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NOVEL MISSENSE MUTATION IN CARDIAC β MYOSIN HEAVY CHAIN GENE FOUND IN A JAPANESE PATIENT WITH HYPERTROPHIC CARDIOMYOPATHY

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Summary: We have analyzed the exon 9, 13, 14, 15, and 16 of cardiac β myosin heavy chain gene in 96 Japanese patients with hypertrophic cardiomyopathy by using PCR-DNA conformation polymorphism analysis. The analysis revealed a sequence variation of the exon 16 in one patient. The sequence variation of a G to C transversion with replacement of Asn by Lys at the codon 615 was confirmed by sequencing and by dot-blot hybridization with an allele-specific oligonucleotide probe. Because the missense mutation was found at the residue conserved through birds to humans, this mutation was suggested to be a cause of hypertrophic cardiomyopathy in the patient. This is the first report of a mutant cardiac β myosin heavy chain gene in the Japanese population.

Hypertrophic cardiomyopathy (HCM) is characterized by myocardial hypertrophy of unknown etiology accompanied with disarray of myocardial fibers (1, 2). The disease is one of the major causes of sudden death in young adults and a large number of patients with HCM show familial occurrence with autosomal dominant inheritance. Recently, linkage studies in large multiplex Caucasian families with HCM showed a close linkage of HCM with the cardiac

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Abbreviations:
DCP; DNA conformation polymorphism; HCM, hypertrophic cardiomyopathy; MHC, myosin heavy chain; PCR, polymerase chain reaction.

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myosin heavy chain (MHC) locus on the chromosome 14 (3-6). Subsequently, missense mutations of the cardiac β MHC gene were found in some Caucasian patients with familial HCM and were suspected to be responsible for the cardiac hypertrophy (4-8). However, no linkage with the MHC locus were reported in several other HCM families (9-11). In addition, linkages with genetic markers on the chromosome 2 (10) and that on the chromosome 18 (12) were suggested from the linkage syudy of other Caucasian and Japanese multiplex families, respectively, implying that the disease is genetically heterogeneous. Therefore it remains to be revealed whether abnormalities of the cardiac β MHC gene will be found in each patient with HCM. In this study, we have screened a sequence variation in the cardiac β MHC gene by polymerase chain reaction (PCR) - DNA conformation polymorphism (DCP) analysis (13-16) to detect a mutation in 96 Japanese patients with HCM. Because the mutations in Caucasians were reported mainly in the head domain of cardiac β MHC, especially in the exons 9, 13, 14, and 16 (5-8), we focused attention on these exons. Subsequently, a missense mutation in the exon 16 was identified in a Japanese HCM patient with a family history.

MATERIALS AND METHODS

Subject: Ninty-six unrelated Japanese patients with HCM were chosen as subjects. These patients were diagnosed by physical examination, electrocardiography, 2-dimensional echocardiography, and cardiac catheterization, based on Maron's criteria (17). Ptients with apical hypertrophy were not included. Forty-two of the patients showed an apparent family history of HCM. Because one patient had a missense mutation of the cardiac β MHC gene, his family members showing no clinical signs of HCM were also analyzed. The patient was a 40 year-old man, diagnosed at 30 years old with the electrocardiogram showing ST-segment depression and T wave inversion in leads V_4 through V_6 and the two-dimentional echocardiogram revealing asymmetrical left ventricular hypertrophy (thickness of the interventricular septum: 3.0 cm, thickness of the left ventricular posterior wall: 1.6 cm) and systolic anterior motion of the mitral valve. His diagnosis was further confirmed to be obstructive hypertrophic cardiomyopathy by cardiac catheterization. His father had died suddenly with unknown cause at 40 years old. The electrocardiogram and two-dimentional echocardiogram showed no abnormal findings in his mother and two children. In addition, 45 healthy Japanese individuals were examined as normal controls. Blood sample was obtained from each individual after obtaining informed consent.

Genomic DNA extraction and PCR: High molecular weight DNA was prepared from peripheral blood leukocytes of patient or normal

Table 1. Nucleotide sequences of primers

Exon	Name	Sequence (5' to 3')
9	BEX 9-5C	TTTAAGCTTCCCCCAACTCATCACCA
	BEX 9-3C	TTTTCTAGAGCAAGGGTGAGCTTA
13	BEX13-5C	GGGAAGCTTACCAACTTTGCTCTTGCC
	BEX13-3	CCTACCCTGCCCACCCAT
14	BEX14-5	CCTGCTCAATATGGGCTCTC
	BEX14-3C	GGGTCTAGAGGAGCGAGTGAGTGATTGTT
15	BEX15-5C	TTTAAGCTTTCTGACTGCTCCCACC
	BEX15-3C	TTTTCTAGATGGAATTCAGGTGGTAAGG
16	BEX16-5a	TGAAGGACACTCAGTGATGCT
	BEX16-5p	GAAGCCTGAAGCCCACTTCT
	BEX16-3a	CCAGCCAATGATGTTGTAGTC
	BEX16-3p	TGTACCGGGAGCCTCAGTC

individuals by the SDS-proteinase K method and phenol/chloroform extraction. Twelve primers flanking exons 9, 13, 14, 15, and 16 of the cardiac β MHC gene (Table 1) were synthesized by cyanophosphamidite method in a DNA synthesizer (Cyclon plus, MilliGen/Biosearch, Burlington) based on the reported sequences of normal human cardiac β MHC gene (18). Two additional oligonucleotide primers, BEX 16-3a and BEX 16-5p (Table 1), were designed for exon 16 so that the exon could be analyzed in relatively narrow ranges, which would facilitate the detection of sequence variations. Amplification of the cardiac β MHC gene was carried out by using PCR (19, 20) in a reaction mixture of 50 μl composed of 100 ng genomic DNA, 10 pmole primers, 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 1.5 mM $MgCl_2$, 0.01% gelatin, 0.8 mM dNTPs, and 1 unit of thermostable DNA polymerase (Ampli-Taq, Perkin Elmer Cetus, obtained via Takara Co. Ltd., Kyoto) via 30 cycles of denaturation (96°C, 1 min), annealing (60°C, 30 sec), and extension (72°C, 1 min) steps in a programmable heat block (DNA Thermal Cycler, Perkin Elmer Cetus Instruments, Norwalk).

PCR-DCP (DNA conformation polymorphism) analysis: Formamide dye (80% formamide, 20 mM EDTA, 0.01% bromophenol blue, pH 8.0) was mixed with an aliquot of PCR products (0.1 - 0.2 μg) to obtain a final concentration of 50% formamide. Samples were heat-denatured at 96°C for 5 min, rapidly chilled in ice-water, and electrophoresed in two 8% polyacrylamide gels (14 x 14 x 0.1 cm, 0.4 x TBE, acrylamide: bisacrylamide = 50:1) with and without 10% glycerol in 0.4 x TBE (1 x TBE: 89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA, pH 8.0) at 13V/cm for 5 to 8 h at room temperature. Single-stranded DNAs, heteroduplex DNAs, and double-stranded DNAs were visualized by silver staining using a commercially available kit (Daiichi Chemical Co. Ltd., Tokyo) according to the manufacturer's instructions.

Sequencing analysis: To identify a mutation in the exon 16, PCR products generated with primers, BEX16-5C (5'-

GGGAAGCTTGAAGGACACTCAGTGATGCT-3') and BEX16-3C (5'-GGGTCTAGATGTACCGGGAGCCTCAGTC-3'), were digested with *Hin*dIII and *Xba*I and cloned into Bluescript SK$^-$ (Stratagene, obtained via Toyobo Co. Ltd., Osaka). DNA sequences were determined by the dideoxy chain termination method (21) using a commercially available sequencing kit (Sequenase ver 2.0, USB, obtained via Toyobo Co. Ltd., Osaka).

Dot-blot hybridization: Oligonucleotide probes specific to normal and mutated sequences at the codon 615 of the cardiac β MHC gene were 5'-TTCCCTCAAGCTGCTCAG-3' and 5'-TTCCCTCAACCTGCTCAG-3', respectively. The probes were end-labeled with [γ-^{32}P] ATP by T4 polynucleotide kinase. Amplified DNA fragments (0.1 μg) were dot blotted onto a nylon membrane (Hybond N plus, Amersham International, Tokyo) and hybridized with radio-labeled oligonucleotide probes (10 pmole/ml) in 3.0 M tetramethylammonium chloride, 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 0.1% SDS, 5 x Denhardt's solution, and 100 μg/ml heat-denatured herring sperm DNA for 30 min at 54°C. The filters were washed twice in 2 x SSPE (0.30 M NaCl, 0.02 M NaH_2PO_4, 2 mM EDTA, pH 7.4) and 0.1% SDS at room temperature for 10 min, once in TMAC solution (3.0 M tetramethylammonium chloride, 50 mM Tris-HCl, 2 mM EDTA, 0.1% SDS, pH 8.0) for 10 min at room temperature. A stringent wash was done twice in TMAC solution for 10 min at 58°C. The filters were exposed to a Kodak XAR5 film at room temperature for 30 min to visualize hybridization signals.

RESULTS AND DISCUSSION

Ninty-six patients were analyzed for mutaions in the exons 9, 13, 14, and 15 of the cardiac β MHC gene by the PCR-DCP analysis and no unusual DNA fragment due to sequence variation was detected (data not shown). Analyzing the exon 16 *in tot* (Figure 1a), in the 5' half (Figure 1b), and in the 3' half (Figure 1c), normal subjects showed several slow-migrating DNA fragments (single-stranded DNAs) and a fast-migrating DNA fragment (double-stranded DNA). These slow-migrating fragments were thought to represent the exon 16-specific single-stranded DNAs, but not the non-specific coamplification products, because more than one tertiary structure could be generated from one single-stranded DNA fragment under the specified condition, as was observed in the other genes (13-16). One patient with HCM showed an additional slow-migrating DNA fragment representing single strand conformation polymorphism (22) (Figure 1a, lane 1 and Figure 1c, lane 1) and an additional fast-migrating DNA fragment representing a mismatched hybrid DNA (23) (Figure 1c, lane 1), suggesting that the patient had a sequence variation in the 3' half of this exon. His healthy family

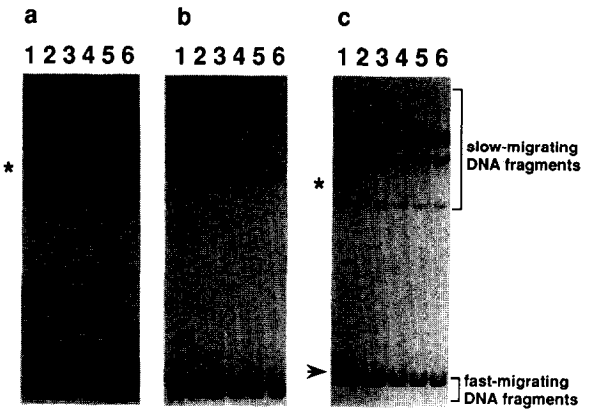
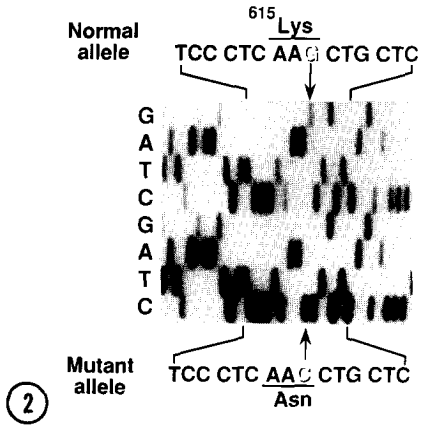


Figure 1. **PCR-DCP analysis of cardiac β MHC gene.** PCR products of the exon 16 of the cardiac β MHC gene were heat-denatured and electrophoresed in an 8% polyacrylamide gel without containing 10% glycerol. Whole exon 16 (amplified with BEX16-5a and BEX 16-3p), 5' half of exon 16 (amplified with BEX16-5a and BEX 16-3a), and 3' half of exon 16 (amplified with BEX 16-5p and BEX 16-3p) were analyzed in **a**, **b**, and **c**, respectively. Lanes: 1, patient with the mutation; 2, mother; 3, elder child; 4, younger child; 5 and 6, normal individuals. **a**; An unusual slow-migrating DNA fragment indicated by an asterisk was found in the patient's sample (lane 1) but not in the other samples including his 3 healthy relatives and 2 healthy individuals. **b**; No unusual fragment was observed. **c**; An unusual slow-migrating DNA fragment indicated by an asterisk and an unusual fast-migrating DNA fragment indicated by an arrow were found only in the patient's sample (lane 1).

members (mother and two children) showed no unusual PCR products (Figure 1, lanes 2-4).

PCR products from the entire exon 16 of the patient were then cloned and sequenced. Sequencing analysis of several independent clones revealed multiple clones with a G to C transversion in the codon 615 from AAG (Lys) to AAC (Asn) (Figure 2). Because several other clones with normal sequences were identified, the patient was heterozygous for the substitution. For further confirmation of the mutation, PCR products of the exon 16 were hybridized with allele-specific oligonucleotide probes. DNA samples from 146 individuals including 3 healthy family members of the patient and the other 95 patients with HCM were hybridized exclusively with the normal probe, while only the patient showed a positive hybridization signal with the mutant probe (Figure 3). Because the missense mutation was found at the evolutionary conserved amino acid residue, as were the cases with the other mutations in the exon 16 reported for Caucasian patients (8) (Figure 4), this mutation was suggested to be the cause of HCM in this patient. The fact that the mutation was not found in the analyzed healthy first degree relatives of the patient and that his father died



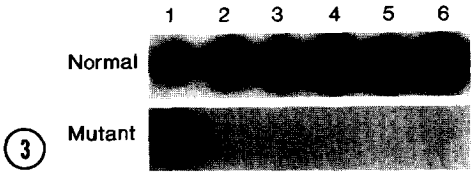


Figure 2. **Nucleotide sequences of normal and mutant cardiac β MHC alleles from the patient.** PCR products of the exon 16 from the patient were cloned and sequenced. The codon 615 of normal allele is AAG and that of mutant allele is AAC, showing that the patient is heterozygous for the Lys to Asn mutation at the codon 615.

Figure 3. **Hybridization with allele-specific oligonucleotide probes.** PCR samples from the patient and normal subjects were hybridized with normal and mutant probes. Only a part of the analysis is shown. The patient (no.1) showed positive hybridization with both normal and mutant probes.

suddenly at age of 40 years-old suggested that the mutation was inherited from his father and not passed to his children.

It was reported that the ^{584}Gly to Arg mutation was associated with a poor and ultimately fatal prognosis, while the disease-related deaths were less frequent with the ^{606}Val to Met mutation (8). It is of interest to note that the ^{584}Gly residue is completely conserved in myosins from amoeba to humans while the ^{606}Val is less conserved (Figure 4). Thus the ^{584}Gly to Arg mutation would be more deleterious than the ^{606}Val to Met mutation. In this respect, it is worth noting that the ^{615}Lys or a similar residue in nature, Arg, is conserved in myosins from birds to humans and that the residue is replaced by Asn, that is the same replacement found in our Japanese patient, in nematodes and dictiosterium (Figure 4). It is therefore possible that the ^{615}Lys to Asn mutation may not be related with a poor prognosis, although the small number of family members of the patient did not allow us to test the possibility.

In conclusion, we found a patient with HCM who had a missense mutation in the exon 16 of cardiac β MHC gene. This is the first report of the mutant cardiac β MHC gene in a non-Caucasian patient with HCM. We could not detect any mutations in the exons 9, 13, 14, 15, and 16 in 95 other patients with HCM by using the PCR-DCP analysis that is a

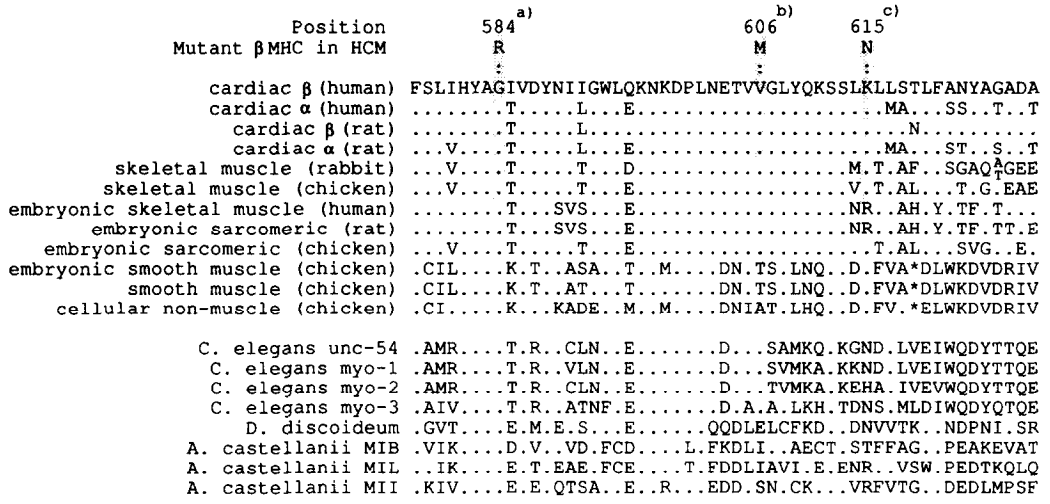


Figure 4. **Comparison of amino acid sequences of myosin chains.** Amino acid sequences of the human cardiac β myosin heavy chain and other myosin chains from various species (12, 24-35) are indicated by one-letter codes. The region encoded for by the exon 16 is shown for comparison and amino acid substitutions deduced from mutant genes found in patients with HCM are indicated at the top. a) and b); Two mutations, ^{584}Gly (G) to Arg (R) and ^{606}Val (V) to Met (M), respectively, are reported in Caucasian patients (8). c); The ^{615}Lys (K) to Asn (N) mutation is identified in the current study. Amino acid residues corresponding to the ^{584}Gly, ^{606}Val, and ^{615}Lys of the human cardiac β myosin heavy chain are shaded.

highly sensitive detection system of a sequence variation, by which more than 90% of one base substitutions in HLA genes could be detected (14-16, and our unpublished observations). These patients need to be analyzed for the other exons of the cardiac β MHC (8, 18) and for other loci suggested to be linked with HCM (10, 12) in order to elucidate the genetic defects causing HCM.

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