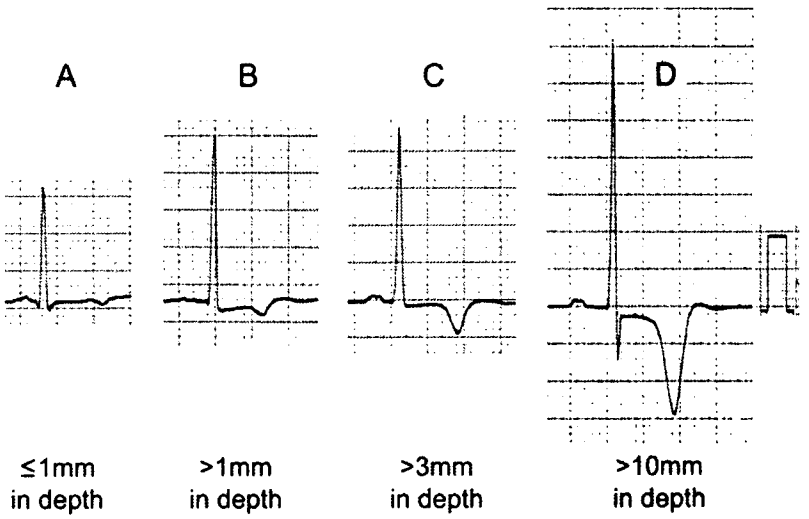

Figure 1 Various depth of negative T waves observed in carriers with disease-causing mutations for HCM

(A) Negatives T wave ⩽ 1 mm in depth; (B) negative T wave > 1 mm in depth; (C) negative T wave > 3 mm in depth; and (D) negative T wave > 10 mm in depth.

particular interest since Japanese investigators described a form of apical HCM with giant negative T waves [3,4]. In addition to the criterion of giant negative T waves (> 10 mm in depth) [5], criteria of conventional negative T waves (> 3 mm in depth) [6] and shallow negative T waves (> 1 mm in depth) [7,8] have been commonly used in clinical practice of HCM, resulting in various definitions of negative T waves being applied in each previous study [3–8]. However, differences in the diagnostic value among various criteria of negative T waves in HCM have not been clarified yet. Furthermore, the diagnostic value of negative T waves in the detection of carriers of HCM using definitive genotyping as the reference standard has not been addressed. Therefore the aim of the present study was to determine the most accurate diagnostic definition of negative T waves for HCM based on a molecular genetic diagnosis.

MATERIALS AND METHODS

Subjects

A total of 20 families with HCM, in which the disease-causing mutations were identified, were studied. After the mutation was identified in the proband with HCM, the family members were examined using a 12-lead ECG and echocardiography, and blood samples were obtained for genetic examination. A total of 173 subjects were included in the present study. Of these, 12 subjects were excluded because of a history of anterior myocardial infarction, pre-excitation, hypertension or conduction disturbance. Subsequently, 161 subjects were analysed. Subjects aged < 30 years were defined as young, and those aged ⩾ 30 years were defined as adult [8]. Informed consent was obtained from all of the subjects or their guardians in accordance with the guidelines of the Bioethical Committee on Medical Research, School of Medicine, Kanazawa University, Kanazawa, Japan.

Detection of mutations

DNA of the probands was isolated from peripheral white blood cells, as described previously [9]. Amplification of genomic DNA was performed using PCR. Oligonucleotide primers were used to amplify exons of *MYBPC3* (cardiac myosin binding protein-C), *MYH7* (β-myosin heavy chain), *TNNT2* (cardiac troponin T) and *TNNI3* (cardiac troponin I) genes, as described previously [10–13]. Mutations were screened using single-strand conformational polymorphism analysis of amplified DNA. The nucleotide sequences of the cloned PCR products were determined on both strands by the dye terminator cycle sequencing method using an automated fluorescent sequencer (ABI PRISM™ 310 Genetic Analyser; PE Biosystems) for abnormal single-strand conformational polymorphism patterns. The sequence variations were confirmed by restriction enzyme digestion. The same method was used to determine the genotype in DNA from family members of the probands.

Definition of negative T waves

A standard 12-lead ECG was recorded in all subjects in the supine position during quiet respiration. The diagnostic value of negative T waves was analysed according to the depth under the isoelectric line (Figure 1). The criteria of negative T waves were defined as follows based on previous studies: Criterion 1, negative T wave

> 10 mm in depth (Figure 1D) in any leads [5]; Criterion 2, negative T wave > 3 mm in depth (Figure 1C) in at least two leads, except aVR, V1 and V2 [6]; and Criterion 3, negative T wave > 1 mm in depth (Figure 1B) in at least two leads, except aVR, V1 and V2 [7]. Negative T waves ⩽ 1 mm were not considered abnormal (Figure 1A). Electrocardiographic abnormalities other than negative T waves were defined as follows: LVH [LV (left ventricular hypertrophy] assessed by a Romhilt–Estes score ⩾ 4 [14], ST-segment depression of an upsloping type > 0.1 mV at 0.08 s after the J point or those of horizontal or downsloping type > 0.05 mV [7], and Q wave > 3 mm in depth and/or > 0.04 s in duration in at least two leads, except aVR [8].

Echocardiographic criteria

Standard M-mode and two-dimensional echocardiographic studies were performed to identify and quantify morphological features of the left ventricle. In addition to parasternal long- and short-axes views, apical two- and four-chamber views were examined carefully in order to avoid misdiagnoses of LVH, particularly in the apex region. LV MWT (maximum wall thickness) ⩾ 13 mm in adults or ⩾ 95% CI (confidence interval) of the theoretical value in children was considered the diagnostic criteria for HCM [15].

Statistical analysis

Sensitivity was defined as true positives/(true positives + false negatives) × 100; specificity was defined as true negatives/(true negatives + false positives) × 100; PPV (positive predictive value) was defined as true positives/(true positives + false positives) × 100; NPV (negative predictive value) was defined as true negatives/(true negatives + false negatives) × 100; and accuracy was defined as (true positives + true negatives)/(true positives + true negatives + false positives + false-negatives) × 100 [16]. Continuous data are expressed as means ± S.D. and were analysed with unpaired two-tailed Student's t tests. Categorical data were compared using χ^2 tests. Sensitivity was compared with McNemar's χ^2 test. Differences were considered to be statistically significant at a P value < 0.05.

RESULTS

Genetic results and characteristics of the subjects

Ten different mutations were identified in 20 families. Genetic analysis revealed that 97 of the 161 subjects enrolled were genetically affected, and 64 subjects were genetically unaffected. Of the 97 genetically affected subjects, 30 were associated with mutations in the *MYBPC3* gene (Arg820Gln, $n = 12$; c.2067 + 1G > A, $n = 14$; and Del593C, $n = 4$), five were associated with mutations in the *MYH7* gene (Ala26Val, $n = 3$; and Glu935Lys, $n = 2$), 21 were associated with mutations in the *TNNT2* gene (Arg92Trp, $n = 8$; Lys273Glu, $n = 10$; Val85Leu, $n = 1$; and Phe110Ile, $n = 2$), and 41 were associated with a mutation in the *TNNI3* gene (Lys183Del; $n = 41$). All mutations have been identified and described elsewhere [9,17–23]. The demographics and the clinical characteristics of the study population are shown in Table 1. There were no statistically significant differences in the mean age and gender distribution between carriers and non-carriers.

Table 1 Clinical characteristics of the study groups

$^*P < 0.01$, $^{**}P < 0.001$ and $^{***}P < 0.0001$ compared with the non-carriers. IVST, interventricular wall thickness; PWT, posterior wall thickness; LVDd, LV end-diastolic dimension; LVDs, LV end-systolic dimension; FS, fractional shortening; LAD, left atrial dimension.

	Carriers	Non-carriers
n	97	64
Age (years)	44.9 ± 21.8	39.8 ± 21.2
Male/female (*n*)	45/52	28/36
Echocardiography		
MWT (mm)	15.8 ± 5.8***	9.5 ± 1.4
IVST (mm)	15.1 ± 5.7***	9.4 ± 1.6
PWT (mm)	10.5 ± 2.1***	9.0 ± 1.5
LVDd (mm)	43.9 ± 5.3*	46.3 ± 4.3
LVDs (mm)	27.9 ± 6.3	28.9 ± 4.1
FS (%)	36.7 ± 9.0	37.8 ± 6.5
LAD (mm)	37.2 ± 8.4**	32.9 ± 5.6
Genes (*n*)		
TNNI3	41	31
TNNT2	21	16
MYBPC3	30	15
MYH7	5	2

Diagnostic value of various criteria of negative T waves in detecting carriers

Table 2 shows the diagnostic value of the various criteria of negative T waves for carriers with disease-causing mutations. In the young and adult populations, Criterion 3 (negative T waves > 1 mm in depth) had the highest sensitivity (21% in young and 52% in adult) while retaining a high specificity (100% in the young and 93% in adults), which resulted in the highest accuracy (57% in young and 67% in adult). All three criteria had higher diagnostic values in the adult population compared with those in the young population, with a higher sensitivity, resulting in a higher accuracy. Overall, in all subjects ($n = 161$), the sensitivity, specificity, PPV, NPV and accuracy of Criterion 3 were 43%, 95%, 93%, 53% and 64% respectively (Table 2). In contrast with Criterion 3, Criterion 1 (giant negative T waves) had a sensitivity of only 5%. Of the five subjects with giant negative T waves, three were associated with mutations in the *TNNT2* gene, one with a mutation in the *TNNI3* gene and one with

Table 2 Diagnostic value of various criteria of negative T waves in carriers with disease-causing mutations

*$P < 0.05$ compared with the sensitivity of Criterion 1; †$P < 0.001$ compared with the sensitivity of Criterion 1; ‡$P < 0.0001$ compared with the sensitivity of Criterion 2; §$P < 0.0001$ compared with the sensitivity of Criterion 1.

Criteria	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
Age < 30years ($n = 51$)					
Criterion 1	4	100	100	46	47
Criterion 2	11	100	100	48	51
Criterion 3	21*	100	100	51	57
Age ⩾ 30years ($n = 110$)					
Criterion 1	6	100	100	39	38
Criterion 2	32†	100	100	47	57
Criterion 3	52‡§	93	92	54	67
All subjects ($n = 161$)					
Criterion 1	5	100	100	41	43
Criterion 2	26§	100	100	47	55
Criterion 3	43‡§	95	93	53	64

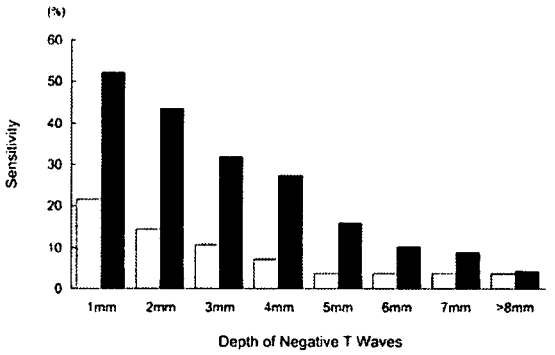

Figure 2 Sensitivity of negative T waves according to the different depth of negative T waves

Open bars, the sensitivity of carriers aged < 30 years; closed bars, the sensitivity of carriers aged ⩾ 30 years.

mutations in the *MYBPC3* gene. None of the five subjects with giant negative T waves had isolated hypertrophy localized to the cardiac apex.

Figure 2 shows the sensitivity of the various definitions of negative T waves for carriers with disease-causing mutations. In both the young and adult populations, the sensitivity of negative T waves decreased according to the depth of the T waves in the criteria. Sensitivities of all of the definitions of negative T waves were higher in the adult population compared with that in the young population.

Negative T waves in clinically affected carriers and pre-hypertrophic carriers

Table 3 shows the diagnostic value of the various criteria of negative T waves in clinically affected carriers

Table 3 Diagnostic value of various criteria of negative T waves in clinically affected carriers with LVH by echocardiography

*$P < 0.001$ compared with the sensitivity of Criterion 2; †$P < 0.0001$ compared with the sensitivity of Criterion 1; ‡$P < 0.001$ compared with the sensitivity of Criterion 2.

Criteria	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	Accuracy (%)
Criterion 1	8*	100	100	51	53
Criterion 2	36	100	100	60	68
Criterion 3	58†‡	95	93	69	76

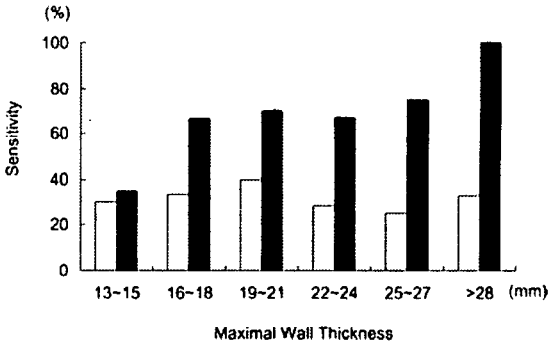

Figure 3 Sensitivity of abnormal Q waves (open bars) and negative T waves (closed bars) according to the different extent of MWT in carrier subjects

with LVH by echocardiography. Of the three criteria, Criterion 3 had the highest sensitivity (58%) while retaining a high specificity (95%), which resulted in the highest accuracy (76%). Differences in the sensitivity of negative T waves were evaluated according to the variable extent of LVH, and the sensitivity of negative T waves was compared with that of abnormal Q waves, which are also one of the major electrocardiographic abnormalities in HCM. Using Criterion 3, the sensitivity of negative T waves increased in a stepwise direction with the increasing extent of MWT ($P < 0.001$), whereas there was less association between the sensitivity of abnormal Q waves and the extent of MWT (Figure 3). MWT was significantly greater in carriers with ($n = 42$) than without ($n = 55$) negative T waves (19.0 ± 5.2 compared with 13.5 ± 5.1 mm respectively; $P < 0.0001$). In contrast, MWT was similar between carriers with ($n = 28$) and without ($n = 69$) abnormal Q waves (16.0 ± 5.7 mm compared with 15.6 ± 5.6 mm respectively; $P =$ not significant).

Negative T waves and abnormal Q waves were also applied to the identification of pre-hypertrophic carriers in clinically healthy subjects without echocardiographic evidence of LVH. Of the 97 genetically affected subjects, 31 did not manifest LVH by echocardiography, and

they were defined as pre-hypertrophic carriers. MWT and age were not significantly different between pre-hypertrophic carriers and non-carriers (MWT, 9.6 ± 1.9 and 9.5 ± 1.4 mm respectively; age, 31.0 ± 22.4 and 39.8 ± 21.2 years respectively). The frequency of negative T waves assessed by Criterion 3 was similar between pre-hypertrophic carriers and non-carriers ($n = 64$) (12.9 % compared with 4.7 % respectively; P = not significant). In contrast, the frequency of abnormal Q waves was significantly higher in pre-hypertrophic carriers than in non-carriers (22.6 % compared with 4.7 %; $P < 0.01$).

DISCUSSION

The results of the present study have demonstrated that Criterion 3 for negative T waves (i.e. negative T wave > 1 mm in depth) was the most accurate in diagnosing carriers of HCM in a genotyped population. Furthermore, the diagnostic value of negative T waves may increase in a stepwise direction with the increasing extent of LVH, whereas abnormal Q waves may be useful in detecting carriers without LVH.

Diagnostic value of various criteria of negative T waves in HCM

In conventional electrocardiographic criteria for HCM, Criterion 2 (> 3 mm in depth) has been used as a definition of negative T waves for HCM [6]. In the present study, in both the young and adult populations, we have demonstrated that Criterion 3 (negative T wave > 1 mm in depth in at least two leads except aVR, V1 and V2) had the highest diagnostic accuracy of the three criteria for detecting not only clinically affected carriers, but also all carriers, including pre-hypertrophic carriers (Tables 2 and 3). Using Criterion 3, we were able to detect 17 additional carriers (14 clinically affected carriers and three pre-hypertrophic carriers) compared with results using the conventional criterion (Criterion 2), with an increase of only three false positives. In this regard, Charron et al. [6] have shown that many false positives were observed with minor European diagnostic criteria, especially for minor repolarization changes, in detecting carriers of HCM. However, the definition of minor repolarization changes was not mentioned in their study. In the present study, Criterion 3 did not include negative T waves ⩽ 1 mm in depth (Figure 1A), which may contribute to a high specificity of 95 % in all subjects (Table 2).

In contrast with negative T waves for Criterion 3, giant negative T waves (Criterion 1) were observed in only five (5.2 %) out of 97 carriers (Table 3). Previous reports from Japan indicate that patients with giant negative T waves have hypertrophy confined to the LV apex [3,4]. However, in the present study, none of the five subjects having giant negative T waves had this form of apical HCM. Similar findings were reported in studies performed on Western patients [5,24]. Racial differences have been postulated to explain the discrepancies in echocardiographic morphology between Japanese and Western patients with giant negative T waves. As three out of the five subjects had mutations in the *TNNT2* gene (Table 3) and as our study subjects were all of Japanese descent, we postulate that differences in the disease-causing genes may also contribute to clinical manifestations of HCM with giant negative T waves. Further study is needed to clarify the genetic determinants of the phenotype in patients with giant negative T waves.

Diagnostic value of negative T waves with respect to phenotypes

In clinical practice, the identification of pre-hypertrophic carriers is important, because sudden death occurs in young asymptomatic patients with HCM, even in the absence of LVH [25]. In this regard, it has been demonstrated previously [7,8] that abnormal Q waves may be observed before the appearance of LVH in genotyped families. In fact, in the present study, the frequency of abnormal Q waves was significantly higher in pre-hypertrophic carriers compared with non-carriers, whereas the frequency of negative T waves was similar between both groups. These findings suggest that abnormal Q waves may be more useful in detecting pre-hypertrophic carriers than negative T waves. On the other hand, in detecting carriers with LVH, the sensitivity of negative T waves increased in a stepwise direction with the increasing extent of LVH (Figure 3), whereas there was less association between the sensitivity of abnormal Q waves and the extent of LVH. Thus we note that the diagnostic value of major electrocardiographic abnormalities, such as negative T waves and abnormal Q waves, may differ according to the clinical manifestations of HCM in a genotyped population.

Study limitations

There may be a significant bias in the subjects being studied, because 41 (42 %) out of the 97 genetically affected subjects had a Lys183Del mutation in the *TNNI3* gene. Furthermore, the present study did not include all disease-causing genes and, therefore, our results may not be applicable to all patients with HCM.

Conclusions

The present study is the first to attempt to define the most accurate diagnostic criterion of negative T waves on the basis of a molecular genetic diagnosis. We propose that Criterion 3 (negative T wave > 1 mm in depth in at least two leads, except aVR, V1 and V2) may be the most accurate diagnostic definition of HCM in genotyped populations. The present study also found that the diagnostic value of negative T waves increased in a stepwise direction

with the increasing extent of LVH, whereas abnormal Q waves had preferable diagnostic value in detecting pre-hypertrophic carriers. We note that negative T waves have a different diagnostic value according to the different criteria and phenotypes in genotyped populations of HCM.

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A novel β-myosin heavy chain gene mutation, p.Met531Arg, identified in isolated left ventricular non-compaction in humans, results in left ventricular hypertrophy that progresses to dilation in a mouse model

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ABSTRACT

Mutations in the βMHC (β-myosin heavy chain), a sarcomeric protein are responsible for hypertrophic and dilated cardiomyopathy. However, the mechanisms whereby distinct mutations in the βMHC gene cause two kinds of cardiomyopathy are still unclear. In the present study we report a novel βMHC mutation found in a patient with isolated LVNC [LV (left ventricular) non-compaction] and the phenotype of a mouse mutant model carrying the same mutation. To find the mutation responsible, we searched for genomic mutations in 99 unrelated probands with dilated cardiomyopathy and five probands with isolated LVNC, and identified a p.Met531Arg mutation in βMHC in a 13-year-old girl with isolated LVNC. Next, we generated six lines of transgenic mice carrying a p.Met532Arg mutant αMHC gene, which was identical with the p.Met531Arg mutation in the human βMHC. Among these, two lines with strong expression of the mutant αMHC gene were chosen for further studies. Although they did not exhibit the features characteristic of LVNC, approx. 50% and 70% of transgenic mice in each line displayed LVH (LV hypertrophy) by 2–3 months of age. Furthermore, LVD (LV dilation) developed in approx. 25% of transgenic mice by 18 months of age, demonstrating biphasic changes in LV wall thickness. The present study supports the idea that common mechanisms may be involved in LVH and LVD. The novel mouse model generated can provide important information for the understanding of the pathological processes and aetiology of cardiac dilation in humans.

Key words: cardiomyopathy, left ventricular non-compaction, mutation, myosin heavy chain, transgenic mouse.

Abbreviations: DCM, dilated cardiomyopathy; FS, fractional shortening; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; gDNA, genomic DNA; H and E, Haemotoxylin and Eosin; HCM, hypertrophic cardiomyopathy; IVST, interventricular septal thickness; LV, left ventricular; LVD, LV dilation; LVEDD, LV end-diastolic diameter; LVESD, LV end-systolic diameter; LVEF, LV ejection fraction; LVH, LV hypertrophy; LVNC, LV non-compaction; MHC, myosin heavy chain; PWT, posterior wall thickness; RT, reverse transcription; SSCP, single-strand conformational polymorphism.

[1] These authors contributed equally to this study.

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INTRODUCTION

HCM (hypertrophic cardiomyopathy) and DCM (dilated cardiomyopathy) are very common types of cardiomyopathy. HCM is characterized by abnormal cardiac hypertrophy, fibrosis and myofibrillar disarray. DCM is defined by ventricular chamber dilation and impaired contractile function. Genetic studies have indicated that approx. half of HCM is familial and caused by a mutation in sarcomeric proteins [1]. Among the causative genes, βMHC (β-myosin heavy chain) is most commonly associated with HCM [2]. Disease penetrance, severe hypertrophy and high risk of sudden cardiac death are more frequently associated with mutations in βMHC than in the other sarcomere protein genes, such as cardiac troponin T, α-tropomyosin and cardiac myosin-binding protein C genes [3]. On the other hand, approx. 25–30 % of idiopathic DCM is caused by a missense mutation or deletion in cardiac genes such as βMHC, cardiac troponin T, cardiac actin, lamin A/C and dystrophin [2,4]. In DCM, βMHC mutations are also relevant to early onset and serious cardiac dysfunction [5,6]. In addition, cases in which HCM has progressed to DCM have been reported [7,8]. This progression occurs in 10–15 % of patients with HCM [9]. However, the mechanisms whereby mutations of the βMHC gene lead to cardiac hypertrophy or dilation remain unclear. Moreover, it is still not clear whether there is a common aetiology for these diseases.

Transgenic mouse models expressing mutant proteins provide a means of gaining insight into the pathophysiological and clinical features of human cardiomyopathy. For example, transgenic mice carrying the missense mutation p.Arg403Gln in the αMHC gene, the murine analogue of the human βMHC gene, recapitulate the characteristics of human HCM [5,10], whereas homozygous mice for the same transgene develop DCM-like disease [11]. The homozygous mutant transgenic mice of another sarcomeric protein, myosin-binding protein C, are also affected with DCM [12]. However, the mechanisms of the primary cardiac dilation caused by the βMHC mutation are still unclear. This is because animal models bearing the analogous mutation within the sarcomeric protein genes identified in human DCM have not so far been investigated.

In the present study we explored mutations in the sarcomere proteins in a patient with isolated LVNC [LV (left ventricular) non-compaction] and found a novel mutation, p.Met531Arg, in the βMHC. We then generated the αMHC transgenic mice with a p.Met532Arg mutation corresponding with the p.Met531Arg in human βMHC. Although these transgenic mice did not develop LVNC, they showed the pathological changes from HCM to DCM. The results of our present study suggest that HCM and DCM may be closely related pathological conditions rather than independent diseases.

MATERIALS AND METHODS

Patients

The study subjects comprised 99 unrelated patients with DCM (27 familial and 72 sporadic or unknown) and five patients with isolated LVNC (one familial and four sporadic or unknown). The diagnosis of DCM was based on the criteria of the Collaborative Research Group of the European Human and Capital Mobility Project on Familial Dilated Cardiomyopathy [13], i.e. echocardiographic demonstration of depressed systolic function of the left ventricle [LVEF (LV ejection fraction) < 0.45 and/or fractional shortening < 0.25) and a dilated left ventricle [LVEDD (LV end-diastolic diameter) > 117 % of the predicted value corrected for age and body surface area) in the absence of other cardiac or systemic causes. The diagnosis of isolated LVNC was based on the following echocardiographic criteria [14] in four patients: (i) the absence of coexisting cardiac abnormalities; (ii) the presence of a two-layer structure in the myocardium, with a compacted thin epicardial band and a much thicker noncompacted endocardial layer of trabecular meshwork with deep endomyocardial spaces showing a maximal end systolic ratio of noncompacted to compacted layers of > 2; (iii) the predominant localization of the non-compaction in the mid-lateral, apical and mid-inferior walls; and (iv) colour Doppler evidence of deep perfused intertrabecular recesses. One patient (with the βMHC mutation) was diagnosed by postmortem examination because echocardiographic evidence of LVNC was lacking at that time.

Informed consent was obtained from all subjects in accordance with the guidelines of the Bioethical Committee on Medical Research, School of Medicine, Kanazawa University. gDNA (genomic DNA) was purified from white blood cells [15].

Detection of mutation

Oligonucleotide primers used for the amplification of the βMHC gene exons were based on published sequences [16] and sequences obtained from GenBank®. PCR was used for amplification of gDNA, and SSCP (single-strand conformational polymorphism) analysis of this amplified DNA was then performed with a slight modification of a method published previously [17,18]. DNA fragments with abnormal SSCP patterns were sequenced by the dye terminator cycle sequencing method using an automated fluorescent sequencer (ABI Prism™ 310 genetic analyser; PE Biosystems). To increase the probability of detecting the presence of any sequence change, SSCP was carried out at two different temperatures for each exon, and the size of fragments for SSCP was kept at less than 300 bp. Sequence analysis results were validated by restriction enzyme digestion with Eco81I. To confirm the paternity of the subjects, five short tandem-repeat systems TH01, vWA, LPL, F13B and FES/FPS were investigated as

previously described [19]. From the allele distributions of each short tandem-repeat locus, the probability of paternity was calculated based on the allelic frequencies in the Japanese population [20]. Screening for mutations in other genes, including dystrophin, myosin-binding protein-C, α-tropomyosin, cardiac troponin C, cardiac troponin T, cardiac troponin I, cardiac α-actin, lamin A/C, G4.5, ZASP and α-dystrobrevin was performed by direct sequencing in the proband.

Transgenic constructs

Murine αMHC (the analogous gene of the human βMHC) cDNA (5.9 kbp) and the transgenic construct, αMHC clone 918 (9.1 kbp), were generously provided by Dr J. Robbins (University of Cincinnati, Cincinnati, OH, U.S.A.). The αMHC cDNA was mutated using site-directed mutagenesis according to the manufacturer's protocol (Stratagene), which resulted in a p.Met532Arg mutation in the protein. The mutagenic primers used were 5′-CCCATGGGCATCAGGTCCATCCTGGAGG-3′ and 5′-CCTCCAGGATGGACCTGATGCCCATGGG-3′. The mutated cDNA was sequenced to confirm the presence of the correct mutation and the absence of undesired errors during mutagenesis. The mutated αMHC cDNA was subcloned into the SalI site of αMHC clone 918 between the murine αMHC promoter and the human growth hormone polyadenylation site. The transgenic construct was purified by caesium chloride ultracentrifugation and digested with EcoRI to release a 12.1 kbp fragment that was used for microinjection. This fragment was purified by agarose gel electrophoresis, dissolved in 10 mmol/l Tris/HCl (pH 7.5) containing 0.2 mmol/l EDTA and injected into the pronucleus of fertilized zygotes from BDF1 mice. The microinjections were performed at Japan SLC Inc.

Generation of transgenic mice

Founder transgenic mice were identified by hybridization of tail DNA to a ^{32}P-labelled DNA probe corresponding to the human growth hormone 3′-untranslated region (a 630 bp HindIII/EcoRI fragment from the transgenic construct). PCR was also used to identify the transgenic mice. A forward (5′-TGCCCACCAGCCTTGTCCTAATAA-3′) and a reverse (5′-CAGGGAAGGGAGCAGTGGTTCAC-3′) primer were derived from the human growth hormone sequence; PCR with these primers produced a 411 bp fragment using DNA of mice harbouring the transgene. Stable transgenic lines were generated by mating founder transgenic mice with non-transgenic BDF1 mice. Male transgenic mice and non-transgenic male littermates were used for analysis. Experiments were conducted according to guidelines for the care and use of laboratory animals in Kanazawa University and safety guidelines for gene manipulation experiments.

RT (reverse transcription)–PCR

RT–PCR was performed to assess the amount of αMHC, βMHC and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) mRNA in wild-type and transgenic hearts. Total RNA was isolated from the heart using the AGTC (acid guanidinium thiocyanate/phenol/chloroform) method [21] and the first strand cDNA was synthesized using standard cDNA synthesis reagents (first strand cDNA synthesis kit for RT–PCR; Roche) according to the manufacturer's protocol. To assess the αMHC transgene expression, PCR cycling was performed at 94 °C for 60 s, 59 °C for 60 s and 72 °C for 60 s for 30 cycles using rTaq (Takara). The forward (5′-GCCGCGCCAGTACTTCATAGGT-3′) and the reverse (5′-TTGCGAGGCTTCTGGAAGTTGTTA-3′) primers were derived from the murine αMHC cDNA sequence. When the PCR product (351 bp) was digested with XhoI, fragments of 248 bp and 103 bp were generated from the endogenous allele, while the 351 bp fragment from the transgene was not digested because the XhoI site was abolished by site-directed mutagenesis. To determine transcript levels of αMHC and βMHC genes, cDNA products were amplified by cycling at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s for 25 cycles using rTaq (Takara). Sequences of primers were as follows: αMHC, 5′-ATCGCCGAGTCCCAGGTCAAC-3′ and 5′-TATTGGCCACAGCGAGGGTCTG-3′; βMHC, 5′-GTGCCAAGGGCCTGAATGAGG-3′ and 5′-AGGGCTGTTGCAAAGGCTCCAG-3′; GAPDH, 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′.

Echocardiography

Echocardiographic studies were performed using a 12-MHz phased array probe and a Sonos 5500 ultrasonograph (Philips Medical Systems). Mice were anaesthetized lightly by intraperitoneal injection of 10 μg/ml of pentobarbital sodium at a dose of 10 μl/g of body mass. Body fur of the upper sternal and subxiphoid areas was shaved and the exposed skin was moistened for better acoustic coupling. M-mode echocardiographs of the left ventricle were recorded at the middle of the left ventricle. IVST (interventricular septal thickness), PWT (posterior wall thickness), LVESD (LV end-systolic diameter) and LVEDD were measured and the percentage FS (fractional shortening) was calculated as (LVEDD−LVESD)/LVEDD.

Histological examination

Mice were anaesthetized, and the hearts were removed while still beating, rinsed in PBS, and fixed in 10% formalin before sectioning. The hearts were dehydrated through a graded alcohol series and embedded in paraffin. Longitudinal 8 μm sections were cut and stained with H and E (Haematoxylin and Eosin or with Azan and examined under an Olympus IX71

microscope. Photomicrographs were obtained with an Olympus DP70 digital camera. For electron microscopic analysis, the hearts were removed while still beating, and immersed in a cardioplegic solution (25 mmol/l KCl and 5 % dextrose in PBS) to ensure complete myocardial relaxation. Blocks of 1 mm^2 were dissected from the left ventricular free wall. The blocks were trimmed, fixed in 2.5 % glutaraldehyde in cacodylate buffer at pH 7.4, postfixed in 2.0 % osmium tetroxide, dehydrated in ethanol in propylene oxide, and embedded in EPOK812 (Oken). Sections were cut at 60 nm, stained with lead citrate and uranyl acetate, and examined with a JEM-1210 transmission electron microscope (JEOL).

Statistical analysis

Statistically significant differences between groups of non-transgenic and transgenic mice were assessed using an unpaired Student's t test. Results are expressed as means $\pm$ S.D. A P value of <0.05 was considered statistically significant.

RESULTS

Baseline characteristics of the study patients

The 99 adult patients with DCM comprised 68 men and 31 women (mean age 58.1 $\pm$ 13.1 years, range 21–82). The three adult patients (aged 29, 57 and 60 years), and two young patients (aged 10 and 13 years) with isolated LVNC comprised two men and three women. In the patient groups with DCM and isolated LVNC, the LVEDD was 64.8 $\pm$ 7.4 mm and 62.4 $\pm$ 12.8 mm respectively, the LVESD was 55.8 $\pm$ 8.2 mm and 54.2 $\pm$ 10.3 mm respectively and the LVEF was 29.1 $\pm$ 9.8 mm and 26.2 $\pm$ 13.7 mm respectively.

A point mutation was found in the βMHC gene of an isolated LVNC patient

SSCP analysis identified polymorphisms in the βMHC gene in 17 patients with DCM (14 with c.189C>T, two with c.732T>C, four with c.1062C>T, one with c.1128C>T and three with c.3027T>C) which have been reported previously [22]. A mutation was found in the βMHC gene derived from the proband, a 14-year-old girl (Figure 1A, II-4) with isolated LVNC. Sequence analysis of the abnormal polymorphism conformer revealed a nucleotide substitution in codon 531, resulting in substitution of a methionine residue by arginine (Figure 1B). This mutation was not detected in 200 control individuals. No other mutations in the βMHC or other genes, including myosin-binding protein-C, α-tropomyosin, cardiac troponin T, cardiac troponin I, actin, lamin A/C, G4.5, ZASP or α-dystrobrevin were identified in this proband.

Cardiac examination of the proband (II-4) revealed left ventricular dilation and diminished contractile function like DCM (Table 1), although she was asymptomatic. Heart failure progressed and she died at the age of 14 in 1999. Figures 1(C) and 1(D) show the ECG and the photograph of the heart at autopsy. The ECG showed left ventricular hypertrophy. In the autopsy, the left ventricle was markedly dilated and prominent numerous trabeculations with intertrabecular recesses were found at the lateral wall, the inferior wall and the apex of the left ventricle. The thickness ratio between the noncompacted and compacted layer was 3–5. Other congenital cardiac malformations were not found. These findings were consistent with isolated LVNC. On microscopic examination, mild vacuolation was evident in the myocytes, and moderate subendocardial fibrosis was observed (results not shown). The endocardium was thickened by fibrous tissue, but no fibroelastosis was identified. Myocyte hypertrophy was not found. She had an identical twin sister (II-3), who had been diagnosed with DCM and also died of heart failure at the age of 13 (Figure 1A).

Table 1 Echocardiographic data in the proband and her identical twin sister

Parameter	II-3	II-4
Sex	Female	Female
Age (years)	13	13
LVEDD (mm)	70	70
LVEDS (mm)	62	58
FS (%)	11	17
IVST (mm)	10	6
PWT (mm)	10	9

We could not find any abnormalities in the parents (Figure 1A, I-1 and I-2), elder sister (II-1) and elder brother (II-2) by clinical examination, and none of them had the p.Met531Arg mutation in the βMHC gene. The presence of this sequence variant was confirmed with restriction enzyme digest analysis. The 201 bp fragment was digested with Eco81I. The T to G transition at nucleotide position 1674 allows cleavage (yielding 16 bp and 185 bp fragments), whereas the wild-type allele is not cut. Only the proband was heterozygous for the T to G base change (Figure 1A). The allele distribution of five short tandem-repeat loci was then examined in the subject's parents to determine the paternity of the proband. The probability of paternity was 0.9873, which was considered to be highly likely. Thus we concluded that the identified p.Met531Arg mutation in the βMHC gene in the proband was *de novo*.

Generation of p.Met532Arg αMHC transgenic mice

To elucidate the importance of p.Met531Arg of βMHC observed in the human patient, we constructed a

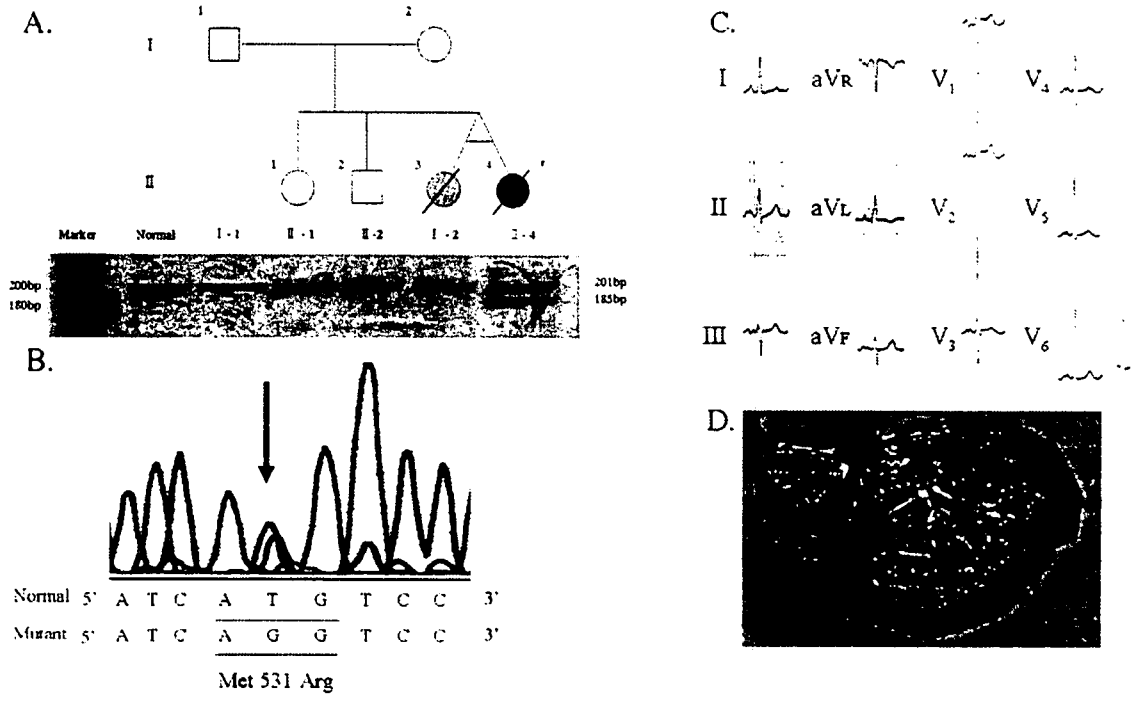

Figure 1 A point mutation was found in the βMHC gene of an isolated LVNC patient

(A) Upper panel: pedigree I and II indicate generations. An asterisk indicates the proband. Open circles and open boxes indicate female and male normal individuals respectively. A closed circle indicates a phenotype-positive, genotype-positive individual. A hatched circle indicates a phenotype-positive, genotype unknown individual. The forward slash indicates deceased individuals. Lower panel: PCR-restriction fragment length polymorphism analysis. Digestion of the PCR products with Eco81I generates polymorphic restriction fragments of 201 bp (wild-type allele) and/or 185 bp (mutant allele). (B) DNA sequence analysis. A single nucleotide transition from thymine to guanine at nucleotide position 1678 of βMHC was identified. This mutation leads to a missense mutation of Met^{531} to an arginine residue. (C) Twelve-lead ECG of the proband (II-4). (D) A photograph of the heart of the proband (II-4) at autopsy. Features of the heart were consistent with isolated LVNC.

transgenic vector based on the mouse αMHC (the analogous gene of the human βMHC) clone 918 (Figure 2A). Nucleotides 1604 and 1614 of the coding region were mutated using site-directed mutagenesis. c.1604T>G resulted in a p.Met532Arg mutation, and c.1614C>G resulted in the deletion of the XhoI site without amino acid change and enabled us to distinguish the mutant cDNA from wild-type cDNA. PCR of gDNA revealed that we could obtain 17 transgenic lines (Figure 2B), and six independent lines expressing transgene-derived αMHC mRNA (Figure 2C). Densitometric analysis of PCR products revealed that each transgenic line had unique ratios of transgene (a 351 bp band) to endogenous (248 bp and 103 bp bands) αMHC, which could be used to distinguish each transgenic line. Among these transgenic lines, we chose line numbers 41 and 23 because they showed severe phenotypes and expressed more αMHC mRNA than the other transgenic lines.

The non-transgenic mice and transgenic mice seemed to grow normally. However, by 12 months of age 19% ($n=33$) and 20% ($n=20$) of mice died in transgenic mice lines 23 and 41 respectively, compared with only one death in 30 (3.3%) non-transgenic mice (Figure 3). Transgenic mice died sporadically without showing prominent organ diseases except cardiac hypertrophy. The most likely cause of death was sudden cardiac arrest.

Echocardiography

Echocardiography was performed in seven transgenic mice of line 41 and six non-transgenic littermates. All mice were 8–10 months old and the mean age was not significantly different between the groups. Representative echocardiograms are shown in Figure 4. The difference in mean values of LVEDD, LVESD and FS were not statistically significant between non-transgenic and transgenic mice of line 41 (Table 2). However, IVST and PWT were significantly greater in transgenic mice of line 41 compared with non-transgenic mice.

Myocardial histopathology and morphology showed that transgenic mice developed HCM and DCM

There were no significant differences between non-transgenic mice and transgenic mice at 1 month of

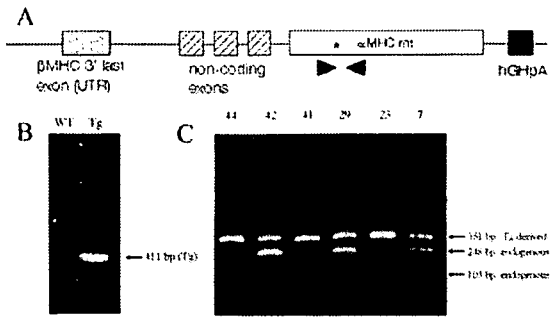

Figure 2 Transgenic construct and transcript expression

(A) A construct used to generate transgenic mice. Arrow heads indicate PCR primers for detection of αMHC cDNA. The asterisk indicates the position of the mutation (c.1604T>G). UTR, untranslated region; hGH pA, human growth hormone polyadenylation signal. (B) Identification of transgenic mice by PCR analysis of gDNA. A 411 bp PCR product was amplified using transgene specific primers for hGH pA. The 411 bp products are present only in the trangenic mice. (C) Analyses of transgene RNA expression by RT–PCR using primers in (A) in the hearts of six transgenic lines. When the PCR product (351 bp) was digested with XhoI, 248 bp and 103 bp fragments were generated from the endogenous allele, while the 351 bp fragment from the transgene was not digested, because the XhoI site was abolished by site-directed mutagenesis. Densitometric analysis of PCR products revealed that six transgenic lines (the number of the transgenic line is shown on each lane) had unique ratios of transgene to endogenous αMHC mRNA.

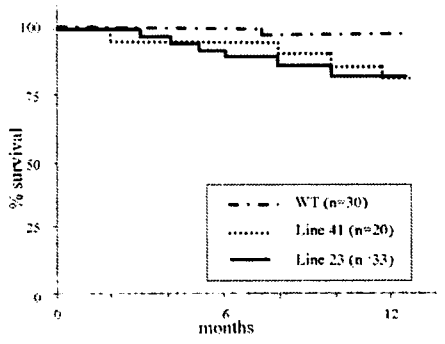

Figure 3 Survival curves of transgenic and non-transgenic mice

Non-transgenic mice (WT), $n = 30$; transgenic mice line 23, $n = 33$; transgenic mice line 41, $n = 20$.

age. Transgenic mice began to display left ventricular hypertrophy at 2–3 months of age, and showed left ventricular hypertrophy at about 12 months (Figure 5D). Striking histological and morphological abnormalities were observed in approx. 50% and 70% of transgenic mice of line 23 and line 41 respectively. When mice were approx. 18 months old, transgenic mice displayed enlarged atria and approx. 25% of transgenic mice progressed to exhibit left ventricular dilation compared with

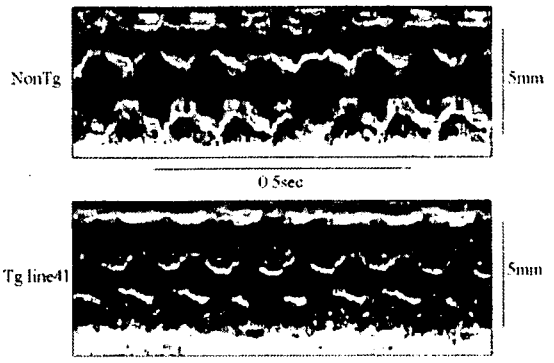

Figure 4 Echocardiographic analysis

Representative M-mode left ventricle images in 8- to 10 month old non-transgenic mice and transgenic mice line 41.

Table 2 Echocardiographic data in 8–10 month old non-transgenic mice and transgenic mice of line 41

Values are means ± S.D. HR, heart rate. $^*P < 0.05$ when compared with non-trangenic mice.

Parameter	Non-transgenic	Transgenic line 41
n	6	7
HR (beats/min)	590 ± 18	600 ± 13
LVEDD (mm)	3.57 ± 0.28	3.38 ± 0.30
LVESD (mm)	1.97 ± 0.18	1.84 ± 0.25
FS (%)	44.7 ± 2.0	45.7 ± 3.4
IVST (mm)	1.02 ± 0.08	1.16 ± 0.13*
PWT (mm)	1.01 ± 0.09	1.14 ± 0.11*

non-transgenic mice (Figures 5A–5C and 5E). However, transgenic mice did not show typical findings consistent with LVNC. Heart-to-body weight ratios at 12–15 months of age were significantly higher in transgenic mice compared with non-transgenic mice (Figure 6).

Histological examination of transgenic hearts revealed mild relative myocyte hypertrophy and myofibrillar disarray starting at 2–3 months of age. These features became more severe with aging. Multiple pleiotropic nuclei were also observed. These histological features were scattered throughout the left ventricular free wall. H and E- and Azan-stained sections of hearts from 15 month old mice showed interstitial fibrosis especially at the endocardium. Non-transgenic mice showed regular arrangement of myofibres and no fibrotic lesions (Figures 5F–5I).

Transmission electron microscopy was performed to examine the ultrastructure of transgenic and non-transgenic mice hearts at 16 months of age. The non-transgenic mice showed normal sarcomeric structure, with regularly aligned Z-bands (Figure 7A). In contrast, transgenic mice showed an abnormal sarcomeric structure. The sarcomere lengths were greatly reduced and the myofilaments were misaligned. The M-lines

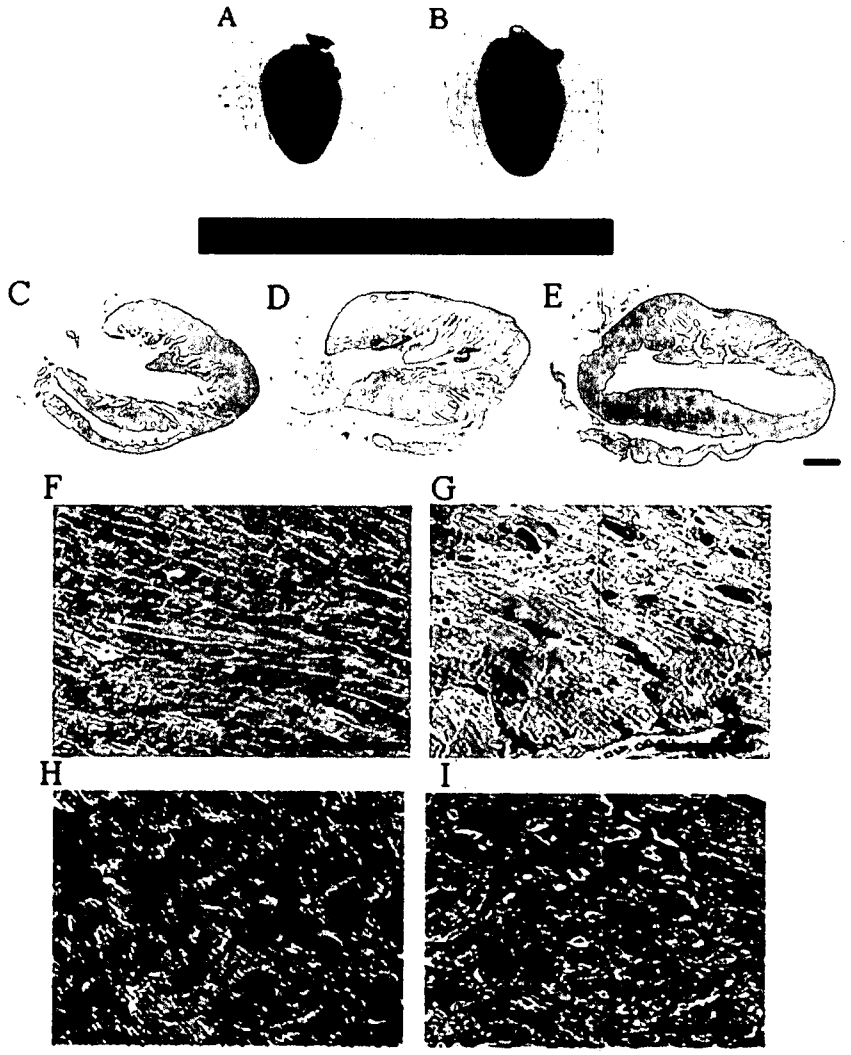

Figure 5 Histological analysis

Hearts were obtained from a non-transgenic mouse (A) and transgenic mouse line 41 (B) at 18 months of age. (C–E) Coronal sections of hearts stained with H and E. (C) Non-transgenic mouse (18 months old). (D) Transgenic mouse line 41 with left ventricular hypertrophy (12 months old). (E) Transgenic mouse line 41 with left ventricular dilation (18 months old). Scale bar represents 1 mm. Higher magnification views of H and E-stained left ventricle sections from a 15 month old non-transgenic (F) and transgenic mouse line 41 (G). Azan-stained sections of ventricles from a non-transgenic (H) and transgenic mouse line 41 (I). Scale bar represents 50 μm.

were indistinct and the Z-bands were thicker than those of non-transgenic mice (Figure 7B).

The expression level of βMHC (corresponding to αMHC in human), which is associated with a cardiac stress response, was compared in the hearts of transgenic mice of line 23 and transgenic mice of line 41 with non-transgenic mice at 4 months of age. Transgenic mice demonstrated significant increases in βMHC compared with age-matched non-transgenic mice (5.6-fold and 4.6-fold in lines 23 and 41 respectively). The amount of αMHC transcripts in transgenic hearts was the same as in age-matched non-transgenic hearts (Figure 8).

DISCUSSION

Generation of a novel transgenic mouse model having a point mutation in the αMHC gene that exhibits HCM developing to DCM

In the present study we have generated the first mouse model with a point mutation in the αMHC gene exhibiting HCM that developed to DCM, and it showed a more malignant phenotype compared with other αMHC mutant mice. At first, we identified a novel *de novo*

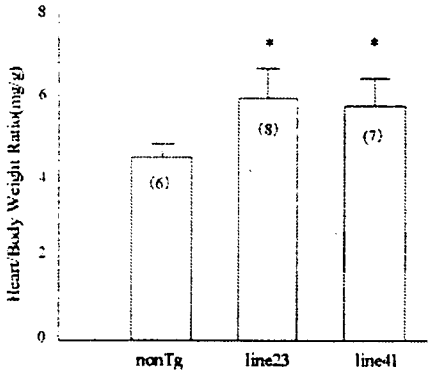

Figure 6 Ratios of heart-to-body weight for non-transgenic and transgenic mice of line 23 and line 41 at 12–15 months old

Bars represent means ± S.D. (*n* in parentheses). $^*P < 0.05$ compared with non-transgenic mice.

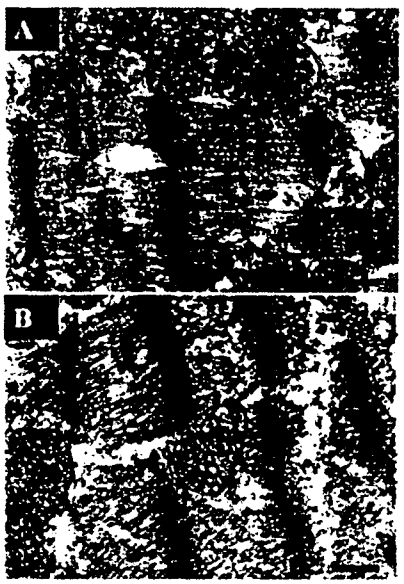

Figure 7 Electron micrographs of longitudinal sections of left ventricular cardiac myocyte cells from a non-transgenic mouse (A) and a transgenic mouse of line 23 (B) at 16 months

Scale bars represent 500 nm.

mutation in the βMHC gene in a patient with isolated LVNC, and generated αMHC transgenic mice with a p.Met532Arg missense mutation that is an analogous mutation of the patient. Although these mice did not develop LVNC, approx. 50–70% of them demonstrated the pathological and clinical features of human HCM after they were 2–3 months old. Moreover, 25% of transgenic mice progressed to exhibit DCM-like dilated phase HCM by 18 months of age. The phenotype of these p.Met532Arg αMHC transgenic mice is similar in

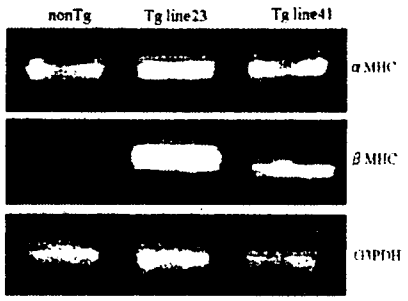

Figure 8 Semi-quantitative RT–PCR analysis of gene expression in hearts of 4-month-old mice

Expression levels of αMHC and βMHC were examined. The expression level of βMHC was increased 5.6-fold and 4.6-fold in transgenic mice line 23 and line 41 respectively. GAPDH was used as an internal control.

part to that of previously constructed αMHC transgenic mice models of HCM. It is not appropriate to compare the consequences of mutations by the severity of cardiac impairment of each mutant mouse because the amount of mutant cDNA expression and strains of mice are different. Nevertheless, our results suggest that the p.Met531Arg mutation may have more malignant consequences for cardiac function than other mutations in αMHC. For example, the mutation, p.Arg403Gln, in human βMHC caused severe HCM associated with early disease onset and short life expectancy in humans [23]. However, the p.Arg403Gln αMHC (analogous to human βMHC p.Arg403Gln) trangenic mice displayed mild cardiac dysfunction and hypertrophy with normal survival [24]. Compared with this p.Arg403Gln αMHC transgenic model, our p.Met531Arg αMHC transgenic mice progressed to severe left ventricular hypertrophy and further to dilated cardiomyopathy with sudden death. These results indicate that cardiac impairment of p.Met532Arg αMHC transgenic mice is significantly increased. In another model, αMHC transgenic mice bearing both the p.Arg403Gln mutation and a deletion in a part of the actin-binding site of αMHC evolved from left ventricular hypertrophy to dilation at 10 months of age even though only a small amount of transgenic protein was expressed (10% of endogenous αMHC protein) [10]. However, this model does not represent the real clinical consequence caused by p.Arg403Gln mutation in humans because the compound heterozygote of this mutation has not been identified to date in individuals with HCM. These results indicate that the phenotype of cardiomyopathy in mutant αMHC transgenic mice may be milder than that in humans.

Approx. 20% of our transgenic mice died by 12 months of age. The cause of death remains unknown because we could not evaluate the transgenic mice electrophysiologically due to technical difficulties. However, a previous study has reported that the degree of ventricular hypertrophy was significantly associated

with increased arrhythmia susceptibility in p.Arg403Gln αMHC transgenic mice [25]. Moreover, dead transgenic mice did not show the findings of heart failure or prominent diseases of other organs, except cardiac hypertrophy. These results suggest that these transgenic mice died of malignant arrhythmia. Further evaluation is necessary to clarify the electrophysiological abnormality in p.Met532Arg αMHC transgenic mice. *In vitro* functional studies may also help to elucidate the pathophysiological mechanisms affected by the p.Met531Arg mutation.

In the present study, p.Met532Arg αMHC trangenic mice did not show the phenotype of LVNC, unlike that found in the human patient. This result demonstrates that there is little impact of αMHC mutation on left ventricular morphogenesis. One explanation is that the expression pattern of αMHC in the murine heart is different from that of βMHC in the human heart. Human heart atria express αMHC and the ventricles express predominantly βMHC. In mouse, αMHC is expressed in both the postnatal atria and ventricles, whereas expression of βMHC in the embryonic ventricle is predominant over αMHC, especially when the process of ventricular myocardium compaction progresses. In mice, at 10.5 days post fertilization, αMHC gene expression begins to decrease in ventricular myocytes and trabeculations begin to form in the ventricles where βMHC is expressed dominantly [26]. Thus we speculate that the effect of βMHC mutation on left ventricle morphogenesis in humans may be much bigger than that of αMHC mutation in mice.

It is of note that the patient's monozygotic twin sister developed DCM. If the Met^{531} of βMHC was mutated to an arginine residue at the 1-cell stage, the twin sister had the same mutation. Although we could not examine this twin sister genetically because she died before the present study, it is possible that the p.Met531Arg mutation in βMHC caused DCM in her heart. Hence the transgenic model might reflect the pathology of human DCM rather than LVNC.

The mechanisms of onset of HCM and DCM

Met^{531} of βMHC is located in the actin-binding site. Replacement of a methionine residue by an arginine residue may impair the α-helix structure and disrupt the interaction between myosin and actin because methionine is a hydrophobic amino acid, whereas arginine is a basic and hydrophilic amino acid. Interestingly, other human DCM-causing mutations of βMHC are located near this region, such as p.Ser532Pro and p.Ala550Val [6,27]. For example, the p.Ser532Pro mutation which changes the charge of the amino acid at this position caused severe DCM. These results suggest that the p.Met531Arg mutation may cause catastrophic cardiomyopathy by a similar mechanism. Mutations in the genes encoding sarcomere proteins may alter contraction of cardiac muscle cells and activate multiple cellular pathways. When sarcomere proteins cannot interact sufficiently with other proteins because of the presence of mutations, cardiac remodelling may develop in order to compensate for the dysfunction, resulting in cardiomyopathy. It remains unclear why mutations of proteins with similar functions can cause two different morphologies, HCM and DCM, and whether these diseases are part of the same progressive pathology.

The results of the present study support the hypothesis that HCM and DCM reflect stages of a single progression pathway of heart disease [2]. Several studies of other mutant mice also support this hypothesis. For example, heterozygous mutant mice for the R403Q mutation developed HCM [25], whereas homozygous mutant mice developed DCM [11,12]. Furthermore, the R403Q mutation combined with a deletion in a part of the actin-binding site caused progression from HCM to DCM [26]. The fact that myohypertrophy is seen in DCM, and that HCM deteriorates into a phase that resembles DCM in human patients also supports the idea of a single pathophysiological progressive pathway.

In conclusion, the p.Met532Arg αMHC transgenic mice demonstrated a severe HCM phenotype with sudden death although they did not recapitulate the LVNC phenotype. In addition, some of the mice progressed to left ventricular dilation. These results indicate that the βMHC p.Met531Arg mutation contributes to malignant cardiomyopathy. This model would help to understand the pathological processes and aetiology of cardiomyopathy caused by MHC mutations.

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Exercise-induced systolic dysfunction in patients with non-obstructive hypertrophic cardiomyopathy and mutations in the cardiac troponin genes

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Short title: Haemodynamics in HCM with troponin mutations

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Abstract

Objectives: This study was to investigate left ventricular (LV) function reserve in hypertrophic cardiomyopathy (HCM) patients with and without cardiac troponin gene mutations before transition to the dilated phase.

Methods: LV ejection fraction (EF) was continuously evaluated in 52 patients with non-obstructive HCM during supine ergometer exercise using radionuclide ventricular function monitoring with a cadmium telluride detector (VEST). On the basis of genetic analysis, patients were divided into 2 groups: 10 with cardiac troponin gene mutations (group A) and 42 without these gene mutations (group B).

Results: Exercise duration, peak exercise load, and heart rate during exercise did not differ between the 2 groups. The differences from baseline to peak exercise of the LV end-diastolic volume decreased similarly in the 2 groups. In contrast, the difference of the LV end-systolic volume in group A increased significantly compared with group B ($p = 0.0031$). Consequently, the difference of LVEF in group A decreased significantly in contrast with group B ($p = 0.0025$). Additionally, the changes in LVEF and stroke volume decreased significantly more in group A than in group B ($p = 0.0017$ and 0.0042, respectively).

Conclusions: These results suggest that HCM patients with cardiac troponin gene mutations may display exercise-induced LV systolic dysfunction more frequently than HCM patients without this abnormality.

Abbreviations: DCM, dilated cardiomyopathy; EDV, end-diastolic volume; EF, ejection fraction; ESV, end-systolic volume; FS, fractional shortening; HCM, hypertrophic cardiomyopathy; IVST, interventricular septum thickness; LV, left ventricle; PCR, polymerase chain reaction; PWT, left ventricular posterior wall thickness; TnI, cardiac troponin I; TnT, cardiac troponin T; VEST, ambulatory radionuclide continuous ventricular function monitoring

Hypertrophic cardiomyopathy (HCM) is a primary cardiac disorder, often transmitted genetically, with heterogeneous clinical and morphological expression. Many patients experience a relatively benign course, whereas others appear to be at high risk for adverse events and premature death. A major challenge in the management of the broad HCM disease spectrum has been the identification of subsets of patients predisposed to sudden and unexpected or heart failure-related death.[1, 2] Although heart failure in HCM is largely the consequence of diastolic dysfunction,[1] some patients with HCM progress to "end-stage" or "burned-out" HCM with left ventricular (LV) dilatation and severe systolic dysfunction characteristic of dilated cardiomyopathy (DCM).[3-5]

Recent molecular genetic studies have revealed that HCM may be caused by mutations in the genes which encode the sarcomeric proteins.[6] Mutations in the troponin genes have been associated with specific clinical phenotypes, such as lesser degrees of hypertrophy and a high proportion of sudden death. In particular, patients with HCM associated with a mutation in the cardiac troponin I (TnI) gene and the cardiac troponin T (TnT) gene frequently develop systolic dysfunction and show progression to DCM.[7, 8]

Exercise-induced LV systolic dysfunction has been observed in about one-half of patients with HCM.[9, 10] Moreover, an abnormal blood pressure response during exercise, which was one of the high risk factors for sudden cardiac death, was associated with exercise-induced LV systolic dysfunction.[9-13] However, little is known about whether LV functional reserve (response of LV to exercise) before transition to the dilated phase differs among patients with and without disease-causing genes. The aim of our study was to assess the differences in the effect of exercise on haemodynamics between HCM patients with cardiac troponin gene mutations and those without them by ambulatory radionuclide continuous ventricular function monitoring (VEST), a method that reliably allows the assessment of LV function.

METHODS

Study patients

The study group consisted of 52 patients with non-obstructive HCM (41 men and 11 women, mean age (SD) of 51.5 (13.8) years, range 19 to 72). All patients were identified at the Kanazawa University Hospital or its related hospitals (from primary to tertiary care centres). With standard clinical assessment of family history, relatedness was excluded through three degrees. The diagnosis of HCM was based on the echocardiographic demonstration of a nondilated, hypertrophied left ventricle in the absence of other cardiac or systemic diseases that could cause LV hypertrophy.[14] Patients with any of the following findings were excluded from this study: (1) echocardiographic evidence of systolic anterior motion of the mitral valve; (2) midsystolic closure of the aortic valve; (3) the presence of a pressure gradient ($\geq$ 20 mm Hg) in the outflow tract or midportion of the left, right, or both ventricles at

baseline; and (4) a peak aortic valve gradient ≥ 50 mm Hg after provocative maneuvers or dobutamine stress.[10] All patients underwent cardiac catheterization and coronary angiography and had no evidence of coronary artery stenosis ≥ 25%. Patients with atrial fibrillation, left bundle branch block (including left anterior hemiblock or left posterior hemiblock), mitral regurgitation, aortic regurgitation, diabetes mellitus, hypertension, or the dilated phase of HCM were also excluded from the study.

VEST

All medications, including beta-blockers and calcium channel blockers, were discontinued at least 24 hours before the study. Informed consent was obtained from each patient. To evaluate ventricular function during exercise, VEST with a cadmium telluride detector (CdTe-VEST RRG-607, Aloka, Tokyo, Japan) was used. This monitoring system consists of a plastic jacket with a socket for holding the sodium iodide main detector and cadmium telluride background detector. It also contains a two-channel electrocardiogram and a nuclear data recorder with a real-time clock. VEST during rest, exercise, and recovery was performed using a previously described protocol.[9] After equilibration of 740 to 925 MBq of technetium 99m-labeled red blood cells, 12 electrocardiographic electrodes were attached to the patient's chest and a vest-like elastic garment was placed over the chest. With the patients in the supine position, the VEST detection device was placed over the LV blood pool under gamma camera visualization in the left anterior oblique position.

After 5 minutes of rest, supine bicycle ergometer exercise was initiated by the patient at a workload of 25 W and increased by 25 W every 2 minutes. Exercise was terminated because of severe chest pain, shortness of breath, and/or leg fatigue. Systolic and diastolic blood pressures were measured with a sphygmomanometer (STBP-680, NIPPON COLIN Co., Tokyo, Japan), and 12-lead electrocardiograms were recorded at 1 minute intervals during the test. LV function monitoring was continued for at least 10 minutes after the termination of exercise. After completion of the study, the position of the detector was reconfirmed with a 20-second static image obtained with the gamma camera.

VEST data analysis

The details and accuracy of VEST data analysis have been published previously.[9] Briefly, the maximum and minimum counts from the LV time activity curve were defined as the end-diastolic and end-systolic counts, respectively. The relative end-diastolic volume (EDV) was defined as 100% at the beginning of the study, and subsequent measurements were expressed relative to this value. LV ejection fraction (EF) was calculated as the difference between the end-diastolic and end-systolic counts divided by the background-corrected end-diastolic counts. All parameters were calculated for each beat and averaged for 20-s intervals. Systemic vascular resistance was calculated as mean blood pressure × 80/(heart rate × stroke volume), where mean blood pressure = diastolic pressure + 1/3 (systolic blood pressure – diastolic blood pressure), and stroke volume = EDV – end-systolic volume (ESV).