

Original Research Article

The Association of Methylenetetrahydrofolate Reductase (MTHFR)/C677T Polymorphisms with The Development of Peripheral Neuropathy in Type 2 Diabetes Mellitus

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Abstract

One of important microvascular complication of patients with T2DM is neuropathy, commonly diabetic peripheral neuropathy (DPN). MTHFR/C677T polymorphisms affect MTHFR enzyme activity with subsequent elevation in the toxic homocysteine concentrations that result in vasculopathy and nerve ischemia. This case control study was planned to estimate the association between the MTHFR/C677T polymorphism and the occurrence of peripheral neuropathy in type 2 diabetic (T2DM) patients in a group of Iraqi people. Eighty three Iraqi subjects were included in this study, 36 had diabetic peripheral neuropathy, 25 without peripheral neuropathy and 22 were healthy control. Genomic DNA was isolated from fresh whole blood and genotyped using the polymerase chain reaction-based restriction fragment length polymorphism assay for the MTHFR gene C677T mutation.

The distribution of MTHFR/ C677T mutated genotypes between DPN and healthy groups was statistically differed with increased risk of occurrence of DPN (OR= 3.1, 95% CI: 1.02-9.4, p=0.05), while this risk was highly increased by seven folds when comparing DPN group with those without DPN (OR= 6.7, CI=2.1-20.8, p=0.0008). MTHFR/C677T polymorphism is highly associated with development of peripheral neuropathy in T2DM Iraqi population.

Key words: diabetes mellitus, diabetic peripheral neuropathy, methylenetetrahydrofolate reductase (MTHFR).

الخلاصة

يعتبر اعتلال الاعصاب المحيطية واحد من اهم مضاعفات الاوعية الدموية الدقيقة التي تصاحب مرض السكري النوع الثاني, كما ان الطفرة الجينية MTHFR / C677T تؤثر سلبا على عمل انزيم MTHFR مما يؤدي الى ارتفاع مستوى الهوموسيستين السام في الدم والذي بدوره يقلل من مستوى الارواء الدموي للاعصاب من خلال تأثيره الضار على وظائف الخلايا المبطنة للاوعية الدموية.

الهدف من الدراسة هو لتقدير ارتباط الطفرة الجينية MTHFR / C677T مع حدوث اعتلال الاعصاب المحيطية لمرضى السكري النوع الثاني. ادرج 83 متبرع في هذه الدراسة, 36 هم مرضى السكري الذين يعانون من اعتلال الاعصاب المحيطية, 25 ايضا هم مرضى السكري لكن لا يعانون من اعتلال الاعصاب المحيطية, اما الباقي (22) فهم اصحاء تاريخهم المرضي سليم. لجميع هؤلاء المتطوعين تم استخلاص الحامض الاميني من الدم الطازج وتمت دراسته باستعمال اساس تفاعل البلمرة المتسلسل الجينات. لقد توضح من خلال هذه الدراسة ان خطر حدوث وتطور اعتلال الاعصاب المحيطية لمرضى السكري النوع الثاني يزداد 7 اضعاف مع وجود الطفرة الجينية (OR= 6.7). MTHFR/ C677T.

الكلمات المفتاحية: داء السكري, اعتلال الاعصاب المحيطية.

Introduction

One of important microvascular complication of patients with T2DM is neuropathy, commonly diabetic peripheral neuropathy (DPN). DPN results in significant disability and morbidity [1] with many complications that include severe pain, immobility, recurrent ulcerations and infection and others [2].

Methylenetetrahydrofolate reductase (MTHFR) gene product is MTHFR enzyme which catalyzes the irreversible conversion of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate that donate methyl group to homocysteine forming adenosylmethionine which is considered as a big source of methyl groups in the body [3]. The damaging effects of homocysteine are arising from their spontaneous chemical reactions and inactivation of proteins and other biologically vital molecules [4].

Up to 24 mutations within MTHFR gene were reported, most of them are unimportant in the impact of activity of the enzyme and few causing deficiency of the enzyme but the only two polymorphisms that had been studied and affect enzyme activity and homocysteine concentrations were; C677T polymorphism on exon 4 and A1298C single nucleotide polymorphisms (SNP) on exon 7 [5].

Materials and Methods

Subjects

A total of 83 Iraqi subjects were included in this study, among them, 61 were patients suffering from type 2 diabetes mellitus (T2DM) that had been selected from those who attended the center of diabetes and endocrinology in Al-Sader and Merjan hospitals in Najaf and Babylon cities, respectively those patients were divided into 2 groups according to the presence or absence of peripheral neuropathy that was diagnosed by electrophysiological study, the remaining 22 subjects were healthy and match the patients in age, gender and geographical area.

Accordingly, the study groups include:

Group 1: 36 patients with DPN

Group 2: 25 patients without DPN

Group 3: 22 healthy control

The patient inclusion criteria:

- 1- All patients should have T2 DM
- 2- Their ages range between 30-60 years.
- 3- They should have BMI range between 18.5-29.5 Kg/cm²
- 4- Both genders was included.
- 5- All patients had negative past medical history apart from DM
- 6- No features suggestive of other causes of neuropathy like nutritional deficiency, endocrine abnormalities and others.

The purpose of this study and the nature of the neurophysiological tests were explained to the participants and verbal consents were taken before the start of the tests.

All patients undergo a full clinical evaluation, if the history of the patients, their BMI and blood pressure fit to the inclusion criteria then after blood sample was taken to do the blood investigation (FBS, or RBS, HbA1c, renal function test, lipid profile & thyroid function test), then NCS is performed to evaluate the presence or absence of peripheral neuropathy by examining sensory and motor nerves in both upper and lower limbs, including ulnar, tibial, and sural nerves using Micromid electromyography device.

Methods

Five to eight milliliters of blood were collected and dispensed in 3 tubes, one is gel tube to obtain sera that used to do some biochemical tests including blood glucose, cholesterol, triglycerides, high density lipoprotein, thyroid function test, blood urea and serum creatinine, and the other two are EDTA tubes. The blood in one of these EDTA tubes is used for fresh determination of HbA1c, while the other freshly collected blood samples in the second EDTA tube was used for extraction the genome DNA for genetic study.

Biochemical measurements

Serum urea, creatinine, total Cholesterol, High density lipoprotein, low density lipoprotein, Triglyceride, HbA1C and fasting blood sugar were done.

Genotyping analysis of MTHFR gene

DNA Extraction

The preparation of high-molecular weight genomic DNA from whole blood for all

participants was carried out by using QiAamp DNA mini kit

Determination of DNA Concentration, Purity & Integrity

The concentration of extracted DNA ($\mu\text{g/ml}$) was measured by adding 50 μl of stock DNA into the plastic disposable cuvette (Eppendorf UVette) and evaluated at 260 nm wavelength, while for measurement the contamination of protein, 280 nm wavelength was used. The purity of DNA was assessed by measuring the ratio of A260/A280 while the integrity of DNA was measured by using 1% agarose gels electrophoresis.

PCR Amplifications

The 198 bp DNA fragment of exon 4 of MTHFR gene containing the C677T polymorphism was amplified by conventional PCR technique using primers 5'-CAT CCC TAT TGG CAG GTT AC-3' and 5'-GAC GGT GCGGTG AGA GTG-3' [13].

PCR- based RFLP analysis

RFLPs was obtained by digestion of 198 bp amplified product with HinfI restriction enzyme (1000 unit (U), Promega/USA) at the restriction site, 5'...G ∇ ANT C...3' 3'...C TNA \blacktriangle G...5'

Detection of Restriction Fragment length polymorphism

3% agarose (run at 60 V for 3 hrs) and 8% non-denaturing polyacrylamide gel electrophoresis had been used to separate RFLPs results.

Statistical analysis

In this case control study, all statistical analyses were performed by using Statistical Package of Social Science software (SPSS) computer program (Version 22, SPSS Inc., Chicago, IL, USA)

The ANOVA test was used to test the level of significance (p-value) for all parameters between the study groups. Chi-square test was used also to determine the percentage and the significant level of the categorical variables. A p-value equal to or less than 0.05 and 0.001 were considered to be significant and highly significant, respectively [6].

Results

Detection of MTHFR genotype:

Genomic DNA was successfully extracted from fresh whole blood of all three groups subjected in the present study and the PCR amplification was efficaciously performed for all presented DNA samples that yield specific 198 bp fragment containing the C-677T polymorphism of MTHFR gene.

MTHFR gene genotyping was done by restriction analysis using the *HinfI* restriction enzyme that determines the C-677T polymorphism present in the specific amplified PCR products. *HinfI* restriction enzyme was used to digest C-677T polymorphism of 198 bp PCR product giving rise to undigested C/wild-type allele (198 bp fragment) and digested T/mutant allele (175 and 23 bp fragments). The resulted Restrictional Fragment length polymorphisms analyzed on polyacrylamide gel represented into: homozygotes wild-type alleles (CC), heterozygotes alleles (CT), and homozygotes mutant alleles (TT) were analyzed on 3% agarose gel and 8% non-denaturated polyacrylamide gel that showed in Fig. 1. A and Fig 1. B, and C respectively.

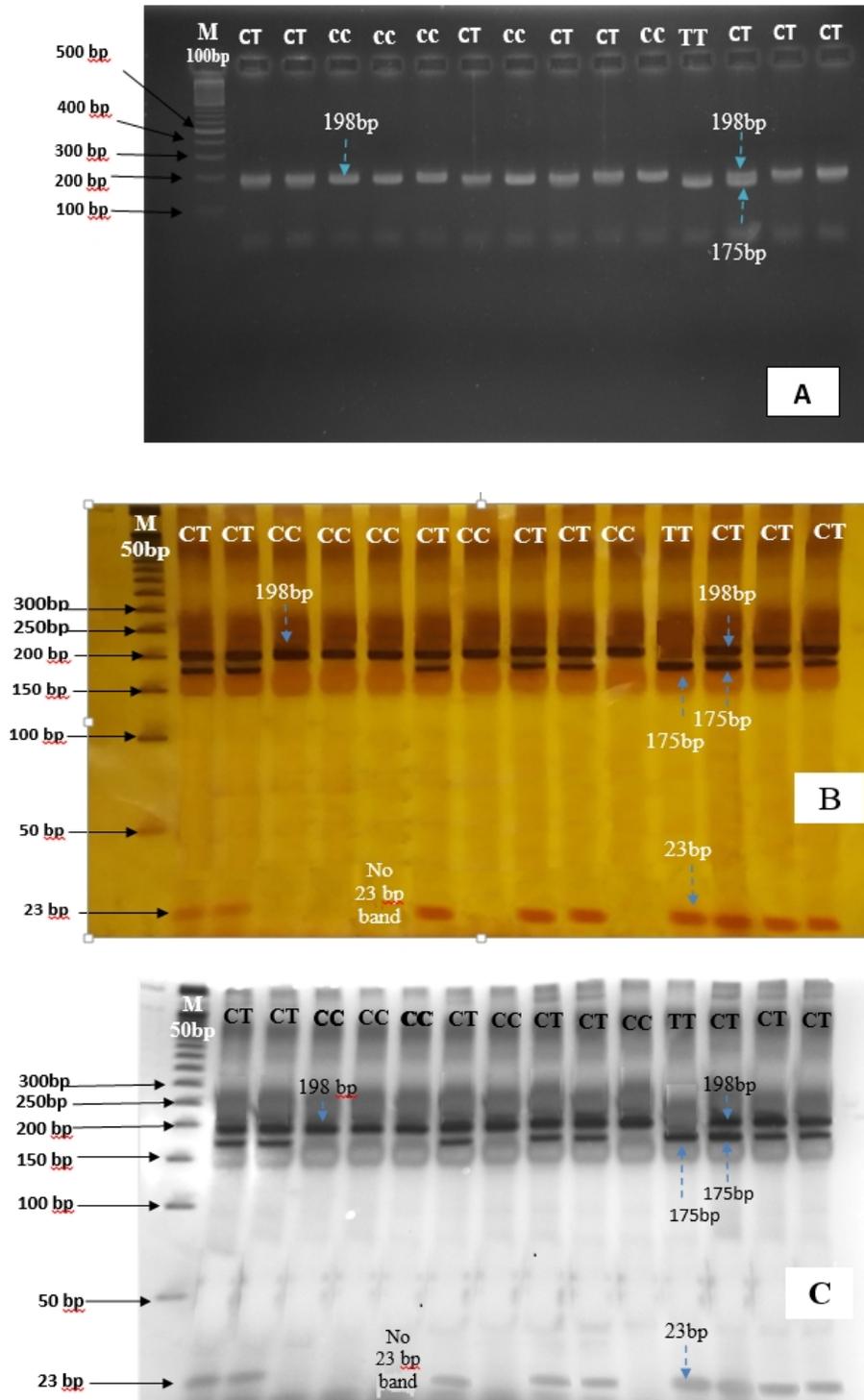


Figure 1:Example of RFLPs Patterns of MTHFR gene/ C-677T polymorphism showing different MTHFR gene genotypes, analyzed on 3% agarose gel (A) and on 8% non-denaturing polyacrylamide photographed by digital camera (B) on 8% non-denaturing polyacrylamide photographed by light and white light converter plate (C).

A: at 60V and room temperature for 3 hour, M: molecular weight marker(100bp), band 23 not recognized.
 B&C: at 180V and room temperature for 2-3 hour, M: molecular weight marker(50bp), band 23 well recognized. A, B, C: Lane CC: homozygotes wild-type, Lane CT: heterozygotes type, Lane TT: homozygotes mutant type.

Distribution of MTHFR gene/ C677T Genotypes

The frequency of the different MTHFR gene/ C677T genotypes and alleles for the three study groups are shown in table (1). MTHFR/ C677T mutated genotypes (included CT and TT) were compared with CC wild type between the DPN group (group1) with healthy control (group3) and with internal control (group 2). The distribution of mutated genotypes between groups 1 & 3 was statistically differed with increased risk of occurrence of DPN (OR=

3.1, 95% CI: 1.02-9.4, p=0.05), while this risk was highly increased by seven folds when comparing groups1 & 2 (OR= 6.7, CI=2.1-20.8, p=0.0008). Statistically significant relation of C677T/T allele was detected with 2.2 and 4.6 risk for DPN occurrence throughout the comparison of group 1 with group 3 and group 2, (OR= 2.2, CI= 1-5.1, P= 0.03 and OR= 4.6, CI= 1.8-11.7, p=0.006), respectively.

Table 1: Distribution of MTHFR /C677T genotypes and allele frequency in DPN patients in comparison with internal and healthy control groups

MTHFR/ C677T	Group 1 N=36	Group 2 N=25	OR (95% CI)	P value	Group 1 N=36	Group 3 N=22	OR (95% CI)	P value
Genotypes								
CC	10 (27.8%)	18 (72%)	6.7 2.1-20.8	0.000 8	10 (27.8%)	12 (54.5%)	3.1 1.02- 9.4	0.05
CT	21 (58.3%)	7 (28%)			21 (58.3%)	10 (45.5%)		
TT	5 (13.9%)	0			5 (13.9%)	0		
Allele frequency								
C allele	41(56.9%)	43(86%)	4.6 1.8-11.7	0.000 4	41(56.9%)	34 (77.2%)	2.2 1-5.1	0.03
T allele	31(43.%)	7 (14%)			31(43.%)	10(22.7%)		

Group 1= patients with DPN, Group 2= patients without DPN, Group 3= healthy control

Discussion

Many studies have been conducted to estimate the association between MTHFR gene polymorphism and the development of T2DM [7-9] as well few other researchers concerned with study of the effect of MTHFR gene polymorphism on the development of diabetic complications like retinopathy, nephropathy and neuropathy [10-13].

To our knowledge, the present study might be the first study concerned with the role of MTHFR/C677T gene polymorphism in development of diabetic neuropathy among Iraqi patients.

In this study MTHFR/C677T genotypes (CC, CT and TT) was detected depending on RFLPs analyzed on polyacrylamide gel

and staining with the silver nitrate due to polyacrylamide gel have the ability to separate DNA fragments much more than the agarose gel. Polyacrylamide gel can separate the DNA fragments from each other even though they differ in their lengths by single nucleotide. Furthermore silver staining is highly sensitive to detect 1pg of DNA providing high band resolution than the Ethidium bromide and cybergreen staining; therefore, the 198 and 175 bp bands were well separated from each other and 23 bp was recognized easily.

Of the 36 diabetic patients with DPN, 26 had MTHFR/C677T polymorphism, 5 had homozygotes mutant alleles (TT) and 21 had heterozygotes alleles (TC), while the remaining 10 patients had wild-type alleles

(CC).The frequency of CT genotype was 58.3% in DPN group which is higher than other groups while TT genotype (13.9%) was presented only in DPN group with Tallele frequency 43% (Table 1).

Yigit et al. [13] evaluate the association of MTHFR/C677T polymorphism with the retinopathy and DPN in T2DM Turkish patients and they found that this polymorphism is related to DPN and to retinopathy.

Our current data revealed that diabetic patients with DPN whom carry CT and TT genotypes had significantly increased risk of DPN by 3 folds when compared to the CT and TT healthy individuals carriers, while interestingly; this risk was highly increased by seven folds during comparison with the diabetic patients without DPN patients (CT and TT genotypes) (Table 1). Similar findings was obtained regarding risk of C677T/T allele frequency to the DPN occurrence when compared diabetic patients with DPN to the healthy individuals and to the diabetic patients without DPN patients (2.2 and 4.6 folds, respectively)(Table 1).

Unfortunately, there are no studies concerned with the development of DPN among diabetic patients in Iraq, however, Wang *et al.* [14] establish the association of MTHFR/C677T polymorphism with development of neuropathy in T2DM Chines patients, further Yigit et al. [13] demonstrated that MTHFR/C677T polymorphism was related to the DPN development in T2DM Turkish patients, and both of these studies are consist with our results.

This may be explained by the fact that C677T polymorphism of MTHFR gene is associated with elevated level of homocysteine [15-17].

Hyperhomocysteinemia affect nerves function through either direct cytotoxic damage or by oxidative injury to the endothelial cells of vasa nervosa. This usually results in nerve ischemia with subsequent development of neuropathy [18-20]. Alternatively, thrombosis of the microvasculature and hypercoagulability

with reduction the perfusion in the arterial microvasculature (and subsequently neuropathy) is shown to be associated with MTHFR 677TT genotype [21].

Taken as a whole, the present result suggests that MTHFR/C677T polymorphism might have a predisposing risk for the DPN development in Iraqi patients suffering from T2DM.

Conclusion: MTHFR/C677T polymorphism is associated with development of peripheral neuropathy in T2DM Iraqi population, and the heterozygous genotype (CT) of MTHFR gene increases the risk of development the DPN by 7 folds.so, screening for MTHFR/C677T polymorphism in type 2 diabetes mellitus patients is important, Otherwise, Folic acid should supplement the treatment of these patients.Also, studying similar parameters with strict limitation of the diabetes duration taking in consideration their glycemic control with increase sample size.

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