Association between -44G/A and +71A/G polymorphisms in the connexin 40 gene and atrial fibrillation in Uyghur and Han populations in Xinjiang, China

J. Hailati*, Y.C. Yang*, L. Zhang*, P.Y. He, M. Baikeyi, W. Muhuyati and Z.Q. Liu

Heart Center, First Affiliated Hospital, Xinjiang Medical University, Urumqi, China

*These authors contributed equally to this study.
Corresponding authors: W. Muhuyati / Z.Q. Liu
E-mail: muhuyati_dr@163.com / zqiangliu@126.com

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ABSTRACT. We aimed to elucidate the association between connexin 40 (Cx40) genetic polymorphisms and atrial fibrillation (AF) in a Chinese population in Xinjiang comprising Uyghur and Han individuals. We enrolled 275 Uyghur and 305 age- and gender-matched Han subjects, and used polymerase chain reaction to detect single nucleotide polymorphisms (SNPs; -44G/A and +71A/G) in the gene encoding Cx40. A mutation screening was performed by direct sequencing and calculation of genotype and allele frequencies among AF patients and control subjects to determine the relationship between these variants and this condition in Uyghur and Han populations. The two SNPs examined were significantly associated with AF in both

ethnic groups. Further analysis showed the SNPs to be in perfect linkage disequilibrium in both AF and control groups among Uyghur and Han individuals. In both populations -44AA genotype and A allele frequencies among AF patients were significantly higher than those in the control group. In addition, under the dominant model (GG *vs* GA+AA), a significant difference in the distribution of Cx40 -44G/A genotypes was detected between patients and controls. Logistic regression analysis revealed that Cx40 genetic polymorphisms increase AF risk in Uyghur and Han residents of Xinjiang. In conclusion, both the -44G/A and +71A/G variants of the gene encoding this protein are associated with AF in Uyghur and Han populations in northern China.

Key words: Atrial fibrillation; Connexin 40; Gene polymorphism

INTRODUCTION

Atrial fibrillation (AF) is the most common form of sustained clinical cardiac arrhythmia. It is characterized by fast atrial rhythm and uncoordinated atrial mechanical activity (January et al., 2014). Although AF is usually associated with cardiac pathology, including hypertensive heart disease, cardiomyopathy, valvular disease, or atherosclerotic cardiovascular disease, recent and increasing evidence points to an important heritable component, with significant genetic determinants. Scholars in China and elsewhere have described the mechanism underlying AF at the genetic level, and have established ethnic differences in the characteristics of genes encoding sodium channels, nitric oxide synthase, angiotensinogen, and gap junction protein 40, thereby revealing polymorphisms related to this condition. It has been suggested that a common single nucleotide polymorphism (SNP) in the promoter of the gap junction protein connexin 40 (Cx40) *GJA5* affects its activity and influences AF risk (Wirka et al., 2011).

Cx40, together with Cx43, is responsible for the electrical coupling of atrial cardiomyocytes. Several SNPs of the gene encoding Cx40 lead to abnormal connexin localization and impaired gap junction channels (Sun et al., 2014). This aberration may result in the development of AF. Two SNPs have been identified in *GJA5* in the immediate upstream promoter region (-44G/A) and the untranslated region (+71A/G; Groenewegen et al., 2003). Some studies have suggested that these SNPs are robustly associated with AF in populations of European ancestry.

Epidemiological investigations have shown that the incidence of AF is 0.77% in China (Hu and Sun, 2008); however, among Uyghur adults of the Xinjiang region of this country, this figure is only 0.25% (Yao et al., 2010). AF incidence in the Uyghur population is distinctly lower than that in other ethnic groups, and their clinical characteristics differ from those of Han Chinese individuals (Mu et al., 2007a,b, 2008). This may be due not only to differences in lifestyle and eating habits, but also in heritable factors affecting AF. However, to date, no reports exist concerning the relationship between the Cx40 gene and AF among Uyghur and Han populations. This study aimed to explore any connection with *GJA5* SNPs among Uyghur and Han patients, and identify potential ethnic differences. Furthermore, this study may provide the theoretical basis for future approaches to the diagnosis and treatment of AF among different ethnic groups in the Xinjiang region.

MATERIAL AND METHODS

Subjects

Informed consent was obtained from all subjects according to the guidelines of the Medical Ethics Committee of the First Affiliated Hospital of Xinjiang Medical University. From January 2012 to January 2014, we recruited 580 patients from this hospital. The AF group consisted of 135 Uyghur (72 men and 63 women, aged 26 to 78 years) and 158 Han (87 men and 71 women, aged 27 to 80 years) patients. The control group included 140 Uyghur (67 men and 73 women, aged 26 to 83 years) and 147 Han (82 men and 65 women, aged 23 to 79 years) individuals with no history or electrocardiographic signs of AF. These subjects were matched to AF patients by ethnicity, age, and gender. AF evaluation was carried out by expert cardiologists according to the standard diagnostic criteria of the ACC/AHA/ESC 2006 guidelines for the management of AF patients (Fuster et al., 2006). Diagnoses were based on the replacement of sinus P waves by rapid oscillations or fibrillatory waves varying in size, shape, and timing, associated with an irregular ventricular response when atrioventricular conduction was intact. Clinical examinations were carried out using rest electrocardiography (ECG) or ambulatory Holter ECG recordings. Exclusion criteria were applied as in our previous study. All volunteers underwent physical examination, routine laboratory tests, ECG, chest X-ray, and echocardiography. In order to exclude subjects with structural heart disease, transthoracic and/or transesophageal echocardiography was performed. Systemic arterial hypertension was defined as a systolic blood pressure ≥140 mmHg and/or a diastolic pressure ≥90 mmHg (European Society of Hypertension-European Society of Cardiology Guidelines Committee, 2003). Participants smoking one cigarette or more per day for more than 1 year were categorized as smokers. Those smoking occasionally or rarely were deemed non-smokers. Drinking was defined as consuming an average 100 mL liquor daily (alcohol concentration above 50%) for more than 1 year; subjects drinking occasionally or rarely were considered to be non-drinkers.

Experimental methods

Biochemical analysis

Venous blood samples (5 mL) were obtained after fasting overnight for at least 10 h, before being centrifuged at 2500 *g* at 4°C for 30 min. Sera were then immediately stored at -80°C until analysis. Measurement of total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C), triglycerides (TG), glucose, and uric acid was performed using standard methods in the Clinical Laboratory Department of the First Affiliated Hospital of Xinjiang Medical University.

Genomic DNA isolation

Peripheral whole blood samples were collected from participants after overnight fasting and stored in vacuum tubes containing 0.4% ethylenediaminetetraacetic acid as an anticoagulant. Genomic DNA was isolated and purified from these samples using a genomic DNA extraction kit (TIANGEN Biotech Corporation, Beijing, China). Extracted DNA was stored at -20°C until use.

SNP genotyping

The primers used are shown in Table 1. Each 50-μL polymerase chain reaction (PCR) included the following reagents: 25 μL 2X Es *Taq* master mix, 1.25 μL forward primer (10 pg/μL), 1.25 μL reverse primer (10 pg/μL), 4 μL template DNA (50 ng/μL), and RNase-free water up to 50 μL. Reactions were thoroughly mixed by slight agitation and amplification was carried out on an Applied Biosystems (Foster City, CA, USA) 2720 thermal cycler.

Table 1. Primers used in connexin 40 (Cx40) -44G/A and +71A/G polymerase chain reactions (PCRs).

Polymorphism	Primer sequences	Annealing temperature (°C)	PCR product (bp)
Cx40 -44G/A	P1: 5'-TGAGGACAAGGACAACAGGCA G-3'	60	400
	P2: 5'-CTTCCTCTGGCTACTTCATATC-3'		
Cx40 +71A/G	P1: 5'-GCTAAAGTCCAGGAAGAG-3'	49.6	200
	P2: 5'-TTGGGAATGAGATAGTTT-3'		

PCR conditions for the Cx40 -44G/A SNP were as follows: initial denaturation at 95°C for 3 min, 34 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 1 min, then a final extension at 72°C for 5 min. The same conditions were used for the +71A/G SNP, but with an annealing temperature of 49.6°C and 30 amplification cycles.

PCR products were stored at 4°C. To determine the success of PCR amplification, products (10 μL) were electrophoresed on a 1% gel. In addition, each PCR sample (40 μL) was sent to Sangon Biotech Co., Ltd. (Shanghai, China) for direct sequencing.

Statistical analysis

Statistical Package for the Social Sciences version 19.0 (IBM Corp., Armonk, NY, USA) was used for statistical analysis. Quantitative data are reported as means ± standard deviations. Differences between AF patients and control subjects were analyzed by the Student *t*-test, and the chi-square test was employed for count data. Hardy-Weinberg equilibrium (HWE) was examined using the chi-square test. Multiple-logistic regression analysis was performed to analyze risk factors among the different groups. P values <0.05 were considered statistically significant.

RESULTS

Characteristics of the study participants

Differences in basic clinical characteristics between Uyghur and Han individuals are shown in Table 2. No statistically significant differences in age or gender were found between the case and control groups ($P > 0.05$). Among Uyghur subjects, AF patients exhibited increased left atrial dimension (LAD) and TC and LDL-C levels ($P < 0.05$) compared to the controls, whereas other factors did not significantly differ between the two groups ($P > 0.05$). Within the Han study group, of the variables examined, there were significant differences in hypertension rate, LAD, TC and LDL-C levels ($P < 0.05$) between AF patients and controls.

Table 2. Comparison of the basic clinical features of Uyghur and Han individuals.

	Uyghur			Han		
	Control (N = 140)	AF (N = 135)	P value	Control (N = 147)	AF (N = 158)	P value
Age (years)	51.51 ± 11.718	53.26 ± 12.539	0.232	53.25 ± 12.019	54.11 ± 12.096	0.536
Male/female	67/73	72/63	0.399	82/65	87/71	0.909
Hypertension	32 (22.9%)	41 (30.4%)	0.174	28 (19.0%)	48 (30.4%)	0.025*
Drinkers	55 (39.3%)	57 (42.2%)	0.626	55 (37.4%)	64 (40.5%)	0.639
Smokers	55 (39.3%)	51 (37.8%)	0.806	68 (46.3%)	74 (47.1%)	0.909
HR (bpm)	76.04 ± 10.407	76.76 ± 11.439	0.645	72.43 ± 13.529	75.16 ± 13.344	1.360
SBP (mmHg)	118.00 ± 9.795	119.85 ± 12.327	0.244	121.93 ± 12.689	118.97 ± 14.193	0.124
DBP (mmHg)	74.35 ± 6.820	75.68 ± 8.951	0.244	74.26 ± 10.472	74.49 ± 10.903	0.879
LAD (mm)	32.384 ± 2.387	35.941 ± 4.846	0.000*	34.358 ± 2.863	36.099 ± 4.024	0.001*
Uric acid (μM)	351.876 ± 56.90	366.726 ± 96.45	0.189	408.907 ± 65.38	388.334 ± 85.76	0.059
FBG (mM)	5.290 ± 0.836	5.497 ± 1.265	0.176	5.468 ± 1.460	5.862 ± 1.467	0.059
TG (mM)	1.436 ± 0.636	1.577 ± 0.759	0.160	1.717 ± 0.669	1.613 ± 0.576	0.242
TC (mM)	3.469 ± 1.114	3.946 ± 1.123	0.003*	4.086 ± 0.907	4.897 ± 1.348	0.000*
HLD-C (mM)	1.309 ± 0.326	1.244 ± 0.473	0.264	1.317 ± 0.267	1.257 ± 0.277	0.121
LDL-C (mM)	2.472 ± 0.793	2.878 ± 0.516	0.000*	2.652 ± 0.919	3.006 ± 1.189	0.020*
Albumin (g/L)	46.26 ± 6.264	45.50 ± 6.635	0.406	45.29 ± 5.657	44.12 ± 6.988	0.198
BMI (kg/m^2)	27.00 ± 4.28	25.95 ± 3.48	0.183	25.01 ± 3.26	24.68 ± 3.85	0.622

AF = atrial fibrillation; HR = heart rate; SBP = systolic blood pressure; DBP = diastolic blood pressure; LAD = left atrial diameter; FBG = fasting blood glucose; TG = triglycerides; TC = total cholesterol; HLD-C = high-density lipoprotein cholesterol; LDL-C = low-density lipoprotein cholesterol; BMI = body mass index. *Significantly different compared to the control group.

PCR amplification

A gel image of the PCR products obtained is shown in Figure 1. The DNA bands were clear and no non-specific bands were observed, suggesting that the amplification conditions were optimal and in line with the requirements of the experiment.

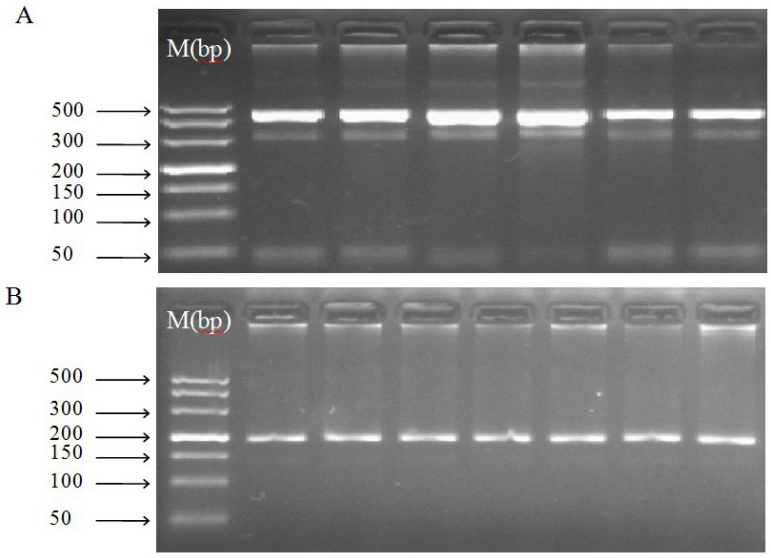

Figure 1. Electrophoresis of connexin 40 (**A**) -44G/A and (**B**) +71A/G polymerase chain reaction products.

HWE test

As shown in Table 3, the genotype distributions of both SNPs were consistent with HWE in the AF and control groups, suggesting that the study sample in the present article was representative of the general population.

Table 3. Hardy-Weinberg equilibrium test of connexin 40 -44G/A genotype frequencies in control and atrial fibrillation (AF) groups.

Genotype	Controls				AF patients			
	Observed number	Expected number	Chi-square	P value	Observed number	Expected number	Chi-square	P value
Uyghur			58	53.29			1.21	0.27
GG	62	57.25			34	30.82		
GA	55	64.55			61	67.37		
AA	23	18.20			40	36.81		
Han			2.64	0.11			1.37	0.24
GG	58	53.29			36	32.35		
GA	61	70.42			71	78.29		
AA	28	23.29			51	47.36		

Cx40 -44G/A and +71A/G genotype and allele frequencies

The -44G/A polymorphism has three possible genotypes (GG, GA, and AA; Figure 2), as does the +71A/G variant (AA, AG, and GG; Figure 3). Pairwise linkage disequilibrium analysis revealed that in Uyghur and Han AF and control groups, these Cx40 polymorphisms were in perfect linkage (D' = 1, $r^2 = 1$). All subjects with allele G at position -44 carried allele A at +71, and those with allele A at position -44 carried allele G at +71, resulting in the genotype combinations -44AA/+71GG, -44GG/+71AA, and -44GA/+71GA. Due to the perfect linkage disequilibrium between these two variants, only the -44G/A SNP was examined further in this study.

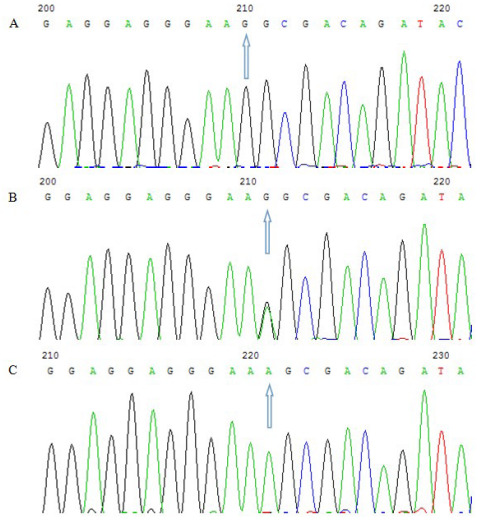

Figure 2. Connexin 40 -44G/A polymorphism sequencing results. **A.** Wild-type GG genotype; **B.** heterozygous GA genotype; **C.** homozygous AA genotype. The arrow indicates the affected residue.

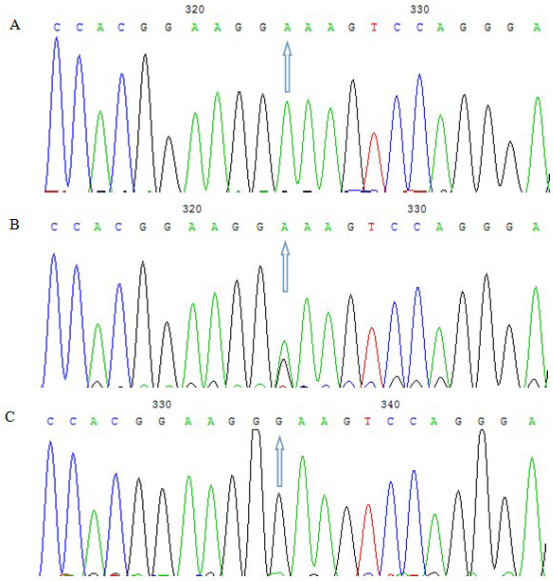

Figure 3. Connexin 40 +71A/G polymorphism sequencing results. **A.** Wild-type AA genotype; **B.** heterozygous GA genotype; **C.** homozygous GG genotype. The arrow indicates the affected residue.

Within the Uyghur group, the distribution of genotypes and alleles significantly differed between controls and AF patients. GG, GA, and AA genotype frequencies were 25.2, 45.2, and 29.6%, respectively, among AF patients and 44.3, 39.3, and 16.4%, respectively, in the control group. The G and A alleles were carried by 47.8 and 52.2%, respectively, of AF patients and 63.9 and 36.1%, respectively, of control subjects ($P < 0.05$). Among Uyghur individuals, the -44AA genotype and A allele were significantly more common in the AF group than the control group. Genotype analysis using several genetic models showed that A allele carriers (GA+AA) had a 2.357-fold increased risk of AF compared to subjects with the wild-type GG genotype [$P = 0.007$, odds ratio (OR) = 2.357, 95% confidence interval (CI) = 1.293-4.298; Table 4]. The frequency of the Cx40 -44A/+71G haplotype was significantly higher among AF patients than control subjects ($P = 0.005$, OR = 1.621, 95%CI = 1.153-2.281).

Table 4. Distribution of connexin 40 -44G/A genotypes and alleles.

	Uyghur					Han				
	Control [N (%)]	AF [N (%)]	Chi-square	OR (95%CI)	P	Control [N (%)]	AF [N (%)]	Chi-square	OR (95%CI)	P
Genotype										
GG	62 (44.3)	34 (25.2)	7.09		0.029*	58 (39.5)	36 (22.8)	12.22		0.002*
GA	55 (39.3)	61 (45.2)				61 (41.5)	71 (44.9)			
AA	23 (16.4)	40 (29.6)				28 (19.0)	51 (32.3)			
Dominant model										
GG	62 (44.3)	34 (25.2)	7.99	2.357 (1.293-4.298)	0.007*	58 (39.5)	36 (22.8)	6.69	2.232 (1.208-4.124)	0.015*
GA+AA	78 (55.7)	101 (74.8)				89 (60.5)	122 (77.2)			
Recessive model										
AA	23 (16.4)	40 (29.6)	5.53	0.444 (0.224-0.881)	0.028*	28 (19.0)	51 (32.3)	4.45	0.498 (0.259-0.957)	0.051*
GG+GA	117 (83.6)	95 (70.4)				119 (81.0)	107 (67.7)			
Co-additive model										
GA	55 (39.3)	61 (45.2)	0.74	0.781 (0.445-1.372)	0.474	61 (41.5)	71 (44.9)	0.18	0.885 (0.506-1.548)	0.776
GG+AA	85 (60.7)	74 (54.8)				86 (58.5)	87 (55.1)			
Allele frequency										
G	179 (63.9)	129 (47.8)	7.74	1.621 (1.153-2.281)	0.005*	177 (60.2)	143 (45.3)	13.65	1.83 (1.326-2.525)	0.0002*
A	101 (36.1)	141 (52.2)				117 (39.8)	173 (54.7)			

AF = atrial fibrillation; OR = odds ratio; CI = confidence interval. *Significantly different compared to the control group.

In the Han study population, the distribution of genotypes and alleles was also significantly different between controls and AF patients. The frequencies of GG, GA, and AA genotypes were 22.8, 44.9, and 32.3%, respectively, in the AF group and 39.5, 41.5, and 19.0%, respectively, among controls. G and A allele frequencies were 45.3 and 54.7%, respectively, among AF patients and 60.2 and 39.8%, respectively, in the control group ($P < 0.05$). The prevalence of the -44AA genotype was significantly higher in the AF group than the control group, as was that of the A allele. Using genotype analysis under several genetic models, A allele carriers (GA+AA) were found to be at a 2.232-fold increased risk of AF compared with individuals carrying the wild-type GG sequence ($P = 0.015$, OR = 2.232, 95%CI = 1.208-4.124; Table 4). Haplotype analysis revealed the frequency of Cx40 -44A/+71G to be significantly higher in the AF group than in the control group ($P = 0.0002$, OR = 1.83, 95%CI = 1.326-2.525).

No significant difference was detected between the Uyghur and Han AF groups in the distribution of genotypes (chi-square = 0.34, $P = 0.84$) or alleles (chi-square = 0.37, $P = 0.54$).

After using an unconditional logistic regression model to adjust for AF risk factors (age, gender, tobacco and alcohol consumption, blood pressure, fasting blood glucose, TG, TC, HDL-C, and LDL-C), our results suggested that Cx40 genetic polymorphisms increase AF risk in both Uyghur and Han populations (Table 5).

Table 5. Logistic regression analysis.

	Uyghur						Han					
	B	SE	Wald	P	OR	95%CI	B	SE	Wald	P	OR	95%CI
Dominant model	1.177	0.467	6.368	0.012*	3.246	1.301-8.100	0.807	0.314	6.599	0.010*	2.240	1.211-4.145
Hypertension	1.693	0.582	8.477	0.004*	5.438	1.739-17.001	-0.306	0.487	0.395	0.530	0.736	0.283-1.914
Smoking	-0.018	0.427	0.002	0.967	0.982	0.426-2.267	-0.157	0.353	0.198	0.656	0.855	0.428-1.706
Drinking	-0.588	0.425	1.911	0.167	0.555	0.241-1.278	-0.154	0.347	0.196	0.658	0.857	0.434-1.693
LAD	0.522	0.095	30.130	0.000*	1.685	1.399-2.030	0.000	0.073	0.000	0.998	1.000	0.867-1.154
TC	0.533	0.169	9.910	0.002*	1.704	1.223-2.375	0.583	0.141	17.044	0.000*	1.791	1.358-2.362
LDL-C	0.627	0.261	5.759	0.016	1.872	1.122-3.125	0.267	0.153	3.055	0.080	1.305	0.968-1.760

SE = standard error; OR = odds ratio; CI = confidence interval; LAD = left atrial dimension; TC = total cholesterol; LDL-C = low-density lipoprotein cholesterol. *Significantly different compared to the control group.

DISCUSSION

In this study, we found that Cx40 gene polymorphisms were correlated with AF among both Uyghur and Han subjects. This is the first investigation to test the association between AF and Cx40 SNPs in the Chinese population.

In the heart, fast and coordinated propagation of cardiac action potentials is mediated by intercellular gap junction channels constructed from membrane-spanning protein subunits called connexins, which are crucial for the conduction of electrical impulses. Connexins are primarily located in the intercalated discs at end-to-end intercellular connections (Severs, 1990). The presence of an arrhythmogenic substrate and initiating triggers is known to determine vulnerability to heart arrhythmias. Initiating triggers of AF most often originate from firing foci in the pulmonary veins and/or superior cava vein (Chaldoupi et al., 2009). Long-term AF gives rise to electrical and structural remodeling that favor the reoccurrence or perpetuation of the condition. Electrical remodeling associated with AF also induces changes in the effective refractory period. Modifications to gap junctions and connexins have also been reported as part of such alterations in AF pathology.

Previous studies have suggested that the Cx40 gene -44G/A and +71A/G polymorphisms are associated with AF in people of European ancestry (Firouzi et al., 2004). The present study showed that these two variants are in perfect linkage disequilibrium, consistent with previous research. Such non-random association at this locus is likely due to the short distance between these two alleles.

In our study, we found that compared with the wild-type GG genotype, GA+AA genotypes showed 2.357- and 2.232-fold increased risk of AF among Uyghur and Han participants, respectively. These findings suggest a strong association between Cx40 polymorphisms and susceptibility to AF in the Uyghur and Han populations of Xinjiang.

Previously published data suggest that Cx40 is mainly expressed in the atria and the cardiac conduction system. Increased atrial vulnerability and propensity to arrhythmias have been reported in Cx40-deficient mice (Hagendorff et al., 1999; Verheule et al., 1999). Using the conscious goat model, van der Velden et al. (2000) found that Cx40 gap junction remodeling may be related to the pathogenesis of sustained AF, and observed decreased Cx40 distribution and quantity during this condition in the goat atrial appendage. However, contrary to these results, some researchers have reported a 2.7-fold increase in Cx40 expression in the atria of human AF sufferers (Polontchouk et al., 2001). Moreover, in another human open-heart surgery study, it was also suggested that conduction velocity during sinus rhythm and atrial pacing is reduced owing to an increase in the proportion of Cx40 (Dupont et al., 2001). Therefore, Cx40 genetic variations may play an important role in promoting AF (Dbouk et al., 2009).

Groenewegen et al. (2003) originally found that two closely linked polymorphisms in the promoter region of the gene encoding Cx40, -44G/A and +71A/G, were strongly associated with familial atrial standstill, and the occurrence of these SNPs in the general population was estimated to be approximately 7%. Firouzi et al. (2004) were the first to connect susceptibility to AF to these Cx40 promoter variations. The study of Hauer et al. (2006) indicated that these polymorphisms are associated with the atrial electrophysiological substrate favoring reentrant mechanisms for initiation of AF. Meanwhile, researchers from Taiwan, China (Juang et al., 2007), demonstrated a significant association between these two SNPs and AF, and suggested that the Cx40 -44A/+71G haplotype is associated with elevated risk of this condition. The present study was undertaken on the basis of our previous epidemiological investigation. After multivariate adjustment, a significant difference between Cx40 polymorphism genotypes in terms of AF risk remained evident. This demonstrates that such SNPs may be involved in the occurrence and development of AF, and may increase susceptibility to this condition among both Uyghur and Han populations in the Xinjiang region of China.

However, Wirka et al. (2011) reported that the -44G/A promoter SNP does not influence Cx40 expression or AF risk. In contrast to our results, they demonstrated a lack of association between this variant and AF with an effect size of 1.18 or greater in a population of European ancestry. Such inconsistency could be partially ascribed to ethnicity-specific genetic effects or differences in baseline patient characteristics. In summary, in Uyghur and Han populations of Xinjiang, frequencies of the -44AA genotype and A allele were significantly higher among AF patients, suggesting that Cx40 gene polymorphisms are associated with this pathology, and that the A allele may be an AF susceptibility factor. However, whether this allele influences Cx40 levels *in vivo* and the manner in which it affects the function of this protein in AF remain unknown. Further studies are therefore required.

Conflicts of interest

The authors declare no conflict of interest.

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