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Original Investigation

677C>T and 1298A>C Polymorphisms of Methylenetetrahydropholate Reductase Gene and Biochemical Parameters in Turkish Population with Spina Bifida Occulta

Spina Bifida Okkultalı Hastalarda Metilentetrahidrofolat Redüktaz (MTHFR) Geninin C677T ve A1298C Polimorfizmlerinin Analizi ve Biyokimyasal Parametrelerinin Değerlendirilmesi

Betul ESER[1]
Murat COSAR[2]
Olcay ESER[3]
Mujgan O. ERDOGAN[4]
Adem ASLAN[5]
Handan YILDIZ[6]
Gazi BOYACI[7]
Sadik BUYUKBAS[8]
Mustafa SOLAK[9]

1 Selcuk University, Meram Faculty of Medicine, Department of Medical Genetics, Konya, Turkey
2 Canakkale 18 March University, Faculty of Medicine, Department of Neurosurgery, Canakkale, Turkey
3,5,7 Kocatepe University, Faculty of Medicine, Department of Neurosurgery, Afyonkarahisar, Turkey
4,6,9 Kocatepe University, Faculty of Medicine, Department of Medical Biology, Afyonkarahisar, Turkey
8 Selcuk University, Meram Faculty of Medicine, Department of Biochemistry, Konya, Turkey

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Correspondence address:
Betul ESER
E-mail: drbetuleser@hotmail.com

ABSTRACT

AIM: This study aimed to investigate the 677C>T and 1298A>C MTHFR gene polymorphisms and their metabolic effects on the levels of folate, vitamin B12 and homocysteine in the serum of Turkish spina bifida occulta (SBO) patients and healthy individuals in disease.

MATERIAL and METHODS: A case-control study was performed to detect 677C>T and 1298A>C MTHFR gene polymorphisms in 39 SBO patients and 34 healthy individuals. The folate, vitamin B12 and homocysteine concentrations in the serum of SBO and healthy individuals were evaluated and compared with MTHFR gene polymorphisms.

RESULTS: 677 CC/CT/TT MTHFR genotype frequency differences between the SBO patients and controls were not significant (x^2=3.325, P=0.068; x^2=1.479, P=0.224; x^2=0.275, P=0.600; respectively). 1298A>C MTHFR genotype frequency differences between the SBO patients and controls were significant (x^2=8.477, P=0.004). The frequencies of the A and C alleles of the 1298A>C polymorphism did not differ in a statistically significant manner between the groups (x^2=0.576, P=0.448). The biochemical parameters were not significantly different between SBO patients and healthy individuals (P>0.05).

CONCLUSION: The 677C>T and 1298A>C polymorphisms of the MTHFR gene cannot be regarded as major risk factors for SBO in the Turkish patients 677TT homozygosity may affect the metabolism of homocysteine.

KEYWORDS: 1298A>C polymorphism, 677C>T polymorphism, Folate, Homocysteine, MTHFR, Spina bifida occulta, Vitamin B12

ÖZ

AMAÇ: Çalışmamızda, SBO ve sağlıklı bireylerde 677C>T ve 1298A>C MTHFR gen polimorfizmlerini ve folat, vitamin B12 ve homosisteinin serum konsantrasyonu üzerine olan metabolik etkilerini araştırdık.

YÖNTEM ve GEREÇ: 677C>T ve 1298A>C MTHFR gen polimorfizmleri 39 SBO ve 34 sağlıklı birey üzerinde vaka kontrol yöntemiyle değerlendirildi.SBO ve sağlıklı bireylerin serum folat,vitamin B12 ve homosistein konsantrasyonları değerlendirildi.

BULGULAR: SBO ve sağlıklı bireyler arasında 677 CC/CT/TT MTHFR genotip frekansları farklılıkları anlamlı değildi. Fakat 1298A>C MTHFR genotip frekanslarının farklılığı anlamlı idi. SBO ve sağlıklı bireyler arasında biyokimyasal parametrelerin konsantrasyonların farklılığı anlamlı değildi.

SONUÇ: MTHFR geninin 677C>T ve 1298A>C polimorfizmlerinin Türk populasyonunda SBO hastalarında majör risk faktörü olduğunu düşünmüyoruz. 677TT homozigositesi homosistein metabolizmasını etkileyebilir.

ANAHTAR SÖZCÜKLER: 1298A>C polimorfizm, 677C>T polimorfizm, Folat, Homosistein, MTHFR, Spina bifida okülta, Vitamin B12

INTRODUCTION

The etiology of neural tube defects (NTDs) has not been fully understood and is believed to involve a complex milieu of genetic and environmental factors. NTDs have a large spectrum of phenotypes ranging from spina bifida occulta (SBO) to spina bifida aperta (SBA) and a prevalence of 30.1 per 10,000 births is reported for Turkey (18). The ratio of spina bifida to anencephaly is 1.20 in Turkey (18). Folate deficiency during pregnancy is considered to be one of the most important risk factors for NTDs (5,8). Current research is focused on clarifying the mechanisms underlying folate metabolism. MTHFR is a key enzyme in folate metabolism (15). Its deficiency usually leads to a significant reduction in serum concentrations of folate, vitamin B12 and methionine, as well as an elevation in homocysteine due to the impairment in folate metabolism (11).

MTHFR is a key enzyme for the reduction of methylenetetrahydrofolate, a single carbon donor that takes part in the synthesis of the nucleotide S-adenosylmethionine (SAM), remethylation of homocysteine to methionine, and the methylation of DNA, proteins, neurotransmitters, and phospholipids (1). The 677C>T allele converts cytosine (C) into a thymine (T); this mutation results in an amino acid substitution (alanine to valine) in the MTHFR enzyme. This mutation on the MTHFR gene makes the enzyme thermolabile, decreases its activity and increases homocysteine concentrations (6). Additionally, the 1298A>C allele converts an adenine (A) into a cytosine (C), causing the substitution of a glutamine to alanine. The central localization of the 677C>T polymorphism affects the capacity of the MTHFR enzyme more than the 1298A>C polymorphism, which is localized at the edge of the gene (13).

Most studies on the genetic tendency of NTDs have been in spina bifida case groups (3,22). Small groups of NTD, anencephaly and spina bifida occulta (SBO) are added to the some studies (12,14,19). Although the prevalence of SBO is 23% of the population (7), only a few SBO cases have been included in genetic studies in the literature.

Some authors have declared that MTHFR gene polymorhisms cannot change the capacity of MTHFR enzyme (2,23) despite the presence of other articles advocating the effects of the 677C>T and 1298A>C polymorphisms of the MTHFR gene on SBO (14,18).

We analyzed the serum concentrations of folate, vitamin B12 and homocysteine and the distribution of the 677C>T and 1298A>C polymorphisms of the MTHFR gene in Turkish adult SBO patients and healthy individuals in the present study.

MATERIALS and METHODS

Patients and Data Collection

We studied 39 adult patients with SBO aged between 19 and 49 years (mean 29.2) seen at the Afyonkarahisar Kocatepe University Hospital (Afyonkarahisar, Turkey). Of the 39 patients with SBO, 20 were female and 19 were male. The control group consisted of 34 unrelated individuals aged between 21 and 35 years (mean 27.0) from the same region. Twenty-four of the 34 healthy individuals were female and the remaining 10 were male. The spina bifida occulta defects of the patients were detected with plain graphies of the lumbar vertebrae. The protocol and the written informed consent obtained from the participants and/or their relatives was approved by the local ethical committee.

To our knowledge, the mothers of the participants of the study had no history of using folic acid or other drugs during their pregnancy. Additionally, there were no risk factors for the members of the study associated with SBO, such as exposure to radiation,

anticonvulsant medication, chemical substances or diabetes mellitus.

Biochemical analysis

Whole blood was directly drawn into a Vacutainer® serum tube that contained no anti-coagulant to prepare serum. Blood was allowed to clot at 4 ± 2ºC for 15-20 minutes and promptly centrifuged at 2000 x g for 10 minutes at 4 ± 2ºC. 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 protocol provided by the manufacturer. BN* II System was used for in vitro diagnostic reagents at the quantitative determination of total homocysteine in human serum by means of particle-enhanced immunonephelometry. The concentration of homocysteine is expressed as μmol/L.

Serum vitamin B12 levels were quantified with the use of a 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. Data are presented as pg/mL.

The levels of serum folate were quantified with the use of an ARCHITECT® System (Abbott Laboratories). The ARCHITECT Folate assay is a chemiluminescent microparticle folate binding protein assay for the quantitative determination of folate in serum, using Chemiluminescent Microparticle Immunoassay (CMIA) technology with flexible assay protocols, referred to as ChemiflexTM.* The levels of folate are presented as ng/mL.

Genetic analysis

The methylenetetrahydrofolate reductase (MTHFR) gene is located on chromosome 1 at 1p36.3(15). DNA was extracted from a 200μl peripheral blood sample by using the High Pure Template Preparation (Roche Diagnostics, Indianapolis, IN, USA) kit. DNA amount and purity were then quantified for each DNA sample by spectrophotometry (Nanodrop ND-100). DNA samples were stored at -20ºC until use.

LightCycler® FastStartPLUS DNA Master Hybridization Probes (Roche Diagnostics, Indianapolis, IN, USA), LightMix® (TIB MOLBIOL, Berlin, Germany) and LightCycler® Instrument 1.2 were used for analyzing the 677C>T and 1298A>C polymorphisms in the MTHFR gene.

A 233 bp fragment of the MTHFR gene was amplified with specific primers and detected with probes labeled with LightCycler® Red 640 (detected in channel 640). The 677C>T polymorphism was determined by running a melting curve with a specific melting point (Tm) of 55°C (±1.5ºC) for the mutant and 62.5°C (±1.5ºC) for the wild type in channel 640. The supplied positive control allowed for the verification of the experiment (TIB MOLBIOL, Berlin, Germany).

Cycling conditions for the 677C>T polymorphism were an initial denaturation at 95°C for 10 min, and followed by 45 cycles with denaturation at 95°C for 5 s. It also annealed at 55°C for 10 s and extended at 72°C for 15 s with a ramping time of 20°C/s. After amplification, melting curves for 677C>T were generated by 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 simultaneously monitoring the fluorecence decline (Figure 1).

A 163 bp fragment of the MTHFR gene was amplified with specific primers and detected with probes labeled with LightCycler® Red 640 (detected in channel 640). The polymorphism was determined by running a melting curve with a specific melting point (Tm) of 59.0°C (±1.5ºC) for the mutant and 65.0°C (±1.5ºC) for the wild type in channel 640. The supplied positive control allowed for the verification of the experiment (TIB MOLBIOL, Berlin, Germany).

Cycling conditions for the 1298A>C polymorphism were an initial denaturation at 94°C for 2 min, followed by 40 cycles with denaturation at 94°C for 10 s, annealing at 55°C 10 s and extension at 72°C for 15 s with a ramping time of 20°C/s. After amplification, melting curves were generated by following denaturation of the reaction at 94°C for 1 s, holding the sample at 40°C for 5 s and then slowly heating the sample to 80°C with a ramp rate of 0.6°C/s and simultaneously monitoring the fluorescence decline (Figure 2).

Statistical analysis

The Chi-square test was used to compare allele frequencies and genotypes for the 677CC/CT/TT and 1298AA/AC/CC positions in the MTHFR gene between SBO patients and healthy individuals. A one-

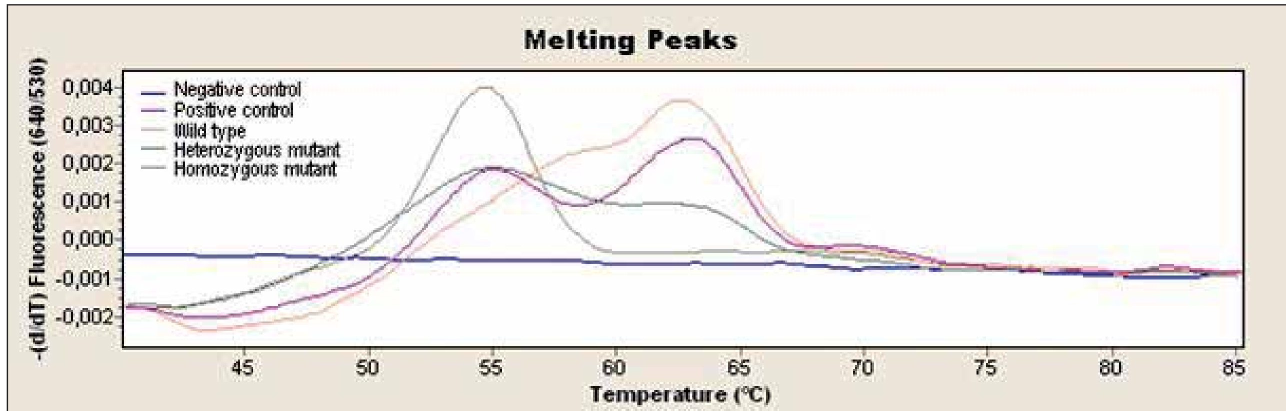

Figure 1. Real Time PCR. LightCycler melting curve analysis was performed to analyze the MTHFR 677C>T polymorphism.

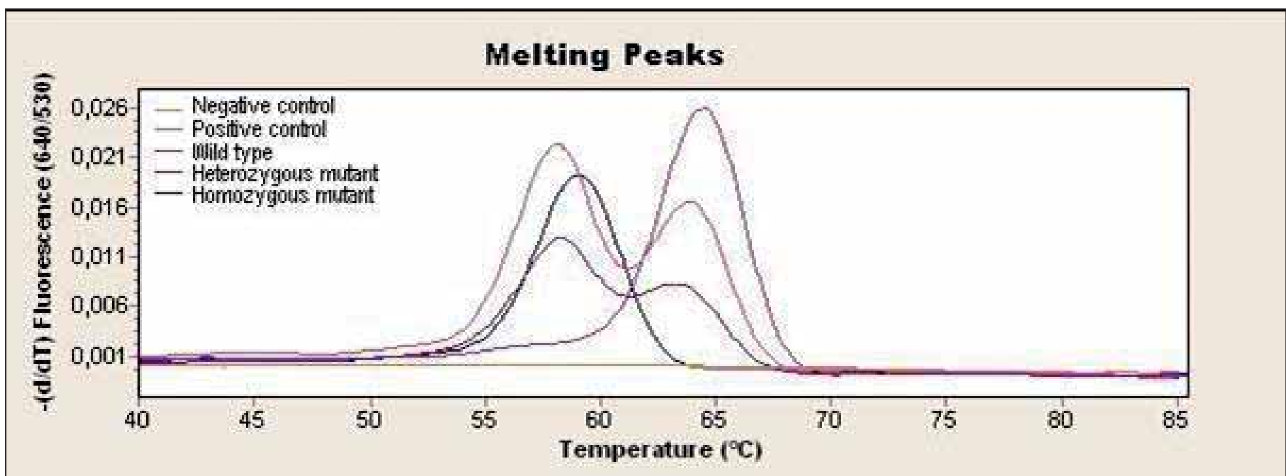

Figure 2. Real Time PCR. LightCycler melting curve analysis was performed to analyze the MTHFR 1298A>C polymorphism.

way analysis of variance was performed to evaluate the effect of the mutation on the variation in biochemical parameters. Tukey's HSD test was used for intra-group comparisons. SPSS for Windows (Version 13.0, SPSS Inc., Chicago, Illinois, USA) was used during statistical analyses. Statistical significance was established at $P<0.05$.

RESULTS

Table I show the distribution of genotype frequencies for the 677C>T and 1298A>C polymorphisms of MTHFR genes in SBO patients and controls.

According to the data, the 677CC/CT/TT MTHFR genotype frequencies between the SBO patients and controls were not significant (x^2=3.325, P=0.068; x^2=1.479, P=0.224; x^2=0.275, P=0.600; respectively). Additionally, the frequencies of the C and T alleles of the 677C>T polymorphism in SBO cases were 64.1% for C and 35.8% for T while the frequencies were 51.4% for C and 48.5% for T in controls. These data did not show a statistically significant difference between the groups (x^2=2.383, P=0.123). The frequencies of the A and C alleles of the 1298A>C polymorphism in SBO cases were 52.5% for A and 47.4% for C while they were 58.8% for A and 41.1% for C in controls. They were not statistically significant between the groups (x^2=0.576, P=0.448).

The statistical study of the 1298AA MTHFR and 1298CC MTHFR genotype frequencies between the SBO patients and controls showed no significant difference (x^2=0.417, P=0.518; x^2=3.246, P=0.072; respectively). The 1298AC MTHFR genotype frequency differences between the SBO patients and controls were significant (x^2=8.477; P=0.004). The frequencies of the A and C alleles of the 1298A>C polymorphism did not show a statistically significant difference between the groups (x^2=0.576, P=0.448).

Table I: Methylenetetrahydrofolate 677C>T and 1298A>C Genotype and Allele Frequencies.

	C677T (%, N)			A1298C (%, N)		
Group (N)	C/C	C/T	T/T	A/A	A/C	C/C
SBO cases (39)	46.1 (18)	35.8 (14)	17.9 (7)	48.7 (19)	7.6 (3)	43.5 (17)
Controls (34)	26.4 (19)	50.0 (17)	23.5 (8)	41.1 (14)	35.2 (12)	23.5 (8)

SBO: Spina Bifida Occulta.

The serum homocysteine, vitamin B12 and folate concentration differences between SBO patients and healthy individuals were not significant (P=0.065, 0.437 and 0.88, respectively) (Table II).

There was no significant difference in serum folate and vitamin B12 concentrations between the 677CC, 677CT and 677TT genotypes in the SBO group (P=0.61 and 0.50, respectively). Serum homocysteine concentration differences between each genotype in the SBO group were significant (P=0.003) (Table II).

The serum homocysteine, folate and vitamin B12 concentration differences between the 1298AA, 1298AC and 1298CC genotypes in the SBO group were not significant (P=0.428, 0.848, and 0.214, respectively).

There was no significant difference in serum homocysteine, folate and vitamin B12 concentrations between the 677CC, 677CT and 677TT genotypes in healthy individuals (P>0.05). The serum homocysteine, serum folate and vitamin B12 concentration differences between the 1298AA, 1298AC and 1298CC genotypes in healthy individuals were similarly not significant (P>0.05).

DISCUSSION

Our study demonstrated that the 1298A>C polymorphisms in the MTHFR gene analysis of SBO patients and healthy individuals did not affect biochemical parameters such as serum homocysteine, folate and vitamin B12 concentrations. However, the 677C>T polymorphisms in the MTHFR gene analysis of SBO patients affects the serum level of homocysteine, while it has no effect on the serum levels of folate and vitamin B12. Additionally, the 1298A>C and 677C>T polymorphisms of MTHFR were not significantly different as regards the allele frequencies of SBO patients and healthy individuals in our study.

Although there is no concensus in the literature about the association between the 677C>T polymorphism and SBO, our results are similar to the studies from Euroasia (2,15). However, some studies from different populations revealed a direct association between the 677C>T polymorphism and SBO (21,23). The difference in 677C>T allele frequencies from different populations could be the reason for this variance. Countries where the MTHFR polymorphism has been implicated in susceptibility to SBO had relatively low frequencies of the 677T allele in the control group.

An association between NTD risk and the 1298A>C polymorphism was reported by Weisberg et al. (24). They found decreased enzyme activity, but observed

Table II: Serum folate (S-folate), vitamin B_{12} (vit-B_{12}) and homocysteine (Hcy) in SBO.

	677CC	677CT	677TT	*P*
S-folate (ng/mL)	12.8±2.2	12.3±3.5	11.5±3.4	0.615
vit B_{12} (pg/mL)	319.0±76.9	367.2±201.2	300.1±119.6	0.506
Hcy (μmol/L)	10.5±4.0	9.7±2.8	19.4±12.2	0.003
	1298AA	1298AC	1298CC	
S-folate (ng/mL)	12.4±3.4	10.8±3.4	12.7±2.4	0.214
vit B_{12} (pg/mL)	323.2±119.3	234.0±68.9	361.2±163.9	0.848
Hcy (μmol/L)	13.4±8.5	88±0.7	10.6±4.5	0.428

SBO: Spina Bifida Occulta.

no difference in the prevalence of polymorphisms in NTD children or their mothers compared to controls. Van Der Put et al. (20) reported that the 1298A>C MTHFR mutation may play a secondary role for NTD risk. In contrast, European and Hispanic populations of US studies showed no difference in 1298A>C mutation frequencies between NTD patients and controls (17,20,23). The 677C>T mutation was shown to produce an enzyme with thermolabile properties and decreased activity, resulting in elevated serum homocysteine concentrations (9). The effect of the 677C>T mutation can be reversed by additional folic acid intake (11). Combined mutations in the MTHFR gene or in additional genes (4) and folate nutritional deficiency may be the possible risk factors (17).

Doolin et al. (4) reported the relationship between variations in genes that are involved in the folate-homocysteine metabolic axis and the risk of spina bifida occulta. Analysis of the data on variants of two genes involved in the homocysteine remethylation/methionine biosynthesis—methionine synthase (MTR) A2756G and methionine synthase reductase (MTRR) A66G—provided evidence that both variants influence the risk of spina bifida via the maternal rather than the embryonic genotype.

Relton et al. (14) divided NTD cases to 4 groups as SBO, SBA, anencephaly and others and studied 3 different genes in each groups. They classified the allele and genotypic frequencies according to the phenotypic features of NTD children. They showed that the 3 investigated genes are a major risk factor in anencephalic cases but the 677C>T gene is borderline in SBO cases. The RFC-1 80G>A variant elevated the risk of SBO and anencephalic pregnancy. The 677C>T mutations of the MTHFR gene were not a risk factor for the SBO cases in our study.

The 677C>T and 1298A>C mutations of the MTHFR gene did not reduce the concentrations of serum folate and vitamin B12 in our study. SBO patients with the 677TT genotype showed increased total homocysteine (P=0.003). This suggests that the 677TT mutation affects homocysteine metabolism, as demonstrated in other studies (16,21). Surprisingly, SBO patients showed increased total serum vitamin B12 concentrations. Among the CC/CT/TT genotypes, patients with the 677C>T genotype showed increased vitamin B12, but this was not statistically significant (P=0.506). Although a higher number of SBO patients need to be studied to confirm these results, minimally increased vitamin B12 concentrations in SBO patients with the 677C>T haplotype did not suggest a positive correlation.

Serum folate, vitamin B12 and homocysteine concentrations did not show any difference between the 1298AA/AC/CC groups (P=0.577, 0.325 and 0.347, respectively). Hanson et al. (9) also did not observe an effect of the 1298A>C 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 a SB offspring (20). Weisberg et al. (24) found decreased enzyme activity but they observed no differences in prevalence of the combined heterozygotes in NTD children or their mothers compared to controls.

Data about homocysteine levels are contradictory. Van Der Put et al. (21) demonstrated that the 1298A>C mutation in the MTHFR gene did not cause elevated homocysteine levels, whereas the 677C>T mutation of the same gene did. According to the study, homocysteine levels alone might not always indicate the possible molecular defect.

In conclusion, our study indicates that the 677C>T and 1298A>C MTHFR gene mutations do not affect folic acid, vitamin B12 and homocysteine metabolism in Turkish adult SBO patients. Homozygosity of the 677TT mutation may affect the metabolism of homocysteine. Maternal folate and vitamin B12 status during the periconceptional period may be critical for the development of SBO. Our data support the hypothesis that the multifactorial etiology of SBOs involves the combination of both genetic and nutritional factors.

REFERENCES

1. Boduroglu K, Alanay Y, Alikasifoglu M, Aktas D, Tuncbilek E: Analysis of the MTHFR 1298 A>C in addition to MTHFR 677C>T polymorphism as a risk factor for neural tube defects in the Turkish population. Turk J Pediatr 47:327-333, 2005
2. Boduroglu K, Alikaşifoglu M, Anar B, Tuncbilek E: Association of the 677C?T mutation on the methylentetrahydrofolate reductase gene in Turkish Patients with neural tube defects. J Child Neurol 14:159-161, 1999
3. De Franchis R, Buoninconti A, Mandato C, Pepe A, Sperandeo MP, Del Gado R, CapraV, Salvaggio E, Andria G, Mastroiacovo P: The 5,10- methylenetetrahydrofolate reductase gene is a moderate risk factor for spina bifida in Italy. J Med Genet 35:1009–1013,1998

4. Doolin M-T, Barbaux S, McDonnell M, Hoess K, Whitehead AS, Mitchell LE: Maternal genetic effects, exerted by genes involved in homocysteine remethylation, influence the risk of spina bifida. Am J Hum Genet 71: 1222–1226,2002
5. Eser O, Yaman M, Cosar E, Konak A, Cosar M, Sahin O, Guney O: Does Botox effect neural tube development in early chick embryos? Eur Spine J 16:235-238, 2007
6. Eskes TK: Neural tube defects, vitamins and homocysteine. Eur J Pediatr 157S:139-141, 1998
7. Fidas A, MacDonald HL, Elton RA, Wild SR, Chisholm GD, Scott R: Prevalence and patterns of spina bifida occulta in 2707 normal adults. Clin Radiol 38: 537–542,1987
8. Güney O, Canbilen A, Konak A, Acar O: The effects of folic acid in the prevention of neural tube defects caused by phenytoin in early chick embryos. Spine 28:442-445, 2003
9. Hanson NQ, Aras O, Yang F, Tsai MY: C677T and A1298C polymorphism of the methylenetetrahydrofolate reductase gene: incidence and effect of combined genotypes on serum fasting and post-methionine load homocysteine in vascular desease. Clin Chem 47:661-666, 2001
10. Homberger G, Linnebank M, Winter C: Genomic structure and transcript variants of the human methylenetetrahydrofolate reductase gene. Eur J Hum Genet 8:725-729, 2000
11. Medical Research Council Vitamin Study Research Group: Prevention of neural tube defects: Results of the Medical Research Council vitamin study. Lancet 338:131-137, 1991
12. Morrison K, Papapetrou C, Hol FA, Mariman EC, Lynch SA, Burn J, Edwards YH: Susceptibility to spina bifida; an association study of five candidate genes. Ann Hum Genet 62: 379–396,1998
13. Rady PL, Tyring SK, Hundnall SD: Methylenetetrahydrofolate reductase: the incidence of mutations C677T and A1298C in the Ashkenazi Jewish population. Am J Med Genet 86:380-384, 1999
14. Relton CL, Wilding CS, Jonas PA, Lynch SA, Tawn EJ, Burn J: Genetic susceptibility to neural tube defect pregnancy varies with offspring phenotype. Clin Genet 64: 424–428,2003
15. Rosenblatt DS: Methylenetetrahydrofolate reductase. Clin Invest Med 24:56-59, 2001
16. Shields DC, Kirke PN, Mills JL: The "thermolabile" variant of methylenetetrahydrofolate reductase and neural tube defects: An evaluation of genetic risk and the relative importance of the genotypes of the embryo and the mother. Am J Hum Genet 64:1045-1055, 1999
17. Stegmann K, Ziegler A, Ngo ET: Linkage disequilibrium of MTHFR genotypes 677C/T-1298A/C in the German population and association studies in probands with neural tube defects. Am J Med Genet 87:23-29, 1999
18. Tuncbilek E, Boduroglu K, Alikasifoglu M: Neural tube defects in Turkey: Prevalence, distrubition and risk factors. Turk J Pediatr 41:299-305, 1999
19. Ou CY, Stevenson RE, Brown VK, Schwartz CE, Allen WP, Khoury MJ, Rozen R, Oakley GP Jr, Adams MJ Jr. 5,10 methylenetetrahydrofolate reductase genetic polymorphisms as a risk factor for neural tube defects. Am J Med Genet 63:610–614,1996
20. Van Der Put NM, Blom HJ: Neural tube defects and disturbed folate dependent homocysteine metabolism. Eur J Obstet Gynecol Reprod Biol 92:57-61, 2000
21. Van Der Put NM, Gabreels F, Stevens EM: A second common mutation in the methylenetetrahydrofolate reductase gene: An additional risk factor for neural tube defects? Am J Hum Genet 62:1044-1051, 1998
22. Van der Put NM, Steegers-Theunissen RP, Frosst P, Trijbels FJ, Eskes TK, van den Heuvel LP, Mariman EC, Heyer M, Rozen R, Blom HJ: Mutated methylenetetrahydrofolate reductase as a risk factor for spina bifida. Lancet 346: 1070–1071,1995
23. Volcick KA, Blanton SH, Tyerman GH: Methylentetrahydrofolate reducrase and spina bifida: Evaluation of level of defect and maternal genotypic risk in Hispanics. Am J Med Genet 95:21-27, 2000
24. Weisberg I, Tran P, Christensen B, Sibani S, Rozen R: A second common mutation in the methylenetetrahydrofolate reductase associated with decreased enzyme activity. Mol Genet Metab 64:169-172, 1998