The significant of C677T methylenetetrahydrofolate reductase MTHFR gene mutation in Iraqi patients with type 2 diabetic nephropathy

Salih M. Al-Khafaji1* Ph.D, MSc, Anwar M. Al-janabi Ph.D, MSc **
Shehab A. Faris MSc.*

*Department of Anatomy & Histology, College of Medicine, Kufa University
**Department of Clinical Biochemistry, College of Medicine, Kufa University

Key words: MTHFR, Diabetus, Nephropathy, C677T, Mutation, Gene, Iraq.

Abstract

This study aimed to address the association of C677T point mutation of MTHFR gene with Diabetic Nephropathy (DN) in Iraqi type 2 diabetes mellitus patients (DM).

We examined age; gender; duration of diabetic, Body mass index, glycosylated hemoglobin (HbAlc), albuminuria, blood sugar (RBS), homocysteine, serum urea and serum creatinine and MTHFR (C677T) gene polymorphism in 65 (DN) and 70 (DM) patients. After DNA extraction from blood samples of all participants the PCR-RFLP technique were applied for detection of MTHFR gene polymorphism. We found the means of serum homocysteine concentration, proteinuria, blood urea, serum creatinine which were significantly increased in DN patients when compared with DM patients (P<0.05). The minor allele frequency of T genotype was significantly increased in DN patients compared to the DM patients (P<0.0001) and the risk of DN was higher by 8.09 folds in homozygous allele genotype (TT) when compared with wild genotype (CC) (OR 8.09, 95% CI= 2.6-24.8, P = 0.0003). We concluded that the TT genotype of MTHFR
Introduction

Diabetic nephropathy (DN) is one of the long term complication of type 2 diabetes mellitus (T2DM) and is the main reason of endstage renal disease (ESRD) [1, 2]. The ESRD become a global health problem that increases the risk of death and disease [1, 2]. The proneness to DN differs among T2DM patients, and its etiology is multifactorial including environmental and genetic risk factors [3, 4]. The first one is characterized by unregularly glycaemic control and hypertension, while the second one was underlined by a genetic predisposition founded on familial clustering of DN [5, 6]. Many studies
weredicated on associations of genetic polymorphisms in candidate genes with the risk of ESRD [6–10].

The newest progresses in genomewide association studies (GWAS) have recognized a numeral of genetic-variants associated with deficiency of renal function [1]. The point mutation C677T of the gene that encoding an enzyme 5, 10-methylenetetrahydrofolate reductase (MTHFR) was newly described.[7]. Furthermore this variant has also been shown to correlate with elevated total plasma homocysteine concentrations.[8]. Serum homocysteine concentrations are augmented in initial renal failure and any further weakening in renal function[9].

(MTHFR) gene is situated on chromosome 1 (1p36.3). The conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate catalysis by MTHFR which is a substrate for remethylation of homocysteine to methionine. The deficiency of MTHFR is correlated with hyperhomocysteinaemia. Homocysteine play important role in the essential amino acid (methionine) metabolism. It is the end point for the formation of cysteine [10]. Higher homocysteine levels were recognized as a hazard
factor for diabetic nephropathy in type 2 diabetes [11, 12].

In Iraq no efforts have been made to shed the light on some of the genetic factors associated with DN in Iraqi population, thus the aim of this study was to explore a probable association of the MTHFR677C>T polymorphism with nephropathy in subjects with type 2 diabetes.

**Patients and method:**

The current study was done in Artificial Kidney Unit (AKU) in Al-Hakeem Hospital in Najaf/ Iraq. The study population was composed of one hundred and thirty five patients with type 2 diabetes mellitus (59 females and 76 males) their age range (46-70 years), they were identified with T2DM based on 1998 WHO classification and diagnostic criteria (WHO). This group divided to two subgroups.

The first subgroup: consist of seventy type 2 diabetic without nephropathy and the second subgroup of type 2 diabetic with nephropathy which consist of sixty five patients. (Those patients diagnosed by physician). Nephropathy was defined by urinary proteinurea level >1.0g/24hrs, increased blood urea and serum creatinine.

The patients group of diabetic without nephropathy (DM, N=70) was defined according to the following criteria: - 1/ duration of DM at least 5 years period,
2/normal albuminuria (i.e. \(<0.15\text{g/24-hrs}\)), 3/Normal values of renal function test which is measured by serum creatinine level \(\leq 1.2\text{mg/dl}\) and blood urea < 45mg/dl. The presence of diabetic nephropathy was determined by the following criteria: 1/ the presence of the albuminuria in urine samples \(>1.0\text{g/24-hrs}\) and abnormal values of renal function test.

Clinical parameters, which include age; gender; duration of diabetic, Body mass index, glycosylated hemoglobin (HbA1c), albuminuria, blood sugar, homocysteine, blood urea and serum creatinine were determined by standard enzymatic technique. Colorimetric method applied to evaluation of blood urea and serum creatinine was using RANDOX kits (United Kingdom BT 29 4QY) with standard procedures. Total plasma homocysteine was analyzed according to the manufacturer procedure for determination by using microplate enzyme immunoassay ELISA kit method of Biorad laboratories.

The specimens were taken after written informed consent obtained from all participants. This study, including the consent protocol, was approved by the Medical ethics committee / Faculty of Medicine / Kufa University.

From all patients a total of 5 milliliter venous blood samples were collected. 1 ml of blood sample was drowning in
EDTA tube for DNA extraction, another 4 ml of blood samples were drown into tubes free of anticoagulant material, these tubes were centrifuged for 10 min at 3000 rpm, and serum were separated and stored at -17°C until the assay was performed. Total plasma homocysteine was analyzed according to the manufacturer procedure for determination by using microplate enzyme immunoassay ELISA kit method of Biorad laboratories. DNA extraction that has been published previously [13]. The amplification of DNA was done by polymerase chain reaction (PCR) using primers mentioned by Alkhafaji S.M. [14]. The primers that used for PCR–RFLP were 5′-TGA AGG AGA AGG TGT CTG CGG GA-3′ forward and 5′-AGG ACG GTG CGG TGA GAG TG-3′ reverse that resulting of 198 bp PCR product. The reaction was carried out in a 25 μl mixture containing (10-100) ng template DNA, 15 pmol of each primer, 12.5 μl master mix contains of (20 mM MgCl2, 10 mM dNTPs, 5 U/μl Taq polymerase with 10X Taq Buffer) (Promega, USA). The reaction volume was completed by addition of nuclease free water. The conditions of PCR were as follows: At 94°C initial denaturation for 6 minutes followed by denaturation of 35 cycles at 94°C for 45 seconds, annealing for 45 seconds at 60°C, extension at 72°C for 45 seconds and final extension at 72°C for 5 minutes. The polymorphism was
detected by enzymatic digestion of the initial polymerase chain reaction product with HinfI at 37°C restrictaseHinfI (Promega, USA) at 37°C for 4 hrs. The resulting DNA fragments was separated on 3% agarose gel, after electrophoresis, the digested products were photographed under UV light. Accordingly, Samples who lack the mutation appeared one 198bp fragment, sample with heterozygous for the mutation revealed both 198bp and 175bp fragments, and homozygous sample revealed one 175bp fragment as shown in Fig.(1).

Statistical analyses.

SPSS software package (revision 20 Inc., Chicago, USA) were used to performed statistical analyses. Means ±SD are conducted for all data. Differences in distribution of genotype or alleles in patients and control were verified using the Chi-square statistic. Also diabetic nephropathy risk was tested and estimated by the use of Odds ratios (ORs) and 95% confidence intervals (95% CI). Values of P < 0.05 were considered statistically significant.

Table 1. Clinical Characteristics of patients and healthy control studied groups
Table 2: The genotype and allele frequency of SNP analysis among diabetic patients without nephropathy and diabetic patients with nephropathy groups.

<table>
<thead>
<tr>
<th>SNP Genotype/Allele frequency</th>
<th>Diabetes without nephropathy</th>
<th>Diabetes with nephropathy</th>
<th>OR (95% CI)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>50 (71.4%)</td>
<td>21 (32.3%)</td>
<td>1</td>
<td>Reference</td>
</tr>
<tr>
<td>CT</td>
<td>15 (21.4%)</td>
<td>27 (41.5%)</td>
<td>2.2</td>
<td>1.90-9.6</td>
</tr>
<tr>
<td>TT</td>
<td>5 (7.1%)</td>
<td>17 (26.2%)</td>
<td>8.0</td>
<td>2.6-24.8</td>
</tr>
<tr>
<td>C</td>
<td>115 (78.57%)</td>
<td>69 (53%)</td>
<td>1</td>
<td>Reference</td>
</tr>
</tbody>
</table>

OR= Odds ratio, CI=Confidence interval, SNP= Single Nucleotide Polymorphisms

Figure 1: The Hinfl restriction digested PCR product profile of MTHFR gene Lane M: DNA Marker, Lane 5 and 6 CC genotype, Lane 1,2, and 4 CT genotype, Lane 3, 7 TT genotype.

Results. Characteristic feature of patients. The clinical features of type 2 diabetic patients with as well as without nephropathy are showed in Table 1. The studied groups were matched for gender, age and body mass index (BMI), blood urea, serum creatinine, proteinuria and the levels homocysteine were considerably increased in patients with nephropathy when matched with patients without nephropathy as appeared in table (1).
substitution at locus 677 in exon 5 region of MTHFR gene is studied using SNP by the use of PCR-RFLP technique. The distributions of genotype and allele frequencies were compared between type2 diabetic patients with nephropathy DN patients and those of type2 diabetic patients without nephropathy DM (Table 2). The genotypes and allele frequency of the C677T MTHFR gene polymorphism in type2 diabetic patients with nephropathy are inconsistent with HWE, P= 0.024 while in type2 diabetic patients T2DM without nephropathy group are consistent with HWE, P= 0.18. The allele frequency and genotype of SNP of the MTHFR gene in DM patients 71.4% for CC, 21.4% for CT and 7.1% for TT respectively, whereas in DN patients group CC, CT and TT variants are at frequency of 32.3%, 41.5% and 26.2% respectively. The allele frequency obtained in the DM patients for C was (78.6%) and for T are (17.9%), whereas in the DN patients group for C was (53%) and for T was (47%). The genotypes frequency of C677T in TT variant 17 (26.2%) which was significantly increased the risk of nephropathy (p<0.05) by eight folds in homozygous genotype of DN patients when compared with wild genotype (OR8.09, 95% CI= 2.6-24.8, p=0.0003). The T allele frequency increased the risk of nephropathy in diabetic patients by four folds when compared with C allele (OR = 4.07, C.I=2.3-7.07, P= 0.0001)

Discussion

In this cross section study we attempted to appraised a possible role of the point mutation C677 T on gene coding for MTHFR enzyme as a risk factor that increasing nephropathy problems in diabetic patients so we summarized possible
association of mutation polymorphism of MTHFR (677 C>T) gene in diabetic patients with nephropathy in contrast to diabetic patients without nephropathy from Iraqi population.

Diabetes mellitus (DM) complicated to develop diabetic nephropathy (DN) and the diabetic nephropathy (DN) is a major factor of renal malfunction[15]. The environment and inheritance elements are involved in its etiology[16-17]. The incidence and prevalence of DN shows large ethnic differences, and 1/3 of diabetic patients progress to renal disease [19]. Indeed, many epidemiologic reports has been established the genetic predisposition and the risk is increased in relatives families with DN [19-21].

Our investigation has been conducted non relative subjects and the results revealed that there are no statistical differences in some of clinical characteristics between DN and DM patients with respect age, sex, diabetes duration, and BMI while other statistical analysis showed that there is a high significant difference in two groups as regard to homocysteine, proteinuria, blood urea, and serum creatinine. In fact, the association between the homocysteine and diabetic nephropathy are detected earlier elsewhere [22].

Homocysteine metabolism is achieved by the enzyme methylenetetrahydrofolate reductase (MTHFR). This enzyme catalyze the remethylation of homocysteine into methionine [23]. Single nucleotide mutation on the gene coding MTHFR enzyme lead to reducing the activity of the enzyme and increased homocysteine levels [24, 25].

Our results also showed that homozygous mutated TT genotypes of C677T and T allele polymorphisms was higher in study group of DN patients compared to DM. Indeed,
homozygous mutation for the 677 G→T in gene for MTHFR, causing decrease production of 5- methyltetrahydrofolate, the main of methyl donor in alteration of homocysteine to methionine which lead to rise of homocysteine in plasma [22]. 677 C→T mutation is responsible for reduced MTHFR activity, and it is found significantly effective only in recessive homozygous state [23]. On the other hand, the association between recessive homozygous 677C in MTHFR gene and DN, and the presence of higher 677C→T mutations in MTHFR gene among patients with DN, compared to DM patients, The TT genotype and T allele frequencies were increased and they were significantly increased in patients with nephropathy than in those without nephropathy. Since our diabetic patients without nephropathy had a higher frequency of the C allele than those with nephropathy (Table 3), supposedly it is possible that the C allele is protective against nephropathy, indeed this finding indicated that mutation in this gene might be a risk factor for DN. This result is consistent with similar studies of Shpitchinetsky et al. [26]. Furthermore, others investigators have been shown this conclusion for both type 1 and type 2 DM [27, 28]. However, not all investigators reach to same conclusion [29]. Indeed these studies were conducted in different populations, and some populations may have genetic factors that are protected from the development of DN in addition to raises in homocysteine. In fact Japanese population was show such a protective effect [30]. Numerous of investigations are proposed ethnicity may play protective effects against C677T mutation or other genetic factors. Although, a bulk of
studies demonstrated always that this mutation is correlated with elevation of total plasma homocysteine.

References


vascular disease: a common mutation in methylenetetrahydrofolate reductase. 


