Correlation between \textit{HCN4} gene polymorphisms and lone atrial fibrillation risk

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\textbf{ABSTRACT}

\textbf{Background and objective:} Atrial electrical remodelling (AER) was significantly associated with atrial fibrillation (AF) development. Polymorphisms in hyperpolarization activated cyclic nucleotide gated potassium channel 4 (\textit{HCN4}) gene might be correlated with AER. In the present study, we explored the association of \textit{HCN4} polymorphisms (rs498005 and rs7164883) with lone AF risk in a Chinese Han population.

\textbf{Methods:} In this case–control study, the Sanger sequencing method was utilized to genotype the \textit{HCN4} polymorphisms. Relative risk of AF was assessed by the $\chi^2$ test, and presented by odds ratios (ORs) and corresponding 95% confidence intervals (CIs). Logistic regression analysis was performed for multivariate analysis. The effects of \textit{HCN4} polymorphisms on AF clinical features were analyzed by the Mann–Whitney U test and adjusted by the Bonferroni method.

\textbf{Results:} C allele of rs498005 was significantly correlated with increased risk of AF (OR = 1.412, 95%CI = 1.012–1.970), and the association still exited after adjustment by age, gender, the status of smoking and drinking, histories of diabetes, hyperlipidaemia and myocardial infarction (adjusted OR = 1.473, 95%CI = 1.043–2.081). G allele of rs7164883 SNP was marginally associated with enhanced AF risk after adjustment by the above clinical parameters (adjusted OR = 1.742, 95%CI = 1.019–2.980). Atrial late potential (ALP), including TP (P wave duration after filtering) and LP\textsubscript{20} (the amplitude of superimposed potential in the final 20 ms of P wave) were significantly associated with rs498005 genotype ($p < .001$).

\textbf{Conclusion:} \textit{HCN4} rs498005 and rs7164883 polymorphisms are significantly associated with AF risk.

\section*{Introduction}

Atrial fibrillation (AF) is one of the most common continuous abnormal heart rhythm [1]. The incidence of AF is increased with age [2]. It is characterized by rapid and irregular beating. Hazard of AF for individual healthy not only depends on itself but also the fatal complications [3]. The occurrence of AF is frequently continuous and based on the organic heart diseases [4–6]. AF without the organic heart diseases is defined as lone AF. Initially, it starts as transitory abnormal beating rhythm, along with the time, the rhythm probably becomes longer and constant [7]. Hypertension and a variety of heart diseases are the alterable risk factors for AF, besides, the genetic factors as irrevocable risk factors are also involved in it [8].

Relevant studies have indicated that atrial remodelling is the main pathogenesis for AF. Atrial remodelling includes atrial electrical remodelling (AER), systolic function reconstruction and atrial structural remodelling. Close association is observed between AF and AER [9]. A part of researches show that AER is caused by AF [10,11], while, other studies suggest that AER is the reason for AF onset [12,13]. Another view regards that AF develops secondary to sustaining atrial structural and electrical remodelling which is induced by AF itself [9]. Multiple theories have been proposed to explain the pathogenesis of AF. Sinoatrial node (SA node) of the heart generates the impulse, thereby, could regulate the electrical conduction system of the heart. Potential changes of SA node cells might lead to the abnormal impulse, finally result in arrhythmia including AF.

Hyperpolarization activated cyclic nucleotide gated potassium channel 4 (\textit{HCN4}) is one subtype of HCN family. HCN family could code the funny current (If) pathway which is the necessary element for cardiac pacemaking process [14,15]. Besides, HCNs are also involved in the spontaneous membrane depolarization process of SA node cells during diastole [16]. Stieber et al. found that \textit{HCN4} was overexpressed in embryonic heart, especially in SA node [17]. Mutations in this gene have been proved to be associated with AF [18]. A meta-analysis based on multiple GWAS samples of European population identified that rs7164883 SNP of \textit{HCN4} gene was a susceptibility loci for AF [19]. Furthermore, the single nucleotide polymorphisms (SNPs) in \textit{HCN4} gene (rs498005 and rs7164883) were with the minor allele frequencies more than 0.05 in CHB (Chinese Han in Beijing) population. Thus, we hypothesize that the two SNPs might be associated with AF in Chinese Han population.
In the present study, we explored the genetic association of rs498005 and rs7164883 polymorphisms with AF susceptibility in a Chinese Han population.

Materials and methods

Participants

Institutional review board of Cangzhou City Central Hospital authorized present study. Study participants have been noticed study process before signing the written informed consent. The study process was consistent with the principles of Helsinki Declaration.

The study subjects were Chinese Han population and recruited from Cangzhou City Central Hospital from January 2014 to January 2016. AF patients were newly diagnosed by two pathologists according to generally acceptable guidelines [20]. Clinical symptoms, electrocardiogram (ECG) and Holter ECG were used to determine AF stages. Only lone AF patients were included in this study. Patients with the histories of hyperthyroidism, severe cardiac valvulopathy, or severe cardiac dysfunction (NYHA class IV) were excluded from present study [21]. AF-free individuals received the same examinations, had sinus rhythm (SR) and without the family history of AF and other cardiac diseases. The case and control groups were matched in age and gender.

Sample collection

About 10 ml peripheral venous blood was collected from the elbow vein of each participant, then, adopted by EDTA-2Na. Blood samples were centrifuged, then leukocytes were collected and stored at −80°C until to use. Genomic DNAs were extracted from leukocytes using the traditional phenol chloroform method.

Genotyping method

HCN4 rs7164883 SNP was amplified by PCR reaction according to previous study [22]. Primers for rs498005 SNP was designed by Primer Premier 5.0. PCR amplification of rs498005 SNP was utilizing the primers of 5'-'ACCCACC TTCCTCTCCA-3' (forward) and 5'-CCACCTGTACGTACCC-3' (reverse). All of the PCR products were sequenced by the Sanger sequencing method.

Statistical analysis

All of the calculations were performed by PASW 18.0 (SPSS, Chicago, IL). The significant level was $p = .05$ (two tailed), while in the multiple testing, it was adjusted by the Bonferroni method. Genotype and allele frequencies were calculated by direct counting. Representativeness of the subjects was analyzed by the Hardy–Weinberg equilibrium (HWE) test. Continuous variables were presented by mean ± SD and assessed by Student’s t test or Mann–Whitney U test. Classified variables were evaluated by the Chi-square test or Fisher’s exact test. Relative risk of AF was estimated by the $\chi^2$ test and revealed by odds ratios (ORs) with corresponding 95% confidence intervals (CIs). Age, gender, smoking, drinking, histories of diabetes, hyperlipidaemia and myocardial infarction were used as the confounding factors to adjust all of the results using the logistic regression method.

Results

Basic and clinical features for subjects

A total of 98 males and 41 females lone AF patients (78 paroxysmal and 61 persistent) were enrolled in the case group with the mean age of 56.12 ± 6.07 years. Controls included 104 males and 42 females, and their mean age was 55.90 ± 5.16 years. Basic features, including age, gender, smoking status and alcohol abuse, had no significant difference between case and control groups (Table 1, $p > .05$ for all). Hyperlipidaemia history exhibited significantly high frequency among AF patients ($p = .035$). Statistical significance were discovered in DBP (diastolic blood pressure), LAD (left atrium diameter), ALP (atrial late potential), including TP (P wave duration after filtering) and LP20 (the amplitude of superimposed potential in the final 20 ms of P wave), between cases and controls (Table 1, $p < .05$ for all). We also found that the value of left ventricular ejection fraction (LVEF) had no significant difference between case and control groups (61.70 ± 7.88 in cases and 62.07 ± 6.56 in controls, $p = .800$).

Association of HCN4 polymorphisms with lone AF risk

Genotype and allele distributions of HCN4 polymorphisms did not deviate from the HWE test (Table 2, $p > .05$), demonstrating the study subjects had well goodness of fit for general population.

| Table 1. Basic and clinical features of the study subjects. |
|-------------------------------- |---------------------------------- |---------------------------------- |---------------------------------- |
| Features                          | Case, $n = 139$ (%)               | Control, $n = 146$ (%)            | $p$                                |
|---------------------------------- |---------------------------------- |---------------------------------- |---------------------------------- |
| Basic features                    |                                   |                                   |                                   |
| Age (mean ± SD, year)            | 56.12 ± 6.07                      | 55.90 ± 5.16                      | .861                              |
| Gender (male)                    | 98 (70.50)                        | 104 (71.23)                       | .892                              |
| Smoking (yes)                    | 35 (25.18)                        | 35 (23.97)                        | .813                              |
| Alcohol abuse (yes)              | 34 (24.46)                        | 27 (18.49)                        | .220                              |
| Paroxysmal/persistent            | 78 (56.12)/61 (43.88)             | –                                 |                                   |
| Disease histories                |                                   |                                   |                                   |
| Diabetes                         | 12 (8.63)                         | 7 (4.79)                          | .194                              |
| Hyperlipidaemia                  | 47 (33.81)                        | 33 (22.60)                        | .035                              |
| Myocardial infarction            | 10 (7.19)                         | 7 (4.79)                          | .393                              |
| Clinical features                |                                   |                                   |                                   |
| SBP (mm Hg)                      | 129.24 ± 10.32                    | 126.36 ± 10.34                    | .207                              |
| DBP (mm Hg)                      | 68.49 ± 6.28                      | 76.38 ± 6.92                      | <.001                             |
| LAD (mm)                         | 50.21 ± 11.51                     | 44.74 ± 6.14                      | .008                              |
| LVDD (mm)                        | 51.68 ± 6.75                      | 54.01 ± 7.55                      | .143                              |
| LVDs (mm)                        | 35.53 ± 3.61                      | 34.93 ± 9.83                      | .758                              |
| LVEF (%)                          | 61.70 ± 7.88                      | 62.07 ± 6.56                      | .800                              |
| ALP                              |                                   |                                   |                                   |
| TP (ms)                          | 130.95 ± 24.04                    | 90.60 ± 18.16                     | <.001                             |
| LP20 (μV)                        | 2.54 ± 0.85                       | 7.43 ± 1.44                       | <.001                             |

Notes: SBP: systolic blood pressure; DBP: diastolic blood pressure; LAD: left atrium diameter; LVDD: left ventricular end-diastolic diameter; LVDs: left ventricular end-systolic diameter; LVEF: left ventricular ejection fraction; ALP: atrial late potential; TP: P wave duration after filtering; LP20: the amplitude of superimposed potential in the final 20 ms of P wave.
TC and CC genotypes of HCN4 rs498005 SNP were more frequently discovered in AF patients, and the CC genotype was marginally associated with increased AF risk. However, the association had no significant alteration if adjusted by confounding factors. Besides, the rs498005 C allele was significantly correlated with enhanced AF risk ($p = 0.042$, OR = 1.412, 95%CI = 1.012–1.970) (Figure 1(A)). Association between rs498005 C allele and AF susceptibility was increased, when adjusted by confounding factors ($p = 0.028$, adjusted OR = 1.473, 95%CI = 1.043–2.081). Although the GA and GG genotypes of rs7164883 SNP had higher frequencies in cases than that in controls (Figure 1(B)), but they had no significant association with the AF risk both in crude and adjusted results (Table 2, $p > 0.05$). Marginally positive association has been observed between rs7164883 C allele and lone AF risk ($p = 0.054$, OR = 1.669, 95%CI = 0.987–2.825). When adjusted by confounding factors, association of rs7164883 C allele and lone AF risk was becoming significant ($p = 0.043$, adjusted OR = 1.742, 95%CI = 1.019–2.980).

**Impacts of rs498005 genotypes for AF clinical features in lone AF patients**

In order to certify the association of HCN4 polymorphisms with AF risk, we explored the effects of rs498005 genotypes on DBP, LAD and ALP (TP and LP20) (Table 3 and Figure 2). Genotypes of rs498005 SNP had no distinct impacts for DBP and LAD ($p > 0.05$). AF patients carried rs498005 CC genotype had significantly higher TP level in comparison with TT carriers (144.39 ± 34.93 ms versus 118.37 ± 15.40 ms, $p < 0.01$). LP20 levels were 3.12 ± 0.97 mV in TT carriers, 2.96 ± 0.86 mV in TC carriers and 2.28 ± 0.74 mV in CC carriers. Respectively, compared with the TT and TC carriers, LP20 level was significantly higher in the CC carriers ($p < 0.001$). These results indicated that significant association existed between rs498005 SNP and ALP in AF patients.

**Discussion**

Lone AF is defined as individuals less than 60 years old without the clinical or ECG findings of cardiopulmonary disease (including hypertension) [20]. Multiple risk factors have been confirmed for lone AF, such as cigarette smoking and alcohol consumption [23–25]. However, we failed to find significant differences of smoking status and alcohol abuse between case and controls. The present study showed that DBP, LAD and ALP clinical features were significantly different between cases and controls. When compared with the controls, AF patients had lower DBP level and higher LAD level. In comparison with healthy controls, TP level of ALP was significantly decreased in AF patients. The results were similar with a previous study. Masuda and colleagues found a close association between ALP and the AF recurrence, the voltage was decreased and P wave duration was increased in the patients with AF recurrence [26]. Besides, genotype and allele distributions of HCN4 polymorphisms did not deviate from the HWE test. All the data revealed that the case group

| SNP      | Case, n = 139 (%) | Control, n = 146 (%) | $p$     | OR (95% CI) | $p^a$ | OR (95% CI)$^a$ |
|----------|------------------|----------------------|--------|------------|------|----------------|
| rs498005 |                  |                      |        |            |      |                |
| TT       | 41 (29.50)       | 59 (40.41)           | 0.113  | 1.533 (0.903–2.605) | 0.453 | 1.549 (0.494–4.858) |
| TC       | 65 (46.67)       | 61 (41.78)           | 0.068  | 1.826 (0.953–3.500) | 0.138 | 2.632 (0.733–9.452) |
| CC       | 33 (23.74)       | 26 (17.81)           | 0.042  | 1.412 (1.012–1.970) | 0.028 | 1.473 (1.043–2.081) |
| C        | 131 (47.12)      | 113 (38.70)          |        |            |      |                |
| T        | 147 (52.88)      | 179 (61.30)          |        |            |      |                |
| rs7164883|                  |                      |        |            |      |                |
| AA       | 106 (76.26)      | 122 (83.56)          |        |            |      |                |
| GA       | 27 (19.42)       | 22 (15.07)           | 0.274  | 1.413 (0.760–2.626) | 0.719 | 0.792 (0.221–2.833) |
| GG       | 6 (4.32)         | 2 (1.37)             | 0.155  | 3.453 (0.682–17.471) | 0.767 | 1.551 (0.085–28.219) |
| A        | 239 (85.97)      | 266 (91.01)          |        |            |      |                |
| G        | 39 (14.03)       | 26 (8.90)            | 0.043  | 1.669 (0.987–2.825) | 0.043 | 1.742 (1.019–2.980) |
| $p_{\text{HWE}}$ | 0.467 | 0.149 | |

**Table 2. Association of HCN4 polymorphisms with AF risk.**

*Adjusted by age, gender, smoking, drinking, histories of diabetes, hyperlipidaemia and myocardial infarction. Bold values had statistical significance.*
had the basic characteristics of AF, and the study subjects had well representativeness for general population. Thus, the results obtained in our study were credible.

HCN belongs to voltage-gated cation channel family, including four subunits (HCN1-4). In the atrial tissues, HCN4 is the main subunit. Voltage dependence and the permeability of K+ and Na+ are the mainly electrophysiological properties of HCN channel. In addition, HCN4 could regulate the funny current. Thus, we considered that the polymorphisms in HCN4 gene might affect the atrial potential, thus influencing the occurrence of AF. We selected two SNPs which were with the minor allele frequencies (MAF) more than 0.05 in the intron region of HCN4 gene to explore the association of HCN4 SNPs with lone AF risk and ALP of the patients.

Higher frequencies of rs498005 TC and CC genotypes have been discovered in AF patients, despite no statistical significance. C allele of rs498005 SNP was significantly correlated with 1.412 times increased risk of AF. After adjusting to the confusing factors, the significant association still existed. No significant difference existed in rs7164883 genotypes between case and control subjects. TP level of ALP was significantly increased in CC genotype carriers when compared with TT carriers, but had no significant difference with TC carriers. The LP20 level was statistical decreased in CC genotype carriers, respectively, compared with the TT and TC carriers. It was suggested that rs498005 C allele was positively associated with the TP level and negatively associated with LP20 level in lone AF patients. All the results demonstrated that genotypes and alleles of rs498005 polymorphism could influence the clinical parameters of AF, suggesting the functional roles of rs498005 in development of AF. As is well known, HCN family plays an important role in controlling neuronal and cardiac excitability. HCN4 serves as a pacemaker channel, which could maintain periodicity of contractions in vertebrate hearts [29]. Rs498005 SNP might influence the activity of HCN4 gene, thus, contributing to initiation of AF. The hypothesis required further verification by animal experiments. However, limited by the short study duration, the animal experiments were not performed in our study. Further analyses are still in urgent need.

Limitations in present study should be recognized. First of all, the sample size was not large enough to obtain a higher test power. Second, gene–gene and gene–environment interactions were not considered in current study. Finally, the present results required replicates in other population. Besides, we acquired the association between rs498005 SNP and lone AF risk and clinical features. Unfortunately, the causality between them was still not clear in this retrospective study. Further well designed studies will be carried out to address the above issues.

In conclusion, rs498005 SNP may be involved in development of AF via impacting the ALP. Rs498005 and rs7164883 polymorphisms of HCN4 gene are obviously associated with the risk of lone AF in a Chinese Han population.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Table 3. Impacts of rs498005 genotypes for AF clinical features in AF patients.

| Genotype | LAD (mm)  | DBP (mm Hg) | TP (ms)  | LP20 (µV) |
|----------|-----------|-------------|----------|-----------|
| TT       | 49.76 ± 11.33 | 68.18 ± 5.96 | 144.39 ± 34.93 | 2.28 ± 0.74 |
| TC       | 50.54 ± 11.78 | 68.29 ± 6.31 | 127.83 ± 23.89 | 2.96 ± 0.86 |
| CC       | 48.79 ± 10.64 | 69.85 ± 6.59 | 118.37 ± 15.40 | 3.12 ± 0.97 |

Notes: *: CC versus TT $p < .01$; **: CC versus TT $p < .001$; #: CC versus TC $p < .05$; #: CC versus TC $p < .001$; the significant level was adjusted by the Bonferroni method, $x = .015$.
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