Mutations of the *SCN4B*-encoded sodium channel β4 subunit in familial atrial fibrillation

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Received October 8, 2012; Accepted April 12, 2013

DOI: 10.3892/ijmm.2013.1355

Abstract. Atrial fibrillation (AF) represents the most common form of sustained cardiac arrhythmia and accounts for substantial morbidity and mortality. Mutations in the cardiac sodium channel α, β1, β2 and β3 subunit genes (*SCN5A*, *SCN1B*, *SCN2B* and *SCN3B*) have been associated with AF, which suggests that mutations in the sodium channel β4 subunit gene, *SCN4B*, are also involved in the pathogenesis of AF. To examine this hypothesis, the coding exons and exon-intron boundaries of *SCN4B* were sequenced in 170 unrelated index patients with familial AF. The available relatives of the probands carrying the identified mutations and 200 unrelated ethnically matched healthy individuals used as the controls were subsequently genotyped. The pathogenic potential of a *SCN4B* sequence variation was predicted using MutationTaster. As a result, 2 novel heterozygous SCN4B mutations, p.V162G and p.I166L, were identified in 2 unrelated families with AF transmitted in an autosomal dominant pattern, respectively. In each family the mutation co-segregated with AF and was absent in the 400 control chromosomes. The mutations altered the amino acids evolutionarily highly conserved across species and were both predicted to be disease-causing. To the best of our knowledge, this is the first study to demonstrate an association of *SCN4B* mutations with AF, suggesting *SCN4B* as a novel AF susceptibility gene.

Introduction

Atrial fibrillation (AF) represents the most common form of sustained cardiac arrhythmia with an estimated prevalence of 1% in the general population (1). The incidence of AF increases markedly with advancing age, ranging from less than 1% in individuals under 60 years of age to approximately 10% of those over 80 years (2). According to the Framingham Heart Study, the lifetime risk for development of AF is approximately 25% for individuals who have reached the age of 40 years (3). AF accounts for substantially increased cardiovascular morbidity and mortality. It is associated with an approximately 5-fold increase in the risk of stroke, and more than 15% of all strokes are ascribed to this disordered heart rhythm (4). The risk of AF-related thromboembolism also increases strikingly with age, rising from 1.5% at age 50-59 years to 23.5% at age 80-89 years (4). The total death rate is roughly doubled among patients with AF compared with people in normal sinus rhythm (5). AF is also responsible for compromised exercise performance, degraded quality of life, impaired cognitive function or dementia, tachycardia-induced cardiomyopathy, and left ventricular dysfunction or even congestive heart failure, conferring a large economic burden on national healthcare system worldwide (6). Despite the significant clinical importance, the molecular mechanisms involved in the pathogenesis of AF remain poorly understood.

Traditionally, AF has been regarded as a complication attributed to miscellaneous adverse cardiac or systemic conditions, including hypertension, coronary artery disease, rheumatic heart disease, valvular heart disease, pulmonary heart disease, cardiomyopathy, cardiac surgery, diabetes mellitus type 2, obstructive sleep apnea, hyperthyroidism, and electrolyte imbalance (1). However, in 30-45% of AF patients, an underlying cause cannot be identified by routine procedures, and such AF is termed 'idiopathic' or 'lone' (1), of which at least 15% have a positive family history, a condition classified as familial AF (7). Increasing evidence has demonstrated the familial aggregation of AF and enhanced susceptibility to AF in the close relatives of patients with AF, suggesting that genetic risk factors play a pivotal role in the initiation and maintenance of AF in a subset of cases (8-14). Genome-wide linkage analyses with microsatellite markers mapped susceptibility loci for AF on human chromosomes 10q22, 6q14-16, 11p15.5, 5p13 and 5p15, of which AF-causative mutations in 2 genes, including *KCNQ1* on chromosome 11p15.5 and *NUP155* on chromosome 5p13, were identified and functionally characterized (15-20). The genetic screening of candidate genes has revealed a great number of AF-associated genes, including *KCNE2, KCNE3, KCNE5, KCNH2, KCNJ2, KCNA5, SCN5A,*

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Key words: atrial fibrillation, genetics, sodium channel, SCN5A, SCN4B

SCN1B, SCN2B, SCN3B, NPPA, GJA1, GJA5, GATA4, GATA5 and *GATA6* (21-44). Nevertheless, AF is of substantial genetic heterogeneity and the genetic basis for AF in an overwhelming majority of patients remains unclear.

Recent studies have highlighted the essential role of the cardiac sodium channel complex in the generation and propagation of the cardiac action potential. The complex comprises multiple protein factors, including the pore-forming α-subunit encoded by *SCN5A*, auxiliary β-subunits, and other accessory proteins, such as MOG1, ankyrin-G, FHF1B, Fyn and PTPH1 (45). In humans, 4 sodium channel β-subunits (β1 to β4, encoded by *SCN1B* to *SCN4B*), which are expressed in both atrial and ventricular cardiomyocytes, have been identified thus far. They share a common protein topology with an extracellular immunoglobulin-like domain, a single transmembrane spanning segment, and an intracellular C-terminal domain, and are implicated in the trafficking of sodium channels to plasma membranes, the modulation of channel gating and voltage dependence, and play a role in cell adhesion and recruitment of cytosolic proteins such as ankyrin-G (45). Mutations in *SCN5A, SCN1B, SCN2B* and *SCN3B* have been implicated in AF (28-33), which prompts us to hypothesize that *SCN4B* is another gene contributing to AF.

To examine this hypothesis, the coding exons and splice sites of *SCN4B* were sequenced in patients with familial AF in contrast to ethnically matched control individuals, and the functional effect of the mutated *SCN4B* gene was analyzed *in silico* by using the online program, MutationTaster.

Materials and methods

Study subjects. A cohort of 170 unrelated index patients with familial AF identified among the Han Chinese population was recruited. The available relatives of the probands harboring the identified mutations were also enrolled in this study. A total of 200 unrelated ethnically matched healthy individuals used as the controls were enlisted. All the participants underwent evaluation by medical history, physical examination, electrocardiography and echocardiography. 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 (7,38). In brief, AF was diagnosed 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 under the age of 60 without other cardiac or systemic diseases by physical examination, electrocardiogram, transthoracic echocardiogram and extensive laboratory tests. Familial AF was defined as that present in a family with more than one first- or second-degree relative affected with AF. Relatives were classified as 'unaffected' if they were asymptomatic and had a normal electrocardiogram. Paroxysmal AF was defined as AF lasting more than 30 sec that terminated spontaneously. Persistent AF was defined as AF lasting more than 7 days and requiring either pharmacological therapy or electrical cardioversion for termination. AF that was refractory to cardioversion or that was allowed to continue was classified as permanent. The study protocol was reviewed and approved by the local Institutional Ethics Committee and written informed consent was obtained from all participants prior to investigation.

Genetic analysis. Genomic DNA from all participants was extracted from blood lymphocytes with the Wizard Genomic DNA Purification kit (Promega, Madison, WI, USA). Initially, the coding exons and intron/exon boundaries of the *SCN4B* gene were sequenced in 170 unrelated index patients with familial AF. Subsequently, genotyping for *SCN4B* in the available relatives of the probands carrying the identified mutations and 200 ethnically matched unrelated healthy individuals used as the controls was performed. The reference genomic DNA sequence of *SCN4B* was derived from GenBank (accession no. NG_011710). With the aid of online Primer3 software (http://frodo.wi.mit.edu), the primer pairs used to amplify the coding regions and splice junctions of *SCN4B* by polymerase chain reaction (PCR) were designed as shown in Table I. PCR was carried out using HotStar Taq DNA Polymerase (Qiagen, Hilden, Germany) on a PE 9700 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 PCR product were sequenced with a BigDye® Terminator version 3.1 Cycle Sequencing kit (Applied Biosystems) under an ABI PRISM 3130XL DNA Analyzer (Applied Biosystems). The sequencing primers were those designed previously for specific region amplifications. DNA sequences were viewed and analyzed with the DNA Sequencing Analysis Software version 5.1 (Applied Biosystems). The variant was validated by resequencing of an independent PCR-generated amplicon from the same subject and met the quality control threshold with a call rate >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 SCN4B protein sequences across species. The multiple SCN4B protein sequences across various species were aligned using the online program, MUSCLE version 3.6 (http://www.ncbi.nlm.nih.gov/homologene?cmd=retrieve&dopt=multipleAlignment&list_uids=18384).

Prediction of the causative potential of a SCN4B sequence variation. The disease-causing potential of a *SCN4B* sequence variation was predicted using MutationTaster (http://www.mutationtaster.org), which automatically gave a probability for the variation to be either a pathogenic mutation or a benign polymorphism. Notably, the P-value is the probability of the prediction rather than the probability of error as used in t-test statistics, i.e., a value close to 1 indicates a high 'security' of the prediction.

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

Table I. The intronic primers used to amplify the coding exons and exon-intron boundaries of *SCN4B*.

Exon	Forward primer (5'→3')	Reverse primer (5'→3')	Size (bp)
1	CTC, TCT, GCC, CGC, TAA, CTT, TC	CTA, TGA, ACC, AGG, CAG, GAA, CC	371
2	TTG, GCA, CTG, AGG, GTG, ATA, GA	CAG, AAG, GGA, CCA, GAG, CGT, AG	372
3	GAG, GAC, CCC, GAT, TCT, TTC, TC	AAA, CAC, CAA, CAC, GGT, CCA, TT	387
4	TGA, TAG, ATG, CCA, TGC, TCT, GC	GGG, GTA, GAT, GAG, AGG, GTG, GT	382
5	TCT, GTA, GAA, GGC, CAG, GGA, GA	GGC, AGG, ACT, CTG, GTT, TCT, TG	361

Table II. The baseline clinical characteristics of the 170 probands with familial atrial fibrillation.

Parameter	Statistic
Age at initial diagnosis of atrial fibrillation (years)	44±9
Age at present study (years)	49±8
Male (n, %)	112 (66)
Body mass index (kg/m^2)	23±3
Left ventricular ejection fraction (%)	61±5
Left atrial diameter (mm)	37±4
Paroxysmal atrial fibrillation (n, %)	98 (58)
Persistent atrial fibrillation (n, %)	51 (30)
Permanent atrial fibrillation (n, %)	21 (12)
Positive family history of atrial fibrillation (n, %)	170 (100)
History of cardioversion (n, %)	88 (52)
Catheter-based ablation for atrial fibrillation (n, %)	76 (45)
History of thromboembolic stroke (n, %)	24 (14)
History of pacemaker (n, %)	9 (5)
Systolic blood pressure (mmHg)	128±10
Diastolic blood pressure (mmHg)	80±5
Fasting blood glucose (mmol/l)	6±1
Total cholesterol (mmol/l)	5±1
Aspirin (n, %)	37 (22)
Warfarin (n, %)	78 (46)
Amiodarone (n, %)	92 (54)
β-blocker (n, %)	33 (19)
Calcium channel blocker (n, %)	27 (16)
Digitalis (n, %)	43 (25)

Results

Characteristics of the study population. A cohort of 170 unrelated patients with familial AF and a total of 200 ethnically matched unrelated healthy individuals used as the controls were registered and clinically evaluated. None of them had apparent traditional risk factors for AF. There were no significant differences between the patient and control groups in baseline characteristics including age, gender, body mass index, blood pressure, fasting blood glucose levels, serum lipid levels, left atrial dimension, left ventricular ejection fraction, heart rate at rest, as well as life style (data not shown). In the present study, 12 patients were also diagnosed with hypertension in accordance to the criterion that the average systolic or diastolic blood pressure (2 readings performed after 5 min of rest in the sitting position) was ≥140 or 90 mmHg, respectively, but at the time of initial diagnosis of AF, their blood pressures were normal. The baseline clinical characteristics of the 170 patients with familial AF are summarized in Table II.

SCN4B mutations. Direct sequencing of the entire coding sequences and flanking intronic sequences of the *SCN4B* gene was performed following PCR amplification of genomic DNA from each of the 170 unrelated patients with familial AF. Two heterozygous *SCN4B* mutations were identified in 2 out of the 170 patients, with a mutational prevalence of approximately 1.18%. Specifically, a substitution of guanine (G) for thymine (T) in the second nucleotide of codon 162 (c.485T>G), predicting the transition of valine (V) into glycine (G) at amino acid 162 (p.V162G), was identified in the proband from family 1. A replacement of adenine (A) by cytosine (C) in the first nucleotide of codon 166 (c.496A>C), equivalent to a transversion of isoleucine (I) into leucine (L) at amino acid 166 (p.I166L), was identified in the proband from family 2. The sequence chromatograms showing the detected heterozygous *SCN4B* mutations in contrast to the corresponding control sequences are shown in (Fig. 1). A schematic linear topology of the *SCN4B*-encoded β4 subunit indicating the locations of the mutations identified in AF patients is presented in (Fig. 2). The missense mutations were not found in the 400 control alleles nor were they reported in the SNP database. A genetic scan of the families of the 2 mutation carriers showed that in each family the mutation was present in all affected living family members, but absent in the unaffected family members examined. Analysis of the pedigrees revealed that each mutation co-segregated with AF transmitted in an autosomal dominant pattern in the family with a complete penetrance. The pedigree structures of the 2 families are illustrated in (Fig. 3). The phenotypic characteristics and genotypic status of the affected family members are listed in Table III.

According to a commonly used criterion to diagnose long QT syndrome (46), the corrected QT interval was defined as normal range (≤440 msec) or prolonged (>440 msec). Using this definition, all 3 AF patients from family 1 had long QT (Table III). The mother of the proband experienced recurrent syncopal episodes that began when she was 26 years old. Since that time, she had experienced >20 syncopal episodes, the majority of which were preceded by emotional or physical stress. The electrocardiogram revealed a markedly prolonged QT interval (corrected QT interval was 542 msec), and the echocardiogram documented a structurally normal heart. Therefore, she was diagnosed with long QT syndrome.

Table III. The phenotypic characteristics and status of SCN4B mutations of the affected pedigree members.

	Subject information			Phenotype	Electrocardiogram			Cardiac echocardiogram		Genotype
Identity	Gender	Age at time of study (years)	Age at initial diagnosis of AF (years)	AF (classification)	Heart rate (beats/min)	QRS interval (msec)	QTc	LAD (mm)	LVEF (%)	SCN4B mutations
Family 1										V162G
I-2	F	62	32	Permanent	71	98	542	38	62	+/-
II-1	M	40	36	Paroxysmal	73	106	445	35	68	+/-
II-3	M	38	30	Paroxysmal	79	90	444	32	60	+/-
Family 2										I166L
I-1	M	69	41	Permanent	68	108	426	37	58	+/-
II-2	F	46	38	Persistent	66	100	404	34	67	+/-
III-1	M	22	22	Paroxysmal	128	86	397	30	64	+/-

AF, atrial fibrillation; F, female; M, male; QTc, corrected QT interval; LAD, left atrial dimension; LVEF, left ventricular ejection fraction; +, presence of mutation; -, absence of mutation.

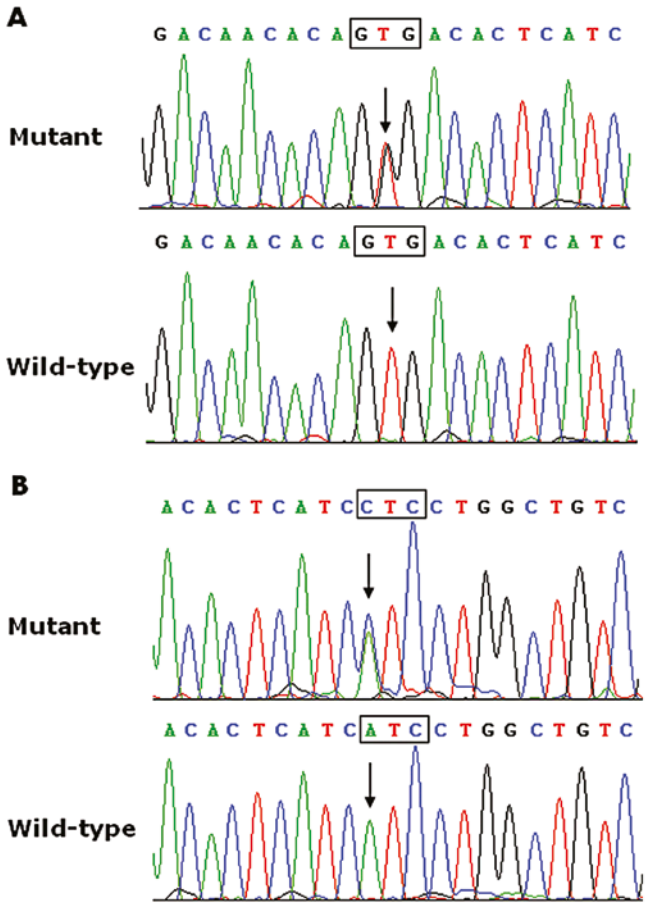

Figure 1. Sequence electropherograms of *SCN4B* in the probands and controls. The arrow indicates the heterozygous nucleotides of (A) T/G or (B) A/C in the proband (mutant) or the homozygous nucleotides of (A) T/T or (B) A/A in the corresponding control individual (wild-type). The rectangle denotes the nucleotides comprising a codon of SCN4B.

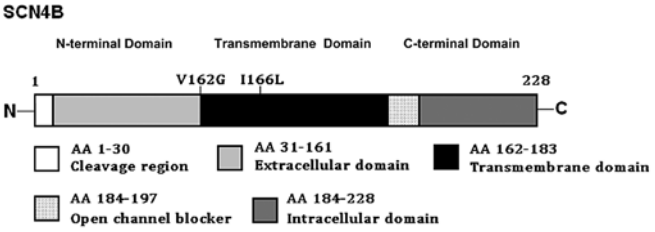

Figure 2. Schematic linear topology of the *SCN4B*-encoded β4 subunit with the mutations related to familial atrial fibrillation indicated. N, amino-terminus; C, carboxyl-terminus; AA, amino acid.

Multiple alignments of SCN4B protein sequences. A cross-species alignment of SCN4B protein sequences displayed that the altered amino acids were evolutionarily highly conserved, as presented in (Fig. 4), suggesting that the amino acids are functionally important.

Causative potential of SCN4B sequence variations. The *SCN4B* sequence variations of c.485T>G and c.496A>C were both automatically predicted to be disease-causing mutations by MutationTaster, with P-values of 0.745474 for c.485T>G

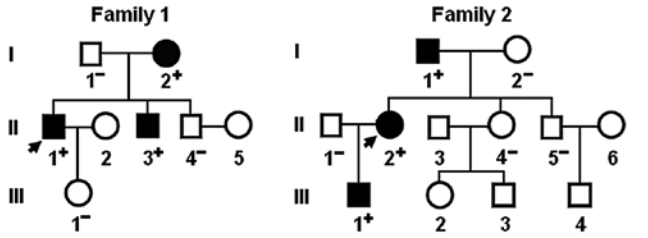

Figure 3. Pedigree structures of families with atrial fibrillation. Family members are identified by generations and numbers. Squares, male family members; circles, female members; closed symbols, affected members; open symbols, unaffected members; arrow, proband; +, carriers of the heterozygous mutation; -, non-carriers.

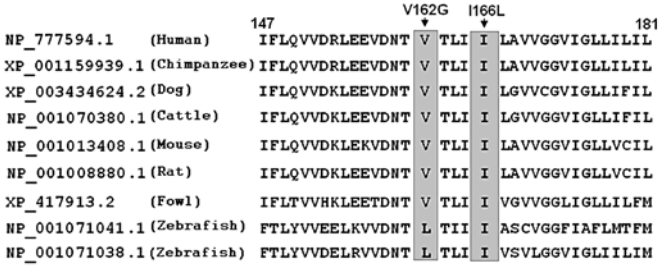

Figure 4. Alignment of multiple SCN4B protein sequences across various species. The altered amino acids of p.V162 and p.I166 are evolutionarily highly conserved across species.

and 0.996496 for c.496A>C. No SNPs in the altered regions were found in the MutationTaster database.

Discussion

In the present study, 2 novel heterozygous SCN4B mutations, p.V162G and p.I166L, were identified in 2 families with AF, respectively. In each family, the missense mutation was present in all the affected family members examined but was absent in the unaffected family members available. These 2 mutations were not detected in the 400 normal chromosomes from an ethnically-matched control population. A cross-species alignment of multiple SCN4B protein sequences exhibited that the altered amino acids were evolutionarily highly conserved. Functional analysis *in silico* demonstrated that the mutations were both disease-causing. Therefore, it is highly likely that mutated *SCN4B* gene contributes to the pathogenesis of AF in these families.

The *SCN4B* gene maps on human chromosome 11q23.3, and is composed of 5 exons, encoding a type 1 membrane protein of 228 amino acids, which forms an auxiliary β4 subunit of voltage-gated sodium channel (47). Quantitative analysis of the tissue distribution of the sodium channel β4 subunit showed that β4 was expressed primarily in excitable tissues, including neuronal, muscular and cardiac tissues from mice, rats and humans (47,48). Similar to the β1-β3 subunits, β4 contains an N-terminal cleaved signal sequence, an extracellular V-type immunoglobulin-like fold, a single transmembrane α helix, and a short intracellular C-terminal tail that may participate in protein-protein interactions. In the immunoglobulin-like fold of the predicted mature β4 protein, there are 3 cysteines, of which the cysteines at positions 23 and 101 are completely conserved across all other β subunits, as well as other V-type immunoglobulin-like folds, and have been proposed to form an intramolecular disulfide bond that stabilizes the structure of the extracellular domain. The β4 subunit is covalently associated with sodium channel α subunit via a disulfide bond to constitute a functional ion channel complexity and functions to increase the expression of sodium channel at the cell surface and modulate its gating kinetics and voltage dependence, which suggests an important role of the β4 subunit in cardiac electrophysiology (45,47).

The findings that the mutated *SCN4B* gene predisposes to AF may be partially attributed to dysfunctional sodium channels. Sodium channels play a pivotal role not only in the initiation of the action potential but also in the maintenance of the action potential dome, and the loss of sodium channel function can result in shortened refractoriness and slowed conduction, which creates an important electrophysiological substrate for reentry in favor of AF (49,50). Additionally, the gain of sodium channel function may give rise to enhanced cellular excitability, increased spontaneous action potential depolarization and reduced threshold for action potential firing, forming an arrhythmogenic matrix prone to AF (51-53). The SCN4B mutations, p.V162G and p.I166L identified in this study, were both located in the transmembrane domain, and thus may be expected to exert a critical effect on the conduction of sodium ions across the membrane and voltage-dependent gating of sodium channel. Therefore, it can by hypothesized that SCN4B is an integral structural component of the cardiac sodium channel complex required for the sodium channel to function adequately, and the mutations, p.V162G and p.I166L, may alter sodium current density and the voltage dependence of sodium channel activation or inactivation. However, the detailed electrophysiological mechanisms by which the mutated *SCN4B* gene confers susceptibility to AF remain to be elucidated.

Of note, all 3 AF patients from family 1, who harbored the SCN4B mutation p.V162G, had a prolonged QT interval, and the mother of the proband had been diagnosed as having long QT syndrome. Since 10-15% of patients with long QT syndrome have a normal QT interval (54), it could not be ruled out that other family members carrying a SCN4B mutation had long QT syndrome. Consistent our results, Medeiros-Domingo *et al* (55) performed a genetic analysis of *SCN4B* in 263 patients with congenital long QT syndrome and found the heterozygous missense mutation, p.L179F, with a mutational prevalence of approximately 0.38%. This mutation was not observed in 800 reference alleles and led to an increase in late sodium current. Tan *et al* (56) genotyped *SCN4B* in 292 cases with sudden infant death syndrome and discovered the heterozygous mutation, p.S206L, with a mutational prevalence of approximately 0.34%. Functional analysis revealed that this mutation accentuated the late sodium current and increased the ventricular action potential duration. These findings indicate that AF may share a common genetic origin with long QT syndrome as well as sudden infant death. Considering that congenital long QT syndrome is potentially lethal secondary to malignant ventricular arrhythmias and that the mutated *SCN4B* gene has been linked to sudden infant death, the present study is of significant clinical importance.

In conclusion, to our knowledge, this is the first study presenting *SCN4B* as a novel AF-susceptibility gene and suggests a common genetic basis for AF and congenital long QT syndrome, as well as sudden infant death. The findings provide significant insight into the molecular mechanisms underlying arrhythmias and provide potential therapeutic strategies for the early prophylaxis and personalized therapy of arrhythmias.

Acknowledgements

We would like to thank the participants for their devotion to the study. This study was supported by grants from the National Natural Science Fund of China (81070153, 81270161 and 30570768), the Natural Science Fund of Shanghai, China (10ZR1428000), the Personnel Development Foundation of Shanghai, China (2010019) and the Key Program of Basic Research of Shanghai, China (10JC1414000, 10JC1414001 and 10JC1414002).

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