Coronary Artery Disease

Association between Endostatin G4349A Polymorphism and Coronary Collaterals in the Chinese Population

Po-Chao Hsu,1,4 Tsung-Hsien Lin,1,3 Ho-Ming Su,1,3 Suh-Hang Juo,2,5,6 Wen-Ter Lai1,3 and Sheng-Hsiung Sheu1,3

Background: Angiogenesis plays a pivotal role in collateral formation and is mediated by a balance of pro-angiogenic and anti-angiogenic factors. Endostatin, an anti-angiogenic factor, was associated with impaired coronary collateral formation. We investigated the association between endostatin polymorphism and coronary collaterals.

Methods: The collateral scoring system developed by Rentrop was used to classify 487 patients according to their collaterals as either poor (grades 0 and 1) or good (grades 2 and 3). Endostatin G4349A polymorphism (rs1248337) was analyzed by polymerase chain reaction.

Results: The frequencies of GG and AG genotypes in the study population were 97.1% and 2.9%, respectively, with no individual AA genotype found. The patients with poor collateral had a lower incidence of family history of coronary artery disease (CAD) (2% vs. 5.9%, p = 0.039), a lower frequency of total occlusive lesions (20.7% vs. 76.5%, p < 0.001), and fewer diseased vessels (1.9 ± 0.9 vs. 2.5 ± 0.7, p < 0.001). There was no significant association between endostatin G4349A polymorphism and coronary collateral grade (p = 0.168). Multivariate analysis showed that total occlusion lesions and the number of diseased vessels were significant independent predictors of good coronary collaterals.

Conclusion: Our data suggest that the endostatin G4349A polymorphism is not associated with coronary collateral grade in the Chinese CAD population.

Key Words: Collateral • Coronary • Endostatin • Gene • Polymorphism

INTRODUCTION

The development of coronary collaterals is an adaptive response to chronic myoschemia, and serves as a conduit bridging the significantly stenotic coronary vessels.1-3 Collateral circulation can therefore protect and preserve myocardium from episodes of ischemia, enhance residual myocardial contractility, and reduce angina symptoms and cardiovascular events.4-6 However, there are inter-individual differences in coronary collateral formation, and the mechanisms for the different individuals’ ability to develop collateral circulation are still unclear.

Angiogenesis plays an important role in collateral vessel formation, and it is mediated by a balance of pro-angiogenic and anti-angiogenic factors. Endostatin, a potent inhibitor of angiogenesis, is a 20-kD protein derived from the carboxyl-terminal fragment of collagen...
It inhibits endothelial cell proliferation, adhesion, and migration, and can induce endothelial cell apoptosis in vitro. Endostatin is encoded by the COL18A1 gene located on chromosome 21, and it significantly inhibits the growth of different tumor types and targets angiogenesis regulatory genes on more than 12% of the human genome. In addition, it has also been reported recently that endostatin levels were higher in patients with poor collaterals than in those with good collaterals.

A single-nucleotide polymorphism (G to A change, G4349A) resulting in an aspartic acid-to-asparagines change (rs1248337) in exon 42 is in the coding region for endostatin. The role of this endostatin G4349A polymorphism has been evaluated in several cancers, including prostate cancer, breast cancer, leukemia and lung cancer. However, there is no research discussing the association between endostatin G4349A polymorphism and coronary collaterals. We previously found vascular endothelial growth factor (VEGF), a pro-angiogenic factor, polymorphisms might affect collateral development. In this study, we examined endostatin, a anti-angiogenic factor, G4349A polymorphism to assess its possible relationships to coronary collaterals in patients with significant coronary artery disease (CAD).

**MATERIALS AND METHODS**

**Study subjects**

From February 2002 to February 2008, we evaluated 950 consecutive patients who underwent diagnostic coronary angiography at Kaohsiung Medical University Hospital (KMUH) in Taiwan. We further excluded patients with coronary artery lumen diameter stenosis < 70%, a history of coronary artery bypass surgery (CABG), a history of percutaneous coronary intervention (PCI), inconclusive genetic restriction digest results, or inadequate angiograms for collateral evaluation. Other analyzed demographic and baseline data included gender, age, and any history of the following: diabetes mellitus (DM), hypertension, hypercholesterolemia, cigarette smoking, CAD duration and medications. CAD duration was defined as the length of time between a patient’s first chest pain symptoms, and the day of the scheduled coronary angiography. The research protocol was approved and registered by the ethics committee (KMUH-IRB-940253) at our institution, and informed consent was obtained from all patients.

**Coronary angiography and collateral scoring**

The coronary artery angiography films were reviewed by two experienced cardiologists, blinded to the clinical and genotype data for all patients. Any differences in interpretation were resolved by a third reviewer, who was blinded to the readings the two reviewers obtained.

Coronary artery stenosis was determined by quantitative coronary angiography. The recorded data also included the number of diseased vessels, the vessel to which the collaterals were connected, and the grade of coronary collateral circulation. Vessels exhibiting a 70% or greater reduction in lumen diameter were classified as a significant lesion. In subjects with more than one significant CAD vessel, the vessel with the highest collateral grade was chosen for analysis. The collateral scoring system developed by Rentrop and Cohen was used. Grades of collateral filling from the contralateral vessel were: 0 = none; 1 = filling of side branches of the artery to be dilated via collateral channels without visualization of the epicardial segment; 2 = partial filling of the epicardial segment via collateral channels; 3 = complete filling of the epicardial segment of the artery being dilated via collateral channels. In subjects with more than one collateral vessel supplying the distal aspect of the diseased artery, the highest collateral grade was recorded. Patients were then classified according to their collateral grades as either poor (grade 0 or grade 1 collateral) or good (grade 2 or grade 3 collateral). The two readers obtained a 96% agreement in the collateral classifications.

**Extraction and amplification of genomic DNA**

Blood samples were collected during angiography. After isolating the buffy coat, which is a leukocyte-enriched fraction of whole blood, genomic DNA was extracted from peripheral blood by using a DNA extraction kit (Puregene Gentra Systems, Minneapolis, MN, USA) according to manufacturer instructions. The extracted genomic DNA was suspended in 10 mmol/l Tris-HCl, 1 mmol/l EDTA pH 8.0, and DNA concentrations were measured by spectrophotometry. Genotypes were determined by TaqMan allelic discrimination assay.
using a commercial kit (Applied Biosystems; ABI, Foster City, CA, USA) according to manufacturer instructions. Genotyping for endostatin G4349A polymorphism (rs12483377) was performed using TaqMan-MGB probes and primers and the Applied Biosystems Assay-on-Demand service. The thermal cycling conditions were 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 90 s. After completing PCR amplification, allelic discrimination was performed using the Applied Biosystems 7900 real-time PCR system. Laboratory personnel were blinded to the disease status of all subjects.

Statistical analysis
All data were expressed as means ± standard deviation. If the minor allele frequency (MAF) for endostatin G4349A polymorphism among the good collaterals was estimated to be 0.12, the study should enroll at least 186 patients with good collaterals to have more than 80% statistical power to detect an association with ORs ≥ 2.5. The genotypic distribution was tested by using the Hardy-Weinberg equilibrium. Independent t test was used to compare continuous variables between the two groups, and Chi-square test was used to compare categorical data. Univariate logistic regression for collateral development as a binary outcome (poor or good) was initially performed using each genotype as a dummy variable, and without assuming a specific genetic model. A model of inheritance was then assumed after this initial result. All p values were two-sided, with a significance level of p < 0.05. The Statistical Package for the Social Sciences 11.0 for Windows (SPSS Inc., Chicago, IL, USA) was used for statistical analysis.

RESULTS

Clinical characteristics
Of the 950 subjects initially enrolled, 463 patients were excluded for the following reasons: coronary artery lumen diameter stenosis < 70%, history of CABG or PCI, genotyping failure, or inadequate angiograms for collateral evaluation. The final study population was 487 subjects (380 male, 107 female; average age, 62.3 ± 12.5 y). Of the 487 patients enrolled, 205 (42.1%) patients had no coronary collaterals. In subjects with collaterals, the coronary grade was distributed as follows: 95 (33.7%) with grade 1, 131 (46.5%) with grade 2 and 56 (19.9%) with grade 3. Table 1 compares the demographic data between the poor group (n = 300) and the good group (n = 187). The patients in the poor group had a lower incidence of family history of CAD (2% vs 5.9%, p = 0.039), lower frequency of total occlusive lesions (20.7% vs. 76.5%, p < 0.001), and fewer diseased vessels (1.9 ± 0.9 vs. 2.5 ± 0.7, p < 0.001).

Endostatin G4349A polymorphism
Among the 487 study subjects, 473 (97.1%) had GG

<table>
<thead>
<tr>
<th>Variables</th>
<th>Poor collateral (n = 300)</th>
<th>Good collateral (n = 187)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>62.7 ± 13.1</td>
<td>61.7 ± 11.6</td>
<td>0.386</td>
</tr>
<tr>
<td>Gender (Male, %)</td>
<td>75.5</td>
<td>81.8</td>
<td>0.116</td>
</tr>
<tr>
<td>DM (%)</td>
<td>44</td>
<td>41.7</td>
<td>0.639</td>
</tr>
<tr>
<td>HTN (%)</td>
<td>65.3</td>
<td>65.8</td>
<td>1.000</td>
</tr>
<tr>
<td>Smoking (%)</td>
<td>58.7</td>
<td>57</td>
<td>0.777</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.6 ± 3.85</td>
<td>25.7 ± 3.55</td>
<td>0.768</td>
</tr>
<tr>
<td>Hypercholesterolemia (%)</td>
<td>52.4</td>
<td>60.5</td>
<td>0.089</td>
</tr>
<tr>
<td>Family history of CAD (%)</td>
<td>2.0%</td>
<td>5.9%</td>
<td>0.039</td>
</tr>
<tr>
<td>CAD duration (month)</td>
<td>11.0 ± 30.2</td>
<td>15.9 ± 32.5</td>
<td>0.102</td>
</tr>
<tr>
<td>Patients with total occlusive lesions (%)</td>
<td>62 (20.7%)</td>
<td>143 (76.5%)</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Number of CAD vessels</td>
<td>1.9 ± 0.9</td>
<td>2.5 ± 0.7</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>One (%)</td>
<td>37.7</td>
<td>10.2</td>
<td></td>
</tr>
<tr>
<td>Two (%)</td>
<td>28.3</td>
<td>31.6</td>
<td></td>
</tr>
<tr>
<td>Three (%)</td>
<td>33</td>
<td>58.3</td>
<td></td>
</tr>
</tbody>
</table>

DM, diabetes; HTN, hypertension; BMI, body-mass index; CAD, coronary artery disease.
genotype, 14 (2.9%) had AG, and no individual had AA genotype. The genotypic distribution was in Hardy-Weinberg equilibrium. There was no evidence of association between endostatin genotype with baseline characteristics. The frequencies of GG and AG genotype in the patients with poor collateral were 98% (294/300) and 2% (6/300), respectively, and the frequencies of GG and AG genotype in the patients with good collateral were 95.7% (179/187) and 4.3% (8/187), respectively. Given a reference group of patients with GG genotype, the OR for the good collateral group was 2.19 (95% CI: 0.748-6.415, p = 0.153) in the AG genotype (Table 2). Allelic analysis of the endostatin G4349A allele, in comparison with the G allele revealed an OR of 2.16 (95% CI: 0.745-6.287, p = 0.156) for good coronary collaterals (Table 2).

Because endostatin level in diabetics was associated with coronary collateral formation, we further analyzed the genetic effect in the diabetic subgroup.18 Among the 210 study subjects with diabetes, 204 (97.1%) had GG genotype, and 6 (2.9%) had AG. The frequencies of the GG and AG genotypes in patients with poor collateral were 97.7% (129/132) and 2.3% (3/132), respectively, and the frequencies of GG and AG genotype in the patients with good collateral were 96.2% (75/78) and 3.8% (3/78), respectively. Given a reference group of patients with GG genotype, the OR for the good group was 1.72 (95% CI: 0.339-8.738, p = 0.513) in the AG genotype (Table 3). Allelic analysis of the endostatin G4349A A allele, in comparison with the G allele revealed an OR of 1.71 (95% CI: 0.340-8.557, p = 0.516) for good coronary collaterals (Table 3).

Logistic regression analysis revealed that the significant independent predictors for good collateral formation were total occlusion lesions (p < 0.001, OR = 13.109, 95% CI = 7.86-21.86) and the number of diseased vessels (p < 0.001, OR = 2.131, 95% CI = 1.54-2.94). Other variables such as gender, age, DM, hypertension, lipidemia, CAD duration, family history of CAD, and endostatin G4349A polymorphism were not statistically significant (Table 4).

**DISCUSSION**

In the current study, we analyzed the association between endostatin G4349A polymorphism and coronary collaterals in 487 Chinese patients, which revealed four major findings. First, there was no evidence of association between endostatin G4349A polymorphism and coronary collaterals in the entire subject population and in the diabetic subgroup. Second, the total occlusive

<table>
<thead>
<tr>
<th>Polymorphism site (4943)</th>
<th>Poor collaterals (n = 132)</th>
<th>Good collaterals (n = 78)</th>
<th>OR (95% CI)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allele frequency</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>261 (98.9%)</td>
<td>153 (98.1%)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>3 (1.1%)</td>
<td>3 (1.9%)</td>
<td>1.71 (0.340-8.557)</td>
<td>0.516</td>
</tr>
<tr>
<td>Genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>129 (97.7%)</td>
<td>75 (96.2%)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>AG</td>
<td>3 (2.3%)</td>
<td>3 (3.8%)</td>
<td>1.72 (0.339-8.738)</td>
<td>0.513</td>
</tr>
<tr>
<td>AA</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Polymorphism site (4943)</th>
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</tr>
</thead>
<tbody>
<tr>
<td>Allele frequency</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G</td>
<td>594 (99%)</td>
<td>366 (97.9%)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>6 (1%)</td>
<td>8 (2.1%)</td>
<td>2.16 (0.745-6.287)</td>
<td>0.156</td>
</tr>
<tr>
<td>Genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GG</td>
<td>294 (98%)</td>
<td>179 (95.7%)</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>AG</td>
<td>6 (2%)</td>
<td>8 (4.3%)</td>
<td>2.19 (0.748-6.415)</td>
<td>0.153</td>
</tr>
<tr>
<td>AA</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

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Table 2. The frequency of endostatin genotype in whole patients

Table 3. The frequency of endostatin genotype in diabetic patients
lesions and the number of diseased vessels were significant independent predictors for good collateral formation. Third, there were no study subjects with homozygous AA genotype in our study population. This result was similar to Liu et al’s study of leukemia.21

Endostatin and angiogenesis
Angiogenesis plays a pivotal role in coronary collateral formation. Growth factors are expressed in response to ischemia or hypoxia and further stimulate angiogenesis.28 There are more than 15 growth factors known to stimulate collateral growth and angiogenesis.29,30 Endostatin is one of the inhibitors of angiogenesis. The anti-angiogenic effects of endostatin might be related to its high affinity for heparin which can interfere with the binding of basic fibroblast growth factor (bFGF) and further inhibit FGF growth signaling.8 In addition, VEGF expression can also be reduced by endostatin and further causes reduced tumor growth and impaired angiogenesis.31 There are some malignancies associated with serum endostatin levels, and the anti-tumor effect of endostatin has been shown in a variety of solid tumors including melanoma, fibrosarcoma, renal cell carcinoma, mammary carcinoma and ovarian carcinoma.32 This has resulted in its evaluation in anti-angiogenic therapy of advanced cancer.33

Endostatin G4349A polymorphism
A single-nucleotide polymorphism (SNP) resulting in an aspartic acid-to-asparagine change (G4349A, rs1248337) in exon 42 is in the coding region for endostatin. Endostatin G4349A polymorphism was reported to possibly affect protein conformation and further impair the ability of endostatin to bind to other molecules.19 The role of this endostatin G4349A polymorphism has been evaluated in several cancers, including prostate cancer, breast cancer, leukemia, lung cancer, and so on.19,25 Iughetti et al initially suggested that G4349A polymorphism was associated with a risk of prostate cancer;19 however, recent studies showed these findings were controversial, and require further study.20,24,25

Endostatin and coronary collaterals
The development of coronary collaterals can reduce angina and infarct size, preserve left ventricular ejection fraction, decrease aneurysmal dilatation, and provide a survival benefit in patients with significant CAD.4-6,34,35 In addition, in the current era, interventional cardiologists can even treat lesions of chronic total occlusion via coronary collaterals by retrograde approach.36 Angiogenesis plays an important role in coronary collateral formation, and is mediated by a balance of pro-angiogenic and anti-angiogenic factors. Endostatin is one of the anti-angiogenic factors and was recently reported to be associated with impaired coronary collateral formation.16-18 Panchal et al. reported that pericardial fluid levels of endostatin, but not VEGF, are associated with the presence or absence of collaterals in patients with CAD, which suggests that endostatin levels may locally modulate coronary collateral formation.16 Mitsuma et al. investigated the dynamics of endostatin and stated that endostatin production within coronary circulation was higher in patients with poorly developed collaterals than in those with well-developed collaterals.17 In addition,
Sodha et al. also reported that endostatin and angiostatin are increased in diabetic patients with CAD, and associated with impaired coronary collateral formation. However, there is no research discussing the association between endostatin G4349A polymorphism and coronary collaterals, and our study is the first study to investigate the genetic effect. However, we did not find any association between endostatin G4349A genotype and coronary collateral formation in this study. In addition, total occlusive lesions and the number of diseased vessels were significant independent predictors for good collateral formation. In a previous study, Sezer et al. reported that the variable of the number of diseased vessels was the only confounding variable of the collateral score after ANCOVA analysis; after the confounding factor was controlled for, the collateral score in the uremic group was found to be significantly lower than those with normal creatinine clearance. Abaci et al. also reported the variables of sex and the number of diseased vessels were the only important confounding variables of the collateral score after ANCOVA analysis; after confounding variables were controlled for, the collateral score in the DM group was significantly different from that in the non-diabetic group.

The possible reasons for the lack of association in our study might be as follows. First, serum endostatin levels were similar between non-carriers and carriers of the variant allele in the previous studies; hence the difference between endostatin level may not be influenced by the endostatin polymorphism like other possible factors involving angiogenesis such as VEGF. Second, racial differences may partially explain the discrepancies. The allelic frequencies and the possible genetic effects of the endostatin gene are considered to be different between Caucasian and Chinese populations. Our results and Liu et al.’s study both showed no subjects with homozygous AA genotype, which is different from those reports involving a Caucasian population.

Limitations of the present study
First, the collateral formation was assessed by coronary angiography in this study. Measuring collateral flow index by intravascular Doppler guidewire may provide a more objective physiological measurement of collateral grade. However, the invasiveness of intravascular ultrasound limits its use in large-scale studies. Second, the effects of endostatin polymorphisms on serum endostatin levels were not examined in the enrolled subjects. Third, since this was only a clinical association study, potential mechanisms were not fully elucidated.

CONCLUSION
In conclusion, our data suggests that the endostatin G4349A polymorphism is not associated with coronary collaterals in the Chinese CAD population.

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