SMARCA5 might be an interesting therapeutic target in gastric cancer presenting this protein overexpression.

The mechanism of SMARCA5 regulation is still unknown. We have previously evaluated the SMARCA5 methylation status in lymphocyte cells. However, we did not observe the presence of this gene promoter methylation in the young and elderly control samples of Alzheimer’s disease patients. Nevertheless, gene methylation status and regulation are frequently tissue-specific or disease-specific (22). The stomach is an organ that presents a normal increased CpG island methylation (23), and gene promoter methylation is commonly reported in the gastric carcinogenesis process (24–29). These genes and tissue characteristics lead us to consider whether epigenetic regulation of SMARCA5 occurs in gastric mucosa.

In the present study, we observed SMARCA5 promoter methylation in about 12.5% and 21.7% of normal and neoplastic gastric samples, respectively. To our knowledge, this is the first report of the presence of methylation in this gene promoter. This is also the first study to describe an association between SMARCA5 promoter methylation and a lack of its protein expression. However, our results suggest that other mechanisms, such as chromatin remodeling, or other protein interactions have a role in SMARCA5 expression control and are responsible for the deregulation observed in our gastric cancer samples.

In conclusion, our data suggest that SMARCA5 immunoreactivity has a potential role in proliferation and malignancy in gastric carcinogenesis. Little is known about the role of SMARCA5 in carcinogenesis and further studies are necessary to evaluate whether this protein could be an interesting therapeutic target or helpful in diagnosis of gastric cancer.

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DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

REFERENCES


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4.2 Artigos submetidos

4.2.1 Epigenetic pattern, mRNA and protein expression of E-cadherin and Caveolin-1 in gastric adenocarcinoma

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Epigenetic pattern, mRNA and protein expression of E-cadherin and Caveolin-1 in gastric adenocarcinoma

Short Title: CDH1 and CAV1 methylation, mRNA and protein expression

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ABSTRACT

Plasma membrane proteins are potential drug targets and diagnostic markers. E-cadherin contributes to cell-cell adhesion and Caveolin-1 is an integral membrane protein. Their respective genes have potential sites for epigenetic regulation by DNA methylation. Epigenetic modifications have a central role in several types of cancer, including gastric adenocarcinoma. Immunostaining, mRNA and DNA methylation were analyzed in neoplastic and non-neoplastic gastric samples. Lack of E-cadherin was associated with gastric
carcinogenesis and with metastasis, whereas Caveolin-1 expression was associated with gastric cancer and with H. pylori infection in diffuse type gastric cancer. Higher CAV1 mRNA levels were also correlated with gastric cancer samples. CDH1 and CAV1 methylation status did not differ between gastric cancer and normal mucosa. Our data showed an inverse relationship between E-cadherin and Caveolin-1 expression. E-cadherin was associated with gastric cancer and with a metastatic phenotype and Caveolin-1 seems to have a role as a pro-tumorigenic factor.

INTRODUCTION

Plasma membrane proteins play a role in cell signalling, adaptation to environment and cell–matrix interactions, and are also involved in the acquisition and maintenance of invasive and metastatic properties of tumour cells. These proteins are potential drug targets and diagnostic markers [1].

E-cadherin is a member of calcium-dependent adherins and contributes to cell-cell adhesion [2]. It has been proposed that loss of E-cadherin-mediated cell-cell adhesion has a role in tumor cell invasion and metastasis [3]. Moreover, E-cadherin was suggested to modulate intracellular signaling, thus promoting tumor growth [4, 5].

The E-cadherin gene (CDH1) is one of the most important tumor suppressor genes in gastric cancer. Genetic alterations such as mutations and chromosomal deletions as well as epigenetic modifications have been reported to be involved in CDH1 inactivation [6-9]. Loss of E-cadherin expression is more frequently associated to methylation of CDH1 promoter region [10], which contains a GC-rich region at -40 from start transcription site [11-13].

Therefore, the molecular mechanism of CDH1 gene silencing may be of great importance in understanding the metastatic process. Lack of CDH1 expression due to methylation has been reported in some human cancer cells [14, 15].

Caveolin-1 is an integral membrane protein in caveolae, which are invaginations in the plasma membrane. It has been implicated in diverse cellular processes such as cholesterol homeostasis, vesicular transport, cell migration, cell cycle, regulation of cell transformation and signal transduction [16].
Associated functions of Caveolin-1 in tumors are controversial depending upon tissue types and stages of cancer [17]. In fact, it is still unknown whether Cav-1 acts as a tumor suppressor [5, 18] or a tumor promoter, increasing invasive and metastatic potentials [17] in gastric carcinogenesis.

A detailed analysis of CAV1 has shown that exons 1 e 2 are embedded within CpG islands, being potential sites for epigenetic regulation by DNA methylation [19]. Indeed, promoter methylation of this gene has already been investigated in several tumors, such as prostate [20, 21], breast [22], colorectal [23] and ovarian [24]. Nevertheless, there are no data of CAV1 promoter methylation in gastric tissue samples.

Gastric cancer is the fourth most common type of neoplasia and the second most prevalent cause of cancer death worldwide [25]. Gastric adenocarcinoma has poor prognosis and high mortality, therefore remaining a health issue. In the state of Pará, northern Brazil, the gastric cancer mortality rates are higher than the national average rate [26]. A better understanding of the biology of this neoplasia progression is crucial for the development of better tests to early neoplasia detection and may help to predict the disease prognosis.

Epigenetic modifications have a central role in several types of cancer, including gastric adenocarcinoma. In tumors, global reduction of DNA methylation (hypomethylation) has been suggested to induce chromosome instabilities and transcriptional activation of oncogenes and prometastatic genes, leading to initiation and propagation of oncogenesis [27]. In contrast, a region- and gene-specific increase of methylation (hypermethylation) of multiple CpG islands has been shown in the carcinogenesis process [28] and is often associated with transcriptional silencing of the associated gene [27].

This study aimed to evaluate E-cadherin and Caveolin-1 protein expression in gastric adenocarcinoma from individuals of North Brazil, and to elucidate the epigenetic mechanism involved in the control of these proteins expression by correlating protein expression with promoter methylation of these genes.

**MATERIAL AND METHODS**

1. Casuistic
All the gastric samples were obtained surgically from João de Barros Barreto University Hospital (HUJBB) in Pará State, Brazil. This population is composed of interethnic crosses among three main origin groups: European (mainly represented by Portuguese), Africans and Amerindians [29]. Informed consent with approval of the ethics committee of HUJBB was obtained. All samples were classified according to Laurén [30] and tumors were staged using standard criteria by TNM staging [31]. 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. Immunohistochemical staining

E-cadherin protein expression was evaluated in formalin-fixed paraffin embedded tissues of 78 samples of gastric mucosa. Among these samples, 58 were sporadic gastric adenocarcinoma and 20 were non-neoplastic and non-infiltretated gastric mucosa. Caveolin-1 protein expression was analyzed in 75 samples of gastric mucosa formalin-fixed, 57 neoplastic and 18 non-neoplastic gastric tissue.

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 minutes to block endogenous peroxidase activity. Sections were then incubated in a humid chamber overnight with E-cadherin primary antibody (NCH-38, DAKO, USA) or Caveolin-1 primary antibody (N20 sc894, Santa Cruz Biotechnology, USA). After the PBS rinse, slides were incubated with secondary antibody and then with streptavidin-biotin-peroxidase complex, both for 30 minutes at room temperature with a PBS wash between each step. Slides were visualized with diaminobenzidine-hydrogen peroxide and counterstained with Harry’s hematoxylin.

Positive protein expression was defined as clear cellular staining, whereas negative immunostaining was considered when no positive cells were observed or in rare cases (less than 25% weakly stained tumor cells) (Figure 1). Normal gastric mucosa was used as an internal control. Two pathologists evaluated the immunostaining results independently.

3. Methylation specific PCR (MSP)
CDH1 and CAV1 methylation pattern were evaluated in 158 and 131 samples of gastric tissue, respectively. Genomic DNA (200 ng) of gastric tissue samples underwent bisulfite modification using EpiTect Bisulfite kit (Qiagen, 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 [32]. MSP for CDH1 was performed as previously described [33]. Specific primers for CAV1 promoter, were as follows: 5’-GAAAATATTTGTTTTTTTGGAT-3’ (sense) and 5’- ACAAAATAAAAACATTTCCTCCACA-3’ (antisense) for the unmethylated reactions; 5’-TTTCGGGACGTTTTTCGTTG-3’ (sense) and 5’-TAAAAACGTTTCTCCCGCCT-3’ (antisense) for the methylated reactions, with PCR products of 116 bp and 96 bp respectively. Briefly, PCR reaction was carried out in a 25 mL volume with 200 mmol/L of MgCl2, 100 ng of DNA, 200 pmol/L of primers and 1.25 units of Taq DNA polymerase. After initial denaturation for 5 min at 94ºC, 40 cycles of 94ºC for 45 s, at 57.8ºC for 45 s, and 72ºC for 30 s were carried out, followed by a final extension for 5 min at 72ºC.

Results were scored when there was a clear and visible band on the electrophoresis gel with the methylated or unmethylated primers. Hypermethylation was considered only in the presence of methylated band (Figure 2).

4. TaqMan quantitative RT-PCR analysis

Complementary DNA was synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) from RNA of approximately 40 gastric tissue extracted with AllPrep DNA/RNA/Protein Mini Kit (Qiagen) according to the manufacturer’s instructions. Taqman RT-PCR for CDH1 (Hs01013953_m1) and CAV1 (Hs00971716_m1) were performed using and ABI 7500 Fast System. The expression levels were normalized to ACTB gene (Hs03023943_g1 ).

5. Statistical analyses

Statistical analyses were performed using the χ2 test or Fisher’s exact test to assess associations between the expression or methylation status and clinicopathological characteristics. χ2 test was also used to correlate CDH1 and CAV1 methylation status with its respective protein expression. Student’s
paired t or Wilcoxon tests were used to compare RNAm expression with clinicopathological characteristics. P-values < 0.05 were regarded as statistically significant.

RESULTS

E-cadherin and Caveolin-1 IHC

E-cadherin immunostaining was observed in all cases of normal gastric mucosa and in 36.2% of tumor samples. Lack of E-cadherin was associated with gastric carcinogenesis (p<0.0001) and with metastasis (p=0.0035). Absence of E-cadherin expression was significantly higher in diffuse than in intestinal type gastric cancer (0.9063 vs 0.3077, p<0.0001) (Table 1).

Only one sample of normal gastric mucosa (5.6%) presented Caveolin-1 expression, while 84.2% of neoplastic samples had positive immunostaining. Hence, Caveolin-1 expression was associated with gastric cancer (p<0.0001). Caveolin-1 immunostaining was also more frequently observed in intestinal than in diffuse type gastric cancer (1 vs 0.6786, p=0.0008). In addition, Caveolin-1 expression was associated with H. pylori infection (p=0.0196) in diffuse type gastric cancer (data not shown).

Taken together, an inverse relationship between E-cadherin and Caveolin-1 expression (p=0.0259) was observed in our gastric samples.

CDH1 and CAV1 gene promother methylation

Methylated CDH1 promoter was observed in 90.4% and 90.6% of normal mucosa and tumor samples, respectively (Table 2). CDH1 methylation status did not differ between gastric cancer and normal mucosa, as well as between the diffuse and intestinal type. CDH1 methylation and protein expression were both evaluated in 56 gastric tumor and in 20 normal gastric mucosa samples. Our findings did not show a correlation between methylation pattern and protein expression, although all samples with E-cadherin expression presented methylated sequences (Table 3).

Presence of CAV1 promoter methylation was observed in 100% of normal gastric mucosa and 97.7% of gastric cancer samples. Hypermethylated CAV1 samples were observed in 27.9% and 34.1% of non-neoplastic and neoplastic samples, respectively. CAV1 methylation frequencies did not differ between non-neoplastic and neoplastic samples (Table 2). CAV1 methylation
and protein expression were both evaluated in 56 gastric cancer and in 17 normal gastric mucosa samples. We observed an association between hypermethylated CAV1 promoter and no Caveolin-1 expression in tumor samples (p=0.0001) (Table 3). No association between CDH1 and CAV1 promoter methylation status was found.

**Quantitative RT-PCR analysis of CDH1 and CAV1 expression.**

We next performed quantitative RT-PCR on complementary DNA from gastric samples. We analyzed 17-paired samples for CDH1 mRNA and differences of expression were observed (Figure 3A). For CAV1 mRNA, 37-paired samples were analyzed, and were significantly increased in tumors samples (5.3781 ± 1.6950) than in normal gastric mucosa (4.2630 ± 1.5015) (p=0.0004) (Figure 3B). Relative quantification of CDH1 and CAV1 mRNA did not show any association with clinicopathological characteristics.

**DISCUSSION**

In the present study, 63.8% of tumor samples did not present E-cadherin immunoreactivity and absence of this protein was associated with gastric carcinogenesis (Table 1). This association has been widely described in gastric cancer from patients from other populations. Decreased expression of E-cadherin has been observed in this neoplasia ranging from 17-92%, depending on the methodology and the definition used [34]. Moreover, our findings also showed that lack of E-cadherin is more frequent in diffuse than intestinal type cancer, confirming previous studies in sporadic gastric adenocarcinomas [35-37]. Here, we also reported a negative correlation between E-cadherin expression and metastasis, supporting the hypothesis that loss of E-cadherin expression is a prerequisite for tumor cell invasion and metastasis [3, 4].

Loss of E-cadherin mediated cell-cell adhesion may also affect the Wnt-signaling pathway, including the modulation of MYC gene [39, 40], an important oncogene in gastric carcinogenesis [41]. In previous studies, we have observed that all gastric cancer samples of individuals from Northern Brazil, including early gastric cancer, presented positive MYC immunoreactivity [42-44].

We did not observed any differences between quantification of CDH1 mRNA from neoplastic and non-neoplastic gastric samples. This may
reflect an unknown regulatory mechanism that affects this protein translation. A recent study reports E-cadherin regulation by miRNA in breast cancer [38].

CDH1 promoter methylation has been indicated as the main mechanism of E-cadherin inactivation in gastric cancer. Several studies have reported a strong association between CDH1 methylation and the decrease/lack of its expression [34, 45, 46] (Table 2). However, we did not observe an association between CDH1 methylation and protein expression, probably due to the higher frequency of promoter methylation in our sample.

The frequency of CDH1 promoter methylation was about 90% in both neoplastic and non-neoplastic specimens. Thus, no association between CDH1 methylation and the carcinogenesis process was found, corroborating data from Zazula et al [47]. In a previous study with a smaller sample size, we had shown that all advanced gastric tumors of individuals from Northern Brazil presented CDH1 methylated sequences [33].

The stomach presents the highest level of methylated CpG island in non-neoplastic cells, along with age-related methylation that reflects increased CpG island methylation frequency in gastric cancer [48]. Moreover, our research group did not observe the influence of aging in CDH1 methylation pattern in peripheral lymphocytes [49], suggesting that increased frequency of CDH1 methylation in the aging process is tissue specific or limited to gastric mucosa. The high frequency of methylation in the stomach might be related to the accessibility of the tissue to exogenous agents, such as dietary factors or heavy metals to which it is directly exposed [50]. Reactive oxygen species – caused, in example, by H. pylori – may also play a role in aging-related methylation in the stomach [51, 52].

Here we describe that Caveolin-1 expression in 84.2% of the gastric adenocarcinomas while only 5.6% of normal gastric tissue presented positive staining, suggesting a pro-tumorigenic action in this malignancy (Table 1). Higher mRNA levels are also observed in tumor samples, when compared to normal gastric mucosa (Figure 3). However, the literature data is controversial. Burgermeister et al [53] and Gao et al [54] described only 7% and 17.9%, respectively, of Caveolin-1 expression in gastric cancer. On the other hand, a more recent study relates 94% of Caveolin-1 expression in neoplastic gastric cells [55], thus similar to our study. It was suggested that Caveolin-1 is down-