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$p27^{kip1}$–838C>A Single Nucleotide Polymorphism Is Associated With Restenosis Risk After Coronary Stenting and Modulates $p27^{kip1}$ Promoter Activity

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Background—The cyclin-dependent kinase inhibitor $p27^{kip1}$ is a key regulator of smooth muscle cell and leukocyte proliferation in vascular disease, including in-stent restenosis. We therefore hypothesized that common genetic variations or single nucleotide polymorphisms in $p27^{kip1}$ may serve as a useful tool in risk stratification for in-stent restenosis.

Methods and Results—Three single nucleotide polymorphisms concerning the *$p27^{kip1}$* gene (−838C>A, rs36228499; −79C>T, rs34330; +326G>T, rs2066827) were determined in a cohort of 715 patients undergoing coronary angioplasty and stent placement. We discovered that the $p27^{kip1}$-838C>A single nucleotide polymorphism is associated with clinical in-stent restenosis; the −838AA genotype decreases the risk of target vessel revascularization (hazard ratio, 0.28; 95% confidence interval, 0.10 to 0.77). This finding was replicated in another cohort study of 2309 patients (hazard ratio, 0.61; 95% confidence interval, 0.40 to 0.93). No association was detected between this end point and the $p27^{kip1}$-79C>T and +326G>T single nucleotide polymorphisms. We subsequently studied the functional importance of the −838C>A single nucleotide polymorphism and detected a 20-fold increased basal $p27^{kip1}$ transcriptional activity of the −838A allele containing promoter.

Conclusions—Patients with the $p27^{kip1}$-838AA genotype have a decreased risk of in-stent restenosis corresponding with enhanced promoter activity of the −838A allele of this cell-cycle inhibitor, which may explain decreased smooth muscle cell proliferation. **(*Circulation*. 2009;120:669-676.)**

Key Words: cardiovascular diseases ■ muscle, smooth ■ polymorphism, single nucleotide ■ Cyclin-Dependent Kinase Inhibitor p27 ■ restenosis

Despite technological and pharmacological advances, the major limitation of percutaneous coronary intervention (PCI) remains (in-stent) restenosis, requiring repeat revascularization procedures.[1] Although drug-eluting stents have been shown to significantly reduce restenosis, these stents may not be effective in all lesions and patient subsets, have a long-term risk of late and very late stent thrombosis, and require prolonged uninterrupted dual antiplatelet therapy.[2,3] The arterial wall injury induced by balloon inflation and stent placement during PCI is followed by a cascade of events, including platelet and leukocyte activation and smooth muscle cell (SMC) proliferation, ultimately resulting in restenosis.[4,5] Several lines of evidence indicate that genetic factors may explain the excessive risk of restenosis independently of conventional clinical and procedural parameters.[6,7] Consequently, additional genetic tests to identify patients at increased risk of restenosis may lead to improved risk stratification and eventually individual, patient-tailored therapy. This concept has resulted in genetic studies designed to identify single nucleotide polymorphisms (SNPs) in genes associated with restenosis.[8] So far, these studies have resulted in the identification of SNPs in genes related predominantly to inflammation, leukocyte recruitment, the renin-angiotensin system, and platelet activation.[9–11] We hypothesized that SNPs in genes critical to cell-cycle regulation are associated with restenosis.

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Clinical Perspective on p 676

Various studies have provided cumulative evidence that $p27^{kip1}$, a cyclin-dependent kinase inhibitor, is a key regulator of vascular SMC and leukocyte proliferation in vascular disease.[12,13] Progression through the cell cycle depends on the sequential activation of several cyclin-dependent kinases, which requires their interaction with regulatory subunits called cyclins. In resting cells, cyclin–cyclin-dependent kinase complexes are inhibited by the reversible association with cyclin-dependent kinase inhibitory proteins of the Ink4 family and Cip/Kip family, eg, $p27^{kip1}$. Studies using both animal models and human vascular tissue revealed a central role of $p27^{kip1}$ in the pathogenesis of vascular diseases.[14] Mice deficient in both $p27^{kip1}$ and apolipoprotein E display increased arterial cell proliferation and accelerated atherogenesis compared with apolipoprotein E–null mice with an intact *p27kip1* gene.[15] Furthermore, reconstitution of sublethally irradiated apolipoprotein E–null mice with $p27^{kip1}$-deficient bone marrow was sufficient to enhance arterial macrophage proliferation and atherosclerosis.[16] Remarkably, a significantly lower level of $p27^{kip1}$ has been detected in primary atherosclerotic and restenotic tissue compared with nondiseased arterial tissue, and $p27^{kip1}$ expression levels and proliferation rates of vascular SMCs and leukocytes are inversely correlated in human atheroma.[17,18] Interestingly, in a recent study, Gonzalez et al[19] describe that the $p27^{kip1}$-838C>A SNP is associated with an increased risk of acute myocardial infarction.

In the present study, we aimed to detect $p27^{kip1}$ polymorphisms that predict human clinical restenosis after stent placement. We report that the homozygous AA genotype of the common genetic variation −838C>A located in the promoter of the *p27kip1* gene is associated with reduced angiographic restenosis and target vessel revascularization (TVR) risk in 2 independent cohort studies. Next, we evaluated the function of this genetic variation on the promoter activity of $p27^{kip1}$, and in line with the clinical data, we detected increased promoter activity of the cell-cycle inhibitor $p27^{kip1}$ when the protective −838A genetic variation was present.

Methods

Study Populations

In the Genetic Risk Factors for In-Stent Hyperplasia Study Amsterdam (GEISHA), patients were included in a single-center, prospective observational cohort; the inclusion period lasted from 1997 until 2001. The study was approved by the local research and ethics committee and conforms to the Declaration of Helsinki. All patients gave written informed consent for follow-up angiograms and were included after successful bare metal stent placement in a native coronary artery. Indications for stent placement were bailout or unsatisfactory results after balloon angioplasty alone, chronic total occlusion, ostial disease, and restenosis after balloon angioplasty. Patients with in-stent restenosis and complex lesions such as saphenous vein graft lesions, bifurcated lesions, lesions >25 mm, reference vessel diameter <2.5 mm, and primary PCI for myocardial infarction were excluded. Statin therapy was recorded at the time of the procedure and at follow-up. Patients were treated with 100 mg aspirin and 250 mg ticlopidine BID or 75 mg clopidogrel daily for 1 month after PCI and 100 mg of aspirin thereafter. Blood was collected from the participants, and genomic DNA was isolated from leukocytes.

The Genetic Determinants of Restenosis Project (GENDER) population has been described previously.[11] In brief, GENDER is a multicenter follow-up study designed to assess the association between various gene polymorphisms and clinical restenosis. Patients eligible for inclusion in GENDER were treated successfully for stable angina, non–ST-elevation acute coronary syndromes, or silent ischemia by PCI in 4 of 13 referral centers for interventional cardiology in the Netherlands. Patients treated for acute ST-elevation myocardial infarction were excluded. Experienced operators, using a radial or femoral approach, performed standard angioplasty and bare metal stent placement. The inclusion period lasted from 1999 until 2001. The study protocol conforms to the Declaration of Helsinki and was approved by the medical ethics committees of the participating institutions. Written informed consent was obtained from each participant before the PCI procedure.

Follow-Up and Study End Points

Patients participating in GEISHA returned for follow-up angiography between 6 and 10 months after stent placement. Clinical follow-up at 1 year was obtained through written questionnaires, review of hospital records, chart review, and telephone contact with the patient, the referring cardiologist, the patient's general practitioner, or the patient's relatives. TVR was defined as revascularization of the stented segment or within 5-mm margins proximal or distal to the stent by either repeat PCI or coronary artery bypass grafting. The primary end points of this study were angiographic late luminal loss in minimal lumen diameter and binary in-stent restenosis (>50% diameter stenosis) at a 6- to 10-month follow-up. The secondary end point was the occurrence of major adverse cardiac events (death, nonfatal myocardial infarction, coronary artery bypass grafting, repeat PCI, and TVR).

In GENDER, follow-up lasted for 9 to 12 months or until a coronary event occurred. Clinical restenosis, defined as TVR by either PCI or coronary artery bypass graft surgery, was the primary end point because it is considered most relevant for clinical practice by regulatory agencies.

Quantitative Coronary Analysis

Quantitative coronary analysis was performed as described[20] offline on images obtained before and immediately after stent placement and at follow-up using a computerized quantitative analysis system (QCA-CMS, version 5.0, MEDIS, Leiden, the Netherlands).[21] All angiograms were analyzed by a local core laboratory. The angiography was performed in at least 2 projections after intracoronary injection of isosorbide dinitrate (0.2 mg). The tip of the 6F or 7F catheter filled with contrast was used for calibration. Minimal lumen diameter was measured at the narrowest point of the lesion or within the stent. Acute gain was defined as the difference between the minimal lumen diameter before and after PCI (in millimeters); late luminal loss was defined as the difference between the minimal lumen diameter at follow-up and after PCI (in millimeters). All continuous variables were calculated as the mean values of 2 orthogonal views using end-diastolic frames.

Genotyping

Three polymorphisms concerning the *p27kip1* gene (−838C>A, rs36228499; −79C>T, rs34330; +326G>T, rs2066827) were determined. The $p27^{kip1}$-838C>A SNP was genotyped using the Custom *Taq*Man Genotyping Assay (Applied Biosystems, Foster City, Calif). The nucleotide sequences of the primers and probes are available on request. Reactions were performed with LC480 (Roche Diagnostics, Pemzburg, Germany). The −79C>T and +326G>T SNPs were determined as described by Sauer and Gut[22] using a matrix-assisted laser desorption/ionization time-of-flight mass spectrometer from Bruker Daltonics (Billerica, Mass). For more details, see the online-only Data Supplement.

Table 1. SNP Frequencies: GEISHA

SNP	Genotype	n (%)	Minor Allele Frequency, %	*P*, HWE χ^2
+326G>T	GG	45 (7.0)		
(rs2066827)	GT	220 (34.2)	G=24.1	0.099
	TT	378 (58.8)		
−79C>T	CC	397 (58.6)		
(rs34330)	CT	239 (35.2)	T=23.8	0.45
	TT	42 (6.2)		
−838C>A	CC	209 (31.5)		
(rs36228499)	CA	331 (48.3)	A=45.3	0.51
	AA	145 (21.2)		

HWE indicates Hardy-Weinberg equilibrium.

Luciferase Assay

A fragment of the p27kip1 promoter (from −1020 to −12 bp relative to the translational start site) was cloned into the pGL3 basic vector (Promega, Madison, Wis) in front of the firefly luciferase gene. Two constructs were generated with either C or A at −838. The constructs were verified by sequencing and did not contain any other sequence variations. HEK293 cells were cultured in DMEM containing 10% FBS and were cotransfected with 1 μg promoter/luciferase construct and 0.1 μg pRL-TK *renilla* reporter vector (Promega) using the CalPhos mammalian transfection kit (Clontech Laboratories Inc, Mountain View, Calif) according to the manufacturer's instructions. The pRL-TK *renilla* reporter, which contains the complete thymidine kinase promoter, was used to correct for transfection efficiency and cell number. For optimal p27kip1 promoter activity, G1 arrest was induced by growth factor deprivation. Growth medium was removed 16 hours after transfection, and the cells were washed with PBS and incubated with serum-free DMEM containing 0.1% BSA. After 48 hours, luciferase activity was determined through the use of the dual-luciferase reporter assay system (Promega) according to the manufacturer's protocol.

Statistical Analysis

Analyses for possible deviations of the genotype distribution from that expected for a population in Hardy-Weinberg equilibrium were done with the χ^2 test. Data are given as mean (SD) or number (proportion). Continuous variables with a Gaussian distribution, as determined by the Shapiro-Wilks test, were compared by the Student's 2-tailed unpaired *t* test; categorical values were compared by the χ^2 or Fisher's exact test when appropriate. Continuous variables with a non-Gaussian distribution were compared by the Mann-Whitney *U* test. Cox proportional regression analysis was performed to determine the association between the SNP genotype and in-stent restenosis, PCI, or TVR. Variables depicted by univariate linear regression analysis to be predictive for in-stent restenosis, PCI, or TVR ($P<0.05$) and known predictive variables were entered into the model (control of confounding). Event-free survival curves were calculated by Kaplan-Meier analysis, and differences between groups were calculated with the log-rank statistic. Statistical analysis was performed with SPSS 16.0 for Windows (SPSS Inc, Chicago, Ill). A value of $P<0.05$ was considered to be significant. Because in all analyses there were no significant differences between the −838CC and −838CA genotypes, these 2 groups were combined for all *P* value calculations. We adjusted for multiple testing with the Bonferroni correction, multiplying the *P* value by the number of independent tests.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

Results

Baseline Characteristics

Of the 715 patients included in GEISHA, DNA was obtained from 688. Failure of genotyping for the p27kip1 SNPs −838C>A, −79C>T, and +326G>T was 1%, 1%, and 7%, respectively. All genotype distributions were in Hardy-Weinberg equilibrium ($P>0.05$; Table 1). The p27kip1-79C>T and +326G>T SNPs were not associated with the primary and secondary end points of the study (data not shown). Comparisons of the clinical and angiographic baseline characteristics among the −838C>A genotypes are listed in Tables 2 and 3. There were no differences observed between the 3 different −838C>A genotypic groups except for current smoking. There were significantly more smokers in the homozygous AA group compared with the other 2 groups.

Clinical and Angiographic Outcomes

Angiographic follow-up was performed in 598 of 688 cases; 90 patients either were lost to follow-up or refused follow-up angiography. The angiographic data are summarized in Table 4. Of the patients with angiographic follow-up, 105 (18%) developed in-stent restenosis. As shown in Table 4, the p27kip1-838AA genotype is associated with an increase in

Table 2. Baseline Clinical Characteristics: GEISHA

Variable	−838CC (n=209; 31%)	−838CA n=331 (48%)	−838AA (n=145; 21%)	*P*
Male, n (%)	155 (74)	258 (78)	114 (79)	0.65
Age, y	58±10	58±11	57±10	0.40
Hypertension, n (%)	78 (37)	107 (32)	53 (37)	0.62
Diabetes mellitus, n (%)	27 (13)	29 (9)	14 (10)	0.87
Current smoking, n (%)	72 (34)	127 (38)	67 (46)	0.044
Family history of coronary artery disease, n (%)	95 (45)	172 (52)	77 (53)	0.45
History of, n (%)				
Myocardial infarction	104 (50)	153 (46)	69 (48)	1.0
Coronary artery bypass grafting	8 (4)	6 (2)	3 (2)	1.0
PCI	54 (26)	78 (24)	30 (21)	0.38
Statin therapy, n (%)	109 (52)	153 (46)	61 (42)	0.19

*Mean±SD.

Table 3. Baseline Angiographic Characteristics: GEISHA

Variable	−838CC (n=209; 31%)	−838CA (n=331; 48%)	−838AA (n=145; 21%)	*P*
Artery treated, n (%)				0.67
Left anterior descending	89 (43)	154 (47)	70 (48)	
Right coronary artery	82 (39)	118 (36)	48 (33)	
Left circumflex	38 (18)	59 (18)	27 (19)	
Restenotic lesion, n (%)	33 (16)	39 (12)	18 (12)	0.89
Chronic total occlusion lesion, n (%)	72 (34)	108 (33)	48 (33)	1.0
Lesion length, mm*	14±7	14±8	13±6	0.43
Stent length, mm*	17±6	17±6	16±5	0.33
Diameter stenosis, %*				
Before stenting	76±20	75±20	76±18	0.81
After stenting	13±8	13±9	14±10	0.15
Reference diameter, mm*				
Before stenting	2.9±0.7	2.9±0.7	3.0±0.7	0.41
After stenting	3.4±0.5	3.3±0.5	3.4±0.5	0.39
Minimal lumen diameter, mm*				
Before stenting	0.7±0.6	0.7±0.6	0.7±0.6	0.88
After stenting	2.9±0.5	2.9±0.5	2.9±0.5	0.95
Acute gain, mm*	2.2±0.7	2.1±0.7	2.2±0.7	0.86

*Mean±SD.

mean minimal lumen diameter at follow-up (2.0 versus 1.8 mm in the combined CC and CA group; $P=0.029$); furthermore, mean late luminal loss and cumulative late luminal loss were significantly lower in the −838AA homozygotes (Table 4 and Figure 1). In contrast, although binary in-stent restenosis was lower in the AA group (15% versus 18% in the combined CC and CA group), this decrease was not significant ($P=0.42$).

Clinical follow-up was completed in all patients. The results are given in Table 5. One patient died during follow-up, and only 4 patients underwent coronary artery bypass grafting in the first year after stent placement. The incidence of repeat PCI (including both TVR and PCI of lesions other than those treated at the time of inclusion) was significantly decreased in patients with the −838AA genotype (6%) compared with the −838 CC and CA genotypes combined (20%; $P<0.001$). In addition, the occurrence of TVR also was significantly lower in patients with the −838AA genotype (3% versus 10% in the CC and CA combined group; $P=0.004$). After Bonferroni correction for multiple testing, these associations remained significant ($P=<0.001$ and $P=0.035$, respectively). To validate our findings, we analyzed the GENDER population for the $p27^{kip1}$-838C>A SNP. Characteristics of the GENDER cohort have previously been described in detail.[11] Briefly, a total of 3146 patients had complete follow-up (99%). Bare metal stents were used in 2309 patients (74%), whereas the rest of the population was treated with balloon angioplasty alone; we excluded these latter patients to better compare our data with the GEISHA population, which includes only patients treated with bare metal stents. The baseline characteristics of the stented GENDER population are shown in Table II of the online-only Data Supplement. In GENDER, the incidence of TVR was significantly lower in patients with the −838AA genotype (6% versus 10% in the CC and CA combined group; $P=0.016$; see Table III of the online-only Data Supplement), confirming our findings in GEISHA.

Although the $p27^{kip1}$-838C>A SNP is not significantly associated with binary in-stent restenosis, Kaplan-Meier estimates of in-stent restenosis-free survival in GEISHA comparing patients with the −838AA genotype with the CC and CA combined group show that restenosis-free survival is significantly higher in patients with the $p27^{kip1}$-838AA genotype than in patients with the −838CC and CA genotypes ($P=0.016$; Figure 2A). Furthermore, when clinical restenosis

Table 4. Angiographic Follow-Up: GEISHA

Variable	−838CC (n=178; 31%)	−838CA (n=291; 48%)	−838AA (n=129; 21%)	*P*
Late luminal loss, mm*	1.1±0.7	1.0±0.7	0.9±0.6	0.016
In-stent restenosis, n (%)	33 (19)	49 (18)	18 (15)	0.42
Diameter stenosis, %*	35±20	34±21	31±18	0.12
Reference vessel diameter, mm*	2.9±0.5	2.8±0.6	2.9±0.5	0.14
Minimal lumen diameter, mm*	1.9±0.7	1.8±0.7	2.0±0.6	0.029

*Mean±SD.

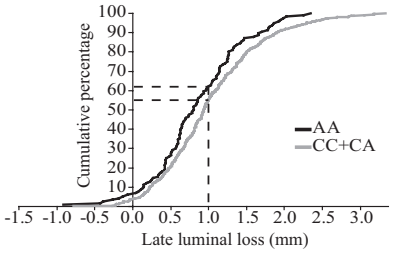

Figure 1. Cumulative frequency distribution of late luminal loss (mm) in patients with a p27kip1-838AA genotype vs the combined p27kip1-838 CA and CC genotypes.

was considered, the TVR-free survival in patients with the p27kip1-838AA genotype is significantly higher than in patients with the −838CC and CA genotypes ($P=0.005$; Figure 2B). In addition, the event-free survival of the secondary combined end point of major adverse cardiac events (death, nonfatal myocardial infarction, coronary artery bypass grafting, repeat PCI, and TVR) is significantly higher in patients with the −838AA genotype ($P=0.013$; Figure I of the online-only Data Supplement). In line with these data, Figure 2C shows that TVR-free survival in the GENDER population is significantly higher in patients with the p27kip1-838AA genotype than in patients with the −838CC and CA genotypes ($P=0.018$).

Multiple Regression Analyses

To explore the strength of the observed associations, we assayed whether these associations persisted after Cox proportional regression analysis including current smoking and other well-known predictive variables such as gender, hypertension, diabetes mellitus, use of statins, lesion length, and stent length (Table 6), showing a decreased risk of repeat PCI (hazard ratio [HR], 0.29; 95% confidence interval [CI], 0.15 to 0.58; $P<0.001$) and TVR (HR, 0.28; 95% CI, 0.10 to 0.77; $P=0.014$) for patients with the −838AA genotype. The risk of angiographic in-stent restenosis was not significantly decreased (HR, 0.74; 95% CI, 0.44 to 1.23; $P=0.24$). Of these, only the association with repeat PCI remained significant after Bonferroni correction for multiple testing ($P=0.004$). In line with published data, multiple regression analysis also identified gender, hypertension, and smoking as independent predictors for in-stent restenosis ($P=0.040$, $P=0.030$, and $P=0.017$, respectively). In our population, lack of statin treatment was a predictor for repeat PCI ($P=0.046$) and TVR ($P=0.001$) but not for in-stent restenosis ($P=0.40$).[23–25] To corroborate these findings, we performed the same multiple regression analysis on the GENDER population, showing a decreased risk of TVR (HR, 0.61; 95% CI, 0.40 to 0.93) for patients with the −838AA genotype (see Table IV of the online-only Data Supplement).

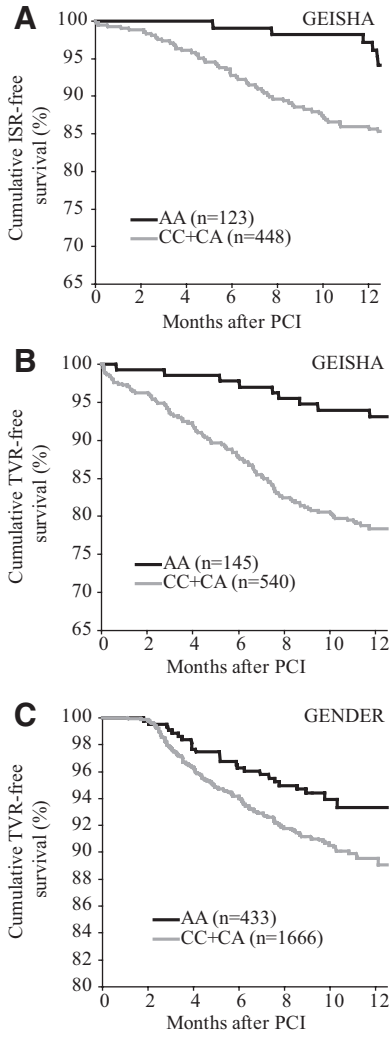

Figure 2. Kaplan-Meier estimates of event-free survival in patients with a p27kip1-838AA genotype vs the combined heterozygous and p27kip1-838CC genotypes. A, Kaplan-Meier curves of in-stent restenosis (ISR)–free survival in the GEISHA population. Log-rank $P=0.016$. B, Kaplan-Meier curves of TVR-free survival in the GEISHA population, Log-rank $P=0.005$. C, Kaplan-Meier curves of TVR-free survival in the GENDER population, Log-rank $P=0.018$.

Functional Effect on p27kip1 Promoter Activity

Because the −838C>A SNP is located in the promoter region of p27kip1 and because this variation is associated with

Table 5. Clinical Outcome: GEISHA

Variable	−838CC (n=209; 31%)	−838CA (n=331; 48%)	−838AA (n=145; 21%)	*P*
Death, n	0 (0)	1 (0)	0 (0)	1.0
Myocardial infarction, n (%)	6 (3)	4 (1)	4 (3)	0.50
Coronary artery bypass grafting, n (%)	2 (1)	2 (1)	0 (0)	0.58
Repeat PCI, n (%)	45 (22)	61 (18)	9 (6)	<0.001*
TVR, n (%)	19 (9)	34 (10)	4 (3)	0.004*

*Significant after Bonferroni correction for multiple testing.

Table 6. Cox Proportional Regression Analysis of Predictive p27^{kip1} −838 Genotype and Other Variables for the Occurrence of In-Stent Restenosis, PCI, and TVR: GEISHA

Characteristic	HR for ISR (95% CI)	*P*	HR for PCI (95% CI)	*P*	HR for TVR (95% CI)	*P*
p27^{kip1}-838C>A	0.74 (0.44–1.23)	0.24	0.29 (0.15–0.58)	<0.001*	0.28 (0.10–0.77)	0.014
Male gender	1.58 (1.02–2.46)	0.040	0.97 (0.62–1.52)	0.89	1.64 (0.92–2.95)	0.097
Hypertension	1.57 (1.05–2.37	0.030	0.93 (0.62–1.37)	0.70	0.79 (0.44–1.41)	0.42
Diabetes mellitus	0.93 (0.49–1.76)	0.82	0.94 (0.51–1.74)	0.84	1.30 (0.51–3.32)	0.58
Current smoking	1.73 (1.11–2.71)	0.017	1.14 (0.77–1.68)	0.52	1.49 (0.83–2.68)	0.18
Statin therapy at time of stenting	0.84 (0.55–1.27)	0.40	0.68 (0.47–0.99)	0.046	0.37 (0.20–0.67)	0.001
Lesion length	1.01 (0.98–1.04)	0.46	1.01 (0.98–1.03)	0.53	1.01 (0.99–1.04)	0.37
Stent length	1.04 (1.00–1.08)	0.088	1.02 (0.99–1.06)	0.23	1.04 (0.99–1.08)	0.14

ISR indicates in-stent restenosis.
*Significant after Bonferroni correction for multiple testing.

in-stent restenosis, we anticipated that the expression of p27^{kip1} is influenced by this SNP. Two constructs were generated in which part of the p27^{kip1} promoter (between −1020 and −12 bp relative to the translational start site) was cloned in front of the firefly luciferase coding sequence. These constructs contained either C or A at position −838. HEK293 cells were transiently transfected with these constructs, and the p27^{kip1} promoter activity was determined after growth factor deprivation by luciferase activity. As shown in Figure 3, the average transcriptional activity of the construct containing −838A was 20-fold higher than that of the construct containing −838C (P=0.001).

To identify potential upstream transcription factors mediating the increased p27^{kip1}-838A promoter activity detected, in silico analyses were performed. First, we analyzed the over-species sequence alignment of the −838C>A SNP flanking regions and revealed a relatively highly conserved 80-bp sequence, indicating that this promoter region is probably of functional importance. Next, we determined the differences in potential transcription factor binding sites between the p27^{kip1}-838A and p27^{kip1}-838C alleles and found that a change at position −838 from C to A resulted in additional binding sites for signal transducers and activators of transcription (STAT) 1, STAT6, early B-cell factor 1, GA-binding protein, and paired box (PAX1) and the loss of a binding site for myc-associated zinc finger (Figure 4).

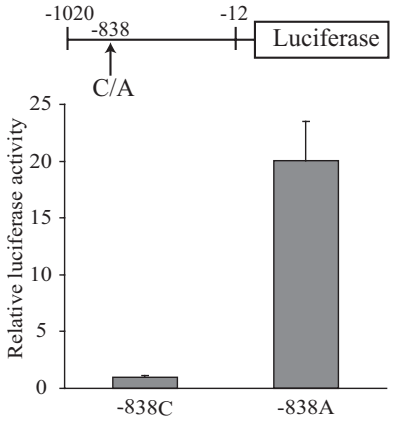

Figure 3. Effect of p27^{kip1}-838C>A polymorphism on the promoter transcriptional activity. HEK293 cells were transfected with constructs in which part of the p27^{kip1} promoter, containing either −838C or −838A, was cloned in front of the firefly luciferase coding sequence. Optimal expression of these promoter fragments was observed under nonserum conditions. Data are expressed as firefly luciferase activity normalized by *Renilla* luciferase and are the means (±SEM) of 3 independent experiments performed in duplicate (P=0.001).

Discussion

p27^{kip1} is a general cell-cycle inhibitor that has been studied extensively in cardiovascular disease. However, an association of p27^{kip1} genetic variations with in-stent restenosis has not been described before. In the present study, we observed that the homozygous AA genotype of the p27^{kip1}-838C>A SNP is associated with reduced risk of clinical in-stent restenosis TVR in 2 independent cohorts. This association is in line with the observation that the −838A allele in the p27^{kip1} promoter results in increased promoter activity. Thus, increased p27^{kip1} expression in −838AA patients may reduce SMC proliferation and explains the antirestenotic profile of the −838C>A genetic variation. Corresponding to the recently published data from Tiroch et al,[26] we did not observe an association of the −79C>T SNP with in-stent restenosis.

To the best of our knowledge, we are the first to describe an SNP in a cell-cycle protein that is associated with TVR rates. In a previous study, the p27^{kip1}-838AA genotype has been associated with an increased risk of myocardial infarction.[19] The apparent discrepancy between the association of the p27^{kip1}-838 AA genotype with a decreased risk of clinical in-stent restenosis versus an increased risk of acute myocardial infarction may be explained by the divergent pathophys-

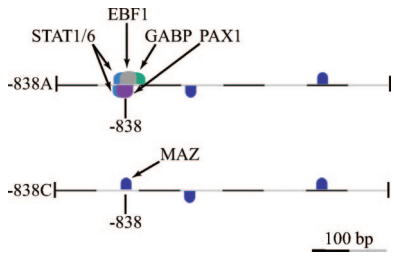

Figure 4. Differences in potential transcription factor binding sites on the p27^{kip1} promoter between the −838A and −838C alleles determined by use of the Genomatix suite (http://www.genomatix.de). EBF1 indicates early B-cell factor 1; GABP, GA-binding protein; PAX1, paired box; and MAZ, myc-associated zinc finger.

iological roles of SMCs in in-stent restenosis and atherosclerosis. In in-stent restenosis, a decrease in SMC proliferation is associated with a reduction in lesion formation. However, in atherosclerotic plaque, a decrease in SMC content may result in a reduction in the fibrous cap volume of the lesion, resulting in a vulnerable plaque that is prone to rupture and causes local thrombosis and subsequent acute myocardial infarction. Furthermore, Gonzalez et al[19] studied the p27kip1 promoter in fully proliferative cells and observed that a promoter fragment containing the −838A variant had a 34% decreased promoter activity compared with the −838C variant. In the present study, we demonstrate a robust 20-fold increase in promoter activity of the −838A over the −838C variant, a difference that may be explained by the setup of the experiment, in which we assayed for optimal p27kip1 promoter activity in quiescent adherent cells.

In silico analyses of the p27kip1 promoter showed differences in potential transcription factor binding sites between p27kip1-838A and p27kip1-838C alleles; a change at position −838 from C to A resulted in additional potential binding sites for STAT1, STAT6, early B-cell factor 1, GA-binding protein, and PAX1 and the loss of a binding site for myc-associated zinc finger (Figure 4). So far, except for a role of STAT1 in angiotensin-induced proliferation of vascular SMCs and GA-binding protein in KIS-mediated SMC proliferation, none of these transcription factors has been described to be functionally involved in SMC biology.[27,28] It recently was shown in mouse embryonic fibroblasts that STAT1 indeed induces p27kip1 expression at a transcriptional level.[29] However, additional studies are required to address whether any of these transcription factors are involved in the differential transcriptional regulation of p27kip1-838A and C promoter sequences.

Our study does have certain limitations. The present study was designed as a nonrandomized single-center cohort study. Nevertheless, the genotypes studied were in Hardy-Weinberg equilibrium, and baseline clinical and angiographic characteristics were equally distributed over the genotypes studied, except for the percentage of smoking patients, which was significantly higher in patients with the p27kip1-838AA genotype. Significantly, despite the higher percentage of current smokers, which is considered a risk factor for in-stent restenosis, the −838A genotype is associated with lower clinical in-stent restenosis rates. In addition, the association with TVR also was found in a second larger multicenter cohort study.

Conclusions

The p27kip1-838C>A SNP is associated with clinical in-stent restenosis; the −838AA genotype gives rise to an ≈2- to 4-fold decreased risk of TVR. Importantly, this polymorphism is associated with differences in basal p27kip1 promoter activity. We propose that increased p27kip1 expression in patients with a −838AA genotype results in decreased SMC proliferation and explains the decreased risk of in-stent restenosis in this patient group. Knowledge of this p27kip1 SNP facilitates the identification of those individuals who are at risk of developing in-stent restenosis in response to a bare metal stent and thus may profit from treatment with a drug-eluting stent. Maybe even more important, after further testing of this SNP in a prospective study, this risk stratification may support the intervention cardiologist in triaging patients with a low risk of restenosis for treatment with a bare metal stent rather than a drug-eluting stent.

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Disclosures

None.

References

1. Serruys PW, Kutryk MJ, Ong AT. Coronary-artery stents. *N Engl J Med*. 2006;354:483–495.
2. Daemen J, Serruys PW. Drug-eluting stent update 2007, part I: a survey of current and future generation drug-eluting stents: meaningful advances or more of the same? *Circulation*. 2007;116:316–328.
3. Daemen J, Serruys PW. Drug-eluting stent update 2007, part II: unsettled issues. *Circulation*. 2007;116:961–968.
4. Costa MA, Simon DI. Molecular basis of restenosis and drug-eluting stents. *Circulation*. 2005;111:2257–2273.
5. Welt FG, Rogers C. Inflammation and restenosis in the stent era. *Arterioscler Thromb Vasc Biol*. 2002;22:1769–1776.
6. Kastrati A, Schomig A, Elezi S, Schuhlen H, Wilhelm M, Dirschinger J. Interlesion dependence of the risk for restenosis in patients with coronary stent placement in multiple lesions. *Circulation*. 1998;97:2396–2401.
7. Weintraub WS, Kosinski AS, Brown CL III, King SB III. Can restenosis after coronary angioplasty be predicted from clinical variables? *J Am Coll Cardiol*. 1993;21:6–14.
8. Kastrati A, Dirschinger J, Schomig A. Genetic risk factors and restenosis after percutaneous coronary interventions. *Herz*. 2000;25:34–46.
9. Horibe H, Yamada Y, Ichihara S, Watarai M, Yanase M, Takemoto K, Shimizu S, Izawa H, Takatsu F, Yokota M. Genetic risk for restenosis after coronary balloon angioplasty. *Atherosclerosis*. 2004;174:181–187.
10. Malik FS, Lavie CJ, Mehra MR, Milani RV, Re RN. Renin-angiotensin system: genes to bedside. *Am Heart J*. 1997;134:514–526.
11. Monraats PS, Pires NM, Agema WR, Zwinderman AH, Schepers A, de Maat MP, Doevendans PA, de Winter RJ, Tio RA, Waltenberger J, Frants RR, Quax PH, van Vlijmen BJ, Atsma DE, van der LA, van der Wall EE, Jukema JW. Genetic inflammatory factors predict restenosis after percutaneous coronary interventions. *Circulation*. 2005;112:2417–2425.
12. Diez-Juan A, Castro C, Edo MD, Andres V. Role of the growth suppressor p27Kip1 during vascular remodeling. *Curr Vasc Pharmacol*. 2003;1:99–106.
13. Sedding DG, Seay U, Fink L, Heil M, Kummer W, Tillmanns H, Braun-Dullaeus RC. Mechanosensitive p27Kip1 regulation and cell cycle entry in vascular smooth muscle cells. *Circulation*. 2003;108:616–622.
14. Boehm M, Nabel EG. The cell cycle and cardiovascular diseases. *Prog Cell Cycle Res*. 2003;5:19–30.
15. Diez-Juan A, Andres V. The growth suppressor p27(Kip1) protects against diet-induced atherosclerosis. *FASEB J*. 2001;15:1989–1995.
16. Diez-Juan A, Perez P, Aracil M, Sancho D, Bernad A, Sanchez-Madrid F, Andres V. Selective inactivation of p27(Kip1) in hematopoietic progenitor cells increases neointimal macrophage proliferation and accelerates atherosclerosis. *Blood*. 2004;103:158–161.
17. Braun-Dullaeus RC, Ziegler A, Bohle RM, Bauer E, Hein S, Tillmanns H, Haberbosch W. Quantification of the cell-cycle inhibitors p27(Kip1) and p21(Cip1) in human atherectomy specimens: primary stenosis versus restenosis. *J Lab Clin Med*. 2003;141:179–189.
18. Tanner FC, Yang ZY, Duckers E, Gordon D, Nabel GJ, Nabel EG. Expression of cyclin-dependent kinase inhibitors in vascular disease. *Circ Res*. 1998;82:396–403.

19. Gonzalez P, Diez-Juan A, Coto E, Alvarez V, Reguero JR, Batalla A, Andres V. A single-nucleotide polymorphism in the human p27kip1 gene (−838C>A) affects basal promoter activity and the risk of myocardial infarction. *BMC Biol.* 2004;2:5.
20. Rittersma SZ, de Winter RJ, Koch KT, Schotborgh CE, Bax M, Heyde GS, van Straalen JP, Mulder KJ, Tijssen JG, Sanders GT, Piek JJ. Preprocedural C-reactive protein is not associated with angiographic restenosis or target lesion revascularization after coronary artery stent placement. *Clin Chem*. 2004;50:1589–1596.
21. van der Zwet PM, Reiber JH. A new approach for the quantification of complex lesion morphology: the gradient field transform: basic principles and validation results. *J Am Coll Cardiol*. 1994;24:216–224.
22. Sauer S, Gut IG. Genotyping single-nucleotide polymorphisms by matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2002;782:73–87.
23. Foley DP, Pieper M, Wijns W, Suryapranata H, Grollier G, Legrand V, de Scheerder I, Hanet C, Puel J, Mudra H, Bonnier HJ, Colombo A, Thomas M, Probst P, Morice M, Kleijne J, Serruys PW, for the MAGIC 5L Investigators. The influence of stent length on clinical and angiographic outcome in patients undergoing elective stenting for native coronary artery lesions; final results of the MAGIC 5L Study. *Eur Heart J*. 2001;22:1585–1593.
24. Kastrati A, Elezi S, Dirschinger J, Hadamitzky M, Neumann FJ, Schomig A. Influence of lesion length on restenosis after coronary stent placement. *Am J Cardiol*. 1999;83:1617–1622.
25. Walter DH, Schachinger V, Elsner M, Mach S, Auch-Schwelk W, Zeiher AM. Effect of statin therapy on restenosis after coronary stent implantation. *Am J Cardiol*. 2000;85:962–968.
26. Tiroch K, Koch W, Mehilli J, Bottiger C, Schomig A, Kastrati A. P27 and P53 gene polymorphisms and restenosis following coronary implantation of drug-eluting stents. *Cardiology*. 2008;112:263–269.
27. Horiuchi M, Cui TX, Li Z, Li JM, Nakagami H, Iwai M. Fluvastatin enhances the inhibitory effects of a selective angiotensin II type 1 receptor blocker, valsartan, on vascular neointimal formation. *Circulation*. 2003;107:106–112.
28. Crook MF, Olive M, Xue HH, Langenickel TH, Boehm M, Leonard WJ, Nabel EG. GA-binding protein regulates KIS gene expression, cell migration, and cell cycle progression. *FASEB J*. 2008;22:225–235.
29. Wang S, Raven JF, Durbin JE, Koromilas AE. Stat1 phosphorylation determines Ras oncogenicity by regulating p27 kip1. *PLoS ONE*. 2008;3:e3476.

CLINICAL PERSPECTIVE

The interventional cardiologist needs to decide for every patient undergoing percutaneous coronary intervention whether that individual is optimally treated with a bare metal stent or may need a drug-eluting stent to minimize the risk for in-stent restenosis. Drug-eluting stents, however, require prolonged treatment with antiplatelet drugs and show enhanced in-stent thrombosis. In-stent restenosis is caused mainly by excessive smooth muscle cell proliferation. The cyclin-dependent kinase inhibitor $p27^{kip1}$ is crucial for the inhibition of the cell cycle and has been reported to be functionally involved in vascular disease. Therefore, we decided to study the association of the $p27^{kip1}$ gene polymorphism with in-stent restenosis. In 2 independent cohorts of patients who received bare metal stents and were clinically followed up, we identified the association of a single nucleotide polymorphism in the $p27^{kip1}$ promoter (−838C>A) with in-stent restenosis. Patients with the −838AA genotype have a decreased risk of target vessel revascularization. This −838A allele corresponds to enhanced $p27^{kip1}$ promoter activity, which may explain decreased smooth muscle cell proliferation. In conclusion, our data provide novel opportunities for risk stratification of individual patients to support the interventional cardiologist in triaging patients with a low risk of restenosis for treatment with a bare metal stent rather than with a drug-eluting stent.