

Study of the C677T and 1298AC polymorphic genotypes of MTHFR Gene in autism spectrum disorderFarida El-baz¹, Mohammed Abd El-Aal², Tarek Moustafa Kamal³, Abdelrahim Abdrabou Sadek⁴, Amr Ahmed Othman⁴¹ Department of Pediatrics, Faculty of Medicine, Ain Shams University, Egypt² Department of Pediatrics, Faculty of Medicine, Sohag University, Sohag, Egypt³ Medical Genetics Research Centre and Genetics Unit, Department of Pediatrics, Faculty of Medicine, Ain Shams University, Egypt⁴ Neurology and Psychiatry Unit, Department of Pediatrics, Faculty of Medicine, Sohag University, Sohag, Egypt**Type of article:** Original**Abstract****Background:** Autism is currently known as "a behaviorally defined syndrome" manifested as impairment in social communication, repetitive routines and restricted interests. There is an increased risk of ASDs associated with common mutations affecting the folate/methylation cycle.**Aim:** The aim of this study was to identify C677T and 1298AC polymorphic genotypes of MTHFR gene among a sample of Egyptian children with autism and to make a phenotype-genotype correlation for the autistic patients.**Methods:** This case-control study was carried out from 2013 through 2015. The study included 31 children with autism and 39 children in a normal control group, the mean age of patients and control was comparable (4.5 years± 2) with males predominant in both groups. We used DSM-V-TR criteria, Stanford-Binet intelligence scale V and childhood autism rating scale (CARS) for assessments. Genotyping for MTHFR gene polymorphic loci C677T and 1298AC was performed on amplified DNA by PCR with subsequent reverse hybridization and restriction fragment length polymorphisms analysis. Data were analyzed by SPSS version 11, using Chi-Square, independent-samples t-test, and ANOVA.**Results:** There was significant relationship between low birth weight and occurrence of autism ($p < 0.01$), and between delayed motor and social milestones in cases of autism compared to controls ($p < 0.01$). Heterozygosity for A1298C polymorphism was highest among patients (41.9%) followed by 35.5% mutant genotype CC and 22.6% normal AA (wild) type and Allele C was detected in patients more than in control (56.45% vs. 11.54%) ($p < 0.001$). For C667T polymorphism, heterozygosity was also highest among patients (48.4%) followed by wild type genotypes C677 (38.7%) and 12.9% for mutant genotypes 667T. Allele T appeared more in patients than control (31.10 %vs. 5.13%) ($p < 0.00$). Heterozygosity for CT and A-C genotypes were detected equally (46.2%) among patients with severe autism (according to CARS).**Conclusion:** There is a significant association between severity and occurrence of autism with MTHFR gene polymorphisms C677T and A1298C. Further studies are needed on a larger scale to explore other genes polymorphisms that may be associated with autism, to correlate the genetic basis of autism.**Keywords:** Autism; MTHFR; Genotype; Phenotype**1. Introduction**

Autism is a complex neuro developmental disorder with a combined genetic and environmental factor (1). Depending on the diagnostic criteria used in each study, the incidence of PDD range is between 4.5 and 59/10.000, (2) with occurrence in males three to four-fold higher than in females (3). Most current studies have found it to be increasing to 110/10.000. The increasing incidence could be actual, or it could be due to the use of broadened criteria in diagnosis (4). Autism is currently defined as "a behaviorally defined syndrome manifested as impairment

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in social communication, repetitive routines and restricted interests. The term "pervasive developmental disorders"(PDDs) refers to five conditions; 1) autistic disorder, 2) Asperger's disorder, 3) Rett's disorder, 4) childhood disintegrative disorder, and 5) PDD not otherwise specified (5, 6). Autism spectrum disorders are characterized by hyperactivity, stereotypic motor behaviors, sensory disturbances, language impairment, restricted interests and self-injury (7). In some patients, it can also be associated with seizure disorder (8), gastrointestinal disturbances (9) and autoimmune disorders (10). A dysfunctional folate methionine pathway has been identified in many individuals with autism, this pathway is crucial for DNA synthesis and methylation. With regard to methylenetetrahydrofolate reductase (MTHFR) gene codes for an essential enzyme in folate metabolism, the gene is located on chromosome 1 P 36.3 in humans (11). There are DNA sequence variants (genetic polymorphism) associated with the MTHFR gene, two of the most investigated are C677T and A1298C as a single nucleotide polymorphisms (SNP) (11). The present study aimed at identification of possibly existing C677T and A1298C polymorphisms within the MTHFR gene among an Egyptian sample of patients with autism, and study of possible phenotype - genotype correlation.

2. Material and Methods

2.1. Patients

Seventy children were included in this case-control study and divided into two groups of 31 patients (case group) and 39 controls. The age of patients ranged from 1.5 to 18 (4.57 ± 2) years and they were diagnosed as autistic by DSM-V-TR criteria (12) at the Psychiatric Clinic, Pediatrics Hospital, Ain Shams University and Psychiatry Clinic, Pediatrics Department, Sohag University Hospital in the period from May 2013 through May 2015. Exclusion criteria included patients with suspected genetic, metabolic or chronic disease. The control group included 39 children not suffering from any psychiatric or behavioral problems with matched age and sex. All patients were subjected to the following: Course and duration of disease, detailed history with particular emphasis on onset, consanguinity of parents and antenatal, natal and postnatal history. Developmental history including mental, language, social and motor development. Family history of any similar condition and other psychological or mental disorders in the family. Thorough clinical examination with special emphasis on neurological examination. Genotyping consent from all patients' and controls' parents/guardians were taken to approve sharing in the study after full description of the steps and aim of the study. The work has been carried out in accordance with the code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans, and the Ethical Committee of Ain Shams University" for the same purpose.

2.2. Methods

Confirmation of diagnosis using DSM- V-TR criteria of autism (12). Intelligence quotient (IQ) was evaluated by use of Stanford-Binet Intelligence Scales V (13). The SBV can be administered to individuals as early as two years of age. There are ten subtests included in this revision including both verbal and nonverbal domains. Five factors which are directly related to Cattell-Horn-Carroll (CHC) hierarchical model of cognitive abilities, are also incorporated in this scale. These factors include knowledge, quantitative reasoning, fluid reasoning, working memory and visual-spatial processing. An assessment for the severity of autistic symptoms was carried out using the childhood autism rating scale (CARS) (14), which rates each patient on a scale from one to four in fifteen areas (listening response, visual response, relation to people, body use, emotional response, imitation, object use, fear or nervousness, verbal communication, nonverbal communication, activity level and consistency of intellectual response, adaptation to change, touch, taste, smell, response and general impression). The test divided patients according to severity as not autistic, mild or moderate autistic, and severe autistic. Genotyping of methylenetetrahydrofolate reductase (MTHFR) gene polymorphism C677T was performed on DNA samples based on polymerase chain reaction (PCR) and reverse hybridization and subsequent conjugation using the streptavidin-alkaline phosphatase (MTHFR StripAssay™), Vienna lab diagnostics® GmbH, Vienna, Austria). Allele specific amplification was done for 1298AC polymorphism using PCR-RFLP analysis as described elsewhere (15, 16). The restriction fragment length polymorphism (RFLP) was done by using a restriction enzyme MboII (Sibzyme™, Russia). The PCR products were all documented by Gel Documentation System and Software for DNA analysis (InGenius Syngene™, UK). Amplified DNA from a patient homozygous for the 1298A allele appeared at a band length of 176 bp in length compared to the size marker, with three smaller fragments of 30, 28, and 22 bp. Presence of the A1298C polymorphism abolishes an MboI cut site; thus, DNA from a patient homozygous for the 1298C allele appeared at a band length of 204 bp with smaller fragments of 30 and 22 bp.

2.3. Statistical analysis

The distribution of genotypes and allele frequencies were all statistically compared in all patients versus healthy controls. The results were analyzed using SPSS version 11.0 (SPSS Inc., Chicago, IL, USA). Qualitative data were presented in the form of frequencies and percentages. Quantitative data were presented as mean \pm SD for normally distributed data and as medians and percentiles for skewed data. Data were analyzed using Pearson's Chi-square test, independent-samples t-test, and one-way analysis of variance (ANOVA). A p-value less than 0.05 was considered statistically significant.

3. Results

Incidence of consanguinity among patients and control group was comparable (80.6% vs. 84.6%) which was statistically not significant ($p=0.661$). Compared to the control group, family history of autism (9.7%), epilepsy (16.1%), prematurity (9.7%) was detected among the patient group ($p<0.05$). Mean birth weight among the patient group was less compared to the control group (2.67 ± 0.389 vs. 3.03 ± 0.309 kg) ($p<0.01$). However, the mean of gestational age was statistically not significant ($p=0.676$) (Table 1). Type of feeding and the mean age of weaning among the patient group compared to the mean age of weaning in the control group was statistically not significant ($p=0.162$ and 0.786 respectively). The developmental milestones in the patient group were delayed in comparison to the control group ($p\leq 0.0001$). The mean level of IQ score among patients (66.13 ± 8.20) was lower than the mean level of IQ among controls (96 ± 8.8) ($p\leq 0.0001$) (Table 1). AC heterozygote genotype was highest among patients (41.9%) followed by CC mutant genotype (35.5%) then AA normal genotype (22.6%) compared to the control group, where the most common polymorphism was AA normal genotype (79.5%) ($p<0.001$) (Table 2). The frequency of C allele was higher in patients (56.45%) than controls (11.54%), while A allele frequency in controls was more (88.46%) than its frequency among patients (43.55%) ($p<0.001$) (Table 2).

Table 1. Characteristics of patients and controls included in the study

Variable		Patients; n (%) / Mean \pm SD	Control; n (%)	Chi-square*/t-test**	p-value
Consanguinity	Yes	6 (19.4)	6 (15.4)	0.192*	0.661 (NS)
	No	25 (80.6)	33 (84.6)		
Family history of autism	Yes	3(9.7)	0 (0)	3.943*	0.047 (S)
	No	28 (90.3)	39 (100)		
Family history of epilepsy	Yes	5(16.1)	0 (0)	6.774*	0.009 (S)
	No	26 (83.9)	39 (100)		
History of prematurity	Yes	3 (9.7)	0 (0)	3.943*	0.047 (S)
	No	28 (90.3)	39 (100)		
Feeding	Breast	20 (64.5)	31 (79.5)	1.958*	0.162 (NS)
	Artificial	11 (35.5)	8 (20.5)		
Mean gestational age (weeks)		39 \pm 1.25	39.1 \pm 0.5	0.408**	0.676 (NS)
Mean birth weight (kg)		2.67 \pm 0.389	3.03 \pm 0.309	4.205**	0.0001 (S)
Mean age for sitting (month)		8.5 \pm 1.1	6.65 \pm 0.87	7.653**	<0.0001 (S)
Mean age at weaning (year)		1.59 \pm 0.3	1.61 \pm 0.31	0.273**	0.786 (NS)
Mean age at 1st spoken word (year)		2.6 \pm 0.69	1.13 \pm 0.18	11.553**	<0.0001 (S)
Mean level of IQ score		66.13 \pm 8.20	96 \pm 8.8	14.654**	<0.0001 (S)

S: Significant; NS: Not Significant

Table 2. A1298C genotype polymorphism and segregation of alleles among patients and control

Group	Patients; n (%)	Control; n (%)	Chi square	p-value
AA genotype	7 (22.6)	31 (79.5)	24.700	<0.001
AC genotype	13 (41.9)	7 (18)		
CC genotype	11 (35.5)	1 (2.5)		
Total	31 (100)	39 (100)		
A-allele	27 (43.55)	69 (88.46)	32.332	<0.001
C-allele	35 (56.45)	9 (11.54)		
Total	62 (100)	78 (100)		

The most common C667 genotype among the patient group was CT heterozygote while in the control group, the most common genotype was the normal wild C667 genotype 35(89.8%) ($p < 0.001$) (Table 3). The frequency T allele in patients was (37.09%), that was more than its frequency in controls (5.13%) and the frequency of C allele in the control group (94.87%) was more than its frequency among patients (62.90%), ($p < 0.001$) (Table 3). CARS assessment mean value (38.091 ± 4.721) in patients with CC mutant genotype of the polymorphic locus A1298C was more than CARS mean value (37.538 ± 5.710) in patients with AC heterozygote genotype and CARS mean value (35.857 ± 5.429) in patients with normal AA genotype ($p = 0.680$). IQ mean value (69.571 ± 7.35) in patients with normal AA genotype was higher than IQ mean value (66.538 ± 7.47) in patients with A-C heterozygote and IQ mean value (63.455 ± 9.29) in patients with CC mutant genotype, this was statistically not significant ($p = 0.306$). Mean of DSM criteria (7.385 ± 1.325) in patients with AC heterozygote was more than mean of DSM criteria (7.091 ± 0.944) in patients with mutant CC genotype and mean of DSM criteria (6.714 ± 0.488) in patients with normal AA genotype ($p = 0.409$) (Table 4). CARS assessment mean value (38.750 ± 4.654) in patients with normal C667 was more than CARS mean value (37.500 ± 5.686) in patients with mutant TT genotype and CARS mean value (36.200 ± 5.570) in patients with C-T heterozygote genotype that was statistically not significant ($p = 0.464$). IQ mean value (65.750 ± 13.376) in patients with mutant TT genotype was lower than IQ mean of patients with normal and C-T heterozygote genotype, again this was statistically not significant ($p = 0.987$). Mean value for DSM criteria in patients (7.333 ± 1.073) with normal C667 genotype was more than mean value for DSM criteria among patients with mutant TT and heterozygote C-T genotype that was statistically not significant ($p = 0.708$) (Table 5).

Table 3. Percentage of C667 genotypes and alleles among patients and control

Group	Case	Control	Chi square	p-value
C667 (normal)	12 (38.6)	35 (89.8)	20.984	<0.001
C667T (hetero)	15 (48.4)	4 (10.2)		
667T (mutant)	4 (13)	0 (0)		
Total	31 (100)	39 (100)		
C allele	39 (62.90)	74 (94.87)	22.679	<0.001
T Allele	23 (37.09)	4 (5.13)		
Total	62 (100)	78 (100)		

Table 4. Correlation between psychological assessment and A1298C genotype in patients

AC	CARS	IQ	DSM
AA (normal)	35.857 ± 5.429	69.571 ± 7.35	6.714 ± 0.488
AC (hetero)	37.538 ± 5.710	66.538 ± 7.47	7.385 ± 1.325
CC (mutant)	38.091 ± 4.721	63.455 ± 9.29	7.091 ± 0.944
ANOVA	0.391	1.236	0.680
p-value	0.680	0.306	0.409

Table 5. Correlation between psychological assessment and C667 genotype

C667	CARS	IQ	DSM
C667 (normal)	38.750 ± 4.654	66.417 ± 7.810	7.333 ± 1.073
C667T (hetero)	36.200 ± 5.570	66.000 ± 7.606	7.000 ± 1.134
667T (mutant)	37.500 ± 5.686	65.750 ± 13.376	7.000 ± 0.817
ANOVA	0.790	0.013	0.350
p-value	0.464	0.987	0.708

4. Discussion

There is an agreement that autism is a neuro developmental disorder characterized by impaired social interaction (17), and is usually diagnosed in children before the age of three years, characterized by deficiency in language skills and social interaction which are associated with repetitive behavior and limited interests (18). In our study, the mean age of patients and control was comparable ($4.5 \text{ years} \pm 2$) with male patients more than females in both groups. This is in agreement with other studies (16, 19). In our study, non consanguinity constituted 80.6% of the studied families compared to 84.6% in the control group. Similar studies reported that the rate of consanguinity is lower among parents of patients with autism than in normal controls (20-24). This may suggest that consanguinity has no role in autism. In the current study, family history of autism was significantly higher in patients compared to control. This is in agreement with El Baz et al., (23), who reported that family history of autism was reported in 16%

of autistic patients versus 1% among the control. We found also that family history of epilepsy among the patient group was significantly higher than the control group. In this study, incidence of low birth weight and prematurity was found to be significantly more prevalent among autistic children than controls. The study by Kolevzon et al. (25) suggested that there was presence of non-heritable prenatal and perinatal risk factors for autism. It is also suggested that there is an association between autism and obstetric complications, prenatal or intra partum use of medications (26). A significant prevalence of low birth weight incidence and use of instrumental tools during delivery were reported among cases with autism compared to controls (23). Perinatal risk factors such as breech presentation, low Apgar score, low birth weight (2500 g), being small for gestational age, and gestational age at birth of less than 35-37 weeks were associated with a statistically significant increase in risk of autism (27). A review in 2007, identified obstetric conditions that included low birth weight, duration of gestation and hypoxia during childbirth as risk factors associated with autism. Studies directed towards single perinatal risk factors have demonstrated a positive association for low birth weight (<2500 g), and gestational age at birth of less than 37 weeks (28). In our study, all developmental and social milestones were significantly delayed among autistic children compared to the control group. This is in agreement with Juneja et al. (29), who reported about 96% of autistic children had motor developmental delay and qualitative impairment in social interaction and communication was also more commonly observed than restricted interests and activities. Children with autism may experience a delay in attaining regular motor skills, such as riding a bicycle. They may have an abnormal gait or posture, poor coordination skills and poor hand writing (30). Some of the noted behavior in autism include delayed speech, pronunciation and other language skills, and a reluctance to point or wave goodbye (31).

In our study, the patients' mean IQ revealed a wide range from mild to severe mental retardation which was lower than the mean IQ among the control group. This is in agreement with previous reports (20). C667T polymorphism genotyping was consistent with the study of Elif et al. (19) that revealed the heterozygote 667CT as the most prevalent genotype among patients. Our study findings were in agreement also with Shawky et al., (20) who reported that homozygote mutant 677TT genotype was present in 15% of the autistic children while the heterozygous 677CT genotype occurred in 50% of the autistic children group. Boris et al., (16) reported that 677CT polymorphisms, whether homozygous or heterozygous, are significantly associated with ASD. In their study, heterozygote CT genotype was more prevalent than mutant TT genotype, which is similar to our study but with different frequencies. As regards the A1298C polymorphism, our study revealed that the most common polymorphism in the patient group was AC heterozygote genotype followed by CC mutant then AA normal genotypes, in comparison to the control group, the most common polymorphism was AA normal genotype ($p < 0.001$). These findings were consistent with Boris et al. (16), who reported that AC heterozygote genotype is the most prevalent among patients, but in their study, the mutant CC genotype in patients (6%) was lesser than ours. The same authors reported that homozygous (TT) individuals have an approximately 50% decrease in MTHFR enzyme activity, the heterozygous (CT) have a 30% decrease in enzyme activity as measured in their lymphocytes and the compound heterozygous state, 677CT/ 1298AC lower enzyme activity by 50 -60%. They also reported that 1298AA normal alleles were more prevalent in the control population than in children with ASD. This is similar to the control group in our study. Our results also agree with Liu et al. (32), regarding the frequency of T allele among patients compared to the control group. It was reported that frequency of the heterozygous 677CT genotype in the ASD group (47.8%) did not differ from that in the controls (43.2%) (32). This differs from our study where heterozygote 677CT genotype in ASD group was more prevalent than in the control group. CARS mean value among the patient group with normal (wild type) AA genotype was lower than that of patients with AC heterozygote and CC mutant genotypes with no significant difference ($p = 0.680$). Again, mean value for IQ in patients with normal (wild type) AA genotype was higher than IQ mean of patients with AC heterozygote and CC mutant genotype with no significant difference ($p = 0.306$). This may indicate that there is no significant correlation between CARS (degree of severity of autism), IQ and A1298 polymorphisms. However, multicenter studies and larger sample sizes are needed to confirm such suggestion. The C-T heterozygote genotype is more prevalent than other MTHFR C667 genotypes among patients with mild to moderate autism and patients with severe autism with no significant difference. CARS mean value in patients with normal C667 was more than CARS mean value in patients with mutant TT genotype and C-T heterozygote genotype, with no significant difference. IQ mean in patients with mutant TT genotype was lower than IQ mean of patients with normal and C-T heterozygote genotype with no significant difference. These findings were in agreement with Shawky et al. (20) who reported similar results with no significant difference for correlation between CARS and different genotypes for the same polymorphic alleles.

5. Conclusions

There is a significant association between severity and occurrence of autism with MTHFR gene polymorphisms C677T and A1298C. Further studies are needed on a larger scale to explore other genes polymorphisms that may be associated with autism to correlate the genetic basis of autism.

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Conflict of Interest:

There is no conflict of interest to be declared.

Authors' contributions:

All authors contributed to this project and article equally. All authors read and approved the final manuscript.

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