

Serum Homocysteine and MTHFR C677T and A1298C gene polymorphisms in Type 2 Diabetic Patients with Nephropathy in Sector of Egyptian Population

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ABSTRACT

Homocysteine (Hcy) is an amino acid, which is a product of methionine demethylation and a precursor to cysteine biosynthesis. Elevations in plasma Hcy (homocysteinemia) are frequently found in increased risk of atherosclerotic, coronary artery disease (CAD), venous thrombosis and stroke. Hcy injures the endothelium and may have a role in microvascular complication of type 2 diabetes mellitus (T2DM). Two common mutations in the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene (MTHFR C677T and A1298C) result in elevated Hcy levels. The aim of the present study was to investigate the relationship between plasma Hcy levels and diabetic nephropathy (DN). Serum Hcy levels, MTHFR genotype, and a panel of variables were evaluated in a sample of 75 T2DM patients with DN, 55 patients without nephropathy and also 95 non-diabetic control Egyptian subjects. Its common genetic polymorphisms (MTHFR C677T and A1298C) were determined for these patients and control subjects together with their correlation with changes in Hcy levels. Biochemical variables (including 24 hours albuminuria, GFR and serum total Hcy, lipogram and HbA_{1c} beside blood urea and serum creatinine) and lifestyle characteristics were investigated. MTHFR genotype was studied by PCR-RFLP analysis, and total Hcy levels were measured by ELISA. The plasma Hcy levels were significantly higher in the diabetic nephropathy ($19.8 \pm 2.3 \mu\text{mol/L}$) than uncomplicated type 2 diabetic patients ($12.7 \pm 2.1 \mu\text{mol/L}$, $P < 0.05$) and also, the control subjects ($11.8 \pm 1.8 \mu\text{mol/L}$, $P < 0.05$). There were no differences between uncomplicated diabetic patients and control subjects with respect to Hcy levels. C677T and T677T were highly prevalent among DN patients, with frequencies of 0.40 and 0.36 respectively. C677T, but not A1298C, SNP, is a risk factor for DN, presumably through elevating serum Hcy level.

Keywords: diabetic nephropathy; homocysteine; methylenetetrahydrofolate reductase; type 2 diabetes

Abbreviations: DN: diabetic nephropathy, GFR: glomerular filtration rate, Hcy: homocysteine, MTHFR: 5, 10-methylenetetrahydrofolate reductase, SNP: single nucleotide polymorphism, T2DM: type 2 diabetes mellitus.

INTRODUCTION

Diabetic nephropathy (DN) is a progressive kidney disease caused by angiopathy of capillaries in the kidney glomeruli. It is due to longstanding

diabetes mellitus⁽¹⁾. The susceptibility to DN varies among T2DM, and its etiology is multi-factorial involving acquired and inherited risk factors⁽²⁾. The former exemplified by poor glycemic control and hypertension⁽³⁾,

while the latter was highlighted by a genetic predisposition based on familial clustering of DN ^(4 & 5).

Plasma homocysteine (Hcy), a non-protein sulfur amino acid that is itself a risk factor for arteriosclerosis and atherothrombosis. It is biosynthesized from methionine by the removal of its terminal methyl group. Hcy can be recycled into methionine or converted into cysteine with the aid of methylene-tetrahydrofolate (MTHF) and vitamins B₆. Plasma tHcy level is a graded and independent risk factor for coronary and other forms of vascular disease. Mild hyperhomocysteinemia is an independent risk factor for several vasculopathies including atherosclerosis, acute myocardial infarction, cerebrovascular disease and carotid artery stenosis ⁽⁶⁾.

Methylene - tetrahydrofolate reductase (MTHFR) is an enzyme involved in the reduction of methylene tetrahydrofolate to methyl tetrahydrofolate which is a carbon donor in remethylation Hcy to methionine. MTHFR deficiency is associated with hyperhomocysteinemia, an independent risk factor for thrombotic disorders including arteriosclerosis and arterial thrombosis^(6,7). Two common mutations within MTHFR gene were reported; first is MTHFR-C677T (alanine to valine substitution at amino acid 226) and second MTHFR-A1298C, (glutamate to alanine at amino acid 429) which were reportedly linked with moderate hyperhomocysteinemia ⁽⁸⁾.

The aim of the current study was to evaluate the serum Hcy level in diabetic nephropathy in Egyptian type

2 DM patients. Its common genetic polymorphisms (MTHFR C677T and A1298C) were determined for 75 T2DM patients with DN, 55 T2DM patients without nephropathy and also 95 non-diabetic control subjects together with their correlation with changes in Hcy levels.

MATERIALS & METHODS

Fifty-five adult patients (36 males and 19 females; mean age 55.7 ± 11.5 years), who were diagnosed as DN by abnormal urinary protein concentration (albuminuria >30 g/24 hours), high serum creatinine levels above 1.3 mg/dl or low glomerular filtration rate (GFR) levels (below 90 ml/minute). Another group was 75 adult patients (47 males and 28 females; mean age 48.7 ± 12.3 years) and was diagnosed T2DM based on 1998 WHO diagnostic and classification criteria (WHO) from outpatient clinic Sohag Faculty of Medicine without renal impairment. None of the patients had ketoacidosis, and treatment for diabetes included diet and/or oral antidiabetic drugs and/or insulin to achieve glycemic control. The control group included 95 non-diabetic subjects (51 males and 44 females; mean age 55.6 ± 14.1 years) without known personal or family history of diabetes. The University of Sohag Ethics Committee approved the study and informed consent was obtained from all participants according to the study protocol.

For all subjects, complete history was recorded, which included age; gender; ethnic origin; age of onset, duration and first-degree family

history of diabetes; dyslipidemia, ischemic 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.

After an overnight fast, venous blood samples were collected for measurement of plasma venous glucose, HbA_{1c}, lipid profile (total cholesterol, triglyceride, LDL-cholesterol, HDL-cholesterol), Hcy, serum urea and creatinine and liver function tests. Twenty-four hours urine sample was collected for measurement of urinary microalbumin. Patients were classified into DN or normoalbuminuria according to their urinary albumin excretion rate (AER) in 24 hours urine collections. DN was defined as AER of >30 mg/24 hours. GFR was calculated according to **Arnadottir et al. (1996)**⁽⁹⁾.

Microalbumin assay:

It was done by competitive ELISA (ALPCO; Catalog Number: 24-MABHU-E01) in which enzyme labeled albumin competes with albumin in samples for limited antibody binding sites on the microplate. After incubation for a fixed time, separation of bound from free is achieved by simple decantation and plate washing. The enzyme activity on the plate is measured using an enzyme substrate and a chromogen. The absorbency of the color developed is read in an ELISA colorimetric reader at 450 nm. The measured absorbance is inversely proportional to the concentration of bound albumin in the microplate. The

concentration of albumin in the urine is determined from a calibration curve.

Glomerular filtration rate (GFR): GFR was calculated using the formula (urine volume x urine creatinine)/ (1440 x plasma creatinine)⁽⁹⁾.

Lipid profile: Serum total cholesterol and triglyceride were determined by enzymatic colorimetric methods. Also, HDL- cholesterol and LDL- cholesterol were measured using the cholesterol –esterase and cholesterol –oxidase enzymes (Randox[®], England902).

Homocysteine assay:

Enzyme Immunoassay (EIA: Alpico cat. No. 66=HCYHU-E01) for the determination of total homocysteine (tHcy) in blood was done. Protein-bound tHcy is reduced to free tHcy by dithiothreitol (DDT) and enzymatically converted to S-adenosyl-L-homocysteine (SAH) by SAH-hydrolase prior to the immunoassay. Solid phase EIA is based on competition between SAH in the sample and immobilized SAH bound to the walls of the microtitre plate for binding sites on monoclonal anti-SAH antibody. After removal of unbound anti-SAH antibody, a second rabbit antimouse antibody labeled with horse radish peroxidase (HRP) is added. The peroxidase activity is measured spectrophotometrically at 450 nm.

MTHFR genotyping:

Total genomic DNA was isolated by the phenol chloroform method. MTHFR genotype analysis was performed by PCR-RFLP analysis using *Hinf*I⁽¹⁰⁾ and *Mbo*II digestion (Boehringer Mannheim, Germany) for

C677T and A1298C detection, respectively. The selected 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⁽¹⁰⁾.

MTHFR A1298C SNP was analyzed using similar conditions to the C677T using the following primer set: forward, 5'-CTT TGC GGA GCT GAA GGA CTA CTA C-3', and reverse, 5'-CAC TTT GTG ACC ATT CCG GTT TG-3'. In wild-type (A1298A) variant, *MboII* can digest the 163 bp amplicon into 56, 31, 30, 28, and 18 bp fragments. In A1298C mutant variant, one *MboII* restriction site was abolished resulting in digestion of the 163 bp amplicon into 84, 31, 30, and 18 bp fragments⁽¹⁰⁾.

Digested fragments were separated by 2% agarose gel electrophoresis and visualized under ultraviolet light following ethidium bromide staining.

STATASTIC ANALYSIS: SPSS software (version 10.0) was used for statistical analysis. Comparison of allele and genotype frequencies between controls, type 2 DM and diabetic nephropathy groups were carried out using the Chi-square test. In addition to the incidence probability of MTHFR-C677T and MTHFR-A1298C in microalbuminuria and HbA_{1c} were analyzed by Odd ratio. *P* values lower than 0.05 were considered statistically significant.

RESULTS

The general characteristics of the studied population are shown in table 1.

Table 1: Characteristics of the participating patients and control subjects

	Control N (95)	T ₂ DM N (75)	DN N (55)
Age (years)	55±14.1	48.7±12.3	55.7±11.5
Sex (males/Females)	51/39	47/28	36/19
BMI (Kg/m ²)	22.5±4.4	29.5±5.4*	28.9±5.1 [#]
Albuminuria (mg/24 h)	19.3±9.7	21.2±8.8	326.7±182.4 ^{#®}
Cholesterol (mg/dl)	195.6±11.2	241.5±9.7	247.6±8.8 ^{#®}
Triglycerides (mg/dl)	140.3±3.7	189.7±4.8	198.7±5.1 ^{#®}
HDL-cholesterol (mg/dl)	44.5±1.9	35.3±2.4	33.1±1.7 ^{#®}
LDL-cholesterol (mg/dl)	119.6±2.8	158±3.5	160.2±3.9 ^{#®}
HbA _{1c} (%)	4.3±1.2	9.5±3.9*	9.7±3.8 [#]
Blood urea (mg/dl)	28.8±3.8	29.5±3.4	75.7±13.4 [#]
Creatinine (mg/dl)	0.7±0.3	0.9±0.2	2.7±1.1 [#]
GFR (ml/min)	98.4± 6.8	97.2± 6.1	61.4± 10.7 ^{#®}
tHcy (µmol/L)	11.8±1.8	12.7±2.1	19.8±2.3 ^{#®}

Data are expressed as mean± SD. * Significant change (*P* value <0.05) between T2DM and control, [#] significant change (*P* value <0.05) between DN and control and [®] significant change (*P* value <0.05) between DN and type 2 DM.

MTHFR- C677T polymorphism: according to figure 1, undigested fragment (fig. 1-B) was detected in MTHFR-C677C wild type (198 bp), digested fragments (23 and 175 bp) were detected in homozygous MTHFR-T677T (fig. 1-C) and digested and undigested fragments (23, 175 and 198 bp) were detected in heterozygous MTHFR-C677T (fig. 1-D).

MTHFR- A1298C polymorphism: As figure 1, MTHFR- A1298A wild type (56, 31, 30, 28 and 18 bp) was detected as three bands (fig. 1-E), homozygous MTHFR- C1298C (84, 31, 30 and 18 bp) were detected as three bands (fig. 1-F) and MTHFR- A1298C heterozygous mutation was detected as (84, 56, 31, 30, 28 and 18 bp) as four bands (fig. 1-G).

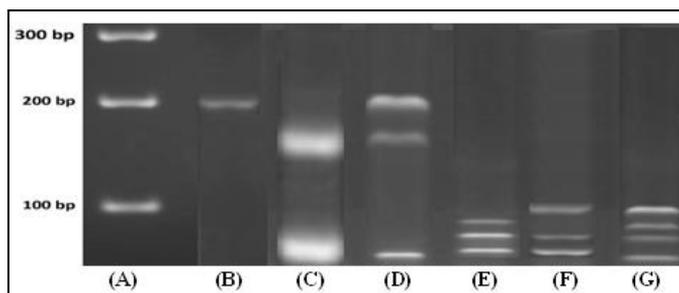


Fig.(1): (A) DNA ladder, (B) MTHFR-C677C wild type, (C) MTHFR-T677T homozygous mutation, (D) MTHFR-C677T heterozygous mutation, (E) MTHFR-A1298A wild type, (F) MTHFR-C1298C homozygous mutation and (G) MTHFR-A1298C heterozygous mutation.

Frequencies of MTHFR-C677T and MTHFR-A1298C genotypes in control, type 2 DM and diabetic nephropathy were tabulated below (table 2).

Table 2: MTHFR genotype in control, type 2 DM and diabetic nephropathy

Genotype		Control	Type 2 DM	DN
MTHFR- 677 N (%)	Wild type (C677C)	73 (76.8%)	39 (52%)	13 (23.6%)
	Heterozygous (C677T)	13 (13.7%)	21 (28%)	22 (40%)
	Heterozygous (T677T)	9 (9.5%)	15 (20%)	20 (36.4%)
MTHFR- 1298 N (%)	Wild type (A1298A)	69 (72.6%)	59 (78.7%)	41 (74.5%)
	Heterozygous (A1298C)	17 (17.9%)	9 (12%)	9 (16.4%)
	Heterozygous (C1298C)	9 (9.5%)	7 (9.3%)	5 (9.1%)

In investigation of polymorphisms odd ratio in microalbuminuria (table 3) and HbA1c as table 4 shown, the odd ratios of MTHFR- C67T7 and MTHFR-

A1298C (one or two allele) polymorphisms in microalbuminuria were 3.74 ($p=0.03$) and 0.98 ($p=0.6$) and HbA1c were 0.98 ($p=0.4$) and 0.72 ($p=0.3$).

Table 3: MTHFR -C677T and A1298C polymorphisms odd ratios in microalbuminuria.

Polymorphism in one or two allele	Odds ratio	P-value
MTHFR- 677 (CT and TT)	3.74	0.03
MTHFR- 1298 (AC and CC)	0.98	0.6

Table 4: MTHFR-C677T and A1298C polymorphisms odd ratios in HbA1c.

Polymorphism in one or two allele	Odds ratio	P-value
MTHFR- 677 (CT and TT)	0.98	0.4
MTHFR- 1298 (AC and CC)	0.72	0.3

DISCUSSION

The identification of plasma Hcy concentration determinants are of clinical relevance⁽¹¹⁾. High concentrations of Hcy have been associated with an increased risk of coronary heart disease, deep vein thrombosis, pulmonary embolism, and stroke⁽¹²⁾. Intracellular Hcy is either converted to cysteine via the vitamin B6-dependent transsulfuration pathway or is remethylated to methionine via cobalamin and tetrahydrofolate (THF) as cofactors. In the current study, plasma mean tHcy level was not higher in uncomplicated diabetic patients than control subjects. However, there was a significant difference in the tHcy mean level between DN and control subjects. There are also a positive correlation between plasma tHcy and macrovascular complications^(13,14) or microvascular complications^(1,9). However, the relationship between

tHcy and diabetic microvascular complications seems stronger than the relationship between tHcy and diabetic macrovascular complications of diabetes. It has been reported that incipient and overt nephropathy is associated with elevations of plasma tHcy in patients with type 2 diabetes⁽⁹⁾. In line with the present study, **Hofmann et al.**,⁽¹⁾ found elevated tHcy levels in diabetic patients with nephropathy, whereas **Robillon et al.**,⁽¹⁵⁾ found reduced tHcy levels in diabetic patients without overt nephropathy. Hyperhomocysteinemia may act to induce DN by inducing the expression of tissue factor (TF) which is an initiator of blood coagulation in vivo⁽¹⁶⁾ or by altering endothelial cell function through upregulation of the expression and secretion of MCP-1 and IL-8 which promote leukocyte recruitment and may contribute to the initiation and progression of vascular disease⁽¹⁾.

Plasma tHcy concentration depends on genetic⁽⁵⁾ and environmental (nutritional) factors such as smoking, alcohol and coffee consumption, diet, usage of vitamins and mineral supplements⁽¹⁷⁾. However, because methyl-THF is the active folate derivative required for remethylation of tHcy and not methylene-THF, the latter is reduced to methyl-THF in the reaction catalyzed by MTHFR⁽¹⁸⁾.

The human MTHFR gene is located on chromosome 1p36.3 and consists of 11 exons⁽¹⁹⁾. There are 2 commonly observed polymorphisms of that gene. One is C-to-T transition in exon 4 at the 677 nucleotide of the transcription start site⁽¹⁹⁾. As a result of that mutation, valine appears instead of alanine in the 226 amino acid of the polypeptide chain. The frequency of that mentioned genotype was investigated in Europe, the Middle East, China, Oceania, Mexico, the United States, and Canada⁽²⁰⁻²²⁾. The prevalence of the homozygous TT genotype was 10% to 12% in several areas in Europe (e.g. France and Hungary). However, there were also areas of a significantly lower frequency of the TT genotype (ie, Helsinki, Finland, the northern Netherlands, and Russia), with frequencies of 4%, 6%, and 7%, respectively^(8,23). On the other hand, in southern Europe, the frequencies observed were as high as 26% and 20% in Campania and Sicily, respectively. In recurrent study, genotype C (homozygous (CC) and heterozygous (CT) forms are 90.5% and higher than that T both homozygous (TT) and heterozygous (CT) forms which are 23.2% in

control group. In diabetic nephropathy, genotype T forms (homozygous and heterozygous) are 76.4% which are higher than C forms (63.6%) and are parallel with microalbuminuria. This substitution leads to the synthesis of a thermolabile form of MTHFR⁽¹⁸⁾. Hence, in subjects presenting the 677TT genotype, there is reduced formation of methyl-THF, and therefore, the individual's ability to methylate tHcy to methionine is lowered, resulting in increased levels of plasma tHcy⁽²⁴⁾. The C677T mutation found within the MTHFR catalytic domain, while the A1298C mutation found in exon7, is located within the enzyme regulatory domain. This was in agreement with previous studies in many ethnic groups including Chinese⁽²⁰⁾, Polish⁽²⁵⁾, and Japanese⁽²⁶⁾, which demonstrated that MTHFR gene may be an aggravating factor for DN in T2DM patients. Others failed to find such an association⁽⁶⁾, and some suggested that gender⁽²⁴⁾ and low folate levels⁽²⁷⁾ may determine to a large extent the pathogenic nature of the C677T mutation.

In addition to the MTHFR C677T polymorphism, there is another one in the same gene resulting in changed enzyme activity. That polymorphism is located in exon 7 of MTHFR at 1298 bp, outside the catalytic domain and on the AdoMet-regulatory domain of the enzyme⁽⁵⁾. The mutation is an A-to-C transversion, which changes glutamic acid into an alanine residue in position 429. The enzyme expressed in polymorphic homozygotes manifests in decreased activity of approximately 60% of

control values (5). In the current study, neither homozygous (CC) nor heterozygous (AC) subjects have a parallel role in elevated plasma tHcy⁽⁵⁾. Nevertheless, numerous studies have found association of that polymorphism with cardiovascular diseases⁽¹⁴⁾ and psychiatric disorders such as depression, schizophrenia and bipolar disorder⁽²⁸⁾.

In conclusion, we found a positive relationship between the MTHFR C677T (not A1298C) mutation, hyperhomocysteinemia, and DN in type 2 DM.

Despite the polymorphisms mentioned above, resulting mainly in mild hyperhomocysteinemia, there are also such mutations in the MTHFR gene that can cause hyperhomocysteinemia because of MTHFR deficiency, such as 559 C-to-T transition (Arg184Ter)⁽²²⁾, 482 G-to-A transition (Arg158Gln)⁽²²⁾, 983 A-G transition (Asn324Ser)⁽²⁹⁾, 1027 T-G transition (Trp339Gly), 1084 C-to-T transition (Arg to Ter), 1711 C-T mutation (Arg to Ter)⁽²⁹⁾, 1081 C-T transition (Arg to Cys)⁽³⁰⁾, and 1141 C-T transition (Arg377Cys)⁽²²⁾. All of these polymorphisms associated with hyperhomocysteinemia may have a role in DN. Also, discovery of these polymorphisms might lead to improve people's health through change in their lifestyles, including nutrition, stop use of supplements as alcohol and smoking or vitamin supplement, to control the prevalence of the DN secondary to type 2 DM, needs further investigation.

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معدل الهيموسيسيتاين بالدم و الطفرة الجينية (MTHFR C677T) و MTHFR A1298C) في حالات اعتلال الكلى الناتج عن مرض البوال السكري النوع الثاني في قطاع من سكان جمهورية مصر العربية

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الهيموسيسيتاين هو احد مشتقات الحامض الامينى ميثيونين والذي يعتبر ارتفاعه في الدم دلالة على خطورة الاصابة بتصلب الشرايين و أمراض الشريان التاجي و الجلطات الدماغية. ونظرا لعلاقة الهيموسيسيتاين بالاوعية الدموية الدقيقة لذا فقد تم اختبار تركيزه بالدم في حالات اعتلال الكلى الناتج عن مرض البوال السكري النوع الثاني وايضا علاقته بالطفرة الجينية MTHFR C677T و MTHFR A1298C. وقع الاختيار على ٧٥ مريضا بالبوال السكري النوع الثاني يصابها اعتلال كلوى و ٥٥ مريضا اخرين بالبوال السكري النوع الثاني ولكن بدون اعتلال الكلى و ٩٥ شخصاخرين اصحاء كمجموعة ضابطة. تم ملاحظة ارتفاع تركيز الهيموسيسيتاين بدم المرضى الذين يعانون اعتلال الكلى المصاحب لمرض البوال السكري النوع الثاني عن مرضى البوال السكري النوع الثاني ولا يعانون اعتلال الكلى وعن الافراد الاصحاء و قد لوحظ ايضا علاقة هذا الارتفاع بوجود الطفرة الجينية MTHFR C677T اكثر من الطفرة الجينية MTHFR A1298C.