

GASTRIC CANCER

Promoter hypermethylation of *CDH1*, *FHIT*, *MTAP* and *PLAGL1* in gastric adenocarcinoma in individuals from Northern Brazil

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Abstract

AIM: To evaluate the methylation status of *CDH1*, *FHIT*, *MTAP* and *PLAGL1* promoters and the association of these findings with clinico-pathological characteristics.

METHODS: Methylation-specific PCR (MSP) assay was performed in 13 nonneoplastic gastric adenocarcinoma, 30 intestinal-type gastric adenocarcinoma and 35 diffuse-type gastric adenocarcinoma samples from individuals in Northern Brazil. Statistical analyses were performed using the chi-square or Fisher's exact test to assess associations between methylation status and clinico-pathological characteristics.

RESULTS: Hypermethylation frequencies of *CDH1*, *FHIT*, *MTAP* and *PLAGL1* promoter were 98.7%, 53.9%, 23.1% and 29.5%, respectively. Hypermethylation of three or four genes revealed a significant association with diffuse-type gastric cancer compared with nonneoplastic cancer. A higher hypermethylation frequency was

significantly associated with *H pylori* infection in gastric cancers, especially with diffuse-type. Cancer samples without lymph node metastasis showed a higher *FHIT* hypermethylation frequency. *MTAP* hypermethylation was associated with *H pylori* in gastric cancer samples, as well as with diffuse-type compared with intestinal-type. In diffuse-type, *MTAP* hypermethylation was associated with female gender.

CONCLUSION: Our findings show differential gene methylation in tumoral tissue, which allows us to conclude that hypermethylation is associated with gastric carcinogenesis. *MTAP* promoter hypermethylation can be characterized as a marker of diffuse-type gastric cancer, especially in women and may help in diagnosis, prognosis and therapies. The *H pylori* infectious agent was present in 44.9% of the samples. This infection may be correlated with the carcinogenic process through the gene promoter hypermethylation, especially the *MTAP* promoter in diffuse-type. A higher *H pylori* infection in diffuse-type may be due to greater genetic predisposition.

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Key words: Gastric adenocarcinoma; DNA hypermethylation; *CDH1*; *FHIT*; *MTAP*; *PLAGL1*

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INTRODUCTION

Gastric cancer (GC) is the most frequent type of cancer and is the second most common cause of cancer death in the world^[1]. In Northern Brazil, the State of Pará has a high incidence of this neoplasia and its capital, Belém, is ranked eleventh in terms of GC incidence rates among all cities in the world with cancer records^[2]. Food may be related to the high incidence of this neoplasia in Pará,

especially the high consumption of salt-conserved food, reduced use of refrigerators and little consumption of fresh fruit and vegetables^[3].

The most successful and widely used classification of GC is that of Laurén^[4], which is divided in two main types: diffuse and intestinal. Intestinal-type GC is usually called "epidemic", is more frequent, more related to environmental factors and associated with precancerous lesions, such as chronic gastritis, gastric atrophy, intestinal metaplasia and dysplasia. Diffuse-type have a poorer prognosis, are not associated with precancerous lesions and show invasive growth patterns^[5].

Epigenetic events play a significant role in cancer development and progression. DNA methylation is the most studied epigenetic alteration, occurring through the addition of a methyl radical to the cytosine base adjacent to guanine. When DNA is methylated in the gene promoter region, genes are inactivated and silenced^[6]. In cancer, epigenetic silencing leads to the aberrant silencing of normal tumor-suppressor functions.

Four genes were chosen for assessment of their functions and/or roles in gastric carcinogenesis: *CDH1*, *FHIT*, *MTAP* and *PLAGL1*.

E-cadherin, *CDH1* product, is a homophilic cell adhesion protein. It has been proposed that loss of E-cadherin-mediated cell-cell adhesion is a prerequisite for tumor cell invasion and metastasis^[7]. E-cadherin also has a possible role in modulating intracellular signaling, thus promoting tumor growth^[8].

FHIT gene was identified in the most common fragile site, FRA3B^[9]. *FHIT* is highly expressed in all normal epithelial tissues. *FHIT* is inactivated in about 60% of human tumors (ranging from 20% to 100%). Thus, *FHIT* is the most commonly altered gene in human cancer and in precancerous conditions^[10]. Despite strong evidence of a *FHIT* tumor suppressor function, the specific signal pathways and mechanisms involved are still unknown.

MTAP is expressed ubiquitously in all normal cells^[11]. Reduced *MTAP* expression may lead to activation of the polyamine biosynthetic enzyme ornithine decarboxylase (ODC)^[12]. In gastric tissue, the ODC activity is elevated in premalignant lesions, which may be useful as biochemical markers of neoplastic proliferation^[13]. Many malignant cells lack *MTAP* activity because of chromosomal loss or epigenetic regulation^[14]. To our knowledge, the methylation status of *MTAP* has not been reported in GC.

PLAGL1 encodes a zinc-finger protein that regulate induction of apoptosis and G1 arrest^[15] and contains transactivation and repressor activities^[16]. Besides *TP53*, the identification of *PLAGL1* provides the first example of a gene which combines concomitant induction of programmed cell death and cell arrest^[17].

Identification of tumor specific epigenetic alterations can be used as a molecular marker of malignancy, which can lead to better diagnosis, prognosis and therapy. In this study, we evaluated the methylation status of *CDH1*, *FHIT*, *MTAP* and *PLAGL1* promoters and hypermethylation frequency as well as their association with clinico-pathological characteristics.

MATERIALS AND METHODS

Samples

The study included 78 samples of gastric tissue. Among them, 13 were nonneoplastic gastric mucosae, including 5 samples from GC patients (distant location of primary tumor) and 65 sporadic GC samples.

Eight normal mucosa samples (samples 1-8) were obtained from patients undergoing upper endoscopy to check for gastritis. All these samples were obtained from the antrum of stomach biopsies. The other nonneoplastic (samples 9-13) and GC samples were surgically obtained in Pará State João de Barros Barreto University Hospital (HUJBB). Patients had never been submitted to chemotherapy or radiotherapy prior to surgery, or had any other diagnosed cancer. All patients signed an informed consent with the approval of the ethics committee of HUJBB and of the Universidade Federal de São Paulo (UNIFESP). All 65 GC samples were classified according to Laurén^[4]: 30 were intestinal (samples 14-43) and 35 were diffuse type (samples 44-78). Tumors were staged using standard criteria by TNM staging^[18]. A rapid urease test was performed on all samples to detect *H pylori*.

Methylation specific PCR (MSP)

Genomic DNA (2 µg) was modified by bisulfite treatment, converting unmethylated cytosines to uracils and leaving methylated cytosines unchanged. MSP was performed on treated DNA as previously described^[19]. Specific primers for MSP (Table 1), located within CpG islands and previously described as associated with their genes expression^[20-23], were designed with the assistance of Methprimer software^[24].

PCR reaction was carried out in a volume of 25 µL with 200 µmol/L of dNTPs, 200 µmol/L of MgCl₂, 100 ng of DNA, 200 pmol/L of primers and 1 unit of AmpliTaq GOLD (Applied Biosystems, Foster City, CA). Initial denaturing was carried out for 3 min at 94°C, 35 cycles at 94°C for 30 s, at different temperatures with the primers for 45 s (Table 1) and 72°C for 30 s. This was followed by a final extension for 5 min at 72°C. PCR products were separated by 3% agarose gel containing 0.0004% ethidium bromide and visualized under UV illumination (Figure 1).

DNA from peripheral lymphocytes of two healthy individuals and water were used as negative controls. MSP results were scored when there was a clearly visible band on the electrophoresis gel with the methylated and unmethylated primers^[19]. Hypermethylation was considered present with methylated sequences for *CDH1* and *FHIT* promoter or only methylated sequences for *MTAP* and *PLAGL1* promoter.

Statistical analysis

Statistical analyses were performed using the χ^2 test or Fisher's exact test to assess associations between the methylation status and frequency, and clinico-pathological characteristics. *P* values less than 0.05 were considered significant.

Table 1 Primer sequences (5'-3') for MSP

Gene	Sense	Antisense	Product size	TM
CDH1	M-ATTCTGAATTTAGTGGAAATTAGAATC	M-CCCAAAACGAAACTAACGAC	125 bp	53°C
	U-GGATTTGAATTTAGTGGAAATTAGAATT	U-CTCCCCAAAACAAAATAACAAC	130 bp	
FHIT	M-TTTTCGTTTTGTTTTAGATAAGC	M-AAAAATATACCCACTAAAATAACCGC	157 bp	52°C
	U-TGGTTTTTGTTTTTGTTTTAGATAAGT	U-AAAAATATACCCACTAAAATAACCACC	159 bp	
MTAP	M-TGTTTTTAGGAATTAAGGAAATAC	M-AACTACAAAATCTAACCCGACGAC	199 bp	52°C
	U-TTTTAGGAATTAAGGAAATATGT	U-CAACTACAAAATCTAACCCAACAAC	197 bp	
PLAGL1	M-GTTTATTTTGGCGGAGATTTC	M-ACTAAACGACACCCACACGTC	147 bp	51°C
	U-GGTTTATTTTGGTGGAGATTTTG	U-AAAAATAAACACACCCACACAT	152 bp	

M: methylated sequences; U: unmethylated sequences; TM: melting temperature.

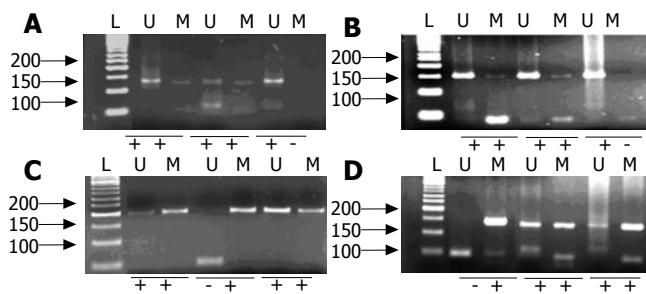


Figure 1 Examples of gel electrophoresis using CDH1 (A), FHIT (B), MTAP (C) and PLAGL1 (D) MSP primers. L: size marker; M: methylated; U: unmethylated.

RESULTS

Clinico-pathological characteristics

Of the 78 patients, 54 were male and 24 were female, and their mean age was 56 ± 12.31 years (range 20-76). According to Laurén's classification, 35 were diffuse and 30 were intestinal types. All GC samples were in advanced stage. Table 2 shows cases with their clinico-pathological characteristics. There was a significant association between diffuse-type and *H. pylori* infection in our sample ($P = 0.0142$).

DNA hypermethylation in gastric tissue

All samples analyzed showed at least one hypermethylated gene promoter. The hypermethylation frequency of CDH1, FHIT, MTAP and PLAGL1 promoter were 98.7%, 53.9%, 23.1% and 29.5%, respectively (Figure 2). Only one sample (sample 4), of a 20 years old patient, did not show CDH1 hypermethylation. The methylation number and frequency of CDH1, FHIT, MTAP and PLAGL1 in nonneoplastic and neoplastic samples are displayed in Table 3. Statistical analysis showed a tendency for increased PLAGL1 hypermethylation in GC samples ($P = 0.0937$) and in diffuse-type ($P = 0.0807$) compared with nonneoplastic samples.

Table 4 shows the number of hypermethylated genes (1-2 or 3-4) in nonneoplastic tissue and GC samples. A tendency for hypermethylation of three or four genes in cancer samples compared to nonneoplastic samples was observed ($P = 0.0959$). Three or four hypermethylated genes were significantly more frequently detected in diffuse-type than in nonneoplastic mucosa ($P = 0.0396$). Neither of two peripheral lymphocyte samples showed hypermethylation (Figure 2).

Association between DNA hypermethylation and clinico-pathological characteristics

We analyzed whether DNA hypermethylation was associated with clinico-pathological characteristics, and detected a tendency for hypermethylation of three or four genes in tissue samples with *H. pylori* infection ($P = 0.0522$). GC samples showed a significant association between higher hypermethylation frequency and *H. pylori* infection ($P = 0.0428$). This association was also observed in diffuse-type samples ($P = 0.0033$). In diffuse-type, hypermethylation of three or four genes tended to be higher in female patients than in male ($P = 0.0529$) and in tumors located in the noncardia stomach region ($P = 0.0805$). MTAP hypermethylation was associated with *H. pylori* infection in gastric samples ($P = 0.0465$). MTAP hypermethylation was also associated *H. pylori* infection in GC samples ($P = 0.0175$), as well as with diffuse-type compared with intestinal-type ($P = 0.0249$). GC samples without lymph node metastasis were associated with FHIT hypermethylation ($P = 0.0018$). An association was observed between absence of lymph node metastasis and FHIT hypermethylation in diffuse-type GC ($P = 0.0408$), as well as between female patients and MTAP hypermethylation ($P = 0.0310$). Absence of lymph node metastasis was associated with FHIT hypermethylation in intestinal-type GC ($P = 0.0104$), as well as a tendency for PLAGL1 hypermethylation and larger tumor extension (T3 and T4) ($P = 0.0637$).

DISCUSSION

DNA hypermethylation in gastric tissue

DNA hypermethylation of CpG islands was detected in all gastric samples, even in nonneoplastic mucosa, which can contain precursor cells for cancer and/or precancerous lesions^[25].

The stomach is the organ that presents more methylated CpG island in nonneoplastic cells, along with age-related methylation that reflects increased CpG island methylation frequency in GC^[26].

In our study, higher hypermethylation frequency was significantly associated with diffuse-type GC compared with nonneoplastic tissue. These findings confirmed those from Leung *et al*^[27], which evaluated the methylation status of 5 genes in 28 samples of GC and their corresponding nonneoplasms.

We detected that 100% of GC and 92.3% of nonneo-



Figure 2 Methylation status of *CDH1*, *FHIT*, *MTAP* and *PLAGL1* promoter in the samples studied. White boxes represent samples that showed only unmethylated sequences, gray boxes represent samples that showed unmethylated and methylated sequences, and black boxes represent samples that showed only methylated sequences. 1-13 samples: nonneoplastic; 14-43 samples: intestinal-type GC; 44-78 samples: diffuse-type GC.

plastic samples were hypermethylated in *CDH1* promoter (Table 3), frequencies higher than those in the literature. *CDH1* methylation status was not significantly different between GC and nonneoplastic samples. This is the first study that has evaluated *CDH1* methylation frequency in

Variable (n)	Samples			P
	Nonneoplastic n (%)	Intestinal n (%)	Diffuse n (%)	
Gender				
Male (54)	9 (16.67)	20 (37.03)	25 (46.3)	0.9176
Female (24)	4 (16.66)	10 (41.67)	10 (41.67)	
Age (yr)				
≤ 50 (23)	7 (30.43)	7 (30.43)	9 (39.14)	0.1056
> 50 (55)	6 (10.91)	23 (41.82)	26 (47.27)	
Smoking status				
Smoker (27)	1 (3.7)	13 (48.15)	13 (48.15)	0.0717
No smoker (51)	12 (23.53)	17 (33.33)	22 (43.14)	
<i>H. pylori</i>				
Present (36)	6 (16.67)	8 (22.22)	22 (61.11)	0.0142 ^a
Absent (42)	7 (16.67)	22 (52.38)	13 (30.95)	
Location				
Cardia (25)	-	11 (44)	14 (56)	0.783
Noncardia (40)	-	19 (47.5)	21 (52.5)	
T stage				
T1, T2 (15)	-	7 (46.67)	8 (53.33)	0.9638
T3, T4 (50)	-	23 (46)	27 (54)	
Lymph node metastasis				
Present (51)	-	23 (45.1)	28 (54.9)	0.7445
Absent (14)	-	7 (50)	7 (50)	
Distant metastasis				
Present (6)	-	4 (66.67)	2 (33.33)	0.4649
Absent (55)	-	23 (41.82)	32 (58.18)	
Unknown (4)	-	3 (75%)	1 (25%)	

^aP < 0.05.

gastric tissue samples from a Brazilian population.

The highest *CDH1* hypermethylation frequency described in the literature was 90% in advanced GC samples^[28], as well as in nonneoplastic tissue^[29]. However, Zazula *et al.*^[29] did not find significant differences between methylation patterns in 84 GC samples and their nonneoplastic controls.

Waki *et al.*^[30] evaluated *CDH1* methylation status in nonneoplastic gastric mucosa samples at autopsies. The authors did not see this gene methylation in nonneoplastic gastric cells from people who were 22 years and younger. However, *CDH1* methylation was found in 86% of nonneoplastic gastric epithelia of people who were over 45 years old. Our findings showed only one nonneoplastic sample without *CDH1* methylation from a 20 years old patient. Thus, our findings confirm those from Waki's study. The incidence of GC rises with age, pointing to an association between age-related methylation and GC development^[30,31]. *CDH1* hypermethylation in nonneoplastic gastric epithelia has also been frequently associated with *H. pylori* infection^[31-33].

In our sample, higher *CDH1* hypermethylation frequency may result from the association of age, *H. pylori* infection, advanced tumor and epidemiological factors, such as genetics constitution and diet.

In our study, *FHIT* hypermethylation frequency was 52.3% in GC samples (Table 2). This value is lower than those from two GC studies in the literature^[34,35]. On the other hand, the *FHIT* hypermethylation frequency was 61.5% in nonneoplastic samples, which is higher than in other studies. Roa *et al.*^[34] reported that *FHIT* was methylated in 62% of 47 GC samples. Schildhaus *et al.*^[35]

Table 3 Methylation number and frequency in gastric tissue samples, *n* (%)

Samples	Methylation status								
	CDH1		FHIT		MTAP		P	PLAGL1	
	M/U	U	M/U	U	M/U	M		M/U	M
Normal tissue (13)	12 (92.31)	1 (7.69)	8 (61.54)	5 (38.46)	11 (84.62)	2 (15.38)		12 (92.31)	1 (7.69)
GC (65)	65 (100)	0 (0)	34 (52.31)	31 (47.69)	49 (75.38)	16 (24.62)		43 (66.15)	22 (33.85)
Diffuse-type GC (35)	35 (100)	0 (0)	17 (48.57)	18 (56.25)	22 (62.86)	13 (37.14)	0.0249 ^a	23 (65.71)	12 (34.29)
Intestinal-type GC (30)	30 (100)	0 (0)	17 (56.67)	13 (43.33)	27 (90)	3 (10)		20 (66.6)	10 (33.33)

U: unmethylated; M/U: methylated and unmethylated; M: methylated. ^a*P* < 0.05 vs Intestinal-type GC.

Table 4 Number of hypermethylated genes in gastric tissue samples

Samples (<i>n</i>)	Number of hypermethylated genes		P value
	1 or 2	3 or 4	
Nonneoplastic gastric tissue (13)	12 (92.31%)	1 (7.69%)	0.0396 ^b
Gastric cancer (65)	44 (67.69%)	21 (32.31%)	
Diffuse-type GC (35)	21 (60%)	14 (40%)	
Intestinal-type GC (30)	23 (76.67%)	7 (23.33%)	

N: number of cases; ^b*P* < 0.05.

observed *FHIT* hypermethylation in all 6 advanced proximal GC and in 40% (2 of 5 samples) of normal gastric tissue, suggesting the *FHIT* hypermethylation is a precursor for GC.

In our study, *FHIT* promoter hypermethylation was detected in 80% (8/10) of nonneoplastic samples with gastritis or from GC patients, whereas none of the 3 nonneoplastic samples from subjects without gastritis and without GC were methylated (data not shown). Our findings suggest that *FHIT* hypermethylation may be associated with the start of gastric carcinogenesis, as well as in other organs^[36].

Unmethylated sequences together with methylated sequences in *CDH1* and *FHIT* promoter may be a result of allelic heterozygosity, clonal heterogeneity or contamination by inflammatory cells or stromal.

The methylation status of the *MTAP* promoter has never been analyzed in gastric tissue samples, while this promoter has been described in other neoplasias. However, methylation in nonneoplastic tissues has never been described^[22,37-40]. Similarly, the *MTAP* promoter hypermethylation has never been analyzed by MSP. We detected methylated sequences for *MTAP* promoter in all samples, including nonneoplastic and lymphocytes samples. Thus, the difference found between *MTAP* methylation status in our work and those from the literature may be due to different CpG islands analyzed, as well as methodological differences.

We considered the *MTAP* promoter hypermethylation when only methylated sequences were observed, comparing with the methylation status in peripheral blood lymphocytes. The *MTAP* promoter hypermethylation was in 24.6% of GC and 15.4% of nonneoplastic samples (Table 3).

In all samples methylated sequences of *PLAGL1* was also observed, including nonneoplastic and lymphocyte

samples. The literature shows only one study that evaluated *PLAGL1* methylation status in gastric samples^[23], where MSP was performed for *PLAGL1* in 5 GC cell lines, 10 primary GC and one nonneoplastic sample. The authors observed methylation sequences in all neoplastic samples, while only one sample showed methylated and unmethylated sequences. The nonneoplastic sample presented only unmethylated sequences.

Our findings did not confirm those of Yamashita *et al*^[23] for the nonneoplastic sample. However, *PLAGL1* has been described as a maternal imprinted gene and hypermethylation leads to transcriptional silencing, as reported in other neoplasias^[41]. Considered an imprinted gene, we found *PLAGL1* hypermethylation in 33.9% of GC and 7.7% of nonneoplastic gastric samples (Table 3).

Association between DNA hypermethylation and clinicopathological characteristics

In our study, a higher hypermethylation frequency of CpG islands was found in *H pylori* infected samples, confirming findings from studies by El-Omar *et al*^[42,43] and Hmadcha *et al*^[44]. El-Omar *et al*^[42,43] reported that interleukin-1 β polymorphism led to an upregulation of interleukin-1 β with *H pylori* infection and was associated with increased risk of GC. Furthermore, Hmadcha *et al*^[44] described that interleukin-1 β might induce gene methylation through the production of nitric oxide and the subsequent activation of DNA methyltransferase. It is possible that *H pylori* induces methylation through the production of interleukin-1 β and hence the downstream activation of nitric oxide and DNA methyltransferase^[31].

A higher hypermethylation frequency was associated with *H pylori* in diffuse-type, which can be related to its higher incidence in our samples of diffuse-type GC. *H pylori* gastritis is the only known precursor for diffuse-type GC^[45].

In our study, a higher *MTAP* hypermethylation frequency was also associated with *H pylori* infection in gastric samples, especially in diffuse-type. *MTAP* hypermethylation in diffuse-type that is associated with *H pylori* infection may be due to a genetic predisposition. An association with female patients was also observed, which may be related to diffuse-type incidence, that is more frequent in female patients^[46].

In our study, GC samples without lymph node metastasis were associated with *FHIT* hypermethylation. However, because of the small number of samples without lymph node metastasis in our study, further studies are

necessary to confirm this association.

This is the first study that has evaluated the methylation status of *CDH1*, *FHIT*, *MTAP* and *PLAGL1* promoters and their hypermethylation frequencies in gastric tissue samples in a population from Northern Brazil. The methylation status of *MTAP* promoter has never been investigated in gastric samples. Our findings show that hypermethylation is associated with gastric carcinogenesis. The *H pylori* infectious agent was present in 44.9% of samples and this infection may be a part of the carcinogenesis process through the gene promoter hypermethylation; especially the *MTAP* promoter in diffuse-type. Increased *H pylori* infection in diffuse-type may also be due to higher genetic predisposition.

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