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Full Length Research Article

Polymorphisms GSTT1, GSTM1 and GSTP1 influence in magnitude of DNA damage induced by cyclophosphamide

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Cancer is one of the major causes of death worldwide and one of the factors associated with this is the therapeutic failure. Recently there has been an increasing interest in designing personalized therapies based on patient's genotype. Glutathione–S-Transferase genes GSTT1, GSTM1 and GSTP1 genes help in detoxification of various genotoxic agents such as cyclophosphamide, an indirect alkylating agent that damages the chemical structure of DNA. It is widely used with other drugs in the treatment of various cancers. Determine whether the extent of DNA damage evaluated by the comet assay performed in vitro by cyclophosphamide in lymphocytes is modulated by polymorphisms of GSTT1, GSTM1 and GSTP1. Lymphocytes from 120 healthy donors were treated with a single concentration of cyclophosphamide and the extent of DNA damage was evaluated by a modified comet assay. Polymorphisms of GSTT1 and GSTM1 were identified by end-point polymerase chain reaction, while GSTP1 alleles were identified by PCR-RFLP. A great variability in the response to cyclophosphamide was found among individuals. Only 12 individuals from all the volunteer donors showed to have the complete wild genotype (GSTT1, GSTM1, GSTP1Ile/Ile105, Ala/Ala114) and coincidentally, this was the group with the lowest cyclophosphamide produced DNA damage. The differences in tail length between this "wild type group" and the other 11 genotypes recognized were statistically significant, suggesting a relation between GST genotype and cyclophosphamide induced DNA damage modulation.

Keywords: GSTT1, GSTM1, GSTP1, Comet assay, Cyclophosphamide, DNA damage

INTRODUCTION

Cyclophosphamide is a drug used to treat some types of cancer. It is an alkylating cytostatic agent, with a broad antineoplastic spectrum. It is frequently used in the therapeutic treatment of breast carcinoma, acute lymphoblastic leukemia, Burkitt's lymphoma, multiple myeloma, ovarian and lung sarcoma, retinoblastoma, neuroblastoma, among others (Moore, 1991). This drug exerts its effect by disrupting DNA, which leads to cell death, resulting on the one hand, in the control of the disease and on the other, in a lot of secondary effects. It

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is nitrogen mustard with the chemical structure of a substituted oxazaphosphorine which requires to be activated by the hepatic microsomal enzyme system in order to be cytotoxic (Garibay et al., 2015).

The liver enzymes convert cyclophosphamide first into 4 hydroxycyclophosphamide, then into aldophosphamide and finally into phosphoramide mustard, which is the metabolite with alkylating effects on DNA (Emadi et la., 2009). During that metabolic activation, involving the alkyl and nucleophilic sites of DNA, adducts are formed. Alkylation products are important in the biological effects caused by this and other alkylating agents. It is a strong bone marrow depressant, which is the biggest toxic effect, leading to a dose related suppression of myelopoiesis, directly attacking the bone marrow. Lymphoid proliferative cells are destroyed, but some resting cells can also be attacked (García et al., 1988; Emadi et al., 2009; Garibay et al., 2015).

Glutathione S-transferases (GSTs), comprise a family of phase II metabolic isozymes best known for their ability to catalyze the conjugation of the reduced form of glutathione (GSH) to xenobiotic substrates for the purpose of detoxification. The GSTs expression plays an important protective effect role on determination the cytotoxicity of chemotherapeutic drugs (Soto et al., 2011). The most studied GST subclasses in mammals are Mu (μ), Pi (π), Theta (θ) and GSTO (ω) (Hayes and Strange, 2000; Hayes et al., 2004). Each class of GSTs has different isoforms codified by several polymorphic genes. The π class by the GSTP1 gene, the θ class by two alleles GSTT1 and GSTT2, and μ class by five genes: GSTM1, GSTM2, GSTM3, GSTM4 and GSTM5 (Pemble et al., 1987; Moore, 1991; Xu et al., 1998).

The GSTT1 and GSTM1 genes have two variants or polymorphisms, the wild type characterized by the presence of the enzyme and the null variant, characterized by the complete lack of enzyme activity (Rossini et al., 2002). The null alleles alter the response and toxicity of chemotherapy in patients with acute lymphoblastic leukemia, breast cancer and metastatic colorectal cancer (Goekkurt et al., 2006; Mossallam et al., 2006; Valladares et al., 2006; Au et al., 2011).

GSTP1 encodes for an enzyme that is highly expressed in epithelial tissue of lung, esophagus and placenta (Chávez, 2011). It presents three different polymorphisms: GSTP1*A, which is considered the wild type allele (Ile105; Ala114); GSTP1*B in which there is a base substitution located in the exon 5 at codon 104, resulting in a mutation of ATC (Ile) to GTC (Val); GSTP1*C presents a base substitution at codon 113 in exon 6 GCG (Ala) to GTG (Val) (Henderson and Wolf, 2005; Custódio et al., 2010; Sharma et al., 2014). These changes reduce the enzyme activity (Chávez, 2011).

It has been observed that patients with the same scheme of treatment show a lot of variability in the effect that treatment causes on them (Gonzalez et al., 2010). Such variability is of concern because chemotherapy can cause life-threatening toxicity.

Pharmacogenomics studies the influence of genetic variations in the individual response to drugs (Steimer et al., 2002; Petros et al., 2005). This is due to the enzymes responsible of drug's metabolism that, depending on the alleles present, may vary in expression, activity and function, therefore determining the cellular response to medical treatment.

It has been demonstrated that a combination with other alleles of GST (GSTM1, GSTT1 and GSTP1) affect the response to treatment in patients with cancer (Bolt and Their, 2006; Oliveira et al., 2010; Wang et al., 2015), a clinical assessment is performed for the evaluation of response to this treatment after two to three cycles, using the system of the World Health Organization (WHO) or the RECIST system. Nowadays, it is impossible to know whether the therapy will be completely effective for the patient after the first application. The evaluation is made usually when half of the treatment has been administered, that is after a period of 4 to 5 months; at this moment patients not responding to the treatment will be detected, along with patients in whom the toxicity was too high to continue the scheme (Garibay et al., 2015). So it is important to clearly understand these mechanisms in order to use them as a platform for personalized therapies.

The comet assay is a relatively inexpensive, fast, sensitive and reliable method to detect DNA breakage in individual cells. The most widely used cell type in this technique in lymphocytes, because they are easy to obtain and handle, and because they are continually exposed to xenobiotics that enter the body. This assay has positioned itself in a privileged position in the battery of biomonitoring studies used as a biomarker capable of demonstrating dose-effect relationships (Silva et al., 2000).

The aim of this investigation is to determine if the differences in the polymorphic genes of GST have an effect on the extent of DNA damage produced by cyclophosphamide in peripheral blood lymphocytes, which is related to the treatment response, in order to support the development and implementation of individualized therapies.

METHODS

Blood samples

In this study, 120 individuals participated under previous informed consent. All of them were apparently healthy and belonged to the university community from Toluca de Lerdo, Estado de Mexico. Volunteers fluctuated from 17 to 56 years old with an average age of 23.71 years; 48 of them were men and 72 women. This study was approved by the institutional ethics committee taking into account the Helsinki declaration.

Lymphocyte isolation

The first part of this work consisted in standardizing the comet assay methodology in order to evaluate lymphocyte DNA damage induced in vitro with cyclophosphamide.

Whole blood samples were aseptically collected by venipuncture in heparinized tubes and labeled for identification. Blood was mixed with an equal volume of Hanks' balanced saline solution (HBSS) and then 5 mL were carefully transferred to 5 mL of Ficol Hypaque 1083 and centrifuged at 600xg for 10 minutes. The interphase ring of nucleated cells was collected, washed twice with HBSS, suspended in RPMI-1640 (Sigma, UK) supplemented with 10% FBS (Gibco, USA) and placed again at 37°C under a 5% CO_2 atmosphere for 12 hours in a 25 cm^2 cell culture flask.

Genotoxicity assessment by means of the comet assay

Cells were counted in a Neubauer chamber and diluted to a cell concentration of $8x10^5$ cells/mL Then, 100 µL aliquots were transferred to microtubes along with 100 µL of S9 microsomal fraction from rat liver treated with phenobarbital and B-naftoflavone; 4.5 mM of cyclophosphamide was added only to one tube. The samples were incubated for three hours at 37°C.

After treatment, cells were spun down, the supernatant was discarded and the pellet was suspended in RPMI-1640 and mixed with an equal volume of 1% low melting point agarose (LMPA) to a final concentration of 0.5%. Then 90 µL were poured on top of slides previously coated with a dried solution of 0.5% normal agarose (to serve as a frost), covered with coverslips and allowed to solidify for 1 minute. Afterwards, the coverslips were retired and the slides were immersed in cold lysis solution (99.5% DMSO, 100% Triton X-100) for one hour at 4°C. Next, the slides were transferred to an electrophoresis chamber, covered with electrophoresis solution (300 mM NaOH, 1 mM EDTA), at 4°C for 30 minutes to allow DNA unwinding and then a 20 V, 300 mAmp current was applied for 24 minutes. Slides were gently rinsed three times with neutralizing buffer (0.4 M Tris, pH7.5) and stained with 60 µL of ethidium bromide (20 µg/mL).

Finally, slides were observed under a fluorescence microscope using a 515–560 nm (green light) excitation filter at 40x magnifying. One hundred cells per slide were examined and comet size was scored by means of the Comet Assay II Analyzer (Perceptive Instruments Inc.) (Silvia et al., 2000).

Genotyping

Genomic DNA was obtained from whole blood by using the Quick-g DNA Mini Prepkit (Zymo Research). GSTM1 and GSTT1 polymorphisms were determined by multiplex PCR. The sequence of the primers used for GSTT1 was: forward 5´TCACCGGATCATGGCCAGCA3´ and reverse 5´TTCCTTACTGGTCCTCACATCTC3´.The primers used for GSTM1 were: forward 5´GAACTCCCTGAAAAGCTAAAGC3´ and reverse 5´GTTGGGCTCAAATATACGGTGG3´ (Zhong et al., 2006). As a positive control, albumin gene was amplified; the sequence used was: forward 5´GCCCTCTGCTAACAAGTCCTAC3´ and reverse 5´GCCCTAAAAAGAAAATCGCCAATC3´. The amplification settings were: initial denaturation 5 minutes at 95°C, denaturation 45 seconds at 95°C, alignment 45 seconds at 57°C, final extension 45 seconds at 72°C for 35 cycles. Subsequently, a horizontal electrophoresis was performed in 2% agarose gel to identify the presence of bands of 480 bp, 215 pb and 352 pb corresponding to GSTT1, GSTM1 and albumin respectively. Absence of DNA fragments of 480 bp and 215bp indicates null genotypes (Abdel et al., 1996).

For GSTP1, polymorphisms were determined by using PCR-RFLP. For the GSTP1 exon 5, the sequence of the primers used was: forward 5'ACCCCAGGGCTCTATGGGAA3' and reverse 5'TGAGGGCACAAGAAGCCCCT3'. PCR settings were: initial denaturation 5 minutes at 95°C, denaturation 45 seconds at 95°C, alignment 45 seconds at 61°C, final extension 45 seconds at 72°C for 35 cycles. For the GSTP1 exon 6,the sequence of the primers was: forward 5´TGGCAGCTGAAGTGGACAGGATT3` and reverse 5´ATGGCTCACACCTGTGTCCATCT3`, PCR settings were: initial denaturation 5 minutes at 95°C, denaturation 45 seconds at 95°C, alignment 45 seconds at 57°C, final extension 45 seconds at 72°C for 35 cycles (Mejia et al., 2013).

The PCR products were verified by horizontal electrophoresis using 1.5% agarose gel. These products were then digested with BsmAI restriction enzyme for GSTP1 exon 5 and AciI for GSTP1 exon 6. These restriction enzymes recognize specifically the sequence variation in the alleles from the wild type, thus generating two fragments. Digestion products were separated by horizontal electrophoresis using a 2% agarose gel and stained with ethidium bromide. Genotype was identified based on the size of DNA fragments; for exon 5, a single 176 bp band correspond to wild GSTP1*A wild type (Ile/Ile105), the 91 bp and 85 bp fragments correspond to GSTP1*B homozygous (Val/Val105) and the 176 bp, 91 bp and 85 bp fragments correspond to GSTP1*B heterozygous (Ile/Val105). As for polymorphisms in exon 6, the 332 bp fragment corresponds to homozygous GSTP1*C (Val/Val114); three fragments, of 332 bp, 174 bp and 158 bp correspond to heterozygous GSTP1*C (Ala/Val114); and two fragments, 158 bp and 174 bp, indicate GSTP1*A wild type (Ala/Ala114) (Mejia et al., 2013).

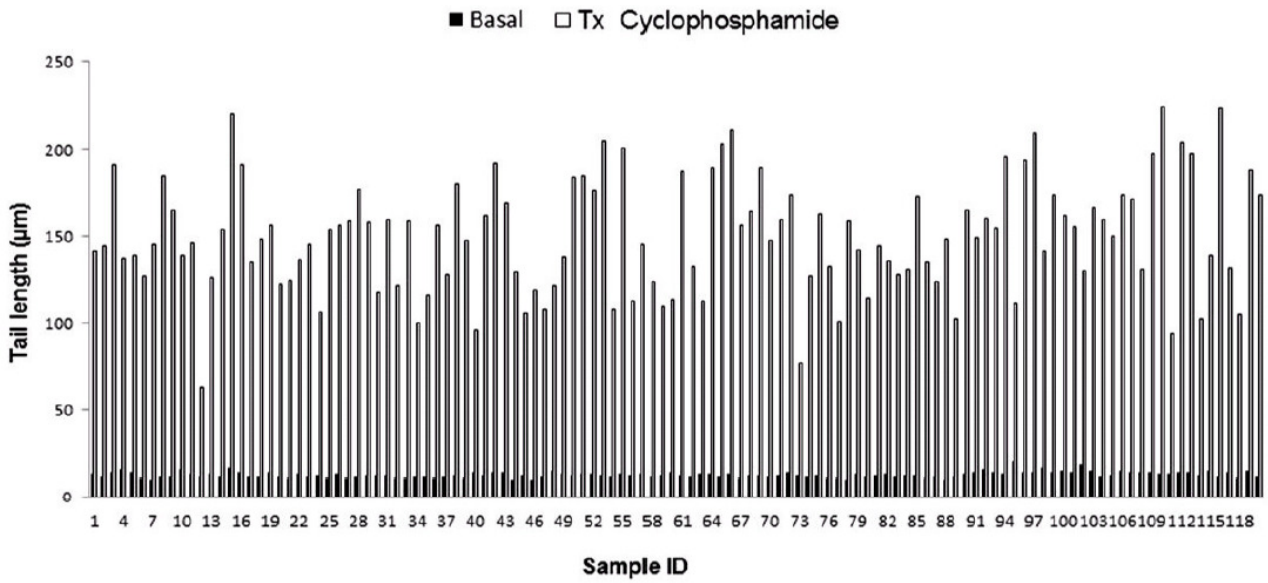

Figure 1. DNA fragmentation level expressed as tail length, in the lymphocytes exposed to a cyclophosphamide.

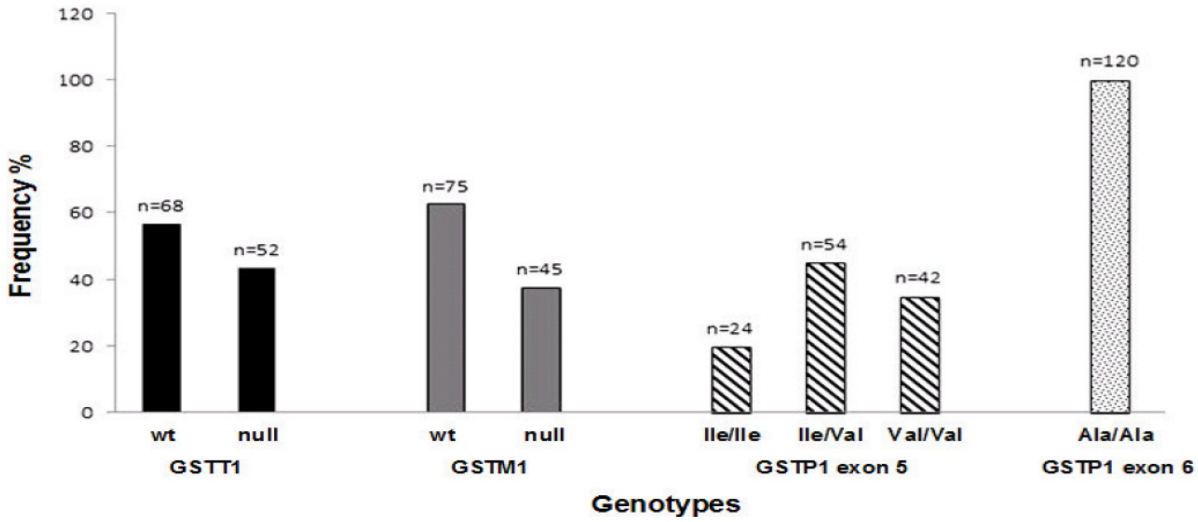

Figure 2. Frequency of the different polymorphisms identified for the genes GSTM1, GSTT1 and GSTP1.

RESULTS

Assessment of the DNA damage produced by cyclophosphamide by means of the comet assay

Figure 1 shows the mean tail length value of the control and treated lymphocytes. Overall, it can be seen that cyclophosphamide produced a large amount of DNA fragmentation in all the samples, however, it is quite evident that there is a great variation among them ranging from 63.25 to 224.23 µm while basal values are very similar from 10.19 to 20.24 µm. Statistical analysis using the t-paired test showed that there is a significant difference between both groups with a significance degree of $p \leq 0.05$.

Allelic frequency

Figure 2 shows the frequency of each polymorphism of the 120 samples. GSTM1 genotypes percentages were 56.67% GSTM1 wild type and 43.33% for GSTM1 null; GSTT1 showed a frequency of 62.5% for the GSTT1 wild type allele, while the GSTT1 null was 37.5%. The allelic frequency for exon 5 of GSTP1 was as follows: 20% wild type (Ile/Ile105); 45% heterozygote (Ile/Val105); 35% homozygote (Val/Val105). Interestingly, for exon 6 GSTP1 all the donors presented the wild type genotype, so it was not possible to analyze the role of the polymorphism GSTP1*C homozygote or heterozygote in the sensitivity to cyclophosphamide.

Table 1. Frequency of the 12 combined genotypes of GSTM1/ GSTT1/GSTP1 in the 120 donors.

Genotype	Frequency (%)	GSTT1	GSTM1	GSTP1 exon 5	GSTP1 exon 6
1	21 (17.5)	wt	wt	Ile/Val	Ala/Ala
2	15 (12.5)	wt	null	Val/Val	Ala/Ala
3	13 (10.8)	wt	null	Ile/Val	Ala/Ala
4	12 (10)	null	wt	Val/Val	Ala/Ala
5	11 (9.2)	wt	wt	Val/Val	Ala/Ala
6	11 (9.2)	null	wt	Ile/Val	Ala/Ala
7	9 (7.5)	wt	null	Ile/Ile	Ala/Ala
8	9 (7.5)	null	null	Ile/Val	Ala/Ala
9	7 (5.8)	null	wt	Ile/Ile	Ala/Ala
10	6 (5)	wt	wt	Ile/Ile	Ala/Ala
11	4 (3.3)	null	null	Val/Val	Ala/Ala
12	2 (1.7)	null	null	Ile/Ile	Ala/Ala

wt: wild type.

Table 2. Statistical comparison of DNA damage between type genotypes of GSTM1, GSTT1 and GSTP1.

Gene	Polymorphism	n	Tail length (µm) ± SD	Statistical test	p
GSTM1	wt	68	150.36 ± 35.73	t-Student	0.800
	null	52	148.88 ± 28.25		
GSTT1	wt	75	141.13 ± 32.38	t-Student	0.077
	null	45	152.29 ± 33.47		
GSTP1 exon 5	Ile/Ile	24	148.45 ± 38.74	Anova	0.955
	Ile/Val	54	150.67± 31.46		
	Val/Val	42	149.21 ± 30.94		
GSTP1 exon 6	Ala/Ala	120	0	-	-

wt: wild type.
Values of p≤0.05 are considered statistically significant

Combined Genotypes of GSTM1/GSTT1/GSTP1

Overall, 12 different genotypes were identified and numbers were assigned to them according to their frequency. Number 1 was the most frequent with 17.5% and it included GSTM1 wild type, GSTT1 wild type, heterozygote GSTP1 exon 5, GSTP1 wild type exon 6. It should be noted that genotype number twelve was only found in two individuals, i.e. 1.7% of the samples. The complete data is shown in Table 1 above.

Relation between DNA damage and GST genotype

The average tail length of the comet induced by cyclophosphamide was calculated individually and associated with the polymorphisms of each allele. The polymorphisms were compared by using the t-Student test. The results show that there are no significant differences between wild and null GSTM1 alleles; the same was true for wild and null GSTT1. For GSTP1 exon 5, an ANOVA was made to analyze the differences in tail length between wild type (Ile/Ile105), heterozygote (Ile/Val105) and homozygote (Val/Val105). No significant differences were found between these allelic variations. Since all the donors have the wild type form of GSTP1 exon 6 alleles, it was not included in the final analysis (Table 2 above).

Interestingly, although there were no variations between the polymorphisms taken individually, differences were found when the full genotypes were analyzed. It was observed that the genotype with the smaller average tail length was number 10, comprising only the wild type alleles GSTM1 wild type, GSTT1 wild type, GSTP1 wild type exon 5, GSTP1 wild type exon 6. Meanwhile, the one with the longest tail length average was number 12 GSTM1 null, GSTT1 null, GSTP1 wild type exon 5, GSTP1 wild type exon 6.

Table 3. Comparison of the magnitude of damage in genotype 10.

ID	Genotype GSTT1/ GSTM1/ GSTP1 exon 5; exon 6	Frequency (%)	Tail length (µm)
10	wt/wt/Ile/Ile;Ala/Ala	6(5)	111.31
3	wt/ null / Ile/Val;Ala/Ala	13(10.8)	144.77*
7	wt / null/ Ile/Ile;Ala/Ala	9(7.5)	145.25*
5	wt/ wt/ Val/Val;Ala/Ala	11(3.3)	148.44*
8	null/ null/ Ile/Val;Ala/Ala	9(7.5)	148.55 *
2	wt / null / Val/Val;Ala/Ala	15(12.5)	148.87
4	null/ wt/ Val/Val;Ala/Ala	12(10)	149.66*
11	null/ null/ Val/Val;Ala/Ala	4(3.3)	151.30
1	wt/ wt /Ile/Val;Ala/Ala	21(17.5)	152.38*
6	null/ wt/ Ile/Val;Ala/Ala	11(3.3)	156.13*
9	null/ wt/ Ile/Ile;Ala/Ala	7(5.8)	172.93*
12	null/ null/ Ile/Ile;Ala/Ala	2(1.7)	188.63

wt: Wild type
*These data are statistically different from wild genotype ID 10, t Student, p≤ 0.05.

Genotype number 10,which is the one with all wild type genes and the one with the highest enzymatic activity, we compared it against all of the remaining 11 using the t Student test.Significant difference was found versus genotypes 3, 7, 5, 8, 4, 1, 6, 9. Genotypes are organized according to tail length. Details of this analysis are shown in table 3 above.

Genotypes were analyzed according to the similarity they presented. Numbers 4, 6, 8 and 9 have the GSTT1 null genotype and genotype GSTP1 exon 6 wild null. Numbers 11 and 12 coincide in having null genotype GSTT1, GSTM1 and GSTP1 wild type exon 6. It should be noticed that these genotypes do not have statistically significant differences compared with the control genotype (GSTT1 wild type, GSTM1 wild type, GSTP1 wild type exon 5, GSTP1 wild type exon 6). Genotypes 2, 3, 7, presented the common genotype GSTT1 wild type, GSTM1 null and GSTP1 wild type exon 6. However, genotype number 2 did not present statistical difference compared to the control genotype (number 10), whereas genotypes 5 and 1 had GSTT1 wild type, GSTM1 wild type and GSTP1 wild type exon 6 (Table 3).

DISCUSSION

The main effect of phosphoramide mustard, the active metabolite of cyclophosphamide, is the formation of adducts and crosslinks (García and Mandina, 2005; Arencibia et al., 2009). If cells were treated in the same conditions, phosphoramide mustard should be producing a similar amount of damage on all the samples, unless genetic differences among the donors would be regulating such damage. In this work, heterogeneity was observed in the extent of DNA damage induced by cyclophosphamide, which suggests that the genetic load of each individual, particularly of the genes involved in the metabolism of cyclophosphamide, regulate its effect.

The genetic variations of genes that encode enzymes involved in the metabolism of cyclophosphamide can influence the response to treatment, as reported by Zhong et al., 2006, who mentioned that individuals who possess the allelic variant GSTP1*B, presented a significant increase in the risk of suffering short-term side effects from high doses of cyclophosphamide.

As for the role of GTS polymorphisms in individuals with respect to their response to cyclophosphamide, statistical tests clearly show that there are no significant differences among all the samples when genes are analyzed individually. However, when the full GST genotype is considered, differences are evident (Wang et al., 2015).

We identified 12 different genotypes in the studied population and, among them, we found one with a complete wild type (genotype number 10) which, according to previous reports would have a higher enzyme activity regarding all the other genotypes with polymorphic genes, where enzyme activities is lower or totally lost, in the case of null alleles. Interestingly, this genotype has the lowest tail length mean value. In individuals with wild type genotypes, the drug is eliminated more efficiently due to an increase in enzymatic activity. This can translate into less therapeutic effect. On the other hand, genotype null GSTM1, GSTT1 null, GSTP1 exon 5 wild type, GSTP1 exon 6wild type (genotype number 12), presented the largest tail length value, meaning that in individuals with this GST alleles the drug would linger for longer, thus producing a larger amount of damage. This genotype could be considered as the most suitable to respond to a medical scheme of

treatment with cyclophosphamide (Soto et al., 2011).

According to our results, patients with the null alleles for GSTM1 or GSTT1 and genotype GSTP1*B would have a better response to a cyclophosphamide treatment. Indeed, it has been reported that patients with ovarian cancer have a better prognosis when the null alleles of GSTM1 and GSTT1 are present (Wang et al., 2015). Other publications about breast cancer and acute lymphoblastic leukemia indicate that null polymorphisms in GSTM1 and GSTT1 are associated with a better response to drug treatments (Stanulla and Schaffeier, 2005; Wang et al., 2015). It is important to consider that multiple enzymes of the GST family such as GSTA1, GSTT1, GSTM1, GSTP1 or other families such as CYP and ALDHs are involved in bioactivation, detoxification and influence the response and toxicity of cyclophosphamide. The results of this investigation demonstrate the heterogeneity in the response to damage induced by cyclophosphamide and, on the other hand, the participation of the genes GSTT1, GSTM1 and GSTP1 as modulators of this damage. However, more preclinical and clinical pharmacogenetic studies are needed to fully understand the mechanism of action, resistance and toxicity of cyclophosphamide in order to design a personalized treatment.

CONCLUSIONS

Cyclophosphamide is part of one of the most used drugs in the treatment against cancer. Response to treatment is often uncertain. This research proposes an option to predict the response to treatment with cyclophosphamide at an early stage of treatment, considering the gene formula of GSTT1, GSTM1 and GSTP1 of each individual and the use of the comet assay. Besides, personalized treatments can be designed according the gene strain.

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