Retrospective Study

Association between *Helicobacter pylori*, Epstein-Barr virus, human papillomavirus and gastric adenocarcinomas

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Abstract

**AIM**

To correlate *Helicobacter pylori* (*H. pylori*), Epstein-
Barr virus (EBV) and human papillomavirus (HPV) with gastric cancer (GC) cases in Pará State, Brazil.

**METHODS**

Tissue samples were obtained from 302 gastric adenocarcinomas. A rapid urease test was used to detect the presence of H. pylori, and the presence of the cagA gene in the HP-positive samples was confirmed by PCR. An RNA in situ hybridization test designed to complement Eber1 RNA was used to detect the presence of EBV in the samples, and the L1 region of HPV was detected using nested PCR. Positive HPV samples were genotyped and analyzed for E6 and E7 viral gene expression. Infections were also correlated with the clinical and pathological characteristics of the patients.

**RESULTS**

The majority of the 302 samples analyzed were obtained from men (65%) aged 55 years or older (67%) and were classified as the intestinal subtype (55%). All three pathogens were found in the samples analyzed in the present study (H. pylori: 87%, EBV: 20%, HPV: 3%). Overall, 78% of the H. pylori-positive (H. pylori+) samples were cagA+ (H. pylori-cagA+), and there was an association between the cytotoxic product of this gene and EBV. Coinfections of H. pylori-cagA+ and EBV were correlated with the most advanced tumor stages. Although only 20% of the tumors were positive for EBV, infection with this virus was associated with distant metastasis. Only the HPV 16 and 18 strains were found in the samples, although no expression of the E6 and E7 oncoproteins was detected. The fundus of the stomach was the region least affected by the pathogens.

**CONCLUSION**

HPV was not involved in gastric tumorigenesis. Prophylactic and therapeutic measures against H. pylori and EBV may prevent the development of GC, especially the more aggressive forms.

**Key words:** Gastric cancer; Gastric adenocarcinomas; Microorganisms; Helicobacter pylori; cagA; Epstein-Barr virus; Human papillomavirus

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Core tip: We investigated the presence of Helicobacter pylori (H. pylori), Epstein-Barr virus (EBV) and human papillomavirus in gastric adenocarcinomas and their relationships with the clinicopathological characteristics of the patients. Despite the fact that all three pathogens were found in the samples, we believe that only H. pylori and EBV contribute to the transformation of tissue associated with carcinogenesis. A significant association between the cagA gene of H. pylori and EBV was also observed. Given this finding, the use of prophylactic and therapeutic measures against both H. pylori and EBV may help to prevent the development of gastric cancer, especially the more aggressive forms.

INTRODUCTION

Though the worldwide incidence of gastric cancer (GC) has declined rapidly over the past few decades, GC is still the world’s fourth leading cause of cancer deaths in both males and females[1]. In Brazil, approximately 20 thousand cases of GC are expected in 2018 and 2019; in the North and Northeast regions of the country, it is the second most common type of cancer in men, and it is the fifth most common in women in the North, Southern and Central West regions[2].

The majority of GC cases have a multifactorial origin, that is, they are determined by a combination of genetic and environmental factors. Just over half of all cases of GC are the result of a gradual accumulation of driver mutations caused by environmental factors[3], such as a diet high in salt and carbohydrates, a high intake of food preserved with nitrates, and reduced consumption of fruit and vegetables[4,5]. Microbial infections have also been shown to contribute to gastric tumorigenesis[6-9], and gastric physiology and immunology are known to be altered by Helicobacter pylori (H. pylori)[10,11]. While more than half of the world’s population is infected with H. pylori, however, only approximately 3% of these individuals will develop GC[12-14]. One of the factors that may be responsible for this discrepancy is the genetic variability of HP, such as the presence of the cagA gene, and interaction with other pathogens[15-19].

The Epstein-Barr virus (EBV) is a second pathogen that may be associated with GC[17]. This virus provokes a disruption of the genes involved in cell cycle regulation, inflammation, and angiogenesis, as well as the loss of tumor suppressor genes by hypermethylation[20,21].

However, in the case of the third pathogen investigated here, the human papillomavirus (HPV), no systematic association has been established[6,22-24]. While the oncogenic properties of HPV have been demonstrated in studies of other portions of the digestive tract[25-28], its possible relationship with GC is still unclear[22,23,24,29,30].

Given the potential influence of H. pylori and EBV on the development of GC[7,8] and the high prevalence of HPV in the general population of Pará State, Brazil[31,32], the present study aimed to elucidate the possible relationship of these microorganisms with the clinical-pathological characteristics of patients with GC in this region.
Materials and Methods

Patients and tissue samples
Tissue samples were obtained from 302 gastric adenocarcinomas collected between 2005 and 2015 in the city of Belém, Pará, Brazil. The patients were between 28 and 92 years old, with a mean age of 62 years. This study was approved by the Ethics Committee of the João de Barros Barreto University Hospital in Belém (No. 142004 and No. 637.233). Written informed consent was obtained from all the patients included in the study prior to the collection of samples. No patient had any previous history of any other type of tumor; and all samples were obtained prior to the administration of chemo- and/or radiotherapy. The tumors were categorized according to the TNM classification system of American Joint Committee on Cancer (AJCC) Cancer Staging[33], the patients’ clinical and histopathological analyses, and Lauren’s histological classification[34].

Sample processing
For the molecular analysis, the biological material was frozen in liquid nitrogen directly after collection. DNA was then extracted from the macerated tumor tissue with phenol-chloroform[35], and RNA was obtained using Tri-reagent® (Thermo, CA, United States), following the manufacturer’s protocol. cDNA was synthesized by incubating the samples at 37°C for 1 h in a buffer containing 50 mmol/L Tris pH 8.4, 75 mmol/L KCl, 23 mol/L MgCl₂, 300 ng of RNA, 0.2 µg of oligo-dT, 10 mmol/L DTT, 0.5 mmol/L dNTP, 10 units of RNase inhibitor, and 50 units of reverse transcriptase. For the anatomopathological analyses, the material was fixed in paraformaldehyde before being embedded in paraffin and stained with hematoxylin and eosin.

Detection of H. pylori and cagA
H. pylori produces urease to convert urea to ammonia, and this property is exploited by the commercially available rapid urease test (Promedical, Juiz de Fora, Minas Gerais, Brazil) to detect the presence of H. pylori in gastric samples. We applied this test to all gastric samples, and when urease was detected, the pH and consequently, the color of the solution, were changed.

The samples were first placed in a tube containing 20 g/L of Christensen’s urea agar and incubated at 37°C for 24 h before being examined for urea hydrolysis. PCR was used to confirm negative results and detect the presence of the cagA gene in the H. pylori-positive (H. pylori+) samples. The oligonucleotides used here were described by Covacci et al[36]. A sample was considered positive if a clear band was visible in the 20 g/L agarose electrophoresis gel in comparison with a molecular weight marker; and all reactions were performed in duplicate.

Detection of EBV
EBV is known to infect lymphocytes, but these cells were not included in the present analysis. In this case, EBV was detected in the gastric samples by using a 30-bp biotinylated probe (5′-AGACACCGTCTCTCACCGGGGACTTGTA-3′) that is complementary to the most abundant viral product in latently infected cells, the EBV-encoded small RNA-1 (Eber1)[37]. For this assay, RNA in situ hybridization (ISH) was used, and the signal was amplified using a mouse anti-biotin antibody (clone BK, 1:20 dilution; DakoCytomation®, CA, United States) and a biotinylated rabbit anti-immunoglobulin antibody (polyclonal, 1:100 dilution; DakoCytomation®). Streptavidin-biotin peroxidase complex (DakoCytomation®) and diaminobenzidine chromogen (DakoCytomation®) were used to detect this reaction. The slides were counterstained with Harris’s hematoxylin, and the cells were examined under light microscopy at 40 x or 20 x magnification by two independent investigators. In this examination, 10 representative microscopic fields containing at least five cells were evaluated. The positive control was an EBV-positive GC sample, and two untreated slides were used as negative controls. Samples were considered positive when 5% or more of the epithelial cells were stained brown/red.

Detection of HPV
Nested PCR was used to classify the samples as HPV positive or negative. The first PCR used the generic PGMY09/11 primers described by Gravitt et al[38], which amplify the L1 region of HPV, and the second PCR used the GPS+/6+ primers described by Jacobs et al[39]. The PCR products were separated on a 2% agarose gel. After identification, the virus was genotyped by sequencing the PCR product with the GPS+ primer using a capillary 3730XL DNA Analyzer (Thermo Fisher Scientific, United States). All the nucleotide sequences obtained in these analyses were compared with the GenBank/NCBI database using the BLAST alignment search tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The E6 and E7 oncoprotein expression of HPV 16 and 18 were investigated by RT-PCR as described by Chang et al[40].

Statistical analysis
The results of the infections were correlated with the clinical-pathological data of the gastric adenocarcinoma patients. The Shapiro-Wilk test was used to evaluate the distribution of the samples. The association between H. pylori, EBV and HPV infections (either separately or combined) and clinical-pathological parameters was evaluated using the chi-square test and logistic regression analysis. A P < 0.05 significance level was considered for all analyses, and a 95% confidence interval was also applied. The results are given as the P value, odds ratio (OR), and confidence interval (CI). The statistical methods of this study were reviewed by André Salim Khayat from Oncology Research Center, Federal University of Pará.

Results
The studied population was divided into two age groups:
Individuals aged 55 years or over (67% of the total) and individuals younger than 55 years (33%). The majority (65%, n = 195) of the 302 samples analyzed in the present study were obtained from men, with the remainder (35%, n = 107) being obtained from women. The clinicopathological data on the GC patients and their association with the presence or absence of *Helicobacter pylori*, *Helicobacter pylori-cagA*, Epstein-Barr virus and human papillomavirus are shown in Table 1.

The GC cases were divided into two groups based on the presence of gastric epithelial stem cells: (1) proximal gastric carcinoma (cardia), and (2) distal carcinoma (noncardia), with specific pathological, clinical, epidemiological and prognostic characteristics. Overall, 35% of the tumors were located in the proximal region, and 65% in the distal region (31% in the antrum, 19% in the fundus, and 15% in the body).

Based on Lauren’s microscopic classification, the most widely used system for gastric adenocarcinomas, there are two principal types of gastric tumor: (1) the diffuse type, which has a worse prognosis and is characterized by invasive growth and the absence of premalignant lesions, and (2) the intestinal type, the development of which is

|     | Gender | Age | Localization | Subtype | TNM  | EBV | HPV |
|-----|--------|-----|--------------|---------|------|-----|-----|
| n (%) |        |     |              |         |      |     |     |
| HP- | 38 (13) | 264 (87) | 0.866 (0.46-1.92) | 0.851 (0.32-2.79) | 0.096 (0.32-2.79) | 0.011 (1.18-3.85) | 0.750 (0.26-6.60) |
| HP+ | 98 (23) | 204 (77) | 0.400 (0.75-2.04) | 0.490 (0.70-2.36) | 0.400 (0.49-1.33) | 0.159 (0.85-2.70) | 0.084 (0.05-1.33) |
| EBV- | 240 (80) | 62 (20) | 0.377 (0.72-2.39) | 0.409 (0.70-2.36) | 0.400 (0.49-1.33) | 0.159 (0.85-2.70) | 0.084 (0.05-1.33) |
| EBV+ | 8 (2) | 7 (3) | 0.372 (0.72-2.39) | 0.409 (0.70-2.36) | 0.400 (0.49-1.33) | 0.159 (0.85-2.70) | 0.084 (0.05-1.33) |
| HPV- | 294 (97) | 8 (3) | 0.105 (0.75-1.36) | 0.000 (1.03-1.15) | 0.438 (0.47-7.92) | 0.048 (0.05-1.33) | 0.000 (1.03-1.15) |
| HPV+ | 8 (3) | 7 (3) | 0.277 (0.01-1.05) | 0.222 (0.42-28.41) | 0.222 (0.42-28.41) | 0.222 (0.42-28.41) | 0.222 (0.42-28.41) |

EBV: Epstein-Barr virus; HPV: Human papillomavirus.

Table 1 Clinicopathological data of patients with gastric cancer and their relation with the presence or absence of *Helicobacter pylori*, *Helicobacter pylori-cagA*, Epstein-Barr virus and human papillomavirus.
dependent on environmental factors and is associated with premalignant lesions, particularly chronic and atrophic gastritis, metaplasia and dysplasia\cite{34,42,43}.

According to this classification, 55% (166) of the samples were of the intestinal subtype, and the other 45% (136) of the diffuse subtype. Based on the stages defined by the AJCC (TNM)\cite{23}, 77% (232) of the samples had the worst prognosis (T3 and T4), 95% (286) involved lymph nodes (N1, N2, N3), and 53% (161) had distant metastasis (M1).

The H. pylori bacterium was detected (H. pylori+) in 87% (n = 264) of the tumor tissue samples, and tumors of the intestinal type were more likely to be infected with H. pylori (92%) than those of the diffuse subtype (82%) (P = 0.016, OR = 2.327, CI: 1.15-4.70). There was no significant relationship between HP infection and the tumor stage, however, nor with the presence of EBV or HPV.

The cagA gene (H. pylori-cagA+) was amplified in 77% (204) of the H. pylori+ samples. The relative frequency of the H. pylori-cagA+ samples did not differ from that of the H. pylori-cagA- samples by sex, age, subtype or tumor stage. However, while EBV was present in 26% (53) of the H. pylori-cagA+ samples, only 9% (9) of the H. pylori-cagA- samples were EBV positive (P = 0.001, OR = 3.471, CI: 1.63-7.37). No association was found between cagA+ and HPV.

Only 20% of the tumors tested were positive for EBV infection (EBV+), and the incidence of the virus did not vary by gender, age, subtype, tumor stage, lymph node invasion or the presence of HPV. There was a significant association between EBV+ and distant metastasis, however; with 68% (42) of the metastasized tumors being EBV+ (P = 0.011, OR = 2.135, CI: 1.18-3.85).

Only 3% (8) of samples were positive for HPV (HPV+). All cases of HPV+ were recorded in young (< 55 years old) patients (P = 0.000, OR = 16.354, CI: 0.87-0.97), and the majority (63%) were female (P > 0.05). The analysis identified two types of high-risk HPV, 16 (50%) and 18 (50%), but with no expression of the E6 and E7 oncoproteins. No significant association was found between HPV+ and sex, lymph node invasion or distant metastasis. A significant relationship was found, however, with the tumor stage, with the virus being significantly more frequent in less aggressive tumors and 62% of the HPV+ samples being classified as T1 and T2 (P = 0.008, OR = 5.872, CI: 1.37-25.22).

Interestingly, only two GC samples had all three pathogens (H. pylori+, EBV+ and HPV+), although there was obviously no significant association in this case. The majority (89%; 55) of the EBV+ cases were coinfection with H. pylori. In addition, 96% (53) of the tumors coinfected with H. pylori and EBV (EBV H. pylori-cagA+) were H. pylori-cagA+ A strains and thus had a six-fold greater chance of having a more aggressive tumor stage (P = 0.010, OR = 6.111, CI: 0.04-0.73, power of the test > 0.90), as 83% (44) of EBV H. pylori-cagA+ tumors were T3 and T4.

The regions of the stomach infected and the relationship with the pathogen and strain are shown in Table 2. While fewer tumors affected the body region, the fundus of the stomach was least affected by the infections, including negative associations with the H. pylori-cagA+ strain (P = 0.00032) and EBV (P = 0.00010, with Bonferroni correction in both cases).

**DISCUSSION**

Many types of cancer are known to be related to infections by microorganisms\cite{1,44}, which may play a role in either the onset of the growth of the cancer cells or their maintenance. While much of the gastrointestinal tract represents a favorable environment for microbial life, this is not the case with the stomach, where any microorganism would need to tolerate extremely acid conditions, antimicrobial compounds, enzymes, and structural barriers\cite{13}. Thus, to colonize the stomach, any pathogen must adapt to an extremely hostile and highly variable environment.

Despite these difficulties, some pathogens are able to establish themselves in the stomach, and their presence has been associated conclusively with the development of GC. H. pylori, for example, produces urease to make the gastric environment more basic, facilitating its survival\cite{34,45}. However, evidence on the involvement of other agents, such as HPV, in the development of gastric

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**Table 2 Ratio, in absolute value and percentage, between pathogen (Helicobacter pylori, Epstein-Barr virus, and human papillomavirus), strain (HP-cagA) and tumor location**

| Infection | Cardia (104) | P (OR, 95%CI) | Fundus (57) | P (OR, 95%CI) | Body (45) | P (OR, 95%CI) | Antrum (95) | P (OR, 95%CI) |
|-----------|--------------|---------------|-------------|---------------|------------|---------------|-------------|---------------|
| HP+ (263) | 91 (34.6%)   | 0.975 (OR = 1.012, 95% CI: 0.494-2.071) | 44 (16.7%)  | 0.012 (OR = 0.395, 95% CI: 0.188-0.831) | 40 (15.2%) | 0.504 (OR = 1.213, 95% CI: 0.447-3.291) | 88 (33.5%)  | 0.064 (OR = 2.214, 95% CI: 0.938-5.228) |
| H. pylori-cagA+ (203) | 67 (33%) | 0.400 (OR = 0.806, 95% CI: 0.488-1.332) | 27 (13.3%)  | 0.000 (OR = 0.361, 95% CI: 0.201-0.648) | 35 (17.2%) | 0.092 (OR = 1.886, 95% CI: 0.894-3.978) | 74 (36.5%)  | 0.009 (OR = 2.087, 95% CI: 1.191-3.656) |
| EBV+ (62) | 23 (37.1%) | 0.621 (OR = 1.188, 95% CI: 0.648-2.069) | 1 (1.6%)    | 0.000 (OR = 0.053, 95% CI: 16 (25.8%) | 0.009 (OR = 2.435, 95% CI: 1.227-4.833) | 22 (35.5%)  | 0.444 (OR = 1.258, 95% CI: 0.699-2.266) |
| HPV+ (8) | 4 (50%) | 0.348 (OR = 1.940, 95% CI: 0.475-7.920) | 0 (0%)     | 0.162 (OR = 0.803, 95% CI: 0.758-0.850) | 2 (25%)    | 0.436 (OR = 1.894, 95% CI: 0.370-9.686) | 2 (25%)    | 0.690 (OR = 0.720, 95% CI: 0.143-3.637) |

EBV: Epstein-Barr virus; HPV: Human papillomavirus; H. pylori: Helicobacter pylori.
tumors is still inconclusive\cite{6,23,24,30}. As in most studies of GC\cite{16,46-49}, the present study recorded a predominance of GC cases in male patients over 55 years of age.

**H. pylori**

The high prevalence of *H. pylori* in GC tumors has been reported widely, both in Brazil and around the world\cite{50-54}. The scenario in Pará is typical\cite{15,47,53}, with the prevalence of *H. pylori* in tumor samples being up to nine times higher than that recorded in healthy tissue samples\cite{16}. In the present study, *H. pylori* was present in a high percentage of tumor samples, with 87% (263) of the patients being affected by the bacterium.

While the exact mechanism through which *H. pylori* may induce gastric carcinogenesis has not yet been fully elucidated, it is known that the inflammatory process caused by this bacterium, coupled with genetic and epigenetic events in the host, is capable of inducing a cascade of morphological events, including both premalignant and malignant transformations (intestinal or diffuse GC)\cite{8,42}. In our study, the presence of the bacterium was more frequent in tumors classified as the intestinal subtype, an association that has also been confirmed in other studies conducted in locations with high GC incidence rates\cite{16,52,56}.

The cagA gene was recorded in 78% (204) of the 263 *H. pylori* samples investigated in this study, a frequency similar to that recorded in other studies in Pará State. Vinagre et al\cite{15} recorded a rate of 73% and found that cagA was associated with a more intense inflammatory response and higher levels of DNA damage in epithelial cells. Souza et al\cite{16} recorded an even higher rate (88%) and found that *H. pylori*-cagA+ was associated with both lymph node metastasis and distant metastasis. In Portugal, Nogueira et al\cite{57} found that 64% of *H. pylori* samples had cagA+, which was more closely related to the progression of gastric adenocarcinoma in comparison with samples infected with *H. pylori*-cagA- strains. This gene has thus been associated with a higher risk of GC development and worse prognosis in a number of studies. In our study, however, while *H. pylori*-cagA+ was more frequent in more advanced stages (T3 and T4) and in patients with distant metastasis, there was no significant difference (P > 0.05) in comparison with the samples that lacked the cagA oncogene.

The cagA gene was also significantly associated with the presence of coinfection with EBV (EBV *H. pylori*-cagA+) and was present in 85% (53) of the EBV+ tumors, a frequency close to that recorded by Souza et al\cite{16}, who recorded coinfection in 100% of EBV+ samples. Minoura et al\cite{58} concluded that the presence of *H. pylori* supports the reactivation of the virus from its latent state in gastric epithelial cells, while Saiki et al\cite{59} proposed that the inflammatory stress generated by this bacterium may attract a greater infiltration of lymphocytes carrying EBV, which increases the possibility of epithelial cells coming into contact with these lymphocytes and thus being infected. EBV may also support *H. pylori*.

Cárdenas-Mondragón et al\cite{60} found that EBV act as a cofactor in triggering gastric inflammation together with *H. pylori* in gastric diseases, and Saju et al\cite{60} recently discovered that host cell SHP1 phosphatase antagonizes the *H. pylori*-cagA virulence factor. However, coinfection with EBV results in methylation of the SHP1 promoter, keeping cagA phosphorylated and thereby allowing the mediation of oncogenic signaling. This evidence indicates that the development and progression of GC in some of the patients analyzed may be influenced by an association between the oncogenic characteristics of EBV and the cytotoxic product of the *H. pylori* cagA gene.

These interactions suggest that the EBV *H. pylori*-cagA+ coinfection favors tissue malignancy, and the higher rate of EBV *H. pylori*-cagA+ coinfection observed in patients with more aggressive tumors further reinforces the role of this interaction in the development and/or progression of gastric adenocarcinoma in the patients analyzed.

**EBV**

This virus is typically found in approximately 10% of GC cases\cite{6,16,61,62}, although in the present study, EBV was present in 26% (62) of the samples, a frequency similar to that found in studies in the United States and Germany (16%-26%)\cite{9,63,64}. Lower frequencies have been found in other countries\cite{16-67}, however, indicating that the prevalence of EBV may vary widely among geographic regions.

Nogueira et al\cite{57}, Lopes et al\cite{61}, and Shibata et al\cite{63} found that EBV was significantly more prevalent in males and that it was associated (but not significantly) with age, although no association was found with tumor subtype. In our study, even though the virus was more prevalent in men, older (> 55 years) patients and patients with the intestinal type of tumor (Table 1), these relationships were not significant (P > 0.05).

It is known that the protein encoded by the EBV BARF1 gene increases the proliferation of virus-infected cancer cells by increasing the expression of NF-κB and reducing p21, thereby facilitating the development of the process\cite{66}. Specifically, monocytes are recruited by vascular endothelial growth factor (VEGF), and tumor associated macrophages (TAM) is induced by granulocyte-macrophage colony-stimulating factor (GM-CSF) in an NF-κB-dependent manner\cite{69}.

Expression of the EBV oncogenic proteins LMP1, LMP2A, and LMP2B has also been shown to have a role in the increased capacity of the cancer to spread and migrate. In other types of tumor, the LMP1 protein activates the ERK-MAPK pathway and is capable of inducing motility and increasing the migration rate of epithelial cells in comparison with LMP1-negative cells, as shown by Dawson et al\cite{70}. LMP2A also increases motility by targeting EGFR\cite{71}, which would account for the lymph node metastasis found in other studies\cite{72,73} and the increased probability of distant metastasis found in the
The present study.

**HPV**

There is no consensus in the literature on the potential association between HPV and GC, and in our study, only 3% of the gastric tumor samples analyzed were infected with HPV. Fakhraei et al. recorded a similar frequency, 5%, in samples of gastric adenocarcinoma in patients from northern Iran. Much higher frequencies, ranging from 37.5% to 52%, have been found in other studies[23,29,75], however. Even so, no HPV was found in the GC samples in some studies[24,30,76]. This disparity between studies may be related in part to the variation in the viral detection methods used (ISH, PCR), as well as the methods of collecting and preserving the samples, in addition to the geographic location of the research.

When the viral DNA of HPV 16 and 18 integrates with the host cell genome, the E2 viral gene product is altered. This product has a regulatory role in the transcription of the viral E6 and E7 oncoproteins, which generate genomic instability, resulting in cellular abnormalities and the abolition of cell cycle checkpoints, thereby increasing the risk of accumulated genetic abnormalities. In addition to the lack of an adequate immune response, these abnormalities represent a favorable condition for the development of cancer[77]. In our study, two types of high-risk HPV, 16 and 18, were found in the tumors analyzed, although no E6 and E7 expression was observed. As these proteins are usually expressed at the beginning of the infection and reflect the persistence of the virus in the host organism[77], this may be an indication that the presence of the virus in the patients’ stomachs was only temporary. These observations, together with previous findings, indicate that the presence of HPV probably had no involvement in the initiation or progression of GC in the patients analyzed in this study.

One important detail from our study is that all HPV+ tumors were found in younger patients (< 55 years). Studies of low-grade intraepithelial lesions (LSIL)[78], cervical cancer[79], and anal cancer[80] have also found a tendency for HPV to occur in younger patients (30-40 years of age), probably indicating a more active sex life, which may increase the chance of exposure to HPV infection.

Furthermore, based on the TNM classification, HPV was found more frequently in less aggressive tumors, with 63% of the HPV+ samples being classified as T1 or T2, similar to the findings of Anwar et al.[81] and Zeng et al.[82]. However, this finding may just be a consequence of the occurrence of less aggressive tumors in younger patients (P = 0.000, OR = 0.113, CI: 0.062-0.206). There was also no significant association with the presence of lymph node involvement, distal metastasis, or the anatomical location of the tumor, as in previous studies[23,29,80].

**Location of the tumor**

Most tumors (65%) were present in noncardia regions, and the stomach body was the least-affected region. By contrast, previous studies in the same geographic location[56,83] found that the stomach fundus was the area least affected by gastric adenocarcinoma. There was no significant difference in infection (H. pylori, EBV, HPV) and the presence of antigens (cagA) between cardia and noncardia regions. However, other studies have found a negative association between HP infection and GC in the cardia[84,85]. Even so, the presence of H. pylori strains that carry the pathogenic island cagPAI may contribute to an increase in the risk of distal GC[8,10,14,84]. In previous studies, the most commonly colonized portion of the stomach was non-cardia region, like the antrum[14,82], which is consistent with our findings (Table 2).

The gastric fundus has one of the lowest rates of infection, and there were negative associations with H. pylori (P = 0.012, OR = 0.395, CI: 0.188-0.831), H. pylori-cagA (P = 0.000, OR = 0.361, CI: 0.201-0.648), and EBV (P = 0.000, OR = 0.053, CI: 0.007-0.388). The body was also associated with relatively few infections, and the lower rates of infection may be related to the fact that these two stomach regions (fundus and body) have a more acidic pH in comparison with the rest of the stomach[86], which protects them against the establishment of infectious microorganisms. Interestingly, EBV was more frequent in tumors of the cardia, in contrast with a study by The Cancer Genome Atlas (TCGA)[8], which showed that most EBV-positive tumors were present in the gastric fundus or body. HPV was not associated with any specific gastric region.

Overall, then, our findings lead us to believe that only H. pylori and EBV contribute actively to the transformation of tissue associated with carcinogenesis. In this case, the systematic application of prophylactic and therapeutic measures against H. pylori and EBV may help prevent the development of GC and more aggressive tumors.

**ARTICLE HIGHLIGHTS**

**Research background**

Gastric cancer (GC) is the world’s fourth leading cause of cancer deaths, and microbial infections have been shown to contribute to gastric tumorigenesis.

**Research motivation**

Gastric physiology and immunology are known to be altered by Helicobacter pylori (H. pylori) and Epstein-Barr virus (EBV), although there is still doubt about the association of GC with some pathogens, such as human papillomavirus (HPV).

**Research objectives**

The present study aimed to elucidate the possible relationship of these microorganisms with the clinical-pathological characteristics of patients with GC in the North region of Brazil.

**Research methods**

A total of 302 gastric adenocarcinomas were collected between 2005 and 2015. Patient samples were categorized according to the TNM classification system and by histology. Molecular techniques were used for pathogen investigations, as they are more sensitive.
Research results
All three pathogens were found in the samples; however, active HPV infection was not identified. Coinfections of H. pylori-cagA and EBV were correlated with tumors at the most advanced stages.

Research conclusions
HPV was not involved in gastric tumorigenesis. On the other hand, H. pylori and EBV seem to be directly related to the development and severity of tumors, especially when coinfections exist.

Research perspectives
Prophylactic and therapeutic measures against H. pylori and EBV may prevent the development of GC, especially the more aggressive forms.

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