Expression of Proto-oncogenes and Gene Mutation of Sarcomeric Proteins in Patients With Hypertrophic Cardiomyopathy

Hisashi Kai,* Akihiko Muraishi,* Yuji Sugiu, Hirohumi Nishi, Yukihiro Seki, Fumitaka Kuwahara, Akinori Kimura, Hirohisa Kato, Tsutomu Imaizumi

Abstract—Several mutations of cardiac β-myosin heavy chain (β-MHC) gene were reported in patients with hypertrophic cardiomyopathy (HCM). Involvement of proto-oncogenes has been shown in the mechanism of experimental cardiac hypertrophy. This study sought to examine the effects of c-H-ras and c-myc expression in the steady-state myocardium on hypertrophic changes and to evaluate the possible interaction between β-MHC mutation and proto-oncogene expression in HCM. Endomyocardial biopsy was performed in 17 HCM patients (5 β-MHC mutations and 1 troponin T mutation) and 7 control subjects (no mutation). Reverse transcription–polymerase chain reaction analysis revealed c-H-ras expression in all members of both groups. Cardiomyocyte size was correlated with the expression level of c-H-ras (P<0.001), and c-H-ras expression was upregulated in HCM patients (P<0.01). HCM patients with a β-MHC mutation had the higher c-H-ras expression than did control subjects or patients without a mutation (P<0.01). c-myc mRNA was expressed in 7 of 17 HCM patients but not in control subjects. Myocyte size was greater in c-myc-positive HCM patients than in control subjects and c-myc-negative HCM patients (P<0.001 and P<0.05, respectively). The proto-oncogene expression did not affect clinical findings, myocardial fibrosis, or disarray. In conclusion, c-H-ras and c-myc expression in the steady-state myocardium may play a role in the hypertrophic mechanism in HCM. It is possible that β-MHC gene mutation has some effect on the regulation of proto-oncogene expression in HCM. (Circ Res. 1998;83:594-601.)

Key Words: proto-oncogene ■ β-myosin heavy chain mutation ■ hypertrophic cardiomyopathy ■ endomyocardial biopsy ■ reverse transcription–polymerase chain reaction

Hypertrophic cardiomyopathy (HCM) often shows a familiar occurrence consistent with autosomal-dominant inheritance with a high, yet variable, degree of penetrance. Since a missense mutation of the β-myosin heavy chain (β-MHC) gene was first identified as the responsible gene,1 a number of mutations of the β-MHC gene have been shown to cosegregate with inheritance of the disease.2 We have reported several point mutations in the β-MHC gene from HCM patients, detected by the polymerase chain reaction (PCR)–DNA conformation polymorphism (DCP) analysis.3–6 In addition, recent studies have shown that gene mutations of other components of the cardiac sarcomere, including cardiac troponin T (TnT) and α-tropomyosin, could be responsible for HCM.7 These findings raise the possibility that the nature of HCM is a “disease” of the sarcomere.

The ras proto-oncogene family encodes low-molecular-weight GTP-binding proteins, which serve as essential transducers of diverse physiological signals.7 Various kinds of hypertrophic stimuli and mechanical stretch have been shown to induce cardiomyocyte hypertrophy and gene expression via the intracellular signaling pathway, including Ras-dependent mitogen-activated protein kinase cascade.8,9 A nuclear proto-oncogene, c-myc, is essential for signal transduction by promoting DNA duplication and driving cells to reenter the cell cycle and is rapidly induced by growth factors or other trophic stimuli as well as by overexpression of proteins located upstream from the cell signaling pathway, including Ras.10,11 Therefore, their potential roles in cardiac growth and hypertrophy are under active investigation. c-H-ras expression is stable throughout cardiac development and after birth,12 whereas c-myc expression is abundant in the rat embryonic cardiocytes, is progressively downregulated during cardiac development, and is not detected after birth.13 Transient upregulation of c-H-ras and reexpression of c-myc are observed in a variety of experimental models of cardiac hypertrophy in adult rats.12,14,15 Although immunoreactivity for Myc was found in the myocardium in some of the HCM patients,16 little is known about the pathogenetic significance...
of the expression of these proto-oncogenes in the myocardium of HCM patients. Furthermore, it is possible that the mutations of the sarcomeric proteins have some effect on the expression of the proto-oncogenes, which might contribute to the development of cardiac hypertrophy in HCM.

The aims of the present study were (1) to examine whether c-H-ras and c-myc are expressed at the mRNA levels in the steady-state myocardium of HCM patients, (2) to determine whether the proto-oncogene expression has some effects on the phenotypic expression of HCM, and (3) to evaluate the possible interaction between the sarcomeric protein mutation and the proto-oncogene expression. We performed histological and reverse transcription–PCR (RT-PCR) analysis of endomyocardial biopsy samples and found that (1) c-H-ras expression was upregulated in HCM patients and the cardiomyocyte hypertrophy was correlated with the c-H-ras expression, and (2) c-myc–positive HCM patients showed greater myocyte hypertrophy than did c-myc–negative patients.

### Materials and Methods

#### Study Group

The study group consisted of 17 HCM patients (10 males, Table). Patients and family members of the patients were evaluated by physical examination, ECG, and 2-dimensional echocardiography. The diagnosis of HCM was based on the echocardiographic findings, including unexplained left ventricular hypertrophy and characteristic ECG changes, and was confirmed by cardiac catheterization. This study enrolled only the HCM patients in whom cardiac catheterization revealed no significant obstruction across the left ventricular outflow tract both spontaneously and after provocative tests, such as postextrasystolic potentiation and the isoproterenol challenge test. Furthermore, these HCM patients did not have either clinical symptoms or the 2-dimensional and Doppler echocardiographic

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FHx indicates family history; IVST, interventricular septal thickness (mm); LVDD, left ventricular diastolic dimension (mm); and LVEF, left ventricular ejection fraction.

*This patient was diagnosed as having Maron type IV HCM (Maron et al 17).
†778Arg>Gly missense mutation in exon 21 of the β-MHC gene.
‡736Ile>Met missense mutation in exon 20 of the β-MHC gene.
§870Arg>His missense mutation in exon 22 of the β-MHC gene.
|

P<0.01 vs control.
findings suggestive of left ventricular outflow tract obstruction during the follow-up period (2 months to 8 years). Moreover, apical hypertrophy was not included in the present study. Patients who had cardiac hypertrophy with a known cause or secondary myocardial disease were excluded. A family history of left ventricular hypertrophy was recognized in 12 cases in the HCM group. The control group consisted of 7 patients (4 males) undergoing elective electrophysiological studies for ventricular arrhythmia who had no ventricular hypertrophy assessed by echocardiographic and histological studies. The interventricular septum was thicker in HCM patients than in control subjects (P<0.01). The left ventricular end-diastolic dimension and the left ventricular ejection fraction did not differ between the 2 groups. Written informed consent was obtained from each subject.

**Mutation Screening of the Cardiac β-MHC and TnT Genes**

PCR-DCP analysis performed by direct DNA sequencing was performed for the mutation screening of the cardiac β-MHC and TnT genes. Briefly, genomic DNA was prepared from peripheral leukocytes of each subject, as reported previously. PCR primers were designed according to the reported normal human cardiac β-MHC and TnT cDNA sequences. The conditions and procedures of PCR-DCP analysis were such as described elsewhere. The PCR primer set contained primers were subjected to sequencing analysis to identify the sequence variations, using a commercial available sequencing kit (Sequenase version 2.0, US Biochemical Co). The PCR-DCP analysis were such as described elsewhere. The PCR primers were designed for the mutation screening of the cardiac β-MHC and TnT genes. Briefly, genomic DNA was prepared from peripheral leukocytes of each subject, as reported previously. PCR primers were designed according to the reported normal human cardiac β-MHC and TnT cDNA sequences. The conditions and procedures of PCR-DCP analysis were such as described elsewhere. The PCR primer set contained primers were subjected to sequencing analysis to identify the sequence variations, using a commercial available sequencing kit (Sequenase version 2.0, US Biochemical Co).

**Endomyocardial Biopsy and RT-PCR Analysis**

All cardiovascular agents were discontinued at least 7 days before cardiac catheterization. Several endomyocardial biopsy samples were obtained from the right side of the interventricular septum using the standard transfemoral technique. Biopsy samples were immediately frozen in liquid nitrogen and stored at −80°C until use.

PCR primers were designed according to the published sequences of c-myc, c-H-ras, β-MHC, and GAPDH cdNA. The nucleotide sequence used for each primer was as follows: for c-myc: 5'-primer (base 6731 to 6750), 5'-CGG-3'; 3'-primer (base 6945 to 6964), 5'-TCTGGATCACCTTCTGCTGG-3'; for c-H-ras: 5'-primer (base 132 to 152), 5'-GTGGGTACATGTCGGGTAGTGTG-3'; 3'-primer (base 378 to 398), 5'-GTCTTCAAGGCCTGCCAGATT-3'; for β-MHC: 5'-primer (base 1262 to 1282), 5'-GAAACGCGAGGTGTTGAGG-3'; 3'-primer (base 1433 to 1453), 5'-ACCTGGGAAAGTGTTGTTG-3'; and for GAPDH: 5'-primer (base 246 to 266), 5'-AAATCCATACCATCATTCCA-3'; 3'-primer (base 537 to 557), 5'-ATGAGTCCCTACGATCA-3'.

The mRNA was isolated from frozen samples (0.6 to 2.5 μg) using a QuickPrep Micro mRNA Purification kit (Pharmacia). RT-PCR was carried out using a high-performance PCR kit (Boehringer-Mannheim Biochemicals) according to the manufacturer's instructions. Briefly, the purified mRNA (300 ng) was reverse-transcribed with cloned Moloney murine leukemia virus reverse transcriptase (50 U) by incubating at 42°C for 60 minutes, followed by heating at 99°C for 5 minutes. The resulting single-stranded cDNA was then amplified using a pair of primers for each target gene (20 pmol) and Taq DNA polymerase (2.5 U) during the denoted cycle number of amplification (2 minutes at 94°C for denaturation, 2 minutes at 54°C for primer annealing, and 3 minutes at 74°C for primer extension). The RT-PCR products were then subjected to 1.5% agarose gel electrophoresis. Semiquantitative RT-PCR analysis of c-H-ras, β-MHC, and GAPDH mRNA expression was performed in the presence of 1 μCi of [α-32P]dCTP using the modified method of Ungerer et al. In preliminary experiments, the linearity of the band intensity against the PCR cycle number was given during 20 to 60 cycles of the PCR amplification of c-H-ras, β-MHC, and GAPDH (data not shown). Thus, 28 cycles for β-MHC and 38 cycles for c-H-ras and GAPDH were adopted for the amplification. The RT-PCR products were electrophoresed in 1.5% agarose gel, and the gel was dried up and exposed to an image plate (BAS-III, Fuji) for 12 hours. The radioactive signals were analyzed quantitatively by an imaging analyzer (BAS 2000, Fuji). The relative amount of c-H-ras and β-MHC expression was indicated as the ratio of the signal intensity for each band to that for GAPDH band as an internal standard.

To verify the validity of the semiquantitative RT-PCR method, quantitative RT-PCR of c-H-ras and GAPDH was performed by the method of Feldman et al in some patients. Briefly, we constructed synthetic DNA templates that contained 2 pairs of primers complementary to those used to amplify c-H-ras and GAPDH. The synthetic templates had sequences for the bacteriophage T7 promoter and polyadenine tracts on their 5' and 3' ends, respectively. Control RNA was generated by an in vitro transcription reaction from the synthetic DNA templates and then separated from control DNA using phenol/chloroform extraction and ethanol precipitation after incubation with RNase-free DNase (Promega). The sizes of the RT-PCR products of the control RNA using primer pairs for c-H-ras and GAPDH were 68 and 69 bp, respectively. When 300 ng sample mRNA and 25 pg control RNA were subjected to RT-PCR in the same reaction tube in the presence of the c-H-ras or GAPDH primer pair, exponential amplification was observed in all cases between cycles 30 and 48. In both primer pairs for c-H-ras and GAPDH, the amplification efficiency appeared to be the same for target and control RNAs, since the yields of the PCR products with increasing concentrations of sample mRNA and control RNA were colinear in the preliminary experiments. Under these conditions, c-H-ras and GAPDH mRNA levels were able to be determined by extrapolation from a standard curve constructed with varying concentrations of the control RNA, as described by Feldman et al. The quantitative RT-PCR analysis was performed in 4 HCM patients and 2 control subjects. Estimated mRNA levels of c-H-ras and GAPDH were 4.9±2.0 and 5.4±1.5 mole (×10^3) of total mRNA (n=6), respectively. The c-H-ras/GAPDH ratio obtained by the method of Feldman et al was plotted against that obtained by our method (Figure 1). A good correlation was found for the c-H-ras/GAPDH ratio between the 2 methods (r=0.975, P<0.01).

**Histological Analysis**

Biopsy samples were fixed in 10% formalin, dehydrated with ethanol, embedded in paraffin, and sectioned at a thickness of 3 μm. Histological analysis was performed in a blind fashion by 2 observers for 3 independent samples from each subject. To determine cardiomyocyte hypertrophy, the shortest transverse diameter of the cardiomyocytes was measured in at least 200 nucleated transverse sections of the
myocytes stained with hematoxylin-eosin. Briefly, cardiomyocytes were selected when cells showed the spindle-shaped transverse section including the elliptical nucleus. The shortest transverse diameter was measured 3 times per cell, and the values were averaged. Usually, 15 to 30 cells that satisfied the selection criteria could be found per 1 observation field at ×200 magnification, and 3 to 4 fields were randomly selected per a sample slide. The measurement was repeated in 3 sections that were obtained from 3 independent biopsy samples from each subject. Finally, the average of the shortest diameter of at least 200 myocytes was calculated. The percent area of myocardial fibrosis was assessed in Mallory-Azan stain samples by the point-counting method. The myocardial disarray was graded semiquantitatively for phosphotungstic acid–hematoxylin stain samples as follows: a parallel arrangement of the myocardial fibers was graded 0, and the disarray was graded 1 to 3, according to the extent of cellular branching and disarrangement. Concordance of each criterion was >0.95 between the 2 observers.

Statistical Analysis

Data were expressed as mean±SD. Unpaired t test or 1-way ANOVA followed by the Scheffé F test was used adequately for the statistical analysis unless otherwise indicated. Comparisons of myocardial disarray grades were performed using the Kruskal-Wallis test followed by the Mann-Whitney U test with the Bonferroni modification. For comparisons of the expression levels of c-H-ras and β-MHC, the data were first subjected to logarithmic transformation and then were analyzed by using 1-way ANOVA followed by the Scheffé F test. Correlation analysis was performed by Spearman rank correlation. A value P<0.05 was considered statistically significant.

Results

Mutation Analysis of the Cardiac β-MHC and TnT Genes

Of 17 HCM patients, 5 and 1 had a mutation of the β-MHC and TnT genes, respectively, whereas no mutation of either gene was found in the control subjects. Figure 2A demonstrates a representative PCR-DCP analysis. The PCR products of exon 21 of the β-MHC gene showed 2 slowly migrating DNA fragments in the HCM patient without a mutation (case 4) and in a control subject, whereas additional distinct DNA fragments were observed in the HCM patients with a mutation (cases 3 and 5). The PCR products containing unusual DNA fragments were subjected to DNA sequencing analysis. A missense mutation of 736 Ile>Met in exon 20 (case 11), 778 Asp>Gly in exon 21 (cases 3, 5, and 14) in the β-MHC gene, or 879 Arg>His in exon 22 (cases 17) and a 92 Arg>Trp mutation in exon 9 of the TnT gene (case 13) were identified (Table). All patients with a β-MHC or TnT mutation had apparent family histories.

Echocardiographic and hemodynamic findings did not differ between the HCM patients with and without a mutation of either gene. The transverse diameter of the cardiomyocytes, a parameter of cellular hypertrophy, was significantly larger in the HCM patients with a β-MHC or TnT mutation versus not only control subjects (P<0.001) but also the HCM patients without either gene mutation (P<0.05), and the HCM patients without a mutation of either gene showed larger cellular diameter than did the control subjects (P<0.05, Figure 3A). Furthermore, the HCM patients with a β-MHC mutation showed significantly greater cellular hypertrophy (23.3±3.9 μm) than did control subjects (13.7±1.3 μm, P<0.001) and the HCM patients without a mutation of either β-MHC or TnT (18.4±3.6 μm, P<0.05).

There was no difference in the myocardial disarray grades between the HCM patients with and without either mutation (1.0±0.6 versus 1.2±0.4), although both groups showed higher degrees of disarray than did control subjects (0.1±0.4; P<0.05 and P<0.01, respectively). The extent of myocardial fibrosis was not different among the 3 groups (10.3±5.0% versus 4.7±3.8% and 9.3±4.7%, respectively).

Proto-oncogene mRNA Expression in Endomyocardial Biopsy Samples

The steady-state expression of c-H-ras and c-myc mRNAs in the endomyocardial biopsy samples was examined using RT-PCR analysis (Figure 2B). In all of the HCM patients and control subjects, the constitutive expression of c-H-ras was observed as a single band with the molecular size of 276 bp on 1.5% agarose gel electrophoresis. In contrast, 7 (41%) of 17 HCM patients showed c-myc expression as a single band of 132 bp, whereas the c-myc band was not detected in control subjects. The expression of cardiac β-MHC and GAPDH was constitutively observed as a single band of 192 and 312 bp in length, respectively, in all of both groups. The molecular size of each RT-PCR product was compatible with that expected from the cDNA sequence of the target gene.

There was no difference in echocardiographic and hemodynamic findings between the c-myc–positive and –negative HCM patients. The HCM patients with and without c-myc expression had significantly greater myocyte transverse diameter than did the control subjects (P<0.001 and P<0.05, respectively), and the cellular diameter was larger in the c-myc–positive than the c-myc–negative HCM patients.
Myocardial fibrosis did not differ among the 3 groups (c-myc-positive, 11.0±4.9%; c-myc-negative, 8.9±4.6%). There was no significant difference in myocardial disarray between the c-myc-positive and -negative HCM patients (1.4±0.5 versus 0.9±0.4), although the 2 groups showed higher degrees of disarray than did the control subjects (P<0.01 and P<0.01).

**Semiquantitative RT-PCR Analysis for c-H-ras and β-MHC mRNAs**

The semiquantitative RT-PCR analysis demonstrated that c-H-ras expression was significantly greater in HCM patients than in control subjects (0.631±0.268 versus 0.271±0.085 [c-H-ras/GAPDH], P<0.01). Furthermore, the extent of myocyte hypertrophy was significantly correlated with the c-H-ras expression level (r=0.826, P<0.001, Figure 4A). The β-MHC mutations had significant effects on the expression levels of not only β-MHC itself but also c-H-ras. c-H-ras expression was increased in the HCM patients with a β-MHC gene mutation compared with control subjects (P<0.001) or the HCM patients without either β-MHC or TnT mutation (P<0.01, Figure 5A). Patients with a β-MHC gene mutation showed increased β-MHC expression compared with that in control subjects (P<0.05) or in HCM patients without a mutation of either gene (P<0.05), whereas β-MHC expression levels were not increased in the HCM patients without either gene mutation (Figure 5B). There was a significant correlation between c-H-ras and β-MHC expression levels (r=0.622, P<0.01, Figure 4B). In contrast, in a patient with a TnT mutation, the expression levels of c-H-ras and β-MHC were equivalent to those seen in control subjects.

As shown in Figure 5C, c-H-ras expression in both the c-myc-positive and -negative HCM patients was significantly greater than that in control subjects (P<0.01 and P<0.05), and there was no significant difference between c-myc-positive and -negative HCM patients. The β-MHC expression did not differ among the 3 groups, although the c-myc-positive HCM patients tended to have higher β-MHC expression than did the control subjects (P=0.068, Figure 5D).

**Discussion**

With the use of myocardial biopsy samples, the present study demonstrated for the first time that c-H-ras mRNA expression was upregulated in the steady-state myocardium of the HCM patients versus control subjects, and the extent of cardiomyocyte hypertrophy was well correlated with the c-H-ras expression level. The HCM patients with a β-MHC mutation showed greater cellular hypertrophy and increased c-H-ras expression than did control subjects and the HCM patients without a β-MHC or TnT mutation. c-myc mRNA was expressed in 7 of 17 HCM patients but not in control subjects, and myocyte hypertrophy was greater in the c-myc-positive HCM patients than in not only the control subjects but also the c-myc-negative patients. These observations suggest a significant role of these proto-oncogene products, as well as mutations of the sarcomeric proteins, in the hypertrophic process of the cardiomyocytes in HCM patients. The echocardiographic parameters of cardiac hypertrophy were not apparently affected by the expression of these proto-oncogenes.
Gene Mutation of Sarcomeric Proteins in HCM

We have reported 5 missense mutations in exons 16, 20, 21, 22, and 23 and a nonsense mutation in exon 3 in the β-MHC gene from Japanese HCM patients. In the present study, missense mutations of the β-MHC and TnT genes were found in 5 and 1 of 17 HCM patients, respectively, and the prevalence of these mutations was compatible with that of the previous observations. In the HCM patients with a β-MHC mutation, cardiomyocyte hypertrophy was significantly greater than that in not only the control subjects but also the HCM patients without a mutation of either gene. These patients had a mutation in exon 20, 21, or 22, which encodes the globular head containing the major functional domains of the β-MHC molecule. Therefore, it is suspected that the mutant β-MHC protein has impaired contractile function that may result in increased fiber stress that leads to compensatory hypertrophy. Furthermore, the defective mutant protein may destabilize the sarcomere through either increased turnover or impaired binding to actin or through the impaired interaction with other proteins necessary for the structural integrity. It was suggested that the increased breakdown of the destabilized sarcomeres may provide a stimulus for compensatory hypertrophy.

The present study revealed the increased β-MHC mRNA levels in the HCM patients with a β-MHC mutation. Since the protein levels of the β-MHC were not evaluated, the meanings of the mRNA increase remain unclarified. reported that the β-MHC protein synthesis was not changed in HCM patients with an Arg>Gln mutation in exon 13. However, this observation may not be applicable to the present study, since this point mutation, which is supposed to occur in the actin binding site, was not found in our patient group. The β-MHC mutations observed in the present study are expected to be located in the essential light chain binding site or the rod portion of the β-MHC molecules. It is plausible that the β-MHC levels in HCM patients are affected by the mutation location in the β-MHC molecules. Furthermore, in HCM patients with a β-MHC mutation, the increased mRNA expression may not necessarily result in the net increase in the β-MHC protein, since it is possible that breakdown of the mutant defective protein is enhanced.

c-H-ras Expression in HCM

ras protein is a member of the low-molecular-weight GTP-binding protein superfamily, which transmits various kinds of growth signals to the nucleus and triggers cell proliferation and hypertrophy in variable cell types. Although c-H-ras expression is stable throughout cardiac development and after birth, c-H-ras is transiently upregulated in rat hearts in response to the pressure overload, preceding the development of cardiac hypertrophy. It has been shown that injection of activated Ras protein into cultured ventricular myocytes induces the expression of c-fos and atrial natriuretic factor genes associated with the hypertrophic response and the morphological changes of the organization of the contractile apparatus. In the present study, c-H-ras expression was upregulated in the steady-state myocardium in HCM patients, and cellular hypertrophy was correlated with the c-H-ras expression level. Taken together, it was suggested that c-H-ras is involved in the mechanism(s) of the hypertrophic process in HCM. It is plausible that upregulation of the components of the intracellular signaling pathways of growth stimuli is involved in the molecular mechanism of cardiac hypertrophy. Our previous observation that HCM patients showed increased expression of myocardin protein kinase C, which mediates various kinds of growth signals by activating c-fos or the Ras–mitogen-activated protein kinase pathway, seems to support this hypothesis.
Increased c-H-ras expression was apparently associated with the presence of a β-MHC mutation. Since the sarcomeres containing the mutant β-MHC are considered to have impaired contractile function, it is likely that in the defective myocardial fibers, mechanical stress would be increased chronically and would directly or indirectly lead to sustained induction and activation of the Ras-dependent signaling pathway. The molecular mechanism(s) regulating c-H-ras expression in the HCM patients with a β-MHC mutation was not examined in the present study but is worthy of future investigation.

**c-myc Expression in HCM**

RT-PCR analysis revealed that c-myc mRNA was expressed in the steady-state myocardium in 7 of 17 intact living HCM patients but not in control subjects. The absence of c-myc expression in control subjects is compatible with the previous observation that c-myc mRNA expression was not detected in the myocardium of healthy human adults. Earlier studies demonstrated that an increase in hemodynamic load triggers the transient reexpression of c-myc preceding contractile protein gene expression and myocyte growth, suggesting a causative role of these immediate-early gene products in the molecular mechanism mediating hypertrophic growth. On the other hand, sustained increase in c-myc expression has been reported in the hearts of spontaneously hypertensive rats with both hypertension and ventricular hypertrophy and in the hearts of cardiomyopathic hamsters. Furthermore, transgenic mice overexpressing c-myc in the heart presented cardiac enlargement as a result of hypertrophy and self-limited proliferation. These observations imply that the sustained c-myc reexpression could mediate hypertrophic mechanism in those animals. In the present study, the c-myc–positive HCM patients showed a greater extent of cellular hypertrophy than did the c-myc–negative patients. Therefore, it is possible that cardiomyocyte hypertrophy is enhanced by the Myc-mediated process at least in some HCM patients. The prevalence of c-myc reexpression was not different between the HCM patients with and without a mutation of the β-MHC or TnT gene. However, it was noteworthy that of 7 HCM patients showing c-myc expression, 3 patients had the same point mutation (Asp>Gly) in exon 21 of the β-MHC gene. This preliminary observation suggests that the genetic background of HCM patients could influence the c-myc reexpression in the myocardium and the phenotypic expression. A future study that includes a larger number of patients is needed to test this hypothesis.

Although c-myc is induced in the myocytes of heart failure models or animals administered thyroid hormone, no patient had either a past history or clinical findings of heart failure or thyroid disease in the present study. In addition, the expression of c-H-ras and c-myc was not associated by the hemodynamic measures, including systemic or pulmonary arterial pressure and the end-diastolic pressure of either ventricle (data not shown).

**Limitations**

Limitations of the present study were as follows: First, The small number of patients limited our discussion of the clinical significance of the observations in the present study. Second, we assessed proto-oncogene expression and phenotypic expression only at the time of endomyocardial biopsy. Thus, we do not deny the possibility that these findings change during the disease process or as the patients age. However, the present study has potential advantages compared with studies using hearts obtained from autopsy or from the recipients of cardiac transplantation, since the influence of postmortem changes or cardiovascular agents given during the end stage, which potentially modulate proto-oncogene expression, can be avoided. Third, the tube-to-tube variabilities in the amplification efficiency would be critical in the present study, since target mRNA and internal standard (GAPDH) mRNA were subjected to RT-PCR in the individual reaction tubes for semiquantitative analysis. Thus, quantitative RT-PCR analysis of c-H-ras and GAPDH mRNAs was performed in 6 subjects in our study group by using the method of Feldman et al. With this method, target mRNA and a known amount of synthesized internal control RNA were reverse-transcribed and amplified using the primer pair for the target mRNA in the same reaction tube. A good correlation was found between the c-H-ras/GAPDH ratios obtained by our and Feldman’s methods. Therefore, the validity of our semiquantitative RT-PCR method was enough to draw our conclusions. Unfortunately, the quantitative RT-PCR analysis was not able to be performed in the other subjects, since the limitation of the amount of the biopsy samples did not allow us to use Feldman’s method. Fourth, semiquantitative analysis of c-myc mRNA expression was not available in the present study, since the PCR amplification condition could not be optimized because of the limited amount of the specimens and probably because of lesser numbers of mRNA copies in the myocardium. Additionally, since some HCM patients without a mutation of either β-MHC or the TnT gene had apparent family histories, it is possible that such patients may have had a mutation of other contractile proteins or a mutation that has not yet been identified. Thus, mutation of other genes may have had different effects on c-H-ras or c-myc expression or phenotypic expression. However, in the patients without a mutation of either gene, the presence of family histories had no effect on the extent of the myocyte hypertrophy or the expression level of c-H-ras or the β-MHC gene. Finally, HCM patients with a TnT mutation have been reported to have a high likelihood for sudden death. Thus, it is of great clinical importance to evaluate the effect of the proto-oncogene expression on not only the phenotypic expression but also the prognosis in these patient populations. Unfortunately, we could not assess this issue because of the infrequent prevalence of the TnT mutation and because of the small size of the patient group in the present study. Therefore, this unsolved problem should be settled in future studies.

In conclusion, we have demonstrated the upregulated expression of c-H-ras mRNA and the reexpression of c-myc mRNA in the biopsy samples retrieved from living intact HCM patients, suggesting that these proto-oncogene products take a role in the process of developing cardiac hypertrophy in HCM. It is also suggested that the gene mutations of the sarcomeric proteins have some interaction with the proto-oncogene expression in HCM patients, although the precise
mechanism still remains to be elucidated. The upregulated expression of c-H-ras or the reexpression of c-myc might be a possible predictor of the phenotypic expression or of the prognosis of the HCM patients, and a follow-up study should enroll larger numbers of HCM patients and their families. The present study may provide an insight into a possible target for gene therapy for HCM by modulating the expression of these proto-oncogenes in the myocardium.

Acknowledgments

This study was supported in part by a Grant-in-Aid for Scientific Research (09770524) from the Ministry of the Education, Science, and Culture, Japan; a research grant from Kaibara Memorial Foundation; and a Kimura Memorial Heart Foundation Research Grant.

References


Expression of Proto-oncogenes and Gene Mutation of Sarcomeric Proteins in Patients With Hypertrophic Cardiomyopathy

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doi: 10.1161/01.RES.83.6.594

_Circulation Research_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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