

Methylenetetrahydrofolate reductase gene polymorphisms in Egyptian Turner Syndrome patients

Manal F. Ismail¹, Waheba A. Zarouk², Mona O. Ruby³, Wael M. Mahmoud⁴ and Randa S. Gad²✉

¹Biochemistry Department, Faculty of Pharmacy, Cairo University, Cairo, Egypt; ²Department of Molecular Genetics, National Research Centre, Cairo, Egypt; ³Department of Clinical Genetics, National Research Centre, Cairo, Egypt; ⁴Department of Cytogenetics, National Research Centre, Cairo, Egypt

Background: Folate metabolism dysfunctions can result in DNA hypomethylation and abnormal chromosome segregation. Two common polymorphisms of the methylenetetrahydrofolate reductase (MTHFR) encoding gene (C677T and A1298C) reduce MTHFR activity, but when associated with aneuploidy, the results are conflicting. Turner Syndrome (TS) is an interesting model for investigating the association between MTHFR gene polymorphisms and nondisjunction because of the high frequency of chromosomal mosaicism in this syndrome. **Objective:** To investigate the association of MTHFR gene C677T and A1298C polymorphisms in TS patients and their mothers and to correlate these polymorphisms with maternal risk of TS offspring. **Subjects and Methods:** MTHFR C677T and A1298C polymorphisms were genotyped in 33 TS patients, their mothers and 15 healthy females with their mothers as controls using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) and sequencing technique. **Results:** Genotype and allele frequencies of both C677T and A1298C were not significantly different between TS cases and controls. There were no significant differences in C677T genotype distribution between the TS mothers and controls ($p=1$). The MTHFR 1298AA and 1298AC genotypes were significantly increased in TS mothers Vs. control mothers ($p=0.002$). The C allele frequency of the A1298C polymorphism was significantly different between the TS mothers and controls ($p=0.02$). The association of A1298C gene polymorphism in TS patients was found to increase with increasing age of both mothers ($p=0.026$) and fathers ($p=0.044$) of TS cases. **Conclusion:** Our findings suggest a strong association between maternal MTHFR A1298C and risk of TS in Egypt.

Key words: chromosomal nondisjunction, DNA methylation, folate, MTHFR gene, Turner Syndrome

Received: 12 January, 2015; revised: 22 May, 2015; accepted: 28 June, 2015; available on-line: 28 July, 2015

INTRODUCTION

Turner Syndrome (TS) is one of the most common aneuploidies in humans, affecting approximately 1/2500 births (Stochholm *et al.*, 2006). In 50% of TS cases, only one X chromosome is inherited as a result of nondisjunction during parental gametogenesis, leaving a monosomy X genotype (45, X) in the fetus (Elsheikh *et al.*, 2002). In 70% to 80% of these monosomic females, the single X chromosome is of maternal origin with the remainder being paternal in origin (Frias & Daven-

port, 2003). The molecular mechanisms which determine chromosomal nondisjunction are not well known so far, but dysfunctions in folate and methyl group metabolism may result in DNA hypomethylation and abnormal chromosome segregation. MTHFR plays a crucial role in the regulation of DNA methylation (De Oliveira *et al.*, 2008) by conversion of 5,10-methylenetetrahydrofolate (5,10-methylene-THF) to 5-methyltetrahydrofolate (5-methyl-THF), the major methyl donor for remethylation of homocysteine to methionine (Steed & Tyagi, 2011). The most studied polymorphisms of the MTHFR gene are C677T within the catalytic domain of the protein (exon 4) and A1298C within the putative regulatory domain (exon 7), they both affect the enzymatic function and consequently lead to a high level of plasma homocysteine. A1298C influences the specific activity of the enzyme and also the folate concentration, but with a lower impact than the C677T polymorphism (Neagos *et al.*, 2012). A reduced MTHFR activity results in an increased requirement for folic acid to maintain homocysteine remethylation, which has been considered a risk factor for nondisjunction (Kaur & Kaur, 2013). The current work aimed to study the MTHFR gene C677T and A1298C polymorphisms in TS patients and their mothers and to correlate these polymorphisms with the maternal risk of TS offspring.

SUBJECTS AND METHODS

Subjects

The study protocol was approved by the Medical Research Ethics Committee of the National Research Centre. The study consisted of 4 groups; 33 patients with TS diagnosed by clinical evaluation and karyotyping, 30 mothers of the TS patients (age from 16 to 39 years), 15 healthy females and 15 healthy mothers. Controls were age matched to cases and mothers. The cases were recruited from the outpatient clinic, Clinical Human Genetics department, National Research Centre, Cairo, Egypt. Written informed consent was obtained from all participants or their parents.

✉ e-mail: randagad83@yahoo.com

Abbreviations: MTHFR, methylenetetrahydrofolate reductase; TS, Turner syndrome; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; THF, tetrahydrofolate; SPSS, Social Science Software program; S.D., standard deviation

Methods

Clinical evaluation. Patients were subjected to pedigree construction up to three generations including consanguinity and similarly affected family member. Clinical examination including systemic examination of all body systems was also performed to exclude congenital heart diseases, kidney affection or others. Special examination was performed to evaluate the characteristic stigmata of Turner Syndrome. Full anthropometric data were obtained for each subject including weight, height and head circumference.

Cytogenetic study. Patients were subjected to standard cytogenetic analysis to establish their karyotypes in addition to FISH analysis to detect hidden mosaicism. Peripheral blood samples were cultured, harvested, and G-banded according to (Gosden *et al.*, 1992; Verma & Babu, 1995).

Molecular investigation. Genomic DNA was isolated from 200 μ l of peripheral blood using a commercial DNA isolation kit (QIAamp DNA Blood Kit; Qiagen).

MTHFR C677T genotyping. The following primers were used: forward: 5'-TGA AGG AGA AGGTGT CTG CGG GA-3', reverse: 5'-AGG ACG GTG CGGTGA GAG TG-3' (Frosst *et al.*, 1995). PCR was performed in a final reaction volume of 50 μ l containing 10 μ l genomic DNA (1 μ g/50 μ l), 20 μ l of Qiagen master mix, 2 μ l of each primer (40 pmol/50 μ l). Amplification was carried out in a thermal cycler (Biometra, Göttingen, Germany) with 5 min denaturation at 95°C, followed by 35 cycles of 1 min at 95°C, 1 min at 61°C and 1 min at 70°C, followed by a final extension for 7 min at 70°C. The amplified products were digested with *Hinf*I restriction enzyme and visualized in a 3% agarose gel with ethidium bromide. Wild types (677CC) produced a single band at 198 base pairs (bp) after *Hinf*I digestions. Heterozygotes (677CT) produced 198-, 175-, and 23-bp bands, while homozygous mutants (677TT) produced 175- and 23-bp bands (Fig. 1).

MTHFR A1298C genotyping. The following primers were used: forward: 5'-CTT TGG GGA GCT GAA GGA CTA CTA C-3', 1298 reverse: 5'-CAC TTT GTG ACC ATT CCG GTT TG-3' (Frosst *et al.*, 1995). The PCR conditions were: 2 min denaturation at 92°C, followed by 35 cycles of 1 min at 92°C, 1 min at 54°C and 30 s at 72°C, followed by a final extension for 7 min at 72°C. A band of 163 bp was generated. Direct sequencing was done to detect different genotypes (Fig. 2).

DNA sequencing. The MTHFR A1298C region amplified products were subjected to DNA sequencing. The

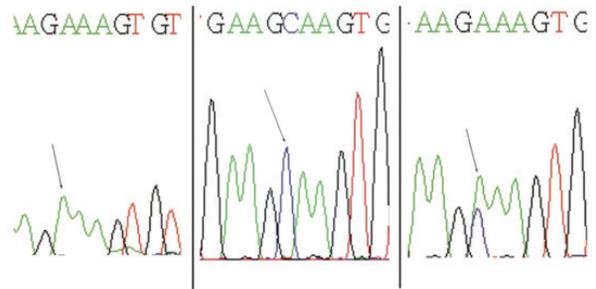


Figure 2. Sequencing chromatograms of A1298C MTHFR gene polymorphism
(a) genotype 1298AA (b) genotype 1298CC, (c) genotype 1298AC

PCR products were purified by QiaQuick PCR purification kit (Qiagen, Valencia, CA, USA), sequenced with ABI Big Dye Terminator Cycle sequencing chemistry and the products were analyzed on an ABI 310 DNA sequencer (Applied Biosystems, Foster City, CA, USA). The sequencing chromatograms were compared with the corresponding wild type using sequence analysis software (Applied Biosystems).

Statistical analysis. Pre-coded data was analyzed statistically by the Statistical Package of Social Science Software program (SPSS), version 21. Data was summarized using frequency and percentage for qualitative data or mean and standard deviation for quantitative ones. Comparison between groups was performed using Chi square test or Fisher's exact test for qualitative data. Spearman correlation coefficient was calculated to clarify the association between ordinal and quantitative variables. P values less than 0.05 were considered statistically significant.

RESULTS

The frequency of MTHFR 677CC, CT and TT genotypes in the patients with Turner Syndrome was 57.6%, 39.4% and 3%, respectively. In the control group, only genotypes 677CC and 677CT were found at 73.3% and 26.7%, respectively. The frequencies of genotypes 1298AA, AC and CC in patients were 24.2%, 69.7% and 6.1%, respectively. In controls, 1298CC was absent while AA and AC were at 26.7% and 73.3%, respectively. The distribution of the 677C allele among patients was 77.3% and 86.7% in controls while the T allele was at 22.7% in patients and 13.3% in controls. The 1298C allele frequency was 40.9% in patients and 36.7% in the control group. No significant difference in the distribution of the risk alleles was found between TS patients and controls. By analyzing the combined effects of both the MTHFR gene variants it was found that the MTHFR 677CC/1298AA genotype was less frequent in patients than in controls (9.1% and 13.3%, respectively). The 677CC/1298AC genotype was also less frequent in patients (42.2%) than in controls (60%). The frequency of double heterozygosity (677CT, 1298AC) was higher in patients (24.2%) than in controls (13.3%). The genotypes 677CT/1298CC, TT/AA and TT/CC were absent in both patients and controls. No significant difference was observed in the distribution of the combined genotypes between TS patients and controls. The distribution and allele frequencies of the MTHFR C677T and A1298C genotypes in the mothers of TS patients and controls is shown in Table 1. There were no significant differences in genotype distributions of the C677T polymorphism between the TS mothers and controls ($p=1$). The

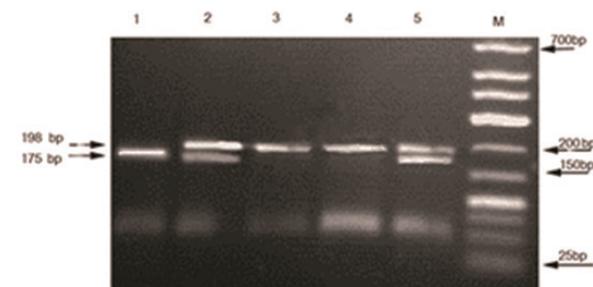


Figure 1. C677T polymorphism analysis of methylenetetrahydrofolate reductase gene (MTHFR) amplicons by agarose gel electrophoresis after restriction endonuclease digestion (RFLP). PCR products were digested with *Hinf*I revealing genotypes CC (Wild type 198 bp), CT (175 bp and 198 bp) and TT (175 bp). Lane 1: homozygous (TT), lanes 2,5: heterozygous (CT), lanes 3,4: homozygous wild type (CC), M: Mwt ladder.

Table 1. MTHFR C677T and A1298C genotypes and allele frequency in TS mothers and controls.

	TS (n=30)		Controls (n=15)		P value
	N	%	N	%	
C677T genotypes					
CC	23	76.7	12	80.0	1.0
CT	7	23.3	3	20.0	1.0
TT	-	-	-	-	1.0
C677T allele					
C	53	88.3	27	90.0	1.0
T	7	11.7	3	10.0	
A1298C genotypes					
AA	5	16.7	4	26.7	0.002
AC	25	83.3	11	73.3	0.0020
CC	-	-	-	-	1.0
A1298C allele					
A	35	58.3	25	83.3	0.02
C	25	41.7	5	16.7	

*Fisher's exact test

1298AA and AC genotypes were significantly more frequent in TS mothers than in control mothers ($p=0.002$). The 1298C allele was significantly more frequent in the TS mothers than in controls ($p=0.02$). The incidence of the risk allele 1298C in TS patients was increased with increasing age of both mothers ($p=0.026$) and fathers ($p=0.044$) while no correlation was found between the MTHFR gene C677T polymorphism and the age of mothers or fathers (Table 2).

DISCUSSION:

The T variant at nucleotide 677 (MTHFR 677C>T) and the C variant at nucleotide 1298 (MTHFR 1298A>C) have been described to reduce MTHFR activity (Finkelstein, 1990). Hobbs *et al.* (2000) reported that pericentromeric DNA methylation is of extreme importance for chromosome stability and normal segregation. Stern *et al.* (2000) investigated whether the MTHFR gene mutation C677T affected genomic DNA methylation and found that individuals with the mutated genotype have a greater capacity to receive the methyl group than normal individuals, indicating hypomethylation in genotype 677TT. Turner Syndrome could be a model for the investigation of MTHFR gene polymorphism associated with somatic chromosomal nondisjunction due to the high frequency of chromosome mosaicism in Turner patients (Santos *et al.*, 2006). They studied 49 Turner Syndrome patients and 200 controls and found a high frequency of the 677T allele, concluding that when homozygous this mutation could enhance somatic chromosomal nondisjunction by the depletion of MTHFR activity in Turner patients. In contrast, Oliveira *et al.* (2008) did not find a positive correlation between the C677T polymorphism of the MTHFR gene and chromosome nondisjunction in Turner Syndrome patients. In contrast, the 1298CC genotype was more frequent in patients with Turner Syndrome, suggesting its involvement in chromo-

Table 2. Correlation between mothers', fathers' age and incidence of risk alleles for both MTHFR gene polymorphisms

	Mother age		Father age	
	rho	P-value	rho	P-value
TS patient 677T allele	0.150	0.438	0.299	0.115
TS patient 1298C allele	0.412	0.026	0.377	0.044

rho = Spearman correlation coefficient.

somal imbalances. Also a study by Kim *et al.* (2011) on Korean patients found that the MTHFR 1298A>C polymorphism was significantly associated with an increased risk for spontaneous abortion with fetal chromosomal aneuploidy. The results obtained in our study are not in accord with the earlier findings; we found that genotype and allele frequencies of both C677T and A1298C were not significantly different between TS patients and controls. Also there were no significant differences in the C677T genotype distribution between the TS mothers and controls. In turn the 1298AA and AC genotypes were significantly more frequent in TS mothers than in control mothers.

Chango *et al.* (2005) studied the C677T MTHFR polymorphism in a French population and, in accordance with some other studies, could not establish a correlation between TT and homocysteine level, suggesting that the high folate intake in France may nullify any effect of this deleterious genotype. Similar results have also been found in an Italian study conducted by Bosco *et al.* (2003). Both studies support the concept that the Mediterranean diet high in folate could nullify a possible effect of MTHFR polymorphism on the risk of chromosomal aneuploidy development. These results are also consistent with the low frequency of neural-tube defects in both countries (James, 2004). The fact that the results of different studies are conflicting and inconsistent can be explained by several factors: (1) de novo mutations affecting natural selection; (2) population migration resulting in genetic imbalance; and (3) genetic drift, whereby random fluctuations can skew allele distribution in a small study group; large cohort studies would be valuable for establishing exact frequency of the MTHFR polymorphisms (Song & Elston, 2006; Sadiq *et al.*, 2011). Some authors suggest that these results are due to differences in linkage disequilibrium in the MTHFR locus and the different diet of the populations studied (Weisberg *et al.*, 1998; Oliveira *et al.*, 2012).

The only well established risk factor for commonly occurring chromosomal syndrome with a variable magnitude is advanced maternal age (Risch *et al.*, 1986; Morton *et al.*, 1988; Hecht & Hook, 1996). Recent studies have shown that the oocyte quality has some age-related dysfunctions which can explain the 'maternal age effect', especially disturbances of the mitochondrial function and thereby a loss of the energy necessary for proper oocyte functioning (Wang *et al.*, 2009). Notably, an absence of a parental age effect has also been reported (Loughlin *et al.*, 1991). A study conducted by Suttur *et al.* (2010) reported that young mothers more frequently give birth to babies with chromosomal syndromes. The results of our study revealed that the association of parental MTHFR gene A1298C polymorphism in the TS is increased with an increasing age of both mothers ($p=0.026$) and fathers ($p=0.044$) of patients.

CONCLUSION

Our findings suggest strong association between maternal MTHFR A1298C polymorphism and risk of having TS offspring in the studied Egyptian cases.

REFERENCES

- Bosco P, Anello G, Barone C, Namour F, Caraci F (2003) Methionine synthase (MTR) 2756 (A→G) polymorphism, double heterozygosity methionine synthase 2756 AG/methionine synthase reductase (MTRR) 66 AG, and elevated homocysteinemia are three risk factors for having a child with Down syndrome. *Am J Med Genet* **121A**: 219–224.
- Chango A, Fillon-Emery N, Mircher C, Bléhaut H, Lambert D, Herbeth B (2005) No association between common polymorphisms in genes of folate and homocysteine metabolism and the risk of Down's syndrome among French mothers. *Br J Nutr* **94**: 166–169.
- Elsheikh M, Dunger DB, Conway GS, Wass JH (2002) Turner's syndrome in adulthood. *Endocr Rev* **23**: 120–140.
- Finkelstein JD (1990) Methionine metabolism in mammals. *J Nutr Biochem* **1**: 228–237.
- Frias JL, Davenport ML (2003) Clinical report: health supervision for children with Turner syndrome. *Pediatrics* **111**: 692–670.
- Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA *et al* (1995) A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat Genet* **10**: 111–113.
- Gosden CM, Davidson C, Robertson M (1992) Lymphocyte culture. In *Human Cytogenetics: A Practical Approach*, Rooney DE, Czepulkowski BH eds, 2nd edn, pp 31–54. IRL Press, Oxford.
- Hecht C, Hook E (1996) Rates of Down syndrome at live birth by one-year maternal age intervals in studies with apparent close to complete ascertainment in populations of European origin: A proposed revised rate schedule for use in genetic and prenatal screening. *Am J Med Genet* **62**: 376–385.
- Hobbs CA, Sherman SL, Yi P, Hopkins SE, Torfs CP, Ne RJ (2000) Polymorphisms in genes involved in folate metabolism as maternal risk factors for Down syndrome. *Am J Hum Genet* **67**: 623–630.
- James S (2004) Maternal metabolic phenotype and risk of Down syndrome: beyond genetics. *Am J Med Genet A* **127A**: 1–4.
- Kaur A, Kaur A (2013) Prevalence of methylenetetrahydrofolate reductase 677 C-T polymorphism among mothers of Down syndrome children. *Indian J Hum Genet* **19**: 412–414. doi: 10.4103/0971-6866.124368.
- Loughlin SA, Redha A, McIver J, Boyd E, Carothers A, Connor JM (1991) Analysis of the origin of Turner's syndrome using polymorphic DNA probes. *J Med Genet* **28**: 156–158.
- Morton NE, Jacobs PA, Hassold T, Wu D (1988) Maternal age in trisomy. *Ann Hum Genet* **52**: 227–235.
- Neagos D, Cretu R, Sfetea R, Mierla D, Bohiltea L (2012) Investigation of the relationship between the risk of spontaneous abortion and C677T and A1298C polymorphisms of the methylenetetrahydrofolate reductase gene. *Revista Română de Medicină de Laborator* **20**: 335–343.
- Oliveira KC, Bianco B, Verreschi ITN, Guedes AD, Galera BB, Galera MF *et al* (2008) Prevalence of the polymorphism MTHFR A1298C and not MTHFR C677T is related to chromosomal aneuploidy in Brazilian Turner Syndrome patients. *Arq Bras Endocrinol Metabol* **52**: 1374–1381.
- Oliveira KC, Verreschi IT, Bianco B, Lipay MV *et al* (2012) C677T and A1298C polymorphisms of MTHFR gene and their relation to homocysteine levels in Turner syndrome. *Genet Test Mol Biomarkers* **16**: 396–400. doi: 10.1089/gtmb.2011.0222.
- Risch N, Stein Z, Kline J, Warburton D (1986) The relationship between age and chromosome size in autosomal trisomy. *Am J Hum Genet* **39**: 68–78.
- Sadiq M, Al-Refai E, Al-Nasser A, Khassawneh M, Al-Batayneh Q (2011) Methylenetetrahydrofolate reductase polymorphisms C677T and A1298C as maternal risk factors for Down syndrome in Jordan. *Genet Test Mol Biomarkers* **15**: 51–7. doi: 10.1089/gtmb.2010.0057.
- Santos K, Lemos-Marini S, Baptista M, Bonadia L (2006) Frequency of 677C →T and 1298A →C polymorphisms in the 5,10-methylenetetrahydrofolate reductase (MTHFR) gene in Turner syndrome individuals. *Genetics and Molecular Biology* **29**: 41–44.
- Shin Young Kim, So Yeon Park, Ji Won Choi, Do Jin Kim, Shin Yeong Lee, Ji Hyae Lim *et al* (2011). Association Between MTHFR 1298A>C Polymorphism and Spontaneous Abortion with Fetal Chromosomal Aneuploidy. *Am J Reprod Immunol* **66**: 252–258. doi: 10.1111/j.1600-0897.2011.00996.
- Song K, Elston RC (2006) A powerful method of combining measures of association and Hardy-Weinberg disequilibrium for fine-mapping in case-control studies. *Stat Med* **25**: 105–126.
- Steed MM, Tyagi SC (2011) Mechanisms of cardiovascular remodeling in hyperhomocysteinemia. *Antioxid Redox Signal* **15**: 1927–1943. doi: 10.1089/ars.2010.3721.
- Stern LL, Mason JB, Selhub J, Choi SW (2000) Genomic DNA hypomethylation, a characteristic in most cancers, is present in peripheral leukocytes of individuals who are homozygous for the C677T polymorphism in the methylenetetrahydrofolate reductase gene. *Cancer Epidemiol Biomarkers Prev* **9**: 849–853.
- Stochholm K, Juul S, Juul K (2006) Prevalence, incidence, diagnostic delay, and mortality in Turner syndrome. *J Clin Endocrinol Metab* **91**: 3897–3902.
- Malini SS, Ramachandra NB (2010) Young mothers produce more chromosomal syndrome babies in Mysore, South India. *Int J Hum Genet* **10**: 105–112.
- Verma RS, Babu A (1995). Tissue culture techniques and chromosome preparation. In *Human Chromosomes. Principles and Techniques*, Verma RS, Babu A, eds. pp 6–71. McGraw-Hill, New York.
- Wang L, Wang D, Zou X, Xu C (2009) Mitochondrial functions on oocytes and preimplantation embryos. *J Zhejiang Univ Sci B* **10**: 483–92. doi: 10.1631/jzus.B0820379.
- Weisberg I, Tran P, Christensen B, Sibani S, Rozen R (1998) A second genetic polymorphism in methylenetetrahydrofolate reductase (MTHFR) associated with decreased enzyme activity. *Mol Genet Metab* **64**: 169–72.