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Gastric and intestinal differentiation in Barrett's metaplasia and associated adenocarcinoma

P. Chaves, C. Cruz, A. Dias Pereira, A. Suspiro, J. C. M. de Almeida, C. N. Leitão, J. Soares

Grupo de Estudo do Esófago de Barrett, Instituto Português de Oncologia Francisco Gentil, Centro Regional de Oncologia de Lisboa SA, Portugal

SUMMARY. **Intestinal metaplasia is a prerequisite criterion for the diagnosis of Barrett's metaplasia and the sole columnar esophageal lining associated with malignancy. It is recognized by the presence of goblet cells, but columnar non-goblet elements, producing gastric or intestinal proteins, are the prevalent cell population. The cellular heterogeneity of Barrett's metaplasia is well documented but the relationship between the distinct cell subtypes and neoplasia is unclear. Our aim was to clarify the relationship between the different metaplastic populations and malignancy in order to investigate putative markers for risk stratification of Barrett's patients. We studied 46 columnar-lined esophageal segments, 15 with associated adenocarcinoma. The presence of the gastric, MUC5AC and MUC6, and the intestinal, MUC2, proteins was evaluated in metaplastic (columnar and goblet) and neoplastic cells. In neoplasia MUC5AC and MUC6 were detected in 100% and 86.6% of the cases, respectively. In metaplasia there were no differences in MUC5AC and MUC6 immunoreactivity, between cases with and without associated neoplasia, except for goblet elements producing MUC6 that were exclusive of metaplasia adjacent to adenocarcinoma ($P < 0.05$). MUC2 was present in 86.6% of the neoplasia. In metaplasia it was restricted to Barrett's cases and was more frequent in areas with intestinal metaplasia. Columnar-lined esophagus without intestinal metaplasia did not express MUC2. Our study suggests a relationship between the metaplastic population with gastric phenotype and malignancy, and points to the involvement of columnar as well as goblet elements in tumorigenesis. The association between goblet cells aberrantly producing MUC6 and the presence of neoplasia suggests they may be useful for risk stratification.**

KEY WORDS: **apomucins, Barrett's adenocarcinoma, Barrett's esophagus, differentiation, malignant transformation.**

INTRODUCTION

Barrett's esophagus, the premalignant condition predisposing to esophageal and cardia adenocarcinoma, is defined as a change in the esophagus, of any length, that is endoscopically recognized and histologically confirmed to have intestinal metaplasia.[1–4]

Columnar-lined esophageal segments include three subtypes of metaplasia but only the intestinal type is associated with malignancy.[5–7] The metaplastic epithelia exhibiting gastric phenotype, the *junctional* or *cardiac* and the *atrophic fundic* or *oxynto-cardiac* types are regarded as carrying no risk of malignant transformation.[7,8]

Address correspondence to: Paula Chaves, MD, PhD, Serviço de Anatomia Patológica, Instituto Português de Oncologia Francisco Gentil, Rua Prof. Lima Basto, 1093-Lisboa Codex, Portugal. Email: pchaves@ipolisboa.min-saude.pt

The metaplastic population includes goblet and columnar non-goblet cells.[7,8] The former are regarded as the histological marker to recognize intestinal differentiation and to select patients for surveillance programs.[4,6] The columnar non-goblet cells are the prevalent metaplastic element of Barrett's epithelium and display a wide range of phenotypic characteristics that are also identified in associated neoplasia.[9–15] This observation supports their involvement in the metaplasia – dysplasia – adenocarcinoma sequence, and leads to the admission that metaplastic elements other than goblet cells could be related to malignancy.[9,13] However, the role of the columnar non-goblet elements in the malignant transformation of Barrett's esophagus remains unclear.[9,13,15]

Mucins are genetically codified high molecular weight glycoproteins with heavily glycosilated proteic cores, the apomucins.[16] They are synthesized by

epithelial cells as membrane-bound or secreted products. MUC2 is present in normal bowel, being the determinant component of mucus in goblet cells. MUC5AC and MUC6 are the apomucins found in normal foveolar and mucopeptic gastric cells, respectively. Alterations of the mucin genes and respective proteic products were identified in gastrointestinal tract tumorigenesis, namely in Barrett's neoplasia.[17–20] Nevertheless, the relationship between these alterations and the distinct cellular subtypes of esophageal metaplasia is unclear and the role of these abnormalities along the process of Barrett's tumorigenesis is unknown.

In this study, we investigated the expression of apomucins MUC2, MUC5AC and MUC6 in columnar-lined esophagus without and with intestinal metaplasia and in associated neoplasia. The immunoreactivity in the two metaplastic elements, columnar non-goblet and goblet, was separately assessed and cases with and without associated neoplasia were compared. Our aim was to evaluate apomucin immunoexpression at the cellular populations of columnar-lined esophagus and to clarify the relationship between the distinct metaplastic cell lineage and Barrett's adenocarcinoma in order to identify markers that could be useful for risk stratification of patients.

MATERIALS AND METHODS

Study population

The histological material of 46 patients was selected from the Barrett's Esophagus Surveillance Program database of the Instituto Português de Oncologia, CROL SA, Portugal, and was organized into four groups:

- Group I: nine endoscopic biopsies from patients with long segments (≥ 3 cm) of columnar-lined esophagus without intestinal metaplasia;
- Group II: 22 endoscopic biopsies from patients with Barrett's esophagus, recognized by the presence of intestinal metaplasia in any extension of red-velvet, gastric-like mucosa endoscopically identified above the esophagogastric junction.[4] Areas without and with intestinal metaplasia were separately assessed as Groups IIA and IIB, respectively;
- Group III: 15 surgical specimens of Barrett's esophagus adjacent to neoplasia (high grade dysplasia or adenocarcinoma). Areas without and with intestinal metaplasia were separately assessed as Groups IIIA and IIIB, respectively;
- Group IV: 15 surgical specimens of Barrett's neoplasia (high grade dysplasia or adenocarcinoma).

Biopsies from patients of Groups I and II were performed according to the following protocol: in long segments, four-quadrant biopsies at 2 cm intervals starting at the esophagogastric junction; in short segments a minimum of five samples were collected. The evaluation was performed in biopsy samples obtained at the first endoscopy (index endoscopy). The absence of intestinal metaplasia in Group I was confirmed through the observation of iterative biopsies obtained during a 5-year period. None of the patients in Groups I and II progressed to either dysplasia or adenocarcinoma during a mean follow-up of 8 years (range: 7–11 years) after the index endoscopy.

Barrett's neoplasia was identified by the recognition, in the esophagus, of intestinal metaplasia adjacent to the tumor.

We studied 72 and 310 mucosal samples in Groups I and II, respectively; in Groups III and IV 42 samples of metaplastic tissue and 32 samples of neoplasia, were analyzed, respectively.

Immunohistochemical analysis

Two monoclonal antibodies reacting against the gastric apomucins MUC5AC (CLH2) and MUC6 (CLH5), kindly provided by L. David MD PhD, IPATIMUP, Portugal, were used. The monoclonal antibody NCL-MUC2 (clone Ccp Novocastra, Newcastle, UK), was used for the identification of the intestinal apomucin MUC2.

Antigen retrieval used a domestic pressure cooker during 1 min at maximum pressure. The optimal dilution of the antibodies CLH2 and CLH5 in TBS were 1 : 50. The optimal dilution of NCL-MUC2 antibody was 1 : 20. Bound antibody was detected using biotinilated rabbit F(ab′)2 antibody directed against mouse immunoglobulin (DAKO, Glostrup, Denmark), and thereafter an avidin-biotin complex linked to horseradish peroxidase (Vector, Burlingame, USA). All incubations were carried out at room temperature and the primary antibodies were incubated overnight at 4°C. A solution of diaminobenzidine was used as chromogenic substrate.

Normal gastric and colon mucosa were used as positive controls for the detection of MUC5AC/MUC6 and for MUC2 antigens, respectively. A result was considered positive whenever at least 5% of the epithelial cells exhibited diffuse cytoplasm or supranuclear/Golgi immunostaining. Immunohistochemical staining for each marker was assessed, in a blinded fashion, by one of the authors (PC).

Statistical analysis was performed using the Fisher and the McNemar exact tests to compare the four groups and the presence versus absence of intestinal metaplasia within the same group, respectively. A statistically significant value was considered at $P < 0.05$.

RESULTS

The results obtained with the antibodies anti–MUC5AC, MUC6 and MUC2 in metaplastic and

Table 1 MUC5AC, MUC6 and MUC2 at metaplastic (columnar and goblet) and neoplastic cells

Groups	Cell type	MUC5AC	MUC6	MUC2
I ($n = 9$) – CLES without IM	Columnar	9 (100%)	9 (100%)	0 (0%)
IIA ($n = 22$) – BE (areas without IM)	Columnar	22 (100%)	21 (95.4%)	10 (45,5%)
IIB ($n = 22$) – BE (areas with IM)	Columnar	22 (100%)	19 (86.3%)	16 (72,7%)
	Goblet	22 (100%)	0 (0.0%)	21 (95,4%)
IIIA ($n = 15$) – BE adjacent to BA (areas without IM)	Columnar	15 (100%)	15 (100%)	6 (40,0%)
IIIB ($n = 15$) – BE adjacent to BA (areas with IM)	Columnar	15 (100%)	14 (93.3%)	13 (86,6%)
	Goblet	15 (100%)	9 (60.0%)	14 (93.3%)
IV ($n = 15$) – BA	Neoplastic	15 (100%)	13 (86.6%)	13 (86,6%)

CLES, columnar-lined esophageal segments. BE, Barrett's esophagus. IM, intestinal metaplasia. BA, Barrett's adenocarcinoma. MUC6: Goblet cells of IIB *versus* Goblet cells of IIIB $P < 0.05$. MUC2: I versus IIA $P < 0.05$; I versus IIIA $P < 0.05$; IIA versus Columnar cells of IIB $P < 0.05$; IIIA versus Columnar cells of IIIB $P < 0.05$.

neoplastic cells of the four groups are summarized in Table 1.

MUC5AC was detected in Barrett's metaplasia as well as in neoplasia in all the cases. In areas of metaplasia (Groups I, II and III) the apomucin was present at both, columnar non-goblet and goblet cells (Fig. 1). Its detection was not related to the presence of concomitant neoplasia. In metaplastic areas MUC5AC was mostly observed at the surface epithelium, with focal expression in the deep glandular structures.

MUC6 was identified in metaplastic columnar non-goblet cells from Groups I, IIA and IIIA and in neoplastic cells (Group IV), with no significant differences. MUC6 expression in metaplastic goblet elements (Fig. 2) was restricted to cases of metaplasia adjacent to Barrett's neoplasia (9/15 cases of Group IIIB), and it was not observed in any case without neoplasia (Group IIB). The difference between the groups was statistically significant ($P < 0.05$). In metaplasia, MUC6 was expressed focally at the surface epithelium and intensely at the deeply seated glandular structures.

MUC2 at the columnar non-goblet elements was exclusive to Barrett's esophageal segments (Groups II and III). The metaplastic elements of columnar-lined segments without intestinal metaplasia (Group I) showed no MUC2 immunoexpression. These differences were statistically significant ($P < 0.05$). The columnar cells in Barrett's segments of Groups II and III (significant at $P < 0.05$), showed more frequent MUC2 positivity in areas with intestinal metaplasia (Group IIB and Group IIIB) than in areas without intestinal metaplasia (Group IIA and Group IIIA), respectively.

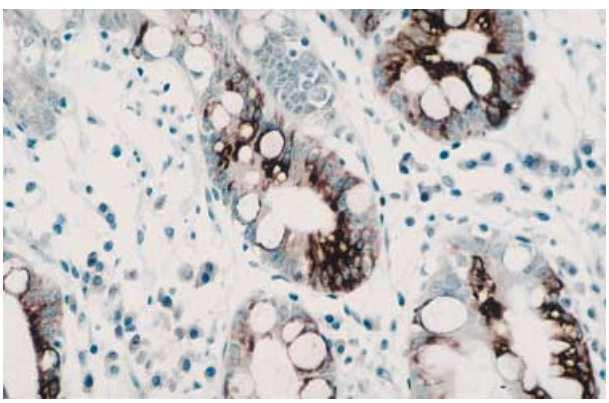

Fig. 1 MUC5AC at columnar non-goblet and goblet cells.

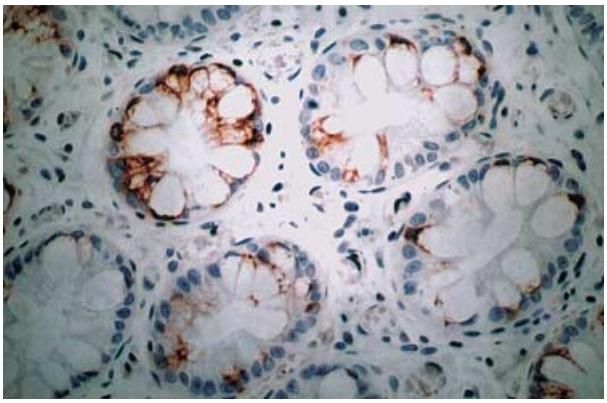

Fig. 2 MUC6 at columnar non-goblet and goblet cells. MUC6 at the goblet elements was restricted to cases with associated adenocarcinoma.

DISCUSSION

Among patients with columnar-lined esophageal segments, only those with intestinal metaplasia, recognized by the presence of goblet cells, carry an increased risk of adenocarcinoma.[4] The risk of adenocarcinoma in Barrett's esophagus is estimated as 0.5% each year, and only a minority of patients progresses to cancer. Epidemiological data support that most of the patients developing neoplasia are male Caucasians over 50 years of age, but there are no morphological markers to identify individual risk.

In this study we addressed the question whether MUC5AC, MUC6 and MUC2 immunoexpression in esophageal metaplasia and associated neoplasia could help to clarify the role of the goblet and the columnar non-goblet metaplastic elements in malignant transformation, and whether they could be used to assess tumor risk stratification.

Our results demonstrate that gastric and goblet phenotypic features, assessed by MUC5AC, MUC6 and MUC2 immunodetection, are both present in the two metaplastic populations as well as in the neoplastic elements. They also show that the metaplastic goblet cells that aberrantly produce the gastric apomucin MUC6 are associated with the presence of carcinoma. These observations strongly favor the association of the two metaplastic cell lineages, goblet and columnar non-goblet, as well as the two cellular phenotypes, gastric and intestinal, with malignancy. They also suggest that the metaplastic goblet cells with aberrant mucopeptic characteristics may be a putative biomarker for the clinical management of Barrett's esophagus patients.

The presence of metaplastic elements with gastric characteristics as part of the histological spectrum of Barrett's esophagus was previously recognized by histological, ultrastructural and immunocytochemical techniques.[7,8,10,11,21–24] Recently, it was also recognized that Barrett's epithelium, unequivocally associated with the development of adenocarcinoma, is a columnar metaplasia extensively expressing gastric markers.[23,24] Nevertheless, it is widely accepted that only the metaplastic lineage with intestinal characteristics is associated with the risk of malignant evolution.[1,2,23–25] This was morphologically suggested by the frequent detection of metaplastic cells with intestinal features adjacent to esophageal adenocarcinoma and is supported by the positive correlation between MUC2 expression and high proliferative rates observed in Barrett's epithelium.[2,24,25] Specific gastric markers were recently identified in cases of esophageal metaplasia but the association between gastric features and malignancy was not recognized, and the role of the elements with gastric differentiation in Barrett's tumorigenesis remains unclear.[23,24] In the present study we showed that gastric characteristics are both present in metaplastic and in neoplastic cells, which strongly suggest the involvement of the metaplastic elements with gastric phenotype in malignant transformation.

Furthermore, we detected the three apomucins at the goblet as well as at the columnar non-goblet elements of Barrett's metaplasia. This was independent of the presence of associated cancer, except for MUC6 positivity in goblet cells that was restricted to areas adjacent to carcinoma. This suggests that gastric characteristics may be observed either in the columnar non-goblet or in the goblet elements of Barrett's epithelium, being the aberrant production of MUC6 by goblet metaplastic cells associated with the presence of neoplasia.

Barrett's epithelium, histologically identified by the presence of goblet elements, is assumed to represent an incomplete form of intestinal metaplasia associated with the risk of malignant progression.[7–9] Columnar-lined esophagus without intestinal metaplasia is regarded as carrying no considerable risk of malignancy. In a previous study using sucrase-isomaltase, we demonstrated that all columnar esophageal lining should be regarded as incomplete forms of intestinal metaplasia regardless the presence or absence of goblet elements.[13] Recently, Reis *et al*. found that intestinal metaplasia of the stomach has two main phenotypes.[26] The non-gastric type, observed in complete intestinal metaplasia reflects a switch in the cell differentiation program. The mixed (gastric and intestinal) type represents an aberrant differentiation program and is related to incomplete intestinal metaplasia.[26] The present study showed that all esophageal columnar metaplastic epithelia have a mixed (gastric and intestinal) phenotype, similar to that observed by Reis *et al*. in the incomplete type of intestinal metaplasia of the stomach.[26] This mixed immunophenotype emerges as non-dependent of the presence of goblet metaplastic cells and is present in metaplasia as well as in associated neoplasia. Furthermore, our results showed that the two cellular metaplastic subtypes, columnar non-goblet and goblet elements, are related to the malignant transformation of Barrett's esophagus.[13]

Recently, Glickman *et al*. verified that the immunoexpression of MUC6 in goblet cells of Barrett's esophagus is distinct from that observed in intestinal metaplasia of the stomach.[27] They found MUC6 positivity in goblet cells in 32% of Barrett's cases while it was not detected in any case of intestinal metaplasia of the gastric antrum. In contrast to our results, these authors did not found any difference regarding clinical and endoscopic features, between cases with MUC6-positive versus MUC6-negative goblet cells. Methodological aspects may explain these apparent discrepancies. In our study Barrett's esophagus cases with and without associated neoplasia were independently assessed, while in Glickman's study there is no explicit mention to the concomitant presence or absence of neoplasia. None of our patients with Barrett's esophagus (Group II) had progressed to dysplasia or cancer after a mean surveillance period of 8 years, while there is no information about patient follow-up in Glickman's paper. This makes impossible any comparison between the results of both studies.

Our study on MUC2, MUC5AC and MUC6 immunoexpression in Barrett's esophagus points to the existence of a biopathological relationship between columnar metaplasia expressing gastric features and neoplasia. This observation challenges the view that the risk for malignancy is exclusively associated with the presence of intestinal differentiation. The involvement of both metaplastic populations, columnar non-goblet and goblet, in Barrett's tumorigenesis is therefore suggested by our results and the goblet population aberrantly producing MUC6 emerges as a useful potential biomarker associated with malignancy.

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