Large scale replication and meta-analysis of variants on chromosome 4q25 associated with atrial fibrillation

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Aims

A recent genome-wide association study identified a haplotype block on chromosome 4q25 associated with atrial fibrillation (AF). We sought to replicate this association in four independent cohorts.

Methods and results

The Framingham Heart Study and Rotterdam Study are community-based longitudinal studies. The Vanderbilt AF Registry and German AF Network (AFNet) are case–control studies. Participants with AF (n = 3508) were more likely to be male and were older than referent participants (n = 12 173; Framingham 82 ± 10 vs. 71 ± 13 years; Rotterdam 73 ± 8 vs. 69 ± 9 years; Vanderbilt 54 ± 14 vs. 57 ± 14 years; AFNet 62 ± 12 vs. 49 ± 14 years). Single nucleotide polymorphism (SNP) rs2200733 was associated with AF in all four cohorts, with odds ratios (ORs) ranging from 1.37 in Rotterdam (95% confidence interval (CI) 1.18–1.59; P = 3.1 × 10−5] to 2.52 in AFNet (95% CI 2.22–2.8; P = 1.8 × 10−10). There also was a significant association between AF and rs10033464 in Framingham (OR 1.34; 95% CI 1.03–1.75; P = 0.031) and AFNet (OR 1.30; 95% CI 1.13–1.51; P = 0.0002), but not Vanderbilt (OR 1.16; 95% CI 0.86–1.56; P = 0.33). A meta-analysis of the current and prior AF studies revealed an OR of 1.90 (95% CI 1.60–2.26; P = 3.3 × 10−13) for rs2200733 and of 1.36 (95% CI 1.26–1.47; P = 6.7 × 10−15) for rs10033464.

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Introduction

Atrial fibrillation (AF) is the most commonly sustained arrhythmia affecting over two million Americans, a number that is expected to increase to between 6 and 12 millions by the year 2050.1,2 AF is a major source of morbidity and mortality. It is associated with a five-fold increased risk of stroke,3 a doubling in risk of dementia,4 and an increased risk of heart failure (HF).5,6

Multiple risk factors for AF exist, including hypertension (HTN), valvular heart disease,7 HF, and a family history of the arrhythmia.8–10 In the past 5 years, data have emerged to support a genetic contribution to AF. Several genetic loci for Mendelian forms of AF have been identified,11–13 although the genes responsible for AF at these loci remain unknown. Mutations in the cardiac sodium channel14,15 potassium channel complexes,16–19 and gap junction proteins20 have been reported to cause AF; however, these ion channel variants appear to account for only a small fraction of AF cases.21 Finally, with the exception of the association between a common variant in KCNQ1 and AF replication in two small cohorts underpowered and have not been replicated.

Recently, a genome-wide association study in Icelanders identified a haplotype block on chromosome 4q25 containing variants that predispose to AF.23 In this block, two single nucleotide polymorphisms (SNPs), rs2200733 and rs10033464, are in strong linkage disequilibrium and define three haplotypes. The haplotype identified by rs2200733 was found to confer a relatively higher risk [odds ratio (OR) = 1.72] of AF or atrial flutter, whereas the haplotype identified by rs10033464 conferred a more modest risk (OR = 1.39) compared with the common sequence. The association of these SNPs with AF was replicated in two small cohorts of Northern Europeans and in a study of people of Asian descent. The 4q25 haplotype block is located in a ‘genomic desert’ of approximately 1.5 million base pairs without any known genes. The closest gene, PITX2, is more than 50 000 bp away from these variants.

PITX2 is a transcription factor critical for determining left–right asymmetry and for the differentiation of the left atrium.24 Furthermore, it is necessary for the development of the pulmonary myocardium,25 the source of ectopic, electrically active foci associated with paroxysmal AF in many individuals. The pulmonary myocardium is the therapeutic target of pulmonary vein ablation procedures that have become increasingly frequent in the management of AF.26

In our study, we sought to determine whether the association between the 4q25 haplotype block and AF replicated in two community-based and two case–control studies with large numbers of AF participants. In an exploratory analysis, we studied whether there was effect modification by age.

Methods

Description of study cohorts

The Framingham Heart Study (FHS) is a longitudinal observational, community-based cohort initiated in 1948 to prospectively investigate cardiovascular disease and its risk factors, as previously described.27 Participants were diagnosed with AF, if AF or flutter was present on an electrocardiogram obtained from the hospital or physician records or from routine Framingham clinic examination (every 2 years in the Original Cohort and every 4–8 years in the Offspring Cohort). AF cases were available through 21 April 2007. For the present project, participants were eligible if they were plated on the standard Framingham DNA distribution plate sets: (i) the unrelated offspring or (ii) the original cohort plate set. All protocols were approved by the Boston University Medical Center Institutional Review Board, and participants provided written informed consent.

The Rotterdam Study (RS) is a community-based study founded in 1991. The Medical Ethics Committee of Erasmus University Rotterdam approved the study, and participants signed consent. Inhabitants of a suburb of Rotterdam (n = 10 275) aged 55 years and older were invited, and 7983 participants (78%) were examined. The participants were interviewed at their home and were examined during two visits at the research centre for baseline data collection. The participants were re-examined twice during three follow-up rounds. The first round was performed between July 1993 and 31 December 1994. The second round started in April 1997 and ended 31 December 1999. The third round started in January 2002 and ended 31 July 2004. Three methods were used to assess cases of AF or atrial flutter, as described previously28 (i) at baseline and during follow-up examinations, 10 s 12-lead ECGs were recorded at the research centre with an ACTA Gnosis IV ECG recorder (Esaote, Florence, Italy), stored digitally, and analysed with the Modular ECG Analysis System (MEANS).29 To verify the diagnosis of AF, all ECGs with a diagnosis of AF or flutter or any other rhythm disorder were re-coded independently by two physicians who were blinded to the MEANS diagnosis. The judgement of a cardiologist was considered decisive in the case of persistent disagreement; (ii) general practitioners information; and (iii) hospital discharge diagnoses were also obtained from the Landelijke Medische Registratie system.

The Vanderbilt AF Registry consists of consecutive patients with documented AF age >18 years, who were prospectively enrolled since October 2002 from the Vanderbilt Cardiology and Arrhythmia Clinics, the emergency department, and in-patient services. At enrolment, participants were asked a detailed medical and drug history and were asked to complete a symptom questionnaire.30 Participants were excluded if AF was diagnosed in the setting of recent cardiac surgery or were unable to give informed consent or report for follow-up. An echocardiogram was obtained on all patients at the time of registry enrolment. The study protocol was approved by the Vanderbilt University Institutional Review Board, and participants were enrolled following informed written consent. Participants with AF (n = 556) were enrolled and age- and sex-matched to referent...
participants (n = 598). The controls were participants who underwent cardiac surgery with no personal or family history of AF and had no AF documented after surgery.

The German Competence Network for Atrial Fibrillation (AFNet) is a national registry of AF patients comprising >10,000 probands. DNA samples are currently collected from patients with AF onset before age 60 years. In this analysis, all samples available by 1 October 2007 (n = 906) from the nation-wide German Competence Network for Atrial Fibrillation were combined with AF patients collected at the Medical Department I of the University Hospital Munich, Campus Grosshadern of the Ludwig-Maximilians University Munich, the Medical Department I of the Technical University Munich Hospital, and the Deutsches Herzzentrum München (in total, n = 1715). Cases were selected if the diagnosis of AF was made on an electrocardiogram analyzed by a trained physician. Patients with signs of moderate-to-severe HF, moderate-to-severe valve disease, or with hyperthyroidism were excluded from the study. Control probands were from a population-based epidemiological survey of persons living in or near the city of Augsburg, Southern Germany (KORA S4), conducted between 1999 and 2001.31 The survey population consisted of German nationality residents born between 1 July 1925 and 30 June 1975 identified through the registration office. A sample of 6640 participants was drawn with 10 strata of equal size according to sex and age and 4261 individuals (66.8%) agreed to participate. Exclusion criteria for control probands were reported history of AF, signs or symptoms of AF on physical examination, or absence of sinus rhythm upon 12-lead resting ECG that all probands received. All studies involving humans were performed according to the declarations of Helsinki and Somerset West, were approved by local medical Ethics Committees, and participants signed informed consent.

Genotyping
SNP genotyping in the FHS and RS was performed using an ABI TaqMan assay.32 Genotyping of samples from Vanderbilt University and AFNet was performed using PCR, iPlex single base primer extension, and matrix assisted laser desorption/ionization—time of flight mass spectrometry in a 384-well format (Sequenom, San Diego, CA, USA), as described previously.23 Subjects were considered to have failed genotyping if we were unable to detect a PCR product or to distinguish between the alleles in at least two separate experiments.

Statistical analysis
Exact Hardy–Weinberg equilibrium (HWE) tests were applied to all SNPs in the referent participants. In the Framingham sample, a subset of unrelated participants was used to test for deviation from HWE. In order to compare our results with those reported previously,23 the association of SNP genotypes with AF was calculated using a log-additive genetic model. SNP × age interactions were assessed using age as a continuous variable. All calculations were performed using R25 (FHS), SPSS (RS), SAS33 (Vanderbilt AF Registry), and STATA SE 8.0 statistical package (AFNet). Logistic regression was used to adjust for participants’ age and sex. In the community-based studies, a multi-variable analysis was performed using logistic regression to adjust for participants’ age, sex, body mass index, and history of HTN, HF, myocardial infarction, and diabetes mellitus.

In addition, familial correlation in FHS was taken into account by applying generalized estimating equation models with each family as a cluster and assuming an independent working correlation matrix. A two-sided P-value of less than 0.05 was considered significant. Only subjects who failed genotyping were excluded from the analysis.

Figure 1
Comparison of the minor allele frequency for single nucleotide polymorphisms rs2200733 in participants either with (filled circle) or without (open circle) AF. 95% confidence intervals are indicated by the error bars.

Meta-analyses of the relations between AF and rs2200733 and rs10033464 were performed using the inverse variance method for pooling log OR estimates with a random-effects estimate based on the DerSimonian–Laird method.26 Given the differences in study design, the community-based samples (FHS and RS) and case–control studies (AFNet and Vanderbilt AF Registry) were analysed separately. A meta-analysis was performed to determine the association between AF and rs2200733 or rs10033464 using all available case–control studies for AF.23

Results
A total of 3508 participants with AF and 12,173 referent participants were available from four cohorts of European descent (Table 1). Participants with AF were older (P ≤ 0.001) and were more likely to be male in the FHS, RS, and AFNet (P ≤ 0.001). By design, there were a similar number of men among those with and without AF in the Vanderbilt AF Registry (67.8% vs. 66.6%; P = NS). Genotype call rates and HWE were similar in subjects without AF (Table 2). Minor allele frequencies for rs2200733 are illustrated in Figure 1.

In an analysis adjusted for age and sex, rs2200733 was strongly associated with AF (Table 3), with ORs ranging from 1.37 (95% CI 1.18–1.59; P = 3.1 × 10−5) in the RS to 2.52 (95% CI 2.22–2.84; P = 1.8 × 10−9) in AFNet. A multi-variable analysis adjusting for age, sex, HTN, HF, diabetes, and body mass index was performed in the population-based studies. The association between rs2200733 and AF remained significant with an OR of 1.47 (95% CI 1.11–1.94; P = 0.0067) in the FHS and 1.36 (95% CI 1.17–1.59; P = 9.3 × 10−5) in the RS. Exclusion of subjects with missing data for at least one co-variate did not alter our findings (FHS n = 28, OR 1.47, 95% CI 1.11–1.94; RS n = 468, OR 1.37, 95% CI 1.18–1.60).

In a meta-analysis of the relations between rs2200733 and AF in the community-based studies (Framingham and Rotterdam), the OR was 1.38 (95% CI 1.21–1.57; P = 1.41 × 10−6), compared
with 2.09 (95% CI 1.41–3.10; \( P = 2.4 \times 10^{-4} \)) in the case–control studies (Vanderbilt AF Registry and AFNet). A meta-analysis of the case–control studies from both the current and prior \(^{23}\) reports reveals an OR of 1.90 (95% CI 1.60–2.26; \( P = 3.3 \times 10^{-13} \)).

The association between AF and SNP rs10033464 was weaker (Table 3) and did not achieve statistical significance in the Vanderbilt AF Registry cohort. In a multi-variable analysis, rs10022464 was not significantly associated with AF in the FHS, with an OR of 1.22 (95% CI 0.92–1.62; \( P = 0.18 \)) or the RS 1.17 (95% CI 0.98–1.39; \( P = 0.09 \)).

### Table 1 Characteristics of the study cohorts

<table>
<thead>
<tr>
<th></th>
<th>Framingham Heart Study</th>
<th>Rotterdam Study</th>
<th>Vanderbilt AF Registry</th>
<th>German AF Network</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of participants</td>
<td>AF (Non-AF)</td>
<td>AF (Non-AF)</td>
<td>AF (Non-AF)</td>
<td>AF (Non-AF)</td>
</tr>
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<td>Age (years) mean ± SD</td>
<td>327 ± 9.9</td>
<td>2006 ± 9.9</td>
<td>910 ± 9.4</td>
<td>5496 ± 11.1</td>
</tr>
<tr>
<td>Men, number (%)</td>
<td>189 (57.8)</td>
<td>833 (41.5)</td>
<td>415 (45.6)</td>
<td>2178 (39.6)</td>
</tr>
</tbody>
</table>

### Table 2 Distribution of genotypes by cohort for single nucleotide polymorphisms rs2200733 or rs10033464

<table>
<thead>
<tr>
<th></th>
<th>CC</th>
<th>CT</th>
<th>TT</th>
<th>Call rate (%)</th>
<th>HWE</th>
<th>GG</th>
<th>GT</th>
<th>TT</th>
<th>Call rate (%)</th>
<th>HWE</th>
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</thead>
<tbody>
<tr>
<td>Framingham Heart Study</td>
<td>AF</td>
<td>229</td>
<td>83</td>
<td>12</td>
<td>99.1</td>
<td>0.15</td>
<td>253</td>
<td>70</td>
<td>2</td>
<td>99.4</td>
</tr>
<tr>
<td>Non-AF</td>
<td>1527</td>
<td>418</td>
<td>29</td>
<td>98.2</td>
<td>0.68</td>
<td>1676</td>
<td>305</td>
<td>24</td>
<td>99.9</td>
<td>0.08</td>
</tr>
<tr>
<td>Rotterdam Study</td>
<td>AF</td>
<td>676</td>
<td>215</td>
<td>19</td>
<td>97.4</td>
<td>0.14</td>
<td>4194</td>
<td>855</td>
<td>52</td>
<td>99.6</td>
</tr>
<tr>
<td>Non-AF</td>
<td>4392</td>
<td>1030</td>
<td>74</td>
<td>97.5</td>
<td>0.04</td>
<td>439</td>
<td>106</td>
<td>7</td>
<td>98.1</td>
<td>0.83</td>
</tr>
<tr>
<td>Vanderbilt AF Registry</td>
<td>AF</td>
<td>398</td>
<td>130</td>
<td>24</td>
<td>98.8</td>
<td>0.01</td>
<td>452</td>
<td>99</td>
<td>4</td>
<td>98.8</td>
</tr>
<tr>
<td>Non-AF</td>
<td>453</td>
<td>99</td>
<td>6</td>
<td>98.4</td>
<td>0.81</td>
<td>432</td>
<td>351</td>
<td>22</td>
<td>99.1</td>
<td>0.83</td>
</tr>
<tr>
<td>German AF Network</td>
<td>AF</td>
<td>1015</td>
<td>598</td>
<td>93</td>
<td>99.5</td>
<td>0.69</td>
<td>3315</td>
<td>675</td>
<td>29</td>
<td>98.7</td>
</tr>
<tr>
<td>Non-AF</td>
<td>3116</td>
<td>853</td>
<td>45</td>
<td>98.6</td>
<td>0.11</td>
<td>—</td>
<td>—</td>
<td>—</td>
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</tbody>
</table>

### Table 3 Association between the single nucleotide polymorphisms rs2200733 and rs10033464 with atrial fibrillation

<table>
<thead>
<tr>
<th></th>
<th>Adjusted for age and sex</th>
<th>Adjusted for age, sex, HTN, HF, MI, BMI, DM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Odds ratio (95% CI)</td>
<td>P-value</td>
</tr>
<tr>
<td>Framingham Heart Study</td>
<td>1.40 (1.07–1.82)</td>
<td>0.01</td>
</tr>
<tr>
<td>Rotterdam Study</td>
<td>1.37 (1.18–1.59)</td>
<td>3.1 × 10^{-5}</td>
</tr>
<tr>
<td>Vanderbilt AF Registry</td>
<td>1.68 (1.29–2.18)</td>
<td>0.0001</td>
</tr>
<tr>
<td>German AF Network</td>
<td>2.52 (2.22–2.84)</td>
<td>1.8 × 10^{-15}</td>
</tr>
</tbody>
</table>

The odds ratio for the association between single nucleotide polymorphisms rs2200733 or rs10033464 and atrial fibrillation in each cohort. The results adjusted for age and sex are provided on the left half of the table and the results adjusted for age, sex, HTN, HF, MI, BMI, and DM are presented on the right. The odds ratios are per allele, thus a patient in the German AF Network that is homozygous for the rs2200733 minor allele will have over a five-fold increased risk of atrial fibrillation compared with an individual homozygous for the major allele. HTN, hypertension; HF, heart failure; MI, myocardial infarction; BMI, body mass index; DM, diabetes mellitus.
Discussion

In the last 2 years, an increasing number of genome-wide association studies have emerged in the literature identifying genetic variants associated with macular degeneration, coronary disease, QT interval and recently AF. \(^2^3\) Although the prior studies provide an opportunity to identify variants associated with apparently complex traits in a population, upon the completion of these studies, two primary issues remain. First, given the inherent limits of multiple hypothesis testing in genome-wide association studies, can the findings from any one study be broadly replicated? Secondly, how do we go from association to mechanism or how precisely do these chromosomal variants lead to AF? In our current report, we address the reproducibility of the findings in the original study.

We found that two SNPs—rs2200733 and rs10033464—were significantly associated with AF in four cohorts of Northern European descent after accounting for age and sex of the participants. Meta-analyses of the relations between AF and both SNPs in all available case–control studies provided a convincing OR of 1.90 (\(P = 0.093\)). A meta-analysis of the association between AF and rs10033464 in the case–control studies, with inclusion of the current and previous reports,\(^2^3\) reveals an OR of 1.36 (95% CI 1.26–1.47; \(P = 6.7 \times 10^{-15}\)).

In an exploratory analysis, we observed a significant interaction between rs2200733 genotype and age in the FHS (\(P = 0.0065\)), but not in the RS (\(P = 0.62\)). There was no significant interaction between rs10033464 genotype and age in the FHS (\(P = 0.24\)) or in the RS (\(P = 0.47\)). In order to compare with prior reports, we have presented these data dichotomized at 60 years of age in Table 4. A higher OR was noted between SNP rs2200733 and AF in participants <60 years in the FHS and AFNet, whereas the opposite was true in the Vanderbilt AF Registry with a higher OR in participants over 60 years.

In the RS, there were 575 cases of incident AF. After adjustment for age and sex, the association between AF and rs2200733 was significant (OR 1.39, 95% CI 1.17–1.65; \(P = 2.1 \times 10^{-4}\)). Among the 2006 participants available in the FHS, there were 184 incident cases of AF. Acknowledging low power to detect an association in Framingham we did not observe a significant odds of incident AF with SNPs rs10033464 or rs2200733 (OR, 95% CI, P-value: 1.11, 0.81–1.52, 0.52; and 1.21, 0.89–1.64, 0.23, respectively).

| Table 4 Relations between age of participants and risk of atrial fibrillation for single nucleotide polymorphisms rs2200733 or rs10033464 |
|---------------------------|---------------------------|---------------------------|---------------------------|---------------------------|
| rs2200733                | Age <60, odds ratio (95% CI), AF/non-AF | Age >60, odds ratio (95% CI), AF/non-AF | rs10033464                | Age <60, odds ratio (95% CI), AF/non-AF | Age >60, odds ratio (95% CI), AF/non-AF |
| Framingham Heart Study   | 2.13 (1.13–4.00), 29/1973 | 1.29 (0.98–1.72), 295/1973 | 1.48 (0.68–3.23), 30/2005 | 1.34 (1.01–1.78), 295/2005 |
| Rotterdam Study          | 1.34 (0.75–2.39), 52/1007   | 1.33 (1.14–1.55), 858/4489 | 1.60 (0.90–2.86), 49/942  | 1.16 (0.97–1.38), 802/4181   |
| Vanderbilt AF Registry   | 1.44 (1.02–2.01), 264/304    | 2.03 (1.34–3.07), 168/254 | 1.12 (0.76–1.64), 264/302 | 1.17 (0.73–1.86), 168/253    |
| German AF Network        | 2.71 (2.34–3.15), 697/2868 | 1.99 (1.68–2.36), 1009/1146 | 1.35 (1.12–1.63), 691/2872 | 1.31 (1.07–1.60), 1010/1147 |

Although the potential mechanism of action of the genetic locus identified by these two non-coding SNPs is unknown and may be mediated through effects of distant genes, it is interesting to note that the closest gene, located ~50 000 bp centromeric, is the transcription factor, PITX2. Mouse knockouts of this gene have demonstrated a critical role for one isoform, PITX2c, in left–right asymmetry\(^2^4\) and specifically the development of the left atrium.\(^4^0,4^1\) The loss of PITX2c leads to right atrial isomerization and a failure to suppress a default pathway for sinus node formation in the left atrium of the embryo.\(^4^2\) Finally, in a recent elegant study, PITX2c has been demonstrated to be necessary for the development of the pulmonary myocardium or the sleeve of cardiomyocytes extending from the left atrium to the initial portion of the pulmonary vein.\(^2^3\) Clinical and animal studies have demonstrated that ectopic foci of electrical activity arising from within the pulmonary veins and posterior left atrium play a substantial role in initiating and maintaining fibrillatory activity.\(^2^6,4^3\) Furthermore, electrical isolation of the pulmonary veins and left atrial region is the goal of catheter ablation procedures that increasingly have been used to treat AF in the last decade.

The association between genetic variants on chromosome 4q25 and AF that we have observed implicates a novel pathway in the genesis of arrhythmia. AF has been reported to be associated with mutations in ion channel proteins, alterations in ion channel flux, and action potential shortening.\(^4^4\) It is interesting to hypothesize that these variants may misregulate PITX2 during cardiogenesis or beyond, thus perturbing the normal structure or function of the left atrium and pulmonary veins and ultimately predisposing to AF.\(^4^5,4^6\) It is important to note that such reasoning is currently speculative and at present, no direct mechanistic relationship between these variants and PITX2 has yet been demonstrated.

Although there was a strong association between rs2200733 and incident AF in the RS, the association was not statistically significant in the FHS. Although the failure to replicate the Rotterdam findings may simply be due to the limited number of incident cases (\(n = 184\)) in the Framingham cohort, the relatively low risk associated with rs2200733, even in the case–control studies, suggests that the broad use of such an SNP in predictive testing is of limited clinical value. Similarly, the variability noted in the ORs for the association between SNP rs2200733 and AF in younger

\[ P = 3.3 \times 10^{-13} \] for rs2200733 or 1.36 (\(P = 6.7 \times 10^{-13}\)) for rs10033464.
vs. older subjects may be due to the limited number of younger subjects available in all studies but the German AF Network. Future studies to determine whether this SNP is associated with the age of onset of AF or other outcomes from AF such as the risk of HF, stroke, mortality, responses to drugs, or catheter ablation procedures will be helpful.

As each of the four studies we sampled consists of participants of European descent, the generalizability of our results to other races and ethnicities is uncertain. In addition, we had low statistical power to test for gene–environment or gene–gene interactions.

In conclusion, we report that two variants on chromosome 4q25 are strongly associated with AF. Although variation at this locus does not appear to be suitable for clinical testing, it does provide a starting point for exploration of a novel pathway for this morbid arrhythmia.

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References


