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Methylation status of *ANAPC1*, *CDKN2A* and *TP53* promoter genes in individuals with gastric cancer

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Gastric cancer is the forth most frequent malignancy and the second most common cause of cancer death worldwide. DNA methylation is the most studied epigenetic alteration, occurring through a methyl radical addition to the cytosine base adjacent to guanine. Many tumor genes are inactivated by DNA methylation in gastric cancer. We evaluated the DNA methylation status of *ANAPC1*, *CDKN2A* and *TP53* by methylation-specific PCR in 20 diffuse- and 26 intestinal-type gastric cancer samples and 20 normal gastric mucosa in individuals from Northern Brazil. All gastric cancer samples were advanced stage adenocarcinomas. Gastric samples were surgically obtained at the João de Barros Barreto University Hospital, State of Pará, and were stored at -80°C before DNA extraction. Patients had never been submitted to chemotherapy or radiotherapy, nor did they have any other diagnosed cancer. None of the gastric cancer samples presented methylated DNA sequences for *ANAPC1* and *TP53*. *CDKN2A* methylation was not detected in any normal gastric mucosa; however, the *CDKN2A* promoter was methylated in 30.4% of gastric cancer samples, with 35% methylation in diffuse-type and 26.9% in intestinal-type cancers. *CDKN2A* methylation was associated with the carcinogenesis process for ~30% diffuse-type and intestinal-type compared to non-neoplastic samples. Thus, *ANAPC1* and *TP53* methylation was probably not implicated in gastric carcinogenesis in our samples. *CDKN2A* can be implicated in the carcinogenesis process of only a subset of gastric neoplasias.

Key words: DNA methylation; Gastric cancer; *ANAPC1*; *CDKN2A*; *TP53*

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Introduction

Gastric cancer is the forth most frequent malignancy and the second most common cause of cancer death worldwide (1). In Northern Brazil, gastric cancer is the second most frequent neoplasia in males (11/100,000) and the third most common in females (6/100,000) (2). Diet may be associated with the high incidence of this neoplasia in the State of Pará, especially because of the high consumption of salt-preserved food, low use of refrigerators and low consumption of fresh fruit and vegetables (3).

DNA methylation is the most studied epigenetic alteration, occurring by the addition of a methyl radical to the cytosine base adjacent to guanine (4). In cancer, DNA methylation of the promoter region of a normal tumor-suppressor gene leads to the aberrant silencing of its functions.

Three genes were chosen for evaluation considering their function and/or their potential role in gastric carcinogenesis: *ANAPC1*, *CDKN2A* and *TP53*.

Our group previously described a line of adenocarcinoma cells in which polyploidization due to endoreduplication was detected (5). Atkin (6) suggested that polyploidization could be a characteristic of carcinogenesis because the aggressiveness of a cancer is related to the degree of genomic instability associated with chromosome selection. This genomic instability is a frequent finding in gastric cancer (7). Accurate segregation of sister chromatids during mitosis is necessary to prevent the aneuploidy found in many cancers. The spindle checkpoint has been shown to be defective in cancers with chromosomal instability.

This checkpoint regulates the anaphase-promoting complex or cyclosome. The product of *ANAPC1* is the largest subunit of the anaphase-promoting complex (8). Thus, *ANAPC1* may be a possible candidate for causing the chromosomal instability seen in gastric cancer. However, the methylation pattern of *ANAPC1* had never been described. The product of *CDKN2A* is a protein that regulates phosphorylation of the retinoblastoma protein and negatively regulates the G1-S transition in the cell cycle (9). *CDKN2A* hypermethylation may play a key role in the progress of gastric cancer (10,11). *TP53* is one of the most studied tumor-suppressor genes and acts especially in cell cycle arrest and induction of apoptosis (12). Inactivation of the *TP53* pathway is a common feature of neoplasia. Dysregulation of the *TP53* pathway has been shown to involve mutations of *TP53*, increased expression of the TP53 inhibitor HDM-2, or epigenetic silencing of the *TP53* promoter (13). However, its methylation status has never been studied in gastric carcinogenesis.

In the present study, we evaluated the methylation status of *ANAPC1, CDKN2A* and *TP53* promoters in gastric adenocarcinoma samples and their possible associations with clinical and pathological characteristics, such as gender, age, histopathology, tumor extension, and presence of lymph node or distant metastasis.

Material and Methods

Samples

The study included 66 samples of gastric tissue. Of 66 patients, 47 were male and 19 were female, and mean age was 59 ± 9.8775 years (range 27-76). Among gastric cancer samples, 20 were non-neoplastic gastric mucosa of gastric cancer patients (distant location of primary tumor) and 46 sporadic gastric adenocarcinoma samples. Gastric samples were surgically obtained at the João de Barros Barreto University Hospital (HUJBB), State of Pará, Brazil, and were stored at -80°C before DNA extraction. Patients had never been submitted to chemotherapy or radiotherapy prior to surgery, nor did they have any other diagnosed cancer. All patients signed an informed consent with the approval of the HUJBB and Ribeirão Preto Medical School Clinical Hospital Institutional Review Board. All 46 gastric cancer samples were classified according to Laurén (14): 20 were diffuse-type and 26 were intestinal-type. Tumors were staged using standard criteria by tumor, node, metastasis (TNM) staging (15).

Methylation-specific PCR

Genomic DNA (2 µg) was modified by bisulfite treatment, converting unmethylated cytosines to uracils and leaving methylated cytosines unchanged. Methylation-specific PCR (MSP) was performed on treated DNA as previously described (16). Specific primers for MSP (Table 1), located within CpG island in the gene promoter region, were designed with the assistance of the Methprimer software (17).

The PCR product was carried out in a volume of 50 µL with 200 µM dNTPs, 200 µM $MgCl_2$, 50 ng DNA, 200 pM primers, and 1 unit 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, 1 min at different temperatures with the primers (Table 1) and 72°C for 40 s were followed by a final extension for 5 min at 72°C. PCR products were separated and visualized by electrophoresis on 8% polyacrylamide gel and stained with 10% silver nitrate (Figure 1). Water was used as negative control. MSP results were scored when there was a clearly visible band on electrophoresis gel with the methylated and unmethylated primers (16).

Table 1. Primer sequences (5'-3') for methylation-specific polymerase chain reaction.

Gene	Sense	Antisense	Product size	Temp.
ANAPC1	M: TTTAAGTTGTAATTCGTCGC	M: TAACAAAAAAACGTACGAAAC	157 bp	52°C
	U: TTTAAGTTGTAATTTGTTGTGG	U: TAACAAAAAAACATACAAAACAT	157 bp	
CDKN2A	M: GTAGGGTTTAGAGTCGTTTCGA	M: AACTACAAACTAAAACCCACGC	162 bp	55°C
	U: CGTAGGGTTTAGAGTTGTTTTGA	U: AACTACAAACTAAAACCCACACA	163 bp	
TP53	M: CGTCGTATTTCGGATTAGATTTC	M: AAAAAAACGTAAACGCTTCTCG	166 bp	57°C
	U: GGTTGTTGTATTTTGGATTAGATTTT	U: AAAAAAAACATAAACACTTCTCACC	170 bp	

M = methylated sequence; U = unmethylated sequence; Temp. = melting temperature.

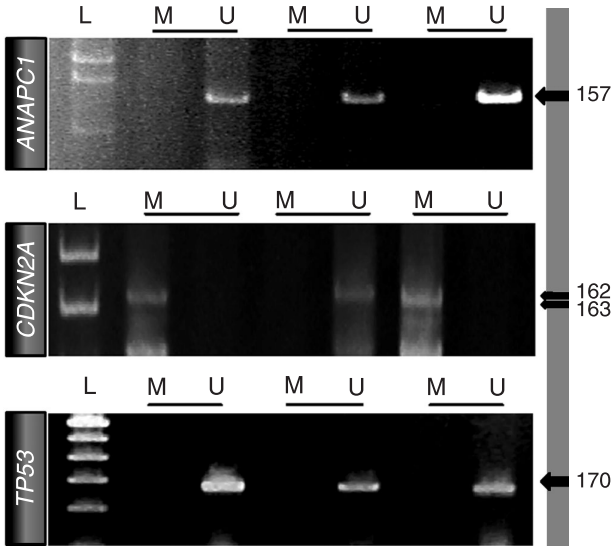

Figure 1. Gel electrophoresis using *ANAPC1*, *CDKN2A* and *TP53* methylation-specific polymerase chain reaction primers. L: size marker; M: methylated; U: unmethylated.

Statistical analysis

Statistical analyses were performed using the Fisher 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 node or distant metastasis. P values less than 0.05 were considered to be significant.

Results

According to Laurén's classification (14), 20 were diffuse-type and 26 were intestinal-type. All gastric cancer samples were in advanced stage: 0% was at pT1, 26.1% were at pT2, 63% were at pT3, and 10.9% were at pT4. It was observed that 34.8% were at N0 stage, 58.7% at N1 and 6.5% at N2. No sample had distant metastasis.

Table 2. Methylation number and frequency of *CDKN2A* in gastric samples for comparison of gastric cancer with non-neoplastic samples.

Samples	Methylation status of *CDKN2A*		P
	M	U	
Normal (N = 20)	0 (0%)	20 (100%)	–
Cancer (N = 46)	14 (30.4%)	32 (69.6%)	0.0009
Diffuse (N = 20)	7 (35%)	13 (65%)	0.0024
Intestinal (N = 26)	7 (26.9%)	19 (73.1%)	0.0137

Data are reported as number of individuals with percent within parentheses. M = methylated; U = unmethylated. Fisher exact test was used for statistical analysis.

None of the non-neoplastic samples showed methylation of any gene promoter. Furthermore, none of the samples showed methylated sequences for the *ANAPC1* and *TP53* promoters. The methylation frequency of *CDKN2A* promoter was 30.43% in gastric cancer samples. *CDKN2A* methylation was associated with gastric cancer samples compared to non-neoplastic samples (P = 0.0009). *CDKN2A* methylation was associated with both diffuse-type and intestinal-type compared to control samples using the Fisher exact test (P = 0.0024 and P = 0.0137, respectively; Table 2).

We analyzed whether *CDKN2A* methylation was associated with clinical and pathological characteristics, and detected a tendency for this gene methylation in intestinal-type gastric cancer with smaller tumor extension (T2) compared to T3 and T4 stages (P = 0.0572).

Discussion

DNA methylation is a stable but reversible epigenetic signal that silences gene expression. Somatic alterations

in genomic methylation patterns contribute to the etiology of human cancers and aging. It is tightly interwoven with the modification of histone tails and other epigenetic signals (18).

Lima et al. (5) developed and characterized cytogenetically a cell line called ACP01 from a gastric adenocarcinoma. In ACP01, polyploidization due to endoreduplication was observed. The spindle checkpoint has been shown to be defective in cancers with chromosomal instability. This checkpoint regulates the anaphase-promoting complex or cyclosome. *ANAPC1* product is the largest subunit of the anaphase-promoting complex (8). To our knowledge, the methylation status of *ANAPC1* has never been evaluated. In the present study, we did not detect *ANAPC1* methylation. Our findings suggest that the *ANAPC1* methylation is probably not important for the control of the chromosomal instability in our samples.

Methylation of the *CDKN2A* promoter was detected in 30.43% of gastric cancer samples, which was not significantly different from frequencies previously described (range 21 to 43%), and was also associated with carcinogenesis process (19-22). Lee et al. (23) proposed that *CDKN2A* methylation may contribute to the malignant transformation of gastric precursor lesions. Tahara et al. (24) also suggested that the evaluation of *CDKN2A* status can help predict gastric cancer risk in non-neoplastic gastric epithelium, because methylation levels of *CDKN2A* seem to accumulate in the progression of gastric mucosa atrophy and intestinal metaplasia, and thus may be associated with the presence of gastric cancer especially for intestinal-type histopathology. In our sample, diet habits may play a role in *CDKN2A* methylation especially in the beginning of intestinal-type gastric cancer.

TP53 is one of the most studied tumor-suppressor genes and acts especially in cell cycle arrest and induction of apoptosis. *TP53* is a gene related to the majority of human cancers. Methylation of *TP53* was reported as a mechanism for its inactivation in some neoplasias, such as acute lymphoblastic leukemia, multiple myeloma, malignant glioma cells, and brain metastases of solid tumors (13,25-27). To our knowledge, the methylation status of *TP53* has never been evaluated in gastric samples. In the present study, we did not detect *TP53* methylation. Thus, methylation of *TP53* is not an important event for gastric carcinogenesis in the population studied.

The identification of epigenetic modifications in tumor suppressor genes and the determination of the frequency of these alterations may be useful in the development of a more specific cancer therapy.

This is the first study that evaluated the methylation status of *ANAPC1*, *CDKN2A* and *TP53* promoters in gastric samples in a population from Northern Brazil. In this study, the methylation pattern of *ANAPC1* and *TP53* promoter was not altered by the gastric carcinogenesis process. However, more studies are necessary to evaluate if the absence of methylation of these genes is a common finding in gastric cancer or if this is a regional characteristic.

CDKN2A promoter methylation was associated with gastric carcinogenesis. However, only about 30% of gastric cancer samples were methylated. *CDKN2A* methylation can be specific to a subset of gastric cancer and probably plays a role in the beginning of intestinal-type gastric cancer.

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