proteins family. In 2004, Nastasi et al. demonstrated the involvement of OZZ in an active E3-ligase complex in which β-catenin serves as substrate in vivo. OZZ knock-out mice show a muscle phenotype with an increased nuclei centralization and misalignment of myofibrils (36).

Mutation scanning

We analyzed all coding exons and flanking introns and verified whether each variation was present in DB-SNP (NCBI) or not. We excluded those described and only listed new gene variants. We identified 79 heterozygous variations classified as:
1. 34 intron changes;
2. 23 silent changes, without aminoacid substitution;
3. 22 missense variations.

We checked the presence of the 22 missense variations in 200 non affected individuals of the same genetic origin. Sixteen variations were not present in normal controls. For most cases conserved aminoacid are changed. Tables 2 below describe all variations identified in our screening gene by gene.

Discussion

Isolated cases of patients affected by rare genetic disorders are not amenable to studies of their Mendelian causes. The genetic nature of the condition can be nevertheless attributed, when there is a precise knowledge of the disease gene(s): There are, however, two main obstacles: genetic heterogeneity and incomplete penetrance. LGMD suffer from both problems, since the heterogeneity seems very complex with a dozen of major genes that explain up to 50-60% of cases and, hypothetically, hun-

Table 5. Kinectin-1 KTN1.

Exon	Status	Variation		Patient alleles	Control alleles
		n.	p.		
2	Het	273A>T	A91A	9	
3	Het	571C>A	Q191K	1	0
7	Het	1127T>C	M376T	1	0
7	Het	1218G>A	M406I	1	0
13	Het	1791C>T	S597S	1	
14	Het	1837G>A	D613N	1	0
15	Het	1972G>C	A658P	1	0
15	Het	1983+113A>C		7	
16	Het	2021+26G>A		2	
18	Het	2104-76G>T		1	
19	Het	2173-12T>A		1	
19	Het	2207+6A>G		1	
23	Het	2496+58A>T		2	
23	Het	2763+13C>G		1	
23	Het	2763+27G>A		1	
27	Het	2741T>C	V911A	1	0
32	Het	3050C>G	S1017C	1	0
35	Het	3336C>T	S1112S	7	
37	Het	3516+32A>G		4	
40	Het	3749T>C	I1250T	1	2
41	Het	3898+26C>G		2	
41	Het	3852G>A	A1284A	2	

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Table 6. Enolase 3 beta, TRIM and SCGZ.

ENO3					
Exon	**Status**	**Variation**		**Patient alleles**	**Control alleles**
		n.	**p.**		
1	Het	64G>A	V22M	2	1
2	Het	86-5delC		21	
2	Het	115G>A	A39T	1	1
8	Het	1102G>A	V332I	1	1
10	Het	1343+3A>G		1	

TRIM11					
Exon	**Status**	**Variation**		**Patient alleles**	**Control alleles**
		n.	**p.**		
3	Het	739+11C>T		7	
5	Het	847G>A	R283Q	1	0

TRIM17					
Exon	**Status**	**Variation**		**Patient alleles**	**Control alleles**
		n.	**p.**		
4	Het	756-18C>T		1	
6	Het	1131G>A	R379R	1	
6	Het	1377G>A	P761P	1	

SGCZ					
Exon	**Status**	**Variation**		**Patient alleles**	**Control alleles**
		n.	**p.**		
2	Het	234C>A	I91I	1	
2	Het	297+30A>T		1	
4	Het	509+32T>C		1	
6	Het	705+19G>T		1	
7	Het	776G>A	S259N	1	0

dreds of other genes involved in the remaining 40%. In addition, there are cases of causative mutations that are not associated with disease.

We selected 11 genes and performed mutation analysis. Each missense variation was then counted in a comparable number of matched controls. We analyzed two plates containing 180 DNA samples for which no mutation was previously found. All heterozygous variations were found in sporadic patients and no segregation analysis could be performed in their families. Thus, the variations we identified cannot be considered as responsible for recessive LGMD phenotype and we can conclude that none of the selected genes can be considered a common cause of recessive LGMD.

Recently, mutations in both ZASP (31) and filamin C (27) have been associated with myofibrillar myopathy with dominant inheritance. Only missense and one nonsense mutations have been identified. We cannot exclude that the variations we identified in these two genes could be responsible for the observed phenotype, since no histological data are available for those patients.

The presence of novel 16 missense variations that were absent in controls can be intriguing on the basis of general considerations about possible modifier variations (37). In particular in Kinectin-1 (32) we identified seven missense alleles in LGMD that were not shared by healthy controls. Larger population studies are required to assess this point.

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