Association of Human Connexin40 Gene Polymorphisms With Atrial Vulnerability as a Risk Factor for Idiopathic Atrial Fibrillation

Mehran Firouzi, Hemanth Ramanna, Bart Kok, Habo J. Jongsma, Bobby P.C. Koeleman, Pieter A. Doevendans, W. Antoinette Groenewegen, Richard N.W. Hauer

Abstract—Alterations in distribution, density, and properties of cardiac gap junctions, which mediate electrical coupling of cardiomyocytes, are considered potentially arrhythmogenic. We recently reported 2 linked polymorphisms within regulatory regions of the gene for the atrial gap junction protein connexin40 (Cx40) at nucleotides −44 (G→A) and +71 (A→G), which were associated with familial atrial standstill. The present study examined whether these Cx40 polymorphisms were associated with increased atrial vulnerability in vivo and arrhythmia susceptibility. In 30 subjects without structural heart disease, of whom 14 had documented sporadic paroxysmal atrial fibrillation (AF) and 16 had no AF history, inducibility of AF was assessed using an increasingly aggressive atrial stimulation protocol. Coefficient of spatial dispersion of refractoriness (CD) was calculated. CD was defined as the SD of 12 local mean fibrillatory intervals recorded at right atrial sites, expressed as a percentage of the overall mean fibrillatory interval. Cx40 genotypes were determined by direct DNA sequencing. Subjects were stratified according to normal or increased CD with a cutoff value of 3.0, because CD > 3.0 was previously shown to be strongly associated with enhanced atrial vulnerability. The prevalence of the minor Cx40 allele (−44A) and −44AA genotype was significantly higher in subjects with increased dispersion (n = 13) compared with those with CD ≤ 3.0 (n = 17; P = 0.00046 and P = 0.025; odds ratios of 6.7 and 7.4) and a control population (n = 253; P = 0.00002 and P = 3.90×10^-3). Carriers of −44AA genotype had a significantly higher CD compared with those with −44GG genotype (6.37 ± 1.21 versus 2.38 ± 0.39, P = 0.018), whereas heterozygotes had intermediate values (3.95 ± 1.38, NS). All subjects with increased CD had a history of idiopathic AF compared with only 1 subject with normal CD. The −44A allele and −44AA genotype were significantly more frequent in subjects with prior AF than in those without (P = 0.019 and P = 0.031; odds ratios 5.3 and 6.2). This study provides strong evidence linking Cx40 polymorphisms to enhanced atrial vulnerability and increased risk of AF. The full text of this article is available online at http://circres.ahajournals.org. (Circ Res. 2004;95:e29-e33.)

Key Words: arrhythmias □ atrium □ electrophysiology □ gap junctions □ genetics

Fast and coordinated propagation of cardiac action potentials is mediated by intercellular gap junction channels, which are composed of membrane-spanning protein subunits called connexins (Cxs).1 In mammalian heart, 3 Cx isoforms, Cx40, Cx43, and Cx45, are expressed in a chamber- and tissue-specific fashion. Current evidence suggests that gap-junctional remodeling in the heart may lead to abnormal electrical coupling and is, therefore, potentially arrhythmogenic.2

Human and animal studies have demonstrated that Cx40 is expressed mainly in the atrium and conduction system. Lack of Cx40 has been reported to result in increased atrial vulnerability and propensity to arrhythmias in the mouse.3,4 In Cx40-deficient mice, atrial conduction was delayed and atrial arrhythmias occurred spontaneously or could frequently be induced by programmed atrial electrical stimulation.3–5 Furthermore, there is accumulating evidence suggesting that changes in expression levels and distribution pattern of atrial Cx40 may form a cellular substrate favoring arrhythmia susceptibility and perpetuation.6,7

Previously, we identified increased spatial dispersion of refractoriness as an electrophysiological substrate for enhanced atrial vulnerability,8 defined as enhanced electrical inducibility of atrial fibrillation (AF), in the absence of remodeling. However, the molecular basis for this increased dispersion is unknown. Recently, we reported 2 closely linked polymorphisms in the promoter region of the Cx40 gene, which were strongly associated with familial atrial
Materials and Methods

Study Population

The study population consisted of subjects with supraventricular tachycardia free of structural heart disease who were referred for catheter ablation. Forty-three subjects participated in our previous study on atrial electrophysiological behavior, performed before ablation, of whom 30 unrelated adults (age <60 years) participated in our genetic study (10 women, 20 men; age 18 to 52 years, mean 32.8 years); 27 with an accessory atrioventricular pathway, of which 2 were concealed and 3 with atrioventricular nodal reentrant tachycardia (AVNRT). Of these 30 subjects, 14 had prior documented sporadic episodes of AF and 16 had no AF history. In subjects with prior AF, the incidence of AF episodes was assessed by questioning them about symptoms of irregular heartbeat. Electrocardiogram (ECG) documentation of ≥1 episode was required for inclusion in the study, and each subject was specifically questioned about the duration and onset of previous episodes. The mean number of AF episodes in the AF group was 1 (range 1 to 5), median duration 1 hour (range 15 minutes to 3 hours), with an AF free interval before the study of 148 days (range 9 to 365 days). Subjects without a history of AF did not have any history of irregular heartbeat.

All participants were white subjects from various parts of the Netherlands. They had a normal physical examination and underwent ECG, echocardiography, chest film, biochemical, and hematological testing to exclude structural heart disease or other potentially arrhythmogenic conditions. Specifically, none of the participants was on antithrombolytic drugs or other medication. Telemetric monitoring for ≥24 hours before the electrophysiological study did not reveal any arrhythmia episodes. In 12-lead ECGs, the mean of 3 manually measured consecutive P-wave durations in standard leads I, II, and III were calculated by 2 physicians who were unaware of the genotyping and the electrophysiological measurements. A population (n=253) of unrelated healthy persons without heart disease or arrhythmias served as a control group for Cx40 genotype frequencies. The study protocol was approved by our hospital review committee, and subjects gave written informed consent.

Electrophysiological Study

The electrophysiological study has been described previously. Briefly, through right femoral venous access, a decapolar catheter (Bard, USCI) was positioned at the right atrial free wall and a quadripolar catheter (Bard, USCI) was placed in the right atrial appendage. Twelve unipolar electrograms were recorded, 10 from the decapolar and 2 from the quadripolar catheter.

Atrial vulnerability was evaluated by a 4-stage atrial stimulation protocol using unipolar pacing (pulse width 2 ms) to assess inducibility of AF. Subsequent stages were performed until AF with a duration of ≥1 minute was obtained. Stage 1 consisted of pacing at 2 times the diastolic threshold current with an 8-pulse drive train and 1 extrastimulus starting at the tip electrode of the decapolar catheter and, if required, was repeated at all other electrodes. The extrastimulus interval S3, S4 of 100 ms was incremented in steps of 5 ms until atrial capture occurred. Stage 2 consisted of pacing at 4 times diastolic threshold current with an 8-pulse drive train and 1 extrastimulus. Stage 3 contained double extrastimuli. Finally, if AF was not induced with the previous stages, stage 4 consisting of multiple 5-second ramp bursts at a cycle length of 100 to 50 ms at 4 times the diastolic threshold current were used. Other induced tachycardias were pace terminated immediately, and the pacing protocol was interrupted for 1 minute after these episodes, as well as after AF episodes lasting <1 minute. AF persisting beyond 5 minutes was terminated by electrical cardioversion. The segment between 15 and 30 seconds after AF onset was used for electrogram analysis.

After AF induction, fibrillatory intervals were measured at all recordings sites and the mean fibrillatory intervals were calculated for each site to serve as an index for the local refractory period. The average and SD values of these indices were calculated. Spatial dispersion of refractoriness was determined by calculating the coefficient of dispersion (CD), defined as the SD of all local mean fibrillatory intervals expressed as a percentage of the overall mean fibrillatory interval. On the basis of our previous study, a CD value of 3.0 or less was considered normal, whereas a CD value of >3.0 was considered enhanced spatial dispersion of atrial refractoriness. All electrophysiological measurements were performed before the genotyping.

Determination of Cx40 Genotypes

Genomic DNA was extracted according to standard protocols, and the Cx40 polymorphisms were detected by direct sequencing as described previously, blinded to the electrophysiological findings.

Statistical Analysis

The χ2 or Fisher exact tests were used to test deviations of genotype distribution from Hardy–Weinberg equilibrium and to compare allele and genotype frequencies between groups. Odds ratios (approximating relative risk) with 95% CIs were estimated (via 2×2 contingency tables). Effects of genotypes on continuous parameters were analyzed by 1-way ANOVA, followed by Bonferroni corrected Fisher least-significant difference test for pairwise comparisons. Because of skewed distribution of the CD, logarithmic transformation of this variable was performed. In this dataset (13 cases with CD >3.0 and 253 controls), the power to reach significance (α-error <0.05) is ~90% under a dominant model and ~80% under a recessive model, assuming a minor allele frequency of 0.21, a prevalence of 0.001, and a strong risk (relative risk 10). Prevalence values of 0.01 and 0.0001 gave similar results (SGDP Statistical Genetics Group, MRC Social, Genetics, and Developmental Psychiatry Research Centre, Kings College, London, UK; http://stagen.iop.kcl.ac.uk/gpc/cc2.html). The SPSS statistical software (version 11.5, SPSS) was used for analyses. Statistical differences were judged significant at P<0.05.

Results

Within the study population, both Cx40 polymorphisms were in complete linkage disequilibrium: all subjects with allele G at position −44 had allele A at +71, and vice versa, ie, they were −44AA/+71GG, −44GG/+71AA, or −44GA/+71GA. This nonrandom association of alleles at this linked locus is probably caused by the short distance between the 2 polymorphisms. Therefore, we report only the data for the −44 polymorphism.

Subjects were classified according to increased or normal CD with a cutoff value of 3.0. There were no significant differences in baseline characteristics (age, sex, left atrial dimensions, baseline heart rate, pacing thresholds, mean fibrillatory intervals), as well as P-wave morphology and duration between the increased and normal CD groups (not shown). The −44 polymorphism was found in Hardy–Weinberg equilibrium, although in the CD >3.0 group there was a trend toward Hardy–Weinberg disequilibrium because of overrepresentation of −44AA genotype (χ² = 3.6,
The prevalence of the minor Cx40 allele and −44AA genotype was significantly higher in subjects with increased dispersion of refractoriness (CD >3.0) compared with those with normal dispersion (CD ≤3.0, \( P = 0.025 \)) and controls (\( P = 0.00002 \) and \( P = 3.90 \times 10^{-7} \); Table 1). Allelic and genotypic frequencies of Cx40 polymorphisms in the normal CD group did not differ from those with normal dispersion (CD >3.0), with the 44AA genotype, as compared with noncarriers, was 6.7 (95% CI: 2.0 to 21.9) or 7.4 (95% CI: 1.0 to 53.6), respectively (Table 1). The electrophysiological parameters of the study population according to Cx40 genotype are presented in Table 2. AF inducibility indicates type of stimulation required to obtain AF. As expected, the frequency of minor Cx40 allele and −44AA genotype was also significantly higher in subjects with prior AF compared with those without (\( P = 0.0019 \) and \( P = 0.031 \) respectively). The odds ratio for AF to carry the minor Cx40 allele or −44AA genotype versus noncarriers was 5.3 (95% CI: 1.7 to 17.2) or 6.2 (95% CI: 0.9 to 44.3), respectively. Consistent with our previous study, all the subject in the current study the mean CD was significantly higher in subjects with a history of AF than in those without (5.96 ± 0.70 versus 1.59 ± 0.18, \( P < 0.001 \)).

**Discussion**

The present study shows that the Cx40 polymorphisms are strongly associated with increased dispersion of refractoriness as the electrophysiological substrate for enhanced atrial vulnerability in man. Previously, we showed a strong association between this increased CD and enhanced electrical inducibility of AF, as well as the risk of spontaneous occurrence of AF in subjects free of structural heart disease.

### Table 1. Comparison of Cx40 Allele and Genotype Frequencies

<table>
<thead>
<tr>
<th>Genotype Frequencies, n (%)</th>
<th>Allele Frequencies, n (%)</th>
<th>P Allele Frequency</th>
<th>Genotype Frequencies, n (%)</th>
<th>P Genotype Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD &gt;3.0 (n=13)</td>
<td>11 (42)</td>
<td>15 (58)</td>
<td>0.00046*</td>
<td>4 (31)</td>
</tr>
<tr>
<td>CD ≤3.0 (n=17)</td>
<td>29 (85)</td>
<td>5 (15)</td>
<td>NS†</td>
<td>13 (76)</td>
</tr>
<tr>
<td>Control (n=253)</td>
<td>399 (79)</td>
<td>107 (21)</td>
<td>0.00002‡</td>
<td>160 (63)</td>
</tr>
</tbody>
</table>

NS indicates not significant.

Only −44 polymorphism data are noted; data for +71 are complementary. \( P \) values for allele frequencies were calculated comparing the 2 allele frequencies in cases with CD >3.0 vs cases with CD ≤3.0, \( \chi^2 \) cases with CD ≤3.0 vs controls, or \( \chi^2 \) cases with CD >3.0 vs controls. \( P \) values for genotype frequencies were calculated accordingly comparing the 3 genotype frequencies.

### Table 2. Electrophysiological Parameters of Study Population According to Cx40 Genotype

<table>
<thead>
<tr>
<th>Cx40 Genotype</th>
<th>−4GG</th>
<th>−4GA</th>
<th>−4AA</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>17</td>
<td>6</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>AF inducibility</td>
<td></td>
<td></td>
<td></td>
<td>0.042*</td>
</tr>
<tr>
<td>One extrastimulus, n (%)</td>
<td>5 (29)</td>
<td>3 (50)</td>
<td>6 (86)</td>
<td></td>
</tr>
<tr>
<td>Two extrastimuli, n (%)</td>
<td>1 (6)</td>
<td>1 (17)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Burst pacing, n (%)</td>
<td>11 (65)</td>
<td>2 (33)</td>
<td>1 (14)</td>
<td></td>
</tr>
<tr>
<td>Recording sites measured, n</td>
<td>9.19 ± 0.28</td>
<td>9.00 ± 0.45</td>
<td>9.17 ± 0.31</td>
<td>NS</td>
</tr>
<tr>
<td>Mean FI, ms</td>
<td>167.12 ± 7.29</td>
<td>169.00 ± 8.54</td>
<td>158.57 ± 9.66</td>
<td>NS</td>
</tr>
<tr>
<td>SD</td>
<td>4.06 ± 0.77</td>
<td>6.45 ± 2.12</td>
<td>9.99 ± 1.96</td>
<td>0.013*</td>
</tr>
<tr>
<td>CD</td>
<td>2.38 ± 0.39</td>
<td>3.95 ± 1.38</td>
<td>6.37 ± 1.21</td>
<td>0.018*</td>
</tr>
<tr>
<td>Atrial conduction time, ms</td>
<td>25.38 ± 2.08</td>
<td>26.67 ± 2.79</td>
<td>26.43 ± 3.22</td>
<td>NS</td>
</tr>
<tr>
<td>P-wave duration on ECG, ms</td>
<td>87.35 ± 2.85</td>
<td>89.17 ± 3.75</td>
<td>87.86 ± 4.61</td>
<td>NS</td>
</tr>
</tbody>
</table>

NS indicates not significant; mean FI, average of the mean fibrillatory intervals from all electrograms.

AF inducibility indicates type of stimulation required to obtain AF.

Atrial conduction time represents the interval from atrial activation in the right atrial appendage to atrial activation at the His bundle electrogram recording position, measured during atrial pacing from the right atrial appendage with cycle length of 600 ms. Only −44 polymorphism data are noted; data for +71 are complementary. Continuous values are presented as mean ± SEM. *P value for comparison between the −44GG vs −44AA genotype.
CD in the study population stratified according to the Cx40 genotype. Probability value was calculated by 1-way ANOVA followed by Bonferroni correction. Values are mean±SEM.

The findings in the present study are in line with those we recently reported. The Cx40 -44AA genotype was strongly associated with atrial standstill, an uncommon arrhythmia characterized by absence of atrial electrical and mechanical activity, when co-inherited with a sodium channel mutation. The minor Cx40 haplotype (-44AA/71GG) significantly reduced (>50%) promoter activity in vitro. If these in vitro data were to be extrapolated to in vivo Cx40 promoter activity levels, homozygous carriers of the minor haplotype (-44AA/71GG) would be expected to have a 50% reduction in promoter activity compared with homozygous carriers of the major haplotype (-44GG/71AA). Similarly, heterozygotes would be expected to have an intermediary level of promoter activity. The final effect on Cx40 protein levels are presently unknown. However, because Cxs have relatively short half-lives (=2 hours) and because levels of Cx40 RNA and proteins in the human heart are closely correlated, any change in promoter activity would be expected to have a concomitant effect on the total Cx40 protein levels in vivo.

The precise mechanism through which these Cx40 gene variations may modulate atrial electrophysiological properties, hence, conferring arrhythmia risk is unknown. Current evidence suggests that abnormalities in gap junction channel distribution, ie, an overall reduction or increased heterogeneity, may lead to increased anisotropy. This would facilitate heterogeneous atrial activation and the occurrence of unidirectional conduction block and delays, which may result in increased atrial vulnerability and propensity to arrhythmias based on a reentrant mechanism.

Because of stringent inclusion criteria and invasive study design, the size of sample population was modest. However, because of the strong association between genotype and CD, our sample size was sufficient to detect a statistically significant difference between the groups, as indicated by power calculations. Our in vivo study design and clear definition of the phenotypes should have reduced the chance of spurious results, a problem inherent in association studies, because electrophysiological measurements were made directly in the atria in relatively young subjects (mean age <33 years) free of identifiable structural heart disease, other proarrhythmic conditions or medications.

Most subjects in the present study had Wolff–Parkinson–White (WPW) syndrome (25 of 30). Branched accessory pathways causing microreentry have been suggested as a causative factor for AF. However, Fujimura et al found that in patients with WPW syndrome, AF usually was initiated in the right atrium regardless of accessory pathway location. In addition, Wathen et al did not find any changes in atrial electrophysiological properties after accessory pathway ablation. In our study, the measurements and induction of AF were performed in the right atrium, whereas the accessory pathway was left-sided in most patients (increased CD group: 12 of 13; normal CD: 12 of 14). Moreover, in the normal CD group, 13 of 17 subjects had WPW syndrome (versus 12 of 13 in CD >3.0 group), low dispersion, and low inducibility, despite their accessory pathway. Notably, subjects with a history of AF had experienced only short lasting and infrequent episodes of idiopathic AF, rendering the possibility of atrial electrical remodeling in these subjects very unlikely. The number of supraventricular tachycardia episodes did not differ among subjects with increased and normal CD.

Therefore, subjects with increased CD had abnormal intrinsic atrial electrophysiological properties not directly attributable to the presence of an accessory pathway.

This study provides considerable direct evidence demonstrating genetic involvement in arrhythmia susceptibility. Although the mechanism responsible for the association of Cx40 genotype with increased dispersion may be explained by atrial electrophysiological parameters, this association does not prove a causal relationship. It is possible that the association described here is attributable to linkage disequilibrium between Cx40 gene polymorphisms and another nearby susceptibility gene. However, this explanation is less likely because Cx40 is a strong candidate gene for atrial vulnerability on the basis of studies of its murine homologue. We also cannot rule out the presence of other polymorphisms or mutations in Cx40 or other genes, not detected by our screening, that confer arrhythmia susceptibility either independently or in synergy with the polymorphisms described here. Discrimination among these between mechanisms requires further investigation.

In conclusion, this is the first study demonstrating that polymorphisms within the Cx40 gene promoter are associated with enhanced atrial vulnerability attributable to increased dispersion of refractoriness, as well as a history of idiopathic AF. To confirm that these polymorphisms are a novel genetic marker for increased atrial vulnerability, investigations in larger well-defined and ethnically diverse populations will be necessary.

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References

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