Inactivation of *COX-2*, *HMLH1* and *CDKN2A* Gene by Promoter Methylation in Gastric Cancer: Relationship with Histological Subtype, Tumor Location and *Helicobacter pylori* Genotype

Markênia Kélia Santos Alves^a Adriana Camargo Ferrasi^b Valeska Portela Lima^a
Márcia Valéria Pitombeira Ferreira^a Maria Inês de Moura Campos Pardini^b
Sílvia Helena Barem Rabenhorst^a

^aGenetic Section, Department of Pathology and Forensic Medicine, Federal University of Ceará, Ceará, and

^bMolecular Biology Laboratory, Botucatu Blood Transfusion Center, Medical School, University of São Paulo State, UNESP, Botucatu, Brazil

Key Words

Gastric cancer · Histological subtypes · Tumor location · Methylation · p16^{INK4A} · *HMLH1* · *COX-2* · *Helicobacter pylori* genotypes

Abstract

Objective: We aimed to evaluate the inactivation of *COX-2*, *HMLH1* and *CDKN2A* by promoter methylation and its relationship with the infection by different *Helicobacter pylori* strains in gastric cancer. **Methods:** DNA extracted from 76 *H. pylori*-positive gastric tumor samples was available for promoter methylation identification by methylation-specific PCR and *H. pylori* subtyping by PCR. Immunohistochemistry was used to determine *COX-2*, p16^{INK4A} and *HMLH1* expression. **Results:** A strong negative correlation was found between the expression of these markers and the presence of promoter methylation in their genes. Among cardia tumors, negativity of p16^{INK4A} was a significant finding. On the other hand, in noncardia tumors, the histological subtypes had different gene expression patterns. In the intestinal subtype, a significant finding was *HMLH1* inactivation by methylation, while in the diffuse subtype, *CDKN2A* inactivation by methylation was the significant finding. Tumors with methylated *COX-2* and *HMLH1* genes were associated with *H. pylori vacA*

s1 ($p = 0.025$ and 0.047 , respectively), and the nonmethylated tumors were associated with the presence of the gene *flaA*. **Conclusions:** These data suggest that the inactivation of these genes by methylation occurs by distinct pathways according to the histological subtype and tumor location and depends on the *H. pylori* genotype.

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Introduction

Gastric carcinoma (GC) is one of the most frequent malignancies in the world and a leading cause of cancer death, therefore representing a major public health problem worldwide [1, 2]. This cancer is a multifactorial disease in which genetic, epigenetic and environmental factors interact and contribute to the origin and progression of these tumors [3]. DNA methylation in normally unmethylated gene promoters is the most epigenetic change in GC, probably due to the easy accessibility to exogenous agents [4], and it has been shown to be an important mechanism in the transcriptional inactivation of certain tumor suppressor genes [4].

CDKN2A, located on the short arm of human chromosome 9, is one the most methylated tumor suppressor

genes in GC. Its product, p16^{INK4A}, acts during the G1 phase of the cell cycle by inhibiting progression to the next cell cycle phase through the selective inhibition of the formation of complexes cyclin D/cyclin-dependent kinase 4 or 6 [5]. The *HMLH1* gene is a human homologue of the gene *mutL* of *Escherichia coli*, located on chromosome 3p21.3. This gene codes for a protein with the same name that belongs to the DNA mismatch repair system [6]. Inactivation of the *CDKN2A* and *HMLH1* genes has been documented in several tumors such as colorectal and kidney, besides GC [4, 5, 7–9]. The restoration of expression of these genes by demethylating agents [10, 11] indicates that these alterations may potentially be therapeutic targets.

Additionally, COX-2 overexpression has been related to the intense inflammatory response and GC development [12], but some studies have shown a low expression of the COX-2 gene in GC associated with gene promoter hypermethylation [11, 13]. This gene, officially called prostaglandin-endoperoxide synthase 2 (*PTGS-2*), is located on chromosome 1q25.2–q25.3 and encodes an enzyme by the same name; PTGS-2 is involved in the synthesis of prostaglandins, which mediate cell signaling and are involved in inflammation process [12]. Although there are studies indicating hypermethylation in promoter regions of genes *COX-2*, *CDKN2A* and *HMLH1* as an important mechanism of gene silencing and suppression of their function in gastric carcinogenesis, there are still many gaps to be considered, such as its importance in each histological subtype, in tumor location and the factors that promote this process.

Studies in vitro and in vivo and of bacterial eradication have associated the presence of *Helicobacter pylori* to the induction of hypermethylation in promoter regions of genes important for GC progression, such as *COX-2*, *CDH1*, *HMLH1*, *CDKN2A* and *RUNX3* [14–16], albeit with controversial results [17–19]. *H. pylori* has a high genetic variability, which has been related to virulence and clinical outcome of gastric diseases [20–22]. Two well-established virulence factors are the presence of the cytotoxin-associated antigen A (*cagA*) gene, located within the right portion of the *cag* pathogenicity island (*cag*-PAI), and vacuolating cytotoxin A (*vacA*), mostly the *vacAs1m1* allelic combination [21, 22]. *cag*-PAI also harbors the *cagE* and *virB11* genes, located in the right and left regions of the island, respectively. These genes are known to play a role in constructing the type IV secretion system and inducing pro-inflammatory, pro-proliferative epithelial cell signaling [20, 23] and show a high frequency in GC [24]. Another important *H. pylori* viru-

lence factor is the presence of flagella, coded by several genes, such as *flaA* and *flaB*, which have been associated with successful colonization and with the process of infection and persistence in the gastric mucosa [25]. In addition to the well-characterized motility role, the flagella have been associated to other functions such as acting as an export apparatus for virulence factors [26], in the sensitivity to medium viscosity [27] and as an important immunogenic protein [28]. *flaA* is the main encoding gene to flagellin of the flagellum filament [29]. So far, few studies have assessed the involvement of *H. pylori* genotypes regarding their methylation inducing potential of gastric carcinogenesis-related genes.

Therefore, studies of the mechanisms by which *H. pylori* may promote gastric carcinogenesis are important. In view of the genetic diversity shown by *H. pylori*, it is important to assess the relationship between specific genotypes and their possible carcinogenic mechanisms. Additionally, tumors of the intestinal and diffuse subtypes need to be considered individually, since they are distinct tumors differing not only in clinical and histopathological features, but in their tumorigenic pathways, with distinct genetic and epigenetic alterations [30, 31].

Materials and Methods

Clinical Specimens

The present study was approved by the Hospital Ethics Committee at the Universidade Federal do Ceará, Brazil, and all subjects signed an informed consent form before inclusion. Samples from 76 patients with gastric adenocarcinoma who had undergone gastrectomy were collected from Walter Cantídio University Hospital and Santa Casa de Misericórdia Hospital, both located in Fortaleza, the capital of Ceará State. The histological classification was done according to Lauren's classification.

DNA Extraction

Genomic DNA was extracted from frozen tumor tissue using the cetyltrimethyl ammonium bromide (CTAB) technique, adapted from Foster and Twell [32]. DNA was extracted from only fragments that showed more than 80% tumor cells. DNA quality was analyzed by 1% agarose gel electrophoresis, and the amount was determined using the Nanodrop® 3300 fluorospectrometer (Wilmington, Del., USA).

Sodium Bisulfite Treatment and Methylation-Specific PCR

DNA extracted from tumor tissue was modified by sodium bisulfite to determine the methylation status of the *CDKN2A*, *HMLH1* and *COX-2* genes by methylation-specific PCR, as previously described by Ferrasi et al. [17]. The primers targeting the promoter gene regions studied and the annealing temperatures are described in table 1. PCR was performed in 25 µl reaction volume, containing 1× Platinum Taq buffer, 3.0 mM MgCl₂ (*CDKN2A*) or 1.5 mM MgCl₂ (*HMLH1* and *COX-2*), 0.4 mM of each

Table 1. PCR primer sets used for MS-PCR

Gene		Primer		Ref.	Annealing temperature °C	Size of PCR product bp
		forward	reverse			
COX-2	M:	TTAGATACGGCGGCGGCGGC	TCTTTACCCGAACGCTTCCG	19	59	161
	U:	ATAGATTAGATATGGTGGTGGTGGT	CACAATCTTTACCCAAACACTTCCA		65	171
CDKN2A	M:	TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCGTAA	33	70	150
	U:	TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCACAACCATAA		70	151
HMLH1	M:	TATATCGTTCGTAGTATTCGTGT	TCCGACCCGAATAAACCCAA	34	66	153
	U:	TTTGATGTAGATGTTTATTAGGGTTGT	ACCACCTCATCATAACTACCCACA		64	124

M = Methylated; U = unmethylated.

dNTP, 0.64 μ M (CDKN2A), 0.24 mM (HMLH1) or 0.4 mM (COX-2) of each primer set, 1 U Platinum Taq DNA Polymerase® (Invitrogen, Carlsbad, Calif., USA), and 50 ng of treated DNA.

DNA methylated in vitro by Sss-I methylase® (New England Biolabs, Beverly, Mass., USA) was used as a positive control. Water and DNA from peripheral lymphocytes of healthy donors were used as negative controls. The PCR products were resolved in a 6% nondenaturing polyacrylamide gel and subsequently submitted to silver staining.

For confirmation of the reaction specificity, MS-PCR products from COX-2, CDKN2A and HMLH1 genes analyzed were cloned with a TOPO TA Cloning Kit (Invitrogen) and both the methylated and unmethylated PCR products were sequenced using an ABI PRISM® BigDye Terminator v.3.0 Cycle Sequencing Kit (Applied Biosystems, Foster City, Calif., USA) and ABI Prism 3100 DNA Sequencer (Applied Biosystems).

Detection of *H. pylori* Infection and *vacA* Alleles and the Presence of *cagA*, *cagE*, *virB11* and *flaA* Genes

H. pylori infection was detected by amplification of the *ureC* gene using primers for PCR, as described by Lage et al. [35]. For the *H. pylori*-positive samples, the presence of the *vacA* alleles, *cagA*, *cagE*, *virB11* and *flaA* genes were identified using the primer sets shown in table 2. PCR mixtures, for amplification of the *cagE*, *virB11* and *flaA* genes, were prepared in a volume of 20 μ l using Green MasterMix® (Taq DNA Polymerase, dNTPs and MgCl₂) according to the manufacturer's instructions (Promega, Madison, Wisc., USA), with addition of 0.8% Tween 20, 0.3 μ M (*virB11*), 0.3 μ M (*cagE*) and 0.3 μ M (*flaA*) of each primer and 50 ng of DNA sample.

The *ureC*, *cagA*, *vacA* s1/s2, *vacA* m1 genes were amplified in a 25 μ l volume containing 2.5 μ l of 10 $\times$ PCR buffer (Invitrogen, Cergy Pontoise, France), 1% Tween 20, 1.5 mM MgCl₂ (Invitrogen), 200 μ M of each dNTP (Invitrogen), 1 U Platinum Taq polymerase® (Invitrogen), 0.4 μ M (*ureC*, *cagA*, *vacA* s1/s2, *vacA* m1), 0.3 μ M (*vacA* m2) of each primer and 50 ng of DNA sample.

The PCR products were resolved by 1% agarose gel electrophoresis with ethidium bromide staining. The sample was considered *H. pylori* positive when a *ureC* fragment of 294 bp was amplified. For confirmation of the reaction specificity, PCR products from the *ureC* gene were cloned in TOPO TA Cloning Kit (Invitrogen)

and sequenced using an ABI PRISM BigDye Terminator v.3.0 Cycle Sequencing Kit (Applied Biosystems) and an ABI Prism 3100 DNA Sequencer® (Applied Biosystems). The *vacA*, *cagA*, *cagE*, *virB11* and *flaA* genes were considered positive when a specific fragment was detected (table 2). DNase-free water was used as a negative control. DNA preservation has also been confirmed by amplification of different genes in other approaches under study in our laboratory. Random samples were reanalyzed to confirm the results.

Immunohistochemistry

The detection of p16^{INK4A} protein was performed using the commercial kit CINTEC Histology® (K5340; Dako, Glostrup, Denmark), according to the manufacturer's recommendations. The proteins HMLH1 and COX-2 were determined by the streptavidin peroxidase method, adapted from the protocol of Hsu et al. [39]. Briefly, for antigen retrieval, deparaffinized sections were pretreated by heating in a microwave oven in 10 mM citrate buffer, pH 6.0, for 20 min. Endogenous peroxidase was blocked by 3% H₂O₂, for 10 min. Sections were incubated in a humid chamber overnight at 4°C with the following primary antibodies: COX-2 (SC-1746; dilution 1:50; Santa Cruz Biotechnology, Santa Cruz, Calif., USA) and HMLH1 (SC-581, dilution 1:100; Santa Cruz Biotechnology). The reaction was detected with the LSAB+ system (DakoCytomation, Carpinteria, Calif., USA), according to the manufacturer's recommendations. Confirmed cases of p16-positive human breast carcinoma, COX-2-positive colon from patients with Crohn's disease and HMLH1-positive tonsil were used as positive controls.

Immunostaining Analyses

The immunohistochemical evaluation was performed independently by two experienced technicians, using direct light microscopy. Any conflicting results were jointly considered for a consensual determination. Protein expression was quantified by manual counting of at least 1,000 tumor cells in 10 different fields at a magnification of $\times 400$. The labeling index expresses the percentage of nuclear or cytoplasmic positive cells in each tumor sample [40]. Only cases with labeling index equal to or greater than 5% were considered positive.

Table 2. PCR primer sets used for genotyping *H. pylori*

Gene	Primer sequence	Ref.	Annealing temperature °C	Size of PCR product bp
<i>ureC</i>	F: 5'-AAGCTTTTAGGGGTGTTAGGGGTTT-3' R: 5'-AAGCTTACTTTCTAACACTAACGC-3'	35	55	294
<i>vacA</i>				
<i>s1/s2</i>	F: 5'-ATGGAAATACAACAAACACAC-3' R: 5'-CTGCTTGAATGCGCCAAAC-3'	36	55	259/286
<i>m1</i>	F: 5'-GGTCAAAATGCGGTCATGG-3' R: 5'-CCATTGGTACCTGTAGAAAC-3'		55	290
<i>m2</i>	F: 5'-GGAGCCCCAGGAAACATTG-3' R: 5'-CATAACTAGCGCCTTGCAC-3'		52	192
<i>cagA</i>	F: 5'-ATAATGCTAAATTAGACAACCTTGAGCGA-3' R: 5'-TTAGAATAATCAACAAACATAACGCCAT-3'	37	56	297
<i>cagE</i>	F: 5'-TTGAAAACTTCAAGGATAGGATAGAGC-3' R: 5'-GCCTAGCGTAATATCACCATTACCC-3'	21	50	509
<i>virB11</i>	F: 5'-TTAAATCCTCTAAGGCATGCTAC-3' R: 5'-GATATAAGTCGTTTTACCGCTTC-3'	21	49	491
<i>flaA</i>	F: 5'-TTCTATCGGCTCTACCAC-3' R: 5'-CTGACCGCCATTGACCAT-3'	38	55	508
F= Forward; R = reverse.				

Statistical Analyses

The statistical analyses were carried out using the SPSS version 15.0 program (SPSS Inc., Chicago, Ill., USA). Statistically significant differences were evaluated by the χ^2 test or Fisher's exact test. Correlations between immunostaining and *COX-2*, *HMLH1* and *CDKN2A* promoter region methylation status were analyzed by Spearman's rank correlation coefficient. The results were considered statistically significant when the p values were less than 0.05.

Results

Study Population

A total of 76 *H. pylori* infection-positive patients participated in this study. The characteristics of the patients and tumors are shown in tables 3 and 4.

Frequency and Correlation between Methylation in Promoter Regions and Expression of Genes *COX-2*, *CDKN2A* and *HMLH1*

The presence of promoter methylation in *COX-2*, *CDKN2A* and *HMLH1* was observed in 50.0% (38/76), 44.7% (34/76) and 32.9% (25/76) of cases, respectively. In 5 cases, it was not possible to determine immunohisto-

chemical expression due to large amount of necrosis in the tumor material analyzed. There was no immunodetection of the proteins studied in 73.2% (52/71) for *COX-2*, 57.7% (41/71) for p16^{INK4A} and 40.8% (29/71) for *HMLH1*. The frequencies of methylation associated to immunohistochemical expression of each gene are shown in table 5. A highly significant negative correlation between immunopositivity and *COX-2*, *CDKN2A* and *HMLH1* promoter region methylation was found. However, a fraction of cases, 34.6% (18/52) for *COX-2*, 31.7% (13/41) for *CDKN2A* and 31.0% (9/29) for *HMLH1*, demonstrated the lack of expression without gene promoter methylation.

Relationship between Promoter Methylation and Expression of the *COX-2*, *CDKN2A*, *HMLH1* Genes as well as Lauren Histological Type and Tumor Location

No significant association was observed between sex, age and tumor staging with promoter methylation as well as expression of the genes *COX-2*, *CDKN2A* and *HMLH1*. Likewise, no difference was observed between the intestinal and diffuse subtype tumors regarding the frequency of

Table 3. Characteristics of 76 subjects

Median age, years	65.5
Sex, male/female	47/29
Lauren classification, intestinal/diffuse	48/28
Tumor location, cardia/noncardia	17/59

Table 4. Stage and differentiation grade of 76 tumors

<i>Grade</i>	
1 Well differentiated	1
2 Moderately differentiated	41
3 Poorly differentiated	34
pTNM stage, I + II/III + IV	22/54
pTNM = Pathologic tumor-lymph-node metastasis.	

Table 5. Correlation between expression and methylation status in promoter regions of genes *COX-2*, *CDKN2A* and *HMLH1*

		Immunodetection		N	p value	R
		(+)	(-)			
<i>COX-2</i>	M	2	34	36	<0.001	-0.486
	U	17	18	35		
Total		19	52	71		
<i>CDKN2A</i>	M	4	28	32	<0.001	-0.546
	U	26	13	39		
Total		30	41	71		
<i>HMLH1</i>	M	3	20	23	<0.001	-0.634
	U	39	9	48		
Total		42	29	71		

M = Methylated; U = unmethylated; + = immunopositive (LI >5%); - = immunonegative (LI <5%).

promoter methylation in these genes and the immunodetection of the *COX-2*, *p16^{INK4A}* and *HMLH1* proteins (table 6). However, when the cases were analyzed according to tumor location, the cardia tumors demonstrated a significant frequency of *p16^{INK4A}*-negative cases ($p = 0.044$), even though no significant difference was found regarding *CDKN2A* promoter methylation. On the other hand, the noncardia tumors were predominantly *HMLH1* methylated ($p = 0.042$) when compared to cardia tumors (table 6).

Table 6. Distribution of *COX-2*, *CDKN2A* and *HMLH1* methylation status and expression according to histological subtype and tumor location

		Intestinal		p values	Cardia		p values	Total
			Dif-fuse			Non-cardia		
<i>COX-2</i>	M	22	16	0.342	8	30	0.783	38
	U	26	12		9	29		38
	+ -	12 34	7 18	0.862	4 12	15 40	1.000	19 52
<i>CDKN2A</i> (<i>p16^{INK4A}</i>)	M	22	12	0.801	9	25	0.440	34
	U	26	16		8	34		42
	+ -	18 28	12 13	0.470	3 13	27 28	0.044*	30 41
<i>HMLH1</i>	M	15	10	0.689	2	23	0.042*	25
	U	33	18		15	36		51
	+ -	27 19	15 10	0.915	9 7	33 22	0.788	42 29

M = Methylated; U = unmethylated; + = immunopositive (LI >5%); - = immunonegative (LI <5%). * Significant results.

Considering both the Lauren histological classification and tumor location, in the intestinal subtype tumors, all tumors except one with the *HMLH1* gene promoter methylated (93.33%; 14/15; $p = 0.040$) were localized in the noncardia region (fig. 1a), while in the cardia tumors the negativity of *p16^{INK4A}* was significantly more frequent ($p = 0.049$) (fig. 1b).

In order to observe the relationship between methylation status and the lack of expression of the studied genes considering the histological subtypes and the tumor location, we analyzed the immunonegative cases separately (table 7). From these analyses, it was evident that in the intestinal tumors, the negativity of *HMLH1* by promoter methylation was predominant in the noncardia tumors ($p = 0.010$). On the other hand, in the diffuse-subtype tumors, all cases *p16^{INK4A}* negative and methylated were in the group of noncardia tumors ($p = 0.021$).

Relationship between Methylation Status, Expression of the *COX-2*, *CDKN2A*, *HMLH1* Genes and *H. pylori vacA* Subtypes, *cagA*, *cagE*, *virB11* and *flaA* Genes

Among the *H. pylori* genes studied, *vacA* s1m1 was the most frequent one (72.4%; 55/76), followed by *flaA* (61.9%; 39/63) and *cagA* (61.8%; 47/76), which had similar fre-

Table 7. Distribution of the COX-2, p16^{INK4A} and HMLH1 negative cases according to histological subtype, tumor location and methylation status

		Intestinal				Diffuse			
		Cardia	Noncardia	p values	total	Cardia	Noncardia	p values	total
COX-2 (-)	M	6	14	0.726	20	1	13	1.000	14
	U	5	9		14	0	4		4
Total		11	23		34	1	17		18
p16 ^{INK4A} (-)	M	8	11	1.000	19	0	9	0.021*	9
	U	3	6		9	2	2		4
Total		11	17		28	2	11		13
HMLH1 (-)	M	1	11	0.010*	12	1	7	1.000	8
	U	5	2		7	0	2		2
Total		6	13		19	1	9		10

M = Methylated; U = unmethylated; - = immunonegative (LI <5%). * Significant results.

Fig. 1. Frequency of promoter region methylation (a) and expression of the COX-2, CDKN2A and HMLH1 genes (b) according to histological subtype and tumor location. M = Methylated; - = immunonegative.

quencies. In 13 cases, it was not possible to amplify the genes *cagE*, *virB11* and *flaA* due of insufficient amount of DNA. Table 8 summarizes the distribution of *H. pylori* genes according to promoter methylation status and expression of the COX-2, CDKN2A and HMLH1 genes. From this table, it can be seen that all except one of the tumors showed the COX-2 gene promoter methylated and that all cases with the HMLH1 gene promoter methylated were significantly associated to the *H. pylori vacA*s1 allele. Additionally, a significant number of nonmethylated CDKN2A cases were related to the *H. pylori flA* gene (82.4%; 29/37; $p = 0.008$).

Discussion

In this study, the data on the methylation and immunoexpression of the genes COX-2, CDKN2A and HMLH1 were analyzed for GC histological subtypes, tumor region and the *H. pylori* genotypes. To our knowledge, there are no studies to date considering all these aspects together. The analysis of the COX-2 gene was included, since its protein, a potent inducer of inflammatory response, has been assigned an important role in gastric carcinogenesis associated with the methylation process [41, 42].

Table 8. Distribution of cases regarding expression and methylation status of genes *COX-2*, *CDKN2A* and *HMLH1* according to *H. pylori* genotype

		<i>vacA</i> s1			<i>vacA</i> m1			<i>cagA</i>			<i>cagE</i>			<i>virB11</i>			<i>flaA</i>		
		+	-	p values	+	-	p values	+	-	p values	+	-	p values	+	-	p values	+	-	p values
<i>COX-2</i>	M	37	1	0.025*	30	8	0.427	21	17	0.238	15	14	0.402	15	14	0.741	15	14	0.124
	U	31	7		27	11		26	12		14	20		19	15		24	10	
	Total	68	8	-	57	19	-	47	29	-	29	34	-	34	29	-	39	24	-
	+	16	3	0.179	14	5	0.645	11	8	0.896	8	9	0.905	12	5	0.128	11	6	0.933
	-	49	3		41	11		31	21		20	21		20	21		27	14	
<i>CDKN2A</i> (p16 ^{INK4A})	M	32	2	0.285	26	8	0.790	19	15	0.336	10	16	0.312	14	12	0.987	12	14	0.008*
	U	36	6		31	11		28	14		19	18		20	17		29	8	
	Total	68	8	-	57	19	-	47	29	-	29	34	-	34	29	-	41	22	-
	+	25	5	0.076	24	6	0.662	18	12	0.901	12	14	0.771	14	12	0.855	19	7	0.542
	-	40	1		31	10		24	17		16	16		18	14		21	11	
<i>HMLH1</i>	M	25	0	0.047*	20	5	0.481	15	10	0.817	12	8	0.129	14	6	0.082	15	5	0.144
	U	43	8		37	14		32	19		17	26		20	23		24	19	
	Total	68	8	-	57	19	-	47	29	-	29	34	-	34	29	-	39	24	-
	+	38	4	1.000	34	8	0.397	24	18	0.678	16	18	0.825	17	17	0.346	24	10	0.334
	-	27	2		21	8		18	11		12	12		15	9		14	10	
Total		65	6	-	55	16	-	42	29	-	28	30	-	32	26	-	38	20	-

M = Methylated; U = unmethylated; + = immunopositive (LI >5%); - = immunonegative (LI <5%). * Significant results.

The literature shows variable frequencies of the immunoexpression of the genes *COX-2* (20.4–74%), *HMLH1* (28.6–81%) and *CDKN2A* (46.8–65%) [13, 43–50]. In this study, only a small fraction (26.8%) of GC samples showed immunohistochemical expression of *COX-2*, while high frequencies were observed for the p16^{INK4A} protein (42.3%) and *HMLH1* (59.2%). Similarly, Huang et al. [13] observed a low frequency of *COX-2* expression (20.4%) in GC, which was validated by Western blotting.

In the present study, a substantial frequency (50.0, 32.9 and 44.7%, respectively) of promoter methylation of *COX-2*, *HMLH1* and *CDKN2A* was detected with a significant negative correlation between the presence of methylation and immunoexpression, confirming the involvement of this event in the inactivation of these genes, as observed by other authors [5, 13, 17, 18, 49–53]. Additionally, gene silencing via methylation could be proven by studies in which expression of *COX-2* gene was restored by treatment in vitro with demethylating agents [11].

However, despite the data pointing to promoter hypermethylation as the main mechanism implicated in the

inactivation of the genes studied, 34.6, 31.7 and 31.0% of the cases which demonstrated negative immunoexpression of *COX-2*, p16^{INK4A} and *HMLH1*, respectively, were not methylated. Other studies also found similar results [5, 18, 52, 53]. The agreement and the representative frequency found in this and other studies indicate that these data are not due to methodological flaws and may be partially explained by the lack of transcriptional activators, as observed by Huang et al. [13] in *COX-2*, who found that inactivation of this gene occurred by both methylation and the absence of transcription activators. In the first process, in 69.0% of the cases, the expression of the enzyme DNMT1, a DNA methyltransferase involved in the methylation process, was related to the negativity of *COX-2*. In addition, alternative mechanisms of inactivation of these genes such as mutations, deletions or microRNAs can contribute to suppress their expression.

Based on studies that demonstrate that genetic and epigenetic changes in gastric adenocarcinomas differ depending on the histological subtype [30, 31], the data were analyzed according to each histological subtype separately. In this study, similar frequencies of expression

Fig. 2. Representation of tumorigenic pathways identified in this study according to histologic subtype, tumor location and immunoexpression as well as promoter methylation of the *COX-2*, *HMLH1* and *CDKN2A* genes. – = Negative immuno-staining; M = methylated tumors.

and promoter methylation of genes *COX-2*, *HMLH1* and *CDKN2A* were found between intestinal and diffuse subtype tumors, corroborating studies by Ferrasi et al. [17], Yamac et al. [45] and Yu et al. [54]. However, it is interesting to note that, although no difference was found related to immunodetection, tumors located in the noncardia region were predominantly *HMLH1* methylated. These data are in accordance with a study by Nakajima et al. [55] and Kim et al. [52]. On the other hand, in the cardia region, we found a significantly greater frequency of p16^{INK4A}-negative cases; however, this negativity was not related to promoter methylation in this gene. Thus, these data together indicate that susceptibility to methylation depends on the location of the tumor.

When tumors were evaluated according to histological subtype and location simultaneously, differences were observed. In intestinal tumors, inactivation of the *HMLH1* gene by methylation was mostly significant in tumors located in the noncardia region. In the diffuse-subtype tumors, although there was a representative number of *HMLH1*-methylated cases in tumors with noncardia location, no significant difference was observed. Studies conducted by Seo et al. [56] and Falchetti et al. [57] showed that most tumors with high microsatellite instability were located distally, belonged to the intestinal subtype and had negative expression of *HMLH1*. However, the authors did not evaluate the pattern of methylation of the promoter of *HMLH1*. Thus, it appears that the higher frequency of methylated *HMLH1* tumors found in the noncardia region is characteristic of intestinal tumors, and probably should determine a specific tumorigenic pathway. The greater number of cases with negative expression of p16^{INK4A} located in the cardia was

typical of the tumors of the intestinal subtype, although the methylation status of *CDKN2A* was not. As in our study, Abbaszadegan et al. [18] also did not find a relation between the methylation of the promoter of *CDKN2A* and the location of the tumor nor histological subtype. The higher frequency of negativity of p16^{INK4A} in cardia tumors was also observed by Kim et al. [58] in a study comparing the expression of several proteins in patients with cardia and noncardia adenocarcinoma, but the authors did not evaluate this expression considering the histological subtypes. Therefore, our data agree with Driesen et al.'s [59, 60] findings, in which they reported that the characteristics of the cardia adenocarcinomas, including clinical data and expression patterns of cytokeratin, were more closely linked to proximal than to distal adenocarcinomas, suggesting the different nature of those tumors in determining tumorigenic pathways.

Therefore, based on the lack of expression, promoter methylation of genes *CDKN2A* and *HMLH1* and the location of the tumor, it was possible to identify distinct tumorigenic pathways in intestinal and diffuse histologic subtypes. Figure 2 summarizes the possible tumorigenic pathways identified in this study according to tumor location and histological subtype. In tumors located in the cardia, the inactivation of p16^{INK4A} was a predominant finding in intestinal subtype tumors. On the other hand, in noncardia tumors, two major events were observed according to histological subtype, in diffuse tumors the inactivation of *CDKN2A* by methylation was a significant finding, while in intestinal subtype; the pathway consists in *HMLH1* inactivation by promoter methylation. Although not significant, the methylation of this gene was also frequent in the diffuse tumors, indicating that the

HMLH1 methylation is an important finding, regardless of histological subtype.

Another important factor in gastric carcinogenesis is its association with *H. pylori* infection and the great genetic variability of this bacterium [20–22]. The relationship between the alteration expression of the *COX-2*, *CDKN2A* and *HMLH1* genes and *H. pylori* infection is controversial [12, 17, 61, 62]. Additionally, there are few studies considering the *H. pylori* genotypes [45, 63]. In our study, no significant relationship was observed between positive immunoexpression of the genes *COX-2*, *CDKN2A* and *HMLH1* and the *H. pylori* pathogenicity genes studied, corroborating studies of Chang et al. [62] and Kim et al. [64].

Recent studies suggest the involvement of *H. pylori* in the inactivation of genes implicated in gastric tumorigenesis via methylation in the promoter regions of these genes [14, 18, 19]. These studies evaluated this process considering each gene independently and grouped according to the number of methylated genes as in tumors with high methylator phenotype of CpG islands of several genes involved in gastric carcinogenesis. Tahara et al. [65] found a strong association between increased number of methylated CpG islands and infection by *H. pylori*. Maekita et al. [66] quantitatively estimated the level of methylation of eight CpG islands in gastric mucosa and observed that the level of methylation of all eight regions of CpG islands analyzed were significantly higher in mucosa infected by *H. pylori* than in noninfected mucosa. Leung et al. [67] observed a decreased degree of promoter methylation of *E-cadherin* after *H. pylori* eradication.

In this study, when *H. pylori* genotypes were considered according to the methylation status in the promoter of genes *COX-2*, *CDKN2A* and *HMLH1*, strains carrying *vacA* s1 allele were found to be significantly associated with tumors with methylated *COX-2* and *HMLH1* genes. Akhtar et al. [19], in an in vitro study, showed that gastric cells exposed for two weeks to *H. pylori* underwent no change in methylation status of *COX-2*, but the authors did not evaluate the genotype of these strains. As in our study, Ferrasi et al. [17] demonstrated no significant association between methylation of the promoter of *COX-2* and *HMLH1* and infection by *cagA* (+) *H. pylori* strains, but the authors did not evaluate the involvement of the *vacA* gene of *H. pylori* in this process. Perri et al. [68] observed that after *H. pylori* eradication in patients with intestinal metaplasia, the frequency of promoter methylation of *HMLH1* did not significantly change, although the genes *CDKN2A*, *CDH1* and *COX-2* did show a significant decrease in methylation status.

Given that recent studies indicate the role of inflammation in inducing methylation through activation of DNA methyltransferases, enzymes that are responsible for methylation reactions [41, 42], it is possible that the more active vacuolizing toxin of *H. pylori* (*vacA* s1) would exacerbate the inflammatory response via activation of free radicals, strengthening the involvement of inflammation in the process of methylation. This hypothesis could be corroborated by an in vitro study performed by Katayama et al. [14], where they identified the association of *H. pylori* with the methylation process, with target being the *RUNX3* gene, one of the most methylated genes in gastric cancer. These authors showed that the presence of *H. pylori* induced nitric oxide production in macrophages, and that nitric oxide was associated with methylation of *RUNX3* in gastric cells. Additionally, some studies have suggested that *H. pylori* can induce cell proliferation, and proliferation itself has been suggested as a factor that promotes the methylation of DNA [69, 70]. Therefore, these data presented in this study add to the evidence that *H. pylori* contributes (directly or indirectly) to the methylation process and that this is dependent on the bacterial genotype. The lack of these analyses could explain conflicting results.

Other significant data in this study include the fact that the strains of *H. pylori* carrying the *flaA* gene were significantly associated with tumors with methylated *CDKN2A*. Despite the few studies correlating *H. pylori* genotype with inactivation of p16^{INK4A} by methylation of the promoter, some studies have correlated infection by *H. pylori* with methylation of *CDKN2A*, but with controversial results [15, 18, 61, 65]. It is plausible to assume that strains lacking flagella would remain in the antral region of the stomach, since they are not actively motile and thus settle in the upper regions of the stomach. As this area has a greater predisposition to methylation of the *CDKN2A* gene, the *flaA*-positive strains would then be indirectly associated with methylation. However, further studies are necessary to explain the association of this gene with *CDKN2A* promoter methylation in a larger number of cases.

In conclusion, the route of inactivation of the genes *COX-2*, *CDKN2A* and *HMLH1* was predominantly methylation in their promoters and depended on the histological subtype and tumor location. Additionally, the promoter methylation of the genes *COX-2*, *CDKN2A* and *HMLH1* seems to depend on the genotype of *H. pylori*; however, this association is independent on the histopathological parameters.

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