Helicobacter pylori and EBV in gastric carcinomas: Methylation status and microsatellite instability

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Abstract

AIM: To verify the methylation status of CDH1, DAPK, COX2, hMLH1 and CDKN2A genes and to evaluate their association with Helicobacter pylori (H. pylori)-cagA+ and Epstein Barr virus (EBV) infections in gastric adenocarcinomas.

METHODS: Methylation-specific PCR (MSP) assay was performed in 89 primary gastric carcinomas (intestinal and diffuse types). Microsatellite instability (MSI) analysis was performed using the BAT26 primer set and PCR products were analyzed with the ABI PRISM 3100 Genetic Analyzer using Genescan 3.7 software (Applied Biosystems). Detection of H. pylori and genotyping were performed by PCR, using specific primers for ureaseC and cagA genes. The presence of EBV was assessed by in situ hybridization. Statistical analyses were performed using the χ2 or Fisher’s exact test.

RESULTS: The most frequent hypermethylated gene was COX-2 (63.5%) followed by DAPK (55.7%), CDH1 (51%), CDKN2A (36%) and hMLH1 (30.3%). Intestinal and diffuse adenocarcinomas showed different methylation profiles and there was an association between methylation of E-CDH1 and H. pylori-cagA+ in the intestinal adenocarcinoma type. MSI was correlated with hMLH1 methylation. There was an inverse correlation between DAPK hypermethylation and MSI.

CONCLUSION: We found a strong association between CDH1 methylation and H. pylori-cagA+ in intestinal-type gastric cancer, association of MSI and better prognosis and an heterogeneous COX-2 overexpression.

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Key words: Gastric cancer; Methylation; Microsatellite instability; Helicobacter pylori; Epstein Barr virus

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INTRODUCTION

Gastric cancer (GC), one of the most common cancer types, is associated with high mortality rates. The prognosis of GC remains poor, especially when the diagnosis is undertaken at advanced stages. Thus, studies to elucidate the mechanisms acting in gastric carcinogenesis and which search for possible markers to assist in both earlier diagnosis and therapeutic approaches are relevant.

Gastric adenocarcinomas can be divided into intestinal or diffuse histological types. Environmental factors appear to be related to the intestinal type, which may play a role in carcinogenesis characterized by precursor lesions of gastric mucosa, followed by intestinal metaplasia that can lead to dysplasia and GC. In contrast, in the diffuse carcinoma no precursor lesions have been identified to date.

Many studies have identified the transcriptional silencing by DNA methylation as a mechanism responsible for tumor suppressor inactivation. Methylation of promoter CpG islands leads to DNA structural changes and, consequentially, gene inactivation. Several cancers, including gastric tumors, show methylation of multiple genes including CDH1, DAPK, COX2, hMLH1 and CDKN2A.

Microsatellite instability (MSI) reflects an erroneous form of DNA replication in repetitive microsatellite sequences and has been considered a hallmark of MSI phenotype. Microsatellite instability (MSI) reflects an erroneous form of DNA replication in repetitive microsatellite sequences and has been considered a hallmark of MSI phenotype. Microsatellite instability (MSI) reflects an erroneous form of DNA replication in repetitive microsatellite sequences and has been considered a hallmark of MSI phenotype.

Heliobacter pylori (H. pylori), carcinogen class I, colonizes the gastric epithelium and causes a severe inflammatory reaction that depends on factors including host genetic susceptibility, immune response, age at the time of initial infection, and environmental and virulence factors such as cytotoxic-associated gene A (cagA). The complex interactions among the different types of H. pylori, inflammation and genetic features of the host could promote a cascade of morphological events leading to GC.

Apart from the accepted role of H. pylori in the pathogenesis of GC, the Epstein Barr virus (EBV) has been associated with gastric carcinoma in at least 10% of cases. Countries with the highest incidences are Japan (19.3%) and Germany (18%). In Brazil, frequencies of EBV infection ranging between 8% and 11% have been described.

Some studies have linked DNA hypermethylation with H. pylori-cagA and EBV infection but these data are not conclusive and the studies did not examine both agents at the same time. By examining 89 primary gastric carcinomas, the present study verifies MSI frequency and the methylation status of the CDH1, DAPK, COX2, hMLH1 and CDKN2A genes and evaluates their association with H. pylori (cagA and cag-A) and EBV infections and also with clinicopathological features of gastric carcinomas.

MATERIALS AND METHODS

Samples

Eighty-nine gastric adenocarcinomas and their corresponding adjacent normal tissue were obtained surgically from Brazilian patients at the Federal University of Ceará State, the Clinical Hospital at the UNESP Medical School in Botucatu, Sao Paulo State and the Amaral Carvalho Hospital, and immediately frozen in liquid nitrogen until micro-dissection and DNA extraction. The Research Ethics Committees of the respective institutions approved this study and each subject signed an informed consent form before tissue was obtained. Histopathological analyses determined that the tumor specimens consisted mainly (> 80%) of tumor tissues and that the adjacent tissue was free of tumor cells. The histological classification was made according to the Lauren classification system and the tumors were staged according to the TNM criteria. DNA was extracted using standard methods.

Bisulfite modification and methylation-specific PCR (MSP)

DNA from both tumoral and normal tissues was subjected to treatment with sodium bisulfite as described by Herman et al. The modified DNA was amplified with primers specific for either the methylated or unmethylated sequences of hMLH1, COX2, DAPK, CDKN2A and CDH1 (Table 1). PCR was individually performed in 25 μL reaction volumes, containing 1 × Platinum Taq buffer, 1.5 mmol/L MgCl₂, 0.2 mmol/L of each dNTP, 0.4 μmol/L of each primer set, 1 U of Platinum Taq DNA Polymerase (Invitrogen) and 1 μL of treated DNA. DNA methylated in vitro by Sss-I methylase (New England Biolabs) was used as a positive control, and water and DNA from peripheral lymphocytes of healthy donors were used as negative controls. PCR products were separated on silver-staining 6% non-denaturing polyacrylamide gels.

Bisulfite sequencing analysis

To confirm reaction specificity, MSP-PCR products from each gene analyzed were cloned with TOPO TA Cloning Kit (Invitrogen) and sequenced using the ABI PRISM® BigDye Terminator v3.0 Cycle Sequencing Kit (Applied Biosystems) and ABI Prism 3100 DNA Sequencer (Applied Biosystems).
Microsatellite instability analysis

MSI analysis was performed using the BAT26 primer set (5'-TGACTAGGGTTAGGATTC-3', sense and 5'- GGATATGAGGATATGAG-3', antisense). Sense primer was labeled with 6-FAM. PCR was performed in a final volume of 25 μL containing 1 × PCR buffer, 3.0 mmol/L MgCl₂, 0.2 μmol/L dNTPs, 0.4 μmol/L of each primer, 2 U of Platinum Taq DNA Polymerase (Invitrogen) and 50 ng of DNA. The thermal conditions were 94 ℃/5 min followed by 40 cycles (94 ℃/1 min, 50 ℃/1 min and 72 ℃/1 min) and a final extension at 72 ℃/7 min. The dye-labeled PCR products were analyzed with ABI PRISM 3100 Genetic Analyzer using Genescan 3.7 software (Applied Biosystems). Both tumoral and normal samples were analyzed. Negative (SW480 cells) and positive (HTC116 cells) controls for MSI had been included in all the analyses. Deletions or insertions of at least 4 bp were required to satisfy the definition of instability[27]. All cases were repeated twice.

H. pylori and CagA detection

Detection of H. pylori in gastric carcinomas was performed by PCR amplification with primers specific to H. pylori ureaseC gene. The primer sequence used (5'-AAGGTTTCATTTGATGTAGATGTTT-3', sense and 5'-AATATTTTAGGTTAGGAGTTAATGTT-3', antisense)[28] amplifies a 294 bp fragment. To detect cag-A, the primer set 5'-GTAAGCTTGAATACGACATTAGAC-3' (sense) and 5'-TTAGGTTAGGATATGAG-3' (antisense) was utilized to amplify a 297 bp fragment. Each primer set (ureaseC and cag-A) was used in an independent PCR reaction in a final volume of 25 μL containing 1 × PCR buffer [20 mmol/L Tris–HCl (pH 8.4) and 50 mmol/L KCl], 1.5 mmol/L MgCl₂, 0.2 mmol/L of each dNTP, 0.4 μmol/L of each primer set, 1 U of Platinum Taq DNA Polymerase (Invitrogen) and 100 ng of DNA, under the following conditions: for ureaseC PCR, initial denaturation at 94 ℃/3 min followed by 35 cycles of denaturation at 94 ℃/30 s, annealing at 58 ℃/30 s and extension at 72 ℃/2 min and final extension at 72 ℃/5 min. The cag-A PCR included an initial denaturation at 94 ℃/3 min followed by 40 cycles of denaturation at 94 ℃/30 s, annealing at 58 ℃/45 s and extension at 72 ℃/2 min and final extension at 72 ℃/5 min. Both tumoral and normal samples were analyzed. Negative and positive controls were assayed in each run. PCR products were separated by silver-stained 6% non-denaturing polyacrylamide gel electrophoresis.[22]

EBER1 in situ hybridization

The presence of EBER was assessed by RNA in situ hybridization reaction with a 30 bp biotinylated probe (5'-AGACCCCGTCCTACACCCCGGGACTTGTA-3') complementary to the RNA EBER1. EBER transcript was shown in high amounts in the nuclei of latently infected cells. Signal amplification was employed with anti-biotin antibody (clone BK, mouse, dilution 1:20; DakoCytomation®) and biotinylated anti-immunoglobulin antibody (polyclonal, rabbit, dilution 1:100; DakoCytomation®). The reaction was detected with the streptavidin-biotinperoxidase complex (DakoCytomation®) and diaminobenzidine chromogen (DakoCytomation®). The slides were counterstained with Harris's hematoxylin. A case of nasopharyngeal carcinoma was used as positive control.

Statistical analysis

For statistical analysis the χ² test or Fisher's exact test was used. P values ≤ 0.05 were considered statistically significant.

RESULTS

Patients and tumor characteristics

The clinicopathological and epidemiological features of patients are shown in Table 2.

Methylation status

Of the five genes analyzed, the COX2 gene was the one most frequently hypermethylated (63.5%) followed by DAPK (55.7%), CDH1 (51%), CDKN2A (36%) and hMLH1 (30.3%). Figure 1 displays representative examples of the MSP products.
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Table 2 Clinicopathological and epidemiological features of patients in the four tumor stages (*n* (%))

| Variables          | Patients | Tumor stage |
|--------------------|----------|-------------|
|                    |          | 0-1 II III IV |
| Age (yr) ≤ 50      | 19       | 3 (15.8) 2 (10.5) 9 (47.4) 5 (26.3) |
| > 50               | 70       | 8 (11.4) 11 (15.7) 30 (42.9) 21 (30) |
| Gender             |          |             |
| Male               | 60       | 8 (13.3) 8 (13.3) 27 (45) 17 (28.3) |
| Female             | 29       | 3 (10.3) 5 (17.2) 12 (41.4) 9 (31) |
| Anatomic subsite   |          |             |
| Proximal (Cardia)  | 13       | 1 (7.7) 1 (7.7) 5 (38.5) 6 (46.1) |
| Distal (Antrum/body)| 46      | 2 (4.3) 9 (19.6) 21 (45.7) 14 (30.4) |
| Mixed              | 3        | 0 (0) 1 (33.3) 2 (66.7) 0 (0) |
| Lauren type        |          |             |
| Diffuse            | 31       | 4 (12.9) 4 (12.9) 12 (38.7) 11 (35.5) |
| Intestinal         | 57       | 7 (12.3) 9 (15.8) 27 (47.4) 14 (24.6) |
| Mixed              | 1        | 0 (0) 0 (0) 0 (0) 1 (100) |

| LDH1            | 1       | M       | M       | U       | 2       | M       | U       | 3       | M       | U       | 4       | M       | U       |
|-----------------|---------|---------|---------|--------|---------|---------|--------|---------|---------|---------|--------|---------|--------|
| CDKN2A          | 1       | M       | U       | 2       | M       | U       | 3       | M       | U       | 4       | M       | U       |
| COX2            | 1       | M       | U       | 2       | M       | U       | 3       | M       | U       | 4       | M       | U       |
| DAPK            | 1       | M       | U       | 2       | M       | U       | 3       | M       | U       | 4       | M       | U       |
| hMLH1           | 1       | M       | U       | 2       | M       | U       | 3       | M       | U       | 4       | M       | U       |
| 150 bp          |         |         |         |         |         |         |         |         |         |         |         |         |
| 100 bp          |         |         |         |         |         |         |         |         |         |         |         |         |
| 50 bp           |         |         |         |         |         |         |         |         |         |         |         |         |
| 200 bp          |         |         |         |         |         |         |         |         |         |         |         |         |
| 15-44           |         |         |         |         |         |         |         |         |         |         |         |         |
| 45-54           |         |         |         |         |         |         |         |         |         |         |         |         |
| 55-64           |         |         |         |         |         |         |         |         |         |         |         |         |
| ≥ 65 yr         |         |         |         |         |         |         |         |         |         |         |         |         |

Figure 1 Example of results from methylation-specific PCR analysis performed in tumoral samples from patients with gastric cancer. The studied gene is given on the right of the each panel. Lane U: Amplified product with primers recognizing unmethylated sequences; Lane M: Amplified product with primers recognizing methylated sequences; Lane L: Ladder 50 bp.

Figure 2 Frequencies of methylation at CDH1, DAPK, hMLH1, COX2 and CDKN2A genes in gastric carcinomas. A: Frequencies of methylation at CDH1, DAPK, hMLH1, COX2 and CDKN2A distributed according to tumor stages in diffuse adenocarcinoma; B: Frequencies of methylation at CDH1, DAPK, hMLH1, COX2 and CDKN2A distributed according to tumor stages in intestinal adenocarcinoma; C: Frequencies of methylation at CDH1, DAPK, hMLH1, COX2 and CDKN2A distributed with regard to age groups.

Microsatellite instability analysis

MSI was observed in 17.2% of GCs. Most of the patients with MSI tumors (86.7%) had an advanced form of the disease and 66.7% of them shown lymph node metastasis. Intestinal and diffuse adenocarcinomas had similar MSI frequencies. There was a significant correlation between MSI and hMLH1 methylation (*P* = 0.001). Conversely, a significant inverse correlation was demonstrated between DAPK methylation and MSI (*P* = 0.012).

Detection of *H. pylori*

The presence of *H. pylori* was identified in 98% of GC patients, of whom 63.2% were *cagA*+. The frequency of *H. pylori-cagA*+ infection was similar between intestinal and diffuse adenocarcinomas (64.5% vs 59.6%, respectively). In the intestinal type, CDH1 methylation was more frequent in *H. pylori-cagA*+ cases (55.8%) than in
Table 3  Associations between gene methylation status and clinicopathological features, age, H. pylori, MSI and EBV a (%)

| Medium age (yr) | Total | CDH1 | P-value | DAPK-kinase | P-value | COX2 | P-value | hMLH1 | P-value | CDKN2A | P-value |
|-----------------|-------|------|---------|-------------|---------|------|---------|-------|---------|--------|---------|
| Age group (yr)  |       |      |         |             |         |      |         |       |         |        |         |
| < 50            | 19    | 1052 | 0.883   | 8 (44.4)    | 0.282   | 9   | 50      | 0.179 | 6       | 33.3   | 0.903   | 3       | 15.8    | 0.035   |
| > 50            | 69    | 3550 | 0.266   | 41 (58.6)   | 0.764   | 45  | 67.2    | 0.304 | 21      | 31.8   | 0.764   | 29      | 42      |
| Gender          |       |      |         |             |         |      |         |       |         |        |         |        |         |
| Male            | 59    | 3050 | 0.938   | 33 (55.9)   | 0.946   | 34  | 59.6    | 0.289 | 21      | 35     | 0.787   | 21      | 35.6    | 0.830   |
| Female          | 29    | 1551 | 0.468   | 16 (55.2)   | 0.297   | 20  | 71.4    | 0.11   | 11      | 37.9   | 0.304   | 11      | 37.9    |
| Nodal metastases|       |      |         |             |         |      |         |       |         |        |         |        |         |
| Absent          | 17    | 8471 | 0.708   | 9 (50)      | 0.586   | 11  | 64.7    | 0.910 | 6       | 33.3   | 0.903   | 6       | 33.3    | 0.746   |
| Present         | 71    | 3752 | 0.468   | 40 (57.1)   | 0.462   | 43  | 63.2    | 0.21   | 21      | 31.8   | 0.764   | 26      | 37.1    |
| Tumor site      |       |      |         |             |         |      |         |       |         |        |         |        |         |
| Cardia          | 13    | 8655 | 0.855   | 9 (69.2)    | 0.427   | 10  | 83.3    | 0.228 | 4       | 30.8   | 0.304   | 8       | 61.5    | 0.266   |
| Antrum          | 38    | 2155 | 0.266   | 20 (52.6)   | 0.262   | 21  | 56.8    | 0.228 | 14      | 37.8   | 0.304   | 8       | 35.9    |
| Body            | 10    | 550  | 0.708   | 7 (70)      | 0.70    | 7   | 70      | 0.11  | 1       | 11.1   | 0.40    | 4       | 40      |
| Mixed           | 3     | 3100 | 0.07    | 2 (66.7)    | 0.14    | 3   | 100     | 0     | 0       | 0      | 0       | 0       | 0       |
| Laurèn          |       |      |         |             |         |      |         |       |         |        |         |        |         |
| Diffuse         | 30    | 1756 | 0.503   | 10 (33.3)   | 0.628   | 19  | 63.3    | 0.973 | 8       | 27.6   | 0.481   | 10      | 33.3    | 0.628   |
| Intestinal      | 57    | 2849 | 0.266   | 22 (38.6)   | 0.628   | 34  | 63      | 0.973 | 19      | 35.2   | 0.481   | 22      | 38.6    |
| Mixed           | 1     | 0100 | 0       | 0          | 0       | 1   | 100     | 0     | 0       | 0      | 0       | 0       | 0       |

H. pylori-cagA cases (39.1%), a phenomenon not observed in the diffuse type. Also, cagA+ among the intestinal cases displayed a higher frequency of hMLH1 methylation than among diffuse cagA+ cases (31.8% vs 15.8%, respectively). On the other hand, methylation of DAPK and COX2 did not vary when the samples were grouped by histotype and cagA status. With regard to MSI and the presence of cagA, MSI was inversely correlated with the cagA gene (P = 0.012), as shown in Table 3.

Detection of EBV

Fifty-four tumors were analyzed for EBV, of which 5 (9.3%) specimens were EBV-positive (EBV+). According to histological classification, 4 patients presented intestinal type adenocarcinoma and one was diffuse. All EBV+ cases were advanced grade (III and IV stages) and presented lymph node metastases. Two cases were EBV/H. pylori. The EBV+ cases were found to be associated with H. pylori, but only one was H. pylori-cagA+. Although the number of EBV+ cases was small, most of them displayed DAPK, COX2 and CDKN2A methylation.

DISCUSSION

Our results show that methylation status tends to increase with age. This finding corroborates the fact that GC occurs at a higher frequency in older individuals[1,2]. Moreover, previous studies showed that the age-related phenomenon of methylation of some tumor-suppressor and tumor-related genes can also be present in various non-neoplastic tissues, suggesting an association between age-related methylation and GC development[30,31].

In this study, we demonstrate that 84.3% of GCs in our sample present methylation for about one in five of the genes analyzed. COX2 was the gene found to be most commonly hypermethylated (63.5%) followed by DAPK (55.7%), CDH1 (51%), CDKN2A (36%) and hMLH1 (30.3%). An interesting observation in this study was related to the difference in methylation profiles between diffuse and intestinal adenocarcinoma types: in diffuse cases, the global methylation status, especially of CDH1, COX2 and CDKN2A, has the highest frequency in early stage tumors (0-I) with a tendency to decrease along with tumor grades; while in the intestinal-type, the methylation status for CDH1, COX2, hMLH1 and CDKN2A tended to increase from the earliest (0-I) to advanced stages (II-IV), as shown in Figure 2A and B. CDH1 methylation was more frequent in the diffuse histotype. In fact, a vast difference was verified in stage I tumors where all diffuse-type tumors presented CDH1 promoter methylation (100%) compared with only a small fraction (28.6%) in the intestinal-type, suggesting that CDH1 methylation is an early event occurring in diffuse-type GCs. Since CDH1 plays a fundamental role in maintaining cell differentiation,

Statistically significant result.

H. pylori-cagA cases (39.1%), a phenomenon not observed in the diffuse type. Also, cagA+ among the intestinal cases displayed a higher frequency of hMLH1 methylation than among diffuse cagA+ cases (31.8% vs 15.8%, respectively). On the other hand, methylation of DAPK and COX2 did not vary when the samples were grouped by histotype and cagA status. With regard to MSI and the presence of cagA, MSI was inversely correlated with the cagA gene (P = 0.012), as shown in Table 3.
polarity and normal tissue architecture\[38\], the diffusely-growing and low cell cohesion characteristics of diffuse-type GC could be a function of CDH1 down-regulation. Differences in the clinicopathological features between the intestinal and diffuse GC histological subtypes may be determined by different pathogenic processes\[35,36\]. The data presented in this study show that methylation in the same crucial genes could be an important pathway for the development of diffuse types. Identification of epigenetic differences between these two pathways could be of great importance in understanding gastric carcinogenesis and useful in the delineation of new therapeutic strategies.

The mechanisms that are implicated in CDH1 promoter methylation are yet to be identified. The role of H. pylori in the regulation of CDH1 expression has been described in recent studies showing that after H. pylori eradication, CDH1 methylation is decreased\[33,36\]. In our study, we observed that in the intestinal adenocarcinoma cases, methylation in the CDH1 promoter was more frequent in the group H. pylori-cagA+ (55.8%) than in those with H. pylori-cagA- (39.1%), indicating that H. pylori-cagA+ may be involved in CDH1 methylation in these tumors.

DAPK methylation has been shown to be associated with aggressive and metastatic phenotypes in some human cancers\[35,37\]. In the present study, we found a substantial frequency of DAPK methylation and observed that positive lymph node cases showed a slightly higher frequency of DAPK methylation than unmethylated cases (57.1% vs 42.9%). An important finding in this study was the inverse correlation observed between DAPK methylation and MSI. Although the mechanisms linking MSI to DAPK methylation are not known, this finding may provide a clue towards a better understanding of the association between MSI and better prognosis, since DAPK participates in the positive control of apoptosis.

Similar to previously reported results\[38\], 36.4% of the cases displayed hypermethylated CDKN2A. The EBV seems to play an important role in CDKN2A methylation\[38,39\]. However, the fact that, in this study, 60% of the EBV+ cases showed methylated CDKN2A should be interpreted cautiously because of the low number of such cases. Methylation in CDKN2A was more frequent in patients over 50 years of age (P = 0.035), and was also present in some samples of non-neoplastic gastric epithelia (data not shown) suggesting a link between aging and cancer via an increase in methylation. However, it is noteworthy that younger patients (< 50 years) who did not present CDKN2A methylation still developed GC, which suggests that other factors may account for the gastric carcinogenesis in these patients. In order to better evaluate in our population the correlation between CDKN2A and EBV it will be necessary to increase the number of tumors analyzed for EBV.

Most of the gastric tumors in our sample (63.5%) exhibit aberrant methylation of COX2. The correlation between COX2 methylation and gene downregulation has been well documented in the literature\[40\], although overexpression of COX2 has also been reported in GCs and some precancerous tissues\[41\]. COX2 overexpression is associated with enhanced proliferation, angiogenesis, resistance to apoptosis and tumorigenesis\[42\]. Despite the apparent selective advantage given by COX2 overexpression, the results from our research group and others\[42\] suggest that COX2 overexpression may not be essential in all cases of gastric tumorigenesis. Recent studies have documented that H. pylori-induced inflammation is linked to COX2 overexpression\[43\] and these findings led us to investigate whether cagA presence was related to the methylation status of COX2. In the present study, no significant correlation between cagA and COX2 methylation status was found. Thus, cagA presence appears not to be the only mechanism involved in the control of COX2 expression in H. pylori+ gastric cells.

Methylation of promoter regions is reported to be the main cause of inactivation of hMLH1\[44\]. The present study revealed a significant relationship between hMLH1 methylation and MSI (P = 0.001), corroborating findings from other studies\[45,46\]. In fact, methylation of this mismatch repair gene has been shown to play a major role in MSI in several cancers. The data from MSI analyses found in our study (17.2%) corroborated other studies in the literature which demonstrated MSI ranging from 13% to 39%\[47\]. The association between MSI and clinicopathological characteristics of GC remains unknown. While some studies reported that MSI gastric tumors are associated with distal tumor location, intestinal histotype, fewer lymph node metastases and better prognosis\[48,49\], others have found the absence of an association among these parameters\[48\]. In our data, 33.3% of MSI patients had no positive lymph nodes (N0) or 18.1% for MSS (stable) patients, suggesting a tendency for a better prognosis. Previous studies detected that MSI frequency in H. pylori-positive groups was significantly higher than in H. pylori-negative ones\[40,51\], indicating that this agent may play an important role in genetic stability. Several studies have suggested that the virulence attributed to the H. pylori-cagA+ strain is associated with a severe inflammatory response. It is known that in the gastric mucosa, reactive oxygen species are released as a result of H. pylori infection\[52\] and that the expression of DNA mismatch repair proteins in mismatch-competent cells might be transiently suppressed in the presence of oxidative stress\[53\]. In the present study, when the cases were divided into two groups (H. pylori-cagA+ and H. pylori-cagA-) we observed that, in contrast to our expectations, MSI was inversely correlated with cagA (P = 0.012), suggesting that, apart from cagA, other factors may contribute to MSI occurrence. These results require further exploration with larger numbers of cases.

In conclusion, our results confirm that methylation is an early epigenetic event in the molecular pathogenesis of GC. The methylation pattern of the genes studied suggests that gastric tumorigenesis can occur through different pathways. It appears that in diffuse adenocarcinoma tumors, methylation can be an early and crucial event in enabling tumorigenesis, where methylation in CDH1 assumes an important role and that MSI is associated with hMLH1 methylation, although this event is infrequent. The inverse association discovered between DAPK methylation
and MSI provides new data for elucidating the mechanisms involved in the association of MSI and better prognosis. Analysis of a larger number of patients is necessary to confirm our findings and to ascertain the significance of the association between promoter methylation, MSI and the presence of infectious agents in gastric carcinogenesis. We observed that H. pylori-cagA may be involved in the methylation process of CDH1 in intestinal adenocarcinoma. Microsatellite instability was inversely correlated with the cagA gene, suggesting that other factors may contribute to MSI occurrence. COX-2 overexpression does not occur in all GC cases.

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