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Interaction effects between *Paraoxonase 1* variants and cigarette smoking on risk of coronary heart disease in a Singaporean Chinese population

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Abstract

Objective—Paraoxonase 1 (PON1) plays an important role in reducing the risk of coronary heart disease (CHD). Smoking is known to reduce PON1 activity. We aimed to investigate the effects of interactions between *PON1* variants and smoking on CHD in the Singaporean Chinese population.

Methods—In a case-control study nested within Singapore Chinese Health Study (N = 1914), subjects with and without CHD were classified into never-smokers and ever-smokers (ever smoked at least one cigarette a day for 1 year or longer). Associations at four independent SNPs at the *PON1* locus (rs3735590, rs3917550, rs662, rs3917481) with CHD were evaluated using logistic regression, before/after stratification on smoking status. Interactions between smoking and

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Disclosures

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.atherosclerosis.2015.01.042>.

PON1 variants were analyzed with likelihood ratio tests, by including the SNP*smoking interaction term in regression analyses.

Results—The T allele at the coding SNP, rs662, was associated with higher risk of CHD in ever-smokers only (OR = 1.35, 95% CI 1.08–1.68; adjusted P = 0.036). At the miR-SNP, rs3735590, carrying at least one copy of minor allele T was associated with increased risk of CHD in a dominant manner in never-smokers only (OR = 1.53, 95% CI 1.11–2.11; adjusted P = 0.036). Significant interactions between two *PON1* SNPs and smoking in relation to CHD risk were identified (adjusted P = 0.012 for rs662; adjusted P = 0.044 for rs3735590). These associations remained significant after adjustment for known CHD risk factors and upon correction for multiple tests.

Conclusions—Two *PON1* SNPs, rs662 and rs3735590, were found to significantly interact with cigarette smoking to modulate the risk of CHD in the Singaporean Chinese population.

Keywords

Coronary heart disease; *Paraoxonase 1*; Cigarette smoking; Interaction; Single nucleotide polymorphism (SNP); miR-SNP; Singaporean Chinese

1. Introduction

Coronary heart disease (CHD) is a complex and multifactorial disorder. Along with its most severe complication, myocardial infarction (MI), it has become a leading cause of death in United States [1] and the number two killer in Singapore [<https://www.moh.gov.sg>]. Human serum paraoxonase 1 (PON1) is a 43-kDa calcium dependent esterase located on high-density lipoprotein cholesterol (HDL-C) that circulates in plasma [2]. It has been reported to reduce oxidative stress and the risk of cardiovascular risk by hydrolyzing the pro-inflammatory lipid-peroxides formed by the oxidized low-density lipoprotein (LDL) [3–5]. A *PON1* polymorphism (rs662) results in a nonsynonymous amino acid substitution (Glutamine to Arginine substitution at position 192), which leads to the difference in the hydrolytic activity towards paraoxon [6,7]. This 192Gln variant hydrolyzes paraoxon at a lower rate than 192Arg [8] and is more effective at impeding the oxidation of LDL than 192Arg [9,10]. The reduced anti-oxidative ability of 192Arg may contribute to its association with a higher risk coronary heart disease (CHD) [11,12].

Cigarette smoking is known to lower PON1 activity in patients with CHD [13], and cigarette smoke extract inhibits PON1 activity in a dose- and time-dependent manner [14]. The study of *PON1*-smoking interaction may be able to identify individuals with a high risk of CHD due to the combination of certain *PON1* polymorphisms and smoking status. For this purpose, previous studies have assessed the interactions between a limited number of coding variants in *PON1* and cigarette smoking status on the risk of CHD. However results have been inconclusive [15,16]. For example, a study in the Costa Rican population showed a significant interaction between the rs662 variant and smoking status to modify CHD risk [15]. In this study, the C allele at rs662 was reported to increase the risk of CHD in non-smokers only. However, another study conducted in a different ethnic group (Asian-Indians)

indicated contrasting results for the same *PON1* SNP (rs662), in which the risk allele (C allele) was associated with increased CHD risk among smokers [16].

In this study we aimed to evaluate whether the association of genetic variants in the *PON1* gene region with CHD risk is modified by cigarette smoking status in the Singaporean Chinese population.

2. Material and method

2.1. Study subjects

This case-control study (N = 1914; 688 cases and 1226 controls) was nested within the Singapore Chinese Health Study (SCHS), a prospective cohort of 63,257 Chinese who were recruited between April 1993 and December 1998 [17]. The study population comprises two major dialect groups of Chinese in Singapore, the Hokkiens and the Cantonese. At recruitment, subjects were interviewed in-person using a structured questionnaire which included socio-demographic information, medical history and life style characteristics [17]. Blood was collected from 28,439 participants mostly between 2000 and 2005. During an average follow-up of 15 years, less than 1% of subjects were lost to follow-up.

Cases who had fatal coronary heart disease (CHD) or suffered from non-fatal myocardial infarction (MI) were identified through the Singapore Registry of Births and Deaths and the Hospital Discharge Database respectively. For all non-fatal cases, medical records were retrieved and reviewed by cardiologists and only those who had confirmed MI using the Multi-Ethnic Study of Atherosclerosis criteria (available at: <http://www.mesa-nhlbi.org/manuals.aspx>), were included. Cases of fatal CHD were only included if there was prior evidence of CHD based on the questionnaire data or the Hospital Discharge Database. For each case, two controls were selected from SCHS participants who were alive and free of CHD at the time of diagnosis or death of the index case and were matched for sex, dialect group, year of birth, year of recruitment and date of blood collection. The Institutional Review Board of the National University of Singapore has approved this study.

2.2. Study variables

Study subjects were classified into never-smokers (N = 1101) and ever-smokers (N = 813) based on their responses (No, Yes) to the following question in the questionnaire, 'Have you ever smoked at least one cigarette a day for 1 year or longer'. An additional variable, pack-year of cigarette smoking, was created and defined as the number of cigarettes smoked per day divided by 20 and multiplied by the number of smoking years to capture the duration and quantity of cigarette smoking, as previously reported [18]. Ever-smokers were classified into light smokers (N = 500) and heavy smokers (N = 313) based on the median of pack-year (median = 30.6). Blood pressure was measured using Omron Automatic Digital Blood Pressure Monitors (HEM 705CP) after the subjects were seated at rest for at least 5 min. Average levels based on three blood pressure measurements were used for the analysis. Hypertension was defined based on subjects' self-report, and/or having a systolic blood pressure (SBP) ≥ 140 mmHg and/or a diastolic blood pressure (DBP) ≥ 90 mmHg [19]. HbA1c was measured by Biorad Variant II using an ion-exchange HPLC method and

diabetes was defined based on subjects' self-report, and/or hemoglobin A1c (HbA1c) $\geq 6.5\%$ [20]. Body mass index (BMI) was determined as body weight (kg) divided by the squared height (m^2). Non-fasting blood lipoproteins (high density lipoprotein-cholesterol (HDL-C) and low density lipoprotein-cholesterol (LDL-C) were measured directly with the Siemens Advia 2400 instrument using an elimination/catalase method.

2.3. Genotyping

The current study population was genotyped on Illumina HumanOmniZhongHua-8 BeadChip. Based on quality control procedures (Supplementary Table 1), we excluded 42 duplicate samples, 35 samples with a call rate $< 98\%$, 20 samples with extreme heterozygosities ($> \text{mean} \pm 3 \text{ standard deviation, SD}$), 25 samples with 1st degree relatedness, identified from identity-by-state analyses, such as monozygotic twins, full-sibling pairs and parent-offspring (only 1 sample from each pair was retained, prioritizing the cases and samples with higher call-rates) and 36 samples with discordant ethnic membership from Singaporean Chinese ethnicity as identified by principal components analysis. Quality control procedures for SNPs excluded 24,461 non-autosomal SNPs, 467 SNPs with poor call-rates ($< 95\%$), 435 SNPs with significant deviations of Hardy–Weinberg Equilibrium (HWE) in controls ($< 1 \times 10^{-6}$) and 67,585 SNPs with minor allele frequencies (MAF) < 0.01 (Supplementary Table 2). Eighty nine samples with missing measurements on HDL-C, LDL-C, hypertension and diabetes were excluded and for final statistical analysis, 1914 samples (688 cases and 1226 controls) and 802,635 SNPs that passed quality control procedures were included.

2.4. Selection of *PON1* variants

A total of 63 SNPs with minor allele frequencies (MAF) $> 1\%$ within the *PON1* gene were selected. From these 63 SNPs, we removed non-informative SNPs by filtering them down to 4 tag SNPs (rs3735590, rs3917550, rs662, rs3917481) that are at low linkage disequilibrium (LD) with each other ($r^2 < 0.25$, Supplementary Table 2).

2.5. Statistical analysis

Statistical analysis of data was carried out with STATA (version 8.2). Differences in means of quantitative traits between cases and controls were determined by the *t*-test. Differences in frequencies for clinical characteristics and *PON1* genotypes between cases and controls were compared by the chi-square test. Logistic regression models were used to examine the associations of the *PON1* genotypes with CHD risk. Based on the regression coefficients, odds ratios (OR) and 95% confidence interval (CI) were calculated. Age, sex, BMI, diabetes, hypertension, HDL-C and LDL-C were considered as potential confounders and thus included as covariates in the logistic regression models. An additive genetic model was applied except for SNPs with low allele frequency, where all subjects harboring the rare variants were combined into a single group. Statistical association results were adjusted for multiple testing using a Bonferroni correction. The above logistic regression analyses were further stratified on smoking status. Next, another logistic regression model was used to test for an association of the gene*smoking interaction with CHD risk as follows:

$$\text{Logit } [p] = \beta_0 + \beta_1 \text{ SMOKING} + \beta_2 \text{ SNP} + \beta_3 \text{ SMOKING} * \text{SNP}$$

The null hypothesis of no interaction effect between this gene and smoking variable on CHD is represented by $\beta_3 = 0$. The gene*smoking interaction were tested by comparing the likelihood of regression models with and without the cross-product interaction variable. This model was applied to all available SNPs while simultaneously accounting for the effects of age, sex, BMI, LDL-C, HDL-C, diabetes and hypertension. Odds Ratios (OR) for each combination of minor variant and smoking status were calculated using the estimated coefficients ($\beta_1, \beta_2, \beta_3$) from the logistic regression model. Conditional probability analysis was conducted to test the independence of associations by including the genotypes of potential dependent SNPs. Adjusted P value of <0.05 (2-sided) was considered statistically significant after correcting for multiple comparisons. The power to detect similar associations that were previously reported by another study [15] was estimated by QUANTO (Version 1.2.4) [21].

3. Results

The characteristics of the Singapore Chinese study participants are presented in Table 1. Cases and controls were matched for age and sex. The prevalence of hypertension, diabetes and ever-smoking was significantly higher in the CHD cases as compared to controls ($P < 4.00 \times 10^{-4}$). Similarly, as compared to the control group, mean BMI and LDL-C levels were significantly higher in the cases ($P < 0.025$) while HDL-C levels were significantly lower ($P = 2.67 \times 10^{-5}$). The distributions of genotype frequencies of the four *PON1* SNPs selected in this study are presented in Table 2.

After adjustments for the known CHD risk factors and multiple testing, none of the four *PON1* SNPs were significantly associated with CHD in the overall study population (Table 3). Stratification for cigarette smoking status however (Table 3), indicated a positive association between the minor allele T of rs662 and CHD risk in ever-smokers only (OR = 1.35, 95% CI 1.08–1.68; adjusted P = 0.036), and the interaction between rs662 and smoking was found to be significant (adjusted P for interaction = 0.012). The trends of an increased CHD risk in ever-smokers and a decreased CHD risk in never-smokers for rs662 T allele are illustrated in Fig. 1A.

A positive association was observed between rs3735590 and CHD risk in never-smokers only, in which subjects carrying at least one copy of minor allele T showed an increased risk (OR = 1.53, 95% CI 1.11–2.11; adjusted P = 0.036), and the interaction between rs3735590 and smoking was observed to be significant (adjusted P for interaction = 0.044). The trends of an increased CHD risk in never-smokers and a decreased CHD risk in ever-smokers for rs3735590 T carriers (CT + TT genotype) are highlighted in Fig. 1B. Conditional probability analysis indicated that the interactions between these two *PON1* variants and smoking status on CHD risk were independent (Supplementary Table 3).

Further analysis for pack-year showed a trend of larger effects of these two *PON1* variants on CHD in heavy smokers than in light smokers, but did not achieve statistical significance.

The interactions between these two *PON1* variants and smoking status (never, light and heavy) were significant (adjusted P for rs662 interaction = 0.022, adjusted P for rs3735590 interaction = 0.032) (Supplementary Table 4).

4. Discussion

Cigarette smoking is recognized as the most important preventable cause of premature death [22] and is a key independent risk factor for CHD [13,23]. Our results indicated that cigarette smoking status and inherent common variants at *PON1* interact to affect the risk of CHD development and that these variants might be differentially associated with CHD risks among ever-smokers and never-smokers.

Similar to other studies conducted in subjects of East-Asian ancestry [12,24,25], we did not detect an association between the *PON1* rs662 index polymorphism and CHD in the overall study population. However, with stratification for smoking status at this same index SNP, we observed a similar association as another group [15] that the T allele was associated with an increased risk of CHD among ever-smokers only.

Studies had suggested that HDL may play a role in the effect of *PON1* on CHD [2,26] and the C allele at *PON1* rs662 was reported to be associated with lower HDL levels [27]. However, similar to other reports [12,15,24,28–33], we did not identify any significant association between *PON1* and HDL (data not shown). In addition, the stratified effect as well as the interaction between *PON1* variants and smoking status on CHD risk observed in our study was not mediated by HDL levels or other known common risk factors for CHD.

Previous genetic association studies of *PON1* have largely been confined to the analysis of a limited number of coding SNPs, principally rs662, because SNPs in the coding region may result in amino acid changes, and subsequently affect *PON1* activity. Despite a modest sample size, we had approximately 88% power in this study to identify similar estimated *PON1* and smoking interaction effects for CHD (OR for interaction = 0.64 [15] at the rs662 variant, MAF = 0.330, alpha = 0.05).

In addition to rs662, we have identified a novel interaction between a SNP at the 3'UTR region of *PON1* (rs3735590) that was significantly associated with increased risk of CHD among never-smokers, but not among ever-smokers. A recent study has shown that the nucleotide change from C to T at rs3735590 reduces binding affinity of the microRNA, miRNA-616, to the 3'UTR region of *PON1*, increasing gene expression and lowering the risk of atherosclerosis [34]. However, never-smoking subjects carrying the T allele were associated with an increased risk of CHD in our study, although it would be difficult to make direct comparisons as the former study did not stratify for smoking status. While there are some biochemical investigations using cigarette smoke extracts to demonstrate its inhibitory effect on plasma *PON1* activity through modification of the enzyme's free thiols [14], there have been no experimental evidence at the molecular level to explain the mechanisms for the interaction between cigarette smoking and *PON1* genetic variants to affect CHD risk. Hence, further *in vitro* and/or *in vivo* studies would be warranted in this regard.

Several strengths of our study are noteworthy. Firstly, as the cases and controls were nested within a cohort study, all the information used in the statistical analysis was captured prior to CHD diagnosis and thus unlikely to be affected by recall bias or by changes due to treatment. Secondly, the use of principal components analysis from genome-wide data would have minimized misclassification of ethnic groups. We were also able to identify and exclude closely related individuals, thus limiting effects of substructure in the results. Thirdly, since diabetes, hypertension and dyslipidemia are major risk factors for CHD [35], their effects on CHD have been considered by including diabetes, hypertension, LDL-C and HDL-C as covariates in the logistic regression model. One limitation of our study is the inability to track changes in the subjects' smoking status from the time they were recruited to the time when they were identified to have CHD. The co-morbidities associated with CHD (e.g. diabetes, hypertension and hyperlipidemia) were more prevalent among cases. Thus, there may have been a greater likelihood for lifestyle changes among the CHD case subjects (such as changes in smoking behavior). Yet, it is unlikely that the expected misclassifications were differential by the genetic factors and thus our findings tended to be conservative. Another limitation is the lack of data for PON1 activity measurement due to limited availability of serum samples and their long storage duration, which is known to compromise on the accuracy of the measurements [36]. Nevertheless, several previous studies on PON1 activity in plasma were supportive of our results. Firstly, variations in serum PON1 activity are dependent on genetic polymorphisms. Rs662 TT genotype is reported to be more effective at impeding the oxidation of LDL than CC genotype [9,10], which may support our observation that the T allele decreased CHD risk among never-smokers in our study. Secondly, PON1 activity was inhibited by cigarette smoke extract in a dose- and time-dependent manner [14]. Our results are consistent with the reported cumulative effect of smoking on PON1 activity in that a trend of larger effects of these two *PON1* variants on CHD in heavy smokers than in light smokers was observed, although it did not achieve statistical significance.

We conclude from our genetic epidemiological study that two SNPs at the *PON1* gene, rs662 and rs3735590, were found to interact with cigarette smoking at a statistically significant level to modulate the risk of CHD in the Singaporean Chinese population. Besides the previously reported *PON1* SNP rs662, we identified a novel, independent interaction between the miR-SNP, rs3735590, and smoking in relation to the risk of CHD at a statistically significant level. Our results may shed additional light on the complex interplay between the cigarette smoking and inherent genotypes to modify risk of developing CHD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Interactions between *PON1* polymorphisms and smoking on risk of coronary heart disease (CHD). (A) Rs662 interactions with smoking. Never-smokers with CC genotype as the reference group. (B) Rs3735590 interactions with smoking. Never-smokers with CC genotype as the reference group.

Table 1

Characteristics^a of Singapore Chinese Health Study stratified by CHD status.

Variable	Controls (N = 1226)	Cases (N = 688)	P	OR (95% CI)
Male% ^b	63.3	64.7	–	–
Hypertension%	59.1	73.7	1.89 × 10⁻¹⁰	1.94 (1.58–2.38)
Diabetes%	20.6	36.6	5.08 × 10⁻¹⁴	2.22 (1.81–2.74)
Ever-smoking%	39.5	47.8	4.00 × 10⁻⁴	1.40 (1.16–1.70)
Age (yr) ^b	66.44 ± 7.76	66.73 ± 7.82	–	–
BMI (kg/m ²)	22.91 ± 3.01	23.24 ± 3.19	0.025	1.04 (1.00–1.07)
HDL-C (mmol/L)	1.34 ± 0.33	1.28 ± 0.30	2.67 × 10⁻⁵	0.52 (0.38–0.71)
LDL-C (mmol/L)	3.17 ± 0.80	3.34 ± 0.87	1.71 × 10⁻⁵	1.28 (1.15–1.44)

CHD: coronary heart disease; OR: odds ratio; CI: confidence interval; BMI: body mass index; HDL-C: high density lipoprotein-cholesterol; LDL-C: low density lipoprotein-cholesterol.

^aData are presented as percentages or as means ± SD. Significant values in bold.

^bCases and controls were matched for age and sex.

Table 2 Genotype and allele frequencies^a for *PON1* variants stratified by CHD status.

rsid	Position	Genotype	Controls (N = 1226)	Cases (N = 688)	P	p ^b
rs3735590	94927495	CC	993 (81.0)	540 (78.5)	0.088	0.352
		CT	217 (17.7)	144 (20.9)		
		TT	16 (1.3)	4 (0.6)		
		MAF	0.102	0.110		
rs3917550	94934573	GG	1064 (86.8)	588 (85.5)	0.697	1.000
		GA	152 (12.4)	93 (13.5)		
		AA	10 (0.8)	7 (1.0)		
		MAF	0.070	0.078		
rs662	94937446	CC	560 (45.7)	296 (43.0)	0.074	0.296
		CT	524 (42.7)	328 (47.7)		
		TT	142 (11.6)	64 (9.3)		
		MAF	0.330	0.331		
rs3917481	94950765	CC	1124 (91.7)	635 (92.3)	0.444	1.000
		CT	100 (8.2)	50 (7.3)		
		TT	2 (0.2)	3 (0.4)		
		MAF	0.042	0.041		

PON1: Paraoxonase 1; CHD: coronary heart disease; MAF: minor allele frequency.^aPresented as N (%).^bAdjusted for multiple testing (for 4 different SNPs).

Table 3

Associations^a of *PON1* variants with CHD by smoking status.

rsid	MA	Model	Genotype	All samples (N = 1914)			Never-smokers (N = 1101)			Ever-smokers (N = 813)			Interaction ^d	
				OR (95% CI)	P	P ^c	OR (95% CI)	P	P ^c	OR (95% CI)	P	P ^c	P	P ^c
rs3735590	T	Dominant	CC	1.000			1.000			1.000				
			CT + TT	1.16 (0.91–1.47)	0.223	0.892	1.53 (1.11–2.11)	0.009	0.036	0.81 (0.56–1.16)	0.255	1.000	0.011	0.044
rs3917550	A	Dominant	GG	1.000			1.000			1.000				
			GA + AA	1.11 (0.84–1.47)	0.452	1.000	1.07 (0.73–1.57)	0.737	1.000	1.16 (0.77–1.74)	0.483	1.000	0.742	1.000
rs662	T	Additive ^b	–	1.05 (0.90–1.21)	0.540	1.000	0.86 (0.71–1.05)	0.137	0.548	1.35 (1.08–1.68)	0.009	0.036	0.003	0.012
rs3917481	T	Dominant	CC	1.000			1.000			1.000				
			CT + TT	0.91 (0.63–1.30)	0.588	1.000	0.96 (0.59–1.55)	0.869	1.000	0.84 (0.49–1.44)	0.524	1.000	0.686	1.000

PON1: *Paraoxonase 1*; CHD: coronary heart disease; OR: odds ratio; CI: confidence interval; MA: minor allele.

^a Adjusted for the covariates (Age, sex, BMI, LDL-C, HDL-C, diabetes and hypertension).

^b Additive model shows the OR associated with each additional minor allele.

^c Adjusted for multiple testing (for 4 different SNPs). Significant values in bold.

^d P value for testing the interaction between *PON1* variants and smoking status for CHD.