

Comparative analysis of glutathione transferase genetic polymorphism, *Helicobacter pylori* and Epstein-Barr virus between the tumor area and the proximal and distal resection margins of gastric cancer.

Análise comparativa do polimorfismo genético da glutathione transferase, do *Helicobacter pylori* e do vírus Epstein-Barr entre a área do tumor e as margens de ressecção proximal e distal do câncer gástrico.

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ABSTRACT

Objective: to compare the polymorphism of the Glutathione S-transferase theta 1 (GSTT1) and Glutathione S-transferase mu 1 (GSTM1) genes from the tumor area with the proximal and distal margins of stomach specimens resected from patients with gastric cancer, and to investigate the presence of Epstein-Barr virus (EBV) DNA and *Helicobacter pylori*. **Methods:** we prospectively collected tissue specimens from the tumor area and from the proximal and distal resection margins of the stomachs of ten patients with gastric adenocarcinoma who underwent gastrectomy with D2 lymphadenectomy, and submitted these specimens to DNA extraction. We compared the tumor area with the proximal and distal margins of the resected stomachs for polymorphism of *GSTT1* and *GSTM1* genes and investigated the presence of EBV-DNA and *H. pylori*. We used the p53 exon 5 gene as an internal control of the multiplex PCR reaction. **Results:** in one patient, we detected null *GSTT1* and *GSTM1* genotypes in the tumor area, in contrast to the presence of both genes in the proximal and distal margins. We found EBV-DNA and *H. pylori* in the tumor area and also in the proximal and distal margins. In another patient, the proximal margin was negative for *GSTT1*, and EBV-DNA was negative in the distal margin. In three

patients, EBV-DNA was negative only in the distal margin. **Conclusion:** this is the first report where different genotypes, EBV-DNA and *H. pylori* infection were observed in the same patient, indicating a probable deletion of these genes in response to tumor progression and intratumoral heterogeneity.

Keywords: Glutathione Transferase. Polymorphism, Genetic. Stomach Neoplasms. *Helicobacter pylori*. Epstein-Barr Virus Infections.

INTRODUCTION

Gastric cancer (GC) is still an important cause of death from cancer throughout the world. In Brazil is the third most common malignancy among men and the fifth among women¹. However, the genetic pathway of this tumor development and progression remains uncertain. It's a multifactorial disease that results from individual genetic predisposition and exposure environmental factors such as diet, alcohol consumption, smoking and chronic infection of *Helicobacter pylori* or Epstein-Barr virus (EBV)²⁻⁴. The Epstein-Barr virus belongs to the genus lymphocryptovirus of the human gamma-herpesvirus family, and infects more than 90% of the world adult population⁵. It is a ubiquitous herpesvirus that is classified as a group 1 carcinogen by the International Agency for Research on Cancer. EBV is associated with various malignancies such as Burkitt lymphoma, Hodgkin lymphoma, nasopharyngeal carcinoma, natural killer cell lymphoma and also gastric cancer^{2,6}. Epstein Barr virus and *H. pylori* co-infection are positively associated with severe gastritis in pediatric patients and gastric cancer^{3,4,7,8}.

Glutathione S-transferases (GST; EC 2.5.1.18), a super gene family of phase II detoxification enzymes, appears to form a protective mechanism against chemical carcinogenesis⁹. Glutathione S-transferase mu 1 (GSTM1) and Glutathione S-transferase theta 1 (GSTT1) are critical enzymes for detoxification of endogenous and environmental carcinogens. The *GSTT1* gene is located in chromosome 22, and the *GSTM1*, in chromosome 1¹⁰. Homozygote deletions or absence of genotype of *GSTT1* and *GSTM1* genes may be associated with increased risk of cancer¹¹. Polymorphism is a term used in genetics to describe multiple forms of a single gene that exists in an individual or among a group of individuals. The *GSTM1* and *GSTT1* isoenzymes exhibit deletion polymorphisms, resulting in a lack of activity, and the null genotypes have been associated with a significantly increased risk of gastric cancer¹².

Our study aims to compare the polymorphism of the *GSTT1* and *GSTM1* genes in the tumor area with the proximal and distal margins of stomach specimens resected from patients with gastric cancer, and to investigate the co-infection of EBV and *H. pylori*.

METHODS

We prospectively studied ten patients with gastric adenocarcinoma who underwent gastrectomy with D2 lymphadenectomy at the Clementino Fraga Filho University Hospital

of the Federal University of Rio de Janeiro, Brazil. We extracted the genomic DNA from fresh tumor tissue and from the proximal and distal resection margins (Figure 1). We isolated it by digestion in 500µl containing 10mM Tris-HCl, pH 7.5, 10mM NaCl, 2% SDS, 10mM EDTA, pH 8.0, and 15µl 10mg/ml proteinase K at 60°C for 2h. Thereafter, we added an equal volume of 1:1 (v/v) phenol: chloroform, followed by vigorous shaking and centrifugation. The aqueous phase was separated and the DNA precipitated with two volumes of absolute ethanol at -20°C overnight. After centrifugation, we washed the pellets with 70% ethanol and resuspended the DNA in Milli-Q water. We submitted the extracted genomic DNA to PCR to confirm DNA integrity using p53 gene exon 5 primers, generating a 274 bp product as reported by Pestaner *et al.*¹³. We detected *GSTM1* and *GSTT1* genotypes by multiplex polymerase chain reaction (PCR) with the p53 gene exon 5 as an internal control for the success of the amplification reaction. The primers used for *GSTM1*, *GSTT1*, and p53 are described in table 1.

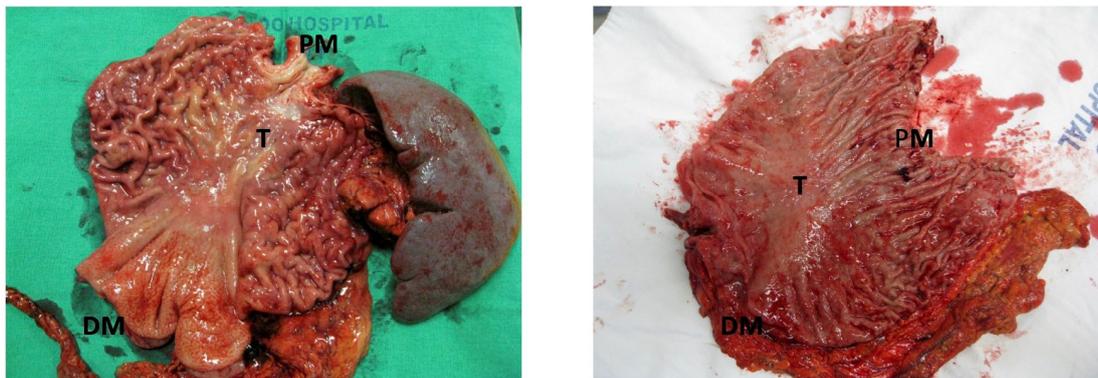


Figure 1. Gastrectomy specimens from patients 1 and 5, showing the tumor area (T), the proximal margin (PM) and the distal margin (DM).

Table 1. List of primers used.

Gene	Sequence of forward (F) and reverse (R) primers	Product size (bp*)
GSTM1**	(F) 5' GAACTCCCTGAAAAGCTAAAGC 3' (R) 5' GTGGGCTCAAATATACGGTGG 3'	220
GSTT1***	(F) 5' TTCCTTACTGGTCCTCACATCTC 3' (R) 5' TCACCGGATCATGGCCAGCA 3'	450
p53 (exon 5)	(F) 5' GCAACCAGCCCTGTCTGTCTCCA 3' (R) 5' GGAATTCTGTTCACTTGTGCCCTGACTTTCAAC3'	274
EBV-TC67#	5' CAG GCT TCC CTG CAA TTT TAC AAG CGG 3'	288
EBV-TC69#	5' CCCAGAAGTATACGTGGTGACGTAGA 3'	
glmM ¹⁷	(F) 5'GGA TAA GCT TTT AGG GGT GTT AGG GG3' (R) 5'GCA TTC ACA AAC TTA TCC CCA ATC3'	140

bp*: base pairs; GSTM1**: Glutathione S-transferase mu 1; GSTT1***: Glutathione S-transferase theta 1; TC67/TC69#: consensus primers; Espinoza *et al*¹⁷.

The PCR conditions included initial denaturation at 94°C for 5 minutes followed by 30 cycles of 95°C for 1min, 64°C for 1min, 72°C for 1min. The final extension was at 72°C for 5min¹⁴. After amplification, we separated the PCR products in 10% polyacrylamide gel electrophoresis and visualized them with silver nitrate. We performed gel staining as follows: one initial step of DNA fixation using a 10% ethanol and 0.37% acetic acid solution for 10min, one step of impregnation by 0.2% silver nitrate solution for 10min followed by a rinse with distilled water for 30s, and one final step with 3% NaOH and 0.4% formaldehyde solution until the DNA bands were clearly visible. We used the initial solution to stop the reaction¹⁵.

Detection of Epstein-Barr Virus (EBV) and *Helicobacter pylori* DNA.

We assessed the presence of EBV and *H. pylori* DNA in the samples using PCR. To detect the EBV-DNA, we employed the consensus primers TC67 and TC69¹⁶, whose product is a 288bp fragment. For the *H. pylori*-DNA detection, we used primer pairs for the *glmM* gene as described by Espinoza *et al.*¹⁷ to obtain a PCR product size of 140bp. We performed the amplification for EBV-DNA as follows: 95°C for 1min, followed by 40 cycles at 94°C for 1min, 55°C for 2min, 72°C for 1min, and a final extension step of 5min at 72°C. The parameters for the amplification of *H. pylori*-DNA were: 5min for the initial denaturing at 96°C, then 35 cycles at 95°C, 55°C and 72°C, being 1min for each temperature during the cycles, and a final elongation step at 72°C for 7min. We stored the products resulting from DNA amplification (amplicons) at 4°C until use, when we analyzed them in 10% polyacrylamide gel stained with silver¹⁵. We used Raji cell lines and a positive sample for *H. pylori* infection as positive reaction controls for EBV and *H. pylori*, respectively. The negative controls were samples containing just the reaction mixture without DNA.

This research complies with the guidelines for human studies and animal welfare regulations. Our institute's committee on human research (Research Ethics Committee/Institutional Review Board) has approved the study protocol (CAAE 43698915.6.0000.5257) and all patients signed an informed consent form.

RESULTS

Ages ranged from 51 to 75 years; six patients were male and four were female. Table 2 shows the tumor characteristics, surgical and pathological findings, as well as staging.

Table 2. Tumor characteristics, surgical and pathological findings, and staging.

Patient	Age	Gender	Tumor location	Histologic type	Surgery	Resection margin	Staging
1	51	M	Cardia III	Poorly differentiated/ diffuse type	Total gastrectomy	Negative	T3N3aM1
2	75	M	Body	Moderately differentiated/ intestinal type	Subtotal gastrectomy	Negative	T2N0M0
3	71	M	Antrum	Moderately differentiated/ intestinal type	Subtotal gastrectomy	Negative	T3N1M0
4	53	M	Cardia III	Poorly differentiated/ diffuse type	Total gastrectomy	Positive	T3N2M0
5	56	F	Antrum	Moderately differentiated/ intestinal type	Subtotal gastrectomy	Negative	T3N3aM0
6	53	F	Fundus	Poorly differentiated/ diffuse type	Total gastrectomy	Negative	T3N1M0
7	52	F	Antrum	Well differentiated/ intestinal type	Subtotal gastrectomy	Negative	T1aN0M0
8	69	M	Antrum	Poorly differentiated/ diffuse type	Subtotal gastrectomy	Negative	T3N2M0
9	61	M	Body + Antrum	Moderately differentiated/ intestinal type	Total gastrectomy	Negative	T3N1M0
10	58	F	Antrum	Poorly differentiated/ diffuse type	Antrectomy	Negative	T3N0M0

Table 3 shows the polymorphism for *GSTT1* and *GSTM1* from the tumor area (T), the distal margin (DM) and the proximal margin (PM).

Table 3. Glutathione S-transferase mu 1 (*GSTM1*) and Glutathione S-transferase theta 1 (*GSTT1*) genotypes.

Genes Patients	<i>GSTT1</i>			<i>GSTM1</i>		
	DM*	T [†]	PM [‡]	DM*	T [†]	PM [‡]
1	POS	NEG	POS	POS	NEG	POS
2	POS	POS	POS	POS	POS	POS
3	POS	POS	POS	NEG	NEG	NEG
4	POS	POS	POS	POS	POS	POS
5	POS	POS	NEG	POS	POS	POS
6	NEG	NEG	NEG	POS	POS	POS
7	POS	POS	POS	NEG	NEG	NEG
8	POS	POS	POS	POS	POS	POS
9	POS	POS	POS	POS	POS	POS
10	POS	POS	POS	POS	POS	POS

DM*: distal margin; T[†]: Tumor area; PM[‡]: proximal margin; POS: positive; NEG: negative.

Table 4 shows the presence or absence of Epstein-Barr Virus (EBV) or *H.pylori* associated with gastric cancer.

Table 4. EBV DNA and *H. pylori* detection.

Patients	EBV			<i>H.pylori</i>		
	DM*	T [†]	PM [‡]	DM*	T [†]	PM [‡]
1	POS	POS	POS	POS	POS	POS
2	NEG	NEG	NEG	NEG	NEG	NEG
3	POS	POS	POS	POS	POS	POS
4	NEG	NEG	NEG	NEG	NEG	NEG
5	NEG	POS	POS	POS	POS	POS
6	NEG	NEG	NEG	POS	POS	NEG
7	NEG	NEG	NEG	POS	POS	POS
8	POS	POS	POS	POS	POS	POS
9	NEG	POS	POS	NEG	NEG	NEG
10	NEG	POS	POS	POS	POS	POS

DM*: distal margin; T[†]: Tumor area; PM[‡]: proximal margin; POS: positive; NEG: negative.

We detected products of *GSTT1* and *GSTM1* genes in both proximal and distal margins in patient 1, but observed null genotypes in the tumor area. In patient 5, the proximal margin was negative for *GSTT1*. The presence of amplification product for the p53 gene in all samples indicates the internal control of the reaction. In the other eight analyzed patients, we observed no such imbalance (Figure 2).

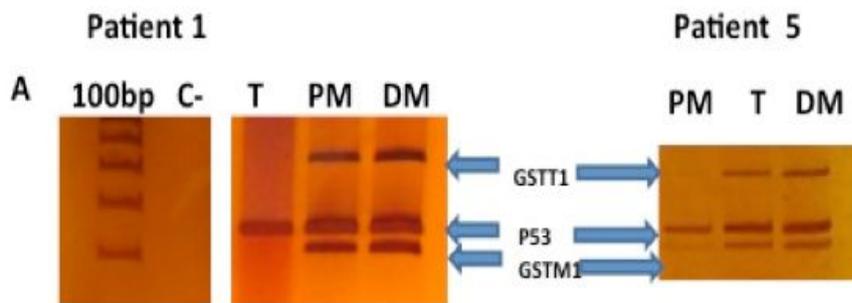


Figure 2. Multiplex PCR of *GSTM1* and *GSTT1* genes and of exon 5 of the p53 protein encoding gene (Tp53), 100 base pairs ladder (bp) negative control (C-). Patient 1: DNA from tumor fragment (T) showing null genotype (absence of 480 and 215 bp) for *GSTT1* and *GSTM1*. PM and DM correspond to proximal and distal tumor margins, showing amplification for *GSTT1* and *GSTM1*. The p53 exon 5, used as an internal control, is detected in all samples. Patient 5: *GSTT1* was absent only in PM.

We found EBV DNA in the tumor area and also in the proximal and distal margins in patients 1, 3 and 8, but not in the distal margin of patients 5, 9 and 10. We observed *H.pylori* and EBV co-infection in 5/10 (50%) of cases (table 4).

DISCUSSION

The review of Knuutila *et al.*^{18,19} describes many chromosomal aberrations, such as losses and gains of tumor DNA sequences, which occur in various malignant tumors. Chromosomal instability and heterogeneous gene amplifications/deletions are associated with many malignancies²⁰⁻²³. For gastric cancer, Noguchi *et al.*²⁴ detected no chromosomal deletions of the *GSTM1* and *GSTT1* genes in their work. The authors, when comparing their results to one performed with Japanese population²⁵, observed differences in the pattern of chromosomal aberrations between European patients and those from high-risk populations, such as from Japan, suggesting different tumor genes in these populations. In our study in Brazilian patients with gastric adenocarcinomas, we noticed a null genotype for *GSTT1* and *GSTM1* only in the tumor area of one patient, and a null genotype for *GSTT1* in the proximal margin in other. These results reflect an intratumoral heterogeneity for the genes studied. In one of our other studies, a multiplex PCR was performed for *GSTT1* and *GSTM1* genes using samples from the three distinct areas of a solid pseudopapillary neoplasm of the pancreas. The results showed a null genotype for *GSTT1* in tumor areas 1 and 3 when compared with area 2. The null genotype for *GSTT1* in areas 1 and 3 of the tumor indicates tumor heterogeneity²⁶. This is the first report where different genotypes for *GSTT1* and *GSTM1*, EBV-DNA and *H. pylori* infection were observed in the same patient, indicating a probable deletion of these genes in response to tumor progression and intratumoral heterogeneity.

Intratumoral heterogeneity has been described as an inherent characteristic in most human cancers²⁷. A major cause of genetic heterogeneity in cancer is genomic instability²⁷. Genomic instability includes increased frequencies of base pair mutation, microsatellite instability, variations such as chromosome number or structure changes, which are also called chromosome instability^{28,29}.

Epstein-Barr virus is associated with several human tumors, and in 1990, EBV genomes were detected in gastric carcinomas³⁰. Wu *et al.*³¹ demonstrated that EBV DNase may induce genomic instability through two mechanisms: directly, by damage to the DNA, and indirectly, by inhibition of DNA repair. *Helicobacter pylori* and EBV infections are well-established risk factors for gastric cancer development. *H. pylori* causes gastritis in humans, with chronic inflammation. This chronic inflammation is thought to be the cause of genomic instability³². *H. pylori* is categorized as a group I carcinogen, since this bacterium is responsible for the highest rate of cancer-related deaths worldwide³³.

In our study, only the specimens from patients 2 and 4 were free of infection by either EBV or *H. pylori*. Probably, other risk factors such as age, gender, smoking, drinking,

or dietary factors may be associated in these patients. Interaction between EBV and *H. pylori* in the host stomach lining may have some synergistic effects in the development of gastric cancer³². In our study, the co-infection between *H. pylori* and EBV may be associated as one of the factors responsible for the onset of patients' gastric cancer. The genomic instability in response to EBV or *H.pylori* infection could also be responsible for the null genotypes of *GSTM1* and *GSTT1* found in the present study.

RESUMO

Objetivo: comparar o polimorfismo dos genes Glutathione S-transferase teta 1 (*GSTT1*) e Glutathione S-transferase mu 1 (*GSTM1*) da área do tumor com as margens proximal e distal de espécimes de estômago ressecados de pacientes com câncer gástrico, e investigar a presença do DNA do vírus Epstein-Barr (EBV) e *Helicobacter pylori*.

Métodos: coletamos prospectivamente amostras teciduais da área do tumor e das margens de ressecção proximal e distal dos estômagos de dez pacientes com adenocarcinoma gástrico submetidos à gastrectomia com linfadenectomia D2 e submetemos esses espécimes à extração de DNA. Comparamos a área do tumor com as margens proximal e distal dos estômagos ressecados para o polimorfismo dos genes *GSTT1* e *GSTM1* e investigamos a presença de DNA do EBV e *H. pylori*. Utilizamos o exon 5 do gene p53 como controle interno da reação de PCR multiplex. **Resultados:** em um paciente, detectamos genótipos *GSTT1* e *GSTM1* nulos na área do tumor, em contraste com a presença de ambos os genes nas margens proximal e distal. Encontramos DNA do EBV e *H. pylori* na área do tumor e também nas margens proximal e distal. Em outro paciente, a margem proximal foi negativa para *GSTT1* e o DNA do EBV foi negativo na margem distal. Em três pacientes, o EBV-DNA foi negativo apenas na margem distal. **Conclusão:** este é o primeiro relato em que diferentes genótipos, infecção por EBV-DNA e *H. pylori* foram observados no mesmo paciente, indicando provável deleção desses genes em resposta à progressão tumoral e heterogeneidade intratumoral. **Descritores:** Glutathione Transferase. Polimorfismo Genético. Neoplasias Gástricas. *Helicobacter pylori*. Infecções por Vírus Epstein-Barr.

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