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Original Article

Immunohistochemical Analysis of KI-67 (MIB-1) Antigen in Columnar Mucosa of the Distal Esophagus in Patients with GERD

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Abstract

Introduction: Lack of intestinal metaplasia in the columnar mucosa of the distal esophagus is characteristic of tissue at the stage preceding progression through the metaplasia-dysplasia-adenocarcinoma sequence. Therefore, it is necessary to seek molecular markers capable of predicting progression to more severe disease stages.**Objectives**: To determine the Ki-67 index of columnar mucosa in patients with gastroesophageal reflux disease. **Methods** Sixty-two patients with columnar mucosa in the distal esophagus were divided into two groups. Group 1 (G1, 30 patients) had columnar epithelia without intestinal metaplasia; group 2 (G2, 32 patients) had columnar epithelia with intestinal metaplasia. All patients were subjected to digestive endoscopy and biopsy. Ki-67 was assessed by immunohistochemistry in all proliferative compartments of the intestinal crypt. **Results**: Proliferative activity (Ki-67 reactivity) within group G1 was limited to the basal epithelial layer in 83.3% of patients, with 3.3% showing reactivity up to the middle layer, and 13.3% showing immunoreactivity in the epithelial surface. The corresponding histological populations within group G2 were 46.9%, 21.9%, and 31.2%, respectively. A significant increase was found in the prevalence of proliferative activity in the compartments above the basal layer of the crypt in patients with metaplastic columnar mucosa ($p<0.001$). **Conclusion**: The presence of proliferative activity above the basal layer in patients with columnar mucosa may assist in the identification of patients more prone to malignant transformation or intestinalization of the gastric mucosa.

Keywords: Ki-67 (MIB-1); Barrett's esophagus; Columnar mucosa; Intestinal metaplasia

Introduction

The current definition of Barrett's esophagus was adopted in 1988, when the American College of Gastroenterology (ACG) defined Barrett's esophagus as the replacement of stratified squamous epithelium that normally lines the esophagus with columnar epithelium, of any extent, that may be endoscopically identified and histologically confirmed by the presence of intestinal metaplasia.[1] This condition is present in more than 10% of patients with long-standing Gastroesophageal Reflux Disease (GERD). The distal esophagus lined by columnar epithelium without intestinal metaplasia is considered a "columnar-lined esophagus", and up to 20% of such cases may be diagnosed as intestinal metaplasia in subsequent examinations. This indicates that the condition is progressive, and therefore requires patient follow-up.[2]

The relationship between GERD symptoms and

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the risk of developing adenocarcinoma is caused by the association of gastroesophageal reflux and Barrett's esophagus.[3] The epithelium with specialized intestinal metaplasia is prone to malignant transformation in 64%-86% of esophageal adenocarcinomas cases originating from Barrett's esophagus.[4-5] Although the exact mechanism through which reflux causes esophageal cancer remains uncertain, it is believed that inflammation and chronic irritation may be the determinant factors in this carcinogenic process.[6-7] Consequently, endoscopic surveillance with subsequent biopsy has become the standard follow-up procedure when this epithelium is found in distal esophagus. Dysplasia is the most accepted histological marker in current clinical practice for identifying patients at a higher risk of developing adenocarcinoma in the metaplasia-dysplasia-carcinoma sequence. However, dysplasia has several limitations as a prognosis marker. One of the most significant limitations is the fact that patients come from heterogeneous groups. Patients with high-grade dysplasia may have occult adenocarcinoma in up to 40% of cases; [5,8] this makes high-grade dysplasia a questionable marker in clinical surveillance of neoplastic progression of Barrett's esophagus.[9] The natural evolution of low-grade dysplasia is also uncertain due to factors such as intra- and inter-observer diagnosis variability, sampling problems and higher regression rates for non-neoplastic epithelium.[1] Therefore, the discovery of new biological markers capable of complementing histological findings and predicting the evolution of Barrett's esophagus is of paramount importance.

The Ki-67 antigen is present in all active stages of the cell cycle (G1, S, G2, and M) except the G0 stage, due to loss of the labile part of this nuclear antigen. [8] The increase of antigen expression occurs as the cell progresses through the cycle, both in normal and malignant epithelia. [10-12] Cell proliferation can be assessed by the number of active cells in cell cycle (proliferation index) and by Ki-67's location in the proliferative compartments of the epithelium.[13] The cell proliferation index, for which Ki-67 is a marker, has been studied as a prognosis factor in various types of cancer, including colorectal, bladder, breast and non-Hodgkin's lymphoma.[10,14-15] The proliferative compartment is normally restricted to the basal layer of the intestinal crypt, in the so-called "neck" of the gland. The expansion of cell proliferation into other proliferative compartments of the intestinal crypt, with marked cells throughout the epithelium from the basal layer to the epithelial surface, characterizes malignant potential of the gastrointestinal tract.[11]

The present study aimed to assess cell proliferation by analyzing Ki-67 (MIB-1) expression in the mucosa of the distal esophagus in patients with GERD, and its expression in the proliferative compartments of the intestinal crypt in columnar epithelia with or without intestinal metaplasia of the esophagus.

Materials and Methods

Patients

Patients aged 30 or over, referred to the Digestive Endoscopy Unit between January 2003 and December 2006, with typical symptoms of gastroesophageal reflux such as pyrosis and or regurgitation (at least once a week for a minimum of five years) were prospectively and consecutively examined. Before the accomplishment of upper gastrointestinal endoscopy and biopsy as per the research protocol, patients were excluded if they had cardiac disease or coagulation disorders, gastro-esophageal varices, hepatopathy, recent upper gastrointestinal hemorrhage, prior diagnosis of Barrett's esophagus, previous radiotherapy or chemotherapy treatment, or hypersensitiveness to iodine. Patients were also excluded if they used anticoagulants and/or AINES, if they had undergone acid suppressor therapy over the last 60 days, or if they had undergone esophageal or stomach surgery. Only those patients with columnar mucosa were included in the study. Patients with normal mucosa, reflux esophagitis visible at upper gastrointestinal endoscopy, or cancer of the esophagus were also excluded.

Upper Gastrointestinal Endoscopy

An orally inserted videoendoscope (Fujinon 2200, Tokyo, Japan) was used for inspection of the esophagus, stomach and duodenum. With the endoscope placed near Z line, chromoscopy was performed with a 3% Lugol solution (12g/L + 24mg/L in 1000mL water), with the purpose of providing better definition of the gastroesophageal junction and highlighting the area of columnar-lined esophagus in distal esophagus. The parameter used to evaluate the size of mucosal lesions was

the open biopsy forceps (7mm). The Z line was defined by the squamous-columnar junction, while the gastro-esophageal junction was defined by the proximal margin of the gastric folds. The detachment of the diaphragm corresponded to the endoscopic impression of the diaphragm muscle. The length of the columnar mucosa of the esophagus was determined by the distance of the proximal and distal margins of the mucosa in relation to the upper dental arch and the end of the gastric folds in the esophagus, respectively. The definition of "short segment Barrett's esophagus" was used when the mucosa extended less than 3 cm and "long segment Barrett's esophagus" was defined by mucosa corresponding to 3 cm or more of metaplastic columnar epithelium.

Biopsy

After chromoscopy with Lugol solution, the visible columnar mucosa in the distal esophagus was biopsied according to the Seattle protocol, with 4 quadrant biopsies every 1-2 cm. [16] Biopsy of the gastroesophageal junction and, subsequently, in the proximal direction, of the entire columnar-lined esophagus was performed with the use of a biopsy forceps with central stylet. The specimens were placed on filter paper, fixed in 10% formalin solution and embedded in paraffin.

Histopathological analysis

For histological examination, all sections were stained with hematoxylin and eosin (HE) and Alcian blue (pH 2.5). The samples were considered adequate if they included two well-oriented intact crypts. The histological findings were classified as follows: adequate; inadequate; columnar mucosa without intestinal metaplasia and columnar mucosa with intestinal metaplasia (defined by positive reactivity of caliciform cells to Alcian blue). Slides were examined and diagnosis was made by two independent pathologists with experience in gastrointestinal pathology. Their agreement was measured using the kappa test. In cases of disagreement, the final result was obtained by consensus between both pathologists.

Immunohistochemical analysis

Biopsy specimens were evaluated by immunohistochemical using Ki-67 (DAKO clone MIB-1) to stain for nuclear antigen. Immunohistochemical analysis was performed using the avidin-biotin-peroxidase complex method. In brief, after dewaxing, inactivating endogenous peroxidase activity and blocking cross-reaction with normal serum, sections were incubated overnight at 4oC with 1:400 diluted solution of MIB-1. Streptavidin-biotin complex was used to locate the primary antibody, and diaminobenzidine-tetrahydrochloride was used as chromogen. Material from cecal appendix was used as a positive control, whereas the negative control was determined by omission of the primary antibody. Cells that displayed a dark brown nuclear stain, similar to the positive control, were considered to be Ki-67-positive. The final result was assessed by the proliferation index for every case, which is calculated from the average of stained cells and the total analyzed cells, with a minimum count of 500 cells, as previously described by Feith et al.[14] The distribution of immunoreactivity (nuclear stain) in the proliferative compartments of the esophageal columnar epithelium (basal layer, middle layer and epithelial surface of the intestinal crypt) was assessed by optical microscope, as previously described by Wada et al.[17] Immunohistochemical reading of the slides was performed by the same pathologists who performed the conventional histopathology. They had no previous knowledge of related clinical and endoscopic information. In case of disagreement, the final result was obtained by consensus between both pathologists.

The Statistical Package for Social Science (SSPS), version 12.0, was used for data processing and storage. The Ki-67 data was shown as mean averages with standard deviations, and compared between categories through variance analysis (ANOVA). The identification of differences, whenever present, was made by the Tukey test. For analysis of the expansion of cell proliferation into the proliferative compartments, the chi-squared test was used. Results were considered significant when $p<0.05$.

Patients were informed about the research procedures and required to sign an informed consent form. The research was approved by the GPPG (Group of Research and Post-Graduation) Ethics Committee of the Hospital de Clínicas, Porto Alegre.

Results

Two hundred and thirty-five patients with long-standing gastroesophageal reflux symptoms (>5 years) were evaluated from January 2003 to December 2006. Of these, 62 patients (26.3%) diagnosed with columnar-lined

distal esophagus were selected. After excluding 58 patients with normal mucosa, 80 patients with chronic esophagitis and 35 patients with adenocarcinoma Siewert's type I and II, the 62 patients were divided into two groups following the anatomo-pathological study of tissue samples: 30 (48.4%) patients with columnar mucosa without intestinal metaplasia and 32 (51.6%) patients with columnar mucosa with intestinal metaplasia.

The index Ki-67 (MIB-1) in the proliferative compartments of the intestinal crypts (basal layer, middle layer, and epithelial surface) was 37±26.3, 16±14, and 6±3 in patients with columnar epithelia without intestinal metaplasia, and was 52±24.6, 12±8, and 4±3, in patients with columnar epithelia with intestinal metaplasia (Table 1).

Table 1 - Ki-67 index for each histologic and for each tissue zone: basal layer, middle layer and epithelial surface

	CM without intestinal metaplasia	CM with intestinal metaplasia	p
Basal layer	37±26.3	52±24.6	<0.05
Middle layer	16±14	12±8	
Epithelial surface	6±3	4±3	

Regarding the distribution of immunoreactivity (Ki-67) in the proliferative compartments in patients with columnar epithelium without intestinal metaplasia, the Ki-67-positive cells were restricted to the basal layer of the gland in 25 cases (83%). In 1 case (3%) positive cells were detected up to the middle layer, and in 4 cases (13%) immunoreactivity was detected up to the epithelial surface of the esophageal crypt (Figure 1).

In patients with columnar mucosa with intestinal metaplasia, there were 15 cases (46.9%) with Ki-67-positive cells restricted to the basal layer of the gland, 7 cases (21.9%) with positive cells up to the middle layer, and 10 cases (31.2%) with immunoreactivity up to the epithelial surface (Figure 2). There was good concordance between the two pathologists regarding Ki-67 immunoreactivity at each proliferative compartment (kappa = 0.6).

The prevalence of Ki-67-positive cells above the basal proliferative compartment, that is in the middle layer and epithelial surface, in patients with columnar epithelium without intestinal metaplasia was 16.6%,

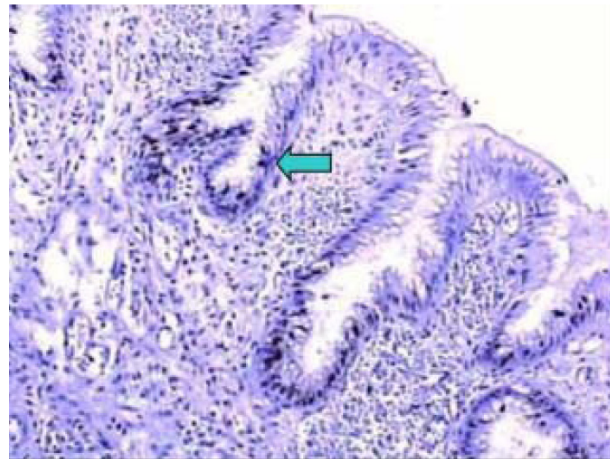

Figure -1 Ki-67 (MIB-1) proliferative activity in the proliferative compartments of the columnar mucosa with intestinal metaplasia (basal layer and middle layer of the gland – green arrow)

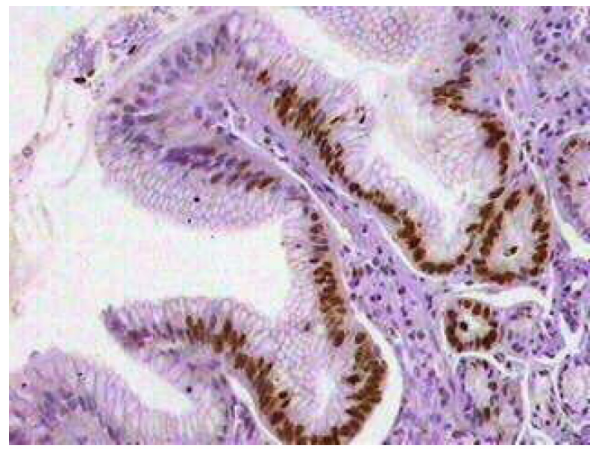

Figure 2 - Ki-67 (MIB-1) proliferative activity in the proliferative compartments of the columnar mucosa with intestinal metaplasia

whereas, in patients with columnar epithelium with intestinal metaplasia, the prevalence of positive cases above the normal proliferative compartment of the intestinal crypt was 53.1% (Figure 3).

Discussion

Over the last 15 years there has been a significant increase in the number of studies on cell proliferation and its role in esophageal carcinogenesis. [4,11-12,18-20]

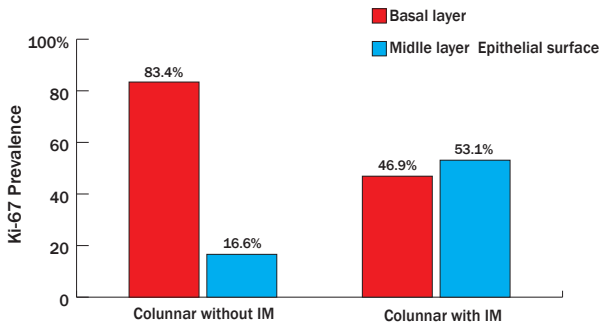

Figure 3 - Stratification of Ki-67 immunoreactivity in patients with columnar mucosa between the middle layer/ epithelial surfaces of the gland ($p<0.001$)

Immunohistochemical determination of cell proliferation, and its location in the proliferative compartments of the intestinal crypt, has been related to progression of the metaplasia-dysplasia-adenocarcinoma sequence.[8,14-15,21] The present study analyzed expression of the proliferation-associated antigen Ki-67 (MIB-1) in the distal esophageal columnar epithelium with and without intestinal metaplasia in patients with GERD. The Ki-67 antigen reflects the level of proliferative activity in active stages of the cell cycle, especially in the mitotic phase.[11] Quantitative assessment of cell proliferation (Ki-67) has been performed by means of expensive and sophisticated techniques that use digitalized images, as well as by flow cytometry, a complex method which requires fresh tissue samples and is not accessible to most histopathological diagnosis centers.[11,22-24] Pathologists usually perform semi-quantitative analysis of cell proliferation (Ki-67) using an optical microscope due to its great feasibility and accuracy. For such analysis, the Ki-67 proliferation index has been used, which considers the percent of marked cells per total number of cells assessed, in a given representative section of a tissue sample.[11] The qualitative study of proliferative activity is based on the type of tissue and the topographic location of marked cells in the proliferative compartments of the epithelium.[10-11,25] In this study, we have used this method to qualitatively and quantitatively qualitatively assess any correlations between histologic subtypes. It is worth stressing the potential limitation of the so-called sampling error in the diagnosis of intestinal metaplasia, and in the presence of dysplastic lesions, which may occur in patients with columnar mucosa of the distal esophagus where no intestinal metaplasia or dysplasia was detected by the biopsy. Such aspects are discussed in many studies that report metaplasia regression after adequate gastroesophageal reflux treatment.[26] In order to minimize this bias, all biopsies in our patients were performed by the same endoscopist and according to the Seattle protocol,[16] in order to ensure that biopsy sites and standard are uniform.

Our results showed that Ki-67 immunoreactivity was located throughout the tissue, in the basal layer, middle layer and epithelial surface of the intestinal crypt, both in patients with and without intestinal metaplasia in the columnar mucosa. This finding indicates that cell proliferation occurs evenly in the stage preceding intestinalization of the columnar mucosa of the distal esophagus. However, there was no statistically significant difference in the percentage of positive nuclei (proliferation index) in each of the crypt's proliferative compartments among patients with and without intestinal metaplasia in the columnar mucosa. Even so, it is possible that those patients who showed cell proliferation at the epithelial surface, even without metaplasia, are more prone to progression to intestinal metaplasia in the columnar mucosa, and may have a worse prognosis concerning the progression of the metaplasia-dysplasia-adenocarcinoma sequence.

Feith et al.[14] reported that Ki-67-positive proliferative activity in the columnar epithelium of patients with intestinal metaplasia without dysplasia, and in squamous epithelium, was restricted to the normal proliferative compartment of each epithelium ("neck" of the gland and basal layer, respectively), where cell proliferation normally occurs. The authors concluded that expansion of cell proliferation into the proliferative compartments represents an initial stage in the progression of Barrett's esophagus to esophageal adenocarcinoma. Kim et al.[27] also found that the increase in expansion of cell proliferation into proliferative compartments, with immunoreactivity in the epithelial surface of the intestinal crypt, indicates disordered cell growth. Hong et al.[11] demonstrated that the Ki-67 proliferation index and its expansion into proliferative compartments correlated with neoplastic progression in the metaplasia-dysplasia-carcinoma sequence, and that Ki-67 immunoreactivity was restricted to the gland zone and the basal layer of the intestinal crypt in samples of Barrett's esophagus without dysplasia. Ishizuka et al.[28] analyzed the immunohistochemical expression of Ki-67 in patients with endoscopic diagnosis of columnar mucosa in the distal esophagus. They showed that Ki-67-immunoreactive cells were localized to the middle layer and epithelial surface, and were more numerous in patients with columnar epithelium with intestinal metaplasia (51% of the cases). The cardiac columnar epithelium group occurred in 25% of the cases with immunoreactive cells above the area where cell proliferation normally occurs. According to the authors, identification of Ki-67 immunoreactivity outside the

normal proliferation zone of the intestinal crypts provides evidence of increased cell proliferation, and also represents a potential for malignant degeneration of intestinal metaplasia in Barrett's esophagus.[11-12,14,28]

Thus, due to the expansion of proliferative activity outside the area where cell proliferation normally occurs, periodic follow-up is recommended for patients with this immunoreactivity pattern, even without significant evidence of increased Ki-67 proliferation index in the respective proliferative compartments. Our findings indicate a predominance of 16.6% and 53% of cases with proliferative activity above the basal layer of the gland in patients with columnar epithelium without and with intestinal metaplasia, respectively. The intestinalization of the columnar mucosa is known to be related to the presence of alkaline reflux. Also, alkaline stimuli are related to proliferative stimuli *in vitro*.[29] Therefore, it is possible that these patients, with non-metaplastic columnar mucosa but with a superficial increase in cell proliferation, may require closer follow-up since their clinical prognosis may be similar to that of patients with Barrett's esophagus. This subgroup of patients, as well as all the other patients included in our study, will be provided with long-term follow-up, and their progress may shed light onto some of these issues.

We found that the distribution of immunoreactivity, as assessed by Ki-67 antigen, was not restricted to the normal proliferative compartment in patients with columnar mucosa in the distal esophagus. In contrast to other studies, we took note of the expansion of proliferative activity from the basal layer of the gland into the epithelial surface in the two study groups. We also found a statistically significant difference in the expansion index between patients with columnar mucosa with and without intestinal metaplasia ($p<0.001$).

The results of the present study reveal an association between cell proliferation into all proliferative compartments of the intestinal crypt (basal layer, middle layer, and epithelial surface), and the process of intestinalization of the columnar mucosa in the distal esophagus in patients with long-standing symptoms of gastroesophageal reflux. Further studies including the use of Ki-67 and other molecular markers may confirm the importance of such markers in predicting the progression of histological alterations in the columnar mucosa in the distal esophagus.

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