Calpainopathy (LGMD2A) in Croatia: Molecular and Haplotype Analysis

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Aim To determine types and frequency of CAPN3 mutations in 29 unrelated Croatian families, analyzed during 6-year prospective and ongoing genetic and epidemiological study of muscular dystrophies in Croatia.

Methods Mutation analysis included allele-specific polymerase chain reaction (PCR) or combination of PCR and restriction fragment length polymorphisms (RFLP) methods. Haplotype analysis was performed by PCR and DNA electrophoresis using 5 highly polymorphic markers flanking CAPN3 gene locus.

Results Mutation analysis revealed the presence of 6 different CAPN3 mutations (550delA, R541W, P82L, delFWSAL, R49H, Y537X), accounting for 94.8% of CAPN3 chromosomes in the studied population. 550delA was the most frequent mutation, found in 43/58 (74%) CAPN3 chromosomes, whereas the frequency of other five mutations ranged from 2-9%. Haplotype analysis of 38 chromosomes carrying 550delA mutation showed the presence of the same haplotype on 66% of analyzed chromosomes.

Conclusions The present data, together with our previously published results, explain the frequency and the distribution of the 550delA mutation in Croatia by founder effect and genetic drift. Results of haplotype study are in favor of the hypothesis that 550delA is an old, rather than a recurrent mutation. The findings are important for effective diagnostic screening of CAPN3 gene in Croatia and neighboring countries, as well as for accurate genetic counseling.

Calpainopathy, limb girdle muscular dystrophy type 2A (LGMD2A; OMIM 253600), or Erb’s muscular dystrophy is an autosomal recessive muscular disorder characterized by symmetrical and selective atrophy of proximal limb muscles. The clinical features have been described by Erb in 1884 (1) as juvenile progressive muscular dystrophy, whereas the name LGMD2A dates from 1995 when Bushby and Beckmann (2) proposed a locus-based classification of LGMDs. In 1991, this muscular dystrophy was linked to chromosome 15q in patients from Reunion Island (3). Further linkage analysis defined 15q15.1-15.3 interval as the one containing LGMD2A disease locus (4). The analysis of genes from this region led to the identification of CAPN3 gene mutations as the cause of LGMD2A (5). CAPN3 gene encodes calpain 3 (p94, OMIM 114240), muscle-specific member of the calpain family, which made LGMD2A the first described muscular dystrophy caused by enzymatic, rather than structural protein defect. Calpainopathy is considered to be the most frequent autosomal recessive limb girdle muscular dystrophy (6) and accounts for 39% of all LGMDs (7).

Since 1995, more than 150 different CAPN3 mutations of all types have been found worldwide (6-12). Although the majority of mutations represent private variants, some mutations have been found more frequently. However, fre-
frequencies and types of CAPN3 mutations found in different populations vary according to the geographic and ethnic origin of the studied population. These findings, together with uncertainty of calpain 3 protein analysis (13,14), show how important accurate knowledge of mutation spectrum in some populations is for developing fast, cheap, and non-invasive diagnostics of LGMD2A.

The aim of our study was to determine the CAPN3 mutation spectrum in Croatia, their frequency, and geographical distribution, as well as to speculate on the origin of some mutations by haplotype analysis.

Materials and Methods

Patients
During a 6-year period, 40 patients from 29 unrelated LGMD2A families (25 male, 15 female) and their 34 healthy relatives were selected for CAPN3 mutation analysis. The selection of patients was done using a specific strategy based on detailed clinical features, progression of disease, creatine phosphokinase (CPK) values, electromyography, computerized transverse tomography (CT scan) of muscles, and genealogical study. Special emphasis was put on intensive search for new secondary cases in the family of the proband in order to determine the mode of inheritance (15,16).

Each patient participating in the study signed the informed consent before giving the blood sample for molecular analysis.

Eighty seven individuals were selected for haplotype analysis: 35 patients from 25 families; 32 healthy family members, 25 of them being heterozygous for one of CAPN3 mutations; 2 healthy 550delA heterozygotes (children of deceased homozygous proband) and; 18 healthy individuals from a population study (16), 2 of them being 550delA carriers. A total of 174 chromosomes were analyzed: 92 with one of 6 CAPN3 mutations, 7 with unknown mutation(s), and 75 wild type chromosomes.

Genomic DNA was isolated from peripheral blood samples by standard salting-out procedure (17).

Mutation Analysis

550delA and Y537X. Polymerase chain reaction (PCR) mixture contained 250 ng of DNA, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 200 μM of each dNTP (Eppendorf, Hamburg, Germany), 1 U Taq polymerase (Eppendorf), 1 pmol of each external primer (5), and two short inner primers. For 550delA mutation, the previously described inner primers were used (18), whereas in case of Y537X, internal primers were generated by our group: 5’-AGG AGC AAA ACC TAC AT-3’ for the amplification of normal size allele (1 pmol) and 5’-TCC CGC ATG TTG ATT TA-3’ for the amplification of a mutated allele (3 pmol). After 5-minute denaturation at 94°C, 550delA thermocycling was performed as follows: first 5 initial cycles of 93°C (1’), 65°C (45”), and 72°C (1’) were followed by 25 cycles of 93°C (1’), 55°C (45”), and 72°C (1’). The Y537X thermocycling conditions differed only in annealing temperatures (62°C in the first 5 cycles, 52°C in the remaining 25 cycles).

R541W, R49H, P82L, and delFWSAL. First, 250 ng of DNA were amplified by PCR in a 25-μL volume containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 200 μM of each dNTP (Eppendorf), 1 Unit of Taq polymerase (Eppendorf), 1 pmol of each primer (5). After 5-minute denaturation at 94°C, thermocycling was performed in 35 cycles as follows: 93°C (1’), 62°C (45”), and 72°C (1’). Subsequently, PCR products were incubated with Bsp LU11 I (Roche, Mannheim, Germany) for R541W, Ita I (Roche) for R49H, orMsp I restriction enzyme (Promega, Mannheim, Germany) for P82L detection, according to manufacturer’s recommendations. DelFWSAL PCR product was electrophoresed on 8% polyacrylamide gel and stained with silver.

Haplotype Analysis

Haplotype analysis was performed using 5 highly polymorphic microsatellite markers, flanking CAPN3 gene locus on chromosome 15q15.1-15.3: D15S514, D15S779, D15S782, D15S780, and D15S778 (4). The marker genotyping consisted of PCR reaction for each marker, DNA-PAGE and silver staining. PCR amplification of DNA (200 ng) was performed in 25 μL reaction mix containing 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.5 mM MgCl₂, 200 μM of each dNTP (Eppendorf), 1 U Taq polymerase (Eppendorf), and 3 pmol of each primer. The primer sequences for each microsatellite marker, as well as marker allele sizes and its frequencies, were found on www.gdb.org and www.cephb.fr. Initial 5-minute denaturation on 94°C was followed by 30 cycles.
of 92°C (30’’), 58°C (D15S514, D15S779, and D15S780) or 60°C (D15S782 and D15S778) (30’’), and 72°C (30’’).

Results

The following mutations were detected in the study sample: 550delA, R541W, P82L, delFWSAL, R49H, and Y537X (Table 1, Fig. 1).

Thirty-eight 550delA chromosomes included in the haplotype study belonged to 12 homozygotes, 11 compound heterozygotes, 1 healthy heterozygote (child of deceased homozygous proband), and two 550delA carriers from population study (16). Five different haplotypes were obtained in 31/38 chromosomes with 550delA mutation (Table 2, Fig. 2). In 7/38 chromosomes belonging to 5 solitary compound heterozygous probands (550delA/R541W; 550delA/R49H; 550delA/delFWSAL; 550delA/unknown mutation) and 2 healthy 550delA heterozygotes, haplotype phases could not be established. However, in 4 solitary patients and both carriers, it was possible to theoretically construct the most frequent haplotype. One patient (550delA/R49H) did not show any of the analyzed haplotypes.

Three families with R541W showed two different haplotypes (Table 2, Fig. 2), with difference only in the first marker (2-9-4-4-2 and 5-9-4-4-2). In additional two families, presented by solitary probands, we were able to theoretically construct a 2-9-4-4-2 haplotype.

Haplotypes obtained for delFWSAL, P82L, and Y537X, as well as 2 haplotypes for 2 chromosomes carrying unknown mutation(s) are presented in Table 2 and Figure 2.

Haplotype analysis also included 75 wild type chromosomes. In 30 of them, haplotype phases could be established (Fig. 2). Their analysis enabled us to compare wild type haplotypes with the most frequent 550delA haplotype.

Discussion

Mutation analysis of 29 Croatian LGMD2A families revealed the presence of 6 different mutations with the predominance of 550delA (16), accounting for 74% of the analyzed CAPN3 chromosomes. The same mutation was described in a few families from France and Netherlands, but its higher frequency was observed in Slavic populations and in several neighboring countries from eastern Mediterranean part of Europe. In Bulgaria, 550delA was found in 9 out of 12 LGMD2A families (19). In Russia, 8 out of 14 families had 550delA homozygous patients (18), whereas in Turkey 550delA mutation was reported on 36% of 44 CAPN3 chromosomes (20). In Italy, 550delA was found on 23% mutated alleles from 56 families (7), but it is worth mentioning that a large proportion of mutant alleles (40%) in one of the northeastern regions of the country.

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Consequence</th>
<th>Exon</th>
<th>Number of CAPN3 chromosomes</th>
<th>Frequency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R49H (146G&gt;A)</td>
<td>missense mutation Arg (R) → His (H)</td>
<td>1</td>
<td>1/58</td>
<td>1.7</td>
</tr>
<tr>
<td>P82L (245C&gt;T)</td>
<td>missense mutation Pro (P) → Leu (L)</td>
<td>1</td>
<td>3/58</td>
<td>5.2</td>
</tr>
<tr>
<td>550delA</td>
<td>frameshift mutation</td>
<td>4</td>
<td>43/58</td>
<td>74.1</td>
</tr>
<tr>
<td>delFWSAL (598-612del)</td>
<td>5 aminoacid deletion (in frame) Phe(F), Trp(W), Ser(S), Ala(A) and Leu(L)</td>
<td>4</td>
<td>2/58</td>
<td>3.5</td>
</tr>
<tr>
<td>Y537X (1611C&gt;A)</td>
<td>nonsense mutation Tyr (Y) → STOP(X)</td>
<td>13</td>
<td>1/58</td>
<td>1.7</td>
</tr>
<tr>
<td>R541W (1621C&gt;T)</td>
<td>missense mutation Arg (R) → Trp (W)</td>
<td>13</td>
<td>5/58</td>
<td>8.6</td>
</tr>
<tr>
<td>Unknown</td>
<td></td>
<td></td>
<td>3/58</td>
<td>5.2</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>58/58</td>
<td>100.0</td>
</tr>
</tbody>
</table>

Table 1. CAPN3 mutations found on 58 chromosomes from 29 unrelated limb girdle muscular dystrophy type 2A (LGMD 2A) families from Croatia

<table>
<thead>
<tr>
<th>Parameter</th>
<th>550delA</th>
<th>R541W</th>
<th>delFWSAL</th>
<th>P82L</th>
<th>Y537X</th>
<th>unknown</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of chromosomes</td>
<td>25</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Marker:</td>
<td>D15S514</td>
<td>2</td>
<td>8</td>
<td>2</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>D15S779</td>
<td>9</td>
<td>9</td>
<td>9</td>
<td>10</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>D15S782</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>D15S780</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>D15S778</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 2. Results for 40/52 CAPN3 chromosomes included in haplotype study
(Friuli) were due to 550delA mutation (6). In addition, 550delA mutation was reported in one family from Greece (8), 2 families from Slovenia (21), 2 Czech compound heterozygous patients (12), and in 3 compound heterozygotes from Croatia originating from Bosnia and Herzegovina. As far as we know, 550delA was not reported in other parts of the world. Based on these data, we would like to propose that the first step in the diagnostics of suspected calpainopathy in patients from eastern Mediterranean and southeastern Europe would be testing for this mutation, using simple and rapid method of allele-specific amplification.

R541W was the second most frequent mutation in Croatia, found in 5 LGMD2A families. It was also found in Italy (7) and Netherlands (www.dmd.nl). Interestingly, 3 families with R541W originated from Bosnia and Herzegovina, suggesting that this mutation would be, like 550delA, important in diagnostics of LGMD2A in that country.

Other 4 mutations, already described in other populations (9,20,22), were found only in one or two Croatian families, with frequency ranging from 2 to 5%. Therefore, the geographic and ethnic origin of LGMD2A patient, as well as geo-

Figure 1. Six CAPN3 mutations found in Croatia. Roman numbers indicate: I) DNA molecular weight marker (Roche, Mannheim, Germany); II) Wild type sample (wt/wt); III) Heterozygous sample (mutation/wt); IV) Homozygous sample (mutation/mutation). A. 550delA: beside the full-size band of 292 bp, a 142 bp band was amplified in the presence of a mutated allele and a 178 bp band in the presence of normal allele. B. Y537X: close to full-size band of 337 bp, a 214 bp band was amplified in the presence of a normal allele and 152 bp band in the presence of a mutated allele. C. P82L: wild type PCR product was cut on 168 and 115 bp bands, in the presence of mutated allele 231 and 115 bp bands were observed. D. delFWSAL: polyacrylamide gel separation of the wild type polymerase chain reaction (PCR) resulted in one normal (292 bp) band, whereas in the presence of mutation, additional band of 277 bp was detected. E. R541W: in the presence of mutated allele PCR product was cut on 194 and 143 bp bands, whereas wild type allele remained the same. F. R49H: in the presence of mutation PCR product was cut on 330 and 230 bp bands, in case of normal allele only 230 bp band was present.
Figure 2. Pedigrees of 16 Croatian LGMD 2A families and results of haplotype analysis. Black squares and circles represent affected men and women. Deceased individuals are presented by diagonal line; horizontal double line corresponds to consanguineous families. Healthy individuals are represented by white squares and circles. Half black squares and circles represent healthy heterozygotes (carriers). Mutations found in each family are indicated above each pedigree. Asterisk indicates unknown mutation(s). Marker loci are listed from centromere to telomere and are presented in front of Family No 1. 550delA haplotype, found in all families, is shown within grey, full-line box. Haplotypes of other mutations are presented within white, full-line boxes. Unboxed haplotypes represent wild type chromosomes. In two individuals, results for markers D15S778 were not presented which is noted with a dash. Arrow indicates the point of the probable crossing-over.
graphical distribution of the mutation should be important in determining the diagnostic approach for these "rare" mutations.

Screening for 6 mentioned mutations, using rapid and simple methods described here, enabled the identification of almost 95% of pathological CAPN3 chromosomes in Croatia. Results of this study are important not only for our population, but show the strategy for developing fast, cheap, and non-invasive diagnostics of LGMD2A.

Haplotype study was used to determine the origin of 550delA and other CAPN3 mutations found among our LGMD2A patients. Linkage analysis of 550delA patients from 8 different countries was found to be associated with the same haplotype, which has suggested 550delA mutation as a representative of the ancestral mutational event (8). Besides on results from Croatia and several other countries, we suggest that 550delA is the typical mutation of eastern Mediterranean and southeastern Europe, from which it had probably spread across Europe (16). To test this hypothesis, haplotype analysis of Croatian LGMD2A families was performed. The 5 polymorphic markers were used: D15S514, D15S779, D15S782, D15S780, and D15S778, where distance between the first and fifth marker is less than 1 cM. CAPN3 gene locus is located between D15S779 and the third D15S782, D15S779 being the closest one to CAPN3 gene (4).

Results of our haplotype study showed the presence of 5 different haplotypes linked to 550delA. The same haplotype, 2-9-3-4-1, was found on 66% of all analyzed 550delA chromosomes. If we include the theoretical results for several solitary patients, this percentage would increase to 82%. In addition, in 2 out of 4 slightly different haplotypes, the difference was observed only for the first marker. Furthermore, haplotype analysis of wild type chromosomes showed distinct haplotypes from those obtained for 550delA (data not shown). These results, together with those from previously population study (16), suggest and favor the hypothesis that Croatian population is rather closed, with founder effect in some parts of the country.

As we have used the same markers as several previous studies (8,18,23), we were able to compare haplotypes from our and other geographical and ethnic origins. It is evident that differences exist, but the core haplotype was preserved in the majority of cases (Table 3). Therefore, our results support the hypothesis that 550delA is an old mutation.

Small number of chromosomes bearing other 5 mutations does not permit any conclusion concerning haplotype. However, it is interesting to mention that in 5 R541W chromosomes from 5 unrelated families almost the same haplotype (2/5-9-4-4-2) has been obtained, suggesting a possible founder effect.

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