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Analysis of methionine synthase (rs1805087) gene polymorphism in autism patients in Northern Iran

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Autism is characterized by impairment in reciprocal communication and speech, repetitive behaviors, and social communication. The genetic and environmental factors play roles in the pathogenesis of autism. It was recently shown that the genes involved in the folate/homocysteine pathway may be risk factors for autistic children. One of the genes that may be the risk factor for autism is Methionine synthase (MTR). MTR is responsible for the regeneration of methionine from homocysteine. The aim of this study was to analyze the association of MTR A2756G gene polymorphism (rs1805087) and the risk of autism in a population in northern Iran. The prevalence of MTR A2756G polymorphism was determined in 108 children with autism and 130 controls in northern Iran. Genotypes and allele frequencies were determined in patients and controls by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). The prevalence of genotype frequencies of AA, AG and GG in autistic children were 57.41%, 22.22% and 20.37%, respectively, while in controls were 61.54%, 32.31% and 6.15%, respectively. There was significant difference between the MTR polymorphism distribution in control and patient groups. The prevalence of allele frequencies of A and G in autistic children were 0.69 and 0.31, respectively and in controls were 0.78 and 0.22, respectively (P=0.03). The MTR G allele conferred a 1.6-fold increased risk to autism relative to the A allele (95% CI=1.06–2.41, P=0.02). The present study suggests that the G allele of *MTR* A2756G polymorphism is associated with an increased risk of autism.

Key words: autism, MTR A2756G, gene polymorphism, PCR-RFLP

INTRODUCTION

Autism spectrum disorders (ASDs) are a collection of neurodevelopmental conditions that are usually of prenatal origin and can be diagnosed in early childhood, when it is severe (Gillberg 2010). The prevalence of ASDs in the United States is currently 1 in 68 children (Centers for Disease Control and Prevention 2014). ASDs affect mainly males, with an estimated 4:1 ratio between males and females, which might be partly related to hormonal involvement in the development of the disease (Lombardo et al. 2012). Although the etiology of ASDs is unknown, many theories support an interaction of environmental and genetic factors (Smalley et al. 1988). The genetic variants participated in ASDs and inherited from parents to affected individuals have been estimated to explain ~40% of ASDs risk. De novo mutations in the patients are thought to contribute to 15–20% of cases (Hallmayer et al. 2011, Devlin and Scherer 2012). Despite the un-success in identifying the candidate genes that are responsible for the most of ASDs cases, epigenetic dys-regulation of genes necessary for normal brain development and growth and cognitive function and behavior are associated with the etiology of ASDs (Liu et al. 2011). Autism is the most severe symbol of a group of neurodevelopmental disabilities known as ASDs and was first described by Leo Kanner (Baird et al. 2006, Kanner 1968). Autism is a heterogeneous neurological disorder defined by three core behavior impairments – for example, fractions in verbal and nonverbal communication, deficits in social interaction, and severe stereotyped behaviors that appear after a period of relatively normal development (American Psychiatric Association 2000). Individuals with Idiopathic autism (IA) have major deficits in temporal information processing (TIP) (Szelag et al. 2004). It has been shown that the genes participated in the folate/homocysteine pathway may be the risk factors for autistic children. Methionine synthase (MTR), methylenetetrahydrofolate reductase (MTHFR), and methionine synthase reductase (MTRR) are key enzymes participated in the folate-mediated one-carbon metabolism, and involves in DNA synthesis, methylation, and repair (Xu et al. 2004). MTR is consisted of five important regions, including homocysteine (HCY) -binding,

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5-methyltetrahydrofolate (5-methylTHF) -binding, cap, cobalamin-binding and SAM-binding domains (Evans et al. 2004, Leclerc et al. 1998). MTR gene is located on chromosome 1q43. MTR, a vitamin B12-dependent enzyme involved in the folate-mediated one-carbon metabolism. It catalyzes the methylation of homocysteine to methionine with simultaneous conversion of 5-methyl-tetrahydrofolate (5-methyl-THF) to tetrahydrofolate (THF). THF is essential for nucleotide synthesis. Methionine is essential for S-adenosyl-methionine (SAM) synthesis and DNA methyltransferases (DNMTs) transfer the methyl group from SAM to the DNA (James et al. 1999). It is reported that a polymorphism in MTR A2756G (rs1805087) leads to a change from aspartic acid to glycine at codon 919 (D919G) and it was initially thought to be associated with the lower enzyme activity followed by homocysteine elevation and DNA hypomethylation (Chen et al. 1997, 1996). However, some other studies revealed a modest inverse association between GG genotype (A2756G MTR) and HCY levels, indicating an increased enzymatic activity of the variant genotype (Goode et al. 2004). The polymorphism in many genes including Forkhead Box P3, SHANK and Vitamin D receptor were shown to be associated with the susceptibility of autism (Safari et al. 2016, Mashayekhi et al. 2016, Schmidt et al. 2015). The aim of this study was to investigate the impact of *MTR* A2756G gene polymorphism on the risk of autism in Iran.

MATERIALS AND METHODS

Subjects

All participants have been given their informed consent in this study. The current study included a total of 108 patients with autism disorder and 130 disease-free control subjects. Controls and patients were selected from the same population that was recruited in 2014. Data on patient characteristics at the study entry for each subject were collected from the Iran Medical diagnostic Center in Rasht, Iran. The diagnosis of ASD was made according to DSM-5 criteria for ASD. Children were investigated in terms of developing to certain genetic diseases in close relatives, neurological disorders and allergy in infancy and intestinal bacterial infections. Children with fragile x syndrome, tuberous sclerosis, a previously identified chromosomal abnormality, dysmorphic features, or any other neurological condition suspected to be associated with autism were excluded. Each subject donated 2 ml blood and drawn into EDTA-Coated tubes (Venoject, Belgium), which was used for genomic DNA extraction. This study has been approved by the local ethical committee (Protocol number: 1392-4, Date: 2013).

Genomic DNA extraction

Subjects were genotyped for the *MTR* 2756 SNP using genomic DNA extracted from peripheral blood leukocytes. Genomic DNA was extracted from peripheral blood samples using the Gpp solution kit (Gen Pajoohan, Iran). Extracted DNA was observed and confirmed by electrophoresis on 0.1% agarose gel containing ethidium bromide.

Analysis of genetic polymorphism

For genotyping of the MTR A2756G polymorphism (rs1805087), polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was used. The PCR primers were synthesized by Shanghai Geneway Biotech. China. The forward and reverse primers of MTR A2756G were 5'-CATCTTTTGCTCATCTATGGCTATC-3' and 5'-TCTAGCACAGCCCCTAACACCT-3', respectively. The primers were designed by Oligo7 software (version 7.54, USA). The amplification procedure was carried out in a total reaction volume of 20 µl, containing 10 µl 2X PCR Master mix (CinnaGen, Iran), 1 µl forward primer, 1 µl Reverse primer, 3 µl sterile deionized water and 5 µl Template DNA. The amplification was performed as follows: initial denaturation at 94°C for 5 min, amplification for 35 cycles at 94°C for 45 s, 58°C for 45 s and 72°C for 45 s, followed by a final elongation step at 72°C for 5 min. The resultant PCR product was visualized on a 2% agarose-ethidium bromide gel under UV illumination. To confirm the accuracy of genotyping results, randomly in 10% of subjects, genotyping was repeated to obtain concordance by minimizing genotyping errors. Then the PCR products were digested for 1 hour at 37°C with 2 unit of *AvaII* (Thermo Scientific Eco47I), and the amplified fragment of 395 bp was cut into fragments of 280 and 115 bp and visualized on a 2% agarose–ethidium bromide gel under UV illumination. This method is able to detect all three possible genotypes for the polymorphism including homozygous wild type (AA: 280 and 115 bp), heterozygous variant type (AG: 395, 280 and 115 bp) and homozygous variant type (GG: 395 bp).

Statistical analysis

Genotype frequencies of MTR polymorphism in patient and control groups were analyzed by χ^2 test. The Hardy-Weinberg equilibrium assumption was assessed by comparing the genotype frequencies with those expected on the basis of the observed frequencies. Logistic regression approach was used to obtain adjusted odds ratio (OR) and 95% confidence interval (CI) for genetic

polymorphisms. The results were considered statistically significant when p<0.05.

RESULTS

This study included 108 children with autism (20 females and 88 males) and 130 controls (34 female and 96 males) in Iran. The undigested PCR product size was 395 bp for MTR A2756G (Fig. 1A). Restriction digestion for the GG genotype generated 395 bp fragment; whereas the AG genotype generated 115, 280 and 395 bp fragments. Moreover, there were two bands (280 and 115 bp) in the presence of homozygous AA (Fig. 1B). The frequency of the A2756G polymorphism of *MTR* was also analyzed. All information about allele and genotype frequencies and associated ORs (95% CI) for patients and controls is presented in Table I. There was significant association in MTR 2756 gene polymorphism was seen between patients and control groups (P=0.002). Moreover, GG genotype (A2756G MTR) seems to be the risk factor in our population (P=0.004, OR 3.54, 95% CI 1.47–8.50). The A and G allele frequencies of this polymorphism were 68.52%, 31.48% in the patient group and 77.69%, 22.31% in the control group, respectively, which was statistically significant (P=0.03). Moreover G allele was shown to be associated with the increased risk of autism (P=0.02).

DISCUSSION

MTR catalyzes the remethylation of homocysteine to form methionine using the methyl group bound to cbl (Födinger et al. 1999). So Cbl(I) state of cobalamin is a very high reactive "supernucleophile", and acts as an

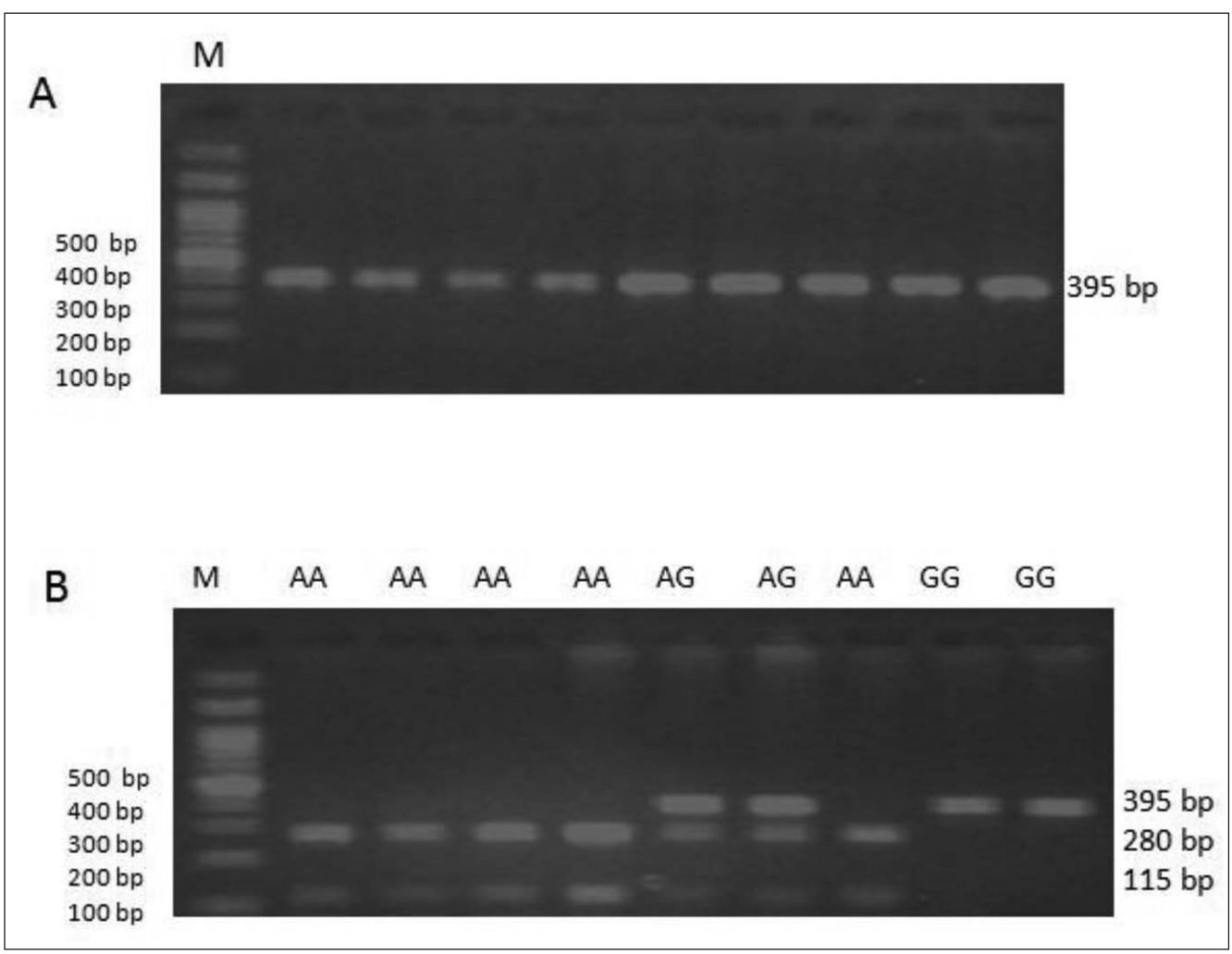

Fig. 1. (A) Agarose gel electerophoresis after PCR amplification of MTR A2756G. "M" represents marker. The PCR product size was 395 bp. (B) Gel picture showing RFLP fragments for MTR A2756G: "M" represents marker. Enzymatic digestion for the GG genotype generated 395 bp fragment; 115, 280 and 395 bp fragments for AG genotype, and 115 and 280 bp for AA genotype.

indicator of the cellular redox environment until it is remethylated (Jensen 2005). However, the cap domain assumes a position above Cbl(I) and partially protecting it from oxidation (Bandarian et al. 2002). Cbl oxidation stops enzyme activity and diverts HCY to transsulfuration pathway, which in turn, increases glutathione (GSH) synthesis until SAM-dependent reductive methylation of Cbl restores MTR activity (Jarrett et al. 1998). Folate and methionine metabolism are required for DNA synthesis and DNA methylation, also their metabolic pathways may play roles in disease susceptibility (Heijmans et al. 2003). Rare genetic defects of MTR, known as the CblG complementation group of cobalamin disorders, which cause in hyperhomocysteinemia, homocystinuria, and megaloblastic anemia without methylmalonic aciduria (Watkins and Rosenblatt 1988). It also indicates that the low activity of MTR results in the hypomethylation of DNA. The AG heterozygotes of the MTR A2756G polymorphism is likely associated with augmented levels of Hcy in Alzheimer's disease and Parkinson's disease patients (Dorszewska et al. 2007). This increase in Hcy is likely due to low MTR activity, caused by excessive oxidation of cobalamin (McCaddon et al. 2002) related to oxidative stress, which is observed in aging and degenerative disorders (Rozycka et al. 2013). Increased oxidative stress has been diagnosed in autistic patients (James et al. 2006). It was demonstrated that MTR status might change during aging and in neurological disorders related with oxidative stress and the level of MTR mRNA revealed a considerable age-dependent decrease. Although MTR mRNA levels were lower in autistic subjects, protein levels of MTR were similar to control. These findings suggested that the prematurely low levels of MTR mRNA in the cerebral cortex were associated with autism (Muratore et al. 2013). Mohammad and others (2009) examined the associations between five gene polymorphisms involved in folate pathway including MTR A2756G, MTHFR C677T, MTHFR A1298C, SHMT1 C1420T, MTRR A66G, and the risk of autism in a cohort of autistic children and nonautistic children from the South India. Their studies show that MTR A2756G polymorphism was not associated with an increased risk of autism (Mohammad et al. 2009). Some studies showed that polymorphisms are important in different cancers. For example, it was found that the MTHFR C677T and MTR A2756G polymorphisms are related with breast cancer susceptibility in a Chinese population in their case-control study, and that folate, vitamin B6, and vitamin B12 intakes influence these associations (Jiang-Hua et al. 2014). The data of Zhu and others (2013) supported the hypothesis that the MTHFR 677TT polymorphism is associated with an increased risk of cervical cancer in Asian females, while reverse association applies to Caucasian females. However, their meta-analysis did not support an association of the A2756G polymorphism of MTR and MTHFR A1298C polymorphism with cervical cancer risk (Zhu et al. 2013). It was shown that MTR A2756G polymorphism is a candidate gene polymorphism for cancer susceptibility (Yu et al. 2010). It was demonstrated that the MTHFR A1298C, the MTHFR C677T, the MTR A2756G, the MTRR A66G, and the thymidylate synthase (TS 2R/3R) polymorphisms have consistent roles in the increased risk of sporadic colorectal adenocarcinoma (SCA) susceptibility among the south and southeastern Brazilian population (Guimarães et al. 2011). However, large sample studies are required to confirm these associations.

Some potential limitations should be considered in this study. First, it was conducted in Iran, and may not be representative of other populations. Second, the numbers of cases and controls is rather small, which may limit the statistical power to detect differences between groups. Third, alterations in laboratory procedures such as methods of data collection and genotyping, could also clarify the inconsistent results.

CONCLUSIONS

The MTR G allele conferred a 1.6-fold increased risk to autism relative to the A allele (95% CI=1.06–2.41, P=0.024). The present study suggests that the G allele of MTR A2756G polymorphism is associated with an increased risk of autism. Larger studies with more patients and controls are needed to confirm the results.

Table I. Allele and genotype frequencies of MTR A2756G polymorphism among patients and controls

	controls (n= 65) n (%)	patients (n= 54) n (%) OR (95 % CI)		P^a	P^b
alleles (A2756G)					
A	202 (77.69)	148 (68.52)	1.00 (reference)	0.03*	–
G	58 (22.31)	68 (31.48)	1.60 (1.06–2.41)		0.02*
genotypes (A2756G)					
AA	80 (61.54)	62 (57.41)	1.00 (reference)	0.002*	–
AG	42 (32.31)	24 (22.22)	0.73 (0.40–1.34)		0.32
GG	8 (6.15)	22 (20.37)	3.54 (1.47–8.50)		0.004*

* – significant at 5% level of significance (P<0.05); [a] – allele and genotype frequencies in patients and controls were compared using χ^2 test; [b] – significance level for allele and genotype frequencies in patients and controls; n – number of subjects.

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REFERENCES

American Psychiatric Association (2000) Diagnostic and Statistical Manual of Mental Disorders (DSM-IV-TR). APA, Washington DC, USA.

Baird G, Simonoff E, Pickles A, Chandler S, Loucas T, Meldrum D, Charman T (2006) Prevalence of disorders of the autism spectrum in a population cohort of children in South Thames: the Special Needs and Autism Project (SNAP). Lancet 368: 210–215.

Bandarian V, Pattridge KA, Lennon BW, Huddler DP, Matthews RG, Ludwig ML (2002) Domain alternation switches B(12)-dependent methionine synthase to the activation conformation. Nat Struct Biol 9: 53–56.

Centers for Disease Control and Prevention (2014) Prevalence of autism spectrum disorder among children aged 8 years -autism and developmental disabilities monitoring network, 11 sites, United States, 2010. MMWR Surveill Summ 63: 1–21.

Chen J, Giovannucci E, Kelsey K, Rimm EB, Stampfer MJ, Colditz GA, Spiegelman D, Willett WC, Hunter DJ (1996) A methylenetetrahydrofolate reductase polymorphism and the risk of colorectal cancer. Cancer Res 56: 4862–4864.

Chen LH, Liu ML, Hwang HY, Chen LS, Korenberg J, Shane B (1997) Human methionine synthase. cDNA cloning, gene localization, and expression. J Biol Chem 272: 3628–3634.

Devlin B, Scherer SW (2012) Genetic architecture in autism spectrum disorder. Curr Opin Genet Dev 22: 229–237.

Dorszewska J, Florczak J, Rozycka A, Kempisty B, Jaroszewska-Kolecka J, Chojnacka K, Trzeciak WH, Kozubski W (2007) Oxidative DNA damage and level of thiols as related to polymorphisms of MTHFR, MTR, MTHFD1 in Alzheimer's and Parkinson's diseases. Acta Neurobiol Exp (Wars) 67(2): 113–129.

Evans JC, Huddler DP, Hilgers MT, Romanchuk G, Matthews RG, Ludwig ML (2004) Structures of the N- terminal modules imply large domain motions during catalysis by methionine synthase. Proc Natl Acad Sci U S A 101: 3729–3736.

Födinger M, Buchmayer H, Sunder-Plassmann G (1999) Molecular genetics of homocysteine metabolism. Miner Electrolyte Metab 25: 269–278.

Gillberg C (2010) The ESSENCE in child psychiatry: Early Symptomatic Syndromes Eliciting Neurodevelopmental Clinical Examinations. Res Dev Disabil 31: 1543–1551.

Goode EL, Potter JD, Bigler J, Ulrich CM (2004) Methionine synthase D919G polymorphism, folate metabolism, and colorectal adenoma risk. Cancer Epidemiol Biomarkers Prev 13: 157–162.

Guimarães JL, Ayrizono Mde L, Coy CS, Lima CS (2011) Gene polymorphisms involved in folate and methionine metabolism and increased risk of sporadic colorectal adenocarcinoma. Tumour Biol 32: 853–861.

Hallmayer J, Cleveland S, Torres A, Phillips J, Cohen B, Torigoe T, Miller J, Fedele A, Collins J, Smith K, Lotspeich L, Croen LA, Ozonoff S, Lajonchere C, Grether JK, Risch N (2011) Genetic heritability and shared environmental factors among twin pairs with autism. Arch Gen Psychiatry 68: 1095–1102.

Heijmans BT, Boer JM, Suchiman HE, Cornelisse CJ, Westendorp RG, Kromhout D, Feskens EJ, Slagboom PE (2003) A common variant of the methylenetetrahydrofolate reductase gene (1p36) is associated with an increased risk of cancer. Cancer Res 63: 1249–1253.

James SJ, Melnyk S, Jernigan S, Cleves MA, Halsted CH, Wong DH, Cutler P, Bock K, Boris M, Bradstreet JJ, Baker SM, Gaylor DW (2006) Metabolic endophenotype and related genotypes are associated with oxidative stress in children with autism. Am J Med Genet B Neuropsychiatr Genet 141: 947–956.

James SJ, Pogribna M, Pogribny IP, Melnyk S, Hine RJ, Gibson JB, Yi P, Tafoya DL, Swenson DH, Wilson VL, Gaylor DW (1999) Abnormal folate metabolism and mutation in the methylenetetrahydrofolate reductase gene may be maternal risk factors for Down syndrome. Am J Clin Nutr 70: 495–501.

Jarrett JT, Huang S, Matthews RG (1998) Methionine synthase exists in two distinct conformations that differ in reactivity toward methyltetrahydrofolate, adenosylmethionine, and flavodoxin. Biochemistry 37: 5372–5382.

Jensen KP (2005) Electronic structure of Cob(I)alamin: the story of an unusual nucleophile. J Phys Chem B 109: 10505–10512.

Jiang-Hua Q, De-Chuang J, Zhen-Duo L, Shu-de C, Zhenzhen L (2014) Association of methylenetetrahydrofolate reductase and methionine synthase polymorphisms with breast cancerrisk and interaction with folate, vitamin B6, and vitamin B12 intakes. Tumour Biol 35: 11895–11901.

Kanner L (1968) Autistic disturbances of affective contact. Acta Paedopsychiatr 35: 100–136.

Leclerc D, Wilson A, Dumas R, Gafuik C, Song D, Watkins D, Heng HH, Rommens JM, Scherer SW, Rosenblatt DS, Gravel RA (1998) Cloning and mapping of a cDNA for methionine synthase reductase, a flavoprotein defective in patients with homocystinuria. Proc Natl Acad Sci U S A 95: 3059–3064.

Liu X, Solehdin F, Cohen IL, Gonzalez MG, Jenkins EC, Lewis ME, Holden JJ (2011) Population- and family-based studies associate the MTHFR gene with idiopathic autism in simplex families. J Autism Dev Disord 41: 938–944.

Lombardo MV, Ashwin E, Auyeung B, Chakrabarti B, Taylor K, Hackett G, Bullmore ET, Baron-Cohen S (2012) Fetal testosterone influences sexually dimorphic gray matter in the human brain. J Neurosci 32: 674–680.

Mashayekhi F1, Mizban N, Bidabadi E, Salehi Z (2016) The association of SHANK3 gene polymorphism and autism. Minerva Pediatr. epub ahead of print.

McCaddon A, Regland B, Hudson P, Davies G (2002) Functional vitamin B(12) deficiency and Alzheimer disease. Neurology 58: 1395–1399.

Mohammad NS, Jain JM, Chintakindi KP, Singh RP, Naik U, Akella RR (2009) Aberrations in folate metabolic pathway and altered susceptibility to autism. Psychiatr Genet 19: 171–176.

Muratore CR, Hodgson NW, Trivedi MS, Abdolmaleky HM, Persico AM, Lintas C, De la Monte S, Deth RC (2013) Age-dependent decrease and alternative splicing of methionine synthase mRNA in human cerebral cortex and an accelerated decrease in autism. PLoS One 8: e56927.

Rozycka A, Jagodzinski PP, Kozubski W, Lianeri M, Dorszewska J (2013) Homocysteine level and mechanisms of injury in Parkinson's disease as related to MTHFR, MTR, and MTHFD1 genes polymorphisms and L-dopa treatment. Curr Genomics 14: 534–542.

Safari MR, Ghafouri-Fard S, Noroozi R, Sayad A, Omrani MD, Komaki A, Eftekharian MM, Taheri M (2017) FOXP3 gene variations and susceptibility to autism: A case-control study. Gene 596: 119–122.

Schmidt RJ, Hansen RL, Hartiala J, Allayee H, Sconberg JL, Schmidt LC, Volk HE, Tassone F (2015) Selected vitamin D metabolic gene variants and risk for autism spectrum disorder in the CHARGE Study. Early Hum Dev 91: 483–489.

Smalley SL, Asarnow RF, Spence MA (1988) Autism and genetics. A decade of research. Arch Gen Psychiatry 45: 953–961.

Szelag E, Kowalska J, Galkowski T, Pöppel E (2004) Temporal processing deficits in high-functioning children with autism. Br J Psychol 95: 269–282.

Watkins D, Rosenblatt DS (1988) Genetic heterogeneity among patients with methylcobalamin deficiency. Definition of two complementationgroups, cblE and cblG. J Clin Invest 81: 1690–1694.

Xu XL, Yu J, Zhang HY, Sun MH, Gu J, Du X, Shi DR, Wang P, Yang ZH, Zhu JD (2004) Methylation profile of the promoter CpG islands of 31 genes that may contribute to colorectal carcinogenesis. World J Gastroenterol 10: 3441–3454.

Yu K, Zhang J, Zhang J, Dou C, Gu S, Xie Y, Mao Y, Ji C (2010) Methionine synthase A2756G polymorphism and cancer risk: a meta-analysis. Eur J Hum Genet 18: 370–378.

Zhu J, Wu L, Kohlmeier M, Ye F, Cai W (2013) Association between MTHFR C677T, MTHFR A1298C and MS A2756G polymorphisms and risk of cervical intraepithelial neoplasia II/III and cervical cancer: a meta-analysis. Mol Med Rep 8: 919–927.