Genetic variants in *CCNB1* associated with differential gene transcription and risk of coronary in-stent restenosis

Silvestre-Roig. SNPs in *CCNB1* and restenosis risk

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ABSTRACT

Background: The development of diagnostic tools to assess restenosis risk after stent deployment may enable the intervention to be tailored to the individual patient, for example by targeting drug-eluting stent's use to high risk patients, with the goal of improving safety and reducing costs. The *CCNB1* gene (encoding cyclin B1) positively regulates cell proliferation, a key component of in-stent restenosis (ISR). We therefore hypothesized that single nucleotide polymorphisms (SNPs) in *CCNB1* may serve as useful tools in risk stratification for ISR.

Methods and Results: We identified 3 SNPs in *CCNB1* associated with increased restenosis risk in a cohort of 284 patients undergoing coronary angioplasty and stent placement (rs350099: TT vs. CC+TC, OR=1.82, 95%CI=1.09-3.03, p=0.023; rs350104: CC vs. CT+TT, OR=1.82, 95%CI=1.02-3.26, p=0.040; rs164390: GG vs. GT+TT, OR=2.27, 95%CI=1.33-3.85, p=0.002). These findings were replicated in another cohort study of 715 patients (rs350099: TT vs. CC+TC, OR=1.88, 95%CI=0.92-3.81, p=0.080; rs350104: CC vs. CT+TT, OR=2.23, 95%CI=1.18-4.25, p=0.016; rs164390: GG vs. GT+TT, OR=1.87, 95%CI=1.03-3.47, p=0.040). Moreover, the haplotype containing all three risk alleles is associated with higher *CCNB1* mRNA expression in circulating lymphocytes and increased ISR risk (OR=1.43, 95%CI=1.00-1.823, p=0.039).The risk variants of rs350099, rs350104 and rs164390 are associated with increased reporter gene expression through binding of transcription factors NF-Y, AP-1 and SP1, respectively.

Conclusions: Allele-dependent transcriptional regulation of *CCNB1* associated with rs350099, rs350104 and rs164390 affects the risk of ISR. These findings reveal these common genetic variations as attractive diagnostic tools in risk stratification for restenosis.

KEYWORDS: restenosis; stent; single nucleotide polymorphism; CCNB1

INTRODUCTION

The use of stents has increased the safety of percutaneous coronary interventions (PCI) and decreased restenosis rates compared with conventional balloon angioplasty.[1] Preclinical studies demonstrate a pivotal role of cell cycle regulatory genes in vascular smooth muscle cell (VSMC) proliferation and neointimal lesion development induced by mechanical injury,[2] and restenosis incidence has been reduced to 5-10% by the use of drug-eluting stents (DES) that locally deliver cytostatic drugs that limit VSMC hyperplasia, compared with 20-30% in patients revascularized with bare metal stents (BMS).[1, 3, 4] However, probably as a consequence of delayed target vessel reendothelialization due to reduced endothelial cell proliferation, DES deployment increases the occurrence of late stent thrombosis, which is associated with a high mortality rate.[1] Patients fitted with DES are therefore submitted to longer duration dual antiplatelet therapy than patients receiving BMS. In addition to these clinical limitations, DES are costlier than BMS. The development of diagnostic tools to assess restenosis risk may enable the intervention to be tailored to the individual patient, for example by targeting DES use to patients at higher risk, with the goal of improving safety and reducing costs.

The incidence of restenosis is highly influenced by clinical, biological, procedural and lesion-related risk factors.[4] However, information on restenosis biomarkers is scarce. Single nucleotide polymorphisms (SNPs) are recognized as suitable markers of disease predisposition.[5] SNPs in the human genome occur on average once every 300 nucleotides (~10 million SNPs), making them the most common type of genetic variation. SNPs reported to influence restenosis risk affect platelet activation, leukocyte recruitment, the inflammatory response, metalloproteinases, lipid metabolism, oxidative stress, nitric oxide, the renin-angiotensin system and cell proliferation (reviewed in).[4, 6, 7] Interestingly, the 838C>A SNP in *CDKN1B* (encoding the tumor suppressor p27^{Kip1}) has been associated with restenosis risk after coronary stenting, which might be due to augmented VSMC proliferation caused by reduced *CDKN1B* promoter activity in patients carrying the risk allele.[8] However, other SNPs in *CDKN1B* or in *TP53* (encoding the tumor suppressor p53) showed lack of association with restenosis risk.[8, 9] In the present study, we investigated a potential association of restenosis risk with SNPs in the *CCNB1* gene (encoding cyclin B1). *CCNB1* is essential for cell proliferation and its ablation in the mouse is embryonically lethal.[10] Several regulatory mechanisms are necessary to ensure that cyclin B1 protein accumulates appreciably only during the G2/M cell cycle transition, and deregulated *CCNB1* transcription leading to aberrantly high levels of cyclin B1 throughout the cell cycle is associated with excessive hyperplasia in several human cancers.[11] Several lines of evidence indicate that *CCNB1* is also important in the context of cardiovascular disease: its expression has been reported in human restenotic tissue obtained by directional coronary atherectomy,[12] it is induced in balloon-injured rat carotid artery,[13, 14] and its inhibition reduces neointimal thickening in this animal model.[15] Herein, we present evidence from two independent cohorts of patients undergoing coronary stent deployment (Clinica Mediterranea and GEnetic risk factors for In-Stent Hyperplasia study Amsterdam: GEISHA)[8] cohorts showing that alleles of the SNPs rs350099, rs350104 and rs164390, located in regulatory regions of *CCNB1*, are associated with higher *CCNB1* mRNA expression and increased risk of in-stent restenosis (ISR) after PCI. We furthermore identify molecular mechanisms that might account for this genotype-disease association.

METHODS

Patients. The Clinica Mediterranea cohort included 434 patients scheduled for PCI in Clinica Mediterranea (Naples, Italy) who were recruited between January- December 2004. Inclusion criteria were elective PCI with planned cobalt-chromium stent, and de novo lesion in a native coronary artery. Of the initial cohort, 101 patients were excluded because of implantation of at least 1 DES (n=77) or inadequate blood sample storage (n=24), leaving a final total of 333 enrolled patients. Of these, 284 (85%) underwent a routine coronary angiographic follow-up 6-9 months after PCI and were included in this study. The cobalt-chromium stents implanted were MultiLink Vision (Abbott Vascular, Abbott Park, Illinois) and Driver (Medtronic CardioVascular, Santa Rosa, California). All patients were treated with 100 mg aspirin and 75 mg clopidrogel daily for at least 1 month after PCI. The local ethics committee approved the study protocol, and all patients gave written informed consent. Peripheral blood samples were taken from all patients before PCI. Samples were collected into trisodium-citrate tubes and immediately placed on ice. Within 1 hour of collection, blood samples were centrifuged (4000 rpm, 20 minutes) and plasma harvested and stored at -80°C until analysis. Plasma total cholesterol, HDL-cholesterol, LDL-cholesterol and triglycerides were measured by enzymatic techniques. Estimated glomerular filtration rate (eGFR) was calculated by applying the modification of diet in renal disease formula. Chronic kidney disease was defined as an eGFR<50 ml/min/1.73m^2. Clinical characteristics of patients are summarized in **Supplemental Table-S1**. Patients received intracoronary isosorbide dinitrate (0.1-0.3-mg) prior to initial and final angiograms to achieve maximal vasodilatation. Angiographic measurements were performed with an automated computer-based system (Cardiovascular Angiographic Analysis Syste; Pie Medical Imaging; Maastricht, The Netherlands). Follow-up restenosis was analyzed by measurement of minimal lumen diameter by independent observers blinded to the genotype data. The following variables were also assessed: acute gain, defined as the difference between the minimal lumen diameter before and after the procedure; late loss, defined as the minimal lumen diameter after the procedure minus the minimal lumen diameter at follow-up; and loss index, defined as the average ratio of late loss to acute gain. Binary angiopraphic ISR was defined as >50% narrowing of the lumen diameter in the target segment (defined as all portions of the vessel that received treatment within the stent zone, including the proximal and distal 5-mm margins). Angiographic characteristics and major adverse coronary events (MACE) are summarized in **Supplemental Table-S2 and -S3**, respectively.

The clinical and angiographic characteristics of the GEISHA patients who were successfully treated for stable angina via BMS placement in a native coronary artery have been described previously.[8] All patients were treated with 100 mg aspirin and 250 mg ticlopidine BID or 75 mg clopidrogel daily for one month after PCI and 100 mg aspirin thereafter. Quantitative coronary angiography was performed between 6 and 12 months after BMS placement as described,[16] and clinical follow-up at 1 year was obtained. The primary endpoints were angiographic binary ISR (>50% diameter stenosis) and late lumen loss in minimal luminal diameter at follow-up. The secondary endpoints were coronary artery bypass grafting (CABG), target lesion revascularization (TLR, defined as repeat revascularization of the stented segment or within 5 mm margins proximal or distal to the stent by either repeat PCI or CABG), repeat PCI, non-fatal myocardial infarction, death, or the combined endpoint of MACE.

Genotyping of SNPs. Blood samples from patients of Clinica Mediterranea cohort were analyzed by SNPlex according to the manufacturer's recommendations (Applied Biosystems, Carlsbad, California, US). Polymorphisms were genotyped in the healthy sample by high resolution melting curves using a 480 LightCycler System and LightCycler 480 High Resolution Melting Master Kit (Roche, Basel, Switzerland). **Supplemental Table-S4** shows the sequence of the primers used to PCR amplify the

genomic regions containing each SNP. The genotype for each curve was identified by direct sequencing of 10 samples with similar curves using a 3730 sequencing analyzer under standard conditions (Applied Biosystems). In the GEISHA cohort, SNPs were genotyped using the Taqman assay (Applied Biosystems). Reactions were performed using the LC480 (Roche Diagnostics).

Statistical analysis. Results are expressed as means±SEM for *in vitro* experiments and as mean±SD for the cohort studies. Differences in continuous values in restenotic versus non-restenotic subjects (as defined by the angiographic lesion progression) were performed by the Student's t test or the Mann-Whitney U test, as appropriate. Categorical variables were analyzed by χ^2 test. In both cohorts, analysis for possible deviations of the genotype distribution from that expected for a population in Hardy-Weinberg equilibrium was performed by χ^2 test. Logistic regression analysis was performed to determine the association between the genotypes and ISR or TLR (repeated PCI or CABG). For genetic association, we first tested the codominant model and then used dominant or recessive models if two genotypes showed a similar effect. The association between the genotypes and TLR was also assessed by Cox proportional regression analysis. Follow-up ended for each individual at the time of the first occurrence of TLR. Patients without TLR were censored at 1 year after stent placement and 1 patient who died during follow-up was censored at the date of death. The proportional hazards assumption was examined by plotting the log(-log(survival)) versus the log of survival time. Event-free survival curves were calculated by Kaplan-Meier analysis, and differences between groups were calculated with the log-rank statistic. Haplotypes in the Clinica Mediterranea cohort were inferred with SNAPStats using the Expectation Maximization algorithm and Odds Ratio was calcutaled by logistic regression.[17] For the GEISHA study, from the obtained unphased SNP genotype data, haplotype frequencies and their effect on risk of TLR were estimated using weighted Cox regression as described.[18] Based on the inferred haplotypes, patients were grouped as those having one or no copies of the risk haplotype (TCG) and as those having two copies of the risk haplotype. Differences were considered statistically significant at p<0.05, as determined by paired 2-sided Student's *t* test (experiments with two groups) or one-way or two-way ANOVA followed by Bonferroni's or Dunnett's test (experiments with more than two groups). Statistical analyses were performed with SPSS (SPSS Inc., Chicago, Illinois), SNPstat[17] and GraphPad-Prism (GraphPad Software, LaJolla, CA).

RESULTS

SNPs rs350099, rs350104 and rs164390 in *CCNB1* are associated with ISR risk. To investigate whether polymorphic variants in *CCNB1* are associated with risk of ISR, we selected SNPs located in the transcriptional regulatory region (promoter and 5'-untranslated region) that have a minor allele frequency greater than 0.2. The 5 SNPs that fulfilled these criteria **(Fig.1A)** were genotyped in a cohort of 284 patients from Clinica Mediterranea who underwent coronary revascularization with BMS and angiographic follow-up **(see Methods and Supplemental Tables-S1,-S2,-S3).** Of these patients, 116 developed ISR. Genotyping was successful in >95% of samples for SNPs rs350099 (-957[T/C]), rs350104 (-475[T/C]), and rs164390 (+102[G/T]), and in 86% of samples for rs352626 (-710[C/T]) and rs8192258 (1055[-/A]). Genotype distribution of the non-restenotic control group was in Hardy-Weinberg equilibrium (p>0.05). Statistical analysis revealed a significant association between -

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957[T/C], -475[T/C] and +102[G/T] allele distribution and the incidence of angiographic (binary) restenosis, which was significant in crude analysis (not shown) and after adjustment for variables including age, gender, hypertension, diabetes mellitus, type of angina, statin therapy and family history of coronary artery disease (CAD) (**Table 1**). Moreover, the false positive report probability test[19] was 0.17 for a prior probability of 0.25, which supports a genuine association of SNPs -957[T/C], -475[T/C] and +102[G/T] with ISR risk in this cohort.

We next analyzed the GEISHA population for the three SNPs that exhibited significant association in our pilot study (-957[T/C], -475[T/C], +102[G/T]). As in Clinica Mediterranea population, BMS were used in the GEISHA cohort. Clinical and angiographic characteristics have previously been described in detail.[8] Of a total of 715 patients, DNA was obtained from 688, and TLR occurred in 55 patients. Failure of genotyping for -957[T/C], -475[T/C] and +102[G/T] was 9%, 1.9% and 1.5%, respectively. All genotype distributions were in Hardy-Weinberg equilibrium (p>0.05). Consistent with the results in the Clinica Mediterranea cohort, we found in GEISHA a statistically significant association between -475[T/C] and +102[G/T] and clinical ISR (based on TLR occurrence) after adjusting for age, gender, hypertension, smoking, diabetes mellitus, statin therapy and family history of CAD (**Table 2**). We also observed increased risk in -957TT compared with -957(TC+CC) patients (Odds Ratio=1.88), which almost reached statistical significance (p=0.080, **Table 2**). Moreover, Kaplan-Meyer analysis revealed reduced cumulative TLR-free survival in carriers of the ISR risk genotypes of each SNP, with differences reaching statistical significance for +102[G/T] (Log-rank p=0.040) or very close to significance (-957[T/C]: Log-rank p-value=0.057; -475[T/C]: Log-rank p=0.056) (**Figure 2**). To explore the strength of the observed associations, we assessed whether these associations persisted after Cox proportional regression analysis including age, gender, hypertension, smoking, diabetes, statin therapy and family history of CAD. Our results demonstrate increased risk of clinical ISR in -957TT (Hazard Ratio [HR], 1.95; CI, 0.99-3.83; p=0.053), -475CC (HR, 2.38; CI, 1.01-5.61; p=0.048) and +102GG (HR, 2.04; CI, 1.04-4.01; p=0.040) patients when considering the interval of time between stent deployment and TLR (**Table 3**).

The haplotype containing all three restenosis-risk allelic variants is associated with augmented risk of ISR and *CCNB1* mRNA expression. Having demonstrated that the -957T, -475C and +102G alleles are associated with higher ISR risk, we analyzed the *CCNB1* haplotype containing these three polymorphic variants. In Clinica Mediterranea cohort, four haplotypes were identified with frequencies above 1% in both the restenotic and non-restenotic groups (Haplotypes 1-4, **Supplemental Table-S5**). Analysis by the D, D' and R^2 methods revealed that -957[T/C], -475[C/T] and +102[G/T] are in linkage disequilibrium (**Supplemental Table-S6**). Haplotype 1, containing the restenosis risk alleles of all three SNPs (T/C/G), was the most frequent (48.8%) in the Clinica Mediterranea population (**Supplemental Table-S5**) and exhibited statistically significant association with increased ISR risk when compared to all other haplotypes (OR=1.43, 95%CI=1.00-1.823, p=0.039) (**Fig.3A**). The analysis of this association was very similar after adjustment for multiple variables, including age, gender, hypertension, diabetes mellitus, statin therapy, type of angina and family history of CAD (OR=1.41, 95%CI=0.99-2.00, p=0.055).

We also performed haplotype analysis in the GEISHA cohort where haplotype 1 was also the most frequent (47.6%, **Supplemental Table-S7**). We found statistically significant association with increased TLR when comparing haplotype 1 versus all other haplotypes (HR=2.87; CI=1.17-7.04; p=0.02, Cox regression analysis with data corrected for age, hypertension, smoking, diabetes mellitus, statin therapy and family history of CAD). In addition, Kaplan-Meyer estimates of TLR-free survival also showed decreased survival in haplotype 1 compared with all other haplotypes with a p-value very close to significance (p=0.058) (**Fig. 3B**).

Taken together, these studies suggest a significant association between haplotype containing all three restenosis-risk allelic variants and increased risk of ISR. We next sought to investigate possible associations between *CCNB1* expression and haplotypes 1 and 2, the most frequent haplotypes in our cohorts (**see Supplemental Table-S5, Table-S7**). Analysis of peripheral blood lymphocytes isolated from healthy volunteers revealed a 2.23-fold increase in *CCNB1* mRNA expression in haplotype 1 versus haplotype 2 carriers (p=0.0044, **Fig.3C; see subjects characteristics in Supplemental Table-S8**).

The restenosis-risk T allelic variant of -957[T/C] generates a binding site for Nuclear Factor-Y (NF-Y). Since -957[T/C], -475[T/C] and +102[G/T] are located in potential regulatory regions in the *CCNB1* gene and are associated with mRNA expression level in circulating lymphocytes, we assessed whether the restenosis-risk allelic variants of these SNPs cause increased *CCNB1* expression through differential transcription factor binding. Previous studies revealed that the *CCNB1* promoter contains two CCAAT boxes at positions -17/-13 and +16/+20 that are essential for gene transcription through binding of NF-Y.[20] Our *in silico* analysis predicted that the restenosis-risk T allelic variant of -957[T/C], but not the C allele, generates an additional CCAAT motif in the *CCNB1* promoter (**Fig.1B**). We evaluated the functionality of this putative CCAAT box by electrophoretic mobility shift assays (EMSAs) using nuclear extracts of HeLa cells and radiolabeled probes (**Supplemental Table-S9**). Incubation with the NF-Y(-25/-7) probe, containing the canonical CCAAT box at position -17/-13 in the *CCNB1* promoter, generated a retarded band (**Fig.4A, lane 2**) that was efficiently competed out by an excess of either unlabeled NF-Y(-25/-7) sequence (**lanes 3,4**) or an oligonucleotide spanning the -957T sequence (**lanes 5,6**). This retarded band was not competed out by an equivalent molar excess of the unlabeled -957C sequence (**lanes 7,8**) or the mutated NF-Y(-25/-7) sequence (NF-Y mutant, **lanes 9,10**). We also generated radiolabeled -957T and -957C probes to assess DNA-binding activity directly associated to these sequences. Consistent with the results of the competition studies, the -957T probe generated a retarded band with the same electrophoretic mobility as that produced by NF-Y(-25/-7), which was specifically supershifted with anti-NF-YB antibody (**Fig.4B, lanes 1-8**), and was not observed in binding reactions with -957C probe (**Fig.4B, lanes 9-12**). Thus, the restenosis-risk -957T allele of rs350099, but not the -957C variant, generates a NF-Y binding site in the human *CCNB1* promoter.

The restenosis-risk C allelic variant of -475[T/C] generates a binding site for activator protein 1 (AP-1) with higher affinity than the T allele. Our *in silico* analyses predicted that the C allelic variant of -475[C/T], but not the T allele, generates an AP-1 binding site (**Fig.1B**). Incubation of nuclear extracts from U2OS cells with probes for -475C (**Fig.5A**, **lanes 6,14**) or -475T (**lanes 10,19**) generated a major retarded band of the same electrophoretic mobility as that obtained with AP-1 consensus probe (**lane 2**). This band was efficiently competed out with an excess of unlabeled -475C, -475T or AP-1 consensus oligonucleotides (**lanes 3, 7, 11, 15, 16, 17, 20, 21, 22**), but not with unrelated NF-Y consensus (**lanes 4,8,12**). We noted that the -475C probe produced a more intense retarded nucleoprotein complex than the -475T probe (**Fig.5A, lanes 6,14 vs. 10,19**). Consistent with this finding, unlabeled -475C oligonucleotide was more efficient than -475T oligonucleotide at competing out DNA-binding activity associated with AP-1 consensus probe (**Fig.5B**).

We next carried out supershift experiments using antibodies specific for the canonical AP-1 family members c-Fos and c-Jun. Anti-c-Fos (**Fig.5C, upper autoradiograph**) and anti-c-Jun (**Fig. 5C, lower autoradiograph**) both produced a supershift when tested against AP-1 consensus, -475C or -475T probes (**lanes 3,6,9,12,15,17**), but not against a probe containing the consensus binding site for specificity protein 1 (SP1) (data not shown). Consistent with the results of **Fig.5A**, the anti-c-Fos and anti-c-Jun supershifted bands were more intense with -475C than with -475T probe (**Fig.5C, lane 6 vs.**

9, and 15 vs. 17). Thus, the ISR-risk -475C allele of rs350104 in the human *CCNB1* promoter binds AP-1 factors with higher affinity than -475T.

The restenosis-risk G allelic variant of +102G[G/T] in *CCNB1* generates a binding site for SP1.
The G allele of +102[G/T] generates a GC-rich sequence very similar to the consensus SP1 binding site.[21] Incubation of U2OS nuclear extracts with an SP1 consensus probe generated a retarded band (**Fig.6A**, **lanes 2,9**) that was efficiently competed by excess unlabeled oligonucleotides corresponding to SP1 consensus (**lanes 3,10**) or to +102G (**lanes 4-7**), but not to +102T (**lanes 11-14**). Moreover, a +102G probe **(Fig.6B, lane 7),** but not the +102T probe **(lane 12),** generated a retarded band with the same electrophoretic mobility as that obtained with the SP1 consensus probe (**lane 2**). The retarded band obtained with SP1 and +102G probes was supershifted with anti-SP1 antibody (**Fig.6B, lanes 4,9**) but not with isotype-matched anti-MEF2C antibody (**Fig.6B, lanes 5,10**). Thus, the ISR-risk +102G allele of rs164390, but not the +102T allele, generates an SP1 binding site in the human *CCNB1* promoter.

The -957T, -475C and +102G restenosis-risk alleles increase reporter gene transcription. We next tested whether the three restenosis-risk alleles supported enhanced transcription by their corresponding putative transcription factors (NF-Y for -957T, AP-1 for -475C and SP1 for +102G). We transiently transfected U2OS cells with pGL3-luciferase reporters driven by three tandem repeats of each of the polymorphic variants of -957[T/C], -475[C/T] and +102[G/T] **(supplemental Table-S10).** The 3x(-957T)-luciferase construct increased reporter gene activity two-fold compared with control pGL3-luciferase (dashed line), whereas 3x(-957C)-luciferase had no effect **(Fig.7A, white bars)**. Importantly, the promoter activity of 3x(-957T)-luciferase was significantly reduced by co-expression of a dominant-negative NF-YA mutant (NF-YAdn), while 3x(-957C)-luciferase activity was unaffected **(Fig.7A, black bars).** We also found that c-Fos overexpression, by transfection with pEGFP-c-Fos, significantly augmented promoter activity associated with 3x(-475C) compared with both pGL3 and 3x(-475T) **(Fig.7B)**. Moreover, additional luciferase activity was achieved with 3x(+102G), but not with 3x(+102T) when SP1 was overexpressed (**Fig.7C**). These results suggest that the NF-Y, AP-1 and SP1 DNA-binding sites generated in the restenosis-risk -957T, -475C and +102G alleles are functional.

DISCUSSION

In this study, we show that the SNPs rs350099 (-957[T/C]), rs350104 (-475[C/T]) and rs164390 (+102[G/T]) located in the 5'-regulatory region of the human *CCNB1* gene are associated with risk of developing restenosis after coronary stent deployment in two independent cohorts and provide molecular insight into these genotype-disease associations. We restricted our analysis to patients who received a BMS, in order to avoid potential effects of antiproliferative drugs used in DES that can significantly reduce cell-cycle gene expression,[13, 22] and may explain negative results observed in other genetic association studies focused on cell-cycle regulators.[9, 23] We find increased ISR risk in patients carrying haplotype 1 (-957T/-475C/+102G), which contains all three restenosis-risk alleles. Interestingly, circulating lymphocytes from individuals carrying haplotype 1 have higher *CCNB1* mRNA levels than those carrying haplotype 2 (-957C/-475T/+102T). Consistent with the notion that disease susceptibility in humans is influenced by common SNPs that affect gene transcription through allelic-dependent transcription factor recruitment,[24] we find that the restenosis-risk alleles of -

957[T/C], -475[C/T] and +102[G/T] generate functional binding sites for NF-Y, AP-1 and SP1, respectively, as revealed by DNA-binding and luciferase reporter assays. Increased recruitment of these transcription factors to the *CCNB1* promoter in individuals with haplotype 1 might therefore augment ISR risk by increasing expression of *CCNB1*, a key positive regulator of cell proliferation and neointimal thickening[10, 15] **(Fig.8).**

Individual patient restenosis risk is to date based on a reduced number of clinical, biological, lesion-related and procedural factors, and efforts to identify high-risk patients based on these variables have been only partially successful until now.[4] Recently, a predictive model utilizing these common variables has been developed to identify patients that will benefit from reduced target vessel revascularization by DES deployment.[25] However, early prediction accuracy of these models may still be improved by using additional individual specific biomarkers such as genetic traits. By using a gene candidate approach, we have identified three polymorphisms in the *CCNB1* associated to increased angiographic restenosis risk in the Clinica Mediterranea cohort of patients who underwent PCI with BMS implantation. Importantly, our studies in the larger GEISHA cohort revealed a statistically significant association with increased clinical ISR (defined as TLR) for SNPs -475[C/T] and +102[G/T], which was close to significance for SNP -957[T/C] (p=0.080) and remained significant after Cox proportional regression analysis. Individual candidate gene studies have limitations,[7] however our validation in two independent cohorts and functional studies support the reliability of the identified associations. Studies in larger cohorts and prospective studies are nevertheless necessary to assess whether diagnostic kits based on genotyping these SNPs in *CCNB1* can improve the safety and cost-effectiveness of stent use. These studies should also evaluate whether prediction can be improved on the basis of combining *CCNB1* SNPs and clinical, biological, lesion-related and procedural factors.

Our functional studies identify likely molecular mechanisms that might underlie the ISR risk associated with -957[T/C], -475[C/T] and +102[G/T]. NF-Y (also called CCAAT-binding factor: CBF) is a ubiquitous heterotrimeric transcription factor formed from NF-YA, B and C subunits that is required for cell proliferation.[26-31] We recently found that NF-Y is induced during atherosclerosis and restenosis in rodent models and humans, and promotes PDGF-BB-dependent *CCNB1* expression, VSMC proliferation and neointimal lesion development in a mouse femoral artery denudation model.[14] The results in the current study demonstrate that the restenosis-risk T allele of -957[T/C], but not the C allele, generates a CCAAT box at position -959/-955 relative to the *CCNB1* transcription initiation site which supports specific binding of NF-Y and drives NF-YA-dependent transcription of luciferase reporter plasmids. It is interesting to note that proper transcriptional regulation of several cell cycle-regulated genes requires multiple promoter CCAAT motifs,[32] possibly by stabilizing DNA-NF-Y complexes[33] and increasing the DNA-binding affinity of neighboring co-activators[34] (and references therein). It is therefore possible that the CCAAT motif at -959/-955 in -957TT homozygotes identified in the present work might cooperate with the -17/-13 and +16/+20 proximal CCAAT boxes to boost NF-Y-dependent *CCNB1* transcription.

Heterodimeric transcription factors of the AP-1 family control cell differentiation, apoptosis and proliferation in response to multiple physiological and pathological stimuli.[35] AP-1 is induced after balloon angioplasty[36-38] and stenting,[39] and gene therapy against AP-1 reduces neointima development in animal models.[36, 40, 41] Our data show that the restenosis-risk C allele of -475[T/C] in the *CCNB1* promoter binds AP-1 with higher affinity that the T variant, and that overexpression of the AP-1 family member c-Fos significantly augments luciferase activity driven by a tandem repeat of -475C, while having no significant effect on a similar construct driven by -475T. Thus, the presence of the -475C sequence in the *CCNB1* promoter may enhance gene transcription through increased recruitment of AP-1 transcription factors.

SP1 is an ubiquitous transcription factor that promotes cell growth by enhancing the expression of pro-proliferative genes whose promoters contain the consensus GC-rich sequence.[21] We found that

the presence of the high-risk ISR +102G allele, but not the +102T variant, generates a sequence that binds SP1 and confers SP1-dependent transcriptional activation in reporter assays. Bearing in mind previous studies reporting that two or more SP1 binding sites can cooperate in gene transactivation,[21] we speculate that enhanced *CCNB1* expression in +102GG homozygous individuals may result from the cooperation between the SP1 binding site generated by +102G and the two SP1 target sequences present at -259/-255 and -140/-136, which have been shown to be required for efficient *CCNB1* transcriptional activation.[21]

In summary, our findings suggest that allele-dependent transcriptional regulation of *CCNB1* associated with the SNPs rs350099, rs350104 and rs164390 affects ISR risk through differential recruitment of NF-Y, AP-1 and SP1 **(Fig.8).** Interestingly, Bouatia-Naji et al. [42] recently reported that the A allele of rs13431652 in the *G6PC2* promoter generates a functional NF-Y-binding CCAAT box, and is strongly associated with elevated fasting plasma glucose in humans. Moreover, a mutation in the CCAAT box of the *TERC* promoter that abrogates NF-Y binding has been associated with human telomere disease, thus providing further evidence that allele-specific differences in the recruitment of NF-Y can contribute to human disorders.[43] Likewise, common functional polymorphisms within the promoter of genes associated with lupus or CAD in diabetic patients influence AP-1 DNA-binding activity and gene expression.[44, 45] Allele-specific SP1 activity may also explain the association between several SNPs and susceptibility to mild fasting hyperglycemia, atherothrombotic stroke, and lung, breast and ovarian cancer.[44, 46-49] Regulation of human gene promoters by polymorphic NF-Y, AP-1 or SP1 sites thus appears to contribute to genetically-determined inter-individual variability in diverse pathophysiological scenarios. Remarkably, van Tiel and colleagues found that the -838C>A SNP in the *CDKN1B* gene (encoding the tumor suppressor p27[Kip1]) modulates promoter activity and is associated with restenosis risk after coronary BMS implantation.[8] Validation in larger cohorts and prospective studies comparing head-to-head BMS and DES is necessary to assess whether genotyping of common SNPs in cell-cycle regulatory genes may assist physicians in targeting DES use to patients at the highest ISR risk in order to improve the safety and cost-effectiveness of stent use. Based on the recent results of a systematic testing of literature reported genetic variation associated with coronary restenosis,[7] these studies should examine a possible joined effect of multiple genetic markers for predicting restenosis compared with individual candidate genes.

CLINICAL PERSPECTIVE

The use of drug-eluting stents (DES) that inhibit vascular smooth muscle cell (VSMC) proliferation significantly reduces in-stent restenosis (ISR). DES also inhibit endothelial cell proliferation, and ISR inhibition with these devices therefore comes at the price of delayed or incomplete reendothelialization, requiring longer-term antiplatelet therapy to prevent late in-stent thrombosis. The development of diagnostic tools to assess ISR risk may enable the intervention to be tailored to the individual patient, for example by targeting DES' use to high risk patients. In the present study, we investigated a potential association of ISR risk with single nucleotide polymorphisms (SNPs) in the *CCNB1* gene encoding cyclin B1, a positive cell cycle regulator which is expressed in human restenotic tissue and is essential for neointimal thickening in the rat carotid artery model of balloon angioplasty. By analyzing two independent cohorts, we show that the SNPs rs350099, rs350104 and rs164390 located in the 5'-regulatory region of the *CCNB1* gene are associated with higher *CCNB1* mRNA expression and elevated ISR risk after coronary stent deployment. Our molecular studies indicate that these SNPs affect *CCNB1* expression and

ISR risk through differential recruitment of the transcription factors NF-Y, AP-1 and SP1. Validation in larger cohorts and prospective studies comparing head-to-head DES and bare metal stents is necessary to assess whether genotyping rs350099, rs350104 and rs164390 may assist physicians in targeting DES use to patients at the highest ISR risk in order to improve the safety and cost-effectiveness of stent use.

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Table 1. Statistical analysis of ISR risk associated with SNPs in the human *CCNB1* gene: Clinica Mediterranea cohort

SNP	Genotype	Total	Binary restenosis	p-value	Odds Ratio	95% CI
-957[C/T] (rs350099)	TT	125	61 (48.8%)	0.023	1.82 (TT vs CC+TC)	1.09-3.03
	CC+CT	141	49 (34.7%)			
-475[C/T] (rs350104)	CC	70	37 (52.8%)	0.040	1.82 (CC vs TT+TC)	1.02-3.26
	CT+TT	188	73 (38.8%)			
+102[G/T] (rs164390)	GG	103	54 (52.4%)	0.002	2.27 (GG vs TT+GT)	1.33-3.85
	GT+TT	164	58 (35.4%)			

The Odds ratios were estimated using a logistic model adjusted for age, gender, hypertension, diabetes mellitus, type of angina, statin therapy at time of stenting and family history of CAD.

Table 2. Statistical analysis of ISR risk associated with SNPs in the human *CCNB1* gene: GEISHA cohort

SNP	Genotype	Total	TLR	p-value	Odds Ratio	95% CI
-957[C/T]	TT	146	17 (11.64%)	0.080	1.88 (TT vs CC+TC)	0.92-3.81
(rs350099)	CC+CT	479	32 (6.68%)			
-475[C/T]	CC	89	13 (14.61%)	0.016	2.23 (CC vs TT+TC)	1.18-4.25
(rs350104)	CT+TT	586	41 (6.99%)			
+102[G/T]	GG	159	19 (11.95%)	0.040	1.87 (GG vs TT+GT)	1.03-3.47
(rs164390)	GT+TT	519	34 (6.55%)			

The Odds ratios were estimated using a logistic model adjusted for age, gender, hypertension, smoking, diabetes mellitus, statin therapy at time of stenting and family history of CAD

Table 3. Cox proportional regression analysis in the GEISHA cohort

SNP	p-value	Hazard Ratio	95% CI
-957[C/T] (rs350099)	0.053	1.95 (TT vs CC+TC)	0.99-3.83
-475[C/T] (rs350104)	0.048	2.38 (CC vs TT+TC)	1.01-5.61
+102[G/T] (rs164390)	0.038	2.04 (GG vs TT+GT)	1.04-4.01

Hazard ratio estimated using a Cox proportional hazard model adjusted for age gender, hypertension, smoking, diabetes, statin therapy at time of stenting and family history of CAD

Figure Legends

Fig.1. Analysis of SNPs in the *CCNB1* gene. (A) Summary of SNPs that were included in the genotyping analysis. Position is defined as SNP location relative to transcription initiation site (+1). MAF: minor allele frequency. **(B)** -957T and -957C are the T or C polymorphic variants of rs350099, respectively. The -957T variant generates a consensus NF-Y binding site (CCAAT box). -475C and -475T are the C and T polymorphic variants of rs350104, respectively. The -475C variant generates a consensus AP-1 binding site (TGAG box). +102G and +102T are the G and T polymorphic variants of rs164390, respectively. The +102G variant generates a consensus SP1 binding site (GGGGCGGGGC box).

Fig.2. Kaplan-Meier estimates of clinical ISR-free survival in the GEISHA cohort. (A) Kaplan-Meier curves of TLR-free survival in patients carrying -957TT genotype vs the combined -957TC+-957CC genotypes. Log-rank p=0.057. **(B)** Kaplan-Meier curves of TLR-free survival in patients carrying -475CC genotype vs the combined -475TC+-475CC genotypes. Log-rank p=0.056. **(C)** Kaplan-Meier curves of TLR-free survival in patients carrying +102GG genotype vs the combined -957GT+-957GG genotypes. Log-rank p=0.040. The number at risk at 0, 2, 4, 6, 8, 10 and 12 months after PCI are shown for each SNP.

Fig.3 Human peripheral lymphocytes from donors carrying the high-risk H1 *CCNB1* haplotype (T/C/G) express increased levels of *CCNB1* mRNA. (A) Statistical analysis of restenosis risk in *CCNB1* haplotypes: H1 (high risk alleles of all three SNPs) vs all other haplotypes (analysis of Clinica Mediterranea cohort). **(B)** Kaplan-Meier curves of TLR-free survival in patients carrying H1 (T/C/G) haplotype vs all other haplotypes (analysis of GEISHA cohort). The p-value is from log-rank test. The number at risk at 0, 2, 4, 6, 8, 10 and 12 months after PCI are shown. **(C)** Relative *CCNB1* mRNA levels in peripheral blood lymphocytes isolated from 31 healthy human donors carrying either H1 haplotype (-957T/-475C/+102G (T/C/G)) or the H2 haplotype (-957C/-475T/+102G (C/T/T)). Results were analyzed by unpaired, 2-sided Student's *t* test.

Fig.4. The -957T restenosis-risk allelic variant of -957[C/T] in *CCNB1* generates a binding site for NF-Y. EMSAs using HeLa cell nuclear extracts and the indicated probes/competitors (see Supplementary Table-S8). Autoradiographs are representative of 3-5 experiments. **(A)** Competition assays using molar excesses of the indicated unlabeled oligonucleotides. The graph shows mean relative intensities of the retarded probe:NF-Y complex (n=3 assays). Black bar: control without competitor (=1). Results were analyzed by one-way ANOVA followed by Dunnet's multiple comparison test. *: p<0.01 and **: p<0.001 versus control without competitor. **(B)** Supershift assay using the indicated antibodies. No Ab: control without antibody.

Fig.5. The -475C restenosis-risk allelic variant of -475[C/T] in *CCNB1* generates a sequence with higher affinity than the -475T variant for binding AP-1. EMSAs using U2OS cell nuclear extracts and the indicated probes/competitors (see Supplementary Table-S8). Autoradiographs are representative of n=3-5 experiments. **(A, B)** Competition assays using molar excesses of the indicated unlabeled oligonucleotides. The graph shows mean relative band intensities of the retarded probe:AP-1 complexes (n=5 assays). Black bar: control without competitor (=1). Results were analyzed by one-way ANOVA followed by Bonferronni's multiple comparison test. *: p<0.01 and **: p<0.001 versus control without competitor. **(C)** Supershift assays using the indicated antibodies. Only retarded bands are shown.

Fig.6. The +102G restenosis-risk allelic variant of +102[G/T] in *CCNB1* generates a binding site for SP1. EMSAs using U2OS cell nuclear extracts and the indicated probes/competitors (see Supplementary Table-S8). Autoradiographs are representative of n=3-5 experiments. **(A)** Competition assays using molar excesses of the indicated unlabeled oligonucleotides. The autoradiograph shows the retarded probe:SP1 complexes and the graph shows mean relative band intensities of retarded probe:SP1 complexes (n=4-7 assays). Black bar: control without competitor (=1). Results were analyzed by one-way ANOVA followed by Dunnett´s multiple comparison test. *: p<0.001 versus control without competitor. **(B)** Binding reactions were incubated with the indicated competitors and antibodies.

Fig.7. The -975T, -475C and +102G restenosis-risk allelic variants increase reporter gene expression via NF-Y, c-Fos and SP1. U2OS cells were co-transfected with pRL-Renilla (control for transfection efficiency) and the indicated reporter vectors (depicted above the graphs). Results are represented as the firefly luciferase/renilla luciferase ratio relative to control pGL3-luciferase (=1). Results were analyzed by one-way ANOVA followed by multiple comparison test. **(A)** Assays were performed without (white bars) or with (black bars) co-transfected expression vector encoding the NF-YAdn dominant-negative mutant (n=12 replicates from 4 independent experiments). Results are expressed relative to control pGL3-luciferase (=1, dashed line). *: p<0.001 versus pGL3-luciferase and 3x(-957C)-luciferase; #: p<0.01 versus control 3x(-957T)- luciferase. **(B)** Assays were performed with co-transfected expression vector encoding c-Fos (n=10 replicates from 5 independent experiments). Results are expressed relative to pGL3-luciferase (=1). **: p<0.01 versus pGL3-luciferase; #: p<0.01 versus 3x(-475T)- luciferase. **(C)** Assays were performed with co-transfected expression vector encoding SP1 (n=15 replicates from 5 independent experiments). Results are expressed relative to pGL3-luciferase (=1). *** : p<0.001 versus pGL3-luciferase; # : p<0.05 versus 3x(+102T)- luciferase.
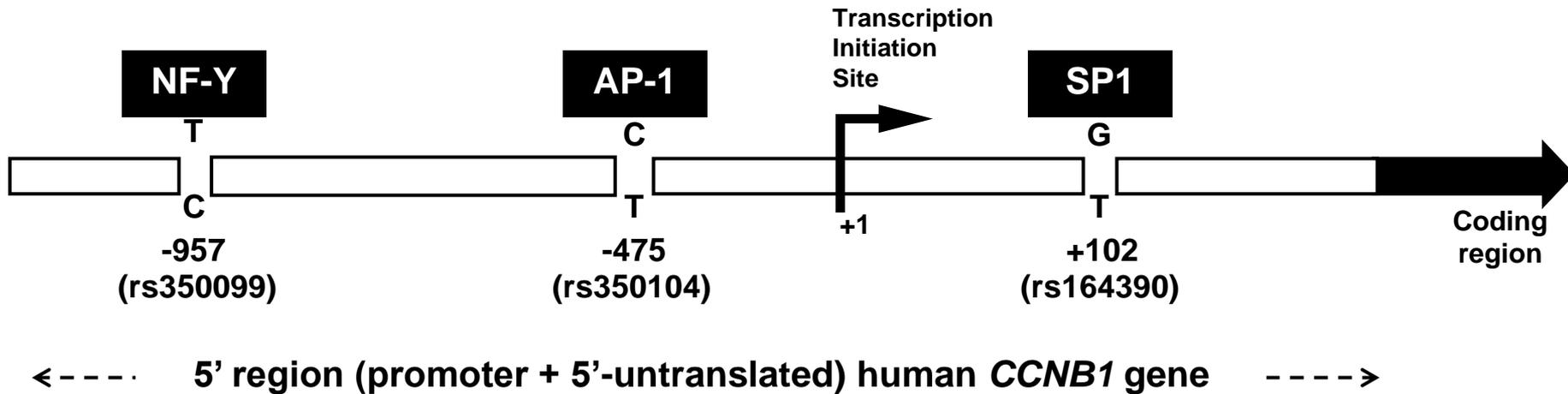
Fig.8. Model of increased risk of ISR through polymorphic transcriptional factor-dependent regulation of *CCNB1* gene transcription. The -957T polymorphic variant of rs350099, the -475C variant of rs350104 and the +102G variant of rs164390 increase recruitment of the transcription factors NF-Y, AP-1 and SP1, respectively, to the human *CCNB1* gene promoter. Increased recruitment of NF-Y, AP-1 and SP1 in subjects bearing the H1 haplotype (-957T/-475C/+102G) may lead to higher *CCNB1* mRNA expression and VSMC proliferation, thus contributing to a higher risk of ISR. In contrast, reduced recruitment of these transcription factors in subjects bearing the H2 haplotype (-957C/-475T/+102T) is associated with lower *CCNB1* expression and reduced ISR risk.

FIGURE 1

A

	SNP	Position	Allelic variants	MAF
Human *CCNB1* gene	**rs8192258 (-1055[-/A])**	-1055	-/A	0.284
	rs350099 (-957[T/C])	-957	T/C	0.320
	rs352626 (-710[T/C])	-710	T/C	0.478
	rs350104 (-475[T/C])	-475	T/C	0.285
	rs164390 (+102[G/T])	+102	G/T	0.427

B

NF-Y   **AP-1**   Transcription Initiation Site   **SP1**

T   C   G

C   T   +1   T

-957   **-475**   **+102**   **Coding region**

(rs350099)   **(rs350104)**   **(rs164390)**

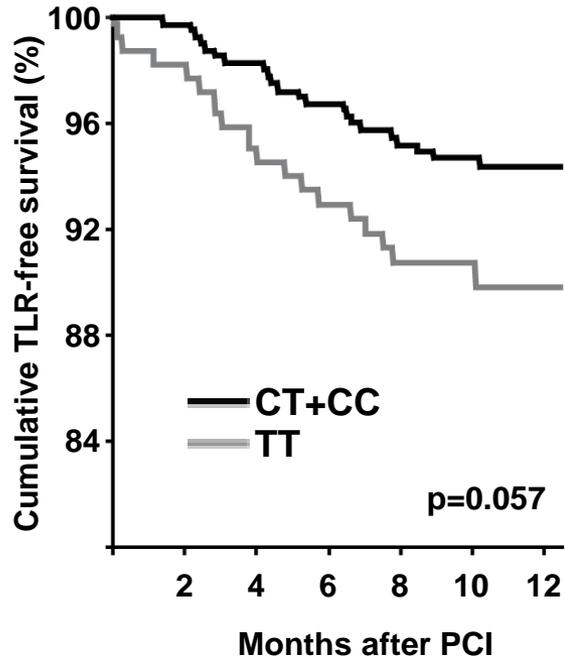**<- - - -   5' region (promoter + 5'-untranslated) human *CCNB1* gene   - - - ->**
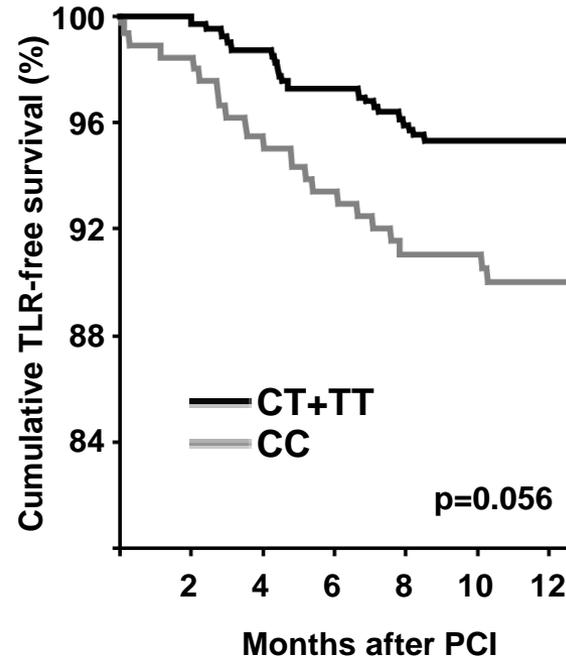
FIGURE 2

A -957[C/T] (rs350099)

B -475[C/T] (rs350104)

C +102[G/T] (rs164390)
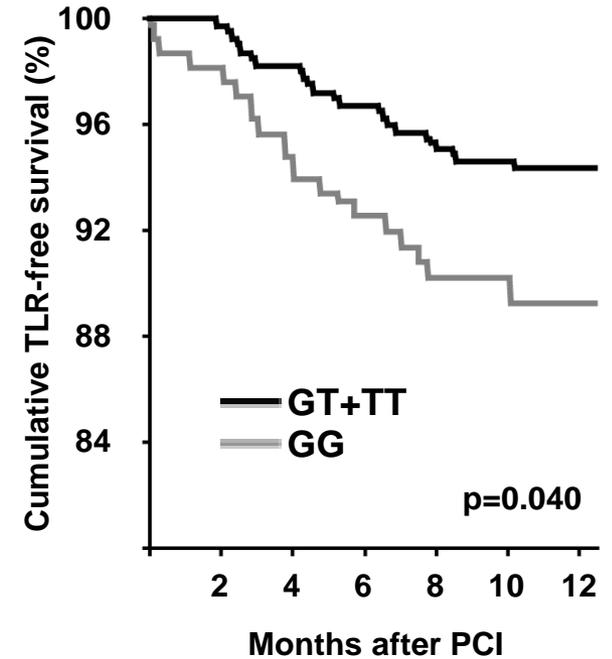
Number at risk

Months after PCI:	0	2	4	6	8	10	12
CT+CC:	457	439	422	408	386	362	171
TT:	142	130	123	118	111	106	51

	0	2	4	6	8	10	12
CT+TT:	561	534	516	496	467	428	206
CC:	86	80	76	72	68	65	31

	0	2	4	6	8	10	12
GT+TT:	495	476	458	443	420	394	185
GG:	155	141	134	128	120	114	55

FIGURE 3

A

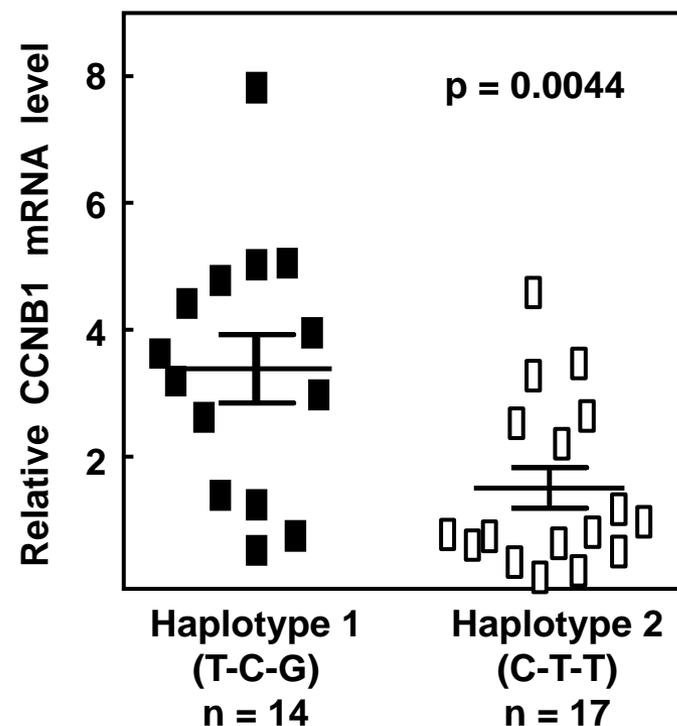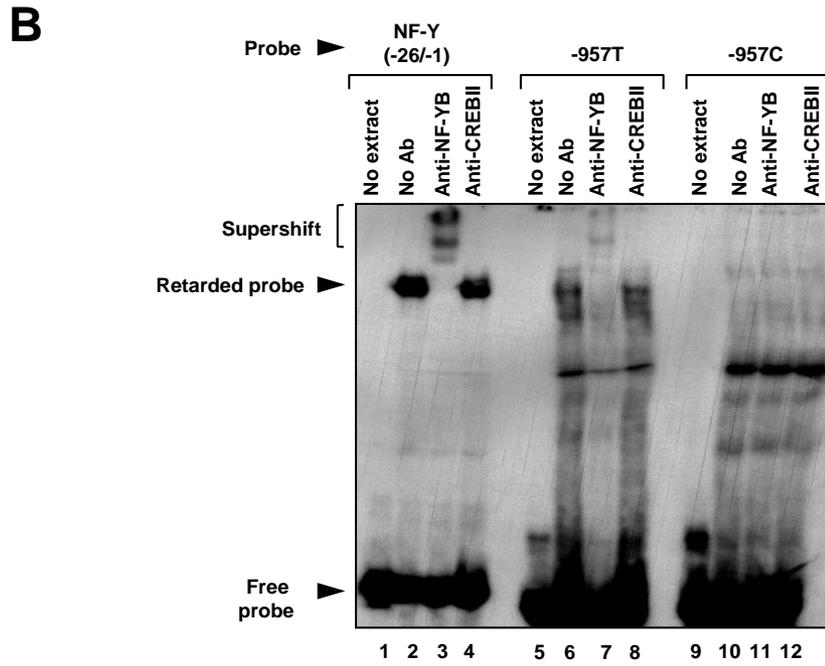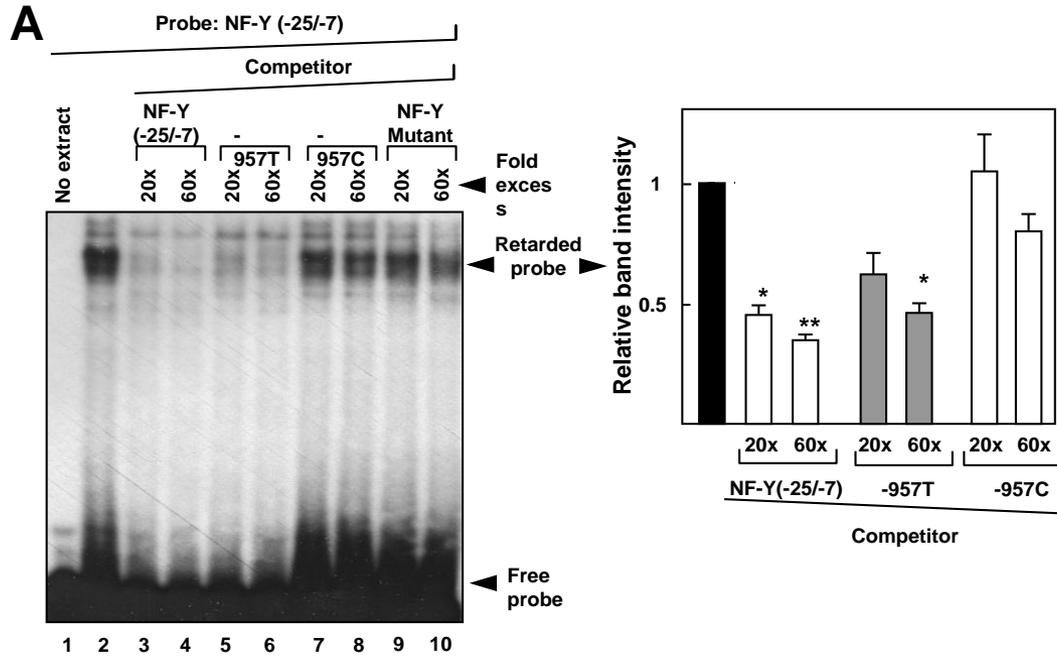| Haplotype	Frequency	No restenosis	Restenosis	P value	Odds Ratio	95% IC
H1 (T-C-G) n = 131	48.8 %	45.7 % n = 60	53.4 % n = 71	0.039	1.43 (H1 vs all other)	1.00-1.82
All other n = 138	51.2 %	54.3 % n = 75	45.6 % n = 63			

B

Cumulative TLR-free survival (%)

— All other haplotypes
— Haplotype 1

p=0.057
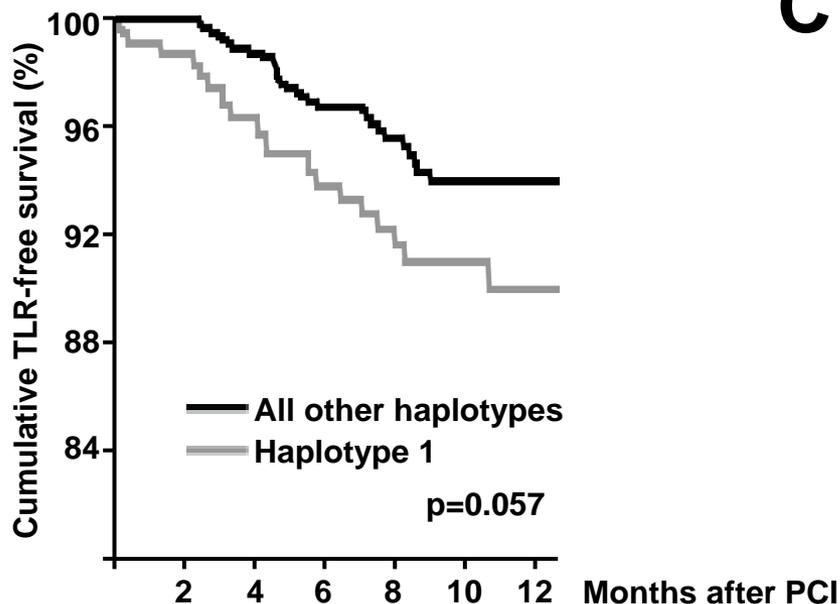
Months after PCI

<u>Number at risk</u>

Months after PCI:	0	2	4	6	8	10	12
All other haplotypes:	457	434	419	402	378	348	168
Haplotype 1:	135	124	118	113	106	101	48

C

Relative CCNB1 mRNA level

p = 0.0044

Haplotype 1 (T-C-G) n = 14

Haplotype 2 (C-T-T) n = 17

FIGURE 4

FIGURE 5

FIGURE 6

A

SP1 consensus probe

Relative retarded probe intensity (y-axis, values 0.5 and 1 marked)

Lanes: 1 2 3 4 5 6 7 8 9 10 11 12 13 14

Top labels: No extract | – | SP1 25 | +102G (100 200 300 600) | No extract | – | SP1 25 | +102T (100 200 300 600) ◄ Fold excess

Bottom labels: No extract | – | SP1 25 | +102G (100 200 300 600) | No extract | – | SP1 25 | +102T (100 200 300 600) ◄ Fold excess

Competitor

B

Probe ► SP1 consensus | + 102G | + 102T

Competitor or antibody: No extract | – | SP1 (100X) | Anti-SP1 | Anti-MEF2C | No extract | – | SP1 (100x) | Anti-SP1 | Anti-MEF2C | No extract | – | SP1(100X) | Anti-SP1 | Anti-MEF2C

Supershift ►
Retarded probe ►
Non-specific
Free probe ►

Lanes: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

FIGURE 7

FIGURE 8

Human *CCNB1* gene 5' regulatory region (promoter + 5'-untranslated)

Haplotype 1

Haplotype 2

rs 350099 T -957

rs 350104 C -475

+1

rs 164390 G +102

NF-Y

AP-1

SP1

rs 350099 C -957

rs 350104 T -475

+1

rs 164390 T +102

AP-1

DNA

Transcription

Transcription

G CODING REGION AAAA
G CODING REGION AAAA
G CODING REGION AAAA

mRNA

G CODING REGION AAAA

Translation

Translation

cyclin B1   cyclin B1   cyclin B1

PROTEIN

cyclin B1

CELLULAR PROLIFERATION

RISK OF RESTENOSIS

CELLULAR PROLIFERATION

RISK OF RESTENOSIS