Methylenetetrahydrofolate reductase C677T gene polymorphism and diabetic nephropathy susceptibility in patients with type 2 diabetes mellitus

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Background Type 2 diabetes mellitus (T2DM) is becoming increasingly prevalent throughout the world. Diabetic nephropathy (DN) is one of the most serious microvascular complications of diabetes mellitus. The C677T polymorphism of the methylenetetrahydrofolate reductase (MTHFR) gene has been reported to cause reduced MTHFR enzyme activity and impaired homocysteine metabolism, leading to hyperhomocysteinemia.

Aim The aim of the study was to evaluate the role of MTHFR C677T gene polymorphism in the susceptibility to DN in type 2 diabetic patients.

Patients and methods The study was conducted on 180 adult Egyptian participants (60 healthy controls, 60 patients with T2DM without nephropathy, and 60 patients with T2DM complicated with nephropathy). C677T genotypes were determined by PCR-RFLP analysis, and homocysteine levels were measured by enzyme-linked immunosorbent assay.

Results The prevalence of polymorphic genotype of CT and TT and T allele was statistically significantly increased in diabetic patients than in controls ($P<0.001$). There was a statistically significant increase in polymorphic genotypes (CT and TT) and T allele in T2DM with nephropathy in comparison to T2DM without nephropathy group ($P<0.001$, 0.05, respectively). Serum homocysteine levels were significantly higher in patients with nephropathy than in patients without nephropathy or controls with *P* less than 0.001. The higher serum homocysteine level was observed with polymorphic genotypes TT and CT compared with CC genotypes ($P<0.001$).

Conclusion The TT genotype and T allele of MTHFR C677T may represent a significant genetic molecular marker to predict the risk of DN in T2DM.

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Introduction

Type 2 diabetes mellitus (T2DM) is becoming increasingly prevalent throughout the world. In 2015, there were an estimated 415 million cases of diabetes mellitus (DM) worldwide, and that the number is expected to rise to 642 million cases by 2040 according to the 7th edition of the International Diabetes Federation Diabetes Atlas [1]. The International Diabetes Federation listed Egypt to be among the world top 10 countries in the number of patients with diabetes [2].

T2DM has a complex etiology which involves numerous environmental risk factors and potentially some genetic risk factors [3].

To diagnose diabetes there are four options according to the American Diabetes Association [4] that include:

(1) Fasting plasma glucose greater than or equal to 126 mg/dl. Fasting is defined as no caloric intake for at least 8 h.
(2) 2-h plasma glucose greater than or equal to 200 mg/dl during an oral glucose tolerance test. The test should be performed as described by the WHO, using a glucose load containing 75 g anhydrous glucose dissolved in water.
(3) Glycated hemoglobin (HbA1C) greater than or equal to 6.5%. The test should be performed in a laboratory using a method that is the National Glycohemoglobin Standardization Program and standardized to the Diabetes Control and Complications Trial reference assay (DCCT).
(4) In individuals with classic symptoms of hyperglycemia or hyperglycemic crisis, a random plasma glucose greater than or equal to 200 mg/dl.

Diabetic nephropathy (DN) is one of the most serious microvascular complications of DM, and is the primary cause of end-stage renal disease worldwide [5].

In recent years, genomic susceptibility to diseases has attracted a growing attention to research the genetic

polymorphisms which have a role in the pathogenesis of diseases. One of these genes that might be associated with type 2 diabetic nephropathy is methylenetetrahydrofolate reductase (MTHFR) gene [6]. The *MTHFR* gene is located on chromosome 1p36.3 and encodes an enzyme MTHFR [7]. MTHFR enzyme is one of the key enzymes in folic acid and homocysteine metabolism [8]. MTHFR enzyme uses the coenzyme flavin adenine dinucleotide and NADPH to convert 5,10-methylenetetrahydrofolate (CH_2-H_4folate) to 5-methyltetrahydrofolate (CH_3-H_4folate) [9]. The 5-methyltetrahydrofolate serves as methyl donor for remethylation of homocysteine to methionine and its dysfunction can lead to hyperhomocysteinemia (HHcy) [8]. This reaction is catalyzed by the methionine synthase enzyme, which requires vitamin B12 (methylcobalamin) as a cofactor [10]. Homocysteine is a sulfur-containing amino acid [11] resulting from the removal of the methyl group of methionine and discovered by the American biochemist Vincent Du Vigneaud in 1932 [12]. HHcy is observed in more than 85% of patients with renal disease [13]. HHcy has been implicated as a risk factor for nephropathy in type 2 diabetes patients [5]. HHcy acts by inducing the expression of tissue factor, an initiator of blood coagulation [14]. HHcy may also act by altering endothelial cell function through upregulation of the expression and secretion of monocyte chemoattractant protein-1 and interleukin-8, which by promoting leukocyte recruitment, may contribute to the initiation and progression of vascular disease [15]. Also, the effects of homocysteine include the promotion of platelet activation, hypercoagulability, oxidative stress, induction of endothelial barrier dysfunction, activation of Factor XII and Factor V, inhibition of surface expression of thrombomodulin and protein C activation, inhibition of von Willebrand factor processing and secretion, increasing release of proaggregation factor thromboxane A2 by altering arachidonic acid metabolism in platelets [16]. Furthermore, the increased production of oxidized low-density lipoproteins (ox-LDL) by homocysteine and the enhanced uptake of ox-LDL by macrophages may lead to the formation of foam cells that play a crucial role in atherosclerotic lesions [17]. Thus, HHcy which promotes the atherosclerotic and thrombotic process by modulating vascular cell proliferation and promoting prothrombotic activities in the vascular wall may contribute to the formation of glomerular lesions [16].

The MTHFR gene is over 20 kilo base (kb) in size. It consists of 13 exons, each of which is 102–432 base pair (bp) in size. The gene structure of the 11 exons was discovered by Goyettte *et al.* [18]. Homberger *et al.* [19] later identified two additional exon sites.

An analysis of the complete genomic structure showed that both alternative initiation and alternative splicing occur in the gene [19]. Such a discovery gives an explanation for previously reported Western blot results, which indicated that human MTHFR can be found in two forms: a 77 kilo dalton (kDa) polypeptide in most tissues and a 70 kDa polypeptide in the liver [20]. The MTHFR C677T single-nucleotide polymorphism (SNP) is a point mutation that involves substitution of cytosine to thymine at nucleotide position 677 in exon 4 in the N-terminal catalytic domain of the enzyme leading to conversion of an alanine residue to a valine (ALA222VAL) and is a common gene variant of MTHFR that includes CC, CT, and TT genotypes and C and T alleles [21,22].

This mutation decreases enzyme activity, such that the activity in individuals with CT and TT genotypes is ~65 and 35%, respectively, in comparison to individuals with the wild type CC genotype [3].

Patients and methods

The present study was done during the period from September 2016 till March 2017. Samples were collected from Al-Zahraa University Hospital (Department of Internal Medicine) and investigations were carried out at the Medical Biochemistry Department, Faculty of Medicine for Girls Al-Azhar University. The protocol of this study was approved by the medical ethics committee in the faculty and informed consent was obtained from all patients and healthy controls. This study was performed on 120 diabetic patients and 60 normal individuals as a control. The participants participating in this study were divided into three groups: control group (included 60 apparently healthy volunteers with no family history of DM, 29 men and 31 women with a mean age of 53.93±7 years, 60 patients suffering from T2DM without nephropathy (T2DM without nephropathy), 29 men and 31 women with a mean age of 53. 36±6.42 years, 60 patients suffering from T2DM complicated with DN (T2DM with nephropathy), 30 men and 30 women with a mean age of 53.73±5.66 years. Patients with T2DM were confirmed according to the criteria from American Diabetes Association [4]. Nephropathy in diabetic patients was determined by the protein in urine in 24 h greater than 150 mg/24 h [23].

Dermographic details of all participants were recorded which included age, sex, family history of diabetes, history of hypertension (HTN), retinopathy, and neuropathy.

The patients and controls were subjected to full history taking, thorough clinical examination with special stress on the measurement of blood pressure and calculation of BMI and fundus examination.Patients with a history of T2DM, tumors, other endocrine diseases, liver diseases, intake of folate, vitamin B6, and vitamin B12 were excluded from the study.

Specimen collection

Morning venous blood samples (8 ml) after 8 h fasting were withdrawn under complete aseptic conditions from all participants. Three milliliter of blood was collected on sterile 'EDTA' (vacutainer) and was used for molecular testing of the MTHFR gene by lymphocyte separation and extraction of DNA for PCR. DNA was extracted and stored at −20°C till the time of assay. Two milliliter was collected on sterile 'EDTA' (vacutainer) for the measurement of HbA1C. The rest was collected in a plain vacutainer and the obtained serum was used for the estimation of serum homocysteine, fasting serum glucose, serum urea, and serum creatinine. Another venous blood sample (2 ml) after 12 h fasting was withdrawn, collected in a plain vacutainer, and the obtained serum was used for the estimation of lipid profile [total cholesterol, triglyceride, high-density lipoprotein (HDL), and LDL].

Another venous blood sample (3 ml) was withdrawn after 2 h from breakfast, on a fluoride vacutainer for the measurement of 2 h postprandial serum glucose.

A 24 h urine sample was collected for t measurement of protein in urine in 24 h.

Methods

Determination of serum glucose level (fasting and postprandial) [23], serum lipid profile including total cholesterol [24], triglyceride [25], HDL [26], LDL [27], HbA1c [28], serum urea [29], serum creatinine [30], protein in urine in 24 h [23], estimation of serum homocysteine by enzyme-linked immunosorbent assay measured by a commercially available enzyme-linked immunosorbent assay kit supplied by Glory Science Co. Ltd (206 Building 6, Chenguang Gardon, Qianjin Street Changchun 130012, China) [31] and genomic DNA extraction from blood leukocytes were analyzed for C677T MTHFR gene polymorphism using the PCR-restriction fragment length polymorphism technique:

(1) Genomic DNA analysis was done for the determination of MTHFR genotype by detecting SNP at position C677T in the coding region of the MTHFR gene using the PCR technique and restriction fragment length polymorphism. The test was done in five main steps:

(a) Extraction of genomic DNA from peripheral blood leukocytes of EDTA anticoagulated blood including:
   (i) Lymphocyte separation.
   (ii) DNA extraction: DNA was extracted using Whole Blood Genomic DNA Purification Miniprep Kit #D3024 (Zymo Research).

(b) Amplification of the extracted DNA (PCR) by primers for C677T MTHFR: the MTHFR C677T genotype was determined by PCR according to the protocol published by Frosst *et al.*[20]: amplification was performed using Taq Red PCR Master Mix (#R0411604; Bio Line Life Sciences, South McDowell Blvd, Petaluma, USA):

(c) Primer sequence:
   (i) Forward primer: 5′-TGAAGGAGAAG GTGTCTGCGGGA-3′.
   (ii) Reverse primer: 5′-AGGACGGTGCG GTGAGAGTG-3′.
   The amplified fragment was 198 bp.
   The computerized thermocycler (Techne Progene, England) was programmed for the following conditions:
   An initial cycle of 95°C for 1 min (for initial denaturation). Thirty-four cycles under the following conditions: denaturation at 95°C for 15 s, annealing at 58°C for 15 s, and extension at 72°C for 10 s with a final extension cycle of 72°C for 7 min.

(d) Detection of PCR amplification products using 2% agarose gel electrophoresis stained by ethidium bromide and ultraviolet (UV) light transillumination as bands at 198 bp:
   (i) The first lane is: a 25 bp ladder (marker).
   (ii) Lanes 2–12: a one band 198 bp PCR product before the action of restriction enzyme Hinf1, performed on 2% agarose, being visualized by UV transillumination.

(e) C677T MTHFR gene polymorphism was identified using specific restriction enzyme: the amplified products were digested with Hinf 1 restriction endonulease enzyme (Catalog # 0411604; Biolab Inc.), and then the digested products were separated by 3% agarose gel electrophoresis stained with ethidium bromide and visualized using a UV transilluminator, where the homozygote (CC) produced one band (198 bp long), the

homozygote (TT) produced two bands (175, 23 bp long), and the heterozygote (CT) produced three bands (198, 75, 23 bp long):

(i) The first lane is 25 bp ladder (marker).
(ii) CC genotype (wild type): lanes 11, 12 (one band 198 bp).
(iii) CT genotype (heterozygous): lanes 3, 4, 6, 7, 8, and 9 (there were three bands 198, 175, and 23 bp).
(iv) TT genotype (homozygous): lanes 2, 5, and 10 (there were two bands 175 and 23 bp).

23 band not seen because the DNA ladder is 25 bp.

Statistical methods

Statistical analysis of the results was done using the Statistical Package for the Social Sciences (SPSS; SPSS Inc., Chicago, Illinois, USA) version 20.0. The differences between groups were analyzed with one-way analysis of variance test. Unpaired Student's *t*-test was used to compare between two groups in quantitative data. Linear correlation coefficient was used for the detection of correlation between two quantitative variables in one group. The χ^2-test is used to determine whether there is a significant difference between the expected frequencies and the observed frequencies in one or more categories. *P* values less than or equal to 0.05 were considered statistically significant [32].

Results

Regarding clinical data of the studied groups, our study showed that T2DM without nephropathy, T2DM with nephropathy, and control groups did not show statistically significant difference regarding age and sex (P_1=0.396 and 0.978, respectively) (Table 1). But they were statistically significantly different regarding BMI, association of HTN, retinopathy, and neuropathy (P_1<0.001).

Table 2 shows a comparison between the duration of diabetes (years) in T2DM without nephropathy and T2DM with nephropathy groups. There was no statistically significant difference between the two groups (*P*=0.940) (Table 3).

Table 1 Clinical data of the studied groups

Items	Groups						
	Control (*N*=60) [*n* (%)]	T2DM without nephropathy (*N*=60) [*n* (%)]	T2DM with nephropathy (*N*=60) [*n* (%)]	P_1	P_2	P_3	P_4
Age (years)							
Mean ±SD	53.93±7	53.36±6.42	53.73±5.66	0.396	0.981	0.391	0.500
Sex							
Male	29 (48.3)	29 (48.3)	30 (50.0)	0.978	1.000	0.855	0.855
Female	31 (51.7)	31 (51.7)	30 (50.0)				
BMI (kg/m^2)							
Mean ±SD	24.06±1.32	30.32±3.01	28.89±2.87	<0.001**	<0.001**	<0.001**	0.005*
Hypertension							
Positive	0 (0.0)	23 (38.3)	27 (45.0)	<0.001**	<0.001**	<0.001**	0.459
Negative	60 (100.0)	37 (61.7)	33 (55.0)				
Retinopathy							
Positive	0 (0.0)	12 (20.0)	13 (21.7)	<0.001**	<0.001**	<0.001**	0.822
Negative	60 (100.0)	48 (80.0)	47 (78.3)				
Neuropathy							
Positive	0 (0.0)	17 (28.3)	26 (43.3)	<0.001**	<0.001**	<0.001**	0.087
Negative	60 (100.0)	43 (71.7)	34 (56.7)				

P_1, between three groups; P_2, control versus T2DM without nephropathy; P_3, control versus T2DM with nephropathy; P_4, T2DM without nephropathy versus T2DM with nephropathy; T2DM, type 2 diabetes mellitus. *P*>0.05, nonsignificant. *$P \leq 0.05$, significant. **$P \leq 0.001$, highly significant.

Table 2 Comparison between the duration of diabetes mellitus in type 2 diabetes mellitus without nephropathy group and type 2 diabetes mellitus with nephropathy group

Groups	Diabetes duration (mean±SD) (years)	*P* value
T2DM without nephropathy	11.25±4.25	0.940
T2DM with nephropathy	11.53±4.51	

T2DM, type 2 diabetes mellitus. *P*>0.05, nonsignificant. *$P \leq 0.05$, significant.

Table 3 The laboratory data of the studied groups

Items	Groups						
	Control (*N*=60) (mean ±SD)	T2DM without nephropathy (*N*=60) (mean±SD)	T2DM with nephropathy (*N*=60) (mean±SD)	P_1	P_2	P_3	P_4
Fasting serum glucose (mg/dl)	89.18±9.85	182.27±25.56	164.97±26.61	<0.001**	<0.001**	<0.001**	<0.001**
2 h postprandial serum glucose (mg/dl)	117.95±8.98	277.37±32.07	278.90±37.18	<0.001**	<0.001**	<0.001**	0.954
HbA1C (%)	4.61±0.43	9.42±1.05	9.75±0.83	<0.001**	<0.001**	<0.001**	0.067
Triglyceride (mg/dl)	127.12±12.17	215.93±23.49	242.13±21.90	<0.001**	<0.001**	<0.001**	<0.001**
Cholesterol (mg/dl)	141.93±11.21	229.62±23.32	248.22±28.40	<0.001**	<0.001**	<0.001**	<0.001**
HDL (mg/dl)	63.50±6.41	44.83±6.17	43.82±4.99	<0.001**	<0.001**	<0.001**	0.611
LDL (mg/dl)	53.14±12.29	141.62±22.61	156.03±27.93	<0.001**	<0.001**	<0.001**	<0.001**
Urea (mg/dl)	27.40±4.44	25.42±5.54	120.62±26.25	<0.001**	0.768	<0.001**	<0.001**
Creatinine (mg/dl)	0.82±0.11	0.81±0.08	3.76±1.19	<0.001**	0.996	<0.001**	<0.001**
Protein in urine in 24 h (mg/24 h)	20.58±3.37	19.20±4.21	843.33±65.30	<0.001**	0.978	<0.001**	<0.001**
Homocysteine (μmol/l)	1.86±3.36	9.93±6.50	13.24±5.46	<0.001**	<0.001**	<0.001**	0.002*

HbA1C, glycated hemoglobin; HDL, high-density lipoprotein; LDL, low-density lipoprotein; T2DM, type 2 diabetes mellitus. *Means significant. **Means highly significant.

Table 4 The correlation between serum homocysteine (μmol/l) and clinicopathological data of all patients

Variables	Homocysteine (μmol/l)	
	r	*P* value
Age (years)	–0.013	0.890
Diabetes duration (years)	–0.095	0.300
BMI (kg/m^2)	0.037	0.687
Fasting serum glucose (mg/dl)	0.020	0.831
2 h postprandial serum glucose (mg/dl)	0.000	0.997
HbA1C (%)	–0.036	0.698
Urea (mg/dl)	0.271	0.003*
Creatinine (mg/dl)	0.249	0.003*
Triglyceride (mg/dl)	0.213	0.020*
Cholesterol (mg/dl)	0.197	0.031*
HDL (mg/dl)	0.150	0.103
LDL (mg/dl	0.122	0.183
Protein in urine in 24 h (mg/24 h)	0.259	0.004*

HbA1C, glycated hemoglobin; HDL, high-density lipoprotein; LDL, low-density lipoprotein. Correlation is significant at *P* value less than or equal to 0.05 and nonsignificant at *P* value greater than 0.05.

Table 5 Methylenetetrahydrofolate reductase C677T genotypic and allelic frequencies in controls and all diabetic patients

Variables	Groups [*n* (%)]		*P* value
	Control	Diabetic patients	
Genotype	*N*=60	*N*=120	
CC	54 (90)	30 (25)	<0.001**
CT	6 (10)	51 (42.5)	
TT	0 (0)	39 (32.5)	
Alleles	*N*=120	*N*=240	
C	114 (95)	111 (46.3)	<0.001**
T	6 (5)	129 (53.8)	

**Means highly significant.

As regards laboratory data of the studied groups, our study showed that T2DM without nephropathy, T2DM with nephropathy, and control groups show a statistically significant difference regarding their fasting serum glucose, 2 h postprandial serum glucose, HbA1C, triglyceride, cholesterol, HDL, LDL, urea, creatinine, protein in urine in 24 h, and homocysteine (P_1<0.001) (Table 4).

There was a statistically significant positive correlation between serum homocysteine level and urea, creatinine, triglyceride, cholesterol, and protein in urine in 24 h *r*=0.271, *P*=0.003; *r*=0.249, *P*=0.003; *r*=0.213, *P*=0.020; *r*=0.197, *P*=0.031; and *r*=0.259, *P*=0.004, respectively. There was no statistical correlation between serum homocysteine level and other variables (age, diabetes duration, BMI, fasting serum glucose, 2 h postprandial serum glucose, HbA1C, HDL, and LDL) (Table 5).

This study showed a statistically significant difference comparing the CC genotype (wild genotype) versus CT and TT genotypes (polymorphic genotypes) in control and diabetic patient groups (*P*<0.001). A statistically significant difference was observed on comparing between the frequency of C allele and T allele in both groups (*P*<0.001) (Table 6).

The results of this study showed that a statistically significant increase in the frequency of CT and TT polymorphic genotypes versus CC genotype (wild type) was observed among T2DM without nephropathy and

Table 6 Methylenetetrahydrofolate reductase C677T genotypic and allelic frequencies in the studied groups

Variables	Groups [*n* (%)]			*P* value
	Control (*N*=60)	T2DM without nephropathy (*N*=60)	T2DM with nephropathy (*N*=60)	
Genotype (*N*=60)				
CC	54 (90.0)	21 (35.0)	9 (15)	$P_1<0.001$** $P_2<0.001$** $P_3<0.001$** $P_4<0.001$**
CT	6 (10)	21 (35.0)	30 (50)	
TT	0 (0)	18 (30.0)	21 (35)	
Allele (*N*=120)				
C	114 (95)	63 (52.5)	48 (40)	$P_1<0.001$** $P_2<0.001$** $P_3<0.001$** $P_4=0.05$
T	6 (5)	57 (47.5)	72 (60)	

T2DM, type 2 diabetes mellitus. **Means highly significant.

Table 7 Comparison between methylenetetrahydrofolate reductase C677T genotypes as regards clinical data of the studied groups and its *P* value

Items	MTHFR C677T gene polymorphism [*n* (%)]			*P* value
	CC (*n*=84)	CT (*n*=57)	TT (*n*=39)	
Age (years)				
Mean±SD	52.87±6.69	53.46±6.24	53.67±6.39	0.515
Sex				
Female	41 (48.8)	30 (52.6)	21 (53.8)	0.840
Male	43 (51.2)	27 (47.4)	18 (46.2)	
BMI (kg/m^2)				
Mean±SD	26.46±3.78	28.79±3.54	29.11±2.37	<0.001**
Family history of diabetes				
Positive	10 (11.9)	10 (17.5)	16 (41)	0.016*
Negative	74 (88.1)	47 (82.5)	23 (59)	
Hypertension				
Positive	13 (15.5)	20 (35.1)	17 (43.6)	0.002*
Negative	71 (84.5)	37 (64.9)	22 (56.4)	
Retinopathy				
Positive	3 (3.6)	11 (19.3)	11 (28.2)	<0.001**
Negative	81 (96.4)	46 (80.7)	28 (71.8)	
Neuropathy				
Positive	8 (9.5)	18 (31.6)	17 (43.6)	<0.001**
Negative	76 (90.5)	39 (68.4)	22 (56.4)	

MTHFR, methylenetetrahydrofolate reductase. *Means significant. **Means highly significant.

T2DM with nephropathy patients when compared with healthy controls (P_2, $P_3<0.001$), respectively. Also on comparing the frequency of polymorphic genotypes (CT and TT) and wild genotype (CC) in T2DM without nephropathy and T2DM with nephropathy groups, there was a statistically significant difference ($P_4<0.001$). On comparing the three groups to each other there were statistical significance differences ($P_1<0.001$).

Also this study showed a statistically significant difference on comparing C allele versus T allele between three groups, between control and T2DM without nephropathy, between control and T2DM with nephropathy, and between two diabetic groups (P_1, P_2, $P_3<0.001$, $P_4=0.05$) (Table 7).

There was statistically significant difference on comparing between different MTHFR genotypes (CC, CT, and TT) with the clinical data of the studied groups (BMI, family history of diabetes, HTN, retinopathy, and neuropathy) ($P<0.001$). But they were no statistically significant difference regarding their age and sex ($P=0.515$, 0.840, respectively) (Table 8).

Also, on comparing MTHFR genotypes with the laboratory findings of the studied groups (fasting serum glucose, 2 h postprandial serum glucose, HbA1C, triglyceride, cholesterol, HDL, LDL, urea, creatinine, protein in urine in 24 h, and homocysteine) there was a statistically significant difference ($P<0.001$).

Discussion

Diabetes is a disorder in which the patients are at all the time at risk of complications [33]. In diabetes, the subsequent problems are grouped under 'microvascular complications' and 'macrovascular complications' [34].

Microvascular complications can lead to neuropathy, retinopathy, and nephropathy. Macrovascular complications can also lead to cardiovascular disease, mainly by accelerating atherosclerosis disorders, which include coronary artery disease, stroke, peripheral vascular disease, which contributes to intermittent claudication as well as diabetic foot [35].

DN is the leading cause of chronic renal disease in patients starting renal replacement therapy [36]. DN develops in about 40% of patients with diabetes. Furthermore, epidemiological and familial studies have demonstrated that genetic susceptibility

Table 8 Comparison between methylenetetrahydrofolate reductase C677T genotypes as regards laboratory findings of the studied groups, and its *P* value

Items	MTHFR C677T gene polymorphism (mean±SD)			*P* value
	CC (*N*=84)	CT (*N*=57)	TT (*N*=39)	
Fasting serum glucose (mg/dl)	121.51±46.74	164.05±37.79	175.18±35.00	<0.001**
2 h postprandial serum glucose (mg/dl)	176.76±81.22	259.16±60.73	277.77±35.84	<0.001**
HbA1C (%)	6.42±2.55	8.87±1.77	9.79±0.74	<0.001**
Urea (mg/dl)	36.48±29.66	75.99±51.73	77.18±52.36	<0.001**
Creatinine (mg/dl)	1.14±0.78	2.35±1.08	2.40±1.14	<0.001**
Triglyceride (mg/dl)	159.85±47.78	221.23±39.74	232.67±27.82	<0.001**
Cholesterol (mg/dl)	173.32±45.49	232.49±40.69	240.38±29.67	<0.001**
HDL (mg/dl)	55.85±12.41	46.50±7.32	44.66±6.00	<0.001**
LDL (mg/dl)	85.50±47.07	141.89±40.04	148.13±26.87	<0.001**
Protein in urine in 24 h (mg/24 h)	110.10±261.36	453.38±417.38	458.87±416.80	<0.001**
Homocysteine (μmol/l)	1.29±1.66	13.00±2.78	16.75±2.24	<0.001**

HbA1C, glycated hemoglobin; HDL, high-density lipoprotein; LDL, low-density lipoprotein; MTHFR, methylenetetrahydrofolate reductase.
**Means highly significant.

contributes to the development of DN in patients with both type I and type II diabetes [37]. Studies of candidate genes have been the most common approach to identify genes involved in susceptibility to DN [38].

A candidate gene implicated to play a major role in DN susceptibility is the MTHFR gene [22]. HHcy have been linked to endothelial dysfunction and arterial stiffness, insulin resistance, prothrombotic inflammation and hypercoagulability, macroangiopathy and nephropathy [3]. Polymorphisms that occur on the MTHFR gene coding MTHFR enzyme lead to HHcy by decreasing the enzyme activity. The best identified polymorphism is C677T which occurs by replacement of cytosine on the 677th position with thymine. This leads to a change in amino acid, deformity in the three-dimensional structure, and a reduction in the catalytic activity of the MTHFR enzyme [39].

In the present study, an association between an SNP at MTHFR C677T was found with diabetes and its DN complications. The polymorphic genotypes CT and TT were statistically significantly increased in diabetic patients compared with the control group ($P<0.001$). MTHFR 677 CC genotype was significantly higher in the controls compared with the diabetic patients, with decreased risk of T2DM; thus, it may have a protective role against susceptibility to T2DM. This agree with Mtiraoui *et al.* [14], who reported that the polymorphic genotypes CT and TT were statistically significantly increased in diabetic patients compared with the control group ($P<0.001$).

There were statistically significant increases in polymorphic genotypes (CT and TT) in T2DM without nephropathy and T2DM with nephropathy groups than the control group ($P_2<0.001$, $P_3<0.001$). Also there was a statistically significant decrease in polymorphic genotypes (CT and TT) in T2DM without nephropathy in comparison to T2DM with the nephropathy group ($P_4<0.001$). This may indicate that the T2DM without nephropathy patients are susceptible to develop DN later on in the course of the disease. The same results were found by Mtiraoui *et al.* [14], who reported that a statistically significant difference was seen when the C677T genotypes were compared in diabetic patients with nephropathy and diabetic patients without nephropathy and found that the CT and TT genotypes were more frequent in the DN group ($P<0.001$, 0.002, respectively). Wang *et al.* [6] agree with our results and reported that a statistically significant increase in genotypic distribution of MTHFR C677T polymorphism was observed among type 2 DN patients in comparison to the control group ($P=0.001$). Also AbdRaboh *et al.* [40] agree with our results and found that there was a statistically significant increase in genotypic distribution of MTHFR C677T polymorphism among type 2 diabetic patients compared with their control group ($P=0.032$). Settin *et al.* [41] agree with our results and found that cases with T2DM showed a significantly higher frequency of the mutant homozygous MTHFR 677TT genotype compared with the control group ($P=0.01$). On studying the alleles frequency of MTHFR C677T, T allele was higher in T2DM without nephropathy and T2DM with nephropathy groups compared with the control group (P_1, P_2, and $P_3<0.001$). Also T allele was higher in T2DM with nephropathy group compared with T2DM without nephropathy group ($P_4=0.05$). Also the T allele was higher in all T2DM patients compared with the control group ($P<0.001$). The same results were found by Mtiraoui *et al.* [14], who reported that the T allele was higher in T2DM patients compared with the control

group ($P<0.001$) and the T allele frequency was higher in diabetic patients with nephropathy compared with diabetic patients without nephropathy ($P<0.001$). Also in accordance with our results AbdRaboh *et al.* [40] found a statistically significant increase in T allele in T2DM patients compared with the control group ($P=0.013$). In the current study on comparing different MTHFR C677T genotypes as regards the clinical data of the studied groups we reported that there was a statistically significant increase in the mean value of BMI in TT genotype followed by the CT genotype when compared with the CC genotype ($P<0.001$). Also there was a statistically significant increase in the frequency of positive family history of diabetes in TT genotype followed by the CT genotype when compared with the CC genotype ($P=0.016$). There was a statistically significant increase in the frequency of HTN in the TT genotype followed by the CT genotype when compared with the CC genotype ($P=0.002$). There was a statistically significant increase in the frequency of retinopathy and neuropathy in TT genotype followed by the CT genotype when compared with the CC genotype ($P<0.001$). But no statistically significant difference was found on comparing genotypes as regards patient age and sex ($P=0.515$, 0.840, respectively). Also in the current study on comparing different MTHFR C677T genotypes as regards the laboratory findings of the studied groups we reported that there was a statistically significant difference in the mean value of fasting serum glucose, postprandial serum glucose, HbA1C, urea, creatinine, triglyceride, cholesterol, HDL, LDL, and protein in urine in 24 h when comparing CC, CT, and TT genotypes ($P<0.001$).

In this study, there was a statistically significant difference in serum homocysteine level on comparing the three groups to each other ($P_1<0.001$). Also there was a statistically significant difference on comparing T2DM without nephropathy and T2DM with nephropathy groups to the control group and in comparing T2DM without nephropathy group to the T2DM with the nephropathy group ($P_2<0.001$, $P_3<0.001$, and $P_4=0.002$).

The same finding was reported by Mtiraoui *et al.* [14], who found that there was a statistically significant difference in serum homocysteine level on comparing the three groups to each other and reported that there was a statistically significant difference on comparing T2DM without nephropathy and T2DM with nephropathy groups to the control group and in comparing T2DM without nephropathy group to T2DM with nephropathy group ($P<0.05$). We studied the association between different genotypes of MTHFR C677T and serum homocysteine level and we found that the higher serum homocysteine level was observed with polymorphic genotypes TT and CT compared with CC genotypes ($P<0.001$). This means that HHcy is related to DN and is associated with renal insufficiency. The C677T polymorphism of the MTHFR gene has been reported to cause reduced MTHFR enzyme activity and impaired homocysteine metabolism, leading to HHcy [40]. Mtiraoui *et al.* [14] go hand in hand with us and reported that plasma homocysteine levels were higher in 677TT carriers as opposed to CT or CC genotype carriers ($P<0.05$).

Conclusion

Our data has shown that there is a relationship between the MTHFR C677T genomic polymorphism, HHcy, and DN. The MTHFR C677T genomic polymorphism can influence the development of DN in patients with T2DM. So the TT genotype and T allele of MTHFR C677T may represent a significant genetic molecular marker for the risk of DN in patients with T2DM. The study of genotype frequency distribution for the SNP in MTHFR might serve as a major achievement in understanding some complex disease states as DM and its DN complication.

Recommendations

A study of other polymorphisms in the MTHFR gene and their relations to diabetes and its microvascular and macrovascular complications is suggested.

Also further researches are required in different ethnic populations, and on large numbers of patients to indicate its usefulness as a potential new genomic indicator and biomarker to screen populations for diabetes and its DN complications.

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Conflicts of interest

There are no conflicts of interest.

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