Genetic Variants in *CCNB1* Associated With Differential Gene Transcription and Risk of Coronary In-Stent Restenosis

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DATA SUPPLEMENT

Expanded Methods

***In silico* analysis.** Putative transcription factor consensus binding sites for rs350099, rs350104 and rs164390 were identified using MatInspector (http://www.genomatix.de/)[1] and Match™, with the core similitude of transcription factor consensus sequence prediction limited to 0.85.

Cell culture. Human HeLa cells (CCL-2) and U2OS cells (HTB-96) from the American Type Culture Collection (Rockville, MD), and were maintained at 37ºC in 5% CO_2 in DMEM (Invitrogen, Breda, The Netherlands) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Sigma, St. Louis, Missouri) and 1% penicillin/streptomycin (Gibco, Invitrogen).

Electrophoretic mobility shift assay (EMSA). Subcellular fractionation and EMSA with 1-3 µg of nuclear extracts was carried out as previously described.[2, 3] Double-stranded oligonucleotides **(see sequences in supplemental Table IX)** were labeled with [^{32}P]dATP using polynucleotide kinase (New England Biolabs, Ipswich, MA) and purified on a Sephadex G-50 column (GE healthcare, UK). For supershift assays, before adding radiolabeled probe lysates were preincubated for 25 minutes with 1 µg of antibodies against NF-YA (sc-10779X), NF-YB (sc-13045X), CREBII (sc-22800X), c-Fos (sc-52), c-Jun (sc-44X), SP1 (sc-59G) and MEF2C (sc-13268) (all from Santa Cruz Biotechnology, Santa Cruz, CA). Binding reactions were resolved by electrophoresis at 4ºC on 5% polyacrylamide gels/0.5X TBE buffer under non-denaturing conditions. Gels were dried and autoradiographed and the intensity of the retarded bands was quantified with Metamorph and ImageQuant v5.2 (GE healthcare).

Luciferase reporter assay. 3x(-957T), 3x(-957C), 3x(-475C), 3x(-475T), 3x(+102G) and 3x(+102T) -luciferase reporter plasmids were generated by subcloning three repeats of a sequence containing the allelic variants from each SNP from the *CCNB1* promoter into KpnI/XhoI-digested pGL3 promoter plasmid (Promega, Madison, Wisconsin). **Supplemental Table X** shows the sequence of each tandem repeat. U2OS cells were transiently transfected by the calcium phosphate method. Cells were co-transfected with 1µg of 3x(-957T), 3x(-957C), 3x(-475C), 3x(-475T), 3x(+102G) or 3x(+102T) and 0.1µg of pRL-TK Renilla (Promega) as a control for transfection efficiency. When indicated, cells were co-transfected with 1µg of pNF-YAm29 (encoding the NF-YA dominant-negative mutant) or the control pSG5 plasmid (gift from R. Mantovani, Milan, Italy), 1µg pEGFP-cFos or empty pEGFP, or 3 µg of pCMV-SP1 or empty pCMV, as indicated. One day after transfection, cell lysates were prepared and luciferase activity was measured with the Dual Luciferase Reporter Assay kit (Promega). Luciferase and GFP expression was measured in a Wallac 1420 Victor luminometer (Perkin Elmer, Waltham, MA).

Quantitative real-time PCR (qPCR). Peripheral blood lymphocytes were obtained from 56 healthy donors who underwent a routine medical examination at Hospital Universitario Miguel Servet (Zaragoza, Spain) (**see clinical characteristics in supplemental Table VIII**).[4] The local research committee approved the study and all subjects gave written informed consent. Cells were genotyped by the high resolution melting curves method in order to detect the two most frequent

haplotypes: -957T/-475C/+102G (H1) and -957C/-475T/+102T (H2) (**see supplemental Table V and Table VII**). In total, 31 individuals had either H1 (n=14) or H2 (n=17). Peripheral blood mononuclear cells obtained by density gradient centrifugation[5] were washed twice with RPMI-1640 (Invitrogen), resuspended in complete RPMI-1640 (with GlutaMAX I, Invitrogen) containing 10% heat-inactivated FBS, Sigma) and 5 µg/ml PHA-M (Sigma), and plated at $2x10^6$ cells/ml. After 24h, adherent cells were discarded and peripheral blood lymphocytes were maintained for 7d in RPMI-1640 supplemented with 10% FBS (37ºC, 5% CO_2). Cell viability assessed by trypan blue exclusion was >95%. Total RNA was isolated from lymphocytes using the RNeasy kit according to the manufacturer's instructions (Qiagen, Valencia, CA). RNA concentration and purity were assessed from the A260nm/A280nm ratio, and integrity was verified by separation on ethidium bromide-stained 1% agarose gels. RNA (0.5 to 2µg) was retrotranscribed using SuperScript III RNase H reverse transcriptase (Invitrogen). qPCR of lymphocytes was performed using FAM dye-labeled TaqMan MGB probes (Assay-on-Demand, RPLP0, Hs99999902_m1 and *CCNB1*, Hs00259126_m1) according to the manufacturer's recommendations (Applied Biosystems). *CCNB1* mRNA levels were normalized to the housekeeping gene RPLP0. qPCR results were analyzed by the comparative Ct method**.**

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SUPPLEMENTAL TABLE I. Clinical characteristics of patients with and without angiographic in-stent restenosis: Clinica Mediterranea cohort

	No restenosis (n = 168)	Restenosis (n = 116)	p-value
Age	59 ± 11	63 ± 9	0.002
Sex	Male: 75%; Female: 25%	Male: 77%; Female: 23%	0.71
Angina pectoris *			
Class I	20.7%	13.6%	0.33
Class II	61.9%	66.2%	
Class III	17.4%	20.2%	
Diabetes mellitus	36.4%	35.8%	0.85
Left ventricular ejection fraction	56±10%	56±90%	0.81
Previous myocardial infarction	48.5%	47.8%	0.91
Family history of CAD	31.5%	38.2%	0.64
Systemic hypertension	64.6%	57.5%	0.15
Hypercholesterolemia	51.2%	49.1%	0.30
Active smokers	23.1%	20%	0.70
Statins	89.4%	89.1%	0.96
eGFR, ml/min/1.73 m^2	67.5 ± 17.4	67.6 ± 17.6	0.93
Plasmatic lipids, mg/dL			
Total cholesterol	181±43	163±38	<0.001
LDL-cholesterol	99±34	89±31	0.035
HDL-cholesterol	46±12	46±15	0.84
Triglycerides	147±74	156±731	0.84

* According to the classification of the Canadian Cardiovascular Society.

CAD: coronary artery disease; **eGFR:** estimated glomerular filtration rate; **HDL:** high-density lipoprotein; **LDL:** low-density lipoprotein.

SUPPLEMENTAL TABLE II. Angiographic characteristics of percutaneous coronary intervention patients with and without angiographic in-stent restenosis: Clinica Mediterranea cohort

	No restenosis (n = 168)	Restenosis (n = 116)	p-value
Extension of CAD			
1-vessel	29.9%	27.2%	
2-vessel	42.1%	41.2%	0.79
3-vessel	28%	31.6%	
Characteristics of the objective vessel			
LAD	45%	44%	
LCx	26%	26.3%	0.21
RCA	29.5%	29%	
Left main	0.5%	0.7%	
Lesion Site			
Ostial	8%	5%	
Proximal	40%	46%	0.73
Midvessel	46%	44.5%	
Distal	6%	4.5%	
Lesion type			
A	12%	13%	
B1	24%	22%	0.48
B2	36%	36%	
C	28%	29%	
Diameter stenosis, %			
Baseline	86 ± 10	86 ± 12	0.57
Post-procedure	1 ± 3	1 ± 3	0.39
Follow-up	25 ± 14	78± 15	<0.001
Reference Vessel Diameter, mm			
Baseline	3.20 ± 0.58	3.20 ± 0.51	0.79
Post-procedure	3.34 ± 0.558	3.32 ± 0.58	0.82
Follow-up	3.31± 0.47	3.24 ± 0.46	0.08
Minimal Lumen Diameter, mm			
Baseline	0.48 ± 0.37	0.45 ± 0.37	0.35
Post-procedure	3.32 ± 0.56	3.31 ±0.54	0.80
Follow-up	3.01 ± 0.70	0.88 ± 0.95	<0.001
Acute gain, mm	2.93 ± 0.50	2.98 ± 0.45	0.74
Late loss, mm	0.55 ± 0.31	2.31 ± 0.15	<0.001
Loss index	0.19 ± 0.11	0.91 ± 0.23	<0.001
Lesion length, mm	18.6 ± 9.8	17.3 ± 9.0	0.26

CAD: coronary artery disease; **LAD:** left anterior descending artery; **LCx:** left circumflex artery; **RCA:** right coronary artery

Table III. Major adverse coronary events (MACE) after 12 months in percutaneous coronary intervention patients with and without angiographic in-stent restenosis: Clinica Mediterranea cohort

	No restenosis (n = 168)	Restenosis (n = 116)	p-value
Cumulative MACE at 12 months	20 (12%)	103 (89%)	<0.001
Death (any cause)	2 (1.2%)	2 (1.7%)	1.00
Myocardial infarction	3 (2.0%)	3 (2.6%)	0.69
Repeat PCI	16 (9.5%)	97 (83.5%)	<0.001
TLR	0	93 (80%)	<0.001
New lesions	16 (9.5%)	11 (9.4%)	1.00

The numbers given for any MACE in the cumulative analysis do not equal the sum numbers given for each single event (death, myocardial infarction, repeat percutaneous coronary intervention: PCI) because some patients had more than one MACE. Myocardial infarction after the intervention was defined as either the development of pathologic Q waves lasting at least 0.4 second in at least two contiguous leads with an elevated creatine kinase MB isozyme level or, in the absence of pathologic Q waves, an elevation in total creatine kinase levels to more than twice the upper limit normal with elevated creatine kinase MB level. Target lesion revascularization (TLR) was defined as repeated revascularization to treat recurrent ischemia owing to stenosis of at least 50 percent of the luminal diameter anywhere within the stent or within the 5-mm borders proximal or distal to the stent. New lesion was defined as new critical (>70%) stenosis occurring in the same vessel (without the criteria for in-stent restenosis) or in other vessels in association with clinical signs or symptoms of ischemia.

SUPPLEMENTAL TABLE IV. Sequences of primers used for PCR amplification of genomic DNA regions containing each SNP and for their analysis by high resolution melting curves: Clinica Mediterranea cohort

SNP	Forward sequence	Reverse sequence
rs350099	5'-AATAACGATCCAAAGAAACCAAATG-3'	5'-AATAACGATCCAAAGAAACCAAATG-3'
rs350104	5'-CCCCGTTGCTAATGTGTGA-3'	5'-GACATTCTTTCATTTGATCGTTGC-3'
rs164390	5'-GAGGCTAGGCTGGCTCTTCTC-3'	5'-CATGGCTTCCTCTTCACCAG-3'

SUPPLEMENTAL TABLE V. Haplotype frequency estimation: Clinica Mediterranea cohort

	SNP			Frequency		
Haplotype	rs350099 -957[T/C]	rs350104 -475[C/T]	rs164390 +102[G/T]	Total	No restenosis	Restenosis
1	T	C	G	0.488	0.457	0.534
2	C	T	T	0.330	0.365	0.280
3	T	T	G	0.119	0.111	0.130
4	T	T	T	0.044	0.047	0.042
5	T	C	T	0.011	0.008	0.015
6	C	C	G	0.006	0.009	NA
7	C	T	G	0.002	0.003	NA
8	C	C	T	0	0	0

NA: Not Available

SUPPLEMENTAL TABLE VI. Linkage disequilibrium analysis: Clinica Mediterranea cohort

D statistic

	rs164390	**rs350099**	**rs350104**
rs164390	-	0.1999	0.184
rs350099	-	-	0.163
rs350104	-	-	-

D' statistic

	rs164390	**rs350099**	**rs350104**
rs164390	-	0.964	0.948
rs350099	-	-	0.964
rs350104	-	-	-

r statistic

	rs164390	**rs350099**	**rs350104**
rs164390	-	0.868	0.756
rs350099	-	-	0.691
rs350104	-	-	-

p-value

	rs164390	**rs350099**	**rs350104**
rs164390	-	0	0
rs350099	-	-	0
rs350104	-	-	-

SUPPLEMENTAL TABLE VII. Haplotype frequency estimation: GEISHA cohort

Haplotype	SNP			Frequency		
	rs350099 -957[T/C]	rs350104 -475[C/T]	rs164390 +102[G/T]	Total	No restenosis	Restenosis
1	T	C	G	0.476	0.500	0.471
2	C	T	T	0.407	0.412	0.406
3	T	T	G	0.096	0.088	0.098
4	T	T	T	0.010	0.012	NA
5	T	C	T	0.009	0.011	NA
6	C	C	G	0.001	0.002	NA
7	C	T	G	0	0	0
8	C	C	T	0	0	0

SUPPLEMENTAL TABLE VIII. Clinical characteristics of subjects included in qPCR studies in lymphocytes

	-975T-475C+102G (n = 14)	**-975C-475T+102T (n = 17)**	**p-value**
Age	46.6 ± 4.33	47.88±3.71	0.82
Sex	Male: 42.80% Female: 57.14%	Male:11.76% Female: 88.23%	0.09
Systemic hypertension	7.14%	17.64%	0.60
Body mass index	24.84±0.97	25.12 ± 0.93	0.83
Active smokers	14.28%	5.88%	0.57
Plasmatic lipids (mg/dL)			
LDL-cholesterol	153.40±14.26	153.6±15.08	0.99
HDL-cholesterol	49.46±4.03	60.06±3.62	0.06
Triglycerides	121.10±38.64	101.80±22.79	0.65

HDL: high-density lipoprotein; **LDL:** low-density lipoprotein.

SUPPLEMENTAL TABLE IX. Sequences of oligonucleotides used for EMSA

Probes	Sequence
-957T	5'-GAGTCTCTATTGGCTCTTATACC-3'
-957C	5'-GAGTCTCTATCGGCTCTTATACC-3'
NF-Y (-25/-7)	5'-GGCAGCCGCCAATGGGAAGG-3'
NF-Y(-26/-1) (NF-Y consensus)	5'-CCGCAGCCGCCAATGGGAAGGGAGTGA-3
NF-Y(-26/-1) mutant (NF-Y mutant)	5'-CCGCAGCCGTTAATGGGAAGGGAGTGA-3'
-475C	5´-TAATGTGTGACCCTGGCAAAG-3´
-475T	5´-TAATGTGTGATCCTGGCAAAG-3´
+102G	5´-TCTGCTGGGTGTAGGTCCTTGGCTGGT-3´
+102T	5´-TCTGCTGGTTGTAGGTCCTTGGCTGGT-3´
AP-1 consensus	5´-CGCTTGATGAGTCAGCCGGAA-3´
SP1 consensus	5′-ATTCGATCGGGGCGGGGCGAGC-3′

Only the upper strand of the double-stranded oligonucleotide is shown. -957T and -957C contain the T and C polymorphic variants of rs350099, respectively. NF-Y(-25/-7) and NF-Y(-26/-1) contain the NF-Y DNA-binding sites (CCAAT box) present in the human *CCNB1* promoter region at position -17/-13. NF-Y(-26/-1) mutant bears a CC→TT mutation that disrupts the CCAAT box in NF-Y(-26/-1) (mutated nucleotides are underlined). -475C and -475T contain the C or T polymorphic variants of rs350104. AP-1 consensus contains the consensus AP-1 DNA-binding site (TGAG box). +102G and +102T contain the G or T polymorphic variants of rs164390. SP1 consensus contains the consensus SP1 DNA-binding site (GC box).

SUPPLEMENTAL TABLE X. Tandem repeat sequences used to drive luciferase reporter expression

Construct	Forward sequence
3x(-957T)	5’-CGAGTCTCTATTGGCTCTTATAC/CGAGTCTCTAT TGGCTCTTATAC/CGAGTCTCTATTGGCTCTTATAC/CC-3’
3x(-957C)	5’-CGAGTCTCTATCGGCTCTTATAC/CGAGTCTCTATC GGCTCTTATAC/CGAGTCTCTATCGGCTCTTATAC/CC-3’
3x(-475T)	5’-CTAATGTGTGATCCTGGCAAAG/TAATGTGTGA TCCTGGCAAAG/TAATGTGTGATCCTGGCAAAG/C-3´
3x(-475C)	5’-CTAATGTGTGACCCTGGCAAAG/TAATGTGTGA CCCTGGCAAAG/TAATGTGTGACCCTGGCAAAG/C-3´
3x(+102G)	5’-TCTGCTGGGTGTAGGTCCTTGGCTGGT/TCTGCTGGGTGTA GGTCCTTGGCTGGT/TCTGCTGGGTGTAGGTCCTTGGCTGGT-3’
3x(+102T)	5’-TCTGCTGGTTGTAGGTCCTTGGCTGGT/TCTGCTGGTTGTA GGTCCTTGGCTGGT/TCTGCTGGTTGTAGGTCCTTGGCTGGT-3’

Only the upper strand of the double-stranded oligonucleotide is shown.