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ORIGINAL PAPER

Methyltetrahydrofolate reductase C677T gene mutation and hyperhomocysteinemia as a novel risk factor for diabetic nephropathy

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Abstract Hyperhomocysteinemia is a well-defined risk factor for endothelial dysfunction and atherosclerosis. A point mutation (677 C-T) of MTHFR gene results in a significant increase at plasma homocysteine levels. In this study we aimed to evaluate the effects of MTHFR gene mutation and consequent hyperhomocysteinemia on the development of diabetic microvascular complications in comparison with the other defined risk factors. Diabetic patients without a history of macrovascular complication or overt nephropathy enrolled into the study. The presence of MTHFR 677 C-T point mutation was evaluated by Real-Time PCR technique by using a LightCycler. MTHFR heterozygous mutation was present in 24 patients over 52. Patients with diabetes were divided into two groups according to the presence of MTHFR gene mutation. Both groups were well matched regarding age and diabetes duration. Metabolic parameters, plasma homocysteine, microalbuminuria, folic acid, and vitamin B12 levels were also studied. Presence of neuropathy and retinopathy were evaluated by specific tests. Duration of diabetes, BMI, systolic and diastolic blood pressure, plasma CRP, HbA1c, and lipid levels were not different between the two groups. Plasma homocysteine (12.89 ± 1.74 and 8.98 ± 1.91 μmol/l; $P < 0.0001$) and microalbuminuria levels (73.40 ± 98.15 and 29.53 ± 5.08 mg/day; $P = 0.021$) were significantly higher in the group with MTHFR gene mutation while creatinine clearance levels (101.1 ± 42.6 and 136.21 ± 51.50 ml/min; $P = 0.008$) were significantly lower. Sixteen over 22 (73%) of the patients with diabetic nephropathy had MTHFR gene mutation, while this was only 27% (8 over 30) in normoalbuminuric patients ($P = 0.017$). There was a significant correlation of plasma homocysteine level with microalbuminuria ($r = 0.54$; $P = 0.031$) in the patients with diabetic nephropathy who had C677T polymorphism. We did not find any specific association of MTHFR gene mutation and hyperhomocysteinemia with retinopathy or neuropathy.

Keywords MTHFR C677T mutation · Type 2 diabetes · Hyperhomocysteinemia · Diabetic nephropathy

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Introduction

Diabetic nephropathy appearing in 20–40% of diabetic patients is the first cause of end-stage renal failure that appears in diabetic patients [1]. Presence of persistent microalbuminuria (30–299 mg/24 h) in type 2 diabetic patients is a very important marker for nephropathy which will develop [1]. Progression of persistent microalbuminuria to macroalbuminuria is a very important marker showing that end-stage renal failure will develop [2, 3]. In

type 2 diabetic patients, the risk of developing nephropathy has been decreased and/or progression has been slowed down by a fine metabolic and blood pressure control, however, it has not been prevented completely, as diabetic nephropathy development is a multifactorial condition which involves multiple environmental and genetic factors [4–6]. Due to this reason, it is not possible to foresee which ones of diabetic patients are more prone to nephropathy development because genetic factors play an important role in nephropathy prognosis as much as metabolic factors do [7]. There is a large body of evidence that genetic abnormalities are involved in the development of diabetic nephropathy [4–7].

Elevated plasma homocysteine levels are associated with diabetic nephropathy [8]. The enzyme methylenetetrahydrofolate reductase (MTHFR) plays a key role in homocysteine metabolism. It catalyzes conversion of composed homocysteine into methionine by remethylation [9]. Polymorphisms which occur on the gene coding MTHFR enzyme lead to hyperhomocysteinemia by reducing enzyme activity [10, 11]. Up to now, many polymorphisms on the gene coding MTHFR has been detected, but most of them have not led to hyperhomocysteinemia since they do not alter the shape and activity of active site of the MTHFR enzyme. The best identified polymorphism is C677T mutation which occurs by displacement of cytidine on 677th position with thymidine. This amino acid change causes deformation in three-dimensional structure and reduction in catalytic activity of MTHFR enzyme in endogenous body temperature and the new form of the enzyme is called thermolabile MTHFR enzyme [12]. Heterozygote MTHFR mutation is thought not to lead a change in plasma homocysteine level and not to increase additional morbidity risk unless there is a folic acid deficiency [12–14]. However, it is not clear whether heterozygote MTHFR mutation increases plasma homocysteine level and microvascular complication risk in diabetic patients. Since poorly metabolically controlled patients with advanced stage nephropathy have also been included in most of the studies in which the relation between MTHFR gene mutation and plasma homocysteine level is evaluated in diabetic patients, it is not clear whether homocysteine elevation is directly related to gene mutation [15, 16].

In this study, we wanted to evaluate the effects of MTHFR gene mutation and consequent hyperhomocysteinemia on the development of diabetic microvascular complications in comparison with the other defined risk factors.

Material and methods

Out patients of our Endocrinology and Metabolic Diseases polyclinic who are diabetic patients without macrovascular complications and overt nephropathy, with type 2 diabetes diagnosis over age 40, with no previous diagnosis/therapy for ketosis or ketoacidosis, and whose blood sugar has been regulated with oral antidiabetics were evaluated. Patients with type 1 diabetes diagnosis, with secondary diseases that may lead to blood sugar disorders, who are pregnant, having stroke history, with peripheral arterial disease, having experience with acute coronary events, having malignancy, with respiratory failure, with renal and liver failure, with heart valve diseases, and thyroid dysfunction were excluded from the study. In addition to these criteria, those who are using drugs for Vitamin B_{12} and folate deficiency, uncontrolled hypertension, alcoholism, methotrexate, phenytoin, carbapazemine, theophylline, cholestipol, and niacin which are the other factors that may elevate plasma homocysteine level were excluded from the study [17]. In conclusion, 52 type 2 diabetic patients were included in the study (32 female and 20 male, mean age: 52.7 $\pm$ 9.9 year). Patients were selected after the study protocol was approved by the local ethics committee as to be in accordance with II. Helsinki Declaration. Consent forms were obtained from the patients after they were informed about the study to be conducted and its outcomes.

Evaluation of macro and microvascular complications

Presence of coronary artery disease (CAD) was defined as previously documented acute myocardial infarction, coronary artery by-pass surgery, or coronary angioplasty. Those individuals who met Minnesota coding [18], based on resting ECG Q-wave or clinical history of angina pectoris, were also included in the total clinical CAD. A history of documented stroke defined cerebrovascular accident. Peripheral arterial disease (PAD) was established by the presence of at least two of the criteria for intermittent claudication, an ankle-brachial pressure index of less than 0.9, and the absence of peripheral pulses. Retinopathy was diagnosed as none, background, or proliferative by an ophthalmology specialist. For microalbuminuria values, $<$30 mg/24 h was accepted as microalbuminuria negative, 30–299 mg/24 h was accepted as microalbuminuria, and $\geq$300 mg/24 h was accepted as macroalbuminuria. Urine creatinine was calculated with creatinine clearance formula [Creatinine Clearance = (urine creatinine mg/dl $\times$ urine volume ml)/(serum creatinine mg/dl $\times$ 1440 min)] by urine volume and serum creatinine values. Nephropathy was established by the presence of at least two criteria of micro/macroalbuminuria, decreased creatinine clearance, and hypertension. Neuropathy was established by examining symptoms and completing neurologic evaluations of the patients.

Biochemical analysis

Tests of all recruited patients were performed in central biochemistry and hematology laboratory. After 10–12 h fasting period, 8 ml blood samples were collected from the patients in fasting. Blood samples taken for routine biochemical tests were put into vacuumed special dry tubes and centrifuged in 2 h (1500×*g*, 10 min). Serum supernatants were transferred to polyethylene tubes by using pipettes. Serum glucose, total cholesterol, triglyceride, high-density lipoprotein cholesterol (HDL-K), and low-density lipoprotein cholesterol (LDL-K) levels were measured via a instrument called Roche Modular autoanalyzer by using Roche Diagnostic original kits (Roche Diagnostics GmbH, D-68298, Mannheim, Germany) with glucose oxidize–PAP, cholesterol oxidize–PAP, glycerol phosphate oxidize–PAP, and homogenous direct enzymatic colorimetric methods. Intraassay and interassay coefficients of variation were 0.9 and 1.8% (serum glucose), 0.8 and 1.7% (total cholesterol), 1.5 and 1.8% (triglycerides), 1.3 and 2.6% (HDL-cholesterol levels), and 0.71 and 1.2% (LDL-cholesterol levels). Lipoprotein (a), Apo A1, and Apo B100 levels were measured via a instrument called Dade Behring autoanalyzer by using Dade Behring original kits (Dade Behring Marburg GmbH d-35041, Marburg, Germany) with nephelometric method, intraassay and interassay coefficients of variation were 2.1 and 2.3%, 2.2% and 5.7%, and 1.9 and 2.4%, respectively. Basal insulin levels were calculated by using Immulite One autoanalyzer with DPC original kits (DPC, CA 90045-5597, LICIN 10123, Los Angeles) and immunometric principle, intraassay and interassay coefficients of variation were 3.8 and 4.2%. Sensitive TSH (sTSH), free T_3, free T_4, cortisol, vitamin B_{12}, and folic acid levels were measured via Roche E170 immunoassay autoanalyzer instrument by using chemiluminescence method, intraassay and interassay coefficients of variation were 2.2 and 5.7%, 1.1 and 1.8%, 1.5 and 2%, 1.9 and 2.2%, 1.3 and 1.7%, and 1.9 and 2.4%, respectively. Peripheral insulin resistance was calculated by HOMA (Homeostasis Model Assessment) method [19] (HOMA = Fasting plasma glucose (mmol/l) × Fasting insulin level (μU/ml)/2.5). For plasma homocysteine level, 2 cc of blood samples from per patient was collected at 08:30 in the morning after an overnight 10–12 h fasting period. Blood samples were collected in tubes with EDTA (Vacutaine, Becton Dickinson, Meylon, France) and put in shipping boxes containing ice. Their plasma was separated immediately in 30 min by centrifuging at +4°C for 15 min at 1600*g*. The obtained plasma was stored at −20°C. Plasma homocysteine was determined via High Performance Liquid Chromatography (HPLC) method by processing the samples on a instrument called Merck–Hitachi L-6200A.

Genetic analysis

DNA required for genetic analyses of patients' MTHFR C677T polymorphisms were obtained from peripheral leukocytes. While DNA extraction was performed, 2 cc of blood samples from per patient were collected into tubes containing EDTA following an overnight fasting. DNA was obtained from peripheral leukocytes with filtered colon system by using Puergene DNA extraction kit (Gentra, Minneapolis, USA) [20]. Obtained DNA concentrations were checked spectrophotometrically at 206 nm. After obtaining of DNA extracts were verified and they were stored at −20°C until all genetic analyses were done at once. The presence of MTHFR C677T mutation was checked via an instrument called LigthCycler (Roche Diagnostics, Mannheim, Germany). Polymorphism was studied with standard Real-Time online PCR protocol. To detect the mutation at MTHFR 677 point, as Forward primer; 5-CGAAGCA GGGAGCTTTGAGGCTG-3, as Reverse primer; 5-AGG ACGGTGCGGTGAGAGAGTG-3, and Fluoresceinprobe 5-TGACCTGAAGCACTTGAAG GAG AAGGTGTC-Fl-3, LCRed640probe:5 LCRed640-CGGG AGCCGATTTCA TCAT-P-3 probes were used. The reactions necessary for polymorphism screening were performed according to Charalampos Aslandis and Gerd Schmitz (Institute for Clinical Chemistry and Laboratory Medicine, University of Regensburg, Regensburg, Germany) protocol in LightCycler Instrument (Roche Applied Science; Mannheim, Germany) with forward primer, reverse primer, Fluoresceinprobe, LCRed640probe, and LightCycler DNA Master Hybridization Probes Kits (Roche Applied Science, Mannheim, Germany). Polymorphic alleles were identified by the specific melting temperature (T_m) of the resulting amplicons. In the analyses done in the consequence of the reactions, copies which carry C/C on both alleles at 677th point specifically gave one melting peak at 61.8°C. Copies which carry both alleles (C/T) at 677th point showed two melting peaks at 61.8 and 53.2°C, respectively.

Statistical analysis

For the comparison of interval data of the groups which both have and do not have the mutation in the study, un-paired student *t*-test for the parameters that fulfill normal distribution and Mann–Whitney U test for the data that does not fulfill normal distribution were used. For the comparison of nominal values between both groups, Chi-Square (Fisher's Exact Test result) test was used. For correlation analyses, bivariate correlation and multivariate regression analyses were used. Data was expressed as mean ± standard deviation and values below $P < 0.05$ were accepted as statically significant. Statistical calculations were done on the computer by using the software called SPSS 10.0.

Results

Data about general characteristics, blood pressures, lipid profiles, metabolic parameters, HOMA-IR, renal functions, vitamin B12, folic acid, creatinine clearance, and plasma homocysteine levels of 52 type 2 diabetic patents (32 female, 20 male; mean age: 52.7 ± 9.9 year) is represented in Table 1. Microvascular complications were identified in 37 (72%) of our type 2 diabetic patients (Table 1). MTHFR C677T polymorphism screening was performed in all patients. Heterozygote MTHFR C677T mutation was identified in 24 (46%) of the patients. No homozygote MTHFR T677T mutation was identified in none of the patients in which polymorphism was screened. After polymorphism screening, the patients were divided into two groups as having and not having heterozygote MTHFR C677T gene mutation, the groups were named as Group CT ($n = 24$, 46%) and Group CC ($n = 28$, 54%), respectively. First of all, demographic characteristics consisting of gender, age, diabetes duration, BMI, hypertension treatment, blood pressure values, metabolic parameters, lipid parameters, vitamin B12, folic acid, plasma creatinine levels, and studied parameters were compared between these two groups (Table 2). Both groups were found similar in terms of demographic and general features (Table 2).

Plasma homocysteine (12.9 ± 1.7 and 8.9 ± 1.9 μmol/l, $P < 0.0001$, Group CT and CC, respectively) (Table 3; Fig. 1) and microalbuminuria level (73.40 ± 98.2 and 19.37 ± 28.4 mg/24 h, $P = 0.021$, Group CT and CC, respectively) in patients with MTHFR C677T polymorphism was found significantly high compared to the group which does not have the mutation (Table 3). Although creatinine clearance was in normal ranges in both groups, it

Table 1 Characteristics of the type 2 diabetic patients

Women (*n*) (%)	32 (62%)
Men (*n*) (%)	20 (38%)
Age (Year)	52.7 ± 9.9
Duration of diabetes (Year)	7.6 ± 6.2
BMI[#] (kg/m^2)	30.6 ± 4.9
Microvascular complication (+)	37 (72%)
Retinopathy (*n*) (%)	25 (45%)
Nephropathy (*n*) (%)	22 (43%)
Neuropathy (*n*) (%)	36 (70%)
Hypertension treatment (*n*) (%)	18 (35%)
Systolic blood pressure (mmHg)	128.2 ± 19.4
Diastolic blood pressure (mmHg)	84.8 ± 10.3
Microalbuminuria (mg/24 h)	43.5 ± 73.1
Glucose (mg/dl)	153.8 ± 50.8
HbA1c (%)	7.4 ± 2.0
Basal Insulin (μIU/ml)	17.4 ± 14.7
HOMA-IR*	4.3 ± 2.8
Total cholesterol (mg/dl)	179.8 ± 40.6
HDL-cholesterol (mg/dl)	48.6 ± 10.9
LDL-cholesterol (mg/dl)	127.5 ± 34.9
Triglyceride (mg/dl)	123.2 ± 34.3
ApoA1 (mg/dl)	134.3 ± 22.0
ApoB100 (mg/dl)	90.4 ± 21.5
Lipoprotein (a) (mg/dl)	26.1 ± 25.7
Plasma homocysteine (μmol/l)	10.8 ± 2.7
Vitamin B12 (pg/ml)	420.3 ± 206.6
Folic acid (ng/ml)	9.5 ± 3.3
Serum creatinine (mg/dl)	0.9 ± 0.3
Creatinine clearance (ml/min)	118.7 ± 52.6

n Number of individuals

[#] Body mass index

* *HOMA-IR* Homeostasis model assessment insulin resistance

Table 2 Comparison of general characteristics, metabolic parameters, vitamin B12, folic acid, and plasma creatinine levels of type 2 diabetic patients according to MTHFR C677T gene mutation presence

	Group CT $n = 24$	Group CC $n = 28$	*P*
Women (*n*) (%)	15 (63%)	12 (43%)	NS*
Men (*n*) (%)	9 (37%)	16 (57%)	NS*
Age (Year)	55.2 ± 12.4	50.6 ± 7.0	NS
Duration of diabetes (Year)	7.5 ± 6.0	7.6 ± 6.5	NS
BMI** (kg/m^2)	29.5 ± 5.1	31.5 ± 4.9	NS
Systolic blood pressure (mmHg)	127.0 ± 18.1	129.6 ± 19.1	NS
Diastolic blood pressure (mmHg)	82.5 ± 9.7	86.5 ± 10.8	NS
Glucose (mg/dl)	147.3 ± 49.3	160.3 ± 52.6	NS
HbA1c (%)	7.03 ± 1.73	7.88 ± 2.29	NS
Basal ınsulin (μIU/ml)	17.4 ± 17.7	17.4 ± 13.2	NS
HOMA-IR[#]	4.04 ± 3.49	4.44 ± 2.42	NS
Total cholesterol (mg/dl)	176.4 ± 42.6	183.1 ± 39.2	NS
HDL-cholesterol (mg/dl)	50.1 ± 10.9	47.3 ± 11.1	NS
Triglyceride (mg/dl)	104.3 ± 19.2	120.8 ± 60.3	NS
LDL-cholesterol (mg/dl)	122.9 ± 35.2	120.6 ± 34.2	NS
ApoA1 (mg/dl)	133.4 ± 25.2	135.3 ± 18.6	NS
ApoB100 (mg/dl)	90.8 ± 22.3	89.8 ± 21.4	NS
Lipoprotein (a) (mg/dl)	30.9 ± 31.5	19.2 ± 12.1	NS
Vitamin B12 (pg/ml)	429.6 ± 219.2	410.6 ± 198.2	NS
Folic acid (ng/ml)	8.7 ± 2.6	10.4 ± 3.3	NS
Plasma creatinine (mg/dl)	0.9 ± 0.3	0.8 ± 0.2	NS

n Number of individuals

* Data were compared between groups by Chi-Square, Fisher's Exact tests

** Body mass index

[#] *HOMA-IR* Homeostasis model assessment insulin resistance

Table 3 Comparison of plasma homocysteine, microalbuminuria, and creatinine clearance levels of type 2 diabetic patients according to MTHFR C677T gene mutation presence

	Group CT $n = 24$	Group CC $n = 28$	P
Plasma homocysteine (μmol/l)	12.9 ± 1.7	8.9 ± 1.9	<0.0001
Microalbuminuria (mg/24 h)	73.40 ± 98.2	19.37 ± 28.4	0.021
Creatinine clearance (ml/min)	101.1 ± 42.6	136.2 ± 51.5	0.008

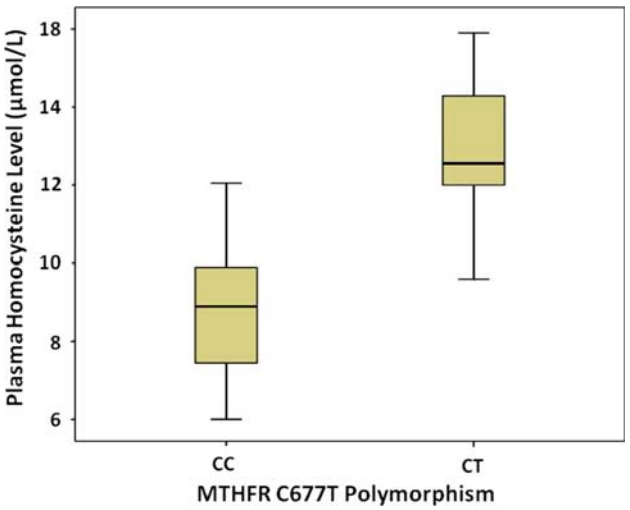

Fig. 1 Homocysteine levels of type 2 diabetic patients according to MTHFR C677T gene mutation presence

was found significantly low in the group having the mutation (101.1 ± 42.6 and 136.2 ± 51.5 ml/min, $P = 0.008$, Group CT and CC, respectively) compared to those which does not (Table 3). In order to evaluate whether MTHFR 677 CT heterozygote gene polymorphism is a novel risk factor for developing microvascular complications in type 2 diabetic patients, we assess complications with mutation frequency. While gene mutation was present in 15 (40.5%) of patients who have at least one microvascular complication, no mutation was observed in 22 (59.5%) of them and the difference between them was not statistically significant (Table 4). In subgroup evaluation of microvascular complications, we determined that the presence of the mutation was not a risk factor for developing retinopathy and neuropathy (Table 4). However, within the group having diabetic nephropathy ($n = 22$), MTHFR C677T gene mutation was present in 16 (73%) of the patients while no mutation was observed in 6 (27%) of them ($P = 0.017$) (Table 4). In correlation analysis that we performed in patient group having diabetic nephropathy with MTHFR C677T mutation, we determined that there was a positive and significant correlation between plasma homocysteine and microalbuminuria levels ($r = 0.54$; $P = 0.031$) (Fig. 2).

Table 4 Genotype distribution of the MTHFR C677T gene polymorphism in type 2 diabetes mellitus patients with and without diabetic microvascular complications, neuropathy, retinopathy, and nephropathy, respectively

Genotype	With microvascular complications ($n = 37$)	Without microvascular complications ($n = 15$)
CT*	15 (41%)	9 (60%)
CC**	22 (59%)	6 (40%)
	With diabetic neuropathy ($n = 36$)	Without diabetic neuropathy ($n = 16$)
CT	18 (50%)	6 (37.5%)
CC	18 (50%)	10 (62.5%)
	With diabetic retinopathy ($n = 25$)	Without diabetic retinopathy ($n = 27$)
CT	11 (44%)	13 (48%)
CC	14 (56%)	14 (52%)
	With diabetic nephropathy[#] ($n = 22$)	Without diabetic nephropathy ($n = 30$)
CT	16 (73%)	8 (27%)
CC	6 (27%)	22 (73%)

Data were compared between groups by Chi-Square, Fisher's Exact tests

n Number of individuals

* CT: C677T heterozygot mutation

** CC: C677C without mutation (Wild type)

[#] $P = 0.017$ (Chi-Square, Fisher's Exact test)

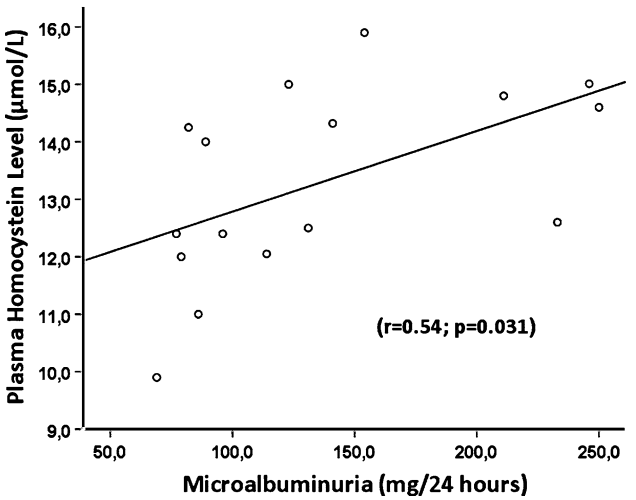

Fig. 2 There was a positive and significant correlation between plasma homocysteine and microalbuminuria levels ($r = 0.54$; $P = 0.031$) in patient group having diabetic nephropathy with MTHFR C677T mutation

Discussion

In our study, we evaluated whether 677 CT mutation on gene sequence coding MTHFR enzyme is a novel risk factor for developing microvascular complications in diabetic patients. MTHFR C677T mutation was determined with the ratio of 46% (24 of 52 diabetic patients) in our study group and it is parallel to conducted studies [21]. We did not determine any homozygote mutation in our study. The most probable reason for that number of our diabetic patients was few. As even in studies with large patient numbers, the ratio of homozygote mutation in diabetic patients varies between 2.4 and 16% [22].

MTHFR enzyme becomes thermolabile as a result of heterozygote mutation and enzyme activity slows down [12]. Also in vitamin B_{12} and folic acid deficiencies that are co-factors of that enzyme, its activity decreases further [12, 13]. Decrease in MTHFR enzyme activity causes an increase in plasma homocysteine level. However, plasma homocysteine level also increases due to environmental factors and drugs such as past macrovascular diseases, thyroid dysfunction, chronic renal disease and failure, vitamin B12 and folate deficiency, uncontrolled hypertension, alcoholism, methotrexate, phenytoin, carbapazemine, theophylline, cholestipol, and niacin use [17]. When we evaluated our diabetic patients by dividing them into two groups as those who have and do not have MTHFR 677 CT mutation, these factors which elevates homocysteine levels were similar in both groups. Despite the patient selection protocol in our study and the homogeneity of our diabetic patient groups which have and do not have heterozygote mutation, plasma homocysteine levels were determined specifically high in diabetic patients with heterozygote mutation (group CT). This result between two groups in which all hyperhomocysteinemia reasons are equal reliably shows that MTHFR 677 CT mutation causes an elevation in homocysteine levels. Since quite heterogeneous patient groups have been compared in most studies in which the relation between gene mutation and plasma homocysteine levels has been studied in diabetic patients [15, 16] we think that this outcome in our study will provide an additional value to the literature. When we evaluated the relation between microvascular complications and MTHFR gene mutation in our diabetic patients, at least one of the microvascular complications was present in 37 (61.5%) of 52 patients and we determined MTHFR gene mutation in 15 patients of this group. Although a difference was seen when compared to the patients without the mutation, it did not reach to a statistically significant difference.

When we evaluated microvascular complications separately, firstly, we did not determine a relation between diabetic neuropathy and heterozygote MTHFR 677 CT gene mutation. To the best of our knowledge, there is no study in the literature which evaluates and/or shows this relation. The first reason for that we were not able to determine this relation may be that neuropathy is the most frequently seen microvascular complication of diabetes and it is also observed frequently in those who do not have the mutation. Our second assumption is that rather than microarterial system disorders such as retinopathy and nephropathy, diabetic neuropathy complication is more affected by that factors such as hyperglycemia, hypoinsulinemia, dyslipidemia, activation of growth factor polyol pathway, and autoimmunity cause neural damage. Metabolic control was moderate (HbA1c = 7.4%) in our patients, nevertheless, neuropathy was determined in 70% of all the patients. The mutation was present in half of the patients with diabetic neuropathy. This result makes us think that neuropathy which we found is more affected by the above mentioned factors. Elevation of homocysteine level in our study might have triggered further microarterial endothelium damage and since this physiopathology has less effect on developing neuropathy, it was concluded to the assumption that neuropathy may be equally and more related to metabolic dysfunction. When we evaluated our patients with diabetic retinopathy, we did not determine any relation between retinopathy and MTHFR heterozygote mutation. However, when Kordonouri et al. [23] classified retinopathy symptoms of patients as without retinopathy, with background and proliferative retinopathy, while they did not find a difference in terms of mutation ration between MTHFR 677 CT gene mutation and patients without complications and with background retinopathy, they did find the mutation frequency higher in patients with proliferative retinopathy compared to healthy people. Only background retinopathy was determined in our patients with retinopathy. There was no proliferative retinopathy. The absence of advanced stage ($\geq$stage 4) diabetic nephropathy in our patients, their metabolic controls were moderate, and their blood pressures were normal ranges may be the reason for that we could not determine proliferative retinopathy in our patients. However, our results match up with the literature.

The most important and striking result of our study is that the presence of MTHFR 677 CT heterozygote gene mutation is related to diabetic nephropathy. This result is important since serum creatinine levels and creatinine clearance, blood pressures of our patents, the factors (vitamin B_{12} and folic acid) that may affect plasma homocysteine levels were in normal ranges. Since >70% of homocysteine metabolism occurs in renal epithelium, normal renal function is important in terms of showing hyperhomocysteinemia that may be caused by the mutation [24]. As there are studies in the literature which show [16] and do not show [15] that MTHFR 677 CT gene mutation contributes to developing diabetic nephropathy. When we

review all of these studies, we see that they are quite heterogeneous and do not give a clear result since they are different in terms of patient selection, diabetes duration, patient ages, nephropathy grades, and renal function tests and vitamin B_{12} and folic acid levels have not been evaluated. One of the most important results of our study is that microalbuminuria and plasma homocysteine levels were significantly high in our diabetic patients with MTHFR 677 CT heterozygote gene mutations compared to those who do not have the mutation. Also we determined a positive relation between plasma homocysteine level and microalbuminuria amount. Since the reasons causing hyperhomocysteinemia were equal in both of our diabetic patient groups with and without the mutation, elevation in homocysteine level determined in the group which has the mutation is clearly due to MTHFR 677 CT mutation.

Diabetic nephropathy most likely is the result of complex genetic and epigenetic factors of which a given polymorphism of the MTHFR gene may be only one component of this disorder. There are some limitations on the current study. First, the most important study weakness is the small size the complex genetic and epigenetic issues in diabetic nephropathy and the failure to use a genome wide search. The sample size is small and so this study can be classified as a pilot project rather than a convincing study. Second, the finding that there is no association between this polymorphism and diabetic neuropathy and diabetic retinopathy may represent a type II error that more patients need to be evaluated. It is possible that patients with impaired renal functions may have hyperhomocysteinemia and microalbuminuria might be a marker of this phenomenon. However, our patient group does not contain stage 4 or 5 nephropathic patients thus hyperhomocysteinemia as a result of impaired renal function do not seem very probable in our patient group.

As a result, in our study plasma homocysteine level was found high in type 2 diabetic patients independently of age, diabetes duration, glysemic control, metabolic parameters, blood pressures variables in the presence of MTHFR 677 CT gene mutation compared to the group which does not have the mutation. Elevated homocysteine level in type 2 diabetic patients with heterozygote mutation shows a linear positive relation with microalbuminuria amount. It is not determined that MTHFR 677 CT heterozygote mutation has any contribution to neuropathy and background retinopathy development. However, further studies are needed by increasing the number of cases.

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