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# Association of the MTHFR C677T polymorphism and fragile X syndrome in an Iranian population

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### *Abstract*

*Background & Objective:* Fragile X syndrome is one of the most common causes of inherited mental retardation in males after Down syndrome. To date less attention was to study secondary genetic factor that may play role in fragile X neuropathology. In central nervous system, folic acid derivatives participate in different process such as neural development and function, synthesis of neurotransmitters and DNA methylation. This study aimed to assess the possible association of three common polymorphisms of folate pathway key enzymes, methylentetra hydrofolate reductase, MTHFR (C677T and A1298C), and methionine synthase reductase, MTRR (A66G), polymorphisms with the development of fragile X syndrome. *Methods:* Genetic polymorphisms were examined in a case-control study of 38 unrelated male patients and 60 age/sex matched unrelated controls using PCR-RFLP method. Allele frequencies and genotypes were calculated by chi-square test. *Results***:** significantly increased frequencies of the 677T allele and the 677CT genotype in patients were observed compared to control ($p$=0.010; OR=2.459 for T allele frequency; $p$=0.028; OR=2.608 for CT genotype frequency). The statistical analysis demonstrated the significant correlation between C677T MTHFR polymorphism and fragile X syndrome in Iranian population (p=0.018). However, no significant case-control differences were found for A1298C MTHFR and A66G MTRR polymorphisms.
*Conclusions:* The association between C677T MTHFR polymorphism and fragile X syndrome has been demonstrated for the first time in Iran population. This study may be important in better understanding of molecular pathology of fragile X syndrome. Further studies need to be undertaken to evaluate these preliminary results in other populations.

## INTRODUCTION

Fragile X syndrome (FXS) is the most common cause of inherited mental retardation, observed in approximately one in 4,000 males and 8,000 females.[1] The diagnosis of FXS was originally based on the expression of a folate-sensitive fragile site at Xq27.3 (FRAXA) induced in cell culture under conditions of folate deprivation. Folate sensitive sites have been located on the X chromosome where FMR1 is located.[2] The mutation responsible for FXS involves expansion of FMR1 CGG repeat segment. The full mutation (>200 CGG-repeats) has been found to be associated with methylation of the promoter region and consequently inactivation of the gene and pathogenesis of FXS.[3]

Although it is clear that methylation status plays a role in phenotype, its effect on clinical severity is somewhat unpredictable, especially in females. Two main approaches are used to diagnose the repeat size and methylation statue; polymerase chain reaction (PCR) and Southern blot analysis. However, cytogenetic analysis of metaphase spreads has demonstrated high degree of variability among individuals. Due to the novel nature of the fragile X mutation, inheritance is less straightforward than in classic Mendelian traits. The penetrance of FXS is about 80% in males and 30% in females and there are some males with a fragile X chromosome appeared to be unaffected. Penetrance varies between sibships, even within the same family. There is great variability in the extent of these physical features, with some retarded males showing few of the physical components such as behavioral disturbances-hyperactivity and autism. There is still no medication for FXS which acts directly on the genetic mechanisms or on the immediate result of the genetic defect. Useful pharmacological treatment consists of central nervous system stimulants, clonidine, folic acid, serotonin reuptake inhibitors, and atypical antipsychotics.[4]

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The variable neuropathology[5] may suggest the mutations that have a secondary etiological or pathogenic roles influence fragile X neuropathy. To date no report in our knowledge addresses the secondary genetic factor to explain FXS variable characteristics. This study for the first time has evaluated autosomal genes elucidated by folate metabolism that have basic roles in cellular functions and are associated with neurons development and function, synthesis of neurotransmitter and gene methylation.

Folate genes mutations, along with dietary declines, have been significantly associated with numerous disorders from birth defects to heart disease and cancer. Folate deficiencies induce DNA hypomethylation, hyperhomocysteinemia, excision DNA repair and DNA synthesis.[6-9] It is necessary for the synthesis of purines, thymidilate and S-adenosylmethionine (SAM).[10,11] Two critical enzymes in folate metabolism pathway are products of methylenetetrahydrofolate reductase (MTHFR) and methionine synthase reductase genes (MTRR). They work in the synthesis of 5-methyltetrahydrofolate and methionine, respectively.[6,7] Methyltetrahydrofolate, the predominant circulating form of folate, acts as the methyl donor for remethylation of homocysteine to methionine by the vitamine B12 dependent enzyme methionine synthase. Severe MTHFR deficiency is characterized by neurological abnormalities, problem in nerve myelination[6,12], and reported to be a risk factor for neural tube defects and chronic neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and Autism.[13,14]

Transition of cytosine (C) to thymidine (T) at nucleotide position 677 causes alanine to valine substitution and correlate with increases in MTHFR thermolability and reduces enzyme activity. The enzyme activity decreases about 35% in heterozygous C/T genotype and about 70% in homozygous T/T genotype, when compared to the normal C/C genotype.[13,14] Second common polymorphism in MTHFR gene, the A1298C leads to glutamate to alanine substitution. This decreases MTHFR activity, mostly in CC genotype with no effect on protein thermolability.[6,7] Methionine synthase reductase is a related flavoprotein that keeps the methionine synthesis enzyme in an active state for the remethylation of homocysteine to methionine, which is the precursor of SAM.[15,16] Homocysteine has neurotoxic effects that may also play a role in the neurologic disturbances that are associated with folate deficiency.[10] MTRR A66G polymorphism decrease the risk of autism whereas MTHFR C677T has been reported to be a risk factor of autism disease[17], the disease that have some similar behavior disorders with FXS. The present study aimed to determine the putative correlation between the MTHFR C677T, A1298C and MTRR A66G polymorphisms and FXS in the Iranian population.

## METHODS

### *Study population*

Extracted DNA from 38 boys with FXS was kindly donated by Dr Najmabadi, Tehran, Iran. All patients had been identified to have fragile site at Xq27.3 (FRAXA) using PCR and Southern blot analysis. Controls were 60 age/sex matched unrelated healthy subjects from the same geographic area. Informed consent was obtained from all participants and the study was approved by the Ethics Committee of the Center.

### *Analysis of MTHFR and MTRR genes polymorphisms*

Thirty-eight FXS male patients and 60 normal control males were tested for common C677T, A1298C polymorphisms of the MTHFR and A66G polymorphism of the MTRR genes using PCR-RFLP (Figures. 1, 2, 3). Pair of primers was designed to amplify 254 bp fragment of MTHFR gene containing codon 677, forward and reverse primers were 5´gcc tct cct gac tgt cat cc3´ and 5´gga gct tat ggg ctc tcc tg3´, respectively. Other primers set for MTHFR A1298C was obtained from previous report[18] and for MTRR 66 was obtained from Dr. Jill James, National Center for toxicological research, USA. PCR thermal cycle was performed in 32 cycles. Each cycle consisted of 95°C denaturation for 30 seconds, 60°C annealing for 1 minute and 72°C extension for 30 seconds. The thermal cycles were started with an initial denaturation of 95°C for 5 minutes and followed by a final extention of 72°C for 10 minutes. For MTHFR C677T, PCR product was exposed to restriction enzyme digestion with *HinfI* (Roche, Germany). The presence of the C677T mutation within the MTHFR gene creates a *HinfI* restriction site that is detected by appearance of a 147 and 108 base pair fragments on a 10% polyacrylamide PAGE gel.

The reaction mixture and PCR condition for MTHFR A1298C and MTRR A66G were the same as that was for the C677T polymorphism except for A1298C annealing temperature that was 55°C. The A1298C amplified 163 bp fragment was

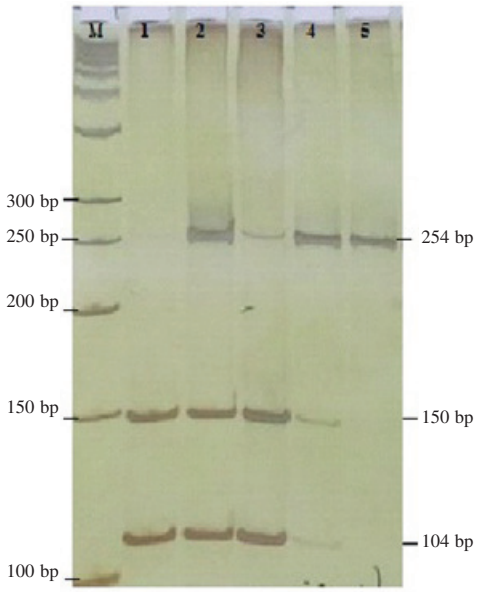


Figure 1. PCR-RFLP pattern of MTHFR C677T polymorphism digested with HinfI restriction enzyme. M, 50 bp DNA ladder; lane 1, 3 homozygous mutant T/T genotype; lanes 2, 4 heterozygous C/T genotype; lane 5, homozygous normal C/C genotype

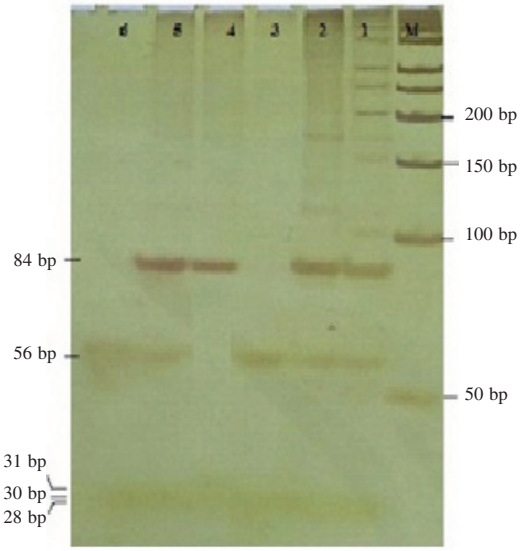


Figure 2. PCR-RFLP pattern of MTHFR A1298C polymorphism digested with MboII restriction enzyme. M, 50 bp DNA ladder; lanes1, 2, 5 heterozygous A/C genotype; lanes 3, 6, homozygous normal A/A genotype; lane 4, homozygous mutant C/C genotype.

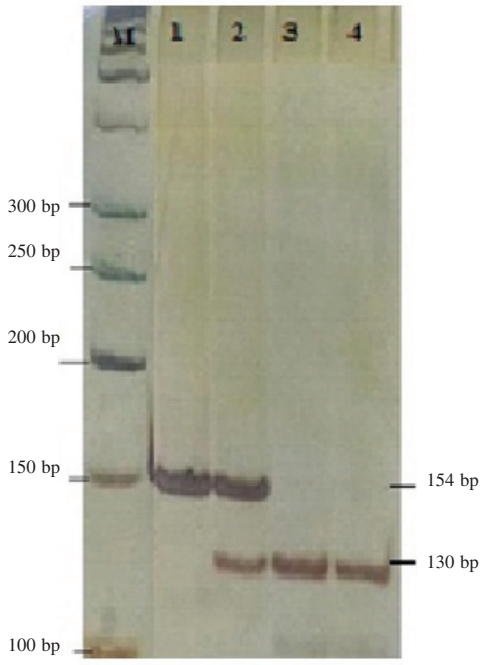


Figure 3. PCR-RFLP pattern of MTRR G66A polymorphism digested with Nde1 restriction enzyme. M, 50 bp DNA ladder; Lanes 1 homozygous mutant A/A; lanes 2 heterozygous G/A genotype; lanes 3, 4 homozygous mutant G/G genotype

digested with MboII (Fermentas, Vilna, Lithuania) in which the variant allele C abolished a *MboII* restriction site. The MTRR A66G amplified 154 bp fragment was digested with *NdeI* (Fermentas, Vilna, Lithuania). The fragments were analyzed by 10% polyacrylamide gel electrophoresis and visualized with silver staining. The accuracy of PCR-RFLP was confirmed by direct sequencing using a Big-Dye Terminator Cycle Sequencing kit (Applied Biosystems).

*Statistical analysis*

Allele frequencies were calculated for each genotype by allele counting. Comparisons of allele frequencies between case and control groups were determined using $\chi^2$ test for genotype and allele frequencies comparison using SPSS for windows version 11.0 (Chicago, Ilinois) software. A level of $P<0.05$ was considered statistically significant. The p values were corrected with the Bonferroni correction using dividing the probability value (0.05) by three comparisons and so the appropriate significance level was 0.05/3 = 0.017. Therefore, we considered chi-square tests being significant only if they had a p value <0.017.

## RESULTS

*MTHFR and MTRR genes polymorphisms*

For C677T polymorphism the CC, CT and TT genotypes were 17 (44.7%), 19 (50.0%), and 2 (5.3%) in patient group respectively whereas they were 42 (70.0%), 18 (30.0%) and 0 (0.0%) in control group. For A1298C polymorphism the AA, AC and CC genotypes were 10 (26.3%), 25 (65.8%) and 3 (7.9%) in patient group respectively whereas they were 21 (35.0%), 28 (46.7%) and 11(18.3%) in control group (Table 1). Statistical analysis showed significant association for C677T polymorphism of MTHFR gene to FXS ($p$=0.018). Moreover, the allele frequency of the C677T and the A1298C in MTHFR mutation among chromosome of patients were 30.3% and 40.8% respectively, while the corresponding frequencies among chromosomes of the control group were 15.0% and 41.7 % respectively.

The heterozygote genotype (CT) were found to be significantly related to occurrence of FXS (P=0.028; OR=2.608). Carriers of the C allele were at a significantly higher risk as compared to control group (p=0.010; OR= 2.459). Frequency of T Allele was 2 fold in patients (30.3%) as compared to controls (15.0%). Allele frequency of C was 69.7% (53) and 85.0% (102) in patients and controls respectively, whereas allele frequency of T was 30.2% (23) and 15.0% (18) respectively in patients and controls (Table.1).

However for 1298 polymorphism, no significant frequency difference was observed in regard to A and C alleles between the patients and controls (P=0.903; OR= 0.964, 95%CI). Allele frequency of A was 59.2% (45) and 58.3% (70) in patients and controls respectively, whereas allele frequency of C was 40.8% (31) and 41.7% (50) in patients and controls respectively (Table.1).

In addition, in the patient group, 14 (36%) subjects were heterozygous and/or mutant homozygous for both SNPs, while it was 12 (20%) subjects in the control group. In the patient group no altered homozygous genotype TT/CC was observed together for 677/1298 polymorphisms, whereas simultaneous CC/AA and CT/AC genotypes were observed respectively in 10% and 28% of the control subjects.

With regards to A66G MTRR polymorphism, AA, AG and GG genotypes were observed respectively in 11 (28.9%), 24 (63.1%), 3 (7.9%) of the patients, whereas they were seen in 28 (46.7%), 27 (45.0%) and 5 (8.3%) of the controls (Table 1). Frequencies of alleles G was significantly different in two groups (P= 0.214; OR= 1.463). As frequency of A allele was 60.5% (46) and 69.2% (63) in patient and control group respectively and frequency of G allele was 39.5% (30) and 30.8% (37) in patient and control groups.

**Table 1. Genotypes and allele frequencies of MTHFR C677T, A1298C and MTRR A66G polymorphisms in 38 patients and 60 controls**

| SNPs | Genotype N (%) | | | Allele Frequencies N (%) | |
|---|---|---|---|---|---|
| C667T | CC | CT | TT | C | T |
| Patients | 17 (44.7) | 19 (50.0) | 2 (5.3) | 53 (69.7) | 23 (30.3) |
| Controls | 42 (70.0) | 18 (30.0) | 0 (0.0) | 102 (85.0) | 18 (15.0) |
| P-value | 1 | 0.028 | 0.999 | 0.010 | |
| OR (95%CI) | | 2.608 (1.108-6.139) | | 2.459 (1.221-4.954) | |
| A1298C | AA | AC | CC | A | C |
| Patients | 10 (26.3) | 25 (65.8) | 3 (7.9) | 45 (59.2) | 31 (40.8) |
| Controls | 21 (35.0) | 28 (46.7) | 11 (18.3) | 70 (58.3) | 50 (41.7) |
| P-value | 1 | 0.183 | 0.461 | 0.903 | |
| OR (95%CI) | | 1.875 (0.743- 4.734) | 0.573 (0.130 - 2.521) | 0.964 (0.538-1.729) | |
| A66G | AA | AG | GG | A | G |
| Patients | 11 (28.9) | 24 (63.2) | 3 (7.9) | 46 (60.5) | 30 (39.5) |
| Controls | 28 (46.7) | 27 (45.0) | 5 (8.3) | 83 (69.2) | 37 (30.8) |
| P-value | 1 | 0.072 | 0.602 | 0.214 | |
| OR (95%CI) | | 2.263 (0.931- 5.499) | 1.527 (0.311- 7.506) | 1.463 (0.802- 2.669) | |

## DISCUSSION

This study was to examine the hypothesis of the existence linkage between impaired folate pathway and FXS. The frequencies of three common polymorphisms C677T and A1298C in MTHFR and G66A in MTRR genes, in two key enzymes of folate pathway, were tested for the first time in 38 males with FXS and 60 normal controls.

Statistical analysis showed a significant correlation between C677T polymorphism and FXS. To the best of our knowledge, this study is the first to demonstrate the association between G677T MTHFR gene polymorphism and FXS. The frequency of altered T allele was significantly higher in patients than in controls.

However, no significant associations were found between A1298C and A66G polymorphisms and FXS in the Iranian patient. The frequency of altered C allele and G allele showed no significant difference in patient and control groups. Although occurrence of both A1298C and C677T polymorphism in MTHFR gene can reflect in enzyme activity, our results showed that the A1298C polymorphism cannot be a major factor in the pathogenesis of FXS. MTHFR enzyme activity in altered homozygouse and heterozygouse status (TT and CT genotypes) are reduced by 50% and 30%, respectively. Simultaneous occurrence of C677T and A1298C polymorphisms reduces 50 to 60 % enzyme activity of MTHFR protein.[19] Haplotype frequency for altered alleles T-/C- of 677/1298 in MTHFR was higher (7.8%) in patients, but it was 18.3% in controls.

Significantly higher frequency of C677T polymorphism in patient group may reveal the likely effects of folate derivatives on neuropathology and methylation process in FXS. Hence, more investigations are required on the role of folate metabolism in this disease. To date, epigenetic effects for MTHFR polymorphisms that could lead to methylation alteration have been recently shown in human and animals.[20-22] Epigenetic mechanisms, such as DNA methylation and modifications to histone proteins, regulate high-order DNA structure and gene expression. Aberrant epigenetic and copy number variation mechanisms are involved in several neurodevelopmental and neurodegenerative disorders including Rett syndrome, FXS, and microdeletion syndromes.[23] On the other hand, methylation of FMR1 gene has essential role in silencing and 70% penetrance of FXS in offspring. Furthermore, folic acid derivatives have an important role in DNA methylation, neural development and synthesis of neurotransmitter.[24] The C677T polymorphism has a significant effect on MTHFR activity[25] and reduces catalysis of 5,10 methylen tetrahydrofolate into 5 methyltetrahydroflate, a carbon donor in the methylation of homocysteine to methionine, and decreases SAM level[26] and Autism.[28] Recent evidence has emerged that children with autism may have altered folate or methionine metabolism, which suggests the folate-methionine cycle may play a key role in the etiology of autism.[28]

Derivatives of folic acid such as biopterins and the SAM synthesis have effects on improvement of depressive disorder[27] and autism.[28] Recent evidence has emerged that children with autism may have altered folate or methionine metabolism, which suggests the folate-methionine cycle may play a key role in the etiology of autism.[28] This study is in accordance with previous investigation with no direct associations between A1298C and A66G polymorphisms and autisms.[17]

In conclusion our study shows the association between T allele of 677 polymorphism and FXS. Further studies with larger sample sizes should be undertaken to confirm these preliminary results. Future research should be directed to the impaired folate pathway in FXS.

## DISCLOSURE

Conflict of interest: None

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