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Association of N-acetyltransferase-2 polymorphism with an increased risk of coronary heart disease in a Chinese population

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ABSTRACT. We investigated the possible correlations between N-acetyltransferase-2 (*NAT2*) gene polymorphisms and the risk of coronary heart disease (CHD). CHD patients (113) and healthy controls (118) were enrolled from the First People's Hospital of Yuhang between January 2013 and June 2014. The patients were divided into mild CHD (N = 72) and severe CHD (N = 41) subgroups. DNA samples were extracted and the distributions of *NAT2* polymorphisms were examined by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Clinical characteristic indexes of severe CHD patients were also examined for relevant statistical analysis. WT, M1, M2, and M3 alleles were observed in both case and control groups. PCR-RFLP identified a wild-type homozygote, WT/WT; a mutant heterozygote, WT/Mx; and a mutant homozygote, Mx/Mx (x = 1, 2, and 3) variant of the *NAT2* genotype. Mx/Mx differed significantly between case and control groups ($P < 0.05$); the frequencies of all four alleles did not differ significantly between case and control groups ($P > 0.05$). Slow acetylator genotype frequencies were notably higher in

the case group than in the control group (P < 0.05). Individuals with the slow acetylator genotype were at 1.97-times higher risk of CHD and also displayed higher triglyceride and lower high-density lipoprotein cholesterol levels than those with the rapid acetylator genotype (P < 0.05). Therefore, the *NAT2* polymorphism was believed to be associated with increased risk of CHD, with the *NAT2* slow acetylator genotype serving as a risk factor for severe CHD in a Chinese population.

Key words: Coronary heart disease; N-acetyltransferase-2; Slow acetylator genotype; Rapid acetylator genotype; Polymorphism; Polymerase chain reaction

INTRODUCTION

Coronary heart disease (CHD) is rapidly becoming a major cause of mortality and morbidity worldwide, placing a large economic burden on the public health systems in many countries, including China (Yu et al., 2011). Evidence from the World Health Organization (WHO) suggests that 3.4 million women and 3.8 million men worldwide die from CHD annually, with more than 60% of the global burden of CHD occurring in developing countries (Jiang et al., 2012). In China, CHD is the second leading cause of cardiovascular mortality and is responsible for 13 and 22% of the cardiovascular mortality in rural and urban areas, respectively (Tian et al., 2012). Since the 1980s, a steady growth in CHD deaths in the Chinese population has been noted in both rural and urban areas (Zhang et al., 2008). Among the well-accepted risk factors, hypertension, obesity, smoking, and diabetes mellitus are mainly attributed to the development and progression of CHD in the Chinese population (Tian et al., 2012). This is because of the close correlation of CHD with these risk factors, as well as the high incidence of hypertension, overweight, and obesity in the Chinese population (Zhang et al., 2008). Most individuals develop CHD through interaction between genetics and environmental factors; therefore, genes involved in the pathogenesis of CHD are increasingly becoming a focus area (Zhou et al., 2012).

Arylamine N-acetyltransferases (*NAT*) catalyze the activation of O-acetylation and deactivation of N-acetylation by reacting with heterocyclic amines and amines with a carbon-only aromatic ring (Hickman et al., 1998). Both NAT isozymes N-acetyltransferase-1 (NAT1) and N-acetyltransferase-2 (NAT2) are believed to be polymorphic in the human population (Hein, 2009). The *NAT2* gene is located on chromosome 8p22, is encoded by a single 870-bp open reading frame, and plays a role in the individual physiological response to a variety of xenobiotic compounds, including a wide range of exogenous chemicals and several clinically useful drugs (Hein et al., 2000b; Sabbagh et al., 2011). Polymorphisms in the *NAT2* gene modify patient susceptibility in humans to cancer, drug response, and the risk of adverse drug reactions, by causing alterations in the structure of the enzyme (Walraven et al., 2008). *NAT2* polymorphisms also generate rapid, intermediate, and slow acetylation genotypes via alterations in the enzyme structure; in fact, humans can be divided into subgroups based on their acetylation capacities (Bozok Cetintaş et al., 2008).

A number of studies have attempted to elucidate the association between the *NAT2* polymorphism and various cancers (including breast, bladder, colorectal, thyroid, pancreatic, and lung cancers), asthma, and allergic/atopic disorders (Ambrosone et al., 2008; Ayaz et al., 2008; Batra and Ghosh, 2008; Guilhen et al., 2009; Cui et al., 2011; Silva et al., 2011; Moore et al., 2011). However, very few reports have documented the correlation between *NAT2* polymorphisms and

heart-related diseases; therefore, in this study, we attempted to quantify the hypothesis that *NAT2* polymorphisms result in increased susceptibility to CHD in a Chinese population.

MATERIAL AND METHODS

Ethics statement

This study was performed in agreement with the Ethics Committee of the First People's Hospital of Yuhang, China. Written informed consent was obtained from all the patients included. This study was conducted in accordance with the guidelines of the Declaration of Helsinki (Holt, 2014).

Study subjects

In this case-control study, 113 CHD patients were enrolled into the case group from the First People's Hospital of Yuhang between January 2013 and June 2014. Disease was identified based on the following CHD diagnostic criteria: left main coronary artery stenosis ≥30%, or coronary artery stenosis ≥50% in at least one of the other three branches, determined by selective coronary arteriography (Fang et al., 2011). Coronary angiographies of the patients were conducted by three interventionalists (together), and recorded and preserved as kinetoscope film and film readings. The interventionalists also evaluated the pathological severity of coronary artery according to the following: 1) scoring based on stenosis degree: 1 point for stenosis ≤25%, 2 for 26-50% stenosis, 3 for 51-75% stenosis, 4 for stenosis >75%; 2) the pathological score of the vessel, comprising the accumulation of scores for multiple stenosis and accumulation of vessel stenosis scores for multiple vessels with stenosis. Based on the scoring, the patients were divided into the mild group (score ≤10; N = 72; 41 male and 31 female individuals; age 28-67 years; average age: 46 ± 7 years) and the severe group (score >10; N = 41; 24 male and 17 female individuals; age 30-68 years; average age: 48 ± 9 years). One hundred and eighteen healthy controls were also selected from the same hospital based on the following inclusion criteria: no history of CHD; no CHD diagnosis after physical, laboratory, and electrocardiogram examinations; and no family history of heredopathia. The height, weight, and blood pressure [mean arterial pressure (MAP)] of patients with severe CHD were measured, and their body mass index was calculated. The serum biochemical indexes [total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), apo-lipoprotein A-I (APO A-I), and apo-lipoprotein B (APO B)] for venous blood were also analyzed by the central laboratory of our hospital. The subjects were unrelated to each other and belonged to the Han population subgroup.

Genomic DNA extraction

Peripheral blood (2 mL) was collected from all subjects (empty stomachs for 10-12 h in the morning); the blood samples were stored in EDTA-anticoagulant tubes at -80°C. Genomic DNA was extracted from the sample and anticoagulated blood using a standard kit (Aidlab Biotechnologies Co., Ltd., Beijing, China); the genomic DNA content in the extracts was determined using an ultraviolet spectrophotometer.

NAT2 polymorphisms

Seven mutant single nucleotide polymorphisms (SNPs) of *NAT2* at bases 191 (G→A),

282 (C→T), 341 (T→C), 481 (C→T), 590 (G→A), 803 (A→G), and 857 (G→A), with 26 alleles, have been identified in humans (Hein et al., 2000a). A previous report indicated that the polymorphism in the 191st bp was found only in the African population (Sakakibara et al., 1991), whereas Asians and Caucasians were shown to express the other six SNPs (Lin et al., 1993). In fact, many studies have reported a specific association among these six mutant SNPs: for example, 341C or 803G is always accompanied with 481T, while 282T is always expressed with 590A; therefore, 481T-M1, 590A-M2, and 857A-M3 were used to examine the polymorphisms in the *NAT2* gene (Aynacioglu et al., 1997).

NAT2 polymorphisms are mainly associated with M1, M2, and M3. Use of an appropriate restriction endonuclease can help identify the different SNPs in the *NAT2* structural gene; therefore, polymerase chain reaction in association with restriction fragment length polymorphism (PCR-RFLP) was employed for this purpose. The PCR-RFLP was performed in a 50-μL reaction mixture containing 5 μL DNA, 5 μL 10X PCR buffer solution (Aidlab Biotechnologies Co., Ltd.), 0.4 μL Taq DNA (5 U/μL; Aidlab Biotechnologies Co., Ltd.), 1 μL each forward and reverse primers (200 μM), and double-distilled water. The following primers used for this reaction were as follows: forward, 5'-CTTCTCCTGCAGGTGACCAT-3'; and reverse, 5'-AGCATGAATCACTCTGCTTC-3'. The PCR conditions were as follows: pre-denaturation at 94°C for 3 min; 35 cycles of denaturation at 94°C for 45 s, annealing at 56°C for 45 s, and extension at 72°C for 1 min; and a final extension at 72°C for 7 min. The PCR products (3 μL each) were individually digested with restriction endonucleases (*Kpn*I, *Taq*I, and *Bam*HI). The reactions were performed in 20-μL reaction mixtures containing the PCR amplification products (13 μL), 10X PCR buffer solution (2 μL), restriction endonucleases (10 U), and double-distilled water. The enzymes *Kpn*I, *Taq*I, and *Bam*HI were incubated in a 37°C water bath for 3 h prior to the reaction. The enzyme-digested products (10 μL) were electrophoresed on a 2% agarose gel and dyed with ethidium bromide; the bands were analyzed using a standard gel imaging system. A positive control was prepared in order to ensure the accuracy of enzyme digestion. A DNA sequencer (ABI370; Applied Biosystems, Foster City, CA, USA) was employed to evaluate the corresponding SNP genotype.

NAT2 gene mutation was observed in the three SNP regions (481, 590, and 857); four alleles (WT, M1-loss of *Kpn*I site, M2-loss of *Taq*I site, and M3-loss of *Bam*HI) were observed. The *NAT2* genotype was divided into the rapid acetylator (WT/WT, WT/M1, WT/M2, and WT/M3) and slow acetylator (M1/M1, M1/M2, M1/M3, M2/M2, M2/M3, and M3/M3) groups.

Statistical analysis

All statistical analyses were performed using the SPSS 17.0 software (IBM, Armonk, NY, USA), and all continuous data are reported as means ± standard deviation (SD). The chi-square test for goodness of fit was employed to assess the conformance of the genotypes in case and control groups with the Hardy-Weinberg equilibrium. Categorical data were analyzed using the chi-square test. Odds ratios (ORs) with 95% confidence intervals (95%CI) exhibited relative risk. P values <0.05 indicated statistically significant differences.

RESULTS

Distribution of *NAT2* polymorphism genotypes

Following PCR amplification and *Kpn*I enzyme digestion, the fragment lengths were as follows: for the wild homozygote (WT/WT), 159 and 656 bp; for the mutant heterozygote (WT/M1),

159, 656, and 815 bp; and for the mutant homozygote (M1/M1), 815 bp (Figure 1A). PCR amplification and *Taq*I enzyme digestion of the wild homozygote (WT/WT), the mutant heterozygote WT/M2, and the mutant homozygote M2/M2 produced fragments with the following lengths: 169, 227, and 378 bp (3 bands); 169, 227, 378, and 396 bp (4 bands); and 378 and 396 bp (2 bands), respectively. Although it was difficult to observe the 41-bp band in the electrophoretogram, the genotype analysis was not affected (Figure 1B). *Bam*HI enzyme digestion of the wild homozygote WT/WT, the mutant heterozygote WT/M3, and the mutant homozygote M3/M3 produced fragments of the following lengths: 280 and 535 bp; 280, 535, and 815 bp; and 815 bp, respectively (Figure 1C). The results indicated that it was not possible for three or more than three mutant alleles to be simultaneously available in one sample. No more than two mutant alleles coexisted in one sample; this was consistent with the results reported by Aynacioglu et al. (1997).

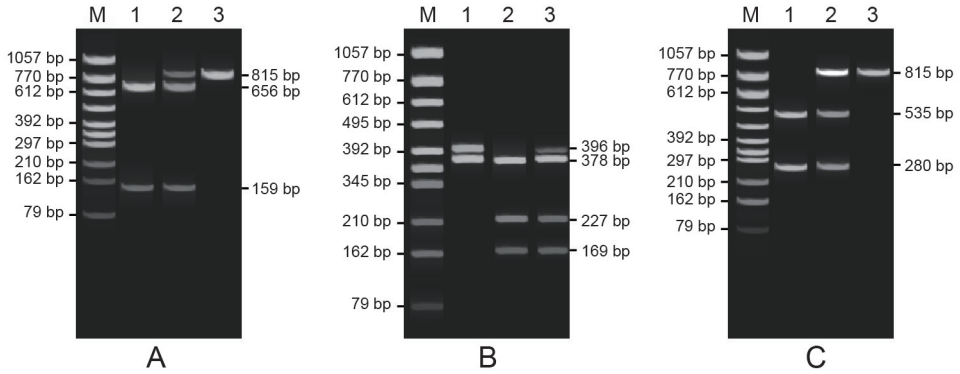

Figure 1. Agarose gel electrophoresis of polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) products for N-acetyltransferase-2 (*NAT2*) polymorphism (**A:** 1, WT/WT; 2, WT/M1; 3, M1/M1. **B:** 1, M2/M2; 2, WT/WT; 3, WT/M2. **C:** 1, WT/WT; 2, WT/M3; 3, M3/M3). *Lane M* = molecular marker.

The frequencies of all alleles were consistent with the Hardy-Weinberg equilibrium (P > 0.05). The distribution frequencies of the mutant homozygote Mx/Mx were 16.10% in the control group and 27.43% in the case group; this difference was statistically significant (P < 0.05; Table 1). The distribution frequencies of the wild homozygote WT/WT and the mutant heterozygote WT/Mx were 21.24 and 51.33% in the control group and 31.36 and 52.54% in the case group, respectively; these differences were not statistically significant (P > 0.05).

Table 1. Distribution frequencies of the *NAT2* genotype and allele [N (%)].

Genotypes	CHD group	Control group	OR (95%CI)	P
WT/WT	24 (21.24)	37 (31.36)	Ref.	
WT/Mx	58 (51.33)	62 (52.54)	1.442 (0.77-2.70)	0.251
Mx/Mx	31 (27.43)	19 (16.10)*	2.515 (1.17-5.42)	0.018
WT	106 (46.90)	136 (57.63)	Ref.	
M1	30 (13.27)	22 (9.32)	1.750 (0.95-3.21)	0.068
M2	57 (25.22)	50 (21.19)	1.463 (0.93-2.31)	0.102
M3	33 (14.60)	28 (11.86)	1.512 (0.86-2.66)	0.154

OR = odds ratio; CI = confidence interval; x = 1, 2, and 3; *P < 0.05 compared to the CHD group.

Distribution of *NAT2* phenotypes

Slow acetylator genotype frequencies were notably higher in the case group than in the control group (27.43 *vs* 16.10%, P < 0.05). Individuals with slow acetylator genotypes showed a 1.97 times greater risk of CHD (95%CI = 1.04-3.74, P < 0.05) and a 2.70 times greater risk of severe CHD than those with the rapid acetylator genotype (P < 0.05; Table 2).

Table 2. Distribution frequencies of the *NAT2* phenotype [N (%)].

Groups	Slow acetylator	Rapid acetylator	OR (95%CI)	P
Control group	19 (16.10)	99 (83.90)	Ref.	
CHD group	31 (27.43)	82 (72.57)*	1.970 (1.04-3.74)	0.037
Mild CHD	17 (15.04)	55 (48.67)	1.611 (0.78-3.35)	0.200
Severe CHD	14 (12.39)	27 (23.89)*	2.702 (1.20-6.08)	0.014

OR = odds ratio; CI = confidence interval; CHD = coronary heart disease; *P < 0.05 compared to the slow acetylator.

Risk of CHD and *NAT2* phenotypes

Individuals with the slow acetylator genotype contained higher TG and lower HDL-C content than those with the rapid acetylator genotype (all P < 0.05), while the risk of CHD by other parameters, such as gender, age, lipid types, and MAP, did not differ significantly among case and control groups (Table 3).

Table 3. Comparison of the risk of CHD with the phenotype.

Clinical parameters	Slow acetylator (N = 14)	Rapid acetylator (N = 27)	P
Gender (F/M)	10/4	14/13	0.228
Age	48 ± 7	49 ± 8	0.695
BMI	23.0 ± 2.8	24.6 ± 3.7	0.164
Lipids (mg/dL)			
TC	160.7 ± 23.0	168.7 ± 32.2	0.415
TG	142.7 ± 19.4	128.9 ± 17.8*	0.028
HDL-C	42.9 ± 8.0	49.8 ± 9.9*	0.030
LDL-C	90.7 ± 11.1	95.9 ± 10.2	0.141
APO A-I (mg/dL)	154.7 ± 24.7	146.7 ± 29.9	0.400
APO B (mg/dL)	68.4 ± 14.8	76.2 ± 14.3	0.110
MAP (mmHg)	94.1 ± 9.8	95.0 ± 10.6	0.793

BMI = body mass index; TC = cholesterol total; TG = triglyceride; HDL-C = high-density lipoprotein cholesterol; LDL-C = low-density lipoprotein cholesterol; APO A-I = apolipoprotein A-I; APO B = apolipoprotein B; MAP = mean arterial pressure; *P < 0.05 compared to the slow acetylator.

DISCUSSION

CHD is a complex and multifactorial disease responsible for a majority of the mortality and morbidity occurring worldwide, and annually results in more than 700,000 deaths in China (Xu

et al., 2011). Previous studies have reported an association between *NAT2* polymorphisms and the risk of human diseases such as breast, bladder, colorectal, thyroid, pancreatic, and lung cancers and asthma and allergic/atopic disorders; however, the correlation between *NAT2* polymorphisms and the risk of CHD has not been extensively studied. Therefore, we hypothesized that the *NAT2* polymorphisms influence the risk of CHD in a Chinese population. We discovered that the mutant *NAT2* homozygote Mx/Mx may be correlated with the risk of CHD. The *NAT2* gene is highly polymorphic and undergoes inter-individual mutations, resulting in the biotransformation of aromatic amines. Polymorphisms in the *NAT2* gene were caused by three mutant alleles (M1, M2, and M3) that modify the restriction enzyme identification sites (Aynacioglu et al., 1997). In this study, the analysis of the distribution of the *NAT2* genotype and allele helped identify the distribution frequencies of the mutant homozygote Mx/Mx (x = 1, 2, and 3) in the CHD patients (27.43%) and healthy individuals (16.10%); these differences were statistically significant.

Additionally, the *NAT2* slow acetylator genotype was identified as a risk factor for CHD. *NAT2* alleles cause alterations in the enzyme activity; specifically, the rapid or slow acetylator phenotypes, arising from variations in the corresponding DNA sequences, have been correlated with changes in the enzyme activity and stability (Zheng et al., 2012). *NAT2* plays a major role in isoniazid metabolism: the elimination rate of isoniazid is trimodally distributed, depending on the *NAT2* phenotype (Kiyohara et al., 2009). Generally, slow acetylators show greater susceptibility to adverse drug effects than rapid acetylators because of the delayed drug elimination, leading to increased concentrations of the drugs (Yalin et al., 2007). In our study, individuals with the slow acetylator genotype were found to be at 1.97 times greater risk of CHD and 2.70 times greater risk of severe CHD than those with the rapid acetylator genotype. Furthermore, individuals with the slow acetylator genotype showed higher TG and lower HDL-C content than those with the rapid acetylator genotype; low HDL-C and high TG concentrations have been strongly associated with the onset and development of CHD (Sarwar et al., 2010). TG is routinely measured during the fasting state, excluding remnant lipoproteins. The results of two long-term prospective cohort studies with large sample sizes, conducted by Bansal et al. (2007) and Nordestgaard et al. (2007) in different populations, also supported the significant role of non-fasting TG levels in the development of CHD and CHD-related events. HDL-C, with its atheroprotective effect, plays a crucial role in reverse cholesterol transport and facilitates the cholesterol transfer from peripheral tissues back to the liver (Li et al., 2012). Medina-Urrutia et al. (2008) reported that HDL-C levels are inversely associated with the development of CHD (Medina-Urrutia et al., 2008). Therefore, the results of our study reflected the conclusions of previous studies that *NAT2* polymorphisms are a risk factor for CHD.

In conclusion, the results of our study primarily demonstrated that *NAT2* polymorphisms may be correlated with increased risk of CHD, and that the *NAT2* slow acetylator genotype may serve as a risk factor for severe CHD in a Chinese population. However, there are two potential limitations to this study: a relatively small number of subjects were employed, and very few outcome measures were utilized, which may affect the results of this study. Therefore, future studies must employ larger sample sizes and more reliable outcome measures in order to better elucidate the association between *NAT2* polymorphisms and risk of CHD.

Conflicts of interest

The authors declare no conflict of interest.

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