ARQGA/1457

METHYLATION STATUS OF *CDH1* GENE IN SAMPLES OF GASTRIC MUCOUS FROM BRAZILIAN PATIENTS WITH CHRONIC GASTRITIS INFECTED BY *Helicobacter pylori*

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ABSTRACT – ***Context*** - Gastric cancer is one of the top list of cancer types that most leads to death in Brazil and worldwide. *Helicobacter pylori* (*H. pylori*) is a class I carcinogen and infect almost 90% of chronic gastritis patients. Some genotypes confer different virulent potential to *H. pylori* and can increase the risk of gastritis development. Methylation of CpG islands can inactivate tumor suppressor genes and therefore, it can be involved in the tumorigenic process. *CDH1* is a tumor suppressor gene that encodes the *E-cadherin* protein, which is important in maintaining cell-cell contacts. The inactivation of this gene can increase the chance of metastasis. Promoter methylation of *CDH1* at early steps of gastric carcinogenesis is not yet completely understood. ***Objective*** - In this study, we investigated the methylation status of *CDH1* in chronic gastritis samples and correlated it with the presence of *H. pylori*. ***Methods*** - Sixty gastric mucosal biopsies were used in this study. The detection of *H. pylori* was performed with the PCR primers specific to *urease C* gene. *H. pylori* genotyping was performed by PCR to *cagA* and *vacA* (s and m region). The methylation status of these gene *CDH1* was analyzed using methylation-specific polymerase chain reaction and direct sequencing of the PCR products was performed using primers methylated and unmethylated in both forward and reverse directions. ***Results*** - *H. pylori* was detected in 90% of chronic gastritis samples; among these 33% were *cagA* positive and 100% *vacA* s1. The genotype *vacA* s2/m1 was not detected in any sample analyzed. Methylation of *CDH1* was detected in 63.3% of chronic gastritis samples and 95% of them were also *H. pylori*-positive. ***Conclusions*** - This work suggests that *CDH1* gene methylation and *H. pylori* infection are frequent events in samples from Brazilian patients with chronic gastritis and reinforces the correlation between *H. pylori* infection and *CDH1* inactivation in early steps of gastric tumorigenesis.

HEADINGS - Helicobacter infections. Gastritis. Cadherins. Methylation.

INTRODUCTION

Gastric cancer comprises one of cancers that most kill in Brazil and worldwide, occupying the third and second position, respectively[20, 35]. Its detection is common in advanced stages of cancer progression and patients rarely survive more than 5 years after this point, moreover surgery in most cases is just palliative[27]. Therefore, the investigation of molecular markers that could help with diagnosis in early steps of gastric carcinogenesis indubitable suits important in gastric cancer. The risk for gastric cancer has been attributed to DNA alterations associated with chronic inflammation, imbalance of epithelial proliferation and apoptosis, and infection by *Helicobacter pylori* (*H. pylori*)[21]. It corresponds to a curved, microaerophilic gram-negative bacterium[25] that infects about 90% of patients with chronic gastritis and it is also associated with the development of peptic ulcer disease, atrophic gastritis and gastric malignancies[42]. It was classified as a class I carcinogen by the World Health Organization International Agency for Research on Cancer. Although almost 50% of the world's population is infected with *H. pylori*[15, 32, 33, 38], only a subset of infected individuals develop *H. pylori* associated gastroduodenal disease and gastric cancer during their life time[11]. Virulence factors of *H. pylori* are attributed by the flagella, bacterial urease and by genes such as *cagA* and *vacA*[11] *cagA* gene (cytotoxin associated gene) is found in 60%-70% of the bacterium's strains. *cagA* positive strains are considered more virulent than negative strains *vacA* gene codifies a vacuolating cytotoxin present in almost all strains[12, 26], it is involved in epithelial cell injury. The s region of *vacA* gene (signal peptide) exists as s1 or s2 allele and the m region (middle) as m1 or m2 allele. Therefore, the variety of *H. pylori* strains

Supported by "Fundação de Amparo à Pesquisa do Estado de São Paulo" (FAPESP) and National Council of Technological and Scientific Development (CNPq).
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could be related with different pathogenic effects. Considering the model which gastric cancer progresses from a normal mucosa[16], we questioned if pathogenic strains of *H. pylori* could play a role in the initial steps of changes that lead to advance from chronic gastritis to gastric carcinogenesis.

Methylation of CpG islands consists in an epigenetic process of gene regulation involved in chromatin conformation changes, genomic imprinting process, inactivation of X chromosome and carcinogenesis[14, 20, 23]. In tumors, methylation is observed in promoter regions impairing gene transcription, and can be considered as an additional way to inactivate tumor suppressor genes[24]. Methylation was previously detected in early steps of gastric carcinogenesis and it was also shown its accumulation over cancer progression[18]. Studies associating methylation in non-neoplasic tissues are relevant.

CDH1 codifies *E-cadherin*, a calcium-dependent transmembrane adhesion glycoprotein[17, 37]. *CDH1* considered a tumor suppressor gene that is expressed in epithelial cells and is important in cell architecture, tissue integrity and is involved in cellular processes including adhesion, morphology, migration, and development[2, 38]. Its inactivation is related with tumor progression through invasion and metastasis and it is found in a variety of cancers, including gastric cancer[4, 30]. Around 50% of diffuse histological type gastric carcinoma carries mutation in *CDH1*, furthermore hypermethylation of *CDH1* was found to be the second cause gene expression inactivation in two kindred harboring a familial gastric cancer and germ line *CDH1* mutation[30].

Therefore, we attempted to correlate the methylation status of *CDH1* in patients with chronic gastritis infected by pathogenic strains of *H. pylori.*

METHODS

Patients and specimens

In total, we analyzed 60 gastric mucosal biopsies, in which 30 samples were from patients with chronic gastritis and other 30 from patients underlining normal mucosa. The mucosa was collected in duplicate in the Endoscopy Surgery Department of Clinical Hospital of Medical University of Botucatu (FMB-UNESP), Botucatu, SP, Brazil, between April 2003 and July 2004. Through microscopic examination, histopathological investigation was carried out for all 60 tissues samples, hematoxylin and eosin-stained histological slides were scored for their histological parameters to the presence of chronic inflammation, acute inflammation, intestinal metaplasia and gastritis. All chronic gastritis included in this work had inflammation activity observed by histopathological analysis, and all the controls had normal mucosa wherein no inflammation activity was observed. Detection of *H. pylori* in histological tissues was performed by Giemsa (400x) (Figure 1). The age ratio was 52 years old, varying between 20-89 and 24-81 years old in chronic gastritis and control groups, respectively. Fifty three percent of the patients were women. All molecular analyses were performed in the Molecular Biology Laboratory of Hemocenter of FMB-UNESP.

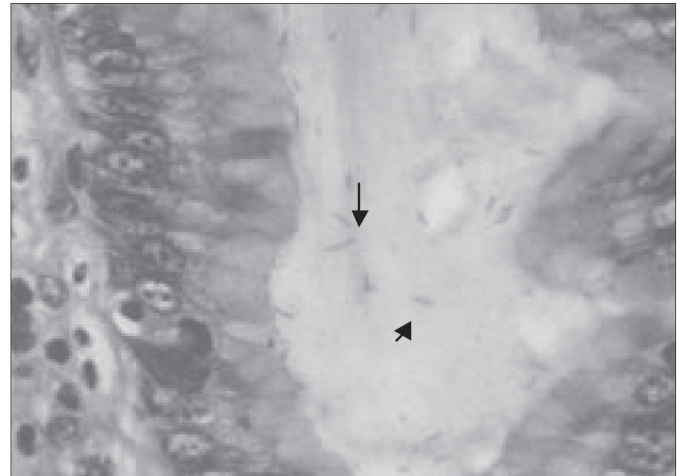

FIGURE 1. Detection of *Helicobacter pylori* in histological tissues was performed by Giemsa (400×), arrows

Committee of Ethics in Research of institutions approved this study (1892/2004) and each subject signed an informed consent term form before tissue was obtained.

DNA extraction

Wizard Kit Genomic DNA Purification (Promega) was utilized to purify the genetic material for further analysis. After DNA preparation, samples were stored at -70°C. Quality of DNA was assessed by amplification of GAPDH by PCR. Primer sequences and the amplicon are showed in Table 1. PCR reactions were performed in a volume of 25 mL containing 1% Buffer, 0.4 mM concentration of the deoxynucleoside triphosphate, 1.5 mM $MgCL_2$, 1 U of Recombinant Taq (Invitrogen), 100 ng of DNA and 0.4 mM of both forward and reverse primers. Amplification was carried out in a thermal cycler with denaturation for 3 minutes at 94°C and for 35 cycles of 40 seconds at 94°C, 40 seconds at 57°C and 2 minutes at 72°C. Final extension was performed for 7 minutes at 72°C. The PCR product was submitted to electrophoresis on 6% non-denaturing polyacrylamide gels and the bands were visualized by silver staining.

H. pylori detection and genotyping

The detection of *H. pylori* was performed with the PCR primers specific to *urease C* gene. Amplification of *cagA* and *vacA* (s and m region) were used for genotyping. Primers sequences and amplicon are illustrated in Table 1.

PCR of *urease C* and *cagA* were carried out in a total volume of 25 mL containing 1% Buffer, 0.2 mM concentration of dNTPs, 1.5 mM $MgCL_2$, 1.5 U of Taq Platinum (Invitrogen), 100 ng of DNA and 0.4 mM of both forward and reverse primers. The cycling conditions were of *urease C*: 3 minutes of preincubation at 94°C followed by 35 cycles of 30 seconds at 94°C, 1 minute at 59°C and 2 minutes at 72°C. Final extension was performed for 7 minutes at 72°C. Annealing temperature was 55°C for 40 seconds.

TABLE 1. Primer sequences, amplicon sizes and the annealing temperature used in this study

Primer	Primer sequence (5´-3´) forward Primer sequence (5´-3´) reverse	Product size (bp)	Annealing T(°C)	Reference
GAPDH	5’- TGGTATCGTGCAAGGACTCATGAC -3’ 5’- ATGCCAGTCAGCTTCCCGTTCAGC -3’	197	57	This study
Urease	5’- AAGCTTTTAGGGGTGTTAGGGGTTT-3´ 5´-AAGCTTACTTTCTAACACTAACGC-3´	294	59	This study
vacA s region	5’ – ATG-GAA-ATA-CAA-CAA-ACA-CAC-3’ 5’- CTG-CTT-GAA-TGC-GCC-AAA-C-3’	259(s1) 286 (s2)	55	(9)
vacA m1 region	5’ –GGT-CAA-AAT-GCG-GTC-ATG-G 5’-CCA-TTG-GTA-CCT-GTA-GAA-AC-3’	290	54	(9)
vacA m2 region	5’- GGA-GCC-CCA-GGA-AAC-ATT-G-3’ 5’-CAT-AAC-TAG-CGC-CTT-GCA-C-3’	352	54	(9)
cagA	5’- ATA-ATG-CTA-AAT-TAG-ACA-ACT-TGA-GCG-A-3’ 5’- TTA-GAA-TAA-TCA-ACA-AAC-ATC-ACG-CCA-T-3’	297	55	(12)
E-cadherin methylated	5’- TTA-GGT-TAG-AGG-GTT-ATC-GCG-T-3´ 5’-TAA-CTA-AAA-ATT-CAC-CTA-CCG-AC– 3’	115	62	(8)
E-cadherin unmethylated	5’- TAA-TTT-TAG-GTT-AGA-GGG-TTA-TTG-T– 3’ 5’- CAC-AAC-CAA-TCA-ACA-ACA-CA– 3’	97	63	(8)

The amplification of *vacA* s region was performed in a volume of 25 mL containing 1% Buffer, 0.4 mM of dNTPs, 3 mM $MgCL_2$, 1 U of Taq Gold (Applied Biosystems), 100 ng of DNA and 0.4 mM of both forward and reverse primers. The cycle conditions of *vacA* s region were: 5 minutes of preincubation at 95°C followed by 35 cycles of 30 seconds at 94°C, 1 minute at 55°C and 2 minute at 68°C. Final extension was performed for 7 minutes at 68°C. The annealing temperature was 54°C. PCR product of *urease C*, *cagA* and *vacA* amplifications were electrophoresed on 6% not denaturized polyacrylamide gels and the bands were visualized by silver staining.

Bisulfite modification

Purified DNA was subjected to bisulfite modification, as described by Herman et al.[17]. Bisulfite treatment converts unmethylated cytosine in uracil and methylated cytosine is unchanged. Briefly, 2 µg of genomic DNA (per sample) and 1 µg of Hering Sperm DNA (10 mg/mL) (Invitrogen) were heated at 97°C for 6 minutes, and then cooled on ice. Fifteen micro liter of 1 M NaOH was added to 35 µl of the denatured DNA solution. Mixture was stored at room temperature for 15 minutes. We added 150 µl of bisulfite (0.22 g) (Sigma-Aldrich, St. Louis, MO, USA) and hydroquinone (0.08 mg) (pH 5.0) (Sigma-Aldrich) and followed to incubation at 55°C for 16 hours. Extraction was carried out with Wizard® SV Gel and PCR Clean Up System – (Promega, USA). DNA was desulphonated with 15 µl of 1 mol/L NaOH. DNA was precipitated in ethanol and resuspended in 50 µl TE buffer (10 mmol/L Tris and 1 mmol/L EDTA (pH 8.0)).

MSP (methylation-specific PCR)

Bisulfite-modified DNA (2 uL) was amplified with primers specific for either the methylated or unmethylated, sequences. All primer sequences, annealing temperatures, cycle numbers and references are summarized in Table 1. PCR was performed in 25-µL reaction volumes, containing 1X PCR buffer, primers (0.4 mM each) and 1 unit of Taq polymerase Gold (Applied Biosystems). Six percent non-denatured polyacrylamide gels and silver nitrate staining were used. Samples showing band intensities approximately equivalent to that of the size marker were scored as methylated. Samples with week positive signals were repeated 3 times and only those samples with consistent positive signals were scored as methylated. Normal peripheral blood lymphocytes DNA, obtained from patients with no evidence of cancer, were used as negative control samples.

To confirm the specificity of MSP-PCR products, amplicons from PCR reactions with methylated and unmethylated primers were purified and sequenced in automatic sequencer ABI Prism 377 (Applied Biosystems) and compared with the expected sequence.

Statistical analysis

To verify the significance of association we utilized chi-square and Fisher tests and to analyze association between the groups studied we used the Goodman test with significance of 5%.

RESULTS

Genotype of *H. pylori*

Detection and genotyping of *H. pylori* was performed in 30 chronic gastritis samples and 30 normal mucosa samples (controls). *H. pylori* was detected by amplification of *urease C* gene and showed to be present in 90% (27/30) of chronic gastritis samples and in 43% (13/30) of controls (Table 2). To genotype *H. pylori* strains we amplified *cagA* and *vacA* genes. Thirty three percent (9/27) of chronic gastritis samples were *cagA* + and 23.1% (3/13) *cagA* + of controls (Table 2). Despite the higher number of *cagA* + strains in chronic gastritis compared with control, we did not observe association between *cagA* + and gastritis (chi-square P = 0.57; Fisher P = 0.391). All chronic gastritis samples (27/27) were *vacA* s1 type and only 54% (7/13) of controls were *vacA* s1 (chi-square and Fisher tests, P = 0.0004). The region m of

vacA was also analyzed. *vacA* m1 was found in 41% (11/27) of chronic gastritis and 31% (4/13) of controls. However *vacA* m2 was more frequent in both groups: 59% (16/27) of chronic gastritis and 69% (9/13) of controls. Combining *cagA* and *vacA* we found the following genotypes: 26% *cagA* + s1/m1, 7% *cagA* + s1/m2, 15% *cagA* - s1/m1 and 52% *cagA* - s1/m2 in chronic gastritis strains (Table 2); and 8% *cagA* + s1/m1, 15% *cagA* + s1/m2, 23% *cagA* - s1/m1, 8% *cagA* - s1/m2 and 46 % *cagA* - s2/m2 in the control samples (Table 2). Strains *cagA* + s2/m1, cagA + s2/m2, *cagA* - s2/m1 and *cagA* - s2/m2 were not identified in chronic gastritis samples and *cagA* + s2/m1, *cagA* + s2/m2 and *cagA* - s2/m1 were not identified in controls. *H. pylori* strains *cagA* - s2/m2 were identified only in controls and not in chronic gastritis samples.

TABLE 2. *Helicobacter pylori* genotypes detected in chronic gastritis and control samples

Group	*H. pylori*	*CagA*		Total
		positive	negative	
Chronic gastritis	27	s1/m1 = 7	s1/m1 = 4	30
		s1/m2 = 2	s1/m2 = 14	
		s2/m1 = 0	s2/m1 = 0	
		s2/m2 = 0	s2/m2 = 0	
		total = 9	total = 18	
Control	13	s1/m1 = 1	s1/m1 = 3	30
		s1/m2 = 2	s1/m2 = 1	
		s2/m1 = 0	s2/m1 = 0	
		s2/m2 = 0	s2/m2 = 6	
		total = 3	total = 10	

MSP for *E-cadherin*

Bisulfite genomic sequencing of the representative PCR products of *CDH1* gene showed that all cytosine at non-CpG sites were converted to thymine. This excluded the possibility that successful amplification could be attributable to incomplete bisulfite conversion. All PCR products analyzed showed extensive methylation of CpG sites, located inside the amplified genomic fragments. The results of both the MSP and bisulfite sequencing analyses were consistent, indicating that it is appropriate to draw inferences from the results of a methylation-specific PCR assay regarding the methylation status of gene promoters. A representative picture of a MSP PCR gel can be seen in Figure 2.

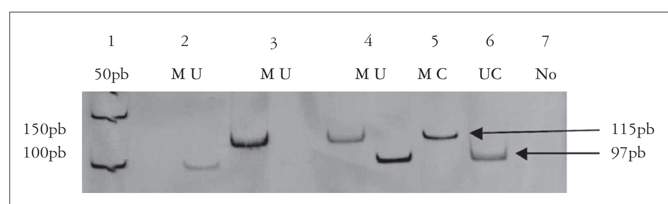

FIGURE 2. Silver stained polyacrylamide gel showing representative MSP PCR products of *CDH1* promoter region. 1. 50pb molecular marker (Invitrogen). 2–4. chronic gastritis samples. 2. just only the unmethylated (U) band is present. 3. just only the methylated band. (M) is present. 4. both bands are present. 5. methylated control (MC). 6. unmethylated control (UC). 7. H_2O (No DNA added)

CDH1 methylation in patients with chronic gastritis

The methylation status of *CDH1* was observed in 63.3% (19/30) of chronic gastritis samples and in just 20% (6/30) of control samples (Table 3). Applying the Goodman test, the difference is statistically significant. There was no difference between the average of age of patients with gastritis and patients with normal mucosa.

TABLE 3. Methylation of *CDH1* promoter detected in chronic gastritis samples and in controls

Methylation status	Chronic gastritis	Controls
Methylated	19 (63.3%)	6 (20%)
Unmethylated	11 (36.7%)	24 (80%)
Total	30 (100%)	30 (100%)

CDH1 methylation and *H. pylori*

Ninety-five percent of chronic gastritis samples which *CDH1* promoter was methylated were also infected by *H. pylori* (18/19), showing association between methylation status and *H. pylori* infection (P = 0.031). Fifty percent of control samples with *CDH1* methylation were infected with *H. pylori* with the following genotypes: *cagA* + s1/m1 (33%), *cagA* + s1/m2 (33%), *cagA* – s2/m2 (33%). The genotype *vacA* s2/m1 was not detected. The small number of samples analyzed in this study did not allow application of statistical tests to evaluate an association between the methylation status of *CDH1* and all possible pathogenic strains of *H. pylori*.

DISCUSSION

In the present study we analyzed 30 samples from chronic gastritis patients and 30 samples from normal mucosa. Ninety percent of chronic gastritis samples were positive for *H. pylori* and in control only 43%, showing a direct correlation between infection by *H. pylori* and chronic gastritis, confirming the importance of this bacterium to the inflammation process in the stomach mucosa. In average, *H. pylori* is found in 50% of the population[30], our data goes in accordance with this statistic.

In evaluation of *cagA* strains and gastritis, we did not observe statistic correlation in our samples. However, *cagA* positive strains were previously linked with chronic gastritis and other gastric diseases[3, 5, 41]. We detected *cagA* positive strains in 33% and 23% of gastritis and control samples, respectively. The presence of *cagA* citotoxin increases the risk of peptic ulcer disease in 1.5 times and gastric ulcer in 1.3 times[28]. Analyzing the *vacA* gene, we observed that all chronic gastritis samples were *vacA* s1 and just 54% of controls presented this strain type. In m region we found *vacA* m2 to be more frequent than m1. We observed that *cagA* + *vacA* s1/m1 are the most frequent in chronic gastritis and *cagA* - s2/m2 in control samples. This data is consistent with those already described, where s1/m1 is found in about 80% of patients[23, 27]. In Brazil, the genotype s1/m1 is the most frequently found[2]. *vacA* s1/m1 genotype is related with high citotoxin liberation and higher virulence[40, 42]. Moreover, these strains might be involved with increase of inflammation in

chronic gastritis and collaboration for Correa's[10] model to gastric cancer progression. Strain *vacA* s1/m2 is the second most virulent and it was found in higher number in gastritis, otherwise the most passive strain (*cagA*- *vacA* s2/m2) was observed only in controls and not in chronic gastritis samples. It corroborates to the fact that virulent strains predispose the mucous to an inflammatory status. The most frequent type of strain was *cagA* – s1/m2 (chi-square, $P = 0.0065$). We did not detect any *cagA* s2/m1 strain among the samples tested. This strain might be rare and related with poor colonization power and survival capacities guarantee to *H. pylori*, as in other studies it was also not detected[8, 9, 29, 34, 42].

Inactivation of *CDH1* was described in advanced and invasive stages of the carcinogenic process and its methylation has been linked with depth of tumor invasion, however recently, *CDH1* methylation process was found in initial stages of diffuse gastric tumors, and was also observed in not cancerous gastric tissues[17]. Here, we verified methylation of *CDH1* in chronic gastritis samples. It suggests *CDH1* inactivation by promoter methylation might be a common process in very early stages of gastric carcinogenesis. Epithelial *CDH1* expression is significantly reduced in *H. pylori* infected gastric tissues[1, 36, 39]. *CDH1* promoter methylation was not detected in a high proportion of chronic gastritis submitted to eradication of *H. pylori*[7, 19, 22, 36]. We demonstrated a positive association between *H. pylori* and *CDH1* methylation. Therefore, patients with chronic gastritis infected by *H. pylori* have a higher pattern of methylation and *H. pylori* could act as an inductor of methylation of *CDH1* in early stages of carcinogenesis. The presence of *H. pylori* is associated with increase of inflammatory mediators, such as *IL-8*, *GRO*-α, MIP-1α, ENA-78 and MCP-1, in gastric mucosa[1, 4]. It has demonstrated that *H. pylori* did not influence activation of methyltransferase genes *DNMT1*, *DNMT3A* or *DNMT3B*[13], thus it might act through inflammatory mediators. Recently, it was demonstrated inducement of *E-cadherin* promoter methylation in gastric cancer cell lines when treated with IL-1 or co-cultured with *H. pylori*[6, 31]. Thus, inflammatory mediators may be involved in stimulus for methylation in chronic gastritis. Still, further studies must be undertaken to understand the role *H. pylori* in gene inactivation by promoter methylation in chronic gastritis.

In conclusion, we showed that *CDH1* gene methylation and *H. pylori* infection are frequent events in samples from Brazilian patients with chronic gastritis and reinforced the correlation between *H. pylori* infection and *CDH1* inactivation in early gastric tumorigenesis.

ACKNOWLEDGEMENTS

We are thankful to the Pathology Department and Dr. Luiz Eduardo Naresse from Surgery Department of FMB-UNESP for the anatomopathological analyses and helpful with samples collections. We also thank Adriana Camargo Ferrasi and Graziela A.P.P.Marafiotti from Hemocenter-FMB-UNESP, Valéria Paixão from LICR for their expert technical assistance. And Otávia L. Caballero from LICR for the discussions.

Kague E, Thomazini CM, Pardini MICM, Carvalho F, Leite CV, Pinheiro NA. Padrão de metilação do gene *CDH1* em amostras de mucosa gástrica de pacientes brasileiros com gastrite crônica infectados pelo *Helicobacter pylori*. Arq Gastroenterol. 2009;46(4):7-12.

RESUMO – ***Contexto*** - O câncer gástrico é uma das principais neoplasias que causam o óbito no Brasil e no mundo. *Helicobacter pylori* é um carcinógeno do tipo I relacionado à gastrite crônica. Diferenças no grau de virulência de suas cepas levam a maior risco de desenvolvimento de doenças gástricas. A metilação de ilhas CpGs está envolvida com o processo de tumorigênese em diferentes tipos de câncer. *CDH1* é um gene supressor tumoral que, quando inativado, pode aumentar as chances de metástase. A metilação deste gene em estágios precoces da carcinogênese gástrica ainda não é totalmente compreendida. ***Objetivo*** – Investigar o padrão de metilação do gene *CDH1* em amostras de gastrites crônicas e correlacionar com a presença do *H. pylori*. ***Métodos*** – Foram usadas 60 biopsias de mucosas gástricas. A detecção de *H. pylori* foi realizada por PCR para o gene da *urease C* e a genotipagem com PCR para os genes *cagA* e *vacA* (região s e m). O padrão de metilação do gene *CDH1* foi analisado usando a técnica de PCR e específica para a metilação e sequenciamento direto dos produtos de PCR. ***Resultados*** - A bactéria *H. pylori* foi detectada em 90% das amostras de gastrites crônicas; destas, 33% portavam o gene *cagA* e 100% *vacA* s1. O genótipo *vacA* s2/m1 não foi detectado nas amostras analisadas. Metilação de *CDH1* foi detectada em 63,3% das amostras de gastrites e 95% delas eram portadoras de *H. pylori*. ***Conclusão*** - Os resultados deste estudo sugerem que a metilação em *CDH1* e a infecção pelo *H. pylori* são eventos frequentes em amostras de pacientes brasileiros com gastrite crônica e reforça a correlação entre infecção por *H. pylori* e inativação do gene *CDH1* em estágios precoces da tumorigênese gástrica.

DESCRITORES – Infecções por helicobacter. Gastrite. Caderinas. Metilação.

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Received 2/9/2008.
Accepted 9/6/2009.