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GSTM1/*GSTT1*-null genotyping role in leukemia development in Brazilian patients

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ABSTRACT. Glutathione S-transferases are detoxification enzymes that protect cells from oxidative stress and help maintain genomic integrity. GSTM and GSTT family genes may be deleted, causing reduced or no glutathione S-transferase activity so that electrophilic carcinogens cannot be eliminated efficiently. This genetic alteration affects cancer incidence and prognosis. We genotyped *GSTM1* and *GSTT1* in 87 Brazilian leukemia patients by multiplex PCR, divided into acute or chronic, and lymphocytic or myeloid type cancers. *GSTM1*-null and *GSTT1*-null frequency was significantly higher in chronicmyeloid leukemia (67.65% and 46.20%) and chroniclymphoid leukemia (29.41% and 12.30%) in relation to controls, respectively, than in non-leukemia controls. More than that, double null genotyping was significantly more present in acutelymphoid leukemia than controls. When individual *GSTM1* and *GSTT1* genotyping were analyzed, again CML and CLL presented significantly diferent in genotyping frequency compared to controls (11.11% and 3.8%). Double null genotypes were significantly more frequent in acute lymphoid leukemia than in controls. Furthermore, CML patients presented a statistically significant higher percentage of double-null and *GSTM1*-null genotypes, and CLL patients had a significantly higher frequency of *GSTT1*-null genotyping when compared to controls.

We suggest that GST genotypes are an important risk factor for leukemia development in the Brazilian population, especially in chronic leukemia due to inefficient detoxification of oxidative stress products.

Key words: Leukemia; Glutathione S-transferases; *GSTM1*; *GSTT1*; Multiplex PCR

INTRODUCTION

Cancer is a disease caused by uncontrolled cellular growth, presenting high infiltration capacity, apoptosis resistance, immune system evasion, leading to tumor formation that can spread throughout the body, generating metastasis (Silva et al., 2020). Leukemias are characterized by a malignant hematological disorientation involving an increase in altered leukocytes in the bone marrow (Farasani, 2019), which become uncontrolled, losing their function and with irregular multiplication. More than 12 types of leukemia are known, the most frequent being Acute Myeloid Leukemia (AML), Chronic Myeloid Leukemia (CML), Acute Lymphocytic Leukemia (ALL) and Chronic Lymphocytic Leukemia (CLL) (Silva et al., 2020).

Acute myeloid leukemia is the most common malignant and aggressive leukemia in adults, being a disease caused by the uncontrolled growth of myeloid or lymphoid cells that lose the power of cell differentiation (Gregory et al., 2019). Chronic myeloid leukemia is given by a reciprocal translocation (9; 22) (q34; q11) that results in a Philadelphia chromosome (Ph+), by a fusion of ABL and BCR genes (Abelson's murine on chromosome 9 and Breakpoint cluster on chromosome 22) leading to an unbalanced proliferation of myeloid lineage cells (Delmond et al., 2021). Acute lymphoid leukemia is a type of leukemia that typically has a clone of immature B or T cells in the bone marrow causing problems with hematopoiesis. Chronic lymphoid leukemia is a type of leukemia defined by a high level of mature B cells, which can also be CD5-positive and involve clonal proliferation (Hallek et al., 2018).

It is known that several environmental factors are related to the tumor's appearance, however there are plenty of defense mechanisms. One of these is the glutathione peroxidase system, which acts to protect against oxidative stress generated by reactive oxygen species. This system is divided into two phases: phase I being given by components of cytochrome P450 and phase II formed by glutathione S-transferases (GSTs) and N-acetyltransferases (NATs) (Pacholak et al., 2021).

Glutathione peroxidases are part of a group of enzymes whose main biological functions involve protecting the body against oxidative stress, which is caused by excess reactive oxygen species, leading to irreversible damage in the membranes, DNA or proteins (Wei et al., 2020; Reis et al., 2021). The main players of the Glutathione family are *GSTM1*, *GSTT1* and *GSTP1* (Liu et al., 2017). In addition, they are also considered as biomarkers, as some of their polymorphisms can be related to increased disease susceptibility, which are related to the detoxification of drugs, xenobiotics and carcinogens (Hollman et al., 2016). Theta1 (*GSTT1*) and mu 1 (*GSTM1*) genes have deletion polymorphisms in the population, as deletion of one of the genes or the homozygous deletion (null genotype) can cause enzyme ineffectiveness (Xiao et al. 2014).

Consequently we examined *GSTM1* and *GSTT1* genotypes in leukemia patients to examine a possible correlation of genotypes with disease development.

MATERIAL AND METHODS

Study population: 87 patients with leukemia were recruited at Erasto Gaertner Hospital, Curitiba, Brazil, after Institutional Ethics Committee approval (CAAE 08809419.0.0000.0098). For all participants, the project was described and a signed informed consent form was obtained. Sample collection and experimental procedures were carried out in accordance with relevant guidelines and Brazilian regulations. The 87 leukemia patients were divided into four groups according to disease type: chronic myeloid leukemia (CML), acute myeloid leukemia (AML), chronic lymphoid leukemia (CLL) and acute lymphoid leukemia (ALL). As a control group data from two Brazilian publications regarding Glutathione S-Transferase genotyping were used with the population presenting a similar ethnic profile.

DNA extraction and multiplex PCR

All the participants had 4 mL of peripheral blood collected, DNA was extracted with QIAmp DNA Blood Mini Kit (QIAGEN) as indicated using 200ul of total blood. A quantification and purity of DNA were performed using NanoDrop One/OneC Microvolume UV Spectrophotometer® (Thermo Scientific). A multiplex PCR was used for *GSTM1* (NG_009246.1) and *GSTT1* (NM_000853.3) amplification with CYP1A1 as an internal control, using primers for *GSTM1* (*Forward*: 5' GAACTCCCTGAAAAGCTAAAGC 3'/*reverse:* 5' GTTGGGCTAAATATACGGTGG 3'), *GSTT1* (*Forward:* 5' TTCCTTACTGGTCCTCACATCTC 3'/*reverse:* 5' TCACCGGATCATGGCCAGCA 3') and CYP1A1 (Forward*:* 5' GAACTGCCACTTCAGCTGTCT 3'*/reverse:* 5' CAGCTGCATTTGGAAGTGCTC 3'). All primers were purchased from Exxtend (Brazil), purified by dessalting and resuspended in nuclease-free water (Thermo Fisher). *GSTM1* and *GSTT1* amplification presents 215 bp and 460bp fragments, while CYP1A1 has a fragment of 312 bp. The absence of PCR products for *GSTM1* and *GSTT1* in the presence of CYP1A1 PCR product indicated the null genotype for each. Multiplex PCR was carried with *HotStar TaqDNA Polimerase* (QIAGEN), using 5µL of 10X PCR Buffer, 10µL 5X Q Solution, 0,25µL de *Hot Star Taq DNA Polimerase*, 1µL de dNTP mix 10mM (ThermoFisher), primers, nuclease free water and 60ng of DNA, in a 50uL reaction. The reaction was performed Pro-Flex PCR System (Applied Biosystems) with an initial denaturation of 95ºC for 15 minutes, followed 35 cycles of 95°C for 1 minute, 53°C for 1 minute and 72°C for 1 minute, and a final extension of 72ºC for 10 minutes. The multiplex PCR amplification visualization was performed with polyacrylamide gel submitted at 100V for 50 minutes and stained with silver nitrate (0.1% w/v).

Statistic

Statistical analysis was performed using GraphPad Prism version7.05. Chi-square was used to compare sex and t-student test was used to compare age. For genotyping

distribution analysis was performed by Chi-square test together with odds ratios (OR), with their corresponding 95% confidence intervals (CI), and p-values were calculated in order to estimate the risk of developing leukemia. All tests were performed using $P < 0.05$ as the level of significance.

RESULTS

The epidemiological data from overall leukemia patients are divided into disease type, shown in Table 1..

Table 1. Epidemiological data describing gender and mean age between patients with leukemia, divided into subgroups by type of leukemia and controls. Number between parentheses represent total number of patients for each group (CML: Chronic myeloid leukemia; AML: Acute myeloid leukemia; CLL: Chronic lymphocytic leukemia; ALL: Acute lymphocytic leukemia).

	Control (179)[#]	Leukemia (87)	CML (34)	AML (18)	CLL (17)	ALL (18)
Male	81	53	23	9	11	11
Female	98	34	11	9	6	7
Age (Mean±SD)	49.98 ± 13.34	51.39 ± 23.79	59 ± 15.81*	51.87 ± 18.98	68.62 ± 8.4**	20.05 ± 18.78***

* P = 0.0010; ** P < 0.0001; *** P < 0.0001. [#] Pinheiro et al., 2017 and Reis et al. 2021.

As described, samples genotyping were performed by multiplex PCR and visualized in polyacrylamide gel electrophoresis silver stained, where internal control CYP1A1 generate a 312 bp fragment, and *GSTT1* and *GSTM1* 460 bp and 215 bp, respectively, as demonstrated in Figure 1.

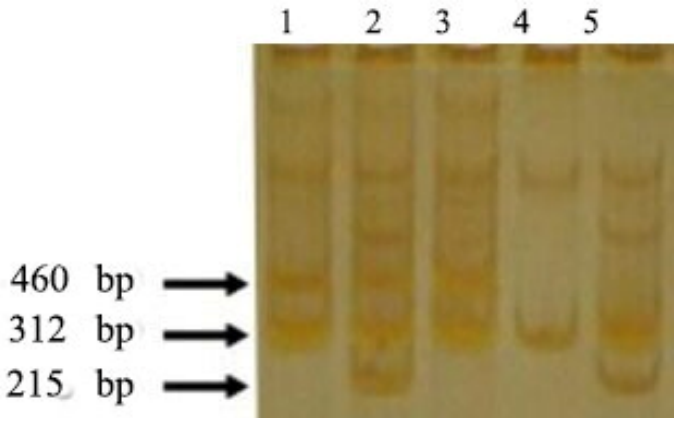

Figure 1. *GSTM1* and *GSTT1* products genotyped by multiplex PCR, visualized in polyacrylamide gel silver stained showing the internal control CYP1A1 (312 bp), *GSTM1* (215bp) and *GSTT1* (460bp). Lanes 1 and 3 represent *GSTM1*-null patients, lane 2 represent a wild type patient, lane 5 a *GSTT1*-null patient and lane 4 a double-null patient.

The data regarding the *GSTM1* and *GSTT1* genotype frequencies in leukemia patients used in this study, together with literature control are shown in figure 2 and table 2. In 87 overall leukemia patients the frequencies of *GSTM1*-null and *GSTT1*-null genotypes

were 56% and 19.5%, respectively, whereas for the control group were 46% and 12%, respectively, with no difference in the genotypic distribution between these groups. When leukemia patients were divided into groups according to disease type, we observed a statistical difference between *GSTM1* and *GSTT1* genotypes in CML and CLL, respectively, when compared to the control group (Table 1). In both cases the genotypes *GSTM1*-null and *GSTT1*-null were found at a higher frequency than in controls. In the multiple logistic regression analysis performed with respect to the risk associated with the *GSTM1*-null and *GSTT1*-null genotypes of developing CML and CLL, OR values of 2.42 (1.33-4.32) and 3.01 (1.44-6.26) were observed.

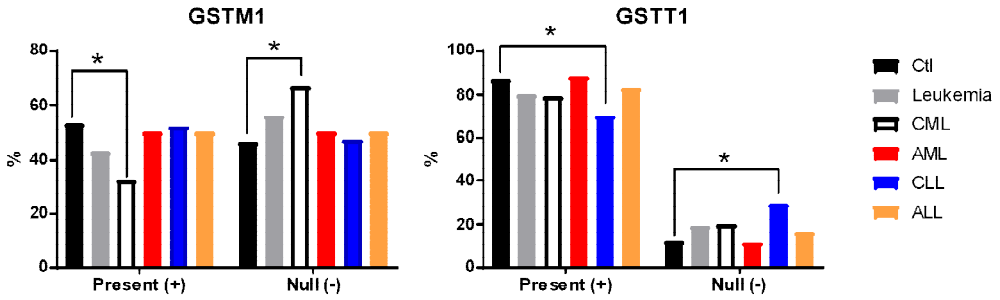

Figure 2. *GSTM1* and *GSTT1* genotype frequencies in leukemia patients, representing presence (+) or null (-) gene in controls, overall and groups leukemia. Statistically difference are represented with *. *GSTM1*-null frequency was significant higher in CML patients than in controls (P =.0026), while *GSTT1*-null frequency was significantly higher in CLL patients than in controls (P = 0.0029).

Table 2. Data for *GSTM1* and *GSTT1* genotype frequencies in leukemia patients and controls used in this study, divided into overall leukemia (87 patients), CML (34 patients), AML (18 patients), CLL (17 patients), ALL (18 patients) and control (179 subjects). *GSTM1* and *GSTT1* positive and null frequency are demonstrated in percentage for all groups, together with chi-square (X2) and odd ratio (OR) result. Statistically difference are represented in bold with * (CML: Chronic myeloid leukemia; AML: Acute myeloid leukemia; CLL: Chronic lymphocytic leukemia; ALL: Acute lymphocytic leukemia).

	Leukemia type (n)	Overall (87)	X2/OR (IC 95%)	CML (34)	X2/OR (IC 95%)	AML (18)	X2/OR (IC 95%)	CLL (17)	X2/OR (IC 95%)	ALL (18)	X2/OR (IC 95%)	Control (179)[#]
GSTM1	Positive	43.68%	0.15/1.50 (0.85-2.57)	32.35 %	0.0026/2.42 (1.33-4.32)*	50.00 %	0.6178/1.15 (0.65-2.03)	52.94 %	0.886/1.04 (0.58-1.84)	50.00 %	0.6178/1.15 (0.65-2.03)	53.80%
	Null	56.32%		67.65 %		50.00 %		47.06 %		50.00 %		46.20%
GSTT1	Positive	80.46%	0.171/1.72 (0.94-3.82)	79.41 %	0.1225/1.83 (0.86-4.04)	88.89 %	0.8245/0.90 (0.39-2.07)	70.59 %	0.0029/3.01 (1.44-6.26)*	83.33 %	0.4146/1.39 (0.63-3.21)	87.70%
	Null	19.54%		20.59 %		11.11 %		29.41 %		16.67 %		12.30%

[#] Pinheiro et al., 2017 and Reis et al., 2021

After that, we analyzed individual *GSTM1* and *GSTT1* genotyping variations, representing presence of both genes (+/+), deletion of one of two genes (*GSTM1*-null/*GSTT1*: -/+; *GSTM1*/*GSTT1*-null: +/-) and double null (-/-). Figure 3 and Table 3 demonstrate comparison between individual genotypes in CML, CLL and control. When all four individual genotypes were analyzed, a significant difference was observed in CML and CLL when compared to controls. As presented in Table 3, in CML patients the percentage

of double-null (-/-) and *GSTM1*-null genotypes (-/+) were statistically significant higher than in control, with an OR of 7.01 (1.75-24.75) and 2.50 (1.33-4.65). This same table presents the data from CLL patients. In this case a statistically significant higher frequency of *GSTT1*-null genotyping (+/-) was observed when compared to control, with an OR of 3.87 (1.61-8.89). Although ALL genotyping frequency was not statistically significant from control (Table 1), double-null (-/-) frequency was statistically significant higher than control, with an OR of 3.66 (0.98-12.82) (Table 3).

Table 3. Individual *GSTM1* and *GSTT1* genotyping variations, representing double null ([-]/[-]), deletion of one of two genes (*GSTM1*/*GSTT1*-null: [+]/[-]; *GSTM1*-null/*GSTT1*: [-]/[+]) and presence of both genes ([+]/[+]). Frequencies are demonstrated in percentage for all groups, together with chi-square (X2) and odds ratio (OR) result. Significant differences are represented in bold with * (CML: Chronic myeloid leukemia; AML: Acute myeloid leukemia; CLL: Chronic lymphocytic leukemia; ALL: Acut lymphocytic leukemia).

Combined *GSTM1/ GSTT1*	**Overall (87)**	**X2/OR (IC 95%)**	**CML (34)***	**X2/OR (IC 95%)**	**AML (18)**	**X2/OR (IC 95%)**	**CLL (17)****	**X2/OR (IC 95%)**	**ALL (18)**	**X2/OR (IC 95%)**	**Control (78)[#]**
[-]/[-]	8.05%	0.0568/3.66 (0.97-13.38)	11.76%	0.0023/7.01 (1.75-24.75)*	0.00%	0.1128/0 (0-1.403)	5.88%	0.2153/2.52 (0.58-10.01)	11.11%	0.0464/3.66 (0.98-12.82)*	3.80%
[+]/[-]	11.49%	0.3022/1.68 (0.59-4.36)	8.82%	0.3313/ 1.7 (0.53-5.05)	11.11%	0.4871/1.41 (0.51-3.62)	23.53%	0.0024/3.87 (1.61-8.89)*	5.56%	0.3204/0.55 (0.19-1.82)	9.00%
[-]/[+]	48.28%	0.1492/1.57 (0.83-2.87)	55.88%	0.0048/2.50 (1.33-4.65)*	50.00%	0.2917/1.37 (0.75-2.54)	41.18%	0.2256/1.48 (0.79-2.82)	38.89%	0.7461/0.90 (0.48-1.68)	42.30%
[+]/[+]	32.18%	Reference	23.53%	Reference	38.89%	Reference	29.41%	Reference	44.44%	Reference	44.90%

* p=0.0047. ** p=0.0211. [#] Pinheiro et al., 2017.

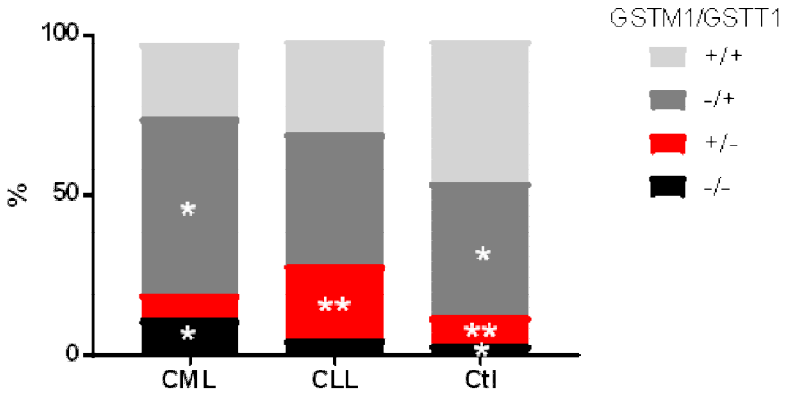

Figure 3. Individual *GSTM1* and *GSTT1* genotypes percentage in chronic myeloid leukemia (CML), chronic lymphocytic leukemia (CLL) and controls (Ctl). In CML patients, *GSTM1*-null/*GSTT1*-present (-/+) and double-null (-/-) have statistically higher frequency than control (represented by *). In CLL patients, *GSTM1*-present/*GSTT1*-null (+/-) have statistically higher frequency than control (represented by **). *GSTM1* and *GSTT1* double presence was set as reference.

DISCUSSION

The present study reports an association between *GSTM1* and *GSTT1*-null genotypes and leukemia development in the Brazilian population. Molecular epidemiology data are extremely variable especially due ethnicity, sample number, groups formation and

susceptibility variants combination (Aydin-Sayitogiu et al., 2006). Another study related the frequencies of deletions in the GSTM and GSTT genotypes in Mexico compared to studies in the literature in different populations, finding variations. (Palma-Cano et al., 2017). Epidemiological data such as ethnicity should be cautiously considered (Gattás et al., 2004). Scientific reports regarding glutathione S-transferases genetic in the leukemia context of Brazilian population are few, however some examples chase for *GSTM1* and *GSTT1* influence in cancer. Cancer development is linked to an imbalance between cellular detoxification enzymatic system and exposure to carcinogenic agents, where glutathione S-transferases system present an essential role (Reis et al. 2021). A classical Brazilian report showed that *GSTM1* and *GSTT1*-null frequency between different Brazilian ethnicities are linked to carcinogenesis (Arruda et al., 1998). Brisson et al. (2015) mention that the polymorphisms of the genes CYP2E1, *GSTM1*, NQO1, NAT2 and ABCB1 (MDR1) had effects when related to investigations such as domestic products, insecticides, among others, these genes were more frequently associated with the risk of leukemia in children. Relation between male gender, advanced age, alcohol and smoking habits and *GSTM1*-null are reported with head and neck cancer (Leme et al., 2010). Additional, *GSTM1*-null was associated with cervical cancer (Tacca et al., 2018) and another study also associated the *GSTP1* Ile105Val genotype may be related to the risk of gastric cancer (Chen et al., 2017). Similar studies using relative data represent controversial results from our findings due to bias such as ethnicity, age, gender, behavioural properties and research time (Crump et al., 2000; Stanulla et al., 2000; Yuille et al., 2002; Aydin-Sayitogiu et al., 2006; Bolufer et al., 2007; Dunna et al., 2013; Kassogue et al., 2015). GST enzyme family deficiency is also investigated as an important player in leukemia prognosis, wherein GST genotyping can be made mandatory in management of acute leukemias so that more aggressive therapy such as allogenic stem cell transplantation may be planned in the case of patients with a null genotype (Dunna et al., 2013).

Here presented, overall leukemia and controls had no significative differences in *GSTM1* and GSTT-null genotypes; however, when a comparison was made with leukemia types we observed a high *GSTM1*-null and *GSTT1*-null frequency in CML and CLL, respectively, while double null frequency was significantly higher in ALL than in controls.

Some reports in international scientific literature reported *GSTM1* and *GSTT1* genetic profiles with CML, however with conflicting results due to study design and population. A recent study (Rostami et al., 2019) reported data that support our finding, where *GSTM*-null frequency was higher in CML than controls, demonstrating a similar frequency between this report and our finding (67,5 Vs 67,65%, respectively). On other hand, Asiatic and European studies showed no association between *GSTM1* and *GSTT1* and CML (Hishida et al., 2005; Al-Achkar et al., 2014; Bənescu et al., 2014). In a similar way, South American studies reported no difference between frequencies in CML and control, however highlighting that in Brazilian report *GSTM1*-null frequency in CML patients was greatly different from here reported (32% vs 67,65%) (Souza et al., 2008; Weich et al., 2016). Another two reports from north of Africa also showed no difference between GST genetic context in CML patients, however one with Moroccan population demonstrated GSTT-null frequency significative higher in male CML patients when compared to male controls, suggesting that this finding may be due to difference in xenobiotic exposure between genders (Kassogue et al., 2014, 2015; Idris et al., 2020). Similar findings were reported by (Taspinar et al., 2008; Bhat et al., 2012; Özten et al., 2012;), where *GSTT1*-null

frequency was significantly higher CML patients than controls. Intriguingly, a Brazilian study reported that *GSTM1*-null frequency was significantly higher in controls than CML patients (Lordelo et al., 2012).

In CLL, the other leukemia type that was accessed with different GST-null frequency, few results are available but also supporting our findings. A report demonstrated that *GSTM1* and *GSTT1* null genotypes were higher in cases than in controls and there was evidence of a trend in increasing risk with the number of putative "high-risk" alleles of the GST family carried (Yuille et al. 2002; Lordelo et al. 2012). A recent data suggest that *GSTT1* positive genotype may represent a protective effect over CLL, however highlighting bias because ethnic heterogeneity in Brazilian population (Reis et al., 2021). A Russiona study demonstrated that the combination of unfavorable polymorphic CYP1A1 variants and *GSTM1* null genotype was found more frequently in B-CLL patients relative to controls (Gra et al. 2008).

Despite ALL no significant difference was observed in *GSTM* and *GSTT* null frequency; we observed a high frequency of double null than in controls. In this context, an Spanish study associated *GSTT1*-null and improved risk for ALL (25.5% vs 13.7%, ALL and controls, respectively) (Bolufer et al., 2007). Again, an Indian study reported that *GSTM1* and *GSTT1*-null genotypes represent a risk factor for ALL and also AML, where enzyme absence can drive to oxidative stress, DNA damage and genomic instability, an acute leukemia characteristic (Dunna et al., 2013). On the contrary way, a Turkish finding showed no correlation between *GSTT1*-null and acute leukemia development, but appoint for *GSTM1*-null genotypes (Aydin-Sayitogiu et al., 2006). Zhou et al. (2013) found an association between the *GSTT1* null genotype and the risk of AML in the Chinese population.. Another study related that another part of the GST enzyme family may be related to t-AML, being related to a deletion of *GSTP* alleles, this may lead to a loophole for further related studies (Allan et al., 2001). Regarding AML, no difference was observed in *GSTM1* and *GSTT1* genotyping, either in individual genotypes, which coincides with other data available (Crump et al., 2000).

CONCLUSIONS

Oncohematological disease such leukemia have a genetic background acting directly, however indirect genetic alteration may be a key player in their incidence. This report provides data for glutathione S-transferase genotyping in Brazilian leukemia patients, demonstrating that CML and CLL showed different GST-null frequencies, suggesting that this genetic deletion could represent a risk factor for these leukemia types. Therefore, despite a limitation of not such a great patient number, we demonstrated all leukemia types which have great use for future studies of glutathione S-transferases genotyping in oncohematological context, following especially CML and CLL incidence and prognosis according to their genetic context.

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AUTHOR CONTRIBUTION

DKW and AMM were responsible for conception of the work, acquisition, analysis and interpretation of data. GSS and ECM were responsible for work viability, samples and clinical data collection, together with interpretation of data. DLZ, LB and MA were responsible for financial support, analysis and interpretation of data. All authors drafted, revised, and approved the final manuscript.

STATEMENTS AND DECLARATIONS

Compliance with Ethics Guidelines

The study was conducted after Erasto Gaertner Hospital, Curitiba, Brazil, Ethics Committee approval (CAAE 08809419.0.0000.0098). All procedures followed were in accordance with Brazilian laws and regulations, together with the Helsinki Declaration of 1975, as revised in 2000. Informed consent was obtained from all patients included in the study.

Consent to participate and for publication

An informed consent form was obtained in writing format from all individual participants included in the study, or from the parents if participant was less than 18 years old. Participants signed informed consent regarding publishing their data.

AVAILABILITY OF DATA AND MATERIALS

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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