H pylori (CagA) and Epstein-Barr virus infection in gastric carcinomas: Correlation with p53 mutation and c-Myc, Bcl-2 and Bax expression

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Abstract

AIM: To investigate the interrelationship between H pylori and Epstein-Barr virus (EBV) infection in the gastric carcinogenesis having in focus the p53 mutation and the c-Myc, Bcl-2 and Bax expression.

METHODS: seventy-one gastric carcinoma tissues were assessed by polymerase chain reaction (PCR) for H pylori and in situ hybridization for EBV. c-Myc, Bcl-2 and Bax expression were detected by immunohistochemistry and single-stranded conformational polymorphism (SSCP) for p53 mutation.

RESULTS: The positivity rates for H pylori and EBV were 94.4% and 8.45%, respectively. The majority of the cases displayed only the H pylori presence. All EBV positive cases were also H pylori positive. None infectious agent was observed in 5.55% of the cases. The intestinal type tumor was more frequent in the co-infected and non-infected groups. The female predominated in the cases displayed only the H pylori positive groups.

CONCLUSION: The frequency of H pylori infection in gastric carcinomas was high. The presented data indicated that gastric carcinogenesis has different pathways depending of the presence of the two investigated infectious agents, suggesting a possible involvement of H pylori with apoptotic process. The low expression of c-Myc and Bax in the EBV-positive groups suggests that EBV may inhibit the expression of these proteins. Nevertheless, p53 mutation shows to be a relevant alteration, independent of both infectious agents.

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Key words: Gastric carcinoma; Helicobacter pylori; Epstein-Barr virus, p53, Bax, Bcl-2, c-Myc

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INTRODUCTION

Gastric cancer is the fourth most common cancer and the second cause of cancer-related death worldwide[1]. There is substantial international variation in the gastric cancer incidence with the highest rates reported from Korea, Japan and Eastern Asia. Other high incidence areas include Eastern Europe and parts of Latin America, while Western Europe and the United States of America generally have low incidence rates. The global burden of gastric cancer is shifting rapidly from the developed world to the developing world[2].

Over 95% of stomach tumors are adenocarcinomas which are subdivided into two main histological types: intestinal type and diffuse type. The intestinal type is...
related to corpus-dominant gastritis with gastric atrophy and intestinal metaplasia, whereas the diffuse type usually originates in pangastritis without atrophy\cite{1,2}.

The two main anatomic sites of gastric adenocarcinoma are proximal (cardia) and distal (non-cardia). The frequency of the theses tumor sites varies in populations from different geographic locations, racial and socio-economic groups. They may differ in genetic susceptibility, pathologic profile, clinical presentation and prognosis. The observed differences between gastric cancers by anatomic sites suggest that they are distinct diseases with different etiologies. Although the distal cancer is usually more frequent, a decreased incidence has been observed in this site while the incidence of the proximal site tumors have increased since the 1970’s especially among males in the Western countries\cite{3,4}.

There are several etiologic factors involved in gastric cancer such as low socioeconomic status, diet, hereditary factors and \textit{H pylori} infection, as the most cited in studies about this issue. Besides the \textit{H pylori}, Epstein-Barr virus (EBV) infection is also associated with development of gastric cancer\cite{5,6}.

The discovery of \textit{H pylori} infection in the early eighties proved a turning point in the understanding of the gastric cancer pathogenesis. While the link between \textit{H pylori} and peptic ulcer was established soon after the successful culture of the bacteria, the association with gastric cancer remained doubtful for almost a decade before credible evidence could be presented. The major reason for this delay was the inability to demonstrate the presence of active infection in gastric cancer tissue\cite{7,8}. Despite the recognition of the association between \textit{H pylori} and gastric cancer the pathogenic mechanism involved in this process is still not understood.

In spite of the wide bibliography regarding to the other tumors, the first report about the association of the \textit{EBV} with gastric carcinomas was in a case of lymphoepithelial-like gastric carcinoma\cite{9}. Soon after, it was identified in common gastric carcinomas\cite{10}. EBV infection is found in approximately 10% of ordinary gastric adenocarcinoma cases; however most of the mechanisms used by the virus to control this process are still unknown. Recent studies have shown that the expression pattern of EBV-encoded genes in gastric carcinoma is different from that in Burkitt’s lymphoma and nasopharyngeal carcinoma (NPC), suggesting that the oncogenic mechanism of the EBV in gastric carcinoma may be unique\cite{11,12}.

In attempt to understand the carcinogenic process triggered by these two infectious agents, some works have looked for alteration in genes/proteins that these agents can target, and therefore, pointing possible pathogenic pathways. In this sense, proteins involved with cell cycle or apoptosis process emerge as a candidate for having a crucial role in the development of the neoplasms. Some of these, like c-Myc, p53 and the apoptotic family members such as Bcl-2 and Bax, deserve attention for playing a key function in the cell proliferation and cell fate, besides they already have shown involvement in the tumorigenesis process in a variety of tumors, including gastric cancer\cite{13,14,15}.

Although the proteins mentioned above have different levels of alteration in gastric carcinoma, the association with \textit{H pylori} or EBV is controversial\cite{16,17,18}. Additionally, there is just one paper related \textit{H pylori} and EBV infection with concomitant analysis, in gastric carcinomas, in which, proteins involved in cell cycle and apoptosis process were also evaluated\cite{19}. So, the aim of the present study was to investigate the interrelationship between \textit{H pylori} and EBV infection in the gastric carcinogenesis having in focus the p53 mutation, and the expression of c-Myc, Bcl-2 and Bax.

**MATERIALS AND METHODS**

**Clinical specimens**

The present study was approved by the ethic committee from Federal University of Ceará and all subjects signed informed consent prior to inclusion. Samples from eighty-two patients with gastric carcinoma were collected from two hospitals of the Ceará State, Brazil: Walter Cantídeo Hospital at Federal University of Ceará and Saint House of Mercy in Fortaleza, both located in Fortaleza, the capital of the State. A representative formalin-fixed tumor specimens embedded in paraffin blocks was selected and histological sections (5 μm) were subjected to immunohistochemistry and \textit{in situ} hybridization (ISH). Clinico-epidemiological data were collected from the medical reports. Upon gastrectomy, fragments of tumor were collected and subjected to DNA extraction and Polymerase Chain Reaction (PCR) to detect \textit{urease C} gene of \textit{H pylori} and p53 mutation.

**DNA extraction**

DNA was extracted from frozen tumor tissue just when the fragment presented more than 80% of tumor cells. The genomic DNA was extracted using cetetyltrimethyl ammonium bromide (CTAB) adapted from the method of Gary\cite{20}. Although we have collected samples from 82 patients, DNA extraction was done in 71 samples because the others did not reach the established percentage of tumor cells.

**Detection of \textit{H pylori} and the presence of \textit{cagA} gene**

The PCR was done to detected \textit{H pylori} infection because this technique is sensitive and specific and it was used by many studies\cite{21,22,23}.

The \textit{H pylori} infection was detected by amplification of \textit{urease C} gene using primers for PCR, described by Lage \textit{et al}\cite{24}. For the \textit{H pylori}-positive samples, the presence of the \textit{cagA} gene was identified using the primers described by Domingo \textit{et al}\cite{25}. PCR mixtures for amplification of both genes were prepared in a volume of 25 μL containing 0.4 μmol/L of each primer; 1.5 μmol/L MgCl₂; 0.8% Tween 20 and 1 μL of DNA sample. PCR products were analyzed by 1% agarose gel electrophoresis with ethidium bromide staining. Sample was considered \textit{H pylori} positive when an \textit{ureC} fragment of 294bp was amplified while \textit{cagA} gene was positive when a fragment of 297bp was detected. As positive control we used DNA known \textit{H pylori} positive and as negative control we used distilled water.

**RNA in \textit{in situ} hybridization**

The presence of the EBV was identified by the expression of EBV-encode small RNA-1 (EBER1), the most...
abundant viral product in latently infected cells. RNA in situ hybridization was performed using a 30bp biotinylated probe complementary to the RNA EBER1 described by Shibata and Weiss[8]. Briefly, after the deparaffinization and rehydration, endogenous peroxidase was blocked with 3% H2O2 solution. Enzymatic digestion was performed with protease K (0.02 µg/µL final concentration) for 13 min. Prehybridization was done with pre-hybridization solution (Denhardt’s solution [3.5X]; SSC [4.5X]; EDTA [0.0075 mol/L]; SDS [0.35%]; NaH2PO4 [0.75 mol/L]; dextran sulfate [10%]) for 60 min at 37°C. Soon after, the slides were incubated overnight at 37°C with hybridization solution containing 0.3 ng/µL of the probe. After washing with 2X SSC buffer, the signal was amplified using anti-biotin antibody (clone BK, mouse, dilution 1:20) and biotinylated anti-immunoglobulin antibody (polyclonal, rabbit, dilution 1:100). Detection was accomplished using streptavidin-biotin-peroxidase method and the reaction was developed with 3, 3’-diaminobenzidine chromogen. All antibodies were purchased from DakoCytomation®. The slides were counterstained with Harris hematoxylin. A case of nasopharyngeal carcinoma was used as positive control for detection of mutation in the p53 gene. The expression of the proteins Bcl-2, Bax and c-Myc was considered positive. Nuclei with a brown or black staining were considered positive.

Immunohistochemistry

The expression of the proteins Bcl-2, Bax and c-Myc was detected according to the method described by Hsu et al[25]. After the deparaffinization and rehydration, antigen retrieval was carried out by microwave-treatment of the slides for 15 min in 10 mmol/L citrate buffer solution (pH 6.0). Endogenous peroxidase activity was blocked with 3% H2O2 solution. Primary antibodies were incubated for 16 h at 4°C-8°C in a humid chamber. All antibodies were purchased from DakoCytomation®. The reaction was detected with LSAB + system (DakoCytomation®) according to the manufacturer’s recommendation.

Histopathological and staining analysis

The histological classification (Laurén) was obtained from the medical reporters and was confirmed by a professional pathologist of the team. The slides were evaluated by three experienced technicians independently using light microscopy at a magnification of 400X. In the in situ hybridization and immunohistochemistry techniques, the results were expressed as percentage of positive cases. In the immunohistochemistry analysis, only the cases with ≥ 5% of stained tumor cells were considered as positive while in the in situ hybridization, any nuclear staining in tumor cells were considered. For all analyses, at least 1000 tumor cells were counted in high power fields.

Analysis of p53 mutation

To detection of mutation in the p53 gene was used Single-Stranded Conformational Polymorphism (SSCP) test. Exons 5-9 of p53 gene were amplified by PCR using four pairs of the primers described by Murakami et al[21]. Each PCR reaction for a total of 25 µL final volume consisted of 0.2 mmol/L of the four deoxynucleotide triphosphate, 1.5 mmol/L MgCl2, 0.4 µmol/L of each primer and 0.5 U of Taq DNA polymerase (Invitrogen®). The cycling temperature included an initial temperature at 95°C for 5 min, followed by 35 cycles of 95°C for 1 min, 1 min for annealing temperature (55°C for exons 5 and 8/9; 63°C for exons 6 and 7), and extension at 72°C for 1 min. For SSCP analysis, five or six microliters of each PCR products were mixed with a equal volume of stop solution (95% of formamide, 20 mmol/L EDTA, 0.05% of xylene cyanol and 0.05% of bromophenol blue), heated at 95°C for 5 min and immediately placed on ice and loaded onto a gel containing 12.5% acrylamide (GenePhor™, Amersham Pharmacia Biotech). Electrophoresis was performed using Electrophoresis Unit GenePhor (GE-Healthcare), at 4°C for 3 h. The patterns of the bands were then visualized by a DNA Silver Staining Kit (Amersham Pharmacia Biotech).

Statistical analysis

The analyses were carried out using the statistical programs EPINFO® 6.04 d version and SPSS® 12.0. Statistically significant differences were evaluated by Chi square test (X²). The results were considered as statistically significant when P-values were less than 0.05.

RESULTS

Among the analyzed cases, 57 were males and 25 were females. The mean age was 56.5 years (range, 23 to 90 years). Among 71 selected patients, males were a majority (70.4% [50/71]) and 49.3% (35/71) were more than 65 years old. Regard to the Lauren’s classification, the intestinal type tumors demonstrated a slightly higher frequency (59.2%).

Detection of H pylori, EBV and markers

The H pylori infection was detected by PCR in 67 out of 71 (94.4%) gastric carcinomas. The correlation with sex showed a statistically significant difference P = 0.041 (Mantel-Haenszel), in this analysis, since almost all males were H pylori positive [98% (49/50)] while the females had a slightly lower prevalence [85.7% (18/21)]. Concerning the gastric regions, the rate of H pylori infection in the antrum was higher [55.22% (37/67)] than that of the cardia [28.35 (19/67)] and body [14.92% (10/67)]. The pathogenic genotype of H pylori (cagA +) was found in 62.7% (42/67) of the cases. Among these cases the intestinal type tumors was predominant [61.9% (26/42)]. The frequency of EBV infection was 8.45% (6/71). All EBV-positive cases were males and half of them were located in the gastric antrum [50% (3/6)]. Concerning the markers, the frequency of Bel-2, Bax, c-Myc and of the p53 mutation was 5.63% (4/71), 54.92% (39/71), 42.25% (30/71) and 73.23% (52/71), respectively. An EBV positive case is shown in Figure 1A, while the Figure 1B-D show the Bel-2, Bax and c-Myc immunodetection, respectively. The Figure 2 is a SSCP gel showing the migration pattern of exons 5 and 7 of p53 gene.

Relationship between H pylori, markers and p53 mutation

The cagA gene was detected in 62.7% (42/67) of the cases with H pylori infection. The relationship between
Bcl-2, Bax and c-Myc was statistically not significant. The relationship of the cagA(+) with the p53 mutation reached statistical significance (P = 0.035).

Relationship between EBV and H pylori
To investigate a possible relation between H pylori and EBV infection in the gastric tumorigenesis, the cases were divided in three groups: Hp(+) EBV(+); Hp(+) EBV(-) and Hp(-) EBV(-). These groups accounted for 8.45% (6/71), 86% (61/71) and 5.55% (4/71) of the total, respectively. No EBV-positive case was found without H pylori infection.

The Table 1 summarizes the clinico-pathological data of the groups. The difference in gender distribution was statistically significant (P = 0.039), and, although overall the males predominated, in the Hp(-)/EBV(-) group the females were more frequent (75%). Furthermore, this group was represented by the most advanced ages. The intestinal type was more frequent in the Hp(+)/EBV(+) and Hp(-)/EBV(-) groups, although the distribution failed to reach statistical significance (P = 0.339), while in the group with only H pylori infection both histological types, intestinal and diffuse, occurred at the same rate.

In the two groups in which the H pylori were present, the cagA genotype predominated in tumors of the intestinal type. In the Hp(+)/EBV(+) group, 2 out of 3 tumors of IV stage were cagA(-), on the other hand, in the group with only H pylori infection, most of the tumors (6/9) in the same stage were cagA(+).

The diffuse type tumors, notably in the Hp(+)/EBV(+) and Hp(-)/EBV(-) groups, were in advanced stages (III B and IV), while this was not the case in the intestinal type tumors. However, the correlation of the Lauren’s classification with tumor stage was never significant. The diffuse type tumors of the Hp(+)/EBV(-) group had similar prevalence of cagA(+) and cagA(-) strains of H pylori. There was not any significant association between diffuse type and the tumor sites.

The Figure 3 shows the tumor stage distribution among the groups. Despite the small size of the groups with both or no infectious agents, it was possible to observe that the IV stage was the most represented stage in all groups. Even so, the earlier stage was present in the three groups. Not statistical significance was obtained in this analysis (P = 0.179).

The distribution of the studied markers according to the groups is presented in Figure 4. Although the expression of Bel-2 was low, it was only observed in tumors with H pylori infection alone. Nevertheless, high expression of Bax and c-Myc was observed in the groups...
and EBV was detected. On the other hand, EBV was detected in almost all tumor cells, indicating that the infection had occurred before malignant transformation and that the tumor cells had been originated from an infected primary cell. The monoclonality of the viral genome and its presence in almost all tumor cells, as shown by strong pieces of evidence such as the presence of the c-Myc oncogene and the apoptotic proteins Bcl-2 and Bax, have a key role in the tumor development. To do so, patients were divided in three groups based on the presence or absence of the infectious agents.

The association between sex and infection observed in other studies showed that males were more susceptible than females to EBV and Hp(-)/EBV(-). None of the markers significantly correlated with any of the groups.

**DISCUSSION**

*H. pylori* is known to be a carcinogen agent of gastric cancer. The major advance in this field came with the recognition that chronic *H. pylori* infection induces physiological and morphological changes within the gastric environment increasing the risk for neoplastic transformation. Now, it is widely accepted that chronic *H. pylori* infection induces hypochlorhydria and gastric atrophy, which are precursors of gastric cancer[1,8]. Recently, it was found that EBV is also linked to the development of a portion of gastric carcinomas. Since then, several studies have been carried out about EBV carcinogenic role in these tumors. The relationship between EBV infection and gastric cancer has been demonstrated by strong pieces of evidence such as the monoclonality of the viral genome and its presence in almost all tumor cells, indicating that the infection had occurred before malignant transformation and that the tumor cells had been originated from an infected primary cell[6,8].

In this study, we assessed the status of EBV by ISH and *H. pylori* infection by PCR in 71 cases of gastric carcinomas. The frequency of *H. pylori* infection (94.4%) was higher than previous studies, where it ranged from 34.1% to 92%. These differences can be due to: (a) the sensitivity of the screening technique, since most previous studies used serology, rapid urease test and/or histological evaluation; (b) and the fact that these studies were performed in different world regions and it is known that the *H. pylori* infection is more frequent in developing countries[8,16,22,24]. On the other hand, EBV was detected in 8.45% of our cases. Although low, this prevalence is in agreement with reported results from various world regions, which vary from 2% to 23.6%[6,9,14,24,25]. The epidemiological data are also in accordance with previous studies. The male-female ratio was 2:1 and the majority of the patients were older than fifty-five[1,2]. Like in other studies, the intestinal type was more frequent than the diffuse type[1,8].

Despite the known importance of *H. pylori* and EBV in the gastric cancer etiology, few studies have focused on the interrelationship of these two agents in gastric cancer cases. Thus, we investigated the presence of both *H. pylori* and EBV, in parallel with the histopathological features, the status of the tumor suppressor p53, the expression of the c-Myc oncogene and the apoptotic proteins Bcl-2 and Bax, which have a key role in the tumor development. To do so, patients were divided in three groups based on the presence or absence of the infectious agents.

In our analysis, we observed a skewed gender distribution among groups, which reached statistical significance (*P* = 0.039). The groups with both infectious agents were composed only by males, while in the group without any agent females were predominant (3:1). The association between sex and infection observed in other studies showed that males were more susceptible than females to EBV and *H. pylori* infection[26,27]. Although the gastric antrum was the prevalent site of gastric cancer,
some studies have shown that EBV is related to the gastric carcinoma in cardia, middle stomach and gastric stump. In our study, we found a slight increase of incidence of tumors located in the cardia in the group with EBV infection compared with patients with \textit{H. pylori} infection alone. However, due to the absence of tumors with only EBV infection, we could not evaluate the real relevance of this finding. In spite of the fact that previous studies failed to show a significant association between cardia tumors and \textit{H. pylori} infection \cite{6,28}, our data point to a possible association between cardia tumors and the presence of either infectious agents, since only the Hp(-)/EBV(-) group did not present tumors located in the cardia \cite{31,32,40}.

The intestinal type was prevalent in all groups, however in the group with \textit{H. pylori} infection alone the prevalence of the diffuse and intestinal type were similar, indicating that \textit{H. pylori} can be involved with the diffuse type. Reports concerning the association of \textit{H. pylori} with histological types of gastric cancers are controversial. Some studies have shown an association between \textit{H. pylori} and intestinal type \cite{6,36}, while others have observed a balanced distribution between the two histological types, like in the present work \cite{15,32}.

Some works have shown an association between Bcl-2 expression and EBV-positive gastric carcinomas \cite{10}. However, the present study did not detect Bcl-2 in the group with EBV infection. On the other hand, Bax was detected in all groups with the highest expression in the group with \textit{H. pylori} infection alone, which also presented Bcl-2 expression as mentioned above. Although some authors have shown an increased Bcl-2 expression in \textit{H. pylori}-positive cases, in the group (Hp+/EBV-) the Bcl-2 expression was very low \cite{15,31}. Although it was not statistically significant, our findings suggest a possible correlation between \textit{H. pylori} and Bcl-2 expression, unlike with EBV.

A few \textit{in vitro} studies have shown that EBV facilitates the tumor development by inducing the expression of the c-Myc protein \cite{39,40}. In present study, the expression of c-Myc was higher in the groups without EBV infection indicating a relationship of c-Myc with gastric carcinogenesis but not with EBV infection \cite{41}. Additionally, some studies point to the involvement of \textit{H. pylori} with c-Myc \cite{38,41}. The biological significance between \textit{H. pylori} and c-Myc is still not understood. A study from Zang \textit{et al} \cite{38} has linked the c-Myc expression with cell proliferation while a study from Yang \textit{et al} \cite{40} on a gastric carcinoma cell line has pointed to a relationship with apoptosis. On the other hand, Kim \textit{et al} \cite{6} have shown a decrease of c-Myc expression after \textit{H. pylori} eradication, but not in its mRNA level, showing the complexity of this process. The data presented here show a high frequency of c-Myc expression in the group with only \textit{H. pylori}, but this was similar to the noninfected group. Therefore, the c-Myc expression may be influenced by the presence of \textit{H. pylori}, but it is also triggered in a \textit{H. pylori} independent way. Nevertheless, it seems that the presence of the EBV may also play a role since the frequency of c-Myc protein was the lowest among the groups, and this may be a disturbing factor when its biologic significance is considered.

The mutation of the \textit{p53} gene is among the major alterations of the multi-step process of gastric carcinogenesis, while it has also been reported in pre-malignant lesions of the stomach, such as chronic gastritis, intestinal metaplasia and dyspepsia. Kodama \textit{et al} \cite{43} have suggested an accumulation of wild-type \textit{p53}, especially in the \textit{H. pylori} infected mucosa probably due to the \textit{H. pylori}-induced DNA damage. The present study shows the highest frequency of \textit{p53} mutation in the groups with \textit{H. pylori} infection, especially among the cagA+ cases, corroborating previous studies \cite{15,32}. The high percentage of \textit{p53} mutation in EBV-associated and EBV-negative gastric carcinomas was observed in our study which was also demonstrated in other studies, demonstrating that the \textit{p53} mutation is a relevant alteration in gastric carcinogenesis independent of the infection \cite{15,44}.

Finally, this study shows the high prevalence of \textit{H. pylori} infection in gastric carcinomas in Ceará State. The groups indicate that there are different pathways according to the presence of infectious agents, with female being predominant in the group without infection. \textit{H. pylori} seems to influence the expression of Bcl-2, since this protein was observed only in the group infected exclusively by this microorganism. The Bax and c-Myc expression was present in all groups. However, the highest expression in the EBV-negative groups suggests that EBV may inhibit their expression. The mutation of the \textit{p53} gene was present in all groups possibly indicating that it was not only a consequence of the infectious agent.
Background

Helicobacter pylori and Epstein-Barr virus are important agents on gastric carcinogenesis. Proteins involved with cell cycle or apoptosis process emerge as a candidate for having a crucial role in the development of the neoplasms. Some of these, like c-Myc, p53 and the apoptotic family members such as Bcl-2 and Bax, deserves attention for having a key function in the cell proliferation and cell fate, besides they already have shown involvement in the tumorigenesis process in a variety of tumors, including gastric cancer. So this article tries to associate H pylori and EBV infection at the same time, with gastric carcinoma and some proteins of the cell cycle.

Research frontiers

Helicobacter pylori and Epstein-Barr virus infection related with gastric carcinogenesis.

Innovations and breakthroughs

Although H pylori and Epstein-Barr virus were well established as gastric cancer etiological agents, works on gastric cancer never associated an analysis of both agents at the same time, so this article did it.

Applications

Although this work had a small number of cases, it may justify further studies on the issue.

Peer review

This paper demonstrates that H pylori infection is very high (94.4%) in Brazil, associated with EBV infection and c-Myc, Bcl-2, Bax and p53 overexpression in some cases. These results have significant implications on the clinical management and on the research on the pathogenesis of this cancer.

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