Analysis of MTHFR 1298A>C in addition to MTHFR 677C>T polymorphism as a risk factor for neural tube defects in the Turkish population

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Maternal folic acid intake in the periconceptional period is strongly related to reduction in recurrence and occurrence of birth defects involving the neural tube.

Among the single nucleotide polymorphisms (SNPs) influencing the folate metabolism, the methylenetetrahydrofolate reductase (MTHFR) gene has been the one most exclusively studied. Many studies have reported significant association between MTHFR 677C>T and increased risk of neural tube defects (NTDs). Our previous study did not support this observation. The present study aimed to determine the prevalence of 1298A>C polymorphism in addition to 677C>T in the same Turkish population as a risk factor for NTDs. We genotyped case (95 offspring with NTDs, 80 mothers, 72 fathers) and control (93 healthy children) populations for MTHFR 677C>T and MTHFR 1298 A>C polymorphisms. The comparison demonstrated a significant increase in the 1298AA/677TT genotype frequency among mothers of offspring with NTDs (OR 5.23 [1.06-25.9]; p=0.067). The 677CT genotype was only 1.35 times higher than controls among mothers when 677C>T polymorphism was evaluated alone, while 677CT/1298AC in the current study demonstrated a 3.8 times increase in this risk. These observations led us to conclude that although not statistically significant, MTHFR 1298AC polymorphism might be a risk factor for the occurrence of NTDs in the Turkish population.

Key words: methylenetetrahydrofolate reductase, MTHFR, neural tube defects, polymorphism, MTHFR 677C>T polymorphism, MTHFR 1298A>C polymorphism.

Neural tube defects (NTDs) are inherited with a multifactorial pattern with both genetic and environmental interactions. The incidence is nearly 1 in 1,000 births, but various numbers have been reported from different countries[1]. The prevalence rates are higher in some parts of the world, and in some ethnic groups, while lower in others[1, 2]. The geographical distribution of cases in our country has confirmed the association with socioeconomic status and environmental factors. The prevalence of NTDs in Turkey has been reported to be 30.1 per 10,000 births[3].

It is now clear that maternal folic acid intake in the periconceptional period is strongly related to reduction in recurrence and occurrence of birth defects involving the neural tube by as much as 50-83%[4,5]. The folate pathway, its enzymes and cofactors have been investigated for some time now to explain the underlying genetic susceptibility. Among the single nucleotide polymorphisms (SNPs) influencing the folate metabolism, the methylenetetrahy drofolate reductase (MTHFR) gene has been the one most exclusively studied. MTHFR is the enzyme responsible for the reduction of methylenetetrahydrofolate, which is a key single-carbon donor taking part in nucleotide synthesis; S-adenosyl-methionine (SAM) synthesis; remethylation of homocysteine to methionine; and the methylation of DNA, proteins, neurotransmitters, and phospholipids. Reduced MTHFR activity results in an increased requirement for folic acid to maintain normal homocysteine remethylation to methionine. In the absence of sufficient folic acid, intracellular

homocysteine accumulates, methionine resynthesis is reduced and remethylation reactions are interrupted[6]. Frosst et al.[7] have shown that a C>T transition at nucleotide 677 (677C>T) on the MTHFR gene renders the enzyme thermolabile, decreases its activity, and increases homocysteine concentrations. Many studies conducted following this observation have reported significant association between MTHFR 677C>T and increased risk of NTDs[8-11]. The association between 677TT homozygosity was supported by de Francis et al.[12] and Shields et al.[13]. Other studies, including one from our center, have led to controversy, failing to support the association[14-17].

A second polymorphism in the MTHFR gene (1298A>C) involving alanine-to-cytosine nucleotide substitution in the MTHFR gene has also been investigated alone, and concomitantly with 677C>T. The MTHFR activity is shown to be lower in individuals with both variants, especially when doubly heterozygous 677CT/1298AC[18]. The MTHFR 1298A>C polymorphism was not found to increase the NTD risk alone[19]. However, combined heterozygosity for both (677C>T and 1298A>C) was associated with a slight increase in homocysteine levels and NTD risk[19]. Two recent studies from Ireland and Brazil, both countries with relatively higher NTD prevalences, have both failed to demonstrate any relationship between 1298A>C alone or combined with 677C>T[20,21]. The only study to date to demonstrate a considerable association between MTHFR 1298C allele and increase in the risk of NTDs was conducted by De Marco et al.[22].

In the present study, we aimed to determine the prevalence of 1298A>C polymorphism in addition to 677C>T in the Turkish population as a risk factor for NTDs. The objective was to determine whether 1298AC has a role independently, or in combination with 677CT, on a group previously shown not to have 677CT or 677TT as a risk factor.

Material and Methods

Study Population

Blood samples were collected from 95 NTD patients, 80 mothers, and 72 fathers. Patients with NTDs admitting to Hacettepe University İhsan Doğramaci Children's Hospital were recruited for the study. Our previous study group was slightly expanded with the addition of four patients with NTD, eight mothers, and nine fathers. The spina bifida (SB) phenotypes included were isolated cases of anencephaly, encephalocele, meningocele, myelocele, and meningomyelocele. Accompanying pes planus and hydrocephalus were not considered as exclusion criteria. Controls were recruited from 93 healthy children admitting to İhsan Doğramaci Children's Hospital during the same time period. Informed consent was obtained from all participants. The Ethics Committee of Hacettepe University Faculty of Medicine approved this study.

Specimen Collection and Genotype Analysis

Blood samples (10 ml) collected from case and control groups were deep frozen at 80°C following DNA isolation procedure described elsewhere [23]. The MTHFR 677C>T and 1298A>C mutations were analyzed by polymerase chain reaction and allele specific restriction digestion with Hinf I and Mbo II, respectively, according to the methods also described elsewhere[19].

Statistical Analyses

Allele frequencies were calculated for each genotype, and the differences in allele frequencies between the NTD groups and the controls were determined using chi-square test.

Odds ratios (OR) and 95% confidence interval (CI) for both the heterozygous and homozygous mutant genotypes, as compared with the wild types, were calculated as a measure of association between the MTHFR genotypes and presence of NTD in an offspring. The interaction between the two MTHFR genotypes was evaluated by calculating the ORs for mutant genotypes, as compared to wild types, for both of the MTHFR genotypes.

All statistical analyses were done using the Statistical Program for Social Sciences (SPSS) software, version 10.01.

Results

Allele Frequencies

The allele frequencies of MTHFR 677C>T and 1298 A>C in case (offspring with NTDs, mothers, fathers) and control populations are listed in Table I. Comparison of allele

Table I. Allele Frequencies of MTHFR 677C>T and 1298A>C in Case (offspring with NTDs, mothers, fathers) and Control Populations

		NTD offspring	Mothers	Fathers	Controls
Genotype	Allele	Allele (%)	Allele (%)	Allele (%)	Allele (%)
677	C	133 (70.0)	103 (64.4)	96 (66.6)	133 (71.5)
	T	57 (30.0)	57 (35.6)	48 (33.4)	53 (28.5)
1298	A	113 (59.5)	101 (63.1)	90 (62.5)	116 (62.4)
	C	77 (40.5)	59 (36.9)	54 (37.5)	70 (37.6)

frequencies of both polymorphisms of the three case groups, i.e. NTD vs control, mothers vs controls, and fathers vs controls, demonstrated no significant difference (p>0.05).

MTHFR 677CT Genotypes

Table II demonstrates the MTHFR 677C>T genotype distribution among case (offspring with NTDs, mothers, fathers) and control populations. Genotypes of each case group (CT, TT, CT+TT) were individually compared with the control group taking CC genotype as reference point. The genotype frequencies showed no significant difference compared with the controls. Although not statistically significant, there was a slight increase in the TT genotype among mothers of offspring with NTDs in comparison with the control group (OR 2.03 [0.70-5.89]; p=0.282).

MTHFR 1298AC Genotypes

Table III demonstrates the MTHFR 1298A>C genotype distribution among case (offspring with NTDs, mothers, fathers) and control populations. Genotypes of each case group (AC, CC, AC+CC) were individually compared with the control group taking AA genotype as reference point. The genotype frequencies showed no significant difference compared with the controls.

MTHFR 677CT and MTHFR 1298AC Genotype Interaction

In Table IV we compared the genotype frequencies between MTHFR 677 CC, CT, TT and MTHFR 1298 AA, AC, CC within case (offspring with NTDs, mothers, fathers) and control populations. The comparison demonstrated a significant increase in the 1298AA/677TT genotype frequency among mothers of offspring with NTDs (OR 5.23 [1.06-25.9]; p=0.067). Although not statistically significant, the 1298AC/677CT and 1298AC/677CC genotype frequencies were found to be increased among the mothers (OR 3.80 [0.95-15.22], p=0.070; and OR 3.66 [0.92-14.57], p=0.073), respectively. The genotype frequencies (1298AA/677CT, 1298AC/677CT, and 1298CC/677CC) were slightly increased among the fathers. These interactions fail to support a strong relation.

Table II. MTHFR677C>T Genotype Distribution Among Case (offspring with NTDs, mothers, fathers) and Control Populations

Genotypes 677	NTD offspring Number (%) (n=95)	Mothers Number (%) (n=80)	Fathers Number (%) (n=72)	Controls Number (%) (n=93)
CC	42 (44.2)	33 (41.2)	28 (38.9)	47 (50.5)
CT	49 (51.6) OR=1.4, 95% CI=.78-2.54	37 (46.3) OR=1.35, 95% CI=.72-2.54	40 (55.6) OR=1.72, 95% CI=.90-3.27	39 (42.0)
TT	4 (4.2) OR=.63, 95% CI=.17-2.33	10 (12.5) OR=2.03, 95% CI=.7-5.89	4 (5.5) OR=.95, 95% CI=.25-3.57	7 (7.5)
CT or TT	53 (55.8) OR=1.29, 95% CI=.72-2.28	47 (58.8) OR=1.45, 95% CI=.79-2.65	44 (61.1) OR=1.60, 95% CI=.86-2.99	46 (49.5)

OR: Odds ratio. 95%, CI: 95% confidence interval.

Table III. MTHFR1298A>C Genotype Distribution Among Case (offspring with NTDs, mothers, fathers) and Control Populations

Genotype 1298	NTD offspring Number (%) (n=95)	Mothers Number (%) (n=80)	Fathers Number (%) (n=72)	Controls Number (%) (n=93)
AA	23 (24.2)	23 (28.8)	25 (34.7)	31 (33.3)
AC	67 (70.5) OR=1.67, 95% CI=.87-3.19	55 (68.7) OR=1.37, 95% CI=.71-2.65	40 (55.5) OR=.91, 95% CI=.47-1.79	54 (58.1)
CC	5 (5.3) OR=.84, 95% CI=.24-2.91	2 (2.5) OR=.34, 95% CI=.06-1.73	7 (9.8) OR=1.08, 95% CI=.34-3.40	8 (8.6)
AC or CC	72 (75.8) OR=1.56, 95% CI=.82-2.96	57 (71.2) OR=1.24, 95% CI=.65-2.37	47 (65.3) OR=.94, 95% CI=.49-1.8	62 (66.7)

OR: Odds ratio. 95%, CI: 95% confidence interval.

Table IV. Genotype Frequencies Between MTHFR 677 CC, CT, TT and MTHFR 1298 AA, AC, CC Within Case (offspring with NTDs, mothers, fathers) and Control Populations

Genotype	677CC	677CT	677TT
NTD offspring			
1298AA	(8)*	(11) OR= 1.16, 95% CI=.34-3.92	(4) OR=.78, 95% CI=.17-3.62
1298AC	(29) OR=1.424, 95% CI=.49-4.06	(38) OR=2.01, 95% CI=.71-5.67	(0)
1298CC	(5) OR=.86, 95% CI=.20-3.63	(0)	(0)
Mothers			
1298AA	(3)*	(10) OR=2.82, 95% CI=.61-12.9	(10) OR=5.23, 95% CI=1.06-25.9
1298AC	(28) OR=3.66, 95% CI=.92-5.57	(27) OR=3.80, 95% CI=.95-15.22	(0)
1298CC	(2) OR=.92, 95% CI=.12-6.82	(0)	(0)
Fathers			
1298AA	(4)*	(17) OR=3.60, 95% CI=.92-13.91	(4) OR=1.57, 95% CI=.29-8.42
1298AC	(17) OR=1.67, 95% CI=.45-6.08	(23) OR=2.43, 95% CI=.68-8.70	(0)
1298CC	(7) OR=2.40, 95% CI=.52-11.10	(0)	(0)
Controls			
1298AA	(11)	(13)	(7)
1298AC	(28)	(26)	(0)
1298CC	(8)	(0)	(0)

* Reference categories. () Number of individuals in each group.

Discussion

The key role played by the enzyme MTHFR in folate metabolism and in the etiology of NTDs has attracted much attention. Numerous studies investigating an association between single nucleotide polymorphisms (SNPs) of the MTHFR gene have been published. Studies conducted in various parts of the world considering the two MTHFR polymorphisms (677C>T and 1298A>C) have yielded conflicting results. This may be attributed to ethnic differences in genotype distribution, heterogeneity of the phenotype, and the multifactorial etiology of the NTDs. There is wide heterogeneity in the prevalences of both 677CT and 1298AC polymorphisms throughout the world.

Our aim in the present study was to investigate whether MTHFR 1298AC mutation has any role in the etiology of NTDs. We kept in mind that our previous results demonstrated no relationship between 677CT polymorphism and NTD risk in the Turkish population[14]. The genotype frequencies of 1298 AC were compared between case and control groups, and a possible interaction of both polymorphisms was evaluated. The rate of consanguinity was 0.20 among the parents of NTD cases, parallel to the Turkish population. Therefore, consanguinity had no additional effect on the homozygosity of the mutant genotype in this population.

The slight increase in the sample group content was not found to change our previous observation considering the 677C>T polymorphism. Both 677TT and 677CT genotypes were not found related to any increased risk. The only notable observation was the slight but insignificant increase in risk in patients carrying 677CT (OR 1.4 [0.78-2.54]; p=0.294).

The 677T allele and 677TT genotype frequencies in the control group of this study were 28.5% and 7.5%, respectively. These are intermediate figures correlating with previous European reports[1]. This supports our efforts in trying to define other factors increasing NTD risk in Turkey, where incidence is as high as 3 in 1,000 live births[3].

The population frequencies of the MTHFR 1298C allele have been documented in a small number of studies. Our control population demonstrated a frequency of 37.6%, slightly higher than the previous reports yielding C allele frequency of 33% in the Dutch[19] and of 30% in the German populations[24]. The frequencies of CC homozygous individuals in these two studies were approximately 9%, very close to our figure (8.6%).

The limited number of studies evaluating the relationship between MTHFR 1298A>C and NTD have yielded different results[19,24-27]. Combined heterozygosity of the two mutant genotypes has been reported to increase the risk of NTD occurrence compared with controls[19,26]. A recent Italian study also reported increased individual risk with the 1298CC genotype found in affected children and their mothers[22]. The demonstrated risk was even higher than that with homozygous mutant genotype of the 677CT mutation, as also reported from Italy by de Francis et al.[12]. The authors concluded that the cases with NTDs unrelated to 677TT mutation could in fact be partly associated with the 1298AC polymorphism. A previous study in the Turkish population has demonstrated 677T allele frequency in control and patient populations as 0.26 and 0.34, respectively, while 1298C allele frequencies were 0.28 and 0.30 in controls and patients, respectively. This study has also shown that mutations in MTHFR have no effect alone, while combined effect of MTHFR 677CT and 1298AC increases the risk of spina bifida[28].

In the current study, the MTHFR 1298AC genotype frequency was higher in the case and mother sample groups compared with controls, but this was not the case with 1298CC. This has been previously observed in two other studies[24]. It may be speculated that the homozygous mutant genotype causes severe metabolic errors leading to fetal loss, and is therefore less likely observed.

This association study on genotype frequencies of both polymorphisms comparing sample groups of cases with NTDs and their fathers with controls failed to demonstrate a statistically significant increase in the risk for any specific group or genotype. Similar to previous reports, we also did not observe two mutant genotypes (CT/CC, TT/CC and AC/TT) in the same allele at the same time[19,21,24].

The present data has enabled us to draw two suggestive conclusions. First, the mothers of children with NTDs carrying 1298AA/677TT

genotype show 5.2 times increased risk (OR [1.06-25.9]; p=0.067). This risk is probably related to the 677TT genotype, and is higher than our previous results (twice the increased risk), which can be explained by the difference in the reference point. The reference group in the previous study was the group with 677CC genotype, versus 677CC and 1298AA in the current study. In other words, the previous group was considered according to 677CC, while 1298AA status was unknown. Therefore, separating the individuals with 1298AC and 1298CC genotype from the control group has enabled us to unmask the role of MTHFR 1298AC and 1298CC mutant genotype as a risk factor in the current study population. An alternative explanation for this may be the small number of patients (n=3) in our reference group.

Second, 677CT genotype was only 1.35-fold higher than controls among mothers when the 677C>T polymorphism was evaluated alone, whereas this risk increased to 3.8-fold when 677CT/1298AC were considered together in the current study (Tables III, IV).

In conclusion, although not statistically significant, these observations led us to conclude that the MTHFR 1298AC polymorphism might be a risk factor for the occurrence of NTDs in the Turkish population. The genetic aspect of the multifactorial origin of NTDs is yet to be elucidated. Further investigation of the genes and micronutrients in folate metabolism in our population, with its relatively high incidence of NTDs, is mandatory. Until then, efforts should be directed towards encouraging women in the childbearing period to consume folic acid periconceptionally.

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