ÖZGÜN ARAŞTIRMA ORIGINAL RESEARCH

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THE MMP9 -1562 C/T POLYMORPHISM IS ASSOCIATED WITH INCREASED RISK OF DIABETIC RETINOPATHY IN TURKISH TYPE 2 DIABETES MELLITUS PATIENTS

MMP9 -1562 C/T POLİMORFİZMİ, TÜRK TİP 2 DİABETES MELLİTUS HASTALARINDA ARTMIŞ DİYABETİK RETİNOPATİ RİSKİ İLE İLİŞKİLİDİR

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Öz

Amaç
Tip 2 diabetes mellitus (T2DM) ve diyabetin mikrokomplikasyonlarından olan diyabetik retinopati (DR) gelişimi genetik faktörlerden etkilenmektedir. Matriks metalloproteinazlar (MMPs), ekstraselüler matriks proteinlerinin yeniden şekillendirilmesi ve anjiyogenez gibi pek çok hücresel proseste rol alırlar. Bu çalışmada Türk toplumunda MMP9 −1562 C/T (rs3918242) polimorfizmi ile T2DM ve DR gelişim riski arasındaki ilişkinin araştırılması amaçlanmıştır.

Gereç ve Yöntem
Mevcut çalışmaya 168 DR'si olan T2DM hastası, 168 DR'si olmayan T2DM hastası ve 174 kontrol olmak üzere toplam 510 birey dahil edilmiştir. Genotipleri belirlemek amacıyla Polimeraz zincir reaksiyonu-Restriksiyon fragment uzunluk polimorfizmi (PCR-RFLP) yöntemi kullanılmıştır.

Bulgular
T2DM gelişimi ile MMP9 −1562 C/T polimorfizmi arasında bir ilişkiye rastlanmazken, CT ve CT+TT genotipleri ile T (p=0,001) alleli artmış DR riski ile ilişkili bulunmuştur. Ayrıca CT (p=0,010) ve CT+TT (p=0,015) genotip sıklığı proliferatif diyabetik retinopati (PDR) hastalarında proliferatif olmayan diyabetik retinopati (NPDR) hastalarına kıyasla yüksek bulunmuş, fakat regresyon analizi sonrasında sadece insülin kullanımının (p=0,003) PDR gelişimi ile ilişkili olduğu belirlenmiştir.

Sonuç
Elde ettiğimiz veriler Türk T2DM hastalarında MMP9 −1562 C/T polimorfizminin DR gelişimi ile ilişkili olduğunu, fakat bu polimorfizm ile DR şiddeti ve T2DM gelişimi arasında anlamlı bir ilişki bulunmadığını göstermektedir.

Anahtar Kelimeler: Diabetik retinopati, MMP9, Polimorfizm, Proliferatif diyabetik retinopati
Tip 2 diabetes mellitus,

Abstract

Objective
Type 2 diabetes mellitus (T2DM) and diabetic retinopathy (DR) development is affected by genetic factors. Matrix metalloproteinases (MMPs) are involved

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in many cellular processes, such as remodeling of extracellular matrix proteins and angiogenesis. In this study, we investigated the relationship between MMP9 −1562 C/T (rs3918242) polymorphism and the development of T2DM and DR in Turkish population.

Material and Method
A total of 510 participants, involving 168 T2DM cases with DR, 168 T2DM cases without DR, and 174 controls, were included in the study. The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was carried out to identify genotypes.

Results
The CT, CT+TT genotypes and the T allele of the MMP9 −1562 C/T polymorphism were associated with increased risk of DR (p=0.001). On the other hand, no relationship was found between the development of T2DM and this polymorphism. In addition, CT (p=0.010) and CT+TT (p=0.015) genotype frequencies were found to be higher in proliferative diabetic retinopathy (PDR) cases compared to non-proliferative diabetic retinopathy (NPDR) cases, but after regression analysis, only insulin use (p=0.003) was found to be associated with the development of PDR.

Conclusion
Our data show that the MMP9 −1562 C/T polymorphism is associated with the development of DR in Turkish T2DM patients, but no significant relationship was found between this polymorphism and the severity of DR and the development of T2DM.

Keywords: MMP9, Diabetic retinopathy, proliferative diabetic retinopathy, type 2 diabetes mellitus, polymorphism.

Introduction

Diabetic retinopathy (DR) is the frequent complication of diabetes mellitus (DM) and it is among the common causes of vision problems and vision loss worldwide (1, 2). The early stage of DR which are observed microaneurysms and hemorrhages is called non-proliferative DR (NPDR). In the later stage of the disease, the formation of new blood vessels begins and the disease is called proliferative DR (PDR) at this stage (3,4). Diabetic macular edema (DME), a complication of DR, is the most prevalent cause of sight loss in DR patients (5). Long duration of DM, ineffective glycemic control, hyperlipidemia, hypertension, and increased body mass index (BMI) are major risk factors for DR (2, 3, 6). However these risk factors do not fully explain the differences between individuals in terms of DR risk and the disease severity (7). For example, between the patients with similar diabetes duration and similar glycemic control, substantial differences are observed in the risk and the severity of disease. In addition, the high risk of disease development in the families of individuals with DR indicates the effect of genetic factors on individual differences in disease development and severity (8).

Matrix metalloproteinases (MMPs) are enzymes belonging to the family of zinc-dependent endoproteases and they contribute to degradation and remodeling of protein components in the extracellular matrix. MMPs take a role in the regulation of various developmental mechanisms such as vascular remodeling, morphogenesis and angiogenesis (3, 9, 10). MMP9 is a member of the gelatinase family of MMPs, and the gene encoding the MMP9 protein is located on chromosome 20 (9). The −1562 C/T polymorphism is a common functional variation located in the promoter region. This polymorphism has been reported to increase the transcription level of MMP9 (9, 11).

Alterations in the blood-retina barrier are one of the most important changes observed in the early stages of DR, and MMP9 is thought to have a primary role in maintaining the integrity of this barrier. The formation of new blood vessels in DR, especially in PDR, is a characteristic feature of the disease. MMP9 is known to be critically important in angiogenesis. (3). MMP9 variations are suggested to have a role in the DR development. There are very few studies in the literature investigating the association between MMP9 polymorphisms and type 2 diabetes mellitus (T2DM) and DR. In these studies, it was reported that −1562 C/T variation may be related with susceptibility to these diseases (12, 13). A similar study was not found in the Turkish population. In the current study, we aimed to investigate the relationship between the MMP9 gene −1562 C/T polymorphism and the development of T2DM, DR, and the clinical features of the disease.

Material and Method

Subjects

The current study complies with the Declaration of

Helsinki and approval was obtained from the Giresun University Faculty of Medicine Clinical Research Ethics Committee. (Approval No: 23.12.2021-08). A total of 510 patients, 168 without DR, 168 with DR and 174 healthy controls, were involved in the present study. T2DM diagnosis was made according to the American Diabetes Association (ADA) standards (14). The diagnosis of DR was made by the ophthalmologists involved in the study, with a detailed ophthalmologic examination with fundus evaluation. Exclusion criteria for the patient group included having a different ocular disease other than DR, poor fundus photo quality, and having a severe systemic disease other than T2DM. In addition, patients with DR were staged as NPDR and PDR according to their examination findings. The control group was selected from individuals who did not have any eye disease, any type of diabetes mellitus and any systemic disease. In addition individuals who have positive family history of DR and diabetes mellitus in their first-degree relatives were excluded from the study. Blood samples were taken from the entire study group for DNA isolation. Moreover, data including age, height, weight, presence of hypertension, fasting blood glucose, HbA1c levels, duration of diabetes and lipid parameters of the patients and the controls were recorded.

DNA Extraction and Genotyping

DNA extraction was carried out using a commercial kit (Roche Diagnostics, Mannheim, Germany) and the manufacturer's protocol was followed. PCR analysis of the region of interest was performed with following primers F-5'-CTCATGCCCGTAATCCTAGC-3', R-5'-CTCCCTCACTCCTTTCTTCCTA-3'. 2% agarose gel electrophoresis was used to display PCR products with a size of 398 base pair (bp). The temperature, time, and cycle numbers of the PCR protocol; 95 °C for 5 min, followed by 35 cycles of 95 °C for 1 min, 65 °C for 1 min, 72 °C for 1 min, and a final extension at 72 °C for 5 min. PaeI enzyme was used for RFLP analysis. For enzyme digestion, 0.3 µl PaeI enzyme, 1.25 µl buffer, 6 µl sterile distilled water and 10 µl PCR product were used and incubated at 37°C for 3 hours. After enzyme digestion, the products were run in 2% agarose gel electrophoresis. A single band of 398 bp for the CC genotype, 3 bands of 398bp, 257bp and 141 bp for the CT genotype, and 2 bands of 257bp and 141 bp for the TT genotype was observed (Figure 1).

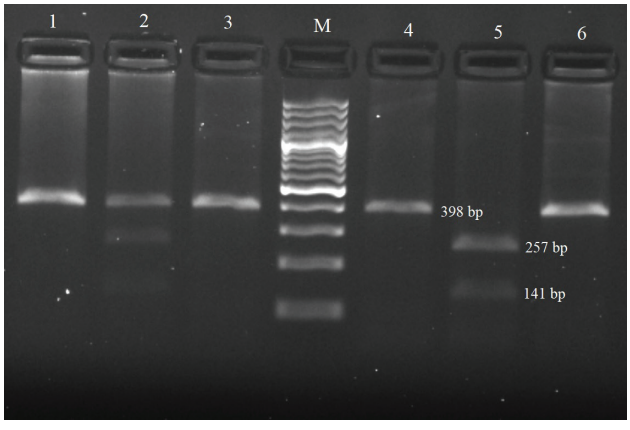

Figure 1
Agarose gel electrophoresis of PCR products. Lane 1, 3, 4, 6: GG genotype, lane 2: CT genotype, lane 5: TT genotype. M: 100 bp marker

Statistical Analyses

Statistical analyzes were performed using the SPSS software program (SPSS, Windows version release 15.0; SPSS Inc.; Chicago, IL, USA). Continuous values were expressed as mean ± SD, and categorical values were expressed as numbers and percentages. The genotype distribution among groups was tested for the Hardy–Weinberg equilibrium (HWE) using the $\chi 2$ test. Comparison of allele and genotype frequencies between groups was performed with the $\chi 2$ test. The distribution of continuous variables between groups was evaluated with Mann–Whitney U and Kruskal Wallis tests. Whether the −1562 C/T polymorphism had an independent effect on the development of DR and PDR was evaluated with binary logistic regression analyzes in which other risk factors were included. P values below 0.05 were considered significant.

Results

Clinical and demographic characteristics of the DR, non-DR and control groups are given in Table 1. Between T2DM patients with and without DR, a significant difference was observed in terms of duration of diabetes, HbA1c, fasting blood glucose level, total cholesterol, LDL, presence of hypertension, insulin use, and presence of DME (Table 1). The genotype distribution in all groups was consistent with the Hardy Weinberg (HWE) equation (Table 2). Allele and genotype frequencies were not different between the T2DM patients and the control group (Table 2). There was a statistically significant difference between the patient groups with and without DR in terms of both genotype and allele frequency. The frequency of CT (p=0.001) and CT+TT (p=0.001) genotypes and the frequency of T allele (p=0.001) were significantly higher in T2DM patients with DR compared to patients without DR (Table 2). In addition, CT (p=0.010) and CT+TT (p=0.015) genotype frequencies were significantly higher in NPDR patients compared to

PDR patients (Table 2). When the DR patient group was separated according to MMP9 rs3918242 genotypes, the mean diabetes duration was found to be higher in those carrying the CC genotype compared to the other genotypes (p=0.023). Also, the mean total cholesterol was found to be higher in those carrying the CC and CT genotypes compared to those carrying the TT genotype (p=0.025) (Table 3). In the logistic regression analysis, long diabetes duration (p<0.001), insulin use (p=0.004), high HbA1c levels (p=0.003) and MMP9 rs3918242 CT genotype (p=0.005) were found as independent risk factors for the DR development (Table 4). On the other hand, only insulin use (p=0.003) was determined as an independent risk factor, for PDR development (Table 5).

Table 1 Demographic and clinical characteristics of study groups.

Characteristic	Controls n=174	Cases (without DR) n=168	Cases (with DR) n=168	P value
Gender (male/female)	90/84	74/94	74/94	1.000
Age (years)	62 ± 11.5	63.3 ± 8.2	62.4 ± 7.5	0.373
BMI (kg/m2)	29.6 ± 4.6	29.6 ± 3.7	30 ± 5	0.612
Duration of diabetes (years)	-	8.9 ± 3.9	15.4 ± 5.6	**<0.001**
HbA1c (%)	4.8 ± 0.7	7.4 ± 1.2	8.2 ± 1.6	**<0.001**
Fasting glucose level (mg/dL)	85.6 ± 9.8	164.5 ± 55.3	193.9 ± 94.2	**0.001**
Total cholesterol (mg/dl)	154.1 ± 30.3	191.9 ± 37.8	208.4 ± 59	**0.009**
LDL (mg/dL)	103.8 ± 25.7	108.5 ± 30.9	118 ± 39.7	**0.029**
HDL (mg/dL)	50.6 ± 8	49.2 ± 12.3	47.8 ± 12.7	0.224
Triglyceride (mg/dL)	131.8 ± 18.7	188.4 ± 144.6	171.1 ± 122.5	0.539
Presence of hypertension (%)	11.5	37.5	54.2	**0.002**
Insulin therapy	-	24.4	65.5	**<0.001**
DME (%)	-	3	74.4	**<0.001**
PDR (%)	-	-	27.4	**-**

*P value compares cases with DR to cases without DR. DR= Diabetic retinopathy, BMI= Body mass index, LDL= Low-density lipoprotein, HDL= High-density lipoprotein, DME= Diabetic macular edema, PDR= Proliferative diabetic retinopathy.

Discussion

The structure and synthesis of the extracellular matrix are altered in DM (15). Disruption of the blood-retina barrier and angiogenesis are very important in the development of DR (3). MMP9 plays an important role in angiogenesis and the degradation and remodeling of extracellular matrix components. (12). As a matter of fact, in a study investigating the relationship between MMP9 polymorphism and T2DM susceptibility in the Indian population, it was reported that −1562 C/T polymorphism may cause T2DM susceptibility (13). In another study, MMP9 279 A/G (rs17576) polymorphism was found risk factor for T2DM susceptibility in Iranian individuals (15). In our study, however, genotype and allele frequencies of MMP9 −1562 C/T polymorphism were not different between T2DM patients and the control group (Table 2). Our results do not seem to be compatible with the other two studies in the literature. The reasons of this inconsistency may include ethnicity differences, differences in T2DM risk factors between the patient and control groups, and the relatively low number of patients included in the studies.

The MMP9 −1562 C/T polymorphism has also been associated with different complications of T2DM. For example, the T allele has been related with a lower risk of developing diabetic nephropathy in Han Chinese (16). Similarly, Shalaby et al., argued that the T allele has a protective role for the development of

Table 2 Comparison of MMP9 genotype and allele frequencies between the groups.

MMP9 rs3918242 Genotypes and alleles	**Controls N (%)**	**T2DM Cases N (%)**	**OR (95% CI)**	**p value**
CC	124 (71.3)	228 (67.9)	Ref	-
CT	48 (27.6)	99 (29.4)	1.122 (0.746-1.687)	0.581
TT	2 (1.1)	9 (2.7)	2.447 (0.521-11.504)	0.342
CT+TT	50 (28.7)	108 (32.1)	1.175 (0.787-1.753)	0.430
C	296 (85.1)	555 (82.6)	Ref	-
T	52 (14.9)	117 (17.4)	1.200 (0.841-1.713)	0.315
	HWE:0.261	HWE:0.653		
MMP9 rs3918242 Genotypes and alleles	**Cases (without DR) N (%)**	**Cases (with DR) N (%)**	**OR (95% CI)**	**p value**
CC	129 (76.8)	99 (58.9)	Ref	-
CT	36 (21.4)	63 (37.5)	2.280 (1.402-3.708)	**0.001**
TT	3 (1.8)	6 (3.6)	2.606 (0.636-10.679)	0.190
CT+TT	39 (23.2)	69 (41.1)	2.305 (1.438-3.696)	**0.001**
C	294 (87.5)	261 (77.7)	Ref	-
T	42 (12.5)	75 (22.3)	2.011 (1.331-3.040)	**0.001**
	HWE:0.791	HWE:0.292		
MMP9 rs3918242 Genotypes and alleles	**NPDR N(%)**	**PDR N(%)**	**OR (95% CI)**	**p value**
CC	65 (53.3)	34 (74)	Ref	-
CT	53 (43.4)	10 (21.7)	0.361 (0.163-0.797)	**0.010**
TT	4 (3.3)	2 (4.3)	0.956 (0.167-5.486)	1.000
CT+TT	57 (46.7)	12 (26)	0.402 (0.190-0.850)	**0.015**
C	183 (75)	78 (84.8)	Ref	-
T	61(25)	14 (15.2)	0.538 (0.284-1.020)	0.055

T2DM=Type II Diabetes Mellitus, OR= Odds ratio, CI= Confidence interval, HWE= Hardy-Weinberg equilibrium, DR= Diabetic retinopathy, PDR= Proliferative diabetic retinopathy, NPDR= Nonproliferative diabetic retinopathy.

diabetic nephropathy (17). In a study investigating the relationship between diabetic foot ulcer and MMP9 profile in T2DM patients, it was observed that MMP9 expression level increased as the wound grade increased. It was also found that the MMP9 promoter was unmethylated in diabetic wounds compared to control wounds. Researchers reported that the −1562 C/T polymorphism CT and TT genotypes combined with unmethylated promoter status caused an increase in MMP9 expression, resulting in chronic non-healing ulcers in the wounds of T2DM patients (18). Singh et al. reported that the MMP9 −1562 C/T polymorphism was a major risk factor for the development of PDR in the Indian population. Researchers claimed that the T allele can induce retinal angiogenesis by causing increased MMP9 production. (12). In another study by Beranek et al. in the Czech Republic, genotype and allele frequencies of MMP9 −1562 C/T and R279Q polymorphisms did not differ significantly between PDR, NPDR and control groups (19). In our study, frequencies of CT genotype, CT+TT genotype, and T allele were significantly higher in T2DM patients with DR compared to those without. In the logistic regression analysis, which also included other risk factors, diabetes duration, HbA1c level and MMP9 CT genotype were determined as independent risk factors for DR development. In terms of comparison between the groups with and without DR, our results are similar to Sing et al.' study (12). On the other hand, our results could not be compared with the

Table 3 Comparison of clinical characteristics stratified by genotypes of MMP9 rs3918242 polymorphism among T2D patients with DR.

Parameters	T2DM patients (with DR)			
	CC	CT	TT	P value
BMI (kg/m2)	30.6±5.3	29±4.7	30.6±3	0.127
Hypertension N(%) No Yes	 46 (60.5) 52 (57.1)	 28 (36.8) 35 (38.5)	 2 (2.6) 4 (4.4)	0.814
Total cholesterol (mg/dl)	211.7±60.1	207.2±57.6	155.2±27.8	**0.025**
LDL (mg/dL)	117.7±37.2	120.9±42.4	82.1±42.3	0.085
HDL (mg/dL)	49.3±13.9	45.9±10.2	41.2±10.6	0.096
Triglyceride (mg/dL)	172.1±144.2	167.5±79.2	190.7±109.8	0.448
Diabetes duration (years)	16.3±5.3	14.1±5.9	14.2±3.8	**0.023**
HbA1c (%)	8.2±1.6	8.2±1.6	9.4±1.9	0.214
Fasting blood glucose level (mg/dL)	191.1±76	185±94	320.2±230.3	0.306
DME N (%) No Yes	 26 (60.5) 73 (58.4)	 15 (34.9) 48 (38.4)	 2 (4.7) 4 (3.2)	0.808

T2DM=Type II Diabetes Mellitus, BMI= Body mass index, LDL= Low-density lipoprotein, HDL= High-density lipoprotein, DME= Diabetic macular edema

Table 4 Risk factors for DR using logistic regression analysis.

Variables	Odds ratio	95% CI	P value
BMI	0,982	0,916-1,053	0,615
Diabetes duration	1,337	1,238-1,445	**<0.001**
Presence of hypertension	1,180	0,645-2,159	0,592
Total cholesterol	1,003	0,991-1,015	0,592
LDL	1,007	0,991-1,023	0,372
HDL	1,000	0,975-1,025	0,973
Triglyceride	0,999	0,996-1,001	0,340
HbA1c	1,458	1,134-1,876	**0,003**
Fasting glucose level	1,002	0,997-1,007	0,426
Insulin therapy	2,549	1,349-4,816	**0,004**
MMP9 CT genotype	2,562	1,334-4,922	**0,005**
MMP9 TT genotype	1,288	0,193-8,614	0,794

OR= Odds ratio, CI= Confidence interval, BMI= Body mass index, LDL= Low-density lipoprotein, HDL= High-density lipoprotein.

Table 5 Risk factors for PDR using logistic regression analysis.

Variables	Odds ratio	95% CI	P value
BMI	1.060	0.982-1.145	0.137
Diabetes duration	1.046	0.976-1.120	0.202
Presence of hypertension	1.175	0.542-2.550	0.683
Total cholesterol	1.002	0.991-1.013	0.732
LDL	1.004	0.,988-1.021	0.607
Insulin therapy	4.887	1.719-13.899	**0.003**
MMP9 CT genotype	0.424	0.179-1.003	0.051
MMP9 TT genotype	0.940	0.147-6.002	0.948

OR= Odds ratio, CI= Confidence interval, BMI= Body mass index, LDL= Low-density lipoprotein

study by Beranek et al. because this study did not include T2DM cases without DR (19). Interestingly, in the comparison between NPDR and PDR patients, the frequency of CC genotype was found to be higher in the PDR group. However, in the logistic regression analysis performed afterwards, it was determined that only insulin use was an independent risk factor for PDR development. In addition, in DR patients classified according to MMP9 genotypes, total cholesterol levels were lower in patients with TT genotype, and diabetes duration was longer in patients with CC genotype. Our data show that the CT and CT+TT genotypes and the T allele increase the risk of developing DR in patients with T2DM. On the other hand, contrary to expectations, the higher frequency of CC genotype in the PDR group compared to the NPDR group may be due to the longer diabetes duration of patients with CC genotype or the poor glycemic control of these patients. Another reason may be the low number of patients in the PDR group.

In conclusion, current study demonstrated that MMP9 −1562 C/T polymorphism increases the risk of DR development in Turkish T2DM patients, but this polymorphism has no effect on the risk of T2DM development. In addition, while insulin use was associated with the development of PDR, no relationship was found between the MMP9 −1562 C/T polymorphism and the development of PDR. Although it has some limitations such as the low number of patients, our study is important in terms of the data obtained on the relationship between MMP9 polymorphism and the development of DR in Turkish population.

Conflict of Interest Statement
The authors have no conflicts of interest to declare.

Ethical Approval
The current study was conducted according to the principles of the Declaration of Helsinki and approved by Giresun University's Faculty of Medicine Clinical Trials Ethics Committee (Approval No: 23.12.2021-08).

Consent to Participate and Publish
Written informed consent to participate and publish was obtained from all individual participants included in the study.

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Availability of Data and Materials
Data available on request from the authors.

Authors Contributions
FMI: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Validation; Visualization; Writing-original draft.

EAk: Conceptualization; Formal analysis; Investigation; Methodology.

EA: Conceptualization; Methodology; Review & editing

SD: Resources; Formal analysis; Review & editing

MAO: Resources; Formal analysis; Review & editing

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