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H558R polymorphism in *SCN5A* is associated with Keshan disease and QRS prolongation in Keshan disease patients

S. Jiang[1], F.L. Li[2], Q. Dong[2], H.W. Liu[2], C.F. Fang[2], C. Shu[2], H. Cheng[2], J. Cui[2], H.X. Ma[3], D.Q. Chen[4], H. Li[2]

[1]Department of Pharmacology, Institute of Medical Sciences, Shanghai Jiao Tong University School of Medicine, Shanghai, China
[2]Department of Biochemistry and Molecular Biology, Harbin Medical University, Harbin, China
[3]Daqing First Hospital, Da Qing, China
[4]Qiqihar First Hospital, Qiqihar, China

Corresponding author: H. Li
E-mail: lihui@ems.hrbhmu.edu.cn

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ABSTRACT. Keshan disease (KSD), a potentially fatal cardiomyopathy, has very high incidence in some selenium-poor regions of China. KSD may be accompanied with a variety of arrhythmia, which is associated with mutations in the gene coding for cardiac voltage-gated sodium channel (*SCN5A*). The molecular mechanism of KSD is still largely obscure. We aimed to determine the association between the H558R polymorphism of *SCN5A* and KSD. We recruited 71 patients with KSD and 80 geographical region-matched control subjects in our study. Vital sign and electrocardiographic (ECG) measurements were performed for heart rate, systolic pressure, diastolic pressure, PR interval, QT interval, QRS duration, ST-T changes and complete right bundle branch block (CRBBB), and H558R polymorphism was genotyped using the polymerase chain

reaction single-strand conformation polymorphism (PCR-SSCP) method and sequencing. A significant association was found between the H558R polymorphism of exon 12 and KSD. Allele C carriers had a decreased risk for KSD with an odds ratio of 0.332 [95% confidence interval (CI), 0.160-0.692] as well as for QRS prolongation in KSD patients with an odds ratio of 0.089 (95%CI, 0.022-0.361). Our results provide support to the association between H558R polymorphism and the decreased risk for KSD. H558R polymorphism might increase susceptibility to KSD, and *SCN5A* containing the polymorphism might be a predisposing gene for QRS prolongation.

Key words: Keshan Disease, *SCN5A*, Polymorphism, H558R, QRS Duration

INTRODUCTION

Keshan disease (KSD), an endemic cardiomyopathy, was named after the outbreak of the disease in Keshan, Heilongjiang, China. Low-selenium (Flores-Mateo et al., 2006; Bellinger et al., 2009) and Coxsackie virus (Ren et al., 2004; Li et al., 2009) have been reported as two important etiological factors of KSD; however, both cannot fully illuminate the clinical and pathological characteristics of KSD patients. Some complicated symptoms involving the digestive and respiratory tracts in KSD patients, such as abdominal pain, diarrhea, and respiratory infection, cannot be explained by low levels of selenium; nonetheless, they are concordant with the symptoms of Coxsackie virus infections, which cannot explain the cardiac symptoms. Because a single etiological hypothesis cannot satisfactorily explain the variety of characteristics displayed by KSD patients, the complicated etiological hypothesis involving genes was embraced by more and more scientists in recent years (Wei et al., 2011).

Cardiac voltage-gated sodium channel (encoded by *SCN5A*) mainly mediate the upstroke stage of action potential (AP) during electrical excitation of myocardial cells. Previous studies have found that *SCN5A* mutations are linked with a variety of arrhythmic diseases (Priori et al., 2002; Lu and Kass, 2010; Neu et al., 2010) [e.g., BrS, long QT syndrome (LQT), and cardiac conduction disease (CCD)], and loss-of-function mutations in *SCN5A* are correlated to a variety of conductive arrhythmia (Tan et al., 2002; Wang et al., 2002) (e.g., bundle branch block and atrioventricular conduction block). KSD patients may complicate a variety of arrhythmia, and many even escalate to sudden cardiac death (SCD). The ECG features of KSD patients include complete right bundle branch block (CRBBB) (52.83%), ST-T changes (13.21%), and left anterior fascicular block (LAFB) (11.32%). The exact molecular mechanism is still largely obscure. Interestingly, KSD has something in common with Brugada Syndrome 1 (BrS1). They share several similar ECG features [e.g., ST-T changes, bundle branch block, and atrial fibrillation (AF)], and BrS1 also has obvious familial and geographical predisposition (Wong et al., 1992; Nademanee et al., 1997; Alings et al., 1999) as KSD.

Therefore, we hypothesized that *SCN5A* may be a candidate susceptible gene for KSD and KSD-related arrythmia. The histidine-558-to-arginine (H558R) polymorphism (dsSNP: rs1805124) in the *SCN5A* gene is a widely reported single-nucleotide polymorphism (SNP) correlated with the modulating electrophysiological properties of myocardial cells (Tan et al., 2005). Although H558R has been reported to occur in many cardiac disorders, no studies have

systematically evaluated the prevalence of H558R in Chinese patients with KSD. Here, we determined the clinical and genetic characteristics of the H558R polymorphism associated with KSD, which is characterized by CRBBB. The study plays a constructive role in the elucidation of the molecular mechanism underlying KSD.

METHODS

Clinical subjects

Patients involved in the study were recruited between September 2006 and March 2011 from the KSD-affected area of Northeast China (Qiqihaer, Heilongjiang Province, China), and 80 control subjects from the same region were recruited during the same period. Twenty men and 51 women with KSD, defined according to *Diagnostic Criteria of KSD GB17021-1997, China*, were recruited in this study. All subjects were Chinese in origin and the average age was 57.8 ± 8.5 years. The lack of manifestation of any cardiac symptom in these patients before migrating to KSD-affected area was verified by reviewing the data obtained from previous physical examinations. The region-matched control group consisted of 80 subjects with an average age of 59.9 ± 9.3 years and who did not meet the below-described criteria of KSD and did not have a history of hypertension, coronary artery disease, structural heart disease, and other cardiac diseases. All subjects of the region-matched control group were Chinese. Blood pressure and electrocardiography (ECG) measurements of the subjects in the case and control groups were carried out by the same group of practitioners. All experimental subjects gave informed consent.

The criteria for diagnosing KSD are 1) resident of a KSD-affected area for at least 6 months; 2) display any one of the following clinical manifestations: a) cardiac enlargement; b) acute or chronic heart function insufficiency; and c) tachyarrythmia or bradyarrythmia; 3) have any one of the following laboratory manifestations: a) ECG changes, or arrhythmia (multiple ventricular extra-systoles, AF, ventricular or supraventricular tachycardia, ECG changes [right or left bundle branch block, prolonged Q-T, ST-T changes and multiple ventricular extrasystoles]); b) chest x-ray changes; c) ECG changes; d) changes in the cardiac markers involved in myocardial injury, and e) pathological changes; and 4) coronary artery disease, dilated cardiomyopathy, rheumatic cardiac disease, and some other cardiac diseases could be ruled out (Oster et al., 1990).

The research protocol was approved by the Regional Ethics Committee. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki 1975 as reflected in a priori approval by the human research committee of the institution.

Mutation analysis

Two-milliliters of whole blood collected from the AF patients and control subjects were preserved in vacuum blood collection tubes (BD, Franklin Lakes, NJ, USA) with EDTA-K2 and stored in -80°C. Primer pairs for PCR were designed via Primer Premier Version 5.0 software, version 5.0 (PREMIER Biosoft, Palo Alto, CA, USA). Primer pairs (forward: 5'-GGC CTC AGC AGG ACT TCT AT-3' and reverse: 5'-TGT CTG GCG GGT GCT CTA-3') were designed for detecting H558R polymorphism in *SCN5A*. DNA extraction was carried out from 2 ml of fresh whole blood by using the TIANamp Genomic DNA Kit (TIANGEN, Beijing, China), according to manufacturer instruction. DNA purity and concentration were determined by electrophoresis

through an 0.8% agarose gel containing ethidium bromide, followed by visualization under UV illumination, and measurement of absorbance at 260/280 nm (GeneQuant pro RNA/DNA Calculator; GE Healthcare, Piscataway, NJ, USA). Around 100 ng of genomic DNA was used for each PCR reaction by PrimeSTAR® HS DNA Polymerase kit (Takara, Otsu, Japan), according to manufacturer instruction. PCR was performed using GeneAmp® PCR System 9700 (PE Applied Biosystems, Foster City, CA, USA). Sequence variants in PCR-amplified DNA fragments were identified by SSCP as follows. For H558R SSCP analysis, PCR reaction products were diluted 1:3 with loading buffer (95% formamide, 0.05% bromophenol blue, and 0.05% xylene cyanol), denatured at 99°C for 15 min, and cooled on ice for 5 min. Electrophoresis of the denatured PCR products was carried out at 2 W at 4°C for 15 h. PCR-SSCP products were visualized by the standard method of DNA silver staining. One-third of the samples yielding abnormal traces were selected for further analysis by sequencing (Beijing Genomic Institute, Beijing, China), and the results of sequencing analysis indicated that the samples randomly selected from each group were regarded representative of all the samples in the group.

Statistical Analyses

Normal distribution data are reported as means ± standard deviation (SD), and the skewness distribution data are reported as median ± quartile range (QR). Statistical analyses were performed using the SPSS software, version 16.0 (SPSS Inc., Chicago, IL, USA). Clinical data with normal distribution between case and control groups were compared using Student's *t* test, and skewness data were compared using Brown-Mood nonparametric chi square test. Genotype frequencies were compared between the case and control groups by using Fisher's exact test, and the allele frequencies were compared using the Chi-square test. Logistic regression was used to calculate the odds ratios (OR) and 95% confidence intervals (CI) of H558R polymorphism for KSD, ECG characteristics, and CRBBB in KSD patients. The normality of distribution was assessed using the Kolmogorov-Smirnov test. $P < 0.05$ was considered to be statistically significant.

RESULTS

Clinical characteristics of KSD cohort

The baseline characteristics of the 71 KSD patients are shown in Table 1. Around 35.2% subjects have ST-T changes and 49.3% have CRBBB in KSD group, and more subjects showed increased QRS duration in the KSD group compared with the region-matched control group ($P < 0.0001$).

Statistical analyses

H558R polymorphism in SCN5A is associated with KSD and QRS prolongation in KSD patients

Genotype frequencies of H558R polymorphism in KSD patients and control subjects were listed in Table 2 and analyzed for deviation from Hardy-Weinberg equilibrium by using the chi-square test ($P = 0.438$ for case group and $P = 0.546$ for control group). We found that at

least 70 samples were required in the case and control groups with a power of 0.9. Significant differences were observed both in genotypic distribution of the 2 groups by using Fisher's exact test (P = 0.0015) and in allele distribution when applying Chi-square test (P = 0.0164). H558R polymorphism TT genotype had a higher frequency in KSD patients (80.0%) than in the controls (57.5%). Logistic regression analysis showed that TC and CC genotypes (C carriers) were significantly associated with the decreased risk for developing KSD (OR = 0.332; 95%CI = 0.160-0.692; P = 0.003) (Table 2). Table 3 presented the genotypic distribution of two typical ECG characteristics and the arrhythmia-induced CRBBB in the KSD group. The TC and CC genotypes (C carriers) were significantly associated with the decreased risk of developing QRS prolongation (OR = 0.089; 95%CI = 0.022-0.364; P = 0.001) in KSD patients by applying logistic regression analysis. However, the H558R polymorphism was not significantly associated with the changes in ST-T and CRBBB in KSD patients.

Table 1. Clinical characteristics of Keshan disease patients and regional matched control subjects.

Characteristics	Cases (N = 71)	Controls (N = 80)	P
Age (year)	57.8 ± 8.5[a]	59.9 ± 9.3[a]	0.871[c]
Male gender (%)	20 (28.2)	18 (22.2)	0.423[d]
Smoking (%)	16 (22.5)	24 (29.6)	0.300[d]
Alcohol (%)	14 (19.7)	18 (22.2)	0.676[d]
Systolic pressure (mmHg)	130.85 ± 23.42[a]	130.40 ± 18.85[a]	0.995[c]
Diastolic pressure (mmHg)	83.78 ± 10.02[a]	83.48 ± 9.50[a]	0.985[c]
Heart rate	79.26 ± 11.33[a]	78.42 ± 12.67[a]	0.972[c]
PR interval (ms)	160 ± 40[b]	140 ± 20[b]	0.10[c]
QT interval (ms)	360 ± 40[b]	360 ± 80[b]	0.07[c]
QRS duration (ms)	120 ± 60[b]	100 ± 20[b]	<0.0001[c]
QRS prolongation (%)	46 (64.8)	0 (0)	<0.0001[d]
ST-T changes (%)	25 (35.2)	0 (0)	<0.0001[d]
CRBBB (%)	35 (49.3)	0 (0)	<0.0001[d]

[a]means ± SD; [b]median ± QR; [c]Student *t*-Test; [d]chi-square test; [e]Brown-Mood nonparametric χ^2 test.

Table 2. Comparison between H558R genotype frequencies in Keshan disease patients and regional matched control subjects.

	Cases (N = 71)	Controls (N = 80)	OR (95%CI)
T/T	57 (80.3%)	46 (57.5%)	1.0[a]
T/C	11 (15.5%)	32 (40.0%)	0.277 (0.126-0.610)[b]
C/C	3 (4.2%)	2 (2.5%)	0.807 (0.155-4.189)
C carriers	14 (19.7%)	34 (42.5%)	0.332 (0.160-0.692)[c]

[a]Reference group. [b]P < 0.05.

Table 3. Comparisons of normal ECG and abnormal typical Keshan disease (KSD) ECG characteristics or CRBBB in KSD patients with respect to genotypes.

Genotype	ECG characteristics and CRBBB								
	QRS prolongation			ST-T changes			CRBBB		
	-[a]	+[b]	OR (95%CI)	-[a]	+[b]	OR (95%CI)	-[a]	+[b]	OR (95%CI)
T/T	14	43	1.0[c]	37	20	1.0[c]	28	29	1.0[c]
			0.089			1.028			0.724
C carriers	11	3	(0.022-0.364)[d]	9	5	(0.303-3.485)	8	6	(0.223-2.354)

[a]Cases without the ECG characteristics of QRS prolongation, ST-T changes or CRBBB. [b]Cases with the ECG characteristics of QRS prolongation, ST-T changes or CRBBB. [c]Reference group. [d]P < 0.05.

DISCUSSION

SCN5A as a candidate gene for KSD

Mutations in *SCN5A* that encodes the α-subunit of voltage-gated sodium channel have been reported to correlate with a variety of cardiac diseases. Generally, the mutations in *SCN5A* can be divided into two categories. Loss-of-function mutations in *SCN5A* result in BrS1, idiopathic ventricular fibrillation, progressive familial heart block type 1 (PFHB1), and congenital sick sinus syndrome, whereas gain-of-function type mutations in *SCN5A* are associated with congenital long-QT syndrome type 3 (LQTS3) and familial AF (Makiyama et al., 2008). The ECG characteristics of loss-of-function mutation-related cardiac diseases, such as BrS1 and PFHB1, include right bundle branch block (RBBB), CRBBB, and ST-T changes, etc. (Brugada et al., 1992; Gussak et al., 1999; Akai et al., 2000; Kyndt et al., 2001; Olson et al., 2005; Hedley et al., 2009; Amin et al., 2010). Interestingly, they are in accordance with the ECG characteristics of KSD, such as bundle branch block, especially CRBBB, and ST elevation as BrS1. Conclusively, these previous studies raised the possibility that mutations in *SCN5A* could cause KSD and arrhythmia in KSD patients.

Biological effect of the H558R polymorphism in KSD patients

To the best of our knowledge, this is the first report about the SNP H558R in KSD patients, which could be found both in the KSD group and the region-matched control group (Viswanathan et al., 2003; Ye et al., 2003; Poelzing et al., 2006; Gui et al., 2010). The present study hypothesized that the H558R polymorphism of the *SCN5A* gene was a predisposing factor for KSD. An increased frequency of the TT genotype and a decrease in the TC and CC genotypes were observed in the KSD group compared with the region-matched control group. Logistic regression analysis revealed that the TT genotype was significantly associated with the increased risk for developing KSD and the increased risk for QRS prolongation, a typical KSD ECG characteristic, in KSD patients. These results indicated that the genetic susceptibility of an individual to KSD was affected by the specific variant of *SCN5A*, and that H558R could increase the incidence of QRS prolongation in KSD patients.

In our study, CRBBB was the only complicated arrhythmia noted in the subjects with KSD. We assessed the association between the complicated arrhythmia CRBBB in these patients and H558R genotypes by using the chi-square test; however, we found no discriminations ($P = 0.5907$). Logistic regression analysis revealed no association between CRBBB and H558R. Moreover, these patients had no cardiac manifestation before migrating to the KSD-affected areas. Thus, our results suggested that the occurrence of complicated arrhythmia CRBBB in KSD patients did not correlate with the distinct genotypes of H558R, and CRBBB was not a confounding factor in the assessment of the relationship between QRS prolongation and H558R.

The possible association between the H558R polymorphism in *SCN5A* and KSD could be explained in view of cardiac channelopathy. As described earlier, conductive arrhythmia such as atrial-ventricular conduction block and bundle branch block are reported to be associated with loss-of-function mutations in *SCN5A*. For example, bundle branch block characterized by prolongation of QRS duration in PFHB1 and BrS1 patients

has been shown to be associated with the downregulation of the function of cardiac sodium channel caused by mutations in *SCN5A* (Tan et al., 2002; Wang et al., 2002). H558R, which is located in the intracytoplasmic linker connecting the first and second domains of the sodium channel, is one of the most common polymorphisms in *SCN5A*. Its functional significance in modulating the concomitant mutated *SCN5A*-induced change in ion channel property has been shown in previous studies. For example, H558R can upregulate the functions of cardiac sodium channels with loss-of-function mutations, such as R282H (Poelzing et al., 2006) and D1275N (Gui et al., 2010). When the two mutants were coexpressed in human embryonic kidney cells (HEK293) with R558, significantly greater currents were produced than those produced by coexpressing the mutants with H558R. Therefore, some loss-of-function mutations in *SCN5A* or some other genes might bring about bundle branch block characterized by QRS prolongation; however, the accompanying SNP H558R could upregulate the electrophysiological property of the sodium channel to counterbalance the influences caused by loss-of-function mutations.

In conclusion, this study provided some evidence for the role of H558R polymorphism of the *SCN5A* gene in reducing the susceptibility to KSD. Further investigations about the molecular mechanism of KSD, as well as prospective and clinical studies, need to be confirmed.

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