Expression of p53 and Ki-67 in distal oesophageal and gastric cardia adenocarcinomas

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Abstract
Lately, a rapid increase in the incidence of distal oesophageal and gastric cardia tumours was noted in the Western countries.

Materials and methods: The present study investigated the p53 and Ki-67 expression in Barrett’s metaplasia and distal oesophageal adenocarcinoma versus the gastric cardia adenocarcinoma, using immunohistochemical methods. The authors chose the immunohistochemical study to identify the genetic changes in Barrett’s metaplasia and gastro-oesophageal adenocarcinoma and to determine whether there was a significant risk pattern for the screening and surveillance of patients with gastro-oesophageal junction adenocarcinoma.

The study included 109 men and women (age range: 31 - 79) with several symptoms: weight loss, persistent heartburn, regurgitation, odynophagia, dysphagia, bleeding. The patients had histologically confirmed Barrett’s metaplasia and junction adenocarcinoma.

Results: The overexpression of p53 was found in 63% of the patients with oesophageal adenocarcinoma and in 49% of the patients with gastric cardia adenocarcinoma. The immunohistochemical expression of the proliferating factor Ki-67 was positive in 45-55% of the Barrett’s metaplasia cases and moderately or strongly positive in 100% of the gastro-oesophageal junction adenocarcinoma cases.

Conclusions: The authors concluded that the p53 and Ki-67 immunoexpression varied among different types of gastro-oesophageal junction adenocarcinomas.

Keywords: Barrett’s metaplasia, dysplasia, distal oesophageal adenocarcinoma, cardia adenocarcinoma, immunohistochemical methods

Introduction
The replacement of the normal epithelium of the lower oesophagus with intestinal metaplasia is considered an important risk factor for the development of dysplasia and gastro-oesophageal junction cancer (K.J. NAPIER & al. [1], A. PENNATHUR & al. [2]).

The specialised intestinal metaplasia of the gastro-oesophageal junction is the result of persistent inflammation under the influence of a chronic gastro-oesophageal reflux (SPECHLER [3]).

Oesophageal and gastric adenocarcinomas are the deadliest of all digestive malignancies (SOUZA & SPECHLER [4]).
The incidence rates of the oesophageal and cardia adenocarcinomas have rapidly increased in the last years, predominantly affecting Caucasian males (M:F=7:1) (W.J. BLOT & al. [5], LAGERGREN [6], S.S. DEVESA & al. [7]). The surgical cure rate is compromised by the fact that patients are usually diagnosed with a late stage cancer, the 5-year survival rate for all patients diagnosed with oesophageal cancer ranging from 15% to 20% (A. RUOL & al. [8]).

This study is based on the detailed analysis of 109 cases of Barrett’s metaplasia, dysplasia, and gastro-oesophageal junction adenocarcinoma.

The immunohistochemical study included the analysis of p53 and Ki-67, possible markers for the early diagnosis of gastro-oesophageal junction adenocarcinomas.

Ki-67 is a double nuclear protein, 345-395 kDa, which plays an important role in maintaining cellular proliferation. Ki-67 is present in all non-G0 phases of the cellular cycle. Starting mid-G1, its level begins to rise, continuing to rise during the S and G2 phases and reaching a peak in the M phase.

At the end of the M phase, this protein is rapidly catabolised. The marking index of Ki-67 represents the percentage of positive tissue cells and indicates a proliferation factor.

The TP53 gene mutations result in an inactive protein viewed as overexpressed in immunohistochemistry. The mutant p53 proteins prevent the cell cycle arrest in the damaged cells, resulting in an increased frequency of mutations and genomic instability.

The inactivation of p53 is the pathway through which lower oesophageal and gastric cardia adenocarcinomas avoid apoptosis. The inactivation of p53 makes the G1 point of the cell cycle useless, allowing the clonal expansion of cells with abnormal karyotype (T.D. McMANUS & al. [9]). The inactivation of p53, with the effect of the loss of heterozygosity – LOH at the 17p chromosome and the mutations of the remaining alleles, has been described in approximately 50-90% of the cases of oesophageal adenocarcinoma and 31-63% of the cases of gastric cardia adenocarcinoma (S.J. MELTZER & al. [10], R. HAMELIN & al. [11], P.C. GALIPEAU & al. [12], M. YANAGI & al. [13]).

C.M. GLEESON & al. [14] described the genetic abnormalities of p53 in the oesophageal adenocarcinoma and Barrett’s dysplasia. The same p53 mutations can be found both in cancerous cell populations, with multiple aneuploidies, and in diploid cell populations of the adjacent mucosa in Barrett’s dysplasia (K. NESHAT & al. [15]).

Materials and methods

Patients

The gastro-oesophageal biopsy samples were collected from the oesophageal junction, at 2 cm levels above and below the junction, and by surgical resection.

We selected 91 patients, men and women, diagnosed with gastro-oesophageal junction carcinoma out of 497 patients diagnosed with gastric cancer between 2005 and 2014. We also selected 18 cases of benign and malignant Barrett’s metaplasia out of 168 cases, diagnosed by oesophageal biopsy during the same time span.

The patients included in this study presented several symptoms: persistent heartburn and regurgitation, odynophagia, progressive dysphagia, occult bleeding, weight loss.

The gastro-oesophageal junction sections were cut to 3 - 4 µm thick and were examined after haematoxylin-eosin (H&E) and van Gieson staining.
For the immunohistochemical analyses, an immune-enzyme polymer method - LSAB/HRP (Universal DAKO Horseradish Peroxidase) was chosen, the indirect tristacial method by S.M. HSU & al. [16].

The tumour and normal tissue samples were fixed in 10% formalin, embedded in paraffin, and microdissected in 3-4 µm slides. The slides were deparaffinised in xylene, rehydrated in alcohol, and incubated in hydrogen peroxide (3%) for 20 minutes to block the non-specific background staining due to the endogenous peroxidase.

After 10 to 20 minutes in the microwave, the antigen retrieval was performed by using 10 mM citrate buffer, pH 6.0. The standard streptavidin-biotin peroxidase complex method was performed on the slides, and the samples were incubated in a secondary antibody solution for 30 minutes. Diaminobenzidine was used as a chromogen and a brown precipitate developed, localising the antigen. The slides were counterstained with Mayer’s hematoxylin. The negative controls were counterstained in the same manner as the tumour samples, using staining methods by omitting the primary antibodies.

The antibodies used in the immunohistological analyses identified the molecular biomarkers targeted for the research of Barrett’s metaplasia and junction cancer; the positive expression was represented by the brown nuclear precipitate.

Table 1 presents the antibodies and working parameters.

<table>
<thead>
<tr>
<th>ANTIBODY</th>
<th>PRODUCER</th>
<th>DILUTION</th>
<th>CLONE</th>
<th>PECULIARITY</th>
</tr>
</thead>
<tbody>
<tr>
<td>p53</td>
<td>Dako</td>
<td>1:50</td>
<td>M7001</td>
<td>Protein gene p53</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IgG2bk</td>
<td></td>
</tr>
<tr>
<td>Ki-67</td>
<td>Dako</td>
<td>1:50</td>
<td>M7240</td>
<td>Proliferating cell nuclear antigen</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>IgG1k</td>
<td></td>
</tr>
</tbody>
</table>

Results

The present study identified 18 cases of Barrett’s metaplasia with benign (14) and malignant lesions (4) out of a total of 109 cases. The gastro-oesophageal junction adenocarcinoma (91 cases) was divided topographically: in the distal oesophagus (4 cases), at the junction (61 cases), and subcardial (26 cases).

The patients’ age ranged between 31 and 79 years. The average age was 46 years for the benign lesions and 65 years for the malignant lesions.

The symptoms were generally of low intensity and frequency, with the onset varying between 3 months and 22 years. Males were predominantly symptomatic (M:F=4.2:1). Hiatus hernia was associated in 11 cases.

The current study identified the main clinical symptoms associated with Barrett’s oesophageal and gastro-oesophageal junction cancer: dysphagia – 83 cases (76.1%), odynophagia – 19 cases (17.4%), chronic gastro-oesophageal reflux – 53 cases (48.6%), haemorrhage/bleeding – 17 cases (15.6%).

Table 2. Clinical symptomatology associated with Barrett’s oesophageal and gastro-oesophageal junction cancer

<table>
<thead>
<tr>
<th>Clinical symptomatology</th>
<th>Number of cases (n(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dysphagia</td>
<td>83 (76.1%)</td>
</tr>
<tr>
<td>Odynophagia</td>
<td>19 (17.4%)</td>
</tr>
<tr>
<td>Chronic gastro-oesophageal reflux</td>
<td>53 (48.6%)</td>
</tr>
<tr>
<td>Haemorrhage/bleeding</td>
<td>17 (15.6%)</td>
</tr>
</tbody>
</table>
The histopathological type of gastro-oesophageal junction adenocarcinoma, in accordance with the classification, was identified in all 91 cases (LAUREN [17]) (Table 3).

The 91 cases of gastro-oesophageal junction adenocarcinoma were divided into three groups: well differentiated adenocarcinoma – G1 (42 cases), moderately differentiated adenocarcinoma – G2 (31 cases), and weakly differentiated adenocarcinoma – G3 (18 cases).

The histopathological types of gastro-oesophageal junction adenocarcinoma were: tubulopapillary type – 71 cases, signet ring type – 11 cases, mucinous type – 6 cases, and undifferentiated type - 3 cases.

<table>
<thead>
<tr>
<th>Histopathological type</th>
<th>No. of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tubulopapillary</td>
<td>71</td>
</tr>
<tr>
<td>Signet ring</td>
<td>11</td>
</tr>
<tr>
<td>Mucinous</td>
<td>6</td>
</tr>
<tr>
<td>Undifferentiated</td>
<td>3</td>
</tr>
</tbody>
</table>

The TP53 gene mutation was detected while searching for the immunohistochemical expression of the p53 mutant protein over Barrett’s oesophagus – junction adenocarcinoma. Because the extended half time allows for increased p53 mutant cell concentrations, the indirect visualisation by the immunohistochemistry technique of the nuclear marking was possible.

The overexpression of p53 was found in 63% of the patients with oesophageal adenocarcinoma and in 49% of the patients with gastric cardia adenocarcinoma (Figs 1 and 2).

Fig 1. Positive p53 immunostaining at the base of the crypt and at the basal layer of the squamous epithelium. Brown nuclear immunostaining that localises the antigen. IHC staining, ABC complex, counterstain with Mayer’s haematoxylin. Ob. 100x
The immunohistochemical expression of the proliferating factor Ki-67 was positive in 45-55% of the Barrett’s metaplasia cases and moderately or strongly positive in 100% of the gastro-oesophageal junction adenocarcinomas (Fig 3).

A large overlap of the Ki-67 staining in samples of neoplastic tissue with different histopathological stages was observed.
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**Discussion**

Several biological characteristics of the patients with Barrett’s oesophagus or cardial intestinal metaplasia were investigated. The distinction between these two lesions is important because the aetiology and the risk of developing cancer are different (H. WANG & al. [18]).

It is very important to distinguish between the oesophageal and cardial adenocarcinomas by knowing the anatomical origin of the biopsy, but it is difficult to distinguish between the anatomical variations of the gastro-oesophageal junction and the squamous - cylinder junction. There is also no significant difference between Barrett’s metaplasia and cardial intestinal metaplasia according to the histological morphology.

In both types of lesions, malignant and benign, the gender distribution presented a male predominance. This investigation also showed a 4.2:1 male – female ratio and an average age of 65 years for the malignant lesions and 46 years for the benign lesions. We underline that our results are in accordance with the current literature (W.J. BLOT & al. [5], POWELL & McCONKEY [19], L.E. HANSSON & al. [20], LAGERGREN [6]).

Out of all the lesions, dysphagia as a dominant symptom was present in 76.1% of the cases and only 48.6% of the cases presented chronic gastro-oesophageal reflux.

Consequently, we have concluded that there is a strong correlation between dysphagia and the lesion’s severity, 84.3% of the cases presenting malignant lesions.

The histopathological marker for the early diagnosis of the gastro-oesophageal junction adenocarcinoma is undoubtedly dysplasia. This is evidenced by the simultaneous presence of the same resection fragments in both dysplasia and cancer.

In the current immunohistochemical study, a strong positive immunoeexpression of p53 in 42.8% of the Barrett’s oesophagus cases and in 46.3% of all gastro-oesophageal adenocarcinoma lesions was observed.

Our research also identified the positive immunoeexpression of p53 in 25 cases of adenocarcinoma with intestinal metaplasia and in 19 cases of adenocarcinoma without intestinal metaplasia (Table 4).

**Table 4.** The immunohistochemical expression of p53 in Barrett’s metaplasia or gastro-oesophageal junction adenocarcinoma.

<table>
<thead>
<tr>
<th>Histological types</th>
<th>p53 pattern</th>
<th>No. of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barrett’s oesophagus</td>
<td>+</td>
<td>6</td>
</tr>
<tr>
<td>Gastro-oesophageal adenocarcinoma with associated Barrett’s metaplasia</td>
<td>++</td>
<td>25 (56.8)</td>
</tr>
<tr>
<td>Gastro-oesophageal adenocarcinoma without associated Barrett’s metaplasia</td>
<td>++</td>
<td>19 (43.2)</td>
</tr>
</tbody>
</table>

No. of adenocarcinomas 95 44

The overexpression of p53 in Barrett’s oesophagus suggests that the p53 gene mutations may occur early along the metaplasia – dysplasia – adenocarcinoma sequence.

These results lead to the idea that the high expression of p53 may be an early marker for malignancies, demonstrated by its presence in the non-dysplasic and metaplasic epithelium of Barrett’s adenocarcinoma.

However, a moderate percentage of p53 junction adenocarcinoma positive cases makes it difficult to evaluate whether the overexpression of p53 is a predictive marker for malignancies, 53.7% of the tumour cases being p53 negative.
The value of the p53 mutations as an early diagnosis marker and the intense positive expression of the p53 mutant protein have been identified both in Barrett’s metaplasia and the gastro-oesophageal adenocarcinoma.

FLÉJOU [21] reported that immunohistochemical studies prove the presence of the p53 overexpression in 5% of the non-dysplasic Barrett’s metaplasia cases, in 20% of the low grade Barrett’s dysplasia cases, and in 60% of the high grade Barrett’s dysplasia and oesophageal adenocarcinoma cases. The author established that the immunohistochemical expression of p53 is useful for the surveillance of Barrett’s dysplasia cases.

Similar results were reported by P.L. BLOUNT & al. [22], A.G. CASSON & al. [23], M.T. BARRETT & al. [24]. They observed the overexpression of p53 in 50-90% of the oesophageal adenocarcinoma cases, in 31-63% of the gastric cardia adenocarcinoma cases, and a positive expression in several Barrett’s metaplasia cases.

In the present study, the immunohistochemical results allow us to consider the p53 expression a useful biomarker for the early diagnosis of malignancy and for the diagnosis of the gastro-oesophageal lesion’s severity, in accordance with another study (A.P. WESTON & al. [25], A.I. KOTZEV & al. [26]).

However, we cannot consider the p53 immunostaining a reliable marker in differentiating tumours according to location, because we observed a positive expression in adenocarcinomas with and without associated Barrett’s metaplasia.

The immunohistochemical studies of Ki-67 have shown cell proliferation. Ki-67 is a marker of the cell cycle progression during the G1 – S phase transition.

It was observed that Barrett’s metaplasia presented Ki-67 positive reactions in the basal layer and at the base of the crypts in 50% of the cases. Moderate or strong positive reactions of Ki-67 were present in all cases of gastro-oesophageal junction adenocarcinoma. N. RIOUX-LECLERQ & al. [27] reported a low expression of Ki-67 (14%) in patients with Barrett’s oesophagus without adenocarcinoma and a relatively high expression (87%) in patients with adenocarcinoma.

The results from various studies showed that the Ki-67 proliferation index is increasing progressively from Barrett’s metaplasia to oesophageal adenocarcinoma (M. BINATO & al. [28], M.K. HONG & al. [29]).

The current study observed the extensive expression of Ki-67 with the same proliferation index in Barrett’s metaplasia and gastro-oesophageal junction adenocarcinoma, with a large overlap of immunostaining in lesions with different histological severities.

The immunohistochemical detection showed increased Ki-67 labelling indices and a disordered proliferative architecture in the gastro-oesophageal adenocarcinoma with no important differentiation among the lesions.

The strong positive expression of Ki-67 represents an aggressiveness predictor of cancer and has been reported as an unfavourable prognostic marker.

Conclusions

In conclusion, the molecular biomarkers identified in the present study, the p53 overexpression and the disordered proliferative architecture of Ki-67, might be considered useful biomarkers to assist in the early diagnosis of the gastro-oesophageal junction adenocarcinoma.

The p53 immunopositivity associated with increased proliferation indices might be needed to confirm and quantify the predictive risk of progression in the early diagnosis of cancer, but cannot represent useful markers in the differentiation of gastro-oesophageal junction adenocarcinomas.
We conclude that the distinction between the distal oesophagus and gastric cardia adenocarcinomas should not be based on a single method, but a combination of clinical symptoms and endoscopic surveillance, with precise endoscopic biopsies, histopathological results, and immunohistochemical features should be used.

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Contribution Note
All authors contributed equally to the manuscript.

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