Gene polymorphisms associated with susceptibility to coronary artery disease in Han Chinese people

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ABSTRACT. This study investigated 5 single nucleotide polymorphism (SNP) haplotypes in susceptibility genes for coronary artery disease (CAD) and the putative involvement of these SNPs in CAD in the Chinese Han population. From March 2008 to June 2009, we selected 119 CAD patients and 115 subjects not related to the CAD of Chinese Han origin as controls. The SNP genotypes were performed by multiplex SNaPshot technology. The *HNRPUL1* gene rs11881940T and *GATA2* gene rs3803T loci were highly correlated with CAD (P < 0.05). rs10757278G increased the risk of CAD in patients indicated by an odds ratio (OR) = 1.242 [95% confidence interval (CI) = 1.04-1.49]; rs11881940T and rs3803T were protective factors for CAD with ORs = 0.767 (95%CI = 0.61-0.97) and 0.53 (95%CI = 0.40-0.72), respectively. Analysis of the rs10757278, rs11881940 and rs3803 loci showed that haplotypes ATC

(OR = 4.26; 95%CI = 2.85-6.40, P < 0.01), GAC (OR = 1.50; 95%CI = 1.25-1.81, P < 0.01) and GAT (OR = 1.53; 95%CI = 1.12-2.09, P < 0.01) were CAD risk factors, whereas GTC was protective (OR = 0.48; 95%CI = 0.32-0.72, P < 0.01). ATC and glucose were positively correlated (OR = 1.91; 95%CI = 1.01-3.61, P < 0.05). GAT was a risk factor for hypertension (OR = 2.86; 95%CI = 1.40-5.83, P < 0.01). In conclusion, polymorphisms and haplotype analysis of susceptibility genes for CAD can improve predicting this disease and will enable early diagnosis of CAD.

Key words: Coronary artery disease; Predisposing genes; Polymorphism; Single nucleotide; Genotyping

INTRODUCTION

Coronary artery disease (CAD) is a complex malady and the leading cause of morbidity and mortality worldwide (Yusuf et al., 2001; Thom et al., 2006; Rosamond et al., 2007). Genetic and environmental factors interact with each other in CAD development and outcome. Genetic factors play a particularly important role in the pathogenesis of CAD and also in myocardial infarction (MI) (Topol et al., 2006). To date, many molecules and genes that play key roles in CAD have not been identified, and especially identifying the genetic risk factors for CAD requires further studies. It is generally accepted by the international community that CAD and other complex diseases are the result of the minor genetic differences that lead to cumulative effects. Indirect detection of gene function mutation and haplotype can be used to better understand the incidence of CAD and its underlying developmental mechanism at the molecular level, because their genetic profiles comprising multiple nucleotide polymorphism haplotypes are better models than single nucleotide polymorphisms (SNPs) in studies of genetic correlation with complex traits (Morris and Kaplan, 2002; Hoehe, 2003). To better understand whether gene variants are pathophysiologically relevant as precipitators of CAD and in other genotype-phenotype relationships, we report here a case-controlled association study in Chinese Han subjects. Selected genes such as the NPPA gene rs5063, the 9p21 regions rs10757274 and rs10757278, HNRPUL1 gene rs11881940, VAMP8 gene rs1010, GATA2 gene rs3803, and rs2713604, representing 7 loci were examined to determine whether genetic variants in these loci were associated with CAD. The aim of this study was to establish locus-specific contributions to the development of CAD within a selected Chinese Han population.

MATERIAL AND METHODS

Study subjects

All individuals included in this study were living in China. We carried out a case-controlled association study involving 238 cases and 230 control volunteers that were selected from Sun Yat-sen Cardiovascular Hospital (Shenzhen, China) from March 2008 to June 2009. CAD diseases had been confirmed by clinical observations in patients' history, including echocardiography and imaging studies. Coronary angiography showed that at least

one coronary artery vessel had >70% luminal stenosis. Control volunteers mainly comprised healthy individuals and non-CAD patients whose past history of physical examination, imaging, and electrocardiography was used to exclude CAD diseases. The Han Chinese population that we selected was mainly from Henan, Hunan, and Guangdong Provinces. The individuals enrolled in this study were unrelated to each other, and written informed consent was obtained.

DNA extraction and purification

In total, 3-mL blood samples were collected at baseline from patients and controls and sodium citrate added to storage at -70°C. A DNA extraction kit was used to isolate and purify DNA from the blood samples.

Primer design

Each SNP site was used for designing 3 primers (Shanghai Health Workers); two of these primers with Tm value of about 60°C were used for amplification of target DNA fragments ranging 200-500 bp in length. Other primers were used for extension of ddNTP that was designed at the upstream of SNP sites or downstream of the reverse.

PCR amplification

Primers were diluted to 100 mM. PCR was performed with the following reagents: 1 µL 10X buffer, 0.4 µL primer (10 mM), 0.3 µL dNTP (10 mM), 0.1 µL HotStar enzyme (5 U/µL), 1 µL DNA, 0.52 µL Mg^{2+} (25 mM), and 6.68 µL ddH_2O to a final volume of 10 µL. Multiplex reactions were performed using a PCR touch-down method for amplification. PCR products were analyzed by agarose gel electrophoresis, and successful PCR amplification assessed by the presence of bands of the predicted size.

Purification of PCR products

PCR products (6 µL) and 2 µL enzyme (including 2 U SAP and 2 U *Exo*I) were mixed by vortexing. This was followed by incubation at 37°C for 1 h and heating to 75°C for 15 min. Purified DNA was stored at 4°C for 24 h or, for long-term storage, at -20°C.

SNaPshot PCR template and purification of hybrid

The PCR product was used as template in a micro-sequencing PCR (SNaPshot PCR). Each primer in the reaction had a final concentration of 0.2 nM. PCR cycling conditions were the following: 25 cycles of 96°C for 10 s, 50°C (53°C) for 5 s; and 60°C, for 30 s, followed by one hold at 60°C for 30 s and storage at 4°C. The SNaPshot PCR product in 5 µL was added to 0.5 U SAP or 1 U CIP, mixed by vortexing, and incubated at 37°C for 1 h, followed by 75°C for 15 min to inactivate the enzymes. Reacted samples were stored at 4°C for 24 h or at -20°C for long-term preservation.

Electrophoresis sample preparation and GeneMapper4.0 software analysis

SNaPshot products (1 µL) were prepared for electrophoresis in 9.25 µL Hi-Di formamide and 0.1 µL GS-120 LIZ, in a total volume of 10.35 µL; the mixture was then subjected to 95°C denaturation for 5 min and then rapidly cooled for 4 min. Each electrophoresis sample was run with LIZ-120 internal standard, to accurately determine the sizes of fragments. The test results were analyzed by the GeneMapper4.0 software.

Statistical analysis

SNP genotypes and allele frequencies were determined with direct-counting methods. Haplotype analysis was performed using the PHASEv2.1 software. Continuous variables are reported as means ± standard deviation. The following describes how statistical analyses were performed using the SPSS 13.0 package. Measurement data were compared between 2 groups using independent-sample *t*-tests. Allelic association of an SNP with a binary disease trait and some count data were assessed using Pearson 2 x 2-contingency table chi-square testing. Odds ratios (OR) and 95% confidence intervals (95%CI) were estimated using SPSS Ver 13.0. Multivariate logistic regression analysis was performed to test correlations of SNPs and haplotypes with CAD accounting for the significant covariates previously described (gender, age, hypertension, diabetes, and others). Empirical probability values were also calculated. A $P < 0.05$ was considered to be statistically significant, and all P values were two-sided.

RESULTS

Basic information of the study

The body mass index of the control group was <25 kg/m². Selected women were >5 years into menopause. To rule out that aging had an impact on CAD, the average age of the CAD group was younger than that of the control group ($P < 0.05$). CAD and control groups were consistent with Hardy-Weinberg equilibrium ($P < 0.05$). The CAD and control groups showed statistically significant differences ($P < 0.01$) in blood pressure, pathoglycemia, smoking history, TC, TG, HDL, LDL, and other test results; gender and family history showed no significant differences between the two groups (Table 1).

Table 1. Basic information of the groups studied.

Related factors	CAD group	Control group	P value
Age (years)	61.22 ± 7.29	63.55 ± 6.52	0.011
Gender (male/female)	186/52	164/66	0.088
Hypertension	174/238	92/230	<0.01
Glucose	80/238	22/230	<0.01
Smoking history	130/238	68/230	<0.01
Family history	16	10	0.428
TC (mM)	5.05 ± 0.94	4.61 ± 0.75	<0.01
TG (mM)	1.76 ± 0.82	1.37 ± 0.59	<0.01
HDL (mM)	1.14 ± 0.32	1.29 ± 0.31	<0.01
LDL (mM)	3.38 ± 0.96	2.95 ± 0.76	<0.01

Age, TC, TG, HDL, and LDL are reported as means ± SD (comparison between the two groups was performed using an independent-sample *t*-test). Gender, blood pressure, pathoglycemia, smoking history, and family history (χ^2 test was used to compare).

CAD susceptibility gene loci

The results of white blood plasma DNA genotyping in 238 cases and 230 controls showed that the 9p21 region rs10757278G and the *HNRPUL1* gene rs11881940T and *GATA2* gene rs3803T sites were highly correlated with CAD ($P < 0.05$). rs10757278G increased the risk of CAD in patients as indicated by an OR = 1.24 (95%CI = 1.04-1.49); rs11881940T and rs3803T were protective factors for CAD, with ORs = 0.77 (95%CI = 0.61-0.97) and 0.53 (95%CI = 0.40-0.72), respectively. The results of our study further showed that the rs5063A, rs10757274G, rs1010G, and rs2713604A loci did not significantly correlate with CAD: ORs and 95%CIs for these 4 loci were 1.10 (0.71-1.67; $P > 0.05$), 1.17 (0.98-1.40; $P = 0.08$), 1.18 (0.95-1.47; $P > 0.05$), and 1.12 (0.54-2.29; $P > 0.05$), respectively (Table 2).

Table 2. Correlation for SNP loci and coronary artery disease (CAD).

Gene	SNP	Allele	Frequence		OR (95%CI)	P-HW	P value
			CAD group	Control group			
ANP	rs5063	A	0.168	0.152	1.10 (0.71-1.67)	0.50	0.64
9p21 district	rs10757274	G	0.550	0.470	1.17 (0.98-1.40)	0.09	0.08
9p21 district	rs10757278	G	0.567	0.457	1.24 (1.04-1.49)	0.14	0.02
HNRPUL1	rs11881940	T	0.324	0.422	0.77 (0.61-0.97)	0.59	0.03
VAMP8	rs1010	G	0.441	0.374	1.18 (0.95-1.47)	0.09	0.14
GATA2	rs3803	T	0.206	0.387	0.53 (0.40-0.72)	0.46	0.00
GATA2	rs2713604	A	0.063	0.057	1.12 (0.54-2.29)	0.17	0.77

P-HW = P value for Hardy-Weinberg disequilibrium analysis; OR = odds ratio; 95%CI = 95% confidence interval.

Haplotype analysis

The PHASEv2.1 software was used to analyze the haplotypes of rs10757278, rs11881940, and rs3803. This analysis showed that haplotypes ATC (OR = 4.26; 95%CI = 2.85-6.40, $P < 0.01$), GAC (OR = 1.50; 95%CI = 1.25-1.81, $P < 0.01$), and GAT (OR = 1.53; 95%CI = 1.12-2.09, $P < 0.01$) were all CAD risk factors. On the other hand, GTC was a protective factor for CAD, indicated by an OR = 0.48 (95%CI = 0.32-0.72; $P < 0.01$) (see Table 3).

Table 3. Susceptibility gene haplotype analysis of SNPs between the two groups.

Haplotype	CAD group (%)	Control group (%)	OR (95%CI)	P value
ATC	44.5	10.4	4.27 (2.85-6.40)	<0.01
AAC	40.3	45.2	0.89 (0.72-1.10)	0.286
GTC	12.6	26.1	0.48 (0.32-0.72)	<0.01
GAC	61.3	40.9	1.50 (1.25-1.81)	<0.01
GAT	31.9	20.9	1.53 (1.12-2.09)	<0.01

Logistic regression analysis of CAD and its related factors

Gender, hypertension, glucose, smoking history, family history, TC, TG, HDL, and LDL were all entered into a variable logistic regression model. This analysis indicated that male gender, hypertension, glucose, and TC were all highly relevant to CAD with adjusted ORs in the range of 3.00-6.07 ($P < 0.05$; Table 4). A forward method based on partial likelihood ratios to the operational rules of gender, high blood pressure, pathoglycemia, smoking history, family history, TC, TG,

HDL, and LDL gradually introduced these factors into a logistic regression model. The results of this analysis showed that male gender, hypertension, pathoglycemia, TC, and TG were highly correlated with CAD with adjusted ORs in the range of 1.89-7.74 (P < 0.05; Table 5).

Table 4. CAD-related factors and logistic regression analysis of all variables.

Related factors	B	P value	OR	95%CI for OR	
				Lower	Upper
Gender	1.80	0.00	6.07	2.57	14.31
Hypertension	1.10	0.00	3.00	1.44	6.23
Pathoglycemia	1.73	0.00	5.63	2.19	14.48
Smoking history	0.40	0.36	1.48	0.63	3.48
Family history	-0.50	0.49	0.61	0.14	2.54
TC	1.26	0.03	3.51	1.12	10.96
TG	0.51	0.10	1.66	0.91	3.02
HDL	-0.66	0.32	0.52	0.14	1.91
LDL	-0.25	0.65	0.78	0.26	2.28

Gender, high blood pressure, glucose, smoking history, family history, TC, TG, HDL, and LDL into all variable logistic regression model; B for the partial regression coefficient; OR for the introduction of the variable OR after correction.

Table 5. CAD-related factors and stepwise logistic regression analysis.

Related factors	B	P value	OR	95%CI for OR	
				Lower	Upper
Gender	2.05	0.00	7.74	3.77	15.88
Hypertension	1.13	0.00	3.10	1.57	6.13
Pathoglycemia	1.74	0.00	5.67	2.25	14.26
TC	0.92	0.00	2.51	1.57	3.99
TG	0.64	0.02	1.89	1.12	3.19

Using forward stepwise (likelihood ratio) the gradual introduction of the rules of gender, high blood pressure, glucose, smoking history, family history, TC, TG, HDL, and LDL; B for the partial regression coefficient; OR for the introduction of the variable odds ratio after correction.

Logistic regression analysis of haplotype and CAD-related factors

Gender, hypertension, glucose, TC, TG, HDL, and LDL were also analyzed in a stepwise logistic regression model. In addition, haplotype ATC, GTC, GAC, and GAT were analyzed by logistic regression analysis to identify possible correlations with CAD. ATC and glucose results showed a positive correlation with CAD and had an OR = 1.91 (95%CI = 1.01-3.61, P < 0.05). GAT was a risk factor for hypertension with an OR = 2.86 (95%CI = 1.40-5.83; P < 0.01) (Table 6).

Table 6. Haplotypes and related factors in stepwise logistic regression analysis.

Haplotype	B	P value	OR	95%CI for OR	
				Lower	Upper
ATC					
Pathoglycemia	0.65	0.05	1.91	1.01	3.61
GAT					
High blood pressure	1.00	0.00	2.86	1.40	5.83

Using forward stepwise (likelihood ratio) rules of gender, high blood pressure, glucose, TC, TG, HDL, and LDL into stepwise logistic regression model; B for the partial regression coefficient; OR for the introduction of the variable odds ratio after correction.

DISCUSSION

Genetic contributions to the risk of CAD are complex, and little attention has been paid to gene-environment interactions. Environmental modification results in heterogeneity of genetic effects and is increasingly recognized as a potential source of variability (Talmud, 2007).

The CAD study and control groups were consistent with Hardy-Weinberg equilibrium (P > 0.05). The two groups showed statistically significant differences (P < 0.01) in blood pressure, glucose, smoking history, TC, TG, HDL, and LDL. In contrast, no such differences were observed for gender and family history (P > 0.05). The samples came from individuals of the Henan, Hunan, and Guangdong Provinces, who live a fast pace of life, face the pressure of survival, and have poor eating and lifestyle habits all of which can affect the incidence of CAD. On the other hand, other characteristics of these samples may also be relevant. When gender, hypertension, glucose, smoking history, family history, TC, TG, HDL, and LDL were all entered into a variable logistic regression model, male gender, hypertension, glucose, and TC were observed to be highly correlated with CAD. This analysis showed that these factors are highly relevant to CAD with an adjusted OR ranging from 3.00-6.06 (P < 0.05), indicating that they are risk factors for CAD in the Chinese Han population.

Shen et al. (2008a), studying populations from South Korea, observed that the rs10757274 and rs10757278 alleles were highly correlated with CAD with ORs of 1.29 (95%CI = 1.06-1.58) and 1.29 (95%CI =1.06-1.57), respectively. rs10757274 and rs10757278 are located near the *CDKN2A* and *CDKN2B* genes, which encode proteins with important roles in the regulation of many cellular processes, including cell proliferation, maturation, senescence, and programmed cell death. However, there is no evidence that *CDKN2A* and *CDKN2B* have effects on the incidence of CAD (McPherson et al., 2007; Helgadottir et al., 2007). Virani et al. (2012) studying Caucasian subjects, observed that rs10757274 and rs10757278 were not associated with recurrent MI and revascularization but that these two alleles may be associated with earlier development of more extensive disease. The results of studies conducted at a number of research centers and including different study populations have shown that rs10757274G and rs10757278G are associated with CAD. However, the mechanisms by which these alleles affect the incidence of CAD remain to be elucidated (Shen et al., 2008b; Ding et al., 2009; Scheffold et al., 2011). Some authors have suggested that these polymorphisms may impair the mechanical properties of the arterial wall and thus influence vascular diseases (Bjorck et al., 2009). In our study, rs10757274G was not significantly associated with CAD in the population we selected (P = 0.08); this observation may be related to sample size and individual susceptibility. rs10757278G was independently associated with an increased risk for the incidence of CAD. Shiffman et al. (2006) in a study of white Americans observed that the rs1010 locus in *VAMP8*, a gene involved in platelet degranulation (OR = 1.75; 90%CI = 1.17-2.62; P = 0.025), and the rs11881940 site in *HNRPUL1*, a gene encoding ribonuclease (OR = 1.92; 90%CI = 1.28-2.86; P = 0.0043), were associated with early-onset MI (Gabler et al., 1998; Polgar et al., 2002). However, we found that the rs1010 locus was not significantly correlated with CAD, a result inconsistent with those reported by Duan et al. (2010), and this inconsistency may be due to differences in individual susceptibility to CAD. rs11881940T was observed to be a protective factor for CAD; this allele may alter a conserved adenine in a putative interferon-regulatory factor-1-binding site, affecting the incidence of CAD.

Connelly et al. (2006), studying a Caucasian population, found that the alleles rs3803 and rs2713604 of the transcription factor *GATA2* (Wang et al., 2007) were associated with

CAD. We observed that rs3803 was a CAD-protective factor. rs2713604 was not significantly correlated with CAD, consistent with observations by Dandona et al. (2010) indicating that rs2713604 does not contribute to the development of CAD.

The results of our ABI PRISM®SNaPshot™ genotyping analysis showed that the 9p21 region rs10757278G, *HNRPUL1* gene allele rs11881940T, and the GATA2 gene allele rs3803T were highly correlated with CAD ($P < 0.05$). rs10757278G increased the risk of CAD in patients with an OR = 1.242 (95%CI = 1.038-1.487) and is an independent risk factor for CAD morbidity in Han Chinese people; rs11881940T and rs3803T were identified as CAD-protective factors, indicated by ORs = 0.767 (95%CI = 0.605-0.973) and 0.532 (95%CI = 0.395-0.717), respectively.

Haplotype analysis of the rs10757278, rs11881940, and rs3803 loci showed that the haplotypes ATC, GAC, and GAT are risk factors for CAD and that GTC is a CAD-protective factor. Analysis of haplotype and CAD-related factors by logistic regression analysis showed that ATC was positively correlated with blood glucose abnormalities with an OR = 1.912 ($P < 0.05$). On the other hand, GAT was significantly associated with hypertension (OR = 2.859; $P < 0.01$). The specific mechanisms responsible for these observations remain to be identified.

With the ongoing completion of the human genome haplotype map (the HapMap), studies of SNPs and haplotypes will certainly play an important role in identifying the underlying genetic mechanisms, the risk of incidence, and drug responses of CAD. But the major genes affecting the incidence and development of CAD still need to be identified, and the haplotypes need to be defined in terms of the number of SNP loci sufficient to explain the pathogenesis of CAD.

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