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Research Paper

Methylenetetrahydrofolate Reductase Gene Polymorphisms in Children with Attention Deficit Hyperactivity Disorder

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Abstract

Objective: The purpose of this study was to evaluate the relationship between 5,10-methylenetetrahydrofolate reductase (MTHFR) polymorphisms and Attention Deficit Hyperactivity Disorder (ADHD) in a sample of Turkish children.

Study Design: MTHFR gene polymorphisms were assessed in 40 patients with ADHD and 30 healty controls. Two mutations in the MTHFR gene were investigated using polymerase chain reactions and restriction fragment length polymorphisms.

Results: Although there were no statistically significant differences in genotype distributions of the C677T alleles between the ADHD and the control groups (p=0,678) but the genotypic pattern of the distributions of the A1298C alleles was different between the ADHD patients and the controls (p=0,033).

Conclusions: Preliminary data imply a possible relationship between A1298C MTHFR polymorphisms and the ADHD.

Key words: Attention deficit-hyperactivity disorder, methylenetetrahydrofolate reductase gene polymorphism, folic acide

Introduction

Attention Deficit Hyperactivity Disorder (ADHD) is a common childhood neurobehavioural disorder defined by symptoms of developmentally inappropriate inattention, impulsivity and hyperactivity. A recent meta-analysis estimated the worldwide prevalence of ADHD to be 5.29% [1], making it the most prevalent psychiatric disorder of childhood. The social and economic costs of childhood ADHD are considerable [2], and difficulties often persist into adulthood. Children with ADHD are at high risk for developing adjustment problems, antisocial behaviour, substance abuse, other psychiatric disorders, and difficulties in education and work [3]. Although the exact aetiology of ADHD has not been determined, the related factors include familial and hereditary factors, prenatal or perinatal factors, chemotoxic factors, sociopsychological stress, structural and functional abnormalities of the brain, and developmental neurobiological factors in the regions of the brain related to ADHD [4]. Family, twin, and adoption studies provide overwhelming evidence for an inherited contribution to the pathogenesis of ADHD, with heritability estimated to be about 70% [5].

Methylenetetrahydrofolate reductase (MTHFR) is important for a chemical reaction involving forms of the vitamin folate (also called folic acid or vitamin

B9). Specifically, this enzyme converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, the predominant circulating form of folate. This reaction is required for the multistep process that converts the amino acid homocysteine to another amino acid, methionine. The body uses methionine to make proteins and other important compounds. The 5,10 methylenetetrahydrofolate reductase gene is located at the end of the short arm of chromosome 1p36.3 [6]. The enzyme plays a central role in folate metabolism by irreversibly converting 5,10-methylenetetrahydrofolate to 5-methylenetetrahydrofolate.

5-Methyltetrahydrofolate is the predominant circulating form of folate, and it donates a methyl group to homocysteine in the generation of *S*-adenosylmethionine, a major source of methyl groups in the brain [7]. MTHFR is a critical component of the 1-carbon cycle, and the polymorphisms of C677T and A1298C affect both nucleotide synthesis and DNA methylation [8]. Heterozygous and homozygous carriers of the 677T allele variant have a 30–40% and 60–70% reduced enzyme activity, respectively, as determined by *in vitro* analysis of the MTHFR activity [9,10]. The effect of the 1298C allele variant is less severe and homozygous carriers of this allele have a more moderate 30–40% reduction of the enzyme activity, yet its function remains controversial [11]. The MTHFR C677T polymorphism is associated with a reduction in the bioavailability of folate and folate metabolites and "mimics" low dietary folate intake [12].

Recent studies showed that genetic polymorphism of MTHFR is related to neuropsychiatric diseases such as schizophrenia, depression, bipolar disorder, Parkinson's disease, Alzheimer's disease, and vascular dementia [13-16]. These studies have generally been carried out in adults and there are insufficient numbers of studies about the relationship of childhood neuropsychiatric problems with folic acid and MTHFR that plays a central role in folate metabolism. In addition, these few studies in pediatric population are related to Autism [17] and ADHD. Studies on the relationship of ADHD with folic acid and folic acid metabolism have focused on the childhood symptoms of folic acid deficiency in antenatal period [18,19]. Although many studies have examined ADHD related genes, only one study has focused on the role of MTHFR. This single study evaluated children with ADHD symptoms after leukemia treatment, rather than children with a diagnosis of ADHD [20]. In our study, we aimed to determine the relationship between common MTHFR gene mutations (C677T and A1298C) and ADHD, a disorder that genetic factors play an important role in its etiology.

Materials and Methods

Participants

Forty patients with ADHD and 30 healthy volunteers were included in the study. The ADHD group consisted of consecutive referrals to the child and adolescent psychiatry outpatient clinic of Konya Research Hospital between October 2009 and March 2010. A total of 72 subjects were approached to be included in the study group. Of those 20 subjects were excluded because of non-consent and 12 subjects were excluded because of exclusion criteria.

Exclusion criteria were as follows: (a) having an IQ below than 80 on Weschler Intelligence Scale for Children-Revised (WISC-R), (b) diagnosis of pervasive developmental disorders, (c) presence of psychosis, drug dependence, and medical or neurological diseases, (d) evidence of a history of physical and/or sexual abuse. Finally a total of 40 subjects were included in the study. The control group consisted of children of hospital employees or members of the general community who became aware of the study via the principal investigators or other participants.

Assessments

The diagnosis of ADHD was made on the basis of DSM-IV criteria. There were 31 male and 9 female patients with ADHD with an age range of 6 to 17 years (mean ± SD: 9.77±2.3), and 23 male and 7 female controls with an age range of 6 to 17 (mean ± SD: 10.5±4.5). All ADHD patients had the symptoms of inattention and hyperactivity/impulsivity, which was recognized by DSM-IV as ADHD-combined type and none of the children have ADHD to the control group.

The clinical assessment included the following parts: (1) The Schedule for Affective Disorders and Schizophrenia for school-age children, lifetime version [21], which is a semi-structured diagnostic interview designed to assess current and past episodes of psychopathology in children and adolescent according to DSM-IV (and DSM-III-R) criteria. KSADS was administered to clinically diagnosed patients with ADHD and their parents to confirm diagnosis of ADHD, (2) The ADHD Rating Scale-IV [22] which contains 18 items referring to the diagnostic criteria of the DSM-IV; 9 items indicating inattention symptoms, and 9 items indicating hyperactivity-impulsivity symptoms. Each item is rated for frequency of symptom on a 4-point Likert scale, ranging from 0 (rarely) to 3 (very often). Scores of 2 or 3 on individual items were regarded as indicating the presence of a symptom. Total scores varied between 0–54. (3) The IQ was assessed by using the Manual for the Weschler Intelligence Scale for Children-Revised [23].

Family and medical/perinatal/developmental histories of the patients were obtained from the parents. A local ethical committee in Konya Research Hospital, Konya, Turkey approved the study. A written informed consent was obtained from the parents.

Determination of The Folic Acid Levels

Totally 4-5 ml of blood samples were drawn after a 12-14 hours of fasting in the morning. Sera of the samples were separated after coagulation and stored at -85^{o}C until the day of analysis. Serum folate levels were determined in Elecsys 2010 auto analyzer (Roche Diagnostics,Indianapolis, IN, USA) by using cobas test kit (lot no: 160-446-02).

Determination of *MTHFR* Gene Mutations

Genomic DNA of patients and controls was isolated from peripheral blood samples using the QI-Aamp DNA Blood Mini Kit (Qiagen, Valencia, CA, USA). The extracted genomic DNA samples then underwent polymerase chain reaction (PCR) to screen for and amplify two *MTHFR* gene mutations; one involving base pair 677 and the other involving base pair 1298. The PCR reaction mix contained 5 µl genomic DNA, 5 µl 10× PCR buffer (P-2192; Sigma-Aldrich, St Louis, MO, USA), 5 µl of deoxynucleotide triphosphate mixture (Promega, Madison, WI, USA) containing 0.2 mM of each nucleotide, 5 µl 1 mM Tris–HCl, 5 µl 5 mM

KCl, 0.2 µl of Taq polymerase enzyme (D- 6677; Sigma-Aldrich) and 4 pmol each of forward and reverse primers for each region. The volume was made up to 50 µl with double distilled water. The primers for each region were: base pair 677 forward 5′-AGG ACG GTG CGG TGA GAG TG-3′, reverse 5′- TGA AGG AGA AGG TGT CTG CGG GA-3′; base pair 1298 forward 5′-CTT TGG GGA GCT GAA GGA CTA CTA C-3′, reverse 5′-CAC TTT GTG ACC ATT CCG GTT TG-3′. The PCR thermal cycling conditions for each region were: base pair 677, an initial denaturation step at 95 °C for 3 min, followed by 35 cycles of 94 °C for 1 min, 61 °C for 1 min, 72 °C for 1 min, and one last extension at 72 °C for 7 min; base pair 1298, an initial denaturation step at 92 °C for 2 min, followed by 35 cycles of 92 °C for 2 min, 60 °C for 1 min, 72 °C for 30 s, and one last extension at 72 °C for 7 min. The PCR products for the base pair 677 and 1298 mutations underwent restriction enzyme digestion: for base pair 677, 15 µl of the 198 base pair PCR product was added into a reaction mixture containing 7 µl of double distilled water, 5 U of Hinfl enzyme (Fermentas UAB, Vilnius, Lithuania) and 2.5 µl of Red (R+) Buffer (Fermentas UAB); for base pair 1298, 20 µl of the 163 base pair PCR product was added into a reaction mixture containing 1 U of Mboll enzyme (Fermentas UAB) and 0.5 µl of Blue (B+) buffer (Fermentas UAB). Both reactions were incubated at 37 °C for 16 h. To visualize the fragments formed after cleavage, 25 µl of digested PCR products for each base pair (677 and 1298) were loaded onto 2% or 3% ultra-pure agarose gel, respectively, and electrophoresed for 90 min to identify the alleles for each base pair mutation. For the base pair 677 mutation, the samples containing only the 198 base pair fragments were identified as 677 CC, the samples containing 198, 175, and 23 base pair fragments were identified as 677 CT, and the samples containing 175 and 23 base pair fragments were identified as 677 TT. For the base pair 1298 mutation, the samples containing 56, 31, 20, 28 and 18 base pair fragments were identified as 1298 AA, the samples containing 84, 56, 31, 30, 28 and 18 base pair fragments were identified as 1298 AC, and the samples containing 84, 31, 30 and 18 base pair fragments were identified as 1298 CC.

Statistical analysis

The data of this study were analysed with SPSS 17 package program. The statistical significance was defined as p value of 0.05 or smaller. Independent sample *t*-tests and chi-square test (Pearson Chi-Square and Fisher's exact test) which is one of nonparametric tests were used.

Results

The age and gender distribution of the patients with ADHD and controls were nearly similar (Table 1). The blood folic acid levels of patients and controls were found to be normal and there was no statistical difference (13,01 ± 2,35 versus 11,82 ± 2,72; p= 0.055). However, there were significant differences between the groups considering the scores of ADHD Rating Scale-IV total (43.2 ± 2,8 versus 17.8 ± 3.9; t = 29.93; p=0.000); ADHD Rating Scale-IV- inattentiveness (21.3 ± 2,1 versus 8.5 ± 2.6; t = 22.14; p=0.000); ADHD Rating Scale-IV-hyperactivity/impulsivity (21.9 ± 1,6 versus 9.2 ± 1.7; t = 31.01; p=0.000).

There were no statistically significant differences in genotype distributions of the C677T alleles between the ADHD and control groups (p=0,678). The genotypic pattern of the distributions of the A1298C alleles was different between the ADHD patients and the controls (p=0,033). We also compared these groups according to the compound heterozygosity. As a result, we could not detect a statistically significant difference (p=0,801). (Table 2)

Table 1. Description of ADHD and Control Groups

	ADHD	Control	p
Age	9.77 ± 2.3	10.5 ± 4.5	0.429
Sex			0.935
Male	31 (%77.5)	23 (%76.7)	
Female	9 (%22.5)	7 (%23.3)	
ADHD Rating Scale-IV Scores			
Inattentiveness	21.3 ± 2.1	8.5 ± 2.6	0.000
Hyperactivity/impulsivity	21.9 ± 1.6	9.2 ± 1.7	0.000
Total	43.2 ± 2.8	17.8 ± 3.9	0.000
Total IQ Score	100.1 ± 7.1	98.3 ± 5.6	0.273

Table 2. MTHFR Gene Alleles of ADHD and Control Groups.

		ADHD		Control		P value
		n	%	n	%	
MTHFR C677T	CC	22	55	15	50	0,678
	CT and TT	18	45	15	50	
MTHFR A1298C	AA	9	22,5	14	46,7	0,033
	AC and CC	31	77,5	16	53,3	
COMBİNED	The compound heterozygous* CT/AC	9	22,5	6	20	0,801
	Other combinations	31	77,5	24	80	

* mutations on different alleles

When MTHFR gene alleles were evaluated with respect to sex, there was no statistically significant difference between ADHD and male control groups according to MTHFR C677T (p=0,61) and A1298C (p=0,173) alleles. In females, no difference was found in respect to C677T between two groups (p=0,949). Although the ratio differed markedly between two groups, the difference did not reach statistical significance for A1298C (p=0,106), as well (Table 3,4).

We could not find a difference between the two groups when we compare the patient and the control groups according to whether they have the risk alleles (Table 5).

Table 3. MTHFR C677T Alleles Respect To Sex

		ADHD		Control		P value
		n	%	n	%	
Male	CC	17	54,8	11	47,8	0,610
	CT and TT	14	45,2	12	52,2	
Female	CC	5	55,6	4	57,1	0,949
	CT and TT	4	44,4	3	42,9	

Table 4. MTHFR A1298C Alleles Respect To Sex

		ADHD		Control		P value
		n	%	n	%	
Male	AA	8	25,8	10	43,5	0,173
	AC and CC	23	74,2	13	56,5	
Female	AA	1	11,1	4	57,1	0,106
	AC and CC	8	88,9	3	42,9	

Table 5. The Distribution of Risky and Non-risky Genotypes

		ADHD		Control		P value
		n	%	n	%	
Male	Risk genotype	13	24,1	6	11,1	0,228
	Non risk genotype	18	33,3	17	31,5	
Female	Risk genotype	5	31,3	3	18,7	1,000
	Non risk genotype	4	25,0	4	25,0	
General	Risk genotype	18	25,7	9	12,9	0,202
	Non risk genotype	22	31,4	21	30,0	

risk genotype* : TT/CC, TT/AC, TT/AA, CT/CC, CC/CC, CT/AC;

non risk genotype*: other alleles

Discussion

MTHFR gene mutations were found related to schizophrenia, depression, and bipolar disorder in studies searching relations between MTHFR gene mutation and neuropychiatric diseases [13]. Roffman et al showed that MTHFR C677T mutation was related to functional deficiency in working memory and negative symptoms in schizophrenia [24].

Folates seem to be of fundamental importance in brain growth, differentiation, development, repair, mood, cognition, and ageing [25-28]. These functions and their breakdown in folate and vitamin-B12 deficiency are probably primarily mediated through nucleotide synthesis, DNA integrity and transcription, and epigenetic mechanisms, including gene expression, relating to DNA methylation [28]. Neuropsychological studies have found general and specific impairments of intellectual function—including attention, episodic and visuospatial memory, and abstract reasoning—that were attributed to folate deficiency [25,29]. In support of this, studies in mice demonstrated that lesser degrees of gestational folate deficiency resulted in a loss of progenitor cells, a net reduction of cells in the fetal brain, reduced brain weight and anxiety-related behaviour in the offspring, suggesting a causal effect of prenatal folate status on neurodevelopment and behavioural functioning later in life [30,31].

As is the norm for the majority of psychiatric phenotypes, traditional aetiological studies have focused primarily on the interplay between genetic and environmental factors. Family, twin, and adoption studies provide overwhelming evidence for an inherited contribution to the pathogenesis of ADHD [5]. Recent years have seen considerable research effort dedicated towards understanding the genetic basis of ADHD, and replicated associations with polymorphisms in several genes have been uncovered [32]. Despite this, we are still a considerable distance from fully understanding the inherited causes of ADHD.

Studies demonstrating multifactorial characteristics in ADHD etiology necessitate considering this point of view in etiological studies assessing this disorder. In that situation, different factors contributing to current predisposition may precipitate the disease. For example, in mothers carrying MTHFR C677T homozygote mutation, presence of folic acid deficiency in antenatal period may cause neural crest defects. Normal folic acid levels found in further assessments complicates determining this multifactorial effect. In our study, normal folic acid levels do not imply that the individual's folic acid levels were in normal range during lifetime. Because nutrition and mineral demands are supplied from maternal circulation in prenatal period, the evaluation of maternal blood folic acid level is critical. Considering that human brain develops rapidly in early prenatal period and most cortical and subcortical structures are formed in 5-25 weeks, evaluation of maternal blood folic acid level becomes more crucial [33]. These findings suggest that maternal folic acid deficiency and fetal or maternal MTHFR mutations (C677T and A1298C) should be assessed together. Correspondingly, del Rio Garcia et al stated that maternal MTHFR polymorphisms (C677T and A1298C) and sufficient folic acid and B12 vitamin intake in early antenatal period is important for the neurobiolological development of the infant [34]. This multifactorial interaction should be considered in studies investigating gene mutations that contribute to intrauterin environmental vulnerability and that are commonly seen in the general population. On this point of view, normal folic acid levels found in the childhood period without the assessment of maternal folic acid levels do not deny this relation because of the reasons we mentioned above. We believe that future studies including large numbers of children along with their mother (particularly in the pregnancy period) can provide more reliable results.

Our study, which aimed to compare children with and without ADHD according to the presence of mutant allele, revealed no difference in respect to MTHFR C677T allele (p=0.678). However, the group with ADHD was shown to carry significantly more mutant A1298C allele than the control group (p=0.033). A previous study by Krull et al [20] has also found a relationship between A1298C polymorphism and the inattentiveness symptoms of ADHD. It is wellknown that inattentiveness symptoms are prominent in female children with ADHD [35]. In the current study, 8 of 9 girls from the ADHD group were found to have A1298C mutation, but no statistical difference was found when compared with the control group (p=0.106). Statistical insignificance despite the great difference between percentages may be due to the very limited sample size. These findings may point to a link between the ADHD and MTHFR gene mutations (C677T and A1298C).

The most important limitation of this study is its small sample size. The literature on the relationship between MTHFR gene mutations (C677T and A1298C) and ADHD is limited. Larger trials with larger patient populations are needed to reach definite conclusions whether this mutations is a noteworthy risk factor for ADHD.

Conflict of Interest

The authors have declared that no conflict of interest exists.

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