



# Lack of association between the methylenetetrahydrofolate reductase gene A1298C polymorphism and neural tube defects in a Turkish study group

S.H. Yildiz<sup>1\*</sup>, M. Ozdemir Erdogan<sup>1</sup>, M. Solak<sup>1</sup>, O. Eser<sup>2</sup>, E.S. Arıkan Terzi<sup>1</sup>, B. Eser<sup>3</sup>, V. Kocabaş<sup>4</sup> and A. Aslan<sup>5</sup>

<sup>1</sup>Department of Medical Genetics, Faculty of Medicine, Afyon Kocatepe University, Afyonkarahisar, Turkey

<sup>2</sup>Department of Surgical Sciences, Faculty of Medicine, Balıkesir University, Balıkesir, Turkey

<sup>3</sup>Department of Medical Genetics, Faculty of Medicine, Balıkesir University, Balıkesir, Turkey

<sup>4</sup>Department of Biochemistry, Meram Medical Faculty, Selçuk University, Konya, Turkey

<sup>5</sup>Department of Neurosurgery, Faculty of Medicine, Afyon Kocatepe University, Afyonkarahisar, Turkey

Corresponding author: S.H. Yildiz

E-mail: handanstart@gmail.com

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**ABSTRACT.** The etiology underlying neural tube defects (NTDs) is not fully understood and is believed to involve a complex milieu of genetic and environmental factors. The A1298C polymorphism in the methylenetetrahydrofolate reductase gene (*MTHFR*) has been associated with mild risk for NTDs. In this study, the genotype distribution of the *MTHFR* gene A1298C polymorphism and the levels

of serum homocysteine, vitamin B12, and folate were evaluated in 33 children with NTDs, their mothers, and 46 healthy controls. Genotyping of the A1298C polymorphism was performed by real-time polymerase chain reaction. The A and C allele frequencies in children with NTDs and their mothers were similar to controls ( $P = 0.160$ ). The 1298AA and 1298CC genotype frequencies ( $P = 0.551$  and  $0.062$ , respectively) in children with NTDs and their mothers did not differ from controls. On the other hand, the 1298AC genotype frequencies in children with NTDs and their mothers were significantly different from controls ( $P = 0.025$ ). The genotype frequency of 1298AC was lower in children with NTDs than in controls. There was no significant association between clinical distribution of NTDs and 1298AA/AC/CC genotypes ( $P > 0.05$ ). Serum vitamin B12 levels were higher in children with NTDs than their mothers and controls ( $P = 0.001$ ). There were no differences among serum homocysteine and folate levels in all groups ( $P = 0.494$  and  $0.141$ , respectively). Both genetic and nutritional factors are important in the etiology of NTDs. Thus, the A1298C polymorphism cannot be regarded as a major risk factor for NTDs.

**Key words:** MTHFR; A1298C; Polymorphism; Neural tube defects

## INTRODUCTION

Neural tube defects (NTDs) are congenital malformations of the brain and spinal cord in neurulation that occur between 21 and 28 days after conception (Christianson, 2006). Anencephaly, encephalocele, and meningocele which are commonly called spina bifida, are the most common and severe forms of the NTDs (De Marco et al., 2000) and affect approximately 3 per 1000 live births in Turkey (Aydinli et al., 1998; Tunçbilek et al., 1999). The cause of NTD is assumed to be multifactorial, involving a large number of unclear genetic and environmental interactions (Copp et al., 1990).

Folate deficiency is one contributor to the etiology of NTDs, and this metabolic pathway has been the basis for many candidate gene studies (Boyles et al., 2005). The current experimental concept related to folate metabolism focuses on the role of methylation in neural tube closure (Afman et al., 2005; Blom et al., 2006; Dunlevy et al., 2006; van der Linden et al., 2008). Polymorphisms in the folate metabolism genes may play an important role in increasing susceptibility for NTDs (Barber et al., 1999). Methylene tetrahydrofolate reductase plays a central role in the metabolism of folate (Perez et al., 2003). Two common polymorphisms, C677T and A1298C, of the *MTHFR* gene show decreased enzyme activity and have been independently associated with NTD (Gonzalez-Herrera et al., 2007). Furthermore, it has been reported that the A1298C polymorphism in the *MTHFR* gene has been associated with MTHFR deficiency and mild risk for NTDs. Homozygosity for this polymorphism results in decreased enzyme activity (van der Put et al., 1998; Weisberg et al., 1998). Decreased enzyme activity usually leads to a significant reduction in plasma concentrations of folate, vitamin B12, and methionine, while levels of homocysteine are increased. The effects of the *MTHFR* polymorphisms on the proper function of the enzyme can be reversed by additional folic acid intake (Eskes, 1998). Periconceptional folic acid supplementation substantially reduces the

risk of NTD-affected pregnancies (MRC Vitamin Study Research Group, 1991). The risk of recurrent NTDs is decreased in women who take folic acid or multivitamins during the periconceptional period (Czeizel and Dudas, 1992).

In this study, we investigated the plasma concentrations of folate, vitamin B12, and homocysteine in mothers and the distribution of the A1298C *MTHFR* gene polymorphism in Turkish children with NTDs.

## MATERIAL AND METHODS

### Patient population

We studied 33 children with NTDs (aged 6 days to 11 years) and their mothers (N = 26, 20-39 years old). Both were seen at the Afyonkarahisar Kocatepe University Hospital (Afyonkarahisar, Turkey). Of 33 children with NTDs, 13 were female and 20 were male. None of the mothers had used folic acid preconceptionally. No additional factors associated with NTD, such as exposure to radiation, anticonvulsant medication, chemical substances, or diabetes mellitus, were identified in any of the subjects. The control group consisted of 48 unrelated healthy individuals from the same region (aged 1 to 40 years). Clinical distribution of lesions in children with NTD is presented in Table 1. All participants (or their responsible next of kin) provided written informed consent and were studied under a protocol approved by the Afyon Kocatepe University Medical Ethics Committee.

**Table 1.** Distribution of lesions between AA/AC/CC genotypes.

Type of lesion	Genotype			P value
	AA	AC	CC	
Myelomeningocele (N)	6	2	6	NS
Meningocele (N)	2	-	2	NS
Spina bifida occulta (N)	5	-	5	NS
Encephalocele (N)	2	-	3	NS

NS = not significant.

### Biochemical analysis

To prepare serum, whole blood was directly drawn into a Vacutainer® serum tube that contained no anti-coagulant. Blood was allowed to clot at  $4^{\circ} \pm 2^{\circ}\text{C}$  for 15-20 min and promptly centrifuged at 2000 g for 10 min at  $4^{\circ} \pm 2^{\circ}\text{C}$ . Serum samples were stored at  $-80^{\circ}\text{C}$  until analysis.

Homocysteine levels in serum samples were quantified with the use of a Siemens Dade Behring BN\* II Nephelometer (USA) according to the manufacturer protocol. *In vitro* diagnostic reagents for the quantitative determination of total homocysteine in human serum were done using particle enhanced immunonephelometry with BN\* II System. The concentration of homocysteine is expressed as  $\mu\text{M}$ . Serum vitamin B12 levels were quantified with the use of a Beckman Coulter Access® Immunoassay System (USA). 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 Systems. Data are presented as pg/mL. The levels of serum folate were quantified with the use of an ARCHITECT® System (Abbott Laboratories, USA). The ARCHITECT folate assay uses chemiluminescent microparticle folate binding protein assay for the quantitative determination

of folate in serum. It is a two-step assay to determine the presence of folate in human serum using chemiluminescent microparticle immunoassay (CMIA) technology with flexible assay protocols referred to as Chemiflex™ (Applied Biosystems, USA). Two pre-treatment steps mediate the release of folate from endogenous folate binding proteins. The levels of folate are presented as ng/mL.

### Genetic analysis

Genomic DNA was extracted from a 200- $\mu$ L peripheral blood sample using a High Pure Template Preparation kit (Roche Diagnostics, Indianapolis, IN, USA). DNA quantity and purity were assessed for each sample using a Nanodrop ND-1000 spectrophotometer V 3.7 (Thermo, USA). DNA samples were stored at  $-20^{\circ}\text{C}$  until use. LightCycler® (Roche Diagnostics), FastStart<sup>PLUS</sup> DNA Master Hybridization Probes (Roche Diagnostics), LightMix® (TIB MOLBIOL, Berlin, Germany), and LightCycler® Instrument 1.2 (Roche, Germany) were used for analyzing the A1298C polymorphism of the *MTHFR* gene. With this technology, real-time detection of the specific product followed by detection of the mutation by identification of the melting behavior of one of the two hybridization oligonucleotides was facilitated. A 163-bp fragment of the *MTHFR* gene was amplified with specific primers and detected with probes labeled with LightCycler® Red 640 (Roche, Germany) (detected in channel 640). The polymorphism was determined by running a melting curve with a specific melting point ( $T_m$ ) of  $59.0^{\circ}\text{C}$  ( $\pm 1.5^{\circ}\text{C}$ ) for the mutant and  $65.0^{\circ}\text{C}$  ( $\pm 1.5^{\circ}\text{C}$ ) for the wild-type in channel 640. The supplied positive control allowed for the verification of the experiment (TIB MOLBIOL). The cycling conditions for *MTHFR* were initial denaturation at  $95^{\circ}\text{C}$  for 10 min, followed by 40 cycles of  $95^{\circ}\text{C}$  for 5 s,  $62^{\circ}\text{C}$  for 10 s, and  $72^{\circ}\text{C}$  for 6 s with a ramping time of  $20^{\circ}\text{C}/\text{s}$ . After amplification, melting curves were generated by denaturation of the reaction at  $95^{\circ}\text{C}$  for 20 s, holding the sample at  $40^{\circ}\text{C}$  for 20 s and then slowly heating the sample to  $85^{\circ}\text{C}$  with a ramp rate of  $0.2^{\circ}\text{C}/\text{s}$  and simultaneous monitoring of fluorescence decline. LightCycler technology is a homogenous system that minimizes polymerase chain reaction (PCR) contamination concerns due to sample handling (closed capillaries throughout the PCR and detection process) and does not require digestion of PCR products with restriction enzymes and/or separation of fragments on gels.

### Statistical analysis

Statistical analyses were performed using SPSS for Windows (Version 13.0, SPSS Inc., Chicago, IL, USA). A chi-square test was used to compare allele frequencies and genotypes for the 1298AA/AC/CC position in the *MTHFR* gene between children with NTDs, their mothers, and healthy individuals. To evaluate the effect of the mutation on the variation of biochemical parameters, a one-way analysis of variance was performed. Tukey's honest significant different test was used for intra-group comparisons. Statistical significance was established at  $P < 0.05$ .

## RESULTS

Table 2 shows the genotype distribution and the relative allele frequencies for the *MTHFR* gene A1298C polymorphism in children with NTDs, their mothers, and healthy controls. A and C allele frequencies in children with NTDs and their mothers were similar to

what was observed in controls ( $P = 0.160$ ). The genotype frequencies for 1298AA and 1298CC did not differ from controls in children with NTDs and their mothers ( $P = 0.551$  and  $P = 0.062$ , respectively). On the other hand, the 1298AC genotype frequencies in children with NTDs and their mothers were significantly different from the healthy controls ( $P = 0.025$ ). The genotype frequency for 1298AC was lower in children with NTDs than controls. Moreover, there was not a significant association between clinical distribution of NTDs and the 1298AA/AC/CC genotypes ( $P > 0.05$ ) (Table 2). Serum folate, vitamin B12 and homocysteine levels in groups were analyzed and it was found that serum vitamin B12 concentrations were higher in children with NTDs than in their mothers and controls ( $P = 0.001$ ). Serum homocysteine and folate levels were not significantly different in children with NTDs and their mothers in comparison to the controls ( $P = 0.494$  and  $P = 0.141$ , respectively; Table 3). Furthermore, we evaluated the effect of the 1298AC genotype on serum folate, vitamin B12, and homocysteine levels in children with NTDs. There was no significant difference in serum folate, vitamin B12, and homocysteine levels between 1298AA, 1298AC and 1298CC genotypes in the children with NTDs group (data not shown).

**Table 2.** *MTHFR* A1298C genotype and allele frequencies.

Group (N)	Genotype frequencies [% (N)]			Allele frequencies [% (N)]	
	AA	AC	CC	A	C
NTD Cases (33)	45.5 (15)	6.0 (2)	48.5 (16)	48.5 (32)	51.5 (34)
Mothers (26)	50 (13)	30.8 (8)	19.2 (5)	65.4 (34)	36.4 (18)
Controls (48)	37.5 (18)	29.2 (14)	33.3 (16)	52.1 (50)	47.9 (46)

NTD = neural tube defects.

**Table 3.** Serum folate (S-folate), vitamin B12 (vit B12), and homocysteine (Hcy) levels in study groups.

	NTD cases (means $\pm$ SD)	Mothers (means $\pm$ SD)	Controls (means $\pm$ SD)	P value
S-folate (ng/mL)	13.22 $\pm$ 2.20	12.06 $\pm$ 2.50	13.15 $\pm$ 2.66	0.141
vit B12 (pg/mL)	408.82 $\pm$ 217.26	281.73 $\pm$ 98.79	292.73 $\pm$ 110.58	0.001
Hcy ( $\mu$ M)	9.66 $\pm$ 3.32	10.66 $\pm$ 3.83	10.31 $\pm$ 3.07	0.494

## DISCUSSION

The *MTHFR* A1298C polymorphism is a missense mutation consisting of a nucleotide transition (1298A $\rightarrow$ C), which results in a glutamate to alanine substitution (van der Put et al., 1998). In this study, we analyzed the genotype distribution and allele frequencies of the *MTHFR* gene A1298C polymorphism in children with NTDs, their mothers, and healthy controls in Turkey. This present study indicates that A and C allele frequencies in children with NTDs and their mothers were similar to what is observed in controls. On the other hand, the 1298AC genotype frequencies in children with NTDs and their mothers were significantly different from the controls. This result cannot be considered as a risk factor because the analysis of the 1298AA and 1298CC genotype frequencies showed similar distribution in children with NTDs and their mothers compared to controls. Similarly, Haggarty et al. (2008) reported that the frequency of the 1299CC genotype was essentially the same in the children and their mothers (9.0 and 9.1%, respectively). Furthermore, Cunha et al. (2002) could not find a direct association between the A1298C polymorphism and occurrence of NTDs. Parle-McDermott et al. (2003) reported that case-control comparisons and log linear analysis did

not reveal an association between the A1298C polymorphism and occurrence of NTDs. Data from Europe and the Hispanic population of the USA showed no difference in the A1298C polymorphism between patients with NTDs and the controls (Stegmann et al., 1999; van der Put and Blom, 2000; Volcik et al., 2000). In contrast, Boduroğlu et al. (1999) concluded that the *MTHFR* 1298AC polymorphism might be a risk factor for the occurrence of NTDs in the Turkish population although it is not statistically significant. De Marco et al. (2002) suggested that the *MTHFR* A1298C polymorphism is a genetic determinant for NTD risk in Italy. Also, Weisberg et al. (1998) reported an association between the A1298C polymorphism and NTD risk. Lesion distribution of NTDs was similar between the genotypes of all three groups in our study. Samson (2003) did not find any statistically significant association between the lesion distribution of NTDs in the consanguineous and non-consanguineous populations. Serum folate and homocysteine levels in our study were not different between groups. Hanson et al. (2001) also demonstrated that there was no effect of the A1298C polymorphism on homocysteine metabolism. Homozygosity for the 1298 *MTHFR* polymorphism (1298CC) does result in decreased enzyme activity but this polymorphism is not associated with elevated homocysteine levels or with an increased risk for NTD in offspring (van der Put and Blom, 2000). Similarly, individuals who are either heterozygous (1298AC) or homozygous (1298CC) for the polymorphism showed decreased enzyme activity although their plasma homocysteine levels were not altered significantly (van der Put et al., 1998; Weisberg et al., 1998).

In our study, mothers of children with NTDs had significantly lower vitamin B12 levels than other groups. Vitamin B12 is a nutritional factor and its deficiency may affect the development of NTDs in offspring. It has been reported that low vitamin B12 concentrations could lead to reduced methylation of homocysteine to methionine, enhancing the impairment of folate metabolism and increasing the risk for development of NTDs (van der Put and Blom, 2000). It has also been reported that the combination of *MTHFR* polymorphisms and low folate and vitamin B12 intake is associated with a greater risk for NTDs than each variable alone, indicating a strong genetic-nutritional interaction (Molloy et al., 1998; Stegmann et al., 1999). Cunha et al. (2002) reported that the mutant C allele was not associated with differences in folate and vitamin B12 concentrations in children with NTDs. Furthermore, the same researchers found higher concentrations of homocysteine in children with NTDs who have the 1289AA *MTHFR* genotype. It is reported that neither the homozygous nor heterozygous genotypes for the 1298AC polymorphism are associated with higher homocysteine nor lower plasma folate levels, although the 1298AC polymorphism has a significant effect on the activity of *MTHFR* (van der Put et al., 1998). Fifty to seventy percent of all NTDs could be prevented by periconceptual folic acid supplementation (Posey et al., 1996). This suggests that polymorphisms in the *MTHFR* gene or other genes involved in folate pathways and low nutritional folate or vitamin B12 intake might have roles in risk of NTDs. van der Put and Blom (2000) suggested that the A1298C *MTHFR* polymorphism may play a secondary role for risk of NTDs. Conflicting results exist regarding the association of the A1298C polymorphism in *MTHFR* and NTD risk. One explanation for this controversy might be that alleles are differently distributed among various populations (Gonzalez-Herrera et al., 2007).

We did not demonstrate an association between the *MTHFR* gene A1298C polymorphism and NTD risk in our study group. Moreover, the *MTHFR* gene A1298C polymorphism does not affect folic acid, vitamin B12, or homocysteine metabolism in Turkish children with NTDs. Low serum vitamin B12 levels in mothers of children with NTDs may be associated with their nutritional status. Maternal folate and vitamin B12 status during the

periconceptional period may be critical for 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.

### Conflicts of interest

The authors declare no conflict of interest.

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