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Original Article

Exploring epistatic relationships of NO biosynthesis pathway genes in susceptibility to CHD

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Aim: To assess the epistatic relationships of nitric oxide (NO) biosynthesis pathway genes in susceptibility to coronary heart disease (CHD).
Methods: A total of 2142 subjects enrolled in two case-control studies was genotyped for 7 single-nucleotide polymorphisms (SNP) within NO biosynthesis pathway genes using TaqMan assays. The association analyses were performed at both SNP and haplotype levels. Two-way SNP-SNP interactions and high-order interactions were tested using multiple unconditional logistic regression analyses and generalized multifactor dimensionality reduction (GMDR) analyses, respectively.
Results: Two alleles (rs1049255*C and rs841*A) were identified that were significantly associated with increased risk of CHD after adjusting for all confounders (OR=1.21, 95% CI: 1.06−1.39, combined P=0.001, P_{corr}=0.007 and OR=1.30, 95% CI 1.12−1.50, combined P<0.001, P_{corr}<0.001, respectively). Significant two-way SNP–SNP interactions were found between SNP rs2297518 and these two significant polymorphisms, affecting the risk of CHD (P<0.001 for both). No significant high-order interactions were identified.
Conclusion: The results suggested that two-way SNP–SNP interactions of polymorphisms within NO biosynthesis pathway genes contribute to CHD risk.

Keywords: coronary heart disease; nitric oxide; genetics; epitasis; interaction; single-nucleotide polymorphism; case control study

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Introduction

Nitric oxide (NO) has numerous important functions that contribute to the maintenance of vascular homeostasis[1]. *L*-arginine and molecular oxygen synthesize three different isoforms of NO: inducible (iNOS), neuronal (nNOS), and endothelial (eNOS)[2–4]. All of these isoforms have been reported to be expressed in human atherosclerotic vascular lesions and play important but separate roles in the development of atherosclerosis[5–7]. In addition, the deficiency of NO activity is involved in the pathogenesis of coronary spasms[8].

Extensive research has been focused on several functionally important polymorphisms in NO biosynthesis pathway genes[9–14]. These polymorphisms could influence individual susceptibility to atherosclerosis by altering levels of NO production. However, the results from these association studies were often not reproducible. For example, studies of the extensively investigated polymorphism rs1799983 (G894T, Glu298Asp) in the eNOS gene have yielded a large number of controversial reports[9–14]. These inconsistent findings might be explained in part by the genetic and environmental heterogeneity among different populations. It is also possible that this locus contributes to atherosclerosis only through its interactions with other genes. Thus, the primary effects of individual loci may be too small to be detected[15]. Alternatively, the effects of the variants under study might be masked by the effects of unstudied polymorphism(s) that affect the phenotype[16]. Therefore, we suggest that searching for susceptibility genes for atherosclerosis risk can be improved by an exploration of gene-gene and/or gene-environment interactions.

In this study, we hypothesized that the complex interactions among polymorphisms within genes involved in the NO biosynthesis pathway may confer variations in risk of coronary heart disease (CHD). To assess the primary effects of these polymorphisms on CHD risk by conventional logistic regression as well as by haplotype analysis, we explored the high-order gene-gene interactions by applying generalized multi-

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factor dimensionality reduction (GMDR) analysis[17]. We also systematically evaluated these approaches for their ability to predict those individuals who were affected by CHD and had any combination of two or more polymorphisms from all of the genotyped markers.

Materials and methods

Study participants and CHD definition

CHD patients (n=557) were consecutively recruited from Tongji Hospital and the Institute of Hypertension (Wuhan, China) between May 2004 and September 2008. The diagnostic criteria for CHD were at least one of the following: (1) the presence of a stenosis >50% in at least one of the major segments of the coronary arteries (right coronary artery, left circumflex, or left anterior descending arteries) on coronary angiography; (2) elevation of cardiac enzymes (troponin T, troponin I, creatine kinase-MB, aspartate aminotransferase, and glutamic pyruvic transaminase), typical ECG changes (Minnesota Code 1.1 or 1.2 in ECG) and clinical symptoms according to the World Health Organization (WHO) criteria; or (3) a documented history of coronary artery bypass graft or percutaneous coronary intervention. Subjects with congenital heart disease, cardiomyopathy, valvular disease, or renal or hepatic disease were excluded from the study. Five hundred fifty-seven ethnically and geographically matched controls were randomly selected either from healthy residents in the community (89.6%) or from inpatients (10.4%) with minor illnesses. All control subjects were free of cardiovascular disease and were subjected to the same exclusion criteria as the patients with CHD. All participants were asked for a detailed medical history and received a physical examination of cardiovascular systems, including evaluation of body mass index.

To confirm the credibility of the results obtained from our first study cohort described above, we obtained another CHD case-control study cohort that comprised 507 CHD patients and 502 unaffected controls recruited from the Tongji Hospital between September 2008 and February 2010 (Wuhan, China). The diagnostic criteria for CHD as well as the inclusion and exclusion criteria for qualified participants were identical to those used in our first CHD discovery sample.

All sensitive personal information was de-identified to protect patient privacy. The institutional review board of Tongji Hospital approved this study. Written informed consent was obtained from all participants. Experiments were conducted according to the principles expressed in the Declaration of Helsinki.

Selection of candidate genes and polymorphisms

With regard to the regulation of the NO biosynthesis pathway, we selected seven single-nucleotide polymorphisms (SNPs) based on previous evidence of potential functionality, validated allele frequency, and sequence-proven allelic variation: Leu608Ser (rs2297518) in iNOS[18, 19], G-84A (rs41279104) in the promoter region of nNOS[20], Glu298Asp (rs1799983) and T-786C (rs2020744) in the promoter region of eNOS[9–11, 21, 22], Tyr72His (rs4673) and C+640T (rs1049255) in the 3′-untranslated region (UTR) of the cytochrome b-245, alpha polypeptide gene (CYBA)[23–26], which encodes the p22 phox subunit of the NADPH oxidase, and G+243A (rs841) in the 3′-UTR of the GTP cyclohydrolase 1 gene (GCH1)[27].

DNA isolation and genotyping

Genomic DNA was isolated from whole blood collected in K_3-EDTA tubes using the QG-Mini80 workflow with a DB-S kit (FUJIFILM Corporation, Tokyo, Japan) according to the manufacturer's instructions. DNA was quantified and diluted to a final concentration of 10 ng/μL.

All samples were genotyped using the Taqman™ 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Each assay was carried out using 10 ng DNA in a 5-μL reaction consisting of TaqMan universal PCR master mix (Applied Biosystems, Foster City, CA, USA), forward and reverse primers and FAM- and VIC-labeled probes designed by Applied Biosystems (ABI Assays-on-Demand (rs1799983, C_3219460_20; rs2297518, C_11889257_10; rs1049255, C_7516913_10; rs4673, C_2038_20) and Assays-on-Design; see Table 1). Allelic discrimination was measured automatically using the Sequence Detection Systems 2.1 software (auto caller confidence level 95%). A total of 10% of all genotypes was repeated in independent PCR reactions to check for consistency and to ensure intraplate and interplate genotype quality control. No genotyping discrepancies were detected between the repeated samples. DNA samples for cases and controls were run in the same batches.

Statistical analysis

Statistical analyses were performed with SPSS 13.0 (SPSS Inc, Illinois, Chicago) for Windows (Microsoft Corp, Redmond, Washington) and SNPassoc for the R statistical package[28].

Table 1. TaqMan primer and probe sequences.

	Primer (5′–3′)	Allele	Allelic probe
rs41279104	Forward AGGCCGAGCGACTGG	G	VIC-CAGAGCCGCCTCCCA-NFQ
	Reverse CCCCTGCCCAAGGCTT	A	FAM-CAGAGCCACCTCCCA-NFQ
rs841	Forward TTTTTGTGGCAATATTAAAGTGAACTGCTAA	G	VIC-TTTGTGCACGTACTTAC-NFQ
	Reverse CAGGCCCCTCTGGTTATCTG	A	FAM-TTGTGCACATACTTAC-NFQ
rs2070744	Forward ACCAGGGCATCAAGCTCTTC	G	VIC-AGGGTCAGCCGGCCAG-NFQ
	Reverse GCAGGTCAGCAGAGAGACTAG	A	FAM-AGGGTCAGCCAGCCAG-NFQ

The level of linkage disequilibrium is indicated here as D' (D-Prime). The presence of Hardy-Weinberg equilibrium (HWE) for each SNP was tested using Haploview 4.0[29], which is based on the χ^2 test.

The normality of the distribution of quantitative variables was assessed using the one-sample Kolmogorov-Smirnov test, and transformations were applied for non-normal variables when necessary. All quantitative variables were generally described as means with standard deviations (SDs). For comparison of the baseline characteristics among different groups, one-way ANOVA tests were performed on quantitative variables, such as age, body mass index, high-density lipoprotein cholesterol (HDL-C), and total cholesterol (TC). The χ^2 test was used for qualitative variables.

For each SNP, differences in allelic frequencies between cases and controls were determined by the χ^2 or Fisher's exact test. Multiple unconditional logistic regression was used to estimate odds ratios (ORs) and 95% confidence intervals (CIs) under the additive model after adjusting for covariates such as gender, age, body mass index, hypertension, diabetes, hyperlipidemia, smoking status and different populations. Two-way SNP–SNP interactions between polymorphisms within the NO biosynthesis pathway genes for CHD were also determined by multiple unconditional logistic regression using allied genotypes after adjusting for significant confounders.

Haplotype frequencies for various SNP combinations were first estimated by haplo.stats[30] (version 1.2.1) in the R statistical package and then verified using Haploview 4.0. Each of these programs applies the Expectation-Maximization (EM) algorithm when constructing the haplotypes. The haplo.stats program was used to compute global scores and haplotype-specific *P* values while allowing for adjusting covariates under the additive model using default settings. To minimize the false-positive results generated from multiple statistical testing in our aforementioned analyses, we adopted the Bonferroni correction method for multiple testing.

The GMDR approach[17], which is an extension of multifactor dimensionality reduction (MDR)[31] for adjustments with covariates based on the score of a generalized linear model, was applied to indentify multi-locus genetic interactions. It computed the maximum-likelihood estimates and the score values of all individuals under the null hypothesis. The cumulative score values were then calculated within each multifactor cell, which were each then labeled either as high-risk if the average score met or exceeded a pre-assigned threshold of 0 or as low-risk if the score was less than 0. We performed an exhaustive search for all possible combinations of one- to seven-locus models for all polymorphisms. The 10-fold cross validation (CV) consistency and the balanced prediction accuracy estimates were calculated for each combination of a pool of polymorphisms. The best model is the one with the highest prediction accuracy and maximal CV. The sensitivity, specificity, odds ratios, and sign tests (to determine *P* values) of the best model were also calculated using the GMDR software.

In CHD patients, population-attributable risks (PARs) were calculated by using the formula[32] PAR (%)=p(OR-1)/[p(OR-1)+1]×100%, where p is the proportion of individuals exposed to a risk gene (proportion or allele frequency of risk allele in CHD patients), and OR is the combined OR when cases and controls are compared in the risk model.

Power calculations to detect genetic associations were estimated using the QUANTO[33] program (Version 1.2.3). Assuming disease prevalence between 0.5% and 1%, our combined sample size can reach >80% power to detect a susceptibility locus with a genotypic relative risk >1.21 at the nominal Type I error rate of <0.05 for SNPs with minor allele frequencies >0.31 under the additive model. This indicates that our cohort sample size is sufficient to generate robust estimates in association analyses.

Results

Association between individual SNPs and the risk of CHD

The demographic details of two case-control studies are shown in Table 2. The observed frequencies of minor alleles and the genotype distributions of seven selected SNPs in cases and controls are summarized in Table 3. For all polymorphisms, the genotype distributions in cases and controls were under Hardy-Weinberg equilibrium (P>0.05). The P_{allele} values for each SNP are shown in Table 3. Two SNPs (rs1049255 and rs841) were statistically significant associated with increased risk of CHD in the first study and were replicated in the second study. Combined analysis of the two studies, comprising a total of 1083 cases and 1059 control subjects, showed even stronger associations between CHD and these two SNPs (combined P_{allele}=0.001 for rs1049255, and combined P_{allele}<0.001 for rs841). Furthermore, these associations remained significant after the Bonferroni correction was applied (P_{corr}=0.007 for rs1049255, and P_{corr}<0.001 for rs841). We further conducted a genotypic association analysis assuming the additive genetic model to investigate how each of these SNPs confers a genetic risk for CHD. We found that same SNPs (rs1049255 and rs841) exerted effects on the disease trait in the additive models after adjusting for covariates such as gender, age, body mass index, hypertension, diabetes, hyperlipidemia, smoking status and different populations (OR=1.21, 95% CI 1.06–1.39, P=0.001, P_{corr}=0.007 and OR=1.30, 95% CI 1.12–1.50, P<0.001, P_{corr}<0.001, respectively; see Table 4). The overall PAR for rs1049255 and rs841 in the CHD patients were 11.52% (95%CI: 3.58%–19.47%) and 10.22% (95%CI: 4.36%–15.95%), respectively.

Haplotype analysis

We subsequently performed haplotype analysis using haplo.stats to study multiple SNPs within the eNOS and CYBA genes (Table 5). Consistent with the single-locus results (rs841 within CYBA), we only observed significant haplotype results in the CYBA gene (global P=0.01, P_{corr}=0.04). To further evaluate the observed genetic effects independent of environmental factors, we conducted haplotype-based hypothesis tests using the software haplo.stats, which allowed for the adjustment of conventional risk factors. When the haplotype CG was chosen as the baseline, haplotype TG displayed a significantly increased risk for CHD (GT *vs* GC, OR=0.97, 95% CI 0.94–1.00,

Table 2. Clinical characteristics of two case-control studies.

Characteristics	First study Controls	First study CHD	Second study Controls	Second study CHD
n	557	576	502	507
Age, y	62.3±9.3	61.0±9.8[b]	62.6±8.7	60.3±10.6[c]
Men, %	62.1	63.9	52.2	76.5[c]
BMI, kg/m²	23.7±3.2	24.4±3.1[c]	23.6±3.8	24.6±3.3[c]
SBP, mmHg	131.3±20.8	145.9±24.2[c]	123.6±17.7	134.4±21.6[c]
DBP, mmHg	78.0±11.1	86.2±13.3[c]	77.9±10.1	80.1±13.7[c]
HDL-C, mmol/L	1.33±0.36	1.03±0.68[c]	1.29±0.53	1.18±0.37[c]
TC, mmol/L	4.56±1.70	4.54±1.27	3.12±1.67	4.86±1.16[c]
Hypertension, %	19.2	30.4[c]	37.8	60.9[c]
Diabetes, %	3.2	18.7[c]	7.8	16.6[c]
Hyperlipidemia, %	21	35.1[c]	30.1	40.2[c]
Smokers, %	37.3	46.5[c]	26.3	57.4[c]

n indicates number of individuals; BMI, body mass index; SBP, systolic blood pressure; DBP diastolic blood pressure; HDL-C, high-density lipoprotein cholesterol; TC, total cholesterol; Values are expressed as mean±SD unless otherwise noted; test for differences between cases and controls, [b]P<0.05, [c]P<0.01.

Table 3. Distribution of genotypes and alleles in patients with CHD and controls.

ID	Gene	SNP rs ID (M>m)	Function	Population	Control MM/Mm/mm	Control MAF	CHD MM/Mm/mm	CHD MAF	P_{allele}	Combined P_{allele}	*Bonferroni*
1	iNOS	rs2297518	Leu608Ser	First	382/162/13	0.169	416/138/18	0.151	0.281	0.013	0.091
	(G>A)			Second	346/144/12	0.167	394/98/15	0.126	0.009		
2	nNOS	rs41279104	promoter	First	366/167/24	0.193	414/140/22	0.160	0.041	0.143	
	(G>A)			Second	346/138/18	0.173	343/152/12	0.174	0.988		
3	eNOS	rs1799983	Glu298Asp	First	446/102/9	0.108	461/107/8	0.107	0.946	0.451	
	(G>T)			Second	398/99/5	0.109	419/81/7	0.094	0.268		
4	eNOS	rs2020744	promoter	First	451/96/10	0.104	457/113/6	0.109	0.735	0.881	
	(T>C)			Second	405/95/2	0.099	410/95/2	0.098	0.941		
5	CYBA	rs1049255	3'-UTR	First	188/261/108	0.428	229/264/83	0.373	0.008	0.001	0.007
	(C>T)			Second	175/221/106	0.431	193/235/79	0.388	0.046		
6	CYBA	rs4673	Try72His	First	471/85/1	0.078	492/83/1	0.074	0.698	0.538	
	(G>A)			Second	426/74/2	0.078	436/70/1	0.071	0.567		
7	GCH1	rs841	3'-UTR	First	267/234/56	0.311	236/253/87	0.373	0.003	<0.001	<0.001
	(G>A)			Second	219/226/57	0.339	194/231/82	0.390	0.018		

M: major allele; m: minor allele; MAF: minor allele frequency; P_{allele}: significance of minor allele frequency differences determined by χ^2 tests, CHD vs control; Combined Pallele from two case-control populations were combined using Mantel-Haenszel test.

Table 4. Association of CYBA and GCH1 variants with CHD.

Gene	SNP	Risk allele	Other allele	Adjusted OR (95% CI) per risk allele	Adjusted *P* value
CYBA	rs1049255	C	T	1.21 (1.06–1.39)	0.006
GCH1	rs841	A	G	1.30 (1.12–1.50)	<0.001

Significance of adjusted OR computed with multivariate unconditional logistic regression analysis, adjusting for age, body mass index, hypertension, diabetes, hyperlipidemia, smoking status and different populations.

P=0.012, P_{corr}=0.048).

Two-way SNP-SNP interactions

After adjusting for all covariates, significant interactions between rs2297518 and either rs1049255 or rs841 for risk of CHD were identified by the logistic regression analyses (P<0.001 for both). Individuals with the rs2297518 polymorphism (GA or AA) and the rs1049255 polymorphism (CT or TT) had a significantly lower risk of CHD (OR=0.49; 95% CI 0.38 to 0.67). In contrast, individuals with the rs2297518 polymorphism (GA or AA) and the rs841 polymorphism GG had a significantly higher risk of CHD (OR=1.79; 95% CI 1.33 to

Table 5. Assessment of association between haplotypes with CHD.

Haplotype	Frequency of Haplotype[a] Control	CHD	Crude P value[b]	Adjusted ORs (95% CI)	Adjusted P value[c]
eNOS rs20207444-rs1799983					
GT	0.792	0.800	0.616	Baseline	
GC	0.099	0.099	0.883	1.00 (0.95–1.14)	0.903
TT	0.106	0.097	0.385	0.98 (0.94–1.16)	0.471
	Over all χ^2=1.26 Global P=0.739				
CYBA rs4673-rs1049255					
GC	0.560	0.612	0.001	Baseline	
GT	0.361	0.315	0.002	0.97 (0.94–1.00)	0.012
AT	0.068	0.064	0.531	0.96 (0.91–1.02)	0.167
	Overall χ^2=11.33 Global P=0.010				

[a] Haplotype frequencies were inferred using the EM algorithm within the haplo.stats R package. Haplotypes are not listed if all the estimated frequencies are <0.02 in controls, patients with CHD;
[b] Crude *P* value based on haplotype-specific score tests;
[c] Odds ratio (95% confidence interval) were adjusted for gender, age, body mass index, hypertension, diabetes, hyperlipidemia smoking status and different populations.

2.40). All of the above results remained significant after correcting for multiple testing (Table 6).

High-order interactions

To detect high-order SNP–SNP interactions, GMDR analyses were performed. The results are presented in Table 7. No significant high-order interactions were detected. However, the combination of rs2297518 and rs1049255 was the strongest among all two-factor models, indicating that some potential interaction exists between rs2297518 and rs1049255, which is consistent with our previous results.

Discussion

In the present study, we examined the relationship between seven genetic polymorphisms within NO biosynthesis pathway genes and the risk of CHD. Single-locus analysis revealed that the C+640T polymorphism (rs1049255) in the 3′-UTR of CYBA and the G+243A polymorphism (rs841) in the 3′-UTR of GCH1 were independently associated with an elevated risk of CHD in a Chinese Han population. Two-way SNP–SNP interaction analyses indicated that the iNOS Leu608Ser polymorphism (rs2297518) has an interaction with the two SNPs mentioned above for risk of CHD. We did not detect any high-order interactions between these SNPs.

One possible explanation for the single-locus analysis is that polymorphisms from these genes are all linked to NO production, which might be associated with the principal pathogenesis process of atherosclerosis. The C+640T polymorphism in the 3′-UTR of the p22 phox gene might modulate the activity and regulation of the NADH/NADPH oxidase, which may lead to a decrease in oxidative stress in the vasculature. Likewise, the common polymorphism G+243A in the 3′-UTR of GCH1 has been shown to predict NO excretion[27]. We also detected two-way SNP–SNP interactions among these candidate genes: iNOS Leu608Ser (rs2297518) with either CYBA C+640T (rs1049255) or GCH1 G+243A (rs841). Interactions among these SNPs are biologically plausible. iNOS expres-

Table 7. Multilocus interaction model by GMDR method.

No of Loci	Best model	Prediction accuracy	Cross-validation consistency	Sign test (*P*)
1	5	52.13	8/10	0.055
2	1, 5	51.80	6/10	0.172
3	1, 4, 5	52.40	7/10	0.623
4	1, 2, 5, 7	52.82	10/10	0.172
5	1, 2, 4, 5, 7	51.31	8/10	0.172
6	1, 2, 3, 4, 5, 7	51.18	10/10	0.623
7	1, 2, 3, 4, 5, 6, 7	50.39	10/10	0.828

1=rs2297518, 2=rs41279104, 3=rs1799983, 4=rs2020744, 5=rs1049255, 6=rs4673, 7=rs841.

Table 6. Interactions among NO biosynthesis pathway genes genotypes for CHD.

Genotype rs2297518	rs1049255	Phenotype Control, *n* (%)	CHD, *n* (%)	Adjusted OR (95% CI)[c]	Adjusted *P* value
GG	CC	251 (46%)	297 (54%)	1.0 (reference)	
	GG	CT or TT	477 (48%)	0.95 (0.74–1.20)	0.647
GA or AA	CC	112 (47%)	125 (53%)	1.01 (0.72–1.44)	0.926
GA or AA	CT or TT	219 (61%)	142 (39%)	0.49 (0.38–0.67)	<0.001
rs2297518	rs841				
GG	GG	333 (51%)	321 (49%)	1.0 (reference)	
GG	GA or AA	451 (51%)	439 (49%)	1.15 (0.65–1.19)	0.375
GA or AA	GG	107 (41%)	155 (59%)	1.79 (1.33–2.40)	<0.001
GA or AA	GA or AA	168 (50%)	168 (50%)	1.03 (0.71–1.51)	0.867

Significance of adjusted OR computed with multiple unconditional logistic regression analysis, adjusting for age, body mass index, hypertension, diabetes, hyperlipidemia, smoking status and different populations.

sion has been localized to vascular smooth muscle cells and mononuclear leukocytes in early and advanced atherosclerotic lesions, which may contribute to lesion formation by increasing oxidative stress in the vessel wall[34]. Given that all of these loss-of-function polymorphisms are functionally involved in the development of atherosclerosis, they may confer risk for CHD though biological interactions with each other. Further in vitro studies are required to identify the molecular and cellular mechanisms that underlie the precise effects of these variants on endothelial function and their interaction with orthodox cardiovascular risk factors.

To control for potential false-positive results, we took several factors into consideration and carefully designed our study. First, we recruited only ethnically and geographically matched subjects from Chinese Han cohorts. Given the homogenous study population, we expect population substructure to be minimal. Second, all selected candidate SNPs have substantial functional effects, which are likely involved in the development of CHD. Third, we used the conservative Bonferroni correction to control for false-positive findings due to multiple testing. Finally, successful replication of the association signals in two independent cohorts as well as in the combined sample warrant the plausibility of our study.

Several limitations of our study should be acknowledged. First, only a limited number of genes and SNPs from the NO biosynthesis pathway were selected. The incomplete gene and SNP coverage likely does not represent the entire pathway and therefore may not fully describe the contributions of these genes. However, our report provides insight for future studies, which will focus on the elucidation of the mechanism of these interactions. Second, population stratification may exist in this study and thus result in a spurious association between a marker and disease. Finally, although we observed an increasing statistical power in most cases when we performed analyses using the combined sample, we noticed that we failed to detect the associations of some SNPs (*eg*, rs4673) with CHD due to their low frequencies of minor alleles, which results in inadequate statistical power. Further validations from larger, independent populations as well as perspective studies are necessary to confirm our results.

In summary, we explored the epistatic relationships of NO biosynthesis pathway genes and CHD susceptibility in two independent case-control cohorts from a Chinese Han population. We have revealed possible interactions between SNPs and a risk for CHD. These results support the hypothesis that common polymorphisms within NO biosynthesis pathway genes modify CHD risk.

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Author contribution

Yuan-chao TU, Hu DING, Yu-jun XU, and Lan ZHANG performed the research; Xiao-jing WANG and Cong-xin HUANG contributed new analytical tools and reagents; Yuan-chao TU and Hu DING analyzed the data; Yuan-chao TU and Dao-wen WANG wrote the paper

References

1 Harrison DG. Cellular and molecular mechanisms of endothelial cell dysfunction. J Clin Invest 1997; 100: 2153–7.
2 Moncada S, Higgs A. The *L*-arginine-nitric oxide pathway. N Engl J Med 1993; 329: 2002–12.
3 Sessa WC. The nitric oxide synthase family of proteins. J Vasc Res 1994; 31: 131–43.
4 Wang Y, Marsden PA. Nitric oxide synthases: Biochemical and molecular regulation. Curr Opin Nephrol Hypertens 1995; 4: 12–2.
5 Esaki T, Hayashi T, Muto E, Yamada K, Kuzuya M, Iguchi A. Expression of inducible nitric oxide synthase in T lymphocytes and macrophages of cholesterol-fed rabbits. Atherosclerosis 1997; 128: 39–46.
6 Yogo K, Shimokawa H, Funakoshi H, Kandabashi T, Miyata K, Okamoto S, *et al*. Different vasculoprotective roles of no synthase isoforms in vascular lesion formation in mice. Arterioscler Thromb Vasc Biol 2000; 20: E96–E100.
7 Wilcox JN, Subramanian RR, Sundell CL, Tracey WR, Pollock JS, Harrison DG, *et al*. Expression of multiple isoforms of nitric oxide synthase in normal and atherosclerotic vessels. Arterioscler Thromb Vasc Biol 1997; 17: 2479–88.
8 Kugiyama K, Yasue H, Okumura K, Ogawa H, Fujimoto K, Nakao K, *et al*. Nitric oxide activity is deficient in spasm arteries of patients with coronary spastic angina. Circulation 1996; 94: 266–71.
9 Shimasaki Y, Yasue H, Yoshimura M, Nakayama M, Kugiyama K, Ogawa H, *et al*. Association of the missense glu298asp variant of the endothelial nitric oxide synthase gene with myocardial infarction. J Am Coll Cardiol 1998; 31: 1506–10.
10 Hingorani AD, Liang CF, Fatibene J, Lyon A, Monteith S, Parsons A, *et al*. A common variant of the endothelial nitric oxide synthase (glu298→asp) is a major risk factor for coronary artery disease in the UK. Circulation 1999; 100: 1515–20.
11 Cai H, Wilcken DE, Wang XL. The glu-298→asp (894g→t) mutation at exon 7 of the endothelial nitric oxide synthase gene and coronary artery disease. J Mol Med 1999; 77: 511–4.
12 Wang CL, Hsu LA, Ko YS, Ko YL, Lee YH. Lack of association between the glu298asp variant of the endothelial nitric oxide synthase gene and the risk of coronary artery disease among taiwanese. J Formos Med Assoc 2001; 100: 736–40.
13 Granath B, Taylor RR, van Bockxmeer FM, Mamotte CD. Lack of evidence for association between endothelial nitric oxide synthase gene polymorphisms and coronary artery disease in the Australian caucasian population. J Cardiovasc Risk 2001; 8: 235–41.
14 Poirier O, Mao C, Mallet C, Nicaud V, Herrmann SM, Evans A, *et al*. Polymorphisms of the endothelial nitric oxide synthase gene — no consistent association with myocardial infarction in the ectim study. Eur J Clin Invest 1999; 29: 284–90.
15 Culverhouse R, Suarez BK, Lin J, Reich T. A perspective on epistasis: Limits of models displaying no main effect. Am J Hum Genet 2002; 70: 461–71.
16 Moore JH. The ubiquitous nature of epistasis in determining susceptibility to common human diseases. Hum Hered 2003; 56: 73–82.
17 Lou XY, Chen GB, Yan L, Ma JZ, Zhu J, Elston RC, Li MD. A generalized combinatorial approach for detecting gene-by-gene and gene-by-

environment interactions with application to nicotine dependence. Am J Hum Genet 2007; 80: 1125–37.

18 Johannesen J, Pie A, Pociot F, Kristiansen OP, Karlsen AE, Nerup J. Linkage of the human inducible nitric oxide synthase gene to type 1 diabetes. J Clin Endocrinol Metab 2001; 86: 2792–6.

19 Wang SS, Davis S, Cerhan JR, Hartge P, Severson RK, Cozen W, *et al*. Polymorphisms in oxidative stress genes and risk for non-hodgkin lymphoma. Carcinogenesis 2006; 27: 1828–34

20 Saur D, Vanderwinden JM, Seidler B, Schmid RM, De Laet MH, Allescher HD. Single-nucleotide promoter polymorphism alters transcription of neuronal nitric oxide synthase exon 1c in infantile hypertrophic pyloric stenosis. Proc Natl Acad Sci USA 2004; 101: 1662–7

21 Alvarez R, Gonzalez P, Batalla A, Reguero JR, Iglesias-Cubero G, Hevia S, *et al*. Association between the nos3 (-786 t/c) and the ace (i/d) DNA genotypes and early coronary artery disease. Nitric Oxide 2001; 5: 343–8

22 Nakayama M, Yasue H, Yoshimura M, Shimasaki Y, Kugiyama K, Ogawa H, *et al*. T-786→c mutation in the 5′-flanking region of the endothelial nitric oxide synthase gene is associated with coronary spasm. Circulation 1999; 99: 2864–70.

23 Guzik TJ, West NE, Black E, McDonald D, Ratnatunga C, Pillai R, *et al*. Functional effect of the c242t polymorphism in the nad(p)h oxidase p22phox gene on vascular superoxide production in atherosclerosis. Circulation 2000; 102: 1744–7.

24 Inoue N, Kawashima S, Kanazawa K, Yamada S, Akita H, Yokoyama M. Polymorphism of the nadh/nadph oxidase p22 phox gene in patients with coronary artery disease. Circulation 1998; 97: 135–7.

25 Li A, Prasad A, Mincemoyer R, Satorius C, Epstein N, Finkel T, *et al*. Relationship of the c242t p22phox gene polymorphism to angiographic coronary artery disease and endothelial function. Am J Med Genet 1999; 86: 57–61.

26 Cahilly C, Ballantyne CM, Lim DS, Gotto A, Marian AJ. A variant of p22(phox), involved in generation of reactive oxygen species in the vessel wall, is associated with progression of coronary atherosclerosis. Circ Res 2000; 86: 391–5.

27 Zhang L, Rao F, Zhang K, Khandrika S, Das M, Vaingankar SM, *et al*. Discovery of common human genetic variants of gtp cyclohydrolase 1 (gch1) governing nitric oxide, autonomic activity, and cardiovascular risk. J Clin Invest 2007; 117: 2658–71.

28 Gonzalez JR, Armengol L, Sole X, Guino E, Mercader JM, Estivill X, *et al*. Snpassoc: An r package to perform whole genome association studies. Bioinformatics 2007; 23: 644–5

29 Barrett JC, Fry B, Maller J, Daly MJ. Haploview: Analysis and visualization of ld and haplotype maps. Bioinformatics 2005; 21: 263–5

30 Schaid DJ, Rowland CM, Tines DE, Jacobson RM, Poland GA. Score tests for association between traits and haplotypes when linkage phase is ambiguous. Am J Hum Genet 2002; 70: 425–34

31 Moore JH, Gilbert JC, Tsai CT, Chiang FT, Holden T, Barney N, *et al*. A flexible computational framework for detecting, characterizing, and interpreting statistical patterns of epistasis in genetic studies of human disease susceptibility. J Theor Biol 2006; 241: 252–61

32 Bruzzi P, Green SB, Byar DP, Brinton LA, Schairer C. Estimating the population attributable risk for multiple risk factors using case-control data. Am J Epidemiol 1985; 122: 904–14.

33 Gauderman W, Morrison J. Quanto 1.1: A computer program for power and sample size calculations for genetic-epidemiology studies, http://hydra.Usc.Edu/gxe. 2006

34 Berliner JA, Heinecke JW. The role of oxidized lipoproteins in atherogenesis. Free Radic Biol Med 1996; 20: 707–27.