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Gastric adenocarcinoma and *Helicobacter pylori*: Correlation with *p53* mutation and p27 immunoexpression

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ABSTRACT

Introduction: Helicobacter pylori infection is an established risk factor for gastric cancer development, but the exact underlying mechanism still remains obscure. The inactivation of tumor suppressor genes such as *p53* and $p27^{KIP1}$ is a hypothesized mechanism, although there is no consensus regarding the influence of *H. pylori cagA*(+) in the development of these genetic alterations. *Goals:* To verify the relationship among *H. pylori* infection, *p53* mutations and $p27^{Kip1}$ Protein (p27) expression in gastric adenocarcinomas (GA) seventy-four tissues were assayed by PCR for *H. pylori* and *cagA* presence. Mutational analysis of *p53* gene was performed by single-strand conformation polymorphism (SSCP). Seventy tissues were analyzed by an immunohistochemical method for p27 expression. *Results:* From the samples examined, 95% (70/74) were *H. pylori* positive, 63% *cagA*(+). Altered *p53* electrophoretic mobility was found in 72% of cases and significantly more frequent in the presence of *cagA*. Considerable reduction in p27 expression (19%) was found with a tendency for association between *cagA*(+) and p27(−), although the results were not statistically significant. Concomitant alterations of both suppressor genes were detected in 60% of cases. In the cases *cagA*(+), 66.7% of them had these concomitant alterations. *Conclusions:* The data suggest that *H. pylori cagA*(+) contributes to *p53* alteration and indicate that concomitant gene inactivation, with reduced p27 expression, may be a mechanism in which *H. pylori* can promote the development and progression of gastric cancer.

1. Introduction

Gastric carcinoma (GC) is the fourth common cancer type and the second cause of cancer-related death worldwide [1]. The majority (95%) of them are gastric adenocarcinomas (GA), divided into two histological types according to Laurén's classification: intestinal and diffuse types [2]. The intestinal type is related to corpus-dominant gastritis with gastric atrophy and intestinal metaplasia, more frequent in elderly men, whereas the diffuse type usually originates in pangastritis without atrophy and occurs in patients under the age of 50 and predominantly women [3,4]. The natural history of GC is complex and not entirely understood. It is a multifactorial disease with significant geographical variations where environmental and host genetic factors are involved.

Helicobacter pylori infection is one of the most important etiological factors established as a risk factor for the development of gastric adenocarcinoma. Although it has been classified as a Group I carcinogen [5] the exact mechanism responsible for the development of GA in *H. pylori*-infected patients still remains obscure [6,7]. Current models have suggested that *H. pylori* is not directly mutagenic, but acts through inflammatory mediators, favoring the formation of mutagenic substances [3,8]. *H. pylori* strains containing the cag pathogenicity island (*cag*PAI), a 40-kb genome segment that encodes approximately 30 genes including the cytotoxin-associated gene A (*cagA*), is associated with a more aggressive phenotype, an intense and extended inflammatory response and alterations of the gastric epithelium [9]. On the other hand, it has also been reported that *H. pylori* can reduce the mismatch repair protein levels, independent of the inflammation, raising the hypothesis that it may also lead to the mutagenic process [10].

Gastric carcinogenesis is a multistep process involving multiple genetic and epigenetic events in the course of the transformation

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of normal epithelium to clinical gastric cancer. In this process, the molecules that are more often altered are involved in the control of the G1/S transition of the cell cycle. At the G1/S check point, the tumor suppressor genes *p53* and $p2^{KIP1}$ play a critical role in cell cycle regulation [11].

The *p53* gene is located on the short arm of chromosome 17 (17p13.1) and encodes a protein that acts basically as a transcription factor, involved in maintaining genomic integrity by activating regulatory molecules of many cellular programs, including the cell cycle, response to DNA damage, apoptosis, cell differentiation and angiogenesis. The main transcriptional target of *p53* is represented by the *WAF1* gene (also known as *CIP1*, *SDI1*, *mda-6* or *CDKN1A*). This gene encodes a phosphorylated 21-kDa protein, which exhibits tumor suppressing activity, called $p2^{WAF1/CIP1}$. The p21 protein acts as a negative regulator of the cell cycle, inhibiting CDK activity in the G1/S transition and controlling DNA synthesis (S phase) [12]. In gastric cancer, *p53* mutations are relatively frequent and have been found in 38–71% of tumors [13].

The $p27^{KIP1}$ gene, located on chromosome 12p13, is another tumor suppressor protein from the CIP/KIP family. $p27^{Kip1}$ Protein (p27) shows 42% structural homology with p21, which explains the similarity of these proteins on the blockade of cell cycle progression through the inhibition of the cyclin D/CDK4, cyclin E/CDK2 and cyclin A/CDK2 complexes [14,15]. Decreased or absent p27 protein expression is a poor prognostic indicator in patients with gastric carcinomas, associated with more aggressive characteristics and tumor proliferation [16–18]. The reduced expression of p27 has been found to be an indicator of aggressive behavior and poor prognosis in a variety of malignant tumors including breast, colon, liver, stomach, lung, brain, prostate and malignant melanoma [14,17,19–23].

It has been suggested that *H. pylori* can initiate gastric cancer through *p53* tumor suppressor mutation [24]. The *p53* abnormalities have been assessed in several studies of gastric cancer associated with *H. pylori* infection [25–28], but there is still some controversy. Moreover, a reduced expression of p27 has been linked to *H. pylori* in gastric cancer [29,30]; however, there is no scientific consensus among studies, and there is a lack of studies linking these two suppressor genes and *H. pylori* [31].

Based on the suggestion that the *p53* and p27 alterations can be due to *H. pylori* infection, and in the absence of more consistent data especially in the simultaneous analysis of the two suppressors, this study aimed to examine the relationship between *H. pylori* infection, presence of *cagA* gene [*cagA*(+)] and the alterations in p27 protein and *p53* gene in gastric adenocarcinomas.

2. Materials and methods

2.1. Clinical specimens

The present study was approved by the Committee on Ethics in Research at the Federal University of Ceará (UFC-CE) and all subjects signed an informed consent form. Samples from patients with gastric cancer were collected from two hospitals, both located in Fortaleza, state capital of Ceará, Brazil: Walter Cantídeo Hospital at Federal University of Ceará (HUWC-CE) and Santa Casa de Misericórdia (SCMF-CE). A total of seventy-four tumor samples classified as gastric adenocarcinomas were selected for study. During the gastrectomy, fragments of tumor were collected and the specimens were subjected to DNA extraction and PCR assay to detect the *ureaseC* (*ureC*) gene of *H. pylori* and *p53* mutation. Representative formalin-fixed tumor specimens embedded in paraffin blocks were selected and histological sections (5 μm) were subjected to an immunohistochemical analysis for p27 protein. General information was collected from the medical reports.

2.2. DNA extraction

DNA was extracted from frozen tumor tissue only when the histopathological analysis determined that the tumor specimens consisted mainly (>80%) of tumor cells from a gastric adenocarcinoma. Genomic DNA was extracted using cetyltrimethyl ammonium bromide (CTAB), adapted from method of Foster and Twell [32]. The quality was evaluated by 1% agarose gel electrophoresis, ethidium bromide staining and by polymerase chain reaction (PCR) amplification of the *MTHFR* gene.

2.3. Detection of H. pylori and the presence of cagA gene

H. pylori infection was detected by PCR amplification of the *ureC* gene using primers described by Lage et al. [33]. For the *H. pylori* positive samples the presence of the *cag*A was identified using the primers described by Covacci and Rappuoli [34]. PCR mixtures for amplification were prepared in a final volume of 25 μL containing 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs, 0.4 μM of each primer set, 1 U of Platinum Taq DNA Polymerase (Invitrogen®), and 100 ng of DNA. The reactions proceeded under the following conditions. The number of cycles was 35 cycles for *Ure*C and 40 cycles for *Cag*A: initial denaturation at 94 °C for 30 seconds (s), annealing at 58 °C (30 s for *Ure*C and 45 s for *Cag*A), and extension at 72 °C for 2 minutes (min). These cycles were followed by a final extension at 72 °C for 5 min. Negative (water) and positive (DNA containing known *H. pylori* positive genes) controls were assayed in each run. PCR products were separated on 6% non-denaturing polyacrylamide electrophoretic gels and silver-stained according to Sanguinetti et al. [35]. The sample was considered *H. pylori* (+) and *cagA*(+) when fragments of 294 bp and 297 bp, respectively, were present.

2.4. Immunohistochemistry

A total of seventy samples were analyzed for p27 expression. The immunohistochemical staining (IH) was performed according to the method described by Hsu et al. [36]. Briefly, after deparaffinization and rehydration, antigen retrieval was carried out by microwave-treatment of the slides for 15 min in 10 mM citrate buffer solution (pH 6.0). Endogenous peroxidase activity was blocked with 3% H_2O_2 solution. Primary antibody (DakoCytomation®) was incubated for 16 h at 4–8 °C in humid chamber. The reaction was detected with the LSAB+ system (DakoCytomation®) according to the manufacturer's recommendation.

2.5. Histopathological and immunostaining analysis

The slides were evaluated by three experienced analysts independently using direct light microscopy, differences in interpretation were resolved by consensus. In the immunohistochemical analysis, the results were expressed as percentage of positive cases. The evaluation was conducted by estimating the percentage of positive cells, which was determined from the number of positive cells per 1000 nuclei in each specimen. Cases with ≥5% stained tumor cells were considered positive, according to the method described by Kudo et al. [37].

2.6. Analysis of p53 mutation

Single-strand conformational polymorphism (SSCP) analysis was used to assess alteration in the *p53* gene [*p53*(+)]. Exons 5–8 of *p53* gene were amplified by PCR using four pairs of primers described by Murakami et al. [38]. PCR was carried out in a 25-μL final volume and consisted of 50 ng of genomic DNA template, 0.2 mM each deoxynucleoside triphosphate, 1.5 mM of magne-