C677T and A1298C Polymorphisms of *MTHFR* Gene and Their Relation to Homocysteine Levels in Turner Syndrome

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Aims: To determine the frequency of C677T and A1298C polymorphisms of the *MTHFR* gene and correlate them with homocysteine serum levels in patients with Turner syndrome (TS) and controls. **Methods:** This case–control study included 78 women with TS and a control group of 372 healthy individuals without personal or family history of cardiovascular disease and cancer. C677T (rs1801133) and A1298C (rs1801131) polymorphisms were detected by polymerase chain reaction–restriction fragment-length polymorphism and the TaqMan system, respectively. Homocysteine serum levels were determined by high-performance liquid chromatography. The results were analyzed statistically, and $p < 0.05$ was considered to represent a significant difference. **Results:** The homocysteine levels change was 13.9 ± 3.3 nM in patients with TS and 8.8 ± 3.2 nM in the control group. No significant difference between groups was found ($p = 0.348$). Single-marker analysis revealed no association between *MTHFR* C677T polymorphism and TS when genotype ($p = 0.063$) or allelic ($p = 0.277$) distribution was considered. Regarding *MTHFR* A1298C polymorphism, a statistical difference was found between the TS group and the control group, for both genotype ($p < 0.0001$) and allele ($p < 0.0001$) distribution. Haplotype analysis of 2 *MTHFR* polymorphisms identified 2 haplotypes—CC and TC—associated with TS ($p < 0.001$ and $p = 0.0165$, respectively). However, homocysteine levels were not higher in patients with haplotype risk. **Conclusion:** The results suggest that the C677T and A1298C polymorphisms of the *MTHFR* gene are not related to homocysteine levels in Brazilian patients with TS, despite the differential distribution of the mutated allele C (A1298C) in these patients. Further studies are needed to investigate the possible genetic interaction with homocysteine levels in TS.

Introduction

TURNER SYNDROME (TS) is one of the most common aneuploidies in humans, affecting approximately 1/2500 births (Stochholm *et al.*, 2006). Cytogenetically, it is characterized by complete (45,X) or partial monosomy of sexual chromosome X in some or all cells. The clinical features are short stature and gonadal dysgenesis (Stratakis and Rennert, 2005; Oliveira *et al.*, 2009).

Cardiovascular disease (CVD) is the leading cause of death in these patients, reducing life expectancy by up to 13 years. In addition, malformations of the cardiovascular system have been well described in this syndrome (Kim *et al.*, 2011). Several risk factors related to vascular abnormalities, such as atherosclerosis, obesity, high blood pressure, diabetes melli-

tus, and dyslipidemia, are also often described (Baguet *et al.*, 2005; Lopez *et al.*, 2008). Data from the general population indicate that only half or two thirds of the CVDs can be explained by these traditional risk factors, suggesting the need to identify new markers for monitoring the onset and progression of the disease (Lloyd-Jones *et al.*, 2006; Kaldmäe *et al.*, 2011).

Plasma levels of homocysteine have shown a strong correlation with the genesis of CVD; they cause important changes in the vascular system, such as smooth-muscle proliferation, progressive arterial stenosis, and hemodynamics alteration (Steed and Tyagi, 2011).

Dudmam (1999) suggested that homocysteine is a natural regulator of leukocytes, including endothelial adhesion and transendothelial migration. That study showed that homocysteine independently activates leukocytes and endothelial

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cells. Neutrophils and monocytes exposed to homocysteine and co-cultured with endothelial cells have evolved mechanisms involving the formation of hydrogen peroxide. Moreover, transendothelial and endothelial migration are mediated by changes in the pattern of protein expression of monocyte chemoattraction and the interleukins, which are responsible for signaling neutrophils and inducing cellular responses. These cells release inflammatory cytokines and agonists, such as tumor necrosis factor- α .

Several factors are considered important for increases in plasma homocysteine, among them age (due to the decline in the metabolism of homocysteine in the kidneys), smoking, sedentary lifestyle, alcohol and caffeine intake, diet rich in lipids, and deficiency of female sex hormones. Estrogen deficiency due to X chromosome monosomy is another physiologic characteristic in patients with TS. However, this deficiency can be compensated by hormone replacement therapy (Oliveira *et al.*, 2009).

Genetic factors, including deficiencies of such enzymes as methylenetetrahydrofolate reductase (MTHFR), are important factors leading to hyperhomocysteinemia. MTHFR catalyzes the synthesis of 5-methyltetrahydrofolate, the major methyl donor for remethylation of homocysteine to methionine (Steed and Tyagi, 2011). The *MTHFR* gene, located on the short arm of chromosome 1 (1p36.3, MIM 607093, Genebank ID 4524) (Goyette *et al.*, 1994), presents 10 polymorphisms as described. The most studied polymorphisms are C677T and A1298C, which present the greatest effect on enzymatic function and consequently lead to a high level of plasma homocysteine.

On the basis of these observations, we sought to determine the frequency of C677T and A1298C polymorphisms of the *MTHFR* gene and correlate them with homocysteine levels in patients with TS and in controls.

Material and Methods

Population

The study included 78 women (mean age 25.0+10.5 years) with clinical and cytogenetic diagnosis of TS from the Gonads and Development Outpatient Clinic of Universidade Federal de São Paulo, Brazil. All patients were assessed for clinical and laboratory measures commonly used to evaluate cardiovascular

risk, such as age, blood pressure, total cholesterol and fractions (low-density lipoprotein, high-density lipoprotein, and very-low-density lipoprotein), triglycerides, and fasting blood glucose (Table 1). A control group of 372 healthy individuals with no personal or family history of CVD or cancer was selected for this study at the Outpatient Clinic of the Universidade de Cuiabá.

The study was approved by the local ethics committee (UNIFESP-EPM #2052/07), and all patients and controls signed an informed consent form.

Karyotype

Peripheral blood samples from the patients with TS were cultured for 72 hours in Roswell Park Memorial Institute 1640 medium supplemented with fetal bovine serum and phytohemagglutinin. Metaphase chromosomes were analyzed by Wright G-banding and C-banding, using standard protocols, and at least 40 peripheral blood metaphases were analyzed for each patient (Hook, 1977). The number of analyzed metaphases was increased to 100 whenever necessary. The karyotype results were described according to the International System for Human Cytogenetic Nomenclature (ISCN 2005). The TS study group karyotypes were distributed as follows: 45,X in 53 women, X isochromosome [including 46,X,i(X)(q) and 45,X/46,X,i(X)(q10)] in 7 patients, structural abnormalities in 9, and mosaic without structural abnormalities [including 45,X/46XX, 45,X/47XXX and other aneuploidies] in 6; in 1 patient, the mosaic karyotype included a lineage with Y chromosome (45,X/46,XY).

Genotyping

Genomic DNA was extracted from peripheral blood of patients and controls according to a standard protocol (Lahiri and Numberger, 1991). The C677T (rs1801133) polymorphism was genotyped by polymerase chain reaction [PCR]-restriction fragment-length polymorphism according to the protocol of Frosst *et al.* (1998). The A1298A polymorphism (rs1801131) was detected by using the TaqMan system by real-time PCR, with commercially available primers and probes provided by Applied Biosystems (Foster City, CA). The assays were performed using TaqMan Universal Master Mix, with 50 ng of DNA per reaction. The PCR conditions were as follows: 40 denaturation cycles of 15 s at 95°C and 1 min annealing/extension at 60°C, as recommended by the manufacturer.

Homocysteine serum levels

Serum levels of homocysteine were measured by high-performance liquid chromatography, with fluorometric detection and isocratic elution, using a substrate-specific thiol group, 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (Wako Chemicals); this technique was previously described by Pfeiffer *et al.* (1999). The technique is based on the use of phosphate-buffered saline (pH, 7.4) for reduction and release of thiols bound to proteins and trichloroacetic acid for deproteinization. After this process the sample is added to a buffer solution containing ethylenediaminetetraacetic acid with ammonium 7 fluorobenzo-2-oxa-1,3-diazole-4-sulphonate and reading in a liquid chromatograph with fluorescence detector (Waters Technologies). Thiols were separated on a Prodigy ODS2 analytical column C18

TABLE 1. CLINICAL AND LABORATORY CHARACTERISTICS OF PATIENTS WITH TURNER SYNDROME EVALUATED FOR BIOCHEMISTRY MEASURES ASSOCIATED WITH CARDIOVASCULAR DISEASE

Variable	Mean value $\pm$ SD	Median value
Age (y)	25.0 $\pm$ 10.5	23.8
Systolic blood pressure (mm Hg)	113.8 $\pm$ 13.9	111.3
Diastolic blood pressure (mm Hg)	74.5 $\pm$ 13.7	76.3
Total cholesterol (mg/dL)	169.4 $\pm$ 30.3	169.5
HDL cholesterol (mg/dL)	53.8 $\pm$ 15.0	52.0
LDL cholesterol (mg/dL)	97.4 $\pm$ 22.3	94.0
Triglycerides (mg/dL)	81.7 $\pm$ 39.6	72.0
Fasting blood glucose (mg/dL)	83.5 $\pm$ 9.5	84.5

HDL, high-density lipoprotein; LDL, low-density lipoprotein; SD, standard deviation.

TABLE 2. DISTRIBUTION OF GENOTYPE AND ALLELE FREQUENCIES OF C677T AND A1298C POLYMORPHISMS OF *MTHFR* GENE AND HOMOCYSTEINE LEVELS IN PATIENTS WITH TURNER SYNDROME AND CONTROLS

Variable	Turner syndrome group (%) (n = 78)	Control group (%) (n = 372)	OR (95% CI)	p value
<i>MTHFR</i> 677				
Genotype				0.063
CC	37 (47.4)	219 (59.0)		
CT	33 (42.3)	107 (29.0)		
TT	8 (10.3)	46 (12.0)		
Allele			1.25 (0.86 – 1.82)	0.277
C	107 (68.6)	545 (73.25)		
T	49 (31.4)	199 (26.75)		
<i>MTHFR</i> 1298				
Genotype				<0.001 ^a
AA	36 (46.2)	239 (64.0)		
AC	28 (35.9)	115 (31.0)		
CC	14 (17.9)	18 (5.0)		
Allele			2.20 (1.51– 3.19)	<0.001 ^a
A	100 (64.1)	593 (80.0)		
C	56 (35.9)	151 (20.0)		
Mean homocysteine $\pm$ SD (μ M)	13.9 $\pm$ 3.3	8.8 $\pm$ 3.2		0.348

CI, confidence interval; OR, odds ratio; SD, standard deviation.

(Phenomenex, Torrance, CA). The detection limit for this technique is 0.16 nM. The reference values of plasma homocysteine are 6–12 nM in women and 8–14 nM in men. Hyperhomocysteinemia was considered moderate with values of 16–30 nM, intermediate with values of 30–100 nM, and severe with values greater than 100 nM.

Statistical analysis

Statistical analyses of the results were done by using SPSS for Windows 11.0 (SPSS, Inc., Chicago, IL). Nonparametric data were expressed as median and range. The chi-square test was used to detect differences in allele and genotype frequencies between patients and controls and to evaluate the Hardy–Weinberg equilibrium. The odds ratio (OR) was used to measure the strength of the association between the frequencies of *MTHFR* genotypes and homocysteine serum levels in TS using a logistic regression model. The association between the combined genotypes of *MTHFR* polymorphisms and homocysteine serum levels was also evaluated by the haplotypes study using Haploview software, version 4.1 (www.hapmap.org). All *p* values were 2-tailed, and 95% confidence intervals were calculated. A *p* value less than <0.05 was considered to represent a statistically significant difference.

Results

The genotype and allele frequencies of the C677T and A1298C polymorphisms of *MTHFR* gene and serum homocysteine values are shown in Table 2. The mean homocysteine level in patients with TS was 13.9 $\pm$ 3.3 nM, with all values within the normal range. The mean value in the control group was 8.8 $\pm$ 3.2 nM; no significant difference between groups was found (*p* = 0.348).

Single-marker analysis revealed no association between *MTHFR* C677T polymorphism and TS, even when genotype (*p* = 0.063) or allelic (*p* = 0.277) distribution was considered. Regarding *MTHFR* A1298C polymorphism, a statistical dif-

ference was found between patients with TS and controls, both for genotype (*p* < 0.0001) and for allele (*p* < 0.0001) frequencies.

The genotypes of C677T were in Hardy–Weinberg equilibrium in the TS group (*p* = 0.987); however, the control group deviated from Hardy–Weinberg equilibrium for this polymorphism (*p* < 0.001). Considering the distribution of A1298C genotypes, patients with TS (*p* = 0.151) and controls (*p* = 0.692) were in Hardy–Weinberg equilibrium.

Combined genotypes of *MTHFR* polymorphisms, C677T and A1298C, identified 2 haplotypes—CC and TC—associated with TS (*p* < 0.001 and *p* = 0.0165, respectively). However, homocysteine levels were not higher in patients with haplotype risk. In contrast, the haplotype CA was more prevalent in controls than in patients (haplotype frequency of 39.3% in patients and 55.7% in controls; *p* < 0.001) (Table 3).

Discussion

We hypothesized that the *MTHFR* C677T and A1298C polymorphisms might be involved in the homocysteine serum levels in patients with TS. To our knowledge, this is the first study of this possible association in TS. The results disclosed no statistical difference between homocysteine serum levels in the TS and control groups. The C677T and A1298C polymorphisms of *MTHFR* gene are not related to homocysteine levels in Brazilian patients with TS, despite the differential distribution of the mutated allele C (A1298C) in these patients. The TS group was in Hardy–Weinberg equilibrium for both C677T and A1298C polymorphisms. Nevertheless, the distribution of C677T genotypes deviated from Hardy–Weinberg equilibrium in the control group.

This fact can be explained by several factors: (1) gene mutations, which can be considered important tools in the natural selection; (2) migration population resulting in imbalanced gametes, which can alter the allelic distribution of certain genes; and (3) genetic drift, in which random fluctuations can cause the disappearance of 1 allele in a small number of people

TABLE 3. HAPLOTYPE ANALYSIS OF 2 *MTHFR* POLYMORPHISMS, C677T AND A1298C, IN PATIENTS WITH TURNER SYNDROME AND CONTROLS

Haplotype	Frequency in control group	Frequency in Turner syndrome group ^a	p value
CA	0.557	0.393	<0.001
TA	0.240	0.248	0.829
CC	0.175	0.293	<0.001
TC	0.028	0.066	0.0165

^aHaplotype order is *MTHFR* 677 C:T e 1298 A:C.

that form a sample (Song and Elston, 2006). Brazil is a heterogeneous nation composed of diverse populations, which may be associated with the imbalance observed in this sample.

Recently, several studies have sought to identify new risk factors and prevent the occurrence of CVD in both the healthy population and patients with TS. Atherosclerosis, a common framework in cardiac patients, is the leading cause of stroke. In 1969, McCully reported an association between atherosclerosis and high concentrations of homocysteine, data subsequently confirmed by other studies (Brattström *et al.*, 1994; Graham *et al.*, 1997; Muniz *et al.*, 2006).

Several studies have attempted to elucidate which mechanisms are responsible for the imbalance in homocysteine metabolism. In 1995, Nygard *et al.* reported a significant difference among plasma homocysteine levels in men and women, confirming the results of earlier, smaller studies and demonstrating a protective role of female hormones on homocysteine levels (Vester *et al.*, 1991; Brattström *et al.*, 1994; Jacobsen *et al.*, 1994).

Menopausal women have plasma homocysteine levels similar to those in men, and low levels are observed during pregnancy in patients who have undergone hormone replacement therapy (Brattström *et al.*, 1992; van der Mooren *et al.*, 1994; Anker *et al.*, 1995). No recent study has addressed the role of hormones in plasma homocysteine. However, this is an important factor in our study because most patients with TS have estrogen deficiency (95% to 98% of women with TS) due to gonadal dysgenesis (Gravholt, 2004). We observed no elevated levels of homocysteine, despite estrogen deficiency, which can be explained by the hormone replacement therapy that such patients receive and by their young age (mean age, 25.0 ± 10.5 years). Age seems to interfere with homocysteine metabolism; significant increases have been reported from the fourth decade of life on.

Some authors have hypothesized the association of *MTHFR* variants, such as C677T and A1298C polymorphisms, with CVD (due to the influence of such polymorphisms on the plasma concentration of homocysteine), with conflicting results. Muniz *et al.* (2006) found an association between high levels of homocysteine and CVD but no relationship between the mutant T allele and plasma levels of homocysteine; these data were later confirmed by Guerzoni *et al.* (2009). On the other hand, Harmon *et al.* (1996) demonstrated a relationship between the 677TT genotype and elevated levels of homocysteine; Spotilla *et al.* later confirmed these findings (2003).

In a previous study conducted to clarify the mechanism of nondisjunction common in TS, our group observed a higher frequency of A1298C polymorphism in these patients, sug-

gesting possible contributions of the C allele in this mechanism (Oliveira *et al.*, 2008). In the present study, there was a significant distinction in the distribution of polymorphic C allele ($p < 0.0001$) between the TS and the control groups, but no change was seen in homocysteine levels. The A1298C polymorphism has previously been associated with decreased enzymatic activity and consequent increase in homocysteine plasma levels, being associated with an increased risk for hypertension and cardiovascular disease. However, the results of different studies are conflicting and inconsistent. Some authors suggest that these results are derived from differences in linkage disequilibrium in the *MTHFR* locus and from the diet of different populations (Weisberg *et al.*, 1998; Ye *et al.*, 2004; Kumar *et al.*, 2005).

In conclusion, our results suggest that C677T and A1298C polymorphisms are not related to high levels of homocysteine in Brazilian patients with TS, despite the differential distribution of the mutated allele C (A1298C) in these patients. Further studies are needed to investigate the possible gene interactions in homocysteine levels and thus in atherosclerosis.

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Disclosure Statement

No competing financial interests exist.

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