Renin–Angiotensin–Aldosterone System Gene Polymorphisms and Coronary Artery Disease: Detection of Gene–Gene and Gene–Environment Interactions

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Key Words

Renin–angiotensin–aldosterone System • Gene-load
• Interaction • Coronary heart disease

Abstract

Objective: The objective of this study was to explore the association between coronary artery disease and genetic polymorphisms of the renin–angiotensin–aldosterone system (RAAS) pathway. In addition, we examined the interactions between demographic and lifestyle risk factors (environmental factors including age, sex, smoking status, alcohol intake) and RAAS polymorphisms on disease risk. **Methods:** A total of 1089 subjects who underwent coronary angiography were enrolled in this study. Eight RAAS polymorphisms were genotyped in this population: the G2350A (rs4343) polymorphism in exon 17 of the angiotensin converting enzyme (ACE) gene, 1166A→C (rs5186) and 573C/T (rs5182) in the angiotensin II type 1 receptor (AGTR1) gene, the -344C→T transversion (rs1799998) in the aldosterone synthase (CYP11B2) gene, and the G-217A (rs5049), G-6A (rs5051), M235T (rs699; T4072C), and T174M (rs4762; C3889T) polymorphisms in the angiotensinogen (AGT) gene. Subjects with coronary heart disease

were defined as those with at least 50% stenosis in at least one major coronary artery, and, the severity of coronary atherosclerosis was defined by the Gensini scoring system. **Results:** Compared to the subjects with AA genotype, the subjects with AG + GG genotype of rs1799998 had significant lower gensini score ($p=0.029$). After adjusting for age, gender, cigarette smoking, and alcohol intake status, the AG genotype (OR 0.717 95%CI 0.541–0.950, $p=0.021$) and the AG + GG genotype (OR 0.730 95%CI 0.559–0.954, $p=0.021$) distributions of rs1799998 were significantly different between the cases and controls compare to the AA genotype. Subjects with three at-risk loci had increased risk of coronary artery disease compared to subjects carrying 0 or 1 risk-associated polymorphism (OR [95%CI]: 1.579 [1.077–2.316], $p=0.019$), and the significance of the association was not reduced after adjusting for age, sex, cigarette smoking, or alcohol intake (adjusted OR [95%CI]: 1.673 [1.116–2.507], $p=0.013$). The results of multifactor-dimensionality reduction analysis revealed an interaction effect of CYP11B2 -344C→T, age, and smoking status on the risk of coronary heart disease (training OR [95%CI]: 3.7685 [2.8463–4.9895], $p<0.0001$; testing OR [95%CI]: 2.7583 [1.2038–6.3203], $p=0.015$).

Conclusions: Subjects who carried the G allele of the rs1799998 polymorphism significantly associated with coronary heart disease and severity of coronary atherosclerosis estimated by the Gensini score in the whole population of the study. And, multiple RAAS gene polymorphisms are associated with coronary artery disease. The interaction of the CYP11B2 -344C→T polymorphism (rs1799998), age, and smoking status is also associated with enhanced risk of coronary artery disease.

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Introduction

Atherosclerotic cardiovascular disease is a major health burden in the United States and throughout the world [1, 2]. Coronary atherosclerosis is a multifactorial and polygenic disorder predisposed by genetic, environmental, and lifestyle factors [3]. Among these predisposing genetic factors, disruption of the renin–angiotensin–aldosterone system (RAAS) is clearly involved in the development of atherosclerosis and coronary artery disease [4]. Genes of the RAAS pathway are therefore promising candidate loci, but several studies on the associations between coronary heart disease and single-nucleotide polymorphisms (SNPs) within RAAS pathway genes have presented conflicting results [5–11]. The reasons for these discrepancies are unknown, but possible confounds and statistical artifacts include insufficient sample size, different population histories or genetic backgrounds, and population stratification. Furthermore, the RAAS pathway is part of a regulatory system with multiple redundancies, so variations in single alleles may only impact disease when other compensatory mechanisms fail [12, 13].

Given that atherosclerosis and coronary heart disease are multigenetic disease, the focus should be on the combined effects of multiple susceptibility genes [14] that interact with demographic, lifestyle, and external factors (environmental or non-genetic risk factors) [15]. We hypothesized that the interactions between multiple genetic variants of the RAAS pathway and several non-genetic risk factors are associated with coronary heart disease. In the present cross-sectional study, we explored the associations between coronary heart disease and combinations of RAAS pathway genetic variants, as well as the interactions between RAAS polymorphisms and several risk factors, in 1089 consecutive subjects with coronary atherosclerosis confirmed by angiography.

Materials and Methods

Study subjects

From 2004 to 2006, 1089 consecutive subjects who underwent coronary angiography for suspected or known coronary atherosclerosis at the First Affiliated Hospital of Nanjing Medical University in China were enrolled in this study. The exclusion criteria for these subjects have been described previously [16]. In brief, subjects with spastic angina pectoris, heart failure, adrenal dysfunction, thyroid dysfunction, or infection within two weeks of the angiogram were excluded. This study was approved by the ethics committee of the First Affiliated Hospital of Nanjing Medical University and all subjects gave written informed consent.

Coronary Angiography

Coronary arteries were cannulated with 6F catheters by the Judkins technique [17]. The presence of stenosis of the coronary arteries was evaluated after direct intracoronary injection of isosorbide dinitrate (ISDN; 2.5 mg in 5 ml solution injected over 20 s). One minute after the ISDN injection, coronary angiography was performed in several projections over one minute at 30 frames/s [16]. Subjects with coronary heart disease were defined as those with at least 50% stenosis in at least one major coronary artery [18]. In addition, the severity of coronary atherosclerosis was defined by the Gensini scoring system. This is based on the hypothesis that the severity of coronary heart disease should be considered to be a consequence of the functional significance of the vascular narrowing and the extent of the area perfused by the involved vessel(s). In this scoring system, a greater reduction of the lumen diameter is assigned a higher score than a distal lesion [19].

Cigarette smoking and alcohol intake

Cigarette smoking status and alcohol intake were assessed with a standardized questionnaire. The subjects' smoking status and alcohol intake were classified as "never smoked" or "smoking" and "never drank" or "drinking" (the positive group included both former and current smokers or drinkers). Smoking was defined as at least 1 cigarette per day within the last month before examination, and drinking was defined as consuming at least 50 g of alcohol per week (5 drinks/week).

Anthropometric measurements

Anthropometric measurements were done after patients removed their shoes and upper garments and donned an examination gown. Each measurement was carried out twice and the mean value used. Height was measured to the nearest 0.1 cm using a wall-mounted stadiometer. Weight was measured to the nearest 0.1 kg using a hospital balance beam scale. Body mass index (BMI) was calculated as weight (kg) divided by the square of height (m²). Blood pressure was measured in the right arm with the participant seated and the arm exposed.

Laboratory measurements

The 12-h fasting blood samples were drawn in the morning. Thereafter, the blood samples were centrifuged, and serums

Variable	Coronary heart disease (n=727)	Control (n=362)	P value
Age, y	63.07±10.63	59.57±10.06	0.000
Gender (male/female)	582/145	234/128	0.000
Smoking	54.9% (399/727)	33.1% (120/362)	0.000
Drinking	23.4% (170/727)	18.5% (67/362)	0.066
Body mass index, kg/m ²	24.82±3.01	25.22±3.16	0.043
Systolic blood pressure, mmHg	130(120-145)	130(120-140)	0.299
Diastolic blood pressure, mmHg	80(70-85)	80(70-90)	0.007
Total cholesterol, mmol/L	4.08(3.52-4.69)	4.07(3.49-4.55)	0.508
Triglyceride, mmol/L	1.39(1.02-2.00)	1.43(0.98-2.04)	0.926
Fasting blood glucose, mmol/L	4.88(4.42-5.87)	4.65(4.29-5.16)	0.000
High-density lipoprotein- cholesterol, mmol/L	0.96(0.83-1.13)	1.04(0.90-1.21)	0.000
Low-density lipoprotein- cholesterol, mmol/L	2.41(1.92-2.96)	2.31(1.90-2.75)	0.041

Table 1. Demographic and biochemical characteristics of the subjects

were separated, collected and analysed. All laboratory measurements were conducted at the central clinical laboratory in the First Affiliated Hospital of Nanjing Medical University. The level of total cholesterol (CH), triglyceride (TG), fasting blood glucose (FBG), fasting high-density lipoprotein-cholesterol (HDL-c), and fasting low-density lipoprotein-cholesterol (LDL-c) were determined by enzymatic procedures on an automated autoanalyzer (AU 2700 Olympus, 1st Chemical Ltd, Tokyo, Japan).

DNA genotyping

Genomic DNA was extracted by the saturated sodium chloride method [20, 21]. Polymerase chain reactions were used to amplify DNA fragments. A total of 8 polymorphisms in 4 genes of the RAAS system were selected as candidate loci, G2350A (rs4343) in exon 17 of the angiotensin converting enzyme (ACE) gene, 1166A→C (rs5186) in the angiotensin II type 1 receptor (AGTR1) gene, 573C/T (rs5182) in the angiotensin II type 1 receptor (AGTR1) gene, -344C→T (rs1799998) in the aldosterone synthase (CYP11B2) gene, and G-217A (rs5049), G-6A (rs5051), M235T (rs699; T4072C), and T174M (rs4762; C3889T) of the angiotensinogen (AGT) gene. TaqMan-MGB Pre-Designed SNP assays purchased from Applied Biosystems (Foster City, CA) were used to genotype the candidate loci. Each PCR reaction was performed in a 5 µl volume containing 5 ng genomic DNA, 2x TaqMan® Universal PCR Master Mix and 0.083 µl 40x Assay Mix. All reactions were run in 384-well plates on an ABI PRISM 7900HT Sequence Detection system (Applied Biosystems, Foster City, CA) at the Chinese National Human Genome Center (Shanghai, China). The PCR program consisted of an initial 10 min denaturation at

95°C, followed by 20 cycles of 15 s at 92°C and 1 min at 60°C, then 30 cycles of 15 s at 89°C and 1.5 min at 60°C. Fluoresce signals were end-point read and automatically analyzed by SDS software 2.3 (ABI). Each 384-well plate had 2 samples from a known carrier as positive controls and 2 blank samples as negative controls for genotyping quality assessment. Result scoring was performed blind to CHD status.

Gene-load score

A gene-load score was calculated to evaluate the combined effect of RAAS pathway genes on the risk of coronary heart disease. The gene-load score can represent an individuals' 'genetic burden' and reflects the combined effect of multiple risk alleles within one pathway [12, 22]. To compute the gene-load score for each patient, we assumed a dominant model for variant alleles where heterozygote and variant homozygote genotypes were combined into one genotype for each of the eight loci (0 = reference genotype; 1 = risk genotypes). The loci of the rs1799998 (-344C→T) polymorphism associates with lower coronary heart disease risk, so a reversed coding was applied; the risk of the genotype AA was coded as 1 and AG+GG was coded as 0. The overall RAAS gene-load score could vary from 0 to 7, reflecting the eight RAAS gene polymorphisms genotyped, each with a weight of 1. Due to the low number of subjects with more than 4 variance alleles, RAAS gene-load score categories 5, 6, and 7 were all reclassified as category 4. Similarly, category 0 was combined with category 1 due to the low number of subjects with no allelic variants. The gene-load score was analyzed using the lowest gene-load score category (one or zero) as the reference category.

No.	dbSNP ID	HW p value	Gene	Relative position or Genomic location	polymorphism	MAF of cases	MAF of controls	% Genotyped
1	rs4343	0.854	ACE	exon 17	G2350A	0.3633	0.3441	98.3
2	rs5186	0.056	AGTR1	1166	1166A→C	0.0546	0.2651	99.6
3	rs5182	0.838	AGTR1	573	573C/T	0.2838	0.2703	97.3
4	rs5049	0.823	AGT	-217	G-217A	0.1634	0.1819	99.1
5	rs5051	0.606	AGT	-6	G-6A	0.1920	0.1849	98.5
6	rs699	0.524	AGT	4072	T4072C (M235T)	0.1945	0.1809	99.8
7	rs4762	0.482	AGT	3889	C3889T (T174M)	0.0976	0.0775	98.9
8	rs1799998	0.355	CYP11B2	344	-344C→T	0.2667	0.3028	99.2

Table 2. Primary information on the selected SNPs within renin–angiotensin–aldosterone system genes. HW, Hardy-Weinberg; MAF, minor allele frequencies; ACE, angiotensin converting enzyme; AGTR1, angiotensin II type 1 receptor; AGT, angiotensinogen; CYP11B2, aldosterone synthase

Statistical analysis

The Statistics Package for Social Sciences (ver. 16.0; SPSS Inc., Chicago) was employed to analyze the data. Due to the normally distributed characteristics, the data of age, and body mass index were presented as mean $\pm$ SD and comparisons between case and control and among group were analyzed by the independent-sample T tests and one way ANOVA respectively. Skewed data, including levels of systolic blood pressure (SBP), diastolic blood pressure (DBP), total cholesterol (CH), triglyceride (TG), fasting blood glucose (FBG), fasting high-density lipoprotein-cholesterol (HDL-c), fasting low-density lipoprotein-cholesterol (LDL-c), and Gensini score were expressed as median and quartile range and comparisons were analyzed using the nonparametric test. Categorical variables, including gender, cigarette smoking, and alcohol drinking, were compared between case and control by chi-squared analysis. The exact test was employed to evaluate the Hardy–Weinberg equilibrium of the polymorphisms. The associations between coronary heart disease risk and individual polymorphisms or gene-load scores were revealed by logistic regression (univariate and multivariate, with adjustment for age, gender, cigarette smoking, and alcohol intake).

The multifactor-dimensionality reduction (MDR) method [22] was employed to evaluate gene-gene interactions and interactions between RAAS genes and external risk factors.

Results

Baseline characteristics of the subjects

A total of 1089 subjects (816 males and 273 females, 61.91 $\pm$ 10.57 years) were enrolled in the study. The clinical and biochemical characteristics of the subjects are presented in Table 1. Significant differences were found between case and control age ($p=0.000$), gender ($p=0.000$), smoking status ($p=0.000$), body mass index ($p=0.043$), diastolic blood pressure ($p=0.007$), fasting blood glucose (FBG) ($p=0.000$), fasting high-density lipoprotein-

cholesterol (HDL-c) ($p=0.000$), and fasting low-density lipoprotein-cholesterol (LDL-c) ($p=0.041$), while the difference in drinking status, systolic blood pressure, total cholesterol, and triglyceride did not reach statistical significance.

Primary information of selected SNPs of the renin–angiotensin–aldosterone system genes

Primary information of the selected SNPs of the renin–angiotensin–aldosterone system pathway genes are presented in Table 2. All genotype distributions in these subjects were consistent with Hardy-Weinberg equilibrium, and none of the SNPs had minor allele frequencies (MAF) < 0.01 in either the case and control groups in this Chinese population.

Association of the renin–angiotensin–aldosterone system genes polymorphisms with clinical and biochemical parameter

The Table 3 shows the statistical results of the association of the renin–angiotensin–aldosterone system genes polymorphisms with clinical and biochemical parameter. Compared to the subjects with AA genotype, the subjects with AG + GG genotype of rs1799998 had significant lower gensini score ($p=0.029$). In addition, a statistically significant association between fasting blood glucose and the rs4762 polymorphism was found (AG+AA genotype vs. GG genotype, $p=0.049$).

Polymorphisms and coronary heart disease

The genotype frequencies of the selected SNPs are presented in Table 4. A statistically significant association between coronary heart disease and the rs1799998 polymorphism was found. After adjusting for age, gender,

Genotype	BMI	SBP	DBP	CH	TG	FBG	HDL-c	LDL-c	Gensini
rs4343									
AA	25.09±3.04	130(120-142)	80(70-87)	4.07(3.45-4.66)	1.47(1.01-2.04)	4.86(4.40-5.66)	0.97(0.83-1.17)	2.37(1.92-2.86)	20(1-57)
AG	24.85±3.05	130(120-145)	80(70-85)	4.09(3.52-4.62)	1.36(1.00-1.98)	4.74(4.36-5.46)	1.00(0.87-1.16)	2.38(1.93-2.85)	24(3-58)
GG	25.04±3.24	130(120-140)	80(70-85)	4.09(3.57-4.64)	1.33(0.92-1.95)	4.65(4.26-5.84)	0.99(0.86-1.14)	2.41(1.85-3.05)	24(4-63)
AG+GG	24.89±3.09	130(120-142)	80(70-85)	4.09(3.52-4.63)	1.36(0.99-1.97)	4.79(4.36-5.49)	1.00(0.87-1.16)	2.38(1.91-2.91)	24(3-59)
rs5186									
AA	24.95±3.06	130(120-142)	80(70-85)	4.08(3.49-4.60)	1.41(1.00-2.02)	4.82(4.37-5.59)	0.99(0.85-1.16)	2.38(1.92-2.86)	21(2-57)
AC	25.09±3.10	130(120-140)	80(70-85)	4.04(3.54-4.71)	1.38(0.98-1.93)	4.77(4.41-5.47)	0.98(0.83-1.13)	2.40(1.86-2.94)	24(2-62)
rs5182									
TT	24.95±3.10	130(120-145)	80(70-86)	4.08(3.54-4.60)	1.42(1.00-2.00)	4.79(4.33-5.22)	0.99(0.86-1.16)	2.37(1.93-2.83)	21(1-56)
CT	24.98±3.09	130(120-140)	80(70-85)	4.07(3.47-4.70)	1.39(0.99-2.03)	4.80(4.40-5.60)	0.98(0.83-1.16)	2.39(1.91-2.94)	24(3-60)
CC	24.75±2.76	130(120-140)	80(70-85)	3.87(3.43-4.44)	1.25(0.89-1.85)	4.77(4.42-5.44)	0.99(0.85-1.17)	2.37(1.82-2.76)	20(3-67)
CC+CT	24.94±3.03	130(120-140)	80(70-85)	4.06(3.45-4.63)	1.37(0.99-2.01)	4.80(4.41-5.56)	0.99(0.84-1.16)	2.38(1.89-2.91)	23(3-60)
rs5049									
CC	24.99±3.13	130(120-140)	80(70-85)	4.08(3.51-4.62)	1.42(0.99-2.05)	4.80(4.40-5.50)	0.98(0.86-1.16)	2.34(1.93-2.85)	22(2-58)
CT	24.90±2.91	130(120-145)	80(70-90)	4.05(3.46-4.67)	1.39(1.00-1.84)	4.82(4.34-5.78)	1.00(0.83-1.18)	2.44(1.89-3.00)	23(2-62)
TT	24.46±2.64	140(120-150)	80(79-90)	4.14(3.54-4.41)	1.35(1.00-2.21)	4.69(4.02-5.47)	0.99(0.79-1.14)	2.57(2.05-2.95)	13(0-48)
CT+TT	24.86±2.88	130(120-145)	80(70-90)	4.06(3.49-4.66)	1.37(1.00-1.85)	4.81(4.31-5.66)	1.00(0.83-1.17)	2.46(1.90-2.99)	22(1-62)
rs5051									
TT	24.91±3.02	130(120-140)	80(70-85)	4.10(3.51-4.60)	1.40(1.00-2.00)	4.76(4.35-5.55)	0.98(0.84-1.16)	2.42(1.91-2.93)	23(2-60)
CT	25.08±3.10	130(120-145)	80(70-88)	4.02(3.48-4.64)	1.42(1.00-2.01)	4.85(4.41-5.67)	0.98(0.87-1.15)	2.30(1.88-2.83)	22(2-57)
CC	24.19±3.06	130(117-140)	80(71-87)	3.91(3.58-4.76)	1.26(0.90-2.15)	4.63(4.28-5.27)	1.04(0.94-1.18)	2.24(1.94-2.72)	9(1-48)
CT+CC	25.00±3.10	130(120-145)	80(70-88)	4.01(3.49-4.68)	1.41(0.99-2.06)	4.83(4.40-5.56)	0.99(0.87-1.15)	2.30(1.89-2.81)	21(2-53)
rs699									
GG	24.91±3.04	130(120-140)	80(70-85)	4.10(3.51-4.59)	1.40(1.01-2.00)	4.77(4.35-5.54)	0.99(0.84-1.17)	2.41(1.91-2.91)	22(2-59)
AG	25.10±3.12	130(120-145)	80(70-89)	4.02(3.49-4.70)	1.42(0.98-2.07)	4.86(4.42-5.70)	0.98(0.87-1.15)	2.30(1.88-2.88)	22(3-58)
AA	24.52±3.11	130(120-140)	80(71-90)	3.84(3.38-4.71)	1.28(0.94-2.15)	4.66(4.39-5.40)	1.01(0.94-1.14)	2.21(1.93-2.72)	9(0-48)
AG+AA	25.04±3.12	130(120-145)	80(70-89)	4.00(3.49-4.70)	1.41(0.98-2.07)	4.84(4.41-5.63)	0.99(0.87-1.15)	2.30(1.89-2.86)	21(2-58)
rs4762									
GG	24.96±3.09	130(120-145)	80(70-85)	4.07(3.49-4.62)	1.40(0.99-2.01)	4.77(4.34-5.52)	0.99(0.85-1.16)	2.39(1.92-2.92)	20(2-58)
AG	24.95±2.95	130(120-140)	80(70-85)	4.08(3.56-4.60)	1.35(0.99-1.91)	4.91(4.53-5.67)	0.98(0.82-1.19)	2.27(1.90-2.76)	26(5-61)
AA	25.59±2.17	140(110-160)	80(70-95)	4.01(3.39-4.45)	1.67(1.21-2.93)	4.71(4.37-6.20)	0.99(0.83-1.14)	2.12(1.85-2.52)	40(5-70)
AG+AA	24.97±2.92	130(120-140)	80(70-85)	4.07(3.55-4.59)	1.36(1.00-1.93)	4.90(4.53-5.68) [†]	0.98(0.83-1.18)	2.26(1.90-2.75)	26(5-61)
rs1799998									
AA	24.83±2.98	130(120-142)	80(70-88)	4.05(3.48-4.68)	1.40(0.99-2.05)	4.86(4.40-5.66)	0.99(0.83-1.17)	2.36(1.88-2.87)	24(3-64)
AG	25.19±3.18	130(120-140)	80(70-85)	4.11(3.54-4.61)	1.40(1.00-1.96)	4.74(4.36-5.46)	0.98(0.85-1.15)	2.43(1.97-2.93)	20(1-49)
GG	24.76±3.01	130(117-146)	80(70-85)	4.05(3.48-4.45)	1.53(1.08-2.13)	4.65(4.26-5.84)	1.02(0.90-1.16)	2.25(1.82-2.77)	22(2-61)
AG+GG	24.81±2.98	130(120-145)	80(70-88)	4.04(3.46-4.67)	1.40(0.99-2.04)	4.86(4.40-5.65)	0.99(0.84-1.17)	2.35(1.88-2.87)	20(1-52)*
Combined genotypes									
0-1 at-risk locus	24.76±3.07	130(120-140)	80(70-85)	4.16(3.54-4.53)	1.42(1.00-1.97)	4.65(4.26-5.27)	0.99(0.88-1.16)	2.41(2.01-2.83)	20(0-56)
2 at - risk loci	25.05±3.14	130(120-145)	80(70-85)	4.16(3.60-4.67)	1.47(1.07-2.09)	4.84(4.37-5.71)	1.02(0.87-1.19)	2.42(1.96-2.97)	18(1-55)
3 at - risk loci	25.13±3.17	130(117-141)	80(70-85)	3.98(3.47-4.65)	1.32(0.98-1.96)	4.82(4.40-5.41)	0.96(0.83-1.14)	2.37(1.87-2.85)	24(5-63)
≥4 at-risk loci	24.82±2.94	130(120-142)	80(70-90)	4.01(3.45-4.72)	1.42(0.99-1.98)	4.80(4.43-5.73)	1.00(0.83-1.18)	2.32(1.88-2.85)	22(2-60)

Table 3. Association of the renin–angiotensin–aldosterone system genes polymorphisms with clinical and biochemical parameter. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; CH, total cholesterol; TG, triglyceride; FBG, fasting blood glucose; HDL-c, fasting high-density lipoprotein-cholesterol; LDL-c,fasting low-density lipoprotein-cholesterol (LDL-c); Gensini, Gensini score ,* $p=0.029$; $^{\dagger}p=0.049$

cigarette smoking, and alcohol intake status, the AG genotype (OR 0.717 95% CI 0.541–0.950, $p=0.021$) and the AG + GG genotype (OR 0.730 95% CI 0.559–0.954,

$p=0.021$) distributions of rs1799998 were significantly different between the cases and controls compare to the AA genotype. In contrast, none of the other seven SNPs

Genotype	Cases, n (%)	Controls, n(%)	Crude OR (95% CI)	Adjusted OR (95% CI)*
rs4343	717	353		
AA	286(39.9)	155(43.9)	1.00	1.00
AG	341(47.6)	153(43.3)	1.208(0.919–1.587)	1.235(0.925–1.650)
GG	90(12.6)	45(12.7)	1.084(0.721–1.630)	1.064(0.694–1.632)
AG+GG	431(59.3)	198(54.7)	1.180(0.912–1.527)	1.195(0.910–1.570)
rs5186	724	361		
AA	645(89.1)	321(88.9)	1.00	1.00
AC	79(10.9)	40(11.1)	0.983(0.657–1.471)	0.947(0.617–1.454)
rs5182	703	357		
TT	359(51.1)	193(54.1)	1.00	1.00
CT	289(41.1)	135(37.8)	1.020(0.629–1.652)	0.979(0.584–1.640)
CC	55(7.8)	29(8.1)	1.151(0.879–1.506)	1.177(0.885–1.565)
CC+CT	344(48.9)	164(45.9)	1.128(0.874–1.456)	1.142(0.871–1.496)
rs5049	719	360		
CC	501(69.7)	242(67.2)	1.00	1.00
CT	201(28.0)	105(29.2)	0.925(0.698–1.225)	0.964(0.717–1.295)
TT	17(2.4)	13(3.6)	0.632(0.302–1.322)	0.689(0.309–1.538)
CT+TT	218(30.4)	118(32.8)	0.886(0.675–1.161)	0.929(0.698–1.236)
rs5051	716	357		
TT	464(64.8)	238(66.7)	1.00	1.00
CT	229(32.0)	106(29.7)	0.907(0.452–1.823)	0.975(0.468–2.032)
CC	23(3.2)	13(3.6)	1.108(0.839–1.464)	1.168(0.870–1.568)
CT+CC	252(35.2)	119(33.3)	1.083(0.829–1.415)	1.145(0.863–1.520)
rs699				
GG	466(64.3)	244(67.4)	1.00	1.00
AG	236(32.6)	105(29.0)	0.926(0.461–1.861)	0.991(0.475–2.066)
AA	23(3.2)	13(3.6)	1.177(0.892–1.553)	1.226(0.914–1.644)
AG+AA	259(35.8)	118(32.6)	1.149(0.880–1.501)	1.200(0.904–1.592)
rs4762	717	361		
GG	582(81.2)	307(85.0)	1.00	1.00
AG	130(18.1)	52(14.4)	1.319(0.254–6.837)	2.153(0.401–11.556)
AA	5(0.7)	2(0.6)	1.319(0.929–1.871)	1.280(0.884–1.853)
AG+AA	135(18.8)	54(15.0)	1.301(0.922–1.835)	1.285(0.894–1.849)
rs1799998	720	360		
AA	394(54.7)	174(48.3)	1.00	1.00
AG	268(37.2)	154(42.8)	0.769(0.589–1.003)	0.717(0.541–0.950)*
GG	58(8.1)	32(8.9)	0.800(0.502–1.277)	0.797(0.481–1.321)
AG+GG	326(45.3)	186(51.7)	0.769(0.598–0.990)‡	0.730(0.559–0.954)†
Combined genotypes	693	348		
0-1 at-risk locus	121(17.5)	77(22.1)	1.00	1.00
2 at-risk loci	182(26.3)	96(27.6)	1.206(0.827–1.760)	1.140(0.764–1.701)
3 at-risk loci	206(29.7)	83(23.9)	1.579(1.077–2.316) ^Δ	1.673(1.116–2.507) [○]
≥4 at-risk loci	184(26.6)	92(26.4)	1.273(0.871–1.861)	1.310(0.876–1.957)

Table 4. Single genotype and gene-load score distribution of the renin–angiotensin–aldosterone system genes between cases and controls. *Adjusted for age, sex, cigarette smoking and alcohol intake. * $p=0.021$; † $p=0.021$; ‡ $p=0.042$; $\Delta p=0.013$; $\circ p=0.019$

(rs4343, rs5186, rs5182, rs5049, rs5051, rs699, and rs4762) were associated with the risk of coronary heart disease in single locus analysis. Therefore, gene-load score analyses were performed assuming the dominant model

for each locus (the genotype with no at-risk allele versus the combination of the heterozygous and homozygous at-risk genotypes). As shown in Table 4, compared to the subjects harboring no at-risk locus or one at-risk locus,

No. model	Training balanced accuracy	Training Odds Ratio	Training p value	Testing balanced accuracy	Testing Odds Ratio	Testing p value	Cross-validation consistency
smoke	0.6087	2.4532 (1.859–3.2373)	P<0.0001	0.6087	2.4532 (1.0675–5.6374)	0.0324	10/10
Smoke, age	0.6436	3.334 (2.5272–4.3982)	P<0.0001	0.6436	3.334 (1.4521–7.6544)	0.0038	10/10
Rs1799998, smoke, age	0.6544	3.7685 (2.8463–4.9895)	P<0.0001	0.6187	2.7583 (1.2038–6.3203)	0.015	7/10
Rs4343, rs5182, smoke, age	0.6802	4.5446 (3.409–6.0585)	P<0.0001	0.5906	2.0826 (0.9268–4.6801)	0.0736	7/10

Table 5. Results of multifactor dimensionality reduction analysis

the subjects carrying 3 at-risk loci had increased risks of coronary heart disease (*OR*, [95%*CI*]: 1.579 [1.077–2.316], $p=0.019$). This significant association was not influenced by adjusting for age, gender, cigarette smoking, and alcohol intake status (adjusted *OR* [95%*CI*]: 1.673 [1.116–2.507], $p=0.013$). However, the association was not reach significant level with regard to the subjects carrying 4 at-risk loci and more than 4 at-risk loci compared to the subjects harboring no at-risk locus or one at-risk locus.

Environment-Gene-Gene Interactions

Statistical analyses of 10-fold cross-validation and configured multifactor-dimensionality reduction were employed to consider one through 12 variables at one time, including the eight SNPs and age, gender, smoking status, and drinking status. Table 5 presents the results of the MDR analysis for each combination of factors. The model with the lowest prediction error and highest cross-validation consistency was selected for each number of factors considered. One 3-factor model had a training balanced accuracy of 0.6544, testing balanced accuracy of 0.6187, and a cross-validation consistency of 7/10 that was significant at $P<0.05$. A 4-factors model also had a significant training balanced accuracy of 0.6802 ($P<0.001$). However, this 4-factor model had a lower testing balanced accuracy of 0.5906 ($P=0.0736$) than the 3-factor model. Therefore, the 3-factor model was selected as the best model. This 3-factors model consisted of rs1799998, smoking status, and age, and the training *OR* [95%*CI*] for coronary heart disease was 3.7685 [2.8463–4.9895] and the testing *OR* [95%*CI*] for coronary heart disease was 2.7583 [1.2038–6.3203].

Discussion

In this hospital-based study of consecutive adult Chinese subjects with angiographic evidence, we found that the -344C→T polymorphism (rs1799998) in the aldosterone synthase (CYP11B2) gene was significantly associated with the risk of coronary heart disease and severity of coronary atherosclerosis estimated by the Gensini score, even after adjusting for age, gender, smoking status, and drinking status. These results further demonstrate that the RAAS gene-load score is a robust model for multilocus effects associated with coronary heart disease where each genetic variant confers only a small change in risk. The presence of three risk genotypes in the RAAS resulted in a 1.6-fold or greater increase in the risk for coronary heart disease compare to the presence one or no risk alleles after adjusting for age, gender, smoking status, and drinking status. In addition, we evaluated the interactions between RAAS gene polymorphisms and the common coronary heart disease risk factors such as age, gender, smoking status, and drinking status. We found significant interactions between age, smoking status, and the rs1799998 SNP (-344C→T) within the aldosterone synthase (CYP11B2) gene.

A recent study found a significant association between RAAS gene-load score and coronary heart disease; patients with a gene-load score of 5 or 6 had a coronary heart disease risk 2.3 times higher than patients with scores of 0 or 1 [12]. The subjects in that study, however, were limited to familial hypercholesterolaemia associated with premature coronary heart disease as defined by clinical criteria. The subjects in the present study were not limited to familial hypercholesterolaemia

and both cases and controls had coronary heart disease as confirmed by coronary angiography. To the best of our knowledge, this is the first study to demonstrate a significant association between RAAS gene-load score and coronary heart disease confirmed directly by angiography.

Despite the recent success of genome-wide association studies in identifying loci consistently associated with coronary artery disease, a large proportion of these loci are associated with metabolic risk factors, including elevated plasma lipids, type 2 diabetes, and elevated body mass index, and have not been linked mechanistically to the etiology of coronary artery disease. Gene-gene and gene-environment interactions might produce a meaningful improvement in quantification of the genetic determinants of coronary heart disease [23]. Recently, 1254 consecutive patients who underwent cardiac catheterization (735 with documented coronary artery disease and 519 without) between 1996 and 2003 were recruited to explore gene-gene and gene-environment interactions. The angiotensin-converting enzyme (ACE) gene insertion/deletion (I/D) polymorphism T174M, the A1166C polymorphism of the angiotensin II type I receptor gene, and the M235T, G-6A, A-20C, G-152A, and G-217A polymorphisms of the angiotensinogen gene were genotyped. The results demonstrated a significant three-locus (G-217A, M235T, and I/D) gene-gene interactions by the multifactor-dimensionality reduction method and many higher-order gene-gene interactions by multilocus genotype disequilibrium tests [24]. Significant interactions between angiotensin II gene haplotypes, gender, and hypertension were detected [25]. The -344C→T polymorphism (rs1799998) in the aldosterone synthase (CYP11B2) gene was not genotyped in that study, however. In the present case-control study of coronary heart disease in a Chinese population, we found, for the first time, an interaction between rs1799998, age, and smoking status associated with the risk of coronary heart disease. These results are compatible with the concept that interaction between gene polymorphisms and various demographic, lifestyle, and environmental factors might contribute to the risk of coronary heart disease.

The exact molecular mechanisms underlying the interaction of rs1799998, age, and smoking status on the development of coronary heart disease remain to be unraveled. Published studies focusing on rs1799998 do suggest several potential roles of this polymorphism. Aldosterone synthase (CYP11B2) catalyses the final step of aldosterone biosynthesis in adrenal glomerulosa and is

sensitive to the effects of angiotensin II. The T/C transversion at 344 base is upstream of the start codon of the CYP11B2 gene. A long-term prognosis study of acute myocardial infarction (AMI) at a young age suggested that polymorphisms in RAAS genes including CYP11B2 polymorphisms can be important in the onset of a first AMI in young patients [26]. Conversely, a prospective study of healthy UK men indicated that the aldosterone synthase genotype was unrelated to overall coronary heart disease risk. A possible interaction with smoking requires confirmation [27]. A prospective study of unrelated, healthy middle-aged Caucasian males concluded that the aldosterone synthase -344C→T polymorphism does not significantly influence the risk of myocardial infarction either directly or through interactions with other risk factors [28].

In the present study, the interaction of rs1799998, age, and smoking status was associated with the risk of coronary heart disease. A significant association between the thymidine to cytosine substitution variant (the -344C→T polymorphism (rs1799998) in the aldosterone synthase gene) and *in vivo* circulating aldosterone levels [29], arterial stiffness [30] and left ventricular mass and dimensions [31] may underlie the association. However, the exact pathophysiological mechanisms that contribute to this association require further study.

In the present study, the subjects carrying 3 at-risk loci had increased risks of coronary heart disease compared to the subjects harboring no at-risk locus or one at-risk locus, however, this significant association was not existed with regard to the subjects carrying 4 at-risk loci and more than 4 at-risk loci compared to the subjects harboring no at-risk locus or one at-risk locus. The exact reason underlying the above difference was unknown, however, the sample-size of the present study is small, and it maybe explain partially the phenomenon. Therefore, a larger sample-size, prospectively epidemiological study needs to be conducted to confirm the finding from the present study in the future.

Conclusions

Subjects who carried the G allele of the rs1799998 polymorphism significantly associated with coronary heart disease and Gensini score in the whole population of the study. And, the present study revealed for the first time that the renin-angiotensin-aldosterone (RAA) system gene-load score is associated with coronary heart disease. While each of the genetic variants analyzed conferred

only a small portion of risk (or no risk), any three risk polymorphisms statistically associated with coronary heart disease. Furthermore, we uncovered a significant association between the interaction of age, smoking status, and the -344C→T polymorphism (rs1799998) in the aldosterone synthase (CYP11B2) gene and the risk of coronary heart disease.

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