

Null polymorphisms in *GSTT1* and *GSTM1* genes and their associations with smoking and cervical cancer

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ABSTRACT. Human papillomavirus infection is the main risk factor for cervical cancer. Other risk factors include smoking and genetic susceptibility. Glutathione-S-transferases are enzymes involved in tobacco carcinogen metabolism, and genes encoding these enzymes are highly polymorphic. We compared the frequencies of *GSTM1* and *GSTT1* null polymorphisms in women with cervical cancer and in a control group, as well as to determine possible associations between such polymorphisms, cigarette smoking and the prognosis of cervical cancer. The series comprised 135 cervical cancer patients and 100 women without cancer. Genotypes were investigated by PCR. The results were compared using the Chi-square test or Fisher's exact test,

and survival analysis by Kaplan-Meier test and Log-rank. Among the cases, the frequency of *GSTM1* gene null polymorphism was 22.2%, and for the *GSTT1* gene it was 48.5%. Among the controls, the frequency of the *GSTM1* gene null polymorphism was 45.0%, while for *GSTT1* it was 56.0%. A significant association was found between smoking and cervical cancer ($P = 0.0062$; $OR = 2.16$). Differently from *GSTM1*, the *GSTT1* null polymorphism was not associated with cervical cancer risk in this study. The *GSTT1* null genotype was significantly associated with worse prognosis. The overall survival rate for the cervical cancer group was 78.5%, and when stratified by genotypes, survival was higher in patients presenting at least one of the alleles, *GSTT1* or *GSTM1*, indicating a higher risk of death for those presenting dual nullity ($P = 0.031$; $RR = 2.458$).

Key words: Cervical cancer; *GSTM1*; *GSTT1*; Smoking

INTRODUCTION

The main risk factor for cervical cancer is infection with Human Papillomavirus (HPV) (Waggoner, 2003; Doorbar, 2012). HPV infection is necessary, but not enough for the development of cervical cancer. Other risk factors have become apparent, such as viral genotype, persistence of HPV infection, genetic factors, immunological status and smoking (Campaner et al., 2010). Genetic susceptibility factors affecting the development of cervical cancer have been studied in many populations (Li et al., 2013; Abbas et al., 2014; Roszak et al., 2014), but little is known about interactions between genetic polymorphism and risk factors for cervical cancer, such as smoking (Roszak et al., 2014).

Chemical substances produced through tobacco burning and absorbed by the lungs can be found in the cervical mucus of smoker women, directly exposing the cervical cells to tobacco carcinogens (Campaner, 2011). Smoking has been reported as an important risk factor to cervical cancer. The duration and intensity of the smoking habit are related to the development of cervical precursor lesions and invasive carcinomas; smoking increases the risk of neoplasia (Roura et al., 2013).

Tobacco contains many carcinogenic substances, such as nitrosamines and aromatic hydrocarbons, which interact with the genetic material of the cells resulting in DNA adducts, which in turn favor the occurrence of mutations that drive the carcinogenic process. Chemical substances present in tobacco are metabolized by enzymes from the P450 cytochrome family and glutathione-S-transferases (*GSTT1* and *GSTM1*) (Leme et al., 2010).

Glutathione is an antioxidant tripeptide, comprised of glycine, cysteine and glutamic acid. It is a cofactor in many enzymes and detoxification processes (Carreiro, 2011). Glutathione-S-transferases (GST) are a family of enzymes involved in the detoxification of xenobiotics, such as the polycyclic aromatic hydrocarbons present in cigarettes, hence protecting cells from deleterious and oxidative effects (Joseph et al, 2006; Singh et al, 2008; Leme et al., 2010). The homozygous deletion or null genotype of *GSTM1* and *GSTT1* results in reduction or absence of the activities of these enzymes, with the possibility of increasing susceptibility to cancer (Joseph et al, 2006; Singh et al, 2008;

Leme et al., 2010). The genes that code these enzymes are highly polymorphic and show great variability of expression levels and activity (Leme et al., 2010).

The Brazilian population is extremely mixed and studies about the genetic factors, such as null polymorphisms in xenobiotic metabolic genes are still scarce. Despite the high potential of cervical cancer prevention, thousands of new cases are registered each year. Therefore, investigation of factors associated with the development and prognosis of cervical cancer can improve prevention and treatment planning for this type of cancer.

We evaluated possible associations between the null polymorphisms of the *GSTT1* and *GSTM1* genes, smoking and the prognosis of cervical cancer patients.

MATERIAL AND METHODS

The study was approved by the Ethics Committee in Human Research of the Association of Cancer Combat in Goiás (ACCG). The selection of subjects with cervical cancer was achieved through active search of the files of the institutional Pathology Laboratory. Cervical cancer cases diagnosed between 2006 and 2007 were histopathologically confirmed and selected for the analysis of *GSTT1* and *GSTM1* null polymorphisms. Clinical and pathological data were collected from the medical files and included: histological type, histological grade, marital status, age at diagnosis, smoking and alcohol consumption history, menstrual status, type of treatment, clinical staging (extension of the lesion, lymph node metastasis and distant metastasis) and patient status at the last visit, with a follow up of 60 months.

The control group included women without cervical cancer history paired by age group who visited the Clinical Laboratory of Pontifical Catholic University of Goiás for routine exams. The women that participated in the study signed the Written Informed Consent, authorizing collection of a blood sample for the molecular analysis of *GSTT1* and *GSTM1* null polymorphisms.

DNA extraction of the cervical tumor samples was performed by using the commercial Wizard Kit (Promega), according to the deparaphinization and extraction protocol. DNA extraction of peripheral blood samples of the control group was performed by the *salting-out* method (Sambrook, Fritsch and Maniatis, 1989).

PCR was used for the amplification of a 75 bp fragment of the *GSTM1* gene and a 70 bp fragment of the *GSTT1* gene, according to the protocol described by Halolu, 2013. The primers used for the amplification of the *GSTM1* fragment were: Forward 5'ATGGTTTGCAGGAAACAAGG3' and Reverse 5'CCTCCATAACACGTGAAGCA3'. Amplification of the *GSTT1* gene employed: Forward 5'TTCCTGGGTGAGCCAGTATC3' and Reverse 5'ACTGCAGGGTCACATCCAA3' primers. All the reactions included positive and negative controls for verification of amplification efficiency. The amplification products were analyzed in 8% polyacrylamide gel and revealed by staining with silver nitrate.

Clinical and pathological data for the two groups were compared in relation to the genetic polymorphisms. Chi-squared or Fisher's exact tests were used, with a

significance level of 5% ($P < 0.05$), using the GraphPad 4 Prisma software. Survival analysis used the Kaplan Meier and Log-rank method.

RESULTS

The case group included 135 women with cervical cancer with confirmed histopathological diagnosis. The age of the group varied from 26 to 85 years, and the mean age was 49.6 years (± 14.3). Cigarette smoking was described by 44.4% of the women and alcohol consumption by 9.6%. Most of the women with cervical cancer were submitted to some type of surgery during treatment, including radical hysterectomy as the most frequent procedure (31.1%). A combination of external radiation + brachytherapy was applied to 40.0% of the women. When we examined histological type, squamous cell carcinoma was found in most of the cases (74.1%), followed by adenocarcinoma (17.0%). Chemotherapy was used only for the metastatic cases. After 60 months, death was registered for 23.0% of the group. The control group included 100 healthy women, with age varying from 24 to 86 years and a mean of 54.3% years (± 15.1). Cigarette smoking was reported by 27% of the women in the control group at a certain period of their lives and 15.0% of the total group were still active smokers.

The frequency of *GSTT1* and *GSTM1* null polymorphisms is described in Table 1. *GSTM1* null polymorphism was less frequent in cases (22.2%) than in controls (45.0%) ($P = 0.0016$). A significant relationship was observed between smoking habit and cervical cancer ($P = 0.0062$), with a risk 2.16 times higher for smokers to develop cervical cancer (Table 2). Overall survival for the subjects with cervical cancer, at the end of 60 months was 78.5% (Figure 1). When adjusted for *GSTM1* genotype, the survival rate was 80.0% for the *GSTM1* positive genotype and 73.3% for the *GSTM1* null genotype carriers (Figure 2) ($P = 0.368$). For *GSTT1*, the adjusted survival for the patients presenting at least allele, was 85.5%, and it was 71.2% for the negative *GSTT1* genotype carriers (Figure 3). A significant difference between the two groups was observed ($P = 0.034$) and the risk for death was 2.2 times higher when the *GSTT1* null genotype was detected.

Table 1. *GSTT1* and *GSTM1* null polymorphisms in the case and control groups.

Gene	Cases (N = 135)		Controls (N = 100)		P	OR
	Null genotype	Present genotype	Null genotype	Present genotype		
<i>GSTM1</i>	22.2%	77.8%	45.0%	55.0%	0.0016	0.4127
<i>GSTT1</i>	48.5%	51.5%	56.0%	44.0%	0.2808	-
<i>GSTM1</i> and <i>GSTT1</i>	13.3%	86.7%	28.0%	72.0%	0.0051	0.3956

OR = odds ratio.

Table 2. Frequency of smoking habit between the case and control groups.

Variable	Cases (n = 135)	Controls (n = 100)	P	OR
Smokers	44.4%	27.0%	0.0062	2.16
Non-Smokers	55.6%	73.0%		

OR = odds ratio.

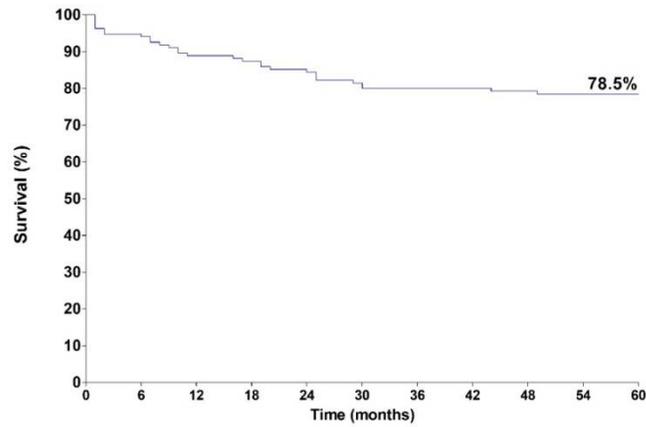


Figure 1. Overall survival of the patients with cervical cancer.

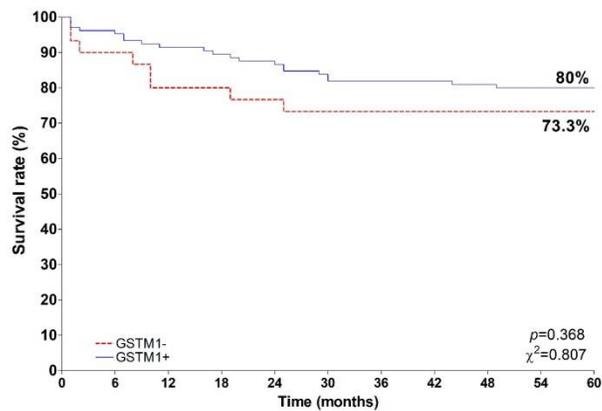


Figure 2. Survival curve adjusted to the *GSTM1* genotype.

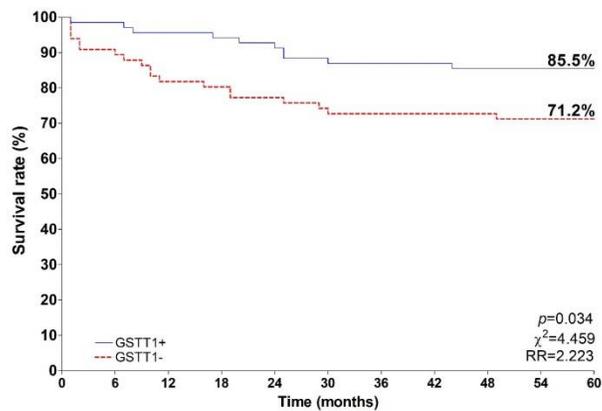


Figure 3. Survival curve adjusted to the *GSTT1* genotype.

When the double null genotypes were combined, the survival of patients carrying the double null polymorphism was 61.1%, while the survival rate for those with at least one of the alleles was 81.2% (Figure 4). A significant difference was found between the groups ($P = 0.031$), with the risk of death 2.5 times higher for those with the double null genotypes.

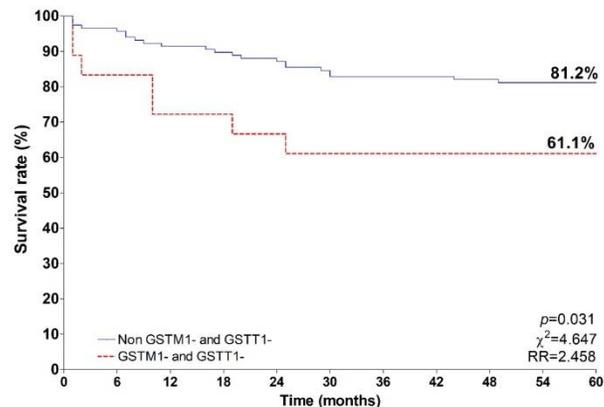


Figure 4. Survival curve adjusted to the double null genotype.

DISCUSSION

We observed a difference in the frequency of *GSTM1* and *GSTT1* null polymorphisms between the group of patients with cervical cancer and controls. *GSTM1* and *GSTT1* null polymorphisms were both less frequent in cases than in controls. These results contradict the initial hypothesis of this study that the absence of these genes, which encode detoxification enzymes, would improve the process of carcinogenesis induced by HPV.

Many studies have investigated the relationship between the *GSTM1* and *GSTT1* null genotypes and different types of cancer, such as bladder, lung, prostate, kidneys, ovary, head and neck and others (Leme et al., 2010; Rodrigues et al., 2010; Halolu et al., 2013; Matic et al., 2013). However, due to the well-established relationship between cervical cancer and HPV, studies investigating these polymorphisms in cervical cancer are still scarce and differences between populations have been described. After reporting carcinogens derived from polycyclic aromatic hydrocarbons in the cervical mucus (Campaner et al., 2007; Kiran et al., 2010), interest in detoxifying enzymes started to increase in studies of cervical cancer. In Brazil only one published study investigated the frequency of *GSTM1* and *GSTT1* null polymorphisms in cervical adenocarcinomas and in controls without cancer (Carvalho et al., 2008). Our study is the first, in Brazil, investigating the frequency of *GSTM1* and *GSTT1* null polymorphisms in cervical cancer in different histological types, such as squamous cell carcinomas, adenocarcinomas, and adenosquamous carcinomas, compared to healthy controls. Brazilian studies about this topic investigated only the frequency of the polymorphism in the general population or the

frequency of null genotypes in other type of cancers (Guembarovski and Cólus, 2001; Gatás et al., 2004; Leme et al., 2010; Olivera et al., 2010).

The relationship between the null polymorphisms of *GSTM1* and *GSTT1* with cervical cancer is controversial. Studies performed in different countries such as India (Joseph et al, 2006), Italy (Palma et al, 2010), and Kazakhstan (Djangusurova et al., 2013), considered the *GSTM1* and *GSTT1* null polymorphism as a risk factor for cervical cancer. On the other hand, studies in countries such as Thailand (Ishida et al, 2009), Turkey (Kiran et al, 2010), and Serbia (Stosic et al., 2014), did not consider *GSTM1* and *GSTT1* null polymorphism as a risk factor for cervical cancer. In a study in Colombia (Sierra-Torres et al, 2006), the *GSTM1* and *GSTT1* null polymorphisms did not affect the risk for high grade intraepithelial lesions, suggesting other factors as more important for cancer evolution, such as high grade infection by HPV, exposition to mutagenic compounds and polymorphisms in other metabolism genes, such as *CYP2E1*. A recent study developed in Thailand concluded that deletion of *GSTM1* is not a risk factor for cervical cancer. This study seems to present higher accuracy, since not only the null genotype for *GSTM1* was studied but also the heterozygous variables for this gene (Natphopsuk et al., 2015).

A meta-analysis investigated this association in the world population and found that the *GSTM1* null genotype confers increased risk for cervical cancer in non-Chinese populations, while *GSTT1* null polymorphism showed no association with cervical cancer (Economopoulos et al., 2010). Another more recent meta-analysis revised and updated the data of the Chinese population and found a significant association between *GSTM1* null polymorphisms and cervical cancer in that population (Sun and Song, 2016). Disagreement between published results can be explained mainly by the different frequencies of this polymorphism in different populations, which can vary from 26% in Africans to 53% in Caucasians (Natphopsuk et al., 2015). Additionally, other factors could be associated with cancer development, such as lifestyle and exposition to different environmental xenobiotics.

Important prognostic factors such as lymph node involvement, extension of the lesion and the number of deaths were less frequent in *GSTM1* positive genotype carriers (data not shown). As mortality, it was observed that most of the living patients, at the end of 60 months (68.9%), had a functional genotype. This means that some enzymatic activity could be retained in these patients, compared to those with double null genotypes. This data is reflected in the survival of the patients, which was higher in all the situations where at least one positive genotype was observed, indicating a higher death risk in the cases with the double null genotype ($P = 0.031$; $RR = 2.458$).

One study evaluated cervical cancer survival associated with the *GSTT1* and *GSTM1* polymorphisms. In that study, the double null *GSTM1* and *GSTT1* genotype was associated with higher survival (Abbas et al, 2015). All the patients in the study were in advanced stages of disease, and all them went through treatment with cisplatin, a drug that is also metabolized by the GST enzymes. Therefore, differences between findings could be explained by the specificity of the group evaluated. It is known that overexpression of *GSTM* and *GSTT* in tumor cells can determine resistance to treatment with cisplatin or derivative compounds; in this case, the genetic null polymorphism would favor these subjects (Abbas et al., 2015).

Concerning the smoking habit, the well-established relationship (Philips and Venitt, 2012; Sobus and Warren, 2014; Wei et al., 2014) between tobacco and the risk for cervical

cancer was also confirmed in our study ($P = 0.0062$; $OR = 2.163$). However, significant difference in survival were not observed between smokers and non-smokers ($P = 0.1845$).

Some limitations for this study include the size of the samples, the lack of information in the patient's files and the molecular techniques employed for the polymorphism analysis. Concerning the size of the sample, despite our efforts, it was not possible to include all the population samples initially expected, resulting therefore in sampling by convenience. In the molecular technique, conventional PCR did not allow differentiation between homozygous and heterozygous genotypes, limiting the results to null or present genotypes.

We conclude that the *GSTM1* null genotype is associated with cervical cancer in our cohort, and that a *GSTT1* positive genotype is significantly associated with a better prognosis in patients with cervical cancer.

Further studies on cervical cancer and genes related to the metabolism of xenobiotic should include analysis of other genes that act synergistically in the metabolism and elimination of toxic and mutagenic compounds. Inclusion of genes of the P450 cytochrome family and a more detailed study of the GST genes, including the heterozygous genotypes, could improve elucidation of factors involved in cervical cancer development and xenobiotic metabolism.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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