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Is the MTHFR C677T variant a genetic risk factor in the etiology of autism spectrum disorder? Is it alone or by combined with rare variants of the PHGDH gene?

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Abstract

Aim: Autism spectrum disorder (ASD) is a group of diseases characterized by restricted interests, speech disorders, lack of reciprocal social communication. Genetic factors are predominantly responsible for the etiology of ASD. ASD genetics is complex and heterogeneous, and a variety of genetic factors has been associated with ASD. The C677T in the methylenetetrahydrofolate reductase (*MTHFR*) gene has been associated with the increased genetic liability for ASD in many studies. In the present study, we asked whether the *MTHFR* C677T variant increases the risk of ASD alone or in combination with rare harmful SNVs (MAF values are ≤ 0.001) on the phosphoglycerate dehydrogenase gene (*PHGDH*) in the genetic etiology of ASD.

Materials and Methods: Two hundred twenty-two patients with ASD and 323 neurotypical controls were included in this study. PCR and Restriction Fragment Length Polymorphism method was used for screening the C677T. The *PHGDH* gene was scanned in the groups by targeted next-generation sequencing carrying C677T variant as homo-or heterozygous. Allele and genotype distributions of the C677T were analyzed by using the chi-square test. Genotype distributions were analyzed according to all four possible genetic models; homozygote (TT vs CC), heterozygote (CT vs CC), dominant (TT+CT vs CC), and recessive (TT vs CT+CC).

Results: There was not any significant difference in terms of C and T allele distributions between ASD and controls (T vs C: OR = 1.16, 95% CI = 0.89-1.5, $p > 0.05$). There were not any significant different distributions of the 4 possible genotypes. In possible combined effects of the C677T and rare and possibly harmful SNVs of the *PHGDH* in the genetic etiology of ASD, we could not find any differences between patients and controls.

Conclusion: No data were found to support that the *MTHFR* C677T variant alone or in combination with rare variants of the PHGDH gene is a genetic risk factor in the etiology of ASD.

Introduction

Autism spectrum disorder (ASD) is a group of diseases that share common core findings include restricted interests, speech disorders, lack of reciprocal social communication and interaction [1]. The prevalence of ASD has increased regularly from its first described by psychiatrist Leo Kanner in 1943 until today and reached 1/54 according to the Centers for Disease Control and Prevention (CDC) of U.S.A [2]. In the etiology of ASD it has been suspected from a variety of environmental risk factors including chemical substances, malnutrition during pregnancy, and infections [3]. But, twin studies performed so far have been shown that genetic/hereditary factors are predominantly responsible for the etiology of ASD [4]. The genetics of ASD is very complex and heterogeneous and a variety of genetic/epigenetic factors have been associated with ASD within the last decades of researches [5, 6]. Consisted with these researches, some chromosomal aberrations, a variety of hereditary and de novo pathologic DNA copy number variations (CNVs), and single nucleotide variations (SNVs) have been associated with ASD [7, 8]. Apart from these robust and rare major genetic factors which explain rare causes of ASD genetic etiology, ASD researchers suspect common genetic risk factors for explaining the increased prevalence of ASD, in recent years [9]. In the finding of common genetic risk

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factors and to explain the shared genetic etiology of ASD patients, genome-wide association studies (GWAS) have been increasingly performed in the recent decades by using thousands of ASD and control samples [10]. With these GWA studies, some genetic risk loci have been associated with ASD, robustly [11]. Hereditary common SNVs that located on some genes of metabolic enzymes such as those catalyzing folate-dependent one-carbon metabolism (1C) have been usually associated with the increased risk of ASD by some ASD researchers. In particular, two SNVs that are known as C677T and A1298C in the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene are generally thought to increase the risk of some common cardiovascular diseases and neural tube defects [12]. At the same time, the same SNVs also have been associated with the increased genetic liability for ASD in the many genetic association studies [13, 14].

When studying the genetic etiology of a family which has a child with ASD, we found that there was apparently balanced chromosomal translocations in the karyotypes of both mother and her male child (that is 46,XY,t(1;5)(p12;p15)mat). By using whole- genome DNA sequencing analysis we successfully determined the chromosomal breakpoint regions and saw that the phosphoglycerate dehydrogenase gene (PHGDH) on the derivative chromosome 1 (chr1) was broken due to translocations (the other breakpoint region was in the intergenic region on derivative chr5) [15]. At the beginning of the study despite all our efforts, we could not find any aberrant genetic data to explain the genetic etiology of this ASD patient (data not shown). But this patient was homozygous in terms of the MTHFR C677T variant (rs1801133) that has been associated with ASD in many studies mentioned above. This single nucleotide polymorphism (SNP) which has a minor allele frequency (MAF) of about 28-30% in the worldwide human population is a loss of function (thermolabile) variant [16]. It was shown by functional studies that it reduces the activity of the MTHFR enzyme by about 50% and associated with elevated plasma homocysteine levels [17]. Also, the protein products of both MTHFR and PHGDH genes were taking place in the 1C metabolism [18] (Figure 1). Therefore, we asked whether the MTHFR C677T variant increases the risk of ASD alone or in combination with rare harmful SNVs on the PHGDH gene in the genetic etiology of ASD.

Materials and Methods

Study design

We performed an observational genetic (allelic) association study to test whether the MTHFR C677T variant increases the risk of ASD alone or in combination with rare harmful SNVs on the PHGDH gene in the genetic etiology of ASD.

Sample size determination

We used PGA software for sample size and power detection [19].

Participants

Informed consent form was obtained from the parents of the patients. This study was approved by the Ethics Committee of Karadeniz Technical University Faculty of Medicine (approval number: 2016/24). Two hundred twenty-two patients with ASD (184 male, 82%; 38 females, 18%) and 323 neurotypical controls (158 male, 49%; 165 female, 51%) were included in this study. The ages of ASD range from 3 to 17 (6,018 ± 3,104 SD). ASD diagnosis was performed by experienced child and adolescent psychiatrists according to The Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV) (20).

Determining of the MTHFR (NM_005957) C677T variant in case and controls

Genomic DNA was obtained from peripheral blood by standard DNA isolation protocol. For screening the C677T SNV in the case and controls the PCR and Restriction Fragment Length Polymorphism (PCR-RFLP) method was used. PCR primers were designed by Primer3 (21). A 388 bp PCR product was obtained by primer pairs of Forward: 5'-AGTCCCTGTGGTCTCTTCATC-3'and Reverse: 5'-GGAGATCTGGGAAGAACTCAG-3'. The PCR reactions and conditions were implemented according to standard PCR protocols on the Applied Biosystems® 9700 Thermocycler. The PCR product was treated by TaqI restriction endonuclease (New England Biolabs®) according to the manufacturer's instruction. Accordingly, the C677T substitution was creating an enzyme cutting site (T'CGA) for TaqI and producing two products of 154 and 234 bp, respectively.

Scanning the rare and probably harmful SNVs on the PHGDH gene in patients with ASD and controls carrying MTHFR C677T variant as homo-or heterozygous

The whole *PHGDH* gene (NM_006623.3) was screened by targeted next-generation sequencing (NGS) in patients with ASD and control subjects carrying *MTHFR* C677T variant as homo-or heterozygous, in terms of rare and probably harmful SNVs. A total of 121 patients with ASD and 125 controls (out of 162 controls carrying the *MTHFR* C677T variant either as homo - or heterozygous) were screened. These NGS processes were carried out through service procurement on Miseq® (Illumina) platform from a private company that has a DNA sequencing facility. The SNVs that MAF values are ≤ 0.001 and thought to be probably harmful were confirmed by Sanger sequencing in our laboratory.

Bio-statistical analysis

All allele and genotype distributions of the *MTHFR* C677T variant between cases and controls were analyzed by GraphPad Prism 7® software using the chi-square (χ^2) test. It was considered statistically significant if two-sided p-value is < 0.05. Odds ratios (OR) and 95% confidence interval (CI) have also been calculated. Genotype distributions were analyzed according to all four possible genetic models; homozygote (TT vs CC), heterozygote (CT vs CC), dominant (TT+CT vs CC), and recessive (TT vs CT+CC). To test whether the allele frequencies of the *MTHFR* C677T variant detected in the control groups were in the Hardy-Weinberg equilibrium, we used a chi-square test which was established by Rodriguez et al (22).

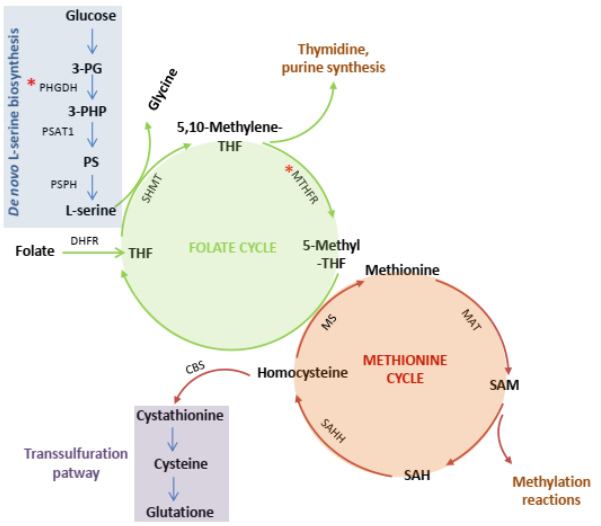

Figure 1. Schematic drawing of the de novo L-serine biosynthesis and folate-dependent one-carbon metabolism relationship. 3-PG: 3-phosphoglycerate; *PHGDH*: 3-phosphoglycerate dehydrogenase; 3-PHP: 3-phosphohydroxypyruvate; PSAT1: phosphoserine aminotransferase1; PS: Phosphoserine; PSPH: phosphoserine phosphatase; DHFR: dihydrofolate reductase; THF: Tetrahydrofolate; SHMT: serine hydroxymethyltransferase; *MTHFR*: methylenetetrahydrofolate reductase; MS: methionine synthase; MAT: methionine adenyltransferase; SAM: S-adenosyl-methionine SAH: S-adenosylhomocysteine; SAHH: S-adenosylhomocysteine hydrolase; CBS: cystathionine β-synthase. Red stars illustrate enzymes involved in this study.

Sanger sequencing

For confirming NGS results the Sanger sequencing was performed by using BigDye terminator® (Applied Biosystems) sequencing kit with standard dideoxy termination reactions according to manufacturer's instruction and, after purification electrophoresed on the ABI 3130 (Applied Biosystems) genetic analyzer and analyzed.

Results

Detection of the *MTHFR* C677T variant. Representative PCR and TaqI restriction endonuclease digested products were shown in Figure 2.

Distributions of the C677T risk allele and genotypes in ASD and control groups. The C677T variant was Hardy-Weinberg equilibrium in the control groups. There was not a significant difference in terms of C and T allele distributions between ASD and controls (T vs C: OR = 1.16, 95% CI = 0.89-1.5, p > 0.05). Again, there were not any statistically significant different distributions of the 4 possible genotypes between ASD and controls (Table 1).

However, in the dominant genotype model, the risk of ASD was increasing about 1.3-fold but not statistically significant manner (dominant (TT + CT vs CC: OR = 1.29, 95% CI = 0.91-1.85, p = 0.14)) (Table 1). Because ASD is predominantly a male disorder we also performed distributions of the C677T risk allele/ TT+CT genotypes only in male ASD and male control groups. Again, we could not find any statistically significant difference between groups in terms of both the risk allele and all four possible genotype distributions (Table 2).

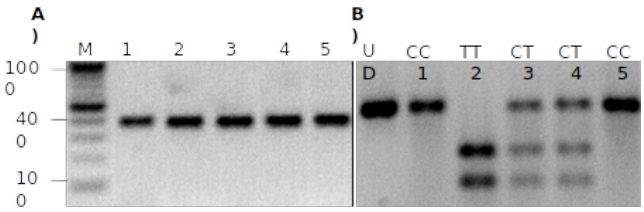

Figure 2. 2 % agarose gel images of PCR-RFLP reactions of *MTHFR*. A) PCR products used for the RFLP reaction; B) RFLP results of TaqI digestion reactions. CC: homozygous reference, TT: homozygous mutant, CT: heterozygous mutant, M: Marker (bp) and UD: Undigested PCR product.

Findings of rare and possibly harmful SNVs on the *PHGDH* gene in patients with ASD and controls carrying *MTHFR* C677T variant as homo-or heterozygous. Findings of rare and possibly harmful SNVs on the PHGDH gene in patients with ASD and controls carrying *MTHFR* C677T variant as homo-or heterozygous. When we analyzed the possible combined effects of the *MTHFR* C677T variant and rare and possibly harmful SNVs (MAF values are $\leq$ 0.001) of the *PHGDH* in the genetic etiology of ASD, we could not find any differences between patients and controls (Table 3). The statistically significant differences between ASD and controls could not be calculated because the sample sizes are not adequately large for statistical analyses in terms of rare variant effects on the common disease like ASD. For such an analysis there is needed to thousands of ASD and control subjects that have *MTHFR* C677T variant as homo-or heterozygous.

Confirming of the rare and possibly harmful SNVs by Sanger sequencing. All rare and probably harmful SNVs found by NGS on the *PHGDH* have been confirmed by Sanger sequencing (Figure 3).

Discussion

It is generally accepted among researchers that ASD predominantly occurs due to abnormalities of the genetically determined neurodevelopmental processes of the central nerves system (CNS) during fetal or neo-natal developmental periods of the fetus [23, 24]. But, there is not a consensus about genetic/hereditary mechanisms by which act on these abnormal CNS developmental processes [25].

More than 150 high confidence genes are associated with the Mendelian forms of ASD (generally known as "syndromic ASD") by using whole exome or whole genome

Table 1. *MTHFR* C677T allele and genotype distributions in ASD and control groups

Allele distributions					
Allele	T	C	Total	p value	OR(95% CI)
SD	141	303	444		
Control	185	461	646	0.26	1.16(0.89-1.5)
Total	326	764	1,090		
Genotype distributions (four genetic models)					
Homozygote	TT	CC	Total	p value	OR(95% CI)
ASD	20	101	121		
Control	23	161	184	0.32	0.72(0.37-1.3)
Total	43	262	305		
Heterozygote	CT	CC	Total	p value	OR(95% CI)
ASD	101	101	202		
Control	139	161	300	0.42	1.15(0.81-1.64)
Total	240	262	502		
Dominant	TT+CT	CC	Total	p value	OR(95% CI)
ASD	141	81	222		
Control	185	138	323	0.14	1.29(0.91-1.85)
Total	326	219	545		
Recessive	TT	CT+CC	Total	p value	OR(95% CI)
ASD	20	202	222		
Control	23	300	323	0.42	1.29(0.67-2.3)
Total	43	502	545		

Table 2. *MTHFR* C677T allele and genotype distributions in male ASD and male control groups

Allele distributions					
Allele	T	C	Total	P value	OR(95% CI)
ASD (male)	117	251	368		
Control (male)	90	226	316	0.34	1.17(0.83-1.62)
Total	207	477	684		
Genotype distributions (four genetic models)					
Homozygote	TT	CC	Total	P value	OR(95% CI)
ASD (male)	15	82	97		
Control (male)	10	78	88	0.41	1.42(0.61-3.35)
Total	25	160	185		
Heterozygote	CT	CC	Total	P value	OR(95% CI)
ASD (male)	87	82	169		
Control (male)	70	78	148	0.45	1.18(0.76-1.82)
Total	157	160	317		
Dominant	TT+CT	CC	Total	P value	OR(95% CI)
ASD (male)	102	82	184		
Control (male)	80	78	158	0.37	1.21(0.79-1.86)
Total	182	160	342		
Recessive	TT	CT+CC	Total	P value	OR(95% CI)
ASD (male)	15	169	184		
Control (male)	10	148	158	0.51	1.31(0.59-2.94)
Total	25	317	342		

Table 3. *MTHFR* C677T and *PHGDH* rare gene variant combinations in patients with ASD and controls

ASD / Control member	*MTHFR* C677T	*PHGDH* SNV	rsID	gnomAD MAF
ASD_1	C / T	c.141C>G; p.D47E / G	*De novo* (new)	-
ASD _2	T / T	c.1559C>A; p.A520E / C	rs151275800	0.0009
ASD _3	C / T	c.1559C>A; p.A520E / C	rs151275800	0.0009
Control_1	C / T	c.1559C>A; p.A520E / C	rs151275800	0.0009
Control_2	C / T	c.806A>T; p.D269V / A	rs762430145	0.000004
Control_3	C / T	c.718G>A; p.V240M / G	rs765276824	0.00002

*: In naming the SNVs it is preferred using known common *MTHFR* variant name whereas for *PHGDH* it was used the HGVS (Human Genome Variation Society) nomenclature. gnomAD: The Genome Aggregation Database

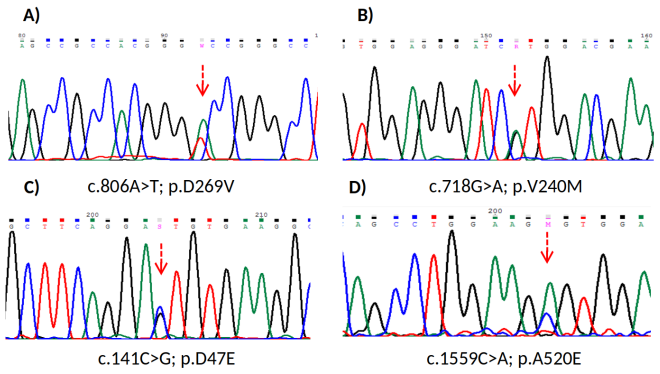

Figure 3. Sanger chromatograms of the rare SNVs found in the *PHGDH* genes in case and control subjects depicted in Table 3. A) c.806A> T; p.D269V; B) c.718G> A; p.V240M; C) de novo c.141C > G; p.D47E; D) c.1559C > A; p.A520E.

sequencing approach between ASD and neurotypical controls (or family design as trio/quad) to date [26]. But, if pathological CNVs are also included in these Mendelian forms of ASD it is generally assumed that they are totally explained only about 15-20% of the ASD genetic liabilities [27]. Thus, in the great majority of cases, genetic factors that being directly causal or increases the risk remains obscure. One well-known and generally accepted explanation of this subject is that the great majority of ASD is a multifactorial (complex/polygenic) disease; i.e. both genetic and environmental risk factors have a role in the etiology of ASD [28]. The genetic part of these complex diseases (maybe a gene, a regulator DNA sequence or a common SNP, etc.) generally has been researched by two methods: One of these is GWAS which is an unbiased approach and requires thousands of case and matched controls to reach statistically significant outcomes. The other approach is the genetic or allelic association studies (an observational study based on suspected genes, regulator DNA sequences or common SNPs) and requires relatively modest case and control numbers [29]. The *MTHFR* C677T allele is one of the most commonly investigated SNP by genetic association studies to date for understanding whether there is a causal relationship or increase the risk of ASD and other psychiatric diseases [30]. About these genetic association studies outcomes concerning ASD, there are very contradictory results in the biomedical literature. Whereas in the reports of some researchers' studies (e.g. Egypt, China, and USA) there were statistically significant associations between *MTHFR* 677T allele / 677TT genotype and the risk of ASD [31–33] , in the other researchers' studies (e.g. Turkey, Brazil, Iran, and Romania) including from our country there were not any significant association [17, 34–36]. It is interesting to note that it has been found statistically significant associations between *MTHFR* 677T allele / TT+CT genotypes and the risk of ASD in all published meta-analytic studies, so far [37]. In the present study, we could not find any statistically significant associations between *MTHFR* 677T allele / TT+CT genotypes and the risk of ASD in comparisons of both total or only in male cases-controls (Table 1, 2). Our results are consistent with the results from our country which was performed by Sener et al. and the results from Brazil, Iran, and Romania, respectively. To our knowledge, from our country there is only one study published so far about this topic (Sener et al.) and, our study is the second one and has a much higher number of cases and controls. We also carried out a statistical analysis by including the results published by Sener et al. in order to see whether there are any statistically significant associations between cases and controls in our population. Again, we could not find any allelic or genotypic associations (data not shown).

There is also multiple genetic-hits hypotheses in the genetic etiology of ASD (i.e., the combination of two or more common SNVs, common SNVs plus rare and possibly harmful SNVs, etc.) [38, 39]. Due to the reason we mentioned before, 1C metabolism genes are strong candidates for such multiple-hit SNVs research (Figure 1). In the present study, we wanted to test this situation in terms of common *MTHFR* 677T allele and PHGDH gene rare (possibly harmful) variant combinations. Although we do not have enough patient and control samples to obtain statistically significant results, if there is a tendency about this combination, we hoped to find some indications (i.e., at least, there would be a greater number of the rare and possibly harmful SNVs of the *PHGDH* gene in the ASD compared to controls). We did not see such a tendency about these combinations in ASD groups compared to controls (Table 3). To our knowledge, such analysis about genetic causes of ASD was performed for the first time by the present study.

Conclusion

In the present study, we performed a genetic association study by using the largest sample numbers in Turkey so far to investigate whether there are statistically significant associations between *MTHFR* 677T allele / 677TT genotype and the risk of ASD. There was not a statistically significant association between the mentioned *MTHFR* allele/genotypes and the risk of ASD in contrast to those of some published research and meta-analytic studies. Our results are consistent with the results published from our country, Brazil, Iran, and Romania, before. We also performed targeted whole PHGDH gene resequencing analysis in patients with ASD and controls carrying *MTHFR* C677T variant as homo-or heterozygous. We did not find any increasing tendency of these rare and possibly harmful SNVs in ASD groups compared to controls. Future studies about the effects of the rare and common SNVs of the 1C metabolism genes (and combination effects of these SNVs) on the pathophysiology of ASD will lead to more obvious results. For these investigations that should have needed possessing an adequate statistical power, it will be needed to use much more patients with ASD and controls.

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