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Synergistic effects of the MTHFR C677T and A1298C polymorphisms on the increased risk of micro- and macro-albuminuria and progression of diabetic nephropathy among Iranians with type 2 diabetes mellitus

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ABSTRACT

Objectives: To find whether polymorphisms of methylenetetrahydrofolate reductase (MTHFR) C677T and A1298C are risk factors for diabetic nephropathy (DN) among type 2 diabetes mellitus (T2DM) patients from Western Iran.

Design and methods: The MTHFR polymorphisms were detected in 72 microalbuminuric, 68 macroalbuminuric and 72 normoalbuinuric T2DM patients by PCR-RFLP.

Results: The possession of both MTHFR 677T and 1298C alleles increase the risk of microalbuminuria to 4.3-fold ($p=0.007$) in T2DM patients. The presence of either MTHFR 677T, 1298C allele is sufficient to increase the risk of macroalbuminuria in T2DM patients by 4.1 and 5.5 times ($p=0.027$, and $p=0.006$, respectively). The concomitant presence of both 677T and 1298C alleles act in synergy to increase the risk of macroalbuminuria by 20.4-fold ($p<0.001$) and progression of DN from microalbuminuria to macroalbuminuria (OR = 4.73, $p=0.01$).

Conclusion: Both MTHFR 677T and 1298C alleles increased the susceptibility to the onset and progression of DN in Iranians with T2DM.

Introduction

Due to the influence of genetic factors and metabolic control in the pathogenesis of diabetic nephropathy (DN), its development varies among diabetic patients [1]. Diabetic nephropathy is a serious microangiopathic complication of diabetes mellitus, and is the leading cause of end-stage renal failure [2,3].

The methylenetetrahydrofolate reductase (MTHFR) is an enzyme catalyses the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a co-substrate for homocysteine remethylation to methionine. Severe MTHFR deficiency is associated with hyperhomocysteinemia [4]. Elevated levels of plasma homocysteine are associated with diabetic nephropathy [5,6]. One of the MTHFR gene polymorphisms is a single point mutation at nucleotide 677, leading to a Val/Ala amino acid exchange at position 222, which has been reported to be associated with moderate hyperhomocysteinemia when folate levels are low in homozygotes (677TT) compared with heterozygotes or non-carriers [2]. This polymorphism is located in the catalytic domain of enzyme and results in a thermolabile protein [2,4]. The A1298C missense mutation is another MTHFR polymorphism located in the regulatory domain of enzyme and is not associated with increased plasma homocysteine level [2,4]. There are conflicting reports related to the association between diabetic nephropathy and MTHFR polymorphism [1–10].

Iran is a land of different ethnic groups, which include Farsis, Turks, Kurds, Lurs, Baluches, Arabs, Bakhtiaris, Azari, Taleshes, Turkmans, Ghashghais, and Armenians. Kermanshah Province is located in Western Iran and Kurds are the prominent ethnic group living in this area [11]. In a population-based study by Azimi-Nezhad et al. [12] a prevalence of 5.5% was established for type 2 diabetes mellitus (T2DM) among Iranians.

Recently, we established a frequency of 27.85% for T allele of MTHFR C677T among healthy individuals from Western Iran [13]. Also, we indicated that this variant is not an independent risk factor for coronary artery disease (CAD) or diabetes in the population of

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Western Iran [14]. There is no sufficient evidence to indicate an association between MTHFR 677TT and risk of cardiovascular disease [15] and MTHFR TT genotype is not a significant risk factor for development of cardiovascular disease in T2DM patients [16]. However, in one study MTHFR C677T allele in T2DM patients with renal failure (diabetic nephropathy) has been indicated as a strong risk factor for atherosclerotic disease [17]. In other studies neither MTHFR C677T nor A1298C in chronic renal disease patients have been associated with cardiovascular complications [18].

The present study was conducted to examine whether MTHFR C677T and A1298C polymorphisms are associated with the onset and progression of diabetic nephropathy in T2DM patients from Western Iran.

Materials and methods

The studied individuals consisted of 68 macroalbuminuric patients (33 male patients and 35 female patients aged 57.1 ± 8.7 years) and 72 diabetic patients with microalbuminuria including 26 males and 46 females, aged 55.3 ± 8.6 years. The control group included 72 age- and sex-matched normoalbuinuric diabetic patients including 23 males and 49 females aged 54.35 ± 7.93 years. All individuals who participated in the study were referred to the Taleghani Diabetes Research Center of Kermanshah University of Medical Sciences. The ethnic background of all individuals was Kurds.

From all individuals detailed demographic, biochemical and medical history including age, sex, diabetic duration, body mass index (BMI), history of hypertension, the presence of nephropathy, retinopathy, neuropathy, coronary artery disease (CAD), percent of HbA_{1C}, the level of lipids and lipoproteins, serum creatinine level, and creatinine and albumin excretion during 24 h were recorded. Informed written consent was obtained from each individual before participation. The study was approved by the Ethics Committee of Kermanshah University of Medical Sciences and was in accordance with the principles of the Declaration of Helsinki II. Microalbumin was measured by immunoturbidimetric assay for urinary microalbumin using available commercially kits (Randox laboratories Ltd, Canada). Microalbuminuria and macroalbuminuria were defined as albumin to creatinine ratio (ACR) of 30–299 mg/g and ≥300 mg/g, respectively in a random spot collection in two of three specimen collected within a 3- to 6-month period. Controls were those diabetic patients with ACR<30 mg/g [19]. Type 2 diabetes mellitus was diagnosed according to WHO criteria [20].

DNA was extracted from the leukocyte fraction of the EDTA-treated whole blood by using the phenol-chloroform method [21].

For detection of MTHFR C677T, a 198-bp region in exon 4 was amplified using the forward primer of 5' TGA AGG AGA AGG TGT CTG CGG GA 3' and the reverse primer of 5' AGG ACG GTG CGG TGA GAG TG 3' as described by Frosst et al. [22]. Amplification was carried for 40 cycles at 94 °C for 0.5 min, 62 °C for 0.5 min, 72 °C for 0.5 min, with a final extension period of 5 min at 72 °C. The 198-bp PCR product (10–15 μl) was digested with 5 units of the restriction enzyme *Hin*fI at 37 °C overnight. The C to T substitution in the 198-bp fragment creates a *Hin*fI recognition sequence which digests the 198-bp fragment into 175 and 23-bp fragments. The *Hin*fI treated PCR fragments were analyzed by 3% agarose gel electrophoresis. For detection MTHFR A1298C the following primers were used: forward, 5′ CTT TGC GGA GCT GAA GGA CTA CTA C 3′, and reverse, 5′ CAC TTT GTG ACC ATT CCG GTT TG 3′ [2]. The 163-bp PCR product was digested with 5 units of the *Mbo*II restriction enzyme. The presence of A1298C mutation produces 84-, 31-, 30- and 18-bp fragments, while in the absence of mutation 5 fragments of 56-, 31-, 30-, 28-, and 18-bp are produced.

Statistical analysis

The allelic frequencies were calculated by the gene counting method. The genotypes and MTHFR allele frequencies in patients were compared to controls using χ^2 test. Odds ratios (OR) were calculated as estimates of relative risk for disease and 95% confidence intervals (CI) obtained by SPSS logistic regression. The interaction between the two polymorphisms of MTHFR C677T and A1298C was determined using logistic regression model. Using Vaisi-Raygani et al. report [23], four categories defined by the presence (+) or absence (−) of a 677T or 1298C allele. The correlation values of biochemical and clinical data with the MTHFR polymorphisms between studied groups were calculated using linear regression and an unpaired t test. Two-tailed Student's *t*-test and ANOVA analysis were also used to compare quantitative data. Statistical significance was assumed at the $p<0.05$ level. The SPSS statistical software package version 16.0 was used for the statistical analysis.

Results

Characteristics of normoalbuminuric, microalbuminuric and macroalbuminuric T2DM patients are demonstrated in Table 1. As could be seen in Table 1, macroalbuminuric patients had significantly higher systolic and diastolic blood pressure values and duration of diabetes than normoalbuminuric and microalbuminuric patients. Total cholesterol level was significantly lower in macroalbuminuric patients than normoalbuminuric ($p=0.001$) and microalbuminuric patients ($p=0.008$). Also, the level of HDL-C was significantly ($p=0.009$) lower in macroalbuminuric patients compared to normoalbuminuric patients. In macroalbuminuric patients with MTHFR CT plus TT genotype compared to normoalbuminuric patients with the same genotype the incidence of neuropathy was higher in the first group (68.3% vs. 48.1%). Only a trend ($p=0.079$) for higher level of HbA_{1C} was found in macroalbuminuric patients carrying MTHFR C677T (677CT: 7.76 ± 1.76% and 677TT: 8.44 ± 1.47%) compared to those with MTHFR 677CC (7.20% ± 1.65). However, this difference was significant ($p=0.022$) comparing 677TT with 677CC genotype.

MTHFR C677T genotypes and alleles

Macroalbuminuric group

The overall frequencies of 677T allele in normoalbuminuric and macroalbuminuric patients were 19.4% and 41.9%, respectively (Table 2). The overall prevalence of C677T in normoalbuminuric patients was 37.5% (the heterozygous state 36.1% and homozygous state 1.4%). In macroalbuminuric patients, 25 heterozygous (36.8%) and 16 homozygous (23.5%) individuals were found for MTHFR C677T polymorphism giving a prevalence of 60.3%. The frequency of T allele in macroalbuminuric patients was 41% in males and 42.9% in females. In normoalbuminuric male and female patients the frequencies of 19.8 and 19.4% were found for T allele, respectively.

The distribution of MTHFR C677T genotypes was found to differ in macroalbuminuric group ($\chi^2=7.27$, $p=0.007$) compared to normoalbuminuric group. In addition, the frequency of MTHFR 677T allele was found to be 2.1 ($p<0.001$) times in macroalbuminuric group than that of normoalbuminuric group (Table 2). Computation of the OR (95%CI) as an estimate of relative risk for nephropathy indicated that individuals with the MTHFR 677T allele were found to be 3.0 times ($p<0.001$) more likely to suffer from diabetic nephropathy (Table 3).

Microalbuminuric group

Table 2 depicted the genotype and allele frequencies of MTHFR C677T in microalbuminuric patients compared to normoalbuminuric patients. The respective frequencies of the CC, CT, and TT genotypes were 45.8, 52.8, and 1.4% in microalbuminuric group, and 62.5, 36.1, and 1.4% in normoalbuminuric group. The allele frequencies for T allele in microalbuminuric patients and in normoalbuminuric group were 27.8 and 19.4%, respectively ($p=0.096$). The frequency of CT + TT genotype compared to CC genotype in microalbuminuric patients was significantly higher (54.2%) than that in normoalbuminuric