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

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ABSTRACT

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.

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 transcription 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].

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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.

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

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

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

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.

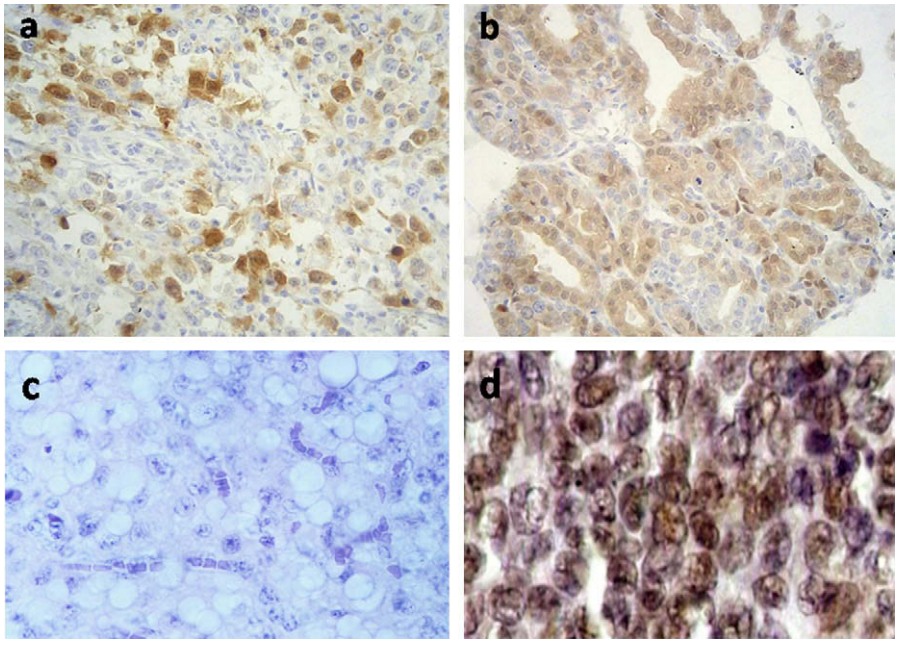

Fig. 1. Immunohistochemical detection of *IGFBP-3* protein. (a) Immunostaining in diffuse-type gastric adenocarcinoma (b) immunostaining in intestinal type gastric adenocarcinoma. (c) No immunostaining in negative control (d) immunostaining in advanced breast cancer used as positive control. Photomicrographs (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 ovarium 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-TTATTTTGGTTTTTATATAGTGGTT-3 (sense) and 5-AACAAAAAACAACTAATCCTCAACA-3 (antisense) for the unmethylated reactions; 5-TTTCGGTTTTTATATAGCGGTC-3 (sense) and 5-AAAAAACGACTAATCCTCAACG-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 $MgCl_2$, 100 ng of DNA, 200 pmol/L of primers and 1.25 U of Taq (LGC, Brazil). After initial denaturating 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 χ^2 test or Fisher's exact test to assess associations between the expression or methylation status and clinicopathological characteristics. χ^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.

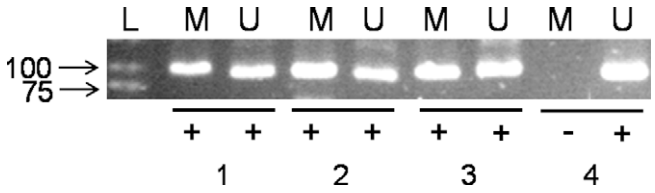

Fig. 2. Methylation analysis by MSP of *IGFBP-3* promoter showing methylated and unmethylated band. Samples 1, 2 and 3 show both bands and sample 4 shows only the unmethylated band. L: size marker; M: methylated; U: unmethylated; +: positive result; −: negative result.

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

	Tumor Methylation		*p* value	Normal		*p* value
	M	*U*		*M*	*U*	
IHQ						
Positive	42 (79.2%)	1 (100%)	0.4577	4 (23.5%)	0	0.4918
Negative	11 (20.8%)	0		13 (76.5%)	1 (100%)	

IHC: immunohistochemistry assay; M: methylated; U: unmethylated.

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],

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 individual 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.

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