Associations between the CDKN2A/B, ADTRP and PDGFD Polymorphisms and the Development of Coronary Atherosclerosis in Japanese Patients

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Aim: Genome-wide association studies have identified a series of susceptibility loci for coronary artery disease (CAD). The present study attempted to replicate the results for eight of these loci, CDKN2A/B (rs1333049), ADTRP (rs6903956), PDGFD (rs974819), TCF21 (rs12190287), COL4A1-A2 (rs4773144), HHIP1 (rs2895811), ADAMTS7 (rs4380028) andUBE2Z (rs46522), in patients with pathologically defined atherosclerosis of the coronary arteries.

Methods: Autopsy cases of elderly Japanese subjects were enrolled in the JG-SNP study (n=1,536). Polymorphisms were genotyped, and their associations with the coronary stenosis index (CSI) and incidence of pathological myocardial infarction (MI) were investigated. The potential combinatorial effects of the susceptibility loci were also assessed.

Results: Among the eight loci tested, three exhibited signs of positive associations. CDKN2A/B showed the most robust associations with CSI and MI (p=0.007 and OR=1.843, 95% CI 1.293-2.629, p=0.001, for CC+CG vs. GG). In addition, ADTRP demonstrated associations with CSI and MI, although the risk allele was opposite from that observed in the original report (p=0.008 and OR=1.652, 95% CI 1.027-2.656, p=0.038 for GG vs. AA+AG). Meanwhile, PDGFD displayed a suggestive association with CSI in women, but not men (p=0.023). CDKN2A/B and ADTRP were also found to be significantly associated with the severity of the CSI in a case-control setting. The cumulative risk allele counting of CDKN2A/B, ADTRP and PDGFD indicated an increased number of risk alleles to be associated with a higher CSI (p=4.61E-05).

Conclusions: The present study confirmed the association between CDKN2A/B and CAD and identified a different associated risk allele of ADTRP. PDGFD was found to exhibit a gender-specific association with CAD. The combination of multiple risk alleles may be associated with a higher risk of CAD.


Key words: Coronary artery disease (CAD), Myocardial infarction (MI), Pathology, Genome-wide association study (GWAS)

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Introduction

Coronary artery disease (CAD), including myocardial infarction (MI), is one of the leading causes of morbidity and mortality in both developed and developing countries. CAD is a complex disease involving underlying genetic and environmental factors and
interactions between them. Approximately 30-60% of the interindividual variation in the risk of CAD is heritability, indicating that genetic risk factors play a critical role in the pathogenesis of CAD. Indeed, genome-wide association studies (GWAS) have identified 46 independent CAD susceptibility loci to date. Some loci appear to confer risk regardless of ethnic differences, while other variants are thought to function in an ethnicity-specific manner.

The locus rs1333049 on chromosome 9p21.3 was first identified as a strongly associated CAD susceptibility locus according to a GWAS in Caucasians. It is located near two cyclin-dependent kinase inhibitors, CDKN2A and CDKN2B. A recent GWAS in a Chinese population identified rs6903956, which lies within intron 1 of the androgen-dependent tissue factor pathway inhibitor regulating protein (ADTRP) gene on chromosome 6p24.1, as a novel susceptibility locus for CAD. At chromosome 11q22.3, rs974819 is located 117 kb downstream of the PDGFD gene, a member of the platelet-derived growth factor family. This variant was originally found to be associated with CAD in a GWAS of Europeans and South Asians. This GWAS also identified rs4380028 as a novel CAD susceptibility locus located 7.6 kb upstream of the gene encoding ADAM metalloproteinase with thrombospondin type 1 motif 7 (ADAMTS7) at 15q25.1. A large-scale association analysis in individuals of European descent identified the following four variants associated with CAD: rs12190287 at 6q23.2, within the 3' untranslated region (3' UTR) of the gene encoding transcription factor 21 (TCF21), rs4773144 at 13q34, in an intron of collagen, type IV, alpha 2 (COL4A1-A2), rs2895811 at 14q32.2, in the gene encoding hedgehog interacting protein-like 1 (HHIPL1) and rs46522 at 17q21.32, in an intron of ubiquitin-conjugating enzyme E2Z (UBE2Z).

We herein evaluated eight chromosomal loci recently found to be significantly associated with CAD and determined whether they are associated with coronary atherosclerosis, an intermediate phenotype of CAD. Although a series of loci associated with CAD have been identified using GWAS examinations, most previous reports were performed in Caucasians. Since there are differences between populations, studies of specific populations are required to confirm the risk of CAD.

In this replication study, our aim was to investigate the association between these previously identified CAD susceptibility loci and the incidence of coronary atherosclerosis in elderly Japanese subjects.

### Study Population

The present study included a total of 1,536 consecutive autopsy cases of elderly Japanese patients registered in the Japanese SNP database for geriatric research (JG-SNP) (http://www.tmghig.jp/jg-snp/english/E_top.html). The autopsies were performed at Tokyo Metropolitan Geriatric Hospital in Tokyo, Japan between 1995 and 2004. The details of the major clinical diagnoses and direct cause of death have been described elsewhere. Regarding the clinical parameters, hypertension was diagnosed as a repeatedly elevated systolic blood pressure of more than 140 mmHg or diastolic blood pressure of more than 90 mmHg. The Japan Diabetes Society defines diabetes as a fasting plasma glucose level greater than 126 mg/dl, a casual plasma glucose level greater than 200 mg/dl or a two-hour blood glucose level on a 75-g oral glucose tolerance test of ≥ 200 mg/dl. In addition, hyperlipidemia was defined as an LDL cholesterol level greater than 140 mg/dl, an HDL cholesterol level less than 40 mg/dl or a triglyceride level greater than 150 mg/dl, according to the Japan Atherosclerosis Society. Among the 1,536 subjects, 1,503 patients (men: 806; women: 697; mean age at death: 80.24 ± 8.87 years) met the criteria of having adequate DNA samples for the PCR experiments, well-determined genotyping results and appropriate availability of their clinical history. Accordingly, the remaining patients did not meet the aforementioned criteria and were therefore excluded. The study protocol obtained the dual approval of the ethics committees of Tokyo Medical and Dental University and Tokyo Metropolitan Geriatric Hospital.

### Assessment of Coronary Artery Disease

Coronary artery disease (CAD) was investigated based on two measurements, the coronary stenosis index (CSI) and the incidence of pathological myocardial infarction (MI). In addition to these two parameters, we assessed the degree of clinical ischemic heart disease and systemic atherosclerosis, defined according to the pathological atherosclerotic index (PAI). First, the CSI was defined as the sum of the stenotic scores in three branches: the left anterior descending branch, the left circumflex branch and the right coronary artery. The degree of atherosclerosis was evaluated based on a macroscopic examination of the luminal surface in formalin-fixed arteries of the coronary circulation. The extent of coronary sclerosis was examined using transverse sections at 5-mm intervals, as previously described. The severity of coronary ste-
nosis was scored as: 0 (no sclerosis), 1 (slight stenosis), 2 (25% stenosis), 3 (50%), 4 (75%) or 5 (100% obstruction)\textsuperscript{9, 10}. Second, MI was defined as the presence of myocardial fibrosis greater than 1 cm in diameter attributable to coronary stenosis. Information regarding clinical ischemic heart disease and clinical MI was retrieved from the subjects’ clinical charts\textsuperscript{12}.

The pathological atherosclerotic index (PAI) was defined as the average value of the atherosclerotic scores evaluated in eight large arteries based on a macroscopic examination. The ratio of the atheroma-occupied area to the entire surface was scored semi-quantitatively on a scale of 0–8 in all arteries\textsuperscript{9, 11}.

**SNP Selection and Genotyping**

Among the 46 independent CAD susceptibility loci that have been primarily studied in Europeans, we prioritized SNPs taking into account a replication study of East Asians, with a minor allele frequency (MAF) of study of East Asians, with a minor allele frequency prioritized SNPs taking into account a replication loci that have been primarily studied in Europeans, we

SNP Genotyping Assays (Applied Biosystems, Foster City, CA, USA) and the LightCycler\textsuperscript{®} 480 system (Roche Diagnostics, Penzberg, Germany), according to the manufacturer’s instructions, except for rs974819, due to non-availability of a commercial kit for this SNP. The PCR conditions of the LightCycler\textsuperscript{®} 480 system were as follows: pre-incubation at 95°C for 10 minutes, followed by 45 cycles of amplification at 92°C for 15 seconds and cooling at 60°C for 1 minute. Following PCR amplification, we determined the genotypes of seven SNPs using a TaqMan allelic discrimination assay.

The remaining SNP, rs974819, was genotyped using a melting curve analysis on a LightCycler 480 system. The PCR conditions were as follows: 95°C for 10 minutes (preincubation), followed by 45 cycles of 95°C for 10 seconds, 55°C for 10 seconds and 72°C for 10 seconds (amplification). After PCR, the melting curve analysis was performed under the following conditions: 95°C for 1 minute, 40°C for 1 minute and 80°C (melting curve), followed by re-cooling at 40°C for 30 seconds. The melting temperature results were confirmed based on direct sequencing using the BigDye\textsuperscript{®} Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on a 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The PCR primers and sequencing primers for direct sequencing were designed using several software programs, including Primer3 (ZMBH, Heidelberg, Germany), Primer Quest (Integrated DNA Technologies, Inc, Coralville, IA, USA) and Gene Fisher (Bielefeld BioInformatics Service, Germany). The PCR products were computationally investigated using Amplify3X (University of Wisconsin, Madison, WI, USA), Operon (Eurofins MWG Operon, Huntsville, AL, USA), the Oligo Analyzer (Integrated DNA Technologies, Inc, Coralville, Iowa, USA) and gel electrophoresis. After selecting the PCR primers, we designed the sequencing primers and performed the direct sequencing method. The pathological assessments and genotyping were carried out at different institutions in a double-blind fashion in order to minimize bias.

**Statistical Analysis**

The statistical analysis was performed using the Statistical Package for the Social Sciences for Windows (SPSS), version 19.0 (IBM, Chicago, IL, USA), and statistical significance was defined as a two-tailed p-value of $p<0.05$. All SNPs were tested for deviation from Hardy-Weinberg Equilibrium using the $\chi^2$ test. Categorical variables were analyzed using the Pearson chi-squared test and expressed as the mean and percentage of cases. Continuous variables are presented as the mean ± S.D (standard deviation) and were analyzed using Student’s t-test and a one-way analysis of variance (ANOVA). Multiple linear regression analyses were performed to assess the associations between the individual SNPs and the severity of the CSI and PAI using three genetic models: a dominant model, a recessive model and an additive model. Potential confounding factors included in the regression models were as follows: age at death; gender; history of hypertension; diabetes mellitus; hyperlipidemia (presence vs. absence); smoking (non-smokers vs smokers); and alcohol consumption status (none vs. habitual). Power calculation of the included SNPs was carried out using OSSE, an Online Sample Size Estimator at the following web address: http://osse.bii.a-star.edu.sg/index.php. Genotyping errors were calculated for all SNPs. The effect of gender on the associations between the genetic polymorphisms and the risk of CAD was also investigated.
Table 1. Characteristics of the 1,503 study subjects stratified by gender

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Total n = 1503</th>
<th>Male n = 806</th>
<th>Female n = 697</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>80.2 ± 8.9</td>
<td>78.9 ± 8.3</td>
<td>81.8 ± 9.3</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>17.3 ± 3.7</td>
<td>17.1 ± 3.5</td>
<td>17.5 ± 3.9</td>
<td>0.044*</td>
</tr>
<tr>
<td>Total serum cholesterol (mg/dl)</td>
<td>166 ± 45</td>
<td>158 ± 43</td>
<td>177 ± 45</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>HDL (mg/dl)</td>
<td>42.6 ± 15.7</td>
<td>41.5 ± 15.4</td>
<td>44.1 ± 16.0</td>
<td>0.005*</td>
</tr>
<tr>
<td>Lp(a) (mg/l)</td>
<td>210 ± 216</td>
<td>207 ± 218</td>
<td>213 ± 212</td>
<td>0.685</td>
</tr>
<tr>
<td>PAI</td>
<td>4.23 ± 1.59</td>
<td>4.35 ± 1.51</td>
<td>4.09 ± 1.66</td>
<td>0.002*</td>
</tr>
<tr>
<td>CSI</td>
<td>8.24 ± 3.68</td>
<td>8.62 ± 3.48</td>
<td>7.79 ± 3.86</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>CSI ≥11.5 (%)</td>
<td>384 (26)</td>
<td>225 (28)</td>
<td>159 (23)</td>
<td>0.024*</td>
</tr>
<tr>
<td>ICAI</td>
<td>2.67 ± 2.10</td>
<td>2.47 ± 1.96</td>
<td>2.90 ± 2.23</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Ischemic heart disease</td>
<td>247 (16)</td>
<td>129 (16)</td>
<td>118 (17)</td>
<td>0.629</td>
</tr>
<tr>
<td>Myocardial infarction</td>
<td>325 (22)</td>
<td>172 (21)</td>
<td>153 (22)</td>
<td>0.774</td>
</tr>
<tr>
<td>Hypertension</td>
<td>440 (29)</td>
<td>227 (28)</td>
<td>213 (31)</td>
<td>0.309</td>
</tr>
<tr>
<td>Diabetes</td>
<td>221 (15)</td>
<td>120 (15)</td>
<td>101 (14)</td>
<td>0.828</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>32 (2)</td>
<td>18 (2)</td>
<td>14 (2)</td>
<td>0.764</td>
</tr>
<tr>
<td>Drinking (%)</td>
<td>498 (33)</td>
<td>422 (52)</td>
<td>76 (11)</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Smoking (%)</td>
<td>716 (48)</td>
<td>565 (70)</td>
<td>151 (22)</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>

The data are presented as the mean ± s.d. or %. The p-value was calculated using Student's t-test or the χ²-test.

BMI, body mass index; HDL, high-density lipoprotein; Lp(a), lipoprotein; PAI, pathological atherosclerotic index; CSI, coronary stenotic index; CSI ≥11.5, 75th percentile; ICAI, intracranial stenotic index.

In order to evaluate the associations between the above variants and risk of CAD, the subjects were classified into two groups based on the CSI score, arbitrarily using the 75th percentile CSI value as a cutoff point: CSI ≥11.5 for the CAD patients and CSI < 11.5 for the controls, according to our previous reports. Multiple logistic regression analyses were used to examine the associations between the CSI values of the patients and controls and the presence of genetic polymorphisms, and the results were expressed as the odds ratio (OR) and 95% confidence interval (95% CI). Two additional CAD parameters, the incidence of MI and clinical ischemic heart disease, were categorized as 0 or 1 (controls or cases) and analyzed using a multiple logistic regression analysis. We did not apply correction for multiple testing in this study.

We further examined whether the effects of multiple potential SNPs increase the likelihood of developing CAD. We first defined the risk score as follows: 2 (homozygous risk allele), 1 (heterozygous) or 0 (homozygous with the other allele). We then summed the number of risk alleles for the individual SNPs and calculated the mean CSI value for each risk score. The association between the risk score and the mean CSI value was evaluated using a linear regression analysis.

Results

Characteristics of the Study Cohort

A total of 1,536 elderly Japanese patients were recruited in this study. We further refined the number to 1,503 subjects based on the quality of the genotyping results and the CSI values. Of the 1,503 subjects, 806 were men and 697 were women. Table 1 shows the clinical characteristics of the population according to gender. There were significant differences between the genders in the following parameters: age at death, BMI, total cholesterol, HDL, PAI, CSI, CSI ≥11.5, ICAI and drinking and smoking habits.

Genotyping Results

The genotyping results for CDKN2A/B (rs1333049), ADTRP (rs6903956), PDGFD (rs974819), TCF21 (rs12190287), COL4A1-A2 (rs4773144), HHIP1 (rs2895811), ADAMTS7 (rs4380028) and UBE2Z (rs46522) are shown in Table 2. Except for UBE2Z (rs46522), the observed genotypic frequencies of the loci were consistent with Hardy-Weinberg equilibrium. Although UBE2Z (rs46522) deviated slightly from the Hardy-Weinberg equilibrium (p = 0.037), we included it in our analysis. The rate of genotyping success was greater than 99.8% for all SNPs, except PDGFD (rs974819), for which the call rate was 96% using a melting curve analysis. We confirmed the
Dechamethakun et al. (rs1333049) and PDGFD (rs974819) were in the same direction as those observed in previous reports, we found a different risk allele for ADTRP (rs6903956).

We then investigated the gender stratification of the associations between the CDKN2A/B (rs1333049), ADTRP (rs6903956) and PDGFD (rs974819) polymorphisms and the CSI values. As shown in Fig. 1A, the association with CSI for CDKN2A/B (rs1333049) was significantly higher in the CC + CG genotype group than in the GG genotype group among women (p = 0.003). In men, we observed a similar trend; however, it was not significant (p = 0.051). As demonstrated in Fig. 1B, the AA + AG genotype of ADTRP (rs6903956) was significantly higher than the GG genotype in men (p = 0.025), but not women (p = 0.219). Meanwhile, the genotype analysis performed under the additive model for PDGFD (rs974819) showed an increase in the mean CSI value in association with an increase in the cumulative number of risk alleles in women (p = 0.023), but not men (p = 0.245) (Fig. 1C).

Next, we dichotomized the subjects according to the severity of CSI and defined the top 75% as cases, accuracy of the melting curve analysis by performing direct sequencing, the results of which were consistent. The SNP call rate of PDGFD (rs974819) was 100% using direct sequencing. The statistical power was greater than 80% for CDKN2A/B (rs1333049), 51.7% for ADTRP (rs6903956) and 23.1% for PDGFD (rs974819), whereas the power of the remaining SNPs was less than 15%.

**Table 2.** Results of genotyping and the association analysis of eight SNPs with CSI using linear regression in the 1,503 subjects

<table>
<thead>
<tr>
<th>SNP Gene</th>
<th>Chr Minor allele MAF Genotype</th>
<th>Genotype Frequency</th>
<th>HWP</th>
<th>Unadjusted</th>
<th>Adjusted</th>
<th>Genetic models</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>p-value</td>
</tr>
<tr>
<td>rs1333049 CDKN2A/B</td>
<td>9 C 0.485 CC/CG/GG</td>
<td>324 670 364 0.648</td>
<td>0.462 0.001*</td>
<td>0.602 0.007*</td>
<td>Dominant CC+CG vs GG</td>
<td></td>
</tr>
<tr>
<td>rs6903956 ADTRP</td>
<td>6 A 0.066 AA/AG/GG</td>
<td>3 173 1187 0.204</td>
<td>0.775 0.007*</td>
<td>0.799 0.008*</td>
<td>Dominant GG vs AA+AG</td>
<td></td>
</tr>
<tr>
<td>rs974819 PDGFD</td>
<td>11 C 0.412 CC/CT/TT</td>
<td>216 630 442 0.738</td>
<td>-0.345 0.019*</td>
<td>-0.195 0.185</td>
<td>Additive TT vs CT vs CC</td>
<td></td>
</tr>
<tr>
<td>rs4380028 ADAMTS7</td>
<td>15 A 0.341 AA/AG/GG</td>
<td>157 600 584 0.878</td>
<td>0.155 0.280</td>
<td>0.120 0.551</td>
<td>Dominant AA+AG vs GG</td>
<td></td>
</tr>
<tr>
<td>rs4773144 COL4A1-A2</td>
<td>13 G 0.367 GG/AG/AA</td>
<td>187 609 545 0.420</td>
<td>-0.081 0.578</td>
<td>-0.071 0.726</td>
<td>Dominant GG+AG vs AA</td>
<td></td>
</tr>
<tr>
<td>rs12190287 TCF21</td>
<td>6 G 0.435 GG/CG/CC</td>
<td>250 667 424 0.665</td>
<td>-0.104 0.468</td>
<td>-0.200 0.352</td>
<td>Dominant GG+CG vs CC</td>
<td></td>
</tr>
<tr>
<td>rs46522 UBE2Z</td>
<td>17 C 0.282 CC/CT/TT</td>
<td>122 512 707 0.037</td>
<td>0.009 0.955</td>
<td>-0.101 0.613</td>
<td>Dominant CC+CT vs TT</td>
<td></td>
</tr>
<tr>
<td>rs2895811 HHIPL1</td>
<td>14 C 0.260 CC/CT/TT</td>
<td>95 506 740 0.505</td>
<td>-0.216 0.177</td>
<td>-0.187 0.351</td>
<td>Dominant CC+CT vs TT</td>
<td></td>
</tr>
</tbody>
</table>

Chr, chromosome; MAF, minor allele frequency; RR, minor homozygous; Rr, heterozygous; rr, major homozygous. *Adjusted for gender, age, hypertension, diabetes, hyperlipidemia, smoking and drinking.

**Associations between Polymorphisms and CSI**

The CC + CG genotype of CDKN2A/B (rs1333049) and the GG genotype of ADTRP (rs6903956) were both found to be associated with a significantly increased risk of a higher CSI, even after adjusting for confounding factors, assuming a dominant model (p = 0.007 and p = 0.008, respectively) (Table 2). The additive model of PDGFD (rs974819) indicated that the TT genotype was significantly associated with the CSI (p = 0.019), although this finding was not statistically significant following adjustment for confounding factors (p = 0.185). We were unable to replicate any significant associations with CSI for the remaining six SNPs. Although the risk alleles for CDKN2A/B (rs1333049) and PDGFD (rs974819) were in the same direction as those observed in previous reports, we found a different risk allele for ADTRP (rs6903956).
Coronary Atherosclerosis and Susceptible Genes

The distribution of individuals carrying different numbers of risk alleles, ranging from 1 to 6, for the three above polymorphisms is shown in Fig. 2. There was a rightward shift of the mean CSI value, which was found to be correlated with the carriage of multiple risk alleles by 0.374 ($p = 4.61E-05$). Therefore, the addition of one risk allele increased the mean CSI by 0.374. We also examined the relationship between the number of risk alleles and the prevalence of MI; however, we found no significant associations.

Associations between Polymorphisms and other CAD Parameters

In order to further investigate the effects of the $CDKN2A/B$ (rs1333049), $ADTRP$ (rs6903956), $PDGFD$ (rs974819), $TCF21$ (rs12190287), $COL4A1-A2$ (rs4773144), $HHIPL1$ (rs2895811), $ADAMTS7$ (rs4380028) and $UBE2Z$ (rs46522) polymorphisms on CAD, we evaluated the incidence of MI, clinical ischemic heart disease and PAI. The CC+CG genotype of $CDKN2A/B$ exhibited a significant association with MI and clinical ischemic heart disease (OR = 1.843; 95% CI 1.293-2.629, $p = 0.001$; and OR = 1.706; 95% CI = 1.148-2.534, $p = 0.008$, respectively) relative to the GG genotype under a logistic model.
regression analysis after adjusting for age, gender, hypertension, diabetes, hyperlipidemia and drinking and smoking habits. A linear regression analysis also demonstrated a significant association between \( \text{CDKN2A/B} \) and PAI \((p = 0.005)\) after adjusting for the conventional confounding factors, as described above. \( \text{ADTRP} \) \((\text{rs6903956})\) exhibited a suggestive association with MI in the \( \text{AA} + \text{AG} \) genotypes, after adjusting for the confounding factors described above \((\text{OR} = 1.652, 95\% \text{ CI} = 1.027-2.656, p = 0.038)\). No other genes were shown to be associated with these three additional CAD parameters.

**Discussion**

In the present study, we investigated the associations between \( \text{CDKN2A/B} \) \((\text{rs1333049})\), \( \text{ADTRP} \) \((\text{rs6903956})\), \( \text{PDGFD} \) \((\text{rs974819})\), \( \text{TCF21} \) \((\text{rs12190287})\), \( \text{COL4A1-A2} \) \((\text{rs4380028})\), \( \text{HHIPL1} \) \((\text{rs2895811})\), \( \text{ADAMTS7} \) \((\text{rs46522})\) polymorphisms and the incidence of CAD in a cohort of 1,536 elderly Japanese subjects. The results showed \( \text{CDKN2A/B} \) \((\text{rs1333049})\) to be the most robust SNP, exhibiting a significant association

### Table 3. Associations between the eight SNPs and the CSI values based on a logistic regression analysis in the 1,503 subjects

<table>
<thead>
<tr>
<th>SNP</th>
<th>Gene name</th>
<th>Minor allele</th>
<th>MAF Case</th>
<th>MAF Control</th>
<th>Dominant model</th>
<th>Recessive model</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Adjusted†</td>
<td>Adjusted†</td>
</tr>
<tr>
<td>rs1333049</td>
<td>CDKN2A/B</td>
<td>C</td>
<td>0.553</td>
<td>0.463</td>
<td>1.683</td>
<td>1.219-2.323</td>
</tr>
<tr>
<td>rs6903956</td>
<td>ADTRP</td>
<td>A</td>
<td>0.044</td>
<td>0.073</td>
<td>1.839</td>
<td>1.172-2.886</td>
</tr>
<tr>
<td>rs974819</td>
<td>PDGFD</td>
<td>C</td>
<td>0.383</td>
<td>0.422</td>
<td>0.864</td>
<td>0.650-1.148</td>
</tr>
<tr>
<td>rs4380028</td>
<td>ADAMTS7</td>
<td>A</td>
<td>0.335</td>
<td>0.343</td>
<td>1.042</td>
<td>0.797-1.363</td>
</tr>
<tr>
<td>rs4773144</td>
<td>COL4A1-A2</td>
<td>G</td>
<td>0.365</td>
<td>0.367</td>
<td>0.948</td>
<td>0.724-1.240</td>
</tr>
<tr>
<td>rs12190287</td>
<td>TCF21</td>
<td>G</td>
<td>0.430</td>
<td>0.437</td>
<td>1.071</td>
<td>0.803-1.427</td>
</tr>
<tr>
<td>rs46522</td>
<td>UBE2Z</td>
<td>C</td>
<td>0.298</td>
<td>0.276</td>
<td>1.117</td>
<td>0.856-1.457</td>
</tr>
<tr>
<td>rs2895811</td>
<td>HHIPL1</td>
<td>C</td>
<td>0.240</td>
<td>0.266</td>
<td>0.849</td>
<td>0.649-1.110</td>
</tr>
</tbody>
</table>

MAF, minor allele frequency. †Adjusted for gender, age, hypertension, diabetes, hyperlipidemia, smoking and drinking. OR, odds ratio; CI, confidence interval; NA, not available.

**Fig. 2.** Distribution of the individuals, according to the risk allele count, with the mean CSI

For each individual, the number of potential CAD associated risk loci: \( \text{CDKN2AB} \) \((\text{rs1333049})\), \( \text{ADTRP} \) \((\text{rs6903956})\), and \( \text{PDGFD} \) \((\text{rs974819})\) was calculated. Sample sizes for each risk allele group are shown by the shaded bars. CSI scores are shown with error bars representing ± 1 standard error. A linear line and equation are also shown.

\[
y = 0.374x + 6.724 \\
p = 4.61\times10^{-05}
\]
with different parameters of CAD, including CSI, MI, clinical ischemic heart disease and PAI. In addition, ADTRP (rs6903956) demonstrated a significant association with both CSI and MI, while PDGFD (rs974819) displayed a suggestive association with CSI after adjusting for gender, age, hypertension, diabetes and hyperlipidemia. Rs974819 showed a possible gender interaction, with a significant effect in women, but not men. The CC genotype of TCF21 (rs12190287) was also found to be significantly associated with CSI in a logistic regression analysis.

Since three independent variants, CDKN2A/B (rs1333049), ADTRP (rs6903956) and PDGFD (rs974819), exhibited a positive association with CAD, we studied the combinatorial effects of these three polymorphisms. The linear regression analysis showed that the individuals carrying more risk alleles had an increased risk of CAD. While each of the three SNPs demonstrated a suggestive association with CAD, the combination of these three loci exhibited only a modest effect on increasing the risk of CAD. Although the increment was small, the relationship between the number of risk alleles and the CSI was statistically significant. The modest effect of individual alleles is indeed consistent with the results of the current common disease variant method, and we expect that unidentified genetic factors underlying this cardiovascular phenotype remain. In addition, we investigated the relationships between CAD and these three variants using Ingenuity pathway analyses (Ingenuity Systems, Inc., Redwood City, CA, USA); however, we found no associated canonical pathways.

In this study, CDKN2A/B (rs1333049) demonstrated a strong association with CAD, which has been previously replicated in several studies across populations from Caucasians to East Asians. Functional analyses of the effects of CAD-associated variation on 9p21 indicate that the CAD risk allele is associated with a lower p15 expression in vascular smooth muscle cells (VSMCs) and a higher level of VSMC proliferation. p15, which is encoded by CDKN2B, is known to play a critical role in the regulation of the cell cycle and inhibits cell proliferation by suppressing the dissociation of the transcription factor E2F from retinoblastoma proteins and consequently inhibiting the E2F-mediated expression of cell proliferation genes. Since VSMC proliferation is involved in the pathogenesis of atherosclerosis, the association between the 9p21 variant and an increased risk of CAD is possibly due to increased VSMC proliferation.

At present, ADTRP (rs6903956) has not been identified to be a CAD susceptibility locus in Caucasians based on GWAS examinations. Although ADTRP was first identified in a Han Chinese population and later replicated in two small Han Chinese cohorts, a recent large GWAS in a Han Chinese population failed to identify whether this gene is a causative susceptibility locus. Although rs6903956 has also been evaluated in the same racial group, the results are controversial.

ADTRP regulates the mRNA expression of the tissue factor pathway inhibitor (TFPI) gene. It is believed that a decreased ADTRP expression results in a decreased TFPI expression, with a subsequently increased rate of thrombosis and higher risk of atherosclerosis and CAD. Expression studies have indicated that the GG genotype of ADTRP is associated with a higher mRNA expression level of ADTRP than the AG or AA genotype, which has been shown to be associated with CAD. Therefore, a decreased expression of ADTRP may be a pathogenic cause of CAD. Interestingly, we found ADTRP to be a significant coronary risk factor, although this risk allele differs between Han Chinese and Japanese populations. As a minor allele, its frequency is low in Japanese subjects; therefore, we performed direct sequencing, which confirmed our genotyping findings. One possible explanation for the associations with alternate alleles between populations is differences in genetic structures and interactions with environmental factors. Further studies are needed to fully explain the differential association between this gene and the incidence of atherosclerosis in these two different populations.

The risk allele of PDGFD (rs974819) has been replicated in Japanese, Korean and Han Chinese populations. A GWAS by Takeuchi et al. identified no significant associations between PDGFD and CAD in a Japanese population. PDGFD is predicted to play an important role in atherosclerosis by influencing the matrix metalloproteinase (MMP) activity and monocyte migration and inhibiting the smooth muscle cell (SMC) gene expression. MMPs are highly expressed in atherosclerotic lesions and play important roles in the turnover of basement membrane type IV collagen and the regulation of cell proliferation and migration. Importantly, a case-control study from China revealed a gender-dependent genetic effect of PDGFD, with a significant difference in women, similar to our results. Although we observed increased CSI values in women in this study, our sample size was comparatively small.

Recently, a GWAS in a Han Chinese population replicated the association between TCF21 and CAD. However, the variant studied in this ethnic group was
rs12524865, which is poorly correlated with the SNP evaluated in the present study, rs12190287\(^2\). In this study, we found a modest positive association, which is consistent with the findings of previous reports in Europeans\(^3\). In fact, two studies of Chinese and Japanese populations failed to replicate the association\(^3, 14\). It has been shown that individuals carrying the risk allele for rs12190287 have an increased TCF21 expression due to increased binding of activator protein 1 (AP-1) complexes to cis regulatory sites. This aberrant upregulation of TCF21 may contribute to increasing the risk of CAD by altering the SMC response to injury in the vessel\(^1\).

The current study was unable to replicate an association with CAD among the other four SNPs, including \(COL4A1-A2\) (rs4773144), \(HHIPL1\) (rs2895811), \(ADAMTS7\) (rs4380028) and \(UBE2Z\) (rs46522). These four loci have been identified to exhibit associations with CAD in Europeans and South Asians\(^7, 8, 14\). The association between \(HHIPL1\) (rs2895811) and CAD was replicated in another GWAS in a Japanese population\(^14\). In addition, the association between \(ADAMTS7\) (rs4380028) and CAD was replicated in a GWAS in a Han Chinese population\(^21\). These results suggest that the associations observed for these four SNPs are population-specific.

Age-related progression in the degree of atherosclerosis is evident in the elderly, and atherosclerosis exhibits sustained advancement in most arteries in elderly patients. Although many studies have investigated the pathogenesis of atherosclerosis, there is little information regarding the development of atherosclerosis in the elderly\(^11\). In this series of autopsy cases of elderly Japanese subjects, we investigated the severity of atherosclerosis, that is, the outcome of sustained progression. The use of consecutive autopsy cases allowed us to directly measure the degree of coronary stenosis using pathological procedures, the most reliable assessments of systemic atherosclerosis\(^10\). Nevertheless, there are some limitations in this study, including both selection and survival bias. First, the subjects included autopsy patients treated at a geriatric hospital. Therefore, the potential for selection bias must be considered when interpreting the results. Oda \textit{et al}. compared the direct causes of death among our subjects and the census data included in the ‘Abridged Life Tables For Japan 2003’ provided by the Ministry of Health, Labour and Welfare of Japan. Almost all death rates were consistent with our autopsy data. Therefore, our study population did not differ significantly from the standard elderly residents of Tokyo, Japan\(^9\). Second, our subjects were old. Death due to atherosclerosis at an early age may have contributed to exclusion from our cohort. Lastly, the effects of conventional risk factors for atherosclerosis, including hypertension, diabetes mellitus, dyslipidemia, chronic kidney disease and smoking, as well as environmental factors, such as a high-calorie diet and physical inactivity, may have a greater effect on the development of CAD than genetic variants in the elderly. However, we adjusted for age, gender, hypertension, diabetes, hyperlipidemia, drinking and smoking in our analysis.

In conclusion, we replicated the associations between the \(CDKN2A/B\) (rs1333049) and TCF21 (rs12190287) polymorphisms and CAD and identified a different associated risk allele of \(ADTRP\) (rs6903956). We also showed that \(PDGFD\) (rs974819) has a specific gender effect in its association with CAD. Cumulatively, \(CDKN2A/B\) (rs1333049), \(ADTRP\) (rs6903956) and \(PDGFD\) (rs974819) were found to have an influence on the mean CSI value. This is the first study to investigate the associations between SNPs and the severity of CAD, as defined by the CSI. A large-scale study is needed to further define the roles of these genetic loci in the pathogenesis of CAD.

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**Conflicts of Interest**

We declare that there are no conflicts of interest.

**References**


