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ORIGINAL ARTICLE

The -174 G > C Interleukin-6 Gene Polymorphism is Associated with Angiographic Progression of Coronary Artery Disease over a 4-Year Period

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SNP

Abstract *Background:* Inflammation is a key process underlying the clinical course of coronary artery disease (CAD). C-reactive protein (CRP) and interleukin-6 (IL-6) contribute to its pathophysiology and act as biomarkers. We sought to examine whether known single nucleotide polymorphisms (SNPs) impact CAD progression, reflecting increased inflammation.
Methods: We retrospectively evaluated coronary angiographies of patients with established CAD who were re-investigated for stable/unstable angina after a time interval of >12 months. We defined progression of CAD as the emergence of a new plaque or a ≥20 % increase of a formerly non-significant lesion. We genotyped patients for the 1846 C>T CRP and -174 G>C IL-6 SNPs. The probability of CAD progression among the Mendelian randomization groups was evaluated using the Kaplan—Meier method. Data were analyzed using a Cox model that included relevant clinical factors.
Results: A total of 157 patients were included. The serum levels of CRP and IL-6 differed significantly between genotypes. The genotype frequencies of IL-6 were consistent with Hardy—Weinberg equilibrium, whereas those for CRP were excluded from our conclusions. At 48

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months, 83 patients (52.9 %) with the IL-6 C allele versus 74 (47.1 %) with the G allele exhibited CAD progression. Patients with the IL-6 C allele had a 52.8 % probability for progression versus 13.3 % for those with the G allele ($p=0.005$). The results were confirmed by multivariate analysis; dyslipidemia, family history, and IL-6 SNP emerged as significant factors.
Conclusion: Patients with established CAD who carried the -174 C allele of the IL-6 gene demonstrated an increased risk for the progression of coronary plaques over a four-year period. Further studies will be needed to validate these findings.

1. Introduction

It is well established that inflammation is a critical component of the process of atherosclerosis that drives the pathogenesis of clinical syndromes.[1–4] Inflammatory cells and lipoproteins interact with biochemical substrates in the vascular wall and lead to atheroma formation, along with further changes in atherosclerotic plaque composition and inflammation, which lead to either stable or unstable disease. Amongst the most studied inflammation-related molecules involved in this process are Interleukin-6 (IL-6) and C-reactive protein (CRP), which both can serve as biomarkers in every-day clinical practice. From a clinical perspective, it is critical to understand the progression of coronary artery disease (CAD) in patients with established lesions. Many studies have suggested that CRP and IL-6 are associated with the long-term risk for recurrent cardiovascular events among patients with established disease and future events among individuals who are at risk for atherosclerosis.[5–10]

In the last two decades, human genetic studies have identified single nucleotide polymorphisms (SNPs) as a natural characteristic of genetic diversity among individuals. With advances in genotyping, several genetic polymorphisms have been linked to phenotypic effects. Many SNPs that are located in promoter regions have a functional impact by causing significant changes in the expression levels of gene products. Accordingly, several studies have linked SNPs in the CRP and IL-6 genes to clinical aspects of cardiovascular disease, such as incidence of asymptomatic carotid, coronary and peripheral artery disease, longer stays in intensive care units after cardiovascular events, and even hard outcomes such as mortality, although results sometimes are conflicting.[11–28]

Based on data that inflammation is a subclinical process that affects vascular biology over a large time-span before its causes clinically obvious outcomes, we sought to evaluate the impact of SNPs in the IL-6 and CRP genes on the progression of CAD in a Mendelian-randomization study through angiographic evaluation of clinical syndromes in patients with previously established CAD.

2. Methods

2.1. Coronary Angiographies

We retrospectively investigated previous coronary angiographies in patients who underwent catheterization in our lab to investigate stable angina or unstable angina/NSTEMI from June 2006 to June 2014. Patients with a prior visit who underwent percutaneous coronary interventions for the treatment of a clinical syndrome were included in the study. We characterized the coronary lesions in each patient (angiographically significant stenoses that caused $\geq$50 % narrowing of an epicardial coronary artery lumen). By comparing two angiographies, we defined the progression of coronary artery disease as the emergence of a new coronary lesion or a $\geq$20 % clinically relevant increase in a formerly known, but non-significant and untreated coronary lesion.

The minimum time interval allowed between two consecutive angiographies was 12 months. Exclusion criteria included patients with STEMI, comorbidities such as severe kidney or liver disease, malignancies, and inflammatory or autoimmune diseases. All patients received therapy according to standard guidelines and were under medication for the secondary prevention for coronary disease. Each patient provided informed consent and the study protocol was approved by the ethics committee of the hospital.

2.2. Blood measurements and genotyping assays

Peripheral venous blood samples were drawn from every patient at the time of the second angiography. The serum IL-6 levels were measured by enzyme-linked immunosorbent assay (Quantine HS-Human IL-6) according to standard techniques. Serum CRP levels were measured using the immunonephelometric method. We performed genotype analyses for the 1846 C>T CRP gene (rs1205, NC_000001.11:g.159712443C>T) and -174 G>C IL-6 gene (rs1800795, NC_000007.13:g.22766645C>G) SNPs. Genomic DNA was extracted from blood samples using a QIAamp Blood Midi Kit (Spin Protocol, Qiagen). Genotyping analyses were performed by the restriction fragment length polymorphism polymerase chain reaction (RFLP-PCR) method. Patients were divided into three groups depending on the SNP that they carried (i.e., homozygous referent or heterozygous or homozygous variant) for each gene (see Appendix for more details).

2.3. Statistical Analysis

The primary study endpoint was the cumulative probability for CAD progression after the first angiography among

different groups of Mendelian randomization polymorphisms for the IL-6 and CRP gene promoter regions (SNPs rs1800795 and rs1205, respectively).

The consistency of genotypes with Hardy–Weinberg equilibrium (HWE) was tested using the χ^2 and exact tests, as described by Wigginton.[21] Continuous variables are expressed as the mean ± sd and categorical variables are presented as percentages %. Differences between groups were assessed using the Kruskal–Wallis test, *t*-test, Mann–Whitney U test, χ^2 or Fisher's exact test with the Bonferroni adjustment, as appropriate.

Follow-up time was considered to be the time interval between the two angiographies (and for patients with progression of CAD, it was considered to be the time-to-progression). Differences in the time-to-progression were evaluated using the Kaplan–Meier method and log-rank test for the three genotypes with two different alleles for each gene. Data were further evaluated by Cox proportional hazards analysis regarding primary endpoints and relevant clinical factors. An initial multivariate (basic effects) model was built that included all significant variables of the univariate analysis (at a level of 0.15 significance), with a step-by-step backwards elimination approach based on the Wald statistic criterion until only significant variables remained. A final model was built by adding clinical variables relevant to CAD progression to the initial model.

The level of statistical significance was two-sided at 0.05 or at a level of 0.017 for multiple comparisons of categorical variables (Bonferroni adjustment). All statistical analyses were performed using the SPSS version 23.0 software package.

3. Results

A total of 190 patients were enrolled in this study. Among these patients, 33 were excluded because they met one or more exclusion criteria or refused to provide consent for genotyping. Ultimately, we included 157 patients, including 81 % men, with a mean follow-up time of 50.9 ± 21.8 months (median: 48 months, minimum: 12 months, maximum: 88 months).

The genotypic distribution of alleles for IL-6 was as follows: GG homozygotes, 30 %; CC homozygotes, 55 %; GC heterozygotes, 72 % (C variant frequency, 58 %), while those for CRP were as follows: CC homozygotes, 70 %; TT homozygotes, 47 %; CT heterozygotes, 40 % (T variant frequency, 43 %). After completion of this study, testing for G and C alleles using survival analysis along with a probability of type I error 0.05 was carried out and yielded for the IL-6 and CRP genes a statistical power of 88.79 % and 88.91 %, respectively. Distributions of IL-6 alleles were consistent with HWE: $\chi^2=0.54542$, $p=0.460$, but the distribution of CRP alleles departed from HWE: $\chi^2=36.06290$, $p<0.001$. We further evaluated consistency with HWE for the CRP gene with the exact test, as described by Wigginton, and obtained similar results ($p=0.00022$), which led us to exclude the CRP SNP from our conclusions (see relevant data presented in Tables AI and II in the Appendix). Baseline characteristics of patients were evenly distributed except for serum IL-6, which differed significantly among genotypes as follows for mean serum IL-6: CC, 5.58±2.52; GG, 2.73±1.08; and GC, 4.04±1.86, $p<0.001$ (Table 1 and Figure 1).

A total of 83 patients (52.9 %) demonstrated progression of CAD, in contrast to 74 patients (47.1 %) that did not have progression. Survival from disease progression differed significantly among GG, GC and CC genotypes. Patients with the IL-6 CC genotype demonstrated 61.8 % cumulative probability for progression, versus 45.8 % for GC and 13.3 % for GG, $p=0.005$. Similar results were obtained when the impact of SNPs was examined per allele: carriers of the C allele had a cumulative probability for progression of 52.8 % versus 13.3 % for the G allele, $p=0.005$ (Table 2 and Figure 2).

Using multivariate analysis with the basic effects model, the significance of the IL-6 C allele on CAD progression was confirmed and the presence of CAD family history emerged as a significant variable. When well established clinical factors of CAD were forced in the final model, the impacts of the IL-6 C allele and family history were preserved. Furthermore, among other clinical factors, the presence of

Table 1 Characteristics of patients among the IL-6 genotypes.

	Total (n=157)	CC (n=55)	GC (n=72)	GG (n=30)	*p*-value	GC+CC (n=127)	*p*-value
Age (years)	66.6±9.4	67.7±8.9	66.8±9.5	64.1±9.8	0.232	67.2±9.2	0.134¶
Men (%)	127 (80.9 %)	45 (81.8 %)	58 (80.6 %)	24 (80.1 %)	0.975	103 (81.1 %)	0.891
BMI (kg/m^2)	28.2±3.7	28.2±4.1	27.8±3.4	28.9±3.5	0.363	27.9±3.7	0.353¶
Ejection Fraction (%)	49.7±7.6	48.5±9.3	50.1±6.5	50.6±6.6	0.925	49.4±7.8	0.773¶
Smoking (%)	47 (29.9 %)	16 (29.1 %)	22 (30.6 %)	9 (30.1 %)	0.984	38 (29.9 %)	0.993
Diabetes (%)	47 (29.9 %)	19 (34.5 %)	20 (27.8 %)	8 (26.7 %)	0.647	39 (30.7 %)	0.664
Hypertension (%)	115 (73.3 %)	41 (74.5 %)	53 (73.6 %)	21 (70.0 %)	0.898	94 (74.1 %)	0.655
Family History (%)	61 (38.9 %)	16 (29.1 %)	33 (45.8 %)	12 (40.1 %)	0.157	49 (38.6 %)	0.886
Dyslipidemia (%)	137 (87.3 %)	47 (85.5 %)	63 (87.5 %)	27 (90.1 %)	0.860§	110 (86.6 %)	0.767§
Serum IL-6 (mg/dl)	4.3±2.3	5.58±2.52	4.04±1.86	2.73±1.08	**<0.001**≠	4.7±2.3	**<0.001**¶
Serum CRP (mg/dl)	2.5±2.4	2.57±2.29	2.33±2.29	2.87±2.95	0.792	2.4±2.3	0.956¶

IL-6, Interleukin-6; BMI, Body Mass Index; CRP, C-Reactive Protein; G, Guanine; C, Cytosine. The *p*-values were calculated using the χ^2 and Kruskal–Wallis tests.

Bold values are statistical significant *p*-values.

≠ among all categories.

§ Fisher's exact test.

¶ Mann–Whitney U test.

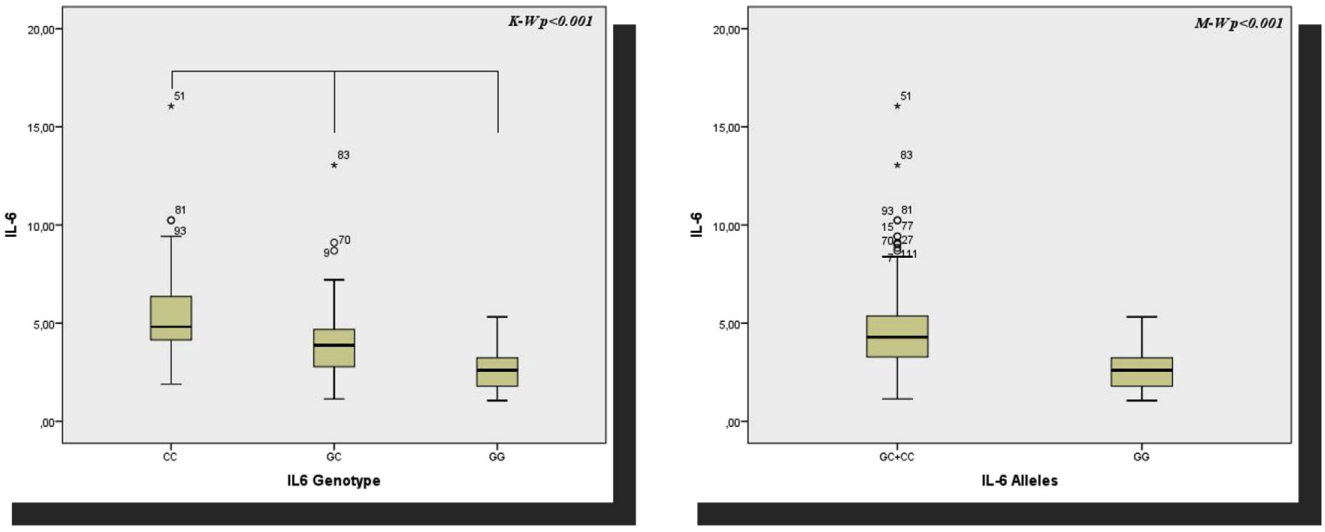


Figure 1 Population-wide distribution of the serum IL-6 levels among three genotypes and two alleles of the rs1800795IL-6 SNP; C, Cytosine; G, Guanine; K—W, Kruskal—Wallis test; M—W, Mann—Whitney U test.

Table 2 Cumulative probability for progression among genotypes during the follow-up period.

Genotype	Cumulative probability	*p*-value	Alleles	Cumulative probability	*p*-value
IL-6 gene		**0.005**	IL-6 gene		**0.005**
GG	13.3 %		GG	13.3 %	
GC	45.8 %		GC+CC	52.8 %	
CC	61.8 %				

IL-6, Interleukin-6; G, Guanine; C, Cytosine. All *p*-values were calculated using the log-rank test.

dyslipidemia showed a significant contribution to the events. The Interleukin-6 C allele showed the strongest impact on CAD progression (Table 3).

4. Discussion

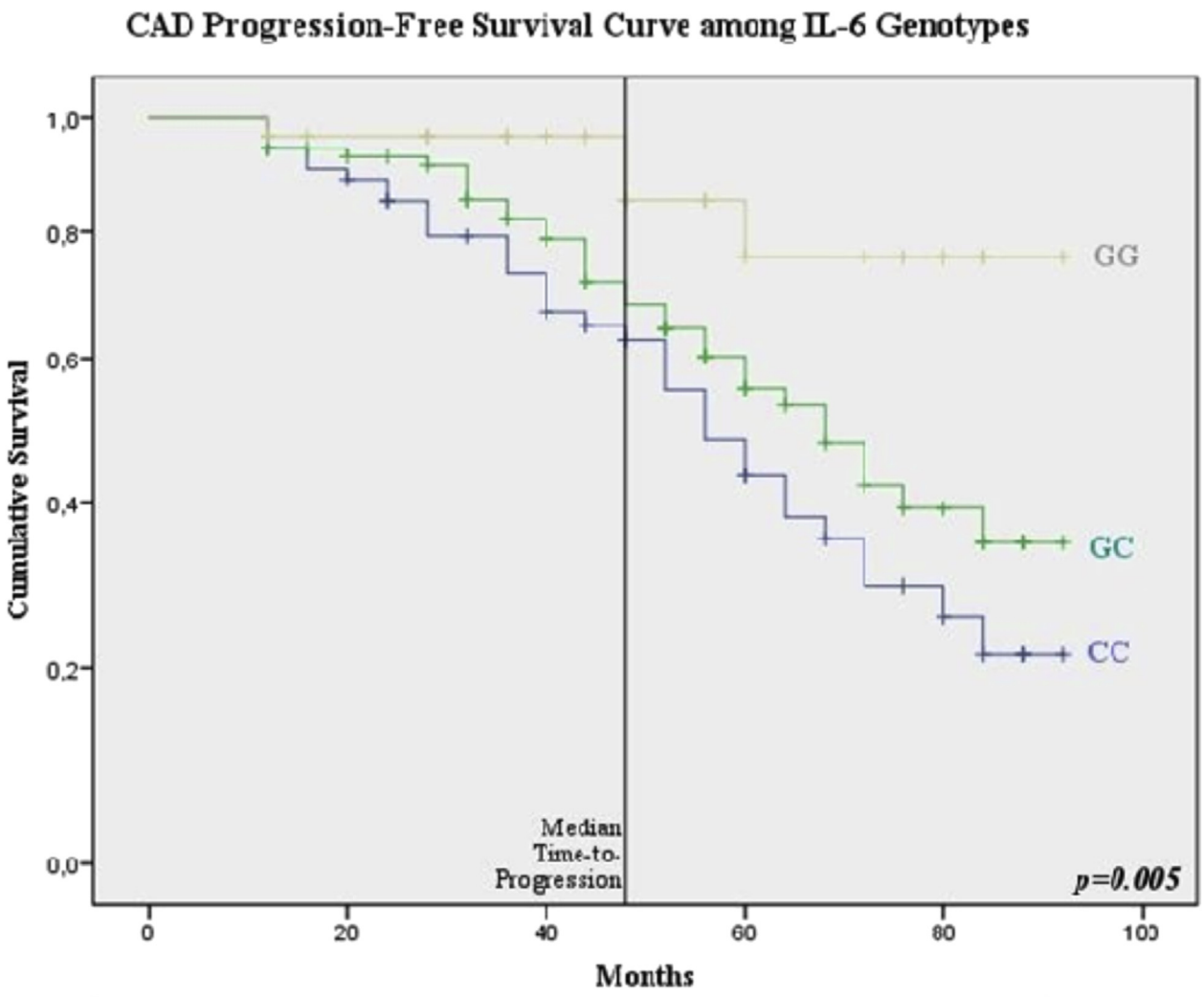
This current study demonstrated that patients with established CAD who carry the -174 C allele of the IL-6 gene promoter demonstrated an increased risk for new clinical events resulting from the angiographic progression of coronary plaques over a 4-year period.

We assessed the impact of specific SNPs of inflammation-related genes on the angiographic progression of CAD, while taking into account the possibility that small changes in serum levels of inflammatory molecules could play a role in the clinical course of disease over a long-term period. Our study groups were formed by genotype, thus by Mendelian randomization the baseline characteristics were evenly distributed, except for differences of the relevant gene products in the serum (i.e., IL-6 and CRP levels). Mendelian randomization studies are useful for adding evidence about the causality of an observed association. Indeed, in the higher concentrations of IL-6 and CRP in patients with already established CAD has been previously reported.[11]

All participants in this study were "real-life" patients with previously known CAD all in secondary prevention; that later demonstrated a new clinical syndrome in a different time frame. As consistency with HWE is a major factor in genotyping studies, we excluded CRP genotype analysis from our results because the CRP allele distribution deviated significantly from HWE. Deviations from HWE can be caused by several reasons, including associations of an allele with the disease state in patient studies; however, small sample, population stratification, and genotyping errors cannot be excluded.[22]

The C allele of the -174 IL-6 SNP has been found more frequently among CAD patients and their first-degree relatives. It has also been associated with higher systolic pressure and adverse cardiovascular outcomes, such as myocardial infarction and survival, in three vessel disease patients in European and Asian populations.[13, 23—26]

In our present study, the different allele groups for each gene resulted in different serum concentrations of the gene products, yielding a measure of the functional differences among genotypes. The IL-6 -174 G>C SNP had a significant impact on the time to progression of CAD. Patients homozygous for the IL-6 -174 G allele demonstrated a higher concentration of IL-6 in the serum compared with patients who were homozygous for the IL-6 -174 C allele. This specific allele would have exposed those patients to constantly higher concentrations of IL-6 over time, reflecting a naturally increased inflammatory response to underlying causal factors. This general innate characteristic could modify the natural course of CAD by increasing the harmful effects and imbalance of the inflammatory response, ultimately leading to instability of plaques through the higher recruitment of inflammation-responsive cells, oxidation of lipids in the plaque core, production of reactive oxygen species, and



Time (months)	0	12	24	36	48	60	72	84
Nr at Risk								
GG	30	29	29	29	27	26	26	26
CC	55	52	47	41	36	28	23	21
GC	72	68	67	60	53	46	41	39

Figure 2 Kaplan—Meier curves for CAD progression among three genotypes of the IL-6 gene; *p*-values were calculated using the log-rank test.

altered necrosis rates. Indeed, patients who inherited the allele that yielded higher concentrations of serum IL-6 showed a significantly higher probability for progression of CAD at 4 years, a finding that was confirmed in multivariate analysis.

The presence of a family history of CAD and dyslipidemia were also important factors that influenced our findings, possibly reflecting the global genetic burden (other than the studied SNPs) and the importance of dyslipidemia in the pathophysiology of coronary disease. Other clinical factors, such as smoking, hypertension and diabetes, did not reach statistical significance in the multivariate analysis of disease progression events (Table 3). This might reflect a suboptimal treatment of hyperlipidemia without a suitable control for other factors in these patients, although we do not have detailed data about compliance to secondary prevention. The effective control of modifiable risk factors through lifestyle changes and medications, along with a typical regular medical follow-up, would likely eliminate the additional risk of these factors on disease progression. Nevertheless, the influence of family history and dyslipidemia is reasonable and, in presence of them, the IL-6 C allele polymorphism represented a considerable hazard in the interpretation of time-to-progression. According to these observations, polymorphisms in inflammatory genes may produce, at least in part, an independent impact on the clinical course of CAD.

4.1. Limitations

Our study is limited by the small sample size, in contrast to desired samples, in Mendelian randomization studies.

Table 3 Multivariate analysis of CAD progression.

Basic effects model			Final model		*p*-value
Variables	HR, 95 % CI	*p*-value	Variables	HR, 95 % CI	
Family history	2.26, [1.35, 3.79]	**0.002**	Family history	2.46, [1.43, 4.23]	**0.001**
IL-6 CC+CG	4.24, [1.33, 13.6]	**0.015**	IL-6 CC+ CG	5.09, [1.55, 16.7]	**0.007**
			Age	0.99, [0.97, 1.02]	0.518
			Smoking	1.03, [0.76, 1.40]	0.840
			Diabetes	0.87, [0.65, 1.21]	0.442
			Hypertension	0.77, [0.55, 1.07]	0.112
			Dyslipidemia	1.47, [1.02, 2.11]	**0.041**

HR, hazard ratio; CI, confidence interval; IL-6, Interleukin-6; G, Guanine; C, Cytosine. The *p*-value was calculated using Cox proportional hazard analysis.
Bold values are statistical significant *p*-values.

There was no control/replication group, as comparisons were performed in an established cohort of CAD patients. Although we did not assess the adherence of patients to secondary prevention measures, the results of this present study represent every-day clinical practice. Furthermore, we lack of multiple measurements of serum IL-6 and CRP in the interval between the two coronary investigations to establish the constant increase of gene products among alleles.

5. Conclusions

Patients with established CAD who carry the -174 C allele of the IL-6 gene promote, demonstrated an increased risk for new clinical events because of the progression of coronary plaques over a 4-year period. Further studies will be needed to validate these findings.

Conflict of interest

No conflict of interest disclosed.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.hjc.2017.02.002.

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