Interleukin-1 gene cluster polymorphisms and risk of coronary artery disease

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Background and Objectives. The pro-inflammatory cytokine interleukin (IL)-1 has been suggested to play a role in atherosclerosis. Several genetic polymorphisms have been described in the genes of the IL-1 cluster and associations with coronary artery disease (CAD) have been reported, although with contrasting results.

Design and Methods. The associations of a variable number tandem repeat (86bp) polymorphism in intron 2 of interleukin-1 receptor antagonist (IL1-RA) and of the -511 C/T polymorphism of IL-1β with the risk of CAD were studied. Three hundred and thirty-five case (CAD+) patients with angiographically documented CAD (stenosis >50% in at least one major coronary artery) were compared with 205 unrelated individuals free of CAD signs at angiogram (CAD- controls). One hundred and two (30.5%) CAD+ patients had single-vessel disease (SVD) and 233 (69.5%) multiple-vessel disease (MVD).

Results. There was no statistically significant difference in either genotype distribution or allele frequency of both IL-1 RA and IL-1β –511 C/T polymorphisms between CAD+ cases and CAD- controls. Moreover, in multivariate analysis, adjusting for multiple comparisons and confounding factors, no difference was found in IL-1 RA genotype distribution between patients with SVD or MVD.

Interpretation and Conclusions. Our study does not support the association between IL-1 RA intron 2 VNTR and IL-1β –511 C/T polymorphisms and the risk of CAD in individuals undergoing coronary angiography.

Key words: interleukin-1β, interleukin-1 receptor antagonist gene, polymorphism, coronary artery disease.

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tion between IL-1 RA polymorphisms and the risk of acute myocardial infarction (MI)\textsuperscript{23,24} In consideration of such contrasting findings additional studies on the relationship between polymorphisms in the IL-1 cluster and CAD risk are needed. The differences can be explained by different populations, different end-points and also by different statistical and methodological approaches. Considering IL-1 RA VNTR as a biallelic polymorphism by omitting less common alleles, and omitting adjustment for multiple comparisons in the analysis may have contributed to the heterogeneity of the findings.

In addition, as the IL-1 pathway might be modulated by both IL-1β and IL-1RA activities, polymorphisms in both genes should be considered in investigating the role of IL-1 gene cluster on CAD. Therefore, in the present study, we evaluated the association of the variable number tandem repeat (86bp) polymorphism in intron 2 of the IL-1 RA gene and the polymorphism at position -511 of IL-1β gene with the risk of angiographically assessed coronary atherosclerosis.

Design and Methods

Subjects

The study population was selected from a cohort of patients undergoing coronary angiography between 1994 and 1996 at the Department of Cardiosurgery of Rome University “La Sapienza”. Coronary artery disease (CAD), defined as the presence of one or more stenoses >50% in at least one major coronary artery, was identified in 335 case patients (mean age 59.5±9.4 years, 280 males and 55 females). Of these 335 patients, 102 (30.5%) had single-vessel disease (SVD) and 233 (69.5%) had multiple-vessel disease (MVD). In the latter group 112 (33.4%) had double- and 121 (36.1%) triple-vessel disease. No patient was enrolled during the acute phase of the ischemic syndrome.

As a control group (CAD-), 209 unrelated patients free of CAD (defined as <10% stenosis) were included. We failed, however, to genotype 4 control patients, so the total number of controls used in the analysis was 205 (mean age 56.6±11.8 years, 96 males and 109 females). The CAD- group consisted of patients evaluated for valvular surgery or other diagnostic purposes and patients suspected of having ischemic heart disease but in whom no significant coronary stenosis was detected. The classification was based on the visual assessment of 15 coronary segments, according to the American Heart Association criteria.\textsuperscript{25} Patients with concurrent thyroid, liver or renal disease were excluded from both cases and controls.

CAD risk factors (smoking, history of hypertension, diabetes mellitus, hyperlipidemia and cardiac events) were assessed by a structured questionnaire. Weight, height, systolic and diastolic blood pressures were measured. Two cardiologists who were unaware that the patients were participating in the study assessed the angiograms. The study was approved by the local institutional ethical committee and was performed according to the Declaration of Helsinki of 1975. Each participant gave written informed consent to the study.

Laboratory measurements and techniques

Fasting venous blood was collected from an antecubital vein in 10mL Na-EDTA. Plasma total cholesterol, triglyceride and lipoprotein fraction levels were measured with a Technicon RA-1000 Autoanalyzer. HDL cholesterol was determined in the whole plasma after precipitation of apoB-containing lipoproteins with phosphotungstic acid/MgCl\textsubscript{2}.\textsuperscript{26} Genomic DNA was extracted from peripheral blood using a salting out method. Enzymatic amplification of DNA was performed by polymerase chain reaction (PCR) using Taq polymerase (Promega, Madison, WI, USA).

Primers for the IL-1 RA variable number tandem repeat (86bp) in intron 2 were 5'-CTACGCAA-CACTCTCAT-3' and 5'-TCTGGTCTGAGTTAA-3'. PCR conditions were as follows: denaturing step at 96°C for 2 min, 35 cycles of 94°C for 1 min and 30 seconds, 60°C for 1 min and 30 seconds and 72°C for 2 min.\textsuperscript{27} Allele 1 (4 repeats) was 412 bp, allele 2 (2 repeats) 404 bp, allele 3 (3 repeats) 326 bp, allele 4 (5 repeats) 498 bp, and allele 6 (6 repeats) 584 bp. Primers for IL-1β-511 C/T polymorphism were TGGCATTGATCTGTTCACT (upstream) and GTTTAG-GAATCTTCCCCACTT (downstream).\textsuperscript{14,16} Thirty amplification cycles run at 95°C for 1 min, 53°C for 1 min and 72°C for 1 min, after 1 min prewarming at 95°C and restriction enzyme digestion (overnight, 37°C) with 3 units of Ava I and Bsu 36I (Promega, USA) of the amplified fragments followed by a run on 2.5% agarose gel were performed for a double confirmation of the genotyping: Ava I produces fragments of 190+114 bp (C allele) and 304 bp (T allele), while Bsu 36I produces fragments of 304 bp (allele C) and 190+114 bp (allele T).

Statistical analysis

According to previous studies, the prevalence of the T allele of -511 IL-1β and of the 2 allele of IL-1 RA polymorphisms was assumed to be 0.32 and 0.23 in healthy subjects.\textsuperscript{21} According to the lowest allele frequency, a minimum sample size of 320 cases was necessary to detect a difference in risk of at least 75% with β=0.10 and α=0.025 two sided (established after Bonferroni’s correction for two polymorphisms).

The frequencies of the alleles were determined by genotype count and compared with the values predicted on the basis of the assumption of Hardy-Weinberg equilibrium performing the exact test of the Hardy-Weinberg proportion for multiple alle-
les. The coefficient of gametic linkage disequilibrium was calculated by likelihood methods in the control sample. The coefficient (D) is reported as the ratio of the unstandardized coefficient to its maximal value.

The problem of multiple testing arises in performing many hypothesis tests on the same data set; in our study such a problem was addressed by using a permutation technique (MULTTEST procedure for SAS). An adjusted for multiple comparison p-value is defined as the probability of observing a raw (unadjusted) p-value at least as extreme as the given p-value when the entire family of tests (generated after permutations) is considered under the complete null hypothesis. Following this approach, we compared the presence of allele 2 against no presence of allele 2 in both polymorphisms, as the method applied only on 2xN tables (with N≥2) in our study related to CVD status – CAD−, CAD+, SVD, MVD). Give the major effect of allele 2 found in previous studies, this was not a limitation in analyzing the IL-1 RA intron 2 VNTR polymorphism.

In order to evaluate the relative effects of the polymorphisms, the following strategy (based on a recent report by Cordell et al.31) was applied: i) the effect of each polymorphism was first coded as \( \beta_0 \times X_0 + \beta_1 \times X_1 \) in a multiple logistic regression model, with \( X_0 = (0, 0, 1) \) and \( X_1 = (0, 1, 2) \) for genotype (11, 12, 22) (for the RA polymorphism in all groups are shown in Table 2. Genotype distribution was in Hardy-Weinberg equilibrium in both case and control groups (p=0.21 and p=0.07, respectively), when only alleles 1 and 2 were considered; however the equilibrium was lacking in controls when calculated for all genotypes (p=0.033). There was no significant difference in genotype distribution (p=0.07, Fisher's exact test) nor in allele frequencies between cases and controls (p=0.08, Fisher's exact test). IL-1 RA allele frequencies did not differ in either SVD or MVD from CAD-controls (p=0.09 and p=0.2, respectively) in Fisher's exact test. Similarly, there

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>CAD− (n=205)</th>
<th>CAD+ (n=335)</th>
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</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>56.6±11.8</td>
<td>59.5±9.4*</td>
</tr>
<tr>
<td>Male sex (%)</td>
<td>46.8</td>
<td>83.6*</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>25.8</td>
<td>45.7*</td>
</tr>
<tr>
<td>Blood pressure - systolic (mmHg)</td>
<td>124.2±14.7</td>
<td>124.6±14.5</td>
</tr>
<tr>
<td>Blood pressure - diastolic (mmHg)</td>
<td>75.1±9.2</td>
<td>75.9±8.8</td>
</tr>
<tr>
<td>Diabetes (%)</td>
<td>3.9</td>
<td>12.8*</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>202.9±45.3</td>
<td>218.4±44.3*</td>
</tr>
<tr>
<td>LDL cholesterol (mg/dL)</td>
<td>130.3±38.1</td>
<td>140.2±39.3*</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>135.7±81.2</td>
<td>191.7±121.3*</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dL)</td>
<td>46±14.8</td>
<td>41.6±11.6*</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.2±4.1</td>
<td>26.9±3.8*</td>
</tr>
<tr>
<td>Blood glucose (mg/dL)</td>
<td>97.1±27.4</td>
<td>88.6±25.4*</td>
</tr>
<tr>
<td>Smoker (%)</td>
<td>24.9</td>
<td>53.1*</td>
</tr>
</tbody>
</table>

*p<0.05, †p<0.001.
was no difference in genotype distribution in MVD and controls \((p=0.2)\). However, there was a difference in genotype distribution between patients with SVD and MVD \((p=0.024)\). Adjusting for the multiple comparison we did not find any statistically significant difference between the groups \((Table 4)\).

When we evaluated the relative effect of the polymorphism on the risk of CAD in a multiple logistic regression model, neither a model including a dominant and a recessive term nor one with a single co-dominant term yielded statistically significant differences \((Table 5)\). Similarly, when we considered subgroups based on number of diseased vessels \((CAD-, SVD, MVD)\) in the multiple logistic regression model, we did not observe statistically significant differences \((data not shown)\).

### IL-1\(\beta\) –511 C/T polymorphism and risk of CAD

The genotype and allele distribution of the –511 C/T promoter polymorphism of IL-1\(\beta\) gene are shown in Table 3. Genotype distributions were in Hardy-Weinberg equilibrium, both in cases \((p=0.48)\) and in controls \((p=0.59)\). There was no difference in genotype and allele distribution between CAD\(^+\) and CAD\(^−\) patients according to either Fisher’s exact test \((Table 3)\) or permutation tests \((Table 4)\).

Among CAD\(^+\) patients, no statistically significant difference in genotype and allele distribution was found between patients with SVD and those with MVD \((Tables 4 and 3)\).

As for IL-1 RA polymorphism, neither a model including a dominant and a recessive term nor one with a single codominant term was statistically significant \((Table 5)\).
Combination of IL-1β -511 C/T and IL-1 RA polymorphisms and risk of CAD

IL-1β (-511) and IL-1 RA intron 2 VNTR polymorphisms were in linkage disequilibrium in both cases (D'=0.32) and controls (D'=0.35, p<0.001 for both).

When terms for both polymorphisms were simultaneously included in a logistic regression model with forced co-variates no additional effects met the 0.025 significance level for entry into the model (stepwise selection procedure): residual $\chi^2 = 2.16$, degrees of freedom= 4, $p = 0.71$ for dominant or recessive effects, and residual $\chi^2 = 1.14$, degrees of freedom = 2, $p = 0.57$ for co-dominant effects (Table 5). No statistically significant associations were found testing for various combinations of models of inheritance (dominant or recessive for one polymorphism mixed with co-dominant effect for the other), or for inclusion of terms for genotype-genotype interaction (data not shown).

Discussion

This study evaluated the potential role on the risk of CAD of the IL-1 gene cluster polymorphisms, in particular related to IL1-RA and IL-1β genes.

IL-1 RA binds to cellular interleukin-1 receptors, without activating them and therefore acts as a competitive inhibitor of interleukin-1. Recently, the association of IL-1RA gene polymorphisms with SVD and risk of restenosis after coronary stenting has been studied, with contrasting results. We were not able to demonstrate any significant association between the IL-1 RA polymorphism and the risk of CAD. This is in agreement with a recently published large prospective study that showed no association between IL-1 RA VNTR gene polymorphism and the risk of future MI and with our previous results on Italian patients with MI at young age. Similarly to other studies, a difference in genotype distribution between SVD and MVD groups was observed in our study using raw analysis not taking into account adjustment for multiple comparisons. To avoid reporting spurious associations and results that occur by chance alone, we performed additional tests dealing with the problem of multiple comparisons. In such analysis we did not confirm evidence of a significant association between IL-1 RA polymorphism and the risk of SVD. Francis et al. associated the presence of allele 2 of the IL-RA polymorphism with an increased risk of SVD. Such an association was found in an English population from Sheffield, but was not confirmed in another from London. Moreover the definition of CAD was different, since patients with coronary stenoses >30% were included as cases. In our study, patients with a stenosis exceeding 50% were considered as CAD.

In contrast, Kastrati et al. studying 1,850 patients, reported a protective effect of allele 2 of IL-1 RA (+2,018) exon 2 polymorphism on the incidence of coronary re-stenosis after stenting. This polymorphism shows a high degree of linkage disequilibrium with the IL-1 RA VNTR polymorphism. The allele 2 has been associated, although not consistently, with higher plasma levels and higher monocyte release of IL-RA. The inhibiting effect of IL-RA on IL-1 activities could be responsible for a slight reduction in the risk of developing SVD. In MVD, in which the inflammatory stimulus is much stronger and the pathogenetic mechanism is complicated by many additional factors, the effect of allele 2 might no longer be evident. Recently, it has been shown that the enhancing effect of IL-1 RA allele 2 on IL-1 RA plasma levels may require the presence of the IL-1β (-511) allele T.

Therefore a combined analysis of the two polymorphisms was performed, assessing the risk of both CAD and SVD. We did not find any substantial difference in risk associated with the combined effect of the two polymorphisms.

Furthermore, we did not find any risk for CAD related to the IL-1β -511 C/T polymorphism. Indeed, there were no differences in the frequencies of the genotypes and alleles between cases and controls. These results are in agreement with our preliminary observations showing no difference in IL-β (-511) polymorphism distribution according to the number of affected vessels in patients with premature MI (unpublished data). In contrast, the polymorphism was associated with the risk of MI at young age, which suggested a possible role of the IL-1β−511 C/T polymorphism in the risk of thrombosis rather than in development of arteriosclerosis.

Our study has some limitations. The use as a control group of patients who had a coronary angiography test with a negative finding of arteriosclerosis may introduce some bias. Targeted polymor-
phisms may be linked to each of the reasons for which control patients underwent the coronaryography test. So we cannot generalize the results of this study to all populations. Our study did not reach sufficient power to assess a possibly slight influence of the polymorphisms on the risk of CAD. Indeed, it was powered to reveal differences of 75% between CAD- and CAD+. Moreover, the IL-1 system is influenced by other cytokines whose expression levels may also be controlled at the DNA level, so the observed effects could be attributable to interaction of the IL-1 family with other cytokines and/or their genes. Genetic association studies require cautious consideration and replication. Heterogeneity in the strength of an association is common even within studies of seemingly similar populations, which may differ in parameters that are not yet known or in parameters that the original studies have not considered. In conclusion, our study does not provide sufficient evidence supporting associations between IL-1β –511 C/T and IL-1 RA intron 2 VNTR polymorphisms with the risk of CAD. Up to now, the data available on this topic do not allow conclusive considerations; more studies, on larger cohorts of patients, are needed to confirm the potential role of the IL-1 gene cluster polymorphisms in the risk of CAD.

References

Pre-publication Report & Outcomes of Peer Review

Contributions
BV: analysis and interpretation of data and drafting of the manuscript; ADC: analysis and interpretation of data and critical revision; RT: conception and design of the study; AD: analysis and interpretation of data; GP: conception and design of the study and critical revision; MA: conception and design of the study; MA: conception and design of the study; Li: conception and design, interpretation of data, drafting of the manuscript, and gave the final approval. The authors thank Dr. Giovanni de Gaetano, Catholic University, Campobasso, for his useful suggestions and Ms Alexandra Cianci who helped prepare the manuscript. They are also particularly indebted to the cardiologists of the Department of Cardiosurgery of the University of Rome “La Sapienza” for allowing them to study their patients. Primary responsibility for the paper: Li; primary responsibility for tables 1-5: Li.

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Disclosures
Conflict of interest: none.
Redundant publications: no substantial overlapping with previous papers.

Manuscript processing
This manuscript was peer-reviewed by two external referees and by Professor Steven S. Witkin, who acted as an Associate Editor. The final decision to accept this paper for publication was taken jointly by Professor Witkin and the Editors. Manuscript received September 3, 2002; accepted November 7, 2002. In the following paragraphs, professor Witkin summarizes the peer-review process and its outcomes.

What is already known on this topic
The interleukin-1 system plays a pivotal role in the regulation of inflammation. It is perhaps not surprising, therefore, that associations between polymorphisms in the genes coding for IL-1β and IL-1RA and a large number of different disease states have been reported. The evidence appears to be most consistent for chronic inflammatory disorders. Investigations relating polymorphisms in these two genes to atherosclerotic changes have yielded inconsistent results.

What this study adds
The present study should serve as a model on how to conduct gene polymorphism research studies. The statistical analysis of the data is comprehensive and other variables that may influence outcome — polymorphisms in other identified or unidentified genes, population and ethnic variability, use of adequate control groups — are succinctly delineated.

Caveats
Although a control group of individuals who did not undergo angiography is lacking, the distribution of the IL-1β and IL-1RA alleles in the controls that were utilized parallel that seen in other populations. The results demonstrate, therefore, that the -511IL-1β and IL-1RA polymorphisms do not have a major influence on the risk of coronary artery disease. The influence of gene polymorphisms on inflammatory events are difficult to interpret conclusively since what is important for the disease is increased production of pro-inflammatory mediators at a specific localized site, and not necessarily a systemic-wide elevation in cytokine levels. Publication of comprehensive studies that show negative results is essential in providing the reader with a balanced analysis of the field.