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Calpain-3 mutations in Turkey

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Abstract Autosomal recessive limb-girdle muscular dystrophies (LGMD2s) are a clinically and genetically heterogeneous group of disorders, characterized by progressive involvement of the proximal limb girdle muscles; the group includes at least 10 different genetic entities. The calpainopathies (LGMD2A), a subgroup of LGMD2s, are estimated to be the most common forms of LGMD2 in all populations so far investigated. LGMD2A is usually characterized by symmetrical and selective atrophy of pelvic, scapular and trunk muscles and a moderate to gross elevation of serum CK. However, the course is highly variable. It is caused by mutations in the CAPN3 gene, which encodes for the calpain-3 protein. Until now, 161 pathogenic mutations have been found in the CAPN3 gene. In the present study, through screening of 93 unrelated LGMD2 families, we identified 29 families with LGMD2A, 21 (22.6%) of which were identified as having CAPN3 gene mutations. We detected six novel (p.K211N, p.D230G, p.Y322H, p.R698S, p.Q738X, c.2257delGinsAA) and nine previously reported mutations (c.550delA, c.19_23del, c.1746-20C>G, p.R49H, p.R490Q, p.Y336N, p.A702V, p.Y537X, p.R541Q) in the CAPN3 gene. There may be a wide variety of mutations, but clustering of specific mutations (c.550delA: 40%, p.R490Q: 10%) could be used in the diagnostic scheme in Turkey.

Keywords LGMD2A · CAPN3 gene · Novel mutations · Mutation frequency

Abbreviations LGMD2s: Autosomal recessive limb-girdle muscular dystrophies · CAPN3: Calpain-3 · DMD: Duchenne muscular dystrophy · IF: Immunofluorescence · WB: Western blot

OMIM numbers: CAPN3 = OMIM: 114240, 253600 (LGMD2A), GenBank: AF209502.1

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Introduction

Limb-girdle muscular dystrophies (LGMD2s) are a clinically and genetically heterogeneous group of diseases that are characterized by progressive weakness of the pelvic and shoulder girdle muscles and highly elevated serum creatine kinase levels [7]. These disorders may be inherited as an autosomal dominant (LGMD1) or autosomal recessive (LG MD2) trait. The autosomal recessive forms represent more than 90% of all LGMDs [6]. At least 10 different genetic loci leading to LGMD2 are recognized: LGMD2A (calpain-3) [26], LGMD2B (dysferlin) [2, 16], LGMD2C (γ-sarcoglycan, γ-SG)) [22], LGMD2D (α-sarcoglycan, α-SG) [30], LGMD2E (β-sarcoglycan, β-SG) [4, 15], LGMD2F (δ-sarcoglycan, δ-SG) [19], LGMD2G (telethonin) [18], LGMD2H (TRIM 32) [13], LGMD2I (fukutin-related protein, FKRP) [5] and LGMD2J (titin) [8].

LGMD2A (MIM# 253600) or calpainopathy is considered to be the most frequent form of recessive LGMD in several populations, accounting for about 30–40% of the identified cases [10, 21, 27]. It is caused by mutations in the human calpain-3 (CAPN3) gene (OMIM# 114240), which is located on chromosome 15q15.1-15.3 [3], consists of 24 exons spanning approximately 53 kb of genomic DNA, and encodes a muscle-specific member of a family of Ca^{++}-activated neutral proteases named calpain-3 (p94) [25]. Calpain-3 in LGMD2A was the first example of an enzymatic, rather than structural, protein defect causing a progressive muscular dystrophy. CAPN3 may play a functional role in the disassembly of sarcomeric proteins, and it may also have a regulatory role in modulation of transcription factors [33]. Although the cascade of pathogenic events leading to calpainopathy is still unknown, functional studies [23] provided strong evidence that LGMD2A results from the loss of proteolysis of substrates by calpain-3, suggesting a novel molecular mechanism for LGMD2A. The disease is characterized by symmetrical and selective proximal atrophy, with no cardiac or facial disturbance and normal intelligence [28]. In most cases, symptoms arise in childhood and progression is usually milder than in Duchenne muscular dystrophy (DMD), leading to a loss of independent ambulation about 10–20 years after onset. However, a marked heterogeneity of inter- and intrafamilial clinical severity has been reported [32]. In addition, there is no direct correlation between the amount of calpain and the severity of the phenotype [1].

According to the Leiden Muscular Dystrophy database http://www.dmd.nl/capn3_home.html), 161 distinct pathogenic mutations in the CAPN3 gene have been described to date. All these mutations are distributed along the entire length of the CAPN3 gene, and small hot spot regions are present in exon 11 and exon 21. Some mutations, such as 550delA, have been found more frequently in some populations (e.g., Russia [24], Croatia [9] and Turkey [31]), but most of them represent private variants. The first extensive CAPN3 mutation study was based on the analysis of 181 families from 19 countries and reports the identification of 97 pathogenic mutations [28]. In addition, 37 different CA PN3 gene mutations were detected in the largest collection of 548 myopathic patients published so far [12]. In this study, we report our 10-year experience in diagnosis of LGMD2A among 93 Turkish LGMD2 families. A total of 15 different CAPN3 gene mutations were detected, 6 of which were novel (p.K211N, p.D230G, p.Y322H, p.R698S, p.Q738X, c.2257delGinsAA).

Methods

Patient selection criteria and clinical examination

The Hacettepe University Children's Hospital in Ankara is a major referral center for neuromuscular disorders in Turkey. In our cohort, there are 93 families with cases diagnosed as LGMD2 and whose combined clinical and biochemical investigations have been completed [10, 11, 31]. The majority of these cases have been reported with entire clinical and genetic data. Our selection criteria for LGMD2 were as follows: (1) a pedigree compatible with an autosomal recessive trait, (2) onset after the child walked, (3) progression of muscle weakness of varying severity showing a limb-girdle distribution with sparing of facial muscles, (4) normal intelligence, (5) muscle biopsy compatible with a muscular dystrophy and (6) exclusion of dystrophinopathy by immunocytochemistry. Manual muscle testing was done according to the British Medical Council (MRC) grading [17]. The following parameters were chosen for the practical purpose of grading the functional stage of dystrophy in a mainly paediatric setting: severe, if onset was in childhood and the disability was similar to DMD; intermediate, if onset was in childhood and the progression or disability was similar to Becker muscular dystrophy (BMD); moderate, if onset was in adulthood and the patient showed physical disability of any grade; and mild, if onset was in adulthood and the patient did not have any disability (i.e., less than grade I) [14]. Chest x-ray and routine ECG recordings were obtained at each follow-up. Each patient had at least two cardiac echograms. Respiratory function assesments were also performed.

Muscle biopsy

At the time of diagnosis, open biopsy from the quadriceps femoris muscle was obtained under local anesthesia after written informed consent from the patients or their parents. Muscle biopsy specimens were frozen in isopentane, cooled in liquid nitrogen and stored at −80°C until processed.

Immunofluorescence (IF) and Western blot (WB) analysis

Immunofluorescence (IF) and Western blot (WB) analysis were performed on skeletal muscle biopsies using a panel of monoclonal antibodies against dystrophin, α, β, γ and δ sarcoglycan (Novocastra).

Genotyping, linkage studies and mutation analysis

The genomic DNA from 93 families was extracted from peripheral blood samples according to standard protocols (after written informed consent). For genotyping, highly polymorphic markers of chromosomes 2p13-p16, 4q12, 5q33-q34, 13q12, 15q15.1-q15.3 and 17q12-q21.33 were used [29]. A total of 29 families with 63 cases matched our genotyping criteria for LGMD2A and probands from these families were selected for mutation screening. Mutations in the CAPN3 gene (GenBank accession #AF209502.1) were detected by SSCP, DHPLC and/or by direct sequencing of PCR products (ABI-PRISM 3700, Applied Biosystems) generated from genomic DNA using specific primers. Additional novel mutations found in patients were checked

based on 600 normal chromosomes to exclude the possibility of polymorphisms. DNA mutation numbering was based on the cDNA sequence.

Results

In our cohort, there were 93 families with cases diagnosed as LGMD2. Combined Western blot, immunofluorescence and linkage analysis were performed. By linkage analysis, 29 of these families were found to be compatible with the LGMD2A locus. This represents a frequency of 31.2%. The vast majority of our families were closely consanguineous. IF and WB analysis of the muscle biopsy specimens revealed normal expression of dystrophin and all SGs in each case from those families.

Probands from these 29 LGMD2A families were subjected to mutation screening. Six novel mutations (p.K211N,

Table 1 Summary of the CAPN3 gene mutations in LGMD2A patients

Case	Allele	Location	Nucleotide change	Amino acid change	Consanquinity	Sex	Phenotype[a]	Age (age of onset) (years)
1	1	Exon 4	c.550delA	–	+	F	Intermediate	20 (10)
	2	Exon 4	c.550delA	–				
2	1	Exon 1	c.19_23del	–	+	M	Intermediate	20 (3)
	2	Exon 1	c.19_23del	–				
3	1	Exon 4	c.550delA	–	+	F	Intermediate	23 (12)
	2	Exon 4	c.550delA	–				
4	1	Exon 7	c.1006T>A	p.Y336N	+	F	Intermediate	27 (10)
	2	Exon 11	c.1469G>A	p.R490Q				
5	1	Exon 19	c.2105C>T	p.A702V	+	F	Intermediate	22 (11)
	2	Exon 19	c.2105C>T	p.A702V				
6	1	Exon 4	c.550delA	–	+	F	Intermediate	24 (2 1/2)
	2	Exon 4	c.550delA	–				
7	1	Exon 4	c.550delA	–	+	M	Intermediate	21 (10)
	2	Exon 4	c.550delA	–				
8	1	Exon 4	c.550delA	–	+	M	Moderate	26 (14)
	2	Exon 4	c.550delA	–				
9	1	Exon 13	c.1611C>A	p.Y537X	+	F	Intermediate	23 (10)
	2	Exon 13	c.1611C>A	p.Y537X				
10	1	Exon 4	c.550delA	–	+	F	Intermediate	31 (10)
	2	Exon 4	c.550delA	–				
11	1	Exon 4	c.550delA	–	+	F	Mild	22 (10)
	2	Exon 4	c.550delA	–				
12	1	Exon 4	c.550delA	–	–	F	Asymptomatic (CK>4000 U/l)	9 (6)
	2	?	?	?				
13	1	Exon 11	c.1469G>A	p.R490Q	–	M	Intermediate	16 (10)
	2	Exon 13	c.1622G>A	p.R541Q				
14	1	Exon 21	c.2212T>C	p.Q738X	+	M	Mild	11 (6)
	2	Exon 21	c.2212T>C	p.Q738X				
15	1	Exon 1	c.146 G>A	p.R49H	–	F	Intermediate	30 (17)
	2	Exon 21	c.2257delGinsAA	–				
16	1	Exon 11	c.1469G>A	p.R490Q	+	M	Intermediate	45 (19)
	2	Exon 11	c.1469G>A	p.R490Q				
17	1	Exon 5	c.689A>G	p.D230G	+	F	Intermediate	24 (13)
	2	Exon 5	c.689A>G	p.D230G				
18	1	Exon 7	c.964 T>C	p.Y322H	+	M	Mild	12 (3)
	2	?	?	?				
19	1	Exon 5	c.633G>T	p.K211N	+	M	Severe	31 (15)
	2	?	?	?				
20	*1*	Exon 4	c.550delA	–	+	F	Intermediate	15 (6)
	2	Exon 19	c.2092C>A	p.R698S				
21	1	Exon 14	c.1746-20C>G	–	+	M	Intermediate	44 (17)
	2	Exon 14	c.1746-20C>G	–				

[a]See Methods section for precise definition of terms

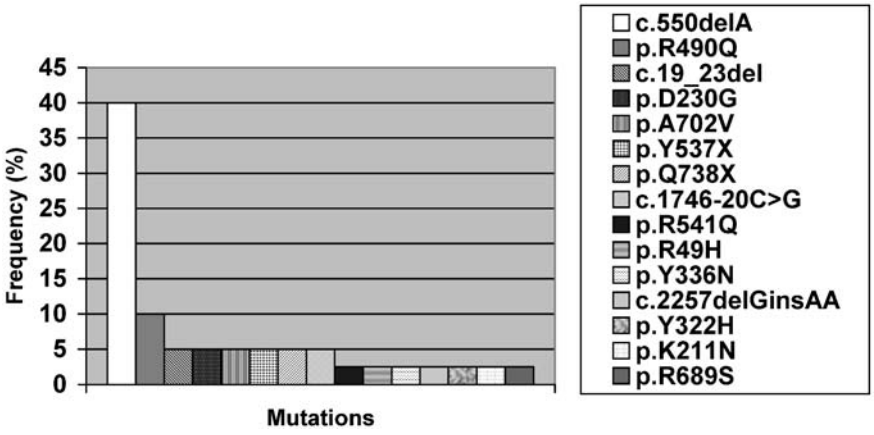

Fig. 1 Frequencies of the CAPN3 gene mutations in Turkey

p.D230G, p.Y322H, p.R698S, p.Q738X, c.2257delGinsAA) and nine previously reported mutations (c.550delA, p.R541Q [28], c.19_23del, p.Y336N, p.A702V, p.Y537X, p.R490Q [10, 27], p.R49H [9], c.1746-20C>G (http://www.dmd.nl/capn3_home.html) have been detected in 21 cases in either homozygous or compound heterozygous form (Table 1). Mutations of the CAPN3 gene were not identified among the eight additional patients. Thus, we were able to identify 67.2% of mutant alleles in 29 patients. c.550delA (40%) and p.R490Q (10%) were detected as the most frequent mutations in our cases. The other mutations were detected with a frequency of 2.5 or 5%. The frequencies of the mutations are reported in Fig. 1.

All of our patients, except cases 15 and 16, were diagnosed by pediatricians. In all LGMD2A-ascertained patients, the onset of symptoms was between 2.5 and 19 years of age. In general, the disease course was mild. The pattern of muscle involvement was similar in all patients. Involvement predominated in the limb girdle and trunk muscles and was usually symmetrical. The most striking feature of the examination was atrophy of the proximal muscles to varying degrees. CK levels were invariably highly elevated and usually measured in several thousands (2,000–8,000 U/l). None of our patients lost ambulation during the time of study. Severity is, of course, age-related, and all muscular dystrophies are progressive. Compared to other forms, such as typical DMD or sarcoglycan deficiency, the evolution in our group overall was slower. Facial, ocular and velopharyngeal muscles never became involved. Cardiac functions of all patients were normal. Also, respiratory functions did not show any abnormalities.

Discussion

This study is devoted to outlining the present advancements in understanding the molecular basis of LGMD2A in Turkey. Since 1994, 93 LGMD2 patients have been diagnosed at the Hacettepe University Faculty of Medicine. It has been suggested that calpainopathy may account for approximately 30–40% of all cases of LGMD2 [10, 21, 27]. In another study in Italy, this proportion was found to be 28–36% [12, 20]. Consistent with these reports of relatively high proportion, considering the number of different types of LGMD2, calpain-3 deficiency constituted at least 22.6% of our LGMD2 cohort.

Most of the patients have consanguineous parents and are homozygous for the same pathogenic mutations as expected. However, in cases 18 and 19 only one mutant allele was identified. Although there is a consanguinity in these families, two different mutant haplotypes were found in each family depending on the non-consanguineous relationships.

A marked inter- and intrafamilial heterogeneity in the severity of the clinical course has been reported in LGMD2A patients [28, 32]. Among our LGMD2A patients, calpain-3 deficiency usually runs with a mild clinical severity especially during childhood, and it would be rare to reach clinical stage VI before age 20. With the exception of one severe case (case 19) and one case of asymptomatic hyperCKemia (case 12), all patients in our cohort had a clinical course graded from mild to intermediate. The intermediate phenotype was found in patients with all mutation types, and the mild and moderate phenotypes corresponded to patients carrying nonsense, missense and frameshift mutations (cases 8, 11, 14 and 18). Therefore, there was no significant correlation between mutation type and clinical phenotype.

Up to this point, 161 distinct mutations have been found in the CAPN3 gene that are associated with calpainopathy (http://www.dmd.nl/capn3_home.html). Here, we report 15 different mutations in the CAPN3 gene, 6 of which are novel (p.K211N, p.D230G, p.Y322H, p.R698S, p.Q738X, c.2257delGinsAA). Whereas p.D230G, p.R698S and c.2257delGinsAA mutations are localized in domain 4, which binds Ca^{++} ions, p.K211N, p.Y322H, p.Q738X mutations are localized in domain 2, corresponding to the proteolytic module of the calpain-3 protein. All of these amino acid residues are conserved throughout all muscle-specific calpains.

Mutations were distributed along the entire length of the CAPN3 gene, and some of these particular mutations have been found more frequently in some populations. Nine of our cases share the same c.550delA mutation with a high frequency of 40%. Also p.R490Q mutation was detected as the second most frequent mutation in Turkey with a frequency of 10% in our study. Thus, investigating these

two mutations may be used as a first step in molecular diagnosis of LGMD2A in Turkish families and may be helpful in generating diagnostic screening strategies.

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