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Cox-2 gene polymorphism and IL-6 levels in coronary artery disease

K.K. Ol[1], B. Agachan[1], U. Gormus[2], B. Toptas[1] and T. Isbir[3]

[1]Department of Molecular Medicine, Institute for Experimental Medicine, Istanbul University, Istanbul, Turkey
[2]Department of Biochemistry, Faculty of Medicine, Istanbul Bilim University, Istanbul, Turkey
[3]Department of Medical Biology, Faculty of Medicine, Yeditepe University, Istanbul, Turkey

Corresponding author: T. Isbir
E-mail: tisbir@superonline.com / turgay.isbir@yeditepe.edu.tr

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ABSTRACT. Coronary artery disease is one of the leading causes of mortality and diabetes mellitus is one of its main risk factors due to microvascular and macrovascular complications, such as atherosclerosis. Atherosclerosis is now known to be an inflammatory process mediated by prostaglandins and several interleukins. As both are important in inflammatory processes, we examined Cox-2 (-765G > C) polymorphism and interleukin-6 levels in coronary artery disease patients compared to healthy controls. We also divided the patients into diabetic and non-diabetic groups to check the effects of diabetes mellitus separately. We found that the GG allele frequency was significantly higher in the patient group. Patients with the GG genotype had an approximately 2.78-fold higher risk of coronary artery disease. We also found that the Cox-2 (-765G > C) polymorphism is associated with lower interleukin-6 levels, which decreased in the order: GG > GC > CC.

Key words: Cox-2 (-765G > C); Polymorphism; Diabetes mellitus; Coronary artery disease; IL-6

INTRODUCTION

Coronary artery disease (CAD) and stroke are multifactorial disorders known to be caused both by genetic predispositions and life styles. One of the main predisposing factors is the existence of diabetes mellitus (DM). DM is one of the leading causes of morbidity and mortality in Western countries due to its microvascular and macrovascular complications. It is known to be associated with cardiovascular diseases, including atherosclerosis, CAD, hypertension, dyslipidemia (Laakso et al., 1985; Barrett-Connor et al., 1991; Biondi-Zoccai et al., 2003; Sjöholm and Nyström, 2005).

Atherosclerosis of coronary arteries is known to be an inflammatory process mediated by prostaglandins (PGs) and PGs are synthesized by the enzyme cyclooxygenases (Cox) (Davis et al., 2006). One of the three known isoforms of Cox enzymes, Cox-1, is constitutively expressed in most human tissues; but Cox-2 expression is primarily induced in response to inflammatory stimuli (Huuskonen et al., 2008). Since they are part of the chronic inflammatory process, Cox-2 levels are generally known to be higher in atherosclerosis (Huuskonen et al., 2008). Cox-2 expression has been detected in endothelial cells, smooth muscle cells, monocytes, and macrophages within human atherosclerotic lesions. Many PGs produced by the Cox-2 enzyme, including thromboxane, stimulate vasoconstriction, platelet aggregation, and leukocyte-endothelial cell adhesion and so contribute to the formation of thrombosis and atherosclerosis (Huuskonen et al., 2008).

Cox-2, which is normally undetectable in most tissues, can be upregulated by bacterial lipopolysaccharides, cytokines, growth factors, and tumor promoters, suggesting its relevance to inflammation (Prescott and Fitzpatrick, 2000; Pisetsky and St. Clair, 2001). Cox-2 was found to be induced in atherosclerotic plaque, but not in normal blood vessels, and is responsible for the increase in PGI_2 biosynthesis seen in patients with atherosclerosis (Belton et al., 2000; Antman et al., 2005).

The human Cox-2 gene is located on chromosome 1q25.2-q25.3, is about 8.3 kbp in size and contains 10 exons (Yokoyama and Tanabe, 1989; Kosaka et al., 1994). The Cox-2 (-765G > C) polymorphism was previously found to be associated with lower promoter activity (Papafili et al., 2002; Orbe et al., 2006).

In this study, according to all those interrelationships, we aimed to examine Cox-2 (-765G > C) polymorphism in relation to IL-6 levels as an inflammation marker in CAD patients with or without DM compared to healthy subjects.

MATERIAL AND METHODS

Patient selection and clinical investigation

One hundred and eighteen CAD patients and 80 healthy control subjects were included in this study. The subjects were followed up by Istanbul University, Faculty of Medicine, Department of Endocrinology and Metabolism. WHO definitions and criteria for diabetes were used during ascertainment (WHO, 1999). The patients received a standard questionnaire regarding the diagnosis time, family history, treatments, and other medical issues. The control group included individuals with normal fasting glucose and negative family history for DM type II among first-degree relatives. All participants signed an informed consent before enroll-

ment and approval was obtained for the study from the Institutional Ethics Committee.

DNA isolation

Blood specimens were collected in tubes containing EDTA. DNA samples were extracted from whole bloods with the salting-out procedure (Miller et al., 1988).

Analysis of Cox-2 (-765G > C) polymorphism

The primers for polymerase chain reation (PCR) amplification of the target region are forward: 5' AGG CAG GAA ACT TTA TAT TGG 3' and reverse: 5' ATG TTT TAG TGA CGA CGC TTA 3' (19). Cox-2 (-765G > C) genotyping was performed using the PCR-restriction fragment length polymorphism (PCR-RFLP) technique. PCRs were conducted in a reaction mixture containing 100 ng genomic DNA that was placed in 10 mM Tris-HCl, 50 mM KCl buffer containing 0.2 mM of each dNTP, 1.5 mM $MgCl_2$, 50 pmol for each primer and 1.0 U Taq DNA polymerase. Amplifications were done in an Applied Biosystems Gene Amp PCR system 9700 Gold Plate Thermocycler with the following process: 95°C for 3 min followed by 35 cycles of 95°C for 45 s, 55°C for 45 s, and 72°C for 45 s, and finally 72°C for 45 s. PCR products were digested with *Aci*I restriction enzyme at 37°C overnight. Digested DNAs were separated on 2% agarose gel in 1X Tris-borate EDTA buffer, followed by staining by an ethidium bromide solution. The Cox-2 (-765G > C) genotypes were typed by visualization under ultraviolet light. G allele results in 209- and 109-bp fragments. C allele remains undigested, 309 bp (Figure 1).

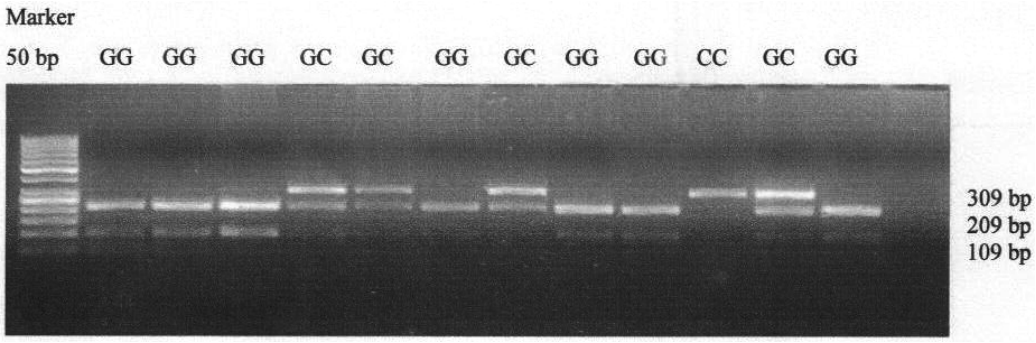

Figure 1. Genotyping for Cox-2 (-765G > C) polymorphism.

IL-6 assay

Serum was separated and frozen at -20°C until serum IL-6 levels were determined by enzyme-linked immunosorbent assay (Cat No. KHC0011 Biosource International, USA).

Statistical analysis

Statistical analyses were performed using the SPSS version 11.0 for Windows (SPSS

Inc. Chicago, IL, USA). Discrete variables were expressed as counts or percentages and compared by the chi-square or the Fisher exact test when appropriate. Analysis of variance (ANOVA) was used to compare concentrations of serum IL-6 levels. Continuous variables are reported as means ± standard error of the mean (SEM) and compared by the Student *t*-test or ANOVA for more than two groups. Results were considered to be statistically significant at $P < 0.05$.

RESULTS

Patient and control groups had similar distributions for gender and age. The total patient group had a significantly higher level of IL-6 compared to healthy controls ($P \leq 0.011$) (Table 1).

Table 1. Clinical characteristics of patients and healthy control group.

	Control (N = 80)	CAD (N = 118)
Gender (female/male)	28/52	38/80
Age (years)	54.80 ± 1.75	58.12 ± 0.91
IL-6 levels (pg/mL)	35.84 ± 7.62	75.97 ± 13.32

Data are reported as means ± SD. N = number of individuals; CAD = coronary artery disease.

CAD patients were also separated into two different groups, diabetic and non-diabetic. We found higher IL-6 levels in non-diabetic patients with CAD than in controls ($P \leq 0.020$) and increased IL-6 levels in diabetic CAD patients compared to the control group but with no statistically significant differences ($P > 0.059$). We observed no significant difference of IL-6 levels between diabetic and non-diabetic CAD groups ($P > 0.74$) (Figure 2).

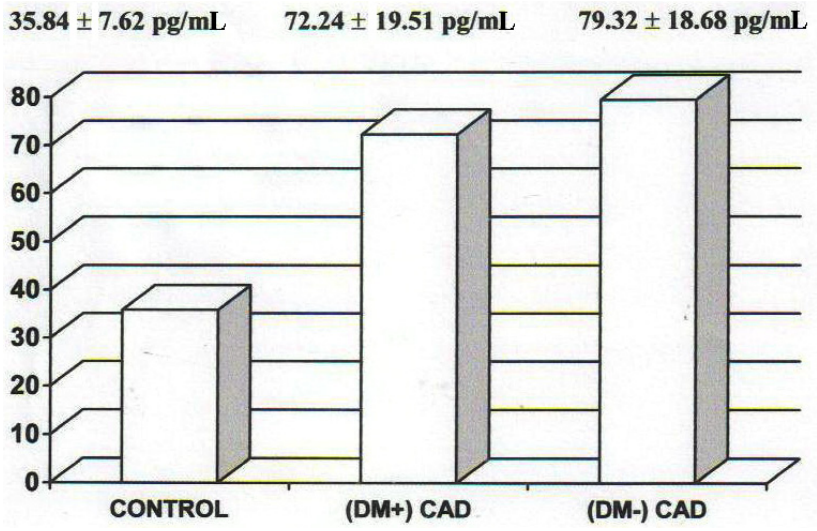

Figure 2. Distribution of IL-6 levels in patient and control groups. (DM+) CAD and (DM-) CAD = diabetic and non-diabetic coronary artery disease patients, respectively.

As shown in Table 2, the genotype distributions and allele frequencies for the Cox-2 (-765G > C) gene were not found to be significantly different between groups ($P > 0.58$, $\chi^2 = 2.81$).

Table 2. Allele and genotype distribution of Cox-2 (-765G > C) in patients and controls.

Cox-2 (-765G > C) genotypes	Control	Total CAD	(DM-) CAD	(DM+) CAD
CC	9 (11.3%)	8 (6.7%)	6 (8.5%)	2 (4.3%)
GG	27 (33.8%)	48 (40.6%)	30 (42.3%)	18 (38.3%)
GC	44 (55.0%)	62 (52.5%)	35 (49.3%)	27 (57.4%)

CAD = coronary artery disease; (DM-) = non-diabetic patients; (DM+) = diabetic patients.

The GG genotype was more frequent in the entire CAD patient group compared to the control group (Table 2). The GG genotype was found to increase the risk of CAD by approximately 2.78-fold.

Cox-2 (-765G > C) GG genotype and IL-6 levels were lower in the control group compared to the diabetic CAD patient group ($P > 0.05$) whose IL-6 levels were decreasing respectively, GG > GC > CC (Table 3).

Table 3. Serum IL-6 levels according to Cox-2 (-765G > C) genotypes.

Cox-2 (-765G > C) genotypes	Control	(DM-) CAD	(DM+) CAD
CC	17.53 ± 3.79	55.2 ± 6.23	18.4 ± 4.36
GG	52.8 ± 34.09	105.2 ± 34.11	75.40 ± 35.18
GC	37.02 ± 8.93	63.84 ± 22.13	46.26 ± 19.44

Data are reported as means ± SEM. (DM-) CAD and (DM+) CAD = non-diabetic and diabetic coronary artery disease patients, respectively.

DISCUSSION

In the pathogenesis of CAD, inflammation plays a crucial role through its contribution to the formation of atheroma, which eventually leads to the evolution of atheromatous injury, plaque rupture and intra-luminal thrombosis. Inflammation is a hallmark in the development and progression of CAD and is mediated by prostaglandins, produced by the enzyme prostaglandin endoperoxide H synthase (cyclooxygenase). A direct role of Cox-2 in atherosclerosis would be inferred from studies showing significiant expression in human atherosclerotic lesions. Cox-2-derived PGE_2 was also found to be increased in subclinical atherosclerosis (Schonbeck et al., 1999; Cipollone et al., 2003; Paramo et al., 2005). Paradoxically, recent evidence also points to a potential protective function of this enzyme in cardiomyocytes subjected to oxidative stress and also in late preconditioning after ischemia/reperfusion injury (Gilroy et al., 1999).

Alterations in prostaglandin expression or activity can significantly modify CAD risks (Davis et al., 2006; Huuskonen et al., 2008). Cox-2, a key regulatory enzyme in prostanoid synthesis, plays important roles in inflammatory processes. Cox-2 (-765G > C) polymorphism has been associated with lower promoter activity (Papafili et al., 2002; Orbe et al., 2006).

In the present study, as both of them are important in inflammatory processes, we examined Cox-2 (-765G > C) polymorphism and IL-6 levels in diabetic and non-diabetic CAD patients compared to healthy controls. We found that GG allele frequency was statistically higher in the entire patient group compared to the control group. Statistically, it seemed that people with the GG genotype had an approximately 2.78-fold higher risk of CAD. We also showed that the Cox-2 (-765G > C) polymorphism was associated with lower IL-6 levels, which increased in the order of the GG > GC > CC genotype. Although we found higher IL-6

levels in non-diabetic patients compared to the control group no such difference was found for the diabetic patient group, and as we observed no significant difference in IL-6 levels between diabetic and non-diabetic CAD groups, it would be prudent to study larger groups before coming to a conclusion on that parameter.

In parallel to our results, Papafili et al. (2002) and Orbe et al. (2006) found that the Cox-2 -765 C allele might provide protective effects for myocardial infarction (MI) and that the C allele might be associated with lower levels of inflammatory markers such as C-reactive protein and IL-6 in cardiovascular patients. Similarly, in some other studies it was also shown that Cox-2 (-765G > C) promoter polymorphism could be an inherited protective factor against MI and stroke (Cipollone et al., 2001, 2003; Rudock et al., 2009). Another study suggested that the C allele of Cox-2 (-765G > C) polymorphism was associated with lower Cox-2 expression, reduced subclinical atherosclerosis and systemic inflammation compared with GG homozygous, thus conferring atherosclerosis protection in populations with cardiovascular risk (Orbe et al., 2006).

But contrary to those results, Hegener et al. (2006) found no evidence of an association of the Cox-2 polymorphisms with risk of MI. Furthermore, Kohsaka et al. (2008) recently reported that the Cox-2 (-765G > C) polymorphism was, in fact, a risk factor for incident stroke in African-Americans and the result was the same for diabetic stroke cases. Huuskonen et al. (2008) reported that the C allele was associated with complicated plaques and severe stenosis at the coronary artery level.

In conclusion, our results showed that there was an association between the Cox-2 GG genotype and higher IL-6 levels in coronary artery disease patients compared to the control group.

The present study has some potential limitations, the small number of patients being the most important one. This could be a reason for some of the results that demonstrated no statistical significance. Statistical significance might be increased with a larger number of patients.

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