Left Ventricle Mass Index and the Common, Functional, X-Linked Angiotensin II Type-2 Receptor Gene Polymorphism (−1332 G/A) in Patients With Systemic Hypertension

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Abstract—A common intronic polymorphism, (−1332 G/A) of the angiotensin-type-2 receptor gene, located on the X-chromosome, has been reported to be functional. The aim of our study was to evaluate this polymorphism for an association with left ventricular hypertrophy. Left ventricle (LV) mass was measured in 197 patients with systemic hypertension and 60 normal volunteers using a 1.5-Tesla Philips MRI system. Genotyping was performed using a restriction enzyme digestion of an initial 310-bp polymerase chain reaction product that included the angiotensin-type-2 (−1332 G/A) locus. The mean LV mass index for the male patients was 94.3±19.6 g/m² (n=125) and for the female patients was 71.2±12.0 g/m² (n=72). Seventy-three (37.1%) of all patients had an elevated LV mass index, defined as the mean LV mass index for normal volunteers plus 2 SD (males 77.8±9.1 g/m²; n=30; females 61.5±7.5 g/m²; n=30). Comparison of LV mass index of the A/G genotype (mean LV mass index=82.4±21.1 g/m²; n=123) against that of the G/G genotype (mean LV mass index=88.1±19.0 g/m²; n=89) as a continuous variable was significant by ANOVA (P=0.044). χ² Comparison between subjects with and subjects without left ventricular hypertrophy revealed an excess of the G/G genotype among the group with LV hypertrophy (P=0.031). We observed an association between the angiotensin type-2 receptor (−1332 G) allele and the presence of left ventricular hypertrophy in hypertensive subjects. (Hypertension. 2004;43:1189-1194.)

Key Words: hypertension ▪ hypotrophy ▪ receptors, angiotensin ▪ magnetic resonance imaging ▪ genetics

The angiotensin II type-2 (AT₂) receptor gene is located on the X-chromosome and its structure and nucleotide sequence in humans was described in 1995. It consists of 3 exons and 2 introns, with the entire open reading frame of the AT₂ receptor located on exon 3. A commonly occurring intronic polymorphism has recently been described at a lariat branch-point in intron 1. Its position, described relative to the translation initiation site of the human AT₂ receptor gene, is (−1332), although it has also been previously described by others as (+1675). In our studies, we use the former nomenclature. It is located 29 bp before exon 2, close to the region that is important for transcriptional activity. Human mRNA studies show that subjects with the G allele have exon 2 missing and that the amount of this abnormally spliced mRNA was markedly reduced. This confirms the functional significance of this point mutation. In the same article, the polymorphism was reported to be associated with congenital anomalies of the kidney and urinary tract in men. Furthermore, Schmieder et al have recently reported a significantly higher left ventricular (LV) mass, measured by M-mode echocardiography, in young mildly hypertensive males with the +1675 A genotype in a study of 120 subjects. However, the accuracy and the reproducibility of M-mode echocardiography, particularly in patients with left ventricular hypertrophy (LVH), have been debated. More recently, cardiac magnetic resonance imaging (MRI) has been used successfully to obtain a more precise and reproducible measure of LV mass in such studies. It is calculated that the number of subjects needed for such studies using cardiac MRI is as little as one-tenth the number required to perform the same study using echocardiography.

The AT₂ receptor is thought to oppose the growth promoting effect of the angiotensin type-1 (AT₁) receptor. Moreover, the balance of AT₁ and AT₂ receptors seems to be important in determining both vascular and cardiac remodeling. Studies show that stimulation of the AT₂ receptor is associated with inhibition of growth of vascular smooth muscle cells and apoptosis. It has also been shown to inhibit growth of cardiomyocytes and fibroblasts. In 2 studies of AT₂ knockout mice, LVH did not develop in response to

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pressure overload, to being induced by constricting the abdominal aorta, or by the infusion of angiotensin II. An obligatory role of AT2 receptor in the development of cardiac hypertrophy was inferred by the authors. To the contrary, Wu et al found that aortic banding caused cardiac hypertrophy in both AT2+ and AT2− mice to a similar degree and that coronary arterial thickening and perivascular fibrosis were more exaggerated in AT2− mice. It is known that receptor subtype redistribution occurs in LVH with AT1 subtype downregulation, resulting in marked increase of the proportion of AT2 receptor. This suggests a key modulating role for the AT2 receptor in the development of LVH. The clinical implication is that regression of hypertension induced LVH by AT1 receptor blockers may in part be caused by unopposed antigrowth effect of angiotensin II mediated via the AT2 receptor.

The aim of our study was to evaluate the AT2 receptor gene polymorphism (−1332 G/A) for an association with LVH in patients with systemic hypertension. LV mass is measured precisely using cardiac MRI.

Methods

Patients and Volunteers
Sixty normal volunteers (30 men) were recruited to establish a normal range for cardiac MRI LV volumes and mass as well as the frequency of the AT2 receptor (−1332 G/A) polymorphism in a normal population. Two hundred five patients with systemic hypertension were recruited from cardiology outpatient clinics; 85% of the patients were known to have a history of systemic hypertension. LV mass is indexed to body surface area (BSA). LVH was defined as elevated LV mass index, based on the mean LV mass index for normal volunteers plus 2 SDs. Because angiotensin-converting enzyme (ACE) inhibitors and angiotensin II receptor blockers (ARBs) are known to influence LV mass regression, we used the test of unpaired proportions to assess for a difference between the A_/AA and G_/GG genotypes (hemizygous males and homozygous females) in drug treatment. We used the Mann-Whitney test to assess for a difference between the means of the doses of those 2 drugs according to genotype. We also used the test to investigate whether there was a difference in BP measurements between the 2 genotypes. The association of the G_/GG alleles with elevated LV mass index, as a continuous variable, was assessed using analysis of variance (1-way ANOVA). χ2 Test was performed to compare the prevalence of A_/AA and G_/GG genotypes, for hemizygous males and homozygous females, based on the presence or absence of LVH.

Results
All 60 healthy volunteers completed the cardiac MRI studies. Their mean age was 43 ± 12 years, range 20 to 65 years, and they had a mean sitting BP of 123/72 mm Hg. Two of the volunteers did not provide a blood sample and were excluded from the genetic part of the analysis. Of the 205 patients recruited, 8 were excluded because of poor DNA quality. The mean age of the 197 remaining patients was 55.4 ± 11.4 years (age range 21 to 79 years); 125 (63.5%) were men, and 168 (85.3%) of the hypertensive patients were using antihypertensive medications (Table 1). To consider whether there was a difference between the A_/AA and G_/GG genotypes in drug treatment with ACE inhibitors and ARBs, we used the test of unpaired proportions. There was no significant difference between the proportion of the A_/AA genotypes on all data sets, one experienced observer manually traced the endocardial and epicardial contours of the LV. Two papillary muscles were outlined separately, excluded from the volume, and included in the mass. LV mass was calculated as LV mass = 1.05 × (epicardial volume − endocardial volume).

Laboratory Methods
After genomic DNA extraction, primers 5’ AGA GAT CTG GTG CTA TTA CG 3’ and 5’ CAC TTG AAG ACT TAC TGG TTG 3’ (Invitrogen) were used to amplify a 310-bp DNA fragment between intron 1 and exon 2 including the A/G polymorphism. Polymerase chain reactions (PCR) were performed at 95°C for 15 minutes to activate the AmpliTaq Gold enzyme (Applied Biosystems), followed by 35 cycles of: 95°C for 30 seconds, 42°C for 30 seconds, and 72°C for 45 seconds. The product (5 μL) was digested for >3 hours with 5 U of HYP 188 III (New England Biolabs), which cuts the G but not the A allele, then subjected to gel electrophoresis (2% agarose) for genotyping (Figure 2). The AT2 receptor G allele gives 2 fragments of 104 and 206 bp, and the AT2 receptor A allele yields a single undigested 310-bp fragment. Genotyping was found to be accurate when confirmed by direct DNA sequencing (BigDye Terminator v3.1 Cycle Sequencing Kit; ABI PRISM, 3100 Genetic Analyzer) for the first 35 patients (Figure 2).

Statistical Methods
A power calculation was used to calculate the sample size with 95% confidence (type I error = 0.05) and 90% power to detect a 10 g/m2 difference in LV mass index between the means (with SD of the sample of 19 g/m2 for men and 9 g/m2 for women). The numbers required were 154 for men and 30 for women. The number of women was doubled (n = 60) as heterozygous females were excluded from the analysis because of the inactivation of one of the X-chromosomes in females.

The results were analyzed using SPSS software (SPSS for Windows, version 11.0). Means and SDs were calculated for LV mass indexed to body surface area (BSA), LVH was defined as elevated LV mass index, based on the mean LV mass index for normal volunteers plus 2 SDs. Because angiotensin-converting enzyme (ACE) inhibitors and angiotensin II receptor blockers (ARBs) are known to influence LV mass regression, we used the test of unpaired proportions for comparison of the A_/AA and G_/GG genotypes (hemizygous males and homozygous females) in drug treatment. We used the Mann-Whitney test to assess for a difference between the means of the doses of those 2 drugs according to genotype. We also used the test to investigate whether there was a difference in BP measurements between the 2 genotypes.

Figure 1. Cardiac MRI short-axis images of the heart in end-diastole derived from (left) a patient with hypertension and LVH and (right) from a normal volunteer. Endocardial, epicardial, and papillary muscles are outlined for the calculation of LV mass.
ACE inhibitors (40/93; 43.0%) compared with the G_/GG genotypes (36/75; 48.0%) \((P=0.518)\). There was no significant difference between the proportion of the A_/AA genotypes on ARBs (17/93; 18.3%) compared with the G_/GG genotype (12/75; 16.0%) \((P=0.698)\). To test whether there was a difference between the means of the doses of those 2 drugs according to genotype, and because the samples were skewed, we used the Mann-Whitney test. No significant difference was observed for the ACE inhibitors \((P=0.604)\) or for the ARBs \((P=0.159)\).

The mean sitting BP for the patients group was 159/93 mm Hg. For those patients who had a 24-hour BP monitor \((n=159)\), the group average of the mean 24-hour BP was 141/87 mm Hg. The group average of the daytime BP (based on the 24-hour BP recording) for the same 159 patients was 146/91 mm Hg. Differences in BP measurements between A_/AA and G_/GG genotypes were considered. The F test confirmed that there were no significant differences between the BP variances for the 2 genotypes. We then used the \(t\) test to test the hypothesis that there was no difference between the means (Table 2).

The mean LV mass indexed to body surface area (BSA) for the male healthy volunteers was 77.8\(\pm\)9.1 g/m\(^2\) \((n=30)\) and for the female healthy volunteers was 61.5\(\pm\)7.5g/m\(^2\) \((n=30).\)\(^{21}\) For the patients with hypertension, the mean LV mass indexed to BSA for the male patients was 94.3\(\pm\)19.6 g/m\(^2\) \((n=125)\) and for the female patients was 71.2\(\pm\)12.0 g/m\(^2\) \((n=72).\) A 2-way ANOVA showed a difference in the mean LV mass according to gender and hypertension status \((P<0.0001).\) Seventy-three (37.1%) patients had LVH (20; 27.8% of the female patients and 53; 42.4% of the male patients). The distribution of genotypes among the normal subjects and the subjects with hypertension with and without LVH was calculated (Figure 3).

We assessed LV mass index as a continuous variable using data from all subjects. When the A_/AA genotype (mean LV mass index=82.4\(\pm\)21.1 g/m\(^2\); \(n=123)\) were compared with the G_/GG genotype (mean LV mass index=88.1\(\pm\)19.0 g/m\(^2\); \(n=89)\), the ANOVA was statistically significant \((P=0.044; 95\% \text{ confidence interval for the difference in means was } -11.3 \text{ g/m}^2 \text{ to } -0.2 \text{ g/m}^2).\) For the female subjects, the mean LV mass index for AA genotype was 67.6\(\pm\)12.8 g \((n=40),\) and for the GG genotype was 68.4\(\pm\)9.8 g \((n=18).\) For the male subjects, the mean LV mass index for the A haplotype was 89.5\(\pm\)20.6 g \((n=83)\) and for the G haplotype was 93.1\(\pm\)17.4 g \((n=71).\)

\(\chi^2\) Test was performed to assess the prevalence of A_/AA and G_/GG alleles between the subjects without LVH (normal volunteers and hypertensives without LVH) and the hypertensive patients with LVH. Heterozygous females were excluded from the analysis because of the inactivation of one of the X-chromosomes in females. The frequency of the G_/GG genotype was found to be higher in the hypertensive patients with LVH \((P=0.031).\) The \(\chi^2\) test was repeated on the females and males separately with \(P=0.67\) and \(P=0.058,\) respectively. The \(\chi^2\) test was also performed to assess the prevalence of A_/AA and G_/GG alleles between the normal volunteers and hypertensive patients with LVH. The frequency of the G_/GG genotype was found to be higher in hypertensive patients with LVH than it was for normal volunteers \((P=0.023).\) When the \(\chi^2\) test was also performed to assess the prevalence of A_/AA and G_/GG alleles between the hypertensive patients without LVH and hypertensive patients with LVH, the frequency of the G_/GG genotype was found to be higher in the patients with LVH. \((P=0.058)\) (Figure 3).
Cardiac MRI provides a spatially defined 3-dimensional data set at multiple levels throughout the heart; hence, the measurement of LV mass does not require geometric assumptions to be made about the LV. The excellent contrast between blood and myocardial tissue and the high resolution mean that the endocardial and epicardial contours are easily defined. MRI measurements of LV mass have been validated in animal studies, shown to be more accurate and reproducible than M-mode and 2-dimensional echocardiography, and is currently accepted as the technique of choice for LV mass measurements. Although M-mode echocardiography is widely used to estimate LV mass, its accuracy is limited, particularly in patients with LVH in whom the geometric assumptions about the structure of the LV may no longer be valid. It is calculated that to perform such studies using echocardiography, one would need 10-times the number of subjects required by cardiac MRI.

Our patients all had systemic hypertension that was either previously diagnosed/treated (85.3%) or newly diagnosed/untreated (14.7%). Consequently, the severity of hypertension also varied within this group. We did not consider it either necessary or appropriate to stop antihypertensives for the purpose of this study. Continued use of antihypertensive drugs was expected to have resulted in attenuation of LVH, the severity of which would, in any case, be expected to vary in this group of patients. Nevertheless, similar frequency of use of both ACE inhibitors and ARBs should have ensured that this was not a confounding factor in our observations. If the use of drugs acted as a confounding factor, the effect would have been to make an association between genotype and LV mass more difficult to detect.

We compared the A_/AA genotype (mean LV mass index=82.4±21.1 g/m²; n=123) to the G_/GG genotype (mean LV mass index=88.1±19.0 g/m²; n=89) by ANOVA and found a difference that was statistically significant (P=0.044). A comparison of subjects with LVH to the subjects without LVH revealed a significant excess of the G_/GG genotype (P=0.031). Furthermore, a comparison of the hypertensives with LVH to the normal volunteers revealed a significant excess of the G_/GG genotype (P=0.023). Additional evidence to support the biological validity of our observations is the trend (P=0.058) of excess G_/GG genotype among the hypertensives with LVH when compared with the hypertensives without LVH (Figure 3). Our findings suggest that the (-1332 G) polymorphism of the AT2 receptor is associated with hypertension related LVH. The LVH observed was present despite the fact that the majority of the patients were using antihypertensive medications. This is of importance because LVH is associated with excess risk of morbidity and mortality. The AT2 receptor polymorphism might serve as a marker for patients who would benefit from a more aggressive control of BP and/or drugs that block the AT1 receptor that have been shown to improve outcomes.

Previously, Schmieder et al studied LV mass as measured by M-mode echocardiography in 120 healthy male students with normal or mildly elevated BP, reporting an association between the AT2 receptor (+1675 A/G) polymorphism and LV mass. The overall allele frequency for this study was comparable to our own (A=57% and G=43%). We have previously shown that the G allele was associated with hypertension and LVH and the G allele was more frequent in hypertensives with LVH when compared with the hypertensives without LVH (P=0.031). Additional evidence to support the biological validity of our observations is the trend (P=0.058) of excess G_/GG genotype among the hypertensives with LVH when compared with the hypertensives without LVH. The LVH observed was present despite the fact that the majority of the patients were using antihypertensive medications. This is of importance because LVH is associated with excess risk of morbidity and mortality. The AT2 receptor polymorphism might serve as a marker for patients who would benefit from a more aggressive control of BP and/or drugs that block the AT1 receptor that have been shown to improve outcomes.

**Discussion**

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G=43%), with subjects being relatively unselected. An excess of the G allele was seen in patients with a casual BP >140/90 mm Hg (A=54% and G 46%) as compared with those deemed to be normotensive (A=59% and G=41%). Given this reported trend toward, and association between, the G allele and the presence of hypertension, it is paradoxical that students in their study with the A geno-type were reported to have higher LV mass. More recently, Herrmann et al investigated the polymorphism in 2 randomly sampled populations28 that were originally used to investigate the prevalence of LV dysfunction. Once again, there was an excess of the G allele among hypertensive men in both subgroups (hypertension, A=47.6% and G=52.4%; normotension, A=52.1% and G=47.9% and hypertension, A=44.9% and G=55.1%; normotension, A=48.7% and G=51.3%). They conclude that the results are not consistent across the cohort and recommended further research.29

Perspectives
Mutations located in noncoding regions, such as those affecting 5' and 3' splice sites or branch sites, are often the cause of hereditary disease.30 The AT2 receptor gene (−1332 G/A) intronic polymorphism has been reported to be located at a lariat branch-point in intron 1. Differently spliced AT2 receptor mRNAs species have already been described and shown to be biochemically functional.31 Elevated LV mass is associated with excess risk of morbidity and mortality. In a prospective study, we have observed a statistical association between the AT2 (−1332 G) allele and the presence of LVH in patients with hypertension. Less effective transcription of the AT2 receptor gene, as a result of the (−1332 G) polymorphism might be expected to result in reduced AT2 receptor mediated effects in patients with the G allele.2 Our results are therefore in keeping with the hypothesis that the AT2 receptor counteracts the myocardial growth promoting effects of the AT1 receptor.14 The AT2 receptor polymorphism might serve as a marker for patients who would benefit from a more aggressive control of BP and/or drugs that block the AT1 receptor, either ARBs or ACE inhibitors, because they have been shown to improve outcomes.20,27

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