A Common Connexin-40 Gene Promoter Variant Affects Connexin-40 Expression in Human Atria and Is Associated With Atrial Fibrillation

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Background—A common single-nucleotide polymorphism (SNP) in the promoter of the Connexin-40 (Cx40) gene GJA5 was suggested to affect Cx40 promoter activity and the risk of atrial fibrillation (AF), but the role of other common Cx40 polymorphisms is unknown.

Methods and Results—Eight SNPs within the Cx40 gene region were tested for association with Cx40 levels measured in atrial tissue from 61 individuals. The previously described Cx40 promoter SNP (rs35594137, −44G→A) was not associated with Cx40 mRNA levels. However, a common SNP (rs10465885) located in the TATA box of an alternative Cx40 promoter was strongly associated with Cx40 mRNA expression (P<0.0001) and displayed strong and consistent allelic expression imbalance in human atrial tissue. A promoter-luciferase assay in cultured murine cardiomyocytes demonstrated reduced activity of the promoter containing the minor allele of this SNP (P<0.0001). Both rs35594137 and rs10465885 were tested for association with early-onset lone AF (≤60 years of age) in 384 cases and 3010 population control subjects. rs10465885 was associated with the AF phenotype (odds ratio, 1.18; P=0.046). This result was confirmed in a meta-analysis including 2 additional early-onset lone AF case-control cohorts (odds ratio, 1.16, P=0.022). rs35594137 was not associated with the lone AF phenotype in any of the cohorts studied or in a combined analysis.

Conclusions—A previously described Cx40 promoter SNP was not found to influence Cx40 expression or risk of AF. We describe an alternate promoter polymorphism that directly affects levels of Cx40 mRNA in vivo and is associated with early-onset lone AF. (Circ Arrhythm Electrophysiol. 2011;4:87-93.)

Key Words: atrial fibrillation ■ ion channels ■ genetics ■ allelic expression imbalance

Cardiac connexin-40 (Cx40) is found exclusively in the atria and conduction system. Along with connexin-43, it comprises the gap junctions that electrically couple atrial myocytes.1 As Cx40 is the only connexin found exclusively in the atria, several studies have investigated the effect of genetic alterations in the Cx40 gene gap junction protein, alpha 5 (GJA5) on the development of atrial conduction abnormalities and atrial fibrillation (AF). Complete absence of Cx40 in Cx40−/− mice leads to altered propagation and ectopic rhythms in the atria2 and arrhythmias on surface ECG.3 In 15 patients with idiopathic AF, Gollob et al4 discovered 3 somatic and 1 germ line mutation in the Cx40 coding region, resulting in dominant-negative Cx40 activity.

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The Cx40 gene GJA5 contains 2 alternative first exons (exons 1A and 1B), using separate promoters (promoters A and B), resulting in 2 alternative transcripts (Cx40 transcripts A and B) that share a common second coding exon.5 In 2003, Groenewegen et al6,7 identified a common single nucleotide polymorphism (SNP) in the Cx40 promoter A (rs35594137, −44G→A)9 that was later associated with decreased Cx40
promoter A activity in vitro, atrial vulnerability and AF. We hypothesized that additional common Cx40 polymorphisms may also affect Cx40 expression in human atrial tissues, and that these polymorphisms may predispose to AF.

We characterized a SNP, rs10465885, in the Cx40 promoter that alters a putative TATA box element and strongly influences expression of Cx40 transcript B and total Cx40 expression in human atra. We further determined that the promoter B SNP acts in cis, influencing expression of the same-strand transcript. The promoter B SNP was significantly associated with early-onset lone AF in a case-control cohort study, and this finding was validated in a meta-analysis including 2 additional cohorts. In contrast to previous reports, we found that the promoter A SNP was not significantly associated with Cx40 expression in vivo, nor was it associated with the AF phenotype.

Methods

Study Subjects
Atrial appendage tissues were obtained from 61 patients undergoing coronary artery bypass grafting, valve surgery, or the Maze surgical AF ablation procedure. Most patients (85.2%) from whom tissues were obtained had a history of AF, and 49.2% of patients had AF at the time of surgery. Supplemental Table 1 (online Data Supplement) provides a detailed description of this cohort.

Three AF case-control cohorts were used to analyze the association of the Cx40 promoter A and B SNPs with the lone AF phenotype. All case subjects had lone AF, defined as AF in the absence of structural heart disease, and all subjects were of European ancestry. In addition, subjects were deemed to have early-onset lone AF if their age at AF diagnosis was ≤60 years.

Subjects from the Cleveland Clinic Lone Atrial Fibrillation GeneBank (CCAF) (n = 596, with 384 early-onset cases) were used as the discovery cohort along with 3010 population control subjects from the Illumina iControl Database. A replication cohort consisting of lone AF subjects (n = 375, with 335 early-onset cases) from the Massachusetts General Hospital Atrial Fibrillation Study (MGH) (n = 7395) was obtained from the Atherosclerosis Risk in Communities (ARIC) study. A second replication case-control cohort consisted of lone AF subjects (n = 596, with 384 early-onset cases) were used as the discovery cohort along with 3010 population control subjects from the Framingham Heart Study (FHS).

Study Subjects

Results
Haplotype Analysis of the GJA5 Gene
Seven contiguous SNPs from the Illumina Hap550 BeadChip located within the GJA5 gene and proximal promoter region, plus rs11552588, a component of the previously described Cx40 promoter A haplotype, were studied. Genotypes at these 8 SNPs in the 61 atrial tissue samples were loaded into Haplovew v.4.1 (Broad Institute) to calculate their LD relationships (Figure 1A). From these data, it was determined that rs12408178 was in perfect LD (r² = 1) with rs11552588, the SNP at +71 in exon 1A, which is in perfect LD with the promoter A SNP rs35594137 (-44G→A). This established rs12408178 as a perfect proxy for the promoter A SNP. In contrast, the promoter A SNP proxies were only moderately linked to the promoter B SNP (r² = 0.33).

The Promoter B SNP Alters A Putative TATA Box
The sequence of the proximal promoter of human Cx40 transcript B, along with the homologous sequence in other mammalian species, is shown in Figure 1B. The promoter B SNP resides at -26 bp relative to the start site of transcription, is denoted. The overlapping TATA box sequences are underlined, and graphical representations of their respective nucleotide frequency matrices are shown above the sequence.
Genotypes at each of the 8 SNPs shown in Figure 1A were measured by separate probes on Illumina Human Ref-8 v2 Expression Bead Chips in human atrial tissue from 61 individuals. Levels of total Cx40 mRNA expression were measured by real-time polymerase chain reaction in 31 atrial tissue samples. As expected, the promoter B SNP was not significantly associated with levels of Cx40 mRNA expression in the 31 atrial tissue samples, with a corresponding quantitative log of the odds ratio (LOD) score of 4.85.

Because none of the probes on the microarray directly measured Cx40 transcript B, quantitative polymerase chain reaction of Cx40 transcript B was performed on cDNA from 31 of the 61 atrial tissue samples. The promoter B SNP genotype was strongly associated with Cx40 transcript B expression in a dose-dependent manner (Figure 2C, \( P<0.0001 \), 1-way ANOVA), with the presence of 1 “G” allele associated with 4.3-fold lower levels of Cx40 transcript B. In a linear regression analysis of the additive genetic model, the promoter B SNP genotype was associated with 49% of the variation in Cx40 transcript B expression in the 31 atrial tissue samples, with a corresponding quantitative log of the odds ratio (LOD) score of 4.85.

The Promoter A SNP Does Not Affect Cx40 Levels In Vivo

The genotype at the promoter A SNP proxy was not significantly associated with levels of Cx40 transcript A in the microarray assay of the 61 tissue samples (Figure 3, \( P=0.55 \), additive genetic model). Similarly, quantitative polymerase chain reaction of Cx40 transcript A in the atrial tissue samples failed to show any association of the promoter A SNP proxy with Cx40 transcript A expression (\( P=0.66 \), additive genetic model, Supplemental Figure 1). In addition, the promoter A SNP proxy was not significantly associated with levels of total Cx40 mRNA (\( P=0.82 \), additive genetic model, data not shown).

The Promoter B SNP Acts in Cis to Affect Cx40 Transcript B Expression In Vivo

Because the promoter B SNP alters the sequence of one of an overlapping pair of TATA box elements, we hypothesized that the effect of the promoter B SNP on Cx40 transcript B expression occurs in cis through modulation of transcription. Therefore, we tested for allelic expression imbalance, comparing Cx40 transcript B levels produced from each allele in atrial tissue samples from subjects heterozygous for both the promoter B SNP and an indicator SNP within Cx40 transcript B. In all of these atrial tissue samples, the amount of mRNA produced from the minor G allele at the promoter B SNP was strongly and significantly decreased compared with the amount produced from the major A allele, with a mean decrease of 3.3-fold and a range of 2.2- to 6.3-fold (Figure 4A). Representative sequence tracings from both cDNA and gDNA in the region surrounding the indicator SNP rs1043806 are shown in Figure 4C, illustrating the differing allelic ratios. As a control, 1 sample homozygous at the

![Figure 2](http://circep.ahajournals.org/)

**Figure 2.** The promoter B SNP genotype is associated with total Cx40 mRNA expression. Levels of total Cx40 and Cx40 transcript A were measured by separate probes on Illumina Human Ref-8 v2 Expression Bead Chips in human atrial tissue from 61 individuals. A, The promoter B SNP genotype was significantly associated with total Cx40 mRNA levels (\( P=0.013 \)). B, The promoter B SNP genotype was not significantly associated with levels of Cx40 transcript A (\( P=0.78 \)). C, Relative levels of Cx40 transcript B were quantified by real-time polymerase chain reaction in 31 atrial tissue samples. The promoter B SNP genotype was significantly associated with Cx40 transcript B levels (\( P<0.0001 \)).

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![Figure 3](http://circep.ahajournals.org/)

**Figure 3.** The promoter A SNP is not significantly associated with Cx40 transcript A expression (\( P=0.55 \)). Levels of Cx40 transcript A were measured on Illumina Human Ref-8 v2 Expression Bead Chips in human atrial tissue from 61 individuals.
promoter B SNP but heterozygous for an indicator SNP was assayed, and was found to have no significant allelic expression imbalance (Figure 4A, first bar marked as (−)).

The Promoter A SNP Is Incompletely Associated With Allelic Expression Imbalance

We measured allelic expression imbalance of Cx40 transcript A in atrial tissue samples from subjects heterozygous for the promoter A SNP and the indicator SNP rs11552588 in exon 1 of transcript A. Most samples exhibited small but significant allelic expression imbalance favoring the −44A allele, with a mean increase in Cx40 transcript A expression of 1.3-fold (Figure 4B). However, significant allelic expression imbalance was not observed in 2 samples, and another 2 samples exhibited significant allelic expression imbalance in the opposite direction. This indicates that the promoter A SNP may be in partial LD with a potential causative genetic variant that has a minor effect on the transcription of Cx40 transcript A.

The Promoter B SNP Affects Promoter Activity in Transfected HL-1 Murine Cardiomyocytes

To exclude the influence of other potential genetic variations in LD with the promoter B SNP on Cx40 transcript B expression, we used a promoter-driven reporter gene assay in cultured cells. HL-1 murine atrial cardiomyocytes were transiently transfected with a promoterless firefly luciferase reporter construct (negative control) or constructs driven by the native Cx40 transcript B promoter containing either the A or G allele at the promoter B SNP site. Each transfection included a plasmid expressing β-galactosidase as a transfection efficiency control. HL-1 cells transfected with the reporter construct containing the G allele at the promoter B SNP site exhibited 2.4-fold lower luciferase activity compared with cells transfected with the construct containing the A allele (Figure 5, P<0.0001, Student t test).

The Promoter B SNP Is Significantly Associated With AF

The promoter A SNP proxy (rs12408178) and the promoter B SNP (rs10465885) were tested for association with the early-onset lone AF phenotype (age of diagnosis <60 years) using 384 CCAF cases and 3010 healthy population control subjects in a logistic regression analysis under a log-additive genetic model. The promoter B SNP was significantly associated with early-onset lone AF status with an odds ratio (OR) of 1.11 (95% confidence interval, 1.00 to 1.39; P=0.046; Figure 6), with the underexpressed “G” allele overrepresented in AF cases. In contrast, the promoter A SNP proxy was not significantly associated with early-onset lone AF (OR=1.11, P=0.26, Figure 6).

Two additional case-control cohorts were used to confirm the findings from the discovery CCAF cohort (Figure 6). The promoter B SNP had an OR of 1.25 for AF in 45 early-onset AF cases and 7300 non-AF control subjects from the ARIC cohort, but because of the small number of cases this was not statistically significant (P=0.31). The promoter A SNP proxy was not significantly associated with early-onset AF in the ARIC cohort (OR=1.06, P=0.81). The promoter B SNP had an OR of 1.09 for lone AF in 334 early-onset AF cases from the MGH cohort and 1067 healthy control subjects from the FHS cohort, which was in the same direction as the other 2 studies, but not statistically significant (P=0.42). The promoter A SNP proxy was not significantly associated with early-onset AF in this group (OR=0.98, P=0.90).
A meta-analysis including all 3 cohorts confirmed the finding of the discovery cohort, revealing a significant association between the promoter B SNP and early-onset lone AF (OR, 1.16; 95% confidence interval, 1.02 to 1.31; \( P = 0.022 \); Figure 6). A similar meta-analysis failed to find a significant association between the promoter A SNP proxy and early-onset lone AF (OR, 1.07; \( P = 0.37 \); Figure 6).

The meta-analyses of the early-onset lone AF case-control studies, including all 3 cohorts, had 80% power to detect a relative risk of 1.16 for the minor allele of the promoter B SNP and a relative risk of 1.18 for the minor allele of the promoter A SNP in the additive genetic model. None of the meta-analyses displayed any significant heterogeneity.

Neither the promoter A nor the promoter B SNP was significantly associated with the lone AF phenotype in any of the cohorts when subjects of all ages were included in their respective AF case cohorts (Supplemental Table 3), demonstrating the expected stronger genetic effects in younger cases.

### Discussion

We used mRNA expression data and analysis of mRNA allelic expression imbalance in human atrial tissue to characterize a novel regulatory polymorphism in the Cx40 gene GJA5. This polymorphism, rs10465885, is located in the promoter region of Cx40 transcript B, where it alters the configuration of 1 of 2 partially overlapping TATA box elements. The strong correlation between the promoter B SNP and Cx40 mRNA levels and its consistent and profound effect on Cx40 transcript B allelic mRNA expression ratios demonstrate that this polymorphism is strongly associated in cis with transcription of Cx40 transcript B in vivo. Its location in a TATA box element and the results of the promoter-luciferase reporter gene assay provide strong evidence that the promoter B SNP directly affects transcription of Cx40 transcript B.

Comparison of the promoter region altered by the promoter B SNP with a nucleotide frequency matrix of the human TATA box sequence revealed 2 possible overlapping TATA box sequences (Figure 1B). The similarity of the 5′-shifted TATA box to the consensus TATA sequence is substantially improved by the G→A transition at the promoter B SNP site. The base affected by the promoter B SNP is not strictly conserved in mammals and, interestingly, the “non-A” allele appears to be present in the sequences of at least 5 other mammalian species. In addition, the chimpanzee genome at this site contains the G allele, suggesting that the A allele could be a gain-of-function mutation unique to humans.

Cx40−/− knockout mice exhibit atrial ectopy and arrhythmias on surface ECG, and rare dominant-negative defects in the Cx40 coding sequence are associated with AF in humans. However, most SNPs associated with complex traits identified by genome-wide association studies to date are not located within gene coding regions or open reading frames and are instead expected to affect gene expression. Therefore, we tested for an association between Cx40 promoter polymorphisms and AF.

We found that the newly characterized promoter B SNP was significantly associated with early-onset lone AF in our discovery cohort. Although the association of the promoter B SNP with AF did not reach statistical significance in the 2 individual replication cohorts, these analyses were based on smaller sample sizes and were not adequately powered to detect the effect size observed in the discovery cohort. However, the effect of the promoter B SNP observed in each of the replication cohorts was consistent with the effect observed in the discovery cohort with increased risk associated with the G allele. Thus, a meta-analysis including all 3 cohorts confirmed the significant association between the promoter B SNP and early-onset lone AF. In addition, the G allele resulting in diminished Cx40 expression was overrepresented in the AF cases, suggesting that lower Cx40 levels may also be associated with AF. This is consistent with previous observations2–4 that have suggested that impaired Cx40 expression or function predisposes to atrial conduction abnormalities and AF. The observed odds ratio of 1.16 per risk allele in the meta-analysis is modest and is expected under natural selection given the high minor allele frequency (0.47) of this SNP. However, the presence of 2 risk alleles is associated with an odds ratio of 1.35, which underscores the potential importance of this SNP in predicting AF risk.

Because each of the 2 replication cohorts did not achieve statistical significance on their own, how strong is the evidence supporting the association of rs10465885 with early-onset lone AF? First, all 3 studies had odds ratios in the same direction, showing increased risk with the underexpressed G allele. Second, the combined meta-analysis with the greatest power was more highly significant than the discovery cohort, demonstrating that as the sample size

### Table 1: Promoter B and A SNP Association with AF

<table>
<thead>
<tr>
<th>Study</th>
<th>AF (n)</th>
<th>No AF (n)</th>
<th>MAF (%)</th>
<th>OR (95% CI)</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCF</td>
<td>384</td>
<td>3010</td>
<td>51.8/46.9</td>
<td>1.18 (1.00-1.39)</td>
<td>0.046*</td>
</tr>
<tr>
<td>MGH</td>
<td>334</td>
<td>1067</td>
<td>48.2/47.8</td>
<td>1.09 (0.88-1.36)</td>
<td>0.42</td>
</tr>
<tr>
<td>ARIC</td>
<td>45</td>
<td>7300</td>
<td>52.3/47.7</td>
<td>1.25 (0.81-1.93)</td>
<td>0.31</td>
</tr>
<tr>
<td>Meta</td>
<td>763</td>
<td>11377</td>
<td>50.2/47.5</td>
<td>1.16 (1.02-1.31)</td>
<td>0.022*</td>
</tr>
</tbody>
</table>

The meta-analyses of the early-onset lone AF case-control studies, including all 3 cohorts, had 80% power to detect a relative risk of 1.16 for the minor allele of the promoter B SNP and a relative risk of 1.18 for the minor allele of the promoter A SNP in the additive genetic model. None of the meta-analyses displayed any significant heterogeneity.

Neither the promoter A nor the promoter B SNP was significantly associated with the lone AF phenotype in any of the cohorts when subjects of all ages were included in their respective AF case cohorts (Supplemental Table 3), demonstrating the expected stronger genetic effects in younger cases.

### Figure 6: Meta-analysis of promoter B and A SNPs for association with early-onset lone AF. Meta-analyses of the results (additive genetic model) from the 3 cohorts were performed using a fixed effect model. Promoter SNP B (top) but not promoter SNP A (bottom) was significantly associated with early-onset lone AF. Gray boxes reflect the weighting (W), based on sample size, of each cohort in the meta-analysis. MAF indicates minor allele frequency for cases/control subjects; OR, odds ratio for lone AF with each additional copy of minor allele; and CI, confidence interval. \( P < 0.05 \).
increased the association grew stronger. Third, other genetic association studies with weak effects (eg, odds ratio of \( \approx 1.2 \)) are often not replicated in the individual validation cohorts, but when combined, the effects are highly significant. For example, the recently identified SNP associated with abdominal aortic aneurysm was assessed in 8 separate follow-up cohorts, and 6 of these failed to find a significant association.\(^{15}\) However, combining these 8 cohorts together yielded a significant association.\(^{15}\) A prior genome-wide association study meta-analysis for lone AF in subjects 65 years of age or younger detected and validated 2 genome-wide significant loci but did not find a genome-wide significant association for the Cx40 gene.\(^{16}\) However, many real associations are present that fail to meet the strict standard of genome-wide significance, which is very stringent because of the correction for hundreds of thousands of SNPs tested. For example, genome-wide significant loci for height, which is a highly heritable trait, account for only 5% of the variance in height in studies with tens of thousands of subjects, but 45% of the variance can explained by considering all SNPs simultaneously.\(^{17}\) Thus, many more SNPs add to the heritability of height that cannot be identified with certainty using current statistical methods. We conclude that the evidence on hand supports the association of the Cx40-B promoter SNP rs10465885 with early-onset lone AF, although we cannot definitively rule out that this finding is a false-positive.

Interestingly, the promoter B SNP was not significantly associated with lone AF when subjects of all ages were included in the analyses. The prevalence of conditions that predispose to AF such as hypertension, coronary artery disease, valve disease, and heart failure increases with age. Therefore, although these analyses were performed in subjects with lone AF, the inclusion of older subjects (with AF caused by other “nongenetic” factors) in the AF case cohort may dilute the effect of a potential genetic variant. We confirmed the lack of association of the promoter B SNP with lone AF in subjects of all ages in a recent meta-analysis of 5 case-control cohorts comprising 1335 lone AF cases and 12 844 unaffected subjects, which overlaps the ARIC and MGH/FHS cohorts of the current study.\(^{18}\) In this study, rs10465885 was not significantly associated with AF (OR, 1.064; 95% confidence interval, 0.93 to 1.22; \( P=0.64 \)). Age-stratified analysis was not performed in this mega-analysis.

A common SNP in the promoter of Cx40 transcript A, first identified cosegregating with an \( SCN5A \) mutation in familial atrial standstill,\(^{6}\) has also been associated with atrial vulnerability and AF in a small sample of 30 individuals.\(^{8}\) The association with AF was later replicated in a Taiwanese population of 173 AF patients and 232 control subjects,\(^{7}\) yielding an odds ratio of 1.54 for the minor allele of the promoter A SNP in the additive genetic model. Promoter-luciferase assays in cell culture showed varying (\(<20\%\) to \(65\%\)) reduction in the activity of the promoter containing the minor allele of the promoter A SNP. A further study suggested that the minor allele of this promoter SNP negatively affected binding of GATA4, a major cardiac-specific transcription factor, to its binding site in the Cx40 transcript A promoter.\(^{18}\) We sought to extend these in vitro observations by studying the effect of this SNP on Cx40 mRNA expression levels in vivo in human atrial tissues. However, we were unable to detect a significant effect of the promoter A SNP on Cx40 transcript A or total Cx40 mRNA expression. Similarly, analysis of allelic expression imbalance in individuals heterozygous at the promoter A SNP revealed minimal and inconsistent allelic expression imbalance of Cx40 transcript A. For a candidate polymorphism to be considered a true cis-acting regulatory polymorphism, allelic expression imbalance must be consistent. Thus, our data argues against a true cis-acting regulatory role for the promoter A SNP.

In contrast to previous reports,\(^{7,8}\) the promoter A SNP was not significantly associated with AF in any of the case-control cohorts, or in the combined meta-analysis. The discordance of our findings with the Taiwanese study could in part be due to race-specific genetic effects or differences in the baseline patient characteristics. However, our meta-analysis had a markedly greater sample size, and was sufficiently powered to detect a relative risk of 1.18 for the minor allele of the promoter A SNP. Our study demonstrated that there was no association between the promoter A SNP and lone AF with an effect size of 1.18 or greater in populations of European ancestry.

In conclusion, we determined that a previously described Cx40 promoter A polymorphism, rs35594137 (\(44G\rightarrow A\)), was not associated with Cx40 mRNA levels, allelic expression imbalance in human atrial tissue, or the lone AF phenotype. However, we identified a different SNP, rs10465885, as a common Cx40 promoter B variant with a direct cis effect on Cx40 gene expression. Importantly, this promoter B SNP was also significantly associated with early-onset lone AF. Additional studies are needed to more fully characterize the effects of this SNP in atrial tissue and its link to AF pathophysiology. However, this study represents an important step toward understanding the genetic determinants of Cx40 expression, as well as the potential role of variation in Cx40 expression as a determinant of AF risk.

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Disclosures

None.

References


CLINICAL PERSPECTIVE

The occurrence of familial atrial fibrillation (AF) caused by rare mutations in genes encoding cardiac ion channels has been known for some time. In 2004, an analysis of the Framingham Heart Study showed increased AF risk for offspring of AF patients in an unselected population and shifted the paradigm of AF genetics from rare familial mutations to focus on potential common genetic factors that predispose to AF. Indeed, this was borne out in 2007 with the completion of the first genome-wide association study of AF, which revealed 2 common single-nucleotide polymorphisms on chromosome 4q25 that conferred risk of AF. The number of common genetic variants associated with AF is slowly growing, with the recent identification of a single-nucleotide polymorphism within the potassium channel gene KCNN3 that is associated with lone AF. In our current study, we show that a common single-nucleotide polymorphism in the promoter of the gene encoding the atrial-specific gap junction protein connexin-40 directly affects the expression of connexin-40 and is associated with early-onset lone AF. Our findings add to the catalogue of genetic variants that may be used in the future to help predict an individual’s risk of developing AF and thus target patients for prevention of this disease. In addition, the identification of a common connexin-40 variant that predisposes to early-onset lone AF highlights gap junction proteins and connexin-40 in particular as attractive potential drug targets for the treatment of AF, especially in individuals with genetic susceptibility to development of AF through this mechanism.
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Methods

Study Subjects

Cleveland Clinic Atrial Tissue Bank. Atrial appendage tissues were obtained from 61 patients undergoing coronary artery bypass grafting, valve surgery or the Maze surgical AF ablation procedure. Supplemental Table 1 describes the clinical characteristics of these subjects.

Cleveland Clinic Lone Atrial Fibrillation GeneBank (CCAF). The discovery lone AF case cohort consisted of individuals from CCAF, which enrolled subjects >18 years of age with a history of AF and no significant coronary artery disease (≤50% coronary artery stenosis by coronary angiography, a normal stress test or no presumed coronary artery disease by clinical criteria; stress testing or coronary angiography was required for inclusion in males >age 50 years or females >age 55 years,) and normal left-ventricular function (left ventricular ejection fraction≥50%). Subjects were excluded from the study if they had significant valvular disease (>2+ mitral regurgitation, >2+ tricuspid regurgitation, >2+ aortic insufficiency, >2+ pulmonic insufficiency or any mitral,
tricuspid, aortic or pulmonic stenosis), coronary artery disease (>50% coronary artery stenosis, prior myocardial infarction, prior percutaneous coronary intervention, or coronary artery bypass surgery), a history of current congenital heart disease (except isolated patent foramen ovale), or a history of or current structural heart disease.

Illumina Controls. Healthy population subjects were identified from the Illumina iControl Database. Search criteria for the iControl Database were (1) Caucasian subjects of any age and any gender; (2) genotyping platform= Hap550 or Hap610. These criteria yielded 3180 subjects from studies 64, 65, 66 and 67 (www.illumina.com) carried out in New York, NY and Philadelphia, PA. Two of these studies contained only adults and two studies contained only children; thus, the age distribution of the population controls was bimodal, with one peak at a mean age of 50.2 years and another peak at a mean age of 9.2 years.

Atherosclerosis Risk in Communities (ARIC) study. Cases of early-onset AF were identified prospectively from the ARIC cohort. Cases were subjects aged less than 66 years with AF documented by electrocardiography, or AF documented on hospital discharge or death certificate in the absence of clinical evidence of structural heart disease. Referent subjects from the ARIC study had no history of AF at baseline or follow up, and no history of MI, heart failure or valve disease at baseline.

The Massachusetts General Hospital Atrial Fibrillation Study (MGH) prospectively enrolled subjects with early-onset AF defined as an age of onset less than 66 years and AF documented by electrocardiography. Subjects with structural heart disease, hyperthyroidism, myocardial infarction (MI) or heart failure were excluded from the MGH cohort.
Framingham Heart Study (FHS). Referent subjects for use with the MGH cohort were selected from the Original and Offspring cohorts of the FHS, and included unrelated subjects 18-74 years of age and no history of AF at blood draw or in follow up, and no history of myocardial infarction, heart failure or valve disease at baseline.

Preparation of genomic DNA, total RNA and cDNA from human atrial tissue and blood
Atrial genomic DNA (gDNA) and total RNA were prepared from 61 atrial tissue samples using Qiagen kits. cDNA was produced with the iScript Select cDNA Synthesis Kit (BioRad) using an oligo(dT)20 primer. Buffy coat gDNA was prepared from blood samples of 500 subjects in the LAFGB using the MasterPure DNA Purification Kit for Blood Version II (Epicentre Biotechnologies).

Genotyping of human atrial tissue genomic DNA
Atrial gDNA was genotyped for rs11552588, a perfect proxy for the promoter A SNP6,7 by the TaqMan (Applied Biosystems) method in 61 human atrial tissue samples. Genotype data for the promoter B SNP rs10465885 and 6 additional SNPs, shown in Figure 1a, were obtained from Illumina HumanHap 550 BeadChip data on the same samples.

Genotyping of human blood DNA
Genotypes for the promoter B SNP and another perfect proxy for the promoter A SNP (rs12408178, r^2=1) were obtained from genome wide SNP genotyping arrays. Genotyping platforms for each case-control cohort are outlined in Supplemental Table 2.
Because the Affymetrix 5.0 array used for the FHS subjects did not contain the promoter A or B SNPs, imputed values were used in the analyses of the MGH/FHS cohort. Imputation was performed using the program Mach with the CEU Hapmap Phase 2 panel. The imputed SNPs used in the analyses had good imputation quality scores (for example the promoter B SNP had an observed/expected variance of 1.019), which was expected due to the existence of good proxies on the Affymetrix 5.0 array. No SNPs deviated from Hardy Weinberg equilibrium using a p-value cutoff of 0.005.

**Measurement of Cx40 mRNA levels**

cDNAs from the 61 human atrial tissue samples were analyzed for total Cx40 and transcript A expression on Illumina Human Ref-8 v2 Expression Beads Chips. The expression data was log2 transformed and quantile normalized. Standard quality control and probe filtering were performed. 31 samples with sufficient RNA remaining were subjected to quantitative real-time polymerase chain reaction (qPCR) for measurement of Cx40 transcript A and B levels. PCR primers for specific amplification of Cx40 transcript B were upper primer 5’-GGAGAACACACAGACAGGCAGAG-3’ and lower primer 5’-CCAGCACGAGCATAATCGGAATA-3’. PCR primers for specific amplification of Cx40 transcript A were upper primer 5’-AAAAAGGATGGGAGGTGTTGGAG-3’ and lower primer 5’-CCAGCACGAGCATAATCGGAATA-3’. The VIC-labeled TaqMan probe for both Cx40 transcripts was 5’-AGGAAGCTCCAATCGC-3’. A commercially designed FAM-labeled TaqMan assay was used to measure cardiac muscle alpha-actin (ACTC1) cDNA as an internal control. Multiplex PCR reactions were performed in an iCycler IQ real-time PCR instrument (BioRad) using the following thermal protocol: 95°C for 9
minutes, then 40 cycles of: 95°C for 15 seconds, 60°C for 1 minute, and 72°C for 45 seconds (image capture step). Raw data for Cx40 transcript A or B and ACTC1 reactions were extracted as threshold cycle numbers. ACTC1-adjusted Cx40 transcript A or B threshold cycle values were calculated relative to the median threshold cycle value, and were used to calculate relative Cx40 transcript A and Cx40 transcript B expression levels using the $2^{-\Delta\Delta Ct}$ method. The association of the promoter B SNP (rs10465885) genotype with Cx40 transcript B levels was confirmed through a LOD score analysis using the residual sum of squares (rss) method, with the equation LOD=$(n/2) \times \log_{10}(\text{rss}_0/\text{rss}_{\text{model}})$, where ‘n’ refers to the number of samples.

*Quantitative sequencing for analysis of allelic expression imbalance*

**Sample selection** - Genotypes at the 8 SNPs within the GJA5 gene region (shown in Fig. 1A) were determined in 61 atrial tissue samples, and unphased genotypes at each SNP from the 61 individuals were assembled into 122 phased haplotypes using PHASE v.2.1.1. For each tissue sample used in the allelic expression imbalance assay, the phased haplotypes were analyzed to determine the allelic relationship between the Cx40 promoter SNPs and the indicator SNPs within the GJA5 coding region.

**Cx40 transcript B** - Cx40 transcript B cDNA-specific PCR amplification primers were 5’-AGACAGGCAGAGGATTACAACACA-3’ and 5’-GGGCCTCCATAGCTGTCATCA-3’. gDNA specific PCR amplification primers were 5’-GGGGCAAGAGCAGAATCCATAT-3’ and 5’-GGGCCTCCATAGCTGTCATCA-3’.

**Cx40 transcript A** - Cx40 transcript A cDNA-specific PCR amplification primers were 5’-GGTGGAAGAGGAACAACTGA-3’ and 5’-CATACGGAATATGAAGAGGACA-
3’. gDNA-specific PCR amplification primers were 5’-
GGTGGAAGAGGAACAACTGA-3’ and 5’-GAAATAGCGGGAGGGGTAAG-3’.
Both gDNA and cDNA for each transcript were PCR amplified for 40 cycles and PCR
products were purified using the QIAquick PCR Purification Kit according to the
manufacturer’s instructions (Qiagen). The purified products were sequenced using
internal primers (5’-TGGCAGTCAGCAAAGGAAGTAAAT-3’ for Cx40 transcript B
products, 5’ - GAAGCAGCCAGAGTGTGAAGA-3’ for Cx40 transcript A products) on
an Applied Biosystems 3730xl DNA analyzer. Raw .ABI sequence files were analyzed
using PeakPicker2, a program that calculates allelic expression ratios based on peak
heights at the indicator SNP site in both cDNA and gDNA PCR products. PeakPicker2
was then used to normalize each allelic cDNA expression ratio to the average of the
allelic gDNA ratios, assuming a gDNA allele ratio of 1:1. This resulted in a normalized
allelic mRNA expression ratio for each sample.

Luciferase reporter gene assay

A promoter fragment ranging from -765bp to +94bp relative to the first base pair of exon
1B was PCR amplified from genomic DNA of patients homozygous for either the A or G
allele at the Cx40 transcript B promoter SNP, using the upper primer 5’-
GGAAGCTTCTGACCCCATTTCCCCATAA-3’ and lower primer 5’-
GCTCGAGTTGCTGCTCTTCTTCTTCCCCATCAA-3’. HindIII and XhoI sites are
underlined in the upper and lower primers, respectively. Using the HindIII and XhoI
restriction enzymes, the A and G allele-containing promoter fragments were then cloned
into a promoter-less pXPII vector directly upstream of the firefly luciferase gene. The
sequence of both the A and G allele-containing vectors was verified by direct sequencing to ensure that the Cx40-B promoter SNP site was the only site of sequence discordance between the two vectors. HL-1 murine atrial cardiomyocytes were cultured at 37°C and 5% CO₂ in Claycomb medium (Sigma) supplemented with 10% fetal bovine serum, 0.1 mM norepinephrine, 2 mM L-glutamine and 100 U/mL each penicillin and streptomycin. HL-1 cells were cultured in antibiotic-free medium in 6-well plates for 24 hours prior to transfection with 5 µL Lipofectamine LTX (Invitrogen). Cells were transiently co-transfected with 1µg of either the A or G allele-containing promoter-luciferase plasmid and 1µg of the β-galactosidase-containing plasmid pCH110. Additional cells were co-transfected with the empty pXPII vector and pCH110 as a background control. The promoter driven luciferase constructs were transfected in sextuplicate (one well of the A allele got contaminated and was excluded from the analysis). 48 hours later, cell lysates were assayed in duplicate for luciferase and β-galactosidase activity with the Dual-Light reporter gene assay system (Tropix), and the mean background-subtracted (wells with no transfection) luciferase/β-galactosidase levels were calculated for each well.

**Statistical Analyses**

We used both one-way ANOVA and linear regression with the additive genotype model to correlate SNP genotypes with Cx40 expression values. The linear regression model included covariates to correct for age, atrial rhythm at the time of surgery, and expression assay batch effects. The promoter A and B SNPs were tested for association with early onset lone AF (including only subjects with age at AF diagnosis ≤60 years) and lone AF (including subjects of all ages) in the case-control cohorts using logistic regression in a
log-additive genetic model. Cohorts from each center were analyzed individually and in a meta-analysis. For the analysis of the CCAF cohort, sex was included as a covariate and the EIGENSTRAT method of population stratification adjustment was used to help account for the different origins of the CCAF case and control sets. The meta-analyses used a fixed-effects model with invariance-variance weights of the genotype regression effects (log odds ratios). Power calculations used a minimum power of 0.80 and an alpha of 0.05. Statistical analyses were carried out using JMP 7, GraphPad PRISM, and R version 2.11 software. Meta-analyses were performed using the ‘metagen’ function of the R package Meta, version 1.6-0. Power calculations were performed using the htPower.cc function of the DGCgenetics software package, version 1.2.
References


**Supplemental Table 1. Characteristics of the 61 Atrial Tissue Donors**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
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<tr>
<td>Mean Age (SD)</td>
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<td><strong>Sex</strong></td>
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<tr>
<td>Male</td>
<td>37 (60.7)</td>
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<td>Female</td>
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<td>Persistent</td>
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<td>Mixed AF</td>
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<tr>
<td>30 (49.2)</td>
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<td>Hypertension</td>
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<td>Statin</td>
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SD, standard deviation; MI, myocardial infarction; CHF, congestive heart failure; AA, Antiarrhythmic
Supplemental Table 2. Characteristics of Lone AF Case-Control Study Cohorts

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<tr>
<th>Study</th>
<th>Genotyping Platform</th>
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<th>ARIC</th>
<th>Massachusetts General Hospital &amp; Framingham Heart Study</th>
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<td>Cases</td>
<td>Controls</td>
<td>Cases</td>
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<td>iControlDB Study 64/65</td>
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<td>iControlDB Study 66/67</td>
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<tr>
<td>Number</td>
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<td>1423</td>
<td>1587</td>
<td>119</td>
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<tr>
<td>Age at enrollment, mean ± SD</td>
<td>59.1 ± 10.7</td>
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<td>9.2 ± 5.5†</td>
<td>52.9 ± 5.4</td>
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<td>Sex, Male, n (%)</td>
<td>450 (75.5)</td>
<td>314 (22.1)</td>
<td>821 (51.7)</td>
<td>64 (53.8)</td>
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<td>1423</td>
<td>1587</td>
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<tr>
<td>Age at AF diagnosis (cases) or enrollment (controls), mean ± SD</td>
<td>47.3 ± 9.9</td>
<td>50.2 ± 11.1</td>
<td>9.2 ± 5.5†</td>
<td>56.7 ± 2.7</td>
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<td>Sex, Male, n (%)</td>
<td>306 (79.7)</td>
<td>314 (22.1)</td>
<td>821 (51.7)</td>
<td>26 (57.8)</td>
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*Platform included the 50K Human Gene Focused Panel
† Two studies from the Illumina database contained pediatric patients
### Supplemental Table 3. Association of promoter A and B SNPs with lone AF, all ages, additive genetic model

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<thead>
<tr>
<th>Study</th>
<th>AF (n)</th>
<th>No AF (n)</th>
<th>Promoter A SNP MAF (%)</th>
<th>OR (95%CI)</th>
<th>P value</th>
<th>Promoter B SNP MAF (%)</th>
<th>OR (95%CI)</th>
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<td>CCF</td>
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<td>3010</td>
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<td>MGH</td>
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<td>1101</td>
<td>22.4/22.1</td>
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<td>7395</td>
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<td>49.1/47.5</td>
<td>1.06 (0.95-1.17)</td>
<td>0.283</td>
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</table>
Supplemental Figure 1. The promoter A genotype is not associated with Cx40 transcript A expression (P=0.66). Relative levels of Cx40 transcript A were quantified by real-time PCR in 31 atrial tissue samples.