HISTOCHEMICAL AND IMMUNOHISTOCHEMICAL STUDY OF BARRET’S OESOPHAGUS SEQUENCE – DYSPLASIA – GASTRO-ESOPHAGEAL ADENOCARCINOMA

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Summary

The etiology of gastro-esophageal adenocarcinomas is related to gastro-esophageal reflux disease and the development of Barrett’s esophagus or cardia intestinal metaplasia, a pre-neoplastic condition which is diagnosed endoscopically with an histological confirmation, an incomplete intestinal metaplasia. Adenocarcinomas of the gastric cardia and distal esophagus are among the most deadly of all gastro-intestinal malignancies, with 5-year mortality rates exceeding 80% (Souza and Spechler, 2005). The medical societies have recommanded endoscopic cancer surveillance routinely for patients with Barrett’s metaplasia or cardiac intestinal metaplasia. The rising incidence and poor prognosis of gastro-esophageal adenocarcinomas have intensified research into earlier methods of detection of this diseases and the relationship to Barrett’s esophagus. In the present study we investigates several histochemical and immunohistochemical features of gastro-esophageal adenocarcinomas, 87 resection specimens of Barrett’s esophagus and adenocarcinoma junction were carefully selected. We chosed the histochemical examination of the intestinal metaplasia lesions of gastro-oesophageal junctions and the immunohistochemical study for COX - 2 for evidence the lesions of Barrett’s oesophagus sequence – dysplasia – adenocarcinoma and the potential of utilization in the early detection of gastro-esophageal adenocarcinomas junction.

Keywords: Barrett’s oesophagus; intestinal metaplasia; dysplasia; adenocarcinoma junction; immunohistochimical methods.

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Introduction

Barrett’s oesophagus is a premalignant condition that predisposes to the development of oesophageal adenocarcinoma. Barrett's esophagus represents the replacement of the normal squamous epithelium of the lower esophagus by specialised intestinal metaplasia, under the influence of chronic gastro-oesophageal reflux (Spechler et al., 1996; Weinstein et al., 1996; Kim et al., 1997; DeMeester et al., 2000).

The intestinal metaplasia of the gastro-oesophageal junction, as a result of a chronic inflammation, irrespective of etiology, is considered an important risk factor for the development of the lesion towards dysplasia and gastroesophageal carcinoma junction.

Dysplasia is a histopathological marker for the early diagnosis of adenocarcinoma at the level of gastro-oesophageal junction, as evidenced by the simultaneous presence of the same resection pieces on both dysplasia and neoplasia (Sampliner 1998; Spechler, 2002).

The distal oesophagus adenocarcinoma and gastric cardia adenocarcinoma seem to predominate in the case of Caucasian men (M:F=7:1) (Blot et al, 1991; Lagergren, 2005), with a similar average age (56-62 years), the diagnosis being late – in advanced stages of disease and the prognosis being unfavorable, with 5-year mortality rates exceeding 80% (Ruol et al, 2000). Surgical cure rates (subtotal esophagectomy and proximal gastrectomy) are compromised by the fact that most
patients are diagnosed at the late stage of disease of the delayed onset of symptoms.

In this study I have made a detailed analysis within 87 cases of Barrett's oesophagus sequence - dysplasia – adenocarcinoma at the level of gastro-oesophageal junction.

In the case of histochemical study of metaplasia lesions in gastro-oesophageal junctions, the histochemical marker pointed out the acid mucin within goblet cells of intestinal metaplasia.

The immunohistochimical study included the analysis for COX - 2, the marker possible useful for the precocious diagnosis of Barrett's oesophagus sequence – dysplasia – junctional adenocarcinoma.

**Material and methods**

**Patients** Between 2000-2009, in the Histopatological Laboratory from Emergency Hospital of Craiova and within the framework of “VICTOR BABES” Institute of Bucharest we recorded 751 cases: reflux esophagitis, motor disorders (achalasia and esophageal diverticula), Barrett’s (premalignant lesion), inflammatory esophagitis (viral, bacterial or fungal), chemical or drug-induced esophagitis or neoplastic lesions (benign tumors, dysplasia or malignancy).

The material for this study is based on the gastroesophagean biopsy specimens collected from the gastro-oesophageal sections and on the surgical resection specimens.

From 587 of cases diagnosed with gastric cancer we selected 68 cases of gastro-oesophageal adenocarcinoma junction. Also from 154 cases diagnosticated in the same periode with oesophageal biopsy, we selected 19 cases of benign and malignant Barrett's oesophagus.

The patients included in this study presented symptoms of gastro-esophageal reflux disease which included: heartburn and regurgitation at least twice within 1 week and persisted at least six months, all the symptoms could be relieved significantly with H2 receptor blocker or proton pump inhibitor, odynophagia with progresiv dysphagia addition decrease weight, occult haemorrhages (seldom).

**Endoscopy and biopsy protocol**

Endoscopic dates included macroscopic changes to the gastro-esophageal junction: a red velvety mucosa it can extend either circumferentially or as one or several tongues, polipoid lesions, ulcerations, presence or absence of hiatus hernia. For each resection specimen a sample of tumor tissue and normal tissue was available in paraffin – embedded slides. The biopsy protocols included four quadrant biopsies at 2 cm levels in distal esophagus above and below the esophago-gastric junction and sample of normal tissue. Specimens were fixed in 10% buffered formalin, embedded in paraffin wax and carefully microdisected.

**Histochemical and immunohistochemical study**

For the histochemical examination, the sections was made at 4 µm and the preparations was examinated using haematoxylin-eosin (H&E) and van Gieson staining, because only the adequate preparations must be reserved. In order to emphasize the complete/incomplete intestinal metaplasia there were used particular staining: Periodic Acid Schiff stain (PAS), PAS/blue alcian, blue alcian at 2,5 and 1,5 pH.

For the immunohistochimical analyses for COX – 2 it was chosen the indirect tristadial immunohistochemical method – IHC- the avidin-biotin - peroxidase Complex (ABC) after Hsu et al. (1981) modified by Bussolati and Guliotta (1983).

Two-micrometer-thick serial sections were cut from each paraffin-embedded block. The sections were deparaffinized in xylene and rehydrated through graded concentrations of alcohol slides were incubated in 3% hydrogen peroxide for 20 min to block nonspecific background staining due to endogenous peroxidase. Antigen retrieval was performed by using 10 mM citrate buffer, ph 6.0 for 10-20 min in microwave. Using the standard streptavidin-biotin peroxidase complex method were performed on all slides, were incubated for
30 minutes in secondary antibody solution. Diaminobenzidine (DAB) was used as a chromogen and developed a brown precipitate, very fine, localizing the antigen. The slides were counterstained with Mayer’s hematoxylin. Negative controls were counterstained with the same tumor samples and staining methods by omitting the primary antibodies.

The antibody used in immunohistological studies have identified the molecular biomarker followed for the research of metaplasia–dysplasia–adenocarcinoma junction; the positive expression was represented by the membranar and cytoplasmic brown precipitate.

**Results**

From 87 cases of the current study it was identified 19 cases of Barrett’s oesophagus with benign (16) and malignant lesions (3).

We observed that: the clinical symptomatology has a low frequency and intensity, it predominate in males (M:F=4:1:1), it has been associated with hiatus hernia only in 7 cases and the symptom's period has varied between 4 month and 28 years (Table 2).

Of the 42 cases with chronic gastro-oesophageal reflux, in 7 cases were identified lesions of chronic associated esophagitis, identified by the presence of a chronic lymphoplasmocitic inflammatory process within the mucosal chorion with intraepithelial lift, fibrous - conjuctive reshuffle, mucosal erosions and exulcerations, hyperplasia papillomatosis of squamous coverage epithelium (Figure 1,2).

Also, there were 68 cases of adenocarcinoma junction divided topographically: at the distal esophagus (3 cases), at the junction (48 cases) and subcardial (17 cases).

Regarding distribution by age groups, it ranged between 33 and 77 years. The average age for benign lesions was 45 years and 63 years for malignant lesions (Table 1).

**Table 1.** Distribution by age groups of Barrett's oesophagus sequence - dysplasia – junctional adenocarcinoma

<table>
<thead>
<tr>
<th>Age Groups</th>
<th>Number of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>30-39</td>
<td>2</td>
</tr>
<tr>
<td>40-49</td>
<td>3</td>
</tr>
<tr>
<td>50-59</td>
<td>32</td>
</tr>
<tr>
<td>60-69</td>
<td>42</td>
</tr>
<tr>
<td>70-79</td>
<td>8</td>
</tr>
</tbody>
</table>

**Table 2.** Clinical symptomatology associated Barrett's oesophagus sequence – dysplasia – adenocarcinoma junction

<table>
<thead>
<tr>
<th>Clinical symptomatology</th>
<th>Number of cases (n(%))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dysphagia</td>
<td>76 (87,3)</td>
</tr>
<tr>
<td>Haemorrhage/bleeding</td>
<td>11(12,6)</td>
</tr>
<tr>
<td>Odynophagia</td>
<td>15(17,2)</td>
</tr>
<tr>
<td>Chronic gastro-oesophageal reflux</td>
<td>42(48,2)</td>
</tr>
</tbody>
</table>

**Figure 1.** Appearance of chronic esophagitis with inflammatory infiltrate in the mucosal chorion and intraepithelial lift, squamous epithelial hyperplasia papillomatosis of squamous coverage epithelium and erosions. Hematoxylin-eosin staininig (H-E). Ob. 40x
The group of patients with benign and malignant Barrett's esophagus lesions presented two diagnostic criteria - the histological identification and delimitation of a Barrett's metaplasia segment at least 3 inches long - changes present in all 19 cases. The lesions were located in the upper esophagus in 4 cases and in the lower esophagus in 15 cases.

Esophagus lined by cylindrical epithelium showed three distinct types of epithelium: gastric cardial epithelium, fundic gastric and intestinal specialized. Cardial gastric and gastric fundic/fundus epithelium resemble the normal epithelium of the stomach excepting the presence of some distortions of the gastric mucosa, glandular atrophy and medium inflammation, identified in 10 and respective 6 cases (Figure 3).

Specialized intestinal epithelium presented a villiform configuration and cryptic glands delimited as villous intestinal type by glands composed of absorptive enterocytes and goblet cells and it was found in 13 cases (Figura 4). Goblet cells were intensely positive for acid mucins histochemical highlighted with Alcian blue at pH 2.5 (Figure 5; 6).

No patient presented only fundic epithelium in biopsy fragments (Table 3).

**Figure 2.** Appearance of chronic esophagitis with chronic inflammatory infiltrate in the mucosal chorion with intraepithelial lift (arrow). Periodic Acid Schiff stain (PAS). Ob. 1000x

**Figure 3.** Appearance of Barrett's metaplasia - cardiac phenotype and fundic gastric similar with gastric epithelium but with glandular distortion and atrophy and with average/medium inflammation. PAS stain Ob.40x

**Figure 4.** Barrett's specialized intestinal metaplasia with mucip cells predominance easily identified with haematoxylin-eosin (arrow). HE stain. Ob.40x

**Figure 5.** Barrett intestinal metaplasia with highlighting of goblet cells riched in acid mucopolysaccharides blue colored with Alcian blue (arrow). Alcian blue stain. Ob. 100x
### Table 3. Barrett metaplasia: epithelial component types and histopathological data

<table>
<thead>
<tr>
<th>Barrett's esophagus: phenotype</th>
<th>benign lesions (no. of cases)</th>
<th>malignant lesions (no. of cases)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intestinal</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Cardial</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Fundic</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Intestinal + cardial</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Cardial + fundic</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>All three types</td>
<td>4</td>
<td>1</td>
</tr>
</tbody>
</table>

In three of the four cases showing all three types of epithelium, the presence of goblet cells was not identified in hematoxylin eosin stained sections, but only after histochemical marker of goblet cells containing acidic mucin with Alcian Blue (pH 2.5).

There were no differences between the pattern epithelium identified in benign lesion comparative to that of malignant lesions.

**Figure 6.** Barrett's esophagus: identification of goblet cells riched in acid mucopolysaccharides stained blue with Alcian blue (arrows). Alcian blue stain Ob. 40x

All biopsies and surgical specimens were analyzed making serial sections to identify dysplasia in Barrett's esophagus. Pursued cytological modifications included nuclear shape and size changes, nucleotide-cytoplasmic ratio increases, hypercromatism. Pursued architectural changes included: distortions and glandular clusters, papillary extensions into gland lumen and villiform mark of the mucosal surface.

To monitor the degree of dysplasia was preferred the classification defined by Riddell et al., (1983).

We identified 5 cases of Barrett's metaplasia "negative for dysplasia". They showed normal architecture without nuclear abnormalities excepting focal nuclear stratification, the largest nuclear alterations accepted being those associated with inflammation, erosions or ulcers. Also, the presence of active regenerative changes was included in the same category.

Barrett's oesophagus with low grade dysplasia presented cytological abnormalities extended to the mucosal surface, with decreased secretion of mucus, nuclear pseudostratification occupying the lower half of the glandular epithelium, mitoses were isolated, moderate pleomorphism and minimal architectural changes (glands with normal contour but larger, reduced mucus secretion) and this type of Barrett's dysplasia was diagnosed in 6 cases (Figure 7).

In high-grade dysplasia of Barrett's oesophagus (2 cases) we have met a marked nuclear pleomorphism, frequent mitoses, decreased mucus secretion, nuclear stratifications extended to the top of the glands, frequent mitoses and severe architectural alterations (distortion of crypts, glands with papillary aspects, branches, intraluminal clusters) (Figure 8).

**Figure 7.** Barrett's oesophagus with low grade dysplasia (LGD). The characteristics include glands with normal contour but widened, mucous depletion, hypercromatisme. HE stain. Ob. 40x
Figure 8. High-grade dysplasia of Barrett's oesophagus (HGD). Deeper issues than LGD: increased nucleus, pleomorphics, pseudostratification extending to the epithelium surface, glands with papillary appearance, intraluminal congestions, decreased mucous secretion. Feature is that this one do not present own lamina invasion. Col H-E. Ob. 40x

Regarding the 68 cases of gastroesophageal junction adenocarcinoma, they were classified separately being considered as tumors, probably developed by a high-grade Barrett's dysplasia at the lower esophagus or by intestinal cardial and fundic metaplasia.

We observed an increased incidence in the 6th decade of age, males being mainly affected. Most cases were localized to the gastroesophageal junction (48 cazuri), subcardial (17 cases) and distal (3 cases) (Table 4).

Table 4. Anatomical location of the cases regarding the Barrett's oesophagus sequence – dysplasia – junction adenocarcinoma

<table>
<thead>
<tr>
<th>Location</th>
<th>Number of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distal esophagus</td>
<td>3</td>
</tr>
<tr>
<td>Gastroesophageal junction</td>
<td>48</td>
</tr>
<tr>
<td>Subcardial gastric area</td>
<td>17</td>
</tr>
<tr>
<td>All cases of adenocarcinoma</td>
<td>68</td>
</tr>
</tbody>
</table>

In 7 cases we observed the presence of goblet cells and cylindrical cells, constituting the metaplastic epithelium. Depending on histopathology, we identified 52 cases of tubulopapillary adenocarcinoma, 7 cases of signet ring adenocarcinoma, 4 cases of mucinous adenocarcinoma and 5 cases of undifferentiated adenocarcinoma. Grading of tumors was achieved by dividing into three groups:

- G1 – well-differentiated,
- G2 – moderately differentiated
- G3 – poorly differentiated (Table 5).

Table 5. The relationship between the degree of differentiation and the number of cases aboard Barrett's oesophagus sequence – dysplasia – junction adenocarcinoma

<table>
<thead>
<tr>
<th>Grading</th>
<th>No. of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>29</td>
</tr>
<tr>
<td>G2</td>
<td>23</td>
</tr>
<tr>
<td>G3</td>
<td>16</td>
</tr>
</tbody>
</table>

In accordance with WHO recommendations, the mucinous, the undifferentiated or the "signet ring" carcinomas, with a tumor component more than 5% were included in G3 category. G1-well differentiated adenocarcinoma was established more than 95% of the tumor component from well-formed glandular structures with reduced stroma (Figure 9).

In moderately differentiated adenocarcinoma - G2 we have identified malignant glandular structures in over 50% of the tumor having cribriform or acinar pattern and a variable amount of stroma.

Poorly differentiated adenocarcinoma - G3 was characterized by poor training of glands (below 50%) and loss of cell cohesivity. Malignant cells have diffusely proliferated, singly or in small groups, often accompanied by a marked fibrous stromal reaction.

Figure 9. Well differentiated adenocarcinoma G1: prevail malignant glandular structures and the stroma is reduced. Col H-E. Ob. 40x
Immunohistochemistry of cyclooxygenase-2 (COX-2)

Immunohistochemical expression of cyclooxygenase-2 (COX 2) was positive in 53 cases of gastroesophageal junction adenocarcinoma and was negative in all cases of benign or malignant Barrett lesions (Figure 10). Immunomarking intensity for COX-2 correlated with tumor histological grade. Regarding immunohistochemical reaction on gastroesophageal adenocarcinomas we have registered a moderately positive reaction for well and moderately differentiated adenocarcinomas G1 and G2 (Figure 11). An intense positive reaction had been registered for poorly differentiated adenocarcinomas G3 (Figure 12).

Figure 10. Negative immunostaining COX2 in Barrett's oesophagus. The absence of cytoplasmic and membranar brown staining that should locate the antigen. IHC staining: ABC complex, contrastained with Mayer hematoxylin. Ob. 100x

Figure 11. Positive COX 2 immunostaining in tumoral cells of G1 junctional adenocarcinoma (intensity + +). Cytoplasmic and membranar brown staining that localized the antigen (arrow). IHC staining ABC complex, contrastained with Mayer hematoxylin. Ob. 40x

Discussion

We investigated several biological characteristics of patients with Barrett’s metaplasia or cardiac intestinal metaplasia because the distinction between these two entities is important, since the etiology and risk of developing adenocarcinoma are different (Wang LD et al, 2003). The best criterion for the distinction between adenocarcinomas is the precise knowledge that the biopsy specimens are from the esophagus or the stomach, but because of the effects of the anatomic variation of gastro-esophageal junction and squamocolumnar junction, inflammation and hiatus hernia, this is not always possible in endoscopy. In histological morphology there is also no significant difference between Barrett’s metaplasia and cardiac intestinal metaplasia.

Dysplazia has been the histopatological marker for the early diagnosis of adeocarcinoma at the level of gastro-esophageal junction, as evidenced by the simultaneous presence of the same resection pieces on both dysplasia and neoplasia.

In this study we sought to identify Barrett’s epithelium by haematoxylin-eosin staining, and the differentiation of intestinal, gastric cardia or fundic metaplasia by special stains for mucins components: stain PAS / Alcian Blue, pH
2.5 and pH 1.0, Toluidine blue staining at pH 0.5 and pH 4 to 4.5.

Using histochemical marking with PAS - Alcian Blue staining, pH 2.5, we identified acidic mucin content, siafo- and sulpho-mucins, (blue colored) and neutral mucins (red regent colored) in cases of columnar metaplasia of lower esophagus. PAS - Alcian Blue staining, pH 1.0, allowed blue selective staining of sulphomucins on the same sections.

We studied the immunohistochemical expression of cyclooxygenase-2 (COX-2), inducible enzyme, normally absent in cells, but expressed as a response to the cytokines, interleukins, tumor promoters and growth factors action. COX-2 seems involved in carcinogenesis prolonging the survival of tumoral cells by apoptosis and cellular adhesion reduction and angiogenesis facilitation.

In all cases of benign or malignant Barrett's metaplasia, COX-2 expression was negative while a significant percentage - 77.9% of cases of gastroesophageal junctional adenocarcinoma presented a positive, a moderate or an intense reaction (Table 6).

COX-2 overexpression was correlated with tumor histological grade, G3 poorly differentiated adenocarcinoma showing the most intense immunomarking. These results led to the idea that COX-2 expression is a useful prognostic marker in advanced stages of disease assessment, high values correlating with advanced neoplasia.

**Table 6. Immunohistochemical expression of cyclooxygenase-2 (COX-2) in the Barrett's oesophagus sequence – dysplasia – adenocarcinoma**

<table>
<thead>
<tr>
<th>LESION</th>
<th>COX-2 expression</th>
<th>No. cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign Barrett's esophagus</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Adenocarcinoma G1</td>
<td>++</td>
<td>8 (15,0)</td>
</tr>
<tr>
<td>Adenocarcinoma G2</td>
<td>++</td>
<td>14 (26,4)</td>
</tr>
<tr>
<td>Adenocarcinoma G3</td>
<td>+++</td>
<td>31 (58,4)</td>
</tr>
<tr>
<td>The total number of adenocarcinomas</td>
<td>68</td>
<td>53</td>
</tr>
</tbody>
</table>

negative; + poorly positive; ++ moderatly pozitive; +++ intensely pozitive

**Conclusions**

The anatomopathological diagnosis is the best indicator for the evolution of Barrett's oesophagus to adenocarcinoma. The most important area of early diagnosis was a squamo-columnar transitional region and the presence of a Barrett metaplasia segment more than 3 cm above the gastro-oesophageal junction.

Dysplasia has been the histopathological marker for the early diagnosis of adenocarcinoma at the level of gastro-oesophageal junction, as evidenced by the simultaneous presence of the same resection pieces on both dysplasia and neoplasia.

By histochemical marking with PAS blue alcian staining, we have differentiated the epithelial origine of gastro-oesophageal adenocarcinoma from Barrett's metaplasia and gastric cardia metaplasia.

We conclude that the immunohistochemical expression of cyclooxygenase-2 (COX-2) is an objectiv marker for lesions agessivity and for unfavorable diagnosis, its strong positive expression being in correlation with advanced neoplastic phases.

We conclude that the distinction of esophageal adenocarcinoma from gastric cardia adenocarcinoma should not be based
on a single method but the combination of clinical characteristics, histological results, immunohistochemical study and especially precise endoscopic biopsy.

References
Hsu S.M, Raine L., Fanger H. "Use of ABC and unlabeled antibody (PAP) procedures" J. Histochem. Cytochem. 29, 577-580, 1981