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Evaluating glutathione S-Transferase (*GST*) null genotypes (*GSTT1* and *GSTM1*) as a potential biomarker of predisposition for developing leukopenia

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SUMMARY

Glutathione S-transferase (GST) enzymes protect cells against xenobiotics and oxidative stress products through an electrophilic conjugation process. We investigated the theta (*GSTT1*) and mu (*GSTM1*) null genotypes in a group of leukopenic subjects and normal subjects from Northeast Brazil, evaluating their use as biomarkers of susceptibility for developing leukopenia. In a sample-based case–control study, we analysed white blood cell (WBC) counts and *GSTT1* and *GSTM1* genotypes. A total of 278 subjects were analysed: 91 with leukopenia and 187 controls. *GSTT1* null genotype conferred a 5.92-fold risk for occurrence of leukopenia [odds ratios (OR) = 5.92, CI_{MLE}: 1.64–26.72, P_{MLE} = 0.002] and a 3.90-fold risk of neutropenia (OR = 3.90; CI_{MLE}: 1.05–13.66; P_{MLE} = 0.02), while *GSTM1* null genotype conferred a 1.78-fold risk for leukopenia (OR = 1.75; CI_{MLE}: 1.04–3.06, P_{MLE} = 0.017) and no risk of neutropenia (OR = 1.71; CI_{MLE}: 0.88–3.35; P_{MLE} = 0.06). The *GSTT1*, but not the *GSTM1* null genotype, was found to be associated with leukopenia and neutropenia. More cellular and molecular studies are needed to evaluate the existence of genotype interactions, and to confirm the appropriateness of using the *GSTT1* and/or *GSTM1* null genotypes as biomarkers of susceptibility to white blood-cell deficiencies.

INTRODUCTION

Leukocytes play an important role in defending the body against xenobiotics through phagocytosis and antibody production. Phagocytes are classified as either granulocytes (e.g. neutrophils, eosinophils, and basophils) or as monocytes/macrophages (Bondurant & Koury, 1998). Exposure to substances which are toxic to bone marrow can result in pancytopenia, a decrease in the circulating number of all three types of blood elements: red blood cells, white blood cells (WBC) and platelets. The diagnosis of this condition is based on peripheral blood cell counts of these elements. In early stages, decreased WBC count can occur in the absence of other characteristic symptoms such as neutropenia or granulocytopenia (Gay & Athens, 1998).

Glutathione is found in virtually all cell types, where it participates in intracellular oxidant defense mechanisms and serves as a key substrate for several intermediate steps of biological metabolism (Mannervik, 1985). *Glutathione S-Transferase* (*GST*) gene family comprises 16 genes in six subfamilies, the alpha (GSTA); mu (GSTM); omega (GSTO); pi (GSTP); theta (GSTT), and zeta (GSTZ) (Mannervik *et al.*, 1992; Nebert & Vasiliou, 2004) that belong to a family of intracellular enzymes that play a major role in the cellular detoxification of electrophilic compounds of endogenous and exogenous origin.

Genetic polymorphisms of *GST* have been described in several populations and the *GSTM1* absence is associated with an increased risk of developing cystic fibrosis, smoking-induced lung and bladder cancers and childhood acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) (Pakakasama *et al.*, 2005; Aydin-Sayitoglu *et al.*, 2006), and some multifactorial diseases (Baranov *et al.*, 1996). The absence of the *GSTT1* gene is associated with a high risk of myelodysplastic syndromes (Chen *et al.*, 1996) and ALL (Krajinovic *et al.*, 1999). These findings are important, as *GSTT1* null genotype carriers probably have a reduced ability to detoxify metabolites of carcinogens such as 1,3-butadiene (Wiencke *et al.*, 1995), methyl bromide and ethylene oxide (Pemble *et al.*, 1994); in addition, *GSTM1* gene products have been shown to detoxify carcinogens such as 1,2-epoxy-3-butene (Sasiadek *et al.*, 1999).

The GSTA, found predominantly in the liver, has been associated with a reduction of lipid hydroperoxides by glutathione peroxidase activity, and is commonly considered to be a marker of hepatocellular damage (Singhal *et al.*, 1992; Nelson *et al.*, 1995a,b; Coles & Kadlubar, 2005). The GSTP enzyme has been associated with resistance to several anti-cancer drugs; studies have shown that tumor cells from different types of cancers over-express this isoenzyme, a discovery which has driven efforts to find a selective inhibitor of this GST class (Oakley *et al.*, 1997; Lecomte *et al.*, 2006).

The GSTO enzyme has been found to play a role in apoptosis and be associated with age (Wang *et al.*, 2005) and the GSTZ class was recently found and related to the catalyse and oxygenation of diclhoroacetic acid and other alpha-haloacids (Tong, Board & Anders, 1998; Board & Anders, 2005). Thus, the presence of the functional *GST* gene has an important role in cellular defense against toxic factors. Here, we test the hypothesis that the presence of the *GSTT1* and/or *GSTM1* null genotype increases the risk of developing leukopenia. We focused on the *GSTM* and *GSTT* subtypes because these are most closely associated with hematologic and neoplastic diseases.

MATERIALS AND METHODS

This study was conducted in the city of Salvador, which has the highest rate of race admixture in Brazil. A subset of those blood samples drawn at a clinical laboratory during a two-month period (May and June of 2001) was selected and data from the patients who provided the blood samples were subsequently collected. This laboratory, LABCHECAP, is a clinical laboratory where a diverse socio-economic cross-section of Salvador undergoes routine clinical laboratory evaluations. All blood samples collected during the 2-month time period that satisfied the definition of leukopenia (WBC$<4.0 \times 10^9$/L) were selected for inclusion as cases (91 samples). Patients with a history of viral infection during the two weeks preceding blood collection were excluded. For each case, two blood samples collected during the same period which had normal leukocyte counts (i.e. between WBC$\geq 4.0 \times 10^9$/L and $\leq 10.0 \times 11^9/L^3$) were selected as controls, and were matched to cases according to age (±5 years), gender, and racial ancestry. The molecular characterization of *GSTT1* and *GSTM1* genotypes was performed at the Laboratory

of Molecular Pathology and Biology, Gonçalo Moniz Research Center (a regional branch of the Oswaldo Cruz Foundation – FIOCRUZ). The subjects were between 1 and 89 years of age (median = 40.0; mean = 38.9; standard deviation = 17.6). Informed consent was obtained from all individuals or official responsible prior to enrollment. The study protocol was submitted to and approved by the Oswaldo Cruz Research Foundation's human research ethics committee.

Five millilitres of peripheral blood was obtained from each individual for haematologic analysis and DNA extraction. All blood samples were anti-coagulated with EDTA. Haematologic data were obtained with an automated cell counter (Celldyn 3000 – Abbott) and by standard procedures. DNA was isolated from leukocytes with a GFX genomic blood DNA purification kit (Amersham Pharmacia Biotech, Piscataway, NJ, USA) according to the manufacturer's instructions. The *GSTT1* and *GSTM1* genotypes were determined by a multiplex PCR technique, using β-globin amplification as an internal reaction control, as described previously (Baranov *et al.*, 1996; Krajinovic *et al.*, 1999). Each set of reactions also included negative controls to verify the purity of all reagents. Briefly, the PCR buffer reaction mix contained Tris-HCl 10 mmole/l (pH 8.4); KCl 75 mmole : l; $MgCl_2$ 3.5 mmole/l; dNTP 200 μmole/l each; 2.5 U of Taq DNA polymerase (Gibco-BRL Life Technologies, Grand Island, NY, USA); 25 pmol of each specific primer, and approximately 0.5 ng of DNA sample. All reactions were carried out in a Perkin-Elmer Thermocycler model 2400 (Perkin Elmer Inc., Waltham, MA, USA). The PCR reaction mix was heated at 94 °C for 10 min, followed by 35 cycles of denaturation (94 °C for 45 s), annealing (58 °C for 45 s), and extension (72 °C for 1 min 30 s). After cycling, the tubes were initially kept at 72 °C for 10 min, then cooled to 4 °C and analysed by gel electrophoresis (1% agarose in TAE 1× stained with ethidium bromide) and UV visualized. Figure 1 shows a representative gel. The primer combination markers were 5′- TTCCTTACTGGTCCTCACATCTC-3′ and 5′-TCACCGGATCATGGCCAGCA-3′ (*GSTT1* sense and anti-sense, product size 480 bp), 5′-CTGCCCTACTTGATTGATGGG-3′ and 5′-CTGGATTGTAGCAGATCATGC-3′ (*GSTM1*, sense and anti-sense, product size 271 bp), 5′-GCCAAGGACAGGTACGGCTGTCATC-3′ and 5′-CCCTTCCTATGACATGAACTTAACCAT-3′ (β-globin sense and anti-sense, product size 700 bp (Figure 1).

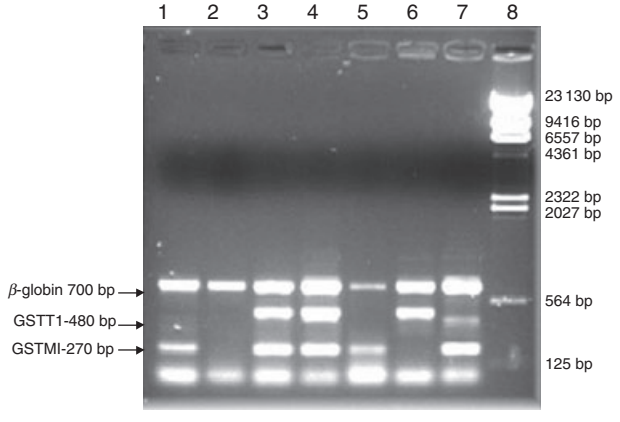

Figure 1. Agarose gel showing PCR fragments of *GST* gene polymorphisms. Lanes 1 and 5: $GSTT1^{NU}$/$GSTM1^{NR}$ genotype; lane 2: $GSTT1^{NU}$/$GSTM1^{NU}$ genotype; lanes 3 and 4: $GSTT1^{NR}$/$GSTM1^{NR}$ genotype; lane 6: $GSTT1^{NR}$/$GSTM1^{NU}$ genotype; lane 7: $GSTT1^{PD}$/$GSTM1^{NR}$ genotype; lane 8: Base pair marker λ *Hind* III.

Statistical analysis

Maximum likelihood (MLE) estimates of odds ratios (OR) and their respective confidence intervals were used to measure the strength of association between the presence of *GST* null genotypes (*GSTT1* and *GSTM1*) and leukopenia/neutropenia in the case-control analysis. The MLE method or maximum likelihood estimation was used because only a limited number of leukopenic subjects, but not the entire population, was included in the in the study. The sample mean was then the maximum likelihood estimator of the population mean, and the sample variation was a close approximation to the maximum likelihood estimator of the population variance. First, the crude risk of leukopenia and/or neutropenia associated with the presence of each null genotype was calculated. Stratified analyses were then conducted to evaluate the presence of effect modification between the two null genotypes (i.e. to evaluate whether the risk of leukopenia/neutropenia associated with the deletion of one gene was influenced by deletion of the other gene). All measures of association were

calculated using EPI Info software, version 6.04 (Centre for Disease Control and Prevention (CDC), Atlanta, GA, USA).

RESULTS

Of the 278 individuals who were recruited into the study, 91 were leukopenic subjects (WBC<4.0×10^9/l) and 187 had normal WBC counts (WBC $\geq 4.0 \times 10^9$/l and <11.0×10^9/l). Analysis of granulocytic neutrophil counts demonstrated that 53.8% of leucopenic subjects had neutrophil counts less then 1.5×10^9/l (indicating the presence of neutropenia). All subjects studied were classified into one of six groups, according to their *GSTT1* and *GSTM1* genotypes: 137 (49.3%) had normal *GST* genotypes for both genes (group 1); 100 (36.0%) had *GSTM1* null genotype and normal *GSTT1* gene (group 2); 8 (2.9%) had normal *GSTM1* and *GSTT1* null genotype (group 3); 6 (2.2%) had null genotype for both genes (group 4); 11 (3.95%) were found to have a *GSTM1* null genotype and a smaller *GSTT1* fragment that we characterized as a partial *GSTT1* deletion (group 5); and 16 (5.75%) had a normal *GSTM1* genotype and partial *GSTT1* deletion (group 6). The distribution of *GST* genotypes in the leukopenic subjects and normal WBC-count groups is summarized in Table 1.

Table 1. Distribution of *GST* genotypes among the leukopenic subjects and normal WBC groups

Group	*GST* genotypes	NR WBC *n* (%)	Leukopenic *n* (%)	Total
01	$T1^{Nr}/M1^{Nr}$	103 (55.1)	34 (37.4)	137 (49.3)
02	$T1^{NR}/M1^{NU}$	64 (34.2)	36 (39.5)	100 (36.0)
03	$T1^{NU}\ M1^{NR}$	02 (1.1)	06 (6.6)	08 (2.8)
04	$T1^{NU}/M1^{NU}$	02 (1.1)	04 (4.4)	06 (2.1)
05	$T1^{PD}/M1^{NU}$	04 (2.1)	07 (7.7)	11 (4.0)
06	$T1^{PD}/M1^{NR}$	12 (6.4)	04 (4.4)	16 (5.8)
Total	-	187	91	278

NR, normal; Nu, null; PD, partial deletion.

Table 2. Age and gender distributions of cases and controls groups

Groups	Gender (%)	Mean age (years)
Cases	Male (45.3)	34.765
91 (32.7%)	Female (54.7)	43.118
Controls	Male (40.8)	34.558
187 (67.3%)	Female (59.2)	39.511

Ten leucopenic subjects had *GSTT1* null genotype and 70 leukopenic subjects had normal *GSTT1* genotype. Four subjects with normal WBC count had *GSTT1* null genotype, and 167 had normal WBC count and normal *GSTT1* genotype. In contrast, 40 leukopenic subjects had *GSTM1* null genotype and 40 leukopenic subjects had normal *GSTM1* genotype. Sixty-six subjects had normal WBC count and *GSTM1* null genotype and 105 had normal WBC count and normal *GSTM1* genotype. The leukopenic subjects and normal WBC count groups were found to have similar age and gender distributions, as shown in Table 2.

The case–control study revealed that both *GSTT1* and *GSTM1* null genotypes were associated with white blood-cell deficiencies. Comparison of the distribution of leukopenia in groups 3 and 4 (*GSTT1* null genotype) with that in groups 1 and 2 (*GSTT1* normal) showed that the *GSTT1* null genotype conferred a 5.92-fold risk of leukopenia (OR = 5.92, CI_{MLE}: 1.64–26.72, $P_{MLE} = 0.002$) and a 3.90-fold risk of neutropenia (OR = 3.90; CI_{MLE}: 1.05–13.66; $P_{MLE} = 0.02$). Partial deletion of the *GSTT1* gene was not found to be significantly associated with leukopenia (OR = 1.64; CI_{MLE}: 0.65–3.98, $P_{MLE} = 0.16$) or with neutropenia (OR = 1.19; CI_{MLE}: 0.33–3.49; $P_{MLE} = 0.46$), as determined by comparing the distributions of leukopenia and neutropenia in groups 4 and 5 with the distribution in groups 1 and 2 (Table 3). As a result, the individuals with partial deletion of *GSTT1* were not included in the calculation of the risk associated with complete deletion of *GSTT1*.

We also found evidence of an association between the presence of *GSTM1* deletion and leukopenia (OR = 1.78; CI_{MLE}: 1.04–3.06; $P_{MLE} = 0.017$), but not with neutropenia (OR = 1.71, CI_{MLE}: 0.88–3.35; $P_{MLE} = 0.06$) (Table 3).

The stratified analysis indicated that the risk of white blood-cell deficiencies associated with *GSTT1* deletion could depend on the *GSTM1* genotype (i.e. that *GSTM1* genotype is potentially an effect modifier of the relationship between *GSTT1* deletion and WBC deficiency), although the associated confidence intervals were too wide to draw definitive conclusions.

Table 3. Associations between *GSTT1* and *GSTM1* genotypes and leukopenia/neutropenia

	Genotypes			OR (95%CI) and MLE (*P*-value)
	$GSTT1^{NU}$	$GSTT1^{NR}$	Total	
Leukopenic	10 (12.5%)	70 (87.5%)	80	5.92 (1.64–26.72)
Normal WBC	04 (2.3%)	167 (97.7%)	171	MLE (P = 0.002)
	$GSTM1^{NU}$	$GSTM1^{NR}$	Total	
Leukopenic	47 (51.6%)	44 (48.4%)	91	1.78 (1.04–3.06)
Normal WBC	70 (37.4%)	117 (62.6%)	187	MLE (P = 0.017)
	$GSTT1^{PD}$	$GSTT1^{NR}$	Total	
Leukopenic	11 (13.6%)	70 (86.4%)	81	1.64 (0.65–3.98)
Normal WBC	16 (8.7%)	167 (91.3%)	183	MLE (P = 0.16)

NR, normal; Nu, null; PD, partial deletion; WBC, white blood cells.

Individuals with both *GSTM1* and *GSTT1* deletion appeared to have a smaller risk of leukopenia (OR = 3.51; CI $_{MLE}$ = 0.48–40.61; P_{MLE} = 0.14) and neutropenia (OR = 1.87, CI_{MLE}: 0.16–14.09; P_{MLE} = 0.39) relative to the crude risk associated with *GSTT1* deletion, while individuals with *GSTM1* normal genotype and *GSTT1* deletion appeared to have increased risk of both leukopenia (OR = 8.92; CI_{MLE}: 1.51–94.41; P_{MLE} = 0.006) and neutropenia (OR = 6.90, CI_{MLE}: 1.17–40.85; P_{MLE} = 0.02) relative to the crude risks associated with *GSTT1* deletion.

The risk of leukopenia associated with *GSTM1* deletion appeared to not vary according to *GSTT1* genotype: individuals with partial *GSTT1* deletion appeared to have not risk for leukopenia (OR = 4.90; CI_{MLE}: 0.77–38.49; P_{MLE} = 0.05) and neutropenia (OR = 2.53, CI_{MLE}: 0.24–36.44; P_{MLE} = 0.31), followed by individuals with normal *GSTT1* (leukopenia: OR = 3.11; CI_{MLE}: 0.93–3.11; P_{MLE} = 0.04) (neutropenia OR = 1.87, CI_{MLE}: 0.88–4.04; P_{MLE} = 0.055) and finally by those with complete *GSTT1* deletion (leukopenia OR = 0.69; CI_{MLE}: 0.03–13.3; P_{MLE} = 0.59) (neutropenia OR = 0.53; CI_{MLE}: 0.03–6.71; P_{MLE} = 0.47); once again, however, the wide confidence intervals prevent us from drawing definitive conclusions from this stratified analysis.

DISCUSSION

Our results suggest that individuals with *GSTT1* null genotype have a higher risk of both leukopenia and neutropenia. Furthermore, the results of the stratified analyses suggest that there is potential for genotype interaction, although the extremely wide confidence intervals prevented us from drawing definitive conclusions. The fact that the risk of leukopenia and neutropenia associated with both *GSTT1* and *GSTM1* null genotypes appears to be greater when the other genotype was normal than when both genotypes were null is an apparently counter-intuitive finding, and merits further investigation. Unfortunately, there was not a prior study that estimates frequencies of *GSTT1* and *GSTM1* deletions in this population and in closed ethnic population groups and thus unable us to calculate the sample size that would have been required to adequately evaluate potential interactions between the two genotypes. Furthermore, the observed frequency of *GSTT1* was much lower than was previously described in others Brazilian groups (Arruda *et al.*, 1998, Gattás *et al.*, 2004), which also contributed to the wide confidence intervals.

The identification of a partial *GSTT1* deletion in this analysis was unexpected. As a result, we were also unable to choose, *a priori*, an appropriate sample size that was adequate for determining whether or not this partial deletion is associated with excess risk of leukopenia. However, this partial deletion did not appear to confer excess risk for white blood cell deficiencies directly.

Several studies have been conducted to investigate whether the deletion of *GST* genes may be a suitable genetic marker of high risk for developing a variety of diseases (Warwick *et al.*, 1994; Nelson *et al.*, 1995a,b; Wiencke *et al.*, 1995; Nedelcheva Kristensen *et al.*,

1998; Lourenco *et al*., 2005; Mondal *et al*., 2005; Rocha *et al*. 2005; Ye & Song., 2005). However, this hypothesis is controversial, and this discrepancy has been attributed to an uneven distribution of enzyme activity in several ethnic groups (Nelson *et al*., 1995a,b; Atoyebi *et al*., 1997; Basu *et al*., 1997; Arruda *et al*., 1998; Krajinovic *et al*., 1999). Chen *et al.* (1997) found a higher frequency of *GSTT1* and *GSTM1* deletion in black children with ALL (8 of 34 or 23.5%) than in black controls (3.9%) ($P = 0.0005$). The authors suggest that the GST genotype, coupled with unidentified additional risk factors, may play a role in childhood ALL in African-Americans. Xiao *et al.* (2008) described that a complete remission rate was higher in AML patients group with normal *GSTM1* and *GSTT1*genes than the *GSTM1*(OR = 1.88; $P = 0.03$) and *GSTT1* (OR = 2.20; $P = 0.02$) null genotypes. Our group (Souza *et al*., 2008) found a higher frequency of *GSTM1/GSTT1* null genotypes among Acute promyelocytic leukemia patients (OR = 3.61; $P = 0.002$). Our finding of a high risk of leukopenia in *GSTT1* null genotypes in this racially mixed population (Azevedo *et al*., 1980; Gonçalves, Souza & Moura Neto, 2000) could be due to a genetic predisposition for low absolute leukocyte counts, such as those described in Ethiopian Jews (Berrebi, Melamed & Van Dam, 1987) and black Africans (Shaper & Lewis, 1971). However, lower counts are less evident in black Americans (Orfanakis *et al*., 1970) and in Africans who consume Western diets (Bain & England, 1975). Alterations in the total number of leukocytes and their relative proportions are known to be significant measures of physiologic reaction to noxious agents (Gay & Athens, 1998). As a result, the relationship between low leukocyte count, ethnicity and *GST* null genotype should be investigated more closely. We also believe that studies of *GST* deletion in leukopenic subjects with xenobiotic exposure history could clarify the observed 5.92-fold increased risk of leukopenia associated with a *GSTT1* null genotype presence. Several *in vitro* experiments have shown differences in cellular responses to chemical exposition to benzene and hydroquinone (Gut *et al*., 1996; Hazel *et al*., 1996).

We are not aware of any studies that have attempted to determine the risk of leukopenia associated with *GST* null genotypes. More detailed studies need to be performed to clarify the value of using null genotypes as markers for leukopenia risk, and to evaluate whether the partial *GSTT1* deletion identified here is unique to Salvador's population.

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