Novel connexin40 missense mutations in patients with familial atrial fibrillation

Yi-Qing Yang1†, Xu Liu2†, Xian-Ling Zhang2, Xin-Hua Wang2, Hong-Wei Tan2, Hai-Feng Shi2, Wei-Feng Jiang2, and Wei-Yi Fang2

1Department of Cardiovascular Research, Shanghai Chest Hospital Affiliated to Shanghai Jiaotong University, 241 West Huaihai Road, Shanghai 200030, China; and 2Department of Cardiology, Shanghai Chest Hospital Affiliated to Shanghai Jiaotong University, 241 West Huaihai Road, Shanghai 200030, China

Aims
This research was aimed at screening connexin40, a cardiac gap junction protein alpha 5, for genetic defects in patients with familial atrial fibrillation (AF).

Methods
The subjects included 218 unrelated families with lone AF and 200 ethnically matched unrelated healthy individuals as controls. The entire coding region of the connexin40 gene was sequenced initially in 218 unrelated probands with familial AF. The relatives of mutation carriers and 200 controls were subsequently genotyped for the presence of mutations identified in probands.

Results
Three novel connexin40 mutations, p.V85I, p.L221I, and p.L229M, were identified in 3 of 218 unrelated AF families, respectively. These heterozygous missense mutations co-segregated with AF in the families and were absent in the 200 unrelated control subjects. A cross-species alignment of connexin40 protein sequences revealed that the altered amino acids were completely conserved evolutionarily.

Conclusion
The findings expand the spectrum of mutations in connexin40 linked to AF and provide new insight into the molecular aetiology involved in the pathogenesis of AF.

Keywords
Atrial fibrillation • Gap junction channel • Genetics

Introduction
Atrial fibrillation (AF) is the most common type of cardiac arrhythmia in clinical practice. The occurrence of AF increases with age, with a prevalence rising from ~0.5% of individuals in their 50s to nearly 10% of the octogenarian population. During their lifetime for men and women over 40 years of age, there is about 25% risk of the development of AF. The anomalous rhythm is not merely associated with a variety of symptoms, such as palpitations, dizziness or shortness of breath, but also is responsible for increased mortality and substantial morbidity. Compared with individuals in sinus rhythm, patients with AF have a six-fold increase in the risk of stroke and a two-fold increase in the risk of death. Although the structural heart diseases or systemic disorders, such as coronary artery disease, rheumatic heart disease, cardiomyopathy, congenital heart defects, pericarditis, congestive heart failure, hypertension, hyperthyroidism, and electrolyte imbalance, predispose atria to fibrillate, AF also occurs in subjects without any known risk factors and increasing evidence suggests a genetic basis for the pathogenesis of AF. Furthermore, the chromosomal loci linked to AF have been mapped and AF-related mutations in several genes, including connexin40 encoding cardiac gap junction membrane channel protein alpha 5, have been identified. Nevertheless, AF is a genetically heterogeneous disorder and the molecular basis for AF in a majority of patients remains elusive.

Gap junctions are intercellular channels accountable for the exchange of ions and small molecules between adjacent cells. These channels mediate the electrical coupling of mammalian cardiomyocytes, which is essential for the fast and coordinated propagation of cardiac action potentials. The functional gap junction channel is composed of two hemichannels, also known as connexons, one provided by each cell. Connexons are hexamers of

[Received 18 May 2010; accepted after revision 25 June 2010; online publish-ahead-of-print 21 July 2010]
membrane-spanning proteins called connexins. Up to date, more than 20 connexin genes have been identified in mouse and human.\textsuperscript{28} In the human heart, myocyte gap junctions are constructed mainly by three different connexin isoforms, connexin40, connexin43, and connexin45. Connexin40 is expressed selectively in the atrial myocytes, atrioventricular node, His-bundle and ventricular conduction system (Purkinje fibres), predominantly contributing to the electrical synchronization of the atrium and the rapid conduction of impulses in the His-Purkinje.\textsuperscript{29} In connexin40-deficient mice, spontaneous or inducible dysrhythmias as well as conduction abnormalities were observed.\textsuperscript{30} In the goat, alterations of connexin40 may constitute a cellular substrate underlying susceptibility and perpetuation of AF.\textsuperscript{31} In human, cardiac connexin40 remodelling may lead to abnormal electrical coupling, forming an electrophysiological matrix with potential arrhythmogenic effect.\textsuperscript{32} By reducing connexin40 protein levels, two closely linked polymorphisms in the promoter region of the connexin40 gene are strongly associated with enhanced atrial vulnerability and increased risk for idiopathic AF.\textsuperscript{33–35} Additionally, the loss-of-function mutations of connexin40 are identified in patients with sporadic AF.\textsuperscript{32} These findings provide a rationale to scan connexin40 as a logical candidate gene for familial AF.

In this study, we analysed the connexin40 gene in 218 unrelated index cases with familial AF and identified three heterogeneous missense mutations, p.V85I, p.L221I, and p.L229M, in 3 of 218 unrelated probands and subsequently in their family members. These novel mutations co-segregated with AF in the families with complete penetrance and were absent in the 400 control chromosomes. Multiple alignment of connexin40 protein sequences across-species displayed that the altered amino acids were completely conserved evolutionarily. To our knowledge, this is the first description of the relationship between missense mutations in connexin40 and susceptibility to familial AF. The findings expand the spectrum of mutations in connexin40 linked to AF and provide new insight into the molecular mechanism involved in AF.

Methods

Ethics

This study was conducted in compliance with the ethical principles of the revised Declaration of Helsinki (Somerset West, Republic of South Africa, 1996). The research protocol was reviewed and approved by the local institutional ethics committee and written informed consent was obtained from all participants before investigation.

Study subjects

A total of 218 unrelated kindreds with familial AF were identified among Chinese population. The controls were 200 ethnically matched unrelated healthy individuals. Peripheral venous blood specimens were prepared and clinical data including medical records, electrocardiogram (ECG) and echocardiography reports were collected. The study subjects were clinically classified using a consistently applied set of definitions.\textsuperscript{37,38} Briefly, diagnosis of AF was made by a standard 12-lead ECG demonstrating no P waves and irregular R–R intervals regardless of clinical symptoms. Lone AF was defined as AF occurring in individuals <60 years of age without other cardiac or systemic diseases by physical examination, ECG, transthoracic echocardiogram, and extensive laboratory tests. Familial AF was defined as the presence of lone AF in one or more first-degree relatives of the proband. Relatives with AF occurring at any age in the setting of structural heart disease (hypertensive, ischaemic, myocardial, or valvular) were classified as ‘undetermined’ for having an inherited form of AF. The ‘undetermined’ classification was also used if documentation of AF on an ECG tracing was lacking in relatives with symptoms consistent with AF (palpitations, dyspnoea, and light-headedness), or if a screening ECG and echocardiogram were not performed, regardless of the symptoms. Relatives were classified as ‘unaffected’ if they were ≥18 years of age, asymptomatic and had a normal ECG. In addition, paroxysmal AF was defined as AF lasting more than 30 s that terminated spontaneously. Persistent AF was defined as AF lasting more than 7 days and requiring either pharmacologic therapy or electrical cardioversion for termination. Atrial fibrillation that was refractory to cardioversion or that was allowed to continue was classified as permanent.\textsuperscript{27}

Genetic studies

Genomic DNA from all participants was extracted from blood lymphocytes with Wizard Genomic DNA Purification Kit (Promega). The candidate gene connexin40 was screened in 218 unrelated probands with familial AF and genotyping connexin40 in the relatives of mutation carriers and 200 ethnically matched unrelated healthy control individuals was conducted subsequently for the presence of mutations identified in probands. The referential genomic DNA sequence of connexin40 was derived from GenBank (accession No. NG_009369). By the aid of on-line Primer 3 software (http://frodo.wi.mit.edu), the primer pairs used to amplify the complete coding region of connexin40 by polymerase chain reaction (PCR) were designed as follows: the forward primer 1 was 5′-GCA, TC T, GTT, CCC, TGG, CTG, TGC-3′ and the backward primer 1 was 5′-CCG, ACC, TCT, TTG, GCC, CTC, TCG-3′ (the PCR product was 404 base pairs in size); the forward primer 2 was 5′-CGG, CAC, AAG, CAG, TGG, TGT, ACA, TGG-3′ and the backward primer 2 was 5′-CCA, GAA, AGC, TGG, CAC, TTA, GCC-3′ (the product was 496 base pairs); the forward primer 3 was 5′-CCT, GGG, CTG, CTA, GAA, GAT, CAG-3′ and the backward primer 3 was 5′-CCG, GAA, ACG, CAG, TGA, CAG, TG-3′ (the product was 500 base pairs). Polymerase chain reaction was carried out using HotStar Taq DNA Polymerase (Qiagen) on a PE 9700 Thermal Cycler (Applied Biosystems). Amplified products were purified with QIAquick Gel Extraction Kit (Qiagen). Both strands of each PCR product were sequenced with a BigDye\textsuperscript{36} Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) under an ABI PRISM 3130 XL DNA Analyzer (Applied Biosystems). DNA sequences were viewed and analysed with DNA Sequencing Analysis Software v5.1 (Applied Biosystems). The variant was validated by re-sequencing an independent PCR-generated amplicon from the subject (work done by the Shensu DNA Sequencing Facility) and met our quality control thresholds with a call rate >99%.

Multiple sequence alignments

The multiple connexin40 protein sequences across various species were aligned using the program of MUSCLE (version 3.6).

Statistical analysis

Data are expressed as means ± SD. Continuous variables were tested for normality of distribution and student’s unpaired t-test was used for comparison of numeric variables between patient and control groups. Comparison of the categorical variables between the two groups was
performed using Pearson’s $\chi^2$ test or Fisher’s exact test when appropriate. A two-sided $P$-value of $<0.05$ was considered to indicate statistical significance.

Results

Characteristics of the study subjects

A total of 218 unrelated kindreds with familial AF were recruited and clinically evaluated in contrast to a cohort of 200 ethnically matched unrelated healthy individuals as controls. None of them had traditional risk factors for AF. There were no significant differences between proband and control groups in baseline characteristics including age, gender, body mass index, blood pressure, fasting blood glucose, serum lipid, left atrial dimension, left ventricular ejection fraction, heart rate at rest, as well as lifestyle (data not shown). At the present study visit, nine probands were also diagnosed with hypertension in accordance to the criterion that the average systolic or diastolic blood pressure (two readings made after 5 min of rest in the sitting position) was $\geq 140$ or $\geq 90$ mmHg, respectively, but at the time of original diagnosis of AF, their blood pressures were normal. The clinical characteristics of the 218 probands with familial AF are shown in Table 1.

Connexin40 mutations

The probands in 218 kindreds with familial AF were genetically evaluated. Direct sequencing of the coding region of the gene encoding the atrial-specific gap junction protein connexin40 was performed after PCR amplification of genomic DNA from the 218 index patients. Three novel heterozygous missense mutations in connexin40 were identified in 3 of 218 unrelated probands. The total population prevalence of connexin40 mutations based on probands was roughly 1.38%. A substitution of A for G in the first nucleotide of codon 85 (c.253G $\rightarrow$ A), predicting the transition of valine (V) into isoleucine (I) at amino acid 85 (p.V85I) was detected in the proband from family 1. The mutations c.661C $\rightarrow$ A and c.685C $\rightarrow$ A, corresponding to the mutations L221I and L229M, were found in two other probands from family 2 and family 3.

Table 1 Clinical characteristics of the 218 probands with familial atrial fibrillation

<table>
<thead>
<tr>
<th></th>
<th>Number or quantity</th>
<th>Percentage or range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male:female</td>
<td>106:112</td>
<td>49:51</td>
</tr>
<tr>
<td>Age of onset</td>
<td>44.8</td>
<td>7–59</td>
</tr>
<tr>
<td>Paroxysmal AF on presentation</td>
<td>165</td>
<td>76</td>
</tr>
<tr>
<td>Progression to permanent AF</td>
<td>35</td>
<td>21</td>
</tr>
<tr>
<td>History of cardioversion</td>
<td>31</td>
<td>14</td>
</tr>
<tr>
<td>History of pacemaker</td>
<td>32</td>
<td>15</td>
</tr>
<tr>
<td>Resting heart rate (bpm)</td>
<td>73.2</td>
<td>48–156</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>126.8</td>
<td>92–184</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>80.3</td>
<td>60–110</td>
</tr>
<tr>
<td>body mass index (kg/m$^2$)</td>
<td>22.0</td>
<td>20–24</td>
</tr>
<tr>
<td>Left atrial dimension (mm)</td>
<td>35</td>
<td>27–40</td>
</tr>
<tr>
<td>left ventricular ejection fraction</td>
<td>0.58</td>
<td>0.51–0.68</td>
</tr>
<tr>
<td>Fasting blood glucose (mmol/L)</td>
<td>4.5</td>
<td>3.8–5.8</td>
</tr>
<tr>
<td>Total cholesterol (mmol/L)</td>
<td>3.6</td>
<td>3.0–5.4</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>1.2</td>
<td>0.5–1.6</td>
</tr>
</tbody>
</table>

Medications

<table>
<thead>
<tr>
<th>Medication</th>
<th>Number or quantity</th>
<th>Percentage or range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspirin</td>
<td>74</td>
<td>34</td>
</tr>
<tr>
<td>Warfarin</td>
<td>65</td>
<td>30</td>
</tr>
<tr>
<td>Beta-blocker</td>
<td>62</td>
<td>28</td>
</tr>
<tr>
<td>Calcium channel blocker</td>
<td>30</td>
<td>14</td>
</tr>
<tr>
<td>Digoxin</td>
<td>71</td>
<td>33</td>
</tr>
</tbody>
</table>

Figure 1 Sequence electropherograms of connexin40 in controls and probands. The arrow indicates the heterozygous nucleotides of G/A (A), C/A (B), and C/A (C) in the probands from families 1, 2, and 3, respectively (mutant), or the homozygous nucleotides of G (A), C (B), and C (C) in the corresponding controls (wild-type). The square denotes the nucleotides comprising a codon of connexin40.
families revealed that the gene variant was present in all affected living family members, but absent in unaffected family members tested in the three kindreds. Analysis of the pedigrees demonstrated that the mutation co-segregated with AF transmitted as an autosomal dominant trait in the three families with a complete penetrance. The pedigree structures of the three families were illustrated in Figure 2. The phenotypic characteristics and results of genetic screening of the affected family members were summarized in Table 2. Specifically, the father of the proband in family 1 and the mother of the proband in family 3 had died of stroke and heart failure, respectively, before this study and had a history of permanent AF with electrocardiographic evidence of AF. The proband in family 1 and the younger brother (II-3) in family 2 had persistent AF and had successful conversion to sinus rhythm with antiarrhythmic agent of amiodarone. They were also evaluated with transoesophageal echocardiography before cardioversion. A 12-lead ECG was recorded 48 h after cardioversion. A 12-lead ECG was recorded 48 h after cardioversion. Individuals III-2 in family 1, II-2 in family 2, and II-2 and II-4 in family 3 had apparent recurrent palpitation (Table 2 and Figure 2).

**Multiple alignment of the connexin40 protein sequences across-species**

A cross-species alignment of connexin40 protein sequences showed that the altered amino acids were completely conserved evolutionarily as shown in Figure 3, suggesting that these amino acids are functionally important.

**Discussion**

In the present study, we report three novel heterozygous missense mutations of connexin40 identified in three AF families. In each family, the mutation is present in all the affected family members alive, but absent in unaffected relatives tested and 200 unrelated control individuals. A cross-species alignment of connexin40 protein sequences shows that the altered amino acids are completely conserved evolutionarily. All the detected substitutions in connexin40 are predicted (by search UniprotKB with a query of P36382 at http://www.uniprot.org/) to occur within or near the transmembrane-spanning regions. Rather, V85 and L221 are located within the second and the fourth transmembrane domains, respectively, and L229 are within cytoplasmic regions near the fourth transmembrane domain. Additionally, all the three gene alterations are automatically predicted (by Mutation Taster at http://www.mutationtaster.org/) to be disease-causing with P-values of 0.9928, 0.9970, and 0.9963 for V85I, L221I, and L229M, respectively. Here the P value is the probability of the prediction, i.e. a value close to 1 indicates a high security of the prediction. Hence, it is likely that the three mutations perturb cardiac connexin40 channel and hereby cause or confer susceptibility to AF in these families.

Our results are supported by the findings of other connexin40 mutations or polymorphisms predisposing to idiopathic AF by impairing or reducing gap junction assembly. Similar to our findings, Gollob et al. identified four novel heterozygous missense mutations of connexin40 in 4 of the 15 patients with lone AF, of which three mutations (p.G38D, p.P88S, and p.M163V) were found in the cardiac tissue specimens but not in the peripheral lymphocytes, one mutation (p.A96S) was detected in both cardiac tissue and lymphocytes. The p.A96S variant was absent in the patient’s three siblings and wife but was present in his two sons without history of AF and in 1 of 120 controls. Functional analysis of mutant connexin40 proteins revealed impaired intracellular transport or reduced intercellular electrical coupling. By sequencing of the 5’ untranslated exon and the proximal promoter region of the connexin40 gene (GenBank accession No. AF246295) in patients with familial atrial standstill, Groenewegen et al. found two closely linked polymorphisms. Luciferase reporter gene assays of the minor connexin40 haplotype (−44A, +71G) showed a more than two-fold decrease in promoter activity compared with the more common haplotype (−44G, +71A), providing an atrial electrophysiological substrate favouring arrhythmia susceptibility. Furthermore, the connexin40 polymorphisms

![Figure 2](https://academic.oup.com/europace/article-abstract/12/10/1421/419381/1424)
were strongly associated with increased spatial dispersion of refractoriness as a marker for enhanced atrial vulnerability and carriers of −44AA genotype had a significantly higher risk of AF compared with those carrying −44GG genotype.34,35

Association of reduced connexin40 with increased predisposition to arrhythmias has been reported in animal models. Targeted gene deletion of connexin40 in mice produced multiple abnormalities including increased sinoatrial node recovery time, decreased conduction velocity of atria, atrioventricular node and bundle branch, and impaired sinoatrial propagation with atrial ectopic pacemakers, which developed arrhythmogenic substrate prone to AF.38,39 In a canine sterile pericarditis model, the gap junction conduction-enhancing antiarrhythmic peptide, Gap-134, improved conduction and reduced AF.40 Similarly, in a dog model of AF due to myocardial ischaemia, administration of ZP123, a gap junction conductance-improving modifier, prevented ischaemia-induced conduction slowing and reduced AF duration.41

It is well known that AF is a complex arrhythmia involved in multiple possible mechanisms. Despite the presence of an inherited defect, a favourable substrate for AF, within the myocardial tissue of affected patients from birth, the onset of genetically based AF often requires a trigger for initiation, presumably by exacerbating the already anomalous cardiac cellular electrophysiology in the existence of mutant protein. One of the most common triggers is the enhanced vagal tone mediated by muscarinic receptors, causing uneven shortening of refractoriness in the atria and, hence, electrophysiological heterogeneity.42 The stimulation of muscarinic receptors has been shown to impair the cell–cell coupling mediated by gap junctions.43 Together with the data mentioned above, this experimental result suggests a potential pathogenic link between increased cardiac parasympathetic nerve activity, impaired myocardial intercellular electrical coupling, and the occurrence of AF.

Table 2 Phenotypic characteristics and status of connexin40 mutations of the affected pedigree members

<table>
<thead>
<tr>
<th>Subject information</th>
<th>Phenotype</th>
<th>Electrocardiogram</th>
<th>Echocardiogram</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AF (Classification)</td>
<td>P wave (ms)</td>
<td>QRS interval (ms)</td>
<td>LAD (mm)</td>
</tr>
<tr>
<td>Identity</td>
<td>Gender</td>
<td>Age at time of study (years)</td>
<td>Age at diagnosis of AF (years)</td>
<td>Permanent</td>
</tr>
<tr>
<td>I-1</td>
<td>M</td>
<td>65a</td>
<td>45</td>
<td>Permanent</td>
</tr>
<tr>
<td>II-1</td>
<td>M</td>
<td>69</td>
<td>39</td>
<td>Permanent</td>
</tr>
<tr>
<td>II-6</td>
<td>F</td>
<td>63</td>
<td>58</td>
<td>Persistent</td>
</tr>
<tr>
<td>III-2</td>
<td>F</td>
<td>42</td>
<td>37</td>
<td>Paroxysmal</td>
</tr>
<tr>
<td>Family 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-2</td>
<td>F</td>
<td>76</td>
<td>35</td>
<td>Permanent</td>
</tr>
<tr>
<td>II-2</td>
<td>F</td>
<td>52</td>
<td>46</td>
<td>Paroxysmal</td>
</tr>
<tr>
<td>II-3</td>
<td>M</td>
<td>48</td>
<td>41</td>
<td>Persistent</td>
</tr>
<tr>
<td>Family 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-2</td>
<td>F</td>
<td>68a</td>
<td>41</td>
<td>Permanent</td>
</tr>
<tr>
<td>II-2</td>
<td>F</td>
<td>56</td>
<td>48</td>
<td>Paroxysmal</td>
</tr>
<tr>
<td>II-4</td>
<td>M</td>
<td>51</td>
<td>43</td>
<td>Paroxysmal</td>
</tr>
<tr>
<td>Family 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I-2</td>
<td>F</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II-2</td>
<td>F</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II-4</td>
<td>M</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

AF, atrial fibrillation; F, female; M, male; N/A, not available or not applicable; LAD, left atrial dimension; LVEF, left ventricular ejection fraction; + indicates present and − denotes absent.
aAge at death.

Figure 3 Multiple alignment of the connexin40 protein sequences across-species. The altered amino acids of p.V85, p.L221, and p.L229 are completely conserved evolutionarily.
system and abnormal expression of connexin40 is responsible for AF. However, functional changes in connexin40 alone may not be sufficient for significantly prolonged P-wave duration, PQ interval, QRS duration, and QTc duration in the surface ECG, as was observed in these AF families and other AF patients. In addition, in connexin40 knockout mice, full deficiency for connexin40 was associated with altered ECG parameters, but in contrast, haploinsufficiency for connexin40 was not.

Theses findings imply that other factors combined with reduced coupling result in AF.

Functionally compromised connexin40 as a genetic determinant of AF is interesting, especially in optimizing AF therapy. It is well known that existing medication with class I or III antiarrhythmic agents, such as dofetilide and sotalol, could lead to a negative feedback impact on ventricular repolarization, which is generally considered a critical risk factor for fatal arrhythmias. These life-threatening adverse effects, attributable to the unselective blockade of the potassium currents in both atrial and ventricular cardiomyocytes, hindered the appropriate application of these antiarrhythmic drugs to patients in clinical practice. Given these limitations, new agents targeting the atrium-selective current may well be an appealing alternative for AF therapy. Functioning restrictedly in the atria, connexin40 is a crucial determinant for electrical coupling between atrial myocytes, which implicates connexin40 as a potential selective target for the medical management of AF.

In conclusion, the present investigation links missense mutations in connexin40 to familial AF and provides novel insight into the molecular mechanisms involved in the pathogenesis of AF, which signifies potential implications for genetic diagnosis and atrium-selective strategy for AF.

Acknowledgements

We are indebted to the participants for their dedication to the study.

Conflicts of interest: none declared.

Funding

This work was supported in part by grants from the Natural Science Fund of Shanghai, China (10ZR1428000); the National Natural Science Fund of China (30570768 and 30871083); and the National Basic Research Program of China (2010CB912604).

References