Positive Association Between rs10918859 of the *NOS1AP* Gene and Coronary Heart Disease in Male Han Chinese

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Westaway *et al.* have revealed a significant association between common variants of calsequestrin-2 (*CASQ2*) and nitric oxide synthase 1 (neuronal) adaptor protein (*NOS1AP*) and the risk of sudden death in patients of coronary heart disease (CHD). In light of the findings, we aim to explore the association between variants of the two genes and CHD risk in Han Chinese. Our results show a significant contribution of rs10918859 of the *NOS1AP* gene to CHD in Han Chinese (genotype: $\chi^2=8.33$, $df=2$, $p=0.015$; allele: $\chi^2=4.00$, $df=1$, $p=0.047$, odds ratio [OR]=1.44, 95% confidence interval [CI]=1.00–2.05). The association of rs10918859 with CHD is seen only in men (genotype: $\chi^2=7.81$, $df=2$, $p=0.02$; allele: $\chi^2=4.49$, $df=1$, $p=0.03$, OR=1.66, 95% CI=1.03–2.66). Moreover, rs10918859 is likely to exert its effect under a dominant model in men ($\chi^2=7.6$, $df=1$, $p=0.005$, OR=2.46, 95% CI=1.29–4.71). No association is observed between *CASQ2* variants and CHD risk. The frequencies of rs12084280-C and rs10918859-A are higher in Han Chinese (36.7% and 41.6%) than those in Europeans (11% and 19.4%, respectively). These ethnic differences imply that further validation of *NOS1AP* in the susceptibility of CHD in other populations is warranted. We confirm that rs10918859 of the *NOS1AP* gene is associated with CHD in Han Chinese. In addition, here we report a gender effect in the association between rs10918859 of the *NOS1AP* gene and CHD.

Introduction

WITH THE HIGH prevalence and mortality rate of coronary heart disease (CHD), it has become one of the leading causes of death in the developed countries and China (Zhang *et al.*, 2008). Compared with the developed countries such as the United States and the countries in the Western Europe, China had a relatively lower CHD mortality level. However, the incidence of CHD in China is predicted to increase sharply in the near future (Zhang *et al.*, 2008). Twin and family studies have demonstrated that the development of CHD has a strong genetic component (Marenberg *et al.*, 1994; Murabito *et al.*, 2005). Although current genome-wide association studies have identified a handful of genetic variants underlying the CHD risk (Peden and Farrall, 2011), over 95% of the genetic variants in disease risk remain uncovered (Peden and Farrall, 2011). Furthermore, these identified variants only explain a small fraction of the total heritability of CHD (Peden and Farrall, 2011).

A recent study has shown that several variants of calsequestrin-2 (*CASQ2*) and Nitric oxide synthase 1 (neuro-

nal) adaptor protein (*NOS1AP*) correlate with the risk of sudden death in CHD patients (Westaway *et al.*, 2011). *CASQ2* is located on 1p13.3-p11, encoding the cardiac calsequestrin, which is the intrasarcoplasmic reticulum calcium-binding protein. Variations of the *CASQ2* gene are shown to be associated with catecholaminergic polymorphic ventricular tachycardia (di Barletta *et al.*, 2006). *NOS1AP* is located on 1q23.3, encoding an adapter protein that serves to physically bridge the neuronal nitric oxide synthase and its targets and modulator proteins (Kao *et al.*, 2009). *NOS1AP* was identified to be a new target that modulates cardiac repolarization in a genome-wide study (Arking *et al.*, 2006). The *NOS1AP* gene variants were shown to influence QT interval in Americans (Post *et al.*, 2007; Lehtinen *et al.*, 2008) and Hollanders (Eijgelsheim *et al.*, 2009b) and alter cardiac repolarization in Finlanders (Raitakari *et al.*, 2009).

Ethnic differences lie in the factors contributing to the risk of CHD (Kuller, 2004; Ferdinand, 2006; Moe and Tu, 2010), which are due to the differences in the allele frequencies and effects of the genetic variants on the risk of CHD. The goal of our study was to evaluate the contribution of the variants of

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CASQ2 and NOS1AP to the risk of CHD in a case-control cohort originated from Ningbo city in Eastern China.

Materials and Methods

Sample collection

All the 273 individuals involved in the current study are Han Chinese originated from Ningbo city in Eastern China. The 161 CHD patients (116 men and 45 women; mean age: 63.29 ± 10.20 years) and 112 controls (59 men and 53 men; mean age: 59.24 ± 9.68 years) were recruited between May 2008 and April 2010 from the Lihuili hospital in Ningbo city of the Zhejiang province, China. All subjects were examined through standardized coronary angiography following Seldinger's method (Higgs *et al.*, 2005). The results of the angiograms were independently judged by at least two physicians. Subjects who had congenital heart disease, cardiomyopathy, severe liver, or kidney disease were excluded from the study. The blood samples of all individuals were collected at the same time and handled by the same researchers. The study protocol was approved by the ethics committee of Lihuili hospital in Ningbo, and the informed consent was obtained from all subjects.

Single-nucleotide polymorphism genotyping

Genomic DNA was isolated from peripheral blood lymphocytes through the conventional phenol/chloroform method (Di Pietro *et al.*, 2011) and was stored in a Tris ethylenediaminetetraacetic acid (Tris-EDTA, TE) buffer. The concentration of DNA was determined using the PicoGreen® double-strand (dsDNA) DNA Quantification Kit (Molecular Probes, Inc., Eugene, OR). Polymerase chain reaction (PCR) amplification was performed on the GeneAmp® PCR System 9700 Dual 384-Well Sample Block Module (Applied Biosystems, Foster City, CA) within a 5- μ L reaction mixture using approximately 1 μ L DNA from each participant with the primers (Supplementary Table S1; Supplementary Data are available online at www.liebertpub.com/gtmb). The thermal cycling parameters were 1 cycle at 94°C for 15 s for an initial

denaturation, followed by 45 cycles of denaturation for 20 s at 94°C, primer annealing at 56°C for 30 s, primer extension for 1 min at 72°C, and a final extension for 3 min at 72°C. Amplified DNA samples were used at 1:10 dilution for genotyping on the SEQUENOM® Mass-ARRAY iPLEX® platform according to the manufacturer's instructions (Gabriel *et al.*, 2009).

Statistical analyses

Genotype and allele frequencies were compared between CHD cases and non-CHD controls using CLUMP22 software with 10,000 Monte Carlo simulations (Sham and Curtis, 1995), and the Hardy-Weinberg equilibrium (HWE) was calculated by the Arlequin program (version 3.5; Bern, Switzerland) (Excoffier and Lischer, 2010). Linkage disequilibrium and haplotype frequencies were inferred using the Arlequin program based on the expectation-maximization algorithm. Frequencies of the inferred haplotypes between cases and controls were tested using CLUMP22 software (Sham and Curtis, 1995). Power analysis was made by Power and Sample Size Calculation software (version 3.0.43; Nashville, TN) (Dupont and Plummer, 1990). A two-tailed $p < 0.05$ was considered to be statistically significant.

Results

We evaluated the allele frequencies of five single-nucleotide polymorphisms (SNPs) in 273 Han Chinese subjects, including rs17500488, rs3010396, and rs7366407 of the CASQ2 gene and rs12084280 and rs10918859 of the NOS1AP gene. Genotype and allele frequencies of these SNPs are shown in Table 1. All of the tested SNPs are in the HWE. Among the five SNPs, only rs10918859 of the NOS1AP gene is shown to be significantly associated with CHD (allele: $\chi^2 = 4.00$, $df = 1$, $p = 0.047$, odds ratio [OR] = 1.44, 95% confidence interval [CI] = 1.00–2.05; genotype: $\chi^2 = 8.33$, $df = 2$, $p = 0.015$). As shown in Table 2, a significant difference of rs10918859 is observed between cases and controls in the dominant model ($\chi^2 = 7.71$, $df = 1$, $p = 0.005$, OR = 2.02, 95% CI = 1.22–3.33). However, no association is found for the other four SNPs (Table 1).

TABLE 1. DISTRIBUTION OF GENOTYPES AND ALLELES IN BOTH GROUPS

Gene	SNP/group	Genotype (n)			χ^2	p (df=2)	Allele (n)		χ^2	p (df=1)	OR (95% CI)	HWE
CASQ2	rs17500488	TT	TC	CC	0.49	0.811	T	C	0.25	0.700	1.13 (0.69–1.85)	1.00
		116	42	3			274	48				
	case	83	28	1	0.44	0.785	194	30	0.005	1.000	1.01 (0.72–1.43)	0.87
		57	80	25			194	130				
	control	38	60	15	0.59	0.729	136	90	0.079	0.793	1.05 (0.74–1.49)	0.24
		62	69	29			193	127				
NOS1AP	rs3010396	GG	GA	AA	0.29	0.849	G	A	0.28	0.645	1.10 (0.77–1.57)	0.86
		64	77	21			205	119				
	case	48	52	13	8.33	0.015	148	78	4.00	0.047	1.44 (1.00–2.05)	0.07
		49	90	22			188	134				
	control	53	45	15			151	75				
		53	45	15			151	75				

All the p -values are uncorrected p -values; bold font indicates p -value < 0.05 .

CASQ2, caldessestrin-2; NOS1AP, nitric oxide synthase 1 (neuronal) adaptor protein; OR, odds ratio; CI, confidence interval; HWE, Hardy-Weinberg equilibrium; SNP, single-nucleotide polymorphism.

TABLE 2. COMPARISON OF THE DOMINANT MODEL AND RECESSIVE MODEL IN RS10918859 BETWEEN CASES AND CONTROLS BY GENDER

SNP/sex	Group	Dominant model					Recessive model				
		GG	GA+AA	χ^2	p	OR (95% CI)	GG+GA	AA	χ^2	p	OR (95% CI)
Male	Case	34	81	7.6	0.005	2.46 (1.29–4.71)	99	16	0.14	0.71	1.20 (0.46–3.10)
	Control	30	29				52	7			
Female	Case	15	31	1.05	0.3	1.53 (0.67–3.48)	40	6	0	1	0.98 (0.31–3.17)
	Control	23	31				46	8			
Male + female	Case	49	112	7.71	0.005	2.02 (1.22–3.33)	139	22	0.01	0.92	1.03 (0.51–2.09)
	Control	53	60				98	15			

All the *p*-values are uncorrected *p*-values; bold font indicates *p*-value < 0.05.

Additionally, we have conducted a breakdown comparison by gender between cases and controls with respect to allele and genotype frequencies (Table 3). In the male subgroup, there are significant differences of rs10918859 in cases and controls (genotype: $\chi^2=7.81$, *df*=2, *p*=0.02; allele: $\chi^2=4.49$, *df*=1, *p*=0.03, OR=1.66, 95% CI=1.03–2.66). In contrast, no significant differences between cases and controls are observed in the female subgroup (*p*>0.05). Furthermore, a strong difference of rs10918859 is revealed between CHD cases and controls in men under the dominant model ($\chi^2=7.6$, *df*=1, *p*=0.005, OR=2.46, 95% CI=1.29–4.71).

There is a strong linkage disequilibrium (LD) among the SNPs (rs17500488, rs3010396, and rs7366407) of the *CASQ2* gene (Supplementary Table S2). The two SNPs (rs12084280 and rs10918859) of the *NOS1AP* gene are about 140 kb away from each other. Interestingly, *D'* values indicate a low LD level between them in the control group (*D'*=0.057) in contrast to a much higher LD level observed in the case group (*D'*=0.365). The comparison between cases and controls of the estimated haplotype frequencies is listed in Supplementary Table S3. No association is observed between the haplotypes of *CASQ2* and the risk of CHD. The two SNPs of the *NOS1AP* gene are not included in the haplotype frequency analysis because of a low LD level in control groups (*D'*=0.057).

As shown in Table 4, there are ethnic differences in the allele frequencies of two SNPs in the *NOS1AP* gene (Americans vs. Han Chinese: 19.4% vs. 41.6% for rs10918859-A; 11.0% vs. 36.7% for rs12084280-C). SNP rs10918859 is shown to be associated with the risk of CHD in both Americans (*p*=0.02) and Chinese Han (*p*=0.04). However, rs12084280 is not a risk factor of CHD in Han Chinese (*p*=0.645). No differences in the allele frequencies of the three SNPs (rs17500488; rs3010396; and rs7366407) of the *CASQ2* gene are observed between Americans and Han Chinese.

Discussion

In the present study, we aim to explore the significant association between the variants of *CASQ2* or *NOS1AP* with the risk of CHD in the Han Chinese. We have tested five significant SNPs shown in Westaway's study (Westaway *et al.*, 2011), and our results show only rs10918859 of the *NOS1AP* gene associated with CHD in Han Chinese. The haplotype analyses reveal no association between *CASQ2* gene variants and CHD. In addition, a much lower LD pattern of the two SNPs of the *NOS1AP* gene is observed in controls (*D'*=0.057) than in cases (*D'*=0.365), and this may explain why not all the two SNPs of the *NOS1AP* gene are positively associated with CHD.

Gender-stratified association study shows different results between men (genotype: *p*=0.02; allele: *p*=0.03) and women (genotype: *p*=0.49; allele: *p*=0.54), suggesting an interplay between rs10918859 of the *NOS1AP* gene and gender. Sexual dimorphism has been observed in the prevalence, symptoms, and severity of cardiovascular diseases (Ober *et al.*, 2008; Wang *et al.*, 2011; Gulati *et al.*, 2012). Significant association of rs702553 is only shown in young men with strokes, but not in women (Liao *et al.*, 2010). Wang *et al.* (2011) have revealed a strong association between rs11053646 and all plaque phenotypes in women, but not in men. Inhibiting IKs (*KCNQ1*) G589D mutation has been confirmed to be a gender-specific QT interval modifier in type-1 long-QT syndrome (LQTS) and be associated with increased severity of disease (Lahtinen *et al.*, 2011).

NOS1AP variants have been shown to be strongly associated with the QT interval and the risk of sudden cardiac death in elderly Hollanders (Aarnoudse *et al.*, 2007), the South African LQTS population (Crotti *et al.*, 2009), and the Rotterdam Study (Eijgelsheim *et al.*, 2009a, 2009b). Interestingly, two

TABLE 3. COMPARISON OF GENOTYPE AND ALLELE FREQUENCIES IN RS10918859 BETWEEN CASES AND CONTROLS BY GENDER

Gender	Group	Genotype (n)			χ^2	p	Allele (n)		χ^2	p	OR (95% CI)	HWE
		GG	GA	AA			G	A				
Male	Case	34	65	16	7.81	0.02	133	97	4.49	0.03	1.66 (1.03–2.66)	0.52
	Control	30	22	7			82	36				
Female	Case	15	25	6	1.42	0.49	55	37	0.36	0.54	1.19 (0.67–2.11)	0.07
	Control	23	23	8			69	39				

All the *p*-values are uncorrected *p*-values; bold font indicates *p*-value < 0.05.

TABLE 4. COMPARISON BETWEEN WESTAWAY'S STUDY AND OURS

Gene	SNP	Reference allele	Westaway's study		Our study	
			Allele frequency	CHD association p-value	Allele frequency	CHD association p-value
CASQ2	rs17500488	C	0.095	0.040	0.149	0.700
	rs3010396	A	0.454	0.007	0.401	1.000
NOS1AP	rs7366407	A	0.286	0.040	0.397	0.793
	rs12084280	C	0.110	0.040	0.367	0.645
	rs10918859	A	0.194	0.020	0.416	0.047

All the *p*-values are uncorrected *p*-values; CHD, coronary heart disease.

independent studies have confirmed that an *NOS1AP* variant is associated with the QT interval duration in Chinese (Lu *et al.*, 2010; Qin *et al.*, 2010). Aouizerat *et al.* (2011) and Westaway *et al.* (2011) have found there is a significant association between the *NOS1AP* gene variants and the risk of CHD in the residents of the United States. In the present study, we confirm that rs10918859 of the *NOS1AP* gene is associated with CHD in Han Chinese, and ethnic differences are found to be in the allele frequency of two *NOS1AP* gene variations. All these pieces of evidence suggest that rs10918859 of the *NOS1AP* gene contributes to the risk of CHD, although further exploration of the mechanism of action between *NOS1AP* variants and the risk of CHD is warranted.

Using Power and Sample Size Calculation software, we estimate the power of our samples to detect the positive association of rs10918859. Our results have shown that the current study has a power of 0.81 to detect the positive association of rs10918859 under the dominant model. To be noted, the *p*-value of rs10918859 of the *NOS1AP* gene is 0.047, which may not retain significance after corrected by the number of tested SNPs. Therefore, we cannot exclude a chance of random positive finding of this SNP. The sample size in the present study is comparatively small, and the number of female participators should be increased in the future study. We cannot exclude the possibility that the negative findings of the four SNPs (rs12084280, rs17500488, rs3010396, and rs7366407) may be due to a lack of power.

In the present study, we fail to validate the association of the *CASQ2* gene variants with the risk of CHD. These findings in Westaway's study (Westaway *et al.*, 2011) are associated with the risk of sudden death in CHD patients, instead of simple CHD risk. This might explain why we do not find the association in our study. Given the complexity of CHD, the discrepancy between the Westaway's study (Westaway *et al.*, 2011) and our study may reflect the population differences in the factors from both the genes and environment (Topol *et al.*, 2006). Our current research supports a positive association of the *NOS1AP* gene with CHD. Given that rs10918859 is located in the intronic region of the *NOS1AP* gene, we hypothesize that there exists a causal variant in high LD with rs10918859. Our replication study in the Eastern Chinese will help to clarify the major genetic factors that are universal to the susceptibility of CHD and thus may contribute to the early diagnosis and prevention of CHD. Further studies are warranted to replicate our findings in various populations and to elucidate the genetic mechanism of the *NOS1AP* gene in the pathogenesis of CHD.

In conclusion, our study supports the significant association between rs10918859 of the *NOS1AP* gene and the risk of

CHD, although we fail to reveal the association between variants of the *CASQ2* gene and the risk of CHD. We identify that there are ethnic differences in the allele frequency of two *NOS1AP* gene variations, and rs10918859-A is likely to increase the risk of CHD in the male Han Chinese under the dominant model.

Acknowledgments

The research was supported by the grants from the National Natural Science Foundation of China (31100919 and 30772155), Qianjiang Scholars Foundation of Zhejiang Province, the Zhejiang Provincial Program for the Cultivation of High-level Innovative Health Talents, Natural Science Foundation of the Zhejiang Province (Y206608), the Scientific Innovation Team Project of Ningbo (No.2011B82014), and Youth and Doctor Foundation of Ningbo (2005A610016). The authors gratefully acknowledge the support of the K.C. Wong Education Foundation, Hong Kong.

Author Disclosure Statement

The authors declare no conflicts of interest.

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