



Clinical and Pathological Heterogeneity of Korean Patients with *CAPN3* Mutations

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Purpose: This study was designed to investigate the characteristics of Korean patients with calpainopathy.

Materials and Methods: Thirteen patients from ten unrelated families were diagnosed with calpainopathy via direct or targeted sequencing of the *CAPN3* gene. Clinical, mutational, and pathological spectra were then analyzed.

Results: Nine different mutations, including four novel mutations (NM_000070: c.1524+1G>T, c.1789_1790inA, c.2184+1G>T, and c.2384C>T) were identified. The median age at symptom onset was 22 (interquartile range: 15–28). Common clinical findings were joint contracture in nine patients, winged scapula in four, and lordosis in one. However, we also found highly variable clinical features including early onset joint contractures, asymptomatic hyperCKemia, and heterogeneous clinical severity in three members of the same family. Four of nine muscle specimens revealed lobulated fibers, but three showed normal skeletal muscle histology.

Conclusion: We identified four novel *CAPN3* mutations and demonstrated clinical and pathological heterogeneity in Korean patients with calpainopathy.

Key Words: Limb girdle muscular dystrophy 2A, calpainopathy, *CAPN3*, lobulated fiber, genetic testing

INTRODUCTION

Limb girdle muscular dystrophy (LGMD) is a clinically and genetically heterogeneous group of disorders characterized by

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progressive weakness of the pelvic and shoulder girdle muscles. Currently, eight autosomal dominant LGMDs (LGMD1) and 22 autosomal recessive LGMDs (LGMD2) have been identified. LGMD2A is the most prevalent form in many European countries.^{1,2} LGMD2A is caused by mutations in the *CAPN3* gene (MIM#114240) on chromosome 15q15.1. Calpain-3, encoded by the *CAPN3* gene, is a muscle-specific member of the calpain superfamily of calcium-activated neutral proteases. Calpain-3 is associated with regulatory processes such as apoptosis, muscle cell differentiation, sarcomere formation, muscle remodeling, and regulation of the cytoskeleton. However, the pathogenesis of calpainopathy remains a subject of discussion.

The typical clinical presentation of LGMD2A includes progressive proximal muscle weakness, ankle contracture, and winged scapula. However, calpainopathy, caused by *CAPN3* mutations, reveals not only LGMD2A phenotypes but also variable clinical phenotypes including pseudometabolic myopa-

thy, eosinophilic myositis, and asymptomatic hyperCKemia.³⁻⁶ There has been only one small case series of calpainopathy in Korea.⁷ To identify the characteristics of Korean calpainopathy, we analyzed mutational, clinical, and pathological spectra in Korean patients with *CAPN3* mutations.

MATERIALS AND METHODS

Subjects

We reviewed our myopathy biobank, which contained 505 muscle and 448 blood samples taken from 896 myopathy patients between March 2004 and August 2011. Firstly, we found four patients from three unrelated families (F1, F2, and F3) who had been previously diagnosed with calpainopathy. F1 and F2 patients were diagnosed with calpainopathy via direct sequencing of *CAPN3*. Two affected members of the F3 family were confirmed via targeted sequencing for 16 LGMD-related genes (*CAPN3*, *CAV3*, *DYSE*, *FKRP*, *FKTN*, *LMNA*, *MYOT*, *POMG1T*, *POMT1*, *POMT2*, *SGCA*, *SGCB*, *SGCD*, *SGCG*, *TCAP*, and *TRIM32*). Secondly, we screened the biobank based on the following inclusion criteria: 1) genetically unconfirmed LGMD or hyperCKemia, 2) autosomal recessive or sporadic inheritance, and 3) serum creatine kinase (CK) levels of more than threefold above the upper limit of normal. We selected 51 myopathy patients and performed targeted sequencing of *CAPN3*. In this way, we identified *CAPN3* mutations in nine patients from seven unrelated families (F4, F5, F6, F7, F8, F9, and F10). Then, we analyzed mutational, clinical, and pathological data in 13 calpainopathy patients from ten unrelated families (Fig. 1A). The research protocol was approved by the Institutional Review Board of Gangnam Severance Hospital, Korea. All participants were previously required to sign an informed consent document for mutational analysis.

Targeted sequencing

Genomic DNA from muscle or blood from 51 myopathy patients was extracted with Qiagen DNeasy blood & tissue kits (Qiagen, Valencia, CA, USA). For mutation analysis, the *CAPN3* gene was enriched via hybridization capture. The target region

included all 24 coding exons and flanking introns of the *CAPN3* gene. The captured library was sequenced using an Illumina HiSeq2500 platform with the 2×150 bp paired-end read module. All samples were pooled into a part of a single lane on a single flow cell and sequenced together. A 6-bp index sequence (Illumina, San Diego, CA, USA) and a 6-bp in-house barcode sequence were used to differentiate between samples. We achieved 497.19× mean (or 471.94× median) coverage for all samples. Exonic coverage of the *CAPN3* gene at 20× or more was plotted in a standard boxplot. For 99.90% of target regions, capture was at least 20× coverage. Almost 99.98% of regions were captured with at least 10× coverage.

Variant analysis

From HiSeq2500 raw data, sequencing data of all samples were sorted by index and barcode sequences. Sorted fastq files were aligned to the hg19 reference genome with the Burrows-Wheeler Aligner (ver. 0.7.5a) algorithm.⁸ Output SAM files were converted to BAM files and sorted with SAMtools (ver. 0.1.18).⁹ Duplicate removal was performed with MarkDuplicates in Picard tools (ver. 1.95). Realignment around known indel sites and base quality score recalibration were performed with the genome analysis toolkit (GATK; ver. 2.6-5) to create the final BAM files.¹⁰

Variants were called with the GATK v2.6 Unified Genotyper algorithm.^{11,12} Called variants were filtered based on the following criteria: 1) loci depth ≥10, variant frequency ≥0.5 and 2) loci depth ≥20, variant frequency ≥0.25. Filtered variants were annotated with Annovar (ver. 2013-06-21) using RefGene, dbSNP 135, and the 1000 Genomes Project SNP (2012 April release) of the Asian population and all-population databases. Among called variants, only non-synonymous single nucleotide variants, frameshift indels, and splicing-site variants were chosen. Polymorphisms in Korean populations (n=276) were filtered out. Variants with low functional scores in Polyphen2 (ver. 2.2.2) were considered to be benign mutations and discarded.¹³ All selected variants were validated by Sanger sequencing.

Clinical assessment

Clinical data included sex; age at symptom onset; family his-

Table 1. *CAPN3* Mutations in Korean Patients with Calpainopathy

Location	Mutation*	Putative effect	Affected family	References
Exon 5	c.706G>A	p.A236T	F4	Krahn, et al. ⁵
Exon 11	c.1468C>T	p.R490W	F3	Fanin, et al. ¹⁵
Intron 11	c.1524+1G>T	Aberrant splicing	F7	Novel
Exon 15	c.1789_1790insA	Frameshift	F4, F7, F8	Novel
Exon 15	c.1795dupA	Frameshift	F1, F2	Chae, et al. ¹⁶
Intron 18	c.2050+1G>A	Aberrant splicing	F3	Chae, et al. ¹⁶
Exon 20	c.2120A>G	p.D707G	F1, F5, F6, F8, F9	Chae, et al. ¹⁶
Intron 20	c.2184+1G>T	Aberrant splicing	F10	Novel
Exon 23	c.2384C>T	p.A795V	F5	Novel

*Mutation was assigned according to the *CAPN3*(NM_000070) mRNA sequences.

tory; initial diagnosis; and presence of winged scapula, lordosis, joint contracture, motor weakness, and muscle wasting. To determine physical disability, we used a Gardner-Medwin and Walton (GMW) scale that had been previously assigned to each patient: grade 0, all normal activities and hyperCKemia; grade 1, normal gait, unable to run freely; grade 2, abnormal gait; grade 3, muscle weakness, climbing stairs with support; grade 4, positive Gower sign; grade 5, unable to rise from floor; grade 6, unable to climb stairs; grade 7, unable to get up from chairs; grade 8, unable to walk independently; grade 9, unable to eat, drink, or sit without support.¹⁴ Laboratory findings included serum CK level, nerve conduction study, needle electromyography, electrocardiography, and echocardiography. Serum CK level was expressed as fold over the upper limit of normal values according to the local reference range in 12 patients, excluding F9 patients. Nerve conduction studies and needle electromyography were performed in all 13 patients. Electrocardiography was performed in eight patients (F3a, F3b, F4, F5a, F5b, F5c, F6, and F8). Echocardiograms were performed in F5a and F5b patients.

Pathological assessment

Muscle specimens were obtained for nine patients, taken from the vastus lateralis (F1, F4, F5c, F7, F9, and F10) and the biceps

brachii (F5b, F6, and F8). Frozen muscle sections (5- μ m thickness) from all muscle specimens were stained with hematoxylin and eosin, modified Gomori trichrome, and reduced nicotinamide adenine dinucleotide-tetrazolium reductase. Protein analysis was performed in seven patients (F4, F5b, F6, F7, F8, F9, and F10). Western blots used antibodies against calpain-3 (Novocastra, Newcastle upon Tyne, UK). Muscle specimens were also analyzed by immunohistochemistry using antibodies against the C-terminus of dystrophin (Leica Microsystems, Newcastle upon Tyne, UK), the rod domain of dystrophin (Leica Microsystems), dysferlin (Leica Microsystems), α -sarcoglycan (Leica Microsystems), β -sarcoglycan (Leica Microsystems), γ -sarcoglycan (Leica Microsystems), δ -sarcoglycan (Leica Microsystems), α -dystroglycan (Millipore, Billerica, MA, USA), and caveolin (BD Biosciences, San Diego, CA, USA).

RESULTS

Identification of *CAPN3* mutations

In the present study, we identified nine different *CAPN3* mutations in 13 patients from ten unrelated families. The nine *CAPN3* mutations consisted of four missense, two small-insertions

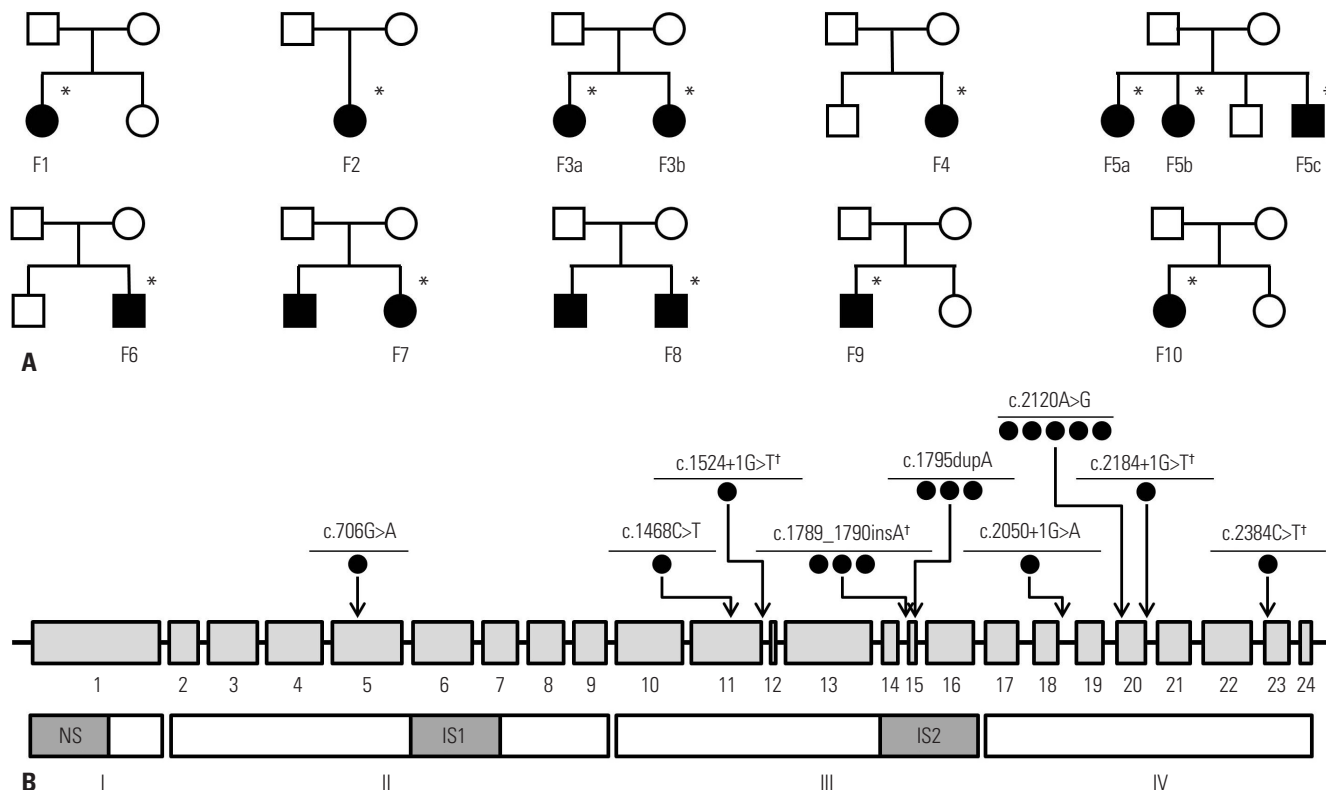


Fig. 1. Pedigrees and localizations of different *CAPN3* mutations in Korean families with calpainopathy. (A) Pedigrees of 10 Korean families with *CAPN3* mutations. Asterisks (*) indicate individuals whose DNA was used for sequencing. (B) Schematic representation of calpain 3 protein (NM_000070). Domain II is a cysteine protease domain, and domain IV is a calcium-binding domain. Muscle-specific sequences of calpain 3 at the N-terminal domain I (NS), protease domain II (IS1), and between domains III and IV (IS2). Black dots (●), numbers of alleles with mutations; dagger (†), four novel mutations.

tion, and three splice-site mutations (Table 1), all of which were mainly located in domains III and IV (Fig. 1B). Five mutations were previously reported in the Leiden Database (http://www.dmd.nl/capn3_home.html) or other reports.^{5,15,16} However, four mutations were not previously reported: c.1524+1G>T, c.1789_1790insA, c.2184+1G>T, and c.2384C>T. Among the 13 patients, two *CAPN3* mutations were identified in 11 patients from eight unrelated families (F1, F2, F3, F4, F5, F6, F7, and F8), and only one mutation was found in two patients from dif-

ferent families (F9 and F10). These two patients with a single mutation showed a total loss of calpain-3 protein via western blots (Fig. 2).

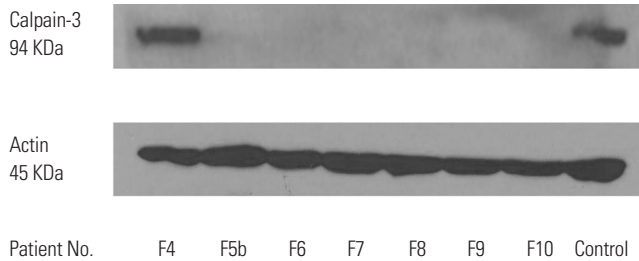


Fig. 2. Calpain-3 band pattern on western blot. Western blot for calpain-3 revealed normal expression in the F4 patient yet nearly total loss in six other patients.

Clinical manifestations

The clinical spectra of 13 patients (4 men and 9 women) with *CAPN3* mutations were summarized in Table 2. Family history was positive in four families (F3, F5, F7, and F8). The median ages at examination and symptom onset were 33 (interquartile range: 20–37) and 22 (interquartile range: 15–28), respectively. In 12 patients (none from F5), the first symptom was proximal muscle weakness, particularly in the lower extremities. Physical examination revealed joint contracture in nine patients, winged scapula in four, and lordosis in one. Calf pseudohypertrophy was identified in only the F1 patient. The median serum CK levels were 20-fold (interquartile range: 11- to 25-fold) above the upper limit of normal. Nerve conduction studies were normal, and needle electromyography revealed short duration, low-amplitude motor unit potentials, and an early recruitment pattern in all 13 patients. Electrocardiography and echocardiography showed no evidence of cardiac

Table 2. Clinical and Laboratory Features of Thirteen Patients with *CAPN3* Mutations

	Mutation*	Sex/ age (yr)	Onset age (yr)	Initial clinical diagnosis	FH	WS	Contracture	CK†	GMW scale	WB
F1	c.1795dupA/ c.2120A>G	F/44	36	Muscular dystrophy	-	-	Ankle	27	3	ND
F2	c.1795dupA/ c.1795dupA	F/22	15	Muscular dystrophy	-	-	Ankle	20	3	ND
F3a	c.2050+1G>A/ c.1468C>T	F/35	23	Muscular dystrophy	+	+	Ankle	58	7	ND
F3b	c.2050+1G>A/ c.1468C>T	F/33	23	Muscular dystrophy	+	+	Ankle	31	7	ND
F4	c.1789_1790insA/ c.706G>A	F/16	12	Muscular dystrophy	-	+	Ankle	20	4	+
F5a	c.2120A>G/ c.2384C>T	F/42	25	Muscular dystrophy	+	-	Ankle	6	8	ND
F5b	c.2120A>G/ c.2384C>T	F/39	33	Muscular dystrophy	+	-	Ankle	15	7	-
F5c	c.2120A>G/ c.2384C>T	M/34	15	Muscular dystrophy	+	-	Ankle	11	6	ND
F6	c.2120A>G/ c.2120A>G	M/21	20	Polymyositis	-	-	-	19	1	-
F7	c.1789_1790insA/ c.1524+1G>T	F/19	8	Emery-Dreifuss muscular dystrophy	+	+	Ankle, elbow	11	ND	-
F8	c.1789_1790insA/ c.2120A>G	M/19	16	HyperCKemia	+	-	-	20	0	-
F9	c.2120A>G/?	M/33	31	Polymyositis	-	-	-	ND	7	-
F10	c.2184+1G>T/?	F/28	22	Muscular dystrophy	-	-	-	3	6	-

FH, family history; WS, winged scapula; GMW scale, Gardner-Medwin and Walton scale; WB, Western blot; ND, no data.

*Mutation was assigned according to the *CAPN3* (NM_000070) mRNA sequences, †Serum creatine kinase (CK) level is expressed as x-fold above the upper limit of the normal range.

dysfunction in any tested patients.

Not only typical features of LGMD2A but also variable clinical features were found in the Korean patients with *CAPN3* mutations. In the F5 family, onset age ranged from 15 to 33 years, even though family members had the same mutations. Additionally, the F5a patient complained of a rapid deterioration in strength during pregnancy. She had four children and recalled a stepwise decline in muscle strength associated with pregnancy. The F7 patient was initially diagnosed with Emery-Dreifuss muscular dystrophy based on early prominent contractures of the elbow and ankle. The F8 patient and his older brother initially visited our hospital for elevated liver enzymes. However, a liver biopsy was normal in his brother, and further laboratory studies showed elevated serum CK level. The patient and his brother did not show clinical signs such as muscle weakness, winged scapula, or ankle contracture. The F9 patient was initially diagnosed with polymyositis based on endomysial inflammatory cell infiltration and relatively abrupt clinical onset.

Pathological assessment

We analyzed muscle biopsies from nine patients in eight families. The muscle specimens of six patients (F4, F5b, F6, F7, F9, and F10) showed nonspecific dystrophic features including varying degrees of fiber-size variability, increased endomysial fibrosis, and degenerative fibers. Among them, four patients (F5b, F7, F9, and F10) had lobulated fibers after staining with nicotinamide adenine dinucleotide tetrazolium reductase (Fig. 3), and the F6 patient had endomysial infiltration of a small number of inflammatory cells. However, three patients (F1, F5c, and F8) showed normal skeletal muscle histology. Muscle specimens were taken from the biceps brachii in F8 and the vastus lateralis in F1 and F5c. When the muscle biopsy was performed, the F8 patient had no clinical symptoms; however, the F1 and F5c patients had mild weakness of the hip and shoulder girdle muscles. Western blot for calpain-3 revealed normal expression in the F4 patient yet nearly total loss in six other patients (Fig. 2). In addition, immunohistochemistry of the muscle specimens showed normal expression

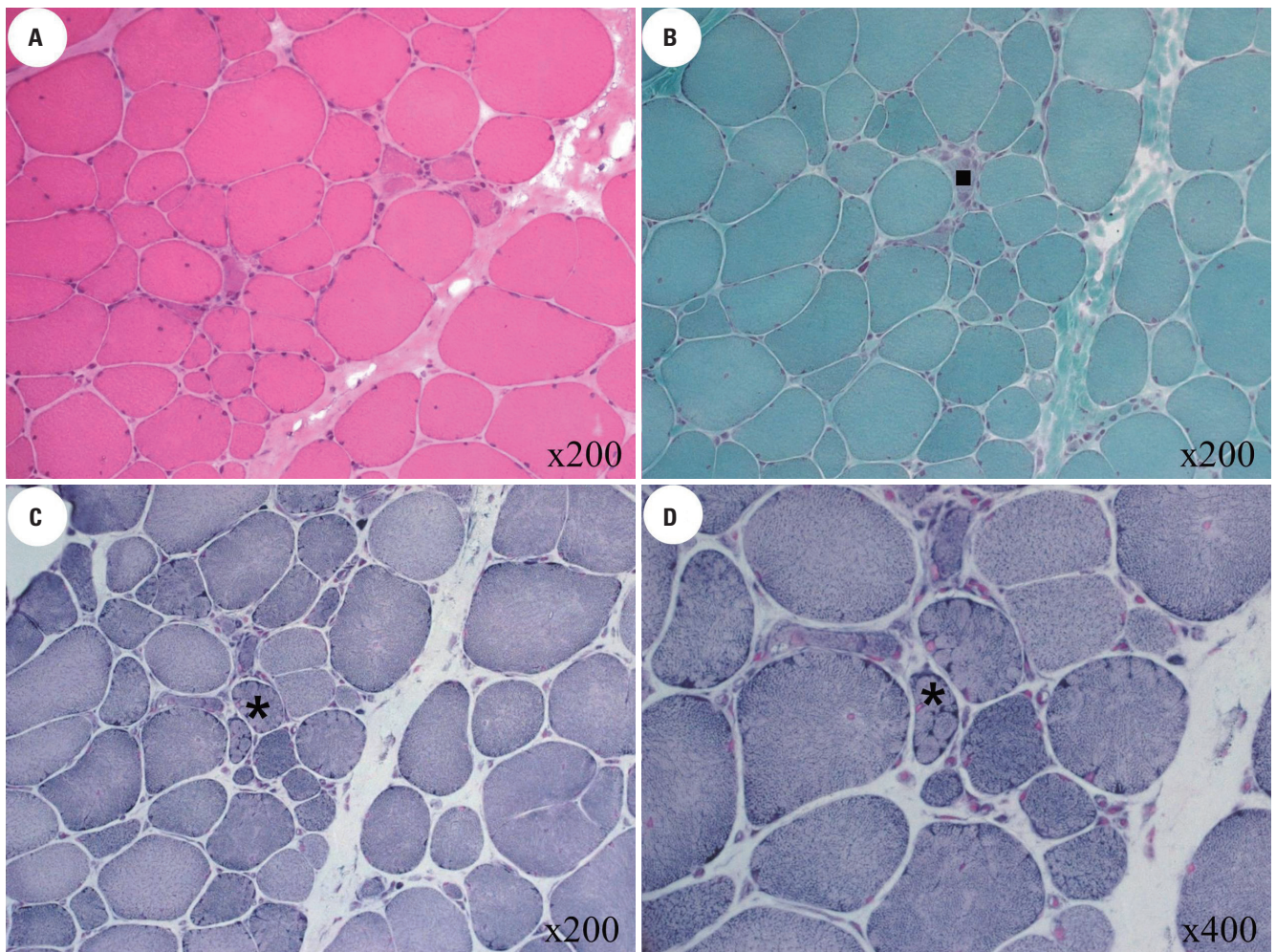


Fig. 3. Pathologic findings of F7 patients. (A) Fiber-size variation and increased endomysial fibrosis on hematoxylin and eosin staining. (B) A small number of degenerative muscle fibers (■) on modified Gomori trichrome staining. (C and D) A small number of lobulated fibers (*) after staining with nicotinamide adenine dinucleotide tetrazolium reductase.

of dystrophin, sarcoglycan (α , β , γ , and δ), dysferlin, α -dystroglycan, and caveolin in all 7 patients.

DISCUSSION

We analyzed the mutational, clinical, and pathological spectra of 13 Korean patients with calpainopathy. Among 13 patients, 11 had genetically-confirmed calpainopathy, and two with only a single mutation were diagnosed based on an analysis of calpain-3 protein.

As diagnostic tools for calpainopathy, both protein analysis and genetic tests are complex and require careful interpretation in the light of clinical findings.¹⁷ Western blots show normal or almost normal amounts of calpain-3 protein in about 20–30% of patients with *CAPN3* mutations.^{15,18,19} Additionally, protein loss of calpain-3 on western blots is not directly correlated with clinical severity.¹⁸ In fact, normal expression of calpain-3 protein was found in the F4 patient with two compound heterozygous mutations. Therefore, mutation analysis of *CAPN3* is the gold standard for the diagnosis of calpainopathy. However, previous reports demonstrated that only a single pathogenic mutation is identified in about 10% of calpainopathy patients,^{2,15,17} as many *CAPN3* mutations are located in a deep intronic position, which can be missed during routine gene sequencing.²⁰ In our study, only one mutation was identified in F9 and F10 patients with complete loss of calpain-3 protein.

We studied the clinical and pathological features of Korean patients with calpainopathy. The first clinical symptom usually appeared in the first or second decade of life, and included proximal muscle weakness, winged scapula, and ankle contracture. Half of the patients who received muscle biopsy had lobulated fibers. It is well-known that lobulated fibers increase as the disease progresses, although this is not a specific finding in calpainopathy.¹⁶ However, highly variable features were also found in Korean calpainopathy patients. F1, F5b, and F9 patients felt muscle weakness in the third decade. F7, F8, and F9 patients were initially diagnosed with Emery-Dreifuss muscular dystrophy, hyperCKemia, and polymyositis, respectively. Additionally, the F5a patient reported rapid deterioration of muscle and weakness in pregnancy. Although this was the first report on calpainopathy, one retrospective study reported permanent worsening in about half (54%) of 15 LGMD pregnancies.²¹ However, the cause of exacerbation during pregnancy is not known. Three muscle specimens from patients in this study revealed normal skeletal muscle histology. A previous pathologic report showed normal findings in three preclinical biopsies except in one focal area of necrosis.²² In the present study, although F1 and F5c patients experienced mild weakness of the hip and shoulder girdle muscles, muscle specimens of the vastus lateralis did not have pathologic abnormalities. This result was likely due to the following reasons. Firstly, the predominantly affected muscles in ambulatory patients with

calpainopathy are in the posterior compartment of the thigh.²³ Secondly, the muscle specimens showed that necrotic and regenerating fibers tended to occur in clusters.¹⁶

We did not find a correlation between genotype and phenotype in Korean patients with calpainopathy. It is known that null mutations are usually associated with a more severe phenotype than missense mutations.^{3,15,24,25} In our study, the F7 patient with two null mutations showed severe phenotype, including early clinical onset and prominent joint contracture. However, the F2 patient with two null mutations did not show a more severe phenotype than other patients with missense mutations. In addition, affected members of the F5 family showed clinical variability including onset age, despite the family members having the same genotype.

The present study suggests the possibility of a relatively low incidence of calpainopathy in Korea compared to other countries. To date, our institute, one of the largest myology centers in Korea, has genetically confirmed *DYSF* mutations, a genetic cause of LGMD2B, in 45 patients (unpublished data). On the other hand, the present study identified *CAPN3* mutations in only nine of 51 tested patients with suspicious calpainopathy. However, the relatively small number of patients limited the interpretability of the present study. Therefore, further studies are needed to identify the clinical and mutational findings in a larger cohort.

In conclusion, we identified nine different mutations including four novel *CAPN3* mutations in Korean patients. The present study confirmed the high variability of clinical and pathological features in Koreans with calpainopathy.

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