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Association study of polymorphisms at A66G (rs1801394) of *MTRR* gene and autism spectrum disorders in a Kurdish population

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ABSTRACT

Autism spectrum disorder (ASD) is a group of neurodevelopmental disorders that are distinguished by the inability in social interaction, communication, and repetitive behavior pattern. One of the etiologic factors of the disease is believed to be methionine synthase reductase (*MTRR*) A66G polymorphism, which participates in homocysteine (Hcy)/folate metabolism. The aim of this study was to explore the link between polymorphism A66G (rs1801394) of *MTRR* and susceptibility of Kurdish autistic children to develop ASD in Iraq. In this study, 200 samples were included and divided into two equal groups: 100 autistic and 100 control children. After extraction of genomic DNA, the allele-specific polymerase chain reaction (AS-PCR) was performed. Our results showed that a significant association between heterozygote and homozygote variants (AG vs. AA: OR = 3.333, 95%CI: 1.723 to 6.449; $P = 0.0004$) and (GG vs. AA: OR = 2.500, 95%CI: 1.362 to 18.35; $P = 0.021$) respectively, when compared with wild homozygote. Also, this study demonstrated a statistical difference in the frequencies of the two alleles in *MTRR* A66G (G vs. A: OR = 1.857, 95%CI: 1.243 to 2.775; $P = 0.003$). Our study revealed that the polymorphism of *MTRR* A66G genotypes and alleles could influence the ASD susceptibility among Kurdish children in Erbil, Iraq.

1. Introduction

Autism spectrum disorder (ASD) is a neuropsychiatric disorder that manifests in children in the early 3 years of life that characterized by an abnormality in social behaviors, eye contact and repetitive behaviors (Mohammad et al., 2009). The autistic children are mentally retarded, there is a defect in brain development postnatally and poor growth during childhood (Hazlett et al., 2011). ASD occurs in male four times more than females (Werling & Geschwind, 2013).

The etiology of ASD, which is still not clear, is divided into non-genetics and genetics factors (Chaste & Leboyer, 2012). Non-genetic factors include environmental factors, which include heavy metals, some microbes, and chemical substances, affect the development of the brain and enhanced oxidative stress in the nervous system (Sung et al., 2005; London, 2000). Besides, environmental factors include some important vitamins such as folic acid, B_{12}, and B_6 that have essential roles in converting detrimental amino acids Hcy into beneficial amino acids such as methionine and cysteine. Hcy is non-standard amino acids

Abbreviation: MTRR, 5-Methyltetrahydrofolate-Homocysteine Methyltransferase Reductase / methionine synthase reductase; ASD, Autism spectrum disorder; AS-PCR, Allele-specific polymerase chain reaction.

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which means don't involve in protein synthesis instead it is regarded as new cholesterol causes cardiovascular abnormality in one hand and it also damages to the neuron in nervous system developing neuropsychiatric disorders such as ASD, Alzheimer's disease (Kieling et al., 2011; da Silva et al., 2006), depression and schizophrenia (James et al., 2004; Permoda-Osip et al., 2013).

Regarding the anabolism of Hcy, the demethylation pathway of methionine, which can be taken from the diet, forms S-adenosylmethionine (SAM). The latter is transmethylated to S-adenosylhomocysteine that finally hydrolyzed into adenosine and Hcy (Gao et al., 2018). The main function of SAM is to donor methyl in the formation of nucleic acids, phospholipids and some neurotransmitters (Almeida et al., 2014). Hcy is a harmful substance to neurons; it must be converted by two pathways. The first pathway involves remethylation reaction by the methionine synthase (*MTR*) that transfer methyl from 5-methyltetrahydrofolate (*5-MTHF*), which is generated by methyltetrahydrofolate reductase (*MTHFR*), to Hcy, this pathway depends on two vitamins: folic acid and B_{12} (Selhub, 1999). The latter is a cofactor for *MTRR* that has an essential role in maintaining *MTR* inactive state; While, the former is essential for the formation of 5-*MTHF* (Puig-Alcaraz et al., 2015). The second pathway implicates a transsulfuration reaction that requires cystathionine B synthase (CBS) as an enzyme and B_6 as a cofactor that results in the production of cysteine from Hcy (Tsiami & Obersby, 2017).

Hyperhomocysteinemia (HHcy) is a disorder which is characterized by the high level of Hcy in the blood; it is believed to be an etiologic factor for many diseases including ASD. HHcy develops either by environmental factors such as disturbance the level of vitamin B_6, B_{12}, and folic acid that have a significant role in the metabolism of Hcy (Brosnan et al., 2004) or by genetic factors such as defect or polymorphism of *MTR, MTRR* and *MTHFR* which are the essential enzymes for catabolism of Hcy (Li et al., 2015).

The second cause for the development of ASD is genetic factors that involve polymorphism of critical enzymes of Hcy metabolism. *MTRR* polymorphism is believed to be one of the genetic etiological factors for the development of HHcy and ASD. *MTRR* gene locates on chromosome 5 (5p15.2–15.3) which consists of 15 exons and 14 introns; it is 3.5 Kb in size (Leclerc et al., 1998). This gene produces an *MTRR* enzyme which contains 698 amino acids and it is 77 kDa in weight (Leclerc et al., 1998). The most common polymorphism of *MTRR* gene is A66G, Ile22Met or rs1801394 which results in substitution of A allele by G allele at position 66 of the *MTRR* gene thereby isoleucine substitutes by methionine in the position of *MTRR* enzyme (Wilson et al., 1999); this substitution leads to decrease the affinity of the enzyme for recycling *MTR* enzyme that consequently causes HHcy. There is a strong association between HHcy and ASD (Ali et al., 2011). Additionally, the *MTRR* gene polymorphism had been selected for this study because we could not find a single study from literature of Iraq while other genes like *MTHFR* (Muftin et al., 2020) and *MTR* (Jabbar & Jebor, 2018) genetic polymorphism have been studied extensively. This study aimed to investigate the relationship between gene polymorphism of *MTRR* A66G and ASD among Kurdish children in Erbil, Iraq.

2. Materials and methods

2.1. Subjects

Hundred children with autism (mean age = 7.005 ± 0.273) and 100 healthy children (mean age = 7.520 ± 0.258) were included in this study, there is no statistical difference in the age of them (Table 1). The ASD cases were diagnosed by pediatric neurologist and psychologist according to DSM-IV criteria (Bell, 1994). Five milliliters of blood was taken from children from the vein and transferred to lavender top tubes. We have stored tubes directly at −20 until DNA extraction would perform.

Table 1
Demographic characteristics of children with ASD and controls.

	Control ($N = 100$)	Autism (N = 100)	*P* value
Age	7.520 ± 0.258	7.005 ± 0.273	0.171
Male	49	40	–
Female	51	60	–
Height	1.254 ± 0.014	1.219 ± 0.015	0.105
Weight	24.21 ± 1.070	27.62 ± 1.143	0.444
BMI	15.04 ± 0.417	18.13 ± 0.537	< 0.0001

2.2. DNA extraction

The DNA has been isolated from the genome by applying spin column (Add prep Genomic DNA extraction Kit; Add bio, Daejeon, South Korea) depending on the recommendation of the manufacturer.

2.3. Allele specific-polymerase chain reaction (AS-PCRS)

The whole blood has been collected, and genomic DNA has been extracted as described above. The AS-PCR was performed to estimate *MTRR* A66G gene polymorphism was amplified with the following primers (Ajabi et al., 2017) and verified their specificity from the website (https://www.ncbi.nlm.nih.gov/tools/primer-blast/).

(5'-TCAAGCCCAAGTAGTTTCGAG– 3') (F1),
(5'–TGTACCACAGCTTGCTCACAT– 3') (RI),
(5'– CTTGTCTACAGGGTTGCACT–3') (F2) and
(5'– TGTACCACAGCTTGCTCACAC– 3') (R2),

The optimized PCRs have been performed in 20 μl reactions comprising two reactions for each sample, 1 μl of F1 and R1 for A allele (367 bp) while, 1 μl of F2 and R2 for G allele (401 s bp) then remaining were the same for both reactions which include 2 μl of genomic DNA, 10 μl of master mix (Ampliqon, Denmark) and 6 μl of deionized distilled water. Amplifications have been carried out on a thermal cycler (Applied biosystem, USA) using the following conditions: initial denaturation at 94 °C for 3 min followed by 35 cycles of the 30s at 94 °C, 30s at 60 °C, 30s at 72 °C and a final extension at 72 °C for 5 min. The PCR products were loaded into 2% agarose gel and run at 130 mV for about 30 min then the gel was stained with ethidium bromide and visualized under gel doc (Bio-Rad, USA), the PCR products have been analyzed (Homberg et al., 2016). If 367 and 401 bp found separated reactions of a sample, it was heterozygote (AG). If 367 bp was seen in both reactions of a sample, it was wild homozygote (AA), If 401 bp was seen in both reactions of a sample, and it was mutant homozygote (GG).

2.4. Statistical analysis

Statistical analysis was performed with GraphPad Prism 6 software (GraphPAd Software, Inc., La Jolla, CA, USA). To compare the results of two variables that participated in the same experiment, it was analyzed using an unpaired student *t*-test. For the statistical analysis of the polymorphism the Chi-square test was used to evaluate the differential hypothesis between the two categorical variables ASD and controls, a contingency table was used where each variable was divided into different categories (A/A, A/G, and G/G alleles). Subsequently, the expected frequencies were calculated.

3. Results

In this case-control study, 100 ASDs patients and 100 controls compared according to SNP on the *MTRR* A66G gene by the AS-PCR method. The genotypes and allele frequencies of SNPs on *MTRR* A66G gene are given in (Table 3). Regarding Hardy-Weinberg equilibrium (HWE), genotype distribution in the case was not in line, but in controls

agreed with it with a *p*-value of 0.0006 and 1.000 respectively (Table 2).

The polymorphism of *MTRR* A66G genotypes and alleles were associated with an increased risk of ASD as compared with the control. Regarding model analysis, we found significant association in dominant model comparison (AA/AG vs. GG: OR = 1.333, 95% CI: 0.6842 to 2.598; $P = 0.498$), and a significant association was also found in the recessive model (AA vs. AG/GG: OR = 3.000, 95% CI: 1.648 to 5.460; $P = 0.0004$). Regarding genotypes, there was a significant association in heterozygote model and homozygote variants (AG vs. AA: OR = 3.333, 95%CI: 1.723 to 6.449; P = 0.0004) and (GG vs. AA: OR = 2.500, 95%CI: 1.362 to 18.35; $P = 0.021$) respectively, if compared to wild homozygote. Moreover, the carrier frequency of the A allele was higher in the patient group in comparison to the G allele. Finally, there was a statistical difference in the frequencies of the two alleles in *MTRR* A66G (G vs. A: OR = 1.857, 95%CI: 1.243 to 2.775; $P = 0.003$) (Table 3). The BMI in ASD group (18.13 ± 0.537) was higher than control group (15.04 ± 0.417).

Table 3
The genotypes and allele distribution of codon MTRR polymorphism in case and control.

Polymorphism	ASD (N = 100)		Control (N = 100)		OR	95% CI	P value
	No	%	No	%			
AA	50	72.22	25	25	1	–	–
AG	30	16.67	50	50	3.333	1.723 to 6.449	0.0004
GG	20	11.11	25	25	2.500	1.362 to 18.35	0.021
AG + GG	50	27.78	75	75	3.000	1.648 to 5.460	0.0004
AA+AG	80	88.89	75	75	1.333	0.6842 to 2.598	0.498
A	130	65	100	50	1.857	1.243 to 2.775	0.003
G	70	35	100	50			

4. Discussion

Prior work has documented the role of polymorphism in A66G of *MTRR* gene in developing ASD. Ajabi et al. (2017) described that the polymorphism in A66G of *MTRR* is related with developing ASD in children who live in the north of Iran; conversely, Mohammad et al. (2009) authenticated that this polymorphism protects children from ASD in Indian children. Finally, Zhang et al. (2018) showed that the ASD among Hans Chinese population did not exhibit significant A66G polymorphism of *MTRR* which means there is no relation between this polymorphism and the disease as done by Smail et al. (Smail et al., 2020).

The polymorphism of *MTRR* at A66G was strongly associated with increased susceptibility to ASD. This result is line with Ajabi et al. (2017) in Iran, but it is inconsistent with Mohammad et al. (2009) in India and Zhang et al. (2018) in China. These results provided evidence that genetics factors such as *MTRR* polymorphism may directly link to the environmental factors such as metabolism of folic acid and B_{12} in autistic children in the north of Iraq that may exuberates the disorder.

One of the neurodevelopmental defects, that affects and appears in children in early 3 years of life and it is characterized by abnormal repetitive behaviors, social isolation and non-verbal contacts such as fears of eye contacts, is ASD. The etiology may be linked to genetics and environmental factors (Homberg et al., 2016; Vijayakumar & Judy, 2016; Modabbernia et al., 2017). The interactions between genetic predisposition and environmental factors have been proposed as the major mechanisms in the pathogenesis of ASD (Modabbernia et al., 2017; Hallmayer et al., 2011; Ozonoff et al., 2011; Bourgeron, 2015; Gaugler et al., 2014).

Hyperhomocysteinemia (HHcy) is an abnormally high level of Hcy in the blood which is non-protein sulfur-containing amino acids (Son & Lewis, 2020). This disorder may be caused by environmental nutritional factors such deficiency in the level of some vitamins including folic acid, B_{12}, and B_6 or may be due to the mutation and polymorphism of some enzymes that are critical in the metabolism of mentioned vitamins and conversion Hcy to protein and beneficial amino acids including methionine and cysteine (Son & Lewis, 2020).

Enzymes such as (*MTRR, MTR, MTHFR,* and *CBS*) have important role metabolism of folic acid, B_{12}, and B_6; *MTRR* enzyme has a role in maintaining B_{12} in the active state which in turn donated methyl group to *MTR* that in turn convert Hcy to methionine amino acids (Weiner et al., 2012). *MTRR* enzyme is produced by the *MTRR* gene; the most common polymorphism of it is A66G in which methionine residue comes from Isoleucine after changing A allele to G allele at position 66, which diminishes the activity of the enzyme (Olteanu et al., 2002). *MTRR* (A66G) genes have also been suggested to increase the concentrations of Hcy (Barbosa et al., 2008).

Table 2
Hardy Weinberg equilibrium (HWE) tests (*p*-Values) for ASD and control groups.

SNP	ASD	Control
rs1801394	0.0006	1.000

One of the etiologies of ASD is HHcy which may be caused by polymorphism of *MTRR* A66G, however this polymorphism and their association with ASD is still not clear, several papers argue their role in pathogenesis which are not consistent with each other; some researchers documented that G carriers alleles have more subjected to HHcy (Gaughan et al., 2001; Kluijtmans et al., 2003). Besides, moderate increased Hcy may be due to AA genotypes, as Gueant-Rodriguez, Juilliere (Gueant-Rodriguez et al., 2005) reported. In contrast, some researchers revealed that this polymorphism has not critical role in the concentration of Hcy in the blood (Brilakis et al., 2003; Jacques et al., 2003; Bosco et al., 2006).

Kaluzna-Czaplinska et al. (2013) Found that HHcy and hyper homocystinuria are more common in autistic children and they are related to a developmental disorder of ASD; they can be regarded as a good diagnostic factor for the disease and they reflect the malnutrition in children which are suffering from the disease. Moreover, the folate/methionine cycle, which determines the level of Hcy in the blood, plays an essential role in ASD and its symptoms (Kałużna-Czaplińska et al., 2013). The HHcy and its role in neuropsychiatric diseases including ASD are supported by many studies (Obeid et al., 2007; Moustafa et al., 2014). Hyper homocystinuria, that is followed by HHcy, reflects the abnormal elevation of Hcy in the blood; in parallel to homocystinuria, penetration of the Hcy to the central nervous system occurs since this amino acid crosses the blood-brain barrier (Obeid et al., 2007). Inside the brain, Hcy acts as glutamate agonist (Puig-Alcaraz et al., 2015), which increases the activity of glutamate in the brain that develops many neuropsychiatric disorders including ASD (Rojas, 2014). Unfortunately, we did not measure the level of Hcy in the study because most of ASD children were under the active folate treatment. As we know taking active folate decreases the level of Hcy in children (Sun et al., 2016) so the result would not be accurate.

Regarding HWE, ASD group deviated from it. The deviation from HWE may be due to that our Kurdish population is not stable; many people migrated to Europe due to economic and political crisis. This deviation may interfere with bias error and interfere with genetic association study (GAS); this problem was tackled by estimating adjusted variances for allele, additive, recessive and dominant models (Schaid & Jacobsen, 1999). This estimate was based on fixation of indices in the case and control (Sato et al., 2006).

The BMI in ASD children would be differ from healthy children because of dietary life styles, genetics and metabolic disorders. The ASD children might eat more food and numerous barriers were found to prevent their physical activity (Obrusnikova & Cavalier, 2011).

Some facts should be taken into considerations, firstly this research has been investigated only in Kurdistan so we cannot generalize it to all countries and other societies. Secondly, due to the low number of cases, it limited our potential to distinguish between the groups precisely. Third, the levels of vitamin B_{12}, folate, and their related metabolites were not examined in these children. Metabolic disorders of the vitamins might interact with certain polymorphisms in these genes to increase the risk of ASD. The role of such interactions was not examined in this study. Lastly, due to depending on different laboratories, gathering information in different processes also may not provide precise results.

5. Conclusion

To best of our knowledge, there is no published paper regarding *MTRR* at A66G polymorphism in the north of Iraq. Our result documented that a highly significant association was found between *MTRR* at A66G polymorphism and ASD in Erbil city in the north of Iraq.

Ethical approval

We have followed all ethical approvals for this study.

Informed consent

All authors have read and approved the contents and manuscript.

Ethical approval and consent to participate

The present study was authorized and approved by the Human Ethics Committee of Salahaddin University-Erbil. Patients provided informed consent.

Patient consent for publication

All patients provided written informed consent for the publication of data in this study.

CRediT authorship contribution statement

Monika Henryka Miasko: Conceptualization, Writing - review & editing. **Shukur Wasman Smail:** Conceptualization, Methodology, Supervision, Validation, Visualization, Writing - review & editing. **Abdulkarim Yasin Karim:** Conceptualization, Writing - review & editing. **Mahdi Khaled Qadir:** Methodology. **Ahmed Abdulrazzaq Bapir:** Methodology. **Shwan Ali Omar:** Methodology.**Iman Idris Ismail:** Methodology, Writing - original draft. **Omer Sardar Taha:** Methodology. **Zhikal Omar Khudhur:** Methodology. **Kovan Faidhalla Jalal** Methodology. **Mohammed Qader Mustafa:** Methodology. **Harem Khdir Awla:** Methodology. **Muhammad Saeed:** Writing - review & editing. **Muhammad Safdar:** Conceptualization, Supervision, Validation, Visualization, Writing - review & editing.

Declaration of competing interest

The authors have declared that no competing interests exist.

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