We report here two heterozygous *BAG3* gene mutations, identified in Japanese patients with familial DCM, which cause abnormal Z-disc assembly and increase the sensitivity to apoptosis in cultured cardiomyocytes. This is the first report demonstrating that the stress-induced apoptotic cell death accompanied by abnormal sarcomerogenesis is associated with DCM.

Materials and Methods

Subjects

A total of 72 genetically unrelated Japanese patients with DCM were included in this study. Each patient had an apparent family history (at least one patient among the first-degree family relatives). The patients were diagnosed based on medical history, physical examination, 12-lead electrocardiogram, echocardiography, and other special tests if necessary. The diagnostic criteria for DCM were described previously [Hayashi et al., 2004] and the patients who manifested with apparent skeletal muscle involvement were excluded from the study. The patients had been analyzed for mutations in 22 known cardiomyopathy-associated genes including genes for titin/connectin (*TTN*), desmin (*DES*), αB-crystallin (*CRYAB*), ZASP/Cypher (*LDB3*), and four-and-half LIM protein 2 (*FHL2*) [Kimura, 2010], and no mutation was found in any of them. Four hundred Japanese healthy individuals served as controls. Blood samples were obtained from each subject after given informed consent. The protocol for research was approved by the Ethics Review Committee of Medical Research Institute, Tokyo Medical and Dental University, Tokyo, Japan.

Mutational Analysis

Genomic deoxyribonucleic acids (DNAs) extracted from peripheral blood of subjects were used to amplify protein-coding exons of *BAG3* (GenBank Accession No. NM_004281.3) by polymerase chain reaction (PCR) in exon-by-exon manner using primer pairs; 5′-CGAGGAGGCTATTTCCAGAC-3′ and 5′-TGCCGTCGAGGTGGCGCCACCGACC-3′ for exon 1, 5′-AGTGTTTCCTCTGCCAGGAG-3′ and 5′-TGGGAAGCACAGCGGCTTGCTC-3′ for exon 2, 5′-CAAGCCAGGGGAGTCATTTG-3′ and 5′-GACATACCACCATAACCAGTC-3′ for exon 3, 5′-CAATTTCTGTGACTTTCAGTCAG-3′ and 5′-GTCAGTCTTCTTGCCTTCAAAG-3′ for the 5′-side half of exon 4, and 5′-ATCCAGGAGTGCTGAAAGTG-3′ and 5′-AAGTCTCTGAAATGCATGCAAC-3′ for the 3′-side half of exon 4. The PCR condition was composed of a denaturing step of 95°C for 2 min, 30 cycles of 95°C for 30 sec, 56°C for 30 sec, and 72°C for 30 sec, followed by an additional extension step of 72°C for 2 min. The PCR products were analyzed by direct sequencing on both strands using Big Dye Terminator chemistry (version 3.1) and ABI3100 DNA Analyzer (Applied Biosystems, CA).

Amino Acid Sequence Comparison of BAG3 from Various Species

Amino acid sequences of human BAG3 protein predicted from NM_004281.3 were aligned with those of rhesus monkey (XM_001104106), cattle (NM_001082471), rat (NM_001011936), mouse (NM_013863), chicken (XM_001233434), xenopus (BC043807), and zebrafish (BC078249).

Indirect Immunofluorescence Microscopy

Complementary DNA (cDNA) of human *BAG3* were obtained by reverse transcriptase PCR from total messenger ribonucleic acid of adult heart. A wild-type (WT) full-length *BAG3* cDNA fragment spanned from bp307 to bp2034 of NM_004281.3 (corresponding to aa1-aa576). Five equivalent mutant cDNA fragments carrying a C to T (MFM-associated Pro209Leu mutation) [Selcen et al., 2009], a C to T (DCM-associated Arg218Trp mutation), a C to T (nondisease-associated Arg258Trp polymorphism), or a T to C (DCM-associated Leu462Pro mutation) substitution were obtained by the primer-directed mutagenesis method. The cDNA fragments of *BAG3* were cloned into pEGFP-C1 vector (Clontech, CA) and they were sequenced to ensure that no errors were introduced.

All care and treatment of animals were in accordance with the guidelines for the Care and Use of Laboratory Animals published by the National Institute of Health (NIH Publication 85-23, revised 1985) and subjected to prior approval by the local animal protection authority. Neonatal rat cardiomyocytes (NRCs) from one-day-old Sprague-Dawley rats were prepared as described previously [Arimura et al., 2009]. NRCs (1×10^4 cells) were plated onto the Collagen Type I Cellware 8-Well Culture Slide (BD Biosciences, MA) in low-glucose DMEM supplemented with 0.01 mg/ml insulin (Sigma-Aldrich, MO), 10% fetal bovine serum (FBS), and 1% penicillin/streptomycin at 37°C with 5% CO_2 for 24 hr. Each pEGFP-based construct (0.3 μg) was transfected into the cells with 0.6 μl of TransFectin Lipid Reagent (Bio-Rad, CA), according to the manufacturer's instructions. Forty-eight hours after the transfection, the NRCs were washed with PBS and fixed for 15 min in 100% ethanol at −20°C. Transfected cells were incubated in blocking solution and stained by primary mouse anti-α-actinin (1:800, Sigma-Aldrich) or anti-desmin (1:200, Dako, Glostrup, Denmark), followed by secondary Alexa fluor 568 goat anti-mouse IgG_1 (1:500, Molecular Probes, OR).

C2C12 cells (8×10^3 cells), a mouse myoblast cell line, were plated onto the gelatin-coated Lab-Tek 2 well Chamber Slide (Nalgen Nunc International, NY) in DMEM supplemented with 20% FBS and 1% penicillin/streptomycin at 37°C with 5% CO_2 for 24 hr. The cells were transfected with each pEGFP-based construct (2 μg) in 4 μl of Turbofect in vitro Transfection Reagent (Fermentas Inc., ML) according to the manufacturer's instructions. Forty-eight hours after the transfection, the cells were cultured in differentiation medium (DMEM with 2% horse serum, 0.01 mg/ml insulin, and 1% penicillin/streptomycin) for 5 days. Differentiated myotubes were washed with PBS, fixed for 15 min in 100% ethanol at −20°C, incubated in blocking solution, and stained by primary mouse anti-MF20 (1:50, DSHB in University of Iowa, IA) monoclonal antibody (Ab), followed by secondary Alexa fluor 568 goat anti-mouse IgG (1:500, Molecular Probes).

All cells were mounted on a cover-glass using Mowiol 4-88 Reagent (Calbiochem, Darmstadt, Germany) with 4′6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich), and images from at least 200 transfected cells were analyzed by using the LSM510 laser-scanning microscope (Carl Zeiss Microscopy, Jena, Germany).

Apoptosis Assay

For the apoptosis assay, 24 hr after the transfection with *BAG3* constructs, the NRCs were cultured under serum-deprived (FBS-free medium) condition for additional 24 hr, washed with PBS, fixed for 1 hr in 4% paraformaldehyde/PBS at room temperature, and permeabilized for 2 min in 0.1% Triton X-100/0.1%

sodium citrate on ice. Apoptosis was evaluated with the terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling (TUNEL) assay using in situ Cell Death Detection Kit, TMR red (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions.

Quantitative analysis of apoptosis was performed with the Cell Death Detection ELISA^{PLUS} kit (Roche Diagnostics) according to the manufacturer's instructions. H9c2 cells, a cell line derived from rat embryonic ventricular myocardial cells, were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin at 37°C with 5% CO_2. The *BAG3* constructs were transfected into H9c2 cells using the TransFectin Lipid Reagent (Bio-Rad) according to the manufacturer's instructions, and transfectants were selected using Geneticin (Life Technologies Japan Ltd., Tokyo, Japan). After establishment of the stable H9c2 transfectants, 4×10^3 cells in each line were plated onto collagen type I-coated 96-well plates. Doxorubicin (1 μM; Sigma-Aldrich) was added to culture media and the cells were cultured for various intervals (24, 48, and 72 hr). Cells were lysed with 0.2 ml of the lysis buffer provided in the kit at room temperature for 30 min. Quantities of histone-associated DNA fragments (mono- and oligonucleosomes) were determined by an absorbance at 405 nm and a reference at 490 nm. Numerical data were arbitrarily expressed as means ± SEM. Statistical differences were analyzed using two-way analysis of variance and then evaluated using a Turkey adjustment for post hoc multiple comparison. A *P*-value of less than 0.05 was considered to be statistically significant.

Results

Identification of BAG3 Mutations in DCM

We searched for *BAG3* variations in 72 proband patients with familial DCM and eight distinct variations were identified (Fig. 1A). Among them, two synonymous substitutions, Pro334Pro (c.1002T>G in exon 4, rs3858339) and Val432Val (c.1296A>G in exon 4, rs196295), and three nonsynonymous variations, Arg258Trp (c.772C>T in exon 3, rs117671123), Asp300Asn (c.898G>A in exon 3, rs78439745), and Pro407Leu (c.1220C>T in exon 4, rs3858340), were known polymorphisms registered in the dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/). In addition, a nonsynonymous variation, Glu553Asp (c.1659A>T in exon 4) found in one patient, was considered to be a polymorphism, because it was found in heterozygous state in nine of the 400 control subjects, that is allele frequency was 0.011 in Japanese patients.

On the other hand, two missense mutations, Arg218Trp (c.652C>T in exon 3) and Leu462Pro (c.1385T>C in exon 4), identified in heterozygous state in two DCM patients (designated II-1 in Fig. 1B and C, respectively) were not observed in the 400 control subjects. A family study suggested a co-segregation of the Leu462Pro mutation with DCM, because the mutation was present in a possibly affected sister, but not present in her father and brother who did not suffer from DCM (Fig. 1C). Most of the *BAG3* sequence variations including polymorphisms were found at the residues that were evolutionary conserved from various species except for zebrafish (Fig. 1D).

Clinical parameters of the patients with *BAG3* mutations are shown in Table 1. The proband patients carrying Arg218Trp or Leu462Pro mutation developed DCM at age 73 or 34, respectively, suggesting that the mutations was associated with DCM of adult onset. It should be noted that a sister of patient carrying the Leu462Pro mutation did not manifest with overt DCM at age 27, but she showed a slight systolic dysfunction of heart. Electrocardiogram findings of the affected individuals demonstrated no primary conduction defect. Serum creatine kinase (CK) level was not increased in both cases with the Leu462Pro mutation. They did not show apparent sign of skeletal myopathy or neuropathy.

Abnormal Assembly of Z-Discs Caused by the DCM-Associated BAG3 Mutations in NRCs

To investigate a possible functional consequence of the *BAG3* mutations, we analyzed cellular distribution of BAG3 proteins by using green fluorescence protein (GFP) chimeras of *BAG3* transfected into NRCs. For this purpose, we constructed GFP-tagged BAG3 of WT and DCM-associated mutations, Arg218Trp and Leu462Pro. We also tested an MFM-associated mutation, Pro209Leu [Lee et al., 2011; Odgerel et al., 2010; Selcen et al., 2009], and a nondisease-related missense variant, Arg258Trp (Fig. 1A), which was found in one patient and 11 controls in this study. Control NRCs transfected with GFP-alone construct showed diffuse localization of GFP signals (data not shown). Western blot analyses showed that the expression of each GFP–BAG3 construct was similar at the protein level in the transfected cells, suggesting that the mutation did not affect the expression of GFP–BAG3 (data not shown). In the mature myofibrils where Z-discs were well organized, GFP-BAG3-WT was assembled in the striated pattern and co-localized with α-actinin and desmin, markers for the Z-disc (Figs. 2A–C and 3A–C, respectively). It was found that most (~90%) of NRCs did not show nuclear localization of GFP-BAG3-WT (Figs. 2A–C and 3A–C). GFP-BAG3-Pro209Leu and GFP-BAG3-Arg258Trp also showed striated pattern co-localized with α-actinin and desmin at the Z-discs and did not show the nuclear localization (Figs. 2D–F and 3D–F, and Figs. 2J–L and 3J–L, respectively). In clear contrast, striated distribution was not found for both GFP-BAG3-Arg218Trp (Figs. 2G–I and 3G–I) and GFP-BAG3-Leu462Pro (Figs. 2M–O and 3M–O) in about 90% of transfected NRCs. Of note was that the Z-disc assembly represented by localization of α-actinin and desmin was impaired in the NRCs transfected with GFP-BAG3-Arg218Trp or GFP-BAG3-Leu462Pro (Figs. 2H and 3H, or Figs. 2N and 3N, respectively). Quite interestingly, these mutant proteins displayed localization within the nuclei in approximately 80% of the transfected NRCs (Figs. 2G and 3G, or Figs. 2M and 3M, respectively). These data suggested that the DCM-associated mutations disturbed the assembly and integrity of Z-discs, along with the nuclear localization of BAG3 protein, while such abnormalities were not observed with the MFM-associated mutations.

Myotube Formation was Affected by the MFM-Associated BAG3 Mutation but Not by the DCM-Associated BAG3 Mutations in C2C12 Cells

The DCM patients carrying *BAG3* mutations in this study did not manifest with apparent skeletal muscle involvement, but some other *BAG3* mutations were reported in patients with MFM. There is a possibility that the DCM-associated mutations might affect the function of BAG3 protein in striated muscles differently from the MFM-associated mutation. To investigate whether the *BAG3* mutations would affect the skeletal muscle differentiation from myoblasts to myotubes, C2C12 myoblast cells were transfected with BAG3 constructs and differentiated into multinucleated myotubes by low-serum culture condition. After 5 days of differentiation, myosin heavy chain positive (recognized by MF20 Ab) myotubes could often be observed in this condition. Control cells transfected with GFP-alone construct (data not shown) and the

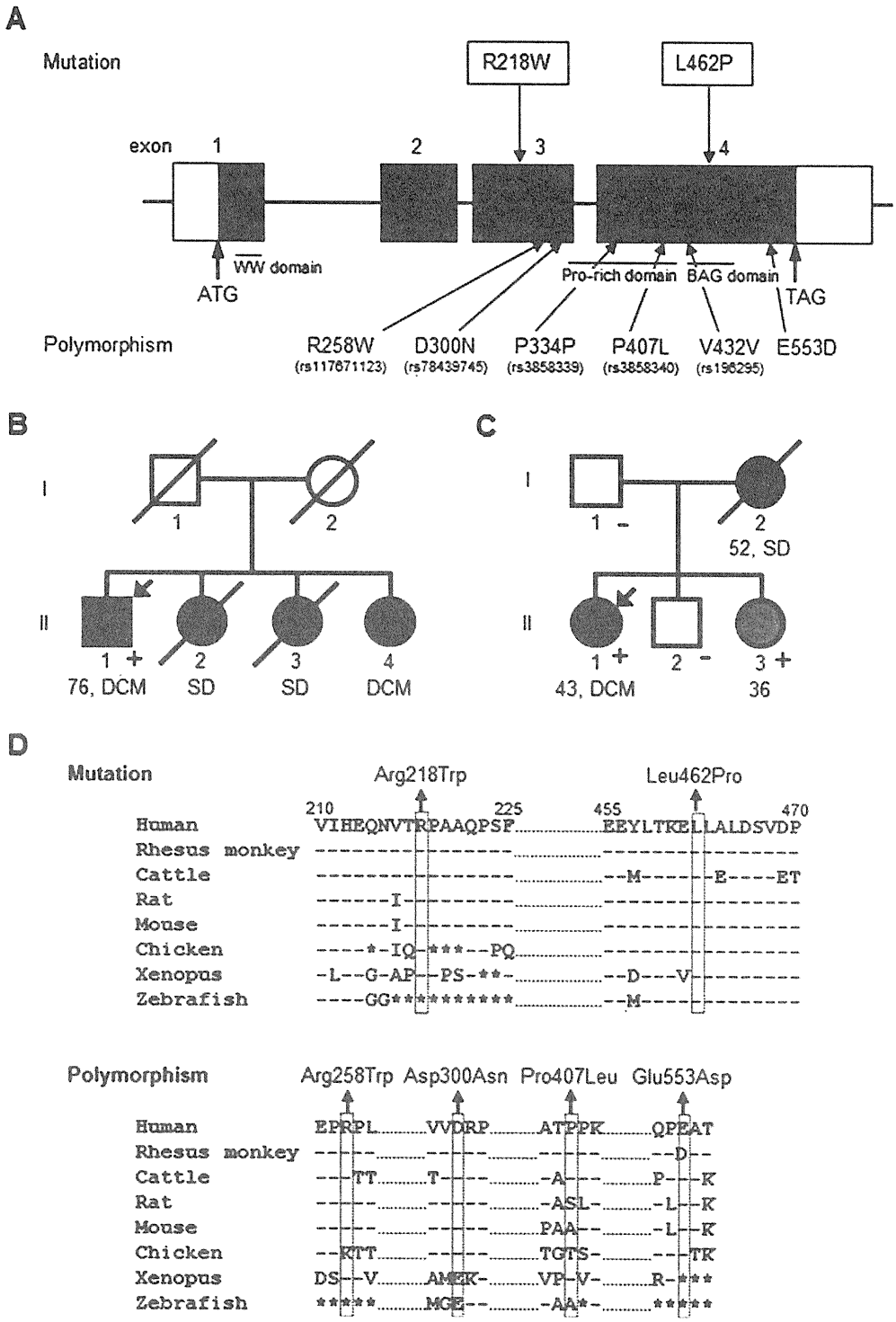

Figure 1. Mutational analysis of *BAG3* in dilated cardiomyopathy. A: Sequence variations found in this study are listed. Single-letter code is used to indicate the amino acid residue. DCM-associated mutations and polymorphisms are indicated above and below the schematic representation of *BAG3* gene, respectively. Known polymorphisms are indicated with reference single nucleotide polymorphism (rs) number in the parentheses. Solid boxes represent coding exons. B and C: Pedigrees of DCM families carrying the R218W mutation (B; CM 2019 family) or L462P mutation (C; CM 2053 family) are shown. Filled square and filled circle indicate affected male and female, respectively. Open square and open circle represent unaffected male and female with DCM, respectively. Arrows indicate the proband patients. Presence (+) or absence (–) of the mutations is noted for analyzed individuals. II-3 in (C), who is represented by a shadowed circle, showed a regional hypokinesia in posterior ventricular wall. SD, sudden death. D: Alignment of amino acid sequences of BAG3 proteins from various species around the DCM-associated mutations, Arg218Trp (R218W) and Leu462Pro (L462P), along with polymorphisms identified in the Japanese populations. Protein sequence of human BAG3 predicted from the nucleotide sequences was aligned with that of rhesus monkey, cattle, rat, mouse, chicken, xenopus, and zebrafish.

Table 1. Clinical Characteristics of Individuals Carrying BAG3 Mutations

ID	Mutation	Age at exam (years) and gender	Age at onset (years)	LVDd (mm)	LVDs (mm)	IVST (mm)	PWT (mm)	%FS	%EF	Other remarks
CM2019 family II-1	R218W	76, male	73	54	48	10	10	11	29	ECG; ectopic atrial rhythm,
CM2053 family II-1	L462P	41, female	34	59	47	6	6	20	40	ECG; premature ventricular contraction, CK = 66 IU/l
CM2053 family II-3	L462P	27, female	27	48	31	7	6	35	64	EchoCG; partial hypokinesia in posterior ventricular wall, CK = 62 IU/l

LVDd, left ventricular end-diastolic dimension; LVDs, left ventricular end-systolic dimension; IVST, interventricular septum thickness; PWT, posterior wall thickness; %FS, percent fractional shortening; %EF, percent ejection fraction; ECG, electrocardiogram; EchoCG, echocardiogram; CK, creatine kinase.

cells transected with GFP-BAG3-WT showed similar morphological differentiation, because about half (40 to 50%) of myotubes transfected with GFP-BAG3-WT were over trinucleation (Fig. 4A–C). Similarly, after 5 days of differentiation, numbers of myotubes with over trinucleation were about half in cells transfected with GFP-BAG3-Arg218Trp (Fig. 4G–I), GFP-BAG3-Arg258Trp (Fig. 4J–L), and GFP-BAG3-Leu462Pro (Fig. 4M–O). In clear contrast, most (~90%) of myotubes transfected with GFP-BAG3-Pro209Leu were in binuclear state (Fig. 4D–F). These observations suggested that the MFM-associated mutation, Pro209Leu, might disturb the multinucleation during the differentiation into skeletal muscle myotubes. It should be noted that GFP-BAG3 proteins showed diffuse localization in the cytoplasm and did not show nuclear localization in the myotubes transfected with GFP-BAG3 constructs, even with the DCM-associated mutations (Fig. 4).

Altered Sensitivity to Apoptosis Caused by the DCM-Associated BAG3 Mutations

Because BAG3 is an antiapoptotic protein, we hypothesized that the *BAG3* mutations might render the cells susceptible to stress-induced apoptosis. To investigate the possible involvement of *BAG3* mutations in the abnormal regulation of cellular apoptosis, we first performed a TUNEL assay on NRCs transfected with GFP chimeras of *BAG3*. There was no difference in the frequency of TUNEL-positive cells among the nontransfected and transfected NRCs under the culture condition without serum starvation; less than 1% of NRCs were TUNEL positive. Under the serum-deprived condition for 24 hr, most (~90%) of NRCs expressing GFP-BAG3-WT showed negative TUNEL staining (Fig. 5A–C). In contrast, about half of NRCs transfected with GFP-BAG3-Arg218Trp (Fig. 5G–I) or GFP-BAG3-Leu462Pro (Fig. 5M–O) demonstrated positive TUNEL staining with disorganized GFP signals under the serum deprivation for 24 hr, albeit most (~90%) of NRCs transfected with GFP-BAG3-Pro209Leu (Fig. 5D–F) or GFP-BAG3-Arg258Trp (Fig. 5J–L) showed negative TUNEL staining with well-organized striated pattern of GFP signals. These observations indicate that the cardiomyopathy-associated *BAG3* mutations, but not the MFM-associated *BAG3* mutation or nondisease-related *BAG3* polymorphism, may increase the susceptibility to stress-induced apoptosis of NRCs.

To confirm the increased sensitivity to stress-induced apoptosis by the DCM-associated *BAG3* mutations by another method, we quantified apoptosis of H9c2 cells stably expressing GFP alone, GFP-BAG3-WT, or GFP chimera of each variant by the cell death ELISA assay. Stable transfected cell lines were treated with doxorubicin at the concentration of 1 μM for 24, 48, or 72 hr, and subjected to the assay. It was demonstrated that doxorubicin induced formation of oligonucleosomes in a time-dependent manner, and there was no significant difference among the nontransfected H9c2 cells and transfected cell lines expressing GFP only, GFP-BAG3-WT, GFP-BAG3-Pro209Leu, or GFP-BAG3-Arg258Trp (Fig. 6). On the other hand, significantly higher amounts of oligonucleosomes were observed in the stable transfectants expressing GFP-BAG3-Arg218Trp or GFP-BAG3-Leu462Pro than the transfectants expressing GFP-BAG3-WT, under the treatment by doxorubicin (Fig. 6). These observations further indicated that the DCM-associated *BAG3* mutations increased the sensitivities to apoptosis under the stressed condition.

Discussion

In the present study, we identified two DCM-associated mutations in a Z/I-band signaling protein, BAG3, which were not found in the controls and caused functional alterations. In addition, we found four other *BAG3* variations with amino acid replacements, but they were not considered to be associated with DCM, because they were present in the healthy individuals, even though evolutionary conserved residues were replaced. The DCM-associated mutations affected the Z-disc assembly of cardiomyocytes and increased the sensitivity to apoptosis under the metabolic stress. The latter functional change might be the reason for that the *BAG3* mutations were found in late-onset DCM. In other words, metabolic stresses to cardiomyocytes might be required to develop overt DCM in the subjects with the *BAG3* mutations found in this study.

We observed no functional alterations caused by the Arg258Trp variant in NRCs, a cardiomyocyte cell line H9c2, and a skeletal muscle cell line C2C12, suggesting that it was not a pathogenic mutation. Although the Arg258Trp mutation was recently reported in a Chinese patient with MFM, it was also found in the unaffected father of the patient and the patient carried another mutation Pro209Leu [Lee et al., 2011]. In this study, we demonstrated that the MFM-associated Pro209Leu mutation impaired the differentiation of skeletal muscle cell line C2C12, although it caused no functional alterations in the NRCs and in a cardiomyocyte cell line H9c2. The observations further suggested that the Arg258Trp variant was a simple polymorphism not associated with the diseases.

BAG3 is a co-chaperone protein and might not be directly involved in the muscle contractile function. Recent genetic studies have revealed that DCM is caused by the gene abnormalities not only in the cytoskeletal/contractile proteins, but also in the noncytoarchitectural molecules distributed in the Z/I-band region [Kimura, 2010]. We previously reported a DCM-associated Arg157His mutation in another chaperone protein, αB-crystallin, and this mutation did not show abnormal localization in the cytoplasm of NRC, whereas a myopathy-associated Arg120Gly mutation formed aggregated cytoplasmic depositions [Inagaki et al., 2006]. BAG3 and αB-crystallin bind with each other and both proteins serve to maintain protein homeostasis against the environmental stress [Hishiya

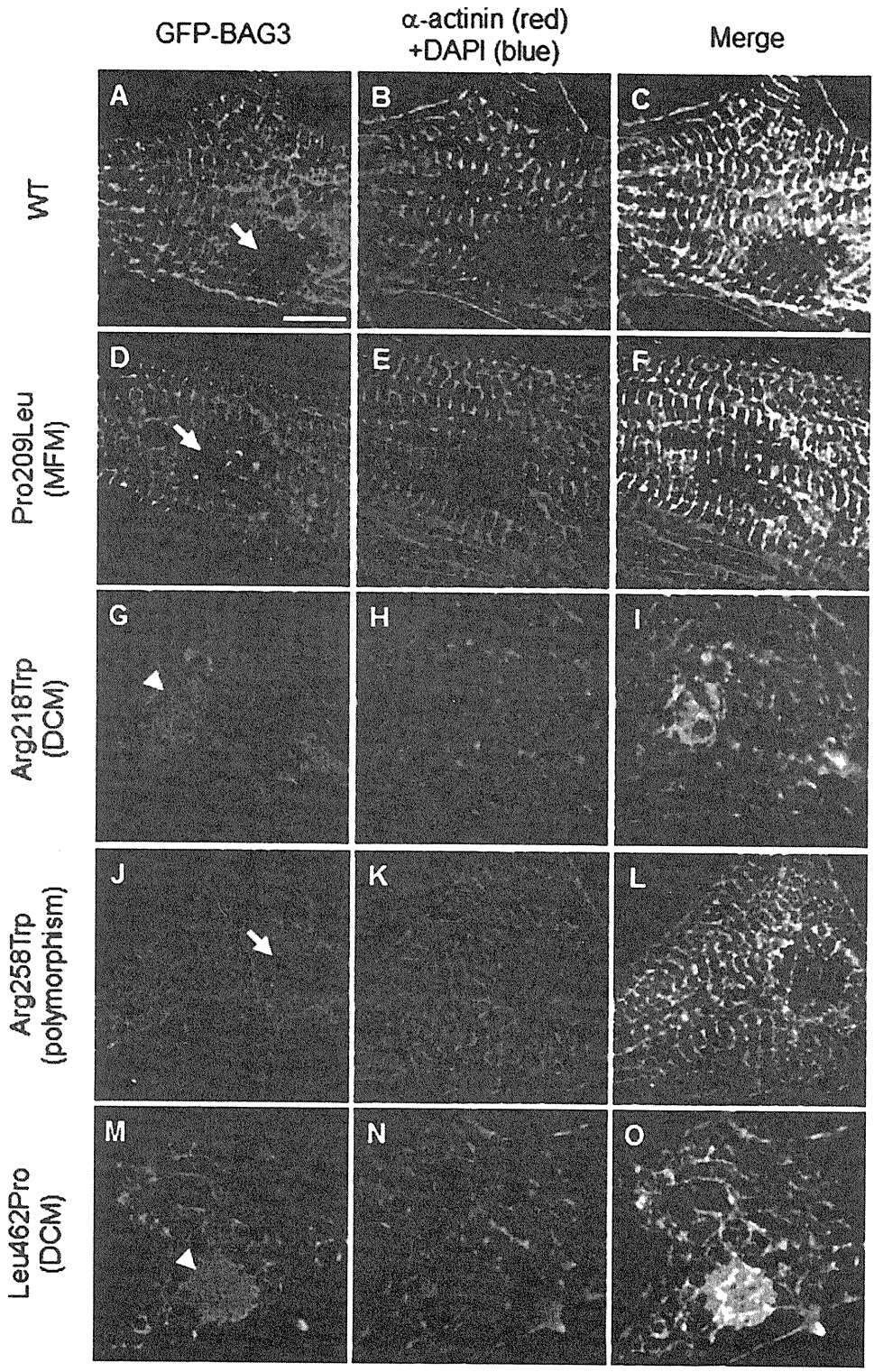

Figure 2. Distribution of α-actinin and transiently expressed GFP chimeras of *BAG3* in NRCs. NRCs transfected with GFP-tagged *BAG3* constructs for WT (A–C) or mutant (P209L, R218W, R258W, or L462P) (D–F, G–I, J–L, or M–O, respectively) were fixed 48 hr after the transfection, and stained with DAPI and anti-α-actinin antibody followed by secondary antibody (B, E, H, K, and N). Merged images are shown in C, F, I, L, and O. In the NRCs showing myofibrils with Z-discs, GFP-BAG3-WT is observed at the Z-discs and cytoplasm (A–C). GFP-tagged BAG3 proteins carrying the MFM-associated mutation, Pro209Leu, and nondisease-related variant, Arg258Trp, showed similar localization to that of WT (D–F and J–L, respectively). In contrast, GFP-tagged BAG3 proteins carrying the DCM-associated mutations, Arg218Trp and Leu462Pro, showed diffused localization that was associated with the disorganization of sarcomeric α-actinin (G–I and M–O). Arrows and arrowheads indicate the absence and presence, respectively, of GFP-BAG3 protein in nuclei. Scale bars = 10 μm.

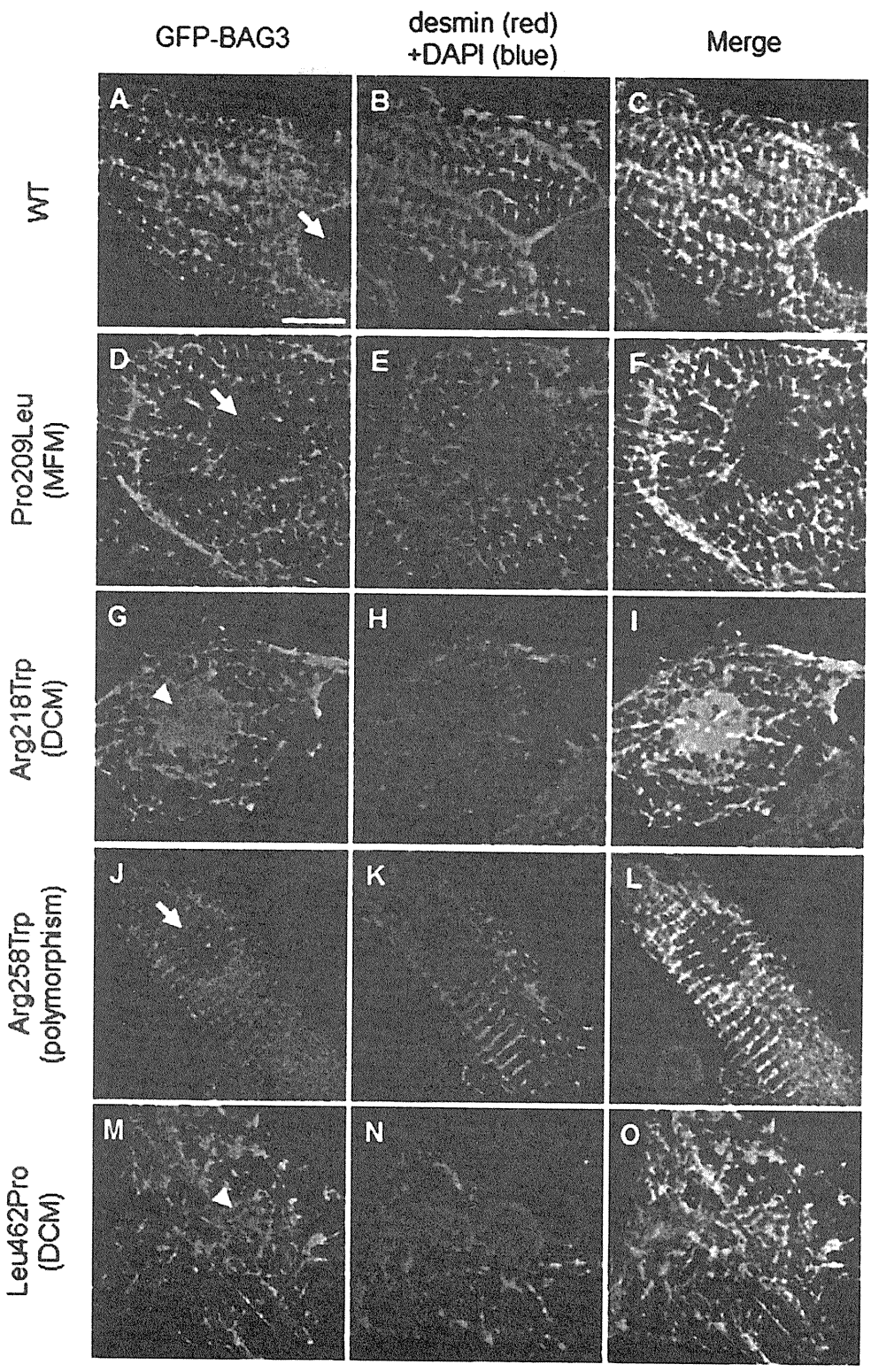

Figure 3. Distribution of desmin and transiently expressed GFP chimeras of BAG3 in NRCs. NRCs transfected with GFP-tagged BAG3 constructs for WT (A–C) or mutant (P209L, R218W, R258W, or L462P) (D–F, G–I, J–L, or M–O, respectively) were fixed 48 hr after the transfection, and stained with DAPI and anti-desmin antibody followed by secondary antibody (B, E, H, K, and N). Merged images are shown in C, F, I, L, and O. In the NRCs showing myofibrils with Z-discs, GFP-BAG3-WT is observed at the Z-discs and cytoplasm (A–C). GFP-tagged BAG3 proteins carrying the Pro209Leu and Arg258Trp, showed similar localization to that of WT (D–F and J–L, respectively). In contrast, GFP-tagged BAG3 proteins carrying the Arg218Trp and Leu462Pro, showed diffused localization that was associated with the disorganization of cytoskeletal desmin (G–I and M–O). Arrows and arrowheads indicate the absence and presence, respectively, of GFP-BAG3 in nuclei. Scale bars = 10 μm.

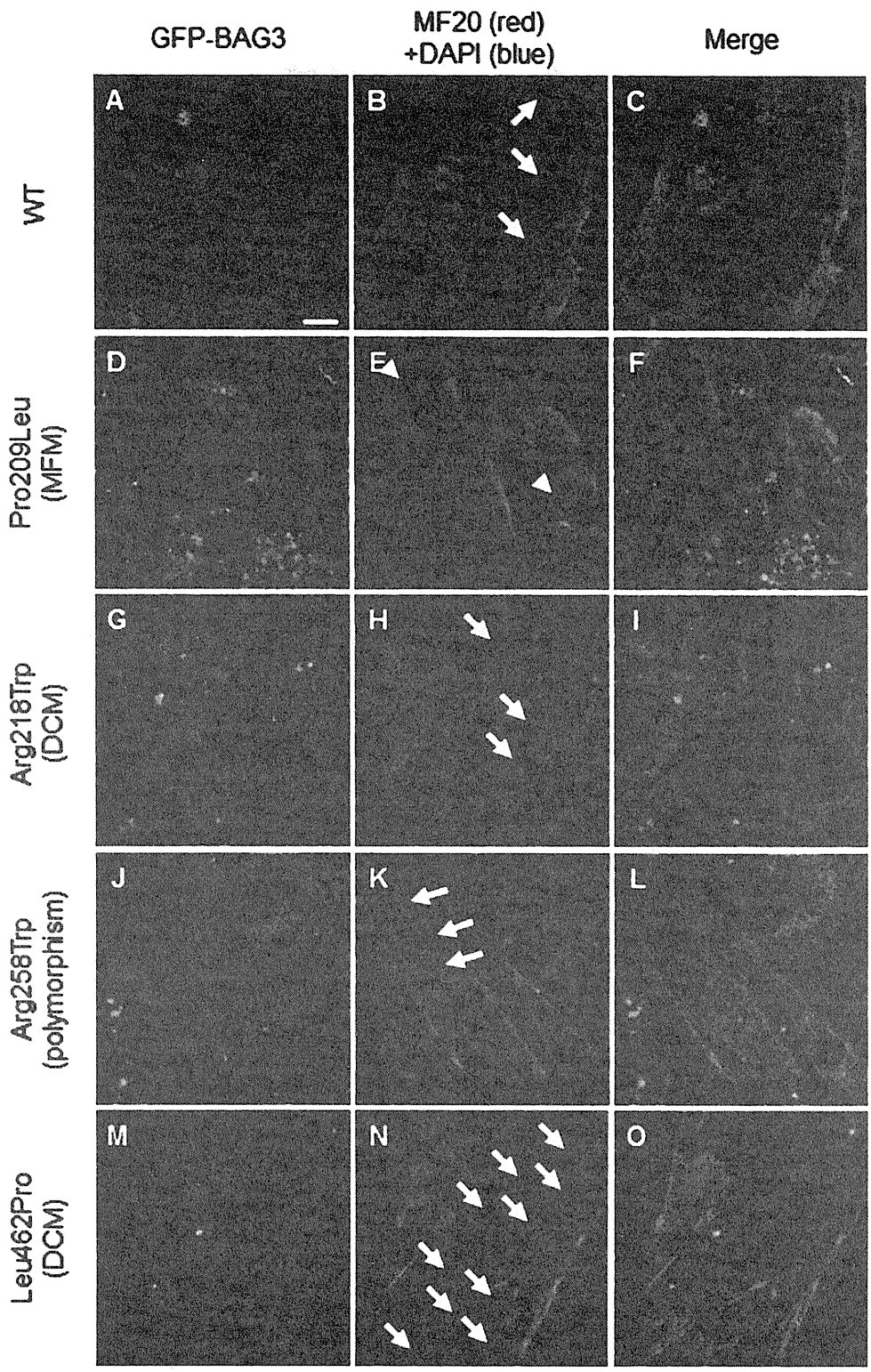

Figure 4. Differentiation of myoblasts into myotubes in C2C12 cells transiently expressed GFP chimeras of BAG3. C2C12 cells transfected with GFP-tagged BAG3 constructs for WT (A–C) or mutant (P209L, R218W, R258W, or L462P) (D–F, G–I, J–L, or M–O, respectively) were differentiated for 5 days in low-serum culture condition, and stained with DAPI and anti-MF20 antibody followed by secondary antibody (B, E, H, K, and N). Merged images are shown in C, F, I, L, and O. In the myotubes positively stained with MF20, GFP-BAG3 proteins were diffusely distributed in cytoplasm (A, D, G, I, and M). Trinucleations (arrows) were observed in the myotubes transfected with GFP- BAG3 WT, Arg218Trp, Arg258Trp, and Leu462Pro, but not in the myotubes transfected with GFP- BAG3 Pro209Leu (arrowheads). Scale bars = 10 μm. [Color figure can be viewed in the online issue, which is available at wiley.com/humanmutation.]

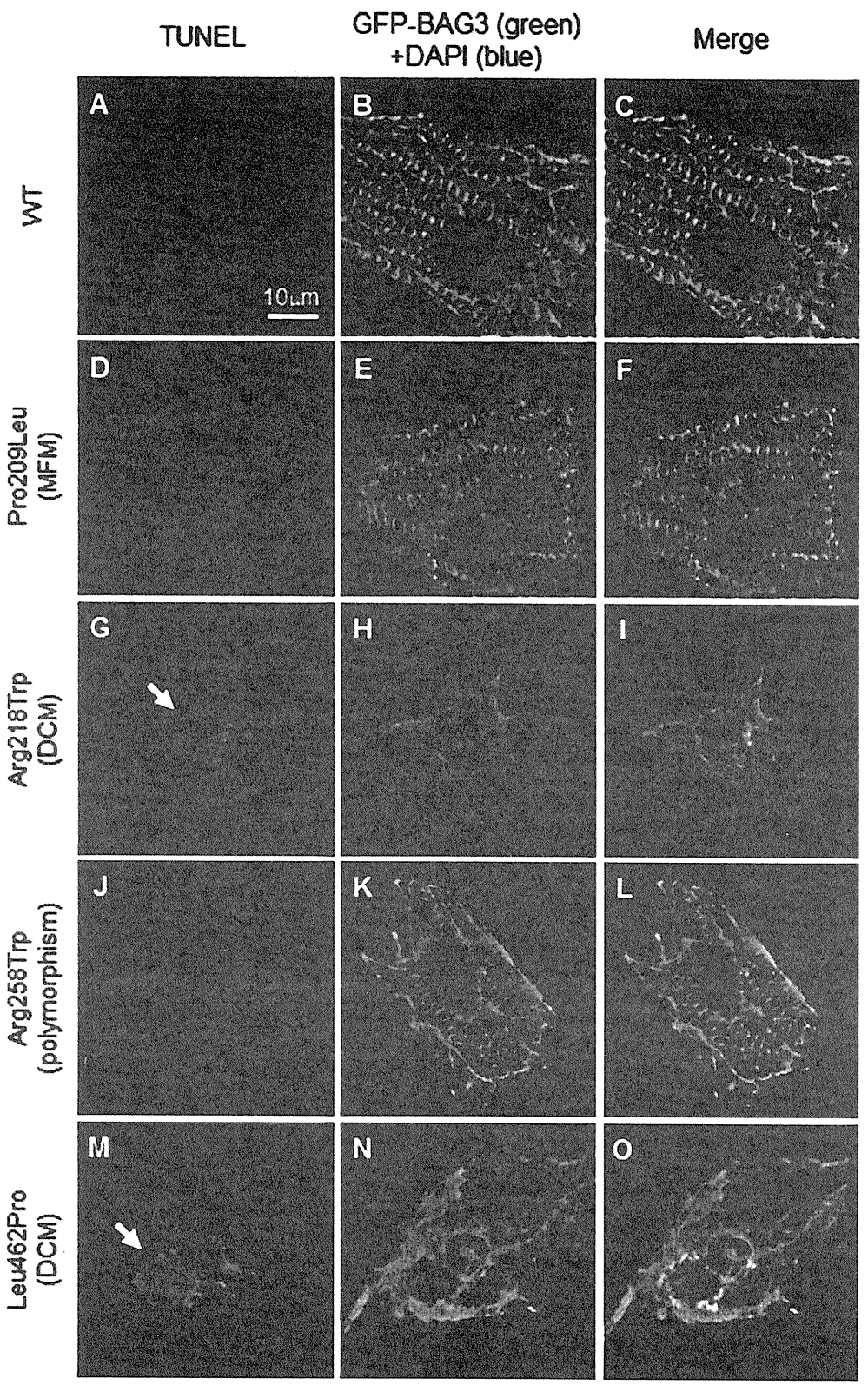

Figure 5. Apoptosis induced by serum deprivation in NRCs transfected with GFP-BAG3. NRCs were transfected with GFP chimeras of WT (A–C) or mutant (P209L, R218W, R258W, or L462P) (D–F, G–I, J–L, or M–O, respectively). The NRCs were cultured under FBS-free condition for additional 24 hr, fixed, subjected to the TUNEL assay, and stained with DAPI (B, E, H, K, and N). Merged images are shown in C, F, I, L, and O. Representative images of TUNEL assays are shown (A, D, G, J, and M). Arrows indicate apoptotic cells with positive TUNEL staining as visualized by red fluorescence; scale bars = 10 μm. [Color figure can be viewed in the online issue, which is available at wiley.com/humanmutation.]

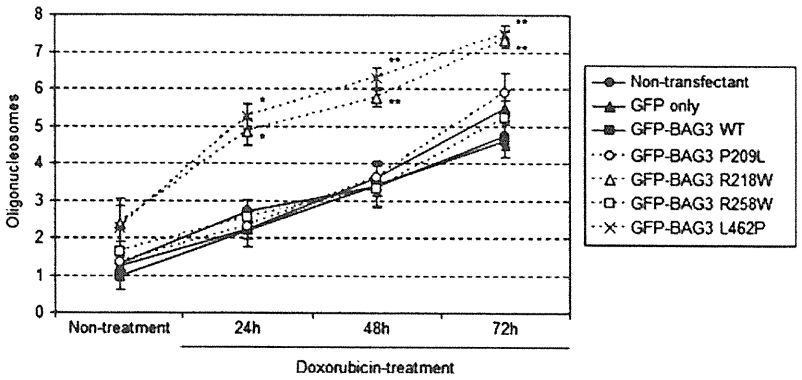

Figure 6. Quantitative analysis of apoptosis induced by doxorubicin in H9c2 cells stably expressing GFP-BAG3. H9c2 cells stably expressing each GFP chimera of *BAG3* in 96-well dishes were treated with 1 μM of doxorubicin for 24, 48, and 72 hr. Cell lysates were subjected to cell death ELISA assay. Amounts of mono- and oligonucleosomes were measured at 405 nm, and referenced by 490 nm. Data are arbitrarily expressed as means ± SEM ($n = 6$ for each case). * $P < 0.01$ versus WT; ** $P < 0.001$ versus WT.

et al., 2011]. In addition, they have crucial roles in protein folding, inhibition of protein aggregation, and degradation of misfolded proteins as chaperone-related proteins [Hishiya et al., 2011]. However, disrupted co-localization of BAG3 and α-actinin was observed for GFP-BAG3-Arg218Trp and GFP-BAG3-Leu462Pro without any cytoplasmic aggregation of mutant BAG3 proteins, indicating that the abnormal Z-disc assembly was directly associated with the *BAG3* mutations. It is worth noting that the functional alteration caused by the DCM-associated *BAG3* mutations was different from that by the DCM-associated αB-crystallin mutation, which was the decreased binding to titin N2-B region without disturbing the Z-disc assembly [Inagaki et al., 2006].

The mechanism of altered Z-disc assembly caused by the DCM-associated *BAG3* mutations is not clear, but a knockdown of *Bag3* in cardiomyocytes induced rapid myofibrillar degeneration and Z-disc disruption under the condition of mechanical stress [Hishiya et al., 2010], suggesting that *BAG3* might play a pivotal role in the Z-disc assembly during the myofibrillogenesis. In the transition from nascent to mature myofibrils, Z-disc precursors, Z-bodies, eventually fuse laterally to form Z-discs at the trunk of myocytes, which is accompanied by the induction of myofibrillar proteins, and this may stabilize the sarcomere structure required for muscle contraction. In this study, it was suggested that the DCM-associated mutations affected the assembly of sarcomere in NRCs. However, because we did not assessed the turnover and/or reorganization of the Z-discs, molecular mechanisms of disturbing the Z-disc organization during myofibrillogenesis should be further investigated in future studies.

The myofibrillar integrity under mechanical stress is maintained by the BAG3–Hsc70 interaction [Hishiya et al., 2010], and Hsc70 is a regulator of a chaperone-dependent E3 ligase CHIP [Murata et al., 2003; Pratt et al., 2010]. It was reported that CHIP-mediated degradation of p53 was involved in the protection against myocardial damage under ischemic condition [Naito et al., 2011]. These observations imply a possible link between the myofibrinogenesis and stress-induced apoptosis of cardiomyocytes. It is well known that serum deprivation and doxorubicin induce apoptosis of cultured cardiomyocytes including NRCs [Chao et al., 2005] and H9c2 cells [Chua et al., 2006], and we demonstrated that the DCM-associated *BAG3* mutations increased the sensitivity to apoptosis of the cardiomyocytes under the stressed conditions. Because BAG3 protein possess antiapoptotic function by enhancing the activity of bcl-2 [Lee et al., 1999], increased number of TUNEL-positive cells and oligonucleosomes in NRCs and H9c2 cells, respectively, expressing the DCM-associated *BAG* mutations might be due to the impaired function of BAG3 protein. Quite interestingly, we observed nuclear localization of GFP-BAG3-Arg218Trp and GFP-BAG3-Leu462Pro proteins in NRCs. Because the abnormal intranuclear accumulation was not observed in the NRCs transfected with GFP-BAG3-WT, GFP-BAG3-Pro209Leu, or GFP-BAG3-Arg258Trp, recruitment of BAG3 protein into nuclei may be a specific phenomenon caused by the DCM-associated mutations, which might be involved in the apoptosis of cardiomyocytes leading to DCM. Because the nuclear localization of mutant GFP-BAG3 proteins was observed in both apoptotic and nonapoptotic cells, we could not conclude whether the apoptosis was a direct consequence of the nuclear localization of mutant GFP-BAG3 proteins. Additional studies will be required to clarify the issue.

A number of skeletal muscle diseases and isolated DCM are caused by mutations in the same genes [Arimura et al., 2007]. The patients with muscular diseases often suffer from cardiac involvement, but most of the patients with isolated DCM do not manifest with the skeletal muscle phenotype. The etiological link between the inherited skeletal muscle diseases and hereditary DCM has raised a question as how the mutations in the genes/proteins expressed in both skeletal and cardiac muscles cause heart-specific phenotypes in the isolated DCM. The most probable explanation was that the phenotypic differences between the skeletal muscle disease and DCM might be due to that mutations in specific and/or different functional domains would affect specific functions. In this study, we found that the MFM-associated mutation, Pro209Leu, did not affect either the Z-disc assembly or the sensitivity to apoptosis. In clear contrast, the DCM-associated Arg218Trp mutations, which located near the Pro209Leu mutation, and the other DCM-associated Leu462Pro mutation caused abnormalities in both Z-disc assembly and sensitivity to apoptosis. It was reported that BAG3 protein with the Pro209Leu mutation was found predominantly in the abnormal form of small discrete granules in the COS-7 cells [Selcen et al., 2009], but such abnormality was not observed in NRCs, H9c2 cells, and C2C12 cells in this study. The reason why the Pro209Leu mutation did not show aggregations in the cardiomyocytes and skeletal muscle cell line is not clear, but the COS-7 cells are used for overexpression of genes from transfected constructs containing the replication origin of SV40, raising a possibility that the aggregation

was caused by the overexpression of mutant BAG3 proteins in the COS-7 cells.

The MFM patients carrying the Pro209Leu mutation were reported to demonstrate cardiac phenotypes of hypertrophic and/or restrictive cardiomyopathy, which are different from DCM. It is notable that the MFM patients manifested with cardiac phenotypes of early onset in childhood. Because the patients carrying the Arg218Trp or Leu462Pro mutations suffered from adult-onset DCM (Table 1), it was speculated that the pathological mechanisms of *BAG3* mutations might be different between the cardiomyopathy accompanied by MFM and isolated DCM. We demonstrated that the MFM-associated Pro209Leu mutation impaired the formation of multinuclear myotubes during the differentiation of C2C12 cells, which was not found with the DCM-associated mutations. However, this functional deficit may not associate with the cardiac phenotype caused by the Pro209Leu mutation, because cell fusion during the differentiation is specific to skeletal muscle cells and not found in cardiomyocytes. On the other hand, disturbance of myotube formation by the Pro209Leu mutation might be an underlying mechanism leading to skeletal muscle phenotypes in MFM, although it is not clear how the Pro209Leu mutation causes axonal neuropathy with giant axons [Odgerel et al., 2010]. Further studies will be required to reveal the difference in the molecular mechanisms of disease phenotypes caused by the *BAG3* mutations.

In conclusion, we report here two heterozygous missense mutations of *BAG3* found in familial DCM, which cause abnormal Z-disc assembly and increase the sensitivity to apoptosis in the cardiomyocytes. We demonstrate here for the first time the association between DCM and increased sensitivity to apoptosis accompanied by the abnormality in myofibrillogenesis. However, the overexpression of mutant proteins in cultured cardiac myocytes has significant limitation to mimic the situation in intact hearts. Further studies on the functional role of BAG3 protein in the cardiac muscle will help understanding the association between the abnormal function of BAG3 protein and DCM.

Acknowledgments

Conflict of Interest: None declared.

References

Arimura T, Bos JM, Sato A, Kubo T, Okamoto H, Nishi H, Harada H, Koga Y, Moulik M, Doi YL, Towbin JA, Ackerman MJ, Kimura A. 2009. Cardiac ankyrin repeat protein gene (ANKRD1) mutations in hypertrophic cardiomyopathy. J Am Coll Cardiol 54: 334–342.

Arimura T, Hayashi T, Kimura A. 2007. Molecular etiology of idiopathic cardiomyopathy. Acta Myol 26: 153–158.

Chao W, Shen Y, Zhu X, Zhao H, Novikov M, Schmidt U, Rosenzweig A. 2005. Lipopolysaccharide improves cardiomyocyte survival and function after serum deprivation. J Biol Chem 280: 21997–22005.

Chua CC, Liu X, Gao J, Hamdy RC, Chua BH. 2006. Multiple actions of pifithrin-alpha on doxorubicin-induced apoptosis in rat myoblastic H9c2 cells. Am J Physiol Heart Circ Physiol 290: H2606–H2613.

Hayashi T, Arimura T, Itoh-Satoh M, Ueda K, Hohda S, Inagaki N, Takahashi M, Hori H, Yasunami M, Nishi H, Koga Y, Nakamura H, Matsuzaki M, Choi BY, Bae SW, You CW, Han KH, Park JE, Knoll R, Hoshijima M, Chien KR, Kimura A. 2004. Tcap gene mutations in hypertrophic cardiomyopathy and dilated cardiomyopathy. J Am Coll Cardiol 44: 2192–2201.

Hishiya A, Kitazawa T, Takayama S. 2010. BAG3 and Hsc70 interact with actin capping protein CapZ to maintain myofibrillar integrity under mechanical stress. Circ Res 107: 1220–1231.

Hishiya A, Salman MN, Carra S, Kampinga HH, Takayama S. 2011. BAG3 directly interacts with mutated alphaB-Crystallin to suppress its aggregation and toxicity. PLoS One 6: e16828.

Homma S, Iwasaki M, Shelton GD, Engvall E, Reed JC, Takayama S. 2006. BAG3 deficiency results in fulminant myopathy and early lethality. Am J Pathol 169: 761–773.

Inagaki N, Hayashi T, Arimura T, Koga Y, Takahashi M, Shibata H, Teraoka K, Chikamori T, Yamashina A, Kimura A. 2006. Alpha B-crystallin mutation in dilated cardiomyopathy. Biochem Biophys Res Commun 342: 379–386.

Kimura A. 2010. Molecular basis of hereditary cardiomyopathy: abnormalities in calcium sensitivity, stretch response, stress response and beyond. J Hum Genet 55: 81–90.

Lee H, Cherk S, Chan S, Wong S, Tong T, Ho W, Chan A, Lee K, Mak C. 2011. BAG3-related myofibrillar myopathy in a Chinese family. Clin Genet, doi: 10.1111/j.1399-0004.2011.01659.x. [Epub ahead of print]

Lee JH, Takahashi T, Yasuhara N, Inazawa J, Kamada S, Tsujimoto Y. 1999. Bis, a Bcl-2-binding protein that synergizes with Bcl-2 in preventing cell death. Oncogene 18: 6183–6190.

Maron BJ, Towbin JA, Thiene G, Antzelevitch C, Corrado D, Arnett D, Moss AJ, Seidman CE, Young JB. 2006. Contemporary definitions and classification of the cardiomyopathies: an American Heart Association Scientific Statement from the Council on Clinical Cardiology, Heart Failure and Transplantation Committee; Quality of Care and Outcomes Research and Functional Genomics and Translational Biology Interdisciplinary Working Groups; and Council on Epidemiology and Prevention. Circulation 113: 1807–1816.

Murata S, Chiba T, Tanaka K. 2003. CHIP: a quality-control E3 ligase collaborating with molecular chaperones. Int J Biochem Cell Biol 35: 572–578.

Naito AT, Okada S, Minamino T, Iwanaga K, Liu ML, Sumida T, Nomura S, Sahara N, Mizoroki T, Takashima A, Akazawa H, Nagai T, Shiojima I, Komuro I. 2010. Promotion of CHIP-mediated p53 degradation protects the heart from ischemic injury. Circ Res 106: 1692–1702.

Norton N, Li D, Rieder MJ, Siegfried JD, Rampersaud E, Zuchner S, Mangos S, Gonzalez-Quintana J, Wang L, McGee S, Reiser J, Martin E, Nickerson DA, Hershberger RE. 2011. Genome-wide studies of copy number variation and exome sequencing identify rare variants in BAG3 as a cause of dilated cardiomyopathy. Am J Hum Genet 88: 273–282.

Odgerel Z, Sarkozy A, Lee HS, McKenna C, Rankin J, Straub V, Lochmüller H, Paola F, D'Amico A, Bertini E, Bushby K, Goldfarb LG. 2010. Inheritance patterns and phenotypic features of myofibrillar myopathy associated with a BAG3 mutation. Neuromuscul Disord 20: 438–442.

Pratt WB, Morishima Y, Peng HM, Osawa Y. 2010. Proposal for a role of the Hsp90/Hsp70-based chaperone machinery in making triage decisions when proteins undergo oxidative and toxic damage. Exp Biol Med 235: 278–289.

Selcen D, Muntoni F, Burton BK, Pegoraro E, Sewry C, Bite AV, Engel AG. 2009. Mutation in BAG3 causes severe dominant childhood muscular dystrophy. Ann Neurol 65: 83–89.

Takayama S, Xie Z, Reed JC. 1999. An evolutionarily conserved family of Hsp70/Hsc70 molecular chaperone regulators. J Biol Chem 274: 781–786.

Villard E, Perret C, Gary F, Proust C, Dilanian G, Hengstenberg C, Ruppert V, Arbustini E, Wichter T, Germain M, Dubourg O, Tavazzi L, Aumont MC, Degroote P, Fauchier L, Trochu JN, Gibelin P, Aupetit JF, Stark K, Erdmann J, Hetzer R, Roberts AM, Barton PJ, Regitz-Zagrosek V, Aslam U, Duboscq-Bidot L, Meyborg M, Maisch B, Madeira H, Waldenstrom A, Galve E, Cleland JG, Dorent R, Roizes G, Zeller T, Blankenberg S, Goodall AH, Cook S, Tregouet DA, Tiret L, Isnard R, Komajda M, Charron P, Cambien F. A genome-wide association study identifies two loci associated with heart failure due to dilated cardiomyopathy. Eur Heart J 32: 1065–1076.

Circulation Journal
Official Journal of the Japanese Circulation Society
http://www.j-circ.or.jp

ORIGINAL ARTICLE
Myocardial Disease

Prevalence and Distribution of Sarcomeric Gene Mutations in Japanese Patients With Familial Hypertrophic Cardiomyopathy

Haruna Otsuka, BSc; Takuro Arimura, PhD; Tadaaki Abe, MD; Hiroya Kawai, MD; Yoshiyasu Aizawa, MD; Toru Kubo, MD; Hiroaki Kitaoka, MD; Hiroshi Nakamura, MD; Kazufumi Nakamura, MD; Hiroshi Okamoto, MD; Fukiko Ichida, MD; Mamoru Ayusawa, MD; Shinichi Nunoda, MD; Mitsuaki Isobe, MD; Masunori Matsuzaki, MD; Yoshinori L. Doi, MD; Keiichi Fukuda, MD; Taishi Sasaoka, MD; Toru Izumi, MD; Naoto Ashizawa, MD; Akinori Kimura, MD

Background: Hypertrophic cardiomyopathy (HCM), which is inherited as an autosomal dominant trait, is the most prevalent hereditary cardiac disease. Although there are several reports on the systematic screening of mutations in the disease-causing genes in European and American populations, only limited information is available for Asian populations, including Japanese.

Methods and Results: Genetic screening of disease-associated mutations in 8 genes for sarcomeric proteins, *MYH7*, *MYBPC3*, *MYL2*, *MYL3*, *TNNT2*, *TNNI3*, *TPM1*, and *ACTC*, was performed by direct sequencing in 112 unrelated Japanese proband patients with familial HCM; 37 different mutations, including 13 novel ones in 5 genes, *MYH7*, *MYBPC3*, *TNNT2*, *TNNI3*, and *TPM1*, were identified in 49 (43.8%) patients. Among them, 3 carried compound heterozygous mutations in *MYBPC3* or *TNNT2*. The frequency of patients carrying the *MYBPC3*, *MYH7*, and *TNNT2* mutations were 19.6%, 10.7%, and 8.9%, respectively, and the most frequently affected genes in the northeastern and southwestern parts of Japan were *MYBPC3* and *MYH7*, respectively. Several mutations were found in multiple unrelated proband patients, for which the geographic distribution suggested founder effects of the mutations.

Conclusions: This study demonstrated the frequency and distribution of mutations in a large cohort of familial HCM in Japan. (*Circ J* 2012; **76:** 453–461)

Key Words: Hypertrophic cardiomyopathy; Genes; Genetics

Hypertrophic cardiomyopathy (HCM) is a prevalent hereditary disease, affecting approximately 1 in 500 of the general population, and a major cause of sudden cardiac death (SCD) in the young, which is characterized by left ventricular (LV) hypertrophy accompanied by diastolic dysfunction and myofibrillar disarrays.[1–3] More than half of HCM patients have apparent family histories of the disease and/or SCD, which is consistent with the autosomal dominant genetic trait, suggesting that genetic abnormalities cause HCM.[1] The etiology of HCM, however, was unknown until

Received August 19, 2011; accepted October 11, 2011; released online November 23, 2011 Time for primary review: 28 days
Department of Molecular Pathogenesis, Medical Research Institute, Tokyo Medical and Dental University, Tokyo (H.O., T. Arimura, A.K.); Department of Pediatric Cardiovascular Medicine, National Cerebral and Cardiovascular Center, Suita (T. Abe); Division of Cardiovascular Medicine, Department of Internal Medicine, Kobe University Graduate School of Medicine, Kobe (H. Kawai); Department of Cardiology, Keio University School of Medicine, Tokyo (Y.A., K.F.); Department of Medicine and Geriatrics, Kochi Medical School, Kochi (T.K., H. Kitaoka, Y.L.D.); Department of Community Health and Medicine, Yamaguchi University School of Medicine, Ube (H.N.); Department of Cardiovascular Medicine, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama (K.N.); Department of Cardiovascular Medicine, Hokkaido Medical Center, Sapporo (H.O.); Department of Pediatrics, Toyama University School of Medicine, Toyama (F.I.); Department of Pediatrics and Child Health, Nihon University School of Medicine, Tokyo (M.A.); Cardiovascular Division, Tokyo Women's Medical University Medical Center East, Tokyo (S.N.); Department of Cardiovascular Medicine, Graduate School of Medical and Dental Science, Tokyo Medical and Dental University, Tokyo (M.I.); Department of Cardiovascular Medicine, Yamaguchi University School of Medicine, Ube (M.M.); Department of Cardiovascular Medicine, Kitasato University School of Medicine, Tokyo (T.S., T.I.); and Department of Cardiovascular Medicine, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki (N.A.), Japan
The first two authors contributed equally to this study (H.O., T. Arimura).
Present address: Division of Cardiology, Japanese Red Cross Nagasaki Atomic Bomb Hospital, Nagasaki (N.A.), Japan
Mailing address: Akinori Kimura, MD, PhD, Department of Molecular Pathogenesis, Medical Research Institute, Tokyo Medical and Dental University, 1-5-45 Bunkyo-ku, Tokyo 113-8510, Japan. E-mail: akitis@mri.tmd.ac.jp
ISSN-1346-9843 doi:10.1253/circj.CJ-11-0876

Table 1. Distribution and Frequency of Disease-Associated Mutations in Patients With HCM

Gene	No. of mutations found in this study (novel mutations)	No. of proband patients with mutations in this study (novel mutations)	% Frequency of mutations in familial HCM patients in this study (n=112)	% Frequency of mutations in the French familial HCM cohort* (n=172)	% Frequency of mutations in the US HCM cohort** (n=389)†
MYH7	12 (2)	12 (2)	10.7	26.2	15.2
MYBPC3	13 (7)	22 (7)	19.6	26.2	18.0
MYL3	0 (0)	0 (0)	0.0	0.0	0.0
MYL2	0 (0)	0 (0)	0.0	0.6	1.8
ACTC	0 (0)	0 (0)	0.0	0.0	0.0
TNNT2	7 (1)	10 (1)	8.9	2.9	2.3
TNNI3	1 (1)	1 (1)	0.9	4.7	1.3
TPM1	4 (2)	4 (2)	3.6	0.0	0.5
Total	37 (13)	49 (13)	43.8	60.6	39.4

*Circulation 2003; **107:** 2227–2232. **J Am Coll Cardiol 2004; **44:** 1903–1910. †120 familial cases and 269 sporadic cases.
HCM, hypertrophic cardiomyopathy.

1990 when a mutation in *MYH7* encoding cardiac β-myosin heavy chain was identified in a multiplex family with HCM by a linkage study and subsequent candidate gene analysis.[4] After the discovery of the *MYH7* mutation in HCM, extensive efforts have been made, using linkage studies and candidate gene analyses, to identify the disease-causing mutations, and hundreds of mutations in several different genes have been reported in HCM.[5]

Editorial p303

The sarcomere is the contractile unit of cardiac and skeletal muscles, composed of highly organized proteins represented by thick and thin filaments, which plays a crucial role in force generation.[6] Numerous HCM-associated mutations have been identified in the genes for components of thick filaments such as cardiac β-myosin heavy chain (*MYH7*), cardiac myosin binding protein-C (*MYBPC3*), ventricular essential myosin light chain (*MYL3*), and ventricular regulatory myosin light chain (*MYL2*), as well as in the genes for the components of thin filaments, including cardiac troponin T (*TNNT2*), cardiac troponin I (*TNNI3*), cardiac troponin C (*TNNC1*), α-tropomyosin (*TPM1*), and cardiac actin (*ACTC*).[5] Familial or hereditary HCM caused by mutations in the genes for components of the sarcomere could be defined as "sarcomere HCM", but there are only a few reports on the systematic screening of sarcomeric gene mutations in a large panel of hereditary HCM, mainly in European and American populations.[7–10] Because the frequency and distribution of disease-causing mutations could vary among the different ethnic groups, it is important for genetic counseling to elucidate the HCM-associated mutations in each ethnic group.

In this study, we performed a systematic screening of mutations in 8 genes for the components of the sarcomere in 112 unrelated proband patients with familial HCM. We report here the frequency and distribution of sarcomeric gene mutations in Japanese familial HCM.

Methods

Subjects

A total of 112 genetically unrelated Japanese patients with familial HCM were the subjects in this study. Each patient had at least one other HCM patient or case of SCD among first-degree family relatives. The patients were diagnosed based on medical history, physical examination, 12-lead electrocardiogram, echocardiogram, and other special tests if necessary. The diagnostic criteria for HCM have been described previously.[11] Two hundred healthy Japanese served as control subjects. When a sarcomeric mutation was found in a proband patient, family relatives were contacted through the proband patient about the possibility of a family study. The family members willing to participate visited the relevant hospital to undergo clinical examination, including electrocardiogram and echocardiogram, and genetic testing if they consented. Informed consent was given by each subject and the research protocol conformed to the ethical guidelines of the Declaration of Helsinki as reflected in a priori approval by the Ethics Review Committee of Medical Research Institute, Tokyo Medical and Dental University, Japan.

Genetic Analyses

DNA samples extracted from the peripheral blood of subjects were used as templates to amplify each coding exon of *MYBPC3*, *MYL2*, *MYL3*, *TNNT2*, *TNNI3*, *TPM1* and *ACTC*, and exons 3–25 of *MYH7* by polymerase chain reaction (PCR). Sequences of primers and the PCR conditions used in this study are available upon request. PCR products were analyzed for sequence variations by direct DNA sequencing of both strands using Big Dye Terminator chemistry (version 3.1) and ABI3100 DNA Analyzer (Applied Biosystems, Foster City, CA, USA). The sequence variations found in the patients were considered to be mutations on the basis of following criteria: (1) presence in all tested affected members of the family of each proband patient, (2) absence from 400 unrelated chromosomes of the control subjects, (3) absence from a public database of polymorphism, dbSNP database (http://www.ncbi.nlm.nih.gov/projects/SNP/), (4) mutations at the evolutionary conserved residues, and/or (5) identification as a HCM-causing mutation in previous reports.

Statistical Analysis

Mean values for the parameter of echocardiography are expressed as mean±SD. Differences between the stratified groups were compared with Student's t-test. A P value less than 0.05 was considered to be statistically significant.

Results

Frequency of Sarcomeric Gene Mutations in Familial HCM

To investigate the distribution of disease-causing genes for HCM in Japan, 112 consecutive proband patients of famil-

Table 2. MYBPC3 Mutations Found in This Study

Exon/Intron	Novelty*	Nucleotide change	Amino acid change	No. of patients**	Co-segregation†	Consequence	Reference††		
							USA	Europe	Asia
Int11	Novel	IVS11+1 g>t		1	NT	Splice donor site			
Ex12	Novel	GAA>TAA	Glu386ter	3	Yes	Termination codon			
Ex15		CGG>CAG	Arg502Gln	1	Yes	Missence	17	18	
Int15	Novel	IVS16–2a>g		1	NT	Splice acceptor site			
Ex16		T>del	Ser593fs/8	5	Yes	Framesift/ter			19
Int17	Novel	IVS17+1–3gtg>del		1	NT	Splice donor site			
Ex23		CGG>CAG	Arg820Gln	3	NT	Missence			20
Ex23		CGC>CTT	Arg835Leu	2	NT	Missence		10	
Ex24		TGG>TGA	Trp890ter	1	Yes	Termination codon	8		
Ex25		CG>del	Arg945fs/108	3	NT	Framesift/ter			19
Ex28	Novel	CGG>CCG	Arg1073Pro	1	NT	Missence			
Ex29	Novel	TGC>CGC	Cys1124Arg	1	NT	Missence			
Ex31	Novel	CTG>CAG	Leu1268Gln	1	Yes	Missence			

*Novel mutations; **no. of unrelated proband patients; †consistent with the co-segregation of mutation with HCM in multiplex family; ††references cited.
Int, intron; IVS, intervening sequence; NT, not tested; Ex, exon; del, deletion; ter, termination; HCM, hypertrophic cardiomyopathy.

ial HCM were searched for mutations in 8 sarcomeric genes: *MYH7*, *MYBPC3*, *MYL2*, *MYL3*, *TNNT2*, *TNNI3*, *TPM1*, and *ACTC*. As shown in **Table 1**, we identified a total of 37 different mutations, including 13 novel mutations, in 49 patients. The mutations were most frequently identified in *MYBPC3*, which accounted for 19.6% of cases. The mutations in *MYH7* and *TNNT2* were found in 10.7% and 8.9%, respectively, of cases. On the other hand, mutations in *TNNI3* and *TPM1* were found in a few cases only, and analysis of *MYL2*, *MYL3* and *ACTC* did not reveal any mutations in this patient cohort (**Table 1**).

The analysis of *MYBPC3* led to the identification of 13 different mutations, including 7 mutations not described previously (**Table 2**). Among the new mutations, 3 were within the consensus sequences of splicing donor or acceptor sites, and the other 4 were missense mutations. One novel missense mutation, Leu1268Gln, was consistent with the co-segregation with HCM in a multiplex family (**Figure 1a**). Although the other mutations were not confirmed for co-segregation in each family, they were not found in 200 healthy controls and the splicing site mutations were suggested to result in the splicing abnormalities generating C-terminal truncated proteins. As for the novel missense mutations, they, except for the Arg1073Pro mutation, were found at the evolutionary conserved amino acid residues among various species, similar to the previously reported HCM-causing *MYBPC3* mutations (**Figure 2a**). The Arg1073Pro mutation was also suggested to be associated with HCM, because the 1,073rd residue is the basic amino acid, arginine (Arg) or lysine (Lys), in various species (**Figure 2a**), which was changed to proline (Pro), leading to a gross structural change.

The mutations found in *MYH7*, *TNNT2*, *TPM1* and *TNNI3* are indicated in **Table 3**. The analysis revealed a total of 25 different missense mutations, including 12 *MYH7*, 7 *TNNT2*, 4 *TPM1*, and 1 *TNNI3* mutations. Among them, 2 *MYH7*, 1 *TNNT2*, 2 *TPM1*, and 1 *TNNI3* mutations were identified for the first time in this study. These novel missense mutations were not observed in the healthy controls and occurred at the evolutionary conserved residues (**Figure 2b**). In addition, co-segregation of each novel mutation with HCM was compatible in the analyzed multiplex family of each patient (**Figures 1b–f**), although we could not test the Asp516Glu mutation in *MYH7* because the family study was denied by the proband patient.

Double Mutation Cases

Of the 49 proband patients with sarcomeric gene mutations, 46 cases carried single heterozygous mutations, while the other 3 cases carried double mutations in *MYBPC3* or *TNNT2*. Two cases were double *MYBPC3* mutations; 1 carried IVS11+1 g>t and Arg835Leu mutations, while the other had Ser593fs/8 and Cys1124Arg mutations. A female patient, who carried IVS11+1 g>t and Arg835Leu mutations, was diagnosed as HCM with ventricular tachycardia at 43 years old, and developed dilated-phase HCM at 61 years old. The other patient, who carried Ser593fs/8 and Cys1124Arg mutations, was affected with HCM of early onset with ventricular fibrillation at 17 years old. Because additional family studies for these mutations were denied by the proband patients, it was not clear whether these mutations were *cis* or *trans*. In addition, because of the lack of family studies, it was unclear whether the presence of missense mutations in these cases would worsen the clinical course, as reported previously for other double mutations.[8]

On the other hand, in another case of *TNNT2* double mutation, the Phe110Ile mutation was inherited from the patient's father and the Pro80Ser mutation from her mother (**Figure 1d**). It should be noted that her father did not manifest clinical findings of HCM, whereas her mother was diagnosed as HCM. Because the Phe110Ile mutation is reported to be a HCM-causing mutation with low penetrance,[12] her father might not develop overt HCM. The clinical manifestation of HCM was more severe in the proband patient than in her mother who possessed a single Pro80Ser mutation, because the proband patient manifested with HCM of early onset at 10 years old, whereas her mother had developed HCM in adulthood. These observations suggested that the relatively benign Phe110Ile mutation might affect the clinical expressivity of the Pro80Ser mutation in double mutation cases.

Geographic Distribution of HCM-Associated Mutations

Japan is an East Asian nation consisting of several islands. There are 4 major islands, from northeast to southwest, the Hokkaido, Honshu, Shikoku, and Kyushu Islands. Among these, Honshu Island is the largest and historically divided into 2 areas, the Honshu-Kanto area and the Honshu-Kansai area. The former includes the Tohoku area and part of the Chubu area, while the latter includes the rest of the Chubu area and

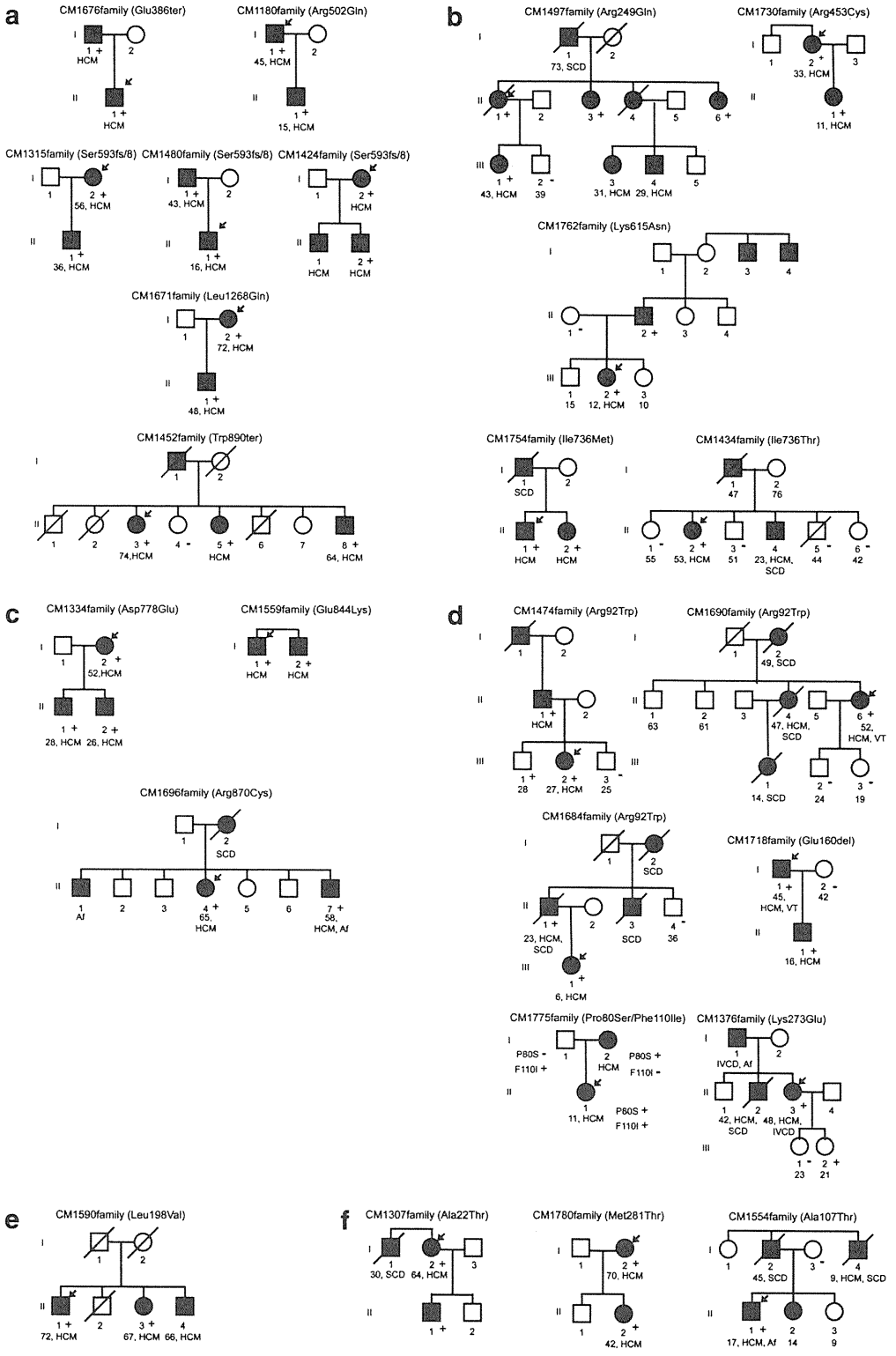

Figure 1. Pedigrees of the HCM families carrying the sarcomeric gene mutations: (**a**) *MYBPC3*, (**b**,**c**) *MYH7*, (**d**) *TNNT2*, (**e**) *TNNI3*, and (**f**) *TPM1*. Only the families of which members other than the proband patients were genotyped for the mutation, are shown. Filled red square and filled red circle indicate affected male and female, respectively. Open square and open circle represent unaffected male and female, respectively. Shadowed square represent affected male with heart disease without detailed information. Red arrows indicate the proband patients. Presence (+) or absence (–) of the mutations is noted for the analyzed individuals. HCM, hypertrophic cardiomyopathy.

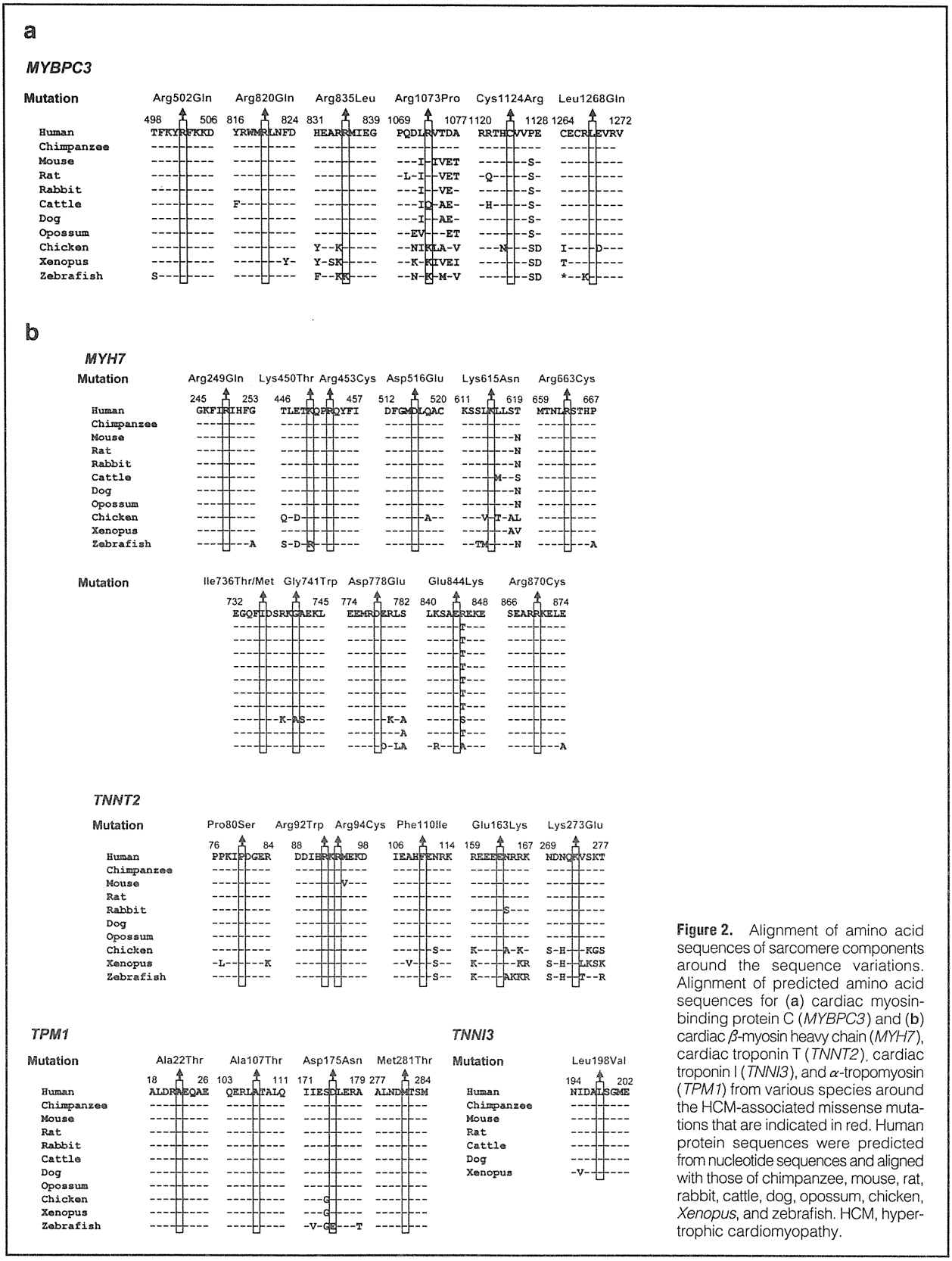

Figure 2. Alignment of amino acid sequences of sarcomere components around the sequence variations. Alignment of predicted amino acid sequences for (**a**) cardiac myosin-binding protein C (*MYBPC3*) and (**b**) cardiac β-myosin heavy chain (*MYH7*), cardiac troponin T (*TNNT2*), cardiac troponin I (*TNNI3*), and α-tropomyosin (*TPM1*) from various species around the HCM-associated missense mutations that are indicated in red. Human protein sequences were predicted from nucleotide sequences and aligned with those of chimpanzee, mouse, rat, rabbit, cattle, dog, opossum, chicken, *Xenopus*, and zebrafish. HCM, hypertrophic cardiomyopathy.

Table 3. *MYH7, TNNT2, TNNI3* and *TPM1* Mutations Found in This Study

Disease-causing gene [Exon/Intron]	Novelty*	Nucleotide change	Amino acid change	No. of patients**	Co-segregation†	Reference††		
						USA	Europe	Asia
MYH7								
Ex9		CGA>CAA	Arg249Gln	1	Yes	21	7	
Ex14		AAG>ACG	Lys450Thr	1	NT			22
Ex14		CGC>TGC	Arg453Cys	1	Yes	21	7	
Ex15	Novel	GAC>GAA	Asp516Glu	1	NT			
Ex16		AAG>AAC	Lys615Asn	1	Yes			22
Ex18		CGC>TGC	Arg663Cys	1	NT	8		19
Ex20		ATT>ACT	Ile736Thr	1	NT		23	
Ex20		ATT>ATG	Ile736Met	1	Yes			24
Ex20		GGG>TGG	Gly741Trp	1	NT		23	25
Ex21		GAC>GAA	Asp778Glu	1	Yes		7	
Ex22	Novel	GAA>AAA	Glu844Lys	1	Yes			
Ex22		CGC>TGC	Arg870Cys	1	Yes			26
TNNT2								
Ex6	Novel	CCC>TCC	Pro80Ser	1	Yes			
Ex8		CGG>TGG	Arg92Trp	3	Yes	27	28	29
Ex8		CGC>TGC	Arg94Cys	1	NT		30	
Ex8		TTT>ATT	Phe110Ile	3	NT	31		29
Ex11		GAG>del	Glu160del	1	Yes	31	7	
Ex11		GAG>AAG	Glu163Lys	1	NT	31		
Ex15		AAA>GAA	Lys273Glu	1	NT			29
TPM1								
Ex1	Novel	GCT>ACT	Ala22Thr	1	Yes			
Ex3	Novel	GCA>ACA	Ala107Thr	1	NT			
Ex5		GAC>AAC	Asp175Asn	1	NT	31	28	32
Ex9		ATG>ACG	Met281Thr	1	Yes	27		
TNNI3								
Ex8	Novel	CTG>GTG	Leu198Val	1	Yes			

Footnotes as in Table 2.

Table 4. Geographic Distribution of Sarcomeric Gene Mutations

Disease-causing gene	Hokkaido Island	Honshu Island		Shikoku Island	Kyushu Island
		Kanto	Kansai		
MYH7	1	1	4	1	5
MYBPC3	4	11	6	1	0
TNNT2	0	5	3	0	2
TNNI3	0	0	0	0	1
TPM1	0	3	0	1	0
NI	3	21	30	2	7
Total	8	41	43	5	15

NI, mutation was not identified in the analyzed sarcomeric genes.

the Chugoku area. Because the migration of people was less frequent and forbidden during the Edo period from the 17th to 19th centuries, especially across the middle of the Chubu area, the HCM-associated mutations might be differently distributed around Japan. We therefore investigated the geographic distribution of the HCM-associated mutations.

As shown in **Table 4**, the most frequent disease-causing gene in the northeastern area (Hokkaido Island and the Honshu-Kanto area including the Tohoku area) was *MYBPC3*, mutations of which were identified in 15 of 49 (30.6%) proband patients, whereas the *MYH7* mutations were identified in only 2 of 49 (4.1%) cases. In clear contrast, the most frequent disease-causing gene in the southwestern area (Shikoku and Kyushu Islands and the Honshu-Kansai area including the Chugoku area) was *MYH7*, mutations of which were detected in 10 of 63 (15.9%) cases, while the *MYBPC3* mutations were found in 7 of 63 (9.5%) cases. The other mutations in *TNNT2*, *TPM1*, and *TNNI3* were relatively infrequent and the frequencies were not largely different between the northeastern and southwestern parts.

We also investigated the geographic distributions of specific mutations, which were observed in several unrelated proband patients analyzed in this study and in our previous study.[13] As shown in **Figure 3**, *TNNT2* Glu163Lys mutation was identified in 5 patients (4 and 1 in the previous and present studies, respectively), who all were residents of Kyushu Island. The *TNNT2* Arg92Trp mutation was found in 9 patients (6 and 3 in the previous and present studies, respectively) and was preferentially identified in the southwestern region in 5 and 3 cases from Kyushu Island and the Kansai area, respectively. In addition, *MYBPC3* Ser593fs/8 mutation was observed in 12 patients (7 and 5 in the previous and present studies, respectively), and was identified in 7 and 3 cases from the Shikoku and Hokkaido Islands, respectively.

Clinical Phenotypes of HCM-Associated Mutations

Genotyping of proband patients and available family members of them allowed us to evaluate the echocardiographic param-

eters of mutation-prone patients grouped by the disease-causing genes, *MYH7*, *MYBPC3*, *TNNT2*, and *TPM1*, as compared with the patients who did not carry any sarcomeric gene mutations (**Table 5**). The ratios of intraventricular septum thickness (IVST) to posterior wall thickness (PWT) were significantly higher in the patients with *MYBPC3*, *MYH7*, or *TPM1* mutations than in the patients without sarcomeric gene mutations. Interestingly, a significantly higher ratio of IVST/PWT was associated with a significantly higher or lower value for IVST in patients with *MYBPC3* mutations or PWT in patients with *MYH7* or *TPM1* mutations, respectively.

Discussion

To ascertain the frequency and distribution of HCM-associated mutations in Japan, we analyzed 8 sarcomeric genes in 112 unrelated proband patients with familial HCM. Mutations were identified in 49 cases (43.8%) as shown in **Table 1**. Prevalence of sarcomeric gene mutations in this Japanese cohort with familial HCM was lower than the reported prevalence in the French cohort with familial HCM[7] and that in the US cohort with familial HCM,[8] in which 60.6% and 54.2%, respectively, of the analyzed patients were found to carry sarcomeric gene mutations, while 10.8% of the US cohort with sporadic HCM had mutations.[8] We have previously reported that 76 (46.9%) and 14 (14.0%) patients, respectively, carried sarcomeric gene mutations in another set of Japanese HCM cohort composed of 162 familial cases and 100 sporadic cases.[13] Because we had screened for gene mutations using single-strand conformation polymorphism method in the previous study,[13] we might have missed several mutations and hence the prevalence of sarcomeric gene mutations might have been underestimated previously. However, we searched for mutations by direct sequencing of PCR products in this study, suggesting that the prevalence of sarcomeric gene mutations in familial HCM is relatively low in Japanese and less than half of cases, whereas in European and American populations it is over 50%. On the other hand, the prevalence of sarcomeric gene mutations in sporadic HCM appeared to be comparable between Japanese[13] and US[8] cohorts.

Study Limitations

First, we did not analyze the patients for mutations in the rod region of cardiac β-myosin heavy chain. In a database of sarcomeric gene mutations in HCM (http://genepath.med.harvard.edu/seidman//cg3/), a total of 194 *MYH7* mutations are registered and among them 21 (10.8%) are found in the rod region corresponding to exons 26–40 of *MYH7*, which we did not investigate in this study. Therefore, the possibility remains that we did not detect several disease-associated *MYH7* mutations. However, such mutations might be present in a few cases, because approximately 90% of *MYH7* mutations are found in exons 3–25 in the mutation database, albeit the previous genetic analyses were mainly focused on the head and neck regions of cardiac β-myosin heavy chain as were we in this study. Second, although the mutations identified in this study were not found in 200 healthy controls, we can not exclude the possibility that they are rare polymorphisms. Further studies are required to demonstrate the effect of these mutations in causing HCM, using both in vitro and in vivo functional analyses that include mutations such as nonsense mutations, splicing mutations or frameshift mutations, which are expected to be deleterious.

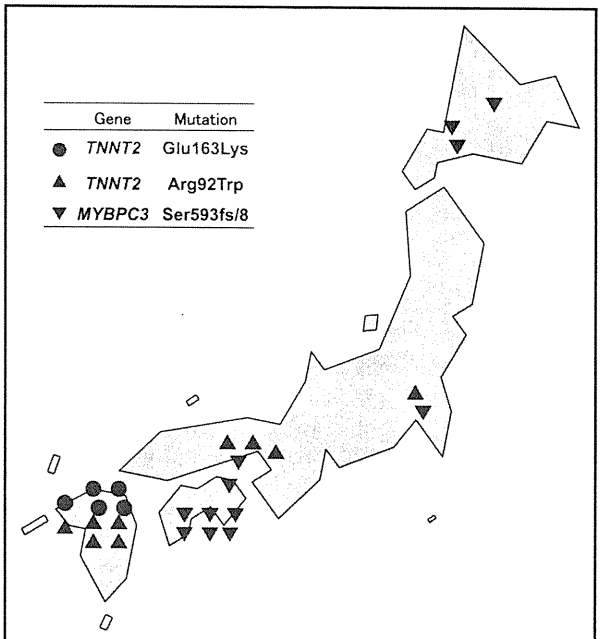

Figure 3. Distribution of same sarcomeric gene mutations found in unrelated patients. Regional distribution of unrelated patients carrying the TNNT2-Glu163Lys, TNNT2-Arg92Trp, or MYBPC3-Ser593fs/8 mutations are schematically shown on the map of Japan.

Conclusions

When we focused on the geographic distribution of mutations,

Table 5. Phenotypes of HCM Patients Carrying Mutations in *MYBPC3*, *MYH7*, *TNNT2* and *TPM1*

Gene	No. of patients	Age at diagnosis (years)	% of men	LVEDD (mm)	LVESD (mm)	IVST (mm)	PWT (mm)	IVS/PW	FS (%)
MYBPC3	14	39±21	64	42.7±5.7	26.7±5.8	21.0±6.3*	11.5±2.8	1.9±0.7*	37.3±8.0
MYH7	7	38±17	57	43.5±5.4	27.5±5.9	16.5±7.0	9.3±1.8*	1.9±1.1*	39.0±9.3
TNNT2	7	34±22	57	45.0±7.9	28.7±9.6	17.7±2.1	11.9±3.4	1.6±0.4	36.8±10.7
TPM1	3	46±19	33	49.0±3.5	31.3±4.2	16.3±4.9	8.3±1.5*	2.2±0.4*	33.3±6.1
NI	30	47±18	60	46.7±5.8	29.0±7.1	14.7±4.8	11.4±2.3	1.3±0.4	38.9±8.3

*P<0.05 vs. mutation not identified group.
HCM, hypertrophic cardiomyopathy; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; IVST, intraventricular septal thickness; FS, fractional shortening; PWT, posterior wall thickness; NI, mutation was not identified in the analyzed sarcomeric genes.

a difference between the northeastern and southwestern regions of Japan was observed, even though we analyzed only patients who resided on the 4 main islands where the population could be considered as a relatively homogeneous ethnic group. It was also demonstrated that relatively common mutations were preferentially found in specific areas, which suggested a founder effect. On the other hand, it was unclear whether the *MYBPC3* Ser593fs/8 mutation in the Hokkaido population originated from the Shikoku population, but most of the present Hokkaido residents are descendants of immigrants from various parts of Japan, including Shikoku Island, after the Edo period. However, we could not exclude the possibility that the *MYBPC3* Ser593fs/8 arose independently in various areas, because more than half of the known mutations found in this study were previously reported in European and/or American populations (**Tables 2,3**), implying that some of the mutations were generated independently in different races or that the origin of mutations might predate the diversification of Caucasoid and Mongoloid populations. These observations suggest that the distribution of disease-causing genes might be influenced by immigration of ancestral mutation carriers. This finding in turn provides clinicians and geneticists with information about possible regional variations in the HCM phenotypes.

There were a few cases of 2 different mutations in the same disease-causing genes. Among them, a girl carrying the Pro80Ser and Phe110Ile mutations of *TNNT2* manifested both HCM and LV non-compaction of early onset. The Phe110Ile mutation, which was identified in her father who was not affected with HCM (**Figure 1d**), has been reported as a HCM mutation associated with low penetrance and favorable prognosis.[12] In contrast, the Pro80Ser mutation, which was identified in her mother who was affected with adult-onset HCM, was identified as a novel HCM-causing mutation in this study, because the Pro80 residue is highly conserved among various species from zebrafish to humans (**Figure 2b**) and this mutation was not found in the 200 healthy control subjects. Previous studies suggested that multiple mutations such as homozygous or compound heterozygous mutations in *MYBPC3* and/or *MYH7* result in more severe clinical phenotypes because of a "double dose" effect.[7,8,14] Although it was reported that homozygous *TNNT2* mutations caused more severe phenotypes than heterozygous mutations,[15,16] a compound heterozygous mutation in *TNNT2* has not been reported previously. Further continuous follow-up of the *TNNT2* double mutation patient is required to reveal the clinical phenotypes caused by the compound heterozygous mutations in *TNNT2*.

In conclusion, we report the results of a systematic screening for mutations in 8 sarcomeric genes in Japanese patients with familial HCM. We demonstrate the prevalence and geographic distribution of the sarcomeric gene mutations in Japan.

Acknowledgments

We are grateful to the patients and their family members for participating in this study. We also thank Drs Michiaki Hiroe, Yoshinori Koga, Fumio Terasaki, Junji Toyama, Hisako Matsuo, Shinichiro Okawa, and other clinicians for their contribution to the blood sampling and clinical examinations of the patients and family relatives. This work was supported in part by Grant-in-Aids from the Ministry of Education, Culture, Sports, Science and Technology, Japan; research grants from the Ministry of Health, Labour and Welfare, Japan and Association Française Contre les Myopathies; grants for the Basic Scientific Cooperation Program between Japan and Korea from the Japan Society for the Promotion of Science and National Research Foundation of Korea. This work was also supported by follow-up grants provided by the Tokyo Medical and Dental University.

Disclosures

Conflict of interest: none declared.

References

1. Maron BJ, Towbin JA, Thiene G, Antzelevitch C, Corrado D, Arnett D, et al. Contemporary definitions and classification of the cardiomyopathies: An American Heart Association Scientific Statement from the Council on Clinical Cardiology, Heart Failure and Transplantation Committee; Quality of Care and Outcomes Research and Functional Genomics and Translational Biology Interdisciplinary Working Groups; and Council on Epidemiology and Prevention. *Circulation* 2006; **113:** 1807–1816.
2. Richardson P, McKenna W, Bristow M, Maisch B, Mautner B, O'Connell J, et al. Report of the 1995 World Health Organization/International Society and Federation of Cardiology Task Force on the Definition and Classification of cardiomyopathies. *Circulation* 1996; **93:** 841–842.
3. Maron BJ. Risk stratification and role of implantable defibrillators for prevention of sudden death in patients with hypertrophic cardiomyopathy. *Circ J* 2010; **74:** 2271–2282.
4. Geisterfer-Lowrance AA, Kass S, Tanigawa G, Vosberg HP, McKenna W, Seidman CE, et al. A molecular basis for familial hypertrophic cardiomyopathy: A beta cardiac myosin heavy chain gene missense mutation. *Cell* 1990; **62:** 999–1006.
5. Kimura A. Molecular basis of hereditary cardiomyopathy: Abnormalities in calcium sensitivity, stretch response, stress response and beyond. *J Hum Genet* 2010; **55:** 81–90.
6. Frank D, Kuhn C, Katus HA, Frey N. The sarcomeric Z-disc: A nodal point in signalling and disease. *J Mol Med* 2006; **84:** 446–468.
7. Richard P, Charron P, Carrier L, Ledeuil C, Cheav T, Pichereau C, et al. Hypertrophic cardiomyopathy: Distribution of disease genes, spectrum of mutations, and implications for a molecular diagnosis strategy. *Circulation* 2003; **107:** 2227–2232.
8. Van Driest SL, Vasile VC, Ommen SR, Will ML, Tajik AJ, Gersh BJ, et al. Myosin binding protein C mutations and compound heterozygosity in hypertrophic cardiomyopathy. *J Am Coll Cardiol* 2004; **44:** 1903–1910.
9. Garcia-Castro M, Coto E, Reguero JR, Berrazueta JR, Alvarez V, Alonso B, et al. Mutations in sarcomeric genes MYH7, MYBPC3, TNNT2, TNNI3, and TPM1 in patients with hypertrophic cardiomyopathy. *Rev Esp Cardiol* 2009; **62:** 48–56 (in Spanish).
10. Millat G, Bouvagnet P, Chevalier P, Dauphin C, Jouk PS, Da Costa A, et al. Prevalence and spectrum of mutations in a cohort of 192 unrelated patients with hypertrophic cardiomyopathy. *Eur J Med Genet* 2010; **53:** 261–267.
11. Hayashi T, Arimura T, Itoh-Satoh M, Ueda K, Hohda S, Inagaki N, et al. Tcap gene mutations in hypertrophic cardiomyopathy and dilated cardiomyopathy. *J Am Coll Cardiol* 2004; **44:** 2192–2201.
12. Anan R, Shono H, Kisanuki A, Arima S, Nakao S, Tanaka H. Patients with familial hypertrophic cardiomyopathy caused by a Phe110Ile missense mutation in the cardiac troponin T gene have variable cardiac morphologies and a favorable prognosis. *Circulation* 1998; **98:** 391–397.
13. Kimura A, Ito-Satoh M, Hayashi T, Takahashi M, Arimura T. Molecular etiology of idiopathic cardiomyopathy in Asian populations. *J Cardiol* 2001; **37**(Suppl 1): 139–146.
14. Ingles J, Doolan A, Chiu C, Seidman J, Seidman C, Semsarian C. Compound and double mutations in patients with hypertrophic cardiomyopathy: Implications for genetic testing and counselling. *J Med Genet* 2005; **42:** e59.
15. Ho CY, Lever HM, DeSanctis R, Farver CF, Seidman JG, Seidman CE. Homozygous mutation in cardiac troponin T: Implications for hypertrophic cardiomyopathy. *Circulation* 2000; **102:** 1950–1955.
16. Lin T, Ichihara S, Yamada Y, Nagasaka T, Ishihara H, Nakashima N, et al. Phenotypic variation of familial hypertrophic cardiomyopathy caused by the Phe(110)→Ile mutation in cardiac troponin T. *Cardiology* 2000; **93:** 155–162.
17. Niimura H, Bachinski LL, Sangwatanaroj S, Watkins H, Chudley AE, McKenna W, et al. Mutations in the gene for cardiac myosin-binding protein C and late-onset familial hypertrophic cardiomyopathy. *N Engl J Med* 1998; **338:** 1248–1257.
18. Cardim N, Perrot A, Santos S, Morgado P, Padua M, Ferreira S, et al. Hypertrophic cardiomyopathy in a Portuguese population: Mutations in the myosin-binding protein C gene. *Rev Port Cardiol* 2005; **24:** 1463–1476.
19. Kubo T, Kitaoka H, Okawa M, Baba Y, Hirota T, Hayato K, et al. Genetic screening and double mutation in Japanese patients with hypertrophic cardiomyopathy. *Circ J* 2011; **75:** 2654–2659.
20. Konno T, Shimizu M, Ino H, Matsuyama T, Yamaguchi M, Terai H, et al. A novel missense mutation in the myosin binding protein-C gene is responsible for hypertrophic cardiomyopathy with left ventricular dysfunction and dilation in elderly patients. *J Am Coll Cardiol* 2003;

41: 781–786.
21. Watkins H, Rosenzweig A, Hwang DS, Levi T, McKenna W, Seidman CE, et al. Characteristics and prognostic implications of myosin missense mutations in familial hypertrophic cardiomyopathy. *N Engl J Med* 1992; **326:** 1108–1114.
22. Song L, Zou Y, Wang J, Wang Z, Zhen Y, Lou K, et al. Mutations profile in Chinese patients with hypertrophic cardiomyopathy. *Clin Chim Acta* 2005; **351:** 209–216.
23. Perrot A, Schmidt-Traub H, Hoffmann B, Prager M, Bit-Avragim N, Rudenko RI, et al. Prevalence of cardiac beta-myosin heavy chain gene mutations in patients with hypertrophic cardiomyopathy. *J Mol Med* 2005; **83:** 468–477.
24. Muraishi A, Kai H, Adachi K, Nishi H, Imaizumi T. Malalignment of the sarcomeric filaments in hypertrophic cardiomyopathy with cardiac myosin heavy chain gene mutation. *Heart* 1999; **82:** 625–629.
25. Arai S, Matsuoka R, Hirayama K, Sakurai H, Tamura M, Ozawa T, et al. Missense mutation of the beta-cardiac myosin heavy-chain gene in hypertrophic cardiomyopathy. *Am J Med Genet* 1995; **58:** 267–276.
26. Anan R, Shono H, Tei C. Novel cardiac beta-myosin heavy chain gene missense mutations (R869C and R870C) that cause familial hypertrophic cardiomyopathy. *Hum Mutat* 2000; **15:** 584.
27. Van Driest SL, Ellsworth EG, Ommen SR, Tajik AJ, Gersh BJ, Ackerman MJ. Prevalence and spectrum of thin filament mutations in an outpatient referral population with hypertrophic cardiomyopathy. *Circulation* 2003; **108:** 445–451.
28. Waldmuller S, Freund P, Mauch S, Toder R, Vosberg HP. Low-density DNA microarrays are versatile tools to screen for known mutations in hypertrophic cardiomyopathy. *Hum Mutat* 2002; **19:** 560–569.
29. Konno T, Shimizu M, Ino H, Fujino N, Hayashi K, Uchiyama K, et al. Phenotypic differences between electrocardiographic and echocardiographic determination of hypertrophic cardiomyopathy in genetically affected subjects. *J Intern Med* 2005; **258:** 216–224.
30. Mogensen J, Bahl A, Kubo T, Elanko N, Taylor R, McKenna WJ. Comparison of fluorescent SSCP and denaturing HPLC analysis with direct sequencing for mutation screening in hypertrophic cardiomyopathy. *J Med Genet* 2003; **40:** e59.
31. Watkins H, McKenna WJ, Thierfelder L, Suk HJ, Anan R, O'Donoghue A, et al. Mutations in the genes for cardiac troponin T and alpha-tropomyosin in hypertrophic cardiomyopathy. *N Engl J Med* 1995; **332:** 1058–1064.
32. Nakajima-Taniguchi C, Matsui H, Nagata S, Kishimoto T, Yamauchi-Takihara K. Novel missense mutation in alpha-tropomyosin gene found in Japanese patients with hypertrophic cardiomyopathy. *J Mol Cell Cardiol* 1995; **27:** 2053–2058.

A 13-YEAR-OLD GIRL WITH PROXIMAL WEAKNESS AND HYPERTROPHIC CARDIOMYOPATHY WITH DANON DISEASE

HUNMIN KIM, MD,[1] ANNA CHO, MD,[1] BYUNG CHAN LIM, MD,[1] MIN JUNG KIM, BS,[1] KI JOONG KIM, MD, PhD,[1] ICHIZO NISHINO, MD, PhD,[2] YONG SEUNG HWANG, MD, PhD,[1] and JONG-HEE CHAE, MD, PhD[1]

[1] Department of Pediatrics, Seoul National University College of Medicine, Seoul, Korea, 110-744

[2] Department of Neuromuscular Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, 4-1-1 Ogawahigashi-cho, Kodaira, Tokyo 187-8502, Japan

Accepted 3 November 2009

ABSTRACT: Danon disease is caused by deficiency of lysosome-associated membrane protein-2 (LAMP-2). It is characterized clinically by cardiomyopathy, myopathy, and mental retardation in boys. Herein we report a 13-year-old female patient with Danon disease who presented with early-onset skeletal myopathy and cardiomyopathy. She had a de novo novel mutation in the *LAMP2* gene, and her muscles showed many autophagic vacuoles with sarcolemmal features and complete absence of LAMP-2 expression. To the best of our knowledge, this girl is one of the earliest-onset manifesting carriers of Danon disease with typical muscle pathology.

Muscle Nerve **41**: 879–882, 2010

Danon disease is a rare X-linked disorder caused by the primary deficiency of lysosome-associated membrane protein-2 (LAMP-2).[1] LAMP-2 deficiency causes accumulation of autophagic vacuoles in a variety of tissues, including skeletal and cardiac muscles.[2] Although the exact pathophysiology of Danon disease is still unknown, accumulation of autophagic vacuoles is a phenomenon that actually contributes to the pathophysiology and/or progression of the disease.[3] Characteristic clinical features of Danon disease include skeletal myopathy, cardiomyopathy, and variable mental retardation. Male patients usually manifest the disease in their teens and die before their 30s from fatal cardiac problems. However, female patients usually present with late-onset dilated cardiomyopathy in their late 20s or later and survive to late adulthood.[4] The extent of organ involvement and degree of clinical severity between patients varies.

We report a 13-year-old female patient with Danon disease who presented with teenage onset, proximal weakness with characteristic muscle pathology, and hypertrophic cardiomyopathy. She had a de novo novel mutation, c.241delG (p.D81MfsX7) in the *LAMP2* gene. To our knowledge, this girl is the earliest-onset female patient with Danon disease with typical muscle pathology.

CASE REPORT

A 13-year-old girl was referred to the pediatric neurology clinic due to an unexplained elevation of serum creatinine kinase (CK). She was born uneventfully at full term. She had normal growth and developmental milestones. When she was 12 years old, she was evaluated for an incidentally detected high level of liver enzymes [alanine transaminase (ALT) and aspartate transaminase (AST)] and total bilirubin. Serologic tests and other laboratory tests for hepatitis were all negative. A liver biopsy was performed because the laboratory abnormalities persisted. It revealed features of chronic hepatitis with mild lymphocytic infiltration in the portal area, which was consistent with autoimmune hepatitis. She received immunosuppressive therapy but there was no improvement. She was referred to the gastroenterology and hepatology clinic in our hospital. CK elevation was detected, and immunosuppressive therapy was discontinued.

Clinical examination revealed no exertional dyspnea, cardiac murmur, or hepatomegaly. Neurologic examination revealed no muscle atrophy, hypotonia, or abnormal muscle tone. She had proximal weakness and a Gower sign. Laboratory abnormalities included: CK 892 mg/dl (reference: 20–270 IU/L); AST 239 IU/L (reference: 0–40 IU/L); ALT 258 IU/L (reference: 0–40 IU/L); and total bilirubin 2.1 mg/dl (reference: 0–1.2 mg/dl). The electromyogram showed small-amplitude, short-duration, polyphasic motor unit potentials, which were consistent with active myopathy. Echocardiography revealed concentric left ventricular hypertrophy (interventricular septum 17 mm, left ventricle frontal wall 19.9 mm) and mild diastolic dysfunction. Wolff–Parkinson–White syndrome was diagnosed by electrocardiography at 14 years of age. Her family members were all healthy without any laboratory abnormalities.

Muscle Biopsy. A muscle biopsy was taken from her left quadriceps femoris muscle, which revealed intracytoplasmic basophilic granules in 15–20% of the muscle fibers on hematoxylin–eosin staining (Fig. 1A). These basophilic granular structures were stained by nonspecific esterase (Fig. 1B), acetylcholinesterase (Fig. 1C), and dystrophin

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; AVSF, autophagic vacuoles with unique sarcolemmal features; CK, creatinine kinase; LAMP-2, lysosome-membrane associated protein-2

Key words: autophagic vacuole; cardiomyopathy; Danon disease; female; LAMP-2

Correspondence to: J.-H. Chae; e-mail: chaeped1@snu.ac.kr

Published online 16 April 2010 in Wiley InterScience (www.interscience.wiley.com). DOI 10.1002/mus.21614