

Mutations of *CAPN3* in Korean Patients with Limb-Girdle Muscular Dystrophy

The limb-girdle muscular dystrophy type 2A (LGMD2A) is a recessively inherited disease caused by a mutation of the calpain 3 gene (*CAPN3*), and is considered one of the most prevalent subtypes of limb-girdle muscular dystrophy (LGMD). In this study, we aimed to identify *CAPN3* mutations and to characterize the phenotype of Korean patients with LGMD2A. Among 35 patients with LGMD, four patients, who showed calpain 3 deficiency on western blot analysis, were analyzed in this study. Total RNA extracted from frozen muscle tissue was amplified by reverse transcriptase polymerase chain reaction (RT-PCR) using six primer pairs covering all coding sequences of *CAPN3*, and direct sequencing was performed. Clinical and pathological features of the patients were also reviewed. We found four different mutations in five alleles from three patients. Of the pathogenic mutations identified, two were novel (c.2125T>C and c.2355-2357delTTC), and the others had been reported elsewhere (c.440G>C, c.1076C>T). All patients showed a high CK level with predominant proximal leg weakness, and the onset was in their childhood except for one patient. Among two novel *CAPN3* mutations, one was a missense mutation (c.2125T>C [p.709Ser>Pro]), and the other was a small in-frame deletion causing omission of a single amino acid (c.2355-2357delTTC [p.786delPhe]). The clinical features of our patients were generally compatible with the characteristics of LGMD2A patients described in the previous studies.

Key Words : *Limb-Girdle Muscular Dystrophy; Calpain 3; Korean*

Jin-Hong Shin^{*,†}, Hyang-Suk Kim^{1,†},
Chang-Hoon Lee^{1,†}, Cheol-Min Kim^{1,§},
Kyu-Hyun Park^{*,†}, Dae-Seong Kim^{*,†}

Departments of Neurology^{*}, Pathology[†], and
Biochemistry[§], Medical Research Institute¹, Pusan
National University School of Medicine, Busan, Korea

[†]These two persons contributed equally to the
preparation of the manuscript.

Received : 13 October 2006
Accepted : 28 November 2006

Address for correspondence

Dae-Seong Kim, M.D.
Department of Neurology, Pusan National University
Hospital, 1-ga 10, Ami-dong, Seo-gu, Busan 602-739,
Korea
Tel : +82.51-240-7672, Fax : +82.51-245-2783
E-mail : dskim@pusan.ac.kr

^{*}This study was supported by Medical Research
Institute Grant (2003-40), Pusan National University.

INTRODUCTION

Limb girdle muscular dystrophies (LGMDs) are a clinically and genetically heterogeneous group of disorders, characterized by progressive weakness of proximal limb girdle muscles. To date, at least seven different forms of autosomal dominantly-inherited LGMD (LGMD1) have been recognized, and 11 in cases of autosomal recessively-inherited ones (LGMD2). LGMD2A (MIM# 253600) consists of 30-60% of all LGMD, and is assumed to be a major form among several ethnic groups (1-7). It is caused by single or small nucleotide changes distributed throughout a gene, named *CAPN3* (MIM# 114240), which encodes the calpain 3 protein (8).

Calpains or calcium-activated neutral proteases are non-lysosomal intracellular cysteine proteases. The mammalian calpains include two ubiquitous proteins, calpain 1 (MIM# 114220) and calpain 2 (MIM# 114230), as well as two stomach-specific proteins, and calpain 3, which is muscle-specific. Unlike dysferlin and sarcoglycans, which are involved in LGMD2B and LGMD2C through 2E, respectively, calpain 3 is not a structural protein and disappears very rapidly in vitro due to autolysis (9), frustrating immunohistochemical staining popular in other muscular dystrophies. The human

CAPN3 covers a genomic region of more than 40 kb and is composed of 24 exons. It is expressed predominantly in the skeletal muscle tissue as a 3.5 kb transcript, driving the translation of a 94 kD protein.

LGMD2A and the mutations in *CAPN3* have not been studied in the Korean population, although it is expected to account for a large proportion of LGMD considering 26% in the Japanese population (6). Here we first report the results of mutational analysis of LGMD2A screened from Korean LGMD patients.

MATERIALS AND METHODS

Selection of the subjects

We selected 35 patients registered in our clinic from 2000 to 2004 with the clinical diagnosis of LGMD, who satisfied the following conditions: slowly progressive mild to moderate limb girdle weakness and elevated serum creatine kinase (CK) level, and various degrees of dystrophic changes on muscle biopsy. The age distribution was in the range of 12-56 yr old. Immunohistochemical stainings for dystrophin, merosin,

dysferlin and sarcoglycans revealed one case of Duchenne muscular dystrophy (DMD; MIM#310200) in carrier state, one case of merosinopathy (MDC1A; MIM#607855), and seven cases of dysferlinopathy (LGMD2B; MIM# 253601), leaving 26 patients to be studied with western blot analysis.

Western blot analysis

In each patient, 10-20 pieces of 6 μm -thick muscle cryosections were obtained, homogenized in 60 μL of sodium dodecyl sulfate (SDS) sample buffer by sonication, and heated at 95°C for 5 min. Two microliters from each sample was loaded to 6% SDS-polyacrylamide gel, and electrophoresed over 20 mA for 1 hr before transferred to polyvinylidene difluoride (PVDF) membrane. The blot was labeled with three kinds of primary antibodies each targeting different epitopes of calpain 3 (Calp3c/11B3, Calp3c/11A2, Calp3d/2C4; Novocastra, U.K.) (10) followed by secondary antibody reaction (Histofine Simple Stain Max Po, Japan) overnight and detection with ECL western blotting detection reagent (Amersham Bioscience, Piscataway, U.S.A.).

Western blotting for dysferlin was then performed on the patients with defective signal on at least one kind of calpain 3 antibody, to exclude secondary calpain 3 deficiency due to dysferlin deficiency (11).

Mutational analysis

In the patients with defective calpain 3 on western blot study, further analysis was performed by screening mutations in *CAPN3*. The purpose and methods were explained in detail and written informed consents were obtained prior to the study using their RNA and DNA. In order to identify mutations, RT-PCR of *CAPN3* and direct sequence analysis were performed. Six sets of primers described before (12) were used to cover the entire coding sequence of *CAPN3*, split into about 600 bp-segments. Each PCR product was directly sequenced using BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, U.S.A.) and 3730xl DNA analyzer (Applied Biosystems). The aberrant sequences detected by sequence analysis were first searched in the Leiden Open Variation Database (13). For the two mutations not found in the database, PCR-RFLP assay or direct sequencing was performed on each 100 alleles from a normal Korean population to exclude normal variation. For c.2125T>C (p.709Ser>Pro), genomic DNA region was amplified with primers CAPN3-ex20.a (5' -GGGGATTTTGCTGTGTGCTGT-3') and CAPN3-ex20.m (5' -ATTCCTGCTCCACCGTCTC-3') (8). The products were digested by restriction endonuclease *Hha* III (Takara Bio, Tokyo, Japan) for 2 hr in 37°C, to make a pattern of 234 bp and 106 bp in wild type, in contrast to the bands of 234 bp and 53 bp in mutant. For c.2355-2357delTTC (p.786delPhe), we could not find an appropriate restriction endonuclease that we amplified exon 22 by

PCR using primers CAPN3-ex22.a (5' -CACAGAGTGG-CCGAGAGGCA-3') and CAPN3-ex22.m (5' -GGAGAT-TATCAGGTGAGATGCC-3') (8), and performed direct sequencing.

Clinical analysis of patients with calpain 3 deficiency

The clinical features of the patients, who showed calpain 3 deficiency on western blot analysis, were reviewed retrospectively based on their clinical records. The clinical history was obtained from each patient in detail with the special interest on the age at onset, first clinical symptom, the speed of progression, current disabling problems, and family history. For clinical examination, presence of muscle atrophy or hypertrophy, and associated musculoskeletal deformities were carefully inspected. We examined changes in their tendon reflexes and individual muscle power according to the Medical Research Council (MRC) grade at first presentation, and at certain points during clinical follow-up. The laboratory tests including complete blood count, liver and renal function tests, thyroid function test, chest radiography, electrocardiogram (ECG), serum electrolytes, and serum creatine kinase (CK) level were done in each patient. Muscle biopsies were obtained from biceps muscles by open biopsy, and were processed for routine histochemistry and reviewed for the diagnosis of muscular dystrophy.

RESULTS

Western blot analysis

Four patients were found to show defective signal with at least one primary antibody. Antibody Calp3c/11B3 was the most sensitive showing defective calpain 3 band in all four patients, while antibody Calp3c/12A2 showed defective bands in patient 1 and 4, and antibody Calp3c/2C4 only in patient 4 (Fig. 1). No patient with deficient calpain 3 was associated with dysferlin deficiency, supporting the evidence that all of them are primarily deficient for calpain 3. None of the others were deficient for either calpain 3 or dysferlin.

Mutation analysis

By direct sequencing of the *CAPN3* mRNA amplified by RT-PCR, five different mutations were identified among the eight alleles, which are summarized in Table 1. We could find only one mutation in cDNA from patient 1 and none from patient 3. Patient 2 showed homozygous mutations. Of the pathogenic mutations identified, the missense mutation c.2125T>C (p.709Ser>Pro) from patient 1 (Fig. 2A) and the in-frame deletion c.2355-2357delTTC (p.786delPhe) from patient 4 (Fig. 2B) were novel, and no same change was observed among 100 control alleles using PCR-RFLP and

the direct sequence analysis technique. Two other mutations (c.440G>C, c.1076C>T) had been reported elsewhere (6, 14).

Clinical features of the patients with calpain 3 deficiency

Patient 1, in whom we found a missense mutation (c.2125T>C [p.709Ser>Pro]) in one allele, was 19 yr old at the time of her first presentation to us. She developed difficulty in climbing upstairs and leg pain at age 12, which slowly progressed to running difficulty at age 15. At presentation, she could not lift up heavy objects or go upstairs without using handrails. Although she did not show waddling gait, a clear self-climbing motion was observed when she was asked to stand up from squatting posture. Neither calf pseudohypertrophy nor muscle atrophy was evident. On muscle power examination, she had MRC grade 3 weakness in leg elevation, and grade 4 in arm abduction, leg extension, leg adduction, leg abduction, knee extension, and knee flexion. The

deep tendon reflexes were hypoactive symmetrically in limbs. She had no family history of similarly affected persons. Her serum CK level was elevated to 1,275 IU/L (normal; 5-275 IU/L), and other laboratory tests including ECG and chest radiography were unremarkable. Muscle biopsy showed marked muscle fiber size variation, increased connective tissue, clusters of regenerating fibers, and scattered whirled fibers with fiber splitting (Fig. 3A).

Patient 2, who had a homozygous missense mutation (c.1076C>T [p.359Pro>Leu]) at exon 8, was a 47-yr-old female at the time of her muscle biopsy. At her age of 30, she first noticed difficulty in climbing stairs, which progressed to limitations in daily activities by age 44. At presentation, she had difficulty in rising up from chair and could not stand up by herself from sitting position. Her muscle power was reduced to MRC grade 1 in leg adduction, grade 2 in hip flexion, hip extension, and leg abduction, grade 3 in shoulder elevation, elbow flexion, knee flexion and knee extension,

Table 1. CAPN3 mutations identified in the study

Patient number	Mutation			Type	Antibodies defective on western blot
	cDNA	Protein	Location		
1	c.2125T>C* Otherwise unidentified	p.709Ser>Pro	Exon 20	Missense	Calp3c/11B3, Calp3c/12A2
2	c.1076C>T, homozygous	p.359Pro>Leu	Exon 8	Missense	Calp3c/11B3
3	Unidentified	-	-	-	Calp3c/11B3, Calp3d/2C4, Calp3c/12A2
4	c.440G>C c.2355-2357delITC*	p.147Arg>Pro p.786delPhe	Exon 3 Exon 22	Missense Small deletion	Calp3c/11B3

*Novel mutations.

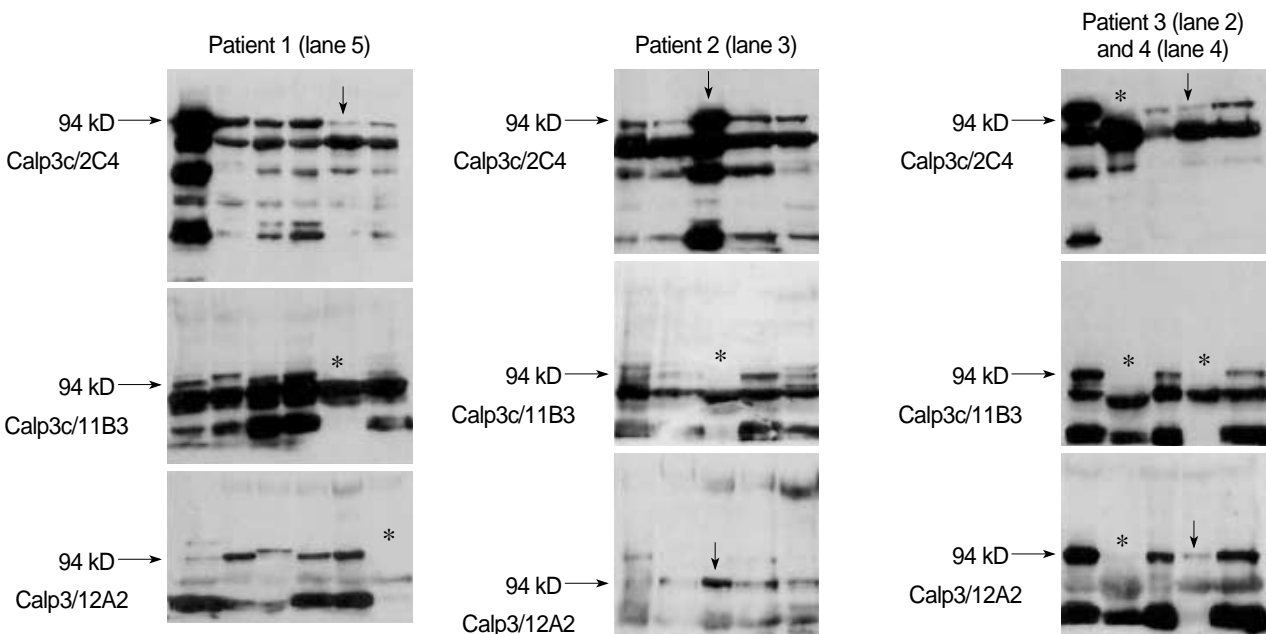


Fig. 1. Western blot analysis of patient 1 (lane 5 of left column panels), patient 2 (lane 3 of middle column panels), and patient 3 and 4 (lane 2 and 4 of right column panels, respectively). Arrows indicate the presence of a band at the appropriate position, and asterisks (*) indicate the absence. The lanes other than described above are from the other samples that have been found to be negative for calpainopathy.

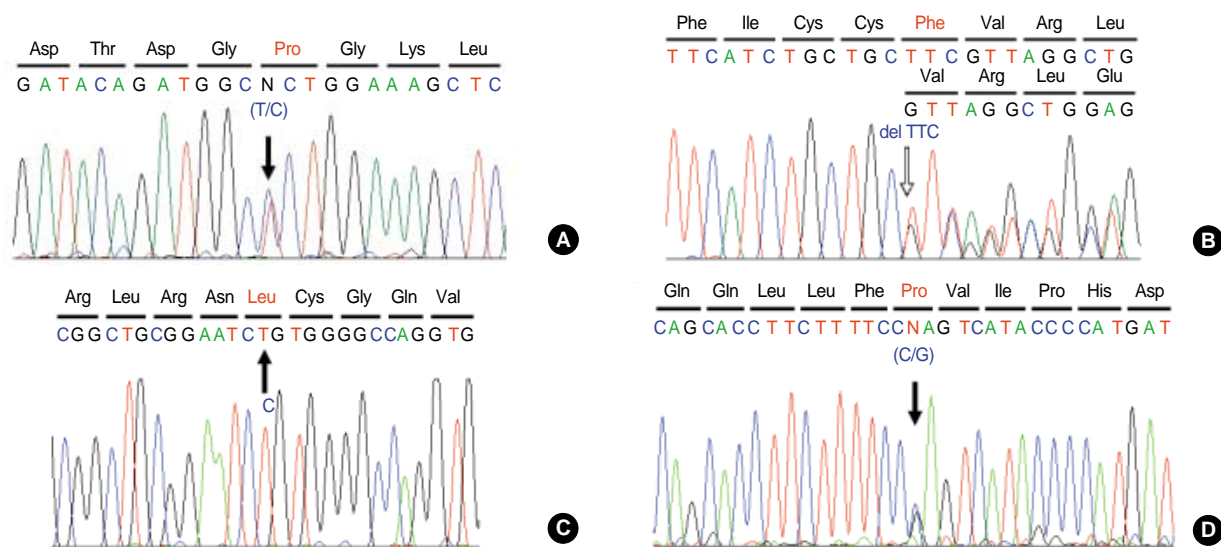


Fig. 2. Mutations identified in the study. (A) Heterozygous c.2125T>C in patient 1 (arrow). Original amino acid serine has been changed into proline by the mutation. (B) c.2355-2357delTTC in patient 4 (open arrow). Mutated allele misses the codon for phenylalanine. (C) Homozygous c.1076C>T in patient 2 (arrow) changes the codon for amino acid proline into leucine. (D) Heterozygous c.440G>C in patient 4 (arrow) changes codon for amino acid arginine into proline.

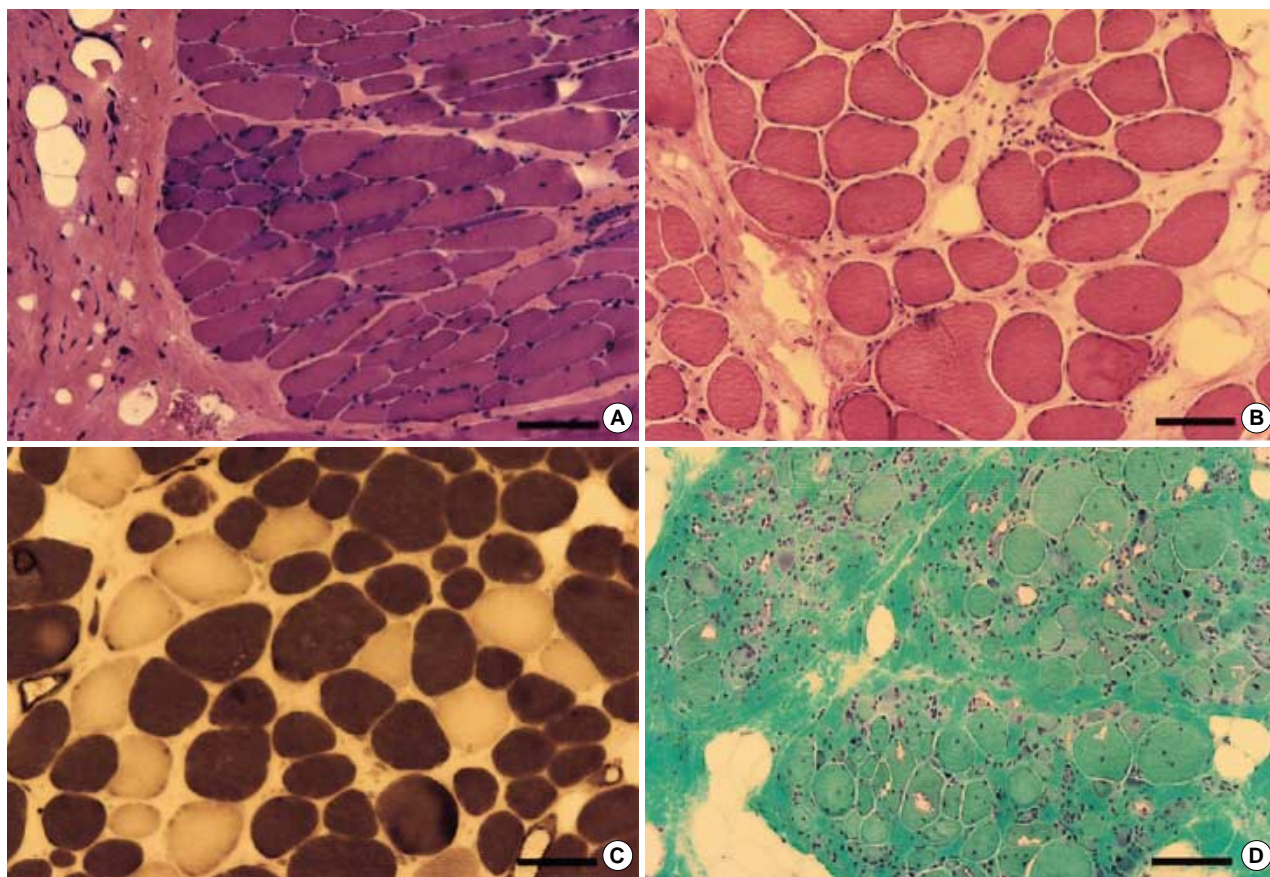


Fig. 3. Muscle biopsy findings of the patients. (A) In patient 1, markedly increased contents of connective tissue and clusters of regenerating muscle fibers are observed. Hematoxylin and eosin (H&E) stain. (B) Marked muscle fiber size variation with many hypertrophic fibers, and markedly increased contents of connective tissue are seen in muscle biopsy from patient 2. H&E stain. (C) In patient 2, type 1 fiber predominance is also observed. ATPase stain at pH 4.3. (D) In patient 3, marked muscle fiber size variation, markedly increased contents of connective tissue, and many fibers with rimmed vacuoles are seen. Modified Gomori-trichrome stain. Size of the bar=100 μ m.

and grade 4 in elbow extension and wrist flexion. Her deep tendon reflexes were lost in limbs except for the ankle jerks. She had no affected family members, and her serum CK level was mildly elevated (496 IU/L). The ECG and chest radiography were normal. Muscle biopsy showed a marked fiber size variation, markedly increased adipose connective tissue, many fibers with internal nuclei, scattered necrotic and regenerating fibers, some fibers undergoing fiber splitting, and type I fiber predominance (Fig. 3B, C).

The patient 3 had been wheelchair-bound for one year when he first presented to us at age 35. He became symptomatic with gait and running difficulty when he was 13 yr old. Since then, his disease was progressively worsened. He started to use canes at age 32 and became wheelchair-bound at age 34. He had a profound muscle weakness on examination. His muscle power was graded as 1 in leg elevation, leg extension, leg adduction, and leg abduction, 2 in distal leg muscles, 3 in upper extremity muscles, and 4 in quadriceps muscles. He also had generalized muscle atrophy, especially in shoulder, pelvic girdle, and intrinsic hand muscles. His deep tendon reflexes were lost except for the knees. He had no affected family members, and serum CK was slightly elevated to 500 IU/L. His ECG and chest radiography were unremarkable. Although he showed loss of immunoreactivity against all the three sets of anti-calpain 3 antibodies, we were not able to find any *CAPN3* mutations. His muscle biopsy showed a marked fiber size variation, markedly increased adipose connective tissue, many fibers with internal nuclei, scattered necrotic and regenerating fibers, many fibers with rimmed vacuoles, and type I fiber predominance (Fig. 3D).

Patient 4, who was compound heterozygous for the mutations c.440G>C (p.147Arg>Pro) and c.2355-2357delTTC (p.786delPhe), was at her age of 21 when she first visited us with progressive difficulty in climbing stairs and rising from chairs. She became symptomatic when she was 8 yr old with running difficulty and tiptoe gait. The tenorrhaphy had been

repeatedly performed due to progressive Achilles contracture. At present, she has gait disturbance with waddling, difficulty in climbing stairs and rising from chairs. Her muscle power was significantly reduced to MRC grade 1 in leg adduction and grade 2 in leg elevation. Muscles in proximal upper limbs and all other lower limb muscles were less severely affected and were graded as 4 to -4. She had a generalized areflexia. Her familial history was significant for her brother and sister who had the same problems with her. Her serum CK level was elevated to 1,104 IU, and ECG and chest radiography were normal. In muscle biopsy, there was a moderate fiber size variation, mildly increased adipose connective tissue, and a few regenerating fibers.

DISCUSSION

This is the first attempt to analyze the *CAPN3* mutations among Korean LGMD patients. We identified four patients with calpain 3 deficiency by western blot analysis, and finally found four different mutations including two novel ones. One of our new mutation, c.2125T>C, changes a codon for polar amino acid serine into nonpolar proline, which is likely to produce a conformational change in the protein structure of calpain 3. Another new mutation c.2355-2357delTTC produces a deletion of single amino acid phenylalanine at codon 786. In multiple alignment analysis, both mutations are located in highly conserved regions of amino acid sequences across several species (Fig. 4), strongly supporting that they are pathogenic mutations. It is also remarkable that c.440G>C mutation had been previously reported in a Japanese population, which is known to be in close ethnic proximity with the Korean population.

In contrast to other reports (1-7), we could find just a small number of patients with *CAPN3* mutations from a LGMD patient pool. This is thought to be partly because we could

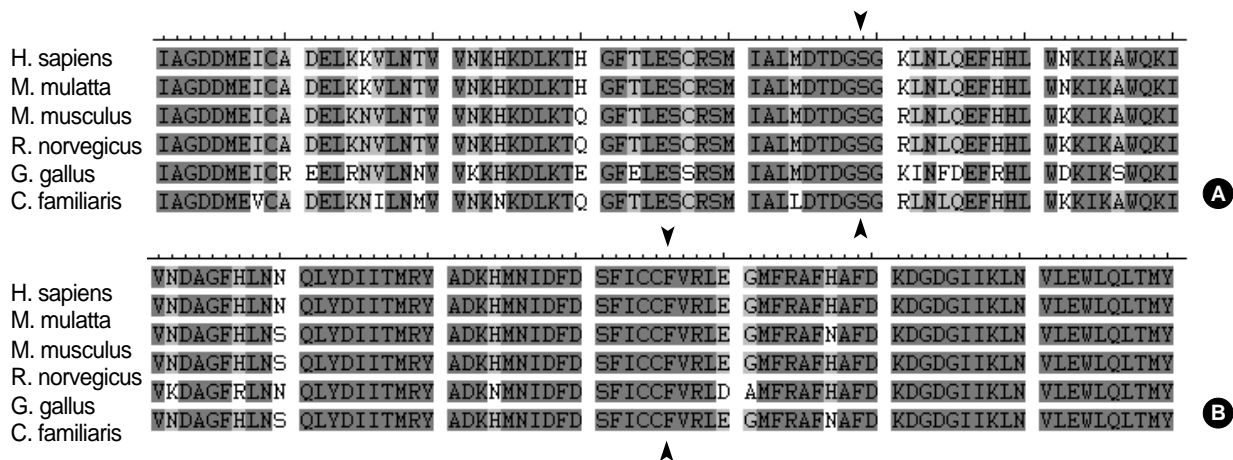


Fig. 4. Multiple alignment analysis of amino acid sequences of calpain 3 in different species. Both mutated amino acids serine (S, marked in panel A) and phenylalanine (F, marked in panel B) are highly conserved across several species. *H. sapiens*: human, *M. mulatta*: rhesus monkey, *M. musculus*: house mouse, *R. norvegicus*: Norway rat, *G. gallus*: chicken, *C. familiaris*: dog.

not reliably exclude autosomal dominant cases, but also that we have screened the patients first by western blot analysis. Although western blot analysis is still considered as a standard tool for the screening of LGMD2A, it seems neither perfectly sensitive nor specific (10, 15-17). According to a study employing a large number of genetically confirmed LGMD2A patients, the diagnostic sensitivity and specificity of western blot analysis were estimated as 52.5% and 87.8%, respectively (17). In our study, we used three types of western blot antibodies in order to maximize the sensitivity, but the antibody Calp3c/11B3 was found to be the most useful, which is known to have the widest reaction profile, binding other calpain molecules even from other species (10). Direct sequencing of the *CAPN3* gene cannot detect all the mutations either, as exemplified by our case, that the two techniques are complementary. It is contradictory that patient 3 in whom we could not find any pathologic mutation, showed the clearest pattern of calpain 3 abnormality on western blot. Overall, we have failed to detect mutations in three alleles. As we have sequenced *CAPN3* by RT-PCR, each segment spanning about 600 bp, a large deletion involving primer site could be the cause, although such mutation has not been reported so far. Mutations may be in the regulatory region, affecting the transcriptional activity, which has not been reported either.

The clinical features of LGMD2A had been studied and characterized in a few previous studies, and following features are now considered as typical: onset between 8-16 yr, early symmetrical weakness of proximal legs, no facial or cardiac involvement, normal intelligence, wheelchair-bound between 20 to 40 yr old, no muscle contracture except for the Achilles tendon, hyporeflexia, and a 5-20 fold increase in the CK level (16, 17, 19, 20). The clinical features of our patients are considered to be typical for LGMD2A. All of our patients showed a high CK level ranging 496-1,275 U/L. The weakness was predominant in their lower extremities, the onset was from childhood except for patient 2, deep tendon reflexes were lost or reduced, and none had mental or cardiac abnormalities.

Although LGMD2A is supposed to account for a major proportion in LGMD, it has been neglected in the Korean populations, partly due to the costly diagnostic procedure. Further studies using a larger number of patients with LGMD2A are needed to fully elucidate the genotypic and clinical characteristics of Korean patients with LGMD2A.

REFERENCES

1. Passos-Bueno MR, Moreira ES, Marie SK, Bashir R, Vasquez L, Love DR, Vainzof M, Iughetti P, Oliveira JR, Bakker E, Strachan T, Bushby K, Zatz M. *Main clinical features of the three mapped autosomal recessive limb-girdle muscular dystrophies and estimated proportion of each form in 13 Brazilian families. J Med Genet* 1996; 33: 97-102.
2. Dincer P, Leturcq F, Richard I, Piccolo F, Yalnizoglu D, de Toma C, Akcoren Z, Broux O, Deburgrave N, Brenguier L, Roudaut C, Urtizberea JA, Jung D, Tan E, Jeanpierre M, Campbell KP, Kaplan JC, Beckmann JS, Topaloglu H. *A biochemical, genetic, and clinical survey of autosomal recessive limb girdle muscular dystrophies in Turkey. Ann Neurol* 1997; 42: 222-9.
3. Richard I, Brenguier L, Dincer P, Roudaut C, Bady B, Burgunder J-M, Chemaly R, Garcia CA, Halaby G, Jackson CE, Kurnit DM, Lefranc G, Legum C, Loiselet J, Merlini L, Nivelon-Chevallier A, Ollagnon-Roman E, Restagno G, Topaloglu H, Beckmann JS. *Multiple independent molecular etiology for limb-girdle muscular dystrophy type 2A patients from various geographical origins. Am J Hum Genet* 1997; 60: 1128-38.
4. Topaloglu H, Dincer P, Richard I, Akcoren Z, Alehan D, Ozme S, Caglar M, Karaduman A, Urtizberea JA, Beckmann JS. *Calpain-3 deficiency causes a mild muscular dystrophy in childhood. Neuropediatrics* 1997; 28: 212-6.
5. Kawai H, Akaike M, Kunishige M, Inui T, Adachi K, Kimura C, Kawajiri M, Nishida Y, Endo I, Kashiwagi S, Nishino H, Fujiwara T, Okuno S, Roudaut C, Richard I, Beckmann JS, Miyoshi K, Matsumoto T. *Clinical, pathological, and genetic features of limb-girdle muscular dystrophy type 2A with new calpain 3 gene mutations in seven patients from three Japanese families. Muscle Nerve* 1998; 21: 1493-501.
6. Chae J, Minami N, Jin Y, Nakagawa M, Murayama K, Igarashi F, Nonaka I. *Calpain 3 gene mutations: genetic and clinico-pathologic findings in limb-girdle muscular dystrophy. Neuromuscul Disord* 2001; 11: 547-55.
7. de Paula F, Vainzof M, Passos-Bueno MR, de Cassia M Pavanello R, Matioli SR, V B Anderson L, Nigro V, Zatz M. *Clinical variability in calpainopathy: what makes the difference? Eur J Hum Genet* 2002; 10: 825-32.
8. Richard I, Broux O, Allamand V, Fougerousse F, Chiannikulchai N, Bourg N, Brenguier L, Devaud C, Pasturaud P, Roudaut C, *et al.* *Mutations in the proteolytic enzyme calpain 3 cause limb-girdle muscular dystrophy type 2A. Cell* 1995; 81: 27-40.
9. Sorimachi H, Toyama-Sorimachi N, Saido TC, Kawasaki H, Sugita H, Miyasaka M, Arahata K, Ishiura S, Suzuki K. *Muscle-specific calpain, p94, is degraded by autolysis immediately after translation, resulting in disappearance from muscle. J Biol Chem* 1993; 268: 10593-605.
10. Anderson LV, Davison K, Moss JA, Richard I, Fardeau M, Tome FM, Hubner C, Lasa A, Colomer J, Beckmann JS. *Characterization of monoclonal antibodies to calpain 3 and protein expression in muscle from patients with limb-girdle muscular dystrophy type 2A. Am J Pathol* 1998; 153: 1169-79.
11. Anderson LV, Harrison RM, Pogue R, Vafiadaki E, Pollitt C, Davison K, Moss JA, Keers S, Pyle A, Shaw PJ, Mahjneh I, Argov Z, Greenberg CR, Wrogemann K, Bertorini T, Goebel HH, Beckmann JS, Bashir R, Bushby KM. *Secondary reduction in calpain 3 expression in patients with limb girdle muscular dystrophy type 2B and Miyoshi myopathy (primary dysferlinopathies). Neuromuscul Disord* 2000; 10: 553-9.
12. Chrobakova T, Hermanova M, Kroupova I, Vondracek P, Mariko-

- va T, Mazanec R, Zamecnik J, Stanek J, Havlova M, Fajkusova L. *Mutations in Czech LGMD2A patients revealed by analysis of calpain3 mRNA and their phenotypic outcome. Neuromuscul Disord* 2004; 14: 659-65.
13. Fokkema IF, den Dunnen JT, Taschner PE. *LOVD: easy creation of a locus-specific sequence variation database using an "LSDB-in-a-box" approach. Hum Mutat* 2005; 26: 63-8.
14. Ginjaar HB, Frankhuizen WS, de Mos M, Verschuuren JJ, Hoogendijk JE, van Doorn PA, van Engelen BG, Faber CG, Anderson LV, de Visser M, Baker E, van der Kooij AJ. *Classification of limb girdle muscular dystrophy types 1C, 2A, and 2B based on protein and/or DNA studies. Neuromuscul Disord* 2002; 12: 731.
15. Minami N, Nishino I, Kobayashi O, Ikezoe K, Goto Y, Nonaka I. *Mutations of calpain 3 gene in patients with sporadic limb-girdle muscular dystrophy in Japan. J Neurol Sci* 1999; 171: 31-7.
16. Fanin M, Fulizio L, Nascimbeni AC, Spinazzi M, Piluso G, Ventriglia VM, Ruzza G, Siciliano G, Trevisan CP, Politano L, Nigro V, Angelini C. *Molecular diagnosis in LGMD2A: mutation analysis or protein testing? Hum Mutat* 2004; 24: 52-62.
17. Saenz A, Leturcq F, Cobo AM, Poza JJ, Ferrer X, Otaegui D, Camano P, Urtasun M, Vilchez J, Gutierrez-Rivas E, Emparanza J, Merlini L, Paisan C, Goicoechea M, Blazquez L, Eymard B, Lochmuller H, Walter M, Bonnemann C, Figarella-Branger D, Kaplan JC, Urtizberea JA, Marti-Masso JF, Lopez de Munain A. *LGMD2A: genotype-phenotype correlations based on a large mutational survey on the calpain 3 gene. Brain* 2005; 128: 732-42.
18. Fardeau M, Hillaire D, Mignard C, Feingold N, Feingold J, Mignard D, de Ubeda B, Collin H, Tome FM, Richard I, Beckmann J. *Juvenile limb-girdle muscular dystrophy. Clinical, histopathological and genetic data from a small community living in the Reunion Island. Brain* 1996; 119: 295-308.
19. Beckmann JS, Bushby KM. *Advances in the molecular genetics of the limb-girdle type of autosomal recessive progressive muscular dystrophy. Curr Opin Neurol* 1996; 9: 389-93.
20. Urtasun M, Saenz A, Roudaut C, Poza JJ, Urtizberea JA, Cobo AM, Richard I, Garcia Bragado F, Leturcq F, Kaplan JC, Marti Masso JF, Beckmann JS, Lopez de Munain A. *Limb-girdle muscular dystrophy in Guipuzcoa (Basque Country, Spain). Brain* 1998; 121: 1735-47.