MYELOPEROXIDASE AND GLUTAMATE-CYSTEINE LIGASE POLYMORPHISMS IN TYPE 2 DIABETES MELLITUS: A PRELIMINARY STUDY

POLIMORFIZMI MIJELOPEROKSIDAZE I GLUTAMAT-CISTEIN LIGAZE U DIABETES MELLITUSU TIPA 2: PRELIMINARNA STUDIJA

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Summary

Background: Oxidative stress can accompany the physiopathology of diabetes mellitus. Myeloperoxidase and glutamate-cysteine ligase catalytic subunit are two important substances which are connected with the maintaining of the redox balance in the body. In this study, the association between diabetes, lipid levels, myeloperoxidase -463G/A, and the glutamate-cysteine ligase catalytic subunit -3506A/G gene polymorphisms have been investigated.
Methods: Frequencies of genotypes and alleles have been assessed using PCR-RFLP techniques in 90 type 2 diabetic patients and 70 healthy controls.
Results: We found an association between myeloperoxidase -463 G/A genotype distributions (p=0.023) and allele distributions (p=0.009) in the study groups. In addition, there is a significant difference for each of the genotype (p=0.014) and allele (p=0.007) distributions for the glutamate-cysteine ligase catalytic subunit -3506A/G polymorphism.
Conclusions: Our study reveals the first data about the myeloperoxidase -463G/A and glutamate-cysteine ligase catalytic subunit -3506A/G gene polymorphisms for type 2 diabetes and we believe that this work will play a pioneering role for further studies.

Keywords: polymorphism, diabetes, oxidant, antioxidant, SNP

Kratak sadržaj

Uvod: Fiziopatologiju diabetes mellitusa može pratiti oksidativni stres. Katalitičke podjedinice mijeloperoksidaze i glutamat-cistein ligaze su dve važne supstance koje su povezane sa održavanjem redoks ravnoteže u telu. U ovoj studiji, ispitivana je veza između dijabetesa, nivoa lipida i genskih polimorfizama -463G/A mijeloperoksidaze i -3506A/G katalitičke podjedinice glutamat-cistein ligaze.
Metode: Učestalost genotipova i alela određena je pomoću tehnika PCR-RFLP kod 90 pacijenata sa dijabetesom tipa 2 i 70 zdravih kontrolnih subjekata.
Rezultati: Otkrili smo povezanost između distribucija genotipa (p=0,023) i distribucija alela (p=0,009) -463 G/A mijeloperoksidaze u proučavanim grupama. Pored toga, postoji značajna razlika za svaku od distribucija genotipova (p=0,014) i alela (p=0,007) za polimorfizam katalitičke podjedinice -3506A/G glutamat-cistein ligaze.
Zaključak: Naša studija otkriva prve podatke o genskim polimorfizmima -463G/A mijeloperoksidaze i -3506A/G katalitičke podjedinice glutamat-cistein ligaze za dijabetes tipa 2 i verujemo da će ovaj rad imati pionirsku ulogu za buduće studije.

Ključne reči: polimorfizam, dijabetes, oksidant, antioksidant, SNP

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Introduction

Type 2 diabetes is characterized by insulin resistance, as well as by being a metabolic disease during which a loss of β cell function is seen. Oxidative stress is an important factor affecting the insulin resistance and the loss of β cell function. It is known that oxidative stress increases as a consequence of states like hyperglycemia and hyperlipidemia, which are observed frequently in diabetic conditions, and suppresses insulin production and secretion (1). High levels of free radicals and insufficiency of antioxidant defense systems can cause damage in cellular organelles and enzymes by increasing the peroxidation and formation of insulin resistance (2, 3).

Myeloperoxidase (MPO) is a phase I enzyme in the metabolism and is also found in the lysozymes of polymorphonuclear leukocytes, which have microbicidal activity. A high quantity of ROS is secreted by myeloperoxidase during the microbicidal effect. Therefore, high myeloperoxidase activity can be a risk factor, as in many other diseases. There have been attempts to clarify whether the MPO enzyme has an effect on diabetes or not. The MPO enzyme causes an increase in the HOCl production, and the HOCl induces lipid peroxidation. As a result of high lipid peroxidation, foam cells are formed, and then atherogenic lesions occur. Considering hyperlipidemia is seen in diabetes, we can say that the MPO enzyme plays a role in diabetic complications. However, the studies conducted are inconsistent. While Wiersma et al. (4) found that the MPO level is meaningfully high, other studies did not find significant results (5, 6). Myeloperoxidase is a product of a single 11 kb gene consisting of 11 introns and 12 exons (7). It is localized on chromosome 17 q12-24 (8–10). MPO -463 G/A polymorphism, which is localized in the promoter region of the myeloperoxidase gene, plays a role in regulating myeloperoxidase activity. While the G allele provides high myeloperoxidase activity, the mutant A allele causes loss of activity (11).

There are a lot of mechanisms that increase oxidative stress and antioxidant defense mechanisms against this stress in the body. One of the most important among these mechanisms is glutathione synthesis. Glutathione is an antioxidant mechanism occurring in almost every mammalian cell. Glutathione (GSH) is present in every mammalian cell and has many functions, such as maintaining reduction of proteins' sulfhydryl groups and protecting against oxidizing agents and electrophilic xenobiotics (12). A decrease in the glutathione levels in cells causes oxidative damage (13). Glutamate-cysteine ligase (GCL) functions as the main enzyme at the beginning of glutathione synthesis (14, 15). GCL catalytic subunit (GCLC) is also a limiting step. It has been detected that the amount of GSH is parallel with the expression of the GCLC gene, of which the expression regulated at the transcriptional level comes first (16–18). On the other

hand, effects of the -3506A/G ve -129C/T polymorphisms which occur independently at the promoter region of the GCLC gene have been investigated in myocardial infarction (MI) and it has been observed that -129C/T polymorphism is increased in individuals who have MI with respect to those who do not (19, 20). Thus, GCLC is an essential enzyme for the glutathione mechanism, and further comprehensive studies are needed in order to understand the effects of alterations in this gene. In a diabetes study, researchers suggested that GCLC promoter polymorphisms may influence GAD65Ab levels and may influence the age at which type 1 diabetes is diagnosed (21).

According to these findings, our aim was to investigate the relation of lipid levels and distributions of MPO and GCLC gene polymorphisms in type 2 diabetes patients, who have undergone a limited number of studies.

Materials and Methods

Patient selection

Two study groups have been selected for this investigation. The first group includes 90 patients, 25–85 years of age (60 female and 30 male) who have been diagnosed with type 2 diabetes at the Haseki Training and Research Hospital, Department of Internal Medicine. The second group includes 70 randomly selected healthy people, 28–75 years of age (54 female and 16 male) who do not have any first-degree relatives with diabetes.

This study was approved by the Ethical Committee of Istanbul University, The Istanbul Faculty of Medicine.

Biochemical parameters

After overnight fasting, blood samples from the participants were drawn in plain tubes and in tubes with EDTA. The samples were centrifuged for 10 min at 1,500 × g at room temperature, followed by the removal of plasma. Serum concentrations of triacylglycerol, total cholesterol, LDL-cholesterol and HDL-cholesterol were determined with an autoanalyzer.

DNA was extracted from the leukocyte pellets by sodium dodecyl sulphate lysis, ammonium acetate extraction and ethanol precipitation (22).

Determination of MPO -463 G/A polymorphism

The polymorphic site at position -463 of the MPO gene was amplified by the use of forward primer (5'-CGG TAT AGG CAC ACA ATG GTG AG-3') and reverse primer (5'-GCA ATG GTT CAA GCG ATT CTT C-3') as described in the literature. Polymerase chain reaction (PCR) was performed with Taq

polymerase; the cycling condition was 95 °C for 2 minutes followed by 35 cycles of 94 °C for 30 seconds, 62 °C for 30 seconds, and 72 °C for 30 seconds. PCR product was 350 bp. Forty μL of PCR products were digested with Aci I restriction enzyme at 37 °C overnight. Fragments were separated using 2% agarose gel. Three possible genotypes were defined by 3 distinct banding patterns: A/A 289 and 61 bp fragments; A/G 289, 169, 120 and 61 bp fragments; and G/G 169, 120 and 61 bp fragments (23).

Determination of GCLC -3506 A/G polymorphism

The promoter site at position -3506 of the GCLC gene was amplified using forward primer (5'-AAGTCCCAGGAAGAATCACA -3'), and the primer was reversed (5'- CGCTCTCCAGGAACCCATCT -3') as described in the literature. The polymerase chain reaction (PCR) was performed with Taq polymerase; the cycling condition was 94 °C for 5 minutes followed by 35 cycles of 94 °C for 45 seconds, 55 °C for 45 seconds, and 72 °C for 5 minutes. PCR product was 874 bp. 10 μL of PCR products was digested with NlaIII restriction enzyme at 37 °C for 2 hours. Fragments were separated via use of 2% agarose gel. Three possible genotypes were defined by 3 distinct banding patterns: A/A 722, 104 and 48 bp fragments; A/G 722, 513, 209, 104 and 48 bp fragments; and G/G 513, 209, 104 and 48 bp fragments (13).

Statistical analysis

The statistical analyses were performed using SPSS 16.0 (Chicago, IL, USA) and PLINK v1.07 statistical software (24). P-values less than 0.05 were assumed to be statistically significant. We checked biochemical data for normality using Kolmogorov-Smirnov and Shapiro-Wilk tests and compared cases and controls for these parameters using the Student's t-test and Mann-Whitney U test. We used the Chi-square and Fisher's exact tests to evaluate the difference in the occurrence of the MPO and GCLC alleles in the case and control groups. In order to determine the relative risks, odds ratios and 95% confidence intervals were used.

Results

Demographical and biochemical information of the study group is given in *Table I*. According to our statistical analysis, there is no significant difference between case and control groups regarding sex and smoking (p>0.05), unlike regarding age (U=589, p<0.05). Cholesterol (p<0.001, 95% CI: 0.45–1.10), LDL-cholesterol (p<0.001, 95% CI: 0.30–0.91), VLDL-cholesterol levels (p<0.05, 95% CI: 0.03–0.24) and body-mass index values (p<0.05, 95% CI: 0.56–

Table I Biochemical and demographical parameters of the study groups.

Groups	Diabetes (N=90)	Control (N=70)
Sex (M/F)	60/30	54 /16
Age (years)	48.8 ± 9.59	43.97 ± 13.67
Body Mass Index (kg/m^2)	26.94 ± 4.23	25.15 ± 3.88
Fasting Blood Glucose (mmol/L)	9.78 ± 3.77	4.53 ± 0.67
Triglyceride (mmol/L)	2.00 ± 1.12	1.69 ± 0.38
Total cholesterol (mmol/L)	5.49 ± 1.41	4.72± 0.59
HDL-cholesterol (mmol/L)	1.01 ± 0.29	1.00 ± 0.26
LDL-cholesterol (mmol/L)	3.55 ± 1.31	2.94 ± 0.60
VLDL-cholesterol (mmol/L)	0.91 ± 0.47	0.77 ± 0.17
Total cholesterol / HDL-cholesterol	5.88 ± 2.33	5.06 ± 1.55
Smokers (%)	14.9	14.3
SBP (mmHg)	134.73 ± 19.31	–
DBP (mmHg)	84.13 ± 12.52	–
Diabetes duration	11.37 ± 8.76	–

n: number of individuals, SBP: systolic blood pressure, DBP: diastolic blood pressure. Differences between groups are evaluated with chi-square and Student-t tests.

Table II Distributions of MPO -463 G/A and GCLC -3506 A/G genotypes and alleles.

MPO -463 G/A	Control (n=70)	Diabetes (n=90)	Total (n=160)
Genotypes	n/%	n/%	n/%
AA	8 (11.4)	5 (5.6)	13 (8.1)
GG	24 (34.3)	50 (55.6)	74 (46.2)
AG	38 (54.3)	35 (38.9)	73 (45.6)
Alleles	n/%	n/%	n/%
A	54 (38.57)*	45 (25)	99 (30.93)
G	86 (61.43)	135 (75)	221 (69.07)
GCLC -3506 A/G	Control (n=70)	Diabetes (n=90)	Total (n=160)
Genotypes	n/%	n/%	n/%
AA	52 (74.3)	50 (55.6)	102 (63.8)
GG	0 (0)	5 (5.6)	5 (3.1)
AG	18 (25.7)	35 (38.9)	53 (33.1)
Alleles	n/%	n/%	n/%
A	122 (87.14)	135 (75)	257 (80.31)
G	18 (12.86)	45 (25)**	63 (19.69)

n: number of individuals. Chi-square and Fisher's Exact tests are used for in-group comparisons.

*(χ^2: 6.789, p=0.009, OR: 0.53, 95% CI: 0.33–0.86)
**(χ^2: 7.344, p=0.007, OR: 2.26, 95% CI: 1.24–4.11)

2.93) are higher in the patient group in relation to the control group. In addition, free blood glucose levels (U=13.5, p<0.001) and cholesterol/HDL proportions (U=2420, p<0.05) are elevated in the patients compared to controls.

Genotypes and alleles of MPO -463 G/A and GCLC -3506 A/G polymorphisms are shown in *Table II*. Each of the case and control groups were checked for all polymorphisms for the Hardy-Weinberg equilibrium and whether they were in the equilibrium was confirmed by PLINK software using exact test (p>0.05). While comparing the patient and control groups for the MPO -463 G/A polymorphism, we found a significant difference between genotype distributions (χ^2=7.569, p=0.023) and allele distributions (χ^2: 6.789, p=0.009, OR: 0.53, 95% CI: 0.33–0.86) of the groups. Thus, a protective effect of the A allele was observed. Also, while searching for the GCLC -3506 A/G polymorphism, we obtained meaningful results for genotypic (χ^2=7.904, p=0.014) and allelic (χ^2: 7.344, p=0.007, OR: 2.26, 95% CI: 1.24–4.11) distributions. The G allele is involved in the increased risk for the disease.

Discussion

Free radicals and ROS have been shown to play an important part in the mammalian glucoregulatory system. Previous studies showed us that hydrogen peroxide production can regulate glucose-stimulated insulin release from β-cells and modulate proximal and distal insulin signaling (25, 26). Also, increased oxidative stress seems to be an important alteration, triggering several complications such as kidney disease, coronary artery disease, etc (27).

The enzyme myeloperoxidase is one of the sources of reactive oxygen species. MPO -463 G/A polymorphism, which is located on the promoter region of the myeloperoxidase gene, has a role in the regulation of myeloperoxidase activity. G allele provides high myeloperoxidase activity, but the mutant A allele causes its loss. There are some studies about the relationship between MPO polymorphisms and diseases. Rudolph et al. (28) studied the effect of MPO -463 G/A polymorphism on left ventricle disorder in 116 patients. Patients were observed during 1050 days, and their MPO plasma levels were recorded. At the end of this period, no meaningful result was found between MPO -463 G/A polymorphism and plasma MPO levels and activity. However, a negative effect of the GG genotype on the left ventricle was identified (hazard ratio 3.16 (95% CI 1.17–8.53, p=0.024)). Katakami et al. (5) reported that MPO -463 G/A, manganese superoxide dismutase (SOD2) Val16Ala, GCLM -588 C/T, endothelial nitric oxide synthase (NOS3)894 G/T and NAD(P)H oxidase p22pkox (CYBA) 242 C/T polymorphisms are not significant individually, but they increase myocardial infarction

risk in type 2 diabetes patients when they are evaluated together (OR: 1.16, 95% CI 1.01–1.34, p=0.034). Nomiyama et al. (29) suggested that the val(16)ala polymorphism of Mn-SOD may be unrelated to the etiology of type 2 diabetes, but it seems to be associated with diabetic nephropathy in Japanese type 2 diabetic patients.

In another study, Lee et al. (30) showed that V16A polymorphism of the Mn-SOD gene is not related to the development of diabetes and progression of diabetic retinopathy, but is associated with diabetic macular edema in Korean type 2 diabetic patients.

In our study, we used 90 type 2 diabetes patients and 70 healthy individuals who do not have any first-degree relatives with type 2 diabetes. As a result, we observed that the genotype distributions of MPO -463 G/A polymorphism in patient and control groups are significantly different from each other (χ^2= 7.569, p=0.023). Also, we found an allelic association and a protective effect of mutant A allele for the MPO -463 G/A polymorphism (χ^2: 6.789, p= 0.009, OR: 0.53, 95% CI: 0.33–0.86). Increased G allele in the patient group indicates increased oxidative activity in patients, as expected.

Glutathione is an antioxidant which is found in almost every mammalian cell and functions as a main enzyme at the beginning of GCL glutathione synthesis (14, 15). GCL has two subunits, a catalytic subunit and a regulator subunit. The catalytic subunit is directly related to glutathione synthesis. It has been determined that the amount of glutathione is parallel with the expression of GCLC gene whose expression is regulated at the transcription level firstly (12, 15). Hence, alterations in the GCLC gene are investigated for many other diseases.

Molecular studies on GCLC -129 C/T polymorphism show that the T allele has lower promoter activity, and thus can cause a decrease in GCLC expression (13). Campolo et al. (31) investigated the association between the GCLC -129 C/T polymorphism and cardiovascular diseases (myocardial infarction, stroke, etc.) and observed that reduced plasma GSH levels are higher with the CT genotype than with the CC genotype. In addition, they found via logistic regression analysis that being male (p=0.027), hypertension (p=0.001) and GCLC CT genotype (p=0.009) factors reveal increased myocardial infarction risk when they are all evaluated together. Another study on a Japanese population obtained similar results and reported that the T allele increases the myocardial infarction risk (24).

We could not find a study which investigated the association between the GCLC gene and type 2 diabetes. However, there are a few studies about the -3506 A/G polymorphism. Koide et al. (13) reported that -129 C/T polymorphism is a risk factor for myocardial infarction, although the -3506 A/G polymorphism is not associated with the disease.

On the contrary, we have found that there is an association between GCLC -3506 A/G polymorphism and type 2 diabetes. There is a significant difference for each of the genotypic (χ^2=7.904, p=0.014) and allelic (χ^2: 7.344, p=0.007, OR: 2.26, 95% CI: 1.24 – 4.11) distributions among the case and control groups for type 2 diabetes. The G allele can be a risk factor for the disease. In addition, as this was a primary study in a Turkish population on the GCLC -3506 A/G polymorphism, we determined the allele frequencies as A: 0.803 and G: 0.197 for our population.

In conclusion, MPO -463G/A and GCLC 3506A/G polymorphisms are suggested as a risk factor for type 2 diabetes patients, according to our results. Furthermore, additional studies are needed to understand how the GCLC -3506A/G polymorphism modifies the gene activity.

Conflict of interest statement

The authors stated that there are no conflicts of interest regarding the publication of this article.

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