Original Article

An Association between *MPO* -463 G/A Polymorphism and Type 2 Diabetes

(*MPO* / oxidation / polymorphism / risk)

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Abstract. Myeloperoxidase (MPO) is an enzyme which is a member of the haem-peroxidase superfamily and plays a role in production of reactive oxygen species. The most common polymorphism in the promoter region of *MPO* gene is -463 G/A. It was shown that carrying the GG genotype means increased activity of the gene approximately 2–3-fold compared to GA and AA genotypes. It was found that hyperglycaemia, modified oxidized proteins and increased advanced glycosylated end products (AGE) are related to oxidative stress in diabetes. Under the hyperglycaemic conditions, production of reactive oxygen radical is elevated in smooth muscle endothelial cells, mesengial and tubular endothelial cells. Especially, elevated lipid oxidation plays an important role in pathogenesis of diabetic complications such as cardiovascular complications. We examined the *MPO* -463 G/A polymorphism by using the PCR-RFLP method in 145 type 2 diabetic patients and 151 healthy controls. We observed that the AA genotype and A allele were protective variants against type 2 diabetes and the GG genotype was a risk factor for diabetes. While we studied the relationship between genotypes and biochemical parameters, we found that patients with the A allele had decreased serum cholesterol, triglyceride, VLDL levels and body mass index. We suggest that the *MPO* gene has an important role in pathogenesis of type 2 diabetes because of the increased frequency of GG genotype, which is related to increased activity and oxidant capacity of MPO in the patients.

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Abbreviations: AGE – advanced glycosylated end products, GSH – glutathione, HOCl – hypochlorous acid, MPO – myeloperoxidase, PCR – polymerase chain reaction, ROS – reactive oxygen species.

Introduction

Type 2 diabetes mellitus, which accounts for 90–95 % of diabetes cases, previously referred to as non-insulin-dependent diabetes, type 2 diabetes, or adult onset diabetes, encompasses individuals who have insulin resistance and usually have relative insulin deficiency. The risk of developing type 2 diabetes increases with age, obesity and lack of physical activity (American Diabetes Association, 2013).

Patients who have type 2 diabetes are at increased risk of developing macrovascular and microvascular complications. Hyperglycaemia, duration of diabetes, possible role of aldose reductase, oxidative stress, hyperlipidaemia, hypertension, non-enzymatic glycosylation and elevated protein kinase C activity are the most significant factors for type 2 diabetes (Cooke et al., 2003).

Free radicals are unstable molecules due to the presence of unpaired electrons. Because of this, they can be highly reactive, although this varies from radical to radical, reacting locally in accepting or donating electrons to other molecules to achieve a more stable state. Reaction of a radical with a non-radical produces a free radical chain reaction with the formation of new radicals, which in turn can react with further macromolecules such as lipids, proteins, etc. (Betteridge, 2000). Chronic hyperglycaemia causes oxidative stress in tissues prone to complications in patients with diabetes. Type 2 diabetic patients have increased lipid peroxidation compared with age-matched control subjects, as well as decreased plasma glutathione (GSH) and GSH-metabolizing enzymes and antioxidant potential, all of which relate directly to the rate of development of complications (Sundaram et al., 1996; Zaltzberg et al., 1999). Besides, elevated free radicals and inadequate antioxidant systems cause cell damage, increased lipid peroxidation and insulin resistance.

A member of the haem peroxidase superfamily, myeloperoxidase (MPO), generates numerous reactive oxidants and diffusible radical species that are capable of initiating lipid peroxidation (Klebanoff et al., 1984; Zhang et al., 2002a,b). The effects of the MPO enzyme on type 2 diabetes has been studied. The major oxidant produced by MPO, hypochlorous acid (HOCl), is able to modify a great variety of biomolecules by chlorination and/or oxidation. In lipids the major sites of attack by HOCl are the double bonds of unsaturated fatty acids and cholesterol, leading to either chlorohydrin formation or peroxidation (Spickett et al., 2000).

Recent advances in diabetic research suggest that reactive oxygen species (ROS) play a key role in hyperglycaemia-mediated endothelial dysfunction and vascular complications (Brownlee, 2001). In another study, it was shown that H_2O_2 and vascular bound MPO are increased in diabetic vessels; MPO/H_2O_2/HOCl/chlorinating species may represent an important pathway in diabetes complications (Zhang et al., 2004). Also, Wiersma et al. (2008) suggested that MPO levels were higher in type 2 diabetic patients.

The *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 this activity (Kantarci et al., 2002).

We aimed to investigate the relationship between the *MPO* -463 G/A gene polymorphism and type 2 diabetes in Turkish patients.

Material and Methods

Sample selection

The patient group consisted of 145 patients (mean age = 57.26 ± 9.54 years; 91 females and 54 males) presenting with type 2 diabetes mellitus. The patients were recruited from the Department of Internal Medicine, Haseki Training and Research Hospital. The control group consisted of 151 healthy individuals (mean age = 54.38 ± 13.57 years, 72 females and 79 males) with a negative family history of type 2 diabetes. All patients and controls were Caucasians. This study was approved by the Ethics Committee of Istanbul University, The Istanbul Faculty of Medicine.

Biochemical parameters

After overnight fasting, blood samples were drawn in plain tubes. The samples were centrifuged for 10 min at 1,500 × *g* at room temperature, followed by the removal of serum. Biochemical parameters were determined using Hitachi 717 autoanalyser (Tokyo, Japan).

DNA isolation

Blood specimens were collected in tubes containing EDTA (Vacuette, Greiner Labor technik, Frickenhausen, Germany) and DNA was prepared from the leukocyte pellets by sodium dodecyl sulphate lysis (Sigma Aldrich, Taufkirchen, Germany), ammonium acetate (Sigma Aldrich) extraction and ethanol (Sigma Aldrich) precipitation (Miller et al., 1988). One tube containing EDTA was used for each subject. DNA samples were stored at 4 °C until polymerase chain reaction (PCR) application.

Determination of MPO -463 G/A polymorphism

The polymorphic site at position -463 of the *MPO* gene was amplified with 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. PCR was performed with *Taq* polymerase; the cycling conditions were 95 °C for 2 min followed by 35 cycles of 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 30 s. The PCR product was 350 bp long. Forty microliters of the PCR product was digested with *Aci* I restriction enzyme at 37 °C overnight. Fragments were separated in 2% agarose gel. Three possible genotypes were defined by three distinct banding patterns: A/A 289 and 61 bp fragments; A/G 289 bp, 169 bp, 120 and 61 bp fragments; and G/G 169, 120 and 61 bp fragments (Nikpoor et al., 2001).

Statistical analysis

The statistical analyses were performed using the SPSS 11.0 statistical software package (SPSS, Chicago, IL). P values lower than 0.05 were assumed to be statistically significant. We compared cases and controls in biochemical parameters using Student's *t*-test. One-way Anova test was used to investigate the biochemical parameters between the genotypes. Allele frequencies were found using the gene counting technique. We used the χ^2-test to evaluate the differences in the occurrence of *MPO* alleles in the case and control groups. In order to determine the relative risks, odds ratios and 95% confidence intervals were used.

Results

Demographical characteristics of the study groups are shown in Table 1. As expected, total-cholesterol (P = 0.001, 95% Cl = 7.59–29.68), LDL-cholesterol (P = 0.001, 95% Cl = 6.29–26.22), VLDL-cholesterol (P = 0.036, 95% Cl = 0.23–6.97), fasting blood glucose (P = 0.001, 95% Cl = 98.11–136.15), systolic blood pressure (P = 0.001, 95% Cl = 9.50–20.34), diastolic blood pressure (P = 0.001, 95% Cl = 4.64–14.55) and body mass index (P = 0.005, 95% Cl = 0.46–2.49) levels were higher in patients compared to controls. Also, patients had increased frequency of hypertension (P = 0.001, OR: 14.14, 95% Cl = 3.25–61.40).

Table 2 shows the distribution of *MPO* -463 G/A genotypes and alleles. We observed that the frequency of carrying GG genotype was increased in patients significantly (P = 0.001, χ^2 = 10.970, OR: 2.20, 95% Cl = 1.37–3.52). In addition, in the control group, we showed elevated frequency of the AA genotype (P = 0.001, χ^2 =

Table 1. Demographic characteristics of the study groups

GROUPS	PATIENT (N = 145)	CONTROL (N = 151)	P value
Gender (F/M)	91/54	72/79	> 0.05
Smoking (%)	19.9	27.2	0.143
Hypertension (%)	43.3*	5.1	0.001
Total cholesterol (mg/dl)	202.90 ± 59.23*	184.26 ± 34.13	0.001
HDL-cholesterol (mg/dl)	38.61 ± 13.90	38.87 ± 11.83	0.867
LDL-cholesterol (mg/dl)	130.78 ± 50.73*	114.52 ± 34.83	0.002
VLDL-cholesterol (mg/dl)	34.36 ± 18.71***	30.76 ± 9.29	0.040
Triglyceride (mg/dl)	169.17 ± 91.86	153.86 ± 46.49	0.077
Body mass index (kg/m^2)	27.18 ± 4.24**	25.69 ± 3.77	0.004
Age (years)	57.26 ± 9.54	54.38 ± 13.57	> 0.05
Fasting blood glucose (mg/dl)	201.10 ± 101.68*	83.86 ± 11.53	0.001
Diastolic blood pressure (mmHg)	83.32 ± 14.64*	73.72 ± 9.36	0.001
Systolic blood pressure (mmHg)	136.64 ± 23.68*	121.67 ± 10.21	0.001

N: number of subjects
* P: 0.001, ** P: 0.005, *** P: 0.036

Table 2. Distribution of MPO -463 G/A genotypes and alleles in the study groups

MPO -463 G/A polymorphism	PATIENTS (N = 145)	CONTROLS (N = 151)
Genotypes		
AA	6 (4.1 %)	24 (15.9 %)*
GG	75 (51.7 %)*	50 (33.1 %)
AG	64 (44.1 %)	77 (51 %)
Alleles		
A	76 (35.5 %)	125 (41.39 %)**
G	214 (64.5 %)	177 (58.61 %)

N: number of subjects
* P: 0.001, ** P: 0.048

11.224, OR: 0.22, 95% Cl = 0.09–0.57) and A allele (P = 0.048, χ^2 = 3.926, OR: 0.62, 95% Cl = 0.39–0.99) compared to the patients. Each of the case and control groups was checked for all polymorphisms by the Hardy-Weinberg equilibrium, and the equilibrium was confirmed by PLINK software using exact test (for patients χ^2 = 0.089 and for controls χ^2 = 0.53) (P > 0.05).

According to genotype distribution, we did not observe any association with biochemical parameters in the study groups. However, we showed that total cholesterol (P = 0.029, 95% Cl = 2.38–42.62), triglyceride (P = 0.038, 95% Cl = 1.78–60.93), VLDL-cholesterol (P = 0.028, 95% Cl = 0.73–12.72) and body mass index (P = 0.001, 95% Cl = 1.14–4.08) levels were higher in patients who had the A allele (Table 3).

Discussion

In this study, we aimed to investigate the relationship between *MPO* -463 G/A gene polymorphism and type 2 diabetes in Turkish patients. Previous studies have demonstrated that ROS represent a crucial factor in the insulin resistance, which especially occurs in the liver, muscle and fat tissues (Baynes, 1991; Kaneto et al., 2009). A high level of ROS is excreted in the environment by myeloperoxidase during the microbicidal effect. The -463 G/A polymorphism located in the promoter area of the myeloperoxidase gene plays a regulatory role in the myeloperoxidase activity. While the G allele provides a high myeloperoxidase activity, the mutant A allele caus-

Table 3. Clinical parameters in the patients according to genotypes and allele distributions

MPO -463 G/A polymorphism	GENOTYPES			ALLELES	
	AA N = 6	GG N = 75	AG N = 64	A N = 70	G N = 139
Triglyceride (mg/dl)	118.00 ± 50.32	180.90 ± 98.10	159.63 ± 85.14	151.38 ± 78.21*	170.64 ± 93.14
Total cholesterol (mg/dl)	164.80 ± 47.10	209.62 ± 53.68	198.14 ± 65.12	190.13 ± 63.96**	204.23 ± 59.66
HDL-cholesterol (mg/dl)	33.60 ± 9.76	38.23 ± 10.92	39.46 ± 17.00	38.42 ± 16.65	38.87 ± 14.07
LDL-cholesterol (mg/dl)	107.72 ± 39.49	135.37 ± 49.46	127.28 ± 52.82	121.97 ± 51.34	131.57 ± 51.37
VLDL-cholesterol (mg/dl)	23.48 ± 10.03	37.12 ± 20.06	32.03 ± 17.11	30.54 ± 15.56***	34.69 ± 18.97
Fasting blood glucose (mg/dl)	180.25 ± 63.86	189.29 ± 102.14	217.50 ± 102.36	217.61 ± 103.08	202.39 ± 103.50
Body mass index (kg/m^2)	29.64 ± 5.39	28.01 ± 4.28	25.97 ± 3.85	25.63 ± 3.64****	27.00 ± 4.16

N: number of subjects
* P: 0.038, ** P: 0.029, *** P: 0.028, **** P: 0.001

es loss of activity. That is why this area has been subject to research regarding many diseases.

In our study, we observed that the *MPO* -463 G/A polymorphism distribution in individuals with the AA genotype was 4.1 %, with the GG genotype 51.7 %, and with the AG genotype 44.1 %. In the control group, the ratios were determined for the AA genotype as 15.9 %, GG genotype as 33.1 %, and for the AG genotype as 51 %. In terms of genotype and allele distributions, we detected that the rate of carrying the GG genotype was higher in the patient group than in the control group. Conversely, compared to the individuals in the patient group, the AA genotype and the rate of carrying the A allele for healthy persons was found to have increased. When we investigated the genotype distribution in the patient group, we found out that the GG genotype caused a rise in the MPO activity and oxidation, and this data confirmed all other studies in the literature which claim that the oxidant mechanism plays a role in diabetes, especially diabetic complications (Gorudko et al., 2012; Rovira-Llopis et al., 2013).

However, we could not investigate the relationship between the diabetic complications and this polymorphism since we do not possess any data of diabetic complications among our patients. One of the rare studies about the relationship between type 2 diabetes and *MPO* polymorphism, by Mäkelä et al. (2008), investigated the relationship between the *MPO* -463 G/A polymorphism and the carotid intima media thickness in diabetic and non-diabetic patients, and the authors have shown that patients with type 2 diabetes who carry the GG genotype compared to those who carry the A allele have a thinner intima media. In another study, it was shown that increased risk of renal damage is associated with the presence of the G allele of the *MPO* -463 G/A polymorphism in type 2 diabetic patients (Katakami et al., 2013).

No meaningful difference regarding the biochemical parameters according to genotype distributions in the working group was observed. Although no significant difference in the control group was seen when evaluating the groups at the allele level, it was found that the serum cholesterol, triglyceride, VLDL levels, and the body mass index in A allele carriers have decreased meaningfully.

Our findings are parallel to those observed in patients carrying the A allele, in whom the MPO activity, and hence the oxidant capacity, decreased as a result of the *MPO* -463 G/A polymorphism. Therefore, when an increased capacity of oxidants is seen at the same time as plasma lipid levels which have increased during diabetes, the way is paved for various diabetic complications. Therefore, in the patient group, the relationship between carrying the A allele causing low enzymatic activity and low plasma lipid levels is a complementary finding.

Our study is one of the first conducted on the *MPO* gene in type 2 diabetes in our country, and we feel that it will find its place in the international literature.

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