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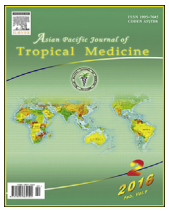

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# E670G polymorphism of *PCSK9* gene of patients with coronary heart disease among Han population in Hainan and three provinces in the northeast of China

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ABSTRACT

**Objective:** To investigate the correlation between E670G polymorphism of proprotein convertase subtilisin/kexin type 9 (*PCSK9*) gene and coronary heart disease (CHD), and contrastively study the regional differences of E670G polymorphism of *PCSK9* gene between patients with CHD among the Han population in Hainan and three provinces in the northeast of China (TPNC), providing scientific basis for prevention and treatment of patients with CHD in different regions.

**Methods:** A total of 233 cases of patients with CHD were selected from the Han population in Hainan and TPNC as the experimental group (118 cases from Hainan, 115 cases from TPNC), and 239 cases with non-CHD were selected among the Han population also in the two regions as control group (125 cases from Hainan, 114 cases from TPNC). The triglyceride (TG), total cholesterol (TC), high density lipoprotein cholesterol and low density lipoprotein cholesterol (LDL-C) levels of plasma were tested and PCR-RFLP method was used to test the E670G polymorphism of *PCSK9* gene. The statistical software package SPSS 21.0 was used for the statistical analysis and $P < 0.05$ was considered as statistically significant.

**Results:** The levels of systolic pressure, diastolic blood pressure, fasting blood sugar, TC, TG, and LDL-C of patients in CHD group were significantly higher than those in non-CHD group, while the high density lipoprotein cholesterol level was lower than that in non-CHD group ($P < 0.05$). In CHD group, the frequencies of AG, GG genotypes of *PCSK9* gene and G allele were higher than those in non-CHD group ($P < 0.05$), and in CHD group, the frequencies of AG, GG genotypes and G allele of patients both in Hainan and TPNC were higher than those in control group ($P < 0.05$). Among the patients with CHD, the frequencies of GG genotype and G allele of patients in Hainan were lower than those in TPNC ($P < 0.05$), and in CHD group, the levels of TG, TC and LDL-C of GG genotype were higher than those of AA genotype ($P < 0.05$). While in non-CHD group, there were no significant differences between the frequencies of GG genotype and G allele of patients in Hainan and TPNC ($P > 0.05$).

**Conclusions:** There was a close correlation between the E670G polymorphism of *PCSK9* gene and CHD with serum lipid level. Among Han population in Hainan and TPNC, the E670G polymorphism of *PCSK9* gene of patients with CHD exhibited regional differences.

## 1. Introduction

The coronary heart disease (CHD) is a common heart disease seriously threatening human health with increasing morbidity and mortality as the changes of social environment and people's living habits. Many researches showed that the CHD is related to various genetic factors, especially the genes involved in lipid metabolism playing a significant role in its occurrence and development. The proprotein convertase subtilisin/kexin type 9 (*PCSK9*) is a newly discovered gene involving in autosomal dominant hypercholesterolemia through a mechanism of that it adjusts the low density cholesterol levels, thereby having an effect on the risk of CHD occurrence [1,2]. Polymorphic site of

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*PCSK9* gene is influenced by the differences of region, race, *etc*. As the discrepancies may exist among distinct regions or races, the risk of CHD may also exhibit differences.

In the present study, the E670G polymorphism of *PCSK9* gene in patients with CHD among Han population in Hainan and three provinces in the northeast of China (TPNC) and the non-CHD people were carried out to identify the correlation between E670G polymorphism and CHD, and to discuss whether there was regional difference in E670G polymorphism among distinct areas and its effects on the serum lipid metabolism.

## 2. Materials and methods

### 2.1. Study objects

A total of 233 cases of patients with CHD were selected from the Han population in Hainan and TPNC as the observation group (118 cases from Hainan, 115 cases from TPNC) with an average age as (63.95 ± 12.35) years old. And 239 cases (non-CHD) having the roughly same condition in gender, age and period of resident, *etc*. were selected among the Han population and the two regions as control group (125 cases from Hainan, 114 cases from TPNC) with an average age as (61.68 ± 13.07) years old. Here, the study objects were the Han nationality.

#### 2.1.1. Inclusion criteria

The diagnostic criteria for CHD were referred to the Coronary Atherosclerotic Heart Disease Diagnosis Standard (WS 319-2010, Health industry standard of the People's Republic of China).

#### 2.1.2. Exclusion criteria

The non-Han population was excluded in this study. The patients with acute or chronic infectious diseases, malignant tumor, autoimmune disease, a family history of hyperlipidemia or other diseases might influence the experiment results were not considered as the study objects. CHD patients without sufficient basic information were also excluded to avoid inaccuracy of the results.

### 2.2. Main reagents and instruments

In the present study, main reagents used in experiments were DNA extraction kit (CWBIO), upstream and downstream primers (Beijing SBS Corporation), 2×EcoTaq PCR SuperMix (+dye) (CWBIO), agarose (Biowest), Ladder Marker (CWBIO), RNase-Free Water (CWBIO), and restriction endonuclease Eam1104I (Fermentas); the main instruments were Biometra PCR (Biometra, Germany), electrophoresis apparatus (Tanon EPS-300, Shanghai Tianneng Technology Co., LTD), nucleic acid analyser (Eppendorf, Germany), fully automatic biochemical analyser (Hitachi 7600, Japan), UV analysis tapping machine (UV-2000, Shanghai Tianneng Technology Co., LTD) and gel image analysis system (Tanon 3500, Shanghai Tianneng Technology Co., LTD).

### 2.3. Methods

#### 2.3.1. Specimen collection

A moderate amount of blood specimens of study objects were collected for the extraction of genomic DNA and the determinations of serum lipid, blood glucose and other biochemical indexes.

#### 2.3.2. DNA preparation of human peripheral blood

The extraction of genomic DNA was conducted strictly complied with the kit introduction. The outside diameter value of DNA was tested approximately at 1.8 (A260 nm/A280 nm), which showed the DNA purity was in accord with PCR requires.

#### 2.3.3. PCR amplification

To find the polymorphic loci gene sequences of E670G of *PCSK9* gene in gene bank, and then the Primer was used for the primer design. Sense primer was 5′-CAC GGT TGT GTC CCA AAT GG-3′. Reverse primer was 5′-GAG AGG GAC AAG TCG GAA CC-3′. The amplified fragment was 440 bp. The PCR primer was synthesized by Beijing SBS Corporation.

The total volume of reactant was 50 μL. Among it, DNA extraction was 10 μL, 2×EcoTaq PCR SuperMix was 13 μL, the upstream and downstream primers were both 2 μL and the rest 23 μL was RNase-free water. These materials were blend and then placed in PCR amplifier for the amplification reaction. The conditions of amplification reaction were as follow: 94 °C for 3 min (initial denaturation); 94 °C for 3 s (denaturation); 55 °C for 30 s (annealing); 72 °C for 30 s (extension); 35 cycles; 72 °C for 5 min (extension); 4 °C (conservation).

Five μL of PCR products were added in 2% agar gel and then conducted to a horizontal electrophoresis for 30 min under a condition of 100 V constant voltages. After that, the imaging was observed by gel imaging system and a fluorescent band occurred in 440 bp was considered as the specific amplifying products.

#### 2.3.4. PCR-RFLP analysis

Enzyme reaction system: a total volume of materials was 25 μL within 10 μL PCR products, 2.5 μL 10 × buffer solution, 0.2 IU restriction endonuclease Eam1104I and 12.3 μL sterilization deionized water. Reaction condition: a warm bath (37 °C) for 8 h was designed. Ten μL enzyme-digested products were added into 2.0% agarose gel and then conducted to an electrophoresis for 30 min with 100 V constant voltages. The band was observed in a gene genius bioimaging system and the experiment results were photographed and recorded. Genotyping: a restriction fragment [(150–440) bp] obtained after restriction endonuclease reaction exhibited two kinds of polymorphism allele (restriction fragment with 400 bp) and A allele (restriction fragments with 150 bp and 290 bp). The PCR products obtained in experiment were sent to the SBS Genetech Co., Ltd for sequencing to confirm the analysis results of restriction endonuclease reaction.

#### 2.3.5. Determination of blood lipid

The determination of blood lipid was carried out by using a fully automatic biochemical analyzer conducted by Inspection Center, the Sanya People's Hospital, Hainan.

### 2.4. Statistical analysis

The data analysis was carried out using statistical software SPSS 21.0. *chi*-Square test was applied for the test of enumeration data; *t*-test was used for the mean comparison of measurement data between two groups; one-way analysis of

variance was applied for the mean comparison of over two groups. For calculation of gene frequency, the gene counting method was utilized and *chi*-square test was also used for testing results and the Hardy–Weinberg equilibrium was used for the detection of representativeness of samples. $P < 0.05$ was considered as statistically significant.

## 3. Results

### 3.1. General conditions and serum lipid levels

The demographics (gender, age, region) in two groups showed a good balance ($P > 0.05$) and there were no significant differences in the number of people smoking and drinking between two groups ($P < 0.05$). The levels of systolic pressure, diastolic blood pressure, fasting blood sugar (FBS), triglyceride (TG), total cholesterol (TC) and low density lipoprotein cholesterol (LDL-C) of patients in CHD group were significantly higher than those in non-CHD group, while the high density lipoprotein cholesterol (HDL-C) level was lower than that in non-CHD group ($P < 0.05$) (Table 1).

There were no significant differences between actual genotype frequency and theoretical frequency of three genotypes in CHD group and non-CHD group ($P > 0.05$), which showed the allele frequency distribution was in accord with Hardy–Weinberg equilibrium (Table 2).

**Table 1**
Comparison of general conditions and serum lipid levels between CHD group and non-CHD group.

| Observable indicator | CHD group | Non-CHD group | $T/\chi^2$ | $P$ value |
|---|---|---|---|---|
| Case ($n$) | 233 | 239 | | |
| Region (Hainan/TPNC) | 118/115 | 125/114 | 0.130 | 0.719 |
| Gender (male/female) | 130/103 | 121/118 | 1.265 | 0.261 |
| Age (years) | 63.95 ± 12.35 | 61.68 ± 13.07 | 1.938 | 0.053 |
| Systolic pressure (mmHg) | 138.39 ± 19.40** | 125.87 ± 10.97 | 8.611 | 0.000 |
| Diastolic pressure (mmHg) | 83.12 ± 10.15** | 77.94 ± 6.72 | 6.511 | 0.000 |
| Smoking (Y/N) ($n$) | 76/157 | 60/179 | 3.247 | 0.072 |
| Drinking (Y/N) ($n$) | 44/189 | 61/178 | 3.006 | 0.083 |
| FBS (mmol/L) | 5.93 ± 1.12** | 5.65 ± 0.47 | 3.558 | 0.000 |
| TC (mmoL/L) | 4.49 ± 0.75* | 4.35 ± 0.75 | 2.075 | 0.039 |
| TG (mmoL/L) | 1.44 ± 0.81** | 1.07 ± 0.45 | 6.148 | 0.000 |
| HDL-C (mmoL/L) | 1.11 ± 0.37** | 1.25 ± 0.31 | –4.447 | 0.000 |
| LDL-C (mmoL/L) | 2.86 ± 0.66* | 2.74 ± 0.62 | 2.163 | 0.031 |

*: Compare to control group, $P < 0.05$; **: Compare to control group, $P < 0.01$.

### 3.2. Dstribution of frequencies of three genotypes and alleles in E670G of PCSK9 gene in CHD group and non-CHD group

The frequencies of AG, GG genotypes in CHD group were higher than those in non-CHD group ($P < 0.01$), and the frequencies of G allele in CHD group were also higher than those in non-CHD group ($P < 0.01$) (Table 3).

### 3.3. Comparison of frequencies of three genotypes and alleles in E670G of PCSK9 gene between CHD groups (Hainan and TPNC) and non-CHD groups (Hainan and TPNC)

The gene frequencies of AG, GG genotypes and G allele in Hainan CHD group were higher than those in Hainan non-CHD group ($P < 0.05$), in TPNC, the gene frequencies of AG, GG genotypes and G allele in CHD group were also higher than those in non-CHD group ($P < 0.01$).

### 3.4. Comparison of frequencies of three genotypes and allele of E670G polymorphism of PCSK9 gene

In CHD group, the frequencies of GG genotype and G allele of patients with CHD in Hainan were both lower than that in TPNC ($P < 0.05$), while in non-CHD group, the frequencies of GG genotype and G allele between people in Hainan and TPNC showed no significant difference ($P > 0.05$) (Table 4).

### 3.5. Comparison between three genotypes of E670G polymorphism in PCSK9 gene and serum lipid levels

In CHD group, the TG, TC and LDL-C levels of GG genotype were higher than those of AA genotype ($P < 0.05$). Although the TG, TC, HDL-C and LDL-C levels between AG and AA, GG and AG showed no significant differences, the TG, TC and LDL-C levels of patients carrying G allele were in a gradually rising trend compared to those of patients with AA genotype (Table 5).

**Table 2**
Genetype distribution of CHD group and non-CHD group detected by Hardy–Weinberg equilibrium law.

| Group | Frequency | Number of people with each genotype ($n$; %) | | | $\chi^2$ | $P$ value |
|---|---|---|---|---|---|---|
| | | AA | AG | GG | | |
| CHD group | Actual frequency | 177 (76.0) | 47 (20.2) | 9 (3.8) | 4.787 | 0.091 |
| | Theoretical frequency | 173 (80.8) | 28 (13.1) | 13 (6.1) | | |
| Non-CHD group | Actual frequency | 215 (90.0) | 21 (8.8) | 3 (1.2) | 2.584 | 0.275 |
| | Theoretical frequency | 213 (93.8) | 13 (5.7) | 1 (0.4) | | |

**Table 3**
Comparison of frequencies of three genotypes and alleles in E670G of the *PCSK9* gene between CHD group and non-CHD group.

| Group | Case | Number of people with each genetype (*n*; %) | | | The number of people with each allele (*n*; %) | |
|---|---|---|---|---|---|---|
| | | AA | AG | GG | A | G |
| CHD group | 233 | 177 (76.0) | 47 (20.2)** | 9 (3.8)** | 401 (86.1) | 65 (13.9)** |
| Non-CHD group | 239 | 215 (90.0) | 21 (8.8) | 3 (1.2) | 451 (94.4) | 27 (5.6) |
| $\chi^2$ | | 16.551 | | | 18.480 | |
| *P* value | | 0.000 | | | 0.000 | |

**: Compare to non-CHD group, $P < 0.01$.

**Table 4**
Distribution of frequencies of three genotypes and allele of E670G polymorphism of PCSK9 gene in two group.

| Group | Region | Case | The number of people with each genotype (*n*;%) | | | The number of people with each allele (*n*;%) | |
|---|---|---|---|---|---|---|---|
| | | | AA | AG | GG | A | G |
| Observation group | Hainan | 118 | 97 (82.2) | 19 (16.1)* | 2 (1.7)* | 213 (90.3) | 23 (9.7)** |
| | TPNC | 115 | 80 (69.6) | 28 (24.3) | 7 (6.1) | 188 (81.7) | 42 (18.3) |
| | $\chi^2$ | | 6.096 | | | 7.036 | |
| | *P* value | | 0.047 | | | 0.008 | |
| Control group | Hainan | 125 | 116 (92.8) | 8 (6.4) | 1 (0.8) | 240 (96.0) | 10 (4.0) |
| | TPNC | 114 | 99 (86.8) | 13 (11.4) | 2 (1.8) | 211 (92.5) | 17 (7.5) |
| | $\chi^2$ | | 2.367 | | | 2.673 | |
| | *P* value | | 0.306 | | | 0.102 | |

*: Compare to TPNC patients with CHD in Observation group, $P < 0.05$; **: Compared to TPNC patients with CHD in Observation group, $P < 0.01$.

**Table 5**
Comparison of three genotypes of *PCSK9* gene E670G polymorphism and serum lipid levels.

| Serum lipid indexes | Serum lipid level of various genotypes (mmoL/L) | | | *P* value | | |
|---|---|---|---|---|---|---|
| | AA (177) | AG (47) | GG (9) | AA/AG | AG/GG | AA/GG |
| T C (mmoL/L) | 4.44 ± 0.76 | 4.58 ± 0.67 | 5.10 ± 0.77 | 0.241 | 0.054 | 0.009 |
| T G (mmoL/L) | 1.40 ± 0.80 | 1.49 ± 0.89 | 1.95 ± 0.09 | 0.473 | 0.124 | 0.048 |
| HDL-C (mmoL/L) | 1.11 ± 0.38 | 1.14 ± 0.35 | 1.04 ± 0.24 | 0.591 | 0.451 | 0.586 |
| LDL-C (mmoL/L) | 2.84 ± 0.89 | 2.50 ± 0.59 | 3.30 ± 0.30 | 0.528 | 0.062 | 0.022 |

## 4. Discussion

The *PCSK9* gene polymorphism is closely related to the cardiovascular and cerebrovascular diseases during its occurrence, development and prognosis. *PCSK9* gene mutations may lead to a change in its normal function and thereby affecting the expressions of a series of metabolite related to it. The *PCSK9* gene polymorphism can result in the lipid metabolism disorder and thus cause the hyperlipidemia which is closely related to the occurrence and development of CHD [3]. CHD has a characteristic of genetic predisposition with different morbidity in distinct nationalities and regions. In population with the same nationality from different areas, the genetic susceptibility of CHD also shows significant difference.

In the present study, the number of patients carrying G allele in CHD group was higher than that in non-CHD group, and either in population from Hainan or TPNC with the significantly statistical difference ($P < 0.05$). The study of Zeng conducted in Sichuan region has revealed that G allele frequency of patients in CHD group is higher than that in non-CHD group, which is consistent with the results of this research [4]. And other present studies have showed G allele frequency of patients with CHD in Japan, the Caucasus, and Guangdong area in our country was between 3.0% and 8.2%, which was lower than that in Africa (26%–37.5%) [5–7]. Our study results revealed that the G allele frequency of E670G in observation group was about 13.9%, also lower than that in Africa. To analyze its causes, it may not only associate with the nationalities but also the regions. Therefore, with consideration of region influence, a comparative study on genotype frequency and allele frequency was conducted in north area and south areas (Hainan, TPNC) and the results showed that in control group (non-CHD), there were no significant differences in GG genotype and G allele frequencies of people between Hainan and TPNC ($P > 0.05$), while in observation group, GG genotype and G allele frequencies of patients with CHD from Hainan were both lower than those from TPNC, there were significant differences ($P < 0.05$). This result possibly correlates with the differences of race, region and environmental condition. China has vast territory, therefore geographical environment in its north and south regions show differences and people also have different living habits. Besides, affected by many external factors and gene characteristics of people in different regions, the gene polymorphisms of people in distinct areas or nationalities would exhibit certain differences. From this study, we also discovered a close correlation between E670G polymorphisms and CHD, resulting different risks of CHD occurrences.

*PCSK9* gene can increase the risk of atherosclerosis by effecting lipid metabolism. It plays a key role in adjusting the

cholesterol level through combining with LDLR, promoting lysosomal degradation, decreasing the uptake of LDL-C and therefore resulting in the increase of LDL-C, thus augmenting the risk of getting CHD [8]. E670G polymorphism of *PCSK9* gene is related to the plasma total cholesterol, apolipoprotein and lipoprotein (A) level [9]. The research of Suet *et al*. has revealed that the E670G mutation of *PCSK9* gene is closely related to the degree of coronary atherosclerosis and can be an independent predictive factor for the increase of LDL-C level, which up and down directly correlate with the risk of getting CHD.

The genetic variation of *PCSK9* gene greatly affects the serum lipid level which expresses differently in various genotypes. In the present study, the results showed that among the patients with CHD, TG, TC, LDL-C levels of GG genotype were higher than those of AA genotype ($P < 0.05$). Although the TG, TC, HDL-C and LDL-C levels between AG and AA, GG and AG showed no significant differences, the TG, TC and LDL-C levels of patients carrying G allele were a gradually rising trend compared to those of patients with AA genotype. The studies of Zhang and Qiu showed that patients carrying G allele had relatively higher levels of LDL-C, TG and TC [10,11], and research by Aung *et al*. revealed E670G polymorphism of *PCSK9* gene was bound up with the serum lipid level of Han population and among the people with G allele, male expressed higher HDL-C, ApoA1 levels [12]. Evans and Beil also found that carrying G allele of *PCSK9* gene E670G occurs in European men was related to the significant increase of LDL-C level, while this correlation did not exist in women, which therefore revealed the effects of E670G mutation of *PCSK9* gene differ between people in distinct regions or group. To analyze the cause of this situation, it was found that a mutation occurred in *PCSK9* gene E670G affected the normal functions and thereby influenced the lipid metabolism; different effects among people in various regions or ethnic groups may caused by their genes. Distinct ethnic backgrounds, living environments, eating habits *etc*. would influence the synthesis and excretion of it [13], which therefore resulted in the pathological manifestations.

Many researches showed a close correlation between mutation of *PCSK9* gene and serum lipid level, the *PCSK9* gene has become a hot issue for the studies on the treatments of hyperlipidemia and CHD [14–16]. This research, by comparatively studying the E670G polymorphism of *PCSK9* gene of Han population in different regions, revealed that there were regional differences existed in E670G polymorphisms of *PCSK9* gene between patients with CHD in Hainan and TPNC and the patients with distinct genotypes would exhibit different serum lipid levels, which reminded us to pay attention to the regional differences during the process of prevention and treatment of hyperlipidemia and CHD. In the future, we will investigate the exact mechanism which result this difference and further discuss the correlation between E670G polymorphism of the *PCSK9* gene and abnormal lipid metabolism, the occurrence and development of CHD as well as the prognosis and its effects, therefore providing the individualized theory basis for prevention and treatment of CHD among population in distinct regions.

## Conflict of interest statement

We declare that we have no conflict of interest.

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