Genetic variation in angiotensin II type 2 receptor gene influences extent of left ventricular hypertrophy in hypertrophic cardiomyopathy independent of blood pressure

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Abstract

Introduction. Hypertrophic cardiomyopathy (HCM), an inherited primary cardiac disorder mostly caused by defective sarcomeric proteins, serves as a model to investigate left ventricular hypertrophy (LVH). HCM manifests extreme variability in the degree and distribution of LVH, even in patients with the same causal mutation. Genes coding for renin–angiotensin–aldosterone system components have been studied as hypertrophy modifiers in HCM, with emphasis on the angiotensin (Ang) II type 1 receptor (AT_1R). However, Ang II binding to Ang II type 2 receptors (AT_2R) also has hypertrophy-modulating effects.

Methods. We investigated the effect of the functional +1675 G/A polymorphism (rs1403543) and additional single nucleotide polymorphisms in the 3' untranslated region of the AT_2R gene (*AGTR2*) on a heritable composite hypertrophy score in an HCM family cohort in which HCM founder mutations segregate.

Results. We find significant association between rs1403543 and hypertrophy, with each A allele decreasing the average wall thickness by ~0.5 mm, independent of the effects of the primary HCM causal mutation, blood pressure and other hypertrophy covariates (p = 0.020).

Conclusion. This study therefore confirms a hypertrophy-modulating effect for AT_2R also in HCM and implies that +1675 G/A could potentially be used in a panel of markers that profile a genetic predisposition to LVH in HCM.

Keywords

Angiotensin II type 2 receptor, cardiac hypertrophy, hypertrophic cardiomyopathy, renin–angiotensin–aldosterone system

Introduction

Hypertrophic cardiomyopathy (HCM), a primary cardiac disorder characterised by left ventricular hypertrophy (LVH), serves as a model to investigate hypertrophy development.[1] HCM is classically caused by mutations in genes encoding sarcomeric proteins, and to date more than 450 HCM causal mutations have been identified within sarcomeric genes.[2] However, the hypertrophic phenotype of HCM is extremely heterogeneous. Not only do distinct disease-causing mutations affect prognostic outcomes differently,[3] but extreme variability in the extent and distribution of hypertrophy is also observed in individuals from the same family with the same disease-causing mutation.[4,5] This, as well as evidence from animal models,[6] suggests that the eventual hypertrophic phenotype of HCM is modified by the primary causal mutation as well as additional genetic and environmental factors.

Previous studies have identified genes encoding renin–angiotensin–aldosterone system (RAAS) components as modifiers of the hypertrophic phenotype of HCM.[7-9] Many of these investigations focussed on the angiotensin (Ang) II type 1 receptor (AT_1R) as binding of Ang II to this receptor

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elicits cellular hypertrophic effects,[10] and because clinical trials concluded that AT$_1$R antagonists reduce LVH and other cardiac morbidities.[11,12] However, Ang II is also capable of binding to Ang II type 2 receptors (AT$_2$R), which results in anti-hypertrophic effects that counter the hypertrophic effects of AT$_1$Rs.[13,14] The AT$_2$R gene (*AGTR2*) is located on the X-chromosome and consists of three exons and two introns, with the entire open reading frame of the gene situated in the third exon.

Studies on hypertensive populations have identified a functional +1675 G/A polymorphism (rs1403543) in *AGTR2* that is significantly associated with LVH indices, but were discrepant regarding which of the alleles increased hypertrophy.[15-17] This discrepancy might be explained by the vulnerability of association studies to the effect of confounding variables that influence the eventual cardiovascular phenotype, if these are not adjusted for in statistical analyses.[18]

While the effect of this +1675 G/A polymorphism on hypertrophy in patients with HCM is unknown, Deinum *et al.*[19] reported an association between a single nucleotide polymorphism (SNP) in the 3' untranslated region (UTR) of *AGTR2*, rs11091046, and left ventricular mass index (LVMI), observed only in female HCM patients. However, this study of ungenotyped cases did not account for differences in the primary HCM causal mutation, which may have had confounding effects on the association.

The present study therefore aimed to investigate the effect of the +1675 G/A polymorphism, rs11091046 and an additional SNP in the 3' UTR of *AGTR2* on a heritable hypertrophy trait in a cohort of HCM families in which either of three HCM founder mutations segregate, while taking the HCM-causing mutation and additional hypertrophy covariates into consideration.

Methods

Subjects

The University of Stellenbosch Health Science Faculty's Institutional Review Board approved this study (N04/03/062), and all subjects entered into the study gave written informed consent. Genomic DNA for all genotypic analyses was extracted from the nuclei of peripheral blood leukocytes as described previously.[5,20] During routine mutation screening

of consecutively referred probands, 22 individuals carrying any of three mutations that occur as founder mutations in South Africa, i.e. R92W$_{TNNT2}$, R403W$_{MYH7}$, and A797T$_{MYH7}$, were identified.[20] Pedigree tracing was performed for these probands and a cohort of 353 genetically and clinically affected and unaffected family members were enrolled in the study. These individuals were all screened for the presence or absence of all three founder mutations.

Clinical evaluation

All participating individuals were clinically characterised by an experienced echocardiographer (MR) who was blinded to the mutation status of each subject. A total of 16 2D-echocardiographic measurements of wall thickness at the mitral valve, papillary muscle and apex levels were taken according to recommendations of the American Society of Echocardiography,[21] as described previously.[3] These measurements were used to generate a composite score of the 16 wall thickness measurements that offered the best representation of the variability in hypertrophy seen in our cohort, by means of principal component analysis.

Blood pressure (BP) measurements were taken twice in the sitting position, after 5 min of rest, and the second measurement used. Subjects were identified as hypertensive if they were on anti-hypertensive medication or if they had a systolic BP ≥ 140 mm Hg or diastolic BP ≥ 90 mm Hg. We derived resting heart rate (HR) from standard electrocardiography performed on a MAC1200ST after 5 min of rest. Medical history and additional covariates of cardiac structure (age, sex and body surface area (BSA)) were recorded for each participant.

AGTR2 *genotyping*

Genotypes were determined on all DNA samples for the variants listed in table 1, using ABI TaqMan Validated SNP Genotyping Assays (Applied Biosystems, Foster City CA, USA). Rs1403543 (+1675 G/A) is located in intron 1 of *AGTR2*, while rs5194 and rs11091046 both occur in the 3' UTR of *AGTR2*.

Polymerase chain reaction (PCR) amplification for each SNP was performed in 5 µl reactions in thermostable

Table 1. Characteristics of polymorphisms chosen for investigation

Gene	Chromosome	SNP ID[a]	Chromosome coordinate[b]	Nucleotide change	Intragenic Position
AGTR2	Xq22-q23	rs1403543	115216220	G/A	Intron 1
		rs5194	115218858	G/A	3' UTR
		rs11091046	115219154	C/A	3' UTR

Key: [a] dbSNP build 126; [b] NCBI build b36; *AGTR2*-Angiotensin II receptor type 2; SNP-single nucleotide polymorphism; UTR-untranslated region

Table 2. Basic characteristics of the study cohort stratified into mutation carrier (MC) and non-carrier (NC) groups according to HCM mutation status

	A797T$_{MYH7}$		R92W$_{TNNT2}$		R403W$_{MYH7}$	
	MC	NC	MC	NC	MC	NC
Total:	71	86	47	74	34	43
Males (%)	37 (52)	39 (45)	23 (49)	30 (41)	21 (62)	41 (44)
Age	44 (27;59)	41 (29;52)	37 (23;47)	42 (28;49)	37 (25;47)	37 (31;50)
BSA (m²)	1.9 (1.7;2.0)	1.9 (1.7;2.1)	1.7 (1.6;1.8)	1.8 (1.6;1.9)	1.8 (1.7;2.0)	2.0 (1.8;2.1)
SBP (mm Hg)	120 (120;140)	120 (110;130)	120 (110;130)	120 (110;125)	123 (115;130)	130 (120;150)
DBP (mm Hg)	80 (70;90)	80 (80;88)	75 (70;80)	80 (70;80)	80 (80;80)	80 (80;90)
HR (bpm)	65 (60;75)	65 (62;75)	68 (61;78)	70 (63;75)	65 (61;75)	74 (65;84)
LVM (g)	201 (135;239)	133 (109;161)	134 (103;188)	114 (96;130)	174 (149;206)	139 (111;199)
PCI	3.36 (0.30;5.69)	−1.31 (−3.02;−0.11)	0.53 (−2.42;2.94)	−2.53 (−4.38;−0.96)	1.61 (−0.20;2.59)	0.30 (−1.85;1.60)

Data summarised as median (interquartile range, Q_1; Q_3) due to the skewness of some distributions
Key: BSA, body surface area; DBP, diastolic blood pressure; HCM, hypertrophic cardiomyopathy; HR, heart rate; LVM, left ventricular mass; MC, HCM mutation carrier; NC, non-carrier; SBP, systolic blood pressure; PCI, first principle component

Table 3. Descriptive (unadjusted) statistics for PCI stratified by *AGTR2* genotypes for the cohort

SNP	Genotype	*n*	Median	(Interquartile range)
rs1403543	G/G	41	0.03	(−2.24;3.36)
	A/G	42	−1.50	(−4.25;1.60)
	A/A	83	0.45	(−1.42;2.56)
rs5194	A/A	80	0.54	(−1.17;2.55)
	A/G	43	−2.10	(−4.71;1.55)
	G/G	43	0.03	(−2.26;2.93)
rs11091046	A/A	79	0.45	(−1.21;2.51)
	A/C	42	−2.01	(−4.71;1.60)
	C/C	45	0.03	(−2.27;3.28)

Key: n - number of individuals typed

384-well plates on an ABI Prism 7900HT Sequence Detection System, following the manufacturer's instructions. Each 5 µl reaction consisted of 2.5 µl ABI TaqMan Universal PCR Master Mix with the passive reference ROX, 20 ng of genomic DNA, 0.25 µl TaqMan primer and probe dye mix and 1.25 µl DNase-free, sterile-filtered water. PCR cycling conditions were 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 s at 92°C and 1 min 30 s at 60°C. Allele discrimination was accomplished by running end-point detection using the ABI Prism 7900HT and SDS v. 2.3 software (autocaller confidence level: 95%).

Statistical analyses

All statistical analyses, unless mentioned otherwise, were done in R and R packages (www.r-project.org).

Validation of input files and Mendelian inheritance within families were verified with Pedstats v. 6.11[22] using X-chromosome settings, and genotyping inconsistencies were resolved by re-genotyping. Pedstats was also used to assess Hardy–Weinberg equilibrium (HWE) on unrelated individuals within the cohort. Haploview v. 4.1[23] was used to estimate minor allele frequencies, linkage disequilibrium (LD) and testing HWE, using X-chromosome settings.

Each wall thickness measurement was transformed to approximate normality using quantile normalisation[24] prior to analysis. Since not only the extent, but also the distribution of hypertrophy in HCM is highly variable, it is difficult to distinguish a single echocardiographic measure that accurately quantifies the extent of hypertrophy in all patients. Although echocardiographically derived LVM is most often used to quantify hypertrophy in HCM, it is known to be an inaccurate measure due to the variable and asymmetric nature of hypertrophy in HCM. However, a single measure that summarises the variability in the 16 quantile-normalised 2D-echocardiographic measurements most effectively is desirable also for reasons of parsimony (to protect against the inflation of type-one errors caused by doing multiple tests on the same cohort, as would happen if multiple measures of hypertrophy were analysed). Principal

Table 4a. Genotype and allele frequencies for the *AGTR2* variants in the entire cohort

	n	Genotype frequencies			Allele frequencies	
rs1403543	331	0.52 GG	0.24 AG	0.25 AA	0.63 G	0.37 A
rs5194	327	0.48 AA	0.24 AG	0.28 GG	0.60 A	0.40 G
rs11091046	327	0.47 AA	0.24 AC	0.29 CC	0.59 A	0.41 C

Key: n - number of individuals typed

Table 4b. Estimated population minor allele frequencies and HWE *p*-values

SNP	Alleles	Minor Allele	f	HWE *p*-value
rs1403543	G:A	A	0.316	0.4985
rs5194	A:G	G	0.362	1.0000
rs11091046	A:C	C	0.368	0.9727

component analysis offers a means to derive a single score that comprehensively describes the person-to-person variability in hypertrophy, regardless of the variability in the distribution of the hypertrophy. The outcome variable analysed here, PC1, is the first principal component. It is the linear combination (weighted sum) of the 16 wall thickness values, which explains the largest proportion of the variation in all 16 measurements combined.

Quantitative transmission disequilibrium testing (QTDT)[25] was used to assess the (broad sense) heritability (ratio of heritable/polygenic variance component to total variance of PC1) of PC1 and LVM after adjusting for hypertrophy confounders.

As QTDT does not have the option to assess association with X-linked variants, association between the *AGTR2* variants and the PC1 score was assessed with specialised mixed-effects models using per-individual random effects which are correlated according to kinship coefficients (R package kinship, function lmekin, www.r-project.org). These models include environmental and polygenic variance components. All analyses were adjusted for covariates that are known to modulate cardiac hypertrophy, namely whether the individual carries a mutation or not, the identity of the primary HCM-causing mutation (R92W$_{TNNT2}$, R403W$_{MYH7}$, or A797T$_{MYH7}$), hypertension diagnosis, systolic BP, diastolic BP, sex, age at clinical assessment, BSA and HR (as a proxy measure for tachycardia). Such modelling results in the effect of the *AGTR2* variant being estimated after the effects of the covariates have been removed from the phenotype. Atrial fibrillation, typically a late consequence of severe LVH in HCM, was present in only six of the 152 mutation carriers and was not included as a covariate in the current analysis.

We initially modelled the genotypes for females as the number of minor alleles (additive) plus a dominance term. As none of the dominance terms were significantly different

from zero, we discarded them from the models. We coded the (hemizygote) men as having 0 or 1 minor alleles. We investigated an alternative coding of 0 or 2 minor alleles, but it resulted in a poorer model fit (results not shown). As we did not detect a significant interaction between sex and any of the *AGTR2* variants (results not shown) on PC1, we did not analyse males and females separately. We also did not detect a significant interaction between mutation status and any of the *AGTR2* variants; therefore mutation carriers did not need to be analysed separately, but could be pooled with non-carriers into a larger group for analysis.

Our models are appropriate for statistical inference (i.e. they deliver valid *p*-values), but provide effect estimates in terms of the change in a weighted sum of quantile-normalised hypertrophy measures, which can not be interpreted clinically. To provide effect estimates in the original units of measurement, mm, we estimated, for each variant, an approximate effect size and standard error, by modelling the mean of the 16 wall thickness measures.

Results

A total of 353 individuals belonging to seven R92W$_{TNNT2}$, three R403W$_{MYH7}$ and 12 A797T$_{MYH7}$ families were genotyped for *AGTR2* variants. Of these, 227 individuals (including all the HCM mutation carriers) consented to clinical investigation. Descriptive statistics (median and interquartile range (first quartile and third quartile), not adjusted for any of the known hypertrophy confounders listed in the methods section), for carriers and non-carriers of each of the three HCM-causing mutations are given in table 2; descriptive statistics (mean and interquartile range) for PC1 for each genotype of the three *AGTR2* SNPs are given in table 3. The allele and genotype frequencies for the complete cohort are depicted in table 4a. All genotype frequencies, in a random sample of unrelated individuals, were in agreement with HWE; the *p*-values and the estimated population (unrelated) minor allele frequencies are shown in table 4b. The two 3' UTR SNPs were found to be in complete LD (D' = 1.00), while incomplete LD exists between rs1403543 and rs5194 (D' = 0.67) and between rs1403543 and rs11091046 (D' = 0.65).

The first principal component, namely our composite hypertrophy score PC1, explained the majority (76%) of the variation in the 16 quantile-normalised wall thickness

Table 5. Weights of wall thickness measures in our hypertrophy score (loadings for the first principal component), PC1

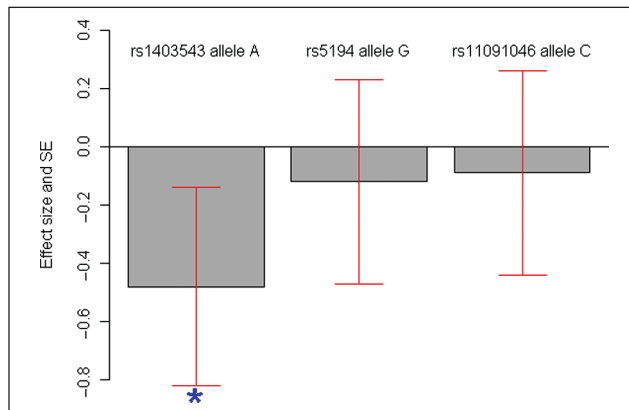| Hypertrophy trait	Loading in PC1
pIVS at mitral valve	0.28
aIVS at mitral valve	0.27
AW at mitral valve	0.27
LW at mitral valve	0.23
IW at mitral valve	0.19
PW at mitral valve	0.20
pIVS at papillary muscle	0.27
aIVS at papillary muscle	0.27
AW at papillary muscle	0.27
LW at papillary muscle	0.27
IW at papillary muscle	0.22
PW at papillary muscle	0.22
IVS at apex	0.28
AW at apex	0.27
LW at apex	0.23
PW at apex	0.23

Key: aIVS, anterior interventricular septal thickness; AW, anterior wall thickness; IVS, interventricular septal thickness; IW, inferior wall thickness; LW, lateral wall thickness; pIVS, posterior interventricular septal thickness; PW, posterior wall thickness.

Figure 1. Bar chart depicting effect size and standard error (SE) for the mean of the 16 hypertrophy measures and *p*-value for test of association between SNPs in *AGTR2* and the PC1 hypertrophy score, after adjusting for covariates and family relatedness. Effect sizes are the average change in mean hypertrophy score (mm) associated with carrying an additional minor allele.
*Each A allele of rs1403543 significantly reduces the average wall thickness by almost 0.5 mm (*p* = 0.020); *p*-values were derived from test of modelling PC1 hypertrophy score.

measurements and is a linear combination of all 16 quantile-normalised traits, with weights (loadings) ranging from 0.19–0.28 (table 5) and scores ranging from -7.9–8.3. The PC1 score ($p = 0.018$, $H^2 = 0.38$) was found to be somewhat more heritable than LVM ($p = 0.028$, $H^2 = 0.29$).

We found significant evidence for association ($p = 0.020$) between PC1 and rs1403543 in a model adjusted for mutation carrier status, the identity of the primary HCM mutation, age, sex, systolic and diastolic blood pressure, BSA, hypertension diagnosis and HR, known hypertrophy covariates (figure 1). The corresponding estimated average reduction in wall thickness for each A allele carried, based on analysis of the mean of 16 wall thickness measures (instead of the PC1 score), is 0.48 mm (figure 1); conversely, each G allele is estimated to lead to an increase of 0.48 mm. No evidence of association was found between hypertrophy and rs11091046 ($p = 0.352$) or rs5194 ($p = 0.307$) (figure 1).

Discussion

We here report a significant association between the previously identified functional +1675 G/A polymorphism (rs1403543) and a heritable composite hypertrophy score (that takes into account extent and distribution of hypertrophy in the left ventricle) in a cohort of genotype-known HCM founder families. The A allele of this +1675 G/A polymorphism was found to significantly decrease our hypertrophy score (PC1). We estimate that, after taking the values of all the relevant confounders (mutation carrying

status, sex, age and others) into account, the average wall thickness will be 0.48 mm lower for each A allele carried by an individual and, conversely, 0.48 mm greater for each G allele carried by an individual. Thus, if all other covariates were equal, one would expect a G/G homozygote's heart wall to be 0.96 mm, almost 1 mm, thicker than that of an A/A carrier. This +1675 G/A polymorphism is located at a lariat branch-point in the first intron, 29 bp before exon 2 in a region that is important for transcriptional activity.[26] Although it had been postulated that this polymorphism may affect pre-mRNA splicing,[27] a later study[28] showed that it modulates AT_2R protein expression, rather than mRNA splicing.

In our statistical modelling, adjustments were made for hypertrophy confounders, and thus this association is independent of known hypertrophy covariates, including sex, BP, HR and the primary HCM causal mutation. However, levels of circulating angiotensin II, which, as a ligand for both AT_1 and AT_2 receptors, may also influence hypertrophy, were not available and thus not included as covariate in this study. As there is complex interplay between the multiple components of the RAAS, eventually a comprehensive analysis which focuses on the compound effect of multiple hypertrophy modifier loci within the RAAS will yield the most accurate understanding of its role in hypertrophy development.

Our results support the finding of Alfakih *et al.*[15] who, in hypertensive patients, reported an association between the G allele of this polymorphism and increased LVMI as determined with magnetic resonance imaging (MRI). MRI is credited with being a more precise and reproducible

measure of LVM than M-mode and 2D-echocardiography, even though echocardiography is still widely used to estimate LVM as it is more readily available as a clinical tool.[29] We circumvented this difficulty by using a composite hypertrophy score derived by principal component analysis, PC1, which best described the variability in the 16 echocardiographically determined wall thickness measurements in the present cohort. Thus we analysed a single trait that takes the extent and distribution of hypertrophy into account without resorting to a geometrically inaccurate estimate of overall hypertrophy, namely echocardiographically derived LVM.

The PC1 score was, however, derived from quantile-normalised data, and the effect size following association analysis of this trait is therefore not interpretable in terms of the original cardiac measurements. Thus, we estimated the effect size from an analysis of the mean of the 16 untransformed cardiac wall thickness measurements originally used to derive PC1. The resulting effect size was modest (0.48 mm), although comparable to effect sizes reported for other RAAS candidate modifiers,[30,31] which suggests that the eventual hypertrophic phenotype of HCM might be the cumulative result of a number of modifier genes of modest effect.

Our results and those of Alfakih *et al.*[15] are in contrast to those previously reported by Schmieder and colleagues[16] as well as by Herrmann *et al.*,[17] who found an association between the +1675 A allele and an *increase* in echocardiographically determined LVM in males only. This finding was, however, not replicated in a larger cohort and those authors subsequently concluded that further research into the role of *AGTR2* in LVH was needed.[17]

Unlike Deinum and colleagues, who previously reported an association between rs11091046 in the 3' UTR of *AGTR2* and LVMI in female patients with HCM,[19] we did not find any significant evidence of an association between either of the two 3' UTR SNPs and hypertrophy. One explanation for this discrepancy could be that, unlike the previous study,[19] this study adjusted all analyses for the primary HCM causal mutation and did not make use of the geometrically inaccurate echocardiographically derived LVMI.[32] Although we specifically tested the interaction between sex and the genotypes generated in this study, we also found no statistically significant interaction with either gender; consequently we did not analyse males and females separately. We did, however, include sex as a covariate in the association analysis due to the known effect of gender on hypertrophy development.

Despite a modest sample size, the use of founder HCM families in this study provided certain benefits over case-control designs as it offers additional quality control measures to ensure genotyping accuracy and the opportunity to circumvent the confounding effect of undetected population stratification. Furthermore, populations carrying founder mutations provide added control over the genetic variability introduced by different HCM causal mutations.

In conclusion, this study confirms the previous suggestion of association between the functional +1675 G/A SNP and cardiac hypertrophy, and is the first to report an association between this genetic variant and hypertrophy in HCM, using a novel approach to quantify the extent and degree of hypertrophy. Previously it had been suggested that the +1675 G/A polymorphism could serve as a marker for hypertensive individuals that would benefit from pharmaceutical intervention;[15] our results further suggest that +1675 G/A SNP could potentially be used in the context of a profile of markers that are indicative of an increased genetic predisposition to LVH development also in patients with HCM.

Conflict of interest

The authors declare that they do not have any conflicts of interest.

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