Association between methylene tetrahydrofolate reductase and glutathione S-transferase M1 gene polymorphisms and chronic myeloid leukemia in a Brazilian population

G.S. Lordelo[1,2], A.L. Miranda-Vilela[1], A.K. Akimoto[1], P.C.Z. Alves[1], C.O. Hiragi[1], A. Nonino[2,3], M.B. Daldegan[2], M.N. Klautau-Guimarães[1] and C.K. Grisolia[1]

[1]Laboratório de Genética, Departamento de Genética e Morfologia, Instituto de Ciências Biológicas, Universidade de Brasília, Brasília, DF, Brasil
[2]Serviços de Hematologia e Hemoterapia do Hospital de Base do Distrito Federal, Brasília, DF, Brasil
[3]Centro de Oncologia e Hematologia de Brasília, Brasília, DF, Brasil

Corresponding author: A.L. Miranda-Vilela
E-mail: mirandavilela@unb.br

ABSTRACT. Chronic myeloid leukemia is a hematopoietic stem cell disorder that causes uncontrolled proliferation of white blood cells. Although the clinical and biological aspects are well documented, little is known about individual susceptibility to this disease. We conducted a case-control study analyzing the prevalence of the polymorphisms *MTHFR* C677T, *MTHFR* A1298C, del{*GSTM1*}, del{*GSTT1*}, and haptoglobin in 105 patients with chronic myeloid leukemia (CML) and 273 healthy controls, using PCR-based methods. A significant association with risk of developing CML was found for *MTHFR* 1298AA (odds ratio (OR) = 1.794; 95% confidence interval (CI) = 1.14-2.83) and *GSTM1* non-null (OR = 1.649; 95%CI = 1.05-2.6) genotypes, while *MTHFR* 1298AC (OR = 0.630; 95%CI = 0.40-

0.99) and *GSTM1* null (OR = 0.606; 95%CI = 0.21-0.77) genotypes significantly decreased this risk. There appeared to be selection for heterozygosity at the *MTHFR* 1298 locus. The considerable range of variation in this and other human populations may be a consequence of distinctive processes of natural selection and adaptation to variable environmental conditions. The Brazilian population is very mixed and heterogeneous; we found these two loci to be associated with CML in this population.

Key words: Chronic myeloid leukemia; Hardy-Weinberg equilibrium; Genetic polymorphisms; Brazilian population; Association study

INTRODUCTION

Chronic myeloid leukemia (CML) was the first cancer to be associated with a specific cytogenetic marker, the Philadelphia chromosome, resulting from a reciprocal translocation between chromosomes 9 and 22 [t(9;22)(q34,q11)]. This translocation transposes the c-abl proto-oncogene from chromosome 9 to the breakpoint cluster region (bcr) on chromosome 22, and this new hybrid *bcr-abl* oncogene encodes a constitutively active tyrosine-kinase that promotes the growth advantage of leukemia cells through enhanced proliferation and reduced apoptosis, generating genomic instability (Nowicki et al., 2004; Kim et al., 2009).

Although these clinical and biological aspects are well documented, little is known about individual susceptibility to CML. Polymorphic variants of several genes, diet, environmental exposure to carcinogens and individual immune system's characteristics are potential factors that increase predisposition to leukemia (Bowen et al., 2003). Association studies have been performed to identify genetic variants associated with CML susceptibility, and among them are methylenetetrahydrofolate reductase (MTHFR) (Hur et al., 2006; Moon et al., 2007; Barbosa et al., 2008), glutathione S-transferases (GSTs) (Hishida et al., 2005; Souza et al., 2008), and haptoglobin (Hp) gene polymorphisms (Nevo and Tatarsky, 1986; Campregher et al., 2004).

MTHFR, a key enzyme in homocysteine (Hcy) and folate metabolism, plays a role in DNA methylation and provision of nucleotides for DNA synthesis (Ulrich et al., 1999). Mutations in the MTHFR gene may result in enzyme deficiency, low plasma folate levels and hyperhomocysteinemia, a risk factor for coronary and peripheral vascular obstructive events and neural tube defects (Franco et al., 1999). However, under situations of decreased MTHFR activity, more MTHFR substrate is available for purine and pyrimidine synthesis, which may prevent imbalances of nucleotide pools during DNA synthesis and potentially oncogenic alterations in DNA methylation (Ma et al., 1997; Ulrich et al., 1999). Associations have been described between the common MTHFR polymorphisms C677T and A1298C and risk of leukemia (Skibola et al., 1999; Franco et al., 2001; Robien and Ulrich, 2003; Robien et al., 2004; Ismail et al., 2009), but few studies with CML have been published, with inconsistent results (Robien and Ulrich, 2003; Robien et al., 2004; Hur et al., 2006; Kim et al., 2007; Moon et al., 2007; Barbosa et al., 2008; Ismail et al., 2009). Therefore, studies in several ethnicities are required to better understand the biological significance of these

polymorphisms in CML susceptibility, since they vary among ethnic groups (Franco et al., 1999; Robien and Ulrich, 2003).

The GSTs M1 and T1 genes encode, respectively, for enzymes GST-μ (mu) and GST-θ (theta), which detoxify environmental carcinogens by glutathione conjugation (Cotton et al., 2000; Souza et al., 2008). Because these genes may be deleted, resulting in deficiency of GST activities (Hengstler et al., 1998) and in diminished ability to detoxify various carcinogens (Crump et al., 2000), environmental exposure to cytotoxic and genotoxic agents could increase CML risk for individuals carrying GST deletions. Indeed, an increased GSTT1 null genotype frequency has been observed in CML patients (Mondal et al., 2005), and GSTM1 polymorphism has been suggested as important in CML etiology (Lourenço et al., 2005). Given interethnic and intra-ethnic differences in GST allele frequencies (Cotton et al., 2000; Landi, 2000), association studies between GSTM1/T1 polymorphisms with CML risk in different ethnicities may help us to better understand the heterogeneity of the disease and of patients' response to treatment.

Susceptibility to leukemia may also be influenced by Hp, a serum glycoprotein that binds free hemoglobin, preventing DNA damage by reactive oxygen species (ROS) (Carter and Worwood, 2007), among other effects. It is known that the *BCR/ABL* expression stimulates ROS production in hematopoietic progenitor cells (Sattler et al., 2000) and studies have suggested that ROS may contribute to increased DNA damage in CML cells (Nowicki et al., 2004). Because the ability of Hp to block hemoglobin-induced oxidative stress and damage is phenotype dependent (Carter and Worwood, 2007), Hp polymorphism has been investigated in association with leukemia, and the Hp1-1 phenotype has been associated with CML, acute myeloid leukemia (AML) and acute lymphoblastic leukemia (ALL) (Nevo and Tatarsky, 1986; Mitchell et al., 1988). However, the frequency distributions of Hp phenotypes and alleles vary among populations worldwide (Carter and Worwood, 2007), so it is important to run further association studies with CML in different ethnic groups to better evaluate these risks.

The Brazilian population originated from five centuries of interethnic crosses among Europeans, Africans and Amerindians (Alves-Silva et al., 2000). Because this miscegenation can influence the distribution of certain polymorphisms, we aimed to investigate, through a case-control study, a possible association between CML and MTHFR C677T, MTHFR A1298C, del{GSTM1}, del{GSTT1} and Hp polymorphisms.

MATERIAL AND METHODS

Study design and participants

This study consisted of 105 adult CML outpatients of both genders, defined by Philadelphia chromosome and/or *bcr-abl* presence, in chronic or accelerated phases (blastic crises or Ph-positive ALL were excluded) and treated in the Hospital de Base (HBDF) in Brasília, Brazil. The control group consisted of 273 non-related healthy volunteers of both genders, recruited in Brasília, among University students, company workers and at HBDF.

This study was conducted under guidelines laid down in the Declaration of Helsinki, and all procedures involving human subjects were approved by the Ethics Committee of the State Health Secretariat of the Federal District (SESDF). Written informed consent was obtained from all registered volunteers.

Procedures and measurements

About 5 mL peripheral blood was collected by venipuncture using Vacutainer tubes with EDTA as anticoagulant for the analyses of gene polymorphisms. Blood samples were centrifuged at 1200 *g* for 5 min at 4°C. The plasma was removed and DNA was isolated from the buffy-coat (leukocyte fraction) by using a Blood genomicPrep Mini Spin Kit (GE Healthcare, Buckinghamshire, England) purification kit. DNA samples were stored below -20°C until analysis and then underwent amplification in an MJ PTC-100 (MJ Research Inc., Waltham, MA, USA).

Genotyping assay

MTHFR C677T and MTHFR A1298C genotypes were determined by multiplex polymerase chain reaction (PCR)-based restriction fragment length polymorphism (RFLP) assays performed as described by Yi et al. (2002). The GSTM1 and GSTT1 fragments were amplified simultaneously as proposed by Chen et al. (1996), using β-globin as positive control. The absence of an amplification product combined with the presence of a positive control band (268-bp DNA fragment of β-globin) indicated the null variants for both polymorphisms. Hp genotypes were determined by allele-specific PCR as described by Yano et al. (1998).

Statistical analyses

To verify possible differences in the genotype frequencies between cases and controls, considering the total groups, gender, age groups and skin color, statistical analyses were carried out using SPSS (Statistical Package for the Social Sciences) version 15.0. The continuous variables were tested for normal distribution with Shapiro-Wilk. Possible differences between the two groups were evaluated by the Mann-Whitney U-test, since the data presented heterogeneous variability; values of $P < 0.05$ were considered to be statistically significant.

For the total group and the genetic markers, possible correlations between the parameters gender/age groups, gender/skin color and age groups/skin color were analyzed through the chi-square (χ^2) correlation test. The odds ratio (OR) with 95% confidence intervals (CI) was calculated and P values were generated by the Pearson chi-square test. Differences were considered to be significant at $P < 0.05$.

Allelic and genotypic frequencies were estimated by gene counting, and the genotype distribution to Hardy-Weinberg equilibrium (HWE) was assessed by the χ^2 test. Values of P > 0.05 indicated HWE. Data for genetic diversity were assessed by comparing the observed and expected heterozygosities; F-statistics, linkage disequilibrium test for MTHFR C677T and A1298C and for MTHFR and GSTM1 polymorphisms, and probability (P) values were generated using the Genepopweb Statistical Program version 4.1 (http://genepop.curtin.edu.au). The χ^2 test was used to compare the genotype frequencies between cases and controls. For dominant markers (GSTM1 and GSTT1), fixation index (F_{ST} - F-statistics) was calculated by Arlequin 3.1.1. Since the PCR method is not suitable for distinguishing between homozygous (+/+, wild type) and heterozygous (+/-), these two groups were considered together (non-null genotypes) and compared with the variant group (null genotypes).

RESULTS

Comparison of the samples from CML and control groups

The distribution frequencies of MTHFR C677T, GSTT1, and Hp genotypes were similar for control and CML groups, but not for MTHFR A1298C and GSTM1. For MTHFR A1298C, the AA genotype frequency was higher in the CML group and the genotypes containing the variant allele (AC and CC) predominated in the control (P = 0.007). For GSTM1, the null genotype presented a significantly higher frequency in the control, while the non-null genotype predominated in the CML group (P = 0.029). For both polymorphisms, these differences were particularly related to females (P = 0.029 for MTHFR A1298C and P = 0.005 for GSTM1) (Table 1).

Table 1. Distribution of MTHFR C677T and A1298C, GSTM1, GSTT1, and haptoglobin (Hp) genotypes by total groups for control and chronic myeloid leukemia (CML) and by gender.

Genetic marker	Total		Female		Male	
	Control	CML	Control	CML	Control	CML
MTHFR C677T						
CC	140 (51.3)	46 (43.8)	68 (24.9)	25 (23.8)	72 (26.4)	21 (20)
CT	114 (41.8)	47 (44.8)	65 (23.8)	22 (21.0)	49 (18.0)	25 (23.8)
TT	19 (7)	12 (11.4)	12 (4.4)	4 (3.8)	7 (2.6)	8 (7.6)
P values	0.125		0.797		0.015	
MTHFR A1298C						
AA	119 (43.6)	61 (58.1)	54 (19.8)	28 (26.7)	65 (23.8)	33 (31.4)
AC	143 (52.4)	43 (41)	86 (31.5)	22 (21.0)	57 (20,9)	21 (20)
CC	11 (4)	1 (1)	5 (1.8)	1 (1.0)	6 (2.2)	0 (0)
P values	0.007		0.029		0.139	
GSTM1						
M1-	176 (64.5)	55 (52.4)	95 (34.8)	22 (21)	81 (29.7)	33 (31.4)
M1+	97 (35.5)	50 (47.6)	50 (18.3)	29 (27.6)	47 (17.2)	21 (20)
P values	0.029		0.005		0.738	
GSTT1						
T1-	65 (23.8)	21 (20)	32 (11.7)	7 (6.7)	33 (12.1)	14 (13.3)
T1+	208 (76.2)	84 (80)	113 (41.4)	44 (41.9)	95 (34.8)	40 (38.1)
P values	0.421		0.196		0.984	
Hp						
1F-1F	16 (5.9)	4 (3.81)	12 (4.4)	1 (0.95)	4 (1.5)	3 (2.86)
1F-1S	18 (6.6)	12 (11.43)	9 (3.3)	7 (6.67)	9 (3.3)	5 (4.76)
1S-1S	28 (10.3)	5 (4.76)	13 (4.8)	3 (2.86)	15 (5.5)	2 (1.9)
1F-2	36 (13.2)	13 (12.38)	22 (8.1)	6 (5.71)	14 (5.1)	7 (6.67)
1S-2	93 (34.1)	32 (30.48)	46 (16.8)	16 (15.24)	47 (17.2)	16 (15.24)
2-2	82 (30)	39 (37.14)	43 (15.8)	18 (17.14)	39 (14.3)	21 (20)
P values	0.402		0.455		0.681	

Data are reported as number with percent in parentheses. P values were generated by the Mann-Whitney U-test, using SPSS (Statistical Package for the Social Sciences), version 15.0.

MTHFR A1298C also showed significant difference between the control and CML groups for age 20-40 years (P = 0.022), as did GSTM1 for those over 60 (P = 0.006) (Table 2). Significant differences in the genotype distribution by skin color were observed only for MTHFR A1298C polymorphism, where AC and CC genotypes were predominant in the white individuals of the control group (P = 0.017) (Table 3).

Table 2. Distribution of MTHFR C677T and A1298C, GSTM1, GSTT1, and haptoglobin (Hp) genotypes of the control and chronic myeloid leukemia (CML) by age groups.

Genetic marker	20-40 years		41-60 years		>60 years	
	Control	CML	Control	CML	Control	CML
MTHFR C677T						
CC	91 (33.3)	15 (14.3)	39 (14.3)	23 (21.9)	10 (3.7)	8 (7.6)
CT	73 (26.7)	16 (15.2)	32 (11.7)	19 (18.1)	9 (3.3)	12 (11.4)
TT	14 (5.1)	5 (4.8)	4 (1.5)	5 (4.8)	1 (0.8)	2 (1.9)
P values	0.219		0.571		0.352	
MTHFR A1298C						
AA	79 (28.9)	23 (21.9)	31 (11.4)	25 (23.8)	9 (3.3)	13 (12.4)
AC	90 (33.0)	13 (12.4)	42 (15.4)	21 (20)	11 (4.0)	9 (8.6)
CC	9 (3.3)	0 (0)	2 (0.7)	1 (1.0)	0 (0)	0 (0)
P values	0.022		0.209		0.367	
GSTM1						
M1-	113 (41.4)	22 (21)	49 (17.9)	27 (25.7)	14 (5.1)	6 (5.7)
M1+	65 (23.8)	14 (13.3)	26 (9.5)	20 (19)	6 (2.2)	16 (15.2)
P values	0.788		0.342		0.006	
GSTT1						
T1-	45 (16.5)	10 (9.5)	15 (5.5)	10 (9.5)	5 (1.8)	1 (1)
T1+	133 (48.7)	26 (24.8)	60 (22)	37 (35.2)	15 (5.5)	21 (20)
P values	0.770		0.866		0.062	
Hp						
1F-1F	14 (5.1)	2 (1.9)	2 (0.7)	2 (1.9)	0 (0)	0 (0)
1F-1S	9 (3.3)	5 (4.76)	9 (3.3)	4 (3.81)	0 (0)	3 (2.86)
1S-1S	22 (8.1)	1 (0.95)	4 (1.5)	4 (3.81)	1 (0.4)	0 (0)
1F-2	27 (9.9)	5 (4.76)	9 (3.3)	6 (5.71)	0 (0)	2 (1.9)
1S-2	55 (20.1)	9 (8.57)	27 (9.9)	14 (13.33)	8 (2.9)	9 (8.57)
2-2	51 (18.7)	14 (13.33)	24 (8.8)	17 (16.19)	7 (2.6)	8 (7.62)
P values	0.591		0.816		0.575	

Data are reported as number with percent in parentheses. P values were generated by the Mann-Whitney U-test, using SPSS (Statistical Package for the Social Sciences), version 15.0.

No correlations were found for CML or control for all analyzed parameters. Significantly increased CML risk was shown for MTHFR 1298AA (OR = 1.794; 95%CI = 1.14-2.83) and GSTM1 non-null (OR = 1.649; 95%CI = 1.05-2.6; P = 0.031). For MTHFR 1298AA, this risk was particularly related to females (OR = 2.052; 95%CI = 1.08-3.92; P = 0.028), age group of 20-40 years (OR = 1.980; 95%CI = 1.06-4.65; P = 0.047) and white skin color (OR = 2.218; 95%CI = 1.09-4.51; P = 0.026); for GSTM1, it was related to females (OR = 2.505; 95%CI = 0.21-0.77; P = 0.031) and the group >60 years (OR = 6.222; 95%CI = 1.63-23.76; P = 0.006). Hp1F-1S individuals of black skin color also presented an increased risk (OR = 7.200; 95%CI = 0.94-54.94; P = 0.037). A decreased CML risk was observed for MTHFR 1298AC (OR = 0.630; 95%CI = 0.40-0.99; P = 0.047) and GSTM1 null (OR = 0.606; 95%CI = 0.21-0.77; P = 0.031), and both were also related to females (OR = 0.520; 95%CI = 0.27-0.99; P = 0.046 for MTHFR, and OR = 0.399; 95%CI = 0.21-0.77; P = 0.005 for GSTM1). For GSTM1 null, it was also related to the group >60 years (OR = 0.161; 95%CI = 0.04-0.61; P = 0.006). For males, a decreased risk was observed only for MTHFR 677CC (OR = 0.495; 95%CI = 0.26-0.95; P = 0.032). A decreased risk was also observed for Hp1S-2 individuals of black skin color (OR = 0.619; 95%CI = 0.44-0.87; P = 0.011).

Table 3. Distribution of MTHFR C677T and A1298C, GSTM1, GSTT1, and haptoglobin (Hp) genotypes of the control and chronic myeloid leukemia (CML) by skin color.

Genetic marker	White		Mulatto ("Pardo")		Black	
	Control	CML	Control	CML	Control	CML
MTHFR C677T						
CC	74 (27.1)	15 (14.39)	46 (16.9)	25 (23.8)	20 (7.3)	6 (5.7)
CT	66 (24.2)	21 (20)	42 (15.4)	24 (22.9)	6 (2.2)	2 (1.9)
TT	15 (5.5)	5 (4.8)	3 (1.1)	7 (6.7)	1 (0.4)	0 (0)
P values		0.215		0.243		0.918
MTHFR A1298C						
AA	68 (24.9)	26 (24.8)	36 (13.2)	29 (27.6)	15 (5.5)	6 (5.7)
AC	79 (28.9)	15 (14.3)	54 (19.8)	26 (24.8)	10 (3.7)	2 (1.9)
CC	8 (2.9)	0 (0)	1 (0.4)	1 (1.0)	2 (0.7)	0 (0)
P values		0.017		0.171		0.295
GSTM1						
M1-	101 (37)	21 (20)	61 (22.3)	31 (29.5)	14 (5.1)	3 (2.9)
M1+	54 (19.8)	20 (19)	30 (11)	25 (23.8)	13 (4.8)	5 (4.8)
P values		0.102		0.139		0.482
GSTT1						
T1-	37 (13.6)	5 (4.8)	24 (8.8)	16 (15.2)	4 (1.5)	0 (0)
T1+	118 (43.2)	36 (34.3)	67 (24.5)	40 (38.1)	23 (8.4)	8 (7.6)
P values		0.106		0.804		0.254
Hp						
1F-1F	7 (2.6)	2 (1.9)	8 (2.9)	1 (0.95)	1 (0.4)	1 (0.95)
1F-1S	9 (3.3)	2 (1.9)	7 (2.6)	7 (6.67)	2 (0.7)	3 (2.86)
1S-1S	11 (4)	2 (1.9)	13 (4.8)	3 (2.86)	4 (1.5)	0 (0)
1F-2	24 (8.8)	5 (4.76)	11 (4)	6 (5.71)	1 (0.4)	2 (1.9)
1S-2	54 (19.8)	13 (12.38)	26 (9.5)	19 (18.1)	13 (4.8)	0 (0)
2-2	51 (18.7)	17 (16.19)	26 (9.5)	20 (19.05)	5 (1.8)	2 (1.9)
P values		0.342		0.209		0.067

Data are reported as number with percent in parentheses. P values were generated by the Mann-Whitney U-test, using SPSS (Statistical Package for the Social Sciences), version 15.0.

Population statistics

Significant deviations from HWE were observed for MTHFR A1298C in both groups (P = 0.0000 for controls and P = 0.0374 for CML), for GSTM1 and GSTT1 (P = 0.000 for both), and for Hp (P = 0.0022) in the control group. For MTHFR A1298C, this was compatible with heterozygote excess (P = 0.000 for control and P = 0.0217 for CML); for GSTs, with heterozygote deficit, probably due to wild-type homozygous and heterozygous being considered together within non-null genotypes. For Hp, deviation from HWE was in conformity with heterozygote deficiency in the control group (P = 0.0374) (Tables 4 and 5). In the two groups a strong linkage disequilibrium was detected between MTHFR C677T and A1298C loci (P < 0.001 for control and CML), but not between MTHFR (C677T or A1298C) and GSTM1 loci.

Results of F-statistics indicated a slight genetic differentiation for MTHFR 1298 (F_{ST} = 0.0166) and GSTM1 (F_{ST} = 0.0238) loci, being in conformity with selection in favor of heterozygotes for MTHFR 1298 locus (F_{IS} = -0.2334; F_{IT} = -0.2130). However, no sub-structuring was observed (F_{ST} = 0.0088) for the population. Homogenous distribution of MTHFR C677T, GSTT1 and Hp genotypes in both groups, and heterogeneous distribution for MTHFR A1298C (P = 0.0221) and GSTM1 (P = 0.0308) loci were observed (Table 5).

Table 4. Distribution of MTHFR C677T and A1298C, GSTM1, GSTT1, and haptoglobin (Hp) allele frequencies, intrapopulation inbreeding coefficient (F_{is}) and Hardy-Weinberg equilibrium (HWE) data for the chi-square (χ^2) test.

Alleles	Allele frequencies		Genotypes	Number of observed individuals		Number of expected individuals		F_{is}		HWE test (P values)	
	Control	CML		Control	CML	Control	CML	Control	CML	Control	CML
MTHFR C677T			MTHFR C677T								
C	0.72	0.66	CC	140	46	142.06	45.89	-0.0375	0.0047	0.6491	1.0000
T	0.28	0.34	CT	114	47	109.89	47.22				
			TT	19	12	21.06	11.89				
MTHFR A1298C			MTHFR A1298C								
A	0.70	0.79	AA	119	61	132.83	64.74	-0.2403	0.2116	0.0000	0.0374
C	0.30	0.21	AC	143	43	115.35	35.53				
			CC	11	1	24.83	4.74				
GSTM1			GSTM1								
M1-	0.64	0.52	Null	176	50	113.35	28.68	-	-	0.0000	0.0000
M1+	0.36	0.48	Non-null	97	55	159.65	76.31				
GSTT1			GSTT1								
T1-	0.24	0.20	Null	65	21	15.39	4.12	-	-	0.0000	0.0000
T1+	0.76	0.80	Non-null	208	84	257.61	100.88				
Hp			Hp								
1F	0.16	0.16	1F-1F	16	4	6.72	2.53	0.0948	0.0459	0.0022	0.1371
1S	0.30	0.26	1F-1S	18	12	26.35	8.53				
2	0.54	0.58	1S-1S	28	5	25.43	6.84				
			1F-2	36	13	46.23	19.42				
			1S-2	93	32	89.78	30.78				
			2-2	82	39	78.49	35.90				

P < 0.05 indicates deviation from HWE. Data were generated using the Genepopweb version 4.1 statistical program (http://genepop.curtin.edu.au). For dominant markers, GSTM1 and GSTT1 genotypes were considered: Null = -/-; Non-null = +/+ and +/-, since the PCR method was not suitable for distinguishing between homozygous (+/+, wild type) and heterozygous (+/-). CML = chronic myeloid leukemia.

Table 5. Genetic diversity data from the control and chronic myeloid leukemia (CML) groups, F-statistics and heterogeneity test of chi-square (χ^2) for MTHFR C677T and A1298C, GSTM1, GSTT1 and haptoglobin (Hp) polymorphisms.

Genetic marker	Observed heterozygosity		Expected heterozygosity		F_{IS}	F_{ST}	F_{IT}	Heterogeneity test (P values)
	Control	CML	Control	CML				
MTHFR C677T	0.4176	0.4476	0.4032	0.4488	-0.0249	0.0053	-0.0195	0.2384
MTHFR A1298C	0.5238	0.4095	0.4200	0.3318	-0.2334	0.0166	-0.2130	0.0221
GSTM1	-	-	-	-	-	0.0238	-	0.0308
GSTT1	-	-	-	-	-	-0.0024	-	0.4288
Hp	0.5385	0.5429	0.5928	0.5704	0.0817	0.0005	0.0822	0.2215
Total					-0.0396	0.0088	-0.0327	

For co-dominant markers, F-statistics data were generated by the Genepopweb version 4.1 statistical program (http://genepop.curtin.edu.au). For dominant markers GSTM1 and GSTT1, F_{ST} data were generated by the Arlequin v. 3.1.1 statistical program. For these markers, Null = -/-; Non-null = +/+ and +/- were considered, since the PCR method was not suitable for distinguishing between homozygous (+/+, wild type) and heterozygous (+/-).

DISCUSSION

Populations worldwide vary considerably in their predisposition to diseases and in the allele frequencies of pharmacogenetically important loci, probably due to genetic drift or adaptation to local selective factors such as climate and available nutrients (Suarez-Kurtz, 2004), and our results corroborate this. MTHFR catalyzes the irreversible conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate (Duthie et al., 2002; Moon et al., 2007), serving as methyl donor in remethylation of Hcy to methionine, which in turn is converted to S-adenosylmethionine (SAM) that methylates specific cytosines in DNA, regulating gene transcription (Duthie et al., 2002).

In this study, the MTHFR 1298 wild-type genotype (AA) was linked to significantly increased risk of developing CML, mainly for young adult females, while 1298AC significantly decreased this risk; no association was found for the C677T polymorphism, although a lower risk was shown for men carrying the 677CC genotype. The A1298C polymorphism lies in the SAM-regulatory domain of the enzyme, and folate affects gene expression, regulating cellular SAM levels (Duthie et al., 2002). Bearing this in mind, results support the idea that, at least for our population, MTHFR 1298 heterozygosis may give an adaptive advantage to individuals, possibly making its carriers less sensitive to folate necessity to regulate gene transcription than the carriers of the wild-type genotype.

Because SAM regulates gene transcription through methylation, as a consequence of folate deficiency, SAM is depleted, inducing DNA hypomethylation and, potentially, proto-oncogene expression leading to cancer (Duthie et al., 2002). Although this study did not assess folate or Hcy levels, neither the 1298CC nor the heterozygous state has been associated with higher plasma Hcy or lower plasma folate concentration, phenomena evident with homozygosity 677TT (van der Put et al., 1998). Moreover, a decreased CML risk in Asians (Hur et al., 2006) and fewer relapses after bone marrow transplant in Caucasians (Robien et al., 2004) have been observed for the 1298AC genotype.

The C allele frequency of MTHFR 1298 ranges from 0.17-0.19 among Asians to 0.27-0.36 in Western Europe. Little is known about A1298C allele frequencies among African or South American populations (Robien and Ulrich, 2003), justifying the importance of this

study. The C allele frequency of our control group (0.30) was within frequencies reported in Western Europe, while for the CML group, this (0.21) was close to those found in South Africa (Robien and Ulrich, 2003), despite an increased risk for white-skinned 1298AA carriers. Miscegenation in Brazil may have influenced distribution of this polymorphism. Consequently, identifying genetic components that can contribute to developing CML in the Brazilian population may help increase understanding of variability in disease course and in patients' response to medication in our population, improving treatment strategies. Because the Federal District was formed by migrants from all Brazilian regions (Queiroz, 2006), its population reflects the overall constitution of the Brazilian population better than populations of other Brazilian regions.

Variation in human populations may reflect natural selection and adaptation to environmental conditions (Barreiro et al., 2008). Deviations from HWE might be explained by natural selection or recent ethnic admixture (Barreiro et al., 2008), but the latter is not the case here. Instead, significant deviation from HWE for the MTHFR 1298 locus was compatible with heterozygote excess, indicating that heterozygosis may provide an adaptive advantage. Moreover, MTHFR C677T and A1298C have been found to be in strong linkage disequilibrium (Robien and Ulrich, 2003), corroborating our results; nevertheless, no association with CML was found for MTHFR 677 locus.

Population growth and positive selection increase the proportion of rare alleles, whereas balancing selection and population substructure increases the proportion of intermediate alleles (Serre and Hudson, 2006). In this study, control and CML groups were genetically homogeneous for MTHFR C677T, GSTT1 and Hp loci. Moreover, the fixation index (F_{ST}), which measures the degree of genetic differentiation of subpopulations (Nei, 1977), indicated no sub-structuring of the Brazilian population under study, despite the slight genetic differentiation for MTHFR 1298 and GSTM1 loci, which reflect the associations found.

Natural selection can act at the level of genes, if particular genotypes allow for increased fitness in specific environments. Genes under positive selection are thought to have an important role in human survival and to affect complex phenotypes of medical relevance (Barreiro et al., 2008). In this study, an association with the risk of developing CML was found for MTHFR 1298AA and GSTM1 non-null genotypes in all statistical tests performed, particularly for women, and these findings are supported by the literature. Because MTHFR directs the flux of intracellular folate toward the conversion of Hcy to methionine at the expense of nucleotide synthesis (Robien et al., 2004), folate deficiency has been associated with uracil misincorporation into DNA and DNA double-strand breaks during uracil excision repair, thus increasing the risk of chromosomal aberrations (Robien and Ulrick, 2003). Moreover, folate deficiency, as observed by alcohol consumption, affects cellular SAM and Hcy levels (Ma et al., 1997; Duthie et al., 2002), and Hcy has been suggested as a marker for cancer (Ozkan et al., 2007). SAM may act as a physiological antidote against estrogen hepatobiliary toxicity in susceptible women (Frezza et al., 1988), and increased total Hcy may decrease folate levels and total glutathione (Ozkan et al., 2007), a tripeptide essential for removal of hydrogen and lipid peroxides from cells by glutathione peroxidases and for detoxifying environmental carcinogens by GSTs (Rover Júnior et al., 2001).

Our study used a PCR method that did not distinguish between non-null homozygous and heterozygous in GST genotyping, but this is a standard method in the literature, with some variations. Moreover, the GSTM1 non-null genotype has already been reported in a higher

frequency in CML patients than in controls among Japanese (Hishida et al., 2005). None of the published articles have performed OR or association studies using GSTM1 non-null geno-type; they have only analyzed the CML risk for the GSTM1 null genotype or in combination with GSTT1 (Hishida et al., 2005; Lourenço et al., 2005; Mondal et al., 2005; Souza et al., 2008). This may be because it is unbelievable that the presence of an enzyme, which operates in carcinogen detoxification, could increase cancer risk, while its lack could protect against it. However, there are also two other alleles at the GSTM1 locus, GSTM1a and GSTM1b, which differ by a substitution in one base pair (Cotton et al., 2000). Although it is believed that there are no functional differences between them (Cotton et al., 2000), these assumptions may be unjustified, since individuals with two positive alleles may be better protected than GSTM1 heterozygotes for null genotypes (Fryer et al., 1993), which could justify the association found in this study. Thus, this needs further investigation.

Moreover, besides interethnic differences in GSTM1 allele frequencies, there are also intra-ethnic differences. In healthy individuals, GSTM1 homozygous nulls have been reported in 22-67% of individuals worldwide in different ethnicities, with lower frequencies in African Americans and Indians and higher frequencies in Caucasians and Asians (Rebbeck, 1997; Hira-gi et al., 2011), although 64-100% have been reported in Oceania (Rebbeck, 1997; Cotton et al., 2000). In Brazilian Afro-descendants, these frequencies are 47-58% (Rebbeck, 1997; Hiragi et al., 2011); in Amerindians, 52-55% (Hiragi et al., 2011), and in urban Brazilian populations, 35-63% (Maciel et al., 2009; Miranda-Vilela et al., 2010; Hiragi et al., 2011).

Although the GSTM1 locus is located near the MTHFR locus, there was no linkage disequilibrium between these loci. However, selection in another gene located close to the GSTM1 gene cannot be discarded. The GSTM1 locus (1p13.3) is close to the NRAS locus (1p13.2), a proto-oncogene in the p21 RAS subfamily of small GTPases, which regulates cell proliferation, cytoskeletal organization, and other signaling networks and is the most frequent target of activating mutations in cancer (Oliveira et al., 2007). The NRAS oncogene activated by a point mutation is implicated in various human tumors, including AML and CML (Chin et al., 1992). Although we have not genotyped the NRAS gene, a linkage disequilibrium between NRAS and GSTM1 loci could be possible. This is mainly because our study also showed in-creased CML risk for >60 years, and the incidence of cancer increases with age, due mainly to longer exposure to carcinogens (Anisimov, 2007). Thus, GSTM1 and NRAS loci need to be investigated together in the context of cancer, concerning possible linkage disequilibrium, to avoid spurious associations.

Because the expression of *BCR/ABL* stimulates ROS production, which may contrib-ute to increased DNA damage in CML cells (Sattler et al., 2000; Nowicki et al., 2004), the Hp polymorphism has been investigated in leukemias, and the Hp1-1 phenotype has been associ-ated with increased risk (Nevo and Tatarsky, 1986; Mitchell et al., 1988; Campregher et al., 2004). Despite the small sample size and large confidence interval, results indicated increased CML risk for Hp1F-1S individuals of black skin color and decreased risk for the same group carrying the Hp1S-2 genotype. Because the deviation from HWE was mainly due to a higher number of 1F-1F and a lower number of 1F-1S and 1F-2 individuals than expected, results indicate that there are differences in biological responses among the Hp[*1] alleles and that they cannot be treated as a single block in the association studies.

In conclusion, an association with a risk of developing CML was found for MTHFR 1298AA and GSTM1 non-null genotypes, while MTHFR 1298AC and GSTM1 null geno-

types decreased this risk in all statistical tests performed. Results also indicated selection in favor of heterozygous for the MTHFR 1298 locus. Because the considerable range of variation in human populations may reflect natural selection, and as the Brazilian population is misce-genated, results indicated that, at least for our population, these two loci are important in the CML context, needing further investigation alongside folate dosages and clinical data.

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