DNA Polymorphisms of the Renin-Angiotensin System and Risk of Restenosis after Coronary Balloon Angioplasty

Kuo-Chin Chen,1 Kwan-Lih Hsu,2 Juey-Jen Hwang,2 Chuen-Den Tseng,2 Yung-Za Tseng2 and Fu-Tien Chiang2

Background and Purpose: DNA polymorphisms of component genes in RAS were reported to modulate the process of post-PTCA restenosis. This study was designed to assess the influence of eight gene polymorphisms involved at different steps of the renin-angiotensin system (RAS) enzymatic cascade on post-percutaneous transluminal coronary angioplasty (PTCA) restenosis simultaneously. We think systemic analysis of predisposing alleles in RAS will clarify our understanding of mechanisms derived from the conflicting results of previous studies.

Methods: This prospective study included 290 Chinese patients who underwent a successful angioplasty procedure and follow-up angiography (mean 42.4 ± 14.5 months) if angina recurred with positive noninvasive stress tests. Restenosis was defined as more than 50% diameter stenosis at the previously treated vessel site.

Results: Among these gene polymorphisms, only ACE D allele was significantly associated with post-PTCA restenosis in univariate (D/D 72.5%, D/I 54.4%, I/I 47.5%, p value test for trend < 0.01) and multivariate (p value test for trend < 0.01) analysis. One novel finding in our study is that the effect of ACE D allele on restenosis is reversed by AGT-M174 allele (p value to test for the interaction term = 0.03).

Conclusions: Our results indicate ACE D allele increases the risk of post-PTCA restenosis, and this risk is modified by the coexistence of AGT-M174 allele in Chinese patients. Gene-gene interactions have increasing importance in the post-genome era and may explain contradictory results in previous studies.

Key Words: Renin-angiotensin system • Polymorphism • PTCA • Restenosis

Percutaneous transluminal coronary angioplasty (PTCA) is a standard treatment for coronary artery disease. However, post-PTCA restenosis is the major limitation. Even in the era of stents, restenosis is still a challenge to interventionists. That vascular remodeling in post-PTCA restenosis occurs has been disclosed in clinical studies, but the exact molecular and cellular mechanisms are still unknown. The renin-angiotensin system (RAS) has been extensively studied as an important mediator of cardiovascular disease since Hilbert et al. first showed in 1991 that it had a role in the pathogenesis of hypertension.1 The major components of RAS include angiotensinogen (AGT), renin, angiotensin-converting enzyme (ACE), angiotensin II and angiotensin II receptors. Binding of the final product of this enzymatic cascade, angiotensin II, to its receptor results in vasoconstriction, and in aldosterone and catecholamine release.2 In addition, angiotensin II has other biologic effects that influence vascular endothelium and smooth muscle cells, and cardiac fibroblasts.3-5 These may play a role in the pathophysiology of coronary artery disease, myocardial infarction, and restenosis after...
percutaneous coronary interventions. The prominent role of RAS in cardiovascular regulation suggests that DNA polymorphisms of component genes may modulate the cardiovascular disease process. However, the results from different studies have been conflicting. Therefore, we think simultaneous analysis of several predisposing alleles in RAS should provide more information about the pathophysiology of atherosclerosis and subsequent interventions and may improve therapeutic management.

The present study was designed to investigate the relation between DNA polymorphisms of RAS (including the AGT-T174M and M235T gene polymorphisms, the AGTS' upstream core promoter region G-6A, A-20C, G-152A, and G-217A gene polymorphisms, the ACE gene insertion/deletion [I/D] polymorphism and the angiotensin II type 1 receptor [AT1R] A1166C gene polymorphism) and risk of restenosis after balloon coronary angioplasty (PTCA) in Chinese. In addition, the potential interactions of these polymorphisms were evaluated.

METHODS

Patient Population
From January 1995 to January 2000, we recruited 350 Chinese patients with coronary artery disease who had undergone a successful angioplasty procedure and obtained their permission to take blood samples. They received clinical follow-up at our outpatient department. If angina recurred with positive non-invasive stress tests including the treadmill exercise test or thallium-201-SPECT, follow-up angiography was performed. Patients with poor left ventricular function (LVEF < 40%), chronic total occlusions and saphenous vein graft stenosis were excluded from this study. Finally, 290 patients with complete clinical and angiographic follow-up were enrolled.

Angiographic Assessment
Coronary angiography was performed in at least two projections. Identical projections of the target lesion were used for follow-up angiograms. PTCA was performed if diameter stenosis was more than 60%. The procedure was considered successful when residual stenosis in the dilated segment was less than 30% and no major complication (myocardial infarction, need for emergent coronary bypass surgery or in-hospital mortality) occurred. Restenosis was defined as a more than 50% diameter stenosis at the previously treated vessel site.

Determination of AGT Gene Polymorphisms
Genomic DNA was extracted by a nonenzymatic method. DNA fragments, including the M235T and T174M variants, were amplified by polymerase chain reaction (PCR). Forward and reverse primers were selected from the genomic sequence of AGT. The forward primer sequence from +921 to +940 in exon 2 of the AGT gene was 5'-GAT GCG CAC AAG GTC CTG TC-3'; the reverse primer from +1255 to +1274 was 5'-GCC AGC AGA GAG GTT TGC CT-3'. The primer mixture consisted of 0.5 μg DNA, 25 pmol of each primer, 0.15 nmol/L dNTP and 1U Taq polymerase in a final volume of 50 μl. The PCR was carried out in a Perkin-Elmer thermal cycler (Model 480; Perkin-Elmer, Norwalk, CT, USA). The reaction condition was achieved first by denaturing for 3 min, and then repeating the following cycle: denaturing at 95 °C for 1 min, annealing at 60 °C for 45 s, and extension at 72 °C for 1 min. This cycle was repeated 30 times with a final extension for 10 min. The 394-bp PCR product was resolved on a 2% ethidium bromide-stained gel and purified by centrifugation through a paper slurry for sequencing. Sequencing for molecular variants M235T and T174M was conducted by using a dye-terminator cycle sequencing method (ABD; Perkin-Elmer, Cetus, CA, USA). Single-strand sequencing was carried out by PCR with a sense primer as the sequencing primer. The PCR reagents and cycling conditions were the same as described above, except that dye-labeled ddNTP replaced unlabeled ddNTP, and AmpliTaq-FS enzyme was used. The product was run in an automatic sequencing apparatus (ABI 373A sequencer) in a 6% denatured polyacrylamide gel at 1500 V and 40 °C. The results were analyzed using incorporated sequencing analysis software (Version 2.01, ABD; Perkin-Elmer, Cetus, CA, USA).

Determination of AGT5' Upstream Core Promoter Region Gene Polymorphisms
The protocol was the same as the above in AGT gene polymorphisms except the forward primer was 5'-CTG TGC TAT TGT TGG TGT T-3' and the reverse
primer was 5’-GCT TAC CTT CTG CTG TAG T-3’.

**Determination of ACE Gene Polymorphism**

The stepdown PCR method was used for amplification of the ACE gene. The PCR condition was optimized at a magnesium concentration of 2 mmol/L. The PCR primers were the same as those used by Rigat et al. Salt concentration from 0.1 to 50 mmol/L of NaCl did not affect the amplification efficiency. All of the amplifications were carried out in the same thermal cycler (Model 480, Perkin-Elmer, Cetus, CA, USA). The reaction condition was achieved by denaturing first for 5 min and then repeating the following cycle: denaturing at 95 °C for 1 min, annealing at 70 °C for 1 min, and extension at 72 °C for 1 min. This cycle was repeated five times, and the annealing temperature was reduced to 65 °C and 60 °C each for five cycles and then to 60 °C for 25 cycles, with a final extension for 10 min. The PCR products were resolved by using 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

**Determination of AT1R Gene Polymorphism**

Leukocyte DNA was extracted by using the OIAmp kit (Quiagen Inc., Valencia, CA). PCR was performed to amplify a fragment encompassing the A→C polymorphic site at 1166 nucleotide position in the 3’ untranslated region of human AT1R gene. The forward primer was 5’-ATA ATG TAA GCT CAT CCA CC-3’ and the reverse primer was 5’-GAG ATT GCA TTT CTG TCA GT-3’. The reaction volume was 30 μL, which contained 100 ng of genomic DNA, 10 pmol of each primer, 250 μmol/L dNTP, 1.0 mmol/L MgCl2, 50 mmol/L KCl, 10 mmol/L Tris-HCl at pH 8.3, and 0.5 U Taq polymerase. After a heating step of 5 min at 94 °C, the main reaction, 94 °C for 30 sec, 55 °C for 45 sec, and 72 °C for 45 sec, was repeated for 40 cycles, and then followed by an extension step at 72 °C for 10 min. PCR products were confirmed to show exact amplification, and were then digested with DdeI (Toyobo, Osaka, Japan) for 3 h at 37 °C. The digested products were visualized on 3% Sea Kem HGT agarose gel (FMC Bioproducts, Rockland, ME, USA) by ethidium bromide staining.

**Statistical Analysis**

Frequencies of genotype and restenosis were cross-tabulated. Odds ratio and Chi-square test were used to analyze their associations. Logistic regression was used to adjust for the effect of potential confounders. Time to restenosis was analyzed using Kaplan-Meier curve. The restenosis curves of patients with different genotypes were compared using the log-rank test. A two-tailed probability value smaller than 0.05 was considered to be statistically significant.

**RESULTS**

**Patient Characteristics**

Table 1 lists baseline characteristics, including athrogenic risk factors, of all patients. Genotype was not available in one case of AGT-T174M polymorphism, 5 cases of AGT upstream core promoter region gene polymorphisms, 8 cases of ACE gene polymorphism and 69 cases of AT1R gene polymorphism because of uninter-
interpretable restriction digest results. Genotype frequencies as shown in Table 2 were in Hardy-Weinberg equilibrium.

**Associations between Genotype and Post-PTCA Restenosis**

The mean follow-up duration was 42.3 months (6-67 months) in the restenosis group and 43.0 months (2-66 months) in the non-restenosis group. Post-PTCA restenosis rates with these genotypes of RAS are shown in Table 2. Among them, ACE D allele was the only one significantly associated with restenosis in the univariate analysis (D/D 72.5%, D/I 54.4%, I/I 47.5%, \( p \) value test for trend < 0.01; D allele vs I allele, odds ratio = 1.70; 95% CI = 1.18-2.45). The result did not change much after adjusting for potential confounders including age, gender, diabetes, dyslipidemia, hypertension, smoking and the number of diseased vessel (\( p \) value test for trend < 0.01; odds ratio = 2.04; 95% CI = 1.32-3.13).

**Interactions among DNA Polymorphisms in RAS**

There was no significant synergistic effect on restenosis.

**Table 2.** Genotype frequencies and post-PTCA restenosis in RAS polymorphisms.

<table>
<thead>
<tr>
<th>Genotype*</th>
<th>n (%)</th>
<th>Restenosis</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>AGT-T174M</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T/T</td>
<td>232 (81.4)</td>
<td>135</td>
<td>97 (58)</td>
</tr>
<tr>
<td>T/M</td>
<td>46 (16.1)</td>
<td>22</td>
<td>24 (48)</td>
</tr>
<tr>
<td>M/M</td>
<td>7 (2.5)</td>
<td>3</td>
<td>4 (43)</td>
</tr>
<tr>
<td>AGT-M235T</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M/M</td>
<td>8 (2.8)</td>
<td>5</td>
<td>3 (63)</td>
</tr>
<tr>
<td>M/T</td>
<td>84 (29.6)</td>
<td>47</td>
<td>37 (56)</td>
</tr>
<tr>
<td>T/T</td>
<td>192 (67.6)</td>
<td>107</td>
<td>85 (56)</td>
</tr>
<tr>
<td>AGT5’-G-6A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G/G</td>
<td>10 (3.6)</td>
<td>6</td>
<td>4 (60)</td>
</tr>
<tr>
<td>G/A</td>
<td>83 (29.6)</td>
<td>43</td>
<td>40 (52)</td>
</tr>
<tr>
<td>A/A</td>
<td>187 (66.8)</td>
<td>107</td>
<td>80 (57)</td>
</tr>
<tr>
<td>AGT5’-A-20C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/A</td>
<td>269 (96.1)</td>
<td>149</td>
<td>120 (55)</td>
</tr>
<tr>
<td>A/C</td>
<td>4 (1.4)</td>
<td>2</td>
<td>2 (50)</td>
</tr>
<tr>
<td>C/C</td>
<td>7 (2.5)</td>
<td>5</td>
<td>2 (71)</td>
</tr>
<tr>
<td>AGT5’-G-152A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G/G</td>
<td>258 (92.2)</td>
<td>144</td>
<td>114 (56)</td>
</tr>
<tr>
<td>G/A</td>
<td>20 (7.1)</td>
<td>11</td>
<td>9 (55)</td>
</tr>
<tr>
<td>A/A</td>
<td>2 (0.7)</td>
<td>1</td>
<td>1 (50)</td>
</tr>
<tr>
<td>AGT5’-G-217A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G/G</td>
<td>204 (72.9)</td>
<td>116</td>
<td>88 (57)</td>
</tr>
<tr>
<td>G/A</td>
<td>67 (23.9)</td>
<td>34</td>
<td>33 (51)</td>
</tr>
<tr>
<td>A/A</td>
<td>9 (3.2)</td>
<td>6</td>
<td>3 (67)</td>
</tr>
<tr>
<td>ACE</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D/D</td>
<td>69 (24.9)</td>
<td>50</td>
<td>19 (72)</td>
</tr>
<tr>
<td>D/I</td>
<td>149 (53.8)</td>
<td>81</td>
<td>68 (54)</td>
</tr>
<tr>
<td>I/I</td>
<td>59 (21.3)</td>
<td>28</td>
<td>31 (47)</td>
</tr>
<tr>
<td>AT1R-A1166C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A/A</td>
<td>200 (92.6)</td>
<td>121</td>
<td>79 (61)</td>
</tr>
<tr>
<td>A/C</td>
<td>15 (6.9)</td>
<td>11</td>
<td>4 (73)</td>
</tr>
<tr>
<td>C/C</td>
<td>1 (0.5)</td>
<td>0</td>
<td>1 (0)</td>
</tr>
</tbody>
</table>

*AGT, angiotensinogen; AGT5’, AGT upstream core promoter region; ACE D/I, angiotensin-converting enzyme deletion/insertion; AT1R, angiotensin II type 1 receptor.*
nosis with the polymorphisms noted in this study. However, one novel finding was the interaction between ACE D/I polymorphism and AGT-T174M polymorphism, as shown in Table 3. Frequencies of restenosis increased with ACE D allele number for patients with AGT-T174 homozygotes, but decreased for patients with AGT-M174 allele. The effect of ACE D allele on restenosis was significantly modified by AGT-M174 allele \((p\) value to test for the interaction term was 0.02). The effect did not change much after adjusting for potential confounders as previously described \((p\) value to test for the interaction term was 0.03). Figure 1 shows the analysis using the Kaplan-Meier curves of the times to restenosis for patients with ACE gene polymorphism, and Figure 2A/2B shows the same for patients with ACE gene polymorphism stratified by AGT-T174M gene polymorphism. The curve pattern was reversed according to ACE gene polymorphism in subgroup with AGT-M174 allele, relative to that in subgroup with AGT-T174 homozygotes.

**DISCUSSION**

Post-PTCA restenosis is the consequence of repair and growth after vessel injury by balloon catheters. The process consists of neointimal hyperplasia, smooth muscle cell proliferation and excessive connective tissue accumulation due to responses to various cytokines. Evidence suggests that RAS and polymorphisms of component genes are involved in the process.\(^{23,24}\) Because the mechanisms of the restenosis process are polygenic and multifactorial, it is important to analyze globally the contribution (including gene-gene interactions) of predisposing alleles to this process. However, this strategy has been rarely studied.\(^{14,25,26}\) Therefore, eight gene polymorphisms involved in the different steps of the RAS enzymatic cascade were analyzed in this study. To our knowledge, this is the most systematic approach to the study of post-PTCA restenosis in relation to gene polymorphisms of RAS.

The genotype frequencies of AGT-T174M, AGT-5'-A-20C and ACE I/D gene polymorphisms in this study are similar to those in other populations.\(^{5,10,11,27,28}\) The frequencies of AGT-T235 and AGT-5'/c162-A(-6) homozygotes in our patient population are higher than those reported for Caucasian populations\(^{6,7,28}\) but similar to those reported for Japanese or other Chinese populations.\(^{27,29,30}\) The frequency of AT1R-C1166 allele is higher than in Caucasian populations and similar to Japanese populations.\(^{31,32}\) The presence of AGT-5'-G-152A and G-217A gene polymorphisms has been noted only in our population so far, and functional studies of these genotypes are ongoing. In this study, we found that the ACE D allele increased the risk of restenosis after PTCA in Chinese patients. Based on a previous study by Rigat et al.,\(^{33}\) ACE D allele is positively associated with the plasma concentration of ACE. Increased plasma ACE may result in increased angiotensin II generation, which stimulates the release of several cytokines such as platelet-derived growth factor and fibroblast-derived growth factor that are potent stimulants for smooth muscle cell proliferation.

**Table 3. Interactions between ACE D/I and AGT-T174M gene polymorphisms**

<table>
<thead>
<tr>
<th>AGT*-T174M</th>
<th>ACE†</th>
<th>n</th>
<th>Yes</th>
<th>No</th>
<th>(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>T/T</td>
<td>D/D</td>
<td>62</td>
<td>47</td>
<td>15</td>
<td>(76)</td>
</tr>
<tr>
<td></td>
<td>D/I</td>
<td>119</td>
<td>67</td>
<td>52</td>
<td>(56)</td>
</tr>
<tr>
<td></td>
<td>I/I</td>
<td>44</td>
<td>20</td>
<td>24</td>
<td>(45)</td>
</tr>
<tr>
<td>T/M + M/M</td>
<td>D/D</td>
<td>6</td>
<td>2</td>
<td>4</td>
<td>(33)</td>
</tr>
<tr>
<td></td>
<td>D/I</td>
<td>30</td>
<td>14</td>
<td>16</td>
<td>(47)</td>
</tr>
<tr>
<td></td>
<td>I/I</td>
<td>14</td>
<td>8</td>
<td>6</td>
<td>(57)</td>
</tr>
</tbody>
</table>

\(*AGT, angiotensinogen\)

\(†ACE D/I, angiotensin-converting enzyme deletion/insertion\)

\(**Wald test for logistic regression**

\(**p**\) value to test for the interaction term = 0.03
proliferation and growth. This notion was also supported by the findings that ACE inhibitors were effective in retarding, preventing, or reversing restenosis. Another interesting finding in this study is that the effect of ACE D allele on restenosis was modified by the presence of AGT-M174 allele. Approximately 19% (25/285) of individuals in our population possessed AGT-M174 allele. Their trend for restenosis with increased ACE D allele was reversed by AGT-M174 allele. Although no significant phenotype with AGT-M174 allele was noted previously, AGT-M174 allele may act as a regulator by an unknown mechanism. The gene-gene interactions between ACE D and AT1R-C1166 homozygotes have been described. An increased risk for CAD was noted among individuals with D/D + C/C genotypes. However, a number of statistical tests used in this study should be reconsidered, and further confirmation of this relationship and its mechanism are definitely required.

In conclusion, we find that ACE D allele increases the risk of post-PTCA restenosis, and this risk is modified by the coexistence of AGT-M174 allele in Chinese patients. In the post-genome era, gene-gene interactions have become a potential subject because more and more genes are identified, and development of microarray results in a global view of gene expression. Understanding these interactions can help us to clarify the genotype-phenotype associations, and may also explain why the results obtained in previous studies were conflicting.

Study Limitations

For reasons of ethics in clinical practice, we only performed follow-up angiography in patients with symptoms and positive noninvasive stress tests. Therefore, the restenosis rate in this study could not represent that of the general population.

We also didn’t collect quantitative coronary angiography (QCA) data. From studies of intravascular ultrasonography (IVUS), we know that QCA is still limited to quantitating the “true” extent or distribution of coronary lesions or identifying vessel wall pathology. IVUS

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**Figures 1.** Kaplan-Meier curves for post-PTCA restenosis in relation to ACE gene polymorphism. Patients with D/D genotype have the highest risk of restenosis (p value test for trend = 0.009).

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**Figures 2A/2B.** Kaplan-Meier curves for post-PTCA restenosis in relation to ACE gene polymorphism stratified by AGT-T174M gene polymorphism. The curve pattern is similar to that of ACE gene polymorphism in patients with AGT-T174 homozygotes (2A), but the reverse of that in patients with AGT-M174 allele (2B) (p value to test for the interaction term = 0.03).
provides a unique insight into the dynamic changes that occur within the vessel wall after intervention. The ethnic background is also an important confounder. The lack of different races in the study population may limit the generalizability of these results.

ACKNOWLEDGEMENTS

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REFERENCES


腎素-血管緊縮素系統之 DNA 多型性對冠狀動脈汽球擴張術後再狹窄之影響

陳國慶1 許寬立2 黃瑞仁2 曾春典2 曾淵如2 江福田2
台北縣 亞東紀念醫院 心臟內科1
台北市 臺大醫院 心臟內科2

背景 研究顯示腎素-血管緊縮素系統組成基因的多型性可以調控冠狀動脈汽球擴張術後再狹窄的過程，本研究同時探討此系統中不同階段的八種基因多型性對冠狀動脈汽球擴張術後再狹窄的影響。希望藉此對再狹窄的機轉有更進一步的了解。

方法 收集 290 位接受成功汽球擴張術的病人，若病人在追蹤過程中有一次發生意絞痛且非侵襲性檢查顯示心肌缺氧，則接受心導管檢查確定有無再狹窄。再狹窄定義為接受汽球擴張術的部位內徑超過百分之五十狹窄。

結果 在八種基因多型性中只有 ACE D allele 在單變項 (D/D 72.5%, D/I 54.4%, I/I 47.5%, p value test for trend < 0.01) 及多變項 (p value test for trend < 0.01) 分析對再狹窄有顯著影響。本研究特別發現 ACE D allele 對再狹窄的效果會受 AGT-M174 allele 的影響而產生相反的結果 (p value to test for the interaction term = 0.03)。

結論 結果顯示在國人 ACE D allele 會增加冠狀動脈汽球擴張術後的再狹窄且此效果會受 AGT-M174 allele 的影響而改變，在後基因體時代，探討基因與基因的交互作用也愈來愈重要。本研究嘗試以此一觀點解釋為何在先前眾多的研究中會有不同的結果。

關鍵詞：腎素-血管緊縮素系統、多型性、冠狀動脈汽球擴張術、再狹窄。