Expression of Ki-67 Antigen and Caspase-3 Protein in Benign Lesions and Esophageal Carcinoma

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Abstract. Background: The present study aimed to evaluate apoptosis and cell proliferation alterations in esophageal benign lesions in comparison to esophageal carcinomas. Materials and Methods: Immunohistochemistry was performed for caspase-3 protein (CPP32) and Ki-67 antigen expression in the esophageal mucosa from patients with Chagas disease (CD) with and without megaesophagus (CM), chronic esophagitis (CE), esophageal carcinoma (ESCC) and in normal mucosa (NM). Results: The Ki-67 labeling index (LI) was similar in all groups (range: 30%-48%), having no statistically significant difference among the groups. Positive CPP32 immunostaining was observed with similar frequency in the CD (30.8%), CM (30.4%) and CE (34.8%) groups, but it was increased in the ESCC group (55.5%); however, it was not statistically different from the other groups. No associations among the levels of CPP32 and Ki-67 expression were observed in the various groups, neither among parameters such as age, gender or alcohol and tobacco consumption. Conclusion: There were no evident changes in cell proliferation and apoptosis in benign lesions studied.

Cancer development involves many genes that control growth, cell proliferation and homeostasis of the tissue by apoptosis (1). After an inflammatory process or the toxic effects of carcinogens, cell proliferation increases the probability of producing new genetically changed cells (2). There is a tendency for the neoplastic population to increase in proliferative capacity and to escape the control mechanisms of normal growth. With increased cell proliferation, mutant progeny arise as a result of genomic instability, where the majority does not survive due to immune selection or apoptotic metabolic changes (3).

Many of the biochemical and morphological alterations that occur during apoptosis is a consequence of a family of cysteine proteases called caspase, which in turn cleave various substrate proteins that account for apoptosis. To date, of all the caspases that have been studied, caspase-3 (CPP32) correlates best with apoptosis. Caspase-3-mediated proteolysis, which is initiated by different stimuli (endogenous and exogenous) that induce apoptosis, is a critical element of the apoptotic process (4). Analysis of the expression of caspase-3 from primary esophageal squamous cell carcinoma (ESCC) showed that 60% of the cases were positive (5), and that caspase-3 was associated with a favourable prognosis. Caspase-3 was suggested as a new prognostic factor in ESCC, indicating that biomarker-related apoptosis can be used as a predictive parameter in the monitoring and prognosis of ESCC (6-8).

The assessment of cell proliferation by the detection of Ki-67 antigen in neoplastic cell populations has been shown to be of prognostic value. There is a strong correlation between the Ki-67 index and the histopathological grading of neoplasms (8). The progressive increase of Ki-67 antigen from dysplasia to ESCC suggests that this antigen is an effective biomarker in the squamous epithelium of esophagus (9-11).

Esophageal carcinogenesis follows the multistep model involving esophagitis, atrophy, dysplasia, carcinoma in situ and, finally, invasive carcinoma (12-14). Thus, it is important to evaluate benign lesions with precancerous potential, such as esophagitis and megaesophagus (esophagus dilation), in order to diagnose the early stages of neoplastic changes (15). Evaluation of Ki-67 and caspase-3 expression in esophageal squamous cell carcinoma (ESCC) suggests that this antigen is an effective biomarker in the squamous epithelium of esophagus (9-11).

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Key Words: Ki-67, CPP32, chagasic megaesophagus, chronic esophagitis, esophageal squamous cell carcinoma.
Materials and Methods

Samples. A total of 78 specimens of paraffin-embedded esophageal tissue were obtained from patients who underwent middle and distal esophageal biopsies, before any chemo- or radiotherapy treatment, at the Pathology Section, Hospital de Base (São José do Rio Preto, SP, Brazil). Twenty-three specimens were obtained from CD patients with CM, 13 from CD patients without CM, 23 from CE patients, and 9 from ESCC patients. Both patients with Chagas disease, and chronic esophagitis were esophageal cancer-free. Esophageal mucosa from 10 healthy individual was obtained and diagnosed as histologically normal (NM). The majority of subjects in the NM group were females, aged between 26 and 67 years (mean±SE=42.4±4.81 years; SE: standard error of the mean); 40% of them had an alcoholic habit and 30% had a smoking habit. Of the 13 chronic chagasic patients without megasphagus (CD), the majority were females, aged between 45 and 79 years (mean±SE=61.3±2.85 years); 30.8% were alcohol drinkers and 38.5% were smokers. The other 23 chronic chagasic patients had CM and most of them were males, aged 57 to 83 years (mean±SE=64.3±2.08 years); 34.8% of them (8/23) were alcohol drinkers and 56.5% of them (13/23) were smokers. In the CE group, about half were females, aged 40 to 70 years (mean±SE=50.5±1.99 years; only 4.3% of them were alcohol drinkers and 17.4% were smokers. In contrast, the ESCC group was formed by 8 males and one female, with ages ranging from 42 to 69 years (mean±SE=56.5±2.68 years). Of those, most were alcohol drinkers and smokers (72.7% and 81.8%, respectively). The study was approved by the Brazilian National Research Ethics Committee (CONEP) and written informed consent was obtained from all patients.

Immunohistochemical assay. Consecutive 4 μm-thick sections were cut from each trimmed paraffin block, and mounted in glass slides pre-treated with 3-aminopropyl-triethoxysilane/acetone solution. In brief, following deparaffinization, sections were re-hydrated, treated with citrate buffer at 96°C for 30 min, and treated with 3% H2O2 in methanol (v/v) for 30 min. to block endogenous peroxidases. The sections were then incubated for 1 hour at room temperature with specific antibodies: CPP32, clone JHM-63; Novocastra, Newcastle, UK; 1:100 and Ki-67 antigen (clone MM1; Novocastra; 1:200). Next, the slides were incubated with secondary antibodies: for CPP32 protein the Novolink polymer were used (Novolink Polymer; Novocastra, Newcastle, UK) and for Ki-67 we used streptavidin-biotin peroxidase Kit (Dako Cytomation Kit, Strept ABComplex/HRP; Dako, Glostrup, Denmark), following the manufacturer’s instructions. The immunostaining was visualised with 3,3’-diaminobenzidine tetrahydrochloride (DAB) containing 0.005% H2O2 and counterstained with hematoxylin. Negative controls were established by replacing the primary antibody with buffer solution. Tonsil tissues were used as positive controls for both antibodies. The polyclonal antibody (NCL-Ki67p) labels Ki-67 antigen in the granular components of the nucleolus during late G1, S, G2 and M phases (9). Positive immunostaining for Ki-67 antigen was brown nuclear staining and the negative was the absence of nuclear staining. Five hundred cells were counted for each sample. Ki-67 was considered positive when >10% of cells showed nuclear reactivity (15, 16). The labeling index (LI) for Ki-67 antigen, defined as the ratio between the cells positively stained for Ki-67 and the total of cells counted for each case (12), was calculated. For CPP32, all tissue extension was examined for each sample. Immunostaining for CPP32 (brown cytoplasm staining) was graded by staining intensity as negative: (–) absent brown staining and (+) weakly stained; or as positive: (++) moderately stained and (+++) strongly stained (4). All analyses were performed under a light microscope (×400 magnification). Areas that were poorly preserved, crushed, catured, folded or retracted were specifically avoided.

Statistical analysis. Descriptive statistics, the Kolmogorov-Smirnov test for normality distribution, the Kruskal-Wallis test for CPP32 and Ki-67 and the analysis of variance (ANOVA) with Tukey-Kramer multiple comparisons post-test for Ki-67 antigen were used to determined statistical significance. The Fisher’s exact test was performed to establish associations between apoptosis and cell proliferation. The level of significance was set as p<0.05. The statistical analysis was performed with GraphPad Instat 3 computer software (GraphPad Software, Inc. La Jolla, CA, USA) (17).

Results

The LI for Ki-67 antigen for each group was as follows: 30.1% for NM, 30.9% for CD, 42.3% for CM, 44.6% for CE and 48.4% for ESCC in 500 cells analysed per case (Table I). The Kolmogorov-Smirnov test indicated normality distribution and then ANOVA with Tukey-Kramer multiple comparison post-test was performed among the groups, but no significant difference was observed. All of the cases in the NM, CD, CE and ESCC groups were classified as Ki-67 positive, since they showed more than 10% of cells positively immunostained for the antigen, while in the CM group 91.3% of cases were Ki-67 positive. The statistical analyses for the positive cases, by the Kruskal-Wallis test, did not indicate any significant difference among the groups (Table I).

Positive cytoplasmatic immunostaining for CPP32 was observed in 20% (2/10) in the normal mucosa group, which was interpreted as normal level of apoptosis. Positive CPP32 immunostaining was observed with similar frequencies in the CD, CM and CE groups, namely 30.8% (4/13), 30.4% (7/23), and 34.8% (8/23), respectively. However, it was increased in the ESCC group, 55.5% (5/9) (Table I). Statistically significant differences were not observed among the groups using the Kruskal-Wallis test.

The establishment of associations between CPP32 and Ki-67 expression were performed by the Fisher’s exact test, indicating that there is no association between the cell regulation processes studied in the groups analysed (Table II). Moreover, significant associations were not observed between altered expression patterns of Ki-67 antigen and CPP32 protein, and parameters such as age, gender, smoking and alcohol consumption (data not shown) at different groups.

Figure 1 illustrates the immunostaining of Ki-67 antigen (nuclear) and CPP32 protein (cytoplasmic) in the esophageal mucosa of benign lesions and carcinoma.
Recent studies have demonstrated that gene abnormalities of cell-cycle regulators that function in the transition from the G1 to S phase are associated with lifestyle factors in ESCC (18), however, associations between these parameters, such as age, gender, smoking and alcohol consumption, and the status of Ki-67 antigen and CPP32 protein were not observed in the studied groups (data not shown).

In the present study, the Ki-67 LI was higher in CM, CE and ESCC groups, than in the other groups, although both LI and positivity for Ki-67 were not statistically significant among the different groups. ESCC showed higher expression of CPP32 than the other groups, indicating a high level of apoptosis in tumour tissue. When the analysis of associations between the molecules (Ki-67 and CPP32) was performed, no associations were found.

Tumour cell cycle analysis has indicated that tumours with a higher proliferation rate (≥40%) show more aggressive clinical behavior compared to tumours with a low proliferation rate (<40%) (19). The Ki-67 LI has been identified as a parameter reflecting tumour proliferation. ESCC patients with a high Ki-67 LI have lower postoperative survival rates; thus, a high Ki-67 LI is one of the prognostic factors of ESCC (18, 20). In the present study, Ki-67 LI was greater than 40% in the CM (14 cases, 60.8%), CE (11 cases, 47.8%) and ESCC (5 cases, 55.5%) groups, but did not show significant difference with the NM and CD groups (with Ki-67 LI ~30%). Despite some

### Table I. Immunohistochemical and statistical analysis for Ki-67 antigen and CPP32 protein in the various groups.

<table>
<thead>
<tr>
<th>Groups</th>
<th>N</th>
<th>Labeling index</th>
<th>Positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>μ±SE (%)</td>
<td>n/N</td>
</tr>
<tr>
<td>NM</td>
<td>10</td>
<td>30.1±2.63</td>
<td>10/10</td>
</tr>
<tr>
<td>CD</td>
<td>13</td>
<td>30.9±2.80</td>
<td>13/13</td>
</tr>
<tr>
<td>CM</td>
<td>23</td>
<td>42.3±3.82</td>
<td>21/23</td>
</tr>
<tr>
<td>CE</td>
<td>23</td>
<td>44.6±3.28</td>
<td>23/23</td>
</tr>
<tr>
<td>ESCC</td>
<td>9</td>
<td>48.4±7.12</td>
<td>9/9</td>
</tr>
</tbody>
</table>

**Statistical analysis**

ANOVA, \( p=0.0395 \)

Kruskal-Wallis test, \( p=0.0305 \)

Kruskal-Wallis test, \( p=0.5731 \)

NM, Healthy individuals; CD, Chagas disease patients without megaesophagus; CM, Chagas disease patients with megaesophagus; CE, patients with chronic esophagitis; ESCC, patients esophageal squamous cell carcinoma; N, total of specimens; n, number of specimens immunostained; μ, average; SE, standard error. Statistical analysis: \( p>0.05 \), there is no statistically significant difference.

### Table II. Association between expression of Ki-67 antigen and CPP32 protein, by Fisher’s exact test (\( p<0.05 \)).

<table>
<thead>
<tr>
<th>Groups</th>
<th>N</th>
<th>Ki-67+</th>
<th>Ki-67−</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CPP32+</td>
<td>CPP32−</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CPP32+</td>
<td>CPP32−</td>
</tr>
<tr>
<td>NM</td>
<td>10</td>
<td>1 (10%)</td>
<td>9 (90%)</td>
</tr>
<tr>
<td>CD</td>
<td>13</td>
<td>1 (7.69%)</td>
<td>12 (92.31 %)</td>
</tr>
<tr>
<td>CM</td>
<td>23</td>
<td>6 (26.08%)</td>
<td>15 (65.22%)</td>
</tr>
<tr>
<td>CE</td>
<td>23</td>
<td>12 (52.17%)</td>
<td>11 (47.83%)</td>
</tr>
<tr>
<td>ESCC</td>
<td>9</td>
<td>5 (55.56%)</td>
<td>4 (44.44%)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Groups</th>
<th>N</th>
<th>Ki-67+</th>
<th>Ki-67−</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CPP32+</td>
<td>CPP32−</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CPP32+</td>
<td>CPP32−</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p-Value</td>
<td></td>
</tr>
<tr>
<td>NM</td>
<td>10</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>CD</td>
<td>13</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>CM</td>
<td>23</td>
<td>0 (0%)</td>
<td>2 (8.70%)</td>
</tr>
<tr>
<td>CE</td>
<td>23</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>ESCC</td>
<td>9</td>
<td>0 (0%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

NM, Healthy individuals; CD, Chagas disease patients without megaesophagus; CM, Chagas disease patients with megaesophagus; CE, patients with chronic esophagitis; ESCC, patients esophageal squamous cell carcinoma; N, total of specimens; NP, analysis was not performed because one row or column is filled with zeros and in this situation the analysis was impossible; ns there is no statistically significant difference.

**Discussion**

Recent studies have demonstrated that gene abnormalities of cell-cycle regulators that function in the transition from the G1 to S phase are associated with lifestyle factors in ESCC (18), however, associations between these parameters, such as age, gender, smoking and alcohol consumption, and the status of Ki-67 antigen and CPP32 protein were not observed in the studied groups (data not shown).

In the present study, the Ki-67 LI was higher in CM, CE and ESCC groups, than in the other groups, although both LI and positivity for Ki-67 were not statistically significant among the different groups. ESCC showed higher expression of CPP32 than the other groups, indicating a high level of apoptosis in tumour tissue. When the analysis of associations between the molecules (Ki-67 and CPP32) was performed, no associations were found.

Tumour cell cycle analysis has indicated that tumours with a higher proliferation rate (≥40%) show more aggressive clinical behavior compared to tumours with a low proliferation rate (<40%) (19). The Ki-67 LI has been identified as a parameter reflecting tumour proliferation. ESCC patients with a high Ki-67 LI have lower postoperative survival rates; thus, a high Ki-67 LI is one of the prognostic factors of ESCC (18, 20). In the present study, Ki-67 LI was greater than 40% in the CM (14 cases, 60.8%), CE (11 cases, 47.8%) and ESCC (5 cases, 55.5%) groups, but did not show significant difference with the NM and CD groups (with Ki-67 LI ~30%). Despite some
studies which indicate that the Ki-67 LI is a powerful prognostic marker for patients with esophageal carcinoma (21-23), the results of the current study did not show increased Ki-67 LI in the ESCC group compared to the others groups analysed.

High expression of tumour proliferation-related factors (Ki-67, PCNA and AgNOR), abnormalities of adhesion molecules (E-cadherin, alpha-catenin), activation of the autocrine mechanisms of growth factor (EGFR, TGFα, EGF), and DNA ploidy pattern, which is thought to be the result of an accumulation of genomic abnormalities, are also prognostic factors for esophageal cancer (24). Ki-67 was significantly higher in patients with erosive esophagitis and in patients with functional heartburn than in controls, indicating that Ki-67 evaluation provides quantitative and objective data on squamous epithelium proliferative activity (9, 11). Meanwhile, the present study did not find any statistically significant difference in the expressions of Ki-67 antigen and CPP32 protein between the benign lesions and ESCC groups, neither among the normal mucosa group. The expression of Ki-67 did not show any significant differences in biomarker expression between carcinoma of the esophagus and its precursor lesions (mild, moderate and severe dysplasia) (19), and this corroborates the present results.

An increased expression of CPP32 protein was observed only in the ESCC group (55.5%), but it was not significant. The positive staining in the ESCC group may be due to the high apoptotic activity in response to the severe injury, whereas in CD, CM and CE (positive in ~30% cases), it may have occurred as a consequence of physiological and inflammatory processes of esophageal epithelium. Therefore, according to some studies that observed a relationship between moderate to strong CPP32 staining and better prognostic value (6), the high expression of CPP32 protein in ESCC in the current study, may indicate a favourable prognostic value. Moreover, caspase-3 revealed a significant increase in the positivity pattern between dysplasia and their corresponding invasive cancer portion and may be involved in the progression from dysplasia to ESCC (25, 26). Thus, the analysis of the apoptotic protein expression patterns may be valuable to develop rational strategies for early detection of lesions at risk in advance as well as for prevention and treatment of ESCC.

The absence of an association between Ki-67 antigen and CPP32 protein could be related to an imbalance in cell defense mechanism, when the organism tries to interrupt the proliferation of damaged cell, decreasing cell proliferation and increasing the apoptosis levels, because close coordination of these two phenomena is essential not only for a regulation and normal physiology, but also for disease prevention (4) and treatment.

In conclusion, the exact role of the Ki-67 antigen in ESCC is unclear, because some studies have shown the increase of Ki-67 expression follows the severity of the lesion, while others have not. In the present study, no difference was found among the groups, indicating the need for further investigations. This study did not show statistically significant difference of the CPP32 expression between the benign lesion groups and ESCC group. In addition there were no observed associations among the levels of CPP32 and Ki-67 expression. Thus it is not evident that significant changes in cell proliferation and apoptosis occurred in the studied esophageal benign lesions, under the tested conditions.
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