A novel mutation in the cardiac myosin-binding protein C gene is responsible for hypertrophic cardiomyopathy with severe ventricular hypertrophy and sudden death

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ABSTRACT
It has been demonstrated previously that clinical phenotypes of HCM (hypertrophic cardiomyopathy) caused by mutations in the cardiac MyBP-C (myosin-binding protein C) gene show late onset, low penetrance and favourable clinical course. However, we have encountered severe phenotypes in several carriers of the MyBP-C gene mutations. The aim of the present study was to screen novel MyBP-C gene mutations in patients with HCM and to investigate the genetic differences in affected subjects with severe phenotypes. The MyBP-C gene was screened in 292 Japanese probands with HCM, and a novel c.2067+1G→A mutation was present in 15 subjects in five families. Clinical phenotypes of carriers of the c.2067+1G→A mutation were compared with those of a previously identified Arg820Gln (Arg820→Gln) mutation in the MyBP-C gene. The disease penetrance in subjects aged ≥30 years was 90% in carriers of the c.2067+1G→A mutation and 61% in carriers of the Arg820Gln mutation. Sudden death occurred in four subjects from three families with the c.2067+1G→A mutation and in two subjects from one family with the Arg820Gln mutation. Two carriers of the c.2067+1G→A mutation had substantial hypertrophy (maximal wall thickness ≥30 mm). In contrast, two carriers of the Arg820Gln mutation had end-stage HCM. In conclusion, the c.2067+1G→A mutation is associated with HCM with substantial hypertrophy and moderate incidence of sudden death, whereas the Arg820Gln mutation is associated with end-stage HCM. These observations may provide important prognostic information regarding the clinical practice of HCM.

INTRODUCTION
HCM (hypertrophic cardiomyopathy) is a primary cardiac disorder, often inherited genetically, with heterogeneous clinical and morphological expression. Mutations in genes encoding sarcomeric proteins are known to account for HCM [1]. Cardiac MyBP-C (myosin binding protein-C) is one of these sarcomeric proteins, and mutations in the MyBP-C gene on chromosome 11 are a frequent cause of HCM [2]. Molecular genetic studies have demonstrated that phenotypic differences in HCM may be characterized by the genetic heterogeneity of the different genes encoding sarcomeric proteins. In families carrying cardiac troponin T gene mutations, the phenotype is similar and is characterized by mild hypertrophy and a high incidence of sudden death [3]. In families

Key words: hypertrophic cardiomyopathy, myosin-binding protein, mutation analysis, sarcomeric protein, sudden death.
Abbreviations: FS, fractional shortening; HCM, hypertrophic cardiomyopathy; LV, left ventricular; LVH, LV hypertrophy; MyBP-C, myosin binding protein-C.
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carrying the cardiac troponin I gene mutation, LV (left ventricular) systolic dysfunction and dilatation (end-stage HCM) is observed after 40 years of age [4]. With regard to MyBP-C gene mutations, several reports have demonstrated a genotype–phenotype correlation in which the clinical features of mutations in the MyBP-C gene include late onset and a favourable clinical course [2,5–7]. These data may provide useful information in genetic counselling of affected subjects with MyBP-C gene mutations. However, in clinical practice, severe phenotypes are observed, such as sudden cardiac death, substantial hypertrophy and end-stage HCM, even in carriers of MyBP-C gene mutations. It is still unclear what kind of genetic differences could affect such severe phenotypes, although previous studies have demonstrated phenotypic differences among various mutations of HCM caused by MyBP-C gene mutations [8]. As for end-stage HCM, the pathogenesis is poorly understood and few studies have performed mutation-based analysis of LV systolic dysfunction in carriers with MyBP-C gene mutations.

In the present study, the first aim was to screen for MyBP-C gene mutations in probands with HCM and to clarify the characteristic phenotype of patients with a novel MyBP-C gene mutation. The second aim was to assess severe phenotypes caused by a novel mutation, c.2067 +1G → A, and a missense mutation identified previously, Arg820Gln (Arg<sup>820</sup> → Gln) [9], from the viewpoint of genetic differences.

**MATERIALS AND METHODS**

**Subjects**
The study subjects comprised 292 unrelated probands with HCM (128 familial and 164 sporadic). All probands were identified at the Kanazawa University Hospital and affiliated hospitals (from primary to tertiary care centres). The diagnosis of HCM was based on the echocardiographic demonstration of a non-dilated hypertrophied left ventricle in the absence of other cardiac or systemic causes of L VH (LV hypertrophy) [10]. If the mutation was identified in the probands with HCM, their family members were studied genetically and clinically.

Informed consent was obtained from all subjects or their guardians, and the study was approved by the Bioethical Committee on Medical Research, School of Medicine, Kanazawa University.

**Detection of the genetic mutation**
DNA was isolated from peripheral white blood cells of all subjects using a DNA extractor 341 Nucleic Acid Purification System (Genepure<sup>TM</sup>; PerkinElmer Biosystems). In vitro amplification of genomic DNA was performed using PCR. Oligonucleotide primers were used to amplify exons of the MyBP-C gene as described previously [11]. At present, 18 out of 34 coding exons in the MyBP-C gene have been screened, and the remaining 16 exons are in the process of being analysed. Single-strand conformational polymorphism analysis of amplified DNA was then performed using a method described previously [12]. For abnormal single-strand conformational polymorphism patterns, PCR products were subcloned into the pCR2.1 vector using the TOPO TA cloning kit (Invitrogen). The nucleotide sequences of the cloned PCR products were determined on both strands by the dye terminator cycle sequencing method with the use of an automated fluorescent sequencer (ABI PRISM<sup>TM</sup> 310 Genetic Analyser; PerkinElmer Biosystems). The sequence variations in the MyBP-C gene were confirmed by restriction enzyme digestion. The presence of the c.2067 +1G → A mutation, which abolishes an RsaI restriction site, was confirmed by digestion of genomic DNA with this enzyme. The same method was then used to determine the genotype of DNA from family members of the probands and in 400 chromosomes of 200 healthy individuals without cardiovascular diseases.

**Detection of aberrant mRNA transcripts**
Total RNA was isolated from lymphocytes to identify the aberrant transcripts that resulted from the c.2067 +1G → A mutation. RNA was subjected to reverse transcription and the DNA products were amplified by PCR using primers 10579F (5′-GGTCCACAAACT-GACCATTG-3′) and 13137R (5′-CTGCTGTGAGA-GACCGAGG-3′). PCR products were subsequently sequenced as described above.

**Clinical evaluation**
Evaluation of the phenotype was completed before determination of the genotype. All subjects underwent echocardiography and 12-lead electrocardiography. Standard M-mode and two-dimensional echocardiographic studies were performed to identify and quantify morphological features of the left ventricle. LV dimensions and the thickness of the septum and posterior wall were measured at the level of the tips of the mitral valve leaflets. FS (fractional shortening) was calculated as the difference in end-diastolic and end-systolic dimensions. LV maximum wall thickness ≥ 13 mm in adults or ≥ 95% CI (confidence interval) of the theoretic value in children were considered as the diagnostic criteria for HCM [5]. LV diastolic dimension ≥ 117% of the predicted value and FS < 25% were defined as HCM with dilated cardiomyopathy–like features (end-stage HCM) [13]. Electrocardiographic abnormalities were defined as follows: Q wave > 3 mm in depth and/or > 0.04 s in duration in at least two leads, except aVR [14]. LVH was assessed by a Romhilt–Estes score ≥ 4 [15], ST-segment depression of an upsloping type > 0.1 mV at 0.08 s after the J point, or those of horizontal or down-sloping type > 0.05 mV, T-wave
RESULTS

Genotype analysis of the probands

Probands (n = 292) with HCM were screened for mutations in the MyBP-C gene. The c.2067+1G→A mutation in the MyBP-C gene was identified in five (1.7%) of these probands with HCM (Figure 1). Besides the c.2067+1G→A mutation, three mutations reported previously were identified. The Arg820Gln mutation was found in eight (2.7%), the c.1777delT mutation in two (0.6%) and the Gln998Glu (Gln998→Glu) mutation in three (1.0%) of the 292 probands [9,17,18].

The c.2067+1G→A mutation was not observed in any of the 400 chromosomes of 200 healthy individuals. This mutation was found in donor splice sequences and predicted to lead to the aberrant transcript. Analysis of mRNA derived from lymphocytes of patients confirmed the presence of the aberrant transcript. The c.2067+1G→A mutation led to an in-frame skipping of exon 21 and a premature stop codon in exon 22 (Figure 2). Relatives of two of these five probands were studied further, giving a total of 23 members from the various families (Figure 3). Of these 23 individuals, 15 (ten men and five women; mean age, 47.3 ± 23.4 years) had the c.2067+1G→A mutation in the MyBP-C gene. Although we screened for all MyBP-C exons in carriers of the c.2067+1G→A mutation, no other sequence variants were identified. Furthermore, mutations in the cardiac β myosin-heavy chain, cardiac troponin T, cardiac troponin I, cardiac actin, α-tropomyosin, myosin ventricular regulatory light chain and myosin ventricular essential light chain genes were not identified in carriers of the c.2067+1G→A mutation.

Clinical phenotypes

All 15 carriers in the five families were evaluated by electrocardiography and echocardiography. The echocardiographic results are shown in Table 1. Asymmetrical
septal hypertrophy was found in six subjects. The mean maximal LV wall thickness was $16.5 \pm 7.0$ mm. None of the carriers showed end-stage HCM. Echocardiographic abnormalities were noted in 11 out of the 15 carriers. The electrocardiographic results are shown in Table 2. All carriers had sinus rhythm and one of them had paroxysmal atrial fibrillation. Abnormal Q waves were observed in four subjects and significant ST-T changes in eight subjects. The disease penetrance in subjects aged $\geq 30$ years was determined to be 90% by echocardiography and ECG, and in those aged $<30$ years this was 40% by echocardiography and 0% by ECG. Sudden death occurred in four subjects from three families (II-2 in HCM-002; III-9 in HCM-033; and I-1 and II-5 in HCM-295) (Figure 3).

**Comparison of the clinical phenotypes**

Table 3 shows a comparison of the clinical phenotypes of the c.2067 + 1G→A mutation with that of a missense mutation, Arg820Gln, which has been reported previously [9]. Substantial ventricular hypertrophy was observed in two carriers with the c.2067 + 1G→A mutation, whereas it was not observed in carriers with the Arg820Gln mutation. In contrast, end-stage HCM was
observed in two carriers with the Arg820Gln mutation, whereas it was not observed in carriers with c.2067 +1G→A mutation. Sudden death occurred in four subjects from three out of the five families with the c.2067 +1G→A mutation, whereas it occurred in two subjects from one of the eight families with the Arg820Gln mutation. The disease penetrance in carriers aged ≥30 years was higher in those with the c.2067 +1G→A mutation than in those with the Arg820Gln mutation, but there was no statistical significance between the two (90% in c.2067 +1G→A compared with 61% in Arg820Gln).

**DISCUSSION**

In the present study, we have demonstrated that a novel mutation in the MyBP-C gene causes severe phenotypes and a high degree of penetrance, findings that have not been observed in previous studies [2,5].

**c.2067 +1G→A mutation and its consequences**

The c.2067 +1G→A mutation was found in clinically affected patients, but was absent in 400 chromosomes...
Table 3 Comparison of the phenotypes between carriers of the c.2067 + 1G → A and Arg820Gln mutations

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<tr>
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<th>c.2067 + 1G → A</th>
<th>Arg820Gln*</th>
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<tbody>
<tr>
<td>n</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>Age (years)</td>
<td>47.3 ± 23.4</td>
<td>55.1 ± 20.0</td>
</tr>
<tr>
<td>MWT (mm)</td>
<td>16.5 ± 7.0</td>
<td>15.1 ± 5.9</td>
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<tr>
<td>PWT (mm)</td>
<td>11.8 ± 2.0</td>
<td>10.7 ± 2.2</td>
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<tr>
<td>LVDd (mm)</td>
<td>42.5 ± 4.8</td>
<td>44.9 ± 6.0</td>
</tr>
<tr>
<td>LVds (mm)</td>
<td>26.0 ± 5.0</td>
<td>28.9 ± 7.7</td>
</tr>
<tr>
<td>FS (%)</td>
<td>38.8 ± 7.9</td>
<td>37.1 ± 8.6</td>
</tr>
<tr>
<td>Substantial LHV (n)</td>
<td>2 (13%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>End-stage HCM (n)</td>
<td>0 (0%)</td>
<td>2 (13%)</td>
</tr>
<tr>
<td>SCD</td>
<td>Four from three families</td>
<td>Two from one family</td>
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Penetration

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<th>Aged ≥ 30 years</th>
<th>Aged &lt; 30 years</th>
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<tbody>
<tr>
<td>Aged ≥ 30 years</td>
<td>90 %</td>
<td>61 %</td>
</tr>
<tr>
<td>Aged &lt; 30 years</td>
<td>40 %</td>
<td>50 %</td>
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from 200 healthy individuals. Carriers with the c.2067 + 1G → A mutation had no other mutations in the MyBP-C gene and in six other sarcomeric genes. These findings suggest that the c.2067 + 1G → A mutation may be associated with the disease. This sequence variant leads to skipping of exon 21, which results in a frameshift and the incorporation of two aberrant residues before a premature stop codon in exon 22, predicted to result in the loss of the C5–C10 domains in the MyBP-C protein [19].

**Clinical phenotypes**

Our analysis has demonstrated that the c.2067 + 1G → A mutation causes HCM with a high degree of penetrance, moderate incidence of sudden death and severe hypertrophy. Molecular genetic data may contribute to the interpretation of the clinical manifestation of HCM observed in the present study. The c.2067 + 1G → A mutation leads to premature protein truncation and subsequently to the loss of functionally important domains, which include not only the titin-binding site (C8–C10 domains) and the myosin-binding site (C10 domain), but also the cardiac-specific region in the C5 domain [20,21]. Moolman-Smook et al. [21] have shown an interesting interaction between the C5 and C8 domains and also between the C7 and C10 domains in an in vitro study. They postulated that MyBP-C molecules trimerize into a collar around the thick filament, with the C5–C7 domains of one MyBP-C overlapping with the C8–C10 domains of another. These findings indicate the functional importance of the C5–C10 domains in the MyBP-C protein. Loss of the C5–C10 domains caused by the c.2067 + 1G → A mutation may disrupt the MyBP-C collar and may alter thick filament structure and regulation [21]. The functional data from this in vitro study [21] may, in part, provide an explanation for the clinical data reported in the present study, which was characterized by a high degree of penetrance and severe phenotypes.

We compared clinical phenotypes between carriers of the c.2067 + 1G → A and the Arg820Gln mutations (Table 3). According to our data, severe phenotypes, such as substantial ventricular hypertrophy and sudden death, were more frequently observed in families with the c.2067 + 1G → A mutation compared with the Arg820Gln mutation. Differences among the various mutations in the MyBP-C gene in terms of the disease severity have not been clarified well. Erdmann et al. [8] indicated that severe hypertrophy and life-threatening arrhythmias were more common in patients with truncation rather than missense mutations in the MyBP-C gene. Severe phenotypes observed in carriers of a truncation mutation, c.2067 + 1G → A, may apply to their observations. In contrast, end-stage HCM was observed in two carriers of the Arg820Gln mutation, whereas it was not observed in carriers of the c.2067 + 1G → A mutation. Both carriers of the Arg820Gln mutation with end-stage HCM were aged ≥70 years. To date, no reports have described the phenotypic differences arising from truncation and missense mutations in the MyBP-C gene relative to end-stage HCM. The Arg820Gln mutation results in a change in the charge of the altered amino acid in the C6 domain (fibronectin type III repeat) [19]. We postulate that a change in the charge of the altered amino acid may be associated with this unfavourable course in later life, although carriers of the Arg820Gln mutation did not show severe phenotypes until middle age [9]. Our data demonstrate the development of LHV and life-threatening arrhythmias in carriers of truncation mutations and also note progression to end-stage HCM in carriers with missense mutations in the MyBP-C gene.

**Study limitations**

Determination of the prevalence of the MyBP-C gene mutations is one of our major interests. A recent study conducted by Van Driest et al. [18] demonstrated that MyBP-C gene mutations were found in 18% of patients with HCM at tertiary referral centres. In the present study, four mutations (one novel and three reported previously) were detected in 17 (6.2%) out of the 292 probands with HCM, which appears to be of lower prevalence when compared with previous studies [2,5,8]. We screened mutations in the MyBP-C gene using single-strand conformational polymorphism analysis, which has
been reported to show a sensitivity of 70–95% [22]. Therefore some mutations may have been missed in the screening process. In addition, because we have not finished screening all coding exons for the 292 probands with HCM, the prevalence of the mutations cannot be concluded at this stage. Further investigations are now underway to clarify the prevalence of the MyBP-C gene mutations in this population.

Conclusions
A novel truncation mutation, c.2067+1G→A, was associated with a high degree of penetrance, moderate incidence of sudden death and severe hypertrophy, whereas a missense mutation, Arg820Gln, was associated with end-stage HCM in elderly carriers. These observations may provide important prognostic information in the clinical practice of HCM.

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REFERENCES