Recurrent Coronary Artery Disease Due to Acetylsalicylic Acid Resistance May Be Related to COX-1 and COX-2 Mutations

Asetilsalisilik Asit Direnci Nedeni ile Oluşan Rekürren Koroner Arter Hastalığı, COX-1 ve COX-2 Mutasyonları ile İlişkili Olabilir

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Objective: Acetylsalicylic acid (ASA) is a commonly used antiplatelet drug for the treatment of coronary artery disease (CAD). However, in some patients recurrent CAD occurs due to ASA resistance (AR). This condition may be related to some genetic factors. Therefore, this study aims to investigate the effects of cyclooxygenase (COX)-1 and COX-2 mutations on recurrent CAD due to AR.

Methods: Hundred CAD patients taking 100 mg ASA daily for 2 years were enrolled to the study. The patients were divided into two groups according to their recurrent CAD status. Forty-eight patients with recurrent CAD due to AR and 52 patients without recurrent CAD were selected to ASA resistant (AR+) and ASA non-resistant (AR-) group, respectively. AR was confirmed by platelet aggregation testing. Risk factors related to recurrent CAD were also obtained. After DNA was isolated from peripheral blood, rs1330344 variation in COX-1 and rs20417 variation in COX-2 were determined using real-time polymerase chain reaction. Results were evaluated statistically.

Results: COX-1 and COX-2 mutations were mostly detected in the AR+ group however these data were not found statistically significant. Nevertheless, C allele of COX-2 was found statistically high in the AR+ group (67.9%) (p=0.023). Additionally statistically significant associations were found between high total cholesterol and low density lipoprotein cholesterol levels with the GC genotype of COX-2.

Conclusion: It was suggested a relation between COX-2 mutations and recurrent CAD due to AR. Similar studies with a large population must explain the mechanisms governing the association of COX-1 and COX-2 genotypes and response to ASA in recurrent CAD patients.

Keywords: ASA resistance, recurrent CAD, COX-1, COX-2

ABSTRACT

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Cite as: Kıraç D, Bayram E, Doran T, Keleş EÇ. Recurrent Coronary Artery Disease Due to Acetylsalicylic Acid Resistance May Be Related to COX-1 and COX-2 Mutations. Med J Bakirkoy 2022;18:46-51

Received: 27.10.2021
Accepted: 10.02.2022

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INTRODUCTION

Coronary artery disease (CAD) is a significant health problem and its complications lead to mortality and disability in the world (1). CAD causes more than 7 million people deaths every year worldwide (2). CAD is a complex disease related to genetic and environmental factors (3).

Acetylsalicylic acid (ASA) is an antiplatelet agent and commonly used for treating cardiovascular disease (4). ASA can decrease mortality and adverse cardiovascular events in patients with CAD (5,6). However, it is thought that approximately half of the patients do not benefit from ASA effectively (7). This condition is known as ASA resistance (AR). AR involves the development of thrombotic vascular events despite aspirin treatment. The heritability of this disorder is thought to be approximately 50% (3). Therefore, investigation of the genetic basis of this disorder due to AR is important for finding new treatment approaches.

Patients who have AR are unable to respond to treatment for CAD as a result of recurrent cardiovascular events are observed in these patients (8-11). ASA irreversibly inhibits platelets by acetylating COX-1 and COX-2 enzymes in platelets (12,13). COX genes are related to ischemic stroke (14,15).

The COX-1 gene contains 10 introns and 11 exons. It is located on chromosomes 9q32-q33.3. COX-1 regulates blood coagulation and platelet function in the body. COX-2 contains 9 introns and 10 exons. It is located on chromosomes 1q25.2-q25.3. COX-2 is found generally in the nuclear membrane and exists in platelets and vascular endothelial cells (16).

Although COX-1 and COX-2 genes are related to AR, studies on this subject are insufficient. Therefore in this study the relation between COX-1 and COX-2 variations with AR in recurrent CAD patients were investigated.

The etiology of AR also includes some other factors such as smoking, poor diet, diabetes mellitus, gender, non-compliance with the amount of drug use, hypertension and excessive COX-2 production in platelets (17-19). There may also be some other factors that lead to unsuccessful ASA therapy (17). Therefore in this study the relation between COX-1, COX-2 variations, and other risk factors with recurrent CAD due to AR was also investigated.

METHODS

Patient Characteristics

A hundred patients with CAD who have applied University of Health Sciences Turkey, Umranıye Training and Research Hospital, Cardiology Polyclinic and taking 100 mg ASA daily for 2 years (2018-2020) were enrolled to the study. The patients were divided into two groups according to their recurrent CAD status. Forty-eight patients with recurrent CAD were selected to the AR+ group and 52 patients without recurrent CAD were selected to the AR- group. Patients diagnosed with recurrent CAD were selected from patients who had previously undergone percutaneous coronary intervention (PCI). The recurrent CAD group was determined as those who needed revascularization again with PCI after the first procedure. Patients who used ASA with other antiaggregant or anticoagulants, were allergic to ASA and stopped ASA for any reason for 2 years were excluded from the study. The information regarding the risk factors such as smoking, alcohol consumption was also obtained. This study is an experimental study. This study protocol was approved by the Institutional Ethics Committee of University of Health Sciences Turkey, Umranıye Training and Research Hospital, Istanbul, Turkey (decision no: 139, date: 24.07.2019). Each individual was informed about the study and written informed consent was obtained from each participant. The study was conducted in accordance with the relevant regulations of the Ministry of Health.

Platelet Aggregation Testing

AR was confirmed by platelet aggregation testing. Platelet aggregation studies were conducted in whole blood lumiaggregometer (Chronolog Corporation, Model 560-Ca). AR was defined as a mean aggregation of ≥20% with 0.5 mg/mL arachidonic acid and a mean aggregation of ≥64% with 5 μM adenosine diphosphate (20).

Blood Sampling and Genotyping

DNA samples of 100 patients were isolated from peripheral blood sample using QIAamp DNA Blood Mini kit (Qiagen, GmBH, Hilden, Germany). DNA concentrations were measured using a Nanodrop spectrophotometer (Thermo Scientific, Foster City, CA, USA). Real-time polymerase chain reactions (PCR) for COX-1 (rs1330344) (-1676 T>C) and COX-2 (rs20417) (-765G>C) were performed using 7500 Fast Real-Time PCR System (Applied Biosystems, Foster
City, CA, USA). The reaction was performed according to the manufacturer’s instructions.

Statistical Analysis
Statistical Package for the Social Science 23.0 was performed for statistical analysis. Normal distribution assumption was checked with the Kolmogorov-Smirnov test. Two independent samples t-tests was used to compare continuous variables’ means between two groups which were normally distributed. Kruskal-Wallis tests were performed to investigate the difference between genotypes and risk factors (which are not normally distributed). If there were statistically significant differences for pairwise comparison, Mann-Whitney U test was performed and Bonferroni correction was applied to p values. P values less than 0.05 (p<0.05) were considered statistically significant.

RESULTS
Study Population
Table 1 shows the baseline characteristics of the study population. When the characteristics were compared between groups; gender, fasting blood glucose level, high density lipoprotein (HDL) and hypertension were found statistically significant in AR+ group (p<0.05).

Table 1. Baseline characteristics of the study population

| Baseline characteristics               | AR- group (n=52) | AR+ group (n=48) | p-value |
|----------------------------------------|------------------|------------------|---------|
| Age (years)                            | 59.44±10.52      | 59.60±10.28      | 0.938   |
| Height (cm)                            | 169.12±28.45     | 166.71±7.37      | 0.414   |
| Weight (kg)                            | 78.09±10.23      | 80.31±10.94      | 0.231   |
| Body mass index                        | 28.66±3.79       | 28.89±3.79       | 0.6     |
| Gender                                 |                  |                  | 0.014*  |
| Female                                 | 29 (55.8%)       | 15 (31.3%)       |         |
| Male                                   | 23 (44.2%)       | 33 (68.8%)       |         |
| Fasting blood glucose level (mg/dL)    | 106.62±36.43     | 117.85±39.01     | 0.002*  |
| Total cholesterol (mg/dL)              | 191.04±42.43     | 203.27±62.25     | 0.588   |
| LDL (mg/dL)                            | 111.69±41.09     | 130.39±54.69     | 0.137   |
| HDL (mg/dL)                            | 51.31±10.91      | 46.19±8.82       | 0.012*  |
| Triglyceride (mg/dL)                   | 144.39±92.75     | 154.67±90.59     | 0.28    |
| Diabetes mellitus (%)                  | 9 (17.3%)        | 11 (22.9%)       | 0.484   |
| Hypertension (%)                       | 24 (46.2%)       | 34 (70.8%)       | 0.012*  |
| Smoking (%)                            | 26 (50%)         | 31 (64.6%)       | 0.141   |
| Alcohol consumption (%)                | 3 (5.8%)         | 5 (10.4%)        | 0.475   |

LDL: Low density lipoprotein, HDL: High density lipoprotein, COX: Cyclooxygenase, AR-: ASA non-resistant, AR+: ASA resistant, ASA: Acetylsalicylic acid, *p<0.05

COX-1 and COX-2 Genotyping
Table 2 shows the genotype distribution of groups. When groups were compared with each other, however heterozygote and homozygote variations were mostly found in the AR+ group for both COX-1 and COX-2, the relation between groups were not found statistically significant.

Allele Frequencies of COX-1 and COX-2 Variations
Table 3 shows allele frequencies of COX-1 and COX-2 genes in study groups. When groups were compared with each other, the presence of C allele of COX-2 was found statistically high in the AR+ group (p=0.023).

Relation Between Risk Factors and Polymorphisms
When the relation between risk factors and variations were investigated, it was found that the heterozygote genotype of COX-2 is associated with high total cholesterol and Low density lipoprotein (LDL) levels (p<0.05). Table 4 shows the relation between risk factors and mutations.

DISCUSSION
CAD is one of the most known heart disease and leads to death in the world. Considering factors such as unhealthy life, CAD rates are also increasing in low and middle income countries (21). Due to recurrent CAD, adverse outcomes
such as death, heart failure, stroke, malignant arrhythmia can be seen more often (22,23). Therefore, it is critical to identify preventable causes of recurrent CAD and to take action against them. The frequency of recurrent cardiovascular events due to AR also varies between countries (24).

A meta-analysis showed a 25% reduction in serious vascular events in high-risk patients with CAD using ASA (10). ASA prevents the conversion of arachidonic acid to thromboxane TXA2 by irreversibly inhibiting COX-1. This inhibition occurs by acetylation of the serin-530 residue located in the active site of COX-1 (25,26). Irreversible enzyme inhibition causes complete COX-1 inhibition with a daily dose of ASA (26). ASA can also reduce the risk of ischemic events by 22% in patients with atherothrombosis (7). However, recurrent cardiovascular events have still seen at a high rate in patients who use ASA (27). Therefore, identification of genetic or environmental factors that may cause recurrent CAD is important for the new therapeutic approaches. Therefore in this study the relation between COX-1, COX-2 variations, and other risk factors related to AR in recurrent CAD patients were investigated.

**Table 2. COX-1 and COX-2 genotyping results of groups**

| Gene names accession number of variations and genotype distributions | AR-group (n=52) | AR+ group (n=48) | p-value |
|---------------------------------------------------------------|----------------|-----------------|---------|
| COX-1 (rs1330344) (-1676 T>C)                                |                |                 |         |
| TT                                                           | 42 (80.8%)     | 32 (66.7%)      | 0.202   |
| TC                                                           | 10 (19.2%)     | 15 (31.3%)      |         |
| CC                                                           | 0 (0%)         | 1 (2.1%)        |         |
| COX-2 (rs20417) (-765G>C)                                    |                |                 |         |
| GG                                                           | 44 (84.6%)     | 32 (66.7%)      | 0.103   |
| GC                                                           | 7 (13.5%)      | 13 (27.1%)      |         |
| CC                                                           | 1 (1.9%)       | 3 (6.3%)        |         |

*p<0.05, COX: Cyclooxygenase, AR: ASA non-resistant, AR+: ASA resistant, ASA: Acetylsalicylic acid

**Table 3. Allele frequencies of COX-1 and COX-2 in study groups**

| Gene names accession number of variations and alleles | AR-group (n=52) | AR+ group (n=48) | p-value |
|------------------------------------------------------|----------------|-----------------|---------|
| COX-1 (rs1330344) (-1676 T>C)                        |                |                 |         |
| T                                                    | 94 (90.4%)     | 79 (82.3%)      | 0.094   |
| C                                                    | 10 (9.6%)      | 17 (17.7%)      |         |
| COX-2 (rs20417) (-765G>C)                            |                |                 |         |
| G                                                    | 95 (91.3%)     | 77 (80.2%)      | 0.023*  |
| C                                                    | 9 (8.7%)       | 19* (19.8%)     |         |

*p<0.05, COX: Cyclooxygenase, AR: ASA non-resistant, AR+: ASA resistant, ASA: Acetylsalicylic acid

**Table 4. Relation between risk factors and mutations**

| Risk factors-variations relationship | Genotype distributions | p-value |
|--------------------------------------|------------------------|---------|
|                                      | Wild type | Homozygous mutation | Heterozygous mutation |
| COX-2 (rs20417)                      |           |                     |                      |
| Total cholesterol                    | 188.89±50.39*        | 188.25±43.57        | 229.1±54.11*        |
| LDL                                  | 112.13±44.57*        | 114.5±41.46         | 154.35±52.75*       |

*p<0.05, LDL: Low density lipoprotein, COX: Cyclooxygenase
COX is an enzyme that is responsible for the synthesis of prostaglandins (PGs) and platelet generation of TXA2. PGs produced by COX-2 can also be synthesized by COX-1 (8). rs1330344 (-1676 T>C) of COX-1 was found to contribute significantly to the occurrence of ischemic stroke. On the one hand, it has been noted that the TT genotype of rs1330344 can reduce ischemic stroke susceptibility and cardioembolic stroke or small vessel occlusion (16). In one study, it was found that the frequency rates of alleles in the COX-1 C50T were 8.6%, therefore this variation may influence the effect of ASA (8). A842G, C22T, G128A, C644A, and C714A are mostly detected variations in the COX-1 and they are related to ASA response (28). In another study, low-dose ASA irreversibly acetylates COX-1 and reduce platelet activity by inhibiting the production of thromboxane A2 (29). In our study, however COX-1 rs1330344 variation was mostly found in the AR+ group, the relation between groups was not found statistically significant (Table 2).

COX-2, induced by cytokines in response to inflammatory stimuli, has been expressed on endothelial cells and macrophages (8). rs20417 SNP is located in the promoter region of COX-2 (-765G>C). This locus mutation in the COX-2 gene can change the promoter activity and affect the expression of COX-2 (16). In a study, a significant relationship was found between rs20417 polymorphism and CAD (30). In another study it was found that the frequency rate of C allele in the COX-2 rs20417 is 21.3% (8). Therefore, it was suggested that the variation of the COX-2 gene influences the effect of ASA. Some metabolic factors (reduced absorption or increased metabolism of ASA) may also cause AR. Some studies have suggested that the COX-2 variant increases the risk of AR (8,31). The COX-2 gene is induced by the activation of the signal transduction pathway and COX-2 protein is covalently acetylated by ASA. ASA activity can be measured using serum TXB2 or urine 11-DH-TXB2 (TXA2 pathway endproducts). Unlike other results, the COX-2 -765G>C variant after ASA treatment causes a high decrease in serum and urine 11-dehydrothromboxane B2 (11-DH-TXB2) levels (28). In our study, COX-2 (33.3%) variation was mostly detected in the AR+ group, however the relation between groups was not found statistically significant (Table 2). When allele frequencies were compared between groups, C allele of COX-2 was found statistically high in AR+ group (p<0.05) (Table 3). Therefore, it was suggested a relation between COX-2 mutations and recurrent CAD due to AR.

One study showed a higher prevalence of AR in patients with acute coronary syndrome than healthy individuals. AR was more prevalent in patients with smokers and low HDL cholesterol. However, no significant difference was found for gender, age and hypertension (11). In other studies any relation was not detected for total cholesterol and LDL cholesterol level with COX-2 variations however in our study, we also found that high total cholesterol and LDL cholesterol levels are related to the GC genotype of COX-2 (Table 4).

**CONCLUSION**

Similar studies with a large population must explain the mechanisms governing the association of COX-1 and COX-2 genotypes and response to ASA in recurrent CAD patients. Detection of AR may be useful in preventing recurrent CAD and reducing mortality and morbidity associated with recurrent CAD.

**ETHICS**

**Ethics Committee Approval:** The study, which is compatible with the Helsinki Declaration, was approved by the Institutional Ethics Committee of University of Health Sciences Turkey, Umraniye Training and Research Hospital, Istanbul, Turkey (decision no: 139, date: 24.07.2019).

**Informed Consent:** All patients and/or legal guardians included in the study provided their written informed consent.

**Authorship Contributions**

Surgical and Medical Practices: E.B., Concept: D.K., E.B., Design: D.K., E.B., E.Ç.K., Data Collection or Processing: D.K., E.B., T.D., E.Ç.K., Analysis or Interpretation: D.K., E.B., T.D., E.Ç.K., Literature Search: D.K., T.D., Writing: D.K., T.D.

**Conflict of Interest:** The authors declare that they have no conflict of interest.

**Financial Disclosure:** The authors declared that this study received no financial support.

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