

DNA mismatch repair gene methylation in gastric cancer in individuals from northern Brazil

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ABSTRACT: Gastric cancer is one of the most common malignancies. DNA methylation is implicated in DNA mismatch repair genes deficiency. In the present study, we evaluated the methylation status of *MLH1*, *MSH2*, *MSH6* and *PMS2* in 20 diffuse- and 26 intestinal-type gastric cancer samples and 20 normal gastric mucosal of gastric cancer patients from Northern Brazil. We found that none of the nonneoplastic samples showed methylation of any gene promoter and 50% of gastric cancer samples showed at least one methylated gene promoter. Methylation frequencies of *MLH1*, *MSH2*, *MSH6* and *PMS2* promoter were 21.74%, 17.39%, 0% and 28.26% respectively in gastric cancer samples. *MLH1* and *PMS2* methylation were associated with neoplastic samples compared to nonneoplastic ones. *PMS2* methylation was associated with diffuse- and intestinal-type cancer compared with normal controls. Intestinal-type cancer showed significant association with *MLH1* methylation. Diffuse-type cancer was significantly associated with *MSH2* methylation. Our findings show differential gene methylation in tumoral tissue, which allows us to conclude that methylation is associated with gastric carcinogenesis. Methylation of mismatch repair genes was associated with gastric carcinogenesis and may be a helpful tool for diagnosis, prognosis and therapies. However, *MSH6* does not seem to be regulated by methylation in our samples.

Introduction

Gastric cancer is the fourth most common malignancy and the second most common cause of cancer death worldwide (Parkin *et al.*, 2002). In Northern Bra-

zil, the state of Pará has a high incidence rate of this neoplasia and its capital, Belém, was ranked eleventh in number of gastric cancers per inhabitant among all cities worldwide with cancer records (INCA, 2005).

DNA methylation is the most studied epigenetic alteration, occurring through the addition of a methyl radical to the cytosine base adjacent to guanine (Bird, 2002). In cancer, DNA methylation of the promoter region of a normal tumor-suppressor gene leads to the aberrant silencing of its functions.

Genetic pathways involved in gastric cancer devel-

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opment are not clearly understood, although several genetic and epigenetic alterations have been implicated in DNA mismatch repair (MMR) genes deficiency, resulting in microsatellite instability (MSI) phenotype. The human MMR system repairs DNA replication errors or physicochemical damage. Microsatellite regions are susceptible to mutation due to slippage of DNA polymerase during DNA replication. Failure to excise these errors may lead to frameshift mutations in many target genes (Bacani *et al.*, 2005). *MLH1*, *MSH2*, *MSH6* and *PMS2* are major MMR genes implicated in genetic stability (Gologan and Sepulveda, 2005).

The MSI phenotype that occurs in tumors has been demonstrated to be due to deficiency of one of the main DNA MMR proteins (*MLH1* and *MSH2*) (Boland, 2000). Hypermethylation of *MLH1* promoter CpG island was found responsible for the development of most gastric cancers exhibiting MSI (Fleisher *et al.*, 1999). *MLH1* may form heterodimers with *PMS2* acting as molecular matchmakers, recruiting other proteins of MMR complex (Kunkel and Erie, 2005). However, *PMS2* methylation status has never been studied in gastric carcinogenesis. *MSH2* has also been implicated in gastric carcinogenesis (Kim *et al.*, 2001; Kitajima *et al.*, 2003). *MSH2* may form heterodimers with *MSH6* repairing single base-base and 1–2 base insertion-deletion mismatches (Kunkel and Erie, 2005). Few studies assessed *MSH2* methylation frequency (Wu *et al.*, 2000; Fang *et al.*, 2004), but *MSH6* methylation status has never been studied in gastric carcinogenesis.

The knowledge of the methylation status of DNA MMR can help understanding a major gastric carcinogenesis pathway. Tumor-specific epigenetic alterations can be used as molecular markers of malignancy, and thus provide more specific therapy. In the present study, we evaluated the methylation status of *MLH1*, *MSH2*, *MSH6* and *PMS2* promoters, as well as their potential association with clinical and pathological characteristics, such as gender, age, histopathology, tumor extension and presence of lymph nodes or distant metastasis.

Materials and Methods

Samples

The study included 66 gastric tissue samples. Of them, 20 were nonneoplastic gastric mucosa of gastric cancer patients (at a distance from the primary tumor) and 46 sporadic gastric cancer samples. Gastric samples were surgically obtained at João de Barros Barreto University Hospital (HUIBB) in the state of Pará. Patients had never been submitted to either chemotherapy or radiotherapy prior to surgery, nor had any other diagnosed cancer. All patients signed an informed consent approved by Research Ethics Committees of HUIBB and Ribeirão Preto Medical School Clinics Hospital. All 46 gastric cancer samples were classified according to Laurén (1965): 20 were diffuse-type and 26 were intestinal-type. Tumors were

TABLE 1.

Primer sequences (5'-3') for MSP

Gene	Sense	Antisense	Product size
<i>MLH1</i>	M-TTACGGGTAAGTCGTTTTGAC	M-CTATACCTAATCTATCGCCGC	154 pb
	U-TATGGGTAAGTTGTTTTGATGT	U-CCTATACCTAATCTATCACCACC	154 pb
<i>MSH2</i>	M-AGTTAAAGTTATTAGCGTGCG	M-CTAATTAATAAAAAAATACGCGAC	199 pb
	U-AGTTAAAGTTATTAGTGTGTGTGG	U-TCCTAATTAATAAAAAAATACACAAC	202 pb
<i>MSH6</i>	M-GGTCGTAATTAATTTGGGGTC	M-CCAATAACCAATCAACAAACG	252 pb
	U-GTTGTAATTAATTTGGGGTTGG	U-CCAATAACCAATCAACAAACAC	251 pb
<i>PMS2</i>	M-GGTCGGTCGGTATAGATGTC	M-CAAATAAAACCCTATCACGAA	252 pb
	U-TTGGTTGGTATAGATGTTGG	U-CCAATAAAACCCTATCACAA	251 pb

M: methylated sequence; U: unmethylated sequences.

staged using TNM staging criteria (Sobin and Wittekind, 2002).

Methylation-specific PCR (MSP)

Genomic DNA was extracted from gastric samples using phenol-chloroform extraction. 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 (Herman *et al.*, 1996). Specific primers for MSP (Table 1), located within CpG islands in gene promoter, were designed using the Methprimer software (Li and Dahiya, 2002).

PCR was carried out in a volume of 50 µl with 200 µmol/L of dNTPs, 200 µmol/L of MgCl₂, 50 ng of DNA, 200 pmol/L of primers and 1 unit of AmpliTaq GOLD (Applied Biosystems, Foster City, CA, USA). After initial denaturing for 2 min at 94°C, 35 cycles at 94°C for 40 s, 50–60°C for 1 min and 72°C for 40 s were carried out, followed by a final extension at 72°C for 5 min. PCR products were separated and visualized using 8% polyacrylamide gel stained with 10% silver nitrate (Fig. 1). Water was used as negative control. MSP results were scored when there was a clearly visible band on electrophoresis gel with methylated and unmethylated primers (Herman *et al.*, 1996).

Sequencing of a methylated and then an unmethylated PCR product of *MLH1* was used as a control for DNA bisulfite treatment. For sequencing analysis, ABI PRISM Big Dye Terminator Cycle Sequencing Kit (Perkin Elmer, Alameda, CA, USA) was used (Fig. 1).

Statistical analysis

Statistical analyses were performed using Fisher's exact test to assess associations between methylation status and frequency, and clinical and pathological characteristics, such as gender, age, histopathology, tumor extension and presence of lymph nodes or distant metastasis. A 5% significance level was set.

Results

Of 66 patients studied, 47 were males and 19 were females, and mean age was 59 ± 9.8775 years (range 27–76). According to Laurén's classification, 20 were diffuse-type and 26 were intestinal-type. All gastric cancer samples were in advanced stage.

Sequencing of a methylated and an unmethylated PCR product of *MLH1*, used as controls for DNA bisulfite treatment, showed methylated and unmethylated (converted) cytosines, respectively, at CpG-sites when compared to non-treated sequence of DNA. At non-CpG sites, all cytosines were converted to thymine (Fig. 1). This excluded the possibility that successful amplification with methylated primers could be attributable to incomplete bisulfite conversion.

Table 2 shows clinical and pathological characteristics and methylation status of *MLH1*, *MSH2*, *MSH6* and *PMS2* promoters of the samples studied. None of the nonneoplastic samples showed methylation of any gene promoter. Of all gastric cancer samples, 50% showed at least one methylated gene promoter. Methy-

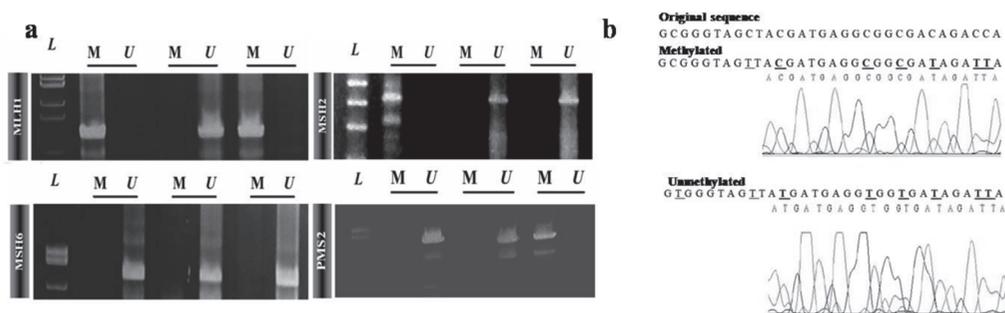


FIGURE 1. a) Examples of gel electrophoresis using *MLH1*, *MSH2*, *MSH6* and *PMS2* MSP primers. L: size marker; M: methylated; U: unmethylated. b) Methylated and unmethylated samples of *MLH1* used as positive control for DNA bisulfite treatment, when compared to untreated sequence.

lation frequencies of *MLH1*, *MSH2*, *MSH6* and *PMS2* promoters were 21.74%, 17.39%, 0% and 28.26% respectively in gastric cancer samples (Table 3).

MLH1 and *PMS2* methylation were associated with gastric cancer samples compared to nonneoplastic samples ($p=0.0256$ and $p=0.0065$, respectively). A tendency for *MSH2* promoter methylation in gastric cancer compared to normal controls was seen ($p=0.0531$) (Table 3).

Higher *PMS2* methylation frequency was associated with diffuse- ($p=0.0471$) and intestinal-type gastric cancer ($p=0.0065$) compared with normal controls. Intestinal-type gastric cancer showed a significant association with higher *MLH1* methylation frequency ($p=0.0287$). In diffuse-type gastric cancer samples, it was detected an association between this cancer type and higher *MSH2* methylation frequency ($p=0.0471$) (Table 3).

TABLE 2.

Clinical and pathological characteristics and methylation status of *MLH1*, *MSH2*, *MSH6* and *PMS2* promoters of the studied samples.

Sample	gender	age	histopathology	TNM	<i>MLH1</i>	<i>MSH2</i>	<i>MSH6</i>	<i>PMS2</i>
1	female	49	nonneoplastic	-				
2	male	59	nonneoplastic	-				
3	female	73	nonneoplastic	-				
4	male	71	nonneoplastic	-				
5	male	55	nonneoplastic	-				
6	male	66	nonneopl	-				
7	female	64	nonneoplastic	-				
8	female	72	nonneoplastic	-				
9	male	62	nonneoplastic	-				
10	male	56	nonneoplastic	-				
11	male	45	nonneoplastic	-				
12	female	58	nonneoplastic	-				
13	male	61	nonneoplastic	-				
14	female	64	nonneoplastic	-				
15	male	71	nonneoplastic	-				
16	male	56	nonneoplastic	-				
17	male	66	nonneoplastic	-				
18	male	62	nonneoplastic	-				
19	female	72	nonneoplastic	-				
20	male	59	nonneoplastic	-				
21	female	52	diffuse	T2N1MX				
22	male	57	diffuse	T4N1				
23	male	42	diffuse	T3N1				
24	male	45	diffuse	T3N0				
25	female	53	diffuse	T3N0				
26	male	76	diffuse	T3N1	■			■
27	female	41	diffuse	T4N1				
28	male	46	diffuse	T2N0	■			
29	male	45	diffuse	T3N0		■		
30	male	62	diffuse	T4N1				
31	female	48	diffuse	T2N0	■	■		■
32	male	60	diffuse	T2N0				
33	male	27	diffuse	T3N1	■			
34	male	76	diffuse	T3N1				■
35	male	61	diffuse	T3N1		■		■
36	male	62	diffuse	T2N0		■		
37	male	55	diffuse	T3N1				
38	male	54	diffuse	T3N0				
39	female	58	diffuse	T3N				■
40	male	45	diffuse	T3N0		■		
41	female	72	intestinal	T3N2	■			
42	male	47	intestinal	T3N2				■
43	male	42	intestinal	T3N0				
44	male	57	intestinal	T2N2MX				
45	male	60	intestinal	T3N1MX				■
46	female	61	intestinal	T3N1	■	■		■
47	male	49	intestinal	T2N0				
48	male	62	intestinal	T3N1	■			■
49	male	70	intestinal	T3N1MX				
50	female	49	intestinal	T2N0	■			
51	male	71	intestinal	T3N1				
52	female	73	intestinal	T3N1				
53	male	66	intestinal	T2N0				
54	male	59	intestinal	T2N1				
55	female	72	intestinal	T3N0	■			
56	female	64	intestinal	T3N1				■
57	male	56	intestinal	T4N1				■
58	male	54	intestinal	T3N0	■			
59	male	63	intestinal	T3N0				
60	male	60	intestinal	T3N1				
61	male	59	intestinal	T2N1				
62	female	63	intestinal	T3N1MX			■	
63	male	65	intestinal	T4N1				
64	male	60	intestinal	T3N1				
65	male	71	intestinal	T3N1		■		
66	male	59	intestinal	T2N1		■		

White boxes: samples that showed unmethylated sequences; black boxes: samples that showed methylated sequences. T: size or direct extent of the primary tumor; N: degree of spread to regional lymph nodes; M: presence of metastasis.

The statistical analysis showed an association between methylation of *MLH1*, *MSH2* or *PMS2* promoter in gastric cancer ($p < 0.0001$), and both diffuse- ($p = 0.0004$) and intestinal-type ($p = 0.0001$) compared to nonneoplastic samples.

We analyzed whether DNA methylation was associated with clinical and pathological characteristics, and detected a tendency for *PMS2* methylation in intestinal-type gastric cancer with larger tumor extension (T3 and T4) ($p = 0.0622$).

Discussion

There are at least two distinct genetic instabilities in gastric tumorigenesis. One is microsatellite instability (or MSI pathway) and the other is chromosomal instability (or suppressor pathway), including tumors with low-frequency MSI (MSI-L) as well as microsatellite stable (MSS) with accumulation of loss of tumor suppressor genes, such as *TP53*, *RB*, *APC*, *MCC* and *DCC*. The MSI pathway consisting of a gastric cancer subset with high-frequency MSI (MSI-H) and the defective repair of mismatched bases induces increased mutation rate at the nucleotide level, and the consequent widespread MSI (Martins *et al.*, 1999; Wu *et al.*, 2000; Fang *et al.*, 2001). In the present study, 50% of gastric cancer samples showed at least one methylated gene promoter and methylation of *MLH1*, *MSH2* or *PMS2* promoter was significantly associated with this cancer, suggesting that this tumor develops by a MSI pathway.

In our study, we found that none of the samples with *MLH1*, *MSH2* or *PMS2* methylation showed unmethylation sequences using MS-PCR assay. MSP is a sensitive method able to detect at least 0.1% of methylated or unmethylated genes. Our results suggest that methylation of these genes is a common event in a subgroup of gastric cancers and may be related to early carcinogenesis.

We evaluated a CpG island of *MLH1* promoter previously described as associated with its gene expression and MSI status (Herman *et al.*, 1998). In the present study, *MLH1* methylation was found in 21.74% of gastric cancer samples, which was not significantly different from previously described frequencies (13–41%) (Leung *et al.*, 1999; Toyota *et al.*, 1999; Fang *et al.*, 2003; An *et al.*, 2005; Truninger *et al.*, 2005). Hypermethylation of the *MLH1* promoter CpG island was found responsible for the development of the majority of gastric neoplasias exhibiting MSI (Fleisher *et al.*, 1999) and was previously associated with high-degree MSI (MSI-H) in gastric cancer (Leung *et al.*, 1999; Toyota *et al.*, 1999; Wu *et al.*, 2000; Fang *et al.*, 2003; An *et al.*, 2005).

We also found an association between *MLH1* methylation and gastric carcinogenesis, especially in intestinal-type samples. Wu *et al.* (2000) described that MSI-H is significantly more common in antral location, intestinal subtype and *H. pylori* seropositivity samples. Thus, our data corroborate Wu *et al.*'s (2000) findings concerning the association between *MLH1* methylation and intestinal-type gastric cancer. Kitajima

TABLE 3.

Methylation number and frequency in gastric tissue samples, n (%), and p-values for comparison of gastric cancer with nonneoplastic samples.

Samples	Methylation status								p
	<i>MLH1</i>		p	<i>MSH2</i>		p	<i>PMS2</i>		
	M	U		M	U		M	U	
Normal (20)	0 (0)	20 (100)		0 (0)	20 (100)		0 (0)	20 (100)	
Cancer (46)	10 (21.74)	36 (78.26)	0,0256 ^a	8 (17.39)	38 (82.61)	0,0531	13 (28.26)	33 (71.74)	0,0065 ^a
Diffuse (20)	4 (20)	16 (80)	0,1060	5 (25)	15 (75)	0,0471 ^a	5 (25)	15 (75)	0,0471 ^a
Intestinal (26)	6 (23.08)	20 (76.92)	0,0287 ^a	3 (11.54)	23 (88.46)	0,2464	8 (30.77)	18 (69.23)	0,0065 ^a

M: methylated; U: unmethylated; ^a $p < 0.05$.

et al. (2003) also described that loss of MHL1 expression is associated with differentiated gastric cancer samples, which is roughly consistent with intestinal-type cancer according to Laurén's classification.

To the best of our knowledge, only one study evaluated *PMS2* methylation status, but no correlation was found between the lack of *PMS2* expression and this gene methylation (0% of methylation) in colorectal cancer (Truninger *et al.*, 2005). The present study it is the first to evaluate *PMS2* methylation status in gastric cancer. We found *PMS2* methylation in 28.26% of gastric cancer samples studied and it was associated with both intestinal- and diffuse-type gastric cancer compared to normal controls. It was also detected a tendency for *PMS2* methylation in intestinal-type gastric cancer with larger tumor extension. However, further studies on *PMS2* methylation are needed to better understand this finding in gastric carcinogenesis.

MSH2 methylation status was only evaluated in two other studies on gastric carcinogenesis (Wu *et al.*, 2000; Fang *et al.*, 2004). All studies, including the present study, evaluated the same CpG island that was previously associated with *MSH2* expression (Wang *et al.*, 2003). Wu *et al.* (2000) have not found *MSH2* methylation in 42 samples studied. Fang *et al.* (2004) performed bisulfite modification and sequencing of *MSH2* in cancerous, paracancerous and non-cancerous tissues from ten patients and found that methylation occurred only at the -166 CpG site. In our samples, we found *MSH2* methylation in 17.39% of samples studied and a tendency for *MSH2* promoter in gastric cancer compared to normal controls. Thus, *MSH2* methylation frequency in our study population was higher than that found in Asian populations.

We also found an association between *MSH2* methylation and diffuse-type gastric cancer samples. Kitajima *et al.* (2003) previously described an association between loss of *MSH2* expression and undifferentiated gastric cancer type, largely corresponding to diffuse-type in Laurén's classification, in a Japanese population.

MSH6 was previously described as a potential transcriptionally silenced gene by cytosine methylation (Bearzatto *et al.*, 2000; Gazzoli and Kolodner, 2003). The 5'-flanking region of *MSH6* gene was found to comprise seven functional Sp1 transcription factor binding sites, each binding to Sp1 and Sp3 and contributing to promoter activity. The Sp1-4, Sp1-5, Sp1-6, and Sp1-7 sites have a paramount role in this activity and allow DNA methylation to inhibit Sp1 binding (Gazzoli and Kolodner, 2003).

The methylation status of *MSH6* has not been reported in any primary tumor. In the present study, we evaluated the methylation status of *MSH6* region which comprises the most important Sp1 sites. However, we did not detect *MSH6* methylation in any gastric specimen, suggesting that *MSH6* methylation is not a major event for gastric carcinogenesis in the samples studied.

The integrity of genetic information depends on the fidelity of DNA replication and the efficiency of several different DNA repair processes. The identification of epigenetic modifications in MMR genes and the determination of the frequency of alterations may be a useful tool for developing more specific cancer therapies. This is the first study evaluating the methylation status of *MLH1*, *MSH2*, *MSH6* and *PMS2* promoters and their hypermethylation frequencies in gastric tissue samples in a population from Northern Brazil. The methylation status of *PMS2* and *MSH6* promoter had never been investigated before in gastric samples. Our findings show that methylation of *MLH1*, *MSH2* and *PMS2* MMR genes is associated with gastric carcinogenesis.

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