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Genetic Polymorphism of Methylenetetrahydrofolate Reductase as a Risk Factor for Lumbosacral Neural Tube Defects

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Abstract: Objectives: Neural tube defects (NTD_S) have a well-established genetic basis, although no single genetic factor has been identified as a major risk factor. The role of C677T and A1298C polymorphisms of the methionine synthase reductase genes are analyzed as risk factors for spina bifida. The study population included 50 mothers and their children with lumbosacral defects and the results were compared with the control group of 50 healthy mothers who have healthy offspring. MTHFR genotypes were assessed by real-time polymerase chain reaction. The results demonstrated a significant increase in the 677 TT genotype frequency among mothers in the study group (OR: 11.43 [1.35-96.89]; p=0.025). A significant increase was also observed in the 677 TT genotype frequency among NTD children, compared to the control group (OR: 12.00 [1.37-105.41]; p=0.025). Homocysteine levels are found to be significantly high among all patients with MTHFR A1298C polymorphism (p= 0.049), but not in those with MTHFR C677T. 677TT genotype should be regarded as an independent risk factor for the occurrence of lumbosacral NTDs in the Turkish population. The study was supported by a grant from Ege University BAP commission.

Key word: Methylenetetrahydrofolate reductase · Neural tube defect · Mutation

INTRODUCTION

There are many evidence suggesting the genetic basis of neural tube defects (NTD_S), with an increased risk of NTD pregnancy in siblings of affected NTD individuals [1]. Investigators have reported a birth incidence of 1.5 to 2.6 NTDs per 1000 in Turkey [2]. In a previous study, 56 newborns with NTD out of 36,331 deliveries was reported in Izmir hospitals in 2000, with an incidence of 1.5 per 1000 births [3].

The defect leads usually to death or life-long handicap in surviving children. These children also need expensive lifelong medical help. The defects are reported to occur due to the failure in closure of the neural tube in one or more of the hypothetical closure sites, between the 21st and the 28th day of embryogenesis, [4].

These birth defects have a multifactorial genesis, with environmental and genetic components. The best-characterized environmental factor is the lack of folate intake and folic acid supplementation has been demonstrated to decrease the occurrence and recurrence of NTDs [1,4]. A second nutrient which has been incriminated is the vitamin B12 (Cobalamin) and several studies have reported that decreased cobalamin levels in the circulation or in the amniotic fluid of mothers of the children with neural tube defects [5-7].

Folate is required in the biochemical step that renders methionine from homocysteine. This step requires the active coenzyme 5-methyl-tetrahydrofolate (MTHFR). MTHFR catalyzes the reduction of 5,10-methylene-tetrahydrofolate into MTHF, the primary circulatory form of folate and the methyl donor in the remethylation of homocysteine to methionine by methionine synthase [4].

Homozygosity for the T allele of the C677T polymorphism of the gene encoding the folate dependent enzyme 5,10-methylenetetrahydrofolate reductase (MTHFR) is a risk factor for neural tube defects [8] and both the homozygous (TT) and heterozygous (CT) genotypes are associated with lower tissue folate concentrations, higher homocysteine concentrations and lower enzyme activity than the wild (CC) genotype and these effects are more obvious in homozygotes. Low folate and raised homocysteine levels in early pregnancy are considered as risk factors for NTDs [9-11]. Several

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studies have demonstrated an increased frequency of the homozygous mutant MTHFR genotype in affected children and their mothers [12-14].

The aim of our study was to evaluate the role of C677T and A1298C polymorphisms of MTHFR, as risk factors for lumbosacral NTDs in a selected population in Izmir, Turkey.

Cases and Methods: The patient group is consisted of 50 children with spina bifida phenotype including deformities like split cord malformation (SCM), meningomyelocele (M) and Lipomeningomyelocele (L) and their mothers, all recruited from Ege University hospital after the approval of the protocol by the Institutional Ethical committee.. The control group included 50 healthy mothers with healthy offspring and not related to the patient group. Blood samples were obtained from mothers and children following the written consent. The MTHFR genotypes and the plasma homocysteine and plasma folate levels were examined in all subjects.

MTHFR Genotyping: Blood specimens were collected in tubes containing EDTA. Genomic DNA was extracted from peripheral leukocytes of the subjects using the High Pure PCR Template Preparation Kit (Roche Applied Science). MTHFR genotypes were revealed by Real-time polymerase chain reaction (LightCycler 2.0). For detection of the C677T polymorphism on the MTHFR gene following primer and probes were used together with the LightCycler - DNA Master Hybridization Probes Kit (Roche Applied Science, Mannheim, Germany): forward primer 5'-CGAAGCAGGGAGCTTTGAGGCTG-3', reverse primer 5'-AGGACGGTGCGGTGAGAGAGTG-3', Fluorescein probe 5'-TGACCTGAAGCAC TTGAAGGAG AAGGTGTC – Fl-3'; LCRed640 probe.

For detection of the A1298C polymorphism on the MTHFR gene following LightMix for the detection of human MTHFR A1298C (Roche Applied Science, Mannheim, Germany) were used together with the LightCycler – FastStart DNA Master Hybridization Probes Kit (Roche Applied Science, Mannheim, Germany).

Experiments were carried out on the LightCycler™ Instrument (Roche Applied Science; Mannheim, Germany) according to the protocol of Charalampos Aslandis and Gerd Schmitz (Institute for Clinical Chemistry and Laboratory Medicine, University of Regensburg, Regensburg, Germany). Polymorphic alleles were identified by the specific melting temperature (Tm) of the resulting amplicons.

In the analysis for A1298C gene polymorphisms on the MTHFR gene, individuals with two copies of the A allele (A/A) showed a single melting peak at 65°C, while individuals with two copies of the C allele (C/C) also showed a single melting peak, but at 59°C and individuals with both alleles (A/C) showed two melting peaks at 59°C and 65°C.

For the C677T analysis; (C/C) and (T/T) alleles showed one single melting peak at 63.1°C and, 55.2°C respectively. Whereas individuals with (C/T) allele showed two melting peaks at 54.6°C and 63.1°C.

The plasma homocysteine and folate levels were assessed with chemiluminescence and electrochemiluminescence methods, respectively.

Statistical Analysis: All statistical analyses were conducted by using the SPSS statistical package, version 15.0. Distributions of continuous variables in groups were expressed as mean ± SD. Categorical variables were compared between groups by use of χ^2 test. Crude odd ratios (OR) and 95% confidence intervals were calculated to estimate the relative risk of the C677T and A1298C mutations for NTD offspring. Compound heterozygosity was calculated with the HelixTree genetic software version 5.0. A value of $p < 0.05$ was considered significant.

RESULTS

The patient group is consisted of 50 children with spina bifida phenotype including deformities like split cord malformation (SCM), meningomyelocele (M) and Lipomeningomyelocele (L). The mean age±SD for children were 7.76±4.93 years, mothers of the offspring with spina bifida were 35.56±6.06 years. The mean age of the control group was 44.16±11.78 years. Clinical and demographic features of the cases are shown in Table 1.

The allele frequencies of MTHFR 677C>T and 1298 A>C are listed in Table 2. Among the control group, 2% of were homozygous for the C677T mutation (TT genotype), 50% were heterozygous (CT genotype) and 48% had no mutation (CC genotype). The genotypic frequencies showed concordance, in control groups for the C677T (χ^2=3.601, p = 0.057) and for the A1298C (χ^2=2.392, p = 0.121) with Hardy-Weinberg equilibrium.

In the mothers of the children with NTDs, the distribution of the TT, CT, CC genotypes were 20%, 38% and 42% respectively. The MTHFR 677 TT mutation genotype were determined as 11-fold higher than the control group (p=0.025). The MTHFR 677 T and C allele

Table 1: Demographic and Clinical Features of Research Groups

		Age		
	n	Mean±SD	Minimum	Maximum
NTD	50	7.76±4.930	1	22
Male	26	7.50±4.630	2	17
Female	24	8.04±5.330	1	22
Mother with NTD	50	35.56±6.060	25	52
Control (Female)	50	44.16±11.78	30	77

	Percent		
Neural Tube Defects	Male	Female	Total
SCM	15.8	29.4	22.2
L	5.3	23.5	13.9
M	78.9	47.1	63.9

SCM: split cord malformation, L: Lipomeningomyeloce, M: Meningomyelocele

Table 2: Genotype distribution of 5,10metylenetetrafolate reductase gene (MTHFR) mutations and polymorphism

Polymorphism	Mother (n=50)	Control (n=50)	OR	95.0% CI	p
C677T Genotype					
CC Wildtype	21 (42%)	24 (48.0%)	1.00	-	
CT Heterozygous	19 (38%)	25 (50.0%)	0.87	0.38 - 2.000	0.741
TT Mutant	10 (20%)	1 (2.0%)	11.43	1.35 - 96.89	0.025
Allele frequency*					
C	61 (61%)	73 (73%)	1.73	0.95 - 3.140	0.048
T	39 (39%)	27 (27%)			
A1298C Genotype					0.180
AA Wildtype	27 (54%)	23 (46,0%)	1.00	-	
AC Heterozygous	20 (40%)	18 (36,0%)	0.95	0.47 - 2.200	0.899
CC Mutant	3 (6%)	9 (18,0%)	0.28	0.07 - 1.180	0.082
Allele frequency*					
A	74 (74%)	64 (64%)	0.63	0.34 - 1.140	0.168
C	26 (26%)	36 (36%)			

**Allele frequency is calculated as two alleles per case*

Table 3: Genotype distribution of 5,10 metylenetetrafolate reductase gene (MTHFR) mutations and polymorphism

Polymorphism	NTD child (n=50)	Control (n=50)	OR	95.0% CI	p
C677T Genotype					0.029
CC Wildtype	16 (32%)	24 (48.0%)	1.00	-	
CT Heterozygous	26 (52%)	25 (50.0%)	1.56	0.68-3.60	0.298
TT Mutant(homozygous)	8 (16%)	1 (2.0%)	12.00	1.37 - 105.41	0.025
Allele frequency*					
C	58 (58%)	73 (73%)	1.96	1.08 - 3.55	0.037
T	42 (42%)	27 (27%)			
A1298C Genotype					0.327
AA Wildtype	25 (50%)	23 (46,0%)	1.00	-	
AC Heterozygous	21 (42%)	18 (36,0%)	1.07	0.46 - 2.50	0.870
CC Mutant (homozygous)	4 (8%)	9 (18,0%)	0.41	0.11 - 1.51	0.180
Allele frequency*					
A	71 (71%)	64 (64%)	0.73	0.40 - 1.32	0.365
C	29 (29%)	36 (36%)			

**Allele frequency is calculated as two alleles per case*

Table 4: Plasma homocysteine and Folic acid levels in relation to MTHFR genotypes in NTD groups

	Homosisteine			Folic Acid		
Gene	Average	SD	p	Average	SD	p
MTHFT C677T						
CC	6.44	3.69	0.798	6.69	3.61	0.080
CT	6.78	2.42		9.69	4.61	
TT	7.32	2.17		7.82	2.62	
Total	6.75	2.83		8.44	4.20	
MTHFR A1298C						
AA	7.08	2.32	0.049	8.67	3.56	0.934
AC*	5.87	2.31		8.20	5.17	
CC*	9.45	5.64		8.27	3.08	
Total	6.75	2.83		8.44	4.20	

*$p<0.05$

Table 5: Compound heterozygous analysis

Compound Genotype	NTD child (n=50)	Control (n=50)	OR	95.0% CI	p
C677T/ A1298C					0.365
CA	15 (30%)	20 (40%)	1.00	-	-
CC	14 (28%)	16 (32%)	1.17	0.44 – 3.11	0.758
TA	20 (40%)	12 (24%)	2.22	0.83 – 5.92	0.110
TC	1 (2%)	2 (4%)	0.90	0.09 – 8.83	0,928
	Mother (n=50)	Control (n=50)	OR	95.0% CI	p
C677T/ A1298C					0.459
CA	18 (36%)	20 (40%)	1.00	-	-
CC	12 (24%)	16 (32%)	0.83	0.31 – 2.23	0.716
TA	19 (38%)	12 (24%)	1.76	0.67 – 4.60	0.250
TC	1 (2%)	2 (4%)	0.56	0.05 – 6.66	0.643

frequency of the mother's were 39% and 61% respectively. The MTHFR 677T allele frequency was 1.7-fold higher than that estimated in the control group (p=0.048).

In the control group, 18% were homozygous for the A1298C mutation (CC genotype), 36% were heterozygous (AC genotype) and 46% had no mutation (AA genotype). In the study group, the distribution of the CC, AC, AA genotypes were 6%, 40% and 54% respectively. The MTHFR 1298C and A allele frequency of mother's were 26% and 74% respectively.

In the children with NTDs, the distribution of the TT, CT, CC genotypes were 16%, 52% and 32% respectively. The MTHFR 677 TT genotype mutation was determined as 12-fold higher than control group (p=0.025). The T and C allele frequency of the NTD group were 42% and 58% and the controls were 27% and 73%; respectively. We revealed a 2-fold higher T allele frequency than that estimated in control group (p=0.037). In the control group, 18% were homozygous for the A1298C mutation (CC genotype), 36% were heterozygous (AC genotype) and 46% showed no mutation (AA genotype). Among the children with NTDs, the distribution between the CC, AC, AA genotypes were 8%, 42% and 50% respectively. The MTHFR 1298 C and A allele frequency of the controls were 36% and 64%, in compared to the NTDs group, which were 29% and 71% respectively (Table 3).

In the children with NTDs, the genotype analysis results were compared with the plasma homocysteine levels and a significant difference is observed in homocysteine levels in the 1298CC genotype (p=0.049) (Table 4). However, MTHFR C677T polymorphism was not found to be related with plasma homocysteine and folate levels. There was no significant difference related to compound heterozygosity for the two polymorphisms of MTHFR (Table 5).

DISCUSSION

Most of the studies related to MTHFR C677T and NTDs have been focused on the risk associated with T allele homozygosity. The possibility that homozygosity might also increase neural tube defect risk has gone as an unrecognized aspect excluding a small study in which an association between CT and these malformations was

thought to be due to the higher than expected proportion of CC control subjects [15]. The 677T mutation in metlylentetrafolate reductase both in children and in their mothers has been associated with increased NTD risk in several studies [12, 16 - 18]. We found that the odds ratios for the homozygous mutation (T/T) were 14 (95% CI 1.6 -122.3) for patients and 13.33 (95% CI 1.58-112.43) for mothers versus controls. Similarly, Van der Put *et al.* [12] showed that a common mutation in the MTHFR gene, C677T, which reduced enzyme activity and impairs homocysteine and folate metabolism, is a risk factor for spina bifida [19]. Based on their data, the odds ratios for the homozygous mutation (T/T) were 3.7 (95% CI 1.5-9.1) for mothers and 2.9 (95% CI 1.5-9.1) for patients versus controls. The odds ratio increased to 6.1 (95% CI 1-35.5) if both mother and her child were homozygous for the mutation.

There are a few published studies related to MHTFR A1298C genotype frequency and spina bifida. In the one of the two reports from Turkey, Boduroglu *et al.* [20] 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 found to be only 1.35 times higher than controls, while 677CT/1298AC demonstrates a 3.8 times increase in this risk.

Polymorphic variation in folate-related genes might be expected to impact equally on all NTD phenotypes. This is supported by the knowledge of the role of folate RNA and DNA synthesis and the methylation of a number of biomolecules, which suggest that a defect in folate metabolism may impact on a wide range of developmental endpoints. Indeed, folate has not only been implicated in the etiology of all forms of NTD, but more recently in Down's syndrome [21].Reports indicate that, maintenance of adequate vitamin levels (cobalamin and folate) should be stressed for women who are considering pregnancy [22].

In our study, the genotyping analysis with homocysteine and folate levels in the presence of A1298C polymorphism and CC homozygous mutant genotypes indicated the homocysteine levels to be increased significantly. (p=0.049).

The findings in this study supported the hypothesis that, C677T polymorphism increases the risk for giving birth to a child with a neural tube defect. In addition to the genetic alteration, other factors like abnormal homocysteine and folate levels may increase the risk for this disease.

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