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A new locus on 3p23-p25 for an autosomal dominant limb-girdle muscular dystrophy, LGMD1H

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Running title: A new locus on 3p23-p25 for AD-LGMD

Abstract

The limb-girdle muscular dystrophies (LGMD) are a genetically heterogeneous group of neuromuscular disorders with a selective or predominant involvement of shoulder and pelvic-girdles. We clinically examined 19 members in a four generations Italian family with autosomal dominant LGMD. Eleven subjects were affected. Clinical findings showed variable expressivity in terms of age at onset and disease severity. Five subjects presented with a slowly progressive proximal muscle weakness, in both upper and lower limbs, with onset during the fourth-fifth decade that fulfilled the *consensus* diagnostic criteria for LGMD. Earlier onset of the disease was found in a group of patients presenting with muscle weakness and/or calf hypertrophy and/or occasionally high CK and lactate serum levels. Two muscle biopsies showed morphological findings compatible with MD associated with subsarcolemmal accumulation of mitochondria and the presence of multiple mitochondrial DNA deletions. A genome wide scan performed by using microsatellite markers mapped the disease on chromosome 3p23-p25.1 locus in a 25-cM region between markers *D3S1263* and *D3S3685*. The highest two-point LOD score was 3.26 ($\theta=0$) at marker *D3S1286* and *D3S3613*, while non parametric analysis reached a p value =0.0004. Four candidate genes within the refined region were analyzed but did not reveal any mutations. Our findings further expand the clinical and genetic heterogeneity of LGMDs.

Key words: Autosomal Dominant Limb-Girdle Muscular Dystrophy; multiple mtDNA deletions; linkage analysis; chromosome 3.

Introduction

The limb-girdle muscular dystrophies (LGMDs) are a heterogeneous group of hereditary neuromuscular disorders with predominant or selective weakness in proximal limb and axial muscles having an estimated incidence of 1:100.000. The clinical phenotype is characterized by a great variability, ranging from early onset, with a severe and rapidly progressive clinical progression to milder forms, with a later onset and a slower progression.¹ To date, seven autosomal dominant (AD) and 12 autosomal recessive (AR) forms of LGMD have been described, with AD families representing less than 10% of the whole group of LGMDs. Among the AD forms, LGMD1A caused by mutations in the *TTID* gene encoding myotilin on chromosome 5q31, is characterized by the presence of dysarthric speech — which probably relates to palatal weakness — in about 50% of the patients.² LGMD1B, due to mutations in *LMNA* encoding lamin A/C on chromosome 1q21, and LGMD1E on 6q23 are also associated with heart conduction system abnormalities, including atrio-ventricular blocks, arrhythmia, and sudden death.³⁻⁵ Mutations in *CAV3* on chromosome 3p25 (LGMD1D) encoding caveolin, are associated with high CK levels with normal strength and distal myopathy, and usually occur in early childhood.⁶ Both the LGMD1D⁷ and the LGMD1F^{8,9} loci map to chromosome 7q and they do not present distinctive clinical features. The LGMD1G form, associated with progressive limitation of fingers and toes flexion, has been positionally cloned on chromosome 4p21 in a Brazilian-Caucasian family.¹⁰ Fewer than ten AD-LGMD families show allelism with Bethlem myopathy and, less frequently, with facioscapulohumeral dystrophy (FSHD), two neuromuscular disorders which are mapped respectively to chromosomes 2q37, 21q22.3, and 4q35 (www.neuro.wustl.edu/neuromuscular/musdist/lg.html). Pedigrees not linked to any of the listed loci warrant further genetic heterogeneity.

We describe the clinical, pathological, and genetic features of a large Italian family with a novel form of AD-LGMD characterized by slowly progressive clinical features and mapped to chromosome 3p23-p25 upon whole genome scan.

Subjects and methods

Clinical and morphological studies

A total of 36 individuals (17 men and 19 women) spanning four generations in a pedigree from southern Italy were investigated. Figure 1 shows a partial pedigree of this kindred with at least two instances of a father-to-son transmission and refers to the branches directly examined in the present study. According to established guideline criteria,¹¹ index subjects were considered affected by LGMD when neurological examination revealed a typical pattern of muscle weakness affecting the upper and lower girdles. However, we recognized a second group of relatives with younger age at onset or milder clinical presentation, or both, and considered them as affected for genome-wide purposes. Muscle strength was appraised using the MRC (British Medical Research Council) Scale. Age-at onset was determined at the time when muscle complains first became manifested. Nineteen subjects (6 men and 13 women) were clinically assessed by two neurologists skilled in the evaluation of neuromuscular disorders. The clinical features of the remaining individuals were obtained by either family recall or consultation of available clinical notes. Routine blood tests were obtained to investigate metabolic causes of the disease. Serum creatin kinase (CK) and lactate levels were determined in all subjects. Electromyography (EMG) was performed in five patients. Four patients (II-1, II-5, II-9 and II-13) underwent a skeletal muscle biopsy. The specimens from the deltoid or *vastus lateralis* were oriented, snap-frozen in liquid nitrogen-chilled isopentane and the cryostat-cut sections were stained using standard histochemical methods. Immunohistochemistry was performed using the following antibodies: desmin and vimentin (Biogenex, CA), ubiquitin (DAKO, DK), Tau protein and Beta-amyloid (Sigma, MO), sarcoglycans, dystrophin, beta-dystroglycan (YLEM s.r.l. Becton Dickinson Company), caveolin-3 (Transduction Laboratories), and the amino terminal of utrophin (DRP2, Novocastra Laboratories, Newcastle upon Tyne, UK).

Having obtained the patients' informed consent, we purified genomic DNA from blood of 19 members of the family and from 6 spouses and from skeletal muscles of II-5 and II-9, using a standard phenol-chloroform procedure. Approximately 6 µg of total DNA from muscle samples were cleaved with the endonuclease *PvuII* to linearize mtDNA molecules, electrophoresed through a 0.8% agarose gel, and blotted onto a nylon membrane (N+ Hybond, Amersham Biosciences, UK). The entire mtDNA was PCR-amplified using four overlapping primer pairs (primers and PCR conditions are

available upon request), labelled with alpha-³²P-dATP by random priming (Random Primed DNA Labelling Kit, Roche Diagnostic Corp., Germany), and used as a probe. Signal intensities were analyzed with a Molecular Imager FX (BioRad, Hercules, CA).

Linkage analysis

A genome wide scan was carried out by using the ABI PRISM Linkage Mapping Set LMS-MD10 (Applied Biosystems, Foster City, CA) which includes 382 highly polymorphic microsatellite markers from the 22 autosomes with an average intermarker distance of 10 cM. Additional, more densely-spaced markers were used to refine chromosomal regions on 3p, 4q, 7q, and 9p according to available genetic maps. Genotyping and fragments analysis was performed as previously described.¹²

Statistical analyses were carried out assuming an autosomal dominant mode of inheritance with a disease allele frequency of 0.001 and assuming equal frequencies and recombination rates between men and women for all markers analyzed. Physical and genetic distances between them were based upon publically available electronic databases from the University of California Santa Cruz (www.genome.ucsc.edu) and the Marshfield genetic map (www.marshfieldclinic.org). Two age-dependent liability classes were considered for at-risk individuals: 0.80 in < 35 years and 0.95 in >35 years subjects. In addition, because of the presence of two different groups of patients — the first made of definitively affected subjects and the second consisting of individuals with younger age at onset or milder clinical presentation, or both — we also conducted data analysis under a more stringent *scenario* where patients belonging to the second group were considered as having an “unknown phenotype”. Data were simulated using SLINK^{13,14} in order to determine the power to detect linkage. Parametric two-point linkage analysis was performed using MLINK from the Linkage Analysis Package II version 5.1, 1996. Non parametric and parametric multipoint analyses were performed by means of the SimWalk2 program version 2.83 that calculates the Maximum Location Score (directly comparable with multipoint LOD score) using Markov chain Monte Carlo (MCMC) and simulates annealing algorithm.¹⁵

Sequencing of candidate genes

The entire coding sequence of *CAV3* was amplified and directly sequenced in subjects II-1 and II-5 using previously published primer pairs and PCR conditions.⁶ The *CMYA1*, *CAPN7* and *MGC15763* genes were screened for mutations in the same

individuals. PCR primers were designed with the Primer 3 software¹⁶ (sequences are available upon request). All PCR fragments were agarose gel-purified and directly sequenced on both strands using the Big Dye Terminator chemistry on an ABI 3100 DNA sequencer (Applied Biosystem, Foster City, CA, USA).

Results

Table 1 summarizes clinical, morphological, and laboratory data in the examined family members. ID numbers refer to the pedigree represented in Figure 1. A subset of severely affected members developed a slowly progressive proximal muscle weakness in both upper and lower limbs; among these, five subjects (II-1, II-3, II-5, II-9, II-13) were considered as affected fulfilling the criteria for a diagnosis of LGMD.¹¹ We also identified a mildly affected group of patients (II:7, III:3, III:4, III:8, IV:1, IV:2) who had calf hypertrophy, and occasional raise of CK and lactate levels. These patients were considered as possibly affected, though to a lesser extent.

On clinical grounds, onset of symptoms started around the fifth decade in five cases with a slowly progressive muscle weakness (median MRC score was 4) initially in the lower limbs, hypotrophy of both upper and lower limb-girdles muscles, reduced deep tendon reflexes, and calf hypertrophy. In one case (I-1), age of onset and clinical phenotype were established by family recall and evaluation of medical records but we could not examine him because he had died at the age of 77 years because of an ischemic heart attack. On average, serum CK levels were elevated (ranging from 997 to 2300 U/L, normal <230) whereas lactate levels were normal. One patient (case II-13) had been defined healthy at age 39 years when examined elsewhere, though he complained of unspecified muscle fatigability, and occasional raise of serum CK. Four-year follow up examination revealed mild weakness in the lower limb-girdle muscles (MRC 4-5) and elevated CK levels (1600 U/L). EMG showed a decreased duration of motor unit potentials, without evidence of denervation in the four limb muscles, in two cases (II-1; II-5). None of the patients presented ultrasound evidence of abnormal heart muscle function. A second, less severely affected group of individuals in this family had a relatively younger age at examination (median age 28 years), displayed calf hypertrophy but not significant muscle weakness. Eight individuals (median age 22

years) are clinically healthy, do not complain of muscular symptoms and have CK values in the normal range.

Histochemistry performed on muscle biopsies in patients II-1, II-5, II-9 and II-13 showed abnormal fiber size and shape variation, increased endo- and perimysial connective tissue on hematoxylin-eosin staining. Central nuclei were occasionally present. Ragged red fibers (RRFs) were observed in patient II-1 and subsarcolemmal accumulation of mitochondria upon succinate dehydrogenase (SDH) staining as well as fibers negative at cytochrome *c* oxidase staining were evidenced in patients II-5 and II-9. Immunohistochemical stains for dystrophin, sarcoglycan, caveolin and δ -sarcoglycan were normal. In addition to the wild-type mtDNA, Southern blotting in skeletal muscle biopsies from patients II-5 and II-9 showed multiple bands of lower size corresponding to deleted mtDNA molecules (Figure 2).

Following the initial 10 cM genome-wide screening, multipoint analysis showed four chromosomal regions (3p23, 4q13.2, 7qter, and 9p13.3) with a maximum location score >2 . Using more densely-spaced markers within each identified region we increased the resolution map to ~ 1.5 cM, and conclusively excluded linkage to the 4q and 7q regions. A maximum location score of 2.36 was obtained for chromosome 9p but the highest value of 3.236 was reached at marker D3S1286, located on chromosome 3p23-p25. A value >3.0 was observed, in the ~ 16 cM interval between markers *D3S3610* and *D3S1266* (Figure 3). Two point analysis showed a MLS=3.23 ($\theta=0.0$) at markers *D3S3613* and *D3S1286* (Table 2). Non parametric multipoint analysis supported this conclusion at both markers, yielding a STAT D score of 2.26 ($p=0.0004$) and a STAT E score of 15.4358 ($p=0.0004$), where “statistic D” is the extent of allele sharing among all affected pairs as measured by their IBD kinship coefficient¹⁵ and “statistic E” is the NPL_all statistic as implemented in GeneHunter¹⁷ (Table 2). When we adopted a more conservative analysis, which considers as “unknown” cases belonging to the group of mildly affected patients, the highest value of LOD score (MLS >1.50) was reached in the interval between markers *D3S3610*-*D3S3726*. Taken together, our results made chromosome 3p23-25p the most likely region where the new LGMD1H locus is potentially located.

Haplotype reconstruction confirmed the results obtained from linkage analysis data (Figure 1). The telomeric boundary of the candidate interval was defined by an obligate

recombination event observed between *D3S1263* and *D3S1259* in subject III-4, whereas the centromeric flanking marker is defined by another recombination event between markers *D3S3521* and *D3S3685* in subject III-3. All the affected individuals shared the same disease haplotype. Two healthy individuals (III-2 and III-6) shared part of the same haplotype between markers *D3S1619* and *D3S1277*, thus further refining the LGMD1H locus to a 25 cM region.

Two presently unaffected individuals (III-1 and III-11) shared the disease haplotypes. Subject III-1 is a 30-year-old man with normal serum CK levels. Subject III-11 is a 14-year-old girl with a history of recurrent migraine who has never had muscle complaints nor high CK. However, she is younger than the median age at onset of her affected relatives. Neurological examinations in both individuals was normal.

The region refined by linkage analysis contains more than 150 genes. Four possible candidates were prioritised for mutational screening because of their function or position: *CAV3*, *CAPN7*, *MGC15763* and *CMYAI*. Although located well outside the interval refined in linkage with this family, the caveolin-3 gene (*CAV3*) was analyzed to firmly exclude allelism with LGMD1C. *CAPN7* encodes an 814-residues protein which contains a calpain-like cysteine protease domain and a C-terminal PBH domain. Expressed mostly in the pancreas and only moderately in skeletal muscle and liver,¹⁸ *CAPN7* shares sequence homologies with *CAPN3*, the LGMD2A causative gene. The *MGC15763* gene encodes for a 312 amino acid long protein which is predicted to be exported to mitochondria (MitoProt II 1.0 α 4 probability is 0.8599; predicted cleavage site at amino acid 44). By primary structure analysis, the gene product has a NADH binding domain but no strong homology with any known protein. *CMYAI* encodes the human orthologue of mXin α , a protein believed to play a regulatory role in cardiac morphogenesis, differentiation, and localized to the adherence junctions of intercalated discs.¹⁹ The entire coding regions of *CAV3*, *CAPN7*, *MGC15763* and *CMYAI* and their flanking intronic sequences were analyzed but no pathogenic mutations were found.

Discussion

We identified a large pedigree from southern Italy segregating a novel AD form of muscular dystrophy that fulfilled the diagnostic criteria for LGMDs.^{11,20} Variable expressivity was observed in term of age at onset and muscular symptoms. With few

exceptions, the adult patients showed the “canonical” clinical features of LGMD, characterised by slowly progressive proximal muscle weakness in upper and lower limbs with onset during the fifth decade and a relatively benign course. Neither ocular-facial-bulbar muscles, nor cardiac muscle involvement, nor early joint contractures were observed. A single individual (I:1) had a likely hypertensive cardiomyopathy, while echocardiography performed in three subjects affected by LGMD (II:1, II:3, II:13) was normal. An earlier onset of the disease was found in a group of patients complaining muscle weakness or calf hypertrophy and occasional raise of CK and lactate levels. Based on the clinical history of the five adult members, this juvenile group is likely to develop later in life the typical pattern of LGMD. Two unaffected subjects (III:1 and III:11) carried the disease haplotype, suggesting an incomplete penetrance for the disease. However, the follow up for the subject III:11 remains unpredictable since she is younger than the mean age of the second generation relatives.

Consistently, immunohistochemistry and linkage analysis excluded any other known dominant form of LGMD. In keeping with the current classification of inherited LGMDs, we propose to define this form of muscular dystrophy as LGMD1H.

Evidence of histochemical features suggestive of a defect of oxidative phosphorylation and multiple mtDNA deletions in skeletal muscle from two patients, appeared to suggest a mitochondrial dysfunction. A similar feature was observed in one patient from a single LGMD1F family thus far reported.⁸ Multiple mtDNA rearrangements usually result from mutations in genes involved in mtDNA replication and maintenance.²¹ At least seven genes have been identified in this subset of mitochondrial disorders (reviewed in Zeviani *et al*²²), but none locate in the 3p23-p25 interval defined in our LGMD1H family. Low levels of multiple mtDNA deletions can also be a secondary event, such as it happens in sporadic and familial Inclusion Body Myosites²³ and in the ageing processes.²⁴ In our patients, mtDNA deletions are likely to be considered as by-products of mitochondrial dysfunction of still unknown significance.

Whole genome scan for the LGMD1H locus showed data compatible with linkage to a 31 cM region on chromosome 3p23-p25. Haplotype reconstruction allowed the refinement of the candidate region to 25 cM between markers *D3S1263* and *D3S1277*. In this interval, *in silico* analyses revealed the presence of more than 150 genes

(www.ensembl.org). However, no evident disease-causative gene emerged, and two of the potentially candidates (*CAPN7* and *MGC15763*) were ruled out by direct sequencing. The function of the human *CAPN7* gene product is presently unknown but the mouse orthologue is thought to be an enzyme calcium-independent with protease activity. The human protein seems to have a nuclear localization and is the most divergent element in the calpain team, sharing only 26-35% of its sequence with the other members of this family.¹⁸ The *MGC15763* gene encodes the hypothetical protein BC008322 which contains a region of NADH -cytochrome *b*- reductase involved in coenzyme transport and metabolism, energy production and conversion, and it is predicted to have a mitochondrial targeting.

In summary, we have presented evidence for a novel form of AD-LGMD mapping on chromosome 3p23-p25.1 characterized by a slowly progression of proximal muscle weakness in both upper and lower limbs. The transmission model of the disease is consistent with an AD pattern of inheritance with variable expressivity and incomplete penetrance. In fact, the symptoms vary in terms of age at onset and clinical severity. The identification of additional LGMD1H families, further refinement of the genetic interval in our kindred or both are needed before considering screening for additional candidate genes.

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Titles and legends to figures

Figure 1. Pedigree of the family affected by the novel AD LGMD form and haplotype analysis of microsatellite markers from chromosome 3p23-p25. The haplotype segregating with the disease is “*boxed*”. Black filled symbols indicate affected subjects; clear symbols indicate the unaffected subjects.

Figure 2. Southern blot performed on DNA extracted from skeletal muscle biopsies.

Figure 3. Diagrammatic representation of the region 3p22-p25.3. (a) Schematic map of the short arm of the chromosome 3 showing markers and genes of interest located between 11 and 42 Mb. (b) Multipoint LOD scores between the disease and markers on chromosome 3p.

Table 1. Principal clinical and laboratory data in family members.

Table 2. Two point LOD score and multipoint non parametric LOD scores between LGMD and microsatellite markers on chromosome 3p23-p25.

3 1 D3S1286
 3 3 D3S3726
 1 2 D3S3659
 3 3 D3S3700
 5 6 D3S2336
 1 1 D3S1266
 8 5 D3S3727
 1 4 D3S3567
 1 1 D3S1619
 2 4 D3S1277
 3 3 D3S3521
 3 3 D3S3685

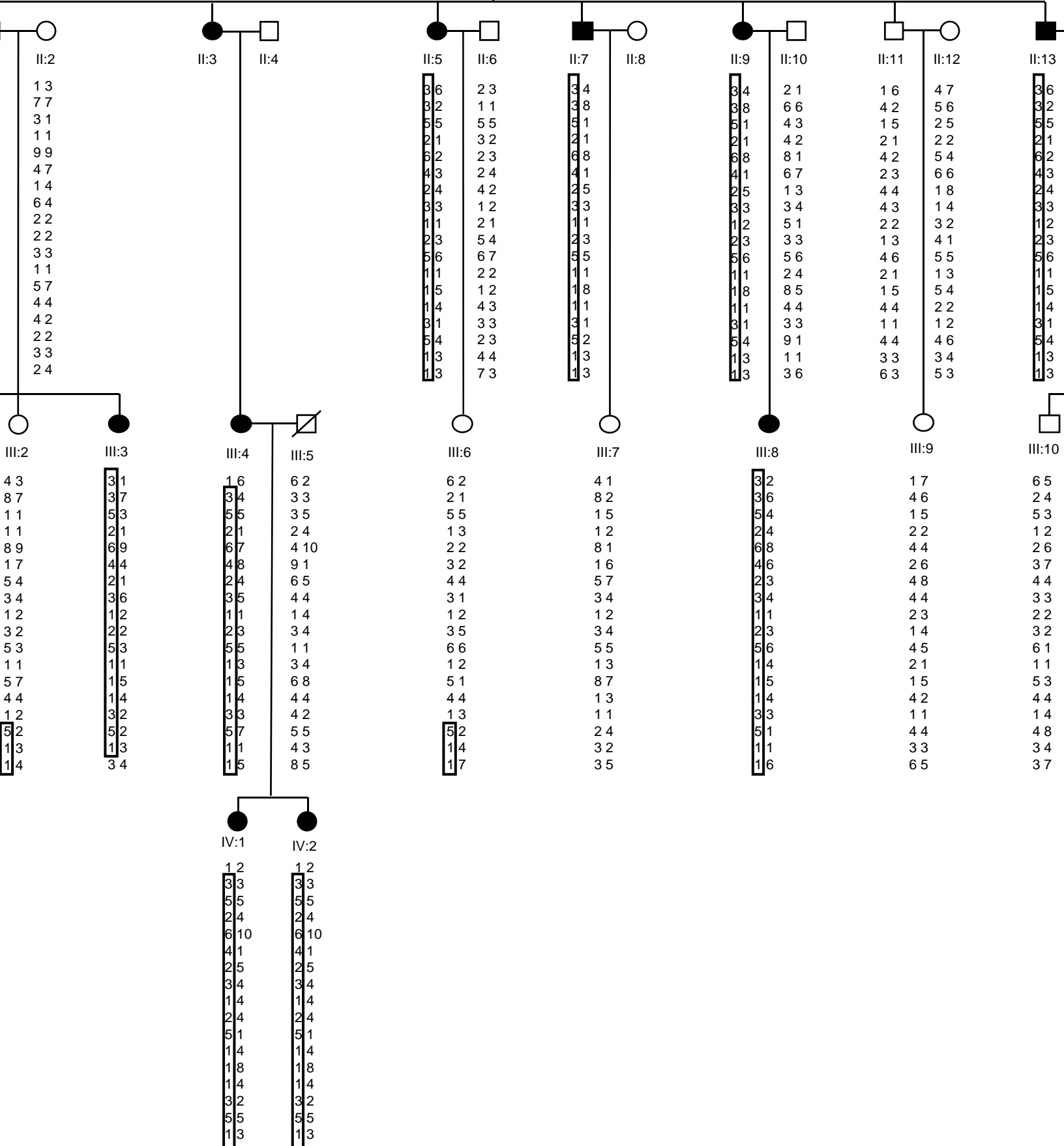
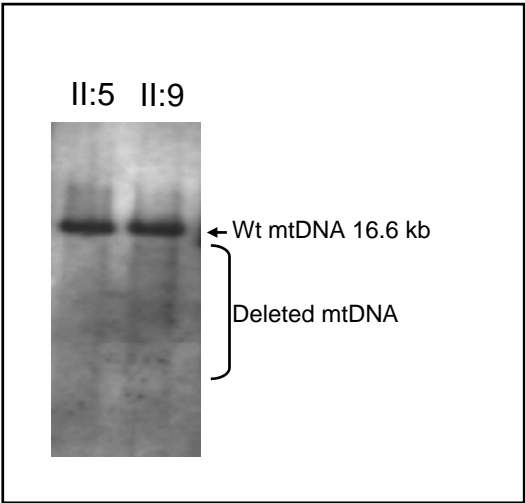


Figure 2



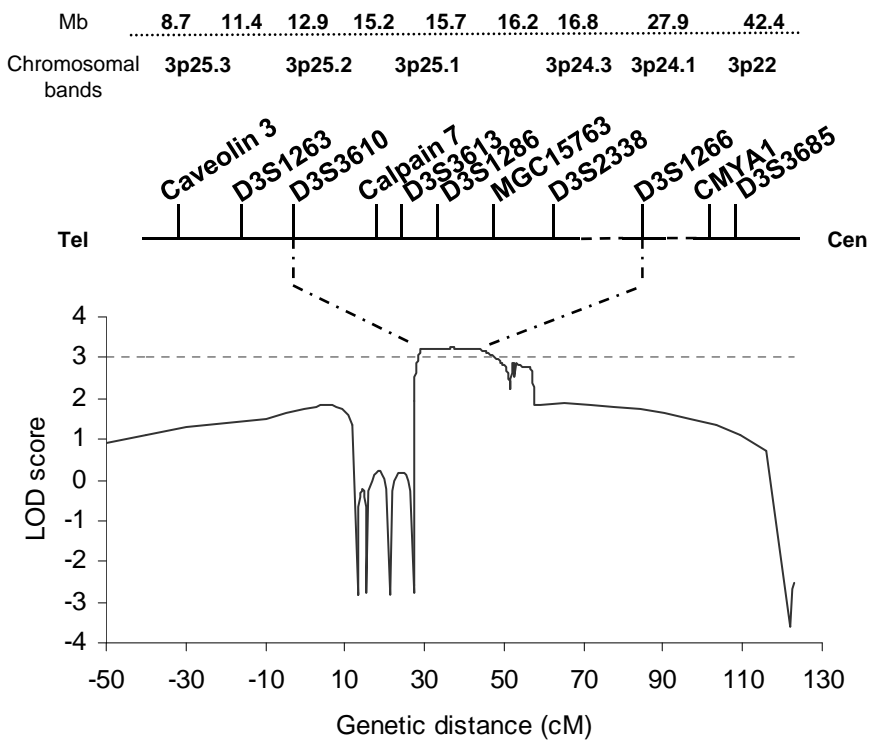


Table 1. Principal clinical and laboratory data in family members.

	Patient	Age at diagnosis	Age at onset	Sex	Biopsy^c	EMG	CK level (U/L)	Lactate level (mg/dl)	Neuromuscular examination	Other symptoms
<i>Severely affected</i>	I:1 ^a	dead at 77 years	-	m	na	na	na	na	calf hypertrophy	Headache, heart problems
	II:1	60	50	m	++	myopathic pattern	2295	13.2	muscle weakness, calf hypertrophy	dyspnea , scoliosis
	II:3 ^b	59	49	f	na	myopathic pattern	1551	11.0	muscle weakness	
	II:5	57	50	f	++	myopathic pattern	997	8.7	muscle weakness	
	II:9	49	45	f	++	myopathic pattern	2139	6	muscle weakness, calf hypertrophy	scoliosis
	II:13	42	39	m	++	normal	1600	10.7	muscle weakness	
	III:3	24	20	f	na	na	2300	11.9	muscle weakness	
<i>Mildly affected</i>	II:7	50	46	m	na	na	153	20.5	calf hypertrophy	
	III:4	37	34	f	na	na	160	18.2	calf hypertrophy	headache
	III:8	22	22	f	na	na	155	38.7	calf hypertrophy	
	IV:1	17	17	f	na	na	67	11.6	calf hypertrophy	
	IV:2	16	16	f	na	na	98	11.3	calf hypertrophy	
<i>Unaffected</i>	II:11	45	-	m	na	na	62	8,2	normal	
	III:1	30	-	m	na	na	185	10,9	normal	
	III:2	27	-	f	na	na	110	12,3	normal	
	III:6	10	-	f	na	na	97	11,1	normal	
	III:7	10	-	f	na	na	183	9,9	normal	
	III:9	24	-	f	na	na	78	11,7	normal	
	III:10	17	-	m	na	na	162	8,1	normal	
	III:11	14	-	f	na	na	130	11,3	normal	recurrent migraine without aura

++: skeletal muscle biopsy showing dystrophic pattern; na: not available; ^aclinical information based on anamnestic recall; ^bconsultation of clinical notes; ^cdescription of bioptical findings in the text.

Table 2. Two point LOD score and multipoint non parametric LOD scores between LGMD and microsatellite markers on chromosome 3p23-p25.

Markers	Position		Two point LOD score at $\theta_{(m=f)} =$							Multipoint NPL			
	cM	Mb	0.0	0.01	0.05	0.1	0.2	0.3	0.4	STAT D	<i>p-value</i>	STAT E	<i>p-value</i>
D3S1263	36.10	11.492	-2.52	0.90	1.47	1.57	1.39	0.97	0.44	1.3998	0.2250	0.1383	0.1527
D3S1259	36.65	12.073	2.32	2.30	2.21	2.06	1.66	1.14	0.53	1.8495	0.1087	8.1269	0.0746
D3S3610	37.20	12.980	0.66	0.66	0.63	0.59	0.46	0.31	0.15	2.2600	0.0010	15.4095	0.0008
D3S1554	39.52	14.342	0.88	0.90	0.92	0.86	0.71	0.49	0.24	2.2591	0.0005	15.3722	0.0004
D3S3613	41.56	15.336	3.23	3.19	3.05	2.83	2.27	1.58	0.76	2.2614	0.0005	15.4394	0.0005
D3S1286	41.56	15.794	3.23	3.19	3.05	2.83	2.27	1.58	0.76	2.2615	0.0004	15.4396	0.0004
D3S2338	42.10	16.824	2.75	2.72	2.57	2.35	1.81	1.16	0.48	2.2615	0.0005	15.4397	0.0004
D3S3726	42.64	19.509	2.21	2.18	2.02	1.81	1.37	0.88	0.36	2.2611	0.0005	15.4268	0.0004
D3S3659	47.44	22.913	1.23	1.21	1.13	1.01	0.73	0.42	0.12	2.2610	0.0002	15.4261	0.0002
D3S3700	48.09	23.885	2.75	2.72	2.57	2.35	1.80	1.15	0.46	2.2624	0.0002	15.4443	0.0002
D3S2336	49.18	24.889	1.75	1.73	1.63	1.44	1.15	0.74	0.29	2.2614	0.0004	15.4163	0.0004
D3S1266	52.60	27.932	2.21	2.18	2.02	1.81	1.37	0.88	0.36	2.2608	0.0007	15.4012	0.0005
D3S3727	56.07	30.652	1.21	1.24	1.28	1.26	1.06	0.74	0.37	2.2622	0.0008	15.4436	0.0007
D3S3567	56.69	30.713	1.20	1.18	1.10	0.97	0.66	0.32	0.07	2.2622	0.0007	15.4436	0.0007
D3S1619	60.98	34.087	2.62	2.60	2.49	2.32	1.87	1.29	0.60	2.3114	0.0005	15.7125	0.0004
D3S1277	61.52	34.627	1.83	1.84	1.84	1.79	1.54	1.11	0.53	2.3241	0.0004	15.7821	0.0003
D3S3521	63.12	38.830	1.23	1.24	1.29	1.28	1.13	0.82	0.37	2.3136	0.0005	15.3653	0.0004
D3S3685	67.94	42.429	-7.75	-1.46	-0.10	0.40	0.70	0.61	0.29	1.7626	0.0321	0.6551	0.0331