Original Article

Methylenetetrahydrofolate Reductase (Mthfr C677T) Gene Polymorphism Effect on Development of Diabetic Nephropathy in Arab Patients with Type 2 Diabetes Mellitus

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ABSTRACT

Background

Genetic susceptibility to diabetic nephropathy (DN) has been well-recognized. The enzyme methylenetetrahydrofolate reductase (MTHFR) plays a critical role in homocysteine metabolism. Variations in the MTHFR gene, particularly the C677T variant, have been implicated in the development of macrovascular and microvascular complications.

Objectives

This study investigates the relationship between the MTHFR C677T genotype and the occurrence of diabetic nephropathy in type 2 diabetes mellitus (T2DM) patients.

Methods

A total of 50 T2DM patients were analyzed for urinary albumin/creatinine ratio, which was used to classify them into two groups: 26 patients without nephropathy and 24 with nephropathy. Additionally, the study included 30 first-degree relatives (FDRs) of diabetic patients and 20 healthy controls. Diagnostic tests performed included fasting blood glucose, HbA1c, and serum creatinine levels. Plasma total homocysteine levels were measured using a chemiluminescent assay, and the MTHFR C677T polymorphism was analyzed using PCR-restriction fragment length polymorphism (RFLP).

Results

Among T2DM patients with nephropathy, the distribution of MTHFR genotypes (CC homozygous, CT heterozygous, and TT homozygous) was 20.8%, 54.2%, and 25%, respectively. The T allele frequency was significantly higher in patients with nephropathy (69.2%) compared to those without nephropathy (23.1%), normal controls (26.7%), and FDRs (30%). There was no significant difference in MTHFR genotype or allele frequency between T2DM patients without nephropathy, FDRs, and healthy controls ($p < 0.05$). The T allele showed a strong association with the development of diabetic nephropathy. Additionally, plasma homocysteine levels were significantly elevated in individuals with TT or CT genotypes compared to those with the CC genotype, with higher levels correlating with the progression of nephropathy.

Conclusion

The findings suggest that the C677T mutation in the MTHFR gene predisposes T2DM patients to diabetic nephropathy. The presence of the T allele may elevate plasma homocysteine levels, contributing to the progression of nephropathy toward end-stage renal failure. These results highlight the potential role of the MTHFR C677T variant as a genetic marker for susceptibility to diabetic nephropathy in T2DM patients.

Keywords

methylenetetrahydrofolate reductase (MTHFR); T2 Diabetes Mellitus; plasma homocysteine (Hcy)

INTRODUCTION

Diabetic nephropathy is characterized by a progressive increase in urinary albumin excretion, accompanied by rising blood pressure, declining glomerular filtration rates, and eventual end-stage kidney failure. It is the leading cause of end-stage renal failure and serves as an independent risk factor for cardiovascular disease. A significant proportion of patients undergoing kidney replacement therapy (KRT) for diabetic nephropathy have type 2 diabetes rather than type 1 diabetes [1-3].

The cumulative incidence of proteinuria in type 2 diabetic patients is comparable to that in type 1 patients, with multiple studies reporting similar rates of microalbuminuria and proteinuria development in both groups [4]. Globally, diabetic nephropathy is now the most common cause of entry into KRT programs [5].

Over the past decade, numerous studies have established a strong association between diabetic nephropathy and cardiovascular disease, with cardiovascular risk increasing alongside albuminuria in both type 1 and type

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2 diabetes. For example, in type 1 diabetes, patients with microalbuminuria face a 1.2-fold higher risk of cardiovascular death compared to normoalbuminuric patients, a risk that rises tenfold in those with proteinuria [6]. This elevated risk is only partially explained by traditional cardiovascular risk factors, suggesting a shared underlying pathology, potentially of genetic origin. While non-diabetic kidney disease also raises cardiovascular risk, the impact is significantly more pronounced in diabetic patients [6].

The exact cause of type 2 diabetes mellitus (T2DM) and its complications remains unclear, but recent research points to elevated plasma homocysteine (Hcy), a sulfur-containing amino acid involved in methionine metabolism, as a contributor to diabetes-related vascular complications [7]. Elevated Hcy levels in diabetes have been linked to insulin resistance [7], nephropathy [8], and increased risk of mortality or coronary events in T2DM patients [9]. However, findings on Hcy levels in diabetes are conflicting, with studies reporting increased, unchanged [10], or decreased [11] levels in T2DM patients.

The candidate gene approach has been widely used to identify genes associated with complex diseases. The 5,10-methylenetetrahydrofolate reductase (MTHFR) gene encodes a crucial enzyme in the folate metabolism pathway, converting dietary folate into 5-methyltetrahydrofolate, which donates a methyl group for remethylating of homocysteine to methionine [12]. A common mutation, C677T, results in a thermolabile enzyme variant with reduced activity [13], leading to elevated Hcy levels and associations with insulin resistance [14]. Elevated Hcy levels negatively impact endothelial and neuronal cells by generating reactive oxygen species (ROS), impairing beta-cell glucose metabolism, reducing insulin secretion, and promoting cell death [14,15].

The C677T polymorphism is considered a potential genetic susceptibility factor for diabetes due to its strong correlation with plasma Hcy levels in a dose-dependent manner [16]. Recent studies have examined the relationship between the MTHFR C677T polymorphism and T2DM or its complications, but inconsistent findings have hindered definitive conclusions [(16,17)]. Located on chromosome 1 (1p36.3), the MTHFR gene harbors several mutations, with C677T being the most studied [18]. This valine-to-alanine substitution at amino acid 226 produces a thermolabile enzyme with reduced activity [19], with homozygous TT individuals exhibiting significantly elevated plasma Hcy levels [20]. These elevated levels are associated with complications such as diabetic retinopathy [20] and diabetic nephropathy (DN) [20,21].

Moreover, an increased prevalence of CT and TT genotypes has been observed in male hemodialysis patients with T2DM, with a correlation between the C677T allele and the progression of renal failure [20].

This study aims to investigate the role of the MTHFR C677T polymorphism in susceptibility to diabetic nephropathy in patients with T2DM.

METHODS AND MATERIALS

For the present study blood samples from Arab Nationals with type 2 diabetes mellitus were obtained mainly from two sources in Dubai, UAE - the Dubai Specialized Medical and Research New Medical Centre – Dubai. All the cases were first examined by the clinician and later confirmed by proper laboratory investigations. Fasting and post prandial blood glucose values were obtained for establishing the diagnosis.

All the patients for type 2 diabetes for this study had fasting and post prandial blood glucose levels greater than 110 mg/dl and 140 mg/dl respectively. Patients who were undergoing insulin treatment and who had a history of hyperglycemic coma were confirmed to be type 1 diabetics. The type 1 cases were found to be positive for ketosis upon with drawl of insulin while never of type 2 diabetics showed ketosis. Samples were collected on every Mondays and Saturdays from the Endocrinology unit of New Medical Centre and Dubai Specialized Medical and Research Center (Dubai). The blood samples were collected in the duration of months from September 2018 - November 2018.The blood from the voluntary first-degree relatives was also collected. Control samples were collected from voluntary blood donors. The control subjects were healthy, age and sex matched and did not have family history of diabetes. All the type 2 diabetic patients, FDRs and control subjects were fasting at the time of blood collection.

The patient's group was classified according to presence of nephropathy into two groups 26 Without DN and 24 with DN as assessed by microalbuminuria in random urine samples. Microalbuminuria is diagnosed if albumin/creatinine ratio (ACR) ranges between 30 and

300 mg albumin/g creatinine. Random urine samples tested for microalbuminuria with measuring ACR. Albumin concentration in urine was measured using an immunoturbidometric assay on a Prospec nephlometry with Albumin-Urine kits (Dade Behring). Fasting venous blood samples were collected; serum was separated for measuring fasting glucose and creatinine, another vacutainer EDTA containing tubes were used for samples collection for homocysteine and HbA1C. Urine, serum creatinine and fasting glucose concentrations were measured on ADVIA 1650 analyzer (Siemens Medical Solutions Diagnostics), glycemic control was assessed by measuring HbA1c using column chromatography [*BioSystems, Middletown, CT, USA*].Homocysteine, was determined by chemiluminescent assay using commercial kits on Immulite , DPC US. A fasting homocysteine concentration above 15mmol/L is the most common definition of hyperhomocysteinemia [21].

Information on age, sex, height, weight, waist, hip, dietary habits, Physical activity was collected from all the subjects as these variables are known to have an impact on serum lipid levels and also on diabetes mellitus. Data on the age at onset of the disease was also collected.

Familial aggregation /clustering of risk factors are implicated in the etiology of diabetes mellitus. Hence details regarding the family history of the diseased condition were collected.

Type 2 diabetic patients and healthy control subjects were requested to fast overnight. Fasting blood samples (min 12-14 hrs) from all the participants were collected in two sterile vial one with anticoagulant for isolation of DNA and PCR analysis and another without anti-coagulant for the collection of serum.

The vial without anticoagulant was left for 1-2 hours at 37°C for the blood to clot. After the clot retraction was complete it was centrifuged for about 10 minutes and serum collected was subsequently used for lipid profile analysis. Samples showing heamolysis were discarded.

Genomic DNA was isolated from 1mL whole blood collected in EDTA anti- coagulated tubes using the Wizard genomic DNA purification kit [*Promega,* Madison, *USA*].

MTHFR C677T genotype analysis was performed by PCR-RFLP analysis using HinfI digestion for C677T. [22]

The primer sequences for C677T were:

Forward, 5´-TGA AGG AGA AGG TGT CTG GGG GA-3´, and

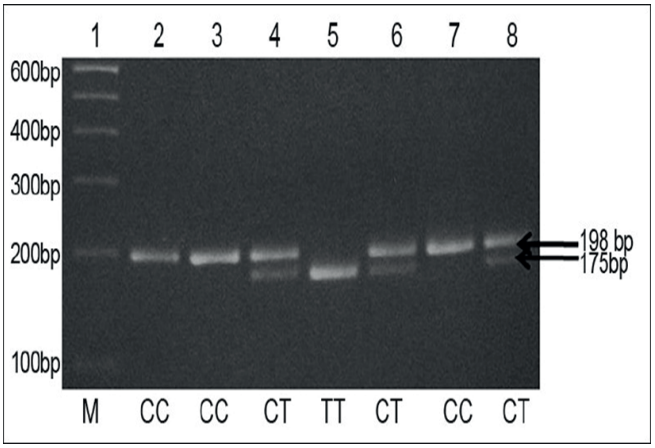
Reverse, 5´-AGG ACG GTG CGG TGA GAG TG-3´.

The C677T mutation introduces a new HinfI restriction site which results in the digestion of the 198 bp amplicon into 175 and 23 bp fragments.

The PCR mixture contained 1µmol/L of each primer, two units of Taq polymerase, 25 mmol/L MgCl2 , 0.2 mmol/L of each dNTP and 1 µg of DNA template in a final volume of 50 µL. The amplication was carried out in a PCR thermal cycler, the cycling parameters were 5 min at 95°C followed by 35 cycles of 45 s at 95°C ,1 min at 55°C ,and 45 s at 72°C followed by a single 10-min extension at 72°C .The 198-bp PCR product (10 µl) was digested with the restriction enzyme HinfI (17) at 37° C for 3–4h in the buffer recommended by the manufacture. HinfI can recognize the C to T substitution in the fragments. This one nucleotide substitute corresponds to a conversion of Ala-to-Val residue in the MTHFR encoding region.

20 µL of each reaction mixture was separated on agarose gel 3% and stained with ethidium bromide and visualized under UV illumination.

The two different alleles were designated T (Val) and C (Ala). The 198-bp fragment derived from the C allele is not digested by HinfI, whereas the fragments of the same length from the T allele are digested by HinfI into 175- and 23-bp fragments. Subjects homozygous for the mutation showed two DNA fragments of 175- and 23-bp, whereas homozygous subjects without it showed a DNA fragment of 198-bp. Heterozygous subjects showed three DNA fragments of 198-, 175- and 23-bp.



RESULTS

The demographic characteristics of the study groups are summarized in Table 1. There were no significant differences between the groups in terms of age, sex, or disease duration. Similarly, fasting blood glucose, HbA1c, and serum creatinine levels showed no significant differences between the two groups of diabetic patients, with or without nephropathy.

Genotype and allele frequencies were compared between diabetic patients and controls (Table 2). While a higher percentage of T allele carriers was observed among diabetic patients and their first-degree relatives compared to controls, this difference was not statistically significant.

A significant difference in the distribution of the **MTHFR C677T** genotype was observed between diabetic patients with and without nephropathy ($\chi^2 = 16.8$, $p < 0.001$) (Table 3). The 677T allele frequency was significantly higher among patients with diabetic nephropathy, with increased frequencies of the C/T (RR = 4.6, CI 1.3–15.5) and T/T (RR = 5.7, CI 1.1–30.8) genotypes.

Plasma homocysteine levels were significantly elevated in patients with nephropathy compared to those without nephropathy and control subjects (Table 1). An association between the **MTHFR C677T** genotype and homocysteine levels was evident, with significantly higher homocysteine levels observed in 677T/T carriers across all groups studied compared to C/T or C/C genotype carriers (Table 5). This effect of the **MTHFR C677T** polymorphism on plasma homocysteine levels was noted not only in diabetic patients but also in their first-degree relatives and healthy controls (Table 4).

There was no significant correlation between glycemic control (HbA1c) and homocysteine levels in diabetic patients ($r = 0.08$, $p > 0.05$). However, a significant correlation was found between plasma homocysteine and serum creatinine levels ($r = 0.47$, $p < 0.01$)

Table 1 : Profile of type 2 diabetes mellitus patients , FDR's and corol subjects

Characteristics	Control	Fdr's	T2DM with nephropathy	T2DM without nephropathy	P value
Age	50.5±10	21.3±2	56.2±6	50.4±8	> 0.05
Duration of the disease	N/A	N/A	8.7±2	7.8±2.4	> 0.05
Fasting glucose(mg\dl)	91.8±8.4	98.9±3.2*	264.2±81.4*	285±71.2*	< 0.05
Hb A1c	4.8 ±0.8	5.4±0.2	8.3±2.2*	8.0±1.4*	< 0.05
Serum creatinine	0.5±0.13	0.5±0.12	0.79±0.32	0.69±0.18	> 0.05
Homocysteine(MmoN)	10.1±2.8	11.9±4.9	18.9±6.7* *	11.8±5.3* *	<0.001

Table 2: Genotype distribution and allele frequency of MTHFR C677t among studied groups

MTHFR C677T genotype	Controls n=30	FDR's n=30	T2DM n=50	P value
CC	19 (63.3%)	15 (50.0%)	23 (46%)	>0.05
CT	8(26.7%)	9 (30.0%)	19 (38%)	
TT	3(10%)	6(20%)	8 (16%)	
C allele	0.76	0.65	0.65	
T allele	0.24	0.35	0.35	

Table 3: Genotype distribution and allele frequency of MTHFR C677t among diseased groups

MTHFR C677T	T2DM without nephropathy n=26	T2DM with nephropathy n=24	P value
CC	18(69.2%)	5(20.8%)	>0.05
CT	6(23.1%)	13(54.2%)	
TT	2(7.7%)	6(25%)	
C allele	81%	48%	
T allele	19%	52%	

Table 4: Relationship between MTHFR C677T genotypes and homocysteine activity (µmol/L) in the studied groups

MTHFR C677T genotype	Controls n=30		FDR's n=30		T2DM n=50	
CC	19 (63.3%)	8.7±1.8*	15 (50.0%)	11.1±3.27*	23 (46%)	11.4±3.4*
CT	8(26.7%)	11.4±2.2*	9 (30.0%)	17.1± 3.7*	19 (38%)	17.3±4.7*
TT	3(10%)	15.5±0.7*	6(20%)	22.9±4.1*	8 (16%)	24.4±4.4*
Test of Significance	F = 13.3		F = 32.5		F = 34.1	
P value	< 0.01				< 0.001	

* Significant difference with other genotypes

Table 5: Relationship between MTHFR C677T genotype and homocysteine activity in the patient groups

MTHFR C677T genotypes	T2DM without nephropathy		T2DM with nephropathy	
	N(%)	Homocysteine level	N(%)	Homocysteine level
CC	18(69.2%)	11.3±2.7	5(20.8%)	11.8±2.4**
CT	6(23.1%)	15.4±3.8*	13(54.2%)	19.4±3.9**
TT	2(7.7%)	21±6*	6(25%)	26.2±2.8**
P value	<0.05		<0.001	

* Significant difference with CC genotype

** Significant difference with other genotypes

DISCUSSION

Diabetic nephropathy is a leading cause of chronic kidney disease (CKD) [23], and it is closely linked to increased cardiovascular mortality [24]. The **MTHFR** gene has two well-documented polymorphisms, **C677T** and **A1298C**, both of which involve single nucleotide substitutions leading to amino acid changes [25]. The C677T polymorphism reduces enzymatic function, elevating plasma homocysteine levels and disrupting the balance of folate metabolites [26]. This polymorphism is more strongly associated with diabetic nephropathy compared to A1298C due to its location in exon 4, which is within the N-terminal catalytic domain of the enzyme, while A1298C is in exon 7, within the C-terminal regulatory domain [27].

Studies have shown higher frequencies of the mutated genotype and allele in diabetic patients and their first-degree relatives compared to controls. For example, in control subjects, the T allele frequency was 24%, with genotype frequencies of 63.3% for CC, 26.7% for CT, and 10% for TT. In diabetic patients, the T allele frequency rose to 35%, with genotype frequencies of 46% for CC, 38% for CT, and 16% for TT. Similarly, in first-degree relatives, the genotype frequencies were 50% for CC, 30% for CT, and 20% for TT.

These findings align with other studies. For instance, among 114 healthy Chinese individuals, the T allele frequency was 38%, with genotype distributions of 55.3% for CC, 27.2% for CT, and 17.5% for TT [28]. In Tunisia, the T allele frequency was 22% among healthy subjects but more prevalent among type 2 diabetes mellitus (T2DM) patients (36%). Genotype distributions were similar, with no significant differences between groups ($\chi^2 = 2.5$, $P > 0.05$) [29]. Other studies also found comparable results, with no significant differences in genotype distributions between T2DM patients and controls ($\chi^2 = 3.67$, $P > 0.05$) [30].

Interestingly, the T allele frequency was higher among patients with nephropathy (19%) compared to those without nephropathy (52%). Our findings suggest that the C677T polymorphism has pathophysiological significance. Patients with the 677T/677T mutation had a significantly higher risk of diabetic nephropathy compared to those with 677C/677C or 677C/677T genotypes. This aligns with a study by Cui et al., which reported a strong association between the 677T allele and diabetic nephropathy (OR = 1.97, 95% CI [1.71, 2.28], $p < 0.00001$), but no association with diabetes mellitus itself (OR = 1.03, 95% CI [0.89, 1.18], $p = 0.70$) [31].

Our data showed a sixfold increase in nephropathy risk among TT genotype carriers (RR = 5.7, CI 1.1–30.8). However, this contrasts with Maeda et al., who reported no significant effect of the 677T/677T genotype on nephropathy risk (OR = 1.17; 95% CI [0.45–3.05]) [32]. These discrepancies may be due to population differences, sample sizes, or gene–environment interactions.

Hyperhomocysteinemia was more prevalent in T2DM patients than in controls and was higher in patients with diabetic nephropathy compared to those without. The C677T/T genotype was associated with elevated plasma homocysteine levels. The molecular mechanism by which MTHFR polymorphism contributes to microvascular diseases remains unclear. However, hyperhomocysteinemia caused by the 677T/677T mutation is thought to initiate and progress microvascular diseases through endothelial dysfunction, triggering various pathological reactions [33]. Heterozygous T allele carriers have a 12% increase in homocysteine levels, while TT individuals exhibit a 30% increase compared to CC genotypes [34]. Elevated homocysteine may induce tissue factor expression, initiating blood coagulation in vivo [35], and upregulate MCP-1 and IL-8 secretion, contributing to vascular disease progression [29].

Finally, no significant relationship was observed between glycemic control (HbA1c) and homocysteine levels in diabetic patients ($r = 0.08$, $P > 0.05$). However, a significant correlation between plasma homocysteine and serum creatinine ($r = 0.47$, $p < 0.01$) indicates a metabolic association between these markers.

CONCLUSION

The 677T/677T mutation in the MTHFR gene may serve as a predictive marker for the development of diabetic nephropathy in diabetic patients. Additionally, homocysteine plays a significant role in this condition. Therefore, the intake of folate and vitamins B6 and B12 can help reduce plasma homocysteine levels in diabetic individuals.

Further Implications

Further studies in more patients are required to establish this polymorphism in diabetic nephropathy.

Declaration of patient consent

Written informed consent was obtained from the patients to use their results for publication. Ethical approval for the research was granted by the Ethical Committee of Dubai Pharmacy College for Girls.

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Conflict of Interest: The authors declare no conflict of interest.

Ethical Clearance: This study was conducted following ethical guidelines and approved by Ethical Committee of Dubai Pharmacy College for Girls.

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- **Data gathering and idea owner of this study:** Gazala Afreen Khan
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- **Writing and submitting manuscript:** Gazala Afreen Khan, Shaima Mazen, Aman Al Hamed, Abir Majed.
- **Editing and approval of final draft:** Gazala Afreen Khan

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