A study of MTRR 66A>G gene polymorphism in patients with autism from northern Iran

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Abstract

Autism is a neurodevelopmental disorders that manifests before 3 years of age, more common in boys. Whereas causes of autism remain uncertain, it is influenced by genetic and environmental factors. Recent studies have shown that the genes involved in the folate metabolism pathway may play an important role in autism. Methionine synthase reductase (MTRR) is a key enzyme that plays an important role in the homocysteine/folate metabolism and has been shown to be implicated in neurological disorders including autism. In this study, 356 subjects were studied, which consists of 142 autistic children and 214 nonautistic control. Genomic DNA was extracted from blood samples. Genotype of MTRR 66A>G gene was performed using polymerase chain reaction-allele specific PCR (AS-PCR). The genotype frequencies of AA, AG and GG in the children with autism were 9.9%, 76.0% and 14.1%, respectively and in control group were 13.1%, 86.0% and 0.9%, respectively. The allele frequencies of A, G in the children with autism were 48.0%, 52.0%, respectively and in control group were 56.0%, 44.0%, respectively. Statistical analysis showed that there is a significant correlation in the genotype between two groups (OR=20, 95% CI=4.1 to 98, P<0.001). It is concluded that MTRR A66G polymorphism is associated with autism in a population in northern Iran. More studies with larger number should be done to confirm this result.

Keywords: Autism; methionine synthase reductase; A66G polymorphism; folate.

INTRODUCTION

Autism is a severe neurodevelopmental disorder. It manifests before 3 years of age and is characterized by impaired social interaction, communication, repetitive and stereotyped behavior and abnormal functioning in symbolism and imaginative play. It has been shown that in autism, the brain volume undergoes an abnormal short period of overgrowth in early postnatal life, followed by growth arrest in later childhood. Previous studies have shown that the prevalence ratio for autism spectrum disorders (ASDs) is four times higher in men than in women. While causes of autism are unknown, it is generally thought to be a developmental disorder that is influenced by genetics, environmental, and immunological factors with increased susceptibility to oxidative stress. Environmental factors such as mercury, lead, measles, rubella virus, retinoic acid, valproic acid and alcohol have been suggested to be involved in the etiology of autism. It has been demonstrated that genetic factors such as methionine synthase reductase (MTRR), methylenetetrahydrofolate reductase (MTHFR), methionine synthase (MTR) and serine hydroxymethyl transferase (SHMT1) are involved in the pathogenesis of autism. Evidence shows that the factors that increase the risk of autism have critical periods of action during pregnancy and embryogenesis. It has also been shown that nutrition such as Vitamin B12, B6 and folic acid plays a significant role in the development and behavior of autistic children. In addition, some children with autism have altered B vitamin metabolism and reduced methylation capacity. However, despite a number of studies to clarify the role of B vitamin deficiency in psychiatric and neurologic diseases, the relationship between B vitamin status and the risk of cognitive or behavioral disorders remains unclear. Vitamins B6, B12 and folic acid are necessary for lowering the level of homocysteine. Insufficient dietary intake can lead to increase in homocysteine level. One-carbon metabolism is a network of interconnected biological reactions and in turn, facilitates the cross-talk between genetic and epigenetic processes. Genes involved...
in the folate metabolic pathway include methylenetetrahydrofolate reductase (MTHFR), methionine synthase (MTR), methionine synthase reductase (MTRR), cystathionine-β-synthase (CBS).\textsuperscript{10,11} Methionine synthase reductase (MSR; gene symbol: MTRR) is a key enzyme in folate metabolism and plays an important role in the homocysteine metabolism as it is responsible for the restoration and maintain methionine synthase regulation by reductive methylation.\textsuperscript{12} MSR gene has about 3.6 kb in length and encodes 698 amino acids and molecular weight of methionine synthase reductase is about 77 kDa.\textsuperscript{13} Furthermore, human MTRR is a housekeeping gene and located at chromosome 5 (5p15.2 – p15.3) and is made of 15 exons.\textsuperscript{14} It has been identified that most common polymorphism in methionine synthase reductase gene is (66A→G) substitution, leading to a change of isoleucine to methionine in codon at position 22 (I22M).\textsuperscript{15} The enzyme required for remethylation of homocysteine (Hcy) to methionine in a Vitamin B12 (cobalamin) and folate dependent reaction. MSR reactivates methionine synthase by reductive remethylation of cob (II) alamin to methylcob (III) alamin using S-adenosylmethionine as the methyl donor.\textsuperscript{13} Thus, MTRR plays a critical role in maintaining cobalamin in an active form and consequently may be an important determinant of homocysteine (tHcy) concentrations. High level of homocysteine generally is associated with neuropsychiatric disorders such as autism.\textsuperscript{16} The aim of this project was to study the association between genotype of polymorphism A66G (rs1801394) of MTRR and autistic children in northern Iran.

**METHODS**

**Participants**

The current study involved a total of 142 patients with Autism disorder and 214 control subjects. Controls and patients were selected from the same population. They were recruited between October 2014 and April 2015 from Iran laboratory, Rasht, Iran. The diagnosis of autism was based on DSM-5 criteria for Autism Spectrum Disorder. Data on genetic diseases in close relatives, neurological disorders and allergy in infancy and intestinal bacterial infections were obtained. 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. The blood was collected from each subject in the EDTA-Coated tubes (Venoject, Belgium) for genomic DNA extraction. This study was approved by the local ethical committee (Protocol number: 7141) in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). All participants have given their informed consent to the study.

**Genomic DNA extraction**

Genomic DNA extraction was done by GPP solution kit (Gen Pajoohan, Iran) as previously described. Extracted DNA was observed and confirmed by electrophoresis on 0.1% agarose gel containing etidium bromide. The concentration and purity of DNA were assessed with a Nanodrop (Thermo) with 260/280 measurement ratio and at the wavelength of 260 and 280 nm. Extracted DNA was stored at -70°C until use.

**Polymorphism analysis**

For genotyping of the MTRR 66A>G polymorphism tetra-primer Amplification Refractory Mutation System-PCR (ARMS-PCR) were used. For MTRR A66G (I22M) genotyping, two independent polymerase chain reaction (PCR) assays for each allele were used. MTRR SNP66 (rs1801394, A>G) genotypes were analyzed using two independent PCR assays for each allele, modified from a technique described. The procedure was performed using primer pairs specific for the two alleles. Primers F1 (5’-TCAAGCCCAAGTAGTTTCGAG– 3’) and R1 (5’–TGTACCACAGCTTGCTCACAT–3’) were used to amplify the 367-bp wild-type allele (A), and primers F2 (5’–CTTGTCTACAGGGTTGCACT–3’) and R2 (5’–TGTACCACAGCTTGCTCACAC–3’) were used to amplify the 401-bp mutant allele (G). The amplification procedure was carried in a total reaction volume of 25 μl, containing 2.5 μl 10X reaction buffer (100 mM Tris–HCl pH 8.3 at 25°C, 500 mM KCl, 15 mM MgCl 2), 2 μl deoxy ribonucleotide triphosphates (1.25 μmol/L), 0.5 μl MgCl2 (25 mmol/L), 1.25 μl of each primer (25 mmol/L), 15.3 μl dH2O, 2 μl of genomic DNA (100 ng/μl) and 0.2 μl Taq DNA polymerase (5 U/μl) (Biflux, Japan). Primer sequences MTRR A66G are summarized in Table 1.

After an initial denaturation at 94°C for 3 min, the DNA was amplified by 35 cycles of 94°C for 30 Sec, 60°C for 30s and 72°C for 30s, with a final extraction at 72°C for 5 min on the Mini PCR (Bio-Rad), then the products electrophoresed on a 2% agarose gel, to allow detection by ethidium
bromide staining. All assays were conducted blindly by two researchers without the knowledge of the case or control status. For quality control, a random of 5% of samples was repeated with 100% concordance.

**Statistical analysis**

All statistical analyses were performed using MedCalc statistical software (Version 12.1, Mariakerke, Belgium). Analysis of difference in allele and genotype frequencies between patients and the controls were calculated by Pearson’s chi-square ($\chi^2$) test. For estimating the association between the $MTRR$ A66G polymorphism variant and the risk of autism, Odds ratios (OR) and 95% confidence intervals (95%CI) were estimated using an unconditional logistic regression model. The results were considered statistically significant when $P<0.05$.

**RESULTS**

This study involved 142 patients with autism (30 females and 112 males) and 214 controls (64 female and 150 males). The length of PCR products for $MTRR$ A66G codon 22 A and G alleles was 401 and 367 bp respectively (Figure 1 and 2). The frequencies of $MTRR$ A66G genotypes were estimated using an allele specific PCR (AS-PCR) that specifically detects either the $MTRR$ A or G allele for codon 22. The allele frequencies of A, G in the children with autism were 48.0%, 52.0% respectively, and in control group were 56.0%, 44.0% respectively ($P=0.04$).

The frequency of AA, AG and GG genotypes of $MTRR$ A66G polymorphism in the controls were 13.1%, 86% and 0.9%, respectively, while those in autism patients were 9.9%, 76% and 14.1%. Statistical analysis showed significant differences in the genotype frequency between two groups (GG vs AA: OR = 20, 95% CI = 4.1 to 98, $P < 0.001$) (Table 2).

**DISCUSSION**

Autism is recognized to have a complex etiology involving both genetic and environmental factors. Studies showed that the combination of specific gene variants within folate metabolism pathways has significantly greater risk for autism in the child. Methionine synthase reductase ($MTRR$) is an enzyme that catalyzes the remethylation of Hcy to methionine via a cobalamin and folate dependent reaction.

<table>
<thead>
<tr>
<th>Primer sequences</th>
<th>AA genotype</th>
<th>AG genotype</th>
<th>GG genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>F: 5’-TCAAGCCCAAGTAGTTTCGAG-3’</td>
<td>367 bp</td>
<td>367 bp</td>
<td></td>
</tr>
<tr>
<td>R: 5’-TGTACCACAGCTTGGCTCACAT-3’</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F: 5’-CTTGCTACAGGGTTGCACT-3’</td>
<td>401 bp</td>
<td>401 bp</td>
<td></td>
</tr>
<tr>
<td>R: 5’-TGTACCACAGCTTGGCTCACAC-3’</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. Agarose gel electrophoretogram showing the detection of $MTRR$ by triplex tetra-primer ARMS-PCR. Products (Lanes 1-7, 367 bp amplicon, Lane M (M = 100 bp ladder, molecular size marker).
Human MTRR gene is localized in chromosome 5 (5p15.2 – p15.3).\textsuperscript{13} MTRR plays a critical role in maintaining cobalamin in an active form and is consequently an important determinant of total plasma Hcy concentration.\textsuperscript{20} However, the 66A > G (rs1801394) polymorphism in the gene MTRR alters an isoleucine into a methionine residue (Ile22Met), which decreases the enzyme activity.\textsuperscript{21}

The MSR A66G (rs1801394) polymorphism has been mostly studied in relation to cancer risk and both alleles have been differently associated with different cancers. The variant G allele-bearing genotype has been significantly associated with an increased risk of hepatocellular carcinoma,\textsuperscript{22} and esophageal squamous cell carcinoma.\textsuperscript{23} According to the investigations conducted there is no association between MTRR A66G and breast cancer.\textsuperscript{24} It was also shown that MTRR A66G polymorphism is associated with the risk of head and neck cancer.\textsuperscript{25} Meanwhile, individuals with the homozygous wild genotype (MTRR 66AA) are at a lower risk for head and neck cancer, confirming that the A allele is protective.\textsuperscript{26} In addition, MTRR gene could be critical in Down syndrome because they are part of the pathway that provides methyl groups for DNA methylation, therefore may influence the regulation of this process and subsequent gene expression. One previous study found an association with Down syndrome preliminary data suggest that the MTRR A66G variant is also of importance.\textsuperscript{27,28}

To our knowledge, no study has been published about the association of MTRR A66G polymorphism with autism in Iranian

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**Table 2: Genotype and allele frequencies of MTRR A66G polymorphism in patients with autism and control groups.**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Cases (n = 142)</th>
<th>Control (n = 214)</th>
<th>Odds Ratio (95% CI)</th>
<th>( P^a )</th>
<th>( P^b )</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/A</td>
<td>14 (9.9 %)</td>
<td>28 (13.1 %)</td>
<td>Ref</td>
<td>&lt;0.001</td>
<td>-</td>
</tr>
<tr>
<td>A/G</td>
<td>108 (76 %)</td>
<td>184 (86 %)</td>
<td>1.17 (0.6, 2.3)</td>
<td>-</td>
<td>0.64</td>
</tr>
<tr>
<td>G/G</td>
<td>20 (14.1 %)</td>
<td>2 (0.9 %)</td>
<td>20 (4.1, 98)</td>
<td>-</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Allele</th>
<th>Cases (n = 284)</th>
<th>Control (n = 428)</th>
<th>( P^a )</th>
<th>( P^b )</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>136 (48 %)</td>
<td>240 (56 %)</td>
<td>-</td>
<td>0.04</td>
</tr>
<tr>
<td>G</td>
<td>148 (52 %)</td>
<td>188 (44 %)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

\( ^a \) allele and genotype frequencies in cases and controls were compared using \( \chi^2 \) test.

\( ^b \) significance level for allele and genotype frequencies in cases and controls
population. Our results showed significant association in the genotype distribution of the MTRR 66A>G polymorphism between autism patients and controls. In a case-control study including 214 controls and 142 patients with autism, MTRR A66G polymorphism genotyping showed significant association between A66G polymorphism with autism. Further studies are needed to confirm the role of MTRR gene in ASD.

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DISCLOSURE
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Conflict of interest: None

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