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Genetic, clinical and experimental aspects of restenosis : a biomedical perspective

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GENETIC INFLAMMATORY FACTORS PREDICT RESTENOSIS AFTER PERCUTANEOUS CORONARY INTERVENTIONS

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Abstract

Background

Percutaneous coronary intervention (PCI) suffers from restenosis. No clinical factors are available that allow good risk stratification. However, evidence exists that genetic factors are important in the restenotic process as well as in the process of inflammation, a pivotal factor in restenosis. Association studies have identified genes that may predispose to restenosis, but confirmation by large prospective studies is lacking. Our aim was to identify polymorphisms and haplotypes in genes involved in inflammatory pathways that predispose to restenosis.

Methods and Results

The GENetic DEterminants of Restenosis (GENDER) project is a multicenter prospective study, including 3,104 consecutive patients after successful PCI. Forty-eight polymorphisms in 34 genes in pathways possibly involved in the inflammatory process were analysed. The 16Gly variant of the beta-2 adrenergic receptor gave an increased risk of Target Vessel Revascularization (TVR). The rare alleles of the CD14 gene (-260T/T), colony stimulating factor 2 gene (117Thr/Thr) and eotaxin gene (-1328A/A) were associated with decreased risk of TVR. However, using multiple testing corrections by means of permutation analysis the probability to find four significant markers by chance was 12%.

Conclusions

Polymorphisms in four genes considered involved in the inflammatory reaction showed an association with TVR after PCI. Our results may contribute to the unravelling of the restenotic process. Given the explorative nature of this analysis, our results need to be replicated in other studies.

Introduction

Restenosis is still the major limitation of percutaneous coronary interventions (PCI), resulting from injury of the vessel wall caused by balloon dilation and stent placement.^(1;2) The vascular damage is characterized by irritation of endothelial and subendothelial structures and injury of medial regions with rupture of the internal elastic lamina. This damage causes segmental thrombus formation and subsequent invasion of macrophages and polymorphonuclear leukocytes, followed by expression and release of numerous growth factors and cytokines from blood cells and stretched smooth muscle cells, leading to proliferation of smooth muscle cells.^(3;4) Vascular inflammation thus plays an important role in this complex multifactorial process.⁽⁵⁻⁷⁾

Identifying patients at increased risk of restenosis may improve stratification of patients to individually tailored treatment. Thus far, however, it has proven difficult to stratify patients with regard to risk of coronary restenosis based only upon clinical or procedural risk factors, since risk factors in relation to restenosis identified so far have not been consistently reported.⁽⁸⁾ There are indications that genetic factors explain part of the excessive risk of restenosis independently of conventional clinical variables. In patients with multivessel disease the incidence of restenosis of a second lesion was 2.5 times higher if the first lesion had restenosis, even after adjustments for well-known patient related risk factors, including diabetes and hypertension.⁽⁹⁾ Inflammatory responsiveness is highly genetically determined. Many studies have demonstrated genetic influences upon the inflammatory response of an individual.^(10;11) Therefore, it is plausible that differences in genetic make-up of inflammatory genes between individuals may explain part of the risk of the at least partially inflammation driven restenotic process. Association studies have identified several candidate genes that may predispose to restenosis, such as the genes for stromelysin-1, IL6, E-selectin, CD18, CD14 and IL1 receptor.^(12;13) However, these studies are mostly inconclusive due to a number of limitations, including limited study size and study design (e.g. not prospective studies or studies using selected patient groups), limiting the clinical value of the observations.

The purpose of this study was to evaluate in a large unselected study sample whether a variety of genetic determinants, considered to be involved in the inflammatory process, can predict the risk of clinical restenosis after PCI.

Methods

Study design

The present study sample has been described previously.⁽¹⁴⁾ In brief, the GENetic DEterminants of Restenosis project (GENDER) was designed to study the association between various gene polymorphisms and clinical restenosis defined in our study by Target Vessel Revascularization (TVR). Patients were eligible for inclusion if they were successfully treated for stable angina, non-ST-elevation acute coronary syndromes or silent ischemia by PCI. Patients treated for acute ST elevation myocardial infarction (MI) were excluded. All patients were treated in four referral centers for interventional cardiology in the Netherlands (Academic Medical Center Amsterdam, Academic Medical Center Groningen, Leiden University Medical Center and Academic Hospital Maastricht). The overall inclusion period lasted from March 1999 until June 2001. In total, 3,104 consecutive patients were included in this prospective multicenter follow-up study.

The study protocol conforms to the Declaration of Helsinki and was approved by the Medical Ethics Committees of each participating institution. Written informed consent was obtained from each participant before the PCI procedure.

PCI procedure

Standard angioplasty and stent placement were performed by experienced operators using a radial or femoral approach. Before the procedure patients received aspirin 300 mg and heparin 7,500 IU. The use of intracoronary stents and additional medication, such as glycoprotein IIb/IIIa inhibitors, was at the discretion of the operator. If a stent was implanted, patients received either ticlopidine or clopidogrel for at least one month following the procedure depending on local practice. During the study no drug-eluting stents were used.

Follow-up and study endpoints

Follow-up lasted at least nine months or until a coronary event occurred. Patients were either seen in the outpatient clinic or contacted by telephone. TVR, either by PCI or coronary artery bypass grafting (CABG), was considered as the primary endpoint, since it is considered most relevant for clinical practice by regulatory agencies. An independent clinical events committee adjudicated the clinical events.

Events occurring within one month after the procedure were excluded from the analysis, since these events were attributable to sub-acute stent thrombosis or occluding dissections.

Data were collected with standardized case-report forms that were completed by the research coordinator at each site who was blinded to the genotype of the patients. Representatives from the data-coordinating center monitored the sites.

Genetic methodology

Blood was collected in EDTA tubes at baseline and genomic DNA was extracted following standard procedures. Genotyping was performed by a validated multilocus genotyping assay to test several markers of inflammation (Roche Molecular Systems).^(15;16) Inflammation is known to play an important role in the development of restenosis, although it is not fully elucidated which factors are exactly involved in the process. Therefore, we intended to examine a very broad spectrum of factors that are considered to have an effect on the inflammatory response. The polymorphisms on the array were chosen based on their relation to inflammation previously described in literature reports of gene associations with inflammatory diseases. Furthermore, polymorphisms were selected also on their known functionality or their known allele frequency. In addition developing the assay for the selected polymorphism had to be possible. A total of 48 markers in 34 genes were examined using these criteria (Table 1). Each DNA sample was amplified in multiple polymerase chain reactions (PCRs) using biotinylated primers. The PCR product was then hybridised to a corresponding panel of sequence-specific oligonucleotide probes that had been immobilized in a linear array on nylon membrane strips.⁽¹⁷⁾ A colorimetric detection method based on incubation with streptavidin-horseradish peroxidase conjugate, using hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine as substrates, was used. Operators blinded to patient data performed genotyping. To confirm genotype assignments, PCR analysis was randomly performed in replicate on 10% of the samples. Two independent observers carried out scoring. Disagreements (<1%) were resolved by further joint reading, and when necessary a repeat genotyping reaction was performed.

Table 1. The 48 different polymorphisms within 34 genes determined by assay

Gene	Chromosome position	SNP
Adrenergic beta-2 receptor (ADRB2)	5q31-q32	Arg16Gly
	5q31-q32	Gln27Glu
	5q31-q32	Thr164Ile
Intercellular adhesion molecule 1 (ICAM1)	19p13.2	Lys56Met
	19p13.2	Gly241Arg
Vascular adhesion molecule 1 (VCAM1)	1p32-p31	-1594T/C
Selectin E (SELE)	1q22-q25	Ser149Arg
Selectin P (SELP)	1q21-24	Ser330Asn
	1q21-24	Val640Leu
Fc fragment of IgE (FCER1A)	11q13	Glu237Gly
CD14 antigen (CD14)	5q22-32	-260C/T
Uteroglobin (SCGB1A1)	11q11-qter	+38G/A
Transforming growth factor beta 1 (TGFB1)	19q13.1	-509C/T
Chemokine receptor 2 (CCR2)	3p21	Val62Ile
Chemokine receptor 3 (CCR3)	3p21.3	Pro39Leu
Chemokine receptor 5 (CCR5)	3p21	deletion
	3p21	-2454G/A
T-cell transcription factor (TCF7)	5q31	Pro19Thr
Interleukin 1, alpha (IL1A)	2q12-q21	-889T/C
Interleukin 1, beta (IL1B)	2q14	-1418C/T
	2q14	105C/T
Interleukin 4 (IL4)	5q23-q31	-589C/T
Interleukin 4 receptor (IL4R)	16p11.2-p12.1	Ile50Val
	16p11.2-p12.1	Ser478Pro
	16p11.2-p12.1	Gln576Arg
Interleukin 5 receptor (IL5R)	3p26-p24	-80G/A
Interleukin 6 (IL6)	7p21-p15	-572G/C
	7p21-p15	-174G/C
Interleukin 9 (IL9)	5q31-q35	Thr113Met
Interleukin 10 (IL10)	1q31-q32	-571C/A
Interleukin 13 (IL13)	5q31	4045 C/T
Complement component 3 (C3)	19p13.3-p13.2	Arg102Gly
Complement component 5 (C5)	9q32-q34	Ile802Val
Colony stimulating factor 2 (CSF2)	5q31.1	Ile117Thr
Leukotriene C4 synthase (LTC4S)	5q35	-444A/C
Cytotoxic T-lymphocyte associated protein 4 (CTLA4)	2q33	-318C/T
	2q33	Thr17Ala
Nitric oxide synthase 2 (inducible) (NOS2)	17q11.2-q12	346C/T
Nitric oxide synthase 3 (endothelial cell) (NOS3)	7q35-36	-948A/G
	7q35-36	Glu298Asp
Small inducible cytokine subfamily A, member 11, alias Eotaxin (CCL11)	17q21.1-q21.2	Ala23Thr
	17q21.1-q21.2	-1328G/A-
Lymphotoxin alpha: (LTA)	6p21.3	1069 A/G
Stromal cell derived factor 1 (CXCL12)	10q11.1	+800G/A
Vitamin D receptor (VDR)	12q13.1	Met1Thr
	12q13.1	Bsml A/G
Group specific component (vit D binding protein) (GC)	4q12-q13	Glu416Asp
	4q12-q13	Thr420Lys

Statistical methodology

Deviations of the genotype distribution from that expected for a sample in Hardy-Weinberg equilibrium were tested using the Chi-squared test with one degree of freedom. Allele frequencies were determined by gene counting. The 95% confidence intervals of the allele frequencies were calculated from sample allele frequencies based on the approximation of the binominal and normal distributions in large sample sizes.

In the first stage we determined the association between each of the 48 polymorphisms and TVR using a Cox proportional regression model. We considered co-dominant, dominant, and recessive inheritance models, and the model with the lowest Akaike information criterion was used.⁽¹⁸⁾ If less than 10 patients were homozygous for a particular allele, the homozygotes and heterozygotes were taken together, thereby assuming a dominant model. No adjustment for covariates was performed at this stage to allow for the assessment of the possible involvement of the polymorphisms in the causal pathway for TVR. Haplotypes were constructed from polymorphisms known to be located in the same gene, or in genes located near each other, and in statistically significant linkage disequilibrium (all p-values of the Pearson Chi-squared test <0.001). The effect of haplotypes on restenosis risk was estimated according to the methods developed by Tanck et al.⁽¹⁹⁾ We considered only one TVR per patient.

The robustness of these 'individual' findings was investigated by a bootstrap study of 1,000 bootstrap samples drawn from the original data set. In each bootstrap sample the optimal inheritance model as well as the association between each of the polymorphisms and TVR was determined, and the percentage of bootstrap samples with a significant association was counted for each polymorphism.

Following the SNP-selection method of Hoh et al. we performed multivariable regression analysis of the TVR risk with all polymorphisms and haplotypes having an individual p-value of 0.10 or less and being significant in at least 40% of the bootstrap samples.⁽²⁰⁾ Firstly, the association between each polymorphism and TVR was adjusted for the confounding effect of the clinical risk factors age and sex and other clinical and intervention related risk factors that were significantly ($p < 0.10$) associated with TVR, being diabetes, stenting, residual stenosis $>20\%$, current smoking, total occlusion and hypertension. If two or more arterial segments were treated we only used intervention related characteristics of the most severely affected segment.

Secondly, polymorphisms with independent prognostic value were selected using the multivariable regression model using a stepwise backward selection algorithm.

No multiple testing correction method was applied to keep power at an accept

able level, and we therefore performed a permutation study to assess the experiment wide error-rate. One thousand permutation samples were created by reshuffling at random the 304 observed TVRs over all available patients. In each permuted (reshuffled) sample the same statistical analysis was performed as described above, and we counted the number of reshuffled samples with zero, one, two, three, four, or five significant polymorphisms in the final multivariable regression model. In reshuffled data no significant association is expected, and the percentage of re-shuffled samples with one or more significant polymorphisms is a quantification of the experiment-wide error rate.

Statistical analysis was carried out using SPSS 11.5 (SPSS Inc., Chicago, IL).

Results

Patient characteristics

The characteristics of this patient sample have been described previously.⁽¹⁴⁾ In summary, a total of 3,146 patients had a complete follow-up (99.3%) with a median duration of 9.6 months (interquartile range 3.9). Out of 3,146 patients 42 had an event in the first 30 days. These patients were excluded from further analysis, according to the protocol. The remaining 3,104 patients had a mean age of 62.1 ± 10.7 years. Of the patients 888 (28.6%) were female, and 453 (14.6%) had diabetes mellitus. The majority of the patients were Whites (97%). 812 patients (26.2%) received glycoprotein IIb/IIIa inhibitors. Stents were used in 2,309 (74.4%) patients. A total of 4,061 lesions were treated in this unselected patient sample. Complex (type C) lesions, classified according to the modified American College of Cardiology and American Heart Association Task Force classification, were treated in 802 patients (25.8%). Other patient characteristics, as well as details of the interventions are summarized in table 2.

Table 2. Demographic, clinical and lesion characteristics of 3,104 patients with (cases) and without TVR (controls) after one-month follow-up

	Cases (n=304)	Controls (n=2,800)	Total (n=3,104)	P-value*
Age (years)	61.7 ± 10.1	62.2 ± 10.8	62.1 ± 10.7	0.56
BMI (kg.m ⁻²)	26.9 ± 3.7	27.0 ± 3.9	27.0 ± 3.9	0.73
Male sex	220 (72.4%)	1,996 (71.3%)	2,216 (71.4 %)	0.73
Diabetes	63 (20.7%)	390 (13.9%)	453 (14.6%)	0.002
Hypercholesterolemia	188 (61.8%)	1,702 (60.8%)	1,890 (60.9%)	0.75
Hypertension	138 (45.4%)	1,121 (40.0%)	1,259 (40.6%)	0.051
Current smoker	62 (20.4%)	700 (25.0%)	762 (24.5%)	0.062
Family history of MI	121 (39.8%)	977 (34.9%)	1,098 (35.4%)	0.13
Previous MI	109 (35.9%)	1,130 (40.4%)	1,239 (39.9%)	0.12
Previous PCI	64 (21.1%)	493 (17.6%)	557 (17.9%)	0.14
Previous CABG	36 (11.8%)	340 (12.1%)	376 (12.1%)	0.97
Stable angina	198 (65.1%)	1,881 (67.2%)	2,079 (67.0%)	0.46
Multivessel disease	148 (48.7%)	1,284 (45.9%)	1,432 (46.1%)	0.26
Peripheral vessel disease	12 (3.9%)	92 (3.3%)	104 (3.4%)	0.34
Lipid lowering medication	171 (56.3%)	1,516 (54.1%)	1,687 (54.3%)	0.53
Restenotic lesions	27 (8.9%)	181 (6.5%)	208 (6.7%)	0.12
Total occlusions	56 (18.4%)	372 (13.3%)	428 (13.8%)	0.048
Type C lesion	94 (30.9%)	708 (25.3%)	802 (25.8%)	0.18
Proximal LAD	70 (23.0%)	619 (22.1%)	689 (22.2%)	0.60
RCX	75 (24.7%)	764 (27.3%)	839 (27.0%)	0.35
Residual stenosis >20%	51 (16.8%)	299 (10.7%)	350 (11.3%)	0.001
Stent length (mm)	10.3 (0-82)	13.0 (0-93)	15 (0-146)	0.80
Diameter stenosis pre-intervention	89% (10%)	89% (10%)	89% (10%)	0.83

BMI: body mass index, MI: myocardial infarction, LAD: left anterior descending branch of the left coronary artery, RCX: circumflex branch of the left coronary artery

** P value, determined by Cox regression analysis*

Follow-up

Of the 3,104 patients, 304 (9.8%) patients underwent TVR during follow-up. Fifty-one patients died (1.6%) and 22 (0.7%) had a myocardial infarction.

Inflammation array results

In the present study genotyping was performed in 3,029 patients of the total sample. Results of the remaining patients ($n=75$, 2.4%) are lacking due to unavailable DNA or inconclusive genotyping. Patients who could not be genotyped did not differ in any characteristic from those who could be genotyped.

The following polymorphisms were associated with TVR ($p<0.05$): beta-2 adrenergic receptor (ADRB2) Arg16Gly, CD14 -260C/T, colony stimulating factor 2 (CSF2) Ile117Thr, and small inducible cytokine subfamily A, member 11, alias Eotaxin (CCL11) -1328G/A (Table 3), being significant in 65.0%, 61.8%, 73.7%, and 69.3% of bootstrap samples, respectively. Interleukin 4 receptor (IL4R) Gl n576Arg and T-cell transcription factor (TCF7) Pro19Thr polymorphisms also showed a tendency of association with TVR ($0.05<P<0.1$), and were significant in 43.6% and 44.2% of the bootstrap samples, respectively. All significant genotype distributions were in accordance to Hardy-Weinberg equilibrium, and markers not in equilibrium were excluded from further analysis.

TVR occurred more often in ADRB2 Gly16 homozygotes (11.3%) than in Arg16Gly heterozygotes (8.7%) or Arg16 homozygotes (9.1%). In contrast, CD14 T/T-genotypes had decreased TVR-risk (7.6%) compared to C/T genotypes (10.5%) and C/C genotypes (10.3%). CSF2 Thr homozygotes (6.6%) and Ile/Thr heterozygotes (8.5%) also showed a decreased risk of TVR compared to Ile genotypes (10.8%). CCL11 -1328 A/A-homozygotes had decreased TVR-risk (4.3%) compared to G/A-heterozygotes (8.6%) and G/G-homozygotes (10.5%) (Table 3). More details are provided in Data Supplement table 1 (<http://circ.ahajournals.org/cgi/content/full/112/16/2417/DC1>).

Table 3. Individual analysis of polymorphisms in association with TVR and the distributions of the polymorphisms

Polymor- phisms	Genotype fre- quencies (%)	TVR (%)	No TVR (%)	Best fitting genetic model	P-value*	P-value**
IL4R 576						
Gln/Gln	1,823 (60.3)	10.6	89.4	Additive	0.077	0.11
Gln/Arg	1,071 (35.5)	8.7	91.3			
Arg/Arg	128 (4.2)	7.8	92.2			
ADRB2 16						
Arg/Arg	1,224 (40.2)	9.1	90.9	Recessive	0.020	0.015
Arg/Gly	1,420 (46.7)	8.7	91.3			
Gly/Gly	397 (13.1)	11.3	88.7			
CD14						
C/C	863 (28.4)	10.3	89.7	Recessive	0.027	0.037
C/T	1,483 (48.8)	10.5	89.5			
T/T	694 (22.8)	7.6	92.4			
TCF7						
Pro/Pro	2,449 (80.6)	10.3	89.7	Additive	0.070	0.065
Pro/Thr	555 (18.2)	7.9	92.1			
Thr/Thr	35 (1.2)	5.7	94.3			
CSF2						
Ile/Ile	1,881 (62.0)	10.8	89.2	Additive	0.013	0.024
Ile/Thr	1,001 (33.0)	8.5	91.5			
Thr/Thr	151 (5.0)	6.6	93.4			
CCL11 (-1328)						
G/G	2,126 (70.1)	10.5	89.5	Additive	0.020	0.014
G/A	813 (26.8)	8.6	91.4			
A/A	92 (3.1)	4.3	95.7			

* P value after individual analysis using Cox regression analysis

** P value after adjustment for clinical confounders using Cox regression analysis

As arterial remodelling is more evident in plain balloon angioplasty and neointima formation is more pronounced in stenting,⁽²¹⁾ we examined whether the association between the polymorphisms and TVR differed between patients who received stents and patients who did not receive stents. Analysing the interaction between balloon angioplasty/stenting and the significantly associated genes with TVR showed no significant difference (CD14, $p=0.34$; ADRB2, $p=0.83$; CSF2, $p=0.30$ and CCL11, $p=0.67$), and neither was there a significant difference between angioplasty and stented patients with respect to the association of any of the other polymorphisms with TVR ($p>0.09$). Since of course the power to find an interaction between type of PCI (angioplasty versus stent) and associations is limited, such interactions cannot be fully excluded. Separately within the balloon angioplasty treated patient group and the stented patient group the hazard ratios are in the same direction, but many of the 95% confidence intervals cross the value of 1.0 (see Data Supplement table 2).

Linkage disequilibrium and haplotypes

The three IL4R-polymorphisms were in linkage disequilibrium (LD) ($p<0.0001$), and the same applied to the polymorphisms of the ADRB2, CTLA4, NOS3, CCL11, IL1A, IL1B, and IL6 genes. Furthermore, LD was found between polymorphisms in the IL4, IL13 and CSF2 genes. Significant LD was observed between SELE and the SELP (Val640Leu) polymorphism and involving the two CCR5- and the CCR2 polymorphisms. Except for a haplotype in the ADRB2-gene, none of the haplotypes were significantly associated with TVR risk ($p>0.13$). ADRB2-haplotypes including the ADRB2 16Gly allele were all associated with increased TVR-risk, whereas haplotypes without the ADRB2 16Gly allele had lower TVR-risk. In subsequent analyses we therefore used the ADRB2 16 genotype, and not these haplotypes.

Multivariable Cox regression

Adjustment of the association between the selected polymorphisms and TVR for the clinical risk factors age, sex, diabetes, stenting, residual stenosis $>20\%$, current smoking, hypertension and total occlusion changed little (last column of Table 3); ADRB2 Arg16Gly, CD14 -260C/T, CSF2 Ile117Thr and CCL11 -1328G/A polymorphisms remained significantly related to TVR.

Subsequently, we included all selected polymorphisms in the multivariable regression analysis and also included the aforementioned clinical risk factors. After stepwise backward selection among the selected polymorphisms the same polymorphisms appeared to be significantly related to TVR (Table 4).

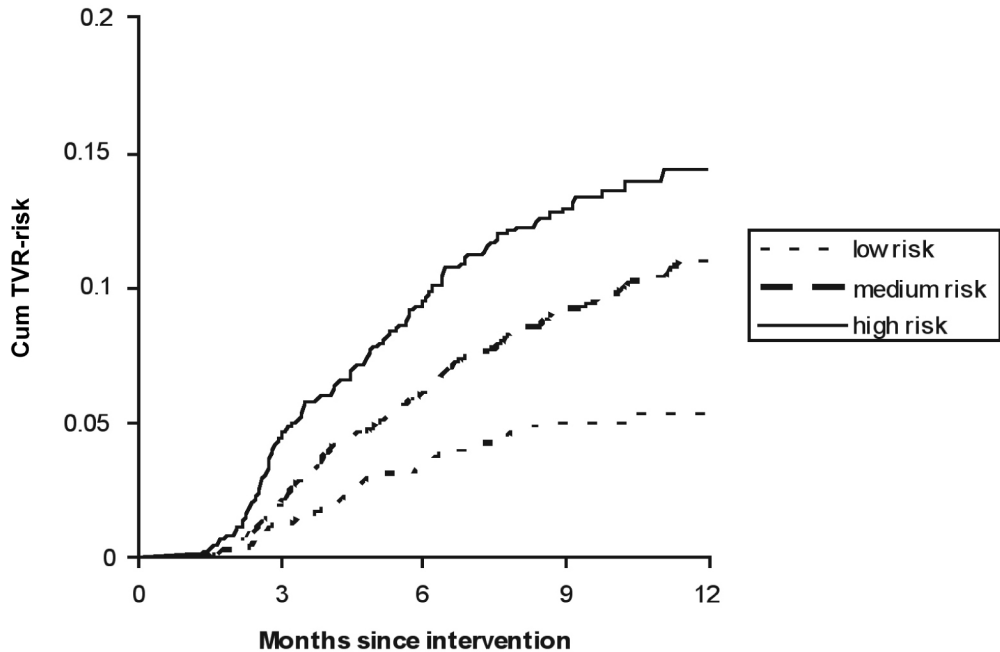
Table 4. Multivariable Cox regression of polymorphisms associated with TVR, including clinical factors

	HR	95% CI		P-value
		Low	High	
Age	0.99	0.98	1.00	0.19
Sex	0.93	0.71	1.21	0.59
Diabetes	1.65	1.25	2.18	0.001
Hypertension	1.19	0.94	1.50	0.14
Current smoker	0.75	0.56	1.01	0.06
Stenting	0.81	0.62	1.07	0.12
Total occlusion	1.50	1.11	2.00	0.01
Residual stenosis>20%	1.51	1.11	2.05	0.01
IL4R (Gln576Arg)	0.84	0.68	1.04	0.10
TCF7 (Pro19Thr)	0.76	0.56	1.02	0.07
CSF2 (Ile117Thr)	0.76	0.61	0.94	0.01
ADRB2 (Arg16Gly)	1.33	1.06	1.68	0.01
CD14 (C-260T)	0.74	0.55	0.99	0.04
CCL11 (G-1328A)	0.73	0.58	0.93	0.01

Finally we considered the association of the 21 possible two-way interaction terms between the 7 selected polymorphisms and TVR. Only one interaction term was found to be statistically significant ($p=0.04$) between TCF7 and CSF2. The relative risk of TVR in CSF2 Thr-carriers versus non-carriers was 0.37 (95% CI 0.17-0.79) in TCF7 Thr-carriers, and 0.83 (95% CI 0.67-1.03) in TCF7 non-carriers.

To illustrate the clinical relevance of our finding, the log-relative risks of the genotypes defined by the 5 polymorphisms with a $p < 0.1$ selected in the Cox model were divided into quartile groups. We calculated the Kaplan-Meier curves in the first quartile-group (low risk, $N=746$), the second and third quartiles (medium risk, $N=1,488$), and the fourth quartile (high risk, $N=749$). At 9 months post-intervention TVR-risks were 5.0%, 9.2%, and 12.9% in these respective groups, and at 12 months 5.3%, 11.0%, and 14.3%, respectively (Figure 1). The high-risk quartile consisted almost exclusively of 435 patients having the TCF7 19Pro/Pro, CSF2 117Ile/Ile, CD14 -260CC/CT and the ADRB2 16Gly/Gly genotype. The low and medium risk quartiles consisted of patients with several genotype combinations.

Figure 1. Cumulative TVR-risks in low, medium, and high risk-groups, based on genotype



Kaplan-Meier curves in the first quartile-group (low risk, N=746), the second and third quartiles (medium risk, N=1,488), and the fourth quartile (high risk, N=749). At 9-months post-intervention TVR-risks were 5.0%, 9.2%, and 12.9%, and at 12 months 5.3%, 11.0%, and 14.3%, respectively. The high-risk quartile consisted almost exclusively of the 435 patients having the TCF7 19Pro/Pro, CSF2 117Ile/Ile, CD14 -260CC/CT and the ADRB2 16Gly/Gly genotype. The low and medium risk quartiles consisted of patients with several genotype-combinations.

Error rate

The experiment-wide type-I error rate of the present study was assessed with a permutation study. Out of 1,000 permuted (reshuffled) datasets there were 97 (10%) in which none of the 48 markers were significantly related with TVR. Thirty-one % of the reshuffled datasets had 1 'significant' marker, 25% of the

reshuffled datasets had 2 ‘significant’ markers, 22% had 3 significant markers, and 12% had 4 or more significant markers. The median false detection rate was only 2 markers.

Discussion

In a prospective multicenter follow-up study, we investigated 48 polymorphisms from 34 different genes. Our assumption that a relationship exists between these genes and the development of restenosis after PCI was based on observations suggesting a role of these genes in the process of inflammation, a well-known determinant in the development of restenosis. After multivariable analysis we identified polymorphisms in the CD14, beta-2 adrenergic receptor (ADRB2), colony stimulating factor 2 (CSF2) and eotaxin (CCL11) genes that were significantly associated with restenosis after PCI. Although neointimal formation is more pronounced after stenting and remodeling is prominent after plain balloon angioplasty, stenting did not give change the associations between the four genes and restenosis.

CD14

The -260 T/T genotype of CD14 was found to be protective against restenosis following PCI. Two previous studies have investigated the role of CD14 in the development of restenosis, one being a prospective study by Zee et al. in 779 patients and the other being a prospective study by Shamada et al. in 129 patients. They found the -260 T/T genotype to be a risk factor for restenosis. ^(22;23) Our data are in conflict with these findings, which may be explained by a biological significance of CD14 that differs between Japanese subjects and Whites, as well as a small sample size. However, the discrepant results of Zee et al. and ours are not yet explained and await further study.

ADRB2

The second gene we found to be associated with TVR is the beta-2 adrenergic receptor gene (ADRB2), located on chromosome 5q31-q32. ADRB2s are cell-surface receptors that upon binding to norepinephrine activate cellular adenylylase via coupling to G-proteins. The ADRB2-gene has a role in the inflammatory response, since adrenoceptors are present on human platelets, and ADRB2 stimulation activates platelet nitric oxide synthase (NOS). ⁽²⁴⁾ NOS catalyzes the formation of NO, which has an inhibitory role on leukocyte adhesion, platelet adhesion and aggregation, smooth muscle cell proliferation and synthesis of ma

trix proteins, and it promotes endothelial survival and proliferation.⁽²⁵⁾ In addition, ADRB2 has an effect on the immune system, since lymphocytes express ADRB2s.⁽²⁶⁾

The polymorphism in the ADRB2 gene that after adjusted analyses showed to be a risk factor for restenosis was the 16A/G polymorphism that results in an amino acid change of glycine to arginine at position 16 (Arg16Gly). Patients with homozygosity for the 16Gly variant had a higher risk of TVR compared to patients with the 16Arg variant (11.3% vs. 9.1%, respectively). Previous *in vivo* and *in vitro* studies have suggested that this Arg16Gly variant may differently affect functional responses to adrenergic stimulation, thereby possibly modulating cardiovascular and metabolic phenotypes. It has been reported that the 16Gly variant of ADRB2 is associated with faster agonist-induced downregulation of the receptor, as compared with the 16Arg variant.⁽²⁷⁾ The higher risk of TVR may be related to less vasodilatation as a result of the downregulation of the receptor containing 16Gly, as compared to the receptor containing 16Arg. Moreover, downregulation of the ADRB2 could result in impaired inhibition of platelet aggregation.⁽²⁴⁾

CSF2

The polymorphism in the colony stimulating factor 2 gene (CSF2) that is significantly associated with restenosis is the 117T/C polymorphism, which results in an isoleucine for threonine substitution on position 117. The Thr117 variant showed a protective association with TVR. The functional effect of this CSF2, also known as granulocyte-macrophage colony stimulating factor, polymorphism still has to be investigated.

CCL11

The fourth polymorphism that was associated with restenosis is eotaxin (CCL11), a CC chemokine that is localized on chromosome 17. The -1328A/A promoter variant of this gene demonstrated a protective association with TVR. Economou et al. reported that eotaxin is elevated in plasma of patients with advanced atherosclerosis. The plasma level of eotaxin in their study rose in the first day after PCI and declined to baseline in the following 3 months.⁽²⁸⁾ In what way the polymorphism determines the expression level on a protein basis is as yet unknown.

Recently, Humphries et al. have put forward the criteria for a genetic variant to be included in clinical risk management of patients with CVD. For candidate variants there should be enough circumstantial evidence from literature

or from theoretical points of view to be involved in the disease and the studies should have enough power to detect a relative risk of 1.25 or more. The genotype must then give a predictive value in carriers over and above established risk factors and the final data set should show no significant evidence for heterogeneity of risk effect. Finally, for each selected gene locus only functional variants (i.e. variants that alter an amino acid or a transcription factor-binding element in a promoter region demonstrated in vitro) should be included.⁽²⁹⁾ With these criteria Humphries et al. referred to genotypes encoding a specific phenotype, such as Factor V Leiden for venous thrombosis. The polymorphisms we examined are explorative and chosen on basis of their known involvement in the multiple pathways of inflammation, being potentially implicated in the development of restenosis. However, they have no strong direct a priori theoretical value in terms of biological plausibility as meant by Humphries et al. However, we believe that the four factors associated with restenosis identified in the present study meet these criteria considerably.

Since it is not only the inflammatory response that causes restenosis, more research and confirmation of our findings are needed before these genetic variants could be used for making a genetic risk profile for patients at increased risk of restenosis.

In addition, circulating protein levels were not assessed in the present study. Basal (pre-PCI) plasma levels of the gene product probably do not reflect genetically determined differences in reaction to a trauma such as PCI. Moreover, local differences in reactions (in the vessel wall at the place of PCI) may not be determined systemically. In the human situation it is impossible to measure gene products locally in the acute phase of treatment or the following days, and several months later the causal trigger has probably already disappeared.

Limitations of the study

The 48 polymorphisms examined in the present study represent only a small proportion of genetic information that is potentially associated with TVR. However, by looking at a broad spectrum of polymorphisms in genes that are considered to be involved in the inflammatory response, we did try to cover a large set of factors that may be associated with restenosis. Furthermore, the candidate gene approach currently remains the most practical approach. Secondly, one or more of the SNPs associated with TVR in our study may be in linkage disequilibrium with other polymorphisms in the gene or with other nearby genes that are actually responsible for the development of this condition.

We did not apply adjustment for multiple testing, which sometimes is consid

ered appropriate for hypothesis-testing studies. Moreover, our experiment-wide error rate was found to be 12% in the permutation analysis. In addition, we performed additional hypothesis testing in our haplotype analysis, which further increases the multiple testing problem. Our results should be primarily seen as hypothesis generating and need independent validation.

In addition, data on our haplotype analysis is limited due to the fact that we do not comprehensively cover the haplotype structure of our selected genes. Thus, individuals who appear identical as our haplotypes are concerned may very well differ when more polymorphisms are taken into account.

Our study has insufficient statistical power to examine whether the genetic associations we observed differed by sex, since there were only 888 female patients of whom 84 with TVR.

Another possible limitation is that we examined TVR as our primary endpoint instead of angiographic outcomes, such as late loss. This could have given a problem with ascertainment. However, in clinical practice clinical restenosis is an endpoint much more valuable than angiographic restenosis.

Furthermore, the four factors we found show a small hazard ratio (0.7–1.3), but it should be taken into account that the process of restenosis is multifactorial involving multiple genes. Thus, relatively small hazard ratios relating to contribution of a single gene to restenosis might be of paramount importance in the overall process, and even a small genetic risk may identify a gene with an important biological role that could reveal new mechanistic insights and provide novel therapeutic targets.

Finally, as our study was conducted in a sample of White patients, extrapolation of the data to other ethnic groups should be done with great caution.

Conclusion

In conclusion, we show that genetic variants in four different genes that are considered to be involved in the inflammatory response may play a role in the development of restenosis. Three of these genotypes have, to our knowledge, not been described before in relation to restenosis. Because of the explorative nature of this analysis, our results need to be replicated in other studies to confirm the data. After this our data could contribute to the unraveling of the process of restenosis and thereby provide novel therapeutic targets as well as contribute to development of improved risk stratification of patients who are scheduled for elective PCI, thereby creating the opportunity to individualize treatment in the future.

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Editorial: Genomics of In Stent Restenosis

Early Insights Into a Complex Disease

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Inflammation is a key component of atherosclerosis. Abundant preclinical data support the hypothesis that atherosclerosis is a chronic inflammatory disorder.^(1,2) Indeed, clinical trial data now provide evidence that inflammation, as reflected in serum markers such as C-reactive protein and interleukin-6, is a strong risk factor for the development and progression of atherosclerosis.^(3,4) The role of genetic factors in determining a predisposition or susceptibility to inflammation that exacerbates atherosclerosis is not fully known.

In-stent restenosis occurs after the deployment of an intravascular stent within an atherosclerotic lesion. The fibroproliferative response to this vascular “injury” typically develops within the first 9 months postprocedure. The response to injury follows a continuum in human arteries; some degree of cell proliferation occurs in all patients and can be thought of as a wound-healing process. In some individuals, however, the wound healing becomes excessive, leading to exuberant vascular smooth muscle cell growth and extracellular matrix synthesis, and encroachment on the arterial lumen, and resulting in a recurrence of clinical symptoms. Molecular and genetic studies suggest that cell cycle proteins, growth factors, and inflammatory cytokines regulate this process.⁽⁵⁾ Drug-eluting stents have dramatically reduced the prevalence of in-stent restenosis because of the local treatment of the fibroproliferation with 2 drugs, sirolimus and paclitaxel, which have antiproliferative and antiinflammatory properties.^(6,7) What is not known, however, is whether there is a genetic susceptibility that determines a patient’s response to stent deployment and development of in-stent restenosis. In this issue of *Circulation*, Monraats et al investigate the genetics of in stent restenosis in a case control association study, using a candidate gene approach.⁽⁸⁾ The authors hypothesize that genetic variation in inflammatory genes is important in individual differences in the vascular wound-healing process, and hence they selected specific genes and polymorphisms hypothesized to be causal. They determined genotypes of 48 single nucleotide polymorphisms (SNPs) in 34 candidate genes in 3,029 patients enrolled prospectively at the time of coronary intervention and studied until either target-vessel revascularization (TVR) oc

curred or 9 months of follow-up were complete. In this cohort, 74.4% of patients received a bare metallic stent as part of their treatment. None received drug-eluting stents. The primary end point of TVR occurred in 9.8% of patients. Using established statistical tests designed to test the association of one marker at a time, 4 SNPs were identified within the genes for *ADRB2*, *CD14*, *CSF2*, and *CCL11*.

In testing many variables for association with disease, multiple testing is a concern because of the increased possibility of false positives. In this study, no multiple testing corrections were applied. Instead, the authors conducted a permutation test on their data set, in which the TVR and no-TVR outcome was shuffled 1000 times among the patients and the association tests were recalculated each time. In this test, any 4 SNPs were identified to be significant in 12% of permutations. To further explore their findings, the authors examined genotypes among patients with postpercutaneous transluminal coronary angioplasty restenosis versus in-stent restenosis. No differences were identified. Haplotype analysis, in which the SNPs assayed were examined for patterns between TVR and no-TVR patients, provided no additional insight. Interactions between SNPs and the association with TVR were considered, and in this analysis, a SNP in the *TCF7* gene was found to interact with the *CSF2* SNP previously identified, with modest significance and no multiple testing corrections. Patients were triaged into 3 risk categories: low, medium, and high, with respective TVR rates of 5.0%, 9.2%, and 12.9%. The high-risk quartile of 749 patients contained 435 patients with a specific pattern of genotypes in the *TCF7*, *CSF2*, *CD14*, and *ADRB2* genes. The *CCL11* gene polymorphism was not indicated to be part of this pattern, despite having been identified as associated with TVR.

During the past several years, we have seen a dramatic evolution in our knowledge of the genome as well as the tools with which to conduct genetic studies. We now know that there are millions of SNPs across the genome, and high-throughput genotyping assays that use SNPs as high-density genetic markers are now available, assaying >100 000 SNPs at a time to identify disease loci.⁽⁹⁾ The study by Monraats et al was initiated in 1999, a time when genetic studies using SNP markers were just coming to the forefront of genetics research. The human genome draft sequence and associated SNP databases were not well developed at that time. Traditional genetic research methods that had been developed to identify disease-causing genes in rare Mendelian disorders relied on microsatellite or other markers to identify diseases in which the effect of a single variant gene was strong. These methods identified a disease locus and further study of the locus was needed to identify specific mutations using techniques such as positional cloning. Today, it is possible to conduct genome-wide association studies

using SNPs as markers across the genome. Exciting new opportunities are on the horizon to identify disease-causing or risk-conferring alleles in complex diseases, in which disease susceptibility is hypothesized to be caused by multiple common variants, each contributing subtly to the disease.

How should we interpret the study by Monraats et al? The authors clearly outline the limitations of their study and acknowledge several important issues. First, the results of statistical testing are of marginal significance, with no multiple testing corrections applied to either the initial per-SNP analysis or the haplotypes analyses. The results of their permutation testing confirm an experiment-wide error rate of 12%, which indicates that their findings are quite possibly the result of chance and the testing considerations outlined. The functional significance of the gene variants reported are not investigated, although the literature does provide some evidence that the genes, not necessarily the SNPs investigated, could plausibly be functionally important in restenosis. Although interesting, the results cannot clearly be linked to the biology of restenosis. Only 48 markers were screened in this study. This analysis could have missed many significant SNPs because of the candidate polymorphism approach taken. Given these considerations, this study can be viewed as a screening analysis, and the results should be viewed as preliminary.

As genomic methodologies continue to evolve at an ever-rapid pace, we can look forward to a new generation of genetic studies aimed at investigating complex genetic diseases. The most common cardiovascular diseases are truly complex, with significant environmental contributions as well as likely multigenic pathogenesis. This is certainly true of vascular injury responses, in which we continue to see treatment failures even after the advent and widespread use of drug-eluting stents. Using the most cutting-edge technologies available, we now have the potential to build on early associative findings such as those reported by Monraats et al and to conduct more definitive investigations into the genetic basis of complex diseases. Importantly, for our patients, these tools are powerful means by which we will better understand the molecular basis of the most common diseases observed in the clinic and develop improved risk stratification tools and treatment modalities for cardiovascular diseases such as atherosclerosis and restenosis.

The opinions expressed in this article are not necessarily those of the editors or of the American Heart Association.

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