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The Relationship of the Peroxisome Proliferator Activated Receptor–Gamma 2 Exon 2 and Exon 6 Gene Polymorphism in Type 2 Diabetic Patients with and without Diabetic Foot Ulcers

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Abstract

We aims investigate Turkish type 2 diabetic patients with/without diabetic foot ulcers and healthy group and examined the contribution of the Pro12Ala exon 2 and C478T exon 6 of the PPARgamma gene polymorphisms to the development of diabetic foot ulcers. The PPARgamma genotypes were determined prospectively in 50 patients with diabetic foot ulcers and 50 without diabetic foot ulcers and a control group of 50 healthy individuals. Genotyping of the Pro12Ala exon 2 and C478T exon 6 of the PPAR-gamma gene polymorphisms for all individuals was performed by PCR-RFLP method. The genotype exon 2 and exon 6 distribution did not differ between the control group and the type 2 diabetes mellitus patients (with and without diabetic foot) (P>0.05). The frequency of the polymorphic G allele in exon 2 was no similar for the groups (6% and 1%, respectively) (p<0.05). The frequency of the polymorphic T allele in exon 6 was similar for the groups (p>0.05). The evaluation of exon 2 and exon 6, genotype and haplotype did not show statistically significant difference between the patient diabetic foot and without diabetic foot (p>0.05). The Pro12Ala exon 2 and C478T exon 6 of the PPARgamma gene polymorphisms are not an independent risk factor for diabetic foot in Turkish type 2 diabetes mellitus patients. Genetic factors in the pathogenesis of diabetic foot may also show any changes in different populations.

Key words: Peroxisome Proliferator Activated Receptor Gene Polymorphism, Type 2 Diabetic Patients, diabetic foot ulcers

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Introduction

Diabetic foot disorder, the major source of disability and morbidity, is a significant burden for the community and a true public health problem [1]. Diabetic foot ulcerations result from different pathophysiological mechanisms; a clear understanding of them is crucial to reduce their incidence, provide early care, and finally delay the amputation risk. The three main diabetes complications involved in foot ulcerations are neuropathy, peripheral artery disease, and infection [2]. Diabetic neuropathy is the most common complication of diabetes, affecting 50% of diabetic patients [3]**.** The most important factor related to the development of foot ulcer is peripheral neuropathy, associated with loss of pain sensation. Neuropathy can be associated with peripheral vascular disease and foot deformities [4].

Peripheral vascular disease is one of the components of the diabetic foot. Diabetic patients should be assessed for lower limb arterial disease. Medical management includes minimization of vascular risk factors, anti-thrombotic therapy, and walking rehabilitation. Vascular testing is required in the presence of a foot wound [5].

Foot infections are common in patients with diabetes and are associated with high morbidity [6]. Infection is always the consequence of a preexisting foot wound whose chronicity is facilitated by the diabetic peripheral neuropathy, whereas peripheral vascular disease is a factor of poor outcome, especially regarding the risk for leg amputation [7].

Peroxisome proliferator-activated receptor gamma (PPARgamma) is an important transcription factor for lipid and glucose metabolism [8]. The peroxisome proliferator-activated receptors (PPARs) are nuclear fatty acid receptors, which contain a type II zinc finger DNA binding motif and a hydrophobic ligand binding pocket. These receptors are thought to play an important role in metabolic diseases such as obesity, insulin resistance, and coronary artery disease. Three subtypes of PPAR receptors have been described: PPARalpha, PPARdelta/beta, and PPARgamma. PPARgamma has high expression in fat, low expression in the liver, and very low expression in the muscle [9].

However, they are also expressed in renal glomerular tissue and in vascular walls, thus participating through various and complex mechanisms, to glomerular and vascular sclerosis and to nephropathy development and progression [10,11].

It is generally accepted that the Pro12Ala polymorphism in peroxisome proliferator-activated receptor-gamma2 (PPAR-gamma2) is associated with an increased risk of type 2 diabetes [12].

We aims investigate Turkish type 2 diabetic patients with/without diabetic foot ulcers and healthy group and examined the contribution of the Pro12Ala (rs1801282) and C478T of the PPARgamma gene polymorphism to the development of diabetic foot.

Material and Method

After getting approval for the Ethics Committee, 50 type 2 diabetic patients with diabetic foot ulcers, 50 type 2 diabetic patients without diabetic foot ulcers and a control group of 50 healthy individuals enrolled in the study. Informed consent was obtained from each participant. A detailed medical history of each patient was obtained. The phenotypic characteristics were determined. Age, sex, duration of diabetes, Body mass index (BMI), blood pressure were recorded. Exclusion criteria were having Type 1 Diabetes Mellitus, serious hepatic, cardiac, renal failure, diseases with acute inflammation, malignancy, thyroid disorders and psychiatric disorders.

Blood samples were taken in the morning after overnight fasting. Serum total-cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides, uric acid, creatinine, thyroid stimulating hormone, glycolizated hemoglobin (4,5-6%), high sensitive C-reactive protein (hs-CRP), erithrocyte sedimentation rate (ESR) and microalbuminuria levels were measured. Biochemical parameters were studied with Roche/Hitachi Moduler autoanalyzer, complete blood count was studied with Roche Symex autoanalyzer.

Foot ulcer is defined as full-thickness skin defect that did not heal within 14 days(16). Wagner classification was used for diabetic foot ulcers. Infection was defined as at least two of the following; purulent discharge, local heat increment, local erythema, lymphangitis, edema, fever and bad odour [13,14].

Retinopathy is diagnosed by ophthalmologic evaluation [15].

Microalbuminuria was diagnosed when albumin excretion rate (AER), measured by radioimmunoassay (RIA), was 30-300 mg/24-hours in at least two out of three 24- hours urine

collections over a three-month period. Cretinine clearance, is calculated by Cockroft-Gault formula [GFR=(140-age) x weight (kg)/ Plasma creatinine x 72 (in women x 0,85)] [16].

Macrovascular disease is defined as; presence of ischemic heart disease, stroke, transient ischemic attack or peripheral artery disease [13,15]. Peripheral artery disease is diagnosed by absence of both feet pulses and/or ankle-brachial pressure index [ABPI] lower than 0,90 [16-21]. ABPI measurement is made by a hand doppler (Hadeco ES-101EX, 8 Mhz).

Peripheral sensory neuropathy was defined as insensitivity to the 5.07 (10-g) Semmes-Weinstein monofilament at any one of ten sites on either foot (dorsal midfoot, plantar aspect of foot including pulp of the first, third, and fifth digits, the first, third and fifth metatarsal heads, the medial and lateral midfoot and the calcaneus).

Genotype Analysis

3 ml peripheral venous blood was taken to the tubes containing EDTA from all of the 150 participants and their genomic DNA was isolated according to the High Pure PCR Template Preparation Kit (Roche Applied Science, Germany) protocole. A 295 bp fragment of PPAR exon2 gene was amplified with LightCycler instrument (Roche Diagnostic) using the following primer pairs for PPAR Pro12Ala genotyping analysis [22]. PPAR exon 2 Forward primer: 5'- CTG ATG TCT TGA CTC ATG GG - 3' . PPAR exon 2 Reverse primer: 5'- GGA AGA CAA ACT ACA AGA GC - 3'.

The 20 µl reaction master mix contained 4 mM $MgCl_2$, 1 µM of each primer, 2 µl Fast Start DNA Master SyberGreen kit 1 and 50 ng genomic DNA. Following an denaturation step at 95^0C for 10 min, DNA was amplified in 45 PCR cycles (95^0C for 10 s; 60^0C for 15 s; 72^0C for 12 sec). The cooling step was 40^0C for 30 sec. After confirmation of PCR with 2% agarose gel, genotype analysis was done according to restriction fragment length polymorphism technique. A measure of 10 µl of the PCR products were digested with *HgaI* (Fermentas) at 37^0C and analysed by gel electrophoresis in a 2% Nusieve-agarose gel which is stained with ethidium bromide and visualized under UV light. The presence of C at codon 12 resulted with undegistion. When G was present, 295 was digested to 117 bp., 178 bp. were observed (Figure 1).

The primers used for PPAR-Y exon6 C478T genotype analysis are;

Forward primer: 5'- CCA GAA AAT GAC AGA CCT CAG ACA- 3'

Reverse primer: 5'- CAG AAT AGT GCA ACT GGA AGA AGG - 3' [22]. We again used LightCycler (Roche, Diagnostic) instrument for PCR with the same reaction protocole as exon2, unless the annealing temparature for exon 6 was set as $63C^0$. to ampify a 181 bp. fragment of PPAR exon6. The sequence C is recognised by the *Pml I* (Fermentas) restriction endonuclease that digested 181 bp. PCR products into 142 bp. and 41 bp. fragments. When there is a T at 478^{th} position, the 181 bp. PCR product remained uncut.

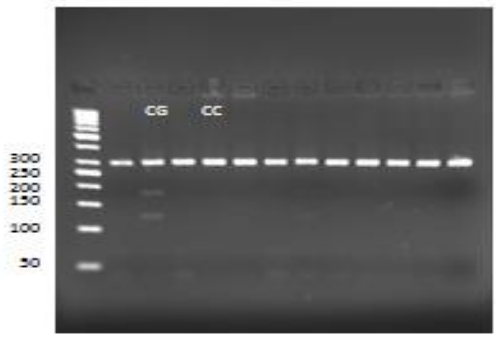

Figure 1. PPAR-gamma Pro12Ala genotype by RFLP-PCR.
Representative agarose gel electrophoresis illustrating PCR products after restriction with Hga I enzyme showed:
Lane 1: DNA marker.
Lanes 2, 4,5, 6, 7, 8, 9, 10, 11, 12, 13: Wild type for CC genotype at 295 bp.
Lanes 3: Heterozygous for CG genotype at 295 bp, 178 bp and 117 bp.

CC genotypes expressed in exon 2 are evaluated as normal, while CG is heterozygous and GG is homozygous mutant. In exon 6, CC is revealed as normal, CT is heterozygous and TT as homozygous mutant (Figure 2).

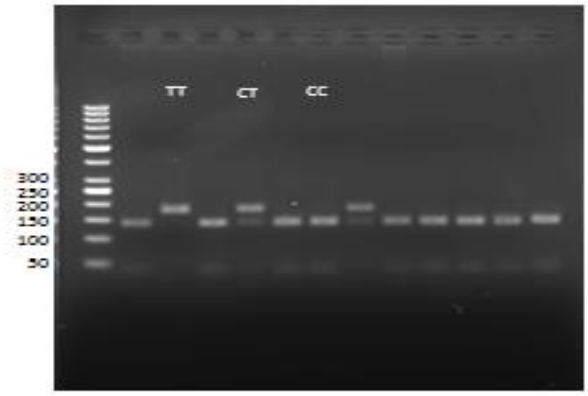

Figure 2. PPAR-gamma C478T genotype by RFLP-PCR.
Representative agarose gel electrophoresis illustrating PCR products after restriction with PmI 1 enzyme showed:
Lane 1: DNA marker.
Lanes 2, 4, 6,7, 9, 10, 11, 12, 13: Wild type for CC genotype at 142 bp and 41 bp.
Lanes 3: Homozygous mutant for TT genotype at 181 bp.
Lanes 5, 8: Heterozygous for CT genotype at 181bp, 142 bp and 41 bp.

Statistical analysis

SPSS 18.0 for windows was used for statistical analysis of results. Distributions of continuous variables in groups were expressed as mean ± S.D. The distribution of alleles and genotypes between groups was compared using χ^2 analysis or Fisher's exact test. Differences between the mean of biochemical parameters were examined by means of ANOVA. A value of $p < 0.05$ was considered to be significant.

Results

Clinical investigation

Retinopathy, neuropathy and infection were statistically different between diabetic patient groups. Systolic and diastolic blood pressures were significantly different between patients and controls groups (Table 1).

Table 1. Anthropometric and clinical features of diabetic patients and healty controls

	Healty Controls (n:50)	**Diabetic Patients without foot ulcers** (n:50)	**Diabetic Patients with foot ulcers** (n:50)	P value
Numbers(M/F)	50(26/24)	50(25/25)	50(26/24)	
Age (years)	56,42±4,60	56,42±8,43	59,82±10,55	
BMI (kg/m^2)	26,86±4,38	29,62±5,45	28,32±5,81	
Diabetes duration (years)	-	11,12±6,70	12,92±6,52	
Smoking (%)	15	22	14,7	
Systolic blood pressure (mm/Hg)	112,40±11,16	125,14±12,94	126,40±15,18	<0,001***
Diastolic blood pressure (mm/Hg)	70,00±7,56	75,10±7,62	75,50±7,30	<0,001***
Retinopathy (%)	-	20	41	<0,05*
Nepropathy (%)	-	78	86	
Macrovascular disease (%)	-	46	62	
Neuropathy (%)	-	60	96	<0,001***
Infection (%)	-	0	50	<0,001***

n= Number of individuals

* Statistically significant difference was determined between two groups (p<0,05).

*** Statistically significant difference was determined between two groups (p<0,001).

HDL-Cholesterol, CRP, ESR levels were statistically different between diabetic patient groups. HDL-Cholesterol and TG levels were statistically different between controls and diabetic patients (Table 2).

Table 2. Laboratory Findings of Diabetic Patients and Healthy Controls

	Healthy Controls (n:50)	**Patients without diabetic foot ulcers** (n:50)	**Patients with diabetic foot ulcers** (n:50)	P value
HbA1c (%)	5,2±0,24	9,6±1,70	9,76±0,31	
Total Cholesterol (mg/dl)	173,94±8,36	180,28±36,32	170,14±51,08	
LDL- Cholesterol (mg/dl)	102,10±22,04	116,45±32,76	106,32±41,65	
HDL- Cholesterol (mg/dl)	45,50±2,44	40,90±12,02	36,12±13,90	<0,01**
Triglyceride (mg/dl)	128,92±6,46	170,10±88,10	165,72±92,74	<0,05*
Creatinine (mg/dl)	0,70±0,14	0,86±0,22	0,98±0,42	
TSH (mIU/ml)	1,14±0,52	1,34±0,88	1,28±0,96	
CRP (mg/dl)	0,24±0,16	1,16±0,97	5,37±3,22	<0,001***
ESR (mm/hr)	10±5,20	34,62±26,56	69,52±30,99	<0,001***

* Statistically significant difference was determined between two groups (p<0,05)

****** Statistically significant difference was determined between two groups (p<0.01)

*** Statistically significant difference was determined between two groups (p<0,001)

Frequencies of C/G exon 2 and C/T exon 6 of the PPARgamma gene polymorphism

Genotyping of the Pro12Ala C/G substitution in exon 2 and C478T C/T substitution in exon 6 of the PPARG gene polymorphism for all individuals was performed by PCR-RFLP method. But for quick results, PCR was done by LightCycler System with FastStart Syber Green Master kit (Roche Applied Science, Germany). The distributions of genotype and allele frequencies were compared between diabetic patients and controls (Table 3,4) as well as between patients with diabetic foot and those without diabetic foot (Table 5,6).

We analyzed the frequency of C/G exon 2 and C/T exon 6 of the PPARgamma gene in type 2 diabetes mellitus and control groups. The genotype exon 2 distribution did not differ between the control group (CC 90%, CG 8%, GG 2%) and the type 2 diabetes mellitus patients (CC 98%, CG 2%, GG 0%) (P>0.05) (Table 3). The frequency of the polymorphic G allele was no similar for the group with type 2 diabetes mellitus as for the control group with 1% and 6%, respectively (p<0.05; Table 3).

Table 3. Genotype distribution and allele frequency of the Peroxisome ProliferatorActivated Receptor–Gamma 2 Exon 2 Gene Polymorphism for the study and control group

Genotype Exon 2	**With and without diabetic foot ulcers** n=100		**Control Group** n=50	
	n	%	*n*	%
CC	98	98	45	90
CG	2	2	4	8
GG	0	0.0	1	2
Allele				
C	198	99	94	94
G	2	1	6	6*

Data were compared between groups by X^2 test (p>0.05). n: number of individuals.
* Statistically significant difference was determined between two groups (p<0,05)

The genotype exon 6 distribution did not differ between the control group (CC 82%, CT 18%, TT 0%) and the type 2 diabetes mellitus patients (CC 73%, CT 19%, TT 8%) (P>0.05) (Table 4). The frequency of the polymorphic T allele was also similar for the group with type 2 diabetes mellitus as for the control group with 17.5% and 9%, respectively (p> 0,05, Table 4).

Table 4. Genotype distribution and allele frequency of the Peroxisome Proliferator–Activated Receptor – Gamma 2 Exon 6 Gene Polymorphism for the study and control group

Genotype Exon 6	**With and without diabetic foot ulcers** n=100		**Control Group n=50**	
	n	%	*n*	%
CC	73	73	41	82
CT	19	19	9	18
TT	8	8	0	0
Allele				
C	173	82.5	91	91
T	37	17.5	9	9

Data were compared between groups by X^2 test (p>0.05). n: number of individuals.

Genotype exon 2 frequencies in diabetic patients with diabetic foot were (G/G) 0%, (C/G) 2%; (C/C) 98% versus (G/G) 0%; (G/C) 2%; (C/C) 98% in those without diabetic foot (p>0.05). The frequency of the polymorphic G allele was 1% in diabetic patients with diabetic foot versus 1% in those without diabetic foot (P>0.05) (Table 5).

Table 5. Genotype distribution and allele frequency of the Peroxisome ProliferatorActivated Receptor–Gamma 2 Exon 2 Gene Polymorphism in type 2 diabetes mellitus patients with and without diabetic foot ulcers

Genotype Exon 2	***with* diabetic foot ulcers** n=50		***without* diabetic foot ulcers** n=50	
	n	%	*n*	%
CC	44	98	44	98
CG	1	2	1	2
GG	0	0	0	0
Allele				
C	99	99	99	99
G	1	1	1	1

Data were compared between groups by X^2 test (p>0.05). n: number of individuals.

Genotype exon 6 frequencies in diabetic patients with diabetic foot were (T/T) 4%, (T/C) 18%; (C/C) 78% versus (G/G) 12%; (G/C) 20%; (C/C) 68% in those without diabetic foot (p>0.05). The frequency of the polymorphic T allele was 13% in diabetic patients with diabetic foot versus 22% in those without diabetic foot (P>0.05) (Table 6).

Table 6. Genotype distribution and allele frequency of the Peroxisome ProliferatorActivated Receptor–Gamma 2 Exon 6 Gene Polymorphism in type 2 diabetes mellitus patients with and without diabetic foot ulcers

Genotype Exon 6	***with* diabetic foot ulcers** n=50		***without* diabetic foot ulcers** n=50	
	n	%	*n*	%
CC	39	78	34	68
CT	9	18	10	20
TT	2	4	6	12
Allele				
C	87	87	78	78
T	13	13	22	22

Data were compared between groups by X^2 test (p>0.05). n: number of individuals.

Discussion

Social deprivation is an important factor, especially for the development of foot ulcers [17]. However, the most important factor related to the development of foot ulcer is peripheral neuropathy. Diabetic peripheral neuropathy (DPN) is one of the complications of type 2 diabetes mellitus (T2 DM) that decreases the quality of life of T2DM patients. Very few studies have found an association between the development and progression of DPN in T2DM [18].

Jurado J et al. analyzed a single angiotensin-converting enzyme (ACE) gene polymorphism (D/I) as a genetic marker of risk of developing DPN. Their findings suggest that heterozygous ACE polymorphism (D/I) stands as a protective factor for DPN development [19]. Vendrell J et al. have studied the relationships between I/D polymorphism of ACE gene and presence of microangiopathic complications in type 2 diabetic patients. There were no significant differences in the frequencies of ACE genotype [20]. Agonism of the peroxisome proliferator-activated receptors (PPARs) has long been an attractive target for antidiabetic therapy due to the role of PPARs in glycemic control and lipid metabolism [21].

There is not study that development of diabetic foot has PPARgamma gene polymorphism. In our study we investigated a possible association of the G/C exon 2 and T/C exon 6 of the PPARgamma gene polymorphism with the development of diabetic foot in Turkish type 2 diabetes patients. PPARgamma exon 2 and exon 6 gene polymorphism was not associated with risk of the development of diabetic foot in our group of type 2 diabetic patients. Our study suggests that polymorphic G allele may play a specific role(s) in determining diabetic susceptibility, but do not seem to be important in the clinical manifestations of development of diabetic foot.

Our study is the first one in a Turkish population which shows that the the C/G exon 2 and C/T exon 6 of the PPARgamma gene polymorphism was not associated with risk of the development of diabetic foot. Thus, we suggest that the C/G exon 2 and C/T exon 6 of the PPARgamma gene polymorphism is not an independent risk factor for diabetic foot in Turkish type 2 diabetes mellitus patients**.** Genetic factors in the pathogenesis of diabetic foot may also show any changes in different populations.

Declaration of interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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