

Multi-locus interactions predict risk for post-PTCA restenosis: an approach to the genetic analysis of common complex disease

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Received: 6 December 2001
Revised: 21 February 2002
Accepted: 21 February 2002

ABSTRACT

The complexity of recognizing the potential contribution of a number of possible predictors of complex disorders is increasingly challenging with the application of large-scale single nucleotide polymorphism (SNP) typing. In the search for putative genetic factors predisposing to coronary artery restenosis following balloon angioplasty, we determined genotypes for 94 SNPs representing 62 candidate genes, in a prospectively assembled cohort of 342 cases and 437 controls. Using a customized coupled-logistic regression procedure accounting for both additive and interactive effects, we identified seven SNPs in seven genes that, together, showed a statistically significant association with restenosis incidence ($P < 0.0001$), accounting for 11.6% of overall variance observed. Among them are candidate genes for cardiovascular pathophysiology (apolipoprotein-species and NOS), inflammatory response (TNF receptor and CD14), and cell-cycle control (p53 and p53-associated protein). Our results emphasize the need to account for complex multi-gene influences and interactions when assessing the molecular pathology of multifactorial medical entities.

The Pharmacogenomics Journal (2002) **2**, 197–201. doi: 10.1038/sj.tpj.6500101

Keywords: restenosis; angioplasty; SNPs; risk factors

INTRODUCTION

The utility of percutaneous transluminal coronary angioplasty (PTCA) is limited by a high incidence of restenosis following the procedure ('post-PTCA restenosis')¹ that occurs in 30–40% of all patients. Like most common diseases, the occurrence of coronary artery restenosis following PTCA is considered a complex multifactorial disease process. Experimental and clinical studies have implicated vascular remodeling,² however, the specific molecular and cellular mechanisms of the processes involved remain largely unknown. None of a large number of clinical risk factors recognized for atherosclerosis or ischemic cardiovascular disease (with the possible exception of diabetes mellitus³) has been found associated with the occurrence of restenosis. To evaluate the possible effects of genetic predisposition, a number of studies focusing on single-gene variants have been carried out to date, but have remained inconclusive due to a number of limitations, including study size and design. In the absence of any known strong predictors for post-PTCA restenosis, we selected this disease entity as a test case for a more comprehensive approach, with regard to both breadth of genotyping and analytical algorithms. In a cohort of post-PTCA patients three times larger than the largest sample studied so far,⁴ a panel of 94 polymorphic markers was tested from 62 'candidate' genes (representing, broadly, genes related to lipid and homocysteine metabolism, vascular tone regulation, cell-matrix adhesion and interaction, matrix integrity, inflammatory and immune responses, thrombosis/coagulation, cellular homeostasis/cytoprotection mechanisms, and cell cycle/apoptotic pathways; see Table 1).

Table 1 Polymorphisms assessed

<i>ADD1</i> [G460W] <i>ADRB2</i> [R16G; Q27E]	<i>FGB</i> [G(-455)A; *R448K] <i>GNB3</i> [C825T]	<i>LPA</i> [C93T; G121A] <i>LPL</i> [T(-93)G; D9N; N291S; S447ter]
<i>ADRB3</i> [W64R]	* <i>HSPA1A</i> [BsrBI site at nucleotide 190]	<i>LTA</i> [T26N]
<i>AGT</i> [M235T]	* <i>HSPA1B</i> [PstI site at nucleotide 1267] * <i>HSPA1L</i> [NcoI site at nucleotide 2437]	* <i>MDM2</i> [NlaIV site in 5' leader region] <i>MMP3</i> [Promoter 5A/6A]
<i>AGTR1</i> [A1166C] <i>APOA4</i> [T347S; E360H]	<i>ICAM1</i> [G214R]	* <i>MMP9</i> [C(-1562)T]
<i>APOB</i> [T71I; R3500Q]	* <i>IL1A</i> [G4845T]	<i>MTHFR</i> [C677T]
<i>APOC3</i> [C(-641)A; C(-482)T; T(-455)C; C1100T; C3175G; T3206G]	* <i>IL1B</i> [AvaI site at -511 bp 5' of start codon; TaqI site in intron 4]	<i>NOS3</i> [E298D]
<i>APOE</i> [C112R; R158C]	* <i>IL1R1</i> [PstI site in exon 1B]	<i>NPPA</i> [G664A(V7M); T2238C]
<i>CBS</i> [I278T and 68 bp-insertion]	* <i>IL1RN</i> [86 bp-repeat in intron 2]	<i>PAI1</i> [G(-844)A; 5G/4G; G11053T]
<i>CCR2</i> [V62I]	<i>IL4</i> [C582T]	<i>PON1</i> [M55L; Q192R]
<i>CCR5</i> [32 bp-deletion]	<i>IL4RA</i> [Q576R; 150V]	<i>PON2</i> [S311C]
<i>CETP</i> [C(-628)A; 1405V; intron 14 G(+1)A; (+3)T insertion in intron 14; D442G]	<i>IL5RA</i> [G482A]	<i>PPARG</i> [P12A]
* <i>CD14</i> [C(-260)T]	<i>IL6</i> [G987C]	<i>SCNN1A</i> [W493R; T663A]
<i>DCP1</i> [D/I in intron 16]	<i>IL9</i> [C4244T]	<i>SCYA11</i> [A23T]
* <i>EDN1</i> [A-insertion at position 138]	<i>ITGA2</i> [G873A]	<i>SELE</i> [S128R; L554F]
* <i>EDNRA</i> [Af/II site in codon 323]	* <i>ITGB2</i> [AvaI site in codon 441]	* <i>TIMP1</i> [Bg/II site; Bg/II site]
<i>F2</i> [G20210A]	<i>ITGB3</i> [L33P]	<i>TNF</i> [G(-308)A; G(-238)A]
<i>F5</i> [R506Q]	<i>LDLR</i> [NcoI site in exon 18]	<i>TNFR1</i> [A845G]
<i>F7</i> [(-323) 10 bp del/ins; R353Q]	<i>LIPC</i> [C(-480)T]	* <i>TP53</i> [16 bp-duplication in intron 3 P72R; MspI site in intron 6]
<i>FCER1B</i> [E237G]		<i>UGB</i> [A587G]

Unless indicated by an asterisk, all markers were screened using Roche Molecular Systems linear array technology. *ADD1*, adducin alpha; *ADRB*, beta adrenergic receptor; *AGT*, angiotensinogen; *AGTR1*, angiotensin receptor 1; *APO*, apolipoprotein; *CBS*, cystathionine beta-synthase; *CCR*, chemokine receptor; *CETP*, cholesterol ester transfer protein; *CD14*, monocyte differentiation antigen; *DCP1*, dipeptidyl carboxypeptidase 1/angiotensin converting enzyme; *EDN1*, endothelin 1; *EDNRA*, endothelin receptor type A; *F2*, factor II; *F5*, factor V; *F7*, factor VII; *FCER1B*, immunoglobulin E receptor 1 beta; *FGB*, fibrinogen beta polypeptide; *GNB3*, guanine nucleotide binding protein beta 3; *HSPA1A*, heat shock protein 70-1; *HSPA1B*, heat shock protein 70-2; *HSPA1L*, heat shock protein 70-hom; *ICAM1*, intracellular adhesion molecular 1; *IL*, interleukin; *IL1R1*, interleukin 1 receptor type; *IL1RN*, interleukin 1 receptor antagonist; *IL4RA*, interleukin 4 receptor alpha; *IL5RA*, interleukin 5 receptor alpha; *ITGA2*, platelet glycoprotein Ia; *ITGB2*, integrin beta 2; *ITGB3*, platelet glycoprotein IIIa; *LDLR*, low density lipoprotein receptor; *LIPC*, hepatic lipase; *LPA*, apolipoprotein(a); *LPL*, lipoprotein lipase; *LTA*, tumor necrosis factor beta; *MDM2*, p53-associated protein; *MMP3*, matrix metalloproteinase 3; *MMP9*, gelatinase beta; *MTHFR-5,10*, methylenetetrahydrofolate reductase; *NOS3*, nitric oxide synthase 3 endothelial; *NPPA*, atrial natriuretic factor; *PAI1*, plasminogen activator inhibitor type 1; *PON*, paraoxonase; *PPARG*, peroxisome proliferator activated receptor gamma; *SCNN1A*, sodium channel epithelial alpha subunit; *SCYA11*, eotaxin; *SELE*, selectin E; *TIMP*, tissue inhibition metalloproteinase; *TNF*, tumor necrosis factor; *TNFR1*, tumor necrosis factor receptor 1; *TP53*, tumor suppressor protein P53; *UGB*, uteroglobin. For a bibliography of these genes with regard to their status as cardiovascular candidate genes please refer to: <http://research.bwh.harvard.edu/rdbook/ca16ref.htm>.

RESULTS

Baseline characteristics of the study population are shown in Table 2. As expected, there were no significant differences between the cases (restenosis) and the controls (no

restenosis) with regard to age, blood pressure status, plasma lipid profile, smoking status, or family history of coronary heart disease. Only diabetes mellitus was significantly more common in cases than controls ($\chi^2_{2df} = 6.66$, $P = 0.011$).

Table 2 Baseline characteristics of the study population

	Cases <i>n</i> = 342	Controls <i>n</i> = 437	<i>P</i>
Age (years \pm SD)	58.9 \pm 9.6	58.2 \pm 10.2	0.34
Male/female	305/37	378/59	–
BMI (kg ² m ⁻¹ \pm SD)	26.7 \pm 3.5	26.9 \pm 3.4	0.53
Hypertension (>160/90 mmHg)	149 (46.5%)	183 (41.8%)	0.22
Hypercholesterolemia (>220 mg dl ⁻¹)	146 (42.8%)	201 (46.0%)	0.38
Smokers	82 (24%)	106 (24.3%)	0.93
Diabetes	68 (19.9%)	57 (13%)	0.011
Family history of MI (<age 60)	78 (22.8%)	87 (19.9%)	0.33
Previous MI	156 (45.6%)	189 (43.2%)	0.51
Stable angina	64 (18.7%)	85 (19.5%)	0.79
Unstable angina	221 (64.6%)	267 (61.1%)	0.31

n, number of subjects; BMI, body mass index; MI, myocardial infarction.

Genotype frequencies for the 94 bi-allelic markers tested were compared by χ^2 analysis. The corresponding uncorrected *P* values are provided (http://research.bwh.harvard.edu/rdbook/SNP_Table.doc). Of note, only four polymorphisms were found to attain a *P*-value of <0.05, and among them only one of <0.01.

Subsequently, we carried out a two-stage analysis⁵ to initially select a set of influential markers, followed by modeling of gene–phenotype effects and gene–gene interactions. In what we believe is the first application of a nested bootstrap (re-sampling) procedure to genetic markers, the selection stage was carried out as follows. For each SNP, χ^2 was computed for a 2 \times 3 table, with case-control status and genotype as the dependent and independent variables. Simply picking a marker based on its significant χ^2 , however, would ignore the joint effects of all markers (each marker by itself does not have a strong effect). With the selection procedure thus based on univariate χ^2 values, the sub-selection of markers to be used for the later stages of the analysis was determined using an approach described in more detail elsewhere.⁵ In brief, a statistic was calculated as the sum of the χ^2 values over all marker loci, where the statistical significance of this sum was evaluated by 1000 bootstrap samples taken under the null hypothesis of no association. Markers were deleted from the sum stepwise, based on ascending χ^2 value. At each step the significance level was re-evaluated for the remaining sum until the significance level of the sum dropped below 5%. This pre-selected a set of influential markers from the original data. To validate this pre-selection procedure, we replicated the original dataset in 499 bootstrap samples retaining the association between markers and disease status. The pre-selection procedure described above was then carried out for each of the 499 replicated samples. Thus, a potentially different set of markers was pre-selected in each sample. Markers that were pre-selected in at least 2/3 of all 500 samples were then selected for modeling. Seven marker loci were thus found (*APOC3* C1100T; *CBS* 1278T; *CD14* C(–260)T; *NOS3* E298D; *TP53* P72R; *MDM2* N1aIV site; *TNFR1* A845G).

In stage 2, modeling was carried out by logistic regression,

a method commonly used to assess the effects of risk factors on outcome variables, but so far applied to genetic analysis only rarely, and only to small datasets.^{6,7} A sequence of backward and forward stepwise regression analyses incorporated two-way and three-way interaction effects into the model. The resulting final model contains the seven markers through linear and quadratic main effects and interaction terms that cumulatively account for 11.6% of the total phenotypic variance (Table 3). The overall significance level of the model is *P* <0.0001. All these stage 2 calculations were carried out with the commercially available S-PLUS[®] 2000 software. Note that one marker, *NOS3*, occurs only through interaction with other markers, that is, its direct main effect is negligible (Table 3).

Under the assumption that non-genetic factors contribute significantly to total variance—given that the phenotype arises in response to procedural intervention—this regression formula likely explains a substantial fraction of overall genetic variance. The fact that the only other known predictor for restenosis, diabetes mellitus, only accounted

Table 3 Results of the coupled-logistic regression

Effect	<i>df</i>	Estimate	SE	<i>P</i>
<i>APOC3</i>	1	1.2127	0.3114	0.0001
<i>CBS</i>	1	–1.3002	0.3039	0.0001
(<i>TNFR1</i>) [*]	1	0.2564	0.0691	0.0002
(<i>TP53</i>) [*]	1	–0.0858	0.0281	0.0023
(<i>CD14</i>) [*]	1	0.1740	0.0769	0.0236
(<i>MDM2</i>) [*]	1	0.2777	0.0851	0.0011
<i>TNFR1</i> \times <i>APOC3</i>	1	–0.5547	0.1606	0.0006
<i>CD14</i> \times <i>MDM2</i>	1	–0.9427	0.2027	0.0001
(<i>CD14</i>) [*] \times <i>MDM2</i> \times <i>CBS</i>	1	0.1848	0.0395	0.0001
(<i>CD14</i>) [*] \times <i>NOS3</i> \times <i>CBS</i>	1	–0.0517	0.0181	0.0042

^{*} Quadratic effect. The two degrees of freedom (*df*) associated with the three genotypes of each marker were split into single *df*'s via dummy variables, one of them representing a linear and the other a quadratic effect. For simplicity, the gene names were used to represent the actual SNPs as mentioned previously. 'Estimate' = estimated regression coefficient; SE, standard error.

for 6.7% of the variance in the present study emphasizes the significance of our finding.

DISCUSSION

Experimental and human studies have indicated plaque retention, vaso-constrictive remodeling, thrombus formation, and neointimal proliferation as the primary effector mechanisms likely to contribute to the pathophysiology of the condition.⁸ Furthermore, inflammatory and immune responses, as well as apoptotic processes have also been implicated^{9–11} in the underlying disease mechanism. Our findings represent the first direct clinical-epidemiological evidence for the actual relevance of these mechanisms by highlighting molecular variants of seven genes that belong to the postulated pathogenic pathways (apolipoprotein CIII, cystathionine beta-synthase, monocyte differentiation antigen CD14, endothelial nitric oxide synthase 3, tumor suppressor protein p53, p53-associated protein, and tumor necrosis factor receptor type 1) and that we found to be significantly associated with the incidence of restenosis. In the absence of data on the functional consequences of the implicated polymorphisms, further speculation is presently not justified; however, our findings prioritize these genes, among all the ones implicated in the literature so far, as candidates for functional-genetic studies.

The present study emphasizes the importance of taking a multiplicity of potentially involved gene variants into account when studying the genetic epidemiology of common complex traits. Concomitantly, it highlights the need to apply powerful, customized analytical algorithms to the data sets thus generated. Of note, although the present findings account only for a fraction of the overall genetic variance of the trait, these results were derived from a modest number of genes, indicating the value of a candidate gene-driven approach. Whereas we recognize that selecting individual markers from a large set may be due to random chance (so-called multiple testing problem), we believe that the validation procedure chosen, using 499 bootstrap replicates, will largely eliminate this problem. At the same time, these results call for a more comprehensive selection of candidate genes and their variants, or for additional approaches not restricted to the bias of pre-selected candidate genes. While the current results will have limited practical applicability, we believe they point the way towards a future feasibility of creating comprehensive risk factor panels, and their application to medical practice.

MATERIALS AND METHODS

Study Design

Between March 1995 and March 1997, 909 consecutive patients who were undergoing PTCA at the Hospital San Carlos, Universidad Complutense in Madrid, Spain, were enrolled in a comprehensive follow-up study specifying symptom-independent angiographic follow-up 6 months later. All patients gave appropriate signed informed consent for both the clinical procedures and genotype testing. A total of 779 consecutive subjects fulfilled the enrollment criteria for the present study: successful PTCA, follow-up car-

diac catheterization, interpretable quantitative coronary angiography (QCA) measurements at baseline (prior to undergoing PTCA), immediately post-PTCA (demonstrating the initial effect of the procedure) and at follow-up cardiac catheterization 6 months later,^{12,13} and the availability of a blood sample for genotype determination. Restenosis was defined as >50% reduction in the luminal diameter at the site of previous PTCA, as compared with the immediate post-PTCA angiographic record; 342 patients were classified as cases. Of note, no intra-coronary stents were deployed in any of the 779 participants in the present investigation.

Genotype Determination

Cardiovascular disease (CVD) and inflammation/asthma linear array panels (Roche Molecular Systems, Alameda, CA, USA)

A general description of the protocol¹⁴ used has previously been reported. In brief, each DNA sample is amplified in multiplex polymerase chain reactions (PCRs) using biotinylated primers. The PCR product pool is then hybridized to the corresponding panel of sequence-specific oligonucleotide probes that have been immobilized in a linear array on nylon membrane strips. A colorimetric detection method based upon incubation with streptavidin-horseradish peroxidase conjugate of hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine as substrates was used. A total of 74 markers within 47 genes were examined (<http://research.bwh.harvard.edu/rdbook/ca16ref.htm>) using this system (Table 1).

Additional polymorphisms examined

An additional 20 polymorphisms (indicated by asterisks in Table 1) were assayed using previously published protocols.^{15–28}

To confirm genotype assignments, the PCR procedure was performed in replicate on all samples. Scoring was carried out by two independent observers. Disagreements (<2%) were resolved by a further joint reading, and where necessary, a repeat genotyping reaction. All results were scored blinded as to case-control status.

Power Calculations

The least significant relative risk was used that we would be able to recognize in the current study, assuming 80% power, based on a univariate model of analysis, on the present sample size, an additive mode of inheritance, and an alpha error of 0.05, for rare and common allele frequencies. If the minor allele frequency is 0.01, the least significant relative risk detectable is >3.3; if the minor allele frequency is 0.50, the least significant relative risk detectable is >1.3.

ACKNOWLEDGEMENTS

Supported by a Grant HG00008 from the National Institute of Health (JO), and a Research Development Award K04-HL-03138-01 from the National Heart, Lung, and Blood Institute (KL).

DUALITY OF INTEREST

None declared.

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