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# Association of the C677T Polymorphism in the Human Methylenetetrahydrofolate Reductase (*MTHFR*) Gene with the Genetic Predisposition for Type 2 Diabetes Mellitus in a Moroccan Population

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*Aims:* Type 2 diabetes mellitus (T2DM) is a major public health problem around the world. The C677T and A1298C polymorphisms of the methylenetetrahydrofolate reductase (*MTHFR*) gene have been reported to be associated with T2DM and its complications. This study aimed to investigate this association in the Moroccan population. *Methods:* A case-control study was performed among 282 Moroccan diabetic patients and 232 healthy controls. The *MTHFR* C677T and A1298C polymorphisms were genotyped by polymerase chain reaction, followed by enzymatic digestion with *HinfI* and *MboII* enzymes, respectively. *Results:* There was a significant association between C677T polymorphism and T2DM in both additive and dominant models. In addition, the 677T allele frequency differed significantly between the diabetic and control groups (26.06% vs. 33.20%, respectively). However, no significant association was found between A1298C polymorphism and T2DM. The frequencies of combined genotypes 677CC/1298AA and 677CT/1298AC differed significantly between the diabetic and control groups (32.62% vs. 20.61% and 9.57% vs. 17.55%, respectively). *Conclusions:* These results show an evident association between the *MTHFR* C677T polymorphism and T2DM in Moroccan patients but no significant association with the *MTHFR* A1298C polymorphism.

## Introduction

Type 2 diabetes mellitus (T2DM) is a major public health problem around the world. It is caused by both decreased insulin sensitivity and impaired insulin secretion due to pancreatic β-cell defects (Permutt *et al*., 2005; Stumvoll *et al*., 2005). The number of people with T2DM is rapidly increasing; by 2030 an estimated 366 million people will have the disease worldwide (Wild *et al*., 2004). In Morocco, as in other developing countries, the prevalence of diabetes has increased dramatically with the adoption of a new lifestyle of overnutrition and reduced physical activity. The frequency of affected persons age 20–76 years is estimated to be about 10% (International Diabetes Foundation, 2009).

T2DM is a complex and multifactorial disease in which multiple genetic variants appear to interact with environmental factors. The genetic component of T2DM was shown by strong familial aggregation and high concordance in twins (Newman *et al*., 1987; Lee *et al*., 2000). Much effort, including genome-wide linkage, candidate-gene, and genome-wide association studies, has been devoted to find common T2DM genes. Numerous variants within several genes that confer an increased susceptibility to T2DM have been identified. However, only a small number of them have been identified as strong candidates (Scott *et al*., 2007; Zeggini *et al*., 2008).

Among these, the methylenetetrahydrofolate reductase (*MTHFR*) polymorphisms have been previously found to be associated with T2DM and some of its complications (Maeda *et al*., 2003; Sun *et al*., 2005; Mehri *et al*., 2010). The *MTHFR* gene, located on chromosome 1 (1p36.3), encodes for methylenetetrahydrofolate reductase enzyme, which plays an important role in folate metabolism (Ralph *et al*., 2001). Some polymorphisms in the *MTHFR* gene reduce enzymatic activity and cause hyperhomocysteinemia (Ksiazek *et al*., 2004; Sun *et al*., 2004). The C677T polymorphism converts an alanine residue to a valine (ALA222VAL), leading to lower enzymatic activity (Yoshioka *et al*., 2004). Compared with the 677CC wild genotype, the 677TT homozygous and 677CT heterozygous genotypes decrease enzyme activity by approximately 70% and 40%, respectively (Frosst *et al*., 1995;

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Weisberg *et al*., 2001). The prevalence of this polymorphism differs among ethnic groups, ranging from 2% to 54.5% (Pepe *et al*., 1998). In the healthy Moroccan population, the 677T allele and the 677TT genotype frequencies were found to be 26.4% and 6 %, respectively (Paluku *et al*., 2009). The second polymorphism of the *MTHFR* gene, A1298C, causes a substitution of glutamate by an alanine residue (GLU429ALA). This polymorphism has also been associated with 30% reduced enzyme activity compared with that seen with the C677T polymorphism with a 30% reduction (Weisberg *et al*., 2001).

In a previous study we reported a positive familial aggregation of T2DM in a Moroccan population (Benrahma *et al*., 2011). In this study, we investigated the association of the *MTHFR* C677T and A1298C polymorphisms with T2DM mellitus in a Moroccan population.

## Materials and Methods

### Patients

In this case-control study we recruited 282 Moroccan patients with T2DM (98 men and 184 women; mean age ± standard deviation, 57.32 ± 11.15 years). All patients were diagnosed at the Department of Metabolic Diseases in the Ibn Rochd University Hospital Center of Casablanca, Morocco, according to World Health Organization criteria. Clinical and anthropometric data were available for all patients. Eighty-six patients had neuropathic complications, 49 had cardiovascular complications, 7 had nephropathic complications, and 101 patients were obese. The control group consisted of 232 unrelated healthy individuals (81 men and 181 women; mean age, 54.70 ± 7.84 years) recruited from the Pasteur Institute of Morocco. The inclusion criteria for the control group were age older than 40 years, no history of diabetes, no diabetes in first-degree relatives, and fasting plasma glucose level less than 1.1 g/L.

The patients and controls had different geographic and ethnic backgrounds. All patients provided informed consent before participation in this study. The institutional ethical committee approved the study.

### Genetic analysis

Genomic DNA was extracted from whole blood by using conventional phenol-chloroform-isoamyl alcohol (Sambrook *et al*., 1989).

To detect the C677T and A1298C polymorphisms, polymerase chain reaction (PCR) conditions and restriction fragment-length polymorphism analyses were performed according to previous published protocols (Fross *et al*., 1995; Van der Put *et al*., 1998).

The C677T polymorphism was identified by the enzymatic digestion of a 198–base pair (bp) PCR amplicon with the *HinfI* restriction endonuclease. The wild-type allele (677C) corresponds to the presence of 1 band of 198 bp, and the mutant allele (677T) corresponds to the presence of 2 bands of 175 bp and 23 bp. The heterozygous genotype (CT) corresponds to the presence of 3 bands of 198, 175, and 23 bp.

The A1298C polymorphism was identified by the enzymatic digestion of a 163-pb PCR amplicon with the *MboII* restriction endonuclease. The wild-type allele (1298A) corresponds to the presence of 5 bands of 56, 31, 30, 28, and 18 bp. The mutant allele (1298C) corresponds to the presence of 4 bands of 84, 31, 30, and 18 bp. The heterozygous (1298AC) corresponds to the presence of 6 bands of 84, 56, 31, 30, 28, and 18 bp.

After digestion, the PCR product was resolved electrophoretically on 3% agarose gel.

### Statistical analysis

After determination of allelic and genotypic distribution, Hardy-Weinberg equilibrium was tested by using HWE2 program, version 2. To test the association between *MTHFR* polymorphisms and T2DM, we compared both allelic and genotypic distributions between the patient and control groups. Further, we tested whether certain genotypic combinations could be associated with an increased risk for T2DM. Odds ratios (ORs) and their 95% confidence intervals (CIs) were computed to assess strength of association. All statistical analyses were performed with STATA software, version 11.0 (Stata Corp, College Station, TX), and CONTING software, version 2.80 (Statistical Genetics Utility programs J. Ott, Beijing Institute of Genomics, Chinese Academy of Sciences). Statistical significance was accepted at a $p$ value less than 0.05.

## Results

In this study we genotyped the C677T and A1298C polymorphisms in 280 patients and in 232 healthy persons. The

Table 1. Genotype Frequency and Hardy-Weinberg Equilibrium of the 2 *MTHFR* Polymorphisms (C677T and A1298C) in Patients with Type 2 Diabetes Mellitus and Healthy Controls

| *SNPs* | *Genotype* | *Patients,* n *(%)* (n = *282*) | *Controls,* n *(%)* (n = *262*) | *Additive model* | | *Recessive model* | | *Dominant model* | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | *OR (95% CI)* | p *value* | *OR (95% CI)* | p *value* | *OR (95% CI)* | p *value* |
| A1298C | AA | 180 (63.83) | 147 (56.10) | 1.32 (0.99, 1.75) | 0.058 | 1.49 (0.67, 3.32) | 0.319 | 1.38 (0.97, 1.95) | 0.066 |
| | AC | 91 (32.27) | 100 (38.17) | [1.31 (0.97, 1.76)][a] | [0.071][a] | [1.48 (0.65, 3.33)][a] | [0.34][a] | [1.36 (0.96, 1.94)][a] | [0.080][a] |
| | CC | 11 (3.90) | 15 (5.73) | | | | | | |
| HWE | | 1 | 0.868 | | | | | | |
| C677T | CC | 160 (56.74) | 114 (43.51) | 1.39 (1.07, 1.81) | 0.011 | 1.13 (0.63, 2.01) | 0.672 | 1.70 (1.20, 2.39) | 0.0021 |
| | CT | 97 (34.90) | 122 (46.56) | [1.46 (1.12, 1.96)][a] | [0.005][a] | [1.22 (0.68, 2.21)][a] | [0.49][a] | [1.79 (1.26, 2.53)][a] | [0.001][a] |
| | TT | 25 (8.87) | 26 (9.92) | | | | | | |
| HWE | | 0.087 | 0.486 | | | | | | |

In the additive model, homozygotes for the major allele (1/1), heterozygotes (1/2), and homozygotes for the minor allele (2/2) were coded to a continuous numeric variable for genotype (0, 1, and 2). The dominant model: was defined as 1/1 + 1/2 vs. 2/2, and the recessive model as 1/1 vs 1/2 + 2/2.

[a]Adjusted for age and sex.

CI, confidence interval; HWE, Hardy-Weinberg equilibrium; OR, odds ratio; SNP, single-nucleotide polymorphism.

Table 2. Allele Frequencies of *MTHFR* C677T and A1298C Polymorphisms in Patients with Type 2 Diabetes Mellitus and Healthy Controls

| *Genotype* | *Allele* | *Patients,* n *(%)* | *Controls,* n *(%)* | *OR (95% CI)* | p *value* |
|---|---|---|---|---|---|
| A1298C | A | 451 (79.96) | 394 (75.19) | | |
| | C | 113 (20.0) | 130 (24.81) | 0.75 (0.57, 1.01) | 0.058 |
| C677T | C | 417 (73.93) | 350 (66.79) | | |
| | T | 147 (26.06) | 174 (33.20) | 0.70 (0.54, 0.95) | 0.009 |

CI, confidence interval; OR, odds ratio.

C677T and A1298C genotypes were in agreement with Hardy-Weinberg equilibrium (Table 1).

The data analysis of distribution of C677T *MTHFR* genotypes (Table 1) revealed that in patients with T2DM, the 677TT genotype was found in 8.84%, the 677CT genotype in 34.90%, and the 677CC genotype in 56.74%. In the control group, 9.92 % had the 677TT genotype, 46.56% had 677CT, and 43.51% had 677CC. The C677T polymorphism was significantly associated with T2DM in both additive (odds ratio, 1.39; 95% CI, 1.07, 1.81; $p=0.011$) and dominant (OR, 1.70; 95% CI, 1.70, 2.39; $p=0.0021$) models, but no association was seen in the recessive model, even after adjustment for age and sex. Furthermore, comparison of 677T allelic frequencies between patient and control groups, presented in Table 2, showed a significant difference (26.06% vs. 33.20%; $p=0.009$).

Analysis of the *MTHFR* A1298C polymorphism with T2DM is presented in Tables 1 and 2. The frequencies of the 1298AA, 1298AC, and 1298CC genotypes in diabetic patients and controls were 63.83%, 32.27%, and 3.90% vs. 56.10%, 38.17%, and 5.73%, respectively. Statistical analyses of the genotype distribution showed no significant association between A1298C and T2DM in different models (Table 1). Allelic frequencies did not significantly differ between patient and control groups ($p=0.58$).

Table 3 shows the combined effect of C677T and A1298C *MTHFR* polymorphisms in development of T2DM. The frequency of the 677TT/1298CC combined genotypes was 35% in patients and 76% in controls; there was no significant association with diabetes (OR, 0.55; 95% CI, 0.07, 4.2; $p=0.52$). However, the frequency of the double homozygous 677CC/1298AA was significantly higher in patients (32.62%) than in controls (20.61%) (OR, 1.85; 95% CI, 1.26, 2.73; $p=0.001$). In addition, double heterozygous 677CT/1298AC combined genotypes were present in 9.57% of patients and 17.55% of controls; this difference was significant ($p=0.006$).

No significant association between patients and controls was seen for *MTHFR* polymorphisms and T2DM complications.

## Discussion

The role of *MTHFR* C677T and A1298C polymorphisms has been widely studied across the world in different populations, but the results are controversial. To our knowledge, our study is the first to assess an association between these polymorphisms and the risk for development of T2DM in a Moroccan population.

We found a significant difference between patients with T2DM and controls regarding the frequency of C677T genotypes in the additive and dominant models. This difference remained significant after adjustment for the age and sex in both models. Several studies have also found an association of C677T and T2DM. Movva *et al*. (2011) reported that the *MTHFR* 677T allele confers a fourfold risk for developing T2DM in an Indian population (OR, 4.0423; 95% CI, 1.8753, 8.7133). Other studies have confirmed the association of this polymorphism with diabetic complications: for example, nephropathy in a Polish population (Moczulski *et al*., 2003) and coronary heart disease in a Chinese population (Sun *et al*., 2005). However, in some previous case-control studies, *MTHFR* C677T polymorphism was not associated with T2DM in different populations: Tunisia (Koubaa *et al*., 2007; Mehri *et al*., 2010), the Czech Republic (Benes *et al*., 2001), Turkey (Eroglu *et al*., 2007), China (Sun *et al*., 2004), Germany (Ndrepepa *et al*., 2008), and Brazil (Helfenstein *et al*., 2005).

We found the 677TT genotype frequency was lowest in Moroccan patients and controls (8.87% and 9.92%, respectively). A similar frequency—approximately 9%—was reported in a Brazilian population (Helfenstein *et al*., 2005). In contrast, a

Table 3. Comparison Between *MTHFR* C677T and A1298C Polymorphisms in Patients with Type 2 Diabetes Mellitus and Healthy Controls

| MTHFR *677* | MTHFR *1298* | *Patients,* n *(%)* | *Controls,* n *(%)* | *OR (95% CI)* | p *value* | $\chi^2$ | *df* |
|---|---|---|---|---|---|---|---|
| CC | AA | 92 (32.62) | 54 (20.61) | 1.85 (1.26, 2.73) | 0.001 | 9.98 | 1 |
| CC | AC | 59 (20.92) | 50 (19.08) | 1.12 (0.73, 1.70) | 0.59 | 0.29 | 1 |
| CC | CC | 9 (3.19) | 10 (3.81) | 0.83 (0.34, 2.04) | 0.69 | 0.16 | 1 |
| CT | AA | 69 (24.46) | 73 (2.23) | 0.83 (0.57, 1.22) | 0.36 | 0.81 | 1 |
| CT | AC | 27 (9.57) | 46 (17.55) | 0.50 (0.30, 0.83) | 0.006 | 7.45 | 1 |
| CT | CC | 1 (0.35) | 3 (1.14) | 0.39 (0.05, 2.62) | 0.28 | 1.16 | 1 |
| TT | AA | 19 (6.73) | 20 (7.63) | 0.87 (0.45, 1.66) | 0.68 | 0.16 | 1 |
| TT | AC | 5 (1.77) | 4 (1.52) | 1.13 (0.32, 4) | 0.82 | 0.05 | 1 |
| TT | CC | 1 (0.35) | 2 (0.76) | 0.55 (0.07, 4.2) | 0.52 | 0.41 | 1 |

CI, confidence interval; df, degrees of freedom; OR, odds ratio.

19.1% frequency of the 677TT genotype was reported in a Chinese population (Sun *et al*., 2004).

The C677T polymorphism of the *MTHFR* gene has been reported to cause reduced enzyme activity and impaired homocysteine/folate metabolism, leading to moderate hyperhomocysteinemia (Boushey *et al*., 1995; Fletcher *et al*., 1998). However, no literature data directly associate the *MTHFR*-linked homocysteine and folate metabolism with T2DM. This hyperhomocysteinemia may damage the vascular endothelium, which is responsible for vasopressant effects (Cugini *et al*., 2002).

On the other hand, our study revealed no association of the *MTHFR* A1298C polymorphism with T2DM or its complications (neuropathy, cardiovascular disease, nephropathy, and obesity). Few studies have reported the association between this polymorphism and T2DM. In a Polish population, the frequency of the A1298C genotypes did not differ among patients with different stages of diabetic nephropathy (Moczulski *et al*., 2003). However, this polymorphism was reported to be associated with the risk for development of neural tube defects (Van der Put *et al*., 1998) and cardiovascular diseases (Szczeklik *et al*., 2001).

Finally, the analysis of the combined genetic profiles of both polymorphisms, C677T and A1298C, showed a significant difference between diabetic and control groups for those found to be double heterozygous for 677CT and 1298AC. In another study, double heterozygous patients had a higher risk for diabetic nephropathy than those with the 677CT genotype (Moczulski *et al*., 2003).

Several studies have reported a closer association between the *MTHFR* C677T polymorphism and T2DM than for the *MTHFR* A1298G polymorphism. This difference could be explained by the fact that the C677T polymorphism decreases the enzyme activity more than does the A1298C polymorphism: −70% vs. −30%, respectively (Weisberg *et al*., 1998). Indeed, the C677T polymorphism is located in the exon 4 coding for the N-terminal catalytic domain of the *MTHFR* enzyme, whereas the A1298C polymorphism is located in the exon 7 coding for the C-terminal regulatory domain (Rozen *et al*., 1996).

In conclusion, these data suggest that the *MTHFR* C677T polymorphism is a risk factor for T2DM in Moroccan patients, but there was no significant association with the *MTHFR* A1298C polymorphism. However, the double homozygous 677CC + 1298AA appears to have protective effect against diabetes.

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## Disclosure Statement

The authors declare that they have no conflicts of interest.

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