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ORIGINAL ARTICLE

Methylenetetrahydrofolate reductase gene polymorphism in type 1 diabetes mellitus: Relationship to microvascular complications

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Abstract In patients with type-I diabetes mellitus folate deficiency is associated with endothelial dysfunction. So, polymorphism in genes involved in folate metabolism may have a role in vascular disease. This study was designed to evaluate the relationship between methylenetetrahydrofolate reductase (MTHFR) gene polymorphism and microvascular complications in adolescents with type I diabetes mellitus.

A total of 99 patients with disease duration of more than 5 years aged 11–18 years participated in the study. History taking, physical, neurological and fundus examinations were performed. Laboratory investigations included mean glycated hemoglobin in the last year, urinary albumin excretion, serum creatinine, nerve conduction velocity and MTHFR genotype determination.

Results revealed that 54 (54.5%) of our patients had normal MTHFR genotype (C/C subgroup), 36 (36.4%) had heterozygous MTHFR gene polymorphism (C/T subgroup) and 9 (9.1%) had homozygous MTHFR gene polymorphism (T/T subgroup). No significant difference was found between the three studied groups as regards age, disease duration or glycemic control. When testing

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for microalbuminuria, the T/T subgroup showed a significantly higher number of patients with microalbuminuria when compared with C/C and C/T subgroups.

Regarding fundus changes, the C/C subgroup showed a significantly lower number of patients with fundus changes when compared with C/T and T/T subgroups .On the other hand the T/T subgroup showed a significantly higher number of patients with fundus changes when compared with the C/T subgroup.

For nerve conduction abnormalities, the T/T subgroup showed a significantly higher number of patients with nerve conduction abnormalities when compared with C/C and C/T subgroups.

Multivariate forward stepwise logistic regression analysis for determination of independent risk factors that best predicts the occurrence of microalbuminuria, fundus changes and nerve conduction abnormalities revealed MTHFR gene polymorphism to be the most important variable.

MTHFR gene polymorphism (T/T) subtype is an important risk factor for the development of micro-vascular complications in patients with type I diabetes mellitus.

1. Introduction

Type 1 diabetes mellitus (T1DM) is an autoimmune disorder resulting in loss of pancreatic insulin-producing-cells that presents in childhood or early adulthood, along with an increased risk of complications including retinopathy, nephropathy, neuropathy, and cardiovascular events [1].

The risk of developing micro-vascular complications is related mainly to the duration of diabetes and the degree of glycemic control achieved over time (i.e., HbA1c 7.0% or less). Genetic factors may also influence the risk of complications. These complications are mainly renal micro-vascular complication (microalbuminuria or diabetic nephropathy), retinopathy and neuropathy (peripheral or autonomic) [2].

5, 10-methylenetetrahydrofolate reductase is a key enzyme in folic acid metabolism. Folic acid has recently gained a great deal of attention as a biologically important molecule. Methylenetetrahydrofolate reductase is the enzyme responsible for reduction of methylenetetrahydrofolate, to methyltetrahydrofolate, which is essential for homocysteine remethylation to methionine. In the absence, deficiency or decreased action of this enzyme homocysteine will not be remethylated to methionine and leads to elevation of its serum level [3,4].

Elevated homocysteine is a known risk factor for vascular disease. So the polymorphism in methylenetetrahydrofolate reductase may have detrimental consequences [5].

In patients with type-I diabetes mellitus folate deficiency is associated with endothelial dysfunction and folate supplementation improves endothelial functions in these patients. The familial clustering of nephropathy and retinopathy found in *diabetes control and complications trial* supports a contribution of genetic factors in their development. So, polymorphism in genes involved in folate metabolism may have a role in vascular disease [5].

This study was designed primarily to investigate the association between methylenetetrahydrofolate reductase gene [MTHFR C677T] polymorphism and microvascular complications in patients with type-I diabetes mellitus.

2. Subjects and methods

2.1. Subjects

The present study included 99 type 1 diabetic patients with a disease duration of more than 5 years recruited from the regular attendants of the Diabetes Specialized Clinic, Children's Hospital, Ain Shams University over a 10 month period (from February to November 2010). The patients are regular attendees at the Clinic which cares for more than 1500 children, adolescents, and young adults with T1DM.

The patients included are those using human insulin in a dose ranging from 0.5 to 2 IU/kg/day with a mean of 1.12 ± 0.40 IU/kg/day. All participants were on intensive insulin therapy. Sixty-eight patients were on pre-prandial injections of short-acting insulin plus intermediate acting insulin (isophane NPH insulin) and 31 patients were on long acting insulin analog (insulin glargine – Aventis Pharma) at bedtime plus pre-prandial short acting insulin analog.

The study has been approved by the Ethics Committee of Ain Shams University. Subjects participated in the study after written information and informed consent from their parents.

2.2. Methods

All subjects underwent the following:

2.2.1. Detailed questionnaire

Complete history was taken including age of the patient at the time of the study, age at the onset and duration of diabetes, and symptoms suggesting diabetic complications.

2.2.2. Clinical assessment

Physical examination included full neurological examination to detect evidence of peripheral neuropathy. Diabetic neuropathy was diagnosed in the presence of typical neuropathic symptoms and positive findings related to neuropathy in neurological examination. Diabetic retinopathy (DR) was diagnosed by doing a complete ocular examination including visual field testing, slit-lamp biomicroscopy and indirect ophthalmoscopy.

2.2.3. Biochemical investigations

Venous blood samples were obtained in the morning from all patients after an overnight fast. A sample of 4 ml of blood was drawn from peripheral veins under complete aseptic conditions and divided among two tubes. One tube contained EDTA for direct assay of HbA1c by high performance liquid chromatography supplied by *Bio-Rad Diagnostic Group. Horcules C.A. Milano, Muchon, Paris* (normal levels in our lab is less than

Table 1 Normal value of motor nerve conduction studies of median and posterior tibial nerves [7].

Nerve	Active electrode	Stimulation site	Latency (ms)	Amplitude	Segment name	Velocity m/s
Median nerve	Abductor pollicis brevis	Wrist–elbow	< 4.2	> 4 (mV)	Wrist–elbow	> 50
Posterior tibial nerve	Abductor hallucis	Ankle–knee	< 6	> 3 (Uv)	Ankle–knee	> 40

Table 2 Age and disease duration in all studied groups.

	CC subgroup	C/T subgroup	T/T subgroup
Age (years)	14.02 ± 3.53	13.06 ± 4.39	14.56 ± 3.9
Disease duration (years)	6.94 ± 3.3	6.72 ± 3.83	9.44 ± 4.28

5.5 g%) and the other tube for MTHFR polymorphism analysis.

Urinary albumin excretion was measured by immuno-turbidimetric method. The kit used was from SERA-PAK (*Bayer Corporation, Bendict Ave, Tarry town, NY, USA*). The result was expressed as albumin to creatinine ratio (ACR) in urine to avoid diurnal variation in albumin excretion. Urinary creatinine was estimated using Synchron Cx7 (*Beckman Instruments Inc., Brea., California, USA*).

Patients with diabetic nephropathy were classified into two groups: group 1 (84) normoalbuminuric patients (with urinary albumin levels of < 29 mg/g); group 2 (15) microalbuminuric patients (with urinary albumin levels of 30–299 mg/g) in at least 2 urine samples, 2 months apart.

2.2.4. Testing for neuropathy

2.2.4.1. Nerve conduction velocity (NCV). Motor nerve conduction studies for both median and posterior tibial nerves bilaterally were done by the Nicollet Viking Quest 4 Channel apparatus a product of Viasys Healthcare Neurocare Group USA [6].

Results were compared with the normal values shown in Table 1.

2.2.5. Methylenetetrahydrofolate reductase gene polymorphism analysis (MTHFR C677T)

For all cases the following were done:

2.2.5.1. DNA extraction. Genomic DNA was extracted using QIAmpl DNA extraction kit (Ca 51104).

2.2.5.2. Polymerase chain reaction (PCR). Polymerase chain reaction (PCR) was performed according to the method described by Frosst et al. [8]. One microliter of genomic DNA was amplified in a 50 μL reaction volume containing 50 mM KCL, 10 mM Tris–HCL pH 8.3, 1.5 mM MCL2, 20 mM dNTPs, 0.5 μM primers and one unit of Taq DNA polymerase enzyme. PCR consisted of initial denaturation at 94 °C for 150 s followed by 35 cycles at 94 °C for 30 s, 57 °C for 60 s and 72 °C for 120 s and a final elongation step at 72 °C for 3 min. PCR product of 198 bp was analyzed on 3% agarose gel electrophoresis.

2.2.5.3. Enzyme digestion. Twenty microliters of PCR product was mixed with 5 units of Hinf1 (promega, Madison) enzyme at 37 °C overnight incubation. The substitution created a Hinf1 recognition sequence that is digested into 175-bp and 23-bp.

2.3. Statistical analysis

Statistical analysis of the data was performed by using SPSS 15 software package under Windows XP operating system. Categorical data parameters were presented in the form of frequency and percentage and analyzed for group differences by using Chi square test (χ^2 value) or Fisher exact test (X^2 value) according to the nature of the data. Continuous data parameters were analyzed for normality by using Shapiro–Wilk and Kolmogorov–Smirnov tests. Central tendency of the data was presented in the form of mean and/or median; and measure of spread was presented as standard deviation, range and/or percentile. Comparative analysis was performed by using Student-*t* test (*t* value) or Mann–Whitney *U* test (*z* value) for two independent samples according to the nature of the data. Multivariate logistic regression analysis was used for determination of best predictor models. Probability level (*P* value) was assumed significant if less than 0.05 and highly significant if *P* value was less than 0.01. *P* value was considered non-significant if greater than or equal to 0.05. Graphic presentation of data was done by using EXCEL 2007 software.

3. Results

Our results revealed that 54 (54.5%) of our patients had homozygous MTHFR genotype (C/C subgroup), 36 (36.4%) had heterozygous MTHFR gene polymorphism (C/T subgroup) and 9 (9.1%) had homozygous MTHFR gene polymorphism (T/T subgroup).

3.1. Age

No significant difference was found between the three studied subgroups as regards age for C/C versus C/T, C/C versus T/T and C/T versus T/T, respectively (Table 2).

3.2. Disease duration

Regarding disease duration, no significant difference was found between the three studied subgroups for C/C versus C/T, C/C versus T/T and C/T versus T/T, respectively (Table 2).

3.3. Glycemic control

As regards HbA1c no significant difference was found when the C/C subgroup was compared with C/T and with T/T subgroups. On the other hand, the T/T subgroup showed

Table 3 Comparison between the three MTHFR subgroups regarding HbA1c (in g%).

	C/C	C/T	T/T
Mean ± SD	8.16 ± 1.71	7.74 ± 0.96	8.72 ± 0.91
Median	8.45	7.95	8.30
Range	(5.10–11.70)	(5.40–10.10)	(8–10.50)
	z value/*t* value	*P* value	Significance
C/C versus C/T	1.516	0.129	NS
C/C versus T/T	0.659	0.510	NS
C/T versus T/T	3.072	0.002	S

NS = non significant.
S = significant.

significantly higher HbA1c when compared with the C/T subgroup (Table 3).

3.4. Microalbuminuria

As regards microalbuminuria no significant difference was found when the C/C subgroup was compared with the C/T subgroup. On the other hand, the T/T subgroup showed a significantly higher number (7 out of 9) of patients with microalbuminuria when compared with the C/C subgroup (2 out of 54) and the C/T subgroup (6 out of 36) (Table 4).

3.5. Fundus changes

As regards fundus changes the C/C subgroup showed a significantly lower number (2 out of 54) of patients with fundus changes when compared with C/T (7 out of 36) and T/T (8 out of 9) subgroups. On the other hand the T/T subgroup showed a significantly higher number of patients with fundus changes when compared with the C/T subgroup (Table 4).

3.6. Nerve conduction abnormalities

As regards nerve conduction abnormalities non-significant difference was found when the C/C subgroup was compared with the C/T subgroup. On the other hand, the T/T subgroup showed a significantly higher number of patients (7 out of 9) with nerve conduction abnormalities when compared with C/C (3 out of 54) and C/T (5 out of 36) subgroups (Table 4).

3.7. Predictors of different microvascular complications

A set of risk factors including age, duration of diabetes, HbA1c and MTHFR gene mutation was used to predict the occurrence of different microvascular complications. Risk factors were analyzed by regression analysis either in single-variable model, double-variable model, triple-variable model, or quad-variable-model.

As a single-variable, it was found that MTHFR gene polymorphism was the best predictor of microalbuminuria, fundus changes and NCV abnormalities with 89.9% of cases correctly classified (Table 5). Sensitivity, specificity, positive and negative predictive values are shown in Tables 6, 8 and 10.

By using the double variable model, MTHFR gene polymorphism and disease duration were the best predictors for the occurrence of microalbuminuria and fundus changes while MTHFR and age were the best predictors for the occurrence of NCV abnormalities. Sensitivity, specificity, positive and negative predictive values are shown in (Tables 7, 9 and 11).

Table 4 Comparison between the three MTHFR subgroups regarding different microvascular complications.

		C/C (n = 54)		C/T (n = 36)		T/T (n = 9)	
		No.	%	No.	%	No.	%
Microalbuminuria	−ve	52	96.3	30	83.3	2	11
	+ve	2	3.7	6	16.7	7	77
		X^2 value		*P* value		Significance	
C/C versus C/T		4.482		0.056		NS	
C/C versus T/T		34.568		< 0.001		S	
C/T versus T/T		13.089		0.001		S	
Fundus changes	−ve	52	96.3	29	80.6	1	11.1
	+ve	2	3.7	7	19.4	8	88.9
		X^2 value		*P* value		Significance	
C/C versus C/T		5.947		0.027		S	
C/C versus T/T		41.921		< 0.001		S	
C/T versus T/T		15.625		< 0.001		S	
Nerve conduction abnormalities	−ve	51	94.4	31	86.1	2	11.1
	+ve	3	5.6	5	13.9	7	77.8
		X^2 value		*P* value		Significance	
C/C versus C/T		1.852		0.258		NS	
C/C versus T/T		30.133		< 0.001		S	
C/T versus T/T		15.028		< 0.001		S	

NS = non significant.
S = significant.

Table 5 Multivariate forward stepwise logistic regression analysis for determination of independent risk factors that best predict the occurrence of different microvascular complications.

	Variables entered	B coefficient			Omnibus X^2 value			*P* value			Percent correctly classified
		Microalbuminuria	Fundus changes	NCV abnormalities	Microalbuminuria	Fundus changes	NCV abnormalities	Microalbuminuria	Fundus changes	NCV abnormalities	
Step 1	MTHFR				25.132	31.948	22.496	< 0.001	< 0.001	< 0.001	89.9%
	MTHFR[1]										
		−4.511	−5.338	4.086							
	MTHFR[1]	−2.862	−3.501	3.077							
Step 2	Duration	0.252	0.229	0.274	9.396	7.592	10.682	0.002	0.006	0.006	88.9%
	MTHFR										
	MTHFR[1]	−4.571	−5.422	1.572							
	MTHFR[1]	2.693	−3.364	3.359							

Variable entered in step 1: MTHFR (constant = 1.253).
Variable entered in step 2: Duration (constant = −0.851) for microalbuminuria and fundus changes and age (constant = −2.485) for NCV abnormalities.

Table 6 Percent of microalbuminuria cases correctly classified based on model 1 of binary logistic regression analysis.

Microalbuminuria (model 1)		Expected			
		Negative		Positive	
Observed	Negative	82	(TN)	2	(FP)
	Positive	8	(FN)	7	(TP)
Sensitivity			46.7%		
Specificity			97.6%		
PPV			77.8%		
NPV			91.1%		
Diagnostic accuracy			89.9%		

TN (true negative), FN (false negative), TP (true positive), FP (false positive).

Table 7 Percent of microalbuminuria cases correctly classified based on model 2 of binary logistic regression analysis.

Microalbuminuria (model 2)		Expected			
		Negative		Positive	
Observed	Negative	82	(TN)	3	(FP)
	Positive	7	(FN)	7	(TP)
Sensitivity			50.0		
Specificity			96.5%		
PPV			70.0%		
NPV			92.1%		
Diagnostic accuracy			89.9%		

TN (true negative), FN (false negative), TP (true positive), FP (false positive).

Table 8 Percent of fundus change cases correctly classified based on model 1 of binary logistic regression analysis.

Fundus changes (model 1)		Expected			
		Negative		Positive	
Observed	Negative	81	(TN)	1	(FP)
	Positive	9	(FN)	8	(TP)
Sensitivity			47.1		
Specificity			98.8%		
PPV			88.9%		
NPV			90.0%		
Diagnostic accuracy			89.9%		

TN (true negative), FN (false negative), TP (true positive), FP (false positive).
Variable entered in step 1, MTHFR (constant = 2.079).
Variable entered in step 2, Duration (constant = 0.231).

4. Discussion

Homozygosity of the mutation (TT genotype of HTHFR gene) predisposes to significantly elevated plasma homocysteine levels [8,9]. Thus MTHFR C677T gene polymorphism associated with increased plasma homocysteine levels may present a genetic risk factor for microangiopathy [10].

Table 9 Percent of fundus change cases correctly classified based on model 2 of binary logistic regression analysis.

Fundus changes (model 2)		Expected			
		Negative		Positive	
Observed	Negative	80	(TN)	2	(FP)
	Positive	8	(FN)	9	(TP)
Sensitivity			52.9%		
Specificity			97.6%		
PPV			81.8%		
NPV			90.9%		
Diagnostic accuracy			89.9%		

TN (true negative), FN (false negative), TP (true positive), FP (false positive).

Table 10 Percent of nerve conduction velocity abnormalities cases correctly classified based on model 1 of binary logistic regression analysis.

Nerve conduction velocity abnormalities (model 1)		Expected			
		Negative		Positive	
Observed	Negative	82	(TN)	2	(FP)
	Positive	8	(FN)	7	(TP)
Sensitivity			46.7%		
Specificity			97.6%		
PPV			77.8%		
NPV			91.1%		
Diagnostic accuracy			89.9%		

TN (true negative), FN (false negative), TP (true positive), FP (false positive).

Table 11 Percent of nerve conduction velocity abnormalities cases correctly classified based on model 2 of binary logistic regression analysis.

Nerve conduction velocity abnormalities (model 2)		Expected			
		Negative		Positive	
Observed	Negative	81	(TN)	3	(FP)
	Positive	8	(FN)	7	(TP)
Sensitivity			46.7%		
Specificity			96.4%		
PPV			70.0%		
NPV			91.0%		
Diagnostic accuracy			88.9%		

TN (true negative), FN (false negative), TP (true positive), FP (false positive).

TN (true negative), FN (false negative), TP (true positive), FP (false positive).

Microangiopathy is the basic pathogenetic event in the occurrences of most diabetic complications like nephropathy, neuropathy and retinopathy [11]. This study was designed to investigate the association between MTHFR gene polymorphism and the occurrence of different microvascular complications in patients with type 1 DM. Ninety-nine diabetic patients were analyzed as regards MTHFR genotype, 54 (54.5%) patients were found to be of the (C/C) genotype, 36 (36.4%) of the (C/T) genotype and 9 (9.1%) of the (T/T) genotype and all subgroups were well matched regarding age.

When we investigated microvascular complications as microalbuminuria, fundus changes and nerve conduction abnormalities in the 3 groups the following interesting results were found. Out of nine patients with the TT genotype, 7 (77.8%) had microalbuminuria and 8 (88.9%) had fundus changes and 7 (77.8%) had nerve conduction abnormalities with significant difference between this group and patients with either the C/C or C/T genotype. Is this a true effect or may be affected by other parameters? When we compared between patients with (T/T) genotype and other groups as regards age, disease duration and HBA1c, we found non significant difference which emphasize that the high occurrence of microvascular complications was caused by the MTHFR gene polymorphism and not by other factors. The last conclusion was emphasized by the multivariate stepwise logistic regression analysis which demonstrated that MTHFR gene polymorphism was an independent risk factor for the occurrence of microalbuminuria, fundus changes and nerve conduction abnormalities. In the literature there are some conflicting data.

A large meta-analysis done by Zintzraras et al. [11] reviewed 15 studies about the relationship between MTHFR gene polymorphism and the occurrence of diabetic nephropathy. These studies involved 8 Caucasians, 5 East Asians, one mixed population and one Arab. Eleven studies involved subjects with type 2 diabetes and 4 with type 1 diabetes. The studies provided 1871 cases and 2150 controls. The main analysis for the investigation was the association between the T/T genotype and diabetic nephropathy which revealed significant heterogeneity. Also in subgroup analysis (Caucasians versus East Asians) and (type 1 versus type 2 diabetes) significant heterogeneity was detected. Overall the meta-analysis results indicated that homozygosity in allele T (T/T genotype) was associated with a 65% increased risk of diabetic nephropathy compared with homozygosity in allele C (C/C genotype). This meta-analysis although did not reach a conclusive final result may be supported by the accumulating evidence of the effect of homocysteine on endothelial cells and blood vessels. In the beginning Fryer et al. [12] documented increased tissue factor activity in patients with elevated homocysteine levels which represented an independent risk factor for premature arterial vascular disease. Following Fryer, Hofmann et al. [13] documented the adverse effect of homocysteine in patients with IDDM mainly the occurrence of cardiovascular disease and diabetic nephropathy.

Also Ragone [14] emphasized the adverse effect of elevated homocysteine on blood vessels and finally Dinelyici et al., [15] found patients with type 1 DM with hyperhomocysteinemia to have a reduced glomerular filtration rate when compared to normohomocysteinemic diabetic patients.

After this important meta-analysis Wiltshire et al. [5] reported that the C/C genotype had a protective effect against the occurrence of microalbuminuria. Finally Ukinc et al. [16] stated a significant correlation between diabetic nephropathy and T/T polymorphism.

The association between retinopathy in type 2 diabetes (DR) and the C677T polymorphism in the MTHFR gene has been investigated in several case–control studies. These

studies rendered contradictory results, some indicating that the polymorphism is associated with the risk of developing DR whereas others concluded there is no association. To shed light on these inconclusive findings, a meta-analysis of all available studies relating the C677T polymorphism to the risk of developing DR was conducted. Four out of five identified studies included populations of East Asian descent, and suggested large heterogeneity between studies ($p = 0.08$, 1(2)–52%) and marginal association between C677T transition and the risk of developing DR random effects odds ratio (OR) = 1.39 [95% CI (1.05 1.83)] [17].

After this meta-analysis was published Maedia et al. [18] and Wiltshire et al.[5] found patients with (T/T) genotype to have reduced survival free of retinopathy. On the other hand Ukinc et al. [16] did not find any association between diabetic retinopathy and MTHFR gene polymorphism.

We found very scarce data about the association between this polymorphism and the occurrence of neuropathy, Ukinc et al. [16], denied any association.

Why these conflicts and contradictory results?

First these studies were done on different ethnic groups and our study may be one of the very few – if any studies were done on Arabs and the first study to be done in Egypt.

Second many studies were done on adults with type 2 DM which may be totally different from the pediatric population with type 1 diabetes.

Finally many results especially negative ones may be unpublished or published in local journals which may add to the conflict.

At the end our study demonstrated a strong association between MTHFR gene polymorphism (T/T genotype) and the occurrence of all microvascular complications (nephropathy, retinopathy and neuropathy) in patients with type 1 diabetes mellitus in Egypt.

The authors declare that there is no conflict of interest.

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