Angiotensin II Type 1 Receptor Gene Polymorphisms in Patients with Cardiac Hypertrophy

Alisher Ishanov, MD, Hiroshi Okamoto, MD, Masashi Watanabe, MD, Keiji Yoneya, MD, Izumi Nakagawa, MD, Hideki Kumamoto, MD, Satoru Chiba, MD, Akira Hata,1 MD, Hideaki Kawaguchi,2 MD, and Akira Kitabatake, MD, PhD

SUMMARY

Chronic mechanical stress of the heart secondary to arterial hypertension is a primary cause of left ventricular hypertrophy (LVH). The renin-angiotensin system (RAS) plays an important role in the cardiovascular system, regulating the expression of cardiac hypertrophy, in part, independent of the effects of systemic hypertension. A major component of RAS is angiotensin converting enzyme (ACE), which is upregulated in pressure overload-induced cardiac hypertrophy as well as heart failure. In a recent study, we found that the T allele of the M235T polymorphism of the angiotensinogen gene in sporadic hypertrophic cardiomyopathy (HCM) patients is associated with LVH. The present study was designed to assess the contribution of the polymorphisms of the angiotensin II type 1 receptor (AGTR A1166C) genes on development of left ventricular hypertrophy. Patients with hypertensive LVH and relatives of HCM without manifesting the disease, showed higher C allele frequency compared to patients with HCM (11.3% vs 4.2%, $\chi^2 = 5.3$, $p < 0.05$ and 10.5% vs 4.2%, $\chi^2 = 5.3$, $p < 0.05$, respectively), but healthy controls did not (11.3% vs 7.5%, $\chi^2 = 1.42$, NS and 10.5% vs 7.5%, $\chi^2 = 1.2$, NS).

The strong interaction between ACE I/D and AGTR A1166C gene polymorphisms has been found in groups of relatives of HCM patients; odds ratio associated with ACE D allele was significant in subjects carrying the AGTR C allele (OR = 7.3, 95% CI 1.6–33.1; $\chi^2 = 7.9$, $p < 0.02$) compared with healthy subjects.

We conclude that the molecular variant of the AGTR A1166C gene is not contributing to the development of cardiac hypertrophy in hypertensive LVH.
and HCM patients, whereas carriers of both C and D alleles had a four-fold increase in the odds ratio for family history of HCM without manifesting the disease. (Jpn Heart J 1998; 39: 87–96)

**Key words:** Renin-angiotensin system, Angiotensin II receptor gene, Left ventricular hypertrophy, Hypertrophic cardiomyopathy

The renin-angiotensin system is an important candidate for susceptibility to LVH, owing to its action on cellular hypertrophy and cell proliferation. It has been demonstrated that components of the RAS such as angiotensinogen, renin, angiotensin-converting enzyme (ACE) and angiotensin II receptors exist within the heart and may function independently from circulating RAS. The variability of the phenotypic expression of LVH in patients with HCM indicates a potential role for additional modifying genes.

In the RAS, angiotensinogen is cleaved by renin to produce the inactive peptide angiotensin I. The ACE then converts angiotensin I to angiotensin II; the latter peptide has various effects including vasoconstriction, aldosterone production, and enhanced noradrenaline release from sympathetic nerve endings. Angiotensin II also has hypertrophic, and possibly hyperplastic, effects on vascular smooth muscle cells and cardiomyocytes, and increases extracellular collagen matrix synthesis. The plasma concentration of angiotensinogen, the substrate for renin, is another clear determinant of angiotensin II levels. In a recent study we found that the T allele of the M235T polymorphism of the angiotensinogen gene in sporadic HCM patients is associated with LVH.

Most of the known actions of angiotensin II, an effector peptide in the renin-angiotensin system, are exerted through the angiotensin II type 1 receptor (AGT_{1}R), which is present in particular vascular smooth muscle cells and the myocardium. Gene variants in the AGT_{1} receptor genes have been found to interact with the ACE gene to increase the risk of myocardial infarction and have been associated with hypertension. Chronic mechanical stress such as hypertension is thought to be a primary cause of LVH, whereas angiotensin II has a trophic action on cardiac myocytes and proliferates fibroblasts leading to LVH independent of mechanical stress. Because of these findings and the importance of the RAS in cardiovascular regulation, the present study was designed to assess the contribution of the polymorphisms of the angiotensin II type 1 receptor (AGT_{1}R A^{166}C) gene on development of LVH with or without systemic hypertension. In addition, we explored interactions between AGT_{1}R A^{166}C and other RAS genes (ACE and angiotensinogen genes, determined previously in this same population) in relation to LVH.
METHODS

One-hundred forty nine patients with LVH (53 with hypertensive LVH and 96 with HCM) were compared with 265 subjects without LVH (healthy subjects without known hypertension and LVH and 105 unaffected siblings and offspring of HCM patients).

All patients were diagnosed by echocardiography in our hospital from 1985 to 1997. All eligible participants gave informed oral or written consent before echocardiographic examination and peripheral blood sampling. To avoid misunderstanding of the results due to incomplete and age-related penetrance, participants under the age of 18 were excluded from this study. Diagnostic criteria adopted for HCM have been published previously.5)

The distribution of LVH was assessed primarily in the parasternal short-axis plane and the long-axis two- and four-chamber views. LVH was diagnosed whether showing local or diffuse hypertrophy in the intraventricular septum, apical wall or free wall of the left ventricle.14)

All subjects were categorized as follows: hypertensive LVHs — 53 patients with pressure-induced hypertrophy, HCMs — 96 HCM patients (43 HCM patients with evident family history and 53 HCM patients without family history), Relatives — 105 unaffected siblings and offspring with a family history of HCM, without manifestating the disease; and Controls — 160 healthy subjects without known hypertension and LVH who were frequency-matched to hypertensive LVH cases by age and sex. In these 4 groups (414 cases, mean age 49.5 ± 14; 59% male) we determined angiotensin II type 1 receptor (AGT1R A1166C) gene polymorphism to detect genetic differences.

Extraction and amplification of genomic DNA: High molecular weight DNA was extracted from peripheral leukocytes as described previously. The region of interest was amplified by the polymerase chain reaction method (PCR), with the use of allele-specific oligonucleotide primers flanking the polymorphic region of the AGT1R A1166C gene to amplify template DNA prepared from peripheral leukocytes (5'AATGCTTGTAGCCAAAGTCACCT3'-sense, and 5'GGCATTGTCTTGTCTTGTGG3'-antisense).

Amplification was carried out in a PCR apparatus, (GeneAMP System 2400, Perkin Elmer Corporation, Norwalk, CT). PCR was performed in a final volume of 25 µl which contained approximately 100 ng of genomic DNA, with 50 pmol of each primer, 50 mmol/l potassium chloride, 2.5 mmol/l magnesium chloride, 10 mmol/l TRIS-hydrochloric acid at pH 8.4, 0.1% Triton X-100 (octoxynol), 100 mmol/l of each deoxynucleotide triphosphate, and 0.5 U of Taq polymerase.

After an initial denaturation step (5 min at 94°C), each of the 30 cycles
A representative sample of patients gel demonstrating the three possible genotypes for the angiotensin II type 1 receptor gene A^{166}C polymorphism. AA and CC denotes homozygotes, AC - heterozygotes. Numbers on the right indicate size in bp of amplified fragments.

Figure. Consisted of 1 min at 94°C, 1 min at 56°C, and 1 min at 72°C. To prevent contamination with other unexpected DNA during PCR, all reaction reagents except for each primer and the genomic DNA of interest were treated in a UV cross linker (Stratalinker UV Cross Linker, STRATAGENE) and a reaction control without primers was prepared for every 5 tubes as a negative control. The 2 µl of unpurified product was diluted to 10 µl in the recommended restriction buffer containing 0.3 units of DdeI (New England Biolabs, Beverly, MA, USA).

After restriction-endonuclease digestion for at least three hours at 37°C, samples were applied to 10 percent polyacrylamide gel, subjected to electrophoresis at 150 V for 60 minutes and visualized by ethidium bromide (Figure).

Statistical analysis: Values are expressed as mean ± SD. Nonparametric tests were used for two group comparisons (Mann-Whitney test) and analysis of variance (Kruskall-Wallis test). A p value < 0.05 was considered statistically significant.

Allele frequencies were deduced from genotype frequencies and differences between groups were tested by chi-squared analysis with one degree of freedom. Odds ratios and their 95% confidence intervals were calculated using the low-risk reference group (healthy subjects) to evaluate the effect of angiotensin II type 1 receptor gene AGT;R A^{166}C polymorphism on the risk of development of car-
diac hypertrophy. We chose this reference group to represent a baseline level of risk, so that we could estimate the effect of having the C allele, as well as the risks posed by homozygotes.

RESULTS

Age, systolic and diastolic blood pressure, and left ventricular maximum wall thickness of the total subject group are shown in Table I. There were no significant differences in sex and age between any groups. Blood pressures showed no significant difference between healthy controls, HCMs patients and HCM relatives by ANOVA (Kruskal-Wallis test). HCM relatives and healthy controls all had normal wall thickness (<11 mm).

**Angiotensin II type 1 receptor gene polymorphism:** As shown in Table II AGT1R genotypes AA, AC, and CC were present in 43, 8, 2 hypertensive LVH patients; 88, 8, and 0 patients with HCM; 139, 18 and 3 healthy controls and 85, 18, and 2 relatives of patients with HCM.

Genotypic proportions were in Hardy-Weinberg equilibrium in HCM rela-

<table>
<thead>
<tr>
<th>Table I. Clinical Characteristics of Subjects in Each Group.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>Sex (male/female)</td>
</tr>
<tr>
<td>Age (years)</td>
</tr>
<tr>
<td>Blood pressure (mmHg)</td>
</tr>
<tr>
<td>Systolic</td>
</tr>
<tr>
<td>Diastolic</td>
</tr>
<tr>
<td>Max.LVWT (mm)</td>
</tr>
</tbody>
</table>

Data are means ± SD. *p < 0.05 versus controls. H-LVHs denotes patients with pressure-induced hypertrophy. Controls — healthy subjects without known hypertension and LVH who were frequency-matched to hypertensive LVH cases by age and sex. HCMs denotes hypertrophic cardiomyopathy patients. Relatives, subjects with normal heart who have family history of HCM. Max. LVWT, maximum left ventricular wall thickness.

<table>
<thead>
<tr>
<th>Table II. Frequencies of the Angiotensin II Type 1 Receptor Genotypes of Hypertensive LVH and Control Subjects, HCM Patients and Their Relatives</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group/Genotype</td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>AA</td>
</tr>
<tr>
<td>AC</td>
</tr>
<tr>
<td>CC</td>
</tr>
<tr>
<td>All</td>
</tr>
<tr>
<td>A allele</td>
</tr>
<tr>
<td>C allele</td>
</tr>
</tbody>
</table>

*Allele frequencies were deduced from genotype frequencies. Numbers of subjects are shown according to the genotypes of the AGTR, A1166C polymorphism.
Table III. Synergistic Effects of RAS Genes on Development of Cardiac Hypertrophy

<table>
<thead>
<tr>
<th>Group and genotype</th>
<th>H-LVH n=53</th>
<th>Controls n=160</th>
<th>HCM n=96</th>
<th>Relatives n=105</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGT/AGC + C/C</td>
<td>2 (3.8)</td>
<td>9 (5.6)</td>
<td>6 (6.3)</td>
<td>18 (17.1)*</td>
</tr>
<tr>
<td>ACE DD + DI</td>
<td>3 (5.6)</td>
<td>11 (6.9)</td>
<td>2 (2.0)</td>
<td>3 (2.9)</td>
</tr>
<tr>
<td>ACE II</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*p < 0.05 versus controls. Allele frequencies were deduced from genotype frequencies. Numbers of subjects are shown according to the genotypes of the AGT/AGC polymorphism.

...tives ($\chi^2 = 0.003$), but deviated in the group of healthy controls ($\chi^2 = 5.73$); however, the power to test deviations from such expected proportions is low, particularly when one of the homozygous classes is rare.

Genotype dissociation was similar in the hypertensive LVH group and controls, relatives and controls, but not in the HCM patients and their relatives. The AA genotype frequency in patients with HCM was higher than in unaffected siblings and offspring (91.6% vs 80.2%, $\chi^2 = 4.96, p < 0.05$). The odds ratio (an estimate of the relative risk of HCM between subjects with AA genotype and subjects with the AC or CC genotype) was 1.6 (95% CI 0.7–3.9, $\chi^2 =$ NS) in patients with HCM.

The frequency of the C allele among unaffected siblings and offspring was similar to that observed in healthy subjects without known hypertension and left ventricular hypertrophy (10.5% vs 7.5%). The C allele frequency in unaffected siblings and offspring was higher than in patients with HCM (10.5% vs 4.2%, $\chi^2 = 5.3, p < 0.05$). The A allele frequency was higher in HCM than in unaffected siblings and offspring (95.8% vs 89.5%, $\chi^2 = 5.3, p < 0.05$).

Relationship between angiotensin II type 1 receptor and RAS genes polymorphisms: ACE D/I and angiotensinogen T235M genotypes were determined previously in this same population.5,10,15 No synergistic interactions have been observed in carriers of both AGT/AGC and angiotensinogen T235 alleles. We observed a significant increase of the C and the D alleles in subjects with a family history of HCM without manifesting the disease (Table III). Nine of 160 healthy subjects without known hypertension and left ventricular hypertrophy were carriers of both C and D alleles, whereas 18 among 105 relatives of HCM patients were both homozygote carriers (OR = 7.3, 95% CI 1.6–33.1; $\chi^2 = 7.9, p < 0.01$). Carriers of both C and D alleles had a four-fold increase in the OR for family history of HCM without manifesting the disease. The number of subjects studied was relatively small and therefore our results might be open to interpretation, but a clear interrelation between genotypes and family history of HCM without manifesting the disease could be seen.
DISCUSSION

This study raised two interesting points concerning AGT1R A1166C polymorphism. First, C allele frequencies were markedly lower in HCM than in hypertensive LVH patients. Second, in subjects with a family history of HCM without manifesting the disease, a significant increase of both the C and D allele carrier states has been observed.

The RAS system, operating through stimulation of AGT1R by angiotensin II, has received intense interest as a mechanism for controlling cardiac hypertrophy.1,2,13) Chronic mechanical stress of the heart is a primary cause of LVH. The peptide hormone angiotensin II plays a principal role in regulating blood pressure and fluid homeostasis. Most of its known effects are mediated by guanine nucleotide-regulatory protein (G protein)-coupled receptor defined as the type 1 angiotensin II receptor or AGT1R. Characterization of cDNA and genomic clones shows that the human AGT1R gene contains five exons and encodes two receptor isoforms as a result of alternative splicing.15) The AGT2R might have a unique signalling regulatory mechanism and its expression is inversely related to the mitogenic activity of cells.16)

The cardiac RAS is often found activated in conditions of increased afterload or mechanical stress of myocytes. Cardiac expression of angiotensinogen and ACE is increased, resulting in elevated cardiac angiotensin II formation.1) This has been demonstrated in stretched cardiac myocytes in vitro as well as in animal models6-19) of pressure overload hypertrophy (supravalvular aortic stenosis and spontaneously hypertensive rats) and in human pressure overload hypertrophy20) (aortic stenosis).

Functional consequences of elevated angiotensin II levels may be vasoconstriction of the coronary vasculature and deterioration of diastolic function of the hypertrophied heart. Local formation of angiotensin II may also have a proliferative effect on connective tissue cells. Angiotensin II may thus be an important factor causing development and progression of LVH itself. The involvement of RAS in remodeling of the heart differs between different animal models of pressure overload-induced hypertrophy and may even differ within the same model for development versus maintenance of cardiac hypertrophy. It has been suggested that pressure overload produces cardiac hypertrophy without angiotensin II.16-19) In the gain-of-function16) study using gene transfer, it was shown that the AGT2R exerts an antiproliferative effect counteracting the growth action of AGT1R. In vivo, the AGT1aR knockout mouse model reverse transcription-PCR analysis using cardiac mRNA revealed that AGT1aR mRNA was abundant in wild type mice but not detected in knockout mice.17) Thus, AGT1aR receptor signalling is not obligate for the cardiac hypertrophic response to hemodynamic
overloading. Factors related to LV end-diastolic pressure and diastolic fiber stretch, rather than AGT1R activation or systolic load, modulate the increased expression of LV ACE mRNA in hypertrophied hearts.19

Hypertrophic cardiomyopathy (HCM) is characterized by myocardial hypertrophy of unknown etiology.6,7) A large number of mutations in the cardiac β-MHC gene, as well as several mutations in the cardiac troponin T, cardiac troponin I, cardiac myosin binding protein C, α and β tropomyosin genes, ventricular myosin essential light chain and ventricular myosin regulatory light chain, have been identified and shown to co-segregate with inheritance of HCM in Caucasian3,4,6-8) and Asian populations.5,8) In addition, mutations in the HCM genes also were found in patients with apical hypertrophy and restrictive cardiomyopathy, providing the evidence for a genetic link between HCM and these cardiomyopathies.

The phenotypic expression of HCM among affected individuals sharing the same mutation varies markedly,4,6-8) indicating a role for environmental factors and it is likely that many genes3-5,10) responsible for HCM remain to be identified. Possible genes modifying the pattern of hypertrophy could be the angiotensin-converting enzyme5,10) and angiotensinogen gene10) and their synergistic interaction.5,10-13,21)

It has been suggested that ACE gene polymorphism and ACE circulating levels might play a role in the myocardial hypertrophic process in HCM,5) especially in HCM with beta-MHC gene mutation.4) In a recent study10) we found that the T allele of the M235T polymorphism of the angiotensinogen gene in sporadic HCM patients is associated with LVH. In an animal model, it has been shown that activation of the AGT1R receptor may not be important for mediating either cardiac hypertrophy17-19) or myocardial fibrosis in the cardiomyopathic hamster.22)

An excess of the AGT1R C allele has been reported in severe hypertensives compared to normotensive subjects,12) which suggests involvement of the AGT1R gene in the predisposition to hypertension.13) However, no significant association between the AGT1R polymorphism and high blood pressure in the population as a whole has been found.11,23-25) It has been suggested that the AGT1R C allele is associated with a modified response of the receptor to angiotensin II.11)

A strong interaction between ACE I/D and AGT1R A1166C gene polymorphisms was found in the group of relatives of HCM patients in this study. We conclude that the molecular variant of the AGT1R A1166C gene is not contributing to development of cardiac hypertrophy in hypertensive LVH and HCM patients, whereas carriers of both C and D alleles had a four-fold increase in the odds ratio for family history of HCM without manifesting the disease.
REFERENCES


22. Kuriihara T, Karita M, Matsuda T, Okuto K, Kagiya T, Kusukawa H. Angiotensin AT1 receptor
antagonism does not reduce either cardiac hypertrophy or myocardial fibrosis in cardiomyopathic hamster (BIO14.6); comparison with the effects of ACE inhibitor. (Abstract) Circulation. 1996; 94; 8. S-I: 657.

