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Research Article

ASSOCIATION OF MTHFR A1298C GENE VARIANT, DNA DAMAGE, AND TOTAL ANTIOXIDANT STATUS WITH THE RISK OF TYPE 2 DIABETES MELLITUS AND ITS COMPLICATIONS

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ABSTRACT

Objectives: We have examined the association of methylenetetrahydrofolate reductase (MTHFR) gene A1298C variant, DNA damage, and total antioxidant status (TAS) in patients with type 2 diabetes mellitus (T2DM) with and without complications and in healthy controls.

Methods: A total of 300 subjects including 100 patients with complications, 100 patients without complications, and 100 controls were included. TAS was assessed by ferric reducing ability of plasma assay. DNA damage was analyzed in lymphocytes using the comet assay. Polymerase chain reaction-restriction fragment length polymorphism analysis was performed to study the MTHFR A1298C gene polymorphism among the study subjects.

Results: The results revealed that the MTHFR 1298 AC+CC genotypes were associated with increased risk (2 fold) for diabetes and its complications. When the effect of DNA damage was analyzed, significant differences between individuals with mutant and normal genotype among the diabetic patients (with and without complications) was observed ($p \leq 0.001$). In contrary, no significant difference was found between TAS and 1298 genotypes (AA *versus* AC+CC) in type 2 diabetic patients (with and without complications), $p=0.338$. We also found a significant difference between the genotypes of the MTHFR A1298C and DNA damage, TAS in T2DM patients (with and without complications) when compared to controls, $p<0.001$.

Conclusions: Our findings suggest that the MTHFR A1298C gene polymorphism is considered as a risk factor for the development of diabetes and its complications among south Indians. Therefore, increased DNA damage and decreased TAS along with the occurrence of a mutant genotype in an individual with diabetes may be at an increased risk for the development of chronic complications.

Keywords: Methylenetetrahydrofolate reductase, Polymorphism, Folate metabolism, Total antioxidant status, DNA damage, Type 2 diabetes mellitus.

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INTRODUCTION

The incidence of type 2 diabetes mellitus (T2DM) is rising at an alarming rate, and the prevalence of this disease has led to different vascular complications [1,2]. Vascular complications may be macrovascular (coronary heart disease, peripheral vascular disease, and stroke), microvascular (neuropathy, retinopathy, and nephropathy), and both micro- and macro-vascular (diabetic foot) [3]. T2DM is a common multifactorial genetic syndrome, which is determined by several different genes and environmental factors [4].

Functional polymorphism in genes involved in the folate metabolic pathway has been associated with low level of folate and a high level of homocysteine (Hcy) [5-7]. Methylenetetrahydrofolate reductase (MTHFR) is the key enzyme that plays an important role in folate metabolism. The MTHFR enzyme, encoded by the MTHFR gene, is responsible for catalyzing the irreversible reaction of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate which is the primary circulating form of folate [8]. The gene encoding MTHFR is located at chromosome 1p36.3. Single-nucleotide polymorphism (SNP) in MTHFR A1298C (rs1801131) leads to glutamate to alanine substitution within the C-terminal regulatory domain of the enzyme due to an A to C transversion that occurs in exon 7, that results in a decrease in MTHFR activity [9]. Low MTHFR activity reduces DNA methylation [10], thereby results in elevated plasma homocysteine (Hcy) [11]. Several studies have also shown that elevated levels of Hcy may induce DNA damage either by an increased production of ROS or by biological mechanisms directly associated with an excessive misincorporation of uracil in DNA and the process of DNA methylation [12-14].

To date, over 40 genetic mutations in MTHFR gene have been identified, of which A1298C and C677T showed the most clinical significance. Few studies have evaluated the relationship between the MTHFR A1298C polymorphism and susceptibility of diabetes, and the results remain inclusive [15]. Hence, the present study was designed to find the association of MTHFR gene A1298C polymorphism with the risk of type 2 diabetes and also to find out the effect of this polymorphism on DNA damage and total antioxidant status (TAS) in type 2 diabetic patients and healthy controls among south Indians.

METHODS

Study subjects

Two hundred type 2 diabetic patients (n=100, with complications & n=100, without complications) and 100 healthy individuals who were of south Indian Tamil ethnicity were included in the study. Complications included in the study were coronary heart disease (18%), peripheral vascular disease (16%), stroke (1%), neuropathy (56%), retinopathy (1%), nephropathy (6%), and foot ulcer (2%). The ethical clearance was obtained from the Institutional Ethical Committee of Asirvatham Hospital and the study was approved by the Institutional Biosafety Committee of Lady Doak College. Samples were collected with the informed consent from all the study subjects.

Ferric reducing ability of plasma (FRAP) assay

Total antioxidant status was measured by FRAP assay according to the method of Benzie and Strain [16].

Single-cell gel electrophoresis (comet assay)

Comet assay was performed to assess the level of DNA damage in peripheral lymphocytes by two-layer method according to Tice *et al.*, 2000 [17] with slight modifications according to Singh *et al.*, 1988 [18].

Genotyping of MTHFR A1298C gene

Genomic DNA was extracted from the whole blood (500 µl) by phenol–chloroform method [19]. The genotyping was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis according to Friedman *et al*., 1999 [20]. PCR amplification was carried out using the following primer set: forward primer 5' CTTTGGGGAGCTGAAGGACTACTAC 3' and reverse primer 5' CACTTTGTGACCATTCCGGTTTG 3' (~10picomoles) (Sigma). The amplified PCR product (163bp) was confirmed by 2% agarose (HiMedia, Mumbai) gel electrophoresis (Fig. 1), using the Gel documentation system. The amplified products were digested with *Mbo*II enzyme (Fermentas Life Sciences, Germany) at 37°C for 5–7 h. The resulting fragments of the digested PCR products were 84, 31, 30, and 18 bp fragments for the A allele and 56, 31, 30, 28, and 18 bp fragments for the C allele (Fig. 2). The products were visualized by performing agarose (3%) gel electrophoresis using a UV transilluminator.

Sequencing of PCR amplified fragments

Selected PCR amplified fragments were completely sequenced both strands in an automated ABI 3100 Genetic Analyzer (Chromous biotech, Bengaluru, India). Sequencing and BLASTN analysis were done to confirm whether the amplified fragment was the MTHFR A1298C gene sequence.

Statistical analysis

All the data were expressed as mean±standard error. Genotype and allele frequencies were calculated by the allele counting method. The Hardy–Weinberg equilibrium was calculated for diabetic patients and controls and was tested using the Chi-square (χ^2) statistics. Odds ratio (OR) and 95% confidence intervals (95% CI) were calculated. Statistical analysis was performed using the Sigma stat 11.0 version software, and $p \leq 0.05$ was considered to be statistically significant.

RESULTS

When BLASTN analysis was performed, 100% high degree of identity was found between the MTHFR A1298C gene and the submitted DNA sequences. The genotype and allele frequencies of MTHFR A1298C gene polymorphism and Hardy–Weinberg equilibrium in type 2 diabetic patients (with and without complications) and controls are shown in Table 1. Among the 200 T2DM patients, 59.5% of the individuals have AC genotype and 22% of the individuals have CC genotype and the remaining 18.5% of the individuals have AA genotype. The calculated genotype frequencies were slightly higher in T2DM patients when compared to controls (40% AC, 39% AA and 21% CC genotype). The most prevalent genotype for MTHFR A1298C gene was heterozygous (AC) in both patients with and without complications and controls. The C allele frequency was found to be slightly higher in patients with complications (0.53) than patients without complications (0.51) when compared to controls (0.41). The genotype distributions in T2DM patients (without complications) have shown a marked deviation from the frequencies predicted on the basis of the Hardy–Weinberg law (χ^2=4.84, p=0.02) and equilibrium was observed in both the patients with complications (χ^2=2.69, p=0.10) and the control group (χ^2=2.99, p=0.22) for MTHFR A1298C polymorphism.

Significant differences were found when type 2 diabetic patients (with and without complications) and controls were compared according to AA genotype *versus* AC+CC genotypes of the MTHFR A1298C gene (patients with complications: p=0.001, OR=2.91, 95% CI=1.52–5.57 & patients without complications: p=0.002, OR=2.72, 95% CI=1.43–5.17). The results revealed that the MTHFR A1298C gene polymorphism is associated with increased risk (2 fold) for diabetes and its complications.

We studied the effect of MTHFR A1298C on TAS and DNA damage in type 2 diabetic patients (with and without complications) and controls (Table 2). When the effect of DNA damage was analyzed, significant differences between individuals with mutant and normal genotype among the diabetic patients (with and without complications) was

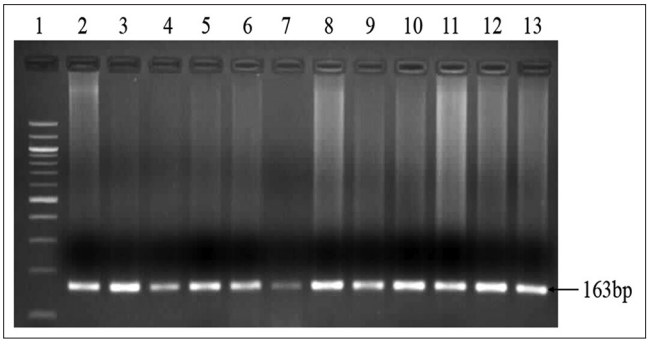

Fig. 1: Polymerase chain reaction (PCR) analysis of the MTHFR A1298C gene. Lane 1: 100 bp DNA marker, Lane 2-13: PCR product (163 bp)

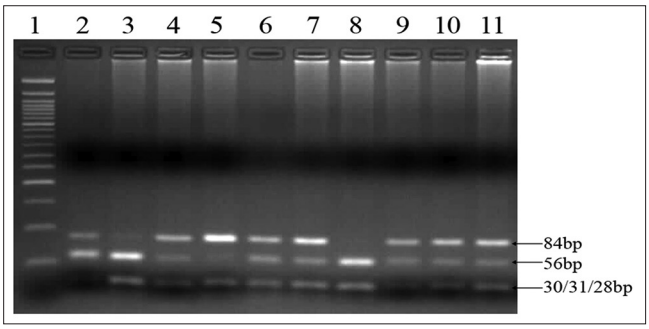

Fig. 2: Restriction digestion analysis of the MTHFR A1298C gene variant. Lane 1: 100 bp DNA marker, Lane 5: wild genotype (AA)- 84, 31, 30 and 18 bp, Lane 2, 4, 6, 7, 9-11: heterozygous genotype (AC)- 84, 56, 31, 30, 28 and 18 bp, Lane 3 and 8: mutant genotype (CC)- 56, 31, 30, 28 and 18 bp

observed ($p \leq 0.001$). In contrary, no significant difference was found between TAS and 1298 genotypes (AA *vs.* AC+CC) in type 2 diabetic patients (with and without complications), p=0.338. We also found a significant difference between the genotypes of the MTHFR A1298C and DNA damage, TAS in T2DM patients (with & without complications) when compared to controls, $p<0.001$.

The association between the MTHFR genotypes *versus* age, duration, sugar level, and HbA1c in patients with and without complications is shown in Table 3. We did not observe any significant association between the wild genotype (AA) and mutant genotypes (A/C & C/C) in both diabetic patients (with and without complications) with regard to their age, duration, sugar level, and HbA1c level ($p>0.05$).

DISCUSSION

Diabetes is a serious illness resulting in chronic complications and growing threat to the health of the global world. It has been suggested that the chronic complications could be prevented by initial understanding of the disease and through better diabetes mellitus treatment [21]. Another finding suggests that the level of glycemic control and the presence of complications are associated with quality of life [22]. In the present study, the association of MTHFR gene (A1298C) variant, DNA damage and TAS with the risk of type 2 diabetes and its complications were analyzed among south Indians.

MTHFR A1298C gene polymorphism and T2DM

The MTHFR 1298C allele frequency observed in the present study was found to be 0.53 for patients with complications and 0.51 for patients without complications. Our findings revealed that the MTHFR A1298C polymorphism is more prevalent in South Indian population and associated with the risk of diabetes and its complications. Moreover, statistical comparison between type 2 diabetic patients and controls

Table 1: MTHFR A1298C gene polymorphism – Genotype and Allele Frequency in type 2 diabetic patients (with and without vascular complications) and controls

Genotype/Allele	Patients with complications (*n*=100)	Patients without complications (*n*=100)	Controls (*n*=100)
Genotypes			
AA	18	19	39
AC	58	61	40
CC	24	20	21
Alleles			
A	0.47	0.49	0.59
C	0.53	0.51	0.41
HWE (χ^2/p)	2.69/0.10	4.84/0.02*	2.99/0.22

Cases *versus* controls	Odds ratio	95% CI	p value
AA *versus* AC+CC			
Patients without complications *versus* Controls	2.72	1.43–5.17	0.002
Patients with complications *versus* Controls	2.91	1.52–5.57	0.001

MTHFR: methylenetetrahydrofolatereductase, n: number of individuals, HWE: Hardy–Weinberg equilibrium, χ^2: Chi-square, CI: Confidence interval, *p<0.05 statistically significant

Table 2: Effect of MTHFR 1298 genotypes on TAS and DNA Damage in type 2 diabetic patients (with and without complications) and controls

Genotypes	TAS (µmol/L) mean±SE			DNA damage (%) mean±SE		
	T2DM patients with complications (*n*=100)	T2DM patients without complications (*n*=100)	Controls (*n*=100)	T2DM patients with complications (*n*=100)	T2DM patients without complications (*n*=100)	Controls (*n*=100)
AA	605.26±35.56 (18)	681.05±43.80 (19)	967.60±22.17 (39)	10.36±1.69 (18)	4.65±1.12 (19)	2.11±0.13 (39)
AC	562.41±20.10 (58)	637.35±24.52 (61)	948.0±35.06 (40)	11.94±1.23 (58)	9.39±0.72 (61)	2.04±0.20 (40)
CC	610.83±48.43 (24)	560.0±55.60 (20)	980.95±53.83 (21)	15.93±1.89 (24)	10.41±1.45 (20)	1.78±0.23 (21)

Data are presented as mean±standard error, MTHFR: Methylenetetrahydrofolate reductase, T2DM: Type 2 diabetes mellitus, n: Number of individuals. SE: Standard error, TAS: Total antioxidant status

Table 3: Association between MTHFR A1298C gene polymorphism *versus* clinical and biochemical parameters in patients with and without complications

Parameters	T2DM patients with complications		p	T2DM patients without complications		p
	A/A (*n*=18)	A/C & C/C (*n*=82)		A/A (*n*=19)	A/C & C/C (*n*=81)	
Age (Yrs)	56.61±1.45	55.0±0.82	0.421	46.05±2.43	48.79±1.01	0.239
Duration (Yrs)	10.33±1.68	10.75±0.73	0.669	5.26±0.80	6.90±0.68	0.670
Sugar Level (mmol/L)	12.49±0.92	12.81±0.43	0.459	14.53±0.90	12.22±0.39	0.017
HbA1c (%)	8.57±0.28	8.76±0.19	0.904	9.03±0.43	8.91±0.20	0.772

Data are presented as mean±standard error, MTHFR: Methylenetetrahydrofolate reductase, T2DM: Type 2 diabetes mellitus, HbA1c: Glycosylated hemoglobin, n: Number of individuals

according to AA *versus* AC+CC genotypes revealed two-fold increased risk for the diabetes and its complications. A previous study conducted in the Tunisian population has reported that the MTHFR A1298C was significantly associated with type 2 diabetes [23]. Another study suggested that MTHFR A1298C polymorphism is a risk factor for T2DM in Egyptian population [24], while other studies did not find any association with diabetes in Taiwanese and Moroccan population [25,26].

Regarding diabetic complications, few studies have found the association of this polymorphism with coronary heart disease in Chinese population [27], ischemic stroke in Tunisian population [28], and retinopathy in Egyptian population [29]. A previous study conducted in a south Indian population has reported that the MTHFR A1298C gene polymorphism might lead to an increased risk for the occurrence of acute myocardial infarction [30]. In contrary, the MTHFR A1298C variant was not associated with the development of type 2 diabetic nephropathy in Chinese population [31] and in Caucasians [32]. The occurrence of the mutant allele frequency varies in different population. This might be due to ethnic variations, geographical background, and interindividual differences of the studied population. The prevalence of the MTHFR A1298C gene polymorphism in healthy controls is shown in Table 4.

Association of MTHFR gene polymorphism, DNA damage, and TAS

When the genotypes (AA, AC, and CC) of MTHFR gene 1298 were related individually to DNA damage and TAS, a statistically significant difference between the diabetic patients (with and without complications) and controls was observed. We found an association between the MTHFR 1298 genotypes and DNA damage in both the diabetic patients with and without complications. This shows that MTHFR A1298C genotypes had an effect on DNA damage in individuals with T2DM. We did not find any association between the MTHFR 1298 genotypes and the levels of TAS among the study subjects. However, TAS was found to be decreased in T2DM patients when compared to controls irrespective of the genotypes. This may be due to hyperglycemia, which has a direct

Table 4: Prevalence of the MTHFR A1298C gene polymorphism in healthy controls

Country	Ethnic population	No. of subjects (N)	Mutant (C) allele Frequency	References
India	South Indian	100	0.41	(Present study)
China	Chinese	302	0.26	[15]
Tunisia	Tunisian	200	0.03	[23]
Japan	Japanese	243	0.20	[37]
Brasil	Brasilian	356	0.26	[38]
UAE	Emirati	169	0.61	[36]
Turkey	Turkish	112	0.40	[39]
Egypt	Egyptian	310	0.32	[29]
China	Chinese	680	0.19	[15]
Egypt	Egyptian	60	0.22	[24]
Pakistan	Pakistani	872	0.55	[7]
Australia	Australian	386	0.35	[40]
Taiwan	Taiwanese	62	0.28	[25]
UK	British	759	0.32	[41]
Tunisia	Tunisian	400	0.20	[42]
India	South Indian	100	0.33	[30]
Italy	Italian	261	0.30	[43]

effect on TAS in T2DM patients. The previous finding also suggests that hyperglycemia contributes to increased oxidative stress and decreased TAS, which would lead to further increase in DNA damage in patients with diabetes and its complications [33].

In the present study, the level of DNA damage was found to be higher in patients with complications when compared to patients without complications. Evidence suggests that patients with diabetic neuropathy have increased oxidative DNA damage than patients with diabetes [34]. Another finding suggests that individuals with diabetes have been shown to have increased oxidative DNA damage and decreased antioxidative defense relative to the overproduction of free radicals [35].

Association of MTHFR 1298 genotypes versus clinical and biochemical parameters in T2DM

The present study shows that the clinical and biochemical variables have no association with the occurrence of genotypes in individuals with diabetes. Similarly, previous studies have found no significant associations between lipid/glucose metabolic indexes with MTHFR 1298 genotypes among T2DM patients [24,25]. Another study also has reported that BMI, hypertension, family history, HbA1c, total cholesterol, and fasting blood glucose of the diabetic patients segregated according to the MTHFR 1298 genotypes genotypes were similar except triglyceride levels [36].

CONCLUSION

The MTHFR A1298C gene polymorphism is considered as a risk factor for the development of diabetes and its complications among south Indians. Therefore, increased DNA damage and decreased TAS along with the occurrence of the MTHFR 1298 mutant genotype in an individual with diabetes may be at an increased risk for the development of chronic complications. Future researchers may focus on the investigation of gene-nutrient interactions and epigenetic interactions for better understanding of the role of folate metabolism genes in the risk of diabetes and its complications among the south Indian population.

CONFLICT OF INTEREST

The authors declare that there were no conflicts of interest for financial interests associated with this manuscript.

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