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Study of polymorphism in methionine synthase gene by RFLP-PCR in middle Euphrates region of Iraq

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Abstract

Background: Autism spectrum disorders (ASDs) are a collection of neuro-developmental conditions that are usually of prenatal origin and can be diagnosed in early childhood when it is severe. Analysis of methionine synthase (rs1805087) gene polymorphism in autism patients in Northern Iran reported that methionine synthase (MTR) is responsible for the regeneration of methionine from homocysteine. Therefore, the aim of current study was to study polymorphism in methionine synthase gene by restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR) in middle Euphrates region of Iraq.

Methods: 70 children with autism and 30 controls in middle Euphrates region of Iraq were involved in current study. Genotypes and allele frequencies were determined in patients and controls by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

Results and conclusion: Current study showed that the highest genotype in control group was AA homozygote genotype (63.33%) followed by mutant homozygote GG (26.66 %) and then heterozygote genotype AG(10%) . In autism disease, the highest genotype was GG mutant homozygote genotype (62.58%). The Rs 18 polymorphism revealed that the homozygote mutant genotype G/G had significant higher risk of autism patients (P= 0.001; OR=13.06; 95% CI= (4.27-39.95) when compared with control group. The A allele frequency was significantly associated with autism patients (P=<0.001; OR=6.72; 95% CI=(3.45-13.11).

Key word: Autism, gene polymorphism, Methionine synthase, RFLP-PCR, Homozygous genotype.

INTRODUCTION

Autism spectrum disorders (ASDs) are a collection of neuro-developmental conditions that are usually of prenatal origin and can be diagnosed in early childhood when it is severe [1, 2]. Although the etiology of ASDs is unknown, many theories support an interaction of environmental and genetic factors, the genetic variants participated in ASDs, and inherited from parents to affected individuals, have been estimated to explain ~40% of ASDs risk [3].

The Methionine synthase (MTR), methylene tetrahydrofolate reductase (MTHFR), and methionine synthase reductase (MTRR) are key enzymes participated in the folate-mediated one-carbon metabolism and involved in DNA synthesis, methylation and repair [4]. MTR consists of five important regions including homocysteine (HCY)-binding, 5-methyltetrahydrofolate (5-methylTHF) binding, cap, cobalamin-binding and SAM-binding domains. On the other hand, MTR gene is located on chromosome 1q43. Methionine synthase, a vitamin B12-dependent enzyme is 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) which is essential for nucleotide synthesis. Methionine is essential for S-adenosyl-methionine (SAM) synthesis and DNA methyltransferases (DNMTs) which transfer the methyl group from SAM to the DNA [5]. 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 [6-8]. 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 [9].

Haghiri et al. [10] showed the analysis of methionine synthase (rs1805087) gene polymorphism in autism patients in Northern Iran and the results reported that methionine synthase (MTR) is responsible for the regeneration of methionine from homocysteine. 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) [10]. 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 was aimed to explore the association between G allele of *MTR* A2756G polymorphism and the increased risk of autism.

MATERIAL AND METHODS

1. Patients and Control groups

A case – control study that was conducted during period from May 2017to May 2018 .The study subjects comprised from 70 Autistic children who were selected from Al-Hilla, Al-Najaf, Diwaniyah and Karbala cities. In addition, blood samples of 30 individuals were collected to compare with case patients. About 2mL of venous blood sample was collected from each subject in the study into EDTA-containing tubes to be used for genetic analysis.

2. Extraction of DNA

Genomic DNA of frozen blood was extracted according to DNA extraction kit protocol (Favorgen).

3. Estimation of DNA Concentration and Purity

The DNA concentration of samples was estimated by using a spectrophotometer (Nanodrop).

4. PCR amplification

Preparing the Primers: The Bioneer® primers in table 1 were prepared depending on manufacturer instruction. To create a stock of primers, the lyophilized primer were dissolved and nuclease-free water was added to each primer to obtain master stock that will be used again to obtain a working stock.

PCR mixtures: The PCR mixtures were brought together according manufacture procedure of the master mix (Promega). All the appending was done in laminar flow cabinet on the frozen cooling blocks and ice when it was necessary. The components of PCR working solutions were representated in table 1.

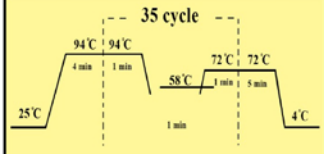
Table 1 The components of PCR working solutions

Component	Amount (µl)	Concentration
Master Mix 2X	12.5	2X
DNA Template	3	30-60 ng/µl
Primers	2	10p moles
DNas free water	Up to 25µl	-
Total volume	25µl	-

5. Genotyping of genes polymorphisms by molecular methods

Genotyping of *rs18* **p**olymorphism: For *rs18* genotyping a set of primers, as mentioned in Table 2 were used. The PCR was performed by thermo cycler system under the conditions shown in Table 2

Table 2 PCR amplification conditions for *rs18* gene

Gene	PCR Cycling Profiles	Expected Product SizeBp	Reference
rs-18		395	Haghiri et al.,2016

PCR product was subjected to gel electrophoresis in 2 % agarose gel, 0.5 X TBE buffer and 5-7 V/cm for 2h and stained with ethidium bromide, photographed and analyzed using gel documentation system. A 100 base-pair ladder was used as a size marker for estimation of fragment sizes.

6. Restriction Fragment Length Polymorphism (RFLP)

The PCR product was cut by using *Taq*$^{\alpha}$ *I* restriction enzyme. The RFLP-PCR was accomplishing according to New BioLabs England company protocol with some modifications (Table 3.)

Table 3 The Restriction Enzyme Digestion reaction for *rs18* protocol

Materials	Volume
PCR product	10
Enzyme	0.25(5 U)
Cut smart buffer 1X	5
dH2O	To 30
Incubation at 2-4 hour	58C°

The digested amplified DNA fragments were electrophoresed on 2 % agarose for 2h at 5-7 V/cm. The bands were visualized after staining with ethidium bromide under UV light. A 100 base-pair ladder was used as a size marker for estimation of fragment sizes.

RESULTS AND DISCUSSION

Gene Polymorphism in Autistic patients

Concentration and purity of DNA

The extraction of DNA from samples of whole blood was successful and the concentration and purity of DNA were 20 to 195µg/ml and 1.7 to 1.9, respectively.

Analysis of RS 18 polymorphism by RFLP-PCR

When specific primers were utilized to amplify the RS 18, a single PCR amplicon, about 395 bp, was created as demonstrated in Figure. The genotypes of the studied subject were distributed into three groups based on the presence or absence of the polymorphism: A/A homozygous (demonstrated 115and 280 bp fragments), G/G homozygous (395 bp) and A/G heterozygous (395,280 and 115 bp). The distribution of fragments described in current study agreed with [10] who demonstrated methionine synthase (MTR) was responsible for the regeneration of methionine from homocysteine and to analyze the association of MTR gene polymorphism (rs1805087) and the risk of autism in a population in northern Iran.

Figure 1 Gel electrophoresis of DNA extracted from blood samples

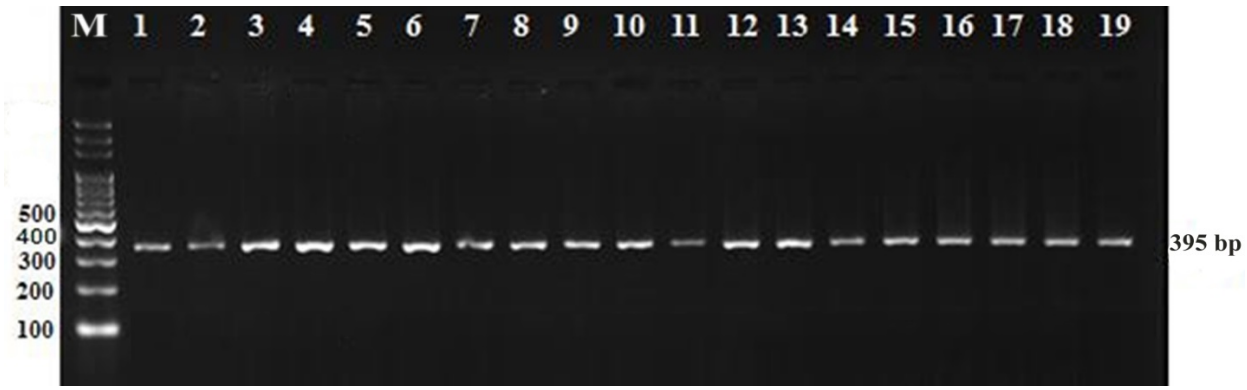


Figure 2: Electrophoresis pattern of PCR product for *RS 18* this amplification product was 395 bp

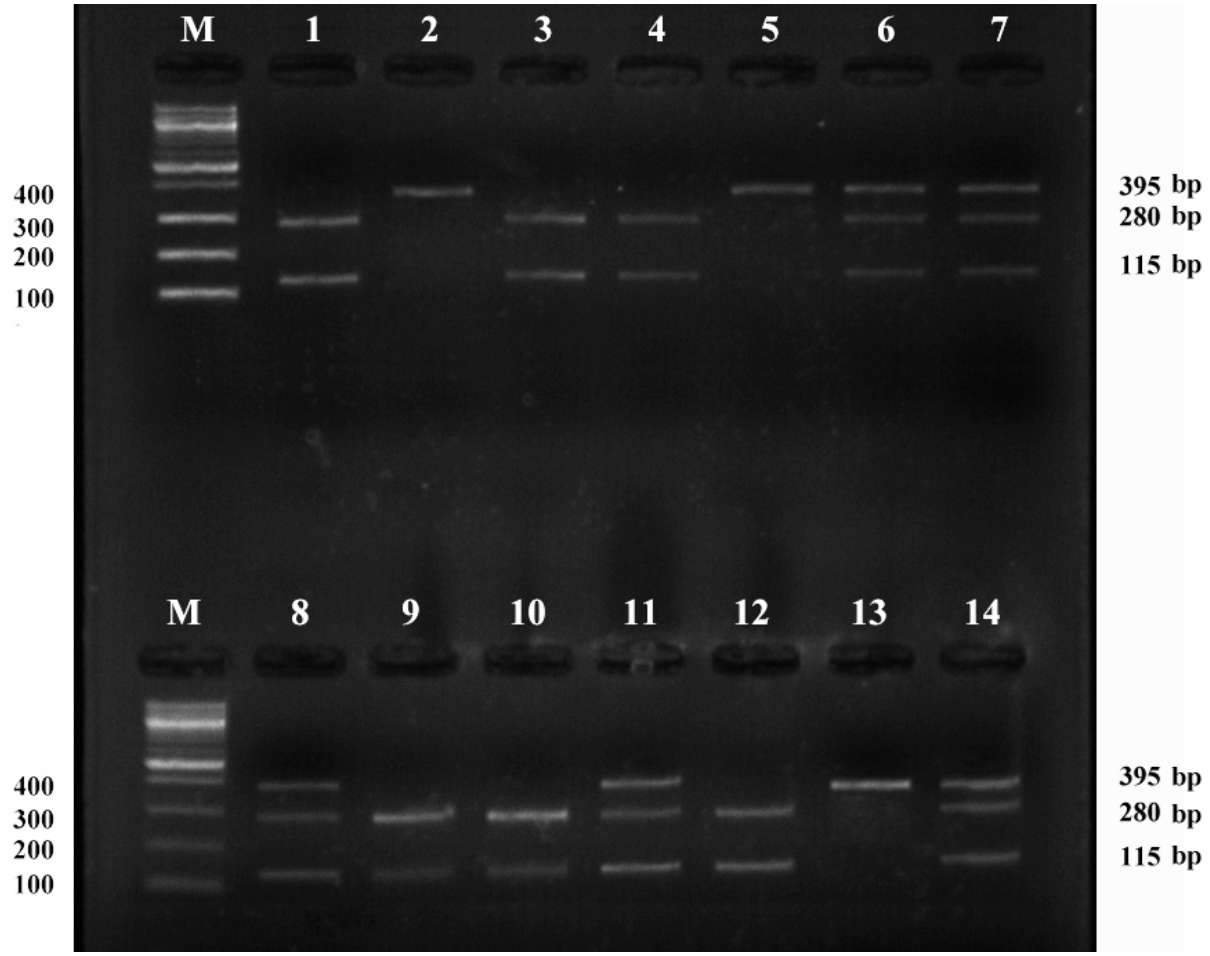


Figure 3 Electrophoresis pattern of PCR- RFLP for PCR product of Rs 18 (395bp) with restriction enzyme (Avall). Lanes 1-4 were controls and lanes 5-14 were cases;

Lane M: DNA ladder 100 bp.
Lane 1, 3, 4, 9, 10 and 12 bands (115 and 280 bp); G/G genotype (homozygotes).
Lane 2, 5 and 13 bands (395 bp); A/A genotype (homozygotes).
Lane 6, 7, 8, 11 and 14:bands (395, 280 and 115 bp); G/A Genotype (heterozygotes).

Table 4 Genotypic distribution of *Rs 18* gene polymorphism with allele frequency and their association in controls and cases

Genotype Rs 18	Patients	Control	P- value	OR (95 CI%)
AA [a]	8(11.42)	19(63.33)		
AG	18(25.71)	3(10)	<0.001	14.25(3.25-62.30)
GG	44(62.85)	8(26.66)	<0.001	13.06(4.27-39.95)
Total	70	30		
A allele	34	41	<0.001	6.72(3.45-13.11)
G allele	106	19		

$P \leq 0.05$; OR= (95%CI); [a] reference

Genotypic distribution of *Rs 18* polymorphism with allele frequency in control and cases

The methionine synthase (rs1805087) gene polymorphism was studied in autism cases and controls. The distribution observed in methionine synthase gene in (rs1805087) control group and cases groups are showed in Table 4. The highest genotype in control group was AA homozygote genotype (63.33%) followed by mutant homozygote GG (26.66 %) and heterozygote genotype AG(10%) . on the other hand, in autism disease, the highest genotype was GG mutant homozygote genotype (62.58%) the genotyping distribution pointed out that heterozygote AG and homozygote AA reached 25.71% and 11.42 %, respectively.

Our results were not in accordance with those reported by [5] who found that MTR A2756G polymorphism was associated with an increased risk of autism. Larger studies with more patients and controls are needed to confirm these results.

Methionine synthase gene is located on chromosome 1q43. Methionine synthase, a vitamin B12-dependent enzyme is 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 [5]. It was 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 lower enzyme activity followed by homocysteine elevation and DNA hypomethylation [7].

Ethical Clearance: Permissions for carrying out the study were obtained from the Scientific Research Committee College of Sciences/ University of Babylon, Iraq.

Financial Disclosure: There is no financial disclosure.

Conflict of Interest: None to declare.

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