Methylenetetrahydrofolate Reductase (Mthfr C677t) Gene Polymorphism Effect on Development of Diabetic Nephropathy in Egyptien Patients with Type 2 Diabetes Mellitus

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Abstract: Introduction: Genetic predisposition has been implicated in diabetic nephropathy (DN). Methylenetetrahydrofolate reductase (MTHFR) is a regulatory enzyme of homocysteine metabolism. The C677T variant of the methylenetetrahydrofolate reductase (MTHFR) gene may play a role in the development of not only vascular disease but also diabetic microangiopathies. In this study, we examined the distribution of the MTHFR genotypes and the association between the C677T variant and diabetic nephropathy. METHODS: 50 type 2 diabetes mellitus patients classified into 2 groups according to presence or absence of nephropathy as measured by urinary albumin /creatinine ratio into 2 groups, 27 patients without nephropathy and 23 with nephropathy and 20 controls were recruited in the study. Fasting blood glucose, HbA1C and serum creatinine were measured. Plasma total homocysteine level was measured using chemilumenecent assay. MTHFR genetic C677T polymorphism was determined with PCR-restriction fragment length polymorphisms (RFLP). RESULTS: The frequency of MTHFR TT genotype and CT heterogenetic type and allele T(30.4%, 43.5%, 52%) was significantly higher in type 2 diabetes mellitus with diabetic nephropathy group than those without nephropathy (7.4%, 25.9%, 20%) or normal controls (10%, 25%, 22%). However, there was no significant difference of MTHFR genotype and allele frequency between type 2 diabetes mellitus without nephropathy and normal controls (χ^2 0.1, p value < 0.05). The presence of T allele appeared to have a stronger association with the development of diabetic nephropathy. The odds ratio was 5.7 and the 95% confidence interval was 1.7-19.3. Moreover, plasma homocysteine levels were markedly higher in patients with TT or CT genotype than those in patients with CC genotype. CONCLUSIONS: Our findings suggest that the C677T mutation in the MTHFR gene predisposes type 2 diabetes patients to the development of diabetic nephropathy. The T allele of this mutation presumably acting by elevating homocysteine levels and seems to be associated with a faster progression of nephropathy to end-stage renal failure.
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1. Introduction:

Diabetic nephropathy (DN) is a serious complication of type 2 diabetes (T2DM), and is the primary cause of end-stage renal failure [1,2]. The etiology of DN is multifactorial and involves both environmental and genetic factors [3,4]. Strong genetic predisposition for nephropathy in type 2 diabetes mellitus is suggested by familial clustering [5].

Homocysteine is a thiol-containing amino acid derived from methionine. It can be catabolized to cystathionine and cysteine by the action of cystathionine b-synthase, in the presence of vitamin B6. An alternative metabolic pathway consists of a remethylation process, primarily by transfer of a methyl group from 5-methyltetrahydrofolate to homocysteine in the presence of vitamin B12, and subsequent formation of methionine [6] This pathway is regulated by the activity of 5,10-methylenetetrahydrofolate reductase (MTHFR), which, in the presence of folate, catalyzes the formation of 5-methyltetrahydrofolate [7].

Elevated homocysteine levels have been identified as a risk factor for diabetic nephropathy in type 2 diabetes [8,9]. In addition, increased plasma homocysteine is an independent risk factor for several vasculopathies including arteriosclerosis, acute myocardial infarction, cerebrovascular diseases, arterial and venous thrombosis [10–13].

The methylenetetrahydrofolate reductase (MTHFR) gene is located on chromosome 1 (1p36.3)[14] . Several mutations within MTHFR gene were reported; the best-characterized being the C677T, a valine-to-alanine substitution at amino acid 226, resulting in a thermo-labile MTHFR variant with reduced catalytic activity. [15,16]. Homozygosity for the mutation (TT genotype) predisposes to significantly elevated plasma homocysteine levels [17,18]. Elevated plasma homocysteine were shown to be associated with predisposition to developing T2DM complications, including diabetic retinopathy [19,20] and diabetic nephropathy (DN) [9-21]. **Noiri *et al.*** [9] reported an increased frequency of the CT and TT

genotypes in male haemodialysed patients with type 2 diabetes as well as a correlation between the presence of the C677T allele and the progression of renal failure. The aim of this study was to evaluate a possible role of the MTHFR C677T polymorphisms in the susceptibility to DN in T2DM patients. The allele and genotype frequencies of the C677T, together with changes in homocysteine levels, were determined for T2DM patients with DN and those without nephropathy, together with non-diabetic control subjects.

2. Subjects and Methods:

The study groups consisted of 50 T2DM patients from the Outpatient Clinic of Zagazig University Hospital diagnosed according to American Diabetes Association revised criteria [22]. These were (30 males, 20 females); 20 healthy individuals (13 males, 7 females) served as the control group. On all subjects, demographic details were recorded, which included age; gender; age of onset and duration of disease; history of hypertension, dyslipidaemia, ischaemic heart disease and other medical illness; history of chronic complications of diabetes, treatment for diabetes including date of initiation and/or discontinuation of oral agents or insulin and history of other medication. Patients with serum creatinine > 2 mg/dL, patients with blood pressure above 130/90, and those taking vitamins or folic acid containing preparation were excluded from the study. The study was approved by the institution Ethics Review Board, and written informed consent was obtained from all participants.

The patients group was classified according to presence of nephropathy into two groups 27 without DN and 23 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 [23]. 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 15 µmol/L is the most common definition of hyperhomocysteinemia [24].

MTHFR C677T genotype:

Genomic DNA was extracted from peripheral blood leukocytes Buffy coat samples using the E.Z.N.A. ™ Blood DNA Kit (Omega – biotek. Inc). Genomic DNA samples were stored at -20°C until genotyping analysis. MTHFR C677T genotype analysis was performed by PCR-RFLP analysis using HinfI (15) digestion for C677T. 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 3–4 h in the buffer recommended by the manufacturer. 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. Twenty 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.

Statistical analysis

Data were expressed as mean ± SD for quantitative variables, number and percentage for qualitative ones. ANOVA, t test, χ^2, and Pearson correlation were used for analysis of results .Relative risk (RR) and 95% confidence interval (CI) were performed to predict the effect of MTHFR C677T genotypes on development of DN. A *P*-value < 0.05 was considered significant. Analysis was performed

with the SPSS statistical package version 10 (SPSS Inc., Chicago, IL).

3. Results:

The demographic characteristics of the studied groups are summarized in Table 1. There was none significant differences between studied groups as regard age, sex or duration of the disease. As regard fasting blood glucose, diabetic control as measured by HbA1c and serum creatinine there was none significant difference between 2 groups of patients with or without nephropathy.

Genotype and allele frequencies were compared between diabetic patients and controls (Table 2). Although there were a higher percentage of T allele carriers among diabetic patients than control the difference was none statistically different. There was a highly significant difference of MTHFR C677T genotype between patients with or without DN (χ^2 16.8, *p* value < 0.001) (Table 3). The frequency of 677T allele was significantly higher among patients with DN, and higher frequency of 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 higher in patients with nephropathy than in patients without nephropathy or control subjects (Table 1). There was an association between MTHFR C677T genotype and homocysteine levels; significantly elevated homocysteine was noted in 677T/T carriers in the three groups of study subjects, as opposed to C/T or C/C genotype carriers (Table 5). The effect of MTHFR C677T polymorphism on plasma homocysteine levels was evident not only in diabetic patients but also in healthy controls (Table4).

Our results showed none significant correlation between glycemic control (HbA1C) and homocystein levels among diabetic patients (r 0.08, *P*>0.05) while there was significant correlation between plasma homocystein and serum creatinine (r 0.47 *p*< 0.01).

Table (1): Profile of T2DM patients and control subjects

Characteristics	Control (n=20)	Type 2DM without nephropathy (n=27)	Type 2DM with nephropathy (n=23)	*P* value
Age (years)	54.6±10	52.4±9	58.4±8	> 0.05
Sex male(%)	13 (65)	15 (55.6)	14(61)	> 0.05
Duration of diseases (years)	N/A	8±2.1	8.9±2	> 0.05
Fasting glucose (mg/dl)	91.2±8.4	285±78.6[a]	228.5±84.1[a]	<0.05
Hb A1C (%)	4.8±0.8	8.1±1.2 [a]	8.4±2.1 [a]	<0.05
Serum creatinine(mg/dl)	0.6±0.13	0.7±0.18	0.78±0.15	>0.05
Homocysteine (µmol/L)	10.1±2.8	12.9±4.9[ab]	19.6±5.8[ab]	<0.001

a: significant difference between T2DM patients and control subjects
b: significant difference between T2DM patients with vs. patients without nephropathy.

Table (2): Genotype distribution and allele frequency of MTHFR C677T among studied groups

MTHFR C677T Genotypes	Control subjects (n=20), No (%)	Type 2DM (n=50), No (%)	χ^2	*P* value
CC	13 (65)	24(48)	1.7	>0.05
CT	5 (25)	17(34)		
TT	2(10)	9(18)		
C allele	0.78	0.65		
T allele	0.22	0.35		

Table (3): Genotype distribution and allele frequency of MTHFR C677T among patients groups

MTHFR C677T Genotypes	Type 2DM without nephropathy (n=27), No (%)	Type 2DM with nephropathy (n=23), No (%)	χ^2	*P* value
CC	18 (66.7)	6(26.1)	9.0	<0.05
CT	7 (25.9)	10(43.5)		
TT	2(7.4)	7(30.4)		
C allele	0.80	0.48		
T allele	0.20	0.52		

Table (4): Relationships between MTHFR C677T genotypes and homocystein activity (µmol/L) in the studied groups.

MTHFR C677T Genotypes	Control (n=20)		Type 2DM patients (n=50)	
	N (%)	Homocysteine levels	N (%)	Homocysteine levels
CC	13(65)	8.7±1.8[a]	24(48)	11.4±3.4[a]
CT	5(25)	11.4±2.2[a]	17(34)	17.3±4.7[a]
TT	2(10)	15.5±0.7[a]	9(18)	24.4±4.4[a]
Test of significance	F = 13.1		F = 34.2	
***P* value**	< 0.01		< 0.001	

a: Significant difference with other genoytpes

Table (5): Relationships between MTHFR C677T genotypes and homocystein activity (µmol/L) in the patients groups.

MTHFR C677T Genotypes	Type 2DM without nephropathy		Type 2DM with nephropathy	
	N (%)	Homocysteine levels	N (%)	Homocysteine levels
CC	18(67)	11.2±3.6	6(26.1)	12.0±2.4[b]
CT	7(26)	15.2±5.3[a]	10(43.5)	18.6±4[b]
TT	2(7)	20±7[a]	7(30.4)	25.6±3[b]
Test of significance	F= 5.2		F = 20.3	
***P* value**	< 0.05		<0.001	

a: Significant difference with CC genotype
b: Significant difference with other genoytpes

4. Discussion:

Diabetic nephropathy is the leading cause of chronic kidney disease in patients starting renal replacement therapy [(25)] and is associated with increased cardiovascular mortality [(26)].

Two common polymorphisms have been described in the MTHFR gene, both single nucleotide substitutions resulting in amino acid changes C677T and A1298C [(17, 27)]. Whereas C677T unequivocally affects enzyme function and has been associated with increased plasma homocysteine concentrations and an altered balance of folate metabolites [(17,28)]. The C677T polymorphism may have a greater association with diabetic nephropathy than A1298C because of the localization of these two variants. The C677T polymorphism is in the exon 4, which is within the N-terminal catalytic domain of the enzyme, whereas the A1298C polymorphism is in the exon 7, which is within the C-terminal regulatory domain. The more dynamic effect of C677T is due to its location within the catalytic region [(29)].

The frequencies of homozygous mutated genotype and the mutated allele is higher in diabetic patients than control group. T Allele frequency was 22% in control healthy subjects while it was 37% in diabetic patients and genotype frequency was 65% for CC, 25% for CT and 10% for TT. Our results were comparable with other studies who performed screening of 114 healthy Chinese people, the allele frequency of the T mutation was 38.0%, comparable to that (33.0%) in a Hong Kong (Chinese) population. The distribution of the three genotypes was as follows: CC genotype 55.3%,; CT genotype, 27.2%; and TT genotype, 17.5% [(30)].In other study done in Tunisia T allele frequency was 22% in healthy subjects ,and more prevalent among T2DM patients, with allele frequencies of 0.36 [(31)].

Genotypes distribution was 44% for CC,38% for CT,18% for TT with none significant difference between two groups ($\chi^2 = 2.5$, $P > 0.05$).This results was similar to other study which showed that the distribution in type 2 diabetic patients in which 44.3% were CC, 34.2% were CT and 21.5% were TT. There were no significant differences in genotype distribution between type 2 diabetic patients and control group ($\chi^2 = 3.67$, $P > 0.05$)[(30)].

T Allele frequency was 20%, 59% in patients with or without nephropathy respectively. These findings indicate that the presence of the C677T polymorphism in the MTHFR gene is of pathophysiological significance, we found that patients with the 677T/677T mutation displayed a frequency of diabetic nephropathy significantly higher than the frequency displayed by those with the 677C/677C or the 677C/677T genotype. This is similar to another study by **Cui *et al.*** who found out that The 677T allele showed significant association with DN (OR = 1.97, 95% CI [1.71, 2.28], p <0.00001), but no relationship with DM (OR = 1.03, 95% CI [0.89, 1.18], p = 0.70) compared with the 677C allele in a Chinese population.[(32)]

Our results showed that risk of nephropathy increased by 6 folds among T/T genotype (RR = 5.7, CI 1.1-30.8) were in contrast to **Maeda *et al.*** [33] who found that the 677T/677T homozygote did not affect the risk for nephropathy (OR=1.17; 95% CI=0.45–3.05). These contrasts are probably due to differences in populations, number of studied cases, or gene–environment interactions.

Our results showed that hyperhomocysteinemia was frequent in T2DM patients as compared with controls, and was also higher than in patients with DN than in patients without DN, with C677T/T carriers having higher plasma homocystein concentration. The molecular mechanism of how MTHFR gene polymorphism promotes microvessel diseases has not been elucidated clearly. One suggestion is that hyperhomocysteinemia resulting from the 677T/677T mutation in the MTHFR gene may lead to the initiation and progression of microvessel diseases through induction of endothelial dysfunction followed by a wide range of pathological reactions [34-36]. Subjects who are heterozygous for the T allele have a 12% increase in homocysteine levels, whereas TT individuals have 30% higher levels, compared to CC genotypes [37].

Another explanations shown that hyperhomocysteinemia may act by inducing the expression of tissue factor (TF), an initiator of blood coagulation *in vivo* [38], by circulating monocytes, which apparently acted independent of peroxide and superoxides, since scavengers of both did not block the expression of homocystein induced TF [39]. Hyperhomocysteinemia may also act by altering endothelial cell function through upregulation of the expression and secretion of MCP-1 and IL-8, which by promoting leukocyte recruitment, may contribute to the initiation and progression of vascular disease [40].

Our results showed none significant difference between glycemic control (HbA1C) and homocystein levels among diabetic patients (r 0.08, $P>0.05$). This in contrast to other studies reported that among diabetics, homocysteine levels may be dependent on the glycaemic control [41]. There was significant correlation between plasma homocystein and serum creatinin (r 0.47 $p< 0.01$), there is a direct metabolic relationship between creatinine and homocysteine. Creatinine originates from metabolism in skeletal muscles, and the amount of released creatinine is therefore determined by muscle mass. The formation of creatine, the precursor of creatinine, depends on a methyl donation by S-adenosylmethionine, leading to the formation of homocysteine. Thus, the level of homocysteine would be expected to reflect both muscle mass and creatinine concentration [42].

We concluded that the 677T/677T mutation in the MTHFR gene could be a predictive marker of the onset of diabetic nephropathy during the initial stage of type 2 diabetes mellitus. Homocystein also has a major role so increased intake of folate and vitamins B6 and B12 can reduce plasma homocysteine levels in patients with diabetic angiopathy further studies in larger number of patients are necessary to establish a role of this interesting polymorphism in the genesis of diabetic nephropathy. It will also be important to study prospectively whether folate supplementation reduces the incidence of DN in type 2 DM in individuals who carry the C677T allele.

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