




Association study of matrix metalloproteinase 3 5A/6A polymorphism with in-stent restenosis after percutaneous coronary interventions in a Han Chinese population

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Abstract

Objective: We aimed to investigate the association between the 5A/6A promoter polymorphism in the matrix metalloproteinase 3 (*MMP3*) gene and in-stent restenosis (ISR) in a regional Chinese population.

Methods: A total of 818 patients who underwent primary implantation of drug-eluting stents were enrolled and received a 6-month follow-up angiography and DNA genotyping of the 5A/6A polymorphism.

Results: ISR was found in 36.9% of all patients (302 ISR vs. 516 no ISR). The genotype proportion of 6A/6A was significantly increased in ISRs (74.2% ISR vs. 66.8% no ISR), whereas the allele frequency of 5A was significantly decreased in ISR patients (25.8%) compared with controls who did not undergo ISR (33.1%).

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Conclusions: Our data indicate that the *MMP3* 6A6A genotype is a genetic susceptibility factor for ISR after coronary stent placement, but the 5A allele can lower the risk for patients within 6 months after stenting. Therefore, genotyping 5A/6A in the *MMP3* promoter is suggested for patients who undergo coronary stent implantation.

Keywords

Percutaneous coronary intervention, in-stent restenosis, extracellular matrix, matrix metalloproteinase-3, 5A/6A promoter polymorphism, Han Chinese population, drug-eluting stent

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Introduction

Cardiovascular disease is a leading cause of global years of life lost. The number of deaths resulting from cardiovascular disease increased by 41% between 1990 and 2013 (from 12.3 million in 1990 to 17.3 million in 2013).¹ Atherosclerosis is the major pathogenesis causing coronary artery disease (CAD). Percutaneous coronary intervention (PCI) with stent implantation is a well-established treatment for coronary atherosclerosis.^{2,3} The major drawback of PCI is restenosis, which is defined as the renarrowing of the vessel lumen to >50% occlusion, usually within 3–6 months after the intervention.⁴ Compared with balloon angioplasty, using intra-coronary stents can significantly reduce the incidence of restenosis.^{5,6} However, 20–40% of such patients with bare-metal stents (BMSs) will undergo a novel pathobiologic process, in-stent restenosis (ISR).⁷ Patients with ISRs are likely to have severe symptoms and high rates of myocardial infarction (MI).⁸ In recent years, drug-eluting stents (DESs) have been widely used in patients to decrease the chances of ISR to <20% after PCI,^{9–11} but even this number is still significant.

The process of ISR is complex and not fully elucidated, involving clinical,

biological, genetic, lesion-related, and procedural risk factors.¹² Histologically, ISR generally results from neointimal (NI) formation.¹³ The causative mechanisms are thought to include inflammation, proliferation, and matrix remodeling. The inflammatory response evoked by vascular damage is the main contributor to restenosis. Intra-coronary stents inevitably cause mechanical injury and elicit a wound healing process to the arterial wall.¹⁴ Smooth muscle cell (SMC) proliferation and migration and excessive extracellular matrix (ECM), prompted by intense inflammatory responses in the artery, comprise severe NI formation.^{15,16}

The ECM substrate breakdown and accumulation has an important role in ISR development.^{17,18} The amount of ECM is regulated by matrix metalloproteinases (MMPs, especially stromelysin) and their endogenous inhibitors (tissue inhibitor of metalloproteinases, TIMPs). MMP3 (stromelysin-1), a key member of the MMP family with a broad catalyzing specificity, is particularly involved in the degradation of ECM components.¹⁹ Thus, MMP3 is suggested to be a crucial candidate for preventing ISR formation. Atherosclerotic plaques were found to contain increased MMP3 expression and matrix degrading activity,²⁰ and antisense

oligonucleotides to *MMP3* mRNA can inhibit vascular SMC migration and proliferation.²¹

In the *MMP3* promoter, a common functional variant (rs3025039) has been reported, in which one allele has a run of six adenosines (6A), while the other has only five (5A).²² *MMP3* promoters containing the 5A allele have approximately 50% higher activity compared with those with the 6A allele because a putative transcriptional repressor protein preferentially binds to the promoter containing the 6A sequence and reduces gene expression.^{23,24} Various studies have demonstrated the association between the 5A/6A *MMP3* polymorphism and ISR after PCI, but there is some debate in the field.^{25–28} In this study, we aimed to investigate the allele frequency of the 5A/6A polymorphisms and estimate their association with the formation of ISR for clinical practice using a regional Han Chinese population.

Materials and methods

Patients and sampling

We enrolled 818 patients who underwent successful coronary artery DES implantation at our hospital (Tianjin Chest Hospital, Tianjin, People's Republic of China) from June 2011 to January 2015. The exclusion criteria are as follows: acute MI with non-target vessel lesion (non-TVL), severe liver and kidney dysfunction, coagulation dysfunction, rheumatic diseases, tumor, recent infection with chronic inflammation, or any other diseases and intervention factors that influence the observation index. Among the 818 patients, sirolimus-eluting stents (Cypher stent, Cordis, Miami Lakes, FL, USA or Firebird stent, MicroPort, Shanghai, China) were used in 109 (13.3%) patients, paclitaxel-eluting stents (Taxus stent, Boston Scientific, San Diego, CA, USA)

were used in 74 (9.0%) patients, and everolimus-eluting stents (Promus stent, Boston Scientific, San Diego, CA, USA) were used for the remaining 635 (77.7%) patients. After PCI, aspirin (100 mg/day) and clopidogrel (75 mg/day) therapy was continued for at least 1 year. All subjects underwent a scheduled 6-month follow-up angiography, and written informed consent was obtained for the intervention. Whole blood samples were collected in 2-mL Vacutainer® EDTA K2 tubes. This study was approved by the Ethics Committee of the Tianjin Chest Hospital, and experiments were conducted in accordance with the approved guidelines.

Coronary angiography

Coronary artery angiography was performed at the same angiography position as the previously targeted angiography for the selected PCI. Assisted by a computer evaluation system for stenosis and restenosis, at least two experienced cardiovascular surgeons were involved in determining whether the target vessels experienced restenosis. The diagnostic standard for ISR was defined as a lumen stenosis exceeding 50% at the site of the stent, inside and at both of its ends within a 5-mm range. Based on the degree of restenosis and other vascular conditions, appropriate treatment was performed.

DNA extraction and genotyping

DNA was extracted and purified from circulating lymphocytes using a QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany), in accordance with the manufacturer's instructions. DNA was quantified using a 1.5 µL DNA sample in solution and a NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). DNA was diluted to a concentration range of 2–5 ng/µL. The target region (129/130 bp fragment) of the *MMP3* promoter was

amplified using the following PCR primers: 5'-GGTTCTCCATTCCCTTTGATGGGGG GAAAAG-3' and 5'-CTTCCTGGAAT TCACATCGAAACCACT-3'. The PCR mixture (25 μ L) contained 5.0 μ L 5 \times PCR buffer, 1.0 μ L of 10 μ M primer mix, 0.5 μ L of 10mM dNTPs, 0.3 μ L (3 U) of *Taq* DNA polymerase, 1 μ L of DNA template, and sterile water. PCR was performed with denaturation at 95°C for 5 minutes, 35 cycles at 95°C for 30 seconds, 63°C for 30 seconds, and 72°C for 1 minute, followed by an additional extension at 72°C for 5 minutes. The DNA restriction enzyme *Tth111I* (Takara, Shiga, Japan) was used to recognize and cut the amplicon sequence of 5'-GACNNN GTC-3' into two fragments of 97 and 33 bp, which is contained by the allele 5A (130 bp) but not 6A (129 bp). After digestion with *Tth111I*, the products were analyzed using 3% agarose gel electrophoresis.

Serum MMP3 level detection

Serum MMP3 protein levels were determined using a commercial enzyme-linked immunoassay (ELISA) kit (MMP3 Human ELISA Kit, Invitrogen, Carlsbad, CA, USA), in accordance with the manufacturer's protocol.

Statistical analysis

Statistical analyses were performed using SPSS 19.0 software (IBM Corp., Armonk, NY, USA). The mean and standard deviation (mean \pm SD) were computed for continuous data. The Hardy-Weinberg Equilibrium (HWE) was assured using a Chi-squared (χ^2) analysis. Analysis of variance (ANOVA) and χ^2 tests were performed for comparison analysis. A stepwise logistic regression was performed for all variables to identify independent predictors. The odds ratio (OR) and 95% confidence intervals (CIs) were estimated for the risk allele. All statistical analyses were conducted at the significance level of $\alpha=0.05$, and the

Bonferroni correction was applied for multiple tests.

Results

Clinical characteristics

Our study included 818 patients with 1037 lesions, with a mean age of 59.1 \pm 10.5 years (range, 32–81 years), who received DES and underwent 6-month follow-up angiography and DNA genotyping. Among these patients, 23.3% were female, 59.8% were current smokers, 59.5% had hypertension, 20.4% had diabetes, 30.4% had hyperlipidemia, 39.4% had unstable angina, 91.7% had acute coronary syndrome (ACS), 54.2% had a previous MI, and 62.2% had multi-vessel CAD. The angiographic and clinical characteristics of the patients are listed in Table 1 and Table 2, respectively. The clinical outcomes of the 6-month follow-up included 83 (10.1%) MI and 134 (16.4%) target lesion revascularization (TLR), and there were no deaths. ISR formation was found in 302 (36.9%) patients (ISR group). The differences in sex, body mass index (BMI), smoking, drinking, stable angina, ACS, MI, left ventricular ejection fraction, multi-vessel disease,

Table 1. Angiographic and PCI characteristics.

Total patients, n	818
Treated vessel, n	874
Left anterior descending branch, n (%)	587 (67.2)
Left circumflex branch, n (%)	125 (14.3)
Right coronary artery, n (%)	162 (18.5)
Stenosis rate (%), mean (SD)	91.5 (6.7)
Lesions treated/Stent, n	1037
Lesion types treated, n (%)	
A	225 (67.2)
B	595 (14.3)
C	217 (18.5)
Stent length (mm), mean (SD)	24.5 (12.3)
Stent diameter (mm), mean (SD)	3.1 (0.5)

Table 2. Clinical characteristics of patients in the in-stent restenosis (ISR) and no ISR groups.

	ISR (n = 302)	No ISR (n = 516)	P value (Total, n = 818)
Age (year)	59.3 ± 11.5	59.0 ± 9.9	0.628 (59.1 ± 10.5)
Female (%)	16.9	26.9	0.003* (23.2)
Body mass index	27.5 ± 4.2	26.1 ± 3.4	0.032* (26.6 ± 3.7)
Hypertension (%/years)	61.2/6.9 ± 9.0	58.5/6.5 ± 8.8	0.454 (59.5)/0.608 (6.7 ± 8.9)
Diabetes mellitus (%/years)	16.7/1.1 ± 2.9	22.6/1.3 ± 3.5	0.055 (20.4)/0.362 (1.2 ± 3.3)
Hyperlipidemia (%/years)	27.6/0.5 ± 0.9	32.1/0.4 ± 0.8	0.201 (30.4)/0.753 (0.4 ± 0.9)
Current smoker (%/years)	67.3/17.5 ± 14.1	55.3/15.9 ± 15.3	0.001* (59.8)/ 0.155 (16.5 ± 14.9)
Alcoholic (%/years)	30.2/4.4 ± 7.8	23.5/3.9 ± 8.4	0.015* (26.0)/0.442 (4.1 ± 8.2)
Stable/Unstable angina (%)	2.7/36.9	7.8/40.9	0.042* (5.9)/0.293 (39.4)
Acute coronary syndrome (%)	96.7	88.8	3.69 × 10 ⁻⁵ * (91.7)
Myocardial infarction (Previous/follow-up, %)	59.7/18.4	50.9/5.5	0.018* (54.2)/ 2.15 × 10 ⁻⁸ * (10.1)
Major adverse cardiac events (%)	1.5	2.0	0.775 (1.8)
Left ventricular ejection fraction (Previous/follow-up, %)	61.9 ± 8.3/ 58.4 ± 9.8	64.4 ± 8.4/ 61.7 ± 9.2	0.003* (63.6 ± 8.4)/ 2.05 × 10 ⁻⁵ * (60.5 ± 9.6)
Number of narrowed coronary arteries			0.181/0.005*
1 (Previous/follow-up, %)	33.8/22.5	40.1/32.8	(37.8/29.0)
2 (Previous/follow-up, %)	32.4/32.8	28.3/27.7	(29.8/29.6)
3 (Previous/follow-up, %)	33.8/44.7	31.6/39.5	(32.4/41.4)
Target vessel revascularization (%)	17.4	5.3	6.00 × 10 ⁻⁷ * (9.8)
Target lesion revascularization (%)	42.3	1.2	3.90 × 10 ⁻²¹ * (16.4)

*Significant *P* value.

target vessel revascularization (TVR), and TLR were significant between the ISR and no ISR groups (all *P* < 0.05, see Table 2).

MMP3 5A/6A polymorphisms

As shown in Figure 1, polymorphism rs3025039 genotypes were detected using electrophoresis, including three fragments of 129 bp/6A, 97 bp/5A, and 33 bp/5A. Overall, 569 patients were 6A6A homozygous, 244 patients were 5A6A heterozygous, and five patients were 5A5A homozygous. The distribution of genotypes was consistent with HWE. Genotype and allele frequency of the 5A/6A polymorphism in the 302 ISR patients and 516 no ISR controls are shown in Table 3. Significant differences were found in the genotype (5A6A: *P* = 0.040; 6A6A: *P* = 0.033) and

allele (5A: *P* = 0.033) frequency distributions between the ISR and no ISR groups. The genotype proportion of 5A6A was significantly decreased (*P* = 0.040, OR: 0.788, 95% CI: 0.626–0.991) in the ISR group compared with the no ISR group. However, 6A6A was significantly increased (*P* = 0.033, OR: 1.109, 95% CI: 1.014–1.214), and 5A5A was slightly decreased (not significant) in the ISR group compared with the no ISR group. Allele 5A occurred in 25.8% of ISR patients vs. 33.1% of no ISR patients (*P* = 0.033, OR: 0.779, 95% CI: 0.621–0.978), and the patient proportions of 6A were almost the same between these two groups. In the above analyses, the significance level (α = 0.05) was not corrected for multiple tests. After Bonferroni correction for genotype (α' = 0.017) frequency comparisons, no significance was found.

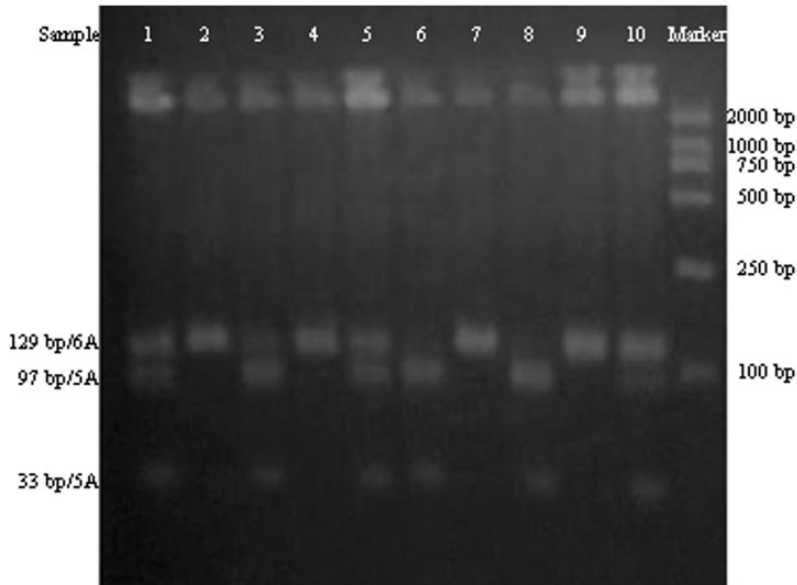


Figure 1. Electrophoresis profile of DNA amplicons digested by the restriction enzyme *TthI111*. The size of the 6A amplicon is 129 bp. The 5A amplicon is 130 bp and can be digested into two fragments with sizes of 97 and 33 bp. The genotypes of samples 2, 4, 7, and 9 are 6A/6A, samples 1, 3, 5, and 10 are 6A/5A, and samples 6 and 8 are 5A/5A.

Table 3. Genotype and allele frequency distributions of the 5A/6A polymorphism between the in-stent restenosis (ISR) and no ISR groups.

	ISR (n = 302, %)	No ISR (n = 516, %)	P value	Odds ratios (95% confidence interval)
Genotype ^a			0.068	
5A5A	1 (0.3)	4 (0.8)	0.657	0.427 (0.047–3.804)
5A6A	77 (25.5)	167 (32.4)	0.040*	0.788 (0.626–0.991)
6A6A	224 (74.2)	345 (66.8)	0.033*	1.109 (1.014–1.214)
Allele				
5A	78 (25.8)	171 (33.1)	0.033*	0.779 (0.621–0.978)
6A	301 (99.7)	512 (99.2)	0.657	1.004 (0.994–1.015)

*Significant *P* value.

Serum MMP3 levels

Serum MMP3 content in the ISR group was slightly lower than that of the no ISR group, but the difference was not significant (ISR: 12.8 ± 2.5 vs. no ISR: 13.2 ± 3.4). No association was found between the 5A/6A

polymorphism and serum MMP3 levels (data not shown).

Discussion

The development of ISR is a complicated pathophysiological process that is caused

by various mechanisms. Currently, two main acknowledged elements are vascular remodeling and intimal hyperplasia.²⁹ Histopathological studies show that ECM components account for 89% of the total components at the restenosis location.^{18,30} As a key member of the MMPs, MMP3 can degrade many ECM components. Several studies suggest that *MMP3* expression may be lower in the vascular wall of patients with the wild-type (6A6A) *MMP3* promoter genotype, which leads to excessive deposition of ECM and the acceleration of atherosclerosis.³¹ Therefore, MMP3 expression is suggested to have an important role in ISR formation.¹⁹ In the current study, we found a weak association between the *MMP3* 5A/6A polymorphism and 6-month follow-up in ISR formation. Our data provide valuable insight to predict ISR occurrence in CAD patients after stent placement to closely follow-up and provide appropriate anti-restenosis treatment. The occurrence of ISR was slightly increased in patients with the 6A6A genotype compared with the other genotypes, but this situation was reversed in patients with the 5A allele. Our findings are in accordance with previous reports that the 6A sequence reduces *MMP3* gene expression, whereas the 5A allele results in increased promoter activity, making 6A6A an independent predictor of ISR.^{23,27} Very few 5A5A (0.6%) genotypes were detected in our CAD patients. Based on population genetic data from Han Chinese (CHB) from 1000 Genomes phase 3, the frequency of 5A5A is 6.7%. This difference suggests that the 5A allele has a prevention role. Our study is similar to a previous study by Hoppmann et al.²⁶ that enrolled over 3000 study subjects and did not find a correlation with such a polymorphism. Statistical significance is borderline in the current study and probably would be negative if more subjects were included.

The mechanism of ISR is complex and involves many predictive clinical, biological,

genetic, and procedure-related risk factors. Our data identified several confounding factors, including sex, BMI, smoking, alcoholism, stable angina, ACS, MI, TVR, TLR, and multi-vessel disease. A high ISR incidence rate (36.9%) was observed in our participants, while most studies show a rate of around 10%.³² Our hospital is one of the largest Centers of Cardiovascular Diseases in northern China, and the admitted patients commonly have more complex conditions than those recruited in previous studies. A high smoking rate is the most outstanding characteristic in the Chinese cohort, especially in males. Our enrolled patients are mainly overweight (BMI > 25.0) male patients (76.8%) with a smoking habit (59.8%), which may account for part of this high ISR rate. Moreover, operator differences (under-expansion of the stent) and restrictiveness of the enrollment criteria for the ISR (50% lumen stenosis) can also influence the results. In the present study, 96.7% of ISR and 88.8% of no ISR patients had accompanying ACS. As reported, binary restenosis was observed in 34% of patients with ACS who received a drug-eluting stent implant at 6 months,³³ which is in accordance with the high ISR frequency (36.9%) among our subjects. ACS may be associated with a high ISR rate at 6 months.

The imbalance between ECM synthesis and degradation is mainly involved in the development of restenosis.³⁴ Recently, a large number of studies have shown that this dynamic balance is maintained by the interactions between MMPs and TIMPs.³⁵ MMP3 is a key member of the MMP family and degrades collagen II, III, IV, V, IX, X, and XI, proteoglycan, laminin, fibronectin, gelatin, elastin, and other ECM proteins. Additionally, it activates MMP-1, -8, -9, and -13 precursors, as well as its own pro-MMP3, to induce a cascade effect on ECM degradation. Regardless, the genetic influence of the functional 5A/6A polymorphism on ISR hovers just around at the

statistically significant level of $\alpha = 0.05$. A variety of MMPs are known to participate in the ECM degradation process, such as MMP-1, -2, -3, -8, -9, -13, -14, and -16. Thus, large-scale genetic screening of those genes may yield a more convincing predictor for ISR after stenting.

Additionally, we explored the relationship between the 5A/6A polymorphism and restenosis by detecting the change in the serum MMP3 content. Our results demonstrated that the content of serum MMP3 in ISR patients was slightly higher than that in no ISR patients, but the difference was not significant between these two groups. No association was found for either genotype or allele frequencies with serum MMP3 content. The reasons for this could include: 1) basic research focused on the effect of polymorphisms in the *MMP3* promoter on gene expression have occurred in isolation, while MMP3 levels *in vivo* are controlled by multiple elements, in addition to regulation at the transcriptional level; and 2) the change in MMP3 content a local plaque may not be enough to cause a change in MMP3 levels in the blood.³⁶ Thus, we propose that further association tests be conducted regionally at the stenting artery. Additionally, an increased concentration of plasma active MMP3 isoforms was reported to be independently associated with ISR.³⁷ MMP3 activity and its correlation with the polymorphism or ISR occurrence should also be evaluated in a future study.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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