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POSSIBLE GENE DOSE EFFECT OF A MUTANT CARDIAC β-MYOSIN HEAVY CHAIN GENE ON THE CLINICAL EXPRESSION OF FAMILIAL HYPERTROPHIC CARDIOMYOPATHY

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Summary: We have examined for a mutation in the cardiac β myosin heavy chain gene from Japanese patients with familial hypertrophic cardiomyopathy. A missense mutation due to a G to A transition in codon 935, leading to a replacement of Glu with Lys, was found in one patient. Family members of this patient were then examined. It was revealed that both the proband and his elder brother, who was also a symptomatic patient, were homozygous for the mutation. The proband eventually died of intractable heart failure, and his brother died suddenly in their thirties. On the other hand, his parents, who were first cousins and heterozygous for the mutation, had cardiac hypertrophy without clinical symptoms. His elder sister was also heterozygous for the mutation, however, she did not manifest with cardiac hypertrophy. These observations suggest a gene-dose-like effect of the mutant cardiac β myosin heavy chain gene on the clinical manifestation of familial hypertrophic cardiomyopathy.

Familial hypertrophic cardiomyopathy (FHCM) is an autosomal dominant inherited disease characterized by left ventricular hypertrophy accompanied by disorganization of cardiac muscle cells (1, 2). Patients with FHCM may manifest with palpitation, dyspnea, chest pain, syncope, arrhythmia, and cardiac sudden death (3). In addition, congestive heart failure is sometimes observed along with the progression of disease (4, 5). On the other hand, there are many asymptomatic patients especially in young relatives of the patients. Thus, it is well known that the clinical course and prognosis of FHCM may vary from an asymptomatic state to sudden death among the patients, even in a family sharing the same major gene for FHCM.

Mutations of the cardiac β-myosin heavy chain (MHC) gene have been identified in several patients with FHCM and were suspected to be the cause of HCM, because the mutations were found in the evolutionarily conserved region of MHC proteins and were linked

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Abbreviations: HCM, hypertrophic cardiomyopathy; β-MHC, β-myosin heavy chain; PCR, polymerase chain reaction; DCP, DNA conformation polymorphism; SSOP, sequence-specific oligonucleotide probe.

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to the disease in respective affected families (6-12). We have previously reported two different missense mutations in exon 16 and 21 of the cardiac β-MHC gene found in Japanese patients with FHCM (13, 14). We report here another missense mutation found in an FHCM family and discuss the relevance of the mutation to the clinical expression of the disease.

MATERIALS AND METHODS

Subject. The proband of this family was diagnosed at 25 year old. He initially manifested with typical asymmetrical septal hypertrophy (ASH), but rapidly progressed to the dilated form within 3 years; he eventually died of intractable heart failure. The brother presented ASH and remained in a stable condition until he died suddenly. The parents revealed ASH, while they were asymptomatic. The proband's sister, who was hypertensive, showed left ventricular hypertrophy in her electrocardiogram, but no abnormalities were found in her echocardiogram. A summary of findings in the echocardiograms and electrocardiograms of the family members is shown in table 1.

PCR. Blood samples were collected from each individual after obtaining informed consent. Genomic DNA prepared from peripheral granulocytes was subjected to PCR (15) in a programmable heat block (Thermal cycler, Perkin Elmer Cetus Instruments, Norwalk, CT) via 30 cycles of denaturation (96 °C, 1 min), annealing (55 °C, 30 sec), and extension (72 °C, 1 min) steps with two primers, BEX23-5C (5'-TTTAAGCTTGAACAGCCTCCCCTCTGT-3') and BEX23-3C (5'-TTTTCTAGACCCGGGCTGGAGCC-3'), flanking exon 23 of the cardiac β-MHC gene (16).

PCR-DNA conformation polymorphism (DCP) analysis. Formamide dye (80% formamide, 20 mM EDTA, 0.01% BPB, pH 8.0) was added to an aliquot (0.1-0.2 mg) of PCR products to obtain a final concentration of 50% formamide. Samples were heat-denatured and electrophorezed in 8% polyacrylamide gels 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 13 V/cm for 5 to 8 hours at room temperature. DNA fragments were visualized by silver staining as described previously (17-19).

Cloning and Sequencing analysis. PCR products containing exon 23 of cardiac β-MHC gene from the patient and his parents, which showed abnormal DCP patterns, were cleaved with *Xba*I and *Hin*dIII, purified in a 1.2% agarose gel and cloned into Bluescript II SK$^-$ (Stratagene, La Jolla, CA). Nucleotide sequences of exon 23 from each subject were determined from at least 6 independent clones by the dideoxy chain termination method (20) using a sequencing kit (Sequenase version 2.0, United States Biochemical Corp., Cleveland, OH) according to the manufacturer's instructions.

Dot blot hybridization with sequence-specific oligonucleotide probes. Sequence-specific oligonucleotide probes (SSOPs) were synthesized in a DNA synthesizer (Cyclone plus, MilliGen/Bioresearch, Burlington, MA) for normal (BEX935N, 5'-ATGAATGCTGAGCTCACT-3') and mutant (BEX935M, 5'-ATGAATGCTAAGCTCACT-3') alleles. These SSOPs were end-labeled with ^{32}P and used in dot-blot hybridization experiments. Procedures of the dot-blot hybridization with SSOPs were described previously (13,14).

Preparation of endomyocardial biopsies and transmission electron microscopy. Endomyocardial tissues were obtained from the right side of the interventricular septum by right ventricular catheterization. After biopsy, the tissues were immersed immediately with 3% glutaraldehyde in 0.1M sodium cacodylate buffer, pH 7.2, at room temperature, then they were fixed for an additional 2 hours at 4 °C and transferred into the cacodylate buffer. The tissues were, then, post-fixed with 1% buffered osmium tetraxide for 1.5 hours at 4 °C, and processed in the conventional manner for observation. Thin sections were observed by a Hitachi H2000 electron microscope.

RESULTS AND DISCUSSION

Heat-denatured PCR products of exon 23 of the β-MHC gene were electrophorezed in two non-denaturing polyacrylamide gels with and without 10% glycerol. The proband patient and his elder brother showed an identical DCP pattern of PCR products, distinct from that observed in normal individuals. His parents and sister showed DCP patterns consisting of both the patient's pattern and the normal pattern. It was suggested that the proband and his brother were homozygous and the other relatives were heterozygous for a sequence variation in exon 23, because a DCP pattern of heterozygotes was identical to a combined DCP pattern of two corresponding homozygotes. This distinctive PCR-DCP pattern was more evident when glycerol was added to the gel matrix (compare Figure 1a with 1b). To identify the sequence variation in exon 23, PCR products from the proband and his parents were cloned and sequenced. All independent clones obtained from the proband had identical sequences with a G to A transition in codon 935, while both mutant and normal sequences were obtained from his father (Figure 2). These sequencing data were consistent with the observations in the PCR-DCP analysis. The transition in codon 935 was a missense mutation replacing a glutamic acid residue (Glu, GAG) with a lysine residue (Lys, AAG). Figure 3 shows amino acid sequence of the cardiac β-MHC protein encoded for by exon 23 (16) in alignment with myosin

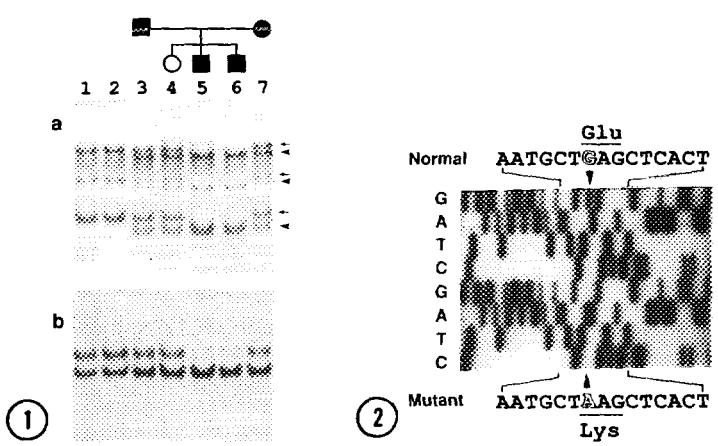

Figure 1. **PCR-DCP analysis of exon 23 of the cardiac β-MHC gene.** PCR products from exon 23 of the cardiac β-MHC gene (amplified with BEX23-5C and BEX23-3C) were heat-denatured and electrophorezed in 8% polyacrylamide gels with (a) and without (b) 10% glycerol. Samples were obtained from control individuals (lanes 1 and 2) and family members with a mutant cardiac β-MHC gene (3, father; 4, sister; 5, brother; 6, proband; 7, mother). Positions of single-stranded DNA fragments found in normal individuals are indicated by arrows and those of abnormal DNA fragments with different electrophoretic mobility are denoted by arrowheads in (a).

Figure 2. **Nucleotide sequence of normal and mutant cardiac β-MHC alleles.** PCR products from exon 23 of the cardiac β-MHC gene from the proband's father were cloned and sequenced by the dideoxy chain termination method. The codon 935 of normal allele is GAG and that of mutant allele is AAG, showing that he is heterozygous for Glu to Lys mutation at codon 935. Same result was obtained from the proband's mother and only the mutant allele was demonstrated from the proband by sequencing.

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                             Position                 908             924        935           949
                   Mutantβ MHC in HCM                 V               K          K             K
                                                      :               :          :             :
                  cardiac β  (human) EQDNLADAEERCDQLIKNKIQLEAKVKEMNERLEDEEEMNAELTAKKRNVEDECSELKRDIDDLELTLAKVEKEKHATENK
                  cardiac α  (human) ................................................KL........K......................
                    cardiac β  (rat) .............................T..................KL.......RK......................
                    cardiac α  (rat) .............................T..................KL........K......................
embryonic skeletal muscle  (human)   .SE..L...........A.F.....I..VT..A.....I.........KL........K......................
       embryonic sarcomeric  (rat)   .SE..L...........A.F.....I..VT..A.....I.........KL........K......................
         skeletal muscle (rabbit)    .A.S.......QD*...T.......I..VT..A.....I.........KL........K......................
   embryonic sarcomeric (chicken)    .A.G.............T.......I..LT..A...............KL........K......................

embryonic smooth muscle (chicken)    .TELY.E...MRVR.AAK.QE..EILH..EA.I.E...RSQQ.Q.E.KKMQQQMLD.EEQLEEE.AARQ.LQL..VTAEG.
     cellular non-muscle (chicken)   .TELY.E...MRVR.AAK.QE..EICHDLEA.V.E...RCQH.Q.E.KKMQQNIQ..EEQLEEE.AA.Q.LQL..VTADG.

              C. elegans unc-54 TKTQ.S.....LAK.EAQQKDASKQLS.L.DQ.A.N.DRT.DVQRA.KKI.A.VEA..KQ.Q...MS.R.A.S..QSKDHQ
               C. elegans myo-1 .KA........NEK.NQL.ATVEKSLNDA.D..SEH..K..D.EKQR.KAQQ.VEN..KS.EAVD.S.R.A.Q..QSRDHN
               C. elegans myo-2 T.GGS.AI..KLTR.NSARQE..G.LADASKK..V..ARAV.INKQ.KL..A..AD..KNCQ.VDGN...SLE..A.K..Q
               C. elegans myo-3 SKGSTREV...MTAMNEQ.VA..S.LSDITGQ...MQ.R.ED.ARQ.KKTDQ.L.DT.KHVQ....S.R...A..N.K.HQ
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Figure 3. **Alignment of amino acid sequences of myosin chains.** Amino acid sequences of human cardiac β-myosin heavy chain (16) and other myosin heavy chains from various species (21-30) are deduced from nucleotide sequences and represented by one-letter codes. The region encoded for by exon 23 is shown for comparison and amino acid substitutions deduced from mutations found in patients with FHCM are indicated at the top. Japanese patients in the current study have a ^{935}Glu (E) to Lys (K) mutation, while the three other mutations at codons 908, 924, and 949 were reported from Caucasian patients (10, 11).

heavy chains from various species (21-30). The mutation is located at the conserved residue of the myosin heavy chains, as were the other missense mutations found in Caucasian patients (10,11). It was, therefore, suggested that this mutation caused FHCM in the proband and his affected relatives. To further confirm the mutation in this family, normal and mutant-specific oligonucleotide probes for codon 935 were synthesized and used in dot-blot hybridization experiments. The proband and his brother showed positive hybridization exclusively with the mutant probe, while the other relatives were positive for both normal and mutant sequences (Figure 4).

It is well known that the penetrance of FHCM is age-dependent, because the cardiac hypertrophy of patients and their relatives often is not manifested in the childhood (31-33). Rosenzweig *et al* (7) reported that 13 out of 14 mutation-prone family members younger than 20 years old did not show abnormal echocardiographic findings, in a Caucasian FHCM family associated with a mutant β-MHC, ^{249}Arg to Glu. Furthermore, Epstein *et al* (11) demonstrated

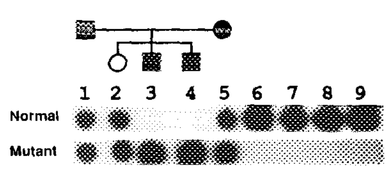

Figure 4. **Hybridization with allele-specific oligonucleotide probes.** PCR samples from the proband, his family members, other patients, and normal controls were hybridized with normal and mutant probes. Only a part of the analysis is shown. The proband (no. 4) and his brother (no. 3) show homozygous positive hybridization with mutant allele and his other family members (no. 1, father; 2, sister; 5, mother) show heterozygous pattern of normal and mutant alleles. Other patients (no. 6 and 7) and healthy individuals (no. 8 and 9) are homozygous for normal allele.

that the penetrance was varied with each mutation in the cardiac β-MHC gene, even in individuals older than 16 years old. Cardiac hypertrophy was observed in 61% and 100% of individuals having the ^{403}Arg to Gln and ^{908}Leu to Val mutations, respectively. It was observed in the current study that the elder sister exhibited left ventricular hypertrophy in her electrocardiogram but had no obvious cardiac hypertrophy (Table 1), *i.e.* she showed less clinical signs of FHCM than the parents. This observation is consistent with the age-dependent penetrance of FHCM.

The observation that the two homozygous patients showed much severer clinical manifestations than the heterozygous family members suggests a gene dose effect of the ^{935}Glu to Lys mutation on the severity and prognosis of the disease. A gene dose effect of dominant disease-causing mutations has been well documented in the familial hypercholesterolemia (34). On the other hand, it has been reported in nematodes that homozygous animals harboring missense mutations in the β-MHC gene (*unc-54*) were inviable (35, 36). In this regard, the ^{935}Glu to Lys mutation identified in this study might not be extensively deleterious than the other missense mutations. Alternatively, the cardiac α-MHC gene might have compensated for the defect in the cardiac β-MHC gene in these two homozygous patients in their earlier lives, as was the case in nematodes in which the lethal phenotype of homozygous missense mutations in the β-MHC gene could be suppressed in part by an elevated expression of the α-MHC gene (35). Because the proband and his elder brother should have no normal cardiac β-MHC protein, it was of interest to examine whether they had more severe pathological change than the other patients who had both variant and normal cardiac β-MHC proteins. A biopsied

Table 1. Clinical findings of family members with the ^{935}Glu to Lys mutant cardiac β-MHC

Family member	Age[1] (Y)	2-D Echocardiogram[2] in 1984	2-D Echocardiogram[2] in 1989	Electrocardiogram in 1989	Initial symptoms and Clinical course
father	67	IVST 14mm PWT 10mm	IVST 18mm PWT 10mm	LVH (SV_1+RV_5=59mV) T-wave inversion in I,aVL	no-symptom
mother	60	N.E.	IVST 20mm PWT 11mm	LVH (SV_1+RV_5=74mV) ST-T change in II,III,aVF	no-symptom
sister	38	N.E.	IVST 10mm PWT 10mm	LVH (SV_1+RV_5=47mV) no ST-T change	no-symptom
brother	32	IVST 26mm PWT 12mm	IVST 25mm PWT 12mm	LVH (SV_1+RV_5=51mV) ST-T change in V3-V6	easy fatigability Sudden death (34 Y)
proband	30	IVST 26mm PWT 13mm LVDd 50mm	IVST 12mm PWT 12mm LVDd 70mm	SV_1+RV_5=32mV	syncope died due to CHF[3] (31 Y)

1) Age at examination in 1989
2) 2-D Echocardiogram: two-dimensional Echocardiogram, IVST: intra-ventricular septum thickness, PWT: ventricular posterior wall thickness, LVDd: left ventricular end-diastolic diameter
3) CHF: congestive heart failure

sample from proband, which had been obtained at the time of first diagnosis when his cardiac function was well-preserved, showed no remarkable difference in the microscopic and electron-microscopic findings from that of another patient who was heterozygous for a ^{778}Asp to Gly mutation (14). The myocardial cells showed marked hypertrophy with abnormal myofibrillar orientation and mitochondriosis which were usually observed in FHCM (Figure 5). These observations also supported that the ^{935}Glu to Lys mutation might be less deleterious than the ^{778}Asp to Gly mutation, because all the individuals heterozygous for the latter mutation and older than 16 years old manifested with apparent cardiac hypertrophy (14), while two of three individuals heterozygous for the ^{935}Glu to Lys mutation exhibited cardiac hypertrophy.

FHCM is basically characterized by left ventricular hypertrophy with normal systolic function. However, several patients have been reported to develop a phase characterized by systolic dysfunction and marked left ventricular dilatation, resembling to the functional feature of a dilated cardiomyopathy (4, 5). In our observation, the proband had initially manifested

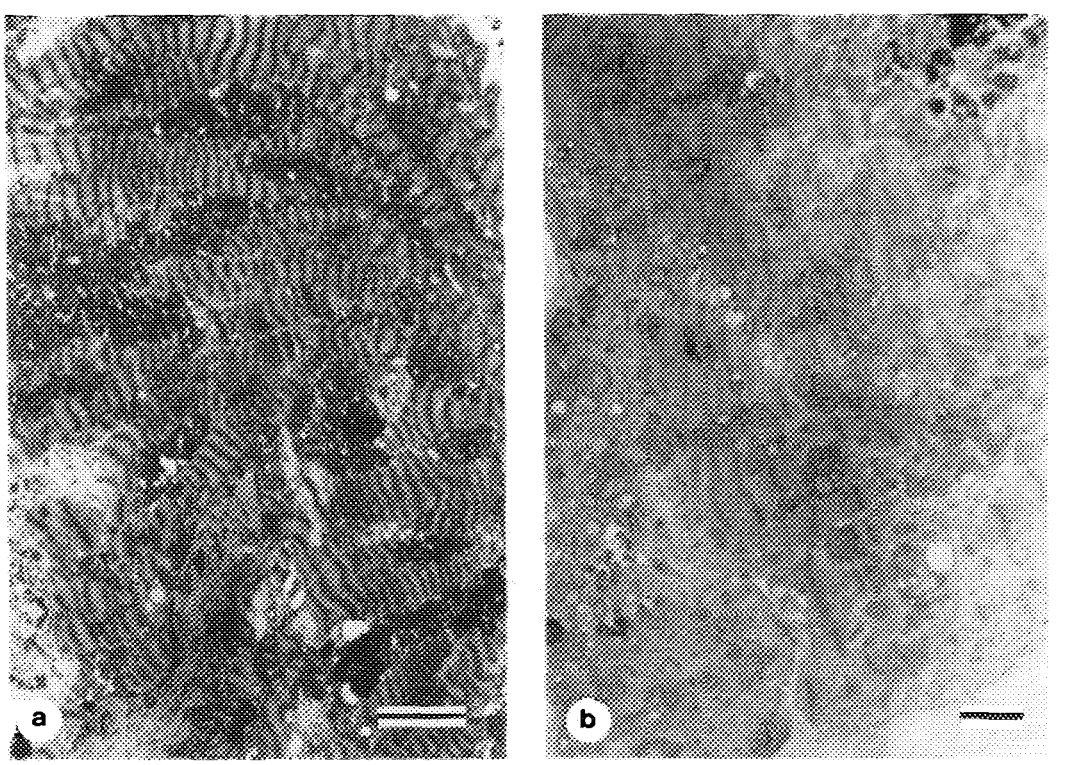

Figure 5. **Electron-microscopic findings of biopsied samples.**
Endomyocardial tissues of patient with ^{935}Glu to Lys mutation (a) and patient with ^{778}Asp to Gly mutation (b) were obtained from the right side of the interventricular septum by right ventricular catheterization. The myocardial cells showed marked hypertrophy with abnormal myofibrillar orientation and mitochondriosis which findings were usually observed in HCM. The bars indicate 5μm in (a) and 2 μm in (b).

with cardiac hypertrophy without left ventricular dilatation, but rapidly progress to the dilated form. On the other hand, the systolic function of his brother had been well preserved without left ventricular dilatation until his unexpected sudden death. Genetic analyses in the current study demonstrated that the cardiac β-MHC proteins should consist of only variant molecule in these affected siblings. Therefore, the development of dilated form of FHCM was suggested to be controlled not only by the cardiac β-MHC gene mutation but also by other genetic or acquired factors.

In conclusion, we have identified a novel missense mutation of the cardiac β-MHC gene in an FHCM family, in which two symptomatic patients were homozygous for the mutation while other three family members were heterozygous for the mutation and asymptomatic. The variability in disease manifestation within the family members observed in this study suggested that the clinical expression of FHCM due to the mutant β-MHC gene could be modified by a gene dose effect of the mutation and by some other non-genetic factors such as aging.

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