Original paper

Immunohistochemical expression analysis of MMP-1, TIMP-2 and p53 in Barrett's esophagus, dysplasia and esophageal adenocarcinoma

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Barrett's esophagus (BE) is the most important risk factor for the development of esophageal adenocarcinoma. It develops through a progressive sequence of histologic and molecular events that begin with metaplasia and then progresses through various stages of dysplasia. Matrix metalloproteinases are involved in the degradation of the extracellular matrix and play an important role in tumor progression.
The immunohistochemical expression of MMP-1, TIMP-2 and p53 in 111 samples from 45 patients diagnosed with BE with and without dysplasia and adenocarcinoma of the esophagus was retrospectively studied, and statistical analysis was conducted to measure the association between their expression and the degree of dysplasia present.
MMP-1 was expressed in 33.3% of the samples studied, mainly in the adenocarcinoma subgroup with up to 40% positive cases ($p = 0.494$). In contrast, TIMP-2 was expressed in 25.2% of the samples, and no positive cases were identified in the adenocarcinoma subgroup ($p = 0.037$). Aberrant p53 expression was observed in 81.4% of the samples diagnosed with some degree of dysplasia ($p < 0.001$).
MMP-1 showed no statistically significant differences between diagnostic entities. A statistically significant loss of TIMP-2 expression was observed in distal esophageal adenocarcinoma samples, which contrasts with the aberrant expression of p53 in dysplastic cases.

Key words: Barrett's esophagus, esophageal adenocarcinoma, matrix metalloproteinases, p53, immunohistochemistry.

Introduction

Barrett's esophagus (BE) affects between 2% and 7% of the adult population in Western countries and is the most important risk factor for developing esophageal adenocarcinoma [1]. The criteria used to establish the diagnosis are not uniform and vary across countries. In the United States, the American College of Gastroenterology requires the presence of intestinal metaplasia for making a diagnosis [2]. The British Society of Gastroenterology requires only metaplastic columnar epithelium, and the presence of goblet cells is not mandatory [3]. In Spain, the Spanish Society of Pathology (SEAP) acknowledges the need to identify goblet cells to diagnose BE [4].

The evolution of BE to adenocarcinoma develops over a series of histological and molecular events that begins with metaplasia and then progresses through various degrees of dysplasia before neoplasia development [1]. In 2020, it is estimated that 18,440 new cases of esophageal adenocarcinoma will occur in the United States, which will cause 2.7% of cancer deaths, with a 5-year survival rate of 20% [5]. Currently, the risk of progression of BE to adenocarcinoma is determined by the presence and histological grade of dysplasia [6]. This correlation is higher when the diagnosis is issued by an expert gastrointestinal pathologist [6]. This risk varies from 0.3% per year for a patient diagnosed with BE without dysplasia to 8% per year for a patient diagnosed with high-grade dysplasia [6]. Medications such as proton pump inhibitors, nonsteroidal anti-inflammatory agents such as aspirin, and statins are associated with a reduced risk of progression [2].

The aggressiveness of malignant tumors is determined by their ability to modify the peritumoral connective tissue, which allows stromal invasion and neoplastic cells to access the vascular and lymphatic system [7]. Among the proteolytic enzymes associated with tumor invasion, one of the most important, due to its ability to cleave most of the components of the extracellular matrix and basement membrane, is the family of matrix metalloproteinases (MMPs) [7]. Currently, 28 different types of MMPs are known in vertebrates, of which at least 23 are expressed in human tissue [8]. Based on their substrate specificity, sequential similarity and domain organization, they can be divided into six subgroups: collagenases, gelatinases, stromelysins, matrilysins, membrane-type metalloproteinases and other metalloproteinases [8]. Alterations in the activity of MMPs can cause numerous diseases, depending on the underlying pathology caused by tissue destruction, fibrosis or weakening of the matrix [8].

The activity of these enzymes is controlled by endogenous inhibitors, some of which are general protease inhibitors, such as α2-macroglobulin, and others are more specific, such as tissue inhibitors of metalloproteinases (TIMPs) [7], numbered sequentially (TIMP-1, -2, -3, -4) [7]. In addition to blocking different MMPs, these inhibitors are directly involved in postischemic apoptotic processes, cell differentiation and proliferation, and tumor growth inhibition, metastasis, and angiogenesis [9].

MMP-1 (collagenase-1) is produced by a variety of normal cells as well as numerous tumors and is associated with many malignancies; there is a negative correlation between its expression and survival [10]. TIMP-2 is a plasma protein that suppresses tumor growth and angiogenesis and inhibits epithelial-to-mesenchymal transition [11].

The aim of this study was to evaluate the immunohistochemical expression of MMP-1, TIMP-2 and p53 in samples from patients diagnosed with BE and adenocarcinoma of the esophagus.

Material and methods

Patient selection

Patients diagnosed with BE and BE-associated esophageal adenocarcinoma were selected after identifying the presence of goblet cells in biopsies from Hospital Universitario Central de Asturias (HUCA) between 2000 and 2014; non-cancer patients had at least three consecutive diagnostic biopsies. The study was restricted to patients in whom there was sufficient histological material for performing immunohistochemical staining. In cases where more than three samples were available, the sample from the initial diagnosis, the last available sample and the sample closest to half the time between the previous two samples were selected. Both endoscopic and surgical resection samples were included. We also included 20 samples of normal squamous epithelium of the distal esophagus.

The follow-up time was calculated from the first diagnosis of BE available to the last sample taken within the time frame provided for the study (180 months). In patients who were diagnosed with adenocarcinoma, the follow-up time was calculated from the diagnosis of malignancy to the completion of the study or the patient's death, whichever occurred first.

Demographic and clinical data were obtained by consulting patient medical records. Age corresponds to the date of the first histopathological diagnosis.

Samples

The tissue samples were fixed in 10% buffered formalin, embedded in paraffin and stained with hematoxylin and eosin (HE) according to the conventional protocol of the Department of Pathology of HUCA for diagnosis. All selected cases were reviewed, and the most representative samples were selected. The selected blocks were cut into 4-μm thick sections, and HE staining was performed in the first and last sections to confirm the presence of sufficient tissue, reserving intermediate sections for immunohistochemical studies. The HE slides were reviewed by one of the authors (AGV) and two independent pathologists (JCG and FFF) to reach a consensus on the presence or absence of dysplasia and its grade.

Immunohistochemistry

The tissue slides were processed using the Agilent Dako Autostainer Link 48. Deparaffination, rehydration and antigen retrieval were carried out in a PT-Link (Agilent Dako) at 97°C for 20 minutes with EnVision FLEX Target Retrieval Solution at pH 6.1 for MMP-1 and TIMP-2 and pH 9 for p53. MMP-1 (polyclonal rabbit antibody; Thermo Fisher Scientific, USA), TIMP-2 (monoclonal mouse antibody; Thermo Fisher Scientific, USA), and p53 (prediluted monoclonal mouse antibody, clone DO-7; Agilent Dako, USA) were used. For concentrated antibodies, dilutions were established based on negative and positive controls with 1 : 100 for MMP-1 and 1:200 for TIMP-2 using the EnVision FLEX Antibody Diluent for this purpose. Endogenous peroxidase activity was blocked by incubating the slides for 5 minutes in the commercial solution intended for this purpose (Agilent Dako). The incubation times were 30 minutes for p53 and 60 minutes for MMP-1 and TIMP-2 at room temperature. EnVision FLEX+ was used as the staining detection system. Finally, the sections were counterstained with hematoxylin, dehydrated and cover slipped with permanent media. The sections were examined and photographed under a light microscope with an Olympus DP70 camera.

Immunohistochemistry assessment

Immunohistochemical staining was evaluated independently by two of the authors (AGV and JSJ). The immunohistochemical signal intensity for MMP-1 and TIMP-2 was assigned according to the microscope magnification used, a method known as the "magnification rule" developed by Ruschoff *et al.* [12]. A four-point scale was obtained: negative expression, weak or barely visible expression confirmed only at × 40, moderate expression that can be observed at × 10/× 20 and strong expression visible at × 2.5/× 5. Cases with weak or no staining at all were considered negative as opposed to cases of moderate and intense expression that were considered positive. Based on the experience of Kastelein *et al.* [13], we evaluated p53 staining as normal expression of the protein (wild-type) or aberrant expression. In nonneoplastic epithelium, normal p53 staining was weak, heterogeneous and localized to the crypts. Aberrant expression included intense nuclear staining as well as complete loss of expression compared with the remaining histologically nondysplastic glands, which served as internal controls. After all scoring had been completed, a consensus score for each case was determined. When there was disagreement, the slides were evaluated by both pathologists simultaneously to reach a consensus score.

Table I. Patient demographics and immunoexpression markers of BE, dysplasia and esophageal adenocarcinoma

	Total N (%)	Barrett's esophagus			Adenocarcinoma N (%)	p-value
		No dysplasia N (%)	Low-grade dysplasia N (%)	High-grade dysplasia N (%)		
Patients	45 (100)	25 (55.6)	4 (8.9)	1 (2.2)	15 (33.3)	
Median age (years)						
≤ 59	23 (51.1)	18 (72)	2 (50)	0 (0)	3 (20)	> 0.05
> 59	22 (48.9)	7 (28)	2 (50)	1 (100)	12 (80)	
Sex						
Male	36 (80)	20 (80)	2 (50)	1 (100)	13 (86.7)	> 0.05
Female	9 (20)	5 (20)	2 (50)	0 (0)	2 (13.3)	
Samples	111 (100)	68 (61.3)	17 (15.3)	6 (5.4)	20 (18)	
MMP-1 expression						
Negative	74 (66.7)	44 (64.7)	14 (82.4)	4 (66.7)	12 (60)	0.494
Positive	37 (33.3)	24 (35.3)	3 (17.6)	2 (33.3)	8 (40)	
TIMP-2 expression						
Negative	83 (74.8)	48 (70.6)	11 (64.7)	4 (66.7)	20 (100)	**0.037**
Positive	28 (25.2)	20 (29.4)	6 (35.3)	2 (33.3)	0 (0)	
p53 expression						
Normal	75 (67.6)	67 (98.5)	6 (35.3)	0 (0)	2 (10)	**< 0.001**
Aberrant	36 (32.4)	1 (1.5)	11 (64.7)	6 (100)	18 (90)	

Bold indicates statistically significant findings.

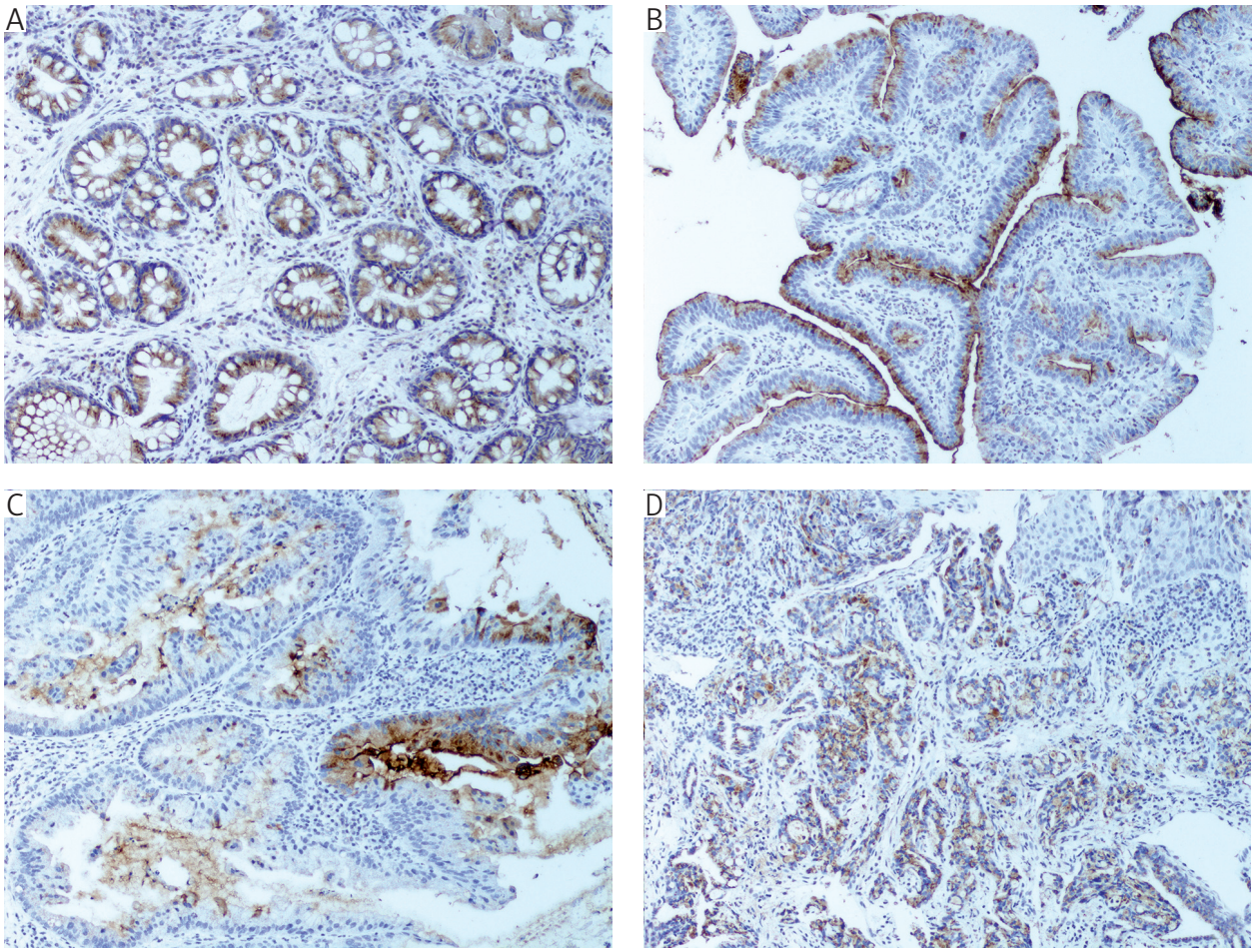

Fig. 1. Immunohistochemical expression of MMP-1. A) BE without dysplasia; B) BE with low-grade dysplasia; C) BE with high-grade dysplasia; D) adenocarcinoma, 100×

Statistical analysis

Standard descriptive statistics were recorded for the baseline demographic and clinical characteristics of each patient and the corresponding pathological data, with quantitative variables expressed as the mean and standard deviation and qualitative variables expressed as the frequency. The association between qualitative variables was determined by a contingency table and χ^2 test. In all cases, a p-value < 0.05 was accepted as statistically significant. All analyses were performed using IBM SPSS Statistics V24.0 for Windows.

The study protocol was approved by the Research Ethics Committee of the Principality of Asturias (Spain) and complied with ethical and data protection standards (approval no. 02/16).

Results

The study included 111 samples from 45 patients who met the inclusion criteria (Table I). Of the 45 patients, 36 (80%) were men. The overall mean age of our patients at the time of the first biopsy was 58.16 ±14.82 years. By group, the mean age of patients with BE without dysplasia (n = 25) was 53.20 ±14.56 years, that of patients with BE with low-grade dysplasia (n = 4) was 56 ±13.29 years, that of patients with BE with high-grade dysplasia (n = 1) was 63 years, and that of patients with adenocarcinoma (n = 15) was 66.67 ±12.83 years. The mean follow-up time was 89 months (range 31-169) for patients with no tumors and 48 months for patients with adenocarcinoma (range 3-113).

The diagnoses in the 111 samples were BE without dysplasia in 61.3% of the biopsies, low-grade dysplasia in 15.3%, high-grade dysplasia in 5.4% and invasive adenocarcinoma in 18%. In the group of patients without dysplasia, 24% progressed to low-grade dysplasia (100 months, range 53-153), 4% to high-grade dysplasia (101 months), and none to adenocarcinoma. Of the patients initially diagnosed with low-grade dysplasia, dysplasia could not be confirmed in 25% in follow-up biopsy studies, 25% maintained low-grade dysplasia, and 50% progressed to high-grade dysplasia (57 months, range 49-64). For the only patient diagnosed with high-grade dys-

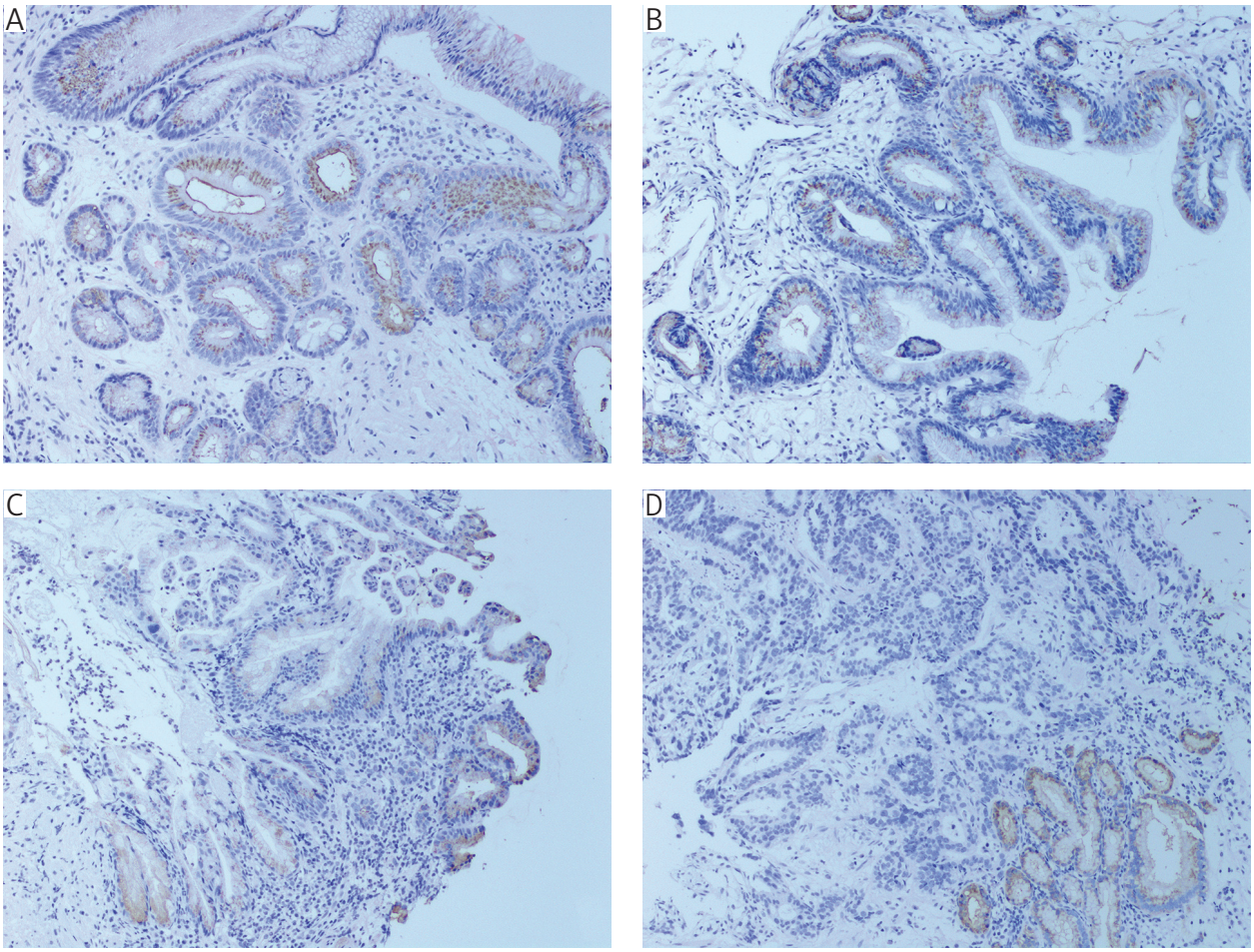

Fig. 2. Immunohistochemical expression of TIMP-2. A) BE without dysplasia; B) BE with low-grade dysplasia; C) BE with high-grade dysplasia; D) adenocarcinoma (note the weak control staining in nontumoral glands). 100 ×

plasia, only low-grade dysplasia and absence of dysplasia could be observed in follow-up biopsies.

The immunohistochemical study was performed on the 111 available samples. In the 20 samples of normal distal esophagus used as a control, no epithelial staining with MMP-1 was observed, and 45% of these samples showed staining with TIMP-2. In positive cases, MMP-1 and TIMP-2 were expressed in the glandular epithelial cytoplasm (Figs. 1 and 2). The expression of p53 was located in the nuclei (Fig. 3). MMP-1 was expressed in 33.3% of the samples studied, and no statistically significant differences in expression were observed among the diagnostic subgroups ($p = 0.494$; Table I, Fig. 1). The immunoexpression of TIMP-2 was constantly negative in the samples of distal esophageal adenocarcinoma, while in the subgroups of BE without dysplasia, BE with low-grade dysplasia and BE with high-grade dysplasia, we observed similar percentages of positivity ($p = 0.037$; Table I, Fig. 2). The aberrant expression of p53 was associated with premalignant and malignant transformation, as it was observed in 73.9% of the samples of BE with dysplasia and in 90% of the samples of distal esophageal adenocarcinoma; this contrasted with 1.5% of samples in which cytologic atypia was not identified ($p < 0.001$; Table I, Fig. 3).

Discussion

In this study, we analyzed the expression of MMP-1 and TIMP-2 in the normal mucosa of the distal esophagus and in BE with different degrees of dysplasia and adenocarcinoma. It should be noted that the diagnosis of BE was based on the histological presence of goblet cells in the mucosa of the distal esophagus. Given the system used for obtaining samples and that our population is similar to those reported in the literature, it is of note that the evolution seen in our patients was similar to that observed by Wani *et al*. [14] and O'Connor *et al*. [15], 18% and 19.2% of whose patients diagnosed with BE developed low-grade dysplasia and 2.7% and 3.2% developed high-grade dysplasia, with a mean follow-up of 66 and 48 months, respectively.

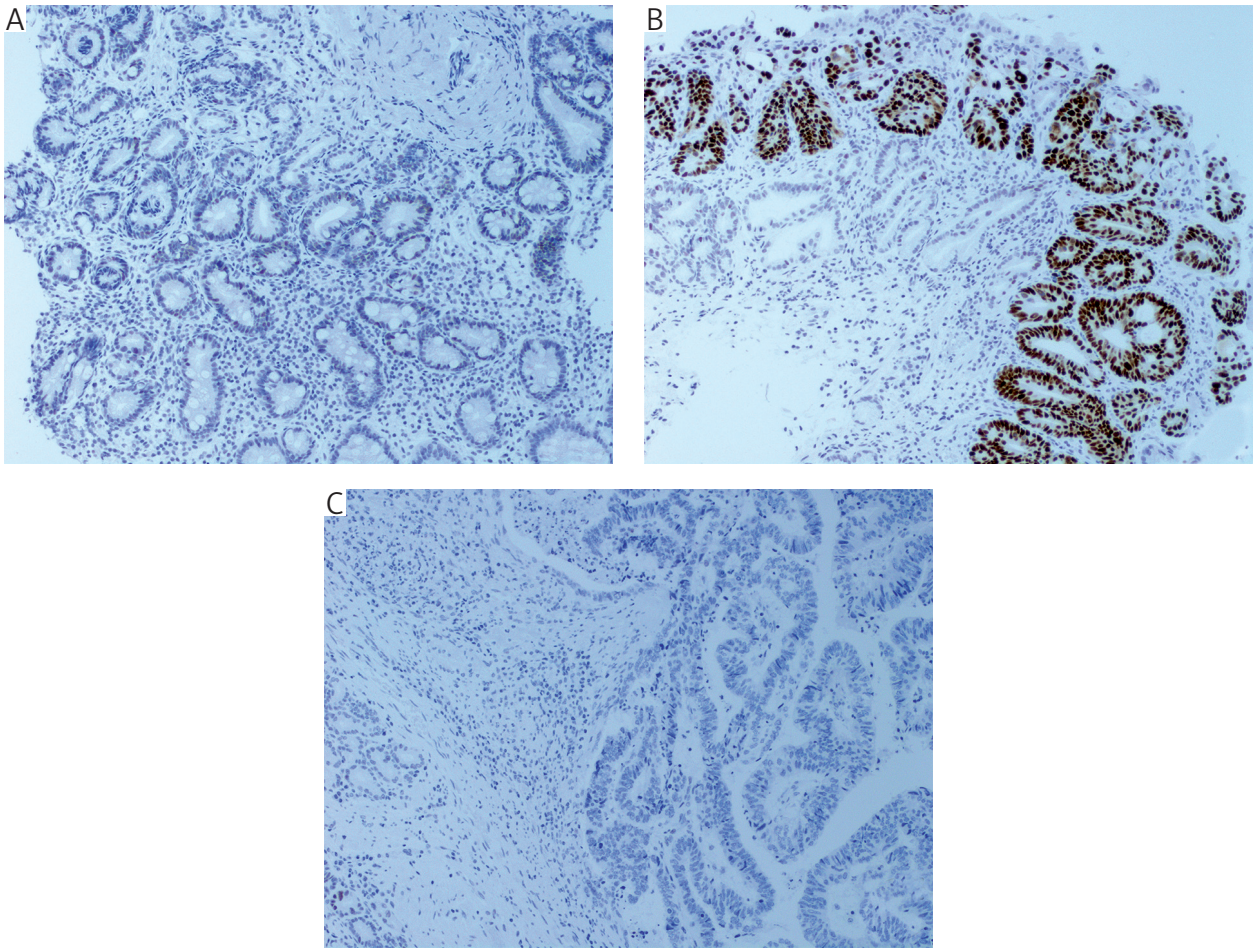

Fig. 3. Immunohistochemical expression of p53. A) normal pattern (wild type); B) aberrant pattern with intense nuclear overexpression; C) aberrant pattern with total absence of staining (note the weak control staining in nondysplastic glands), 100 ×

The expression of various MMPs and TIMPs has been extensively researched over the past 30 years. For BE and esophageal adenocarcinoma, the studies have been limited; most published studies on MMPs have been on MMP-1 [16, 17, 18], MMP-7 [16, 19], MMP-9 [20], MMP-10 [16], MMP-12 [16], MMP-13 [16, 18, 20], TIMP-1 [16] and TIMP-3 [16].

Regarding the expression of MMP-1 in samples with BE and adenocarcinoma, our results are superior to those reported by Salmela *et al.* [16], who published a series using in situ hybridization techniques, which included 4 samples from 5 patients diagnosed with BE without dysplasia. They did not detect any specific signals from a relevant number of cells obtained from these patients. In addition, 19 samples from 15 patients diagnosed with adenocarcinoma were evaluated; for these samples, they reported specific signals in a moderate or high number of cells in 21% of them. The differences can be attributed to the fact that the techniques used were different and that the number of samples in the nondysplastic BE group was much lower than that used in our series.

In our study, 40% of the adenocarcinoma cases expressed MMP-1, similar to what Murray *et al.* [17] found; their study also concluded that this expression was associated with a worse prognosis in patients diagnosed with esophageal carcinoma, both squamous and adenocarcinoma. One of the studies with the highest numbers of patients, carried out by Grimm *et al*. [18], identified immunohistochemical expression of MMP-1 in 56% of patients with BE without dysplasia and in 95% of patients with BE-associated adenocarcinoma. These results are superior to those found in our series, differences that we attributed to the dissimilar methodologies used. For positive cases, this study included those whose weak staining was perceived only with a ×40 objective. Using a similar methodology in our series, the expression of MMP-1 was recognized in 85% of the samples of adenocarcinoma associated with BE.

For TIMP-2, we did not find studies that performed immunohistochemical staining in samples from patients with BE. The few studies published

on tumor esophageal biopsies are limited to those on squamous cell carcinoma or those that do not discriminate between tumor types.

TIMP-2 was expressed in 45% of normal mucosa samples, consistent with the findings of the study by Sharma *et al.* [21]. They observed the expression of TIMP-2 in 50% and 72% of samples with squamous dysplasia and squamous cell carcinoma of the esophagus, respectively, which was not associated with tumor staging. Plum *et al*. [22] presented results without discriminating between squamous cell carcinomas or adenocarcinomas that were limited to cases with invasion circumscribed to the submucosa. Despite these limitations, they concluded that TIMP-2 expression is an independent risk factor for increased tumor infiltration depth (pT1a vs. pT1b). In the biopsies with adenocarcinoma from our series, no expression was found in the tumor epithelium, and we have not found any published study with which to compare our results. Groblewska *et al*. [23] published a study measuring the serum levels of TIMP-2 in 53 patients with esophageal cancer, and although patients diagnosed with squamous cell carcinoma predominated in this group, they concluded that there was no correlation between the serum concentration of TIMP-2 and tumor stage.

Regarding the rest of the gastrointestinal tract studies, Lu *et al.* [24] investigated the immunohistochemical expression of TIMP-2 in patients diagnosed with adenocarcinoma of the gastroesophageal junction and reported tumor cytoplasmic and adjacent stromal cell expression in 52.5% of their cases, although they did not find a significant association of this expression with clinicopathological parameters. The differences from our study may be due to the different locations and etiopathogenic mechanisms. Alakus *et al.* [25] described low expression in 65.5% of their cases of gastric adenocarcinoma, which was associated with an advanced pathologic stage and poor clinical outcome. Joo *et al.* [26] found weak or negative tumor cell staining in their cases of gastric adenocarcinoma and described strong positivity only in peritumor stromal cells. Similarly, Ring *et al.* [27] published results indicating weak or negative epithelial staining of TIMP-2 in all of their cases of colorectal carcinoma. Comparing colorectal tumor samples with paired adjacent noncancerous tissues, Wang *et al.* [28] found that TIMP-2 expression was much lower in tumor tissue, revealing a significant positive association between low expression and pathological classification, depth of invasion, lymph node metastasis and TNM stage.

With regard to the p53 protein expression analysis, we had similar findings to those observed in numerous studies, such as those published by Kastelein *et al.* [13] and Kaye *et al*. [29], who determined that the aberrant expression of p53 is more common in dysplastic cases. In our study, the percentage of patients diagnosed with BE with low-grade dysplasia showing aberrant p53 expression was higher than that found by Kastelein *et al.* [13] (64.7% vs. 38%). This difference could lie in the fact that our biopsies were diagnosed by a consensus of gastrointestinal experienced pathologists, thus confirming what Skacel *et al*. [30] stated, after finding aberrant p53 expression in 56% of the samples of BE with low-grade dysplasia, that a consensus diagnosis and the concomitant use of p53 are useful for identifying patients with a higher risk of progression to high-grade dysplasia and adenocarcinoma.

Our results support the use of p53 and are in line with the recent finding of van der Wel *et al.* [31] that adding p53 immunostaining significantly improved the proportion of indefinite for dysplasia diagnoses, the interobserver agreement and the diagnostic accuracy between dedicated GI pathologists. Also, the systematic review and meta-analyses by Snyder *et al.* [32] suggest that the immunohistochemical assessment of p53 in BE biopsies can be useful for risk stratification and assignment of patients to groups that might benefit from intensive surveillance or endoscopic eradication therapy.

It must be said that not everybody recommends the use of p53 for routine evaluation. Srivastava *et al.* [33], on behalf of the Rodger C. Haggitt Gastrointestinal Pathology Society, stated that although p53 is a promising marker for identifying high-risk patients, existing data are insufficient to recommend it for routine use as a prognostic marker.

Our study has several limitations, including potential biases due to its retrospective nature, the absence of standardized staining and evaluation schemes that limit the comparison of our study with others (in our case, we used an easy evaluation system), the lack of previous research studies on TIMP-2 to make comparisons, the fact that the study was performed at a single center and that the study did not include a second sample group to validate the hypothesis generated.

Conclusions

No MMP-1 staining was observed in the mucosa of the distal esophagus, and 45% of the samples showed TIMP-2 staining. MMP-1 showed no statistically significant differences between BE samples with different degrees of dysplasia and adenocarcinoma. TIMP-2, which is described for the first time in patients diagnosed with BE with and without dysplasia and adenocarcinoma, was negative in all BE-associated adenocarcinoma samples; therefore we think it might serve as a progression marker. Finally, our results indicate, as in previous publications, that a con-

sensus diagnosis and the complementary use of p53 can be useful for determining the presence of dysplasia in BE.

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The authors declare no conflict of interest.

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