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Association of an Endothelial Nitric Oxide Synthase Gene Polymorphism with Diabetic Retinopathy

Sibel Inan[1], Nazmi Zengin[2], Banu Turgut Öztürk[3], Hürkan Kerimoglu[2], Ali Ünlü[4] and Ismet Dogan[5]

[1]Afyon Kocatepe University Medical School Department of Ophthalmology, Afyonkarahisar, Turkey
[2]Necmettin Erbakan University Medical School Department of Ophthalmology, Konya, Turkey
[3]Selçuk University Medical School Department of Ophthalmology, Konya, Turkey
[4]Selçuk University Medical School Department of Biochemistry, Konya, Turkey
[5]Afyon Kocatepe University Medical School Department of Biostatistics, Afyonkarahisar, Turkey

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ABSTRACT The association of the endothelial nitric oxide synthase (eNOS) G894T gene polymorphism with the severity of diabetic retinopathy (DRP) and macular edema (DME) was investigated. One hundred-seven patients with type-2 diabetes mellitus were included. Forty-five patients served as a control group. eNOS G894T gene polymorphism was analysed by polymerase chain reaction. The mean age was 55.8±9.4 years in the study group and 51.8±9.7 years in the control group. There was no significant difference in the genotypes between the diabetic and the control group, or between the non-DRP group and the DRP group. The frequency of the G allele was higher in the proliferative DRP group than that in the non-proliferative DRP group. The GG genotype of G894T gene polymorphism was associated with macular edema and hyperglycaemia. The eNOS G894T gene polymorphism seems to be associated with the DME and unregulated hyperglycaemia.

INTRODUCTION

Diabetic retinopathy (DRP) is one of the most common causes of blindness and occurs between 20 and 74 years of age. Currently, diabetic retinopathy is the leading cause of blindness within the working-age population and the second most common cause of blindness after age-related macular degeneration among all age groups in developed countries. Diabetic maculopathy or macular edema (DME) occurring in any stage of diabetic retinopathy is the most significant cause of visual impairment (Gross and Wolin 1995; Antcliff and Marshall 1999; Pelzek and Lim 2002).

The pathogenesis of diabetic retinopathy has not been clearly established, and there are several ongoing studies on this subject (American Diabetes Association 1999). Antioxidant defence impairment, increased free radical products and lipid peroxidation product involvement in vascular injury associated with DRP have been suggested in patients with diabetic retinopathy (Cerillo et al. 1998). In addition, in recent years, the involvement of nitric oxide (NO), a substance involved in proliferative vitreoretinal diseases, has been proposed in proliferative diabetic retinopathy (Becquet et al. 1997; Taverna et al. 2005)

The "Diabetic Control and Complication Trials" study showed that genetic factors influence the onset of diabetic retinopathy in patients with diabetes (The Diabetes Control and Complications Trial Research Group 1997).

Previously, several candidate genes were reported to be associated with DRP. Various polymorphisms have been identified in the endothelial NOS gene (eNOS) and have also been associated with an increased risk of DRP (Taverna et al. 2002; Awata et al. 2004; Srivastava et al. 2005; Chen et al. 2007; Bazzaz et al. 2010; Angeline et al. 2011; Cheema et al. 2012; Qian-Qian et al. 2014; Bregar et al. 2017)

Objectives

Taken together with the results of previous papers, defective NOS expression in the retina may be a contributing factor to DME develop-

Address for correspondence:
Sibel Inan
Assistant Professor
Afyon Kocatepe University Medical School
Department of Ophthalmology03200, Afyonkarahisar
Phone: +90 5058736689
Fax: +90 2722463333
E-mail: drinan33@gmail.com

ment. In the present paper, the researchers' aim was to investigate the correlation between the G894T exon 7 gene polymorphism in the endothelial nitric oxide synthase gene locus and the severity of DRP and DME in patients with diabetic retinopathy.

MATERIAL AND METHODS

A total of 107 patients with Diabetes Mellitus (DM) and 45 healthy individuals who visited the Necmettin Erbakan University Meram Faculty of Medicine, Ophthalmology Polyclinic and/or were under follow-up at the Retina Division were included in the study. Informed consent was obtained from each patient before blood samples were collected for the gene polymorphism tests. The study was reviewed and approved by the Institutional Ethics Committee. The study was conducted in accordance with the Declaration of Helsinki. The study group consisted of 54 females (50.5%) and 53 males (49.5%) whose age ranged between 34 and 82 years (55.8±9.4 years). The control arm consisted of 45 healthy individuals including 25 males and 20 females (51.8±9.7 years) selected from subjects who visited the researchers' hospital for a regular ocular examination and whose age ranged between 36 and 72 years (51.8±9.7 years). Patients with no hypertension or other systemic pathology other than type II diabetes; patients with no history or diagnosis of any type of glaucoma; patients with no media opacity preventing fundus imaging (corneal leukoma, cataract, etc.); patients with phakia; and patients with no retinal pathology other than DRP (age-related macular degeneration, retinitis pigmentosa, retinal scar, degenerative myopia, etc.) were included in the study.

The patients underwent a complete ophthalmologic examination. Corrected and non-corrected visual acuity were determined using Snellen charts. Goldman applanation tonometry was used to measure intraocular pressure. A slit-lamp anterior segment examination was performed. Pupil dilation was achieved with cyclopentolate one percent drops. A detailed fundus examination was performed using slit-lamp binocular stereoscopic indirect ophthalmoscopy. Fundus fluorescein angiography (FFA) was performed with a digital fundus camera (Topcon ImageNet, NJ, USA).

Patients

The patients were divided into 3 groups based on diabetic retinopathy. The first group included 36 patients without retinopathy (NRP); the second group included 41 patients with non-proliferative diabetic retinopathy (NPDR); and the third included 30 patients with proliferative diabetic retinopathy (PDR). Capillary loss and dilation, arterial abnormalities, retinal and macular edema and haemorrhage, presence of hard and soft exudate, macular cystoid changes, retinal haemorrhages, the presence of neovascularization in the disc and/or retina regions other than the disc, and the presence of fibro-proliferative material in the vitreous and in front of the retina were evaluated by reviewing the modified Airlie House classification. The patients in the non-proliferative and proliferative stages were also divided into two subgroups, A and B, based on the presence or absence of DME. The diagnosis of DME was established by optical coherence tomography (Stratus OCT-III, Zeiss Meditec, Germany). The patients were divided into the following groups based on the classification criteria specified above: Group 1: 36 diabetic patients with no pathological fundus changes were included in the NRP arm. Group 2A: 19 patients with microaneurysms, microhemorrhages, hard and/or soft exudates, arterial and venous deformation without disc or retinal neovascularization (NVD and NVE) or fibrovascular proliferation or vitreous haemorrhage were included in the NPDR arm. Group 2B: 22 patients with group 2A criteria and DME were included in this group. Group 3A: 10 patients with NVD or NVE without DME were included in the PDR arm. Group 3B: 20 PDR patients who met the Group 3A criteria with DME were included in this group. Group 4: 45 healthy controls with no diagnosis of systemic disease were included in the control arm. In patients with two different stages of DRP in their eyes, the higher stage was accepted as the DRP stage.

Demographic Characteristics

The duration of DM was defined as the time elapsed from the time of diabetes diagnosis. Treatment modality, diet, oral antidiabetic drug (OAD) and insulin use were taken into account. Patients who smoked >100 cigarettes during their lifetime were considered smokers. Arterial

pressure was measured three times in the sitting position at rest, and the arithmetic mean value was obtained to record systolic and diastolic blood pressure results. Body mass index (BMI) was defined as the body weight in kg divided by the height squared in m.

Biochemical Measurements

Following 12 hours of fasting, the biochemistry measurements were performed with a 5-cc blood sample obtained from the antecubital vein. Haemoglobin (Hb) and haematocrit (Htc) quantities were detected with a Gen-S device using the laser flowcell method. Serum lipid levels were measured with a Synchron LX20 device using enzymatic analysis. Serum Na, K, Cl, Ca levels were measured with the Synchron LX20 device using the ISE method. Fructosamine level was measured with a Synchron LX20 device employing the calorimetric method. Fasting blood glucose (FBG) was measured with a Synchron LX20 device using the glucosidase method. HbA1c level was detected with a BIORAD D10 device using the HPLC method.

Assessment of the eNOS Point Mutation

DNA isolation was performed using a High Pure PCR (polymerase chain reaction) template kit with the 2-cc whole blood samples in tubes containing EDTA. The method of PCR is based on using the ability of DNA polymerase to synthesize new strand of DNA complementary to the offered template strand. The eNOS G894T polymorphism detection was performed by real-time fluorescence PCR using Light Cycler 2.0 (Roche Diagnostic, Manheim, Germany) device. The Light Cycler® 2.0 instrument allowed the monitor amplification of 32 PCR products simultaneously, in real-time and online, with six different detection channels in multiplex experiments. This system also allowed the observation of fluorometric measurements in micro volumes in real time and online. A high-speed genotyping method was performed. PCR and melting curve determination were performed in a final volume of 20 L with 10 pmol of each primer NOSF (5=-CACTCCCACAGCTCTGCAT-3=) and NOSR (5=-CAATCCCTTTGGTGCTCACG-3=), and 4 pmol of each probe (anchor probe and sensor probe). The anchor probe (5=-LC Red 640-CCTTCTGCCCCCCGAGCTGGTCC-3=-P) was 5= labelled with the LC-Red 640 fluorophore and phosphorylated (P) at its 3= end to prevent probe elongation by the Taq polymerase. The sensor probe (5=-CCCCAGATGATCCCCCAGAACTC-3= FLU) was labelled with fluorescein. DNA was amplified at an annealing temperature of 62°C. The typical melting curve pattern is a single melting peak at a temperature of 65.5°C. For the wild type, the plot is a single melting peak at a temperature of 60.5°C. Heterozygous patients show two melting peaks (60.5°C and 65.5°C) (Sticchi et al. 2004).

Statistical Assessment

SPSS 18.0 package software was used for the statistical analysis. The statistical power (1-β) of the study for sample size was set at 0.80. Genotype differences were analysed with χ^2 (Chi-square test) tests. Biochemical differences between the groups were compared using post hoc-corrected analysis of variance (ANOVA) and student's t-test. Correlations between the biochemical parameters were investigated with Pearson correlation analysis. The frequency of alleles in all groups was in Hardy-Weinberg equilibrium. ($p>0.05$). Statistical significance was assessed at $\alpha=0.05$ ($p<0.05$).

RESULTS

In the present paper, the diabetic cases were within the age range of 34-82 years and comprised 53 males and 54 females. Among the patients with diabetes, the duration of the disease ranged between 1 and 41 years. Thirty-four patients were receiving insulin treatment. Among the insulin users, the treatment duration ranged between 1 and 25 years. The fasting blood glucose levels were between 59 mg/dL and 562 mg/dL. The age, height, weight, body mass index (BMI), visual acuity, intraocular pressure and blood pressure values of the groups are presented in Table 1.

There was a significant difference between the diabetic group and the control arm in terms of age. The diabetic group and the control arm were different in terms of FBG, fructosamine, cholesterol, HbA1c, VLDL, LDL, TG, Na, and K. The group with retinopathy and the retinopathy-free group were different with respect to age, while there was no such difference between the retinopathy-free group and the control group.

Table 1: Demographic characteristics and biochemical parameters in the groups

	Whole study population (Mean±SD)	*Control (Mean±SD)*	*N-DRP (Mean±SD)*	*N-PDR (Mean±SD)*	*PDR (Mean±SD)*
Age (years)	54.4± 9.7	51.0± 9.7	50.3± 7.6	59.8± 9.1	58.0± 8.8
DDM (years)	12.0± 7.5	-	5.5± 4.4	15.3± 6.9	15.7± 5.7
Weight (Kg)	75.1± 11.8	74.8± 11.7	78.1± 12.2	71.1± 9.8	76.4± 12.8
Height (cm)	165.7± 8.5	166.4± 7.9	164.7± 8.3	163.3± 8.9	164.0± 8.9
Body Mass Index	27.8± 4.7	26.9± 3.7	28.9± 4.3	26.8± 4.2	28.7± 6.2
BCVA (Right Eye)	0.79± 0.3	0.99± 0.01	0.98± 0.04	0.67± 0.3	0.44± 0.3
BCVA (Left Eye)	0.79± 0.3	0.99± 0.01	0.98± 0.04	0.66± 0.3	0.43± 0.3
IOP-Right (mmHg)	15.0± 2.5	14.5± 2.2	14.5± 2.4	15.2± 2.7	16.0± 2.7
IOP-Left (mmHg)	15.1± 2.4	14.8± 2.2	14.7± 2.3	15.2± 2.8	15.9± 2.4
SBP (mmHg)	123.0± 10.4	120.9± 8.3	119.8± 6.5	125.3± 11.5	127.2± 13.3
DBP (mmHg)	79.1± 5.9	77.3± 5.6	78.6± 6.0	79.6± 6.2	81.5± 5.5
FBS (mg/dl)	169.3± 90.3	88.8± 16.1	166.5± 75.6	227.7± 95.9	209.0± 82.8
Fructosamine (µmol/l)	441.7±105.9	377.5± 77.9	445.1± 107.4	488.5± 102.3	469.1± 10.8
HbA_{1C}	8.09± 2.4	5.5± 0.6	8.1± 2.5	9.8± 1.6	9.5± 1.7
Cholesterol	192.4± 45.2	174.0± 31.8	184.4± 42.3	198.5± 47.8	220.1± 47.6
VLDL	35.8± 27.5	22.4±11.8	37.1± 24.2	39.2± 35.3	47.6± 30.5
HDL	46.8± 12.7	45.8±11.6	45.2± 11.2	50.0± 15.7	46.6± 12.2
LDL	111.1± 35.6	100.8± 29.8	104.9± 29.6	116.3± 33.4	126.3± 46.3
TG	165.5± 114.2	102.9± 53.2	180.4±122.5	173.5±108.2	218.3±135.3
HB	13.5± 1.4	13.7± 1.4	13.7± 1.5	13.3± 1.5	13.3± 3.7
HCT	39.4± 4.2	40.6± 4.2	39.9± 4.3	38.3± 4.2	38.3± 4.2
NA	136.9± 3.3	138.7± 2.4	136.4± 3.7	136.4± 3.3	136.1± 1.1
K	4.3± 0.4	4.1± 0.3	4.2± 0.3	4.5± 0.5	4.4± 0.5
Ca	9.2± 0.4	9.2± 0.3	9.2± 0.4	9.3± 0.4	9.2± 0.4

N-DRP: Non-Diabetic Retinopathy, N-PDR: Non-Proliferative Diabetic Retinopathy, PDR: Proliferative Diabetic Retinopathy, DDM: Duration of Diabetes Mellitus, BCVA: Best-Corrected Visual Acuity (Snellen), IOP: Intraocular Pressure, FBS: Fasting Blood Sugar, SBP: Systolic Blood Pressure, DBP: Diastolic Blood Pressure

Visual acuity was different between the group with retinopathy and the retinopathy-free group, while there was no significant difference between the retinopathy-free group and the control group. HbA1c was different across all three groups. Cholesterol levels showed asignificant difference in the group with retinopathy relative to those in the retinopathy-free group and the control group. VLDL differed significantly between the diabetic group and the control group. There was no difference between the groups in terms of HDL. LDL showed a significant difference only in the retinopathy group compared with that in the other groups. There was a significant difference in terms of TG between the control group and the 2 diabetic groups.

There was no significant difference between the patients with diabetes and the control arm with respect to the GG, GT and TT genotypes of the endothelial NOS G894T gene polymorphism. Similarly, there was no significant difference between the non-proliferative DRP and proliferative DRP groups or between the retinopathy and retinopathy-free groups with respect to the G894T gene polymorphism ($p>0.05$, χ^2 test) (Table 2).

The evaluation of the G and T allele frequency revealed no difference between the patients with diabetes and the control group with respect to the G or T alleles [p=0.267, odds ratio=0.805, 95 percent confidence interval for the G allele (CI 95%)=0.811-1.093, confidence interval for the T allele=0.778-1.760]. For the patients with diabetes, the following results were reported: The G and T allele frequencies were 80.6 percent (n=58) and 19.4 percent (n=14), respectively, in patients without retinopathy; the G and T allele frequencies were 59.8 percent (n=49) and 40.2 percent (n=33), respectively in the non-proliferative diabetic retinopathy group; the G and T allele frequencies were 71.7 percent (n=43) and 28.3 percent (n=17), respectively, in the proliferative diabetic retinopathy group. There was a statistically significant difference between the groups with respect to allele frequency (Table 3).

In the researchers' paper, the presence of macular edema was defined using the interna-

Table 2: Distribution of genotypes in the groups

		Genotype			Total
		GG	GT	TT	n (%)
		n(%)	n (%)	n (%)	
DM Group		52 (48.6)	46 (43.0)	9 (8.4)	107 (100.0)
N-DRP		22 (62.9)	13 (37.1)	-	35 (100.0)
DRP		30 (41.7)	33 (45.8)	9 (12.5)	72 (100.0)
	NPDR	15 (36.6)	19 (46.3)	7 (17.1)	41 (100.0)
	PDR	15 (50.0)	13 (43.3)	2 (6.7)	30 (100.0)
Control Group		26 (57.8)	15 (33.3)	4 (8.9)	45 (100.0)
Total		78 (51.3)	61 (40.1)	13 (8.6)	152 (100.0)

DM: Diabetes Mellitus, N-DRP: Non-Diabetic Retinopathy (Diabetic patients without retinopathy), DRP: Diabetic Retinopathy, N-PDR: Non-Proliferative Diabetic Retinopathy, PDR: Proliferative Diabetic Retinopathy. There was no statistical difference with respect to the GG, GT or TT genotype polymorphism between the groups (p>0.05)

tional criteria determined by ETDRS, and 19 of the 41 patients with NPDR (46%) and 20 of the 30 patients with PDR (66%) had macular edema. While the GG genotype was detected in 52.6 percent of the patients with DME in the non-proliferative retinopathy group, it was detected in 22.7 percent of patients without macular edema. The GT genotype was detected in 47.4 percent and 45.5 percent of the patients with DME in the non-proliferative retinopathy group and the patients without macular edema, respectively. While the TT genotype was not observed in the patients with DME, it was detected in 31.8 percent of the patients without edema (n=7). While the GG and TT genotypes were detected in eighty percent (n=8) and twenty percent (n=2) of patients with DME in the PDR group, respectively, the GT genotype was not found. In the patients without edema, the GG and GT genotypes were observed in thirty-five percent (n=7) and sixty-five percent (n=13) of the patients, respectively, and the TT genotype was not detected. The GG genotype polymorphism was associated with DME in both the NPDR group and the PDR group (χ^2, p<0.05) (Table 3).

When patients with and without DME in the PDR group were compared with respect to the G and T alleles, no significant difference was detected (Chi-square test, p=0.24, odds ratio=1.92, 95% CI for the G allele=0.872-1.611 and 95% CI=0.230-1.646 for the T allele). For the NPDR group, a statistically significant difference was found between the patients with and without DME with respect to the G and T allele frequencies. The G allele and the T allele were detected in 76.3 percent (n=29) and 23.7 percent (n=9) of the patients with macular edema, respectively, while these alleles were detected in 45.5 percent (n=20) and 54.5 percent (n=24) of macular edema-free subjects, respectively (Chi-square test, p: 0.004, odds ratio: 3.86, 95% CI 0.872-1.611 for the G allele and 95% CI=0.230-1.646 for the T allele). The G allele appeared to be associated with macular edema in patients with NPDR (Table 3). While the GG, GT and TT genotypes were detected in 62.1 percent, 31 percent and 6.9 percent of all patients with retinopathy and macular oedema, respectively, these genotypes were detected in 28.6 percent, 54.8 percent and 16.7 percent of all patients with retinopathy and without

Table 3: Relationship between frequencies of genotypes /alleles and macular edema in the groups

Sub-groups	G	T	Total	GG	GT	TT	Total
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)
N-DRP	58 (80.6)	14 (19.4)	72 (100)				
N-PDR-DME (+)	29 (76.3)	9 (23.7)	38 (100)	10 (52.6)	9 (47.4)	-	19 (100)
N-PDR-DME (-)	20 (45.5)	24 (54.5)	44 (100)	5 (22.7)	10 (45.5)	7 (31.8)	22 (100)
PDR-DME (+)	16 (80)	4 (20)	20 (100)	8 (80)	-	2 (20)	10 (100)
PDR-DME (-)	27 (67.5)	13 (32.5)	40 (100)	7 (35)	13 (65)	-	20 (100)
Control Group	67 (74.9)	23 (25.6)	90 (100)				
Total	217 (71.4)	87 (28.6)	304 (100)				

N-DRP: Non-Diabetic Retinopathy (Diabetic patients without retinopathy), N-PDR: Non-Proliferative Diabetic Retinopathy, PDR: Proliferative Diabetic Retinopathy, DME: Diabetic Macular Edema.
The GG genotype polymorphism was associated with DME in the diabetic retinopathy group, NPDR and PDR groups (p: 0.017, p: 0.008 and p: 0.029, respectively; Chi-square test)

DME, respectively. There was a significant correlation between DME and the GG genotype in patients with diabetic retinopathy (p=0.017, χ^2).

Classification of all patients based on the genotypes showed that FBG was significantly higher in subjects with the GT and TT genotypes than in those with the GG genotype, while HbA1c was higher in individuals with the TT genotype. When patients with diabetes were classified based on the genotypes, FBG was higher in patients with diabetes and the GT and TT genotypes, while patients with diabetes and the GT genotype had higher HbA1c levels relative to other patients with diabetes (Table 4).

Classification of patients with diabetes based on HbA1c levels <8 and >8 showed that the proportion of individuals with the GG genotype was higher in the group with HbA1c levels above 8 compared with that in the group with HbA1c levels below 8, while the GT genotype was detected at a higher frequency in the lower HbA1c group (p=0.002). Comparison of patients with diabetes based on their HbA1c levels with respect to the G and T alleles revealed that the G and T alleles were detected in 82.8 percent (n=53) and 17.2 percent (n=11) of patients with a HbA1c level >8, respectively, while the alleles were observed in 64.7 percent (n=97) and 35.3 percent (n=53) of patients with a HbA1c level <8, respectively; and the difference was statistically significant. In patients lacking long-term regulation of blood sugar levels, a significant correlation was detected between the GG genotype and the G allele.

The distribution of eNOS G894T genotypes, dominant model (TT vs GG + GT), recessive model (GG vs TT + GT) did not show significant differences between patients with DM and control participants (p > 0.05) or between patients with DRP and control participants (p>0.05) (Table 5).

Table 4: Mean fasting blood glucose, HbA1C, HDL, DM duration and BCVA in the GG, GT and TT genotype groups

Genotype	*FBG*	*HbA1c*	*HDL*	*DDM*	*BCVA*	
					Right eye	*Left eye*
Diabetic Patients						
GG	178.5± 79.6	8.5±2.1	43.8± 9.6	12.1±10.1	0.56±0.3	0.53±0.3
GT	215.7± 96.3	9.8±2.2	51.7±15.3	11.8± 7.2	0.63±0.3	0.64±0.4
T T	251.6± 55.5	8.8±0.7	44.5±15.2	13.0± 4.1	0.81±0.2	0.79±0.3
ANOVA	p: 0.035	p: 0.013	p: 0.015	p: 0.005	p: 0.019	p: 0.048
	F: 3.487	F: 4.521	F: 4.379	F: 0.912	F: 4.116	F: 3.138
Whole Study Population						
GG	150± 78.2	7.5±2.2	45.0±11.0	-	0.87±0.2	0.85±0.2
GT	190.6±100.8	9.0±2.6	49.9±14.6	-	0.71±0.3	0.72±0.3
T T	193.2± 91.9	7.5±1.7	44.7±12.2	-	0.79±0.3	0.68±0.3
ANOVA	p: 0.033	p: 0.003	p: 0.042			
	F: 3.478	F: 4.002	F: 4.312	-	-	-

FBG: Fasting Blood Glucose, DDM: Duration of Diabetes Mellitus, BCVA: Best-Corrected Visual Acuity
Statistical significance between GG and GT genotypes for FBG, HbA1C and HDL was p: 0.023, p: 0.010 and p: 0.012, respectively (Tukey posthoc test)

Table 5: The distribution of eNOS G894T genotypes, dominant model (TT vs GG + GT), recessive model (GG vs TT + GT)

	DM	*Control*	*p*	*DRP*	*Control*	*P*
Dominant Model						
GG+GT	98	41		63	41	
T T	9	4	0.92	9	4	0.71
Recessive Model						
GT+TT	55	19		42	19	
GG	52	26	0.19	30	26	0.09

DM: Diabetes Mellitus, DRP: Diabetic Retinopathy

DISCUSSION

While, in the present paper, the researchers found no correlation between the severity of diabetic retinopathy and the eNOS G894T gene polymorphism, a potential correlation was observed between DME occurrence and the G894T gene polymorphism. The researchers also observed that the gene polymorphism investigated in this paper had a potential correlation with chronic hyperglycaemia and uncontrolled blood sugar levels. Chronic hyperglycaemia leads to diabetic retinopathy in most patients. Diabetic retinopathy shows a very rapid progression in some individuals and a silent course in others, which suggests that other factors apart from blood sugar levels may also be involved in the development and progression of retinopathy. While multiple factors are involved in the progression of DRP, genetic factors have also been recently considered to play a role, and various studies have reported relevant findings (Awata et al. 2004; Taverna et al. 2005; Chen et al. 2007; Bazzaz et al. 2010). Genetic studies are investigating chromosomal/genetic alterations in some factors associated with the biochemical mechanisms involved in DRP pathogenesis. Nitric oxide is one of these factors. Increased oxidative stress caused by free radicals and increased NO production are suggested to play a major role in the pathogenesis of microvascular complications (West 2000). NO production is reported to be either increased or decreased at high glucose concentrations. Increased or commonly decreased NO levels have been associated with diabetic complications in clinical and experimental settings, suggesting a potential dual function of NO (Chan et al. 2000). In general, factors such as hyperglycaemia, poor metabolic control, cytokines, vasoactive hormones and insulin resistance are responsible for the progression of diabetic microvascular disease. Increased blood flow, selective loss of retinal pericytes, thickening of the basal membrane, endothelial cell loss and obstruction in minor capillaries lead to retinopathy through complementary effects (Warpeha et al. 1999). However, chronic hyperglycaemia is insufficient to induce severe diabetic retinopathy in some cases, suggesting an additional potential role of genetic factors (Klein et al. 1984). This hypothesis was evaluated in the Diabetes Control and Complications Trial (DCCT), which showed a strong familial retinopathy transition in patients with severe diabetic retinopathy, while there was no such transition in those without severe retinopathy (The Diabetes Control and Complications Trial Research Group 1997).

The pathophysiological step that plays the most important role in development of diabetic macular edema is the impairment of the blood retina barrier (Antcliff and Marshall 1999; Pelzek and Lim 2002). Both the inner retinal barrier, formed by retinal capillary endothelial cells, and the outer retinal barrier, formed by the tight junctions of retinal pigment epithelial cells, may be affected during the development of macular oedema. Nitric oxide is a multi-functional molecule that plays a key role in vascular tonus and the anti-proliferative regulation of vascular smooth muscle cells (Gross and Wolin 1995). Impaired eNOS expression in the retina has been indicated to significantly contribute to the development of macular edema where impairment of the blood-retina barrier is significantly involved. The impairment in eNOS may further accelerate hyperglycaemia-associated endothelial cell injury and thereby disrupt the inner blood retina barrier. eNOS activity may also lead to hypoxia by impairing vascular tonus and increased vascular permeability induced by vascular endothelial growth factor (VEGF).

Increased NO synthase activity was observed in rat retinas with induced retinopathy (doCarmo et al. 1998). High nitric oxide levels were reported in the aqueous humour of diabetic patients (Hattenbach et al. 2000). Patients with type 1 diabetes who develop retinopathy have increased NO synthase activity, which serves as an indicator of endothelial dysfunction (Tsai et al. 2003). Inhibition of nitric oxide synthase protects the eye against ischaemic changes. Excessive release of nitric oxide into the retina directly or indirectly induces oxidative injury, ischaemia and neoangiogenesis, suggesting that it may have an important role in the development and severity of diabetic retinopathy. In fact, excessive NO production may lead to oxidative stress in retinal endothelial cells. The loss of pericytes and increased blood flow in the retinal bed that pioneer the development of diabetic retinopathy may be partially related to the excessive production of nitric oxide. Another finding is the contribution of NO to the angiogenic features of VEGF, which is strongly involved in PDR development. In eNOS-deprived mice, vas-

cular permeability and angiogenesis caused by VEGF were inhibited (Aiello and Wong 2000; Shibuki et al. 2000; Abu El-Asrar et al. 2001; Brooks et al. 2001; EI-Remessy et al. 2003).

Various polymorphisms have been identified in the eNOS gene. Particularly, the T-786C polymorphism in the promoter region, the 27-bp repeat in intron 4, and the Glu298Asp polymorphism in the exon 7 have been noted. In type 1 diabetes, the "b" allele of the 27-bp repeat was associated with an increased risk of DRP (Taverna et al. 2002). In a group of patients with type 2 DM, an eNOS gene polymorphism was reported to be directly associated with DME development (T-786C, 27-bp repeat in the promoter region and intron 4). Among the identified eNOS gene polymorphisms, the T786C polymorphism (-T786C) in the promoter region, the 27-base pair repeat in intron 4 (in4a/b) and the G894T polymorphism in exon 7 (G894T) were reported to affect NO levels (Tsukada et al. 1998; Ohtoshi et al. 2002). The correlation of T786C with insulin resistance and the correlation of G894T with DM have been reported in controlled studies (Ohtoshi et al. 2002). Thus, the human eNOS gene is regarded as a candidate gene for DRP (Tsukada et al.1998; Ohtoshi et al. 2002; Monti et al. 2003; Awata et al. 2004; Srivastava et al. 2005; Chen et al. 2007; Bazzaz et al. 2010; Angeline et al. 2011; Cheema et al. 2012; Qian-Qian et al. 2014). There are inconsistent results reported in the literature regarding the correlation between DRP and the G894T polymorphism, a common variant located in the exon 7 of eNOS that results in a translocation between glutamate and aspartate in codon 298 (Glu298Asp). Since the previous papers reported inconsistent results, and a limited number of studies have investigated the potential correlation between the NOS G984T polymorphism and DRP and DME, the researchers believe that the present paper contributes to the current literature.

The functional effects of the G894T eNOS gene variant on the eNOS protein were previously described. Accumulating clinical and experimental data have shown that DRP pathogenesis is associated with a heterogeneous and complex combination of retinal disorders of the NO pathway, such as aberrant retinal NO consumption, impaired NO-mediated vasodilation, oxidative stress, dysregulation of nitric oxide synthase isoforms and eNOS coupling (Aiello and Wong 2000; Shibuki et al. 2000; Abu El-Asrar et al. 2001; Brooks et al. 2001; EI-Remessy et al. 2003; Tsai et al. 2003). However, inconsistent results have been reported regarding the potential associations between diabetic microvascular complications and the eNOS gene (Taverna et al. 2002; Awata et al. 2004; Srivastava et al. 2005; de Syllos et al. 2006; Chen et al. 2007; Uthra et al. 2007; Nishevitha et al. 2009; Bazzaz et al. 2010; Angeline et al. 2011; Cheema et al. 2012; Santos et al. 2012; Ma et al. 2014; Qian-Qian et al. 2014). In an Indian paper, the eNOS-894 mutant genotype rate was reported to be higher in patients with type 2 DM relative to the control arm (Angeline et al. 2011). Bregar et al. (2017) reported that NOS3 rs869109213 polymorphism may be associated with DR in Slovenian patients with type 2 DM.

Awata et al. (2004) reported that an eNOS gene polymorphism (T786C in the promoter region and the 27-bp repeat in intron 4) was associated with DME in Japanese patients with type II DM. Defective NOS expression in the retina may contribute to DME occurrence. Based on the paper, the researchers believe that the GG genotype in the G894T polymorphism may be associated with DME. However, even though Awata et al. (2004) reported an association between the other 2 polymorphisms and DME, they did not show a correlation between the G894T polymorphism and DME. The authors reported that none of the 3 polymorphisms that they evaluated were associated with diabetic retinopathy. Another paper of 577 patients without retinopathy findings, including 172 patients with type 2 DM and 405 with PDR, reported no association between G894T (Glu298Asp) and PDR (Cilensek et al. 2012).

A paper by Taverna et al. (2005) reported that two other polymorphisms (T786C and C774T) of the eNOS gene had no influence on severe DRP. However, the authors observed that C786C carriers were treated with panretinal photocoagulation 5 and 3.5 years earlier than T786T and T786C carriers, respectively. Furthermore, this association was more prominent in patients with HbA1c levels>eight percent. On the other hand, T774T carriers needed panretinal photocoagulation treatment 6 years later than C774C and C774T carriers (Awata et al. 2004). These data indicate that differences in the eNOS gene affect DRP severity. In another paper, the correlation between the G894T polymorphism and diabetic vascular complications was evaluated in 574

patients with type II DM, and no correlation was detected (Cai et al. 1998). The researchers' paper also confirms the absence of a correlation between the G894T polymorphism and DRP severity.

In a paper investigating the association between all 3 polymorphisms of the nitric oxide synthase gene and long-term glycaemic outcome in patients with impaired glucose tolerance, glycaemic levels were monitored for 5 years after the NOS genotypes were determined in 256 cases; and while normal glucose tolerance was detected in 40.2 percent of the cases at 5 years, impaired glucose tolerance persisted in thirty-nine percent of the patients, and 19.9 percent developed DM. A significant gene effect of exon 7 G894T polymorphism on glycaemic course was demonstrated over a period of 5 years. Compared with individuals with the GG genotype, T894 carriers were observed to have persistent hyperglycaemia. The presence of the T allele was observed to be a significant risk factor for persistent hyperglycaemia. The other 2 gene polymorphisms were reported to not have any significant effect on the 5-year glycaemic status. This paper also reported that the eNOS G894T polymorphism is an indicator of persistent hyperglycaemia (Tso et al. 2006). For the first time, Monti et al. (2003) described a significant correlation between the G894T polymorphism and type II DM to demonstrate a new genetic predisposition factor for patients with type II DM, hyperinsulinaemia and insulin resistance. However, when the presence of the G894T polymorphism in patients with type 2 DM was assessed, no difference was seen between patients with diabetes and the control subjects with respect to allele frequency (Ukkola et al. 2001). While Taverna et al. (2002) determined that the eNOS4 a/a homozygous deletion and its 4a allele were associated with non-severe DRP in patients with type I DM, the eNOS4b/b homozygous deletion and its 4b allele were associated with severe DRP.

While the duration of diabetes, HbA1c levels, HT and albuminuria were significantly different between the study group and the control arm, regression analysis showed that the influence of the eNOS 4a/a genotype on DRP was not affected by these variables. In the researchers' paper, they detected an association between the G894T gene polymorphism and a HbA1c level >8 in patients. In other words, although the G894T gene polymorphism was not associated with DRP severity in the researchers' paper, there may be a causal relationship between hyperglycaemia and/or non-regulated hyperglycaemia and the G894T gene polymorphism.

Taverna et al. (2005) reported that the "b" alleles and bb genotypes were associated with severe DRP. Similarly, Frost et al. (2003) suggested that the bb genotype of the 27-bp repeat was associated with DRP. However, Neugebauer et al. (2000) failed to detect any association between DRP and the 27-bp repeat. Tavakkoly-Bazzaz et al. (2003) indicated that the -786C allele, which showed an unbalanced connection with the "a" allele, had a significant correlation with DRP. In patients with type 2 diabetes, an eNOS gene polymorphism (T-786C, 27-bp repeat in the promoter region and intron 4) was found to be directly associated with DME occurrence. Considering the researchers' present results together with the previous results of other studies, defective eNOS expression in the retina may contribute to DME development. In a meta-analysis, a major protective effect against DRP was demonstrated with the 4b/a polymorphism in the intron 4a allele, particularly in patients with type 2 diabetes. Additionally, it was reported that the T786C polymorphism C allele may be a protective factor for PDR and that no significant association existed between the G894T polymorphism and PDR risk. In terms of the limitations of the meta-analysis, the authors indicated that no subgroup analyses, such as insulin recipients vs. non-insulin recipients or subgroups based on DM duration, were evaluated; and potential factors, such as age, gender and BMI, were not adjusted in all studies included. Moreover, several environmental factors that affect diabetes, such as diet, lifestyle and habits, were not considered, and most of the studies that reported negative results were not published (Zhao et al. 2012).

Several studies have shown that breakdown of the blood retina barrier by impaired eNOS expression in the retina may contribute to DME. Impaired eNOS activity may disrupt vascular tonus regulation and may also directly increase microvascular permeability. In this respect, demonstrating whether gene polymorphisms affect macular oedema occurrence may be of value. While a significant correlation was detected between the G894T gene polymorphism and DME, results obtained from a greater number of subjects are needed to clearly demonstrate that this

gene polymorphism contributes to DME. In addition to environmental and individual factors, such as living conditions, dietary habits, age, gender, and BMI, that affect the usual course of diabetes, differences in genetic features may also be involved in the incidence and severity of microvascular complications.

CONCLUSION

Polymorphisms alone may functionally lead to an increased risk of disease or represent a marker for the relevant genes (linkage disequilibrium) or may reflect the impact of a potential gene-environment relationship on the risk factor. The researchers' paper suggests that the eNOS G894T gene polymorphism seems to be associated with the presence of DME and unregulated hyperglycaemia. Further studies are required to determine these roles.

RECOMMENDATIONS AND FUTURE STUDIES

The researchers' paper was conducted in Middle Anatolia. There may be significant heterogeneity between different papers due to the underlying ethnic subgroup differences in the study samples. Larger scale papers of various ethnic groups may provide us with insight regarding the genotype-phenotype connection. An important point to evaluate is determining whether statistically evaluated results have the same value in clinical practice.

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