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ARTICLE

cDNA analyses of *CAPN3* enhance mutation detection and reveal a low prevalence of LGMD2A patients in Denmark

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Calpainopathy or limb-girdle muscular dystrophy type 2A (LGMD2A) is generally recognized as the most prevalent form of recessive LGMD and is caused by mutations in the *CAPN3* gene. Out of a cohort of 119 patients fulfilling clinical criteria for LGMD2, referred to our neuromuscular clinic, 46 were suspected to have LGMD2A, based on western blot results. Four of these patients were shown to have LGMD2I upon molecular analysis, whereas 16 of the remaining 42 patients harbored mutations in *CAPN3* by both direct genomic sequencing and cDNA analyses. In 10 patients, we identified both mutant alleles. In three other, only one heterozygous mutation could be identified on the genomic level; however, *CAPN3* cDNA analyses demonstrated homozygosity for the mutant allele, indicating the presence of an unidentified allele that somehow compromise correct *CAPN3* RNA processing. In the three remaining patients, only a single heterozygous mutation could be identified both at the genomic level and on full-length *CAPN3* cDNA. All three patients exhibited a highly abnormal western blot for calpain-3 and clinical characteristics of LGMD2A. Only three of the genetically confirmed LGMD2A patients were of Danish origin, indicating a five- to sixfold lower prevalence in Denmark compared to other European countries. A total of 16 different *CAPN3* mutations were identified, of which 5 were novel. The present study demonstrates the value of cDNA analysis for *CAPN3* in LGMD2A patients and indicates that calpainopathy is an uncommon cause of LGMD in the Denmark.
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Introduction

Recessively inherited limb-girdle muscular dystrophy (LGMD2) comprises a heterogeneous group of patients that share the following features: (1) proximal limb weakness, (2) elevated plasma creatine kinase levels, and (3) dystrophic changes on muscle biopsy. These common features together define the diagnostic criteria for LGMD2.[1] Among the LGMD2s, LGMD type 2A (LGMD2A, MIN no. 253600) is the most common and has been reported to account for at least 30% of LGMDs.[2,3] The prevalence has been estimated to be around 1:100 000 although with regional differences.[3,4] Age of onset for LGMD2A ranges between 2 and 40 years of age, but usually manifests itself in the second or third decade of life with atrophy and weakness of pelvic and shoulder muscles. Facial and ocular muscles are spared, intellectual function is normal, and, in contrast to many other LGMDs, there seems to be no significant cardiac involvement.[5,6]

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LGMD2A is caused by mutations in the *CAPN3* gene,[7] which encodes calpain-3; a skeletal-muscle-specific member of the calpain superfamily.[8,9] Western blot for calpain-3 is usually performed as a primary diagnostic test. However, mutation screening of the *CAPN3* gene is the most reliable way to verify the diagnosis, as up to 20% of molecularly confirmed LGMD2A patients show normal western blot for calpain-3.[6,10,11] Normal calpain-3 possess an autolytic activity which can be used to identify LGMD2A patients with normal western blot for calpain-3.[12,13] On the other hand, reduced level or absence of calpain-3 can be secondary to other genetic defects.[6] The nature of this reduction is largely unknown.

The *CAPN3* gene is large, consisting of 24 coding exons with an open reading frame of 2466 nucleotides, making mutation screening quite laborious and expensive. Presently, >300 *CAPN3* variants have been associated with LGMD2A.[14,15] The mutations are located throughout the coding sequence, and are for most part private, although a minor subset of mutations are frequent in some populations.[4,6,16,17] There is no obvious phenotype–genotype correlation, and patients with identical mutations can display quite discordant phenotypes, and vary in onset of disease.[6,10,18] Moreover, African-Brazilian LGMD2A patients seem to be more severely affected than Caucasians.[19] The basis for this variability remains enigmatic, but has been explained by epigenetic modulating factors[10] or a second modifying allele.[2]

Reports from several European countries on mutation identification in *CAPN3* substantiate that LGMD2A accounts for at large fraction of LGMD2s in Caucasians.[4,20–23]

We report here the molecular analyses of 46 patients suspected of LGMD2A, and show that the disease has an unexpected low prevalence in Denmark. In total 31 patients were analyzed for the *CAPN3* cDNA sequence. Among these, the LGMD2A diagnosis was confirmed genetically in 13 patients, although some of the causative mutations remained undetected. In three other patients only one mutation could be identified on both genomic and cDNA. Thus, subset of LGMD2A patients appeared only to be heterozygous for one single *CAPN3* mutation.

Methods and materials

Subjects

All patients were referred to our neuromuscular research unit from all over Denmark for clinical evaluation. Three were referred from clinics in Sweden. Investigations were performed upon receipt of informed consent. Initially, 119 fulfilled the diagnostic criteria for LGMD2, and of these, 59 were assigned a specific LGMD2 diagnosis other than LGMD2A, or a diagnosis of Becker muscular dystrophy (14 patients), based on molecular genetic or western blot investigations.[24] The remaining 46 patients were considered candidates for LGMD2A based on clinical and/or western blot findings. Of the 119 patients, 105 were of ethnic Danes and three were Swedes. The remaining 11 patients, who originated from different parts of the world, all belonged to the group of 46 patients who were potential candidates for LGMD2A.

Western blot

A single muscle biopsy, obtained from the vastus lateralis or tibialis anterior muscles, was snap-frozen in liquid nitrogen-cooled isopentane, stored at −80 °C, and subsequently processed according to standard laboratory techniques. A section of each sample was tested for the presence of dystrophin, merosin, α-sarcoglycan, β-dystroglycan, telethonin, dysferlin and calpain-3 (94, 60 and 30 kDa fragments), using antibodies as previously described.[24] The remaining of the muscle biopsy was stored for subsequent genetic analysis.

Genetic analyses

DNA was isolated from an EDTA blood sample by standard methods. All 24 coding exons and exon–intron boundaries (minimum 35 bp intronic sequence) were amplified in 22 PCR reactions (see Supplementary Table 1 for primer sequences and conditions), and either analyzed by denaturing high-pressure liquid chromatography (dHPLC) using a Wave 2100 system (Transgenomics, Elancourt, France) and/or purified on a JetQuick spin column (Genomed, St Louis, USA), directly sequenced on the forward strand using BigDyeTerminator V1.1 and subsequently resolved on an ABI3100 (Applied Biosystems, Foster City, USA). NM_000070 was used as the reference sequence for *CAPN3*.

Total muscle RNA was extracted from a needle biopsy using TRIzol (Invitrogen, Paisley, UK), and cDNA was generated using Superscript II™ (Invitrogen) in combination with random hexamer primers, both according to the manufacturer's description. The entire *CAPN3* cDNA sequence was either amplified as one single fragment or as overlapping fragments (see Supplementary Table 1 for primer sequences and conditions) using the Expand Long Template PCR system (Roche, Basel, Switzerland).

Primers and conditions for testing mutations in *FKRP* have been described elsewhere.[25]

Results

Out of a cohort of 119 LGMD2 patients referred to our neuromuscular clinic, 46 were initially considered candidates for a LGMD2A diagnosis, based on their phenotypic appearance and/or western blot analysis. In total, 105 of the 119 patients were of Danish origin, 3 were from Sweden and the remaining 11 patients were of other ethnic background. The latter 11 all belonged to the group of 46 patients considered as potential candidates for LGMD2A. As part of the diagnostic workup, all 46 patients were tested

for the common *FKRP* mutation (c.826A>G), which was found in a homozygous state in four of the patients.[24] These four LGMD2I patients all had abnormal western blots for calpain-3 and were ethnic Danes.

The remaining 42 patients were screened for mutations in the *CAPN3* gene; 18 were initially screened by dHPLC, which confirmed the diagnosis in 4 patients. In the remaining 14 patients, the entire coding and intron flanking sequences of *CAPN3* were sequenced. The other 24 patients were investigated by RT–PCR and *CAPN3* cDNA sequencing. Seven of the patients, in whom no mutations were found by dHPLC/exon sequencing, were also sequenced for their *CAPN3* cDNA. Mutations initially identified on the cDNA level were all confirmed by genomic sequencing.

In total, 16 different mutations were identified of which 5 were previously unknown (Table 1). The c.1117T>C mutation was absent from 200 normal control chromosomes (data not shown). In 10 patients, both mutant alleles were identified, of which 3 initially were missed by dHPLC due to homozygosity. Three other patients, who appeared to carry only one mutation on genomic DNA, were shown to be homozygous on cDNA. Subsequently, the 3′- and 5′-UTR region of these patients (nos. 4, 11 and 14) were sequenced (genomic DNA). No mutations were found. In the three remaining patients, only a single mutation was found by demonstration of heterozygosity on both genomic and cDNA (Figure 1).

The c.550delA mutation, which is relatively common in the southeastern European population,[3,21–23] was not detected.

In total, 15 of the 16 mutation-positive patients had a decrease in the 94 kDa fragment of calpain-3 on western blot (Table 1), whereas 20 of the 26 *CAPN3* mutation-negative patients displayed normal western blot for calpain-3 (data not shown).

In total, 10 of the 16 mutation-positive LGMD2A patients had an ethnic background other than Danish

Table 1 The two horizontal rows in each patient referrers to the two alleles

No	*Western blot*	*Ethnicity*	*Genomic*	*mRNA (cDNA)*	*Exon*	*Protein[a] (predicted)*	
1	Absent	Somalia	c.1117T>C	r.1117u>c[b]	9	(Trp373Arg)	New
			c.1117T>C	r.1117u>c[b]			
2	Absent	Peru	c.853G>T	r.853g>t[b]	6	(Glu285X)	New
			c.853G>T	r.853g>t[b]			
3	Highly reduced	Denmark	c.257C>T	ND	1	Ser86Phe	Known[26]
			c.1505T>G		11	Ile502Ser	New
4	Absent	Denmark	c.1336G>A	r.1336g>a	10	(Gly446Ser)	Known[15]
			Not found	r.0		—	
5	Highly reduced	The Netherlands	c.865C>T	ND	6	Arg289Trp	Known[15]
			c.1981delA		17	—	Known[20]
6	Highly reduced	Denmark	c.1715G>C	r.1715g>c	13	Arg572Phe	Known[3]
			Not found	Not found		?	
7	Absent	Turkey	c.1309C>G	ND.	10	(Arg437Gly)	New
			c.1309C>G				
8	Absent	Israel	c.1800+2T>C	ND.	IVS15	—	Known[15]
			c.1800+2T>C				
9	Absent	Romania	c.380−13T>A	r.380_498del119[b,c]	IVS2	(p.126fs)	New
			c.380−13T>A	r.380_498del119[b,c]			
10	Highly reduced	Denmark	c.643_663del21	r.643_663del21	5	p.215_221del	Known[26]
			Not found	Not found		?	
11	Highly reduced	Denmark	c.2393C>A	r.2393c>a	23	Ala798Glu	Known[15]
			(c.1801−51G>A)[d]	r.0		—	
12	Normal	Palestine	c.1381C>T	r.1381c>u[c]	11	Arg461Cys	Known[35]
			c.1381C>T	r.1381c>u[c]			
13	Highly reduced	Denmark	c.643_663del21	r.643_663del21[c]	5	p.215_221del	Known[26]
			Not found	Not found		?	
14	Absent	Sweden	c.2362_2362delAGinsTCATCT	r.2362_2363delAGinsUCAUCU[c]	22	(p.788fs)	Known[26]
			Not found	r.0		—	
15	Absent	Russia	c.1699G>T	r.1699c>u[c]	13	(Gly567Trp)	Known[26]
			c.1699G>T	r.1699c>u[c]			
16	Absent	Sweden	c.2362_2363delAGinsTCATCT	r.2362_2363delAGinsUCAUCU[c]	22	(p.788fs)	Known[26]
			c.2362_2363delAGinsTCATCT	r.2362_2363delAGinsUCAUCU[c]			

Abbreviation: ND, not determined.
[a]Only patient no. 12 had a normal western blot for calpain-3, all other mutations ultimately lead to a quantitatively defect of functional calpain-3.
[b]Full-length cDNA could not be generated.
[c]Mutation was initially identified on cDNA and subsequently confirmed on genomic DNA.
[d]Previously identified as a polymorphism.
The notion 'r.0' in the cDNA column indicate that no cDNA (RNA) was detected from the corresponding allele, eg the patient was hemizygous for a mutation on the cDNA sequence but heterozygous on genomic DNA. The term 'not found' denotes a heterozygous state of the other mutation.

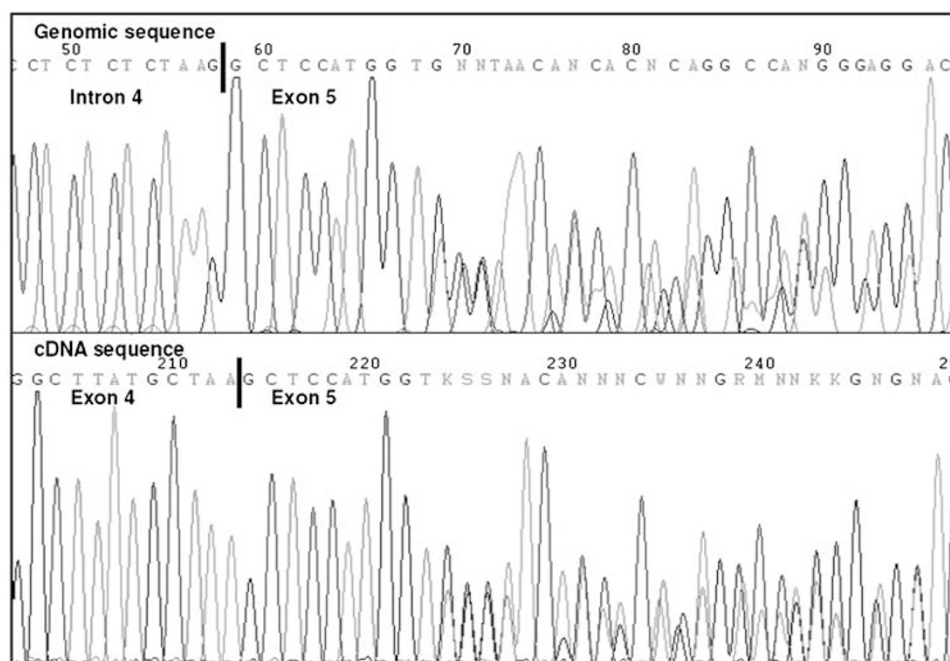

Figure 1 Electropherogram of the sequence of patient no. 13 heterozygous for c.643_663del21. Upper panel shows the genomic sequence of exon 5, whereas the lower panel depicts the cDNA sequence covering the c.643_663del21 mutation. Note that the electropherogram from the genomic and cDNA sequences are highly similar with respect to exon 5.

(Table 1). In the remaining 103 patients with a LGMD2 phenotype, only 1 patient had a non-Danish ethnic background, indicating that the prevalence of LGMD2A in ethnic Danes is much lower than reported elsewhere in the world.

Discussion

Apart from expanding the mutation spectrum leading to LGMD2A with five new mutations, two major messages can be drawn from the present study: (1) The prevalence of LGMD2A is unexpectedly low in the ethnic Danish population, (2) cDNA analysis of *CAPN3* in LGMD2A patients allows comprehensive mutation detection and can uncover the presence of mutations that escapes detection by standard genomic analysis.

Calpainopathy is recognized as the most prevalent form of recessive LGMDs and generally accounts for >30% of LGMD2 in Caucasians.[2,3] Out of a cohort of 119 LGMD2 patients (105 ethnic Danes), we found 16 carrying mutations in *CAPN3*. Surprisingly, only 6 were of a ethnic Danish origin, whereas the remaining 10 originated from different parts of the world, indicating that LGMD2A has a five- to sixfold lower prevalence in Denmark compared to other European countries.[4,16,21,22,26] The reason for this remains unknown, but LGMD2I is very prevalent in Denmark, and accounts for approximately 40% of all recessive LGMD, which is also at variance with other European countries.[24] Among the 46 primary candidate patients, four were found to be homozygous for the common *FKRP* mutation (c.826A>G) resulting in LGMD2I. A similar proportion of LGMD2I patients among LGMD2A referrals has been recently found in a large European cohort.[6]

Numerous reports have described protocols for mutation screening for the *CAPN3* gene by dHPLC,[3,10,16,22,23] and we initially adapted such an approach, but the failure to detect mutations in obvious candidate patients and suspicion of consanguinity, promoted us to shift to direct sequencing. Indeed, half of the molecularly confirmed LGMD2A patients turned out to be homozygous for a mutation on genomic DNA. Three of these mutations were initially missed by the dHPLC screening but could potentially have been detected by mixing DNA from the patient and a normal control (or other patent DNA) prior to dHPLC. Direct genomic sequencing is considered the gold standard for mutation detection, but as the *CAPN3* gene is large, and a muscle biopsy usually is available, we changed to sequence the *CAPN3* cDNA as the primary genetic examination. cDNA analysis has previously aided in the identification of *CAPN3* mutations[23,27] and is becoming a more widespread screening approach.[28,29] To our knowledge, the majority of diagnostic laboratories perform a muscle biopsy as part of the diagnostic workup of LGMD2 patients. It should therefore be straightforward to use the biopsy for genetic analysis. cDNA analysis, however, cannot substitute genomic analysis, and identified mutations must be confirmed on genomic DNA.

The combined genomic and cDNA sequencing led to the identification of 16 different mutations. Two of the five novel mutations (c.1309C>G and c.1505T>G) change the amino-acids Arg437 and Ile502, respectively, which have been found to be mutated in other LGMD2A patients[20] indicating pathogenicity of the mutation, whereas two other mutations (c.853G>T and c.380-13T>A) led to premature transcript termination due to creation of a stop codon and out of frame aberrant splicing of exon 3, respectively. How the c.380-13T>A mutation interferes with the splicing machinery is unknown, but the change from a pyrimidine to a purine suggest an obstruction of the pyrimidine track. *In silico* analysis indicate that c.380-13T>A weakens the 5′ acceptor site of IVS2,[30] supporting this suggestion. The c.1117T>C mutation, found in patient no. 1, leading to the amino-acid change Trp373Arg, potentially interferes with correct splicing. The c.1117T>C is located next to the splice site of exon 9, and according to the exonic splicing enhancers finder,[31] the mutation changes the site preferences for various Ser-Arg-rich proteins. This *in silico* prediction is supported by the fact that full-length *CAPN3* cDNA could not be generated, and the total absence of calpain-3 on western blot in this patient. Patient no. 1 had an affected sib that subsequently was shown to be homozygous for c.1117T->C. Thus, the five identified novel variations are all highly likely to be pathogenic.

The single c.1336G>A mutation identified in patient no. 4 has not previously been described in the literature, but is listed in the Leiden database.[15] The mutation changes the highly conserved hydrophobic amino-acid glycine at position 446 to a hydrophilic serine.

In 10 patients, we identified both mutant alleles. In another three, only one mutation could be identified on the genomic level (nos. 4, 11 and 14); however, *CAPN3* cDNA analyses demonstrated that these patients only expressed the mutant allele, indicating that all three patients harbor an unidentified allele interfering with correct *CAPN3* RNA processing. The *CAPN3* transcript is targeted by the nonsense-mediated mRNA decay pathway, and a recent study has demonstrated that in LGMD2A patients, compound heterozygous for two frame-shift mutations, only trace amounts of the corresponding transcripts can be identified.[32] Thus, cDNA sequencing of patients compound heterozygous for a missense, and a frame-shift/nonsense mutation will occasionally only reveal the missense mutation as the transcript carrying the premature stop-codon, depending on its location,[33] will be quickly degraded. However, hemizygosity for a mutation on the cDNA level is sufficient to confirm the diagnosis. Thus, cDNA sequencing can indirectly disclose partial *CAPN3* gene deletions, large genomic rearrangement, intronic or promoter mutations affecting correct *CAPN3* RNA maturation/transcription, all of which are likely to be missed by traditional exon sequencing. In fact, in about 10% of LGMD2A patients, only one heterozygous mutation is identified by a genomic screening approach,[2] indicating a high prevalence of deep intron mutations. In patient no. 11, we detected an intronic variation (c.1801-51G>A), which has previously been classified as a polymorphism.[15] This variation is absent from the single nucleotide polymorphism database and to our knowledge not mentioned in the literature concerning *CAPN3* mutation detection.

In three patients, we only identified a single variation (nos. 6, 10 and 13). Two of these were heterozygous for a 21 bp in-frame deletion previously identified as a mutation.[26] The deletion does not appear to compromise RNA maturation, as it was clearly present on full-length cDNA (Figure 1). The third patient was heterozygous on both genomic and cDNA for the known mild mutation c.1715G>C.[3] All three exhibited a highly abnormal western blot for calpain-3 and clinical characteristics of LGMD2A. The parents of patients nos. 6 and 10, who carried the respective mutation, were asymptomatic, arguing against a dominant-negative effect as has been indicated for Ser86Phe.[26] The lack of a normal western blot for calpain-3 is somewhat enigmatic and could point to a potential defect in translational control. To address this, both the 3′- and 5′-UTR sequences of patient no. 6 were sequenced, but no aberration could be identified. It is quite common only to find one heterozygous mutation in LGMD2A patients,[2] but to our knowledge only one patient has been described being heterozygous for a single mutation on cDNA.[27,32] Interestingly, this patient carried an in-frame deletion (c.598_612del15) close to the location of the deletion found in our patients 10 and 13. A single family has been described were five male members were heterozygous for a single 3 bp deletion in *CAPN3*, and all showed variable degrees of muscular dystrophy. Interestingly, all four also carried a mutation in the XK gene leading to McLeod's syndrome.[34] We therefore sequenced the XK gene in patient nos. 6, 10 and 13 but did not detect any mutations. To what extent the LGMD2A pathogenesis is related to their single *CAPN3* mutation alone or in combination with an unidentified mutation/locus awaits to be seen.

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