The significant ofC677Tmethylenetetrahydrofolate reductase MTHFR gene mutation in Iraqi patients with type 2 diabetic nephropathy

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Abstract

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

We examined age; gender; duration of diabetic, Body mass index,glycosylated hemoglobin (HbAIc), 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 serumhomocysteine concentration,proteinuria, bloodurea, serum creatinine which were significantlyincreased inDN patients when compared withDM patients ($P<0.05$). The miner allele frequency of T genotype was significantly increased in DN patientscompared to the DMpatients($P<0.0001$) and the risk of DN was higher by 8.09 folds in homozygous allele genotype (TT) when compared with wild genotype (CC) (OR8.09, 95% CI= 2.6-24.8 P= 0.0003).We concluded that the TT genotype of MTHFR

gene is increasedthe risk of DN in type2 diabetic nephropathy patients of Iraqi population.

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Introduction

Diabetic nephropathy (DN) is one oflong term complication of type 2 diabetes mellitus (T2DM) and is the mainreason of endstage renal disease (ESRD) [1, 2].The ESRD become a global health problem that increasesthe risk of death and disease [1, 2].The proneness to DN differs among T2DM patients, and its etiology is multifactorial includingenvironmental and genetic risk factors [3, 4]. The first one ischaracterized byunregularlyglycaemic control and hypertension, while the second one wasunderlined by a genetic predisposition founded on familial clustering of DN [5, 6].Many studies

weredevoted on associations of genetic polymorphisms in candidate genes with the risk of ESRD [6–10]. The newestprogresses in genomewide association studies (GWAS) have recognized a numeral of genetic-variants associated with deficiency of renal function [1]. Thepoint mutation C677T of the gene that encoding an enzyme 5, 10-methylenetetrahydrofolate reductase (MTHFR) was newly described.[7].Furthermore thisvariant 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-metyltetrahydrofolatecatalysisby MTHFR which is a substrate for remethylationof homocysteine to methionine. The deficiencyof MTHFR iscorrelated with hyperhomocysteinaemia. Homocysteine play important role in theessential 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 ofthis study was to explore a probableassociation of the MTHFR677C>T polymorphism with nephropathy in subjects withtype 2 diabetes.

Patients and method:

The current study wasdone in Artificial Kidney Unit (AKU) in Al-Hakeem Hospital in Najaf/ Iraq. The study population was composed of one hundred and thirty fivepatients with type2 diabetesmellitus(59 femalesand 76 males)their age range (46-70 years),they were identifiedwith T2DM based on 1998 WHO classification and diagnostic criteria (WHO). This group divided to two subgroups.

The first subgroup: consist of seventy type2diabeticwithout nephropathy and the second subgroup oftype2diabetic withnephropathy which consist of sixty five patients. (Those patients diagnosed by physician).Nephropathy was defined by urinary protienurealevel >1.0g/24hrs,increased blood urea and serum creatinine.

The patients group of diabetic without nephropathy (DM, N=70) was defined according to thefollowing criteria:- 1/ duration of DM at least 5 yearsperiod,

2/normal albuminuria (i.e. <0.15g/24-hrs), 3/Normal values of renal function test which is measured by serum creatinine level ⩽1.2mg/dl and blood urea < 45mg/dl. The presence of diabetic nephropathy was determinedby the following criteria: 1/ the presence of the albuminuriain urine samples>1.0g/24-hrs and abnormal values of renal functiontest.

Clinical parameters, which include age; gender; duration of diabetic, Body mass index,glycosylated hemoglobin (HbAlc), albuminuria, blood sugar,homocysteine, blood urea and serum creatinine weredetermined 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 specimenswere 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 patientsa total of 5 milliliter venous blood samples were collected. 1 ml of blood sample wasdrowning 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 10min at 3000 rpm, and serum were separated and stored at -17°Cuntilthe assayed 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 polymerasechain 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′reversethat resultingof 198bp PCR product. The reaction was carried out in a 25 µl mixture containing (10-100) ng template DNA, 15pmol of each primer, 12.5 µl master mix contains of (20 mM MgCl2, 10 mM dNTPs, 5 U/ µlTaq polymerase with 10X Taq Buffer) (Promega, USA). The reaction volume was completed by addition of nuclease free water. Theconditions ofPCR were as follows: At 94°Cinitial denaturation for 6 minutes followed by denaturation of 35 cycles at 94°C for 45 seconds, annealing for 45 secondsat 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 of DNAfragments 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 performedstatistical analyses.Means ±SDare conducted for all data.Differences in distribution of genotype or alleles in patients and control were verified using the Chi-square statistic. Alsodiabetic nephropathyriskwas tested and estimatedby 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

Variable	DM Patients No. 70 means ±SD	DN Patients No. 65 means ±SD	P Value
Gender(Male/Female)	35/30	41/29	
Age at study (years)	48±4	47 ±3.4	0.12
Duration of diabetes (Years)	7 ± 3.1	8 ± 4.2	0.11
Glucose Levels (mg/dl.)	153.8 ± 28	146 ± 18.8	0.09
HbA1C (%)	8 ± 1.9	9.4 ± 1.7	0.1
Body mass index (kg/m2)	26 ± 2.0	27 ± 2.7	0.15
Blood Urea(mg/dl.)	118 ± 12.3	48 ± 3.6	P < 0.0001
Creatinine(mg/dl.)	8.8± 2.5	1.3± 0.2	P < 0.0001
Proteinuria (0.3g/24 h)	8.3± 2.0(g/24 h)	0.17 ±0.1 (g/24 h)	P < 0.0001
Homocysteine (µM)	16.7±2.1	6.0±0.9	P < 0.0001

Table 2: The genotype and allele frequency of SNP analysis among diabetic patients withoutnephropathy and diabetic patients with nephropathy groups.

SNPs Genotype/Allele frequency	Diabetes without nephropathy No=70	Diabetes with nephropathy No=65	OR	(95%C.I)	P-Value
CC	50 (71.4%)	21 (32.3%)	1	Reference	-------
CT	15 (21.4%)	27 (41.5%)	2.29	1.90-9.6	0.0004
TT	5 (7.1%)	17 (26.2%)	8.09	2.6-24.8	0.0003
C	115 (78.57%)	69 (53%)	1	Reference	-------
T	25 (17.9%)	61(47%)	4.07	2.3-7.07	0.0001

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

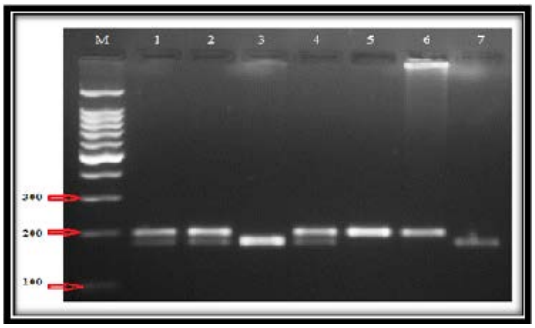

Figure 1: The HinfI restriction digested PCR product profile of MTHFR gene Lane M: DNA Marker, Lane 5 and 6 CC genotype, Lane 1,2, and 4 CT genotype, Lane 3, 7 TT genotype.

Results.

Characteristicfeature of patients.

The clinical featuresof type 2 diabetic patients with as well as without nephropathy areshowed in Table1. The studiedgroups were matched for gender,age and body mass index (BMI)blood urea, serum creatinine, proteinuria and the levels homocysteine wereconsiderablyincreased in patients with nephropathy when matchedwith patients without nephropathy as appeared in table (1).Nucleotide

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 diabeticpatients 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= 0.024while intype2 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,CTand TT variants are at frequency of 32.3%, 41.5% and 26.2% respectively. The allele frequency obtained in the DM patients for Cwas (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 toappraiseda possible role of the point mutationC677 T on gene coding for MTHFR enzyme as a risk factor that increasing nephropathyproblems in diabetic patients so we summarized possible

association of mutation polymorphism of MTHFR (677 C>T) gene in diabetic patients with nephropathy incontrast to diabetic patients without nephropathy from Iraqi population.

Diabetes mellitus (DM) complicated to developdiabetic nephropathy (DN) andthe diabetic nephropathy (DN) is a major factorof 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 1/3of diabetic patientsprogressto renal disease [19]. Indeed, many epidemiologic reports has been establishedthe 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 thehomocysteine anddiabetic nephropathy are detected earlier elsewhere [22]. Homocysteine metabolism is achieved by the enzyme methylenetetrahydrofolate reductase (MTHFR). This enzyme catalyze theremethylation of homocysteine into methionine [23].Single nucleotide mutation on the gene coding MTHFR enzyme lead to reducingthe activity of the enzymeand 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 C→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 significantlyeffective only in recessive homozygous state [23].On the other hand, the association between recessive homozygous 677C T in MTHFR gene and DN, and the presence ofhigher 677C→T mutations in MTHFR gene among patients withDN, 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 investigatorsreach to sameconclusion[29]. Indeed these studies were conducted in different populations, and some populations may have geneticfactors thatare 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 mutationor other genetic factors .Although, a bulk of

studies demonstrated always that this mutation is correlatedwith elevation of total plasma homocysteine.

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