Prevalence and spectrum of GJA5 mutations associated with lone atrial fibrillation

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Abstract. Atrial fibrillation (AF) is the most common form of cardiac arrhythmia observed in clinical practice and a major contributor to cardiovascular morbidity and mortality. Accumulating evidence indicates a substantial genetic basis for AF. However, AF is genetically heterogeneous and the hereditary components responsible for AF remain to be identified in the majority of patients. The cardiac gap junction protein α 5 (GJA5) is specifically expressed in atrial myocytes and is associated with the coordinated electrical activation of the atria, providing a rationale to screen *GJA5* as a logical candidate gene for AF. A cohort of 310 unrelated patients with lone AF and their available relatives were included in this study. A group of 200 unrelated healthy individuals matched for age, gender and race were also included as controls. The entire coding region and splice sites of the *GJA5* gene were initially sequenced in 310 unrelated AF patients. The relatives of mutation carriers and 200 controls were subsequently genotyped for the presence of identified mutations. As a result, 4 novel heterozygous GJA5 mutations, p.K107R, p.L223M, p.Q236H and p.I257L, were identified in 4 of 310 unrelated AF patients, respectively, with a prevalence of ~1.29%. Genetic analysis of the carriers' families showed that in each family the missense mutation was present in all the affected family members. Absent in the 400 reference alleles, these mutations altered the amino acids highly conserved among various species, with the exception of p.I257L. In conclusion, this study expands the spectrum of GJA5 mutations associated with AF and provides novel insights into the molecular basis of AF, suggesting potential implications for the improved, gene-specific rhythm control strategies.

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Introduction

Atrial fibrillation (AF) is the most common type of cardiac arrhythmia encountered in clinical practice, responsible for ~1/3 of hospitalizations for cardiac arrhythmias. This condition shows a marked increase in prevalence with advancing age, ranging from ~0.4% of the whole population to ~10% of the octogenarian population (1,2). According to the Framingham Heart Study (3), during the lifetime of subjects >40 years of age, there is a ~25% risk for the development of AF. The chaotic heart rhythm is not merely associated with a variety of symptoms, such as palpitations, dizziness, syncope or shortness of breath, but is also accountable for significantly increased morbidity and mortality (1). In comparison with individuals in sinus rhythm, patients with AF have a 6-fold increase in the risk of stroke, and >15% of all strokes are ascribed to AF (4). Notably, the risk of AF-related thromboembolism also significantly increases with age, rising from 1.5% at the age of 50-59 years to 23.5% at the age of 80-89 years (4). The incidence of death is estimated to have doubled among patients with AF compared with individuals with normal heart rhythm (5). AF also contributes to degraded quality of life, compromised exercise performance, impaired cognitive function or dementia, tachycardia-induced cardiomyopathy, and left ventricular dysfunction or even congestive heart failure, inflicting a large economic burden on the National Healthcare Systems worldwide (6). Despite the significant prevalence and therapeutic challenge, the molecular mechanisms involved in the pathogenesis of AF remain poorly understood.

Traditionally, AF has been considered as a complication derived from miscellaneous adverse cardiac or systemic conditions, including hypertension, coronary artery disease, rheumatic heart disease, valvular heart disease, pulmonary heart disease, cardiomyopathy, cardiac surgery, pericarditis, congestive heart failure, type 2 diabetes mellitus, obstructive sleep apnea, hyperthyroidism and electrolyte imbalance (1,6-10). However, in 30-45% of AF patients, no underlying causes are identified by routine procedures, where AF is termed

'idiopathic' or 'lone' (1), and ≥15% of AF patients have a positive family history, a condition defined as familial AF (11). Mounting evidence has substantiated the familial aggregation of AF and enhanced susceptibility to AF in the close relatives of patients with AF, suggesting an important genetic basis for AF (12-18). Genome-wide linkage analyses with polymorphic microsatellite markers mapped susceptibility loci for AF on human chromosomes 10q22, 6q14-16, 11p15.5, 5p13 and 5p15, of which AF-causing mutations in 2 genes, including *KCNQ1* on chromosome 11p15.5 and *NUP155* on chromosome 5p13, were identified and functionally characterized (19-24). Genetic scan of candidate genes unveiled a long list of AF-associated genes, including *KCNE2*, *KCNE3*, *KCNE5*, *KCNH2*, *KCNJ2*, *KCNJ8*, *KCNA5*, *SCN5A*, *NPPA*, *GATA4*, *GATA5* and *GATA6* (25-41). Nevertheless, AF is a genetically heterogeneous disorder and the genetic determinants for AF in the majority of patients remain to be identified (11).

A previous study has underscored the essential roles of gap junction channels in heart electrophysiology, particularly in cardiac action potential propagation (42). Gap junctions are intercellular channels responsible for the exchange of ions and small molecules between adjacent cells. The functional gap junction channel is composed of two hemichannels, known as connexons, one provided by each cell. Connexons are hexamers of membrane-spanning proteins called connexins. At present, >20 connexin genes have been identified in mouse and human (43). In the human heart, myocardial gap junctions are constructed mainly by the connexin isoforms 40, 43 and 45. Connexin40, also designated gap junction protein α 5 (GJA5), is selectively expressed in the atrial myocytes, atrioventricular node, His-bundle and ventricular conduction system (Purkinje fibers), and is crucial in the electrical synchronization of the atrium and the rapid conduction of impulses in the His-Purkinje (44). In GJA5-deficient mice, spontaneous or inducible arrhythmias as well as conduction abnormalities have been observed (45). In the goat, alterations in expression levels and the distribution pattern of atrial GJA5 may constitute a cell substrate underlying susceptibility and perpetuation of AF (46). In human, cardiac GJA5 remodeling may lead to abnormal electrical coupling, forming an electrophysiological matrix with potential arrhythmogenic effect (47). By reducing GJA5 protein levels, several closely linked polymorphisms in the promoter region of the *GJA5* gene have been strongly associated with enhanced atrial vulnerability and increased risk for lone AF (48-52). Furthermore, multiple somatic and germline mutations in GJA5 have been reported to underlie AF (53-55). These findings provide a rationale to scan *GJA5* as a logical candidate gene for AF.

In this study, sequence analysis of the *GJA5* gene was performed in a cohort of 310 unrelated patients with lone AF in contrast to a total of 200 ethnically matched, unrelated healthy individuals, in order to evaluate the prevalence and spectrum of *GJA5* mutations associated with lone AF.

Materials and methods

Study subjects. A cohort of 310 unrelated patients with lone AF were included in this study from the Chinese Han population. The available relatives of the probands were also included. A total of 200 unrelated healthy individuals matched for age, gender and race were included as controls. Peripheral venous blood specimens were prepared and clinical data including medical records, electrocardiogram and echocardiography reports were collected. The study subjects were clinically classified using a consistently applied set of definitions (11). Briefly, diagnosis of AF was performed by a standard 12-lead electrocardiogram demonstrating no P-waves and irregular R-R intervals irrespective 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, electrocardiogram, transthoracic echocardiogram and extensive laboratory tests. Relatives with AF occurring at any age in the setting of structural heart disease (hypertensive, ischemic, myocardial or valvular) were classified as 'undetermined' for having an inherited form of AF. The 'undetermined' classification was also used when documentation of AF on an electrocardiogram tracing was absent in relatives with symptoms consistent with AF (palpitations, dyspnea and light-headedness), or when a screening electrocardiogram and echocardiogram were not performed, regardless of the symptoms. Relatives were classified as 'unaffected' when they were asymptomatic and had a normal electrocardiogram. In addition, paroxysmal AF was defined as AF lasting >30 sec that terminated spontaneously. Persistent AF was defined as AF lasting >7 days and requiring either pharmacologic therapy or electrical cardioversion for termination. AF that was refractory to cardioversion or that was allowed to continue was classified as permanent (1). The study protocol was reviewed and approved by the local Institutional Ethics Committee and written informed consent was obtained from all the participants prior to investigation.

Genetic studies. Genomic DNA from the participants was extracted from blood lymphocytes with the Wizard® Genomic DNA Purification kit (Promega, Madison, WI, USA). The candidate gene *GJA5* was screened in 310 unrelated patients with lone AF and genotyping of *GJA5* in the relatives of mutation carriers and 200 unrelated control individuals was subsequently performed for the presence of mutations identified in index patients. The referential genomic DNA sequence of *GJA5* was derived from GenBank (accession number: NG_009369). With the aid of on-line Primer3 software (http://frodo.wi.mit.edu), the primer pairs used to amplify the complete coding region and splice junctions of *GJA5* by polymerase chain reaction (PCR) were designed as previously described (54,55). PCR was performed using HotStar Taq DNA Polymerase (Qiagen, Hilden, Germany) on a Veriti® Thermal Cycler (Applied Biosystems, Foster, CA, USA) with standard conditions and concentrations of reagents. Amplified products were purified with the QIAquick® Gel Extraction kit (Qiagen). Both strands of each amplicon were sequenced with a BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) under an ABI PRISM 3130 XL DNA Analyzer (Applied Biosystems). Primer sequences were those previously designed for specific region amplifications. DNA sequences were viewed and analyzed with the DNA Sequencing Analysis Software v5.1 (Applied Biosystems). The sequence variant was validated by resequencing of an independent PCR-generated amplicon from the same subject and met the quality control threshold

Table I. Clinical characteristics of the 310 unrelated patients with lone atrial fibrillation.

Characteristics	No. or quantity	Percentage or range
Male:female	142:168	71:84
Age of onset (years)	45.2	18-59
Paroxysmal AF on presentation	245	79
Progression to permanent AF	54	17.4
History of cardioversion	31	14
History of pacemaker	18	5.8
Resting heart rate (bpm)	72.5	50-158
Systolic blood pressure (mmHg)	128.4	90-138
Diastolic blood pressure (mmHg)	82.6	60-88
Body mass index (kg/m^2)	23.0	19-26
Left atrial dimension (mm)	35	22-40
Left ventricular ejection fraction	0.6	0.5-0.7
Fasting blood glucose (mmol/l)	4.4	3.6-5.8
Total cholesterol (mmol/l)	3.5	3.0-5.8
Triglycerides (mmol/l)	1.3	0.5-1.7
Medications		
Aspirin	86	27.7
Warfarin	115	37.1
β-blocker	102	32.9
Calcium channel blocker	35	11.3
Digoxin	110	35.5

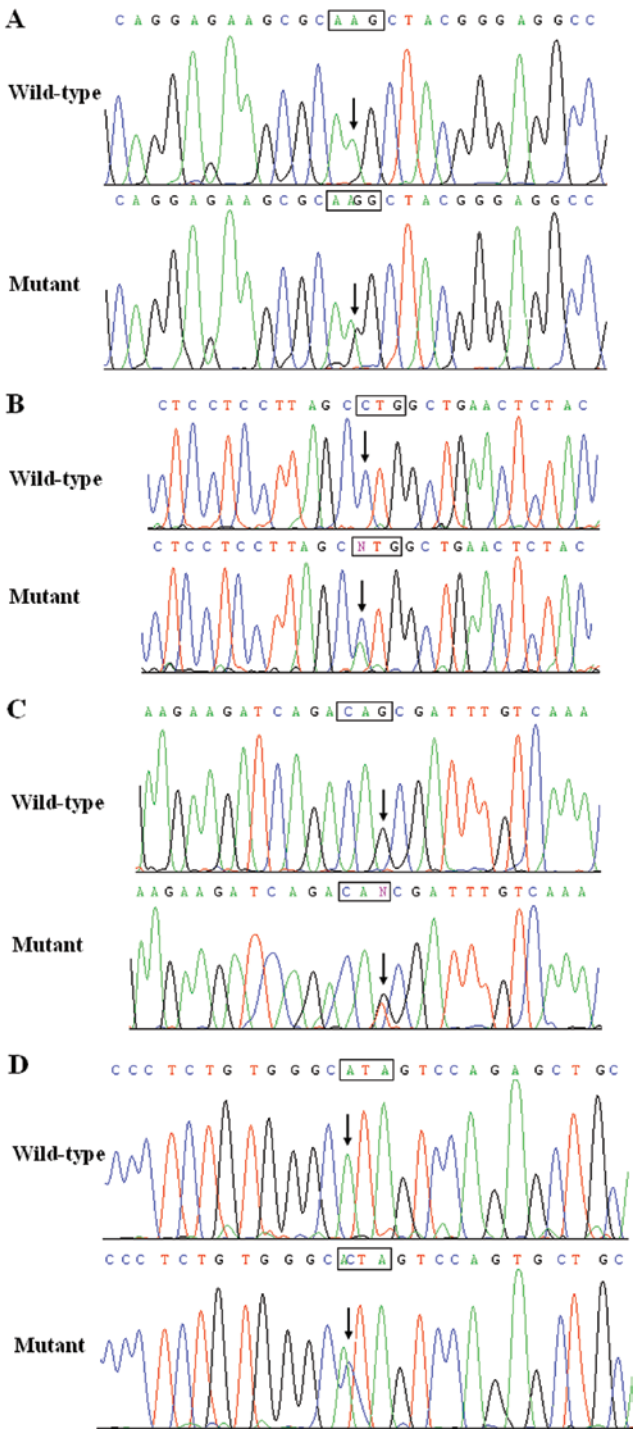

Figure 1. Sequence electropherograms of *GJA5* in the probands and controls. The arrow indicates the heterozygous nucleotides of (A) A/G, (B) C/A, (C) G/T and (D) A/C, in the probands from families 1, 2, 3 and 4, respectively (mutant) or the homozygous nucleotides of (A) A, (B) C, (C) G and (D) A, in the corresponding controls (wild-type). The rectangle denotes the nucleotides comprising a codon of GJA5.

with a call rate of >99%. Additionally, an identified variant was searched in the single nucleotide polymorphism (SNP) database from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/SNP) to confirm the novelty.

Alignment of multiple GJA5 protein sequences across species. The multiple GJA5 protein sequences across various species were aligned using the online program MUSCLE, version 3.6 (http://www.ncbi.nlm.nih.gov/).

Statistical analysis. Data were expressed as the means ± standard deviation (SD). Continuous variables were tested for normality of distribution and the Student's unpaired t-test was used for comparison of numeric variables between patient and control groups. Comparison of the categorical variables between the 2 groups was performed using Pearson's χ^2 or Fisher's exact tests when appropriate. A two-sided P-value of <0.05 was considered to indicate statistically significant difference.

Results

Characteristics of the study subjects. A cohort of 310 unrelated patients with lone AF were included in this study and clinically evaluated in contrast to a total of 200 matched, unrelated healthy individuals. None of the subjects had documented traditional risk factors for AF. There were no significant differences between lone AF 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 life style (data not shown). The basic clinical characteristics of the 310 patients with lone AF are summarized in Table I.

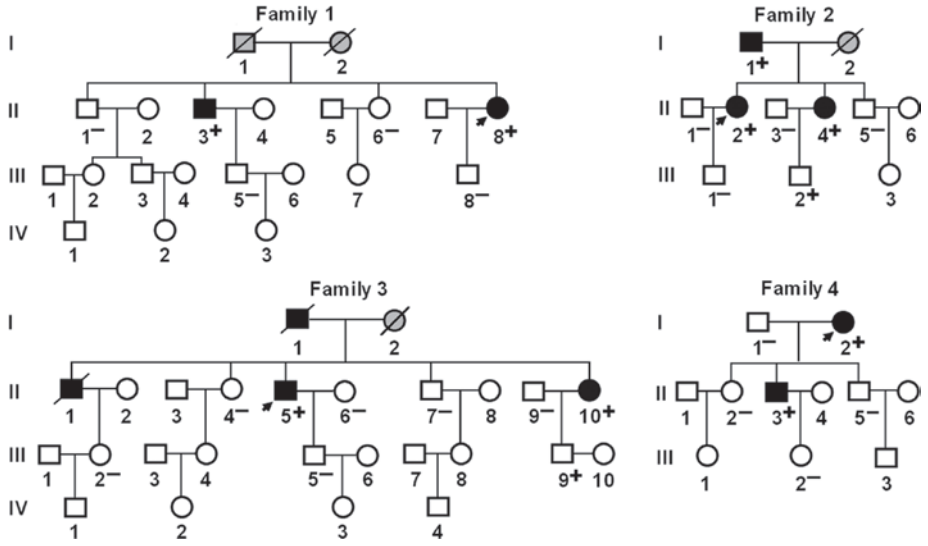

Figure 2. Pedigree structures of families with atrial fibrillation (AF). Families are designated as 1, 2, 3 and 4, respectively. Family members are identified by generations and numbers. Squares, male family members; circles, female members; symbols with a slash, the deceased members; closed symbols, affected members; open symbols, unaffected members; stippled symbols, members with phenotype undetermined; arrows, probands; '+', carriers of the heterozygous mutations; and '-', non-carriers.

GJA5 mutations. A total of 310 unrelated patients with lone AF were genetically evaluated. Direct sequencing of the entire coding region and exon-intron boundaries of the *GJA5* gene was performed after PCR amplification of genomic DNA from the 310 AF patients. Four heterozygous missense mutations in *GJA5* were identified in 4 of 310 unrelated index patients, respectively. The total population prevalence of *GJA5* mutations based on probands was ~1.29%. Specifically, a substitution of guanine for adenine in the second nucleotide of codon 107 (c.320A>G), predicting the transition of lysine into arginine at amino acid position 107 (p.K107R) was detected in the proband from family 1. A replacement of cytosine by adenine in the first nucleotide of codon 223 (c.667C>A), resulting in the transversion of leucine into histidine (H) at amino acid 223 (p.L223H) was observed in the proband from family 2. A change of guanine into thymine in the last nucleotide of codon 236 (c.708G>T), corresponding to the displacement of glutamine by H at amino acid 236 (p.Q236H) was identified in the proband from family 3. An adenine-to-cytosine conversion in the first nucleotide of codon 257 (c.769A>C), equivalent to an isoleucine-to-leucine shift at amino acid 257 (p.I257L) was identified in the proband from family 4. The sequence chromatograms showing the identified heterozygous *GJA5* mutations of c.320A>G, c.667C>A, c.708G>T and c.769A>C in comparison to corresponding control sequences are shown in Fig. 1.

The missense mutations were not found in either the 400 control chromosomes or reported in the SNP database. Genetic scanning of the families demonstrated that in each family, the gene variant was present in all the affected living family members, while it was absent in unaffected family members examined with the exception of individuals III-2 in family 2 and III-9 in family 3, suggesting that the long-term follow-up of asymptomatic subjects harboring the variations is needed to confirm its clinical significance. Analysis of the pedigrees showed that each mutation co-segregated with AF transmitted in an autosomal dominant pattern in the family with an incomplete penetrance. The pedigree structures of the 4 families are shown in Fig. 2. The phenotypic characteristics and results of genetic screening of the affected family members are presented in Table II.

Multiple alignments of GJA5 protein sequences across species. A cross-species alignment of GJA5 protein sequences demonstrated that the altered amino acids were highly and evolutionarily conserved with the exception of p.I257 (Fig. 3).

Discussion

In the present study, four novel heterozygous GJA5 mutations, p.K107R, p.L223M, p.Q236H and p.I257L, were identified in four unrelated families with AF, respectively, with an estimated mutational prevalence of 1.29%. In each family, the missense mutation was present in all the affected family members examined, while it was absent in the unaffected family members, with the exception of individuals III-2 in family 2 and III-9 in family 3. These mutations were absent in 400 normal chromosomes from an ethnically-matched control population. A cross-species alignment of GJA5 protein sequences demonstrated that the altered amino acids were highly and evolutionarily conserved among species, with the exception of p.I257. Therefore, it is likely that mutated GJA5 caused or conferred susceptibility to AF in these families.

Two carriers of GJA5 mutations, including individual III-2 in family 2 who carried the p.L223M mutation and individual III-9 in family 3 who harbored the p.Q236H mutation, did not have AF during a 24-h electrocardiographic monitoring. This observation may be explained by the following reasons. Firstly, AF occurs as rarely as a few times in a lifetime for some patients with AF (56); considering the performed electrocardiographic monitoring for only 24 h, a longer duration of monitoring may be required to record paroxysmal AF in these patients. Secondly, AF occurs more commonly in older patients; thus, these carriers may not be old enough to develop

Table II. Phenotypic characteristics and status of GJA5 mutations of the affected pedigree members.

Subject information				Phenotype	Electrocardiogram		Echocardiogram		Genotype
Identity	Gender	Age at time of study (years)	Age at diagnosis of AF (years)	AF (Classification)	P-wave (ms)	QRS interval (ms)	LAD (mm)	LVEF (%)	GJA5 mutations
Family 1									K107R
II-3	M	56	50	Paroxysmal	100	98	30	70	+/-
II-8	F	48	42	Persistent	106	92	30	65	+/-
Family 2									L233M
I-1	M	65	50	Permanent	98	94	38	62	+/-
II-2	F	41	38	Paroxysmal	92	90	36	56	+/-
II-4	F	36	36	Paroxysmal	110	84	32	66	+/-
Family 3									Q236H
I-1	M	70[a]	55	Paroxysmal	N/A	90	N/A	N/A	N/A
II-1	M	64[a]	53	Paroxysmal	N/A	92	N/A	N/A	N/A
II-5	M	60	58	Paroxysmal	114	114..	38	64	+/-
II-10	F	54	54	Paroxysmal	92	88	32	60	+/-
Family 4									I257L
I-2	F	64	45	Paroxysmal	102	90	36	65	+/-
II-3	M	42	40	Paroxysmal	112	92	33	67	+/-

[a]Age at death. AF, atrial fibrillation; LAD, left atrial dimension; LVEF, left ventricular ejection fraction; M, male; F, female; +, presence of mutation; -, absence of mutation; N/A, not available or not applicable.

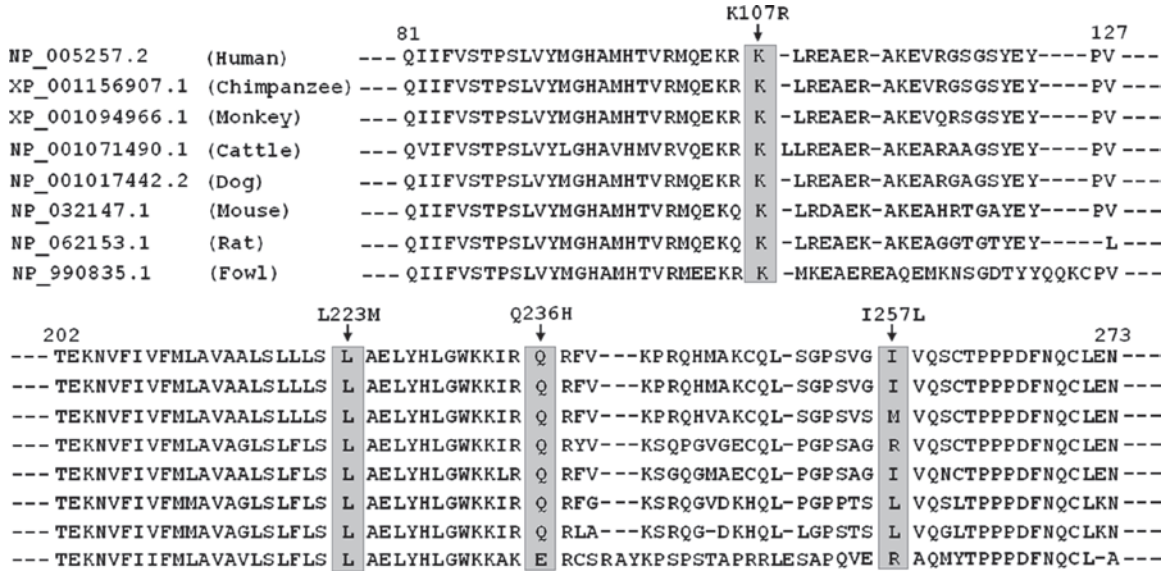

Figure 3. Multiple alignments of GJA5 protein sequences across species. The altered amino acids of p.K107, p.L223 and p.Q236 are highly and evolutionarily conserved among species.

the disease. Thirdly, familial AF caused by the mutation p.L223M or p.Q236H may have a low or incomplete penetration. Additionally, p.L223M or p.Q236H may be only a genetic risk factor predisposing to AF, rather than a direct cause of AF, and environmental risk factors may be required for the onset of AF.

Multiple *GJA5* mutations or polymorphisms have been previously involved in AF (48-55). Similar to the present findings, Yang *et al* (54,55) have previously performed a sequence analysis of the *GJA5* gene in a total of 344 index patients with lone AF, and identified four novel heterozygous missense mutations (p.Q49X, p.V85I, p.L221I and p.L229M), with a mutational prevalence of ~1.16%. Gollob *et al* (53) performed the first scan of GJA5 in patients with lone AF and identified four novel heterozygous missense mutations in 4 of 15 AF patients, of which 3 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, with a germline mutational prevalence of ~6.67%. The p.A96S

variant was absent in the patient's 3 siblings and wife, while it was present in the patient's 2 sons without history of AF and in 1 of 120 controls. Functional analysis of mutant GJA5 proteins demonstrated impaired intracellular transport or reduced intercellular electrical coupling (53). By sequencing the 5' untranslated exon and the proximal promoter region of the *GJA5* gene (GenBank accession no. AF246295) in patients with familial atrial standstill, Groenewegen *et al* (48) found two closely linked polymorphisms, of which one was a G to A transition at 44 nucleotides upstream of the transcription start site (-44G>A), and the other was a substitution of G for A in exon 1 at 71 nucleotides downstream of the transcription start site (+71A>G). Luciferase reporter gene assays of the minor *GJA5* haplotype (-44A, +71G) in GJA5-expressing rat arterial smooth muscular cells showed a >2-fold decrease in promoter activity compared with the more common haplotype (-44G, +71A). The reduced GJA5 expression may lead to a reduction of the total amount of GJA5 protein *in vivo*, providing an atrial electrophysiological substrate favoring arrhythmia (48). Furthermore, the *GJA5* polymorphisms have been strongly associated with increased spatial dispersion of refractoriness as a marker for enhanced atrial vulnerability and carriers of the -44AA genotype had a significantly higher risk of AF compared with those carrying the -44GG genotype (49). In a larger case-control study, the rare haplotype frequency of *GJA5* (-44A, +71G) was statistically higher in the AF compared with the control group, and also functional studies using luciferase as the reporter have demonstrated that *GJA5* (-44A, +71G) had significantly lower promoter activity compared with GJA5 (-44G, +71A) in atrial myocytes from mice (50). A novel common *GJA5* gene promoter variant has recently been associated with reduced GJA5 expression in human atria and increased vulnerability to AF (51). These results highlight the pivotal role of GJA5 for atrial electrophysiology and indicate that dysfunctional *GJA5* may be an important molecular mechanism involved in the pathogenesis of AF.

The association of abnormal *GJA5* with enhanced susceptibility to arrhythmias has been substantiated in animal models. Targeted gene deletion of *GJA5* 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 an arrhythmogenic substrate prone to AF (57,58). In a canine sterile pericarditis model, the gap junction conduction-enhancing antiarrhythmic peptide, Gap-134, improved conduction and reduced AF (59). Similarly, in a dog model of AF due to myocardial ischemia, administration of ZP123, a gap junction conductance-improving modifier, prevented ischemia-induced conduction slowing and reduced AF duration (60). Notably, in experimental swine, gene therapy with adenovirus expressing GJA5 improved cardiac conduction and reduced AF, demonstrating the viability of gene therapy for the prevention of atrial arrhythmias (61).

It is well known that AF is a complex arrhythmia ascribed to multiple possible mechanisms. Despite the presence of an inherited defect, a favorable 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 increased vagal tone mediated by muscarinic receptors, causing uneven shortening of refractoriness in the atria and, thus, electrophysiological heterogeneity (62). The stimulation of muscarinic receptors has been shown to impair the cell-cell coupling mediated by gap junctions (63). 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.

Notably, GJA5 is an important determinant for impulse propagation in the atrium as well as the specialized conduction system and abnormal expression of GJA5 predisposes to AF. However, functional changes in GJA5 alone may not be sufficient for significantly prolonged P-wave duration, PQ interval, QRS duration and QT duration in the surface electrocardiogram, as observed in these AF families and other AF patients (48-55). Additionally, full deficiency for GJA5 has been associated with altered electrocardiographic parameters in *GJA5* knockout mice, in contrast to haploinsufficiency for GJA5 (57). These findings suggest that additional factors combined with reduced coupling lead to AF.

In conclusion, the present investigation links novel *GJA5* mutations to AF, which provides novel insight into the molecular mechanisms associated with the arrhythmogenesis and ultimately may result in improved, patient-specific rhythm control strategies.

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