Prognostic value of two polymorphisms in non-sarcomeric genes for the development of atrial fibrillation in patients with hypertrophic cardiomyopathy

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Summary

Background: Several non-sarcomeric genes have been postulated to act as modifiers in the phenotypic manifestations of hypertrophic cardiomyopathy (HCM). The development of atrial fibrillation (AF) in HCM has adverse prognostic implications with increased thromboembolism and functional class impairment.

Aim: We tested the hypothesis that 2 non-sarcomeric genes [CYP11B2 (-344T>C) and COL1A1 (2046G>T)] are associated with the development of AF.

Design: Prospective study.

Methods: Two polymorphisms in non-sarcomeric genes [CYP11B2 (-344T>C) and COL1A1 (2046G>T)] were analysed in 159 HCM patients (49.3 ± 14.9 years, 70.6% male) and 136 controls. All subjects were clinically stable and in sinus rhythm at entry in the study, without ischemic heart disease or other significant co-morbidities that could mask the effect of the analysed polymorphisms (i.e. previous AF). Thirty-nine patients (24.4%) developed AF during a median follow-up of 49.5 months.

Results: Patients with the -344T>C polymorphism in CYP11B2 gene had a higher risk for AF development [HR: 3.31 (95% CI 1.29–8.50); P = 0.008]. In a multivariate analysis, the presence of the C allele in CYP11B2 gene [HR: 3.02 (1.01–8.99); P = 0.047], previous AF [HR: 2.81 (1.09–7.23); P = 0.033] and a left atrial diameter of ≥42 mm [HR: 2.69 (1.01–7.18); P = 0.048] were independent predictors of AF development. The presence of the polymorphic allele was associated with higher aldosterone serum levels.

Conclusion: We have shown for the first time that the CYP11B2 polymorphism is an independent predictor for AF development in HCM patients. This highlights the importance of non-sarcomeric
Introduction

Hypertrophic cardiomyopathy (HCM) is a mono-genetic cardiac disease with an autosomal dominant pattern of heritability and different penetrance characterized by cardiomyocyte hypertrophy, myofibrillar disarray and fibrosis. The phenotypic expression of HCM is multi-factorial with majority of cases occurring secondary to mutations in genes encoding the sarcomere proteins. However, a group of non-sarcomeric genes have been postulated to act as modifiers in HCM. In particular, polymorphisms in genes encoding proteins of the renin–angiotensin–aldosterone system (RAAS) and proteins associated with collagen synthesis have attracted much interest.

The RAAS system contributes to ventricular hypertrophy through effects mediated by circulating angiotensin as well as local activation of RAAS in the myocardium. Aldosterone secretion is regulated primarily by the RAAS and it is synthesized from deoxycorticosterone by a mitochondrial cytochrome P450 enzyme, aldosterone synthase (CYP11B2). On the other hand, activated fibroblasts are the most important cells depositing type I collagen in all tissues and its excessive synthesis results in fibrosis. Indeed, transcription of collagen type I, alpha 1 (COL1A1) gene is increased 3- to 10-fold in activated fibroblasts.

Atrial fibrillation (AF) is the most common arrhythmia in patients with HCM. The onset of AF represents an adverse prognostic feature for HCM patients, conferring an increased mortality and morbidity. AF independently increases the risk of ischemic stroke by approximately 4-fold, and is also associated with heart failure and death from any cause.

In this study, we tested the hypothesis that two non-sarcomeric genes [CYP11B2 (–344T>C), SNP ID Number: rs1799998; and COL1A1 (2046G>T), SNP ID Number: rs1800012] are associated with the development of AF.

Methods

We prospectively recruited 159 HCM patients (49.3 ± 14.9 years, 70.6% males), included after performing a cardiac magnetic resonance (CMR) and 136 healthy controls from three referral centres in southeastern Spain. Patients and controls were clinically stable at entry in the study, without ischemic heart disease or presence of any other significant co-morbidities (i.e. valvular disease or severe hypertension) that could mask the effect of the analysed polymorphisms. The diagnosis of HCM was based on the presence of unexplained left ventricular hypertrophy, or in accordance with criteria for the diagnosis of familial disease in patients with at least one first-degree relative who has an unequivocal diagnosis. A complete history and clinical examination was performed, including 12-lead electrocardiogram (ECG), standard echocardiography, symptom limited treadmill exercise (Bruce protocol), 24-h electrocardiogram-Holter monitoring and a blinded magnetic resonance study. All patients were included in the study after CMR, so patients with pacemakers and ICDs were excluded because it is not recommended to perform CMRs in patients with implantable devices. All recruited subjects gave their informed consent to participate in the study, which was approved by the local Research Committees (Ethic Committee of Clinic Investigation of the Hospital Universitario Virgen de la Arrixaca on 24 April 2009 for the project entitled ‘Hypertrophic myocardiopathy and tissue remodelling: role of biomarkers’) in accordance with the Declaration of Helsinki, as amended in Edinburgh in 2000.

AF was documented by 12-lead ECG when patients attended our hospital with symptoms of palpitation, dyspnoea and/or chest discomfort, and if asymptomatic, during regular follow-up visits at the dedicated HCM clinics. An episode of AF of 2 min or more was considered as ‘AF development’ and patients were followed up for a median of 49.5 [interquartile range (IQR) 25.8–77.0] months.

Blood samples and laboratory assays

Venous blood was collected without trauma or stasis in the morning, by specialized staff, with the patient fasting for >12 h. Buffy coat was obtained from plasma samples by centrifugation for 15 min at 3500 g. Aliquots were stored at −80°C to allow batch analysis in a blinded fashion. DNA from peripheral blood was extracted using DNeasy Blood and Tissue kit and the QIAcube system (Qiagen, Valencia, CA, USA). After DNA extraction, PCR products were separated by electrophoresis on 3% agarose gels and visualized by ethidium bromide. The analysis of the SNPs rs1799998 (CYP11B2) and rs1800012 (COL1A1) was approached by allelic discrimination using TaqMan probes on real time PCR (TaqMan SNP Genotyping Assays,
myocardium. All CMR images were analysed on a 1.5-T system (Gyroscan NT; Philips Medical Systems, Best, The Netherlands) in conjunction with a phased-array body coil and electrocardiogram gating. Scout images were obtained in three orthogonal planes to determine the exact position and axis of the left ventricle. Delayed contrast-enhanced images were acquired 10 min after the injection of the contrast medium was highly hyperintense and persists in the same slice after swapping the phase encoding in order to exclude artefact images.

Aldosterone measurement

Serum aldosterone levels were measured in the 159 patients using a competitive radioimmunoassay (Immunotech SAS, Marseille, France). In brief, 50 μl of serum samples and calibrators were incubated using antibody coated tubes with 500 μl of tracer labelled with t125. After 3 h of incubation with continuous shaking, the content of the tubes were sucked and then, the radioactivity was determined using a gamma counter. A standard curve was developed and aldosterone values were determined by interpolation with the curve.

Cardiac magnetic resonance

All CMR images were obtained with a 1.5-T system (Gyroscan NT; Philips Medical Systems, Best, The Netherlands) in conjunction with a phased-array body coil and electrocardiogram gating. Scout images were obtained in three orthogonal planes to determine the exact position and axis of the left ventricle. Delayed contrast-enhanced images were acquired 10 min after the injection of the contrast material according to a previous report, with an inversion recovery T1-weighted sequence (repetition time/echo time ms, 8/4.5, flip angle 15°, (Field of view), 400 mm; matrix, 144 x 256; and section thickness, 10 mm) in three short-axis views taken at the base, midpapillary muscles. The time of inversion was adjusted for each patient between 200 and 400 ms to achieve optimal suppression of normal myocardium. All CMR images were analysed on a satellite workstation console with commercial image post-processing software (EasyVision, version 4.0; Philips Medical Systems and Mass Suite 6.1; MEDIS Medical Imaging Systems, Leiden, The Netherlands) by two observers with experience in cardiac MR imaging, whose joint opinion was reached by consensus. The CMR studies were read by experienced observers blinded to the clinical information. The American Heart Association 17-segment model for the left ventricle was used to analyse wall thickness, contractile function and delayed enhancement per segment. Late gadolinium enhancement (LGE) was considered present when the signal intensity of any area within the myocardium was highly hyperintense and persists in the

Statistical analysis

Continuous variables were tested for normal distribution by the Kolmogorov–Smirnov test. The normal distributed continuous variables are shown as mean ± standard deviation, and those non-parametrically distributed are shown as median (IQR). The comparison of discrete variables was done by χ² test or Fisher test (as appropriate). Comparisons of the groups for continuous variables were performed with analysis of variance test or the Kruskal–Wallis test (as appropriate). Receiver operating characteristic (ROC) curves analyses were generated to test the predictive discrimination of different continuous variables (i.e. age and left atrial diameter) to identify AF development during follow-up. We constructed areas under the ROC for sensitivity, specificity, positive predictive value and negative predictive value. The cut-off point with the best sensitivity and specificity was chosen for each case, as assessed by receiver–operator characteristic curves. We analysed those variables associated with AF development. The overall AF occurrence-free survival rates were calculated using the Kaplan–Meier method, and differences determined using the log-rank test. The effect on prognosis was calculated for several clinical, echocardiographic, effort test, Holter and CMR variables by using a Cox proportional hazards regression. Only variables showing P-values <0.15 were included in the multivariate Cox hazard model. All P-values <0.05 were accepted as independently predictive for the AF outcome. Statistical analyses were performed using SPSS 19.0 for Windows (SPSS, Inc., Chicago, IL).

Results

A total of 159 HCM patients (49.3 ± 14.9 years, 70.6% males) (Table 1) and 136 controls were included. Data on genotype frequencies in patients and controls of CYP11B2 (−344T>C) and COL1A1 (2046G>T) polymorphisms are listed in Table 2. There were no significant deviations from the Hardy–Weinberg equilibrium in the HCM patient and control group (P > 0.05). Allele and genotype frequencies did not differ markedly between patients and controls and were similar to previously reported numbers in normal Caucasian populations.

AF development during follow-up

During follow-up, 39 patients (24.4%) developed AF, with an annual rate of 9.75%/year. Survival
analysis (Cox regression) showed that the presence of the polymorphism in the CYP11B2 gene promoter region was significantly associated with a higher risk of developing AF in HCM patients [HR: 4.41 (95% CI 2.22–8.67); P < 0.001] (Figure 1C), a left atrial diameter of ≥42 mm [HR: 2.89 (95% CI 1.31–6.34); P = 0.008] (Figure 1D) and age [HR: 1.03 (95% CI 1.00–1.05); P = 0.030] were also significantly associated with AF development. The presence of LGE or sex was not associated with AF development. Both gene polymorphisms were not significantly associated with left atrial diameter (both P > 0.05; data not shown).

On multivariate analysis (Table 3), the presence of the polymorphism in the CYP11B2 (–344T>C)
gene promoter region [HR: 3.02 (95% CI 1.01–8.99); P=0.047], previous AF [HR: 2.81 (95% CI 1.09–7.23); P=0.033] and left atrial diameter of ≥42 mm [HR: 2.69 (95% CI 1.01–7.18); P=0.048] were independent predictors for AF development.

Aldosterone levels are influenced by CYP11B2 polymorphism

An increase in aldosterone levels was associated with the presence of the polymorphic allele CYP11B2 (−344C) (P<0.001) in 159 HCM patients (72.5% men, mean age 49.8±15.3). Patients carrying the wild type allele (TT) showed a median concentration of aldosterone of 52 pg/ml (IQR 31–60), whereas patients carrying one polymorphic allele (TC) showed a median of 95.5 pg/ml (IQR 70.2–127.5). Patients with the two polymorphic alleles (CC) showed the highest levels of aldosterone in serum (median 209.5, IQR 155.7–268.7) (Figure 2). Kruskal–Wallis test was performed to confirm that aldosterone levels were significantly associated with the presence of the polymorphic allele CYP11B2 (Figure 2).

Discussion

This is the first study to show that CYP11B2 gene polymorphism (−344T>C) in the aldosterone synthase gene is an independent predictor of AF development in HCM patients. This polymorphism is located in the transcriptional regulatory region of CYP11B2, 344 nucleotides before the start of the protein coding sequence. This position can be
either a thymine or cytosine (–344T>C). Of note, this polymorphism in the CYP11B2 promoter region has been previously associated with atrial remodelling in a series of hypertensive patients or with AF development in heart failure patients. Indeed, our observations highlight the role of the CYP11B2 gene polymorphism in the secretion of serum aldosterone and confirm the results obtained in the multivariate analysis regarding the role of the polymorphic gene and AF development.

One recent study, investigating polymorphisms of RAAS genes in patients with different variants of HCM and their effects on morphofunctional parameters of the heart, failed to find a relationship between CYP11B2 gene (–344T>C) polymorphism and the development of AF in a cohort of HCM patients.

As a member of the RAAS, aldosterone is the principal mineralocorticoid hormone and has been shown to play a major role in cardiac fibrosis and remodelling. Aldosterone can directly induce myocardial cell hypertrophy via activation of the mineralocorticoid receptors, a member of the steroid/thyroid/retinoid nuclear receptor family of ligand-dependent transcription factors. More specifically, aldosterone is produced in the heart, particularly in HCM subjects because their cardiac aldosterone synthase (CYP11B2) mRNA levels are 7-fold increased as compared with age- and gender-matched normal donor hearts. Recent reports suggest that aldosterone may also be involved in atrial structural and electrical remodelling by increasing collagen biosynthesis and cardiomyocytes apoptosis. In addition, blockade of the aldosterone receptor using spironolactone prevents the incidence of AF, perhaps offering a new therapeutic approach for this arrhythmia.

Although there are conflicting data about the functional significance and clinical implications, the role of the CYP11B2 gene polymorphism in the secretion of serum aldosterone and AF development is supported by our findings and others. Further studies are needed to clarify the specific mechanisms by which this polymorphism interacts with other genetic and environmental factors to influence AF development.

Table 3 Association of different demographic, clinical and genetic variables with AF development during follow-up

<table>
<thead>
<tr>
<th>Variable</th>
<th>Univariate</th>
<th>Multivariate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HR (95% CI)</td>
<td>P</td>
</tr>
<tr>
<td>Age</td>
<td>1.03 (1.00–1.05)</td>
<td>0.030</td>
</tr>
<tr>
<td>Sex</td>
<td>1.18 (0.58–2.39)</td>
<td>0.642</td>
</tr>
<tr>
<td>Previous AF</td>
<td>4.41 (2.22–8.67)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Atrial diameter ≥ 42 mm</td>
<td>2.89 (1.31–6.34)</td>
<td>0.006</td>
</tr>
<tr>
<td>Late gadolinium enhancement</td>
<td>1.01 (0.92–1.12)</td>
<td>0.785</td>
</tr>
<tr>
<td>Presence COL1A1 polymorphism</td>
<td>0.55 (0.28–1.08)</td>
<td>0.078</td>
</tr>
<tr>
<td>Presence CYP11B2 polymorphism</td>
<td>3.31 (1.29–8.50)</td>
<td>0.008</td>
</tr>
<tr>
<td>Hypertension</td>
<td>1.54 (0.79–2.98)</td>
<td>0.199</td>
</tr>
<tr>
<td>Non sustained ventricular tachycardia</td>
<td>0.99 (0.51–1.92)</td>
<td>0.982</td>
</tr>
<tr>
<td>Abnormal blood pressure response</td>
<td>0.81 (0.40–1.64)</td>
<td>0.550</td>
</tr>
<tr>
<td>Maximum left ventricular thickness</td>
<td>1.02 (0.95–1.09)</td>
<td>0.617</td>
</tr>
<tr>
<td>Left ventricular ejection fraction (%)</td>
<td>0.98 (0.95–1.01)</td>
<td>0.164</td>
</tr>
<tr>
<td>Obstructive (gradient &gt;30 mmHg)</td>
<td>0.96 (0.50–1.85)</td>
<td>0.902</td>
</tr>
<tr>
<td>ACE inhibitors</td>
<td>1.08 (0.33–3.53)</td>
<td>0.903</td>
</tr>
<tr>
<td>ARBs</td>
<td>0.42 (0.09–1.93)</td>
<td>0.266</td>
</tr>
<tr>
<td>Disopiramide</td>
<td>0.29 (0.04–2.34)</td>
<td>0.244</td>
</tr>
</tbody>
</table>

LV, left ventricle; ACE inhibitors, angiotensin-converting enzyme inhibitors; ARBs, angiotensin II receptor blockers. Continuous variables are expressed as mean ± standard deviation or median (25th–75th percentiles). "On effort testing.

Figure 2. Influence of aldosterone syntase gene (CYP11B2) on aldosterone serum levels. Aldosterone serum levels in 159 patients with HCM. Patients were segregated depending on the polymorphism in aldosterone syntase gene (CYP11B2) (TT, wild type; TC, heterozygote; CC, polymorphic gene). Median and IQRs (25th and 75th) are represented for each genotype.
some studies suggest that −344T>C CYP11B2 polymorphism is associated with increased constitutive aldosterone production, which subsequently leads to cardiac fibrosis and remodelling, being a molecular target for the pathogenesis of left ventricular hypertrophy, amongst other cardiovascular diseases. The −344T>C polymorphism in the aldosterone synthase gene is located immediately adjacent to a binding site for a transcription factor, SF-1, that is thought to be essential for expression of steroid biosynthetic enzymes in the adrenal cortex. In vitro, the −344C allele binds SF-1 approximately 4-fold more strongly than does the −344T allele and has been demonstrated to affect the transcription of CYP11B2 increasing aldosterone production as well as serum aldosterone level. Importantly, high expression of the aldosterone synthase gene induced by the presence of the 344C allele may cause a high tissue aldosterone concentration, which subsequently causes atrial fibrosis, conduction heterogeneity and increased the substrate for the development of AF. Importantly, we have shown in this study that the levels of serum aldosterone are significantly associated with the presence of the polymorphic allele in the CYP11B2 gene in our cohort of HCM patients (Figure 2).

We found that a left atrial diameter of ≥42 mm was also independently associated with AF development in HCM patients, consistent with previous reports indicating that left atrial size is an independent risk factor of AF development, even in HCM patients. The most common form of AF is associated with structural and electrical changes, given that the longer the AF persists, the more difficult it is to restore sinus rhythm and to prevent recurrence. Indeed, ‘AF begets AF’, so that AF alters atrial electrophysiology in a way that favours AF initiation and maintenance. In this setting, we confirm that the presence of previous AF is another variable associated with AF development during follow-up. It is important to remark that, in the multivariate analysis the association of the C allele in CYP11B2 gene with the occurrence of AF was of a borderline significance. This decreasing of significance of this genetic variable as compared with the univariate analysis can be assumed as previous AF is a more powerful predictor of AF development as previously described in the literature.

We have observed that the presence of the COL1A1 (2046G>T) polymorphism was associated with a non-significant trend towards a protective role for AF development in HCM patients on the univariate analysis. This polymorphism was identified in a regulatory region of COL1A1 (2046G>T) at a recognition site for the transcription factor Sp1 that is significantly related to collagen synthesis. TT homozygotes have significantly lower bone mineral density, reflecting lower collagen synthesis due to the presence of the T allele.

This study has several limitations. Although our study was conducted in three different hospitals, all patients and controls were Caucasians, and the data should not be extrapolated to other ethnic groups. Indeed, the frequency of the C allele in the CYP11B2 gene is lower in people of African origin than white and South Asian subjects. Second, this study is relatively small, and larger studies are required to confirm our observations. Moreover, patients and control in our cohorts were not comparable in age and sex; however, all the analyses were developed in the patient cohort and this limitation shows less importance. Third, patients could not be included consecutively because only patients with HCM and with CMR were selected for this study, which could result in a selection bias. Finally, as asymptomatic AF detection may be extremely difficult, although a complete history and clinical examination was performed including not only routine 12-lead ECG but also a 24-h ECG Holter, some asymptomatic AF patients can be missed in this study.

Conclusion
We have shown for the first time that the CYP11B2 polymorphism is an independent predictor for AF development in HCM patients. This highlights the importance of non-sarcomeric genes in the phenotypic heterogeneity of HCM. This is a pilot study and despite the promising results, further analyses with larger cohorts are needed to assess the importance of this polymorphism.

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