Table 3 Annual changes in parameters

	%VC						%FVC						FEV (L)						PEF (L)					
Years	1	2	1 + 2	*P*: 1 vs 2	*P*: 1 vs 1 + 2	*P*: 2 vs 1 + 2	1	2	1 + 2	*P*: 1 vs 2	*P* 1 vs 1 + 2	*P* 2 vs 1 + 2	1	2	1 + 2	*P*: 1 vs 2	*P*: 1 vs 1 + 2	*P*: 2 vs 1 + 2	1	2	1 + 2	*P*: 1 vs 2	*P*: 1 vs 1 + 2	*P*: 2 vs 1 + 2
Patient 1	5.8	−1.1	4.7				21.9	−19.1	2.8				0.6	−0.4	0.21				0.55	−0.43	0.1			
Patient 2	16.4	5.2	21.6				4.9	14.9	19.8				0.3	0.2	0.47				2.68	−0.91	1.8			
Patient 3	3.3	1.9	5.2	0.043	0.773	0.057	3.2	3.6	6.8	0.248	1.000	0.248	0.3	−0.1	0.17	0.200	0.306	0.043	0.17	0.07	0.24	0.020	0.468	0.430
Patient 4[a]	Not tested						Not tested						Not tested						Not tested					
Patient 5	5.4	2.9	8.3				7.4	2.7	10.1				0.21	0.05	0.26				0.39	0.07	0.46			

PCF (L)							Grip power (kg)						Pinch power (N)						Occlusal force (kgf)					
1	2	1 + 2	*P*: 1 vs 2	*P*: 1 vs 1 + 2	*P*: 2 vs 1 + 2		1	2	1 + 2	*P*: 1 vs 2	*P*: 1 vs 1 + 2	*P*: 2 vs 1 + 2	1	2	1 + 2	*P*: 1 vs 2	*P*: 1 vs 1 + 2	*P*: 2 vs 1 + 2	1	2	1 + 2	*P*: 1 vs 2	*P*: 1 vs 1 + 2	*P*: 2 vs 1 + 2
0.4	−0.05	0.35					0.7	0.3	1.0				6.4	−5.6	0.8				8.6	0.9	9.5			
2.39	−0.1	2.29					3.1	1.4	4.5				14.2	2.7	16.9				50	5.2	55.2			
0.22	0.03	0.25	0.028	0.885	0.020		3.2	−0.9	2.3	0.083	0.905	0.142	18.8	0.1	18.9	0.009	0.69	0.016	18.7	3.5	22.2	0.021	0.886	0.021
Not tested							Not tested		0.7				8	−3.3	4.7				Not tested					
0.77	0.21	0.98					6.4	1.1	7.5				4.2	−1.2	3				65.8	−1.8	64			

%VC Percent vital capacity, *%FVC* percent force vital capacity, *FEV1.0* forced expiratory volume in the first second, *PEF* peak expiratory flow, *PCF* peak cough flow

[a] Patient 4 could not be evaluated at 1 year after ERT initiation due to severe pneumothorax

grip, and pinch powers were relatively spared in all patients, except patient 1. Four of five patients could write, use utensils, fasten a button, or bite foods as efficiently as healthy people, although their data revealed some decrements compared to normal controls. Cranial muscle involvement is thought to be rare, but we found that occlusal force was mildly reduced in patients with advanced Pompe disease. This suggests that occlusal force is a sensitive parameter for assessing the response to ERT.

Conclusions

The present study showed that ERT improved respiratory function and muscle power for 2 years even in adult patients with advanced GSDII. Improved muscle strength resulted in better ADL and quality of life during the long follow-up period. Taking our results into consideration, we recommend the initiation of ERT in GSDII patients, irrespective of age and disease severity.

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Case report

Acid phosphatase-positive globular inclusions is a good diagnostic marker for two patients with adult-onset Pompe disease lacking disease specific pathology

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Abstract

Diagnosis of adult-onset Pompe disease is sometimes challenging because of its clinical similarities to muscular dystrophy and the paucity of disease-specific vacuolated fibers in the skeletal muscle pathology. We describe two patients with adult-onset Pompe disease whose muscle pathology showed no typical vacuolated fibers but did show unique globular inclusions with acid phosphatase activity. The acid phosphatase-positive globular inclusions may be a useful diagnostic marker for adult-onset Pompe disease even when typical vacuolated fibers are absent.

Keywords: Pompe disease; *GAA*; Globular inclusion; Acid phosphatase

Introduction

Pompe disease (glycogen storage disease type 2; acid maltase deficiency; OMIM #232300) is an autosomal recessive disease caused by mutations in the gene encoding acid α-glucosidase (GAA, OMIM #606800), a lysosomal enzyme involved in glycogen degradation [1]. Based on age of onset and clinical severity, which depends on residual GAA activity, the disease can be classified into infantile, childhood-onset, and adult-onset forms.

Most of the infantile and childhood-onset forms exhibit disease-specific skeletal muscle pathology, which shows fibers occupied by huge vacuoles that contain basophilic amorphous materials. However, diagnosis of the adult-onset form is sometimes challenging due to clinical similarities to muscular dystrophy and the paucity of typical vacuolated myofibers. We diagnosed 37 patients with Pompe disease including 11 infantile, 16 childhood-onset, and 10 adult-onset forms in the muscle repository of the National Center of Neurology and Psychiatry (NCNP), Japan, based on a deficiency of GAA enzyme activity assayed using biopsied muscles, as previously described [2]. Among these 37 patients, two unrelated Japanese patients did not have disease-specific vacuolated muscle fibers but did have unique cytoplasmic inclusions. Here, we report the diagnostic utility of acid phosphatase (ACP)-positive globular inclusions for adult-onset Pompe disease.

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Case report

Clinical summary

Patient 1: A 44-year-old man had been well until the age of 41 years when he started having difficulty in running. He was admitted to the hospital because of progressive muscle weakness. His parents were first cousins, but there was no family history of neuromuscular disorders. He was clinically suspected to suffer from muscular dystrophy because of slowly progressive muscle weakness and elevated creatine kinase levels of around 800 IU/L (normal, <171 IU/L). On examination, he had grade 4-muscle weakness on medical research council (MRC) scale and marked atrophy in his thighs. He did not have apparent respiratory impairment. Electromyography (EMG) showed myopathic changes with fibrillation and increased polyphasic motor unit potentials (MUPs).

Patient 2: A 62-year-old woman first noticed difficulty in climbing stairs at the age of 35 years, and needed a stick to walk at 45 years. Muscle weakness gradually worsened predominantly in her proximal limbs, and she became wheelchair-bound at 55 years. A muscle biopsy was performed at the age of 61 years. On examination, she had muscle weakness and atrophy predominantly in the proximal upper and lower limbs at the grade 3–4 on MRC scale. Serum CK level was 70 IU/L (normal, <142 IU/L). An EMG showed myopathic changes with increased polyphasic MUPs and myotonic-like repetitive discharges. She had been on non-invasive positive-pressure ventilation since the age of 62 years when the respiratory insufficiency appeared.

Skeletal muscle pathology

The skeletal muscle pathology from the vastus lateralis of patient 1 and from the biceps brachi of patient 2 showed nonspecific myopathic changes with moderate fiber size variation, mild endomysial fibrosis, and some fiber splitting (Fig. 1A). No necrotic or regenerating fibers were seen. No vacuoles containing amorphous materials were observed. Importantly, both muscles contained red–purple globular inclusions on modified Gomori-trichrome (mGT) stain (Fig. 1A and B). The average percentages of fibers with globular inclusions in the whole mGT-stained section were 0.5% in patient 1 and 2% in patient 2. These inclusions were invariably highlighted by ACP stain but not stained by periodic acid Schiff (PAS) (Fig. 1C). Inclusions were stained only faintly on menadione-linked α-glycerophosphate dehydrogenase (MAG) without substrate (Fig. 3A). Fibers with ACP-positive globular inclusions were also found in 15 of 16 childhood-onset and seven of eight adult-onset patients with disease-specific pathology in varying proportions (0.1–10%). The rate of fibers with inclusions was not significantly different between the childhood-onset and adult-onset forms. Fibers carrying inclusions did not have typical vacuoles with amorphous materials inside. In the infantile cases, more than 90% of the fibers were vacuolated, whereas non-vacuolated fibers with inclusions were hardly recognizable.

Double immunostaining was performed using primary antibodies against a lysosomal marker, lysosomal associated membrane protein-2 (LAMP-2; Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA, USA) and an autophagosomal marker, microtubule-associated protein 1 light chain 3 (LC3; Novus Biologicals, Littleton, CO, USA). In fibers with ACP-positive inclusions, immunoreactivity for LAMP-2 and LC3 were accumulated focally in inclusions and surrounding area (Fig. 1D). We also examined another samples from adult-onset patients with typical vacuoles. Fibers with typical vacuoles were entirely positive for LAMP-2 and LC3 (data not shown).

On PAS staining, performed on epon-embedded sections (Epon-PAS) to detect glycogen more sensitively, PAS was negative in globular inclusions but positive in the surrounding area (Fig. 1E).

Electron micrography was performed as previously described using a Tecnai spirit transmission electron microscope (FEI, Hillsboro, OR, USA) [3]. The inclusions consisted of homogeneous electron-dense globules surrounded by increased glycogen particles and autophagic vacuoles (Fig. 1F). The globules contained neither dotted glycogen particles nor a filamentous structure.

GAA enzymatic analysis and genetic analysis

Presence of globular inclusions led us to suspect Pompe disease, and GAA enzymatic activity analyses revealed 7.5% of normal control activity in patient 1 and 12.3% in patient 2.

Genomic DNA was extracted from peripheral lymphocytes or biopsied muscle using a standard protocol for mutational analysis of *GAA*. All exons and their flanking intronic regions of *GAA* were amplified by PCR and directly sequenced with an ABI PRISM 3100 Automated Sequencer (Applied Biosystems, Foster City, CA, USA). Both patients carried the homozygous *GAA* mutation at the last codon of exon 2 (c. 546G > T). RT-PCR and direct sequencing were performed using RNA extracted from biopsied muscles. This novel mutation causes aberrant splicing by skipping exon 2 (Fig. 2). This homozygous c. 546G > T mutation was also found in another patient with the adult-onset form, whose muscle pathology showed typical skeletal muscle pathology with vacuolated fibers.

Discussion

ACP-positive globular inclusions were a good diagnostic marker for the two patients with adult-onset Pompe disease lacking typical vacuolated fibers. Among 12,103 muscle biopsies in the NCNP repository from 1979 to 2010, ACP-positive globular inclusions were not reported, except for Pompe disease.

The globular inclusions are most likely the same as "reducing body-like globular inclusions in late-onset Pompe disease" reported by Sharma et al., as the pathological features are

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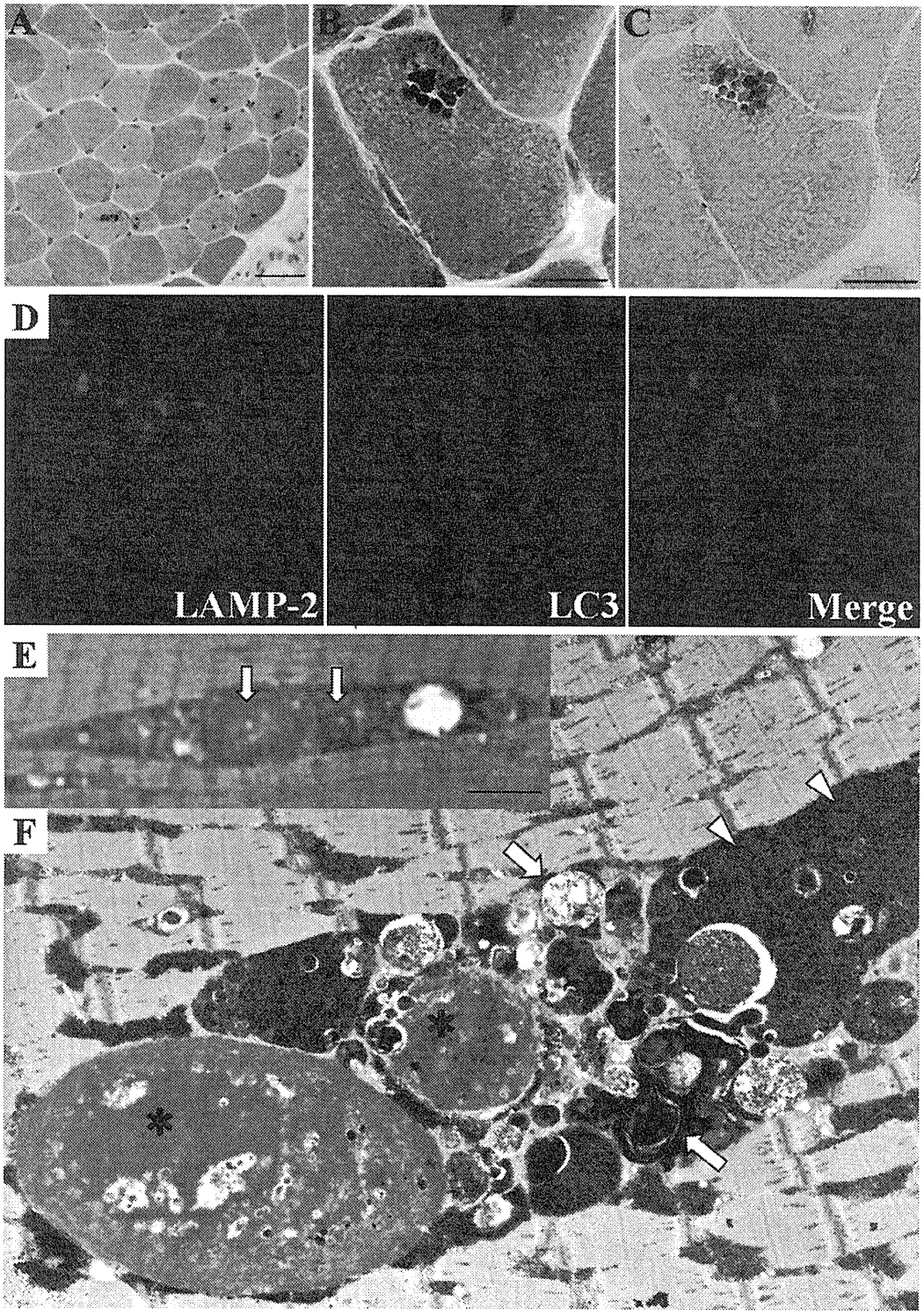

Fig. 1. Acid phosphatase-positive globular inclusions in patient 2. (A and B) Biopsied skeletal muscle showed nonspecific myopathic changes with scattered red–purple colored globular inclusions on modified Gomori-trichrome stain. (C) The inclusions have intense activity on acid phosphatase stain. Bar = 20 μm. (D) Double immunostaining for LAMP-2 (green) and LC3 (red) demonstrates colocalization of positive immunoreactions in the inclusions and surrounding area (B–D; serial sections). (E) On epon-embedded section, periodic acid Schiff stain is negative in inclusions (arrows). Bar = 5 μm. (F) On electron microscopy, globular inclusions (asterisks) lack Z-line structure, which differs from cytoplasmic bodies. Autophagic vacuoles (arrows) and glycogen particles (arrow heads) are seen in the vicinity of globular inclusions (12000×). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

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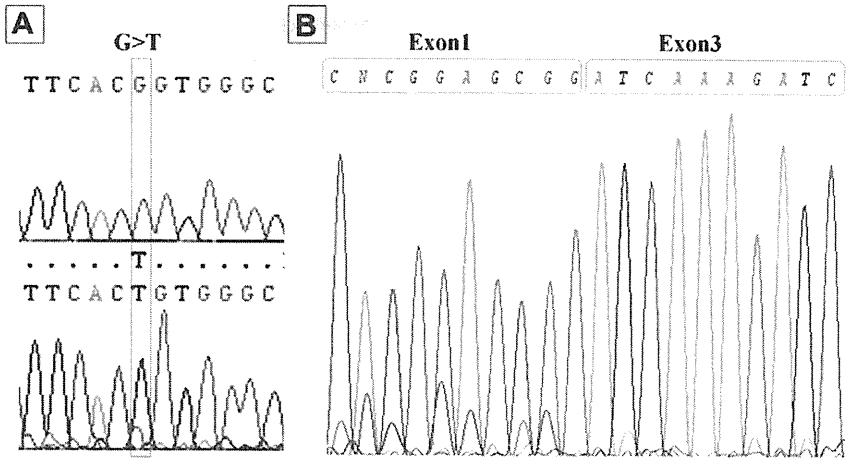

Fig. 2. Mutational analysis of *GAA*. Both patient have a homozygous c. 546G > T mutation at the last codon of exon2 (A upper: control, lower: patient), which creates mRNA with skipping exon 2 (B).

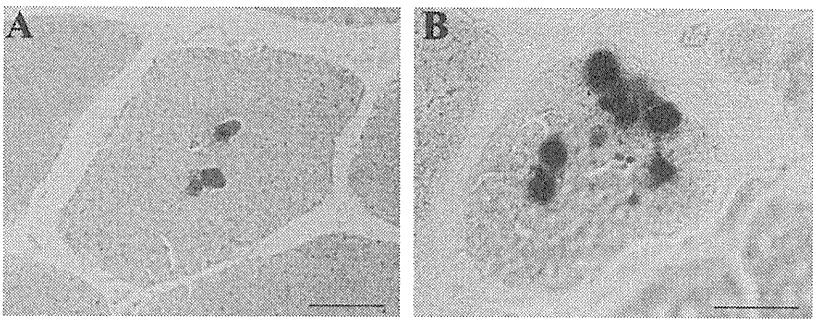

Fig. 3. Inclusions on menadione-linked α-glycerophosphate dehydrogenase (MAG) without substrate. Globular inclusions in Pompe disease (A) are only faintly stained comparing reducing bodies in reducing body myopathy with *FHL1* mutation (B). Bar = 20 μm.

rather similar [4]. However, globular inclusions showed much fainter staining on MAG without substrate than genuine reducing bodies seen in reducing body myopathy with *FHL1* mutations (Fig. 3). More importantly, ACP positivity has not been clearly described previously.

These globular inclusions are reminiscent of cytoplasmic bodies, which are nonspecific findings reflecting degeneration of the Z-disk in various neuromuscular diseases, particularly myofibrillar myopathies. However, the nature of the globular inclusions differs essentially from cytoplasmic bodies because of positive ACP staining and the lack of associated Z-disk components. Although it remains unclear how the ACP-positive globular inclusions are formed, the absence of glycogens in the globular inclusions suggest that they differ from glycogen accumulations in lysosomes. Fibers with typical vacuoles were diffusely positive for both lysosomal and autophagosomal markers as shown previously [5,6]. On the other hand, immunoreactivities of these markers accumulated more focally in fibers with inclusions. Further study should be needed to clarify what causes these pathological differences.

In conclusion, ACP-positive globular inclusions may be a hallmark of Pompe disease and a useful diagnostic marker for adult-onset Pompe disease lacking typical vacuolated fibers. Since enzyme replacement therapy is effective, albeit not fully, in adult-onset patients, early diagnosis is necessary for a better prognosis.

Ethical approval

All clinical materials used in this study were obtained for diagnostic purposes with written informed consent approved by the Ethical Committee of NCNP.

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Contribution of Genetic Factors to the Pathogenesis of Dilated Cardiomyopathy

– The Cause of Dilated Cardiomyopathy: Genetic or Acquired? (Genetic-Side) –

Akinori Kimura, MD, PhD

Dilated cardiomyopathy (DCM) is characterized by dilated ventricles and systolic dysfunction. Its etiology is not fully unraveled, but both extrinsic and intrinsic factors are considered to be involved. The intrinsic factors include genetic variations in the genes (ie, disease-causing mutations and disease-associated polymorphisms), which play key roles in controlling the susceptibility to the disease by affecting the performance, regulation, and/or maintenance of cardiac function. DCM can be classified into 2 types: hereditary and non-hereditary. The genetic variations, or disease-causing mutations, contributing to the pathogenesis of hereditary DCM can be found in various genes, especially those for sarcolemma elements, contractile elements, Z-disc elements, sarcoplasmic elements, and nuclear lamina elements of cardiomyocytes. On the other hand, disease-associated polymorphisms, which control the susceptibility to non-hereditary DCM, may be found in genes expressing not only in cardiomyocytes but also other non-cardiac cells involved in the immune system. Because functional alterations caused by these genetic variations can be classified into several categories, it is necessary to understand the pathogenesis and hence to develop diagnostic and therapeutic strategies for both hereditary and non-hereditary DCM from the viewpoint of genetic factors. (*Circ J* 2011; **75:** 1756–1765)

Key Words: Cardiomyopathy; Dilated cardiomyopathy; Genetics; Stiffness; Stress

Cardiomyopathy is a heterogeneous disease caused by functional abnormality of cardiac muscle and classified as primary or secondary cardiomyopathy.[1] Secondary cardiomyopathy is caused by extrinsic factors, including infection, ischemia, hypertension and metabolic disorders, whereas the diagnosis of primary cardiomyopathy is based on exclusion of secondary cardiomyopathy and there are several different clinical types.[2,3] Dilated cardiomyopathy (DCM) and hypertrophic cardiomyopathy (HCM) are 2 major ones. DCM is characterized by a dilated ventricular cavity with systolic dysfunction and the clinical symptom of DCM is heart failure, which is often associated with arrhythmia and sudden death. On the other hand, HCM, a major cause of sudden death in the young and heart failure, is characterized by left ventricular hypertrophy, often asymmetric, accompanied by myofibrillar disarrays and reduced compliance (diastolic dysfunction) of the cardiac ventricles.

The etiology of familial DCM had been unknown until 1993 when a mutation in the dystrophin gene (*DMD*) was found in male sibling cases with X-lined DCM.[4] Family history of the same disease is indicative that genetic factors contribute to the etiology and pathogenesis of the disease. The genetic factor can be classified into 2 categories: a disease-causing gene mutation as the etiology of monogenic disease or a disease-associated gene polymorphism that is involved in the pathogenesis of multifactorial disease.

Contribution of Genetic Factors in DCM

The concept of genetic factors involved in the disease is shown schematically in **Figure 1**. It is conceivable that full biological function in healthy subjects is impaired to the threshold for the development of disease by both genetic factors and environmental factors. In the monogenic disease, most of the dysfunction is caused by a disease-causing gene mutation, although additional genetic factors or modifier genes might also contribute to the pathogenesis, and other environmental factors including gender, age, and life-style factors may be involved in disease development. The ratio of disease development among carriers of the disease-causing mutation is defined as penetrance, which is determined by factors other than the disease-causing mutation. In clear

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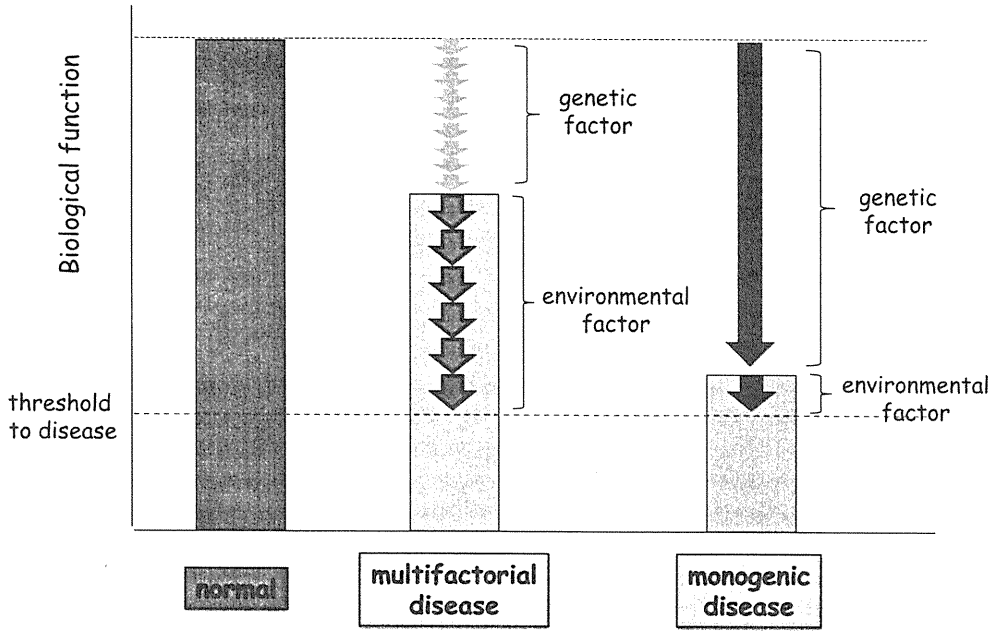

Figure 1. Concept of genetic and environmental factors in dilated cardiomyopathy. Disease can develop when biological function is impaired by genetic and environmental factors reaching a threshold. In multifactorial disease many genetic factors in combination with many environmental factors are required to reach the threshold, whereas only one genetic factor and a few environmental factors may be sufficient to develop the monogenic disease. Orange and red arrows indicate genetic factors, whereas light blue and blue arrows represent environmental factors.

contrast, the genetic factor is composed of multiple disease-associated gene polymorphisms in the multifactorial disease. In general, the contribution of genetic factors to the disease is approximately 60–100% in the monogenic disease, whereas it is approximately 20–30% in the multifactorial disease. Therefore, the disease-causing mutation can determine the development of disease by 60–100%, although it depends on each mutation. On the other hand, each disease-associated polymorphism can determine the disease at a few percent, because there should be multiple polymorphisms involved in the multifactorial disease. Family history of the same disease is indicative of genetic factors in the disease, and family history or aggregation of the disease can be seen in both the monogenic and multifactorial disease. The main difference is that the family history of monogenic disease can be explained by the Mendelian rule of inheritance, whereas the aggregation of multifactorial disease does not follow this rule. However, it is sometimes difficult to distinguish monogenic disease from multifactorial disease when the size of the family is small or the penetrance of the disease is low.

Mutations in Hereditary Cardiomyopathy

It has been reported that as much as 35% of DCM patients have a family history, mainly consistent with autosomal dominant inheritance, although some familial cases can be explained by autosomal recessive or X-linked recessive trait.[5] This report is based on the extensive analysis of family members of the DCM patients, and hence is considered to be the maximal estimation of familial cases in DCM. In general, a family history can be found in approximately 10–20% of DCM patients. In clear contrast, more than half of HCM patients have a family history consistent with an autosomal dominant genetic trait. Therefore, a certain proportion of primary cardiomyopathy is monogenic disease caused by a single disease-causing mutation. However, family history may not be found in patients with hereditary cardiomyopathy, and then sporadic cases might also be with hereditary cardiomyopathy, which cannot easily be distinguished from patients with cardiomyopathy of multifactorial etiology.

Molecular genetic approaches have recently been taken to unravel the disease genes. As shown in **Table 1** many different disease genes (ie, disease-causing mutation in the analyzed gene) have been identified for different clinical types of primary cardiomyopathy.[6] Most of the disease genes encode for sarcomere proteins (**Figure 2**). It should be noted here that each patient or each multiplex family usually carries only one mutation in the disease gene, although there are exceptional cases harboring mutations in 2 or more disease genes. Another noteworthy issue is the overlapping of disease genes for different clinical types (ie, mutations in the same disease gene can be found in different cardiomyopathies).

Frequency of Disease-Causing Mutations in DCM

Disease-causing mutations in Japanese patients stratified into familial or sporadic cases based on family history have been investigated (**Table 2**). Although not all the known disease genes were analyzed, approximately 21% of familial DCM patients possessed one. Of note was that there was no major disease gene for Japanese DCM patients, because the mutations were very heterogeneous and each mutation was found in only one proband patient. Although titin gene (*TTN*) mutations were relatively frequent, this might be due to *TTN* being quite large, encompassing at least 383 exons.[7] On the other hand, approximately 7% of sporadic DCM patients

Table 1. Disease Genes for Primary Cardiomyopathy

Clinical types	Inheritance	Gene symbol	Coding protein
HCM/DCM/RCM	AD	*MYH7*	Cardiac β myosin heavy chain
HCM/DCM	AD	*TNNT2*	Cardiac troponin T
HCM/DCM	AD	*TPM1*	α tropomyosin
HCM/DCM	AD	*MYBPC3*	Cardiac myosin binding protein C
HCM	AD	*MYL3*	Ventricular myosin essential light chain
HCM	AD	*MYL2*	Ventricular myosin regulatory light chain
HCM/DCM/RCM	AD, AR	*TNNI3*	Cardiac troponin I
HCM/DCM	AD	*ACTC*	α cardiac actin
HCM/DCM	AD	*TTN*	Titin/connectin
HCM/DCM	AD	*TNNC2*	Cardiac troponin C
HCM	AD	*MYH6*	Cardiac α myosin heavy chain
HCM/DCM	AD	*CSRP3*	Muscle LIM protein
HCM	AD	*CAV3*	Caveolin-3
HCM/DCM	AD	*TCAP*	Telethonin (Tcap)
HCM/DCM	AD	*VCL*	Metavinculin
HCM	AD	*JPH–2*	Junctophilin
HCM	AD	*OBSCN*	Obscurin
HCM	AD	*MYOZ2*	Calsartin 1/myozenin 2
HCM/DCM	AD	*ANKRD1*	CARP
DCM/RCM	AD	*DES*	Desmin
DCM	AD	*LMNA*	Lamin A/C
DCM	AD	*SAGD*	δ sarcoglycan
DCM	AD	*ACTN2*	α actinin 2
DCM/LVNC	AD	*LDB3*	ZASP/Cypher
DCM	AD	*PLB*	Phospholamban
DCM	AD	*ABCC9*	K_{ATP} channel
DCM	AD	*SCN5A*	Cardiac Na channel
DCM/HCM	AD	*CRYAB*	αB crystallin
DCM	AD	*FHL2*	FHL2
DCM	AD	*LMNA4*	Laminin α4
DCM	AD	*MYPN*	Myopalladin
DCM	AD	*RBM20*	RNA binding motif protein 20
DCM/HCM	AD	*MEXN*	Nexillin
DCM	AD	*BAG3*	bcl2-associated athanogene 3 protein
DCM	XR	*DMD*	Dystrophin
DCM	XR	*EMD*	Emelin
DCM/LVNC	XR	*TAZ*	Tafazzin/G4.5
DCM	XR	*FKTN*	Fukutin
DCM/ARVC	AR	*DSP*	Desmoplakin
DCM/ARVC	AR, AD	*JUP*	Plakoglobin
ARVC	AD	*PKP2*	Placophilin 2
ARVC	AD	*TGFB3*	TGF β3
ARVC	AD	*RYR2*	Ryanodine receptor 2
ARVC	AD	*DSG3*	Desmoglein 3
ARVC	AD	*TMEM43*	LUMA
LVNC	AD	*DTNA*	α dystrobrevin

HCM, hypertrophic cardiomyopathy; DCM, dilated cardiomyopathy; AD, autosomal dominant; AR, autosomal recessive; XR, X-linked recessive; TGF, transforming growth factor.

also carried a gene mutation, most frequently in *DMD*. These observations strongly suggest that there are other disease genes for hereditary DCM and that a certain proportion of sporadic DCM cases is of hereditary DCM. The reason why gene mutations were found in several sporadic cases might be due to the small size of the patient's family, de novo mutation, and/or low penetrance of the disease-causing mutation.

Functional Aspects of Sarcolemmal Mutations in DCM

The first identification of a disease-causing mutation for DCM was the *DMD* mutation in X-linked DCM. Mutations in *DMD* are well known to cause Duchenne-type and Becker-type muscular dystrophy, both of which are often complicated by cardiac dysfunction in the later phase of the clinical course. In X-linked DCM caused by *DMD* mutations, skeletal muscle phenotypes are usually subtle and the difference

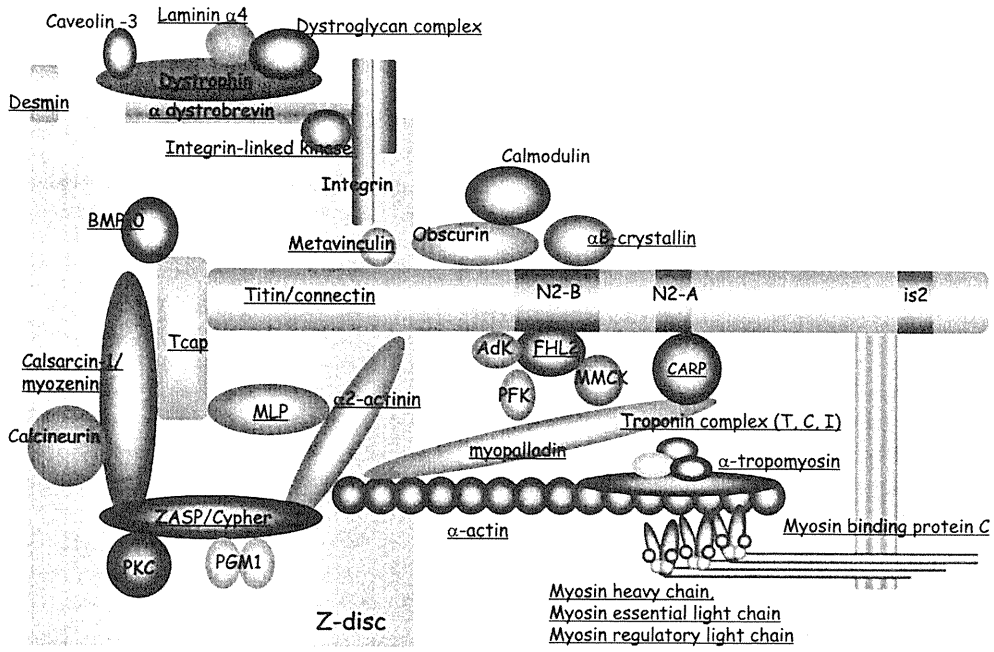

Figure 2. Schematic representation of sarcomere components. A half sarcomere is schematically shown. Elements for which gene mutation causes dilated cardiomyopathy are underlined.

Table 2. Mutation Rate in Japanese Patients With Dilated Cardiomyopathy

Gene symbol	Coding protein	% familial cases (n=72)	% sporadic cases (n=100)
ACTC	α cardiac actin	0.0	0.0
DES	Desmin	1.4	0.0
DMD	Dystrophin	0.0	5.0
LMNA	Lamin A/C	0.0	NT
SAGD	δ sarcoglycan	0.0	NT
MYH7	Cardiac β myosin heavy chain	5.6	0.0
TNNT2	Cardiac troponin T	0.0	0.0
TNNI3	Cardiac troponin I	0.0	0.0
TPM1	α tropmyosin	0.0	0.0
TTN	Titin/connectin*	>4.2	>2.0
CSRP3	Muscle LIM protein	0.0	0.0
VCL	Metavinculin	0.0	0.0
CRYAB	αB crystallin	1.4	0.0
MYBPC3	Cardiac myosin binding protein C	0.0	0.0
TCAP	Telethonin (Tcap)	1.4	0.0
ACTN2	α actinin 2	0.0	0.0
LDB3	ZASP/Cypher	1.4	0.0
FKTN	Fukutin	1.4	0.0
FHL2	FHL2	1.4	0.0
LMNA4	Laminin α4	0.0	0.0
MYPN	Myopalladin	0.0	0.0
ANKRD1	CARP	0.0	0.0
NBLT	Nebulette	0.0	0.0
BAG3	bcl2-associated athanogene 3	2.8	0.0
	Sum	>20.8	>7.0

*Approximately 25% of titin/connectin, Z-region, N2-B region, N2-A region, kinase and adjacent regions, Novex3 region and is2 region, was analyzed.
NT, not tested.

in severity and distribution of affected muscles may in part be determined by which domain of *DMD* is affected. Dystrophin is a sarcolemmal protein that plays a key role in the anchoring of muscle cells. As shown in **Figure 1**, other proteins such as dystrobrevin, integrin, integrin kinase and metavinculin form a sarcolemma complex linking dystrophin and the Z-disc. In addition, the laminin and dystroglycan complex links muscle cells to the extracellular matrix. Furthermore, fukutin is involved in the glycosylation of α-dystroglycan. Mutations in genes for these sarcolemmal proteins are known to cause DCM (**Table 1**), which may lead to disruption of anchoring and hence dysfunction in transmission of the force generated by muscle contraction.

Muscle contraction, which is caused by the interaction between an actin filament and myosin heavy chain, is regulated by the concentration of intracellular Ca^{2+} that is released from the sarcoplasmic reticulum (SR) via the ryanodine receptor and re-uptake to the SR via SR Ca^{2+}-ATPase (SERCA), which is regulated by phospholamban (*PLB*). When the concentration of Ca^{2+} is increased or decreased, muscle contracts or relaxes, respectively. It has been reported that DCM is caused by a *PLB* mutation,[8] by which uptake of Ca^{2+} to the SR is impaired such that release of Ca^{2+} from the SR per beat is decreased.

There are 2 sensing systems for fine tuning of Ca^{2+}-dependent muscle contraction: regulation by the troponin complex, and regulation by myosin light chain. The fine-tuning system controls the magnitude of muscle contraction at the same concentration of Ca^{2+}, which is also defined as calcium sensitivity. The calcium sensitivity in skeletal muscle is mainly controlled by the troponin complex via conformational changes of troponin T and troponin I depending on the capture or release of Ca^{2+} by troponin C, disruption of which can cause DCM in animal models.[9,10] On the other hand, calcium sensitivity in smooth muscle is mainly regulated by myosin light chain through its phosphorylation. As for cardiac muscle, both the troponin complex system and myosin light chain system regulate the calcium sensitivity. Any impairment in the regulation of calcium sensitivity can be associated with dysfunction of muscle contractility and hence would cause cardiomyopathy.

Functional Aspects of Contractile Element Mutations in DCM

Mutations in contractile element genes were initially identified as the cause of HCM,[11] but recently were found in DCM cases (**Table 1**). Because some HCM patients develop systolic dysfunction similar to DCM, which is called dilated-phase HCM, DCM patients carrying contractile element mutations might indeed suffer from HCM. However, it was demonstrated that there are apparent functional differences related to the disease-causing mutations found in DCM and HCM. A typical example is reported for mutations in cardiac troponin T gene (*TNNT2*).

Troponin T binds troponin C and troponin I to form the troponin complex, which regulates the calcium sensitivity of muscle contraction via conformational change in the interaction between cardiac actin and myosin heavy chain. An in vitro assay of muscle contraction through replacement of normal troponin T with the DCM-causing *TNNT2* mutation, del_Lys210, showed that the mutant troponin T caused a rightward shift of the calcium-tension curve, which indicated a decreased calcium sensitivity of the muscle contraction.[12] It was reported that the del_Lys210 mutation altered the calcium sensitivity in the hearts of *TNNT2* mutant knock-in mice, which exhibited the cardiac phenotype of DCM.[13] On the other hand, HCM-linked *TNNT2* mutations were shown to increase the calcium sensitivity in vitro[14] and in the in vivo model of transgenic mice that developed HCM.[15] These observations indicate that the altered calcium sensitivity of muscle contraction because of *TNNT2* mutations is the direct cause of primary cardiomyopathy and the opposite functional alterations are associated with DCM and HCM.

The decreased calcium sensitivity was also reported for DCM-causing mutations in α-tropomyosin (*TPM1*)[16] and cardiac troponin C (*TNNC1*),[17] whereas the increased calcium sensitivity was noted for various HCM-causing mutations in the contractile element genes, including *TPM1*,[16] cardiac troponin I gene (*TNNI3*),[18] ventricular myosin regulatory light chain gene (*MYL3*)[19] and *MYBPC3*.[20] Therefore, the altered calcium sensitivity may at least in part explain the functional alterations caused by the contractile element gene mutations.

Functional Aspects of Z-Disc Element Mutations in DCM

A unit of striated muscle is called a sarcomere, which aligns in tandem to maximize the generation of power. Because the generated power of muscle contraction is transmitted to adjacent sarcomeres through the Z-disc (**Figure 2**), mutations in Z-disc elements may cause hereditary cardiomyopathy (**Table 1**). Identification of a HCM-causing mutation in the Z-disc region of titin (*TTN*) was the first example of the Z-disc element mutation, which increased the binding of titin and α-actinin.[21] On the other hand, 2 different disease-causing *TTN* mutations in the Z-disc region were identified in DCM: one at the actinin-binding domain and the other at the Tcap-binding domain.[22] Functional analyses demonstrated that the former mutation decreased the binding to α-actinin, which was the opposite functional alteration to that caused by the HCM-causing mutation, whereas the latter mutation decreased the binding to Tcap, suggesting that decreased binding of titin and Z-disc elements was a common functional alteration caused by the DCM-causing *TTN* mutations (**Figure 3**).

In addition, 2 DCM-causing mutations in the Tcap gene (*TCAP*), which decreased the binding of Tcap to titin, MLP and calsarcin-1 (myozenin-2), have been found.[23] Moreover, a DCM-associated mutation, Trp4Arg, in the MLP gene (*CSRP3*), was reported to decrease the binding of MLP to Tcap.[24] Because this mutation was found not only in the DCM patients but also in the general population, it may be a polymorphism not a DCM-causing mutation.[25] However, a knock-in mouse line carrying this mutation showed cardiac hypertrophy and heart failure phenotype under the catecholamine-induced cardiac stress.[26] Because *CSRP3* knock-out mice develop the DCM phenotype,[27] along with a wide Z-disc and loss of stretch response,[24] the Z-disc may play a role as a stretch sensor and its dysfunction leads to the DCM phenotype.[24] Furthermore, another DCM-causing *CSRP3* mutation and α-actinin gene (*ACTN*) mutations were reported to decrease the binding of MLP and α-actinin.[25] These observations suggest that the decreased binding among the Z-disc elements could develop into DCM because of the decreased stretch response. In this regard, it can be hypothesized that DCM is a disease of "loose sarcomeres" (**Figure 3**). In clear contrast, HCM-causing mutations in *TCAP* increase the binding of Tcap to titin and carsarcin-1,[23] leading to a hypothesis that HCM may be a disease of "stiff sarcomeres" (**Figure 3**). Loose and stiff sarcomeres would decrease and increase passive tension upon stretch of the sarcomere, respectively. Because the change in passive tension is associated with a change in the calcium sensitivity,[28–30] it is speculated that

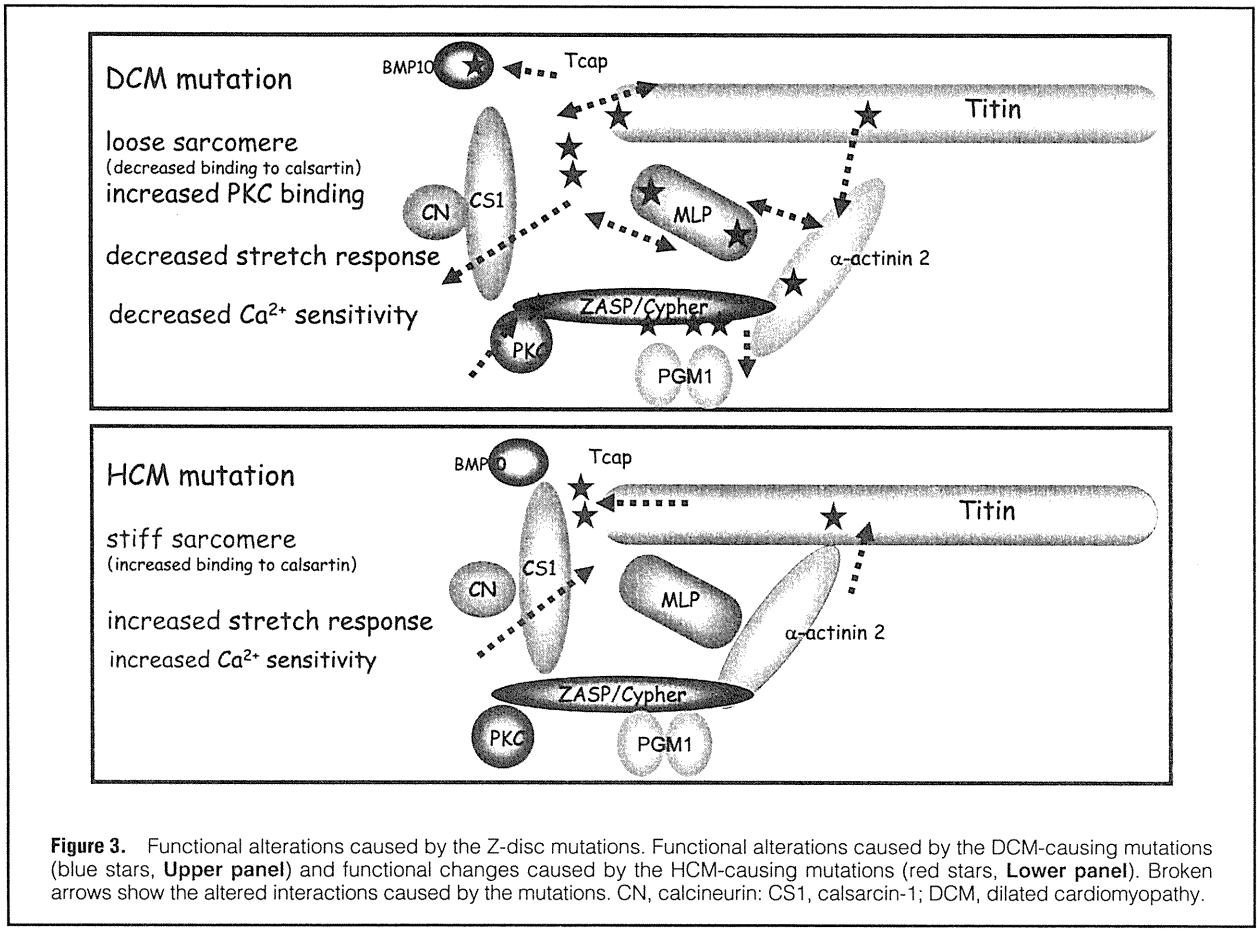

Figure 3. Functional alterations caused by the Z-disc mutations. Functional alterations caused by the DCM-causing mutations (blue stars, **Upper panel**) and functional changes caused by the HCM-causing mutations (red stars, **Lower panel**). Broken arrows show the altered interactions caused by the mutations. CN, calcineurin: CS1, calsarcin-1; DCM, dilated cardiomyopathy.

abnormality in both the Z-disc elements and contractile elements cause the abnormal calcium sensitivity.

There are several other Z-disc elements, desmin (*DES*) and metavinculin (*VCL*), of which mutations have been found in DCM. The *VCL* mutation impaired the binding to actin,[31] whereas the *DES* mutations resulted in the disruption of cytoplasmic desmin network.[32] In addition, mutations in the myopalladin gene (*MYPN*)[33] and nebulette gene (*NBLT*),[34] which impair myofiblinogenesis,[36,37] have recently been reported in DCM. These findings suggest that the Z-disc also plays a role in myofibrinogenesis.

ZASP/Cypher is another Z-disc element connecting calsarcin and actinin[35] (**Figure 2**). Calsarcin binds calcineurin,[36] a Ser/Thr phosphatase involved in the hypertrophic progress of cardiomyocytes.[37] The functional significance of calcineurin anchorage to the Z-disc is not fully understood, but it may be involved in the stress-induced calcineurin–NFAT activation, because heterozygous *CSRP3* knock-out mice show a reduction in NFAT activation along with a dislocation of calcineurin from the Z-disc.[38] In addition, ZASP/Cypher is known to bind protein kinase C (PKC),[35] and as a DCM-causing mutation in the PKC binding domain of ZASP/Cypher increased the binding,[39] it has been suggested that phosphorylation/dephosphorylation of Z-disc elements might be involved in the stretch response.

In addition, several other ZASP/Cypher gene (*LDB3*) mutations not in the PKC interacting domain are reported in DCM and LVNC.[40] Phosphoglucomutase-1 (PGM1) was recently identified as a novel binding protein to ZASP/Cypher[41] (**Figure 2**). PGM1 is a metabolic enzyme involved in glucose–glycogen metabolism. The functional significance of the binding between PGM1 and ZASP/Cypher remains unclear, but the DCM-causing mutations decrease the binding between ZASP/Cypher and PGM1.[41] Because PGM1 localizes at the Z-disc under the stressed culture conditions, a role for PGM1 in energy metabolism at the Z-disc may be required for the response against metabolic stress.[41] These observations suggest that an impaired stress response due to abnormality in the Z-disc elements might be involved in the pathogenesis of DCM.

Functional Aspects of Sarcoplasmic Element Mutations in DCM

Nebulette is anchored to the Z-disc at the C-terminal portion, and the other side is positioned in the sarcoplasm where it binds actin (**Figure 2**). A polymorphism in the actin-binding motif of nebulette associated with DCM has been reported.[42] On the other hand, myopalladin is anchored to the Z-disc at the N-terminal portion, while the other side is positioned in the sarcoplasm to bind a transcriptional cofactor, CARP.[43] CARP is known to shuttle between the sarcoplasm and nucleus to regulate gene expression associated with the stretch response, cardiac remodeling, and myofibrinogenesis.[44] Several DCM-causing CARP mutations that impair myofibrinogenesis were recently reported.[45]

There are several other sarcoplasmic proteins of which gene mutations have been found in DCM. Four and half LIM protein 2 gene (*FHL2*) and αB-crystallin gene (*CRYAB*) are

examples. They bind titin at the N2-B region, where a DCM-causing mutation has been found, and functional studies revealed that the DCM-causing mutation decreased the binding of titin to both proteins.[46,47] In addition, a DCM-causing *FHL2* mutation, which decreased the binding of FHL2 to titin, has been found.[48] Because FHL2 is tethering muscle-specific metabolic enzymes (ie, adenylate kinase, phosphofructokinase, and muscle type creatinine kinase[49]), the DCM-causing *FHL2* mutation would impair the recruitment of these metabolic enzymes to titin. Moreover, a DCM-causing *CRYAB* mutation decreased the binding to titin.[50] Although the functional significance of binding between titin and αB-crystallin remains unclear, it might be involved in the αB-crystallin-mediated protection of cardiac muscle from ischemic stress.[50] These observations suggest that DCM-causing mutations in these sarcoplasmic protein genes would render cardiac muscle susceptible to the metabolic stress. It should be noted here that αB-crystallin is phosphorylated and translocated to the Z-disc under ischemic conditions, suggesting a role of the Z-disc in the stress response.[51]

Functional Aspects of Nuclear Lamina Mutations in DCM

Part of hereditary DCM is caused by mutations in the genes for components of the nuclear lamina, emerin (*EMD*) and lamina A/C (*LMNA*). It is well known that patients with skeletal muscle disease, such as muscular dystrophy or myofibrillar myopathy, often suffer from cardiac dysfunction. Because both skeletal muscle and cardiac muscle are striated muscle, there are several genes specifically expressed in both muscle types and mutations in such genes can be found in both skeletal muscle disease and cardiomyopathy. *EMD* mutations were reported in X-linked[52] Emery-Dreifuss muscular dystrophy, and *LMNA* mutations were found in autosomal-dominant[53] and autosomal-recessive[54] Emery-Dreifuss muscular dystrophy. It is known that Emery-Dreifuss muscular dystrophy is often accompanied by conduction defects and heart failure in the later clinical phase,[52–54] so there is a possible link between Emery-Dreifuss muscular dystrophy and DCM. Indeed, upon screening for mutations in X-linked[55] and autosomal-dominant[56] DCM accompanied by conduction defects without severe skeletal muscle phenotype, *EMD* and *LMNA* mutations were discovered, respectively. As discussed for *DMD* mutations, the difference in clinical phenotype (ie, skeletal muscle disease or cardiomyopathy) may be determined by which domain of the elements encoded by the disease gene was affected. However, there is no definite difference in the distribution of *LMNA* mutations found in the muscular diseases and cardiomyopathy.[57,58] Molecular mechanisms for developing DCM by mutations in nuclear lamina genes remain unknown, but might be involved in the altered regulation of gene expression in the heart as reported for knock-in mice with a *LMNA* mutation.[59]

On the other hand, altered calcium sensitivity may not be responsible for the development of DCM caused by the *LMNA* mutation, because no significant change in calcium sensitivity was found in the hearts of knock-in mice.[60] However, treatment by a calcium sensitizer, SCH00013, which had no activity for inhibiting phosphodiesterase, can delay the development of the DCM phenotype and prevent DCM-associated changes, including pathological fibrosis as well as cardiac remodeling-related gene expression in the heart,[60] suggesting that the decreased calcium sensitivity might be secondarily involved in the pathogenesis of DCM in the *LMNA* mutation knock-in mice.

Polymorphisms Associated With DCM

The contribution of a genetic factor to the pathogenesis of DCM largely depends on whether it is a disease-causing mutation or a disease-associated polymorphism. In most cases, disease-associated polymorphisms would increase the risk for disease by a factor of approximately 1.2, and at most of 3.0, suggesting that there is limited significance of their applications in the diagnosis and/or prediction of the disease. However, information on the disease-associated polymorphism, even if its contribution is small, should be useful in unraveling and understanding the molecular mechanisms of pathogenesis.

Polymorphisms in the genes expressed in cardiomyocytes can be associated with DCM. As described in the previous section, sequence variations in the Z-disc elements, Trp4Arg of *CSRP3* and Asn654Lys of *NBLT*, can be found in the general populations of Europeans and Japanese, respectively. Therefore, they are DCM-associated polymorphisms and not DCM-causing mutations. However, both are rare polymorphisms because of their low carrier frequency, 0.2% in Whites[24] and 1.2% in Japanese,[42] and hence their detection in DCM patients might have, albeit limited, a diagnostic significance. Indeed, Trp4Arg of *CSRP3* proved to have a definite functional significance under certain conditions in the mouse model.[26] Another example of a rare polymorphism associated with DCM is Thr326Ile of BMP10, which was found in 2 out of 46 Japanese patients with DCM accompanied by hypertension.[61] BMP10 is a member of the TGF family and specifically expressed in the cardiomyocyte.[62] This variant is a rare polymorphism, 0.13% in healthy Japanese, but is a significant risk factor for DCM in the presence of hypertension.[61] In addition, BMP10 binds to Tcap and the variant decreases the binding (**Figure 3**), which in turn results in the increased secretion of BMP10.[61] That BMP10 facilitates hypertrophy and maturation of rat cardiomyocytes in primary culture has also been demonstrated.[61] These observations suggest a pivotal role of the Z-disc in cardiac remodeling.

Apart from the Z-disc proteins, sarcolemma proteins are also important for the contractile performance of cardiac muscle and include the adrenergic receptors. There are several polymorphisms in the genes for β1-, β2-, and α2c-adrenergic receptors. Among them, Ser49Gly and Arg389Gly of β1-adrenergic receptors, Arg16Gly, Gln27Glu, and Thr164Ile of β2-adrenergic receptors, and Ins/Del322-325 of α2c- adrenergic receptors are reported to have functional differences,[63] and these polymorphisms are noteworthy with regard to responsiveness to β-blocker therapy in chronic heart failure (CHF) including DCM.[64,65] Although a few initial studies, in which relatively small numbers of cases and controls were examined, reported possible associations of DCM or CHF with adrenergic-receptor gene polymorphisms, subsequent studies could not replicate the association.[64–66] Therefore, the contribution of the adrenergic-receptor genes to the susceptibility to DCM or CHF might not be large enough to be readily proven. However, several independent clinical studies have reported that carriers of β1-adrenergic receptor alleles, Gly49 or Arg389, showed a better response to β-blocker therapy.[63] Although these β1-adrenergic receptor alleles are in strong linkage disequilibrium,[60] both are considered to participate in the beneficial effect.

On the other hand, polymorphisms in genes expressed in cells other than cardiomyocytes can also be associated with susceptibility to the DCM or CHF phenotype. Because immune responsiveness against foreign antigens such as viral

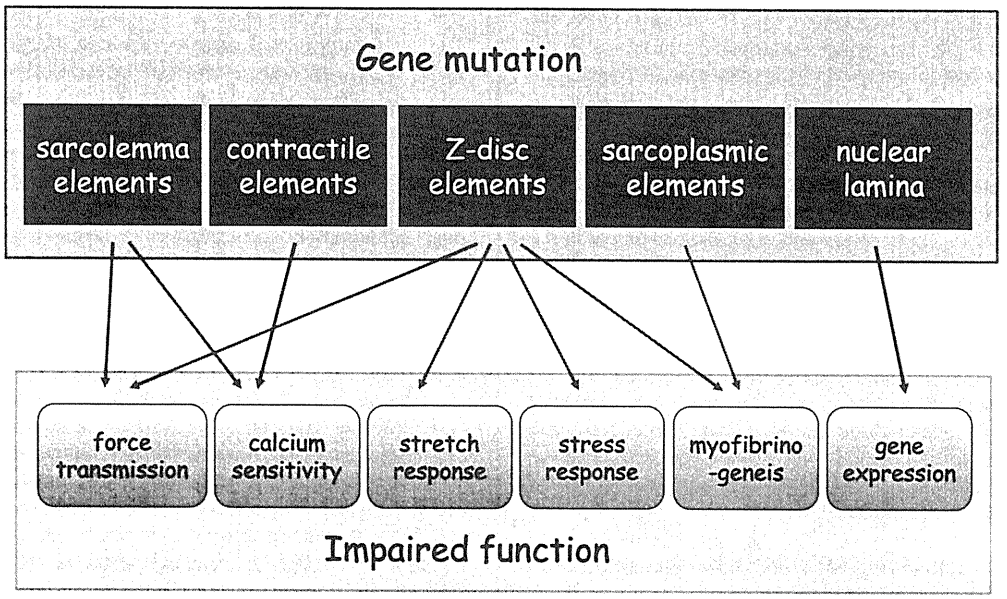

Figure 4. Categories of mutations in sarcomere components and consequent dysfunction. Mutations found in patients with hereditary dilated cardiomyopathy can be categorized by which sarcomere elements are affected. Functional alteration caused by the mutations may be categorized by which function of the sarcomere is impaired. Arrows indicate the causes and consequences.

proteins and against auto-antigens is controlled by genome diversity in the HLA genes, susceptibility to viral myocarditis, which often precedes DCM, and autoimmunity to sarcolemmal or sarcoplasmic proteins accompanied by DCM may be associated with specific HLA alleles. Although the molecular mechanisms and/or antigenic peptides remain unknown, it has been reported that specific HLA alleles or haplotypes such as the DRB1*1401-DQB1*0503 haplotype are significantly associated with DCM in Japanese.[67] As for the susceptibility to DCM accompanied by hepatitis C virus (HCV), HLA-linked susceptibility within the class III–I boundary region where the *NFKBIL1*, *ATP6V1G2*, *BAT1*, *MICB*, and *MICA* genes reside has been mapped.[68] It is unclear which of these genes is responsible for the susceptibility to HCV–DCM, but the data indicate that the association is not found with specific HLA alleles and that a non-HLA gene was involved in the pathogenesis. It is interesting to note that MIC molecules, MICA and MICB, are the ligands for an activating receptor of NK cells, NKG2D.[69] On the other hand, a significant association with HLA-DPB1*0401 and -DPB1*0901 was found for HCM accompanied by HCV,[70] indicating that the HLA-linked genetic background for susceptibility differs between HCV–DCM and HCV–HCM even though both are accompanied by the same virus. Autoimmunity against sarcolemmal or sarcoplasmic elements, such as β-adrenergic receptor or myosin heavy chain, respectively, may worsen the disease severity of DCM.[71,72] It is suggested that the weak association of DCM with HLA-DR4 in Caucasian populations might reflect an association between the production of specific autoantibody and HLA-DR4.[73] However, such associations have not been proven yet, possibly due to the small number of DCM patients with autoantibodies to specific antigen (ie, autoimmunity was directed various different auto-antigens in each DCM patient) and a large cohort study will be required.

Concluding Remarks

In this review, I have focused on the contribution of genetic factors in the pathogenesis of DCM, but environmental factors, including viral infection and secondary autoimmune mechanisms, which ultimately lead to loss of cardiomyocytes, may also play a considerable role in the pathogenesis of DCM. The etiology of DCM is quite heterogeneous even in hereditary DCM (ie, disease-causing mutations and moreover disease genes themselves are different in individual patients). However, because the pathological pathways of hereditary DCM can be classified into several categories (**Figure 4**), such information is helpful in assessing the prognosis of patients and possibly in the evaluation of therapeutics.

Acknowledgments

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Authors' Comments on the Acquired-Side Author

In this issue of the Journal, Dr Yoshikawa nicely summarizes the acquired conditions involved in the pathogenesis of the DCM phenotype.[74] Viral infection and autoimmunity are emphasized. I fully agree with the concept that both viral infection and the immune response against it contribute to the pathogenesis of DCM in a certain proportion of patients, especially for non-familial or sporadic cases, although there might be a few familial cases because of shared genetic factors for the susceptibility and the increased chance of infection by the same virus. Indeed, we experienced a DCM patient who carried a DMD mutation and showed infiltration of immune-related cells in the heart tissue, suggesting that some disease-causing mutations may increase the susceptibility to infection by some viruses. As for autoimmunity, it might be an immune response against damaged cardiomyocytes. It is speculated that the damage was induced by acquired factors, but certain disease-causing mutations, such as DMD mutations, can render cardiomyocytes susceptible to death stimuli. The most important issue is how to differentiate hereditary DCM and acquired DCM and whether different therapies are required. I propose that the presence of a family history is a marker for hereditary DCM, whereas the presence of HLA class II molecules in the cardiac tissue is a potential marker for acquired DCM, although these markers may not clearly distinguish the 2 types. In addition, β-blocker therapy may be applicable irrespective of the etiology of DCM, although additional therapies such as calcium sensitizer and immune-absorption therapy may be required for each patient.

RESEARCH ARTICLE

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Dilated Cardiomyopathy-Associated *BAG3* Mutations Impair Z-Disc Assembly and Enhance Sensitivity to Apoptosis in Cardiomyocytes

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ABSTRACT: Dilated cardiomyopathy (DCM) is characterized by dilation of left ventricular cavity with systolic dysfunction. Clinical symptom of DCM is heart failure, often associated with cardiac sudden death. About 20–35% of DCM patients have apparent family histories and it has been revealed that mutations in genes for sarcomere proteins cause DCM. However, the disease-causing mutations can be found only in about 17% of Japanese patients with familial DCM. Bcl-2-associated athanogene 3 (BAG3) is a co-chaperone protein with antiapoptotic function, which localizes at Z-disc in the striated muscles. Recently, *BAG3* gene mutations in DCM patients were reported, but the functional abnormalities caused by the mutations are not fully unraveled. In this study, we analyzed 72 Japanese familial DCM patients for mutations in BAG3 and found two mutations, p.Arg218Trp and p.Leu462Pro, in two cases of adult-onset DCM without skeletal myopathy, which were absent from 400 control subjects. Functional studies at the cellular level revealed that the DCM-associated *BAG3* mutations impaired the Z-disc assembly and increased the sensitivities to stress-induced apoptosis. These observations suggested that BAG3 mutations present in 2.8% of Japanese familial DCM patients caused DCM possibly by interfering with Z-disc assembly and inducing apoptotic cell death under the metabolic stress.

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KEY WORDS: dilated cardiomyopathy; DCM; *BAG3*

Introduction

Dilated cardiomyopathy (DCM) is a primary heart muscle disorder caused by functional abnormalities in the cardiomyocytes, which is characterized by ventricular chamber dilation and diminished cardiac contractility. DCM is a major cause of chronic heart failure and the most common indication for cardiac transplantation [Maron et al., 2006]. Various etiologies including gene mutations, viral infections, toxins such as alcohol, mitochondrial abnormalities, and metabolic disorders cause DCM [Maron et al., 2006]. Because 20 to 35% of DCM patients have family histories mostly consistent with autosomal dominant inheritance, linkage studies in multiplex families and/or candidate gene approaches have been taken to identify the disease genes and it has been revealed that DCM can be caused by various genetic abnormalities [Kimura, 2010]. The majority of genetic causes are heterozygous mutations in genes for sarcomere proteins including contractile elements, sarcolemma elements, Z-disc elements, and Z-I region components, which play key roles in the generation and/or transmission of contractile force. On the other hand, it has recently been demonstrated by extensive whole-genome analyses that sequence variations in the gene for Bcl-2-associated athanogene 3 (*BAG3*; MIM# 603883) were associated with DCM (CMD1HH; MIM# 613881) [Norton et al., 2011; Villard et al., 2011], although molecular mechanisms of DCM caused by the *BAG3* mutations are not fully unraveled.

BAG3 is a member of antiapoptotic BAG protein family. BAG3 protein binds heat shock protein 70 (Hsp70; MIM# 140550) within the C-terminal BAG domain, which is an evolutionary conserved domain among the BAG family, and serves as a co-chaperone factor controlling the chaperone activity of Hsp70 [Takayama et al., 1999]. It was reported that *BAG3* prominently expressed in the striated muscle and localized at the Z-discs [Homma et al., 2006]. In addition, *Bag3* knockout mice displayed degeneration of muscle fibers with apoptotic nuclei in the striated muscles, resulting in a severe form of skeletal myopathy and cardiomyopathy, which lead to a hypothesis that BAG3 protein might play a role as a Z-disc signaling molecule [Homma et al., 2006]. In accordance with the hypothesis, apart from the association with DCM described above [Norton et al., 2011; Villard et al., 2011], a heterozygous Pro209Leu mutation was found in patients with myofibrillar myopathy (MFM) accompanied by cardiomyopathy (MFM6; MIM# 612954) [Lee et al., 2011; Odgerel et al., 2010; Selcen et al., 2009]. Moreover, it was demonstrated that knockdown of *bag3* in a zebrafish model developed heart failure resembling to human DCM [Norton et al., 2011].

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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