

C677T polymorphism of the methylenetetrahydrofolate reductase gene does not affect folic acid, vitamin B₁₂, and homocysteine serum levels in Turkish children with neural tube defects

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ABSTRACT. Association between neural tube defects (NTDs) and C677T polymorphism of the methylenetetrahydrofolate reductase (MTHFR) gene was suspected, because the MTHFR gene codes for a key enzyme in folate metabolism. Its deficiency usually leads to significant reductions in plasma concentrations of folate, vitamin B₁₂ and methionine, whereas homocysteine levels are

increased. We examined folate, vitamin B₁₂ and homocysteine serum concentrations and polymorphism of the C677T MTHFR gene in Turkish children with neural tube defects. Thirty-three children with NTDs, 26 mothers and 48 healthy individuals were studied. C677T MTHFR polymorphism was determined by melting curve analyses (LightCycler®). The levels of folate, vitamin B₁₂ and homocysteine serum concentrations in NTDs were evaluated and compared, along with information concerning alleles of the MTHFR gene. C677T allele frequencies in NTD children and their mothers were similar to those found in controls. Serum folate and vitamin B₁₂ concentrations were significantly higher in NTD children than that of controls. Serum homocysteine concentrations were not significantly higher in NTD children and mothers. We concluded that C677T MTHFR gene polymorphism does not affect folic acid, vitamin B₁₂ and homocysteine metabolism in Turkish children with NTDs. C677T polymorphism of the MTHFR gene cannot be regarded as a major risk factor for NTDs in Turkish children.

Key words: C677T polymorphism; Folate; Homocysteine; MTHFR; Vitamin B₁₂

INTRODUCTION

Neural tube defects (NTDs) that occur due to failure of neural tube closure are among the most prevalent and severe of all birth defects. A multifactorial mode of inheritance, including genetic predisposition, maternal nutritional deficiencies and other environmental factors, is implicated in the etiology of NTDs (Eskes, 1998; Melvin et al., 2000). NTDs, which encompass a broad spectrum of phenotypes ranging from spina bifida to anencephaly, affect approximately 3 per 1000 live births in Turkey (Aydinli et al., 1998; Tunçbilek et al., 1999). Folate deficiency during pregnancy is considered to be one of the most important risk factors for NTDs and its supplementation before and during early pregnancy can significantly reduce the prevalence of NTDs (Eskes, 1998).

The methylenetetrahydrofolate reductase (MTHFR) gene is located on chromosome 1 at 1p36.3 (Goyette et al., 1998). MTHFR is a key enzyme in folate metabolism. Its deficiency usually leads to a significant reduction in plasma concentrations of folate, vitamin B₁₂ and methionine, whereas homocysteine is increased (Eskes, 1998). Impairment in folate metabolism is implicated as a risk factor for NTDs. The MTHFR gene with a substitution in nucleotide 677 (C to T), which allows the generation of a thermolabile enzyme with decreased activity, has been implicated in the pathogenesis of NTDs in some populations (Eskes, 1998; van der Put and Blom, 2000; Melvin et al., 2000). On the other hand, the association between C677T MTHFR gene polymorphism and NTDs could not be demonstrated in other studies (Boduroglu et al., 1999; Volcick et al., 2000).

In the present study, we analyzed the concentration of folate, vitamin B₁₂ and homocysteine and the distribution of C677T MTHFR gene polymorphism in Turkish children with NTDs, their mothers and healthy individuals.

MATERIAL AND METHODS

Patient population

We studied 33 children aged between 6 days and 11 years with NTDs, and mothers of NTD children (20-39 years old) both seen at the Afyonkarahisar Kocatepe University Hospital, Afyonkarahisar, Turkey. The control group consisted of 48 unrelated individuals (25-40 years old), from the same region. Clinical distribution of lesions in NTD children is presented in Table 3. The local Ethics Committee approved the protocol and written informed consent was obtained from the participants and their relatives.

Biochemical analysis

Whole blood was directly drawn into a Vacutainer[®] serum tube without anti-coagulant, then allowed to clot at $4 \pm 2^\circ\text{C}$ for 15-20 min and promptly centrifuged at 2000 g for 10 min at $4 \pm 2^\circ\text{C}$. Serum samples were stored at -80°C until analysis.

Homocysteine levels in serum samples were quantified with the use of a Dade Behring BN* II Nephelometer according to the manufacturer protocol. *In vitro* diagnostic reagents for the quantitative determination of total homocysteine in human serum was performed by means of particle enhanced immunonephelometry with the BN* II System. The concentration of homocysteine is reported as $\mu\text{mol/L}$.

Serum vitamin B₁₂ levels were quantified with the Beckman Coulter Access[®] Immunoassay System. The Access Vitamin B₁₂ assay is a paramagnetic particle, chemiluminescent immunoassay for the quantitative determination of vitamin B₁₂ levels in human serum using the Access Immunoassay System. Data are reported as pg/mL.

The levels of serum folate were quantified with the use of the ARCHITECT[®] System (Abbott Laboratories). The ARCHITECT Folate assay is a Chemiluminescent Microparticle Folate Binding Protein assay for the quantitative determination of folate in serum. The ARCHITECT Folate assay is a two-step assay to determine the presence of folate in human serum using the Chemiluminescent Microparticle Immunoassay (CMIA) technology with flexible assay protocols, referred to as Chemiflex[™]. Two pretreatment steps mediate the release of folate from endogenous folate-binding protein. The levels of folate are reported as ng/mL.

Genetic analysis

DNA was extracted from a 200- μL peripheral blood sample by a High Pure Template Preparation (Roche Diagnostics, Indianapolis, IN, USA) kit. Then, DNA amount and DNA purity were quantified for each DNA sample by spectrophotometry (Nanodrop ND-1000). DNA samples were stored at -20°C until use.

LightCycler[®] FastStart^{PLUS} DNA Master Hybridization Probes (Roche Diagnostics), LightMix[®] (TIB MOLBIOL, Berlin, Germany) and LightCycler[®] Instrument 1.2 were used for analyzing the C677T polymorphism in the MTHFR gene.

A 233-bp fragment of the MTHFR gene is amplified with specific primers and detected with probes labeled with LightCycler[®] Red 640 (detected in channel 640). Cycling conditions for MTHFR were initial denaturation at 95°C for 10 min, followed by 45 cycles with

denaturation at 95°C for 5 s, annealing at 55°C for 10 s and extension at 72°C for 15 s with a ramping time of 20°C/s. After amplification, melting curves have been generated following denaturation of the reaction at 95°C for 20 s, holding the sample at 40°C for 20 s and then slowly heating the sample to 85°C with a ramp rate of 0.2°C/s and simultaneous monitoring of fluorescence decline (Figure 1). Polymorphism is determined by running a melting curve with a specific melting point (T_m) of $55 \pm 1.5^\circ\text{C}$ for the mutant and $62.5 \pm 1.5^\circ\text{C}$ for the wild type in channel 640. The supplied positive control allows the verification of the experiment (TIB MOLBIOL).

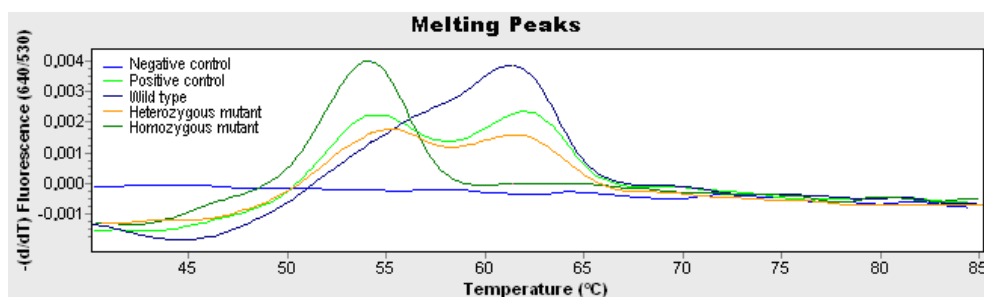


Figure 1. Melting-curve analysis was performed to analyze the MTHFR C677T polymorphism.

Statistical analysis

Statistical analyses were performed using SPSS for Windows (version 13.0, SPSS Inc., Chicago, IL, USA). The chi-square test was used to compare allele frequencies and genotypes for the 677CC/CT/TT position in the MTHFR gene between NTD children, mothers and healthy individuals. To evaluate the effect of the polymorphism on the variation of biochemical parameters, a one-way analysis of variance was performed. The Tukey HSD test was used for intra-group comparisons. Statistical significance was established at $P < 0.05$.

RESULTS

Of 33 children with NTDs, 13 were girls and 20 were boys. None of the mothers had used folic acid preconceptionally. There were not any factors associated with NTD, such as exposure to radiation, anticonvulsant medication, chemical substances, or diabetes mellitus.

Table 1 shows the distribution of genotype and allele frequencies for C677T MTHFR polymorphisms in NTD children, NTD mothers, and controls. According to the data, the C677T MTHFR genotype frequencies in NTD children and NTD mothers were similar to those of the controls ($\chi^2 = 0.702$, $P = 0.704$). Similarly, the 677TT genotype frequency was not higher than controls in NTD cases and mothers ($\chi^2 = 2.081$, $P = 0.353$). Frequencies of C and T alleles between the three groups were not statistically significant ($\chi^2 = 3.894$, $P = 0.143$).

We evaluated the effect of the C677T genotype on vitamin concentrations. There was a significant difference in serum folate and vitamin B₁₂ concentrations between groups ($P = 0.041$ and 0.002 , respectively) (Table 2).

Serum homocysteine concentration was not significantly higher in NTD children and mothers as compared to controls ($P = 0.226$) (Table 2).

Table 1. Methylenetetrahydrofolate C677T genotype and allele frequencies.

Group (N)	Genotype frequency			Allele frequency	
	C/C	C/T	T/T	C	T
NTD cases (33)	13 (39.4%)	16 (48.5%)	4 (12.1%)	42 (63.6%)	24 (36.4%)
Mothers (26)	5 (19.2%)	14 (53.8%)	7 (26.9%)	24 (46.2%)	28 (53.8%)
Controls (48)	18 (37.5%)	21 (43.8%)	9 (19.6%)	57 (59.4%)	39 (40.6%)

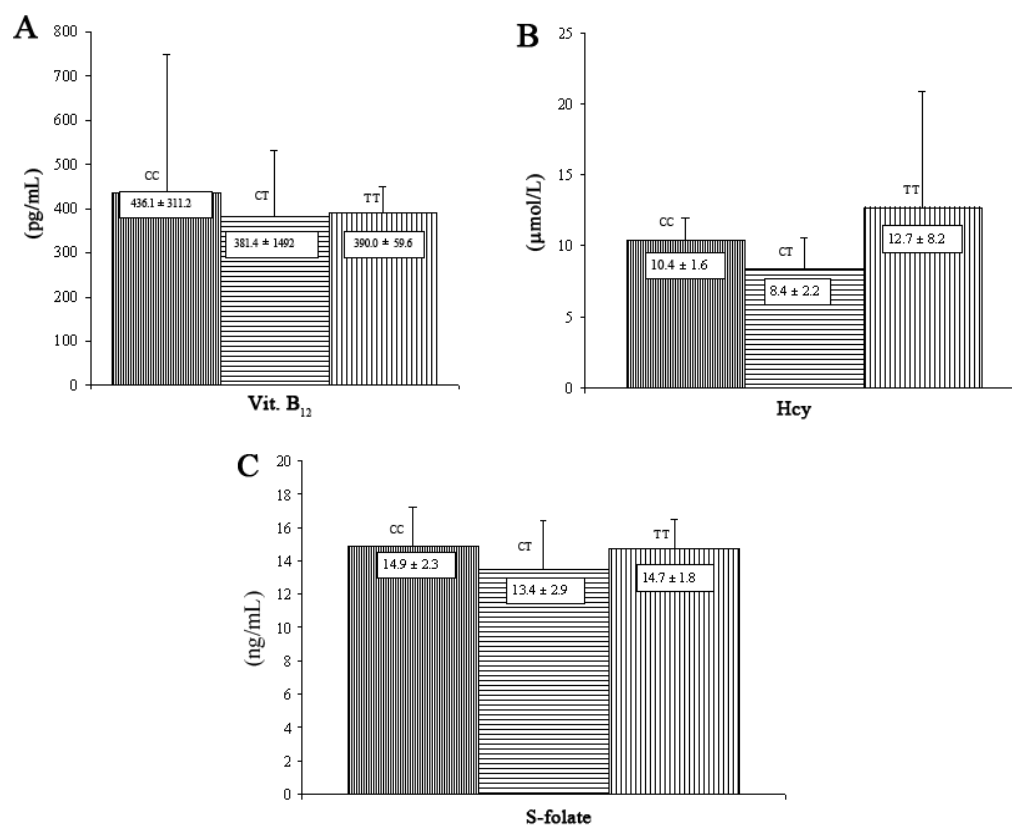
Data are reported as number of individuals with percent in parentheses. NTD = neural tube defects.

Table 2. Serum folate (S-folate), vitamin B₁₂ (Vit. B₁₂) and homocysteine (Hcy) in the groups studied.

	NTD cases	Mothers	Controls	P
S-folate (ng/mL)	14.17 ± 2.61	12.49 ± 2.52	12.97 ± 2.78	0.041
Vit. B ₁₂ (pg/mL)	404.00 ± 218.59	278.88 ± 98.31	299.10 ± 110.11	0.002
Hcy (μmol/L)	9.70 ± 3.44	11.09 ± 3.41	10.36 ± 2.49	0.226

Data are reported as means ± SD.

There was no significant difference in serum folate and vitamin B₁₂ concentrations between 677CC, 677CT and 677TT genotypes in the NTD group ($P > 0.05$) (Figure 2).

**Figure 2.** Homocystein (Hcy), serum folate (S-folate) and vitamin B₁₂ (Vit. B₁₂) concentrations (means ± SD) in neural tube defect children with different MTHFR genotypes (CC, CT, TT).

Serum homocysteine concentration between each genotype in the NTD group was significant ($P = 0.045$). There was no significant association between clinical distribution of NTDs and 677CC/CT/TT genotypes ($P > 0.05$) (Table 3).

Table 3. Distribution of lesions between CC/CT/TT genotypes.

Type of lesion	Genotype			P
	CC	CT	TT	
Myelomeningocele	7	7	-	NS
Meningocele	2	2	-	NS
Spina bifida oculata	2	5	3	NS
Encephalocele	2	2	1	NS

Data are reported as number of individuals. NS = non-significant.

DISCUSSION

In our study, the results of C677T polymorphism of MTHFR gene analysis did not show an association between NTD children, mothers and controls. The frequency of T allele in affected children was similar to those found in NTD patients from Europe and Turkey (Boduroglu et al., 1999; van der Put and Blom, 2000; Volcick et al., 2000). However, the direct association between C677T polymorphism of the MTHFR gene and NTDs found in other populations could not be demonstrated in our study (Eskes, 1998; Melvin et al., 2000; Cunha et al., 2002; Karalti et al., 2007). The reason for the variance between different studies could be the difference of 677T allele frequency among different populations. Countries where MTHFR polymorphism has been implicated in the susceptibility to NTDs had relatively low frequency of the 677T allele in the control group. The C677T polymorphism was shown to induce an enzyme with thermolabile properties and with decreased activity, resulting in elevated plasma homocysteine concentrations (Frosst et al., 1995). The effect of the C677T polymorphism can be reversed by additional folic acid intake (Anonymous, 1991; Medical Research Council Vitamin Study Research Group).

Lesion distribution of NTDs was similar between the genotypes of three groups in our study. The study of Samson (2003) did not find any statistically significant association between the lesion distribution of NTDs in consanguineous and non-consanguineous populations.

In the NTD patients of our study, the C677T polymorphism did not reduce the concentrations of serum folate and vitamin B₁₂. Surprisingly, NTD children showed increased total serum folate and vitamin B₁₂ concentrations. Among the CC/CT/TT genotypes, children with the 677CC genotype showed increased vitamin B₁₂, but this was not significant ($P = 0.802$). Those with C677T polymorphism had significantly decreased homocysteine levels ($P = 0.045$). Decreased serum folate and vitamin B₁₂ concentrations in mothers in this study could be an important factor working synergistically with another genotype of MTHFR. Genetic-nutrient interaction (MTHFR polymorphism and low folate status) is associated with a greater risk for NTDs than each variable alone (Christensen et al., 1999). Thirteen percent of NTDs were attributed to the C677T MTHFR polymorphism but 50-70% of all NTDs could be prevented by periconceptional folic acid supplementation (Posey et al., 1996). This suggest that other polymorphisms in the MTHFR gene or other genes involving in folate pathways might have roles in NTDs. Low vitamin B₁₂ concentrations could also reduce methylation of homocysteine to methionine, enhancing the impairment of folate metabolism and increasing the risk for NTDs (Van der Put and Blom, 2000).

Homocysteine levels of mothers in our study were minimally increased but this was not significant. Polymorphism in genes involving homocysteine metabolism could affect the plasma levels. Data on the homocysteine levels were contradictory. Van der Put et al. (1998) demonstrated that the C677T polymorphism in the MTHFR gene caused elevated homocysteine levels. According to the study, homocysteine levels alone might not always show the possible molecular defect.

In conclusion, our study indicates that C677T MTHFR gene polymorphism does not affect folic acid, vitamin B₁₂ or homocysteine metabolism in Turkish children with NTDs. Maternal folate and vitamin B₁₂ status during the periconceptional period may be critical in the development of NTDs, and our data support the hypothesis of the multifactorial etiology of NTDs, involving the combination of both genetic and nutritional factors. A higher number of NTD patients need to be studied to confirm these results.

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