result using these assays. Although poor correlation between hTERT protein expression and methylation was observed in gastric cancer samples, the clear difference in the frequency of hTERT expression and methylation between cancerous and non-cancerous tissues still might be useful for diagnosis of gastric cancer and may have an impact on the anti-telomerase strategy for cancer therapy.

Acknowledgements

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4.1.2 *Insulin-like growth factor binding protein-3* gene methylation and protein expression in gastric adenocarcinoma
Insulin-like growth factor binding protein-3 gene methylation and protein expression in gastric adenocarcinoma

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A B S T R A C T

Objective: The aim of this study was to evaluate IGFBP-3 protein expression, its correlation with gene promoter methylation pattern in gastric carcinogenesis and with clinicopathological characteristics.

Design: Forty-three normal gastric mucosa and 94 adenocarcinoma samples were investigated through methylation specific PCR, after bisulfite modification. Immunohistochemistry was analyzed using peroxidase in 54 gastric cancer and 20 normal gastric mucosa samples.

Results: IGFBP-3 expression was higher in tumor samples than in normal mucosa (\(p < 0.0001\)). Intestinal type presented a higher frequency of protein expression than diffuse type (\(p = 0.0412\)). Methylation frequency of IGFBP-3 promoter in gastric samples revealed, respectively, 95.7% and 97.7% in neoplastic and non-neoplastic samples. The frequency of IGFBP-3 methylation did not differ between tumor and normal samples (95.7% versus 97.7%, \(p = 0.7810\)). We did not observe a significant correlation between IGFBP-3 promoter methylation and protein expression.

Conclusion: In summary, our study did not observe any influence of IGFBP-3 promoter methylation on protein expression. Moreover we propose that IGFBP-3 immunostaining in gastric tissue may be a useful marker for malignancy.

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1. Introduction

Insulin-like growth factors (IGF) modulate growth and development, and promote cellular proliferation, survival and differentiation [1]. IGF interaction with its receptors is modulated by the family of IGF binding proteins. Among this family, the most abundant binding protein in human serum is IGFBP-3, which circulates associated with IGF and a glycoprotein, called acid labile subunit [2]. IGFBP-3 is also present in several tissues [3]. IGFBP-3 inhibits IGF action by competitively binding IGFs that prevents their binding to the IGF receptor (IGF-R) [4]. Therefore, IGFBP-3 is known to promote apoptosis and this fact suggests a protective against carcinogenesis [5]. While, IGFBP-3 actions independent of IGF include the capacity to maintain growth stimulatory effects [6]. IGFBP-3 gene is located at 7p13 [7] and its transcriptional regulation is mediated by alterations in activity or properties of transcriptional factor or by epigenetic modifications, such as DNA methylation or histone acetylation [8]. DNA methylation of Cpg islands at IGFBP-3 promoter has been described in renal, ovarian, liver, gastric, colorectal and breast cancer, and the expression of IGFBP-3 protein appears to be inhibited by promoter methylation [9]. Furthermore, it is known that IGFBP-3 expression is induced by the tumor suppressor p53 [5].

Tomii et al. [10] suggest that the role of IGFBP-3 varies during carcinogenesis in different organs, and that inter-ethnic comparison of the methylation pattern of this gene has to be analyzed. It is well known that hypermethylation of the Cpg island in tumor-related genes is one of the most important epigenetic alterations in cancer development. Thus, aberrant methylation could be used as diagnostic marker to identify cancer cells from normal samples and as a prognostic marker indicating tumor biological behavior [11].

Gastric cancer is still the second most prevalent cause of cancer death worldwide [12]. In the state of Pará, Northern Brazil, gastric cancer mortality rates are higher than the national average rate [13].
The aim of this study was to evaluate IGFBP-3 protein expression and gene promoter methylation pattern in gastric carcinogenesis and their correlation with clinicopathological characteristics.

2. Materials and methods

2.1. Samples

IGFBP-3 protein expression was evaluated in formalin-fixed paraffin embedded tissues of 74 patients with sporadic gastric adenocarcinoma. Twenty of those patients also had non-neoplastic and non-infiltrated gastric mucosa also evaluated.

IGFBP-3 methylation pattern was evaluated in 137 gastric samples. Forty-three of these were non-neoplastic gastric mucosa and 94 sporadic gastric adenocarcinomas. IGFBP-3 methylation and protein expression were both evaluated in 54 gastric cancer samples and 20 normal gastric mucosa. All samples were classified according to Lauren [14] and tumors were staged using standard criteria by TNM staging. Table 1 shows the clinicopathological characteristics of the studied samples.

In this study, all gastric samples were obtained surgically in João de Barros Barreto University Hospital (HUJBB), Pará State. Informed consent with approval of the ethics committee of HUJBB was obtained. All patients had negative histories of exposure to either chemotherapy or radiotherapy before surgery and there was no other co-occurrence of diagnosed cancers.

2.2. Immunohistochemical staining

Antigen retrieval was performed by microwave treatment 20 min at 900 W in a citrate buffer, pH 6.0. After cooling, sections were immersed in 0.3% hydrogen peroxide in phosphate-buffered saline (PBS) for 10 min to block endogenous peroxidase activity. Sections were then incubated in a humid chamber overnight with IGFBP-3 primary antibody (HPA013357, Sigma–Aldrich, USA). After the PBS rinse, slides were incubated with secondary antibody and then with streptavidin–biotin-peroxidase complex, both for 30 min at room temperature with a PBS wash between each step. Slides were visualized with diaminobenzidine–hydrogen peroxide and counterstained with Harry’s hematoxylin.

Table 1
Gender, age and clinicopathological characteristics and immunohistochemistry results of gastric tissue samples, n (%).

<table>
<thead>
<tr>
<th>Variable</th>
<th>HHC</th>
<th>Total</th>
<th>Positive</th>
<th>Negative</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>52</td>
<td>32 (61.5%)</td>
<td>20 (38.5%)</td>
<td>0.7807</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>22</td>
<td>15 (68.2%)</td>
<td>7 (31.8%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Tissue</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NGM</td>
<td>20</td>
<td>4 (20%)</td>
<td>16 (80%)</td>
<td>&lt;0.001</td>
<td></td>
</tr>
<tr>
<td>GC</td>
<td>54</td>
<td>43 (79.6%)</td>
<td>11 (20.4%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Onset</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;45y</td>
<td>5</td>
<td>3 (60%)</td>
<td>2 (40%)</td>
<td>0.2662</td>
<td></td>
</tr>
<tr>
<td>&gt;45y</td>
<td>49</td>
<td>40 (81.6%)</td>
<td>9 (18.4%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>H. pylori</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>40</td>
<td>32 (80%)</td>
<td>8 (20%)</td>
<td>0.9964</td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>14</td>
<td>11 (78.6%)</td>
<td>3 (21.4%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Lauren classification</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diffuse</td>
<td>28</td>
<td>19 (67.9%)</td>
<td>9 (32.1%)</td>
<td>0.0412*</td>
<td></td>
</tr>
<tr>
<td>Intestinal</td>
<td>26</td>
<td>24 (92.3%)</td>
<td>2 (7.7%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Tumor location</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cardia</td>
<td>11</td>
<td>7 (63.6%)</td>
<td>4 (36.4%)</td>
<td>0.2057</td>
<td></td>
</tr>
<tr>
<td>Non-cardia</td>
<td>43</td>
<td>36 (83.7%)</td>
<td>7 (16.3%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Stage</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>I/II</td>
<td>4</td>
<td>2 (50%)</td>
<td>2 (50%)</td>
<td>0.1805</td>
<td></td>
</tr>
<tr>
<td>III/IV</td>
<td>50</td>
<td>41 (82%)</td>
<td>9 (18%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Lymph node metastasis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>50</td>
<td>41 (82%)</td>
<td>9 (18%)</td>
<td>0.1805</td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>4</td>
<td>2 (50%)</td>
<td>2 (50%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Distant metastasis</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>17</td>
<td>15 (88.2%)</td>
<td>2 (11.8%)</td>
<td>0.7029</td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>34</td>
<td>28 (82.4%)</td>
<td>6 (17.6%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

IHC: immunohistochemistry assay; NGM: normal gastric mucosa; GC: gastric cancer. *p < 0.05

Fig. 1. Immunohistochemical detection of IGFBP-3 protein. (a) Immunostaining in diffuse-type gastric adenocarcinoma (b) immunostaining in intestinal type gastric adenocarcinoma. (c) No immunostaining in negative control (d) immunostaining in advanced breast cancer used as positive control. Photomicrographs (a and b) were taken in median-powered 200×; (c and d) were taken in 400×.
Positive IGFBP-3 expression was defined as clear cellular staining in 50% or more of the cells, whereas negative IGFBP-3 immunostaining was considered when no positive cells were observed or in rare cases (less than 25% weakly stained tumor cells) (Fig. 1). Normal gastric mucosa was used as an internal control. Positive control was obtained by staining tissue of advanced breast cancer, whereas negative control was normal ovarian stromal cells, a tissue known to be negative for IGFBP-3 [15] (Fig. 1). Two pathologists evaluated the immunostaining results independently.

### 2.3. Methylation specific PCR (MSP)

Genomic DNA (200 ng) of all samples underwent bisulfite modification using EpiTect Bisulfite kit (Qiagen, Germany) according to the manufacturer’s instructions, to convert unmethylated cytosines to uracils and leaving methylated cytosines unchanged. MSP was performed on treated DNA as previously described [16]. Specific primers for MSP, located within the IGFBP-3 promoter, about –100 from start transcription site, were 5-TTATTTTTGGTTTTTTATATAGCGGTC-3 (sense) and 5-AAAAAACGACACA-3 (antisense) for the methylated reactions, with 5-TATATAGTGGTTTACCA-3 (sense) and 5-AACAAAAAACAACTAATCCTCAACA-3 (antisense) for the unmethylated reactions; 5-TTATTTTTGGTTTTTTATATAGCGGTC-3 (sense) and 5-AAAAAACGACACA-3 (antisense) for the methylated reactions, with PCR products of 90 bp and 84 bp, respectively. Briefly, PCR reaction was carried out in a 25 µL volume with 200 µmol/L of MgCl2, 100 ng of DNA, 200 µmol/L of primers and 1.25 U of Taq (LGC, Brazil). After initial denaturing for 5 min at 94 °C, 40 cycles at 94 °C for 45 s, at 54 °C for 45 s, and at 72 °C for 30 s were carried out, followed by a final extension for 5 min at 72 °C. Results were scored when there was a clear and visible band on the electrophoresis gel with the methylated and unmethylated primers (Fig. 2).

### 2.4. Statistical analyses

Statistical analyses were performed using the $\chi^2$ test or Fisher’s exact test to assess associations between the expression or methylation status and clinicopathological characteristics. $\chi^2$ test (Phi correlation) was performed to evaluate the relationship between IGFBP-3 gene methylation and its protein expression. Non-parametric Mann–Whitney U test was used to compare patient age and methylation status or protein expression. $p$ values less than 0.05 were considered significant.

### 3. Results

IGFBP-3 expression was more frequently observed in tumor samples of both types of gastric cancer than in normal mucosa ($p < 0.0001$). The intestinal type presented a higher frequency of protein expression than the diffuse type ($p = 0.0412$). IGFBP-3 expression was more frequently observed in diffuse-type tumors located in the non-cardia region (fundus, body and pylorus regions of the stomach) than in the cardia region (gastroesophageal transition), although not significant ($p = 0.0841$) (Table 1).

Methylation analysis of IGFBP-3 promoter gene in gastric samples revealed hypomethylation of only 4.3% of neoplastic and 2.3% of non-neoplastic samples. The frequencies of IGFBP-3 methylation did not differ between tumor and normal samples (95.7% versus 97.7%, $p = 0.7810$), as well as between diffuse and intestinal type (97.9% versus 93.5%, $p = 0.3563$). We did not observe a significantly correlation between IGFBP-3 promoter methylation and protein expression (Table 2).

There was also no association between IGFBP-3 expression and gene promoter methylation with clinical and pathological characteristics as age, gender, Helicobacter pylori infection, tumor extension and presence of distant metastasis.

### 4. Discussion

IGFBP-3 has been described as a direct cell growth inhibitor and may be protective against the development of gastrointestinal carcinogenesis [4].

In the present study, we found IGFBP-3 protein expression in 80% of gastric cancer samples. Moreover, intestinal type gastric cancer samples presented a significantly higher frequency of IGFBP-3 expression than diffuse-type samples. Zhang et al. [4] described 55.4% of tumor samples presenting positive staining for IGFBP-3. Furthermore, patients with well- or moderately-differentiated tumors also showed significantly higher percentage of positive staining of IGFBP-3 than those with poorly-differentiated tumors [4].

We also observed that IGFBP-3 expression showed a tendency to be more frequent in diffuse-type tumors located in the non-cardia region than in the cardia. Therefore, these results indicated that IGFBP-3 expression may contribute to a better prognosis in gastric cancer patients. High expression of IGFBP-3 was described in breast, renal and lung cancer [17–20]. Furthermore a high level of IGFBP-3 was observed in breast cancer with poor prognostic features [18].

IGFBP-3 expression was also described in 45% of gastric cancer cell lines [21] and the upregulation was associated with the inhibitory effects of anticancer drugs, such as paclitaxel and etoposide [22].

Hanafusa et al. [23] hypothesize that IGFBP-3 basal level of expression is essential for cell survival, but upon induction by p53, high levels of expression of IGFBP-3 induce apoptosis. It was also suggested that the loss of p53 expression leads to the suppression of IGFBP-3 in tumor cells [23]. In our population, we previously described that all gastric samples presented TP53 allelic deletions [24]. Thus, the feedback between p53 and IGFBP-3 may be lost in these samples and other pathways may be inducing IGFBP-3 expression.

We previously observed that only intestinal type gastric cancer samples presented p53 immunoreactivity [24]. Increased immunostaining of p53 can depend on either increased synthesis of wild-type protein or accumulation of mutated protein in the cell [25].

---

**Fig. 2.** Methylation analysis by MSP of IGFBP-3 promoter showing methylated and unmethylated band. Samples 1, 2 and 3 show both bands and sample 4 shows only the unmethylated band. L: size marker; M: methylated; U: unmethylated; +: positive result; –: negative result.

**Table 2** IGFBP-3 promoter methylation and protein expression results in tumor and normal gastric samples.

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>Methylation</th>
<th>p value</th>
<th>Normal Type</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>U</td>
<td>M</td>
<td>U</td>
<td></td>
</tr>
<tr>
<td>IHC</td>
<td>Positive</td>
<td>42 (79.2%)</td>
<td>1 (100%)</td>
<td>0.4577</td>
</tr>
<tr>
<td>Negative</td>
<td>11 (20.8%)</td>
<td>0</td>
<td>13 (76.5%)</td>
<td>1 (100%)</td>
</tr>
</tbody>
</table>

IHC: immunohistochemistry assay; M: methylated; U: unmethylated.
which explains the significant difference in IGFBP-3 staining between intestinal (92.3%) and in diffuse type (67.9%).

In the present study, IGFBP-3 promoter hypermethylation of the studied CpG island was observed in 96.3% of our total samples, without difference of methylation between normal and tumors. This observation may be due to the increased CpG island methylation related in stomach [26]. In our population, we previously detected DNA promoter methylation of other tumor suppressor genes in normal gastric mucosa [27,28]. This gene may be commonly methylated in the stomachs of individuals from Northern Brazil. To our knowledge this is the first study that associated IGFBP-3 promoter methylation with its protein expression in primary gastric carcinomas. However, the presence of promoter methylation did not influence IGFBP-3 protein expression.

Tomii et al. [10] observed frequent methylation of IGFBP-3 promoter in Japanese with gastric cancer, which was not observed in American patients (75% versus 15%, p < 0.0001) [10]. In a different Japanese population, IGFBP-3 methylation was found in 90% of gastric neoplastic samples and in 83% of gastric cancer cell lines [7]. These results, along with ours, demonstrated that the frequency of IGFBP-3 promoter methylation differs among populations.

IGFBP-3 methylation has been described in several types of cancer with different frequencies. In hepatocellular carcinoma, 33% of samples presented a correlation between hypermethylation and protein silencing [29]. This study also showed that hypermethylation of four selective sites of p53 bind in IGFBP-3 promoter can suppress protein expression in cell lines [23]. Similar methylation frequency was observed in epithelial ovarian cancer (44%) and, in these samples, methylation was associated with disease progression and mortality [30].

In American patients with colorectal cancer, only 29% of samples presented IGFBP-3 promoter methylation [31]. In colorectal carcinoma, no correlation between this gene methylation and patient survival was observed in American patients under treatment [32]. Poor prognosis was observed in another American patients with non-small cell lung cancer presenting IGFBP-3 hypermethylation, about 61.5% of patients [33]. In urogenital cancer, a frequency similar to ours was observed in German non-advanced tumor samples (86%), and only 36% of advanced tumor presented IGFBP-3 methylation. This gene methylation was correlated with higher risk for recurrence [34].

Several mechanisms are known to regulate IGFBP-3 expression, such as p53, TGF-β, IL-1, IL-6, TNF-α, among others [6]. TGF-β is reported as a potent stimulator of IGFBP-3 production [35]. Overexpression of TGF-β was previously observed in gastric cancer [36], especially in intestinal type adenocarcinomas [37]. Thus, our related findings that intestinal type gastric cancer presented IGFBP-3 staining more frequently than the diffuse-type samples are consistent with those previously reported.

The IGFBP-3 increased expression in our gastric cancer samples may also be due to the ability of IL-1 and TNF-α in inducing IGFBP-3 production [6]. IL-1 and TNF-α are proinflammatory cytokines produced in response to H. pylori infection [38], a bacterium highly associated with gastric carcinogenesis [39]. However, this theoretical possibility was not supported by our findings (Table 1).

In summary, our study did not observe any influence of IGFBP-3 promoter methylation on suppressing protein expression. Moreover we propose that IGFBP-3 immunostaining in gastric tissue may be a useful marker for malignization.

Acknowledgments

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References

4.1.3 *SMARCA5* methylation and expression in gastric cancer
Here, we first evaluated SMARCA5 expression and promoter DNA methylation in gastric carcinogenesis. Immunohistochemistry and methylation-specific PCR were analyzed in 19 and 48 normal mucosa and in 52 and 92 gastric cancer samples, respectively. We observed higher immunoreactivity of SMARCA5 in gastric cancer samples than in normal mucosa. Moreover, SMARCA5 promoter methylation was associated with the absence of protein expression. Our findings suggest that SMARCA5 has a potential role in proliferation and malignancy in gastric carcinogenesis.

**Keywords:** Epigenetic; DNA methylation; Protein expression; IHC; SMARCA5

**INTRODUCTION**

Gastric cancer is the fourth most prevalent cancer in the world. However, due to its poor prognosis, gastric cancer is the second most common cause of death from cancer (1). The early detection of gastric cancer is very important for a good prognosis.

SMARCA5 (SNF2H), the human homologue of Drosophila imitation switch gene (ISWI), is a member of the SWI/SNF chromatin remodeling family of proteins, which has helicase and ATPase activity (2). SMARCA5 mediates DNA accessibility by sliding the histone octamer and, thus, is important for gene expression, DNA replication, DNA repair, and the maintenance of higher order chromatin structure (2, 3).

Chromatin remodeling complexes use the energy of ATP hydrolysis to change nucleosomal positions, so that specific regions of the genome become accessible for interaction with regulating factors (4). These chromatin remodeling complexes have been shown to promote both transcriptional activation and repression (5, 6). Not surprisingly, these complexes have been associated with malignant transformation (7).

There is a 1 kb CpG island with CG content up to 60% in SMARCA5 promoter and exon 1 (8). This CpG island contains binding sites of methylation-sensitive transcription factors such as Sp1, Myb, CREB, AP1, and MZF1 (9). These data suggest that SMARCA5 expression may be regulated by DNA methylation. The mechanism of SMARCA5 regulation is still unknown. DNA methylation is the most common epigenetic alteration and occurs by addition of a methyl radical on a deoxyctosine, frequently found in CpG sites. DNA methylation is usually associated with gene silencing (10).

The aim of this study was to investigate the protein expression and methylation pattern of SMARCA5 in gastric carcinogenesis and their correlation with clinicopathological characteristics.

**METHODS**

**Samples**

SMARCA5 protein expression was evaluated in 52 formalin-fixed paraffin-embedded tissue samples of patients with sporadic gastric adenocarcinoma, in which 19 samples also had adjacent nonneoplastic and noninfiltrated gastric mucosa. Among these samples, the mean age was 56 ± 6.9 years and 70.4% were male.

SMARCA5 methylation pattern was evaluated in 48 nonneoplastic gastric mucosa of patients (distant location of primary tumor) and in 92 sporadic gastric adenocarcinoma. In the studied samples, the mean age was 55 ± 10.35 years and 68.6% were male. SMARCA5 methylation and protein expression were both evaluated in 47 gastric cancer and 16 normal gastric mucosa samples.

All samples were classified according to Lauren (11) and tumors were staged using standard criteria by TNM staging (12). Table 1 shows the clinicopathological characteristics of the studied samples.

All gastric samples were obtained surgically in Pará State João de Barros Barreto University Hospital (HUJBB).
Informed consent with the approval of the ethics committee of HUJBB was obtained. All patients had negative histories of exposure to either chemotherapy or radiotherapy before surgery and there were no other co-occurrence diagnosed cancers.

**Methylation-specific PCR (MSP)**

Genomic DNA (200 ng) of gastric tissue samples, extracted by the phenol-chloroform method, underwent bisulfite modification with the EpiTect Bisulfite kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions, converting unmethylated cytosines to uracils and leaving methylated cytosines unchanged. MSP was performed on treated DNA as previously described (13). Specific primers for MSP, located within the SMARCA5 promoter, were as follows: 5-AGAAGATATAGTGAGATTGTGAGTTGT-3 (sense) and 5-ATAAACAAAACCTCCAAAAAACAC-3 (antisense) for the unmethylated reactions; 5-GAAGATATAGTGAGATTGTGAGTTGT-3 (sense) and 5 AACGAAACCTCCAAAAAACAC-3 (antisense) for the methylated reactions, with PCR products of 120 bp and 114 bp, respectively (14). Briefly, PCR reaction was carried out in a 25 µL volume, with 200 µmol/L of dNTPs, 200 µmol/L of MgCl2, 100 ng of DNA, 200 pmol/L of primers, and 1.25 units of Taq DNA polymerase. After initial denaturing for 5 min at 94°C, 40 cycles at 94°C for 45 s, at 55°C for 45 s, and at 72°C for 30 s were carried out, 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(d)). Results were scored when there was a clear visible band on the electrophoresis gel with the methylated and unmethylated primers (13).

**Statistical analyses**

Statistical analyses were performed using the χ² test or Fisher’s exact test to assess associations between the expression, methylation status, and clinicopathological characteristics. χ² test was performed to evaluate the relationship between SMARCA5 methylation and its protein expression.

**RESULTS**

SMARCA5 expression was more frequently observed in gastric cancer samples of both histological types than in
Figure 1. (a) Immunostaining in diffuse-type gastric adenocarcinoma. (b) Immunostaining in intestinal-type gastric adenocarcinoma. (c) No immunostaining in normal gastric tissue; magnification: 40×. (d) Methylation analysis showing four unmethylated samples, the last sample presents methylated and unmethylated bands. L: Low molecular weight DNA ladder (New England BioLabs, Ipswich, USA); U: unmethylated; M: methylated.

The frequency of SMARCA5 expression did not differ between diffuse and intestinal-type gastric cancer (0.26 versus 0.1667, p = .4080). In gastric cancer, SMARCA5 methylation was associated with the absence of its protein expression (p = .0104) (Table 2).

There was no clear association between the frequency of SMARCA5 expression or methylation and age, gender, tumor location, tumor extension, and presence of distant metastasis.

DISCUSSION

Cancer is a chronic proliferative disease with multiple genetic and epigenetic alterations, which lead to gene expression alteration. Chromatin structure is related to the gene expression regulation. The chromatin structure is determined by several mechanisms, including DNA methylation, histone modifications, and ATP-dependent chromatin remodeling (3).

In the present study, we investigated the regulation and the expression of SMARCA5, a protein involved in chromatin remodeling, in gastric carcinogenesis. Here, we observed that SMARCA5 overexpression is associated with gastric cancer. To our knowledge, no study in literature has evaluated SMARCA5 protein expression, as well as promoter gene methylation status in this pathology.

Little is known about the role of SMARCA5 in carcinogenesis. SMARCA5 expression has been observed in all tested immortal or tumor-derived cell lines (2, 15–17). Stopka et al. (15) observed that both in vitro and in vivo induction of erythroid differentiation is followed by downregulation of SMARCA5 expression that delays replication and stops proliferation of hematopoietic progenitor cells. These authors also described that SMARCA5 was upregulated in CD34+ hematopoietic progenitors of acute myeloid leukemia patients. Moreover, SMARCA5 expression was decreased in acute myeloid leukemia CD34+ progenitors after the patients achieved complete hematologic remission. The authors suggested that SMARCA5 overexpression may thus deregulate the genetic program required for normal differentiation. SMARCA5 also seems to be important in prostate carcinogenesis. Mohamed et al. (2) described that ISWI immunoreactivity was higher in prostatic neoplasia than in benign prostatic hyperplasia samples. Thus, our results corroborate previous studies in different neoplasias and suggest that the detection of SMARCA5 overexpression may have a role in gastric carcinogenesis.

SMARCA5 is generally associated with cell proliferation and viability (15, 18, 19). One previous study with Smarca5-null mice embryos clearly demonstrated that this gene is required for cell proliferation by the attenuation of mitosis and consequent early lethality in these mice (3). SMARCA5 has also been associated with transcription-independent modulation of cell cycles, affecting G1-S transition (20).

SMARCA5, through chromatin remodeling, does not allow the normal differentiation of malignant cells (18, 19). Moreover, ATP-dependent remodeling of a nucleosome is an early step in the regulatory process and it precedes the binding of most transcription factors to DNA (21). Thus,