**OLR1, PON1 and MTHFR Gene Polymorphisms, Conventional Risk Factors and the Severity of Coronary Atherosclerosis in a Chinese Han Population**

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**Key Words**
Coronary atherosclerosis • Gensini score • Polymorphisms • Interaction

**Abstract**

**Aims:** To explore the association between six single-nucleotide polymorphisms in *OLR1, PON1, MTHFR* gene and the angiographical characteristics of coronary atherosclerosis to determine if any of the conventional factors correlate with genetic polymorphisms in the advent of the disease. **Methods:** We examined rs1801131, rs1801133, rs3736232, rs3736234, rs854563 and rs662 by TaqMan® SNP Genotyping Assays in 1075 subjects that underwent angiography. The angiographical characteristics of coronary atherosclerosis were defined by the Gensini Score system. **Results:** The T allele of rs1801133 was associated with coronary atherosclerosis severity with the \( OR = 1.49, 95\% CI = 1.04-2.14 \). In *MTHFR* gene, haplotype T-A was a susceptibility haplotype to coronary atherosclerosis \( (OR = 1.27, 95\% CI = 1.06-1.51) \) whereas haplotype C-A had a protective effect \( (OR = 0.83, 95\% CI = 0.70-0.99) \). In addition, several synergistic effects between rs1801133 and conventional risk factors such as diabetes and smoking were found. **Conclusions:** Collectively, the results demonstrate that the T allele of rs1801133 conferred an increased risk for coronary atherosclerosis. The MTHFR C-A haplotype was a protective haplotype, while T-A haplotype was a susceptibility haplotype. The presence of the T allele of rs1801133 increases the odds of coronary atherosclerosis severity when associated with conventional risk factors.

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Introduction

Cardiovascular disease, which is multifactorial and caused by complex interactions of genetic and environmental factors, represents the main cause of death all over the world [1, 2]. Traditional risk factors for coronary atherosclerosis include age, smoking, male gender, hypertension and diabetes. Newly defined risk factors such as hyper-homocysteine [3], elevated plasma levels of oxidized low-density lipoprotein (OxLDL) and oxidative stress are also emerging [4].

Methylenetetrahydrofolate reductase (MTHFR), a key enzyme in folate metabolism, has been implicated in risk for cardiovascular disease (source). Two common functional MTHFR polymorphisms, rs1801131 and rs1801133, have been well-studied [5]. The CC genotype of rs1801131 reduces MTHFR enzyme activity by 40% in vitro when compared to the AA genotype [6] and the TT genotype has about 80% less enzymatic activity than the CC genotype in vitro [7]. Individuals who have a C-to-T substitution in the rs1801133 polymorphism have reduced enzyme activity, higher homocysteine (Hcy) levels and lower folate levels than individuals without this substitution [8, 9]. Observational studies have found that individuals with low folate intake or low folate levels have a higher risk of coronary artery disease (CAD) [10]. It has also been observed that mild elevation or even moderate plasma Hcy is correlated with atherosclerosis [11]. Elevated plasma levels of Hcy injures endothelial cells [12, 13], which increases oxidative modification of low-density lipoprotein-cholesterol (LDL-c) [14].

In addition to polymorphisms in MTHFR, the lectin-like oxidized low-density lipoprotein receptor (LOX-1/OLR1) has also been implicated in the development of atherosclerosis [15]. There is evidence suggesting that OLR1 causes endothelial dysfunction, impairs the production of nitric oxide and induces proatherogenic genes, endothelial–leukocyte adhesion molecules, and smooth muscle growth factors [16]. As a result, OLR1 is considered a suitable candidate gene for atherosclerosis risk. Among all the polymorphisms, rs3736232 and rs3736234 are much less studied but demonstrate high linkage disequilibrium. The outcomes, however, have been controversial [17, 18].

Other factors have been shown to have protective effects on cardiovascular disease risk. One candidate, paraoxonase 1 (PON1), is associated with high-density lipoproteins (HDLs) and has been shown to have anti-oxidant and anti-inflammatory potential, mainly by protecting lipids of HDLs and low-density lipoproteins (LDLs) from oxidative modifications [19]. In the past several years, there have been several case-control studies investigating the associations between polymorphisms of PON1 and susceptibility to CAD. Among the various single nucleotide polymorphisms (SNPs) of PON1, two common functional polymorphisms, rs662 and rs854563, have been identified in the coding region. Although these polymorphisms are well studied, the results have been inconsistent [20].

To better address the risk of cardiovascular disease, we selected six polymorphisms (rs1801131 and rs1801133 in MTHFR; rs3736232 and rs3736234 in OLR1; rs854563 and rs662 in PON1) in three genes for the present study. Although the three genes belong to different pathways of CAD, it was reported that Hcy was positively correlated with LDL-c, CRP, creatinine and BUN (Blood urea nitrogen), and negatively with HDL-C. In addition, PON1 displays HcyT hydrolase activity, decreasing the toxicity of Hcy [21], and has been suggested to protect against the atherosclerotic effects of Hcy-thiolactone. The main focus was to analyse the six polymorphisms, alone or combined in haplotypes to clarify their potential association with atherosclerosis and determine a possible interaction between the six gene polymorphisms and several conventional CAD risk factors.
Materials and Methods

Study population
A total of 1075 patients (20 to 85 years old) that underwent coronary angiography at the first affiliated hospital of Nanjing Medical University in Nanjing from 2004-2006 were enrolled in this study. Patients with a history of the following conditions were excluded from this study: hyperlipidemia with statin treatment, cerebrovascular disease, peripheral arterial disease, infection within the 2 weeks preceding catheterization, heart failure (Killip Class ≥ 2 after acute myocardial infarction) or severe hepatic or renal disease. All patients filled in a personal history questionnaire which addressed age, sex, hypertension, diabetes mellitus, smoking habits, weight, sedentary habits, alcohol ingestion and family medical history. Patients also provided blood samples for genotype analysis and biochemical measurements. Written informed consent was obtained from all enrolled participants and this study was approved by the ethics committee of the First Affiliated Hospital of Nanjing Medical University.

Determination of biochemical parameters
Venous blood was drawn from each participant after an overnight fast. Serum and plasma were centrifuged immediately and stored at -80°C. Levels of total cholesterol (TCH; mmol/L), triglyceride (TG; mmol/L), fasting blood glucose (FBG; mmol/L), fasting high-density lipoprotein cholesterol (HDL-c; mmol/L) and fasting low-density lipoprotein cholesterol (LDL-c; mmol/L) were determined by enzymatic procedures on an automated autoanalyzer (AU 2700 Olympus; 1st Chemical, Tokyo, Japan). Blood pressure, height and weight were measured by trained nurses according to standardized protocols.

SNP genotyping
Genomic DNA was isolated from peripheral blood and was amplified by the polymerase chain reaction (PCR) using the Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems) according to the manufacturer’s instructions. The numbers of the genotyping assays from Applied Biosystems were as follows: Assay ID for rs1801131: C____850486_20; Assay ID for rs1801133: C___1202883_20; Assay ID for rs3736232: C___3130875_1_; Assay ID for rs3736234: C___3130873_20; Assay ID for rs854563: C___8952841_10; Assay ID for rs 662: C___2548962_20. Amplifications were performed in a 384-well format and post-PCR analysis performed with SDS 2.3 automated software. Four blank controls were included in each plate to ensure accuracy of the genotyping. Approximately 10% of samples were randomly selected for repeat assays and results were in agreement with the results of the initial assays.

Statistical analysis
Patients were classified into two groups according to their Gensini scores using the median as a cutoff point: scores ≥22 were placed in the high Gensini score group and <22 were placed in the low Gensini score group. Genotypes were tested for Hardy-Weinberg equilibrium among all participants using a two-sided χ² test. Distributed variables were presented in the form of mean ± standard deviation (SD) whereas skewed data were expressed as medians and interquartile ranges. Differences in selected demographic continuous variables among groups were evaluated by using one-way analysis of variance (ANOVA) or the Kruskal-Wallis H test. Categorical variables were compared among the genotypes using a χ² test. A binary logistic regression procedure was used for association analyses of Gensini score-related phenotypes and association of the six SNP genotype variants. Angiographical characteristics of coronary atherosclerosis risk were generated by computing the OR and their 95% CI with and without adjustments for age, sex, BMI, and other confounding risk factors. A dominant model (major homozygotes versus heterozygotes plus minor homozygotes) was used to analyze possible positive or negative interactions between classical risk factors of CAD and genetic polymorphisms. Several conventional risk factors between the low and high Gensini score groups were estimated by computing the OR and their 95% CI with a Bonferroni correction. Zero refers to unexposed individuals or those without a susceptibility genotype for a certain background risk for disease while one refers to exposed individuals or with the susceptibility genotype to disease risk. All the above statistical analyses were performed using the SPSS 16.0 statistical software (SPSS, Chicago, IL, USA).

The haplotypes and haplotype frequencies were constructed and calculated using the online SHEsis software [22] (http://analysis.bio-x.cn/myAnalysis.php). A p < 0.05 (two-tailed) was considered statistically significant.
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Results

General characteristics of the study population

General clinical characteristics of all participants are shown in Table 1. Subjects that fell into the higher Gensini score were older males and were more frequently diabetic and smokers. Subjects with a higher Gensini score also had lower concentration of HDL (p < 0.001) and significant higher fasting blood glucose (Glu) (p < 0.001). However, participants with a lower Gensini score had a higher level of cholesterol (CH) (p = 0.890) and triglycerides (TG) (p = 0.982), although this did not reach statistical significance.

Genotype distribution and association with coronary atherosclerosis risk

Primary information on the six selected SNPs is shown in Table 2. There was no deviation of genotype distributions of each SNP from the Hardy-Weinberg equilibrium in both Gensini groups (p > 0.05, Table 2). A univariate logistic regression analysis (Table 3) indicated that the frequencies of CC, CT and TT genotypes of rs1801133 from MTHFR in the lower (≤22) Gensini score group (24.07%, 48.32% and 27.61%, respectively) differed from those in the higher (≥22) Gensini score group (19.85%, 47.12% and 33.02%, respectively).
respectively). When the CC genotype was used as the reference group, the homozygote TT \((p = 0.03, \text{OR} = 1.45, \text{95\% CI} = 1.04-2.03)\) was associated with a significantly increased risk of higher Gensini score even after adjusting for confounding risk factors. When we combined the heterozygote CT and homozygote TT as a pooled risk genotype comparison group, no significance was found. After adjusting for age, gender, diabetic status, hypertension, smoking and alcohol intake, the CC genotype \((p = 0.045, \text{OR} = 1.80, \text{95\% CI} = 1.01-3.18)\) and the CG+CC genotype \((p = 0.042, \text{OR} = 1.39, \text{95\% CI} = 1.01-1.69)\) were associated with an increased risk for a higher Gensini score. However, the significance was eliminated after applying a Bonferroni correction.

### Haplotype association analysis

Using the SHEsis online software, separate haplotypes were reconstructed for \(PON1\), \(MTHFR\) and \(OLR1\). In \(MTHFR\), the frequent haplotype C-A showed a statistically significant reduction in risk for coronary atherosclerosis severity (Table 4) \((p = 0.0342, \text{OR} = 0.83, \text{95\% CI} = 0.70-0.99)\).
CI = 0.702-0.987), with a higher frequency in the low score group than in high score group (47.8% versus 43.3%). For the MTHFR T-A haplotype, we observed a statistically significant increase in risk for CAD severity ($p = 0.0086$, $OR = 1.265$, 95% CI = 1.061-1.507), with a higher frequency in the high score group than in low score group. As for other haplotypes of the OLR1 and PON1 genes, no significant haplotype was discovered although a strong disequilibrium was observed between them ($D' = 0.918$ and 0.754, respectively).

### Interaction between polymorphisms and classical risk factors

The analysis of the potential positive or negative association between genotypes and classical risk factors is summarized in Tables 5 and 6. The $OR$ estimating the effect of dual exposure to diabetes or smoking and rs1801133 was significantly higher than the $OR$s estimating the effect of drinking alcohol or the presence of hypertension. In the overall population analysis, the rs1801133 genotype interacts with a smoking habit to develop coronary atherosclerosis, with an $OR = 3.15$ and 95% CI = 1.77-4.74. In the whole population

<table>
<thead>
<tr>
<th>Classical risk</th>
<th>Alleles</th>
<th>rs1801133 whole population</th>
<th>rs1801131 whole population</th>
<th>Rs376232 whole population</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetes</td>
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<td>31.00 (26.45-35.55)</td>
<td>32.00 (27.42-36.58)</td>
<td>33.00 (28.45-37.55)</td>
</tr>
<tr>
<td></td>
<td>low</td>
<td>28.00 (23.45-32.55)</td>
<td>29.00 (24.42-33.58)</td>
<td>29.00 (24.45-33.55)</td>
</tr>
</tbody>
</table>

Table 5. Synergistic effect of genotype (rs1801133, rs1801131, rs376232) and classical risk factors in high and low Gensini score groups. * $p < 0.05$  ** $p < 0.01$

<table>
<thead>
<tr>
<th>Classical risk</th>
<th>Alleles</th>
<th>rs376234 whole population</th>
<th>rs854563 whole population</th>
<th>rs662 whole population</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diabetes</td>
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<td>25.00 (20.45-30.55)</td>
<td>26.00 (21.42-31.58)</td>
<td>26.00 (21.45-31.55)</td>
</tr>
<tr>
<td></td>
<td>low</td>
<td>22.00 (17.45-27.55)</td>
<td>23.00 (18.42-28.58)</td>
<td>23.00 (18.45-28.55)</td>
</tr>
</tbody>
</table>

Table 6. Synergistic effect of genotype (rs376234, rs854563, rs662) and classical risk factors in high and low score group. * $p < 0.05$  ** $p < 0.01$
analysis, the presence of diabetes increased the risk of cardiovascular disease with the rs1801133 (OR = 2.78 and 95% CI = 1.77-4.74) and rs854563 (OR = 5.06, 95% CI = 1.09-23.54) genotypes. When we performed the same analysis with hypertensive individuals, no significant results were found.

Discussion

Coronary atherosclerosis is generally considered a multifactorial disease involving polygenetic and environmental factors. In the past few years, a large number of genes have been implicated in contributing to the development of CAD by genetic association studies [23]. In the present study, we selected six polymorphisms from three common CAD susceptibility genes and investigated their associations with coronary atherosclerosis severity in a Chinese population that had undergone angiography. Although all selected SNPs have been studied, previous results were conflicting. In our study, only the T allele of rs1801133 was found to be associated with coronary atherosclerosis, as defined by a Gensini score ≥22. A higher Gensini score was more prevalent in the TT genotype carriers of rs1801133 when compared with the CC genotype (48.2% vs 43.4%). Compared with the CC genotype, the TT genotype confers a higher risk for CAD severity with an OR = 1.49 and 95% CI = 1.04-2.14. This confirmed that the T allele was the predisposing allele for CAD and that it was probably associated with CAD severity. This result is in agreement with a meta-analysis involving 2981 Chinese Han subjects [5]. In 2008, Mager et al. [24] reported that a 5.84-fold increase in risk of early CAD development was associated with the TT genotype of MTHFR rs1801133 polymorphisms among non-Oriental women compared to Ashkenazi women. Numerous studies have identified homozygotes for the T allele of rs1801133 in MTHFR as having fasting tHcy two times higher than homozygotes for the C allele [25]. However, another meta-analysis revealed no evidence for an increased risk of CAD in TT versus CC homozygotes for the MTHFR rs1801133 polymorphism [7]. The conflicting findings from these studies may be explained by the different populations studied, by population stratification, or by small sample sizes. In association studies of complex disease, it is necessary to incorporate multiple small effects of different genes, haplotypes, environmental exposures, and quantitative traits [26]. Therefore, we performed haplotype analysis, gene and environmental (e.g. diabetes, hypertension, smoking and drinking habits) interaction analyses to study the combined effects of multiple factors on the development of CAD. By haplotype analysis, no significant haplotype in OLR1 and PON1 was found to be associated with coronary atherosclerosis even these SNPs were in high linkage disequilibrium. This finding was consistent with the results of a single SNP analysis. Although the rs1801131 polymorphism in MTHFR was negatively associated with coronary atherosclerosis severity, the common haplotype C-A provided a protective effect for coronary atherosclerosis, with a higher carrier frequency in the low Gensini score group than in the high Gensini score group. However, the T-A haplotype conferred an increased risk for coronary atherosclerosis severity, with a frequency of 40% in the high Gensini score group compared with 34.6% in the low Gensini score group. These results are in agreement with the finding by L. Ghazouani et al. [27] where the C-A haplotype also indicated a protective role against CAD with an OR = 0.39 and 95% CI = 0.29-0.52. The T-A haplotype provided an increased risk for CAD with an OR = 2.35 and 95% CI = 1.62-3.38, which is also consistent with our findings. In our study, however, the C-C and T-C haplotype frequency showed no difference between the two groups, whereas the Ghazouani et al. [27] study indicated that they conferred an increased risk for CAD. Differences in ethnicity, dietary intake, exposure to environmental risk factors and sample size may be responsible for the discrepancies in the results. Our haplotype analysis further confirmed that the T-A haplotype was a CAD-susceptible haplotype. In another study [28], however, no significant haplotypes of rs1801131 and 1801133 were found to be associated with CAD. These results suggest that some polymorphisms are linked and that certain haplotypes may have a stronger impact on diseases when compared with SNPs alone.
It is a widely accepted hypothesis that both environmental and genetic factors contribute to the pathogenesis of CAD [29]. Genetic predisposition for cardiovascular disease appears to be the effect of various polymorphisms, which do not act isolation. The risk they confer can be increased or decreased by gene-gene and gene-environmental interactions [30, 31]. In most cases, CAD has a multifactorial genetic basis, involving a number of genes and environmental factors interacting to determine the pathogenesis of the disease. Therefore, the inherited genes determine the susceptibility to CAD, but it is the environmental factors (e.g., cigarette smoking, diabetes, hypertension) interacting with the individual's genotype that determine whether coronary atherosclerosis will develop. In this study, we performed further analysis in order to determine whether the presence of genetic polymorphisms concomitant with well-established risk factors – hypertension, smoking habit and diabetes – would enhance the effect on coronary atherosclerosis onset. As summarized in Table 4, there was a synergistic interaction between the \textit{MTHFR} rs1801133 and smoking habit and diabetes status. Additionally, we identified a synergistic interaction between rs854563 and diabetes. In previous studies, synergistic interactions between a gene and environment were also found [32-34]. In 2011, a case control study found that there was significant interaction between gene and conventional risk factors such as smoking or hypertension [34]. Our results suggest that polymorphisms do not act in isolation, but rather that the risk they confer can be increased by the presence of other polymorphisms or environmental factors.

\textbf{Conclusion}

Our data suggest that T allele of rs1801133 from \textit{MTHFR} was associated with CAD severity, which was observed in three analyses: single SNP analysis, haplotype analysis and gene-gene and gene-environmental interactions analysis. By haplotype analysis, we found that the T-A haplotype was a susceptibility haplotype for CAD. There was a synergistic interaction of the rs1801133 polymorphism with smoking and diabetes. Besides, a synergic interaction between rs854563 and diabetes was also found.

\textbf{Acknowledgements}

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\textbf{References}


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