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Original Article

Association of atrial fibrillation with gene polymorphisms of connexin 40 and angiotensin II receptor type 1 in Chongming adults of Shanghai

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Abstract: Objective: To characterized the gene polymorphisms of connexin 40 (cx40) and angiotensin II receptor type 1 (AT1R) in Chongming adults with atrial fibrillation (AF) and to explore their relationships with AF. Methods: 82 patients with AF, and 82 subjects without AF were enrolled. Polymorphisms of cx40 G-44A and AT1 A1166C were detected. Moreover, several samples were randomly selected to validate the gene polymorphisms of cx40 and AT1. Results: Genotypes AA, AG and GG of cx40 G-44A were found in both AF patients and controls. The frequencies of genotypes AA, AG and GG were 39%, 29% and 32%, respectively, in AF patients and 31%, 35% and 34%, respectively in controls. The frequencies of alleles A and G were 54% and 46%, respectively in AF patients and 48% and 52%, respectively, in controls ($P < 0.05$). The risk for AF in patients with allele A increased 1.31 times (OR = 1.31, $P < 0.05$). The frequencies of genotypes AA, AC and CC were 88%, 8% and 4%, respectively in AF patients and 93%, 6% and 1%, respectively in controls. The frequencies of alleles A and C were 92% and 8%, respectively in AF patients and 96% and 4%, respectively in controls ($P < 0.05$). More AF patients had allele C as compared to controls. The risk for AF increased by 1.43 times in patients with allele C (OR = 1.43, $P < 0.05$). Conclusion: There were relationships between gene polymorphisms of cx40 and AT1 and AF in Chongming adults. Allele A of cx40 G-44A and allele C of AT1 A1166C significantly increase the risk for AF.

Keywords: Atrial fibrillation, connexin, angiotensin ii receptor type 1, gene polymorphism

Introduction

Atrial fibrillation (AF) is a common atrial tachyarrhythmia in clinical practice and has a prevalence of 0.77%, and the percentage of people with AF increases with age [1]. During AF, the heart rate will be 350-600 beats per minute and the atrium loses its systolic and diastolic capabilities, resulting in blood stasis in the atrium, severe arrhythmia, and cardiac dysfunction. Moreover, it may cause thrombosis and subsequent severe complications such as thromboembolism and stroke. Thus, it has high disability and high mortality [1-3]. Framingham study shows AF increases the risk for stroke by 5 times [4]. AF significantly threatens the human health, but there is no ideal strategy for the therapy of AF. Although the pathogenesis of AF has been studied for more than 100 years, the specific mechanism is still poorly understood [5, 6].

In recent years, studies have reported cases of familial AF, and thus increasing investigators focus on the role of genetic factors in the pathogenesis of AF. The molecular and genetic mechanism has been one of hot topics in the studies on the cardiovascular diseases [7-9]. In a majority of patients, AF usually occurs following coronary heart disease, valvular heart disease, pulmonary heart disease, hypertension, heart failure, hyperthyroidism and major surgery, but these diseases or surgery do not necessarily cause AF. This suggests that there is still genetic heterogeneity in the non-familiar AF. There is evidence showing that the single nucleotide polymorphisms (SNP) of ion channels and connexins are closely related to the occurrence of AF [10-13]. Connexin 40 (cx40) is a major type of connexins expressed in the myocardium, is essential to the propagation of action potential and plays an important role in the electrical remodeling and structural remodeling of the

Table 1. Primers used in this study

Gene	Primers	
Cx40 G-44A	Forward	5'-CCCTCTTTTTAATCGTATCTGTGGC-3'
	Reverse	5'-GGTGGAGGGAAGAAGACTTTTAG-3'
AT1R A1166C	Forward	5'-CACCATGTTTTGAGGTTG-3'
	Reverse	5'-CGATTTCTGACATTGTTC-3'

Table 2. Reagents used for PCR of cx40 G-44A (volume: 25 μl)

Reagents	Volume
10× CoralLoad PCR Buffer	2.5 μl
Genomic DNA	2 μl
25 mM $MgCl_2$	0.5 μl
2.0 mM dNTPs	2 μl
Forward primer	1 μl (10 pmol)
Reverse primer	1 μl (10 pmol)
Sterilized distilled water	15.5 μl
TaqDNA polymerase	0.5 μl

atrium [14]. Cx40 is encoded by CJA5 gene mapped to 1q21.1 and contains 3 exons (A1, 1B and encoding exon 2). Studies have confirmed that SNPs of CJA5 gene (such as -44 G→A) is related to AF [15]. Renin-angiotensin system (RAS) is a key humoral regulation system in humans, of which angiotensin II (Ang II) is the most important. In the cardiovascular system, Ang II acts on the angiotensin receptor (ATR), which plays an important role in the regulation of electrical stability of the atrium. ATR type 1 (AT1R) gene is mapped to 3q21-25 and about 2.2 kb in length, and has an exon and no intron. The SNPs of AT1R gene include A1166C, A1166G, T537C, G1517T and A1878G [16]. Of these SNPs, A1166C is closely associated with some diseases including hypertension and coronary heart disease [17, 18]. However, few studies have been conducted to investigate the SNPs of AT1R and AF, especially in Shanghai, China.

In respect of the differences in the gene polymorphism among regions and races, this study was undertaken to investigate the association of gene polymorphisms of cx40 and ATIR with AF in Chongming adults on the basis of an epidemiological survey on AF. In the present study, the characteristics of gene polymorphisms of cx40 G-44A and AT1R A1166C were investigated, aiming to elucidate the molecular and genetic pathogenesis of AF and provide evidence for the gene therapy of AF.

Materials and methods

Subjects

From May 2011 to April 2013, an epidemiological study was conducted in 18 towns of Chongming, and one community was randomly selected from each town. Thus, a total of 18 communities were selected for this cross-sectional survey. Subjects aged ≥ 20 years were recruited into this epidemiological survey on AF after obtaining the informed consent. In this survey, 122 subjects were diagnosed with AF. Patients with concomitant cardiomyopathy, pulmonary heart disease, valvular disease, hyperthyroidism, fever, tumors and other systemic diseases were excluded from this study. Finally, 82 patients with AF were enrolled into present study. There were 47 males and 35 females. In the same period, 82 subjects without AF were recruited as controls. There were 47 males and 35 females. There were no sibship, no history of intermarriage and no history of family racially mixed marriage among these subjects. The AF patients and controls were matched at a ratio of 1:1 according to age, gender, race, smoking status, blood uric acid, CRP, region and concomitant diseases (diabetes, hypertension, coronary heart disease).

Methods

Epidemiological survey: This was a cross-sectional survey.

Blood sampling: Peripheral venous blood (about 5 ml) was collected in the morning from each patient and subject. Blood was divided into 2 parts: one was not anti-coagulated and processed for the detections of blood glucose, lipids, liver and kidney function and CRP; the other was anti-coagulated with ethylenediaminetetraacetic acid, and plasma, red blood cells and white blood cells were separated by centrifugation at 3000 rpm for 10 min. The plasma was stored at -80°C for DNA extraction.

DNA extraction and detections of DNA purity and concentration: DNA detection kit (QIAGEN) was used to extract DNA from the peripheral white blood cells and the extracted DNA was stored at -20°C for use. UV spectrophotometry

Table 3. Reagents used for PCR of AT1R A1166C (volume: 25 μl)

Reagents	Volume
10× CoralLoadPCRBuffer	3.5 μl
Genomic DNA	2 μl
25 mM $MgCl_2$	1 μl
2.0 mM dNTPs	2 μl
Forward primer	1 μl (10 pmol)
Reverse primer	1 μl (10 pmol)
Sterilized distilled water	14.5 μl
TaqDNA polymerase	0.5 μl

Table 4. Clinical characteristics of controls and patients

Variables	AF patients	Controls	P
Gender (M/F)	47/35	47/35	
Age (Years)	66.1±15.2	59.4±10.4	0.05
SBP	120±15.6	117±7.2	0.07
DBP	72±13.6	73.5±5.9	0.06
LAD	42±6.5	27.1±3.7	0.21
LVEF	63.5±9.3	67.0±5.3	0.17
TC	4.35±0.72	4.60±0.67	1.06
TG	1.24±0.95	1.12±0.45	1.17
CRP	4.76±5.90	3.78±4.25	1.62
UA	141±12.86	128±22.73	0.06
n	82	82	

Note: SBP: systolic blood pressure; DBP: diastolic blood pressure; LA: left atrial diameter; LVEF: Left ventricular ejection fraction; TC: Total cholesterol; TG: Triglycerides; CRP: C reaction protein; UA: uric acid.

was performed to detect the DNA concentration and purity, and the extracted DNA was further identified by being subjected to 2% agarose gel electrophoresis.

Primer design and synthesis: According to previously reported and data in GenBank, primers for cx40 G-44A and AT1R A1166C were designed with Primer 5 [19, 20], and synthesized in Shanghai Sangong Biotech Co., Ltd (**Table 1**).

Polymerase chain reaction (PCR): Primers were used to prepare 20 pmol/μl working solution. A mixture was prepared with primers for Cx40 G-44A and other reagents presented in **Table 2** for PCR. PCR conditions were as follows: predenaturation at 94°C for 5 min, and a total of 38 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s and extension at 72°C for 30 s, and a final extension at 72°C for 6 min. The products were stored at 4°C [21].

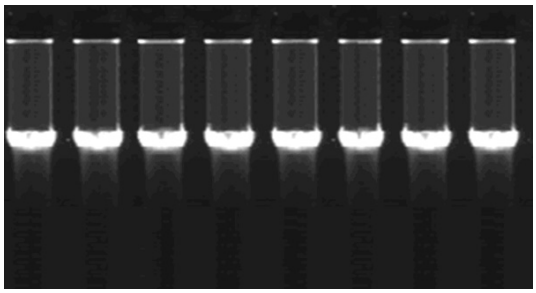

Figure 1. Electrophoresis of genomic DNA extracted from peripheral blood.

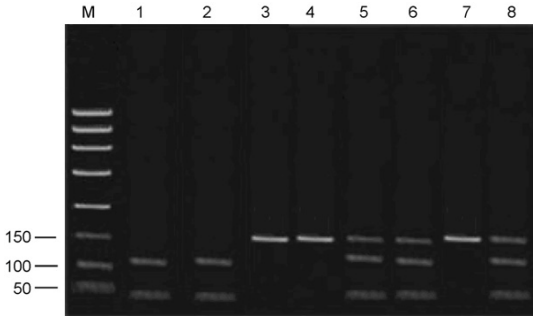

Figure 2. Electrophoresis of cx40 G-44A. M: mark, GG genotype: 1, 2; AG genotype: 5, 6, 8; AA genotype: 3, 4, 7.

A mixture was prepared with primers for AT1R A1166C and other reagents presented in **Table 3** for PCR (final volume: 25 μl). PCR conditions were as follows: pre-denaturation at 94°C for 4 min, and a total of 35 cycles of denaturation at 94°C for 1 min, annealing at 57°C for 1 min and extension at 72°C for 1 min, and a final extension at 72°C for 8 min. The products were stored at 4°C [22].

Restriction fragment length polymorph (RLFP): The PCR products of cx40 G-44A were subjected to digestion with HaeII restriction endonuclease with a final volume of 20 μl (10× buffer: 2 μl; PCR product: 12 μl; HaeII restriction endonuclease 1 μl; sterilized distilled water: 5 μl). Reaction was done at 40°C for 10 h [21]. The PCR products of AT1R A1166C were subjected to digestion with DdeI restriction endonuclease with a final volume of 20 μl (10× buffer: 2 μl; PCR product: 10 μl; DdeI restriction endonuclease 0.5 μl; sterilized distilled water: 7.5 μl). Reaction was done at 37°C for 8 h [22, 23].

Table 5. Hardy-Weinberg equilibrium test of allele frequency of cx40 G-44A in controls and AF patients

Groups		Genotypes			X^2	P
		AA	AG	GG		
AF patients	Actual number	32	24	26	13.88	0.1
	Actual frequency	0.39	0.29	0.32		
	Theoretical number	23.61	40.78	17.61		
	Theoretic frequency	0.29	0.50	0.21		
Controls	Actual number	25	29	28	6.98	0.07
	Actual frequency	0.31	0.35	0.34		
	Theoretical number	19.03	40.95	22.03		
	Theoretic frequency	0.23	0.50	0.27		

Detection of gene polymorphism: After digestion, the PCR products of cx40 G-44A and AT1R A1166C were subjected to low melting point agarose gel electrophoresis at 120 V for about 40 min. Gel image analysis system was used for genotyping of cx40 G-44A and AT1R A1166C.

After genotyping, 20 samples were randomly selected for validation of above results. The concordance rate was as high as 100%. Then, the PCR products were subjected to sequencing with ABI3730XL, and their sequences were compared with those obtained from database (http://www.ncbi.him.nih.gov/).

Statistical analysis

Data were input into Excel and double-checked. Statistical analysis was done with SPSS version 17.0. Quantitative data are expressed as mean ± standard deviation. Comparisons between groups were done with student t test. Qualitative data and Hardy-Weinberg equilibrium were compared and tested, respectively, with chi square test. Logistic regression model was employed to screen potential confounding factors among gender, age, smoking status, history of drinking, region, blood biochemical parameters, and concomitant cardiovascular diseases (hypertension, coronary heart disease and diabetes). The paired design was employed in our study, and thus confounding factors related to AF were not identified. Then, logistic regression model was used to evaluate the relationships between gene polymorphism of cx40 G-44A and AT1R A1166C and AF. A value of $P < 0.05$ was considered statistically significant.

Results

Subjects' characteristics of patients and controls

Of 18 towns, one community was selected from each town, and thus cluster sampling was done in 18 communities. A total of 14885 subjects aged ≥ 20 years were recruited into this epidemiological study. There were 7277 males and 7608 females. In addition, 14802 were Han Chinese and 83 were of Chinese minority. A total of 122 subjects were diagnosed with AF. Subjects with concomitant cardiomyopathy, pulmonary heart disease, valvular disease, hyperthyroidism, fever, tumors and other systemic diseases. Finally, 82 patients with AF were included in the present study. There were 47 males and 35 females. In addition, 82 subjects without AF were also recruited as controls. There were 47 males and 35 females.

Variables in both groups were compared with t test. Results showed there were no marked differences in the age, gender, blood uric acid, CRP, left atrial size, left ventricular ejection fraction, blood pressure, blood lipids, coronary heart disease, diabetes, and hypertension ($P > 0.05$). This suggests that both groups match in these factors and results may not biased by these confounding factors (**Table 4**).

Peripheral blood DNA

UV spectrophotometry was done to determine the concentration and purity of genomic DNA extracted from peripheral white blood cells of controls and AF patients, and measurements were done at 260 nm and 280 nm (OD260 and OD280). The ratio of OD260 to OD280 was calculated. The DNA purity and concentration were calculated on the basis of OD260 and OD260/OD280, respectively. Results showed the DNA concentration of samples was 20-50 mg/ml, and OD260/OD280 ranged from 1.8 to 2.0. This suggests that extracted DNA meets the requirements in following experiments. Blood samples were then randomly selected for 2% agarose gel electrophoresis. Results

Table 6. Distributions of alleles and genotypes of cx40 G-44A in controls and patients

G-44A	Genotype/frequency			X^2	P	Allele frequency		X^2	P
	AA	AG	GG			A	G		
AF patients	32/0.39	24/0.29	26/0.32	2.96	0.04	88/0.54	76/0.46	1.98	0.01
Controls	25/0.23	29/0.50	28/0.27			79/0.48	85/0.52		

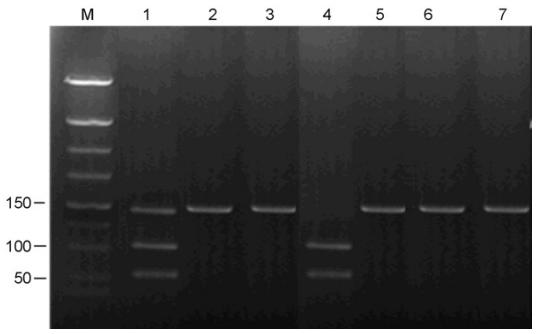

Figure 3. Electrophoresis of AT1R A1166C. AA genotype: 2, 3, 5, 6, 7; AC genotype: 1; CC genotype: 4.

showed the DNA bands were clear, and no other confounding bands were found (**Figure 1**).

Allele frequency and genotypes

After PCR-RLFP, products were subjected to agarose gel electrophoresis (**Figure 2**). Results showed 3 genotypes of cx40 G-44A: AA, AG and GG. Hardy-Weinberg equilibrium test (**Table 5**) showed *P* value was > 0.05, suggesting that the distribution of cx40 G-44A genotypes is in accordance with genetic equilibrium law and samples collected in the present study are representative. The distributions of genotypes and alleles were significantly different between controls and AF patients. The frequencies of genotypes AA, AG and GG were 39%, 29% and 32%, respectively, in AF patients and 31%, 35% and 34%, respectively, in controls; the frequencies of alleles A and G were 54% and 46%, respectively, in AF patients and 48% and 52%, respectively, in controls ($P < 0.05$) (**Table 6**). These findings indicate that AF patients are more likely to have allele A. Logistic regression analysis showed allele A increased the risk for AF by 1.31 times (OR = 1.31, $P < 0.05$).

After PCR-RLFP, products were subjected to agarose gel electrophoresis (**Figure 2**). Results showed 3 genotypes of AT1R A1166C: AA, AC and CC (**Figure 3**). Hardy-Weinberg equilibrium test (**Table 7**) showed the distribution frequency of AT1R A1166C genotypes possessed genetic stability and met the genetic equilibrium law ($P > 0.05$). This suggests that samples selected into present study are representative. Significant differences were observed in the distribution of genotypes and alleles between controls and AF patients. The frequencies of AA, AC and CC were 88%, 8% and 4%, respectively, in AF patients, and 93%, 6% and 1%, respectively, in controls; the frequencies of alleles A and C were 92% and 8%, respectively, in AF patients and 96% and 4%, respectively, in controls ($P < 0.05$) (**Table 8**). AF patients are more likely to have allele C. Further logistic regression analysis showed allele C increased the risk for AF by 1.43 times (OR = 1.43, $P < 0.05$).

Discussion

The pathogenesis of AF has involvement of trigger and maintenance of AF. The multiple wavelet reentry theory and atrial remodeling theory have been accepted by cardiac electrophysiologists as mechanisms underlying the pathogenesis of AF. Connexins are intercellular channels and may form ascendant coupling regions. Cxs play important roles in the electrical remodeling and structural remodeling of the atrium [24, 25]. Animal studies found Cx43 and Cx40 gene therapy were able to inhibit the electrical remodeling of the atrium to prevent AF [26]. In rats with Cx40 knock out, atrial conduction velocity reduced significantly, which increased the susceptibility to atrial injury [27, 28]. The differential distribution of Cx40 may increase the difference in the intercellular conduction and then form small reentries, which is helpful for the onset and maintenance of AF [29]. In 2003, Groenewegen et al found that polymorphisms within regulatory regions of the gene for the atrial-specific gap junction protein connexin40 (Cx40) at nucleotides -44 (G-->A) and +71 (A-->G) were closely related to arrhythmia [30]. Thereafter, other studies confirm that the SNPs of cx40-44 may significantly reduce cx40 protein expression and increase the risk for AF [31]. The present study was undertaken on the basis of our epidemiological study and results

Table 7. Hardy-Weinberg equilibrium test of allele frequency of AT1R A1166C in controls and AF patients

Groups		Genotypes			χ^2	P
		AA	AC	CC		
AF patients	Actual number	72	7	3	14.13	0.09
	Actual frequency	0.88	0.08	0.04		
	Theoretical number	71.37	10.26	0.37		
	Theoretic frequency	0.87	0.13	0.00		
Controls	Actual number	76	5	1	5.28	0.06
	Actual frequency	0.93	0.06	0.01		
	Theoretical number	75.15	6.70	0.15		
	Theoretic frequency	0.92	0.08	0.00		

Table 8. Distributions of alleles and genotypes of AT1R A1166C in controls and patients

A1166C	Genotype/frequency			χ^2	P	Allele frequency		χ^2	P
	AA	AC	CC			A	C		
AF patients	72/0.88	7/0.08	3/0.04	5.01	0.03	151/0.92	13/0.08	5.37	0.04
Controls	76/0.93	5/0.06	1/0.01			157/0.96	7/0.04		

showed cx40-40 had three genotypes: AA, AG and GG, and the frequency of allele A in AF patients was significantly higher than in controls (88% vs. 79%). This suggests that the SNPs of cx40-44 are involved in the occurrence and development of AF and they may increase the risk for AF.

RAS is involved in multiple pathophysiological processes of some cardiovascular diseases and plays important roles in the electric remodeling and structural remodeling of the atrium. The bioactivity of RAS is dependent on the binding of AT1R to Ang II [32]. It may activate the ERK of tyrosine kinase pathway and then increase the expressions of collagen and fibrin as well as the proliferation of fibroblasts; it may inhibit the matrix metalloproteinases (MMPs), key enzymes involved in the collage degradation, to suppress the degradation of collagens and promote myocardial fibrosis; it activates L-type and T-type calcium channels in the myocytes and then increases calcium overload (available studies confirm that calcium overload is an important initiator of AF; it may stimulate the aldosterone release, which causes myocyte necrosis and secondary myocardial fibrosis due to repair and further increases AT1 density and the activity of focal Ang II. In 1994, a French investigator Bonnardeaux reported that there was difference in the frequency of SNP of AT1R A 1166-C [33], and it is a SNP of AT1R gene with the closest relationship with clinical diseases. Sarah et al found the SNPs of AT1R A 1166-C were associated with some ventricular arrhythmias [34]. Belenkov et al found that the SNPs of AT1R A1166C could increase the risk for AF [35]. In the present case-control study, the number of AF patients was identical to that of controls. Results showed subjects with genotypes AC and CC of AT1R A 1166-C had increased risk for AF as compared to subjects with genotype AA, and allele C increased the risk for AF, which were consistent with previously findings.

Taken together, on the basis of our epidemiological study conducted in 18 towns of Chongming, Shanghai, this case-control study was undertaken, in which the number of patients was identical to that of controls. Our results demonstrated that the SNPs of cx40-44 and AT1R-1166 were closely related to AF, and cx40-44 A and AT1R1166C significantly increased the risk for AF.

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Disclosure of conflict of interest

None.

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