Abstract. Background: Models have suggested esophageal carcinogenesis can result from the alteration of sequences, leading to esophagitis, atrophy, dysplasia, carcinoma in situ and invasive carcinoma. While numerous genetic alterations have been reported in esophageal carcinogenesis, studies of benign lesions with precancerous potential are scarce. Materials and Methods: Immunohistochemistry was performed for p53, p16 and Fhit proteins in the esophageal mucosa from patients with Chagas disease (CD), chagasic megaesophagus (CM), chronic esophagitis (CE), esophageal squamous cell carcinoma (ESCC) and in normal mucosa (NM). Results: The proportion of p53-positive cases increased progressively according to the severity of the pathology CD (7.7%), CM (26.1%), CE (52.2%) and ESCC (100%). However, p16 and Fhit did not show any statistically significant differences among the groups. Conclusion: p53 overexpression is involved in the initial steps of esophageal carcinogenesis, supporting further evaluation of its utility as a marker in precursor lesions, conversely, losses of Fhit and p16 expression may not be significant.

The development of human esophageal cancer is a multi-step, progressive process. An early indicator of this process is increased proliferation of esophageal epithelial cells morphologically including basal cell hyperplasia, dysplasia, carcinoma in situ and invasive carcinoma (1). Patients with histological evidence of esophagitis, a promoter of the inflammatory process in the esophageal epithelium, may have a moderately increased risk of esophageal squamous cell carcinoma (ESCC) (1-3). Similarly, one of the serious late consequences of chagasic megaesophagus (CM) (esophagus dilation) due to achalasia in Chagas disease (CD) patients is the increased risk, around 3% to 8%, of these patients developing esophageal carcinoma (4, 5).

The process of tumorigenesis at the cellular level is related to disorders of cell proliferation control, differentiation and apoptosis (6). Genetic changes associated with ESCC development include alterations in the tumor suppressor genes TP53 (17p13.1), CDKN2A/P16 (9p21) and FHI T (3p14.2), and the oncogenes c-MYC (8q24), CCND1 (11q13), EGFR (7p11-15), ERBB2 (17q11.2-q12) and HGF (7q21.1), such as the regulation of their respective protein expressions (1, 7, 8-20).

While numerous genetic and epigenetic alterations have been reported in the initial and advanced steps of esophageal carcinogenesis, studies are scarce regarding benign lesions with precancerous potential (5, 21-23). The aim of this study was to evaluate the levels of p53, p16 and Fhit protein expression in precursor lesions, such as CM and chronic esophagitis (CE) in cancer-free patients, to determine the relationship between these lesions and tumor development.

Materials and Methods

Samples. A total of 80 specimens of paraffin-embedded esophageal tissue were obtained from patients who had undergone middle and distal esophageal biopsies, before any chemo- or radiotherapy induction, 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 patients with CD but without CM, 23 from CE patients and 11 from ESCC. Both patients with CD and CE were esophageal cancer free. Esophageal mucosa from 10 healthy individuals were obtained and diagnosed as histologically normal (NM). The study was approved by the Brazilian National Research Ethics Committee (CONEP).

Immunohistochemical assay. Consecutive 4 µm-thick sections were cut from each trimmed paraffin block and mounted on glass slides...
pre-treated with 3-aminopropyl-triethoxysilane/acetone solution. In brief, following deparaffinization, the 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 16 hours at 4°C with specific antibodies: anti-p53 (clone DO-7, 1:200; Novocastra, Newcastle, UK), anti-p16 (clone 6H12, 1:50; Novocastra) or anti-Fhit (clone SPM472, 1:200; Abcam, Cambridge, USA). The slides were incubated with biotinylated secondary antibody, then with streptavidin-biotin peroxidase, following the manufacturer’s instructions (Super ABC Universal Kit, Easy Path; Erviegas, São Paulo, Brazil to anti-p53; Dako Cytomation Kit, Strept ABComplex/HRP; Dako, Glostrup, Denmark to anti-p16 and anti-Fhit). The immunostain was visualized 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. Breast carcinoma, anterior pituitary and normal breast tissues were used as positive controls for p53, p16 and Fhit antibodies, respectively.

For p53 protein, approximately 500 cells were counted for each sample. For p16 and Fhit proteins, all the prepared tissue was examined for each sample. All the analyses were conducted under light microscopy (×400 magnification). The index of positivity for p53 protein (p53+) was defined as >3% of cells showing strong brown nuclear staining (5 times the maximum value observed in NM).

Immunostaining for p16 (brown nuclear and cytoplasmic staining) and Fhit (brown cytoplasmic staining) proteins was graded by intensity of staining as negative i.e. (−) absence of brown staining, or (+) weakly stained; or as positive i.e. (+++) strongly stained as described previously (9, 24, 25). Positive scores (++/++++) corresponded to normal protein expression, while negative immunostaining (−/−) was associated with loss of protein expression. Areas that were poorly preserved, crushed, cauterized, folded, or retracted were specifically avoided.

Statistical analysis. Descriptive statistics, Kruskall-Wallis test with Dunn post-test, Fisher’s exact test and paired Student t-test were used to determined statistical significance. The statistical analysis was carried out with GraphPad Instat 3 computer software (26). The level of significance was set as p<0.05. The Kappa test was used to examine the agreement among the three proteins (altered or normal expression) in each patient in the groups evaluated. It was performed using an online tool (27).

Results

The demographic and clinicopathological data for all the groups are shown in Table I. Kruskal-Wallis test with Dunn post-test, Fisher’s exact test and paired Student t-test were used to determined statistical significance. The statistical analysis was carried out with GraphPad Instat 3 computer software (26). The level of significance was set as p<0.05. The Kappa test was used to examine the agreement among the three proteins (altered or normal expression) in each patient in the groups evaluated. It was performed using an online tool (27).

There was no agreement in simultaneous altered expression of the three proteins (Agreement Analysis by Kappa) in each studied group, but associations between protein expressions, comparing two by two, were found (p53 and p16; p53 and Fhit; p16 and Fhit) by Fisher’s exact test (Table II). Moreover, no significant associations were observed between altered expression of p53, p16 and Fhit proteins and demographic and clinicopathological parameters such as age, gender, smoking and or (data not shown) in the different groups.

Discussion

The age difference between the groups was highly significant. The age of patients with CD, CM and ESCC was greater than those with NM and CE, corroborating the finding that patients with CD can be asymptomatic for many years up to late diagnosis (28). Megaoesophagus affects individuals between the second and fourth decades of life and the diagnosis of carcinoma is hampered by a number of factors that mask its symptoms (21, 28). CE is only differentiated from ESCC when smoking and alcohol habits are compared, in both subgroups, indicating that the esophagitis can be caused by several factors (28), independent of alcoholism and or smoking habit. However, associations between these parameters and altered expression of p53, p16 and Fhit proteins were not observed in the studied groups.

In the present study, an increased expression of p53 protein was progressively observed according to the severity of the lesion (CM, CE and ESCC). The strong and diffuse nuclear staining in the ESCC group probably arose from the high expression of mutant p53 protein, whereas in CE (with
diffuse staining) and CM (strong and focal staining), it was not possible to indicate p53 as mutated protein. It may also have been due to the expression of wild-type p53 that accumulates in the cells as a consequence of the physiological and inflammatory processes in the esophageal epithelium.

Fagundes et al. (11) also detected a progressive increase of p53 expression from normal esophageal mucosa (12%), through moderate esophagitis (22%), severe esophagitis (33%), dysplasia (36%) to carcinoma (100%), most often in smokers and alcoholics. Thus they suggested that patients with CE and p53 protein overexpression have an increased risk of esophageal cancer.

Recently, p53 expression was examined by immunohistochemistry in patients with esophageal carcinoma and achalasia in regions of non-malignant epithelia, showing esophagitis and or dysplasia. The authors observed increased expression of p53 in tumors, but not in non-neoplastic epithelia, such as esophagitis and dysplasia. However, histological changes were observed in the achalasia epithelia, from esophagitis due to the stagnation of food, leading to high epithelial proliferation and increased expression of mutant p53 (29). Chagasic megasphagus patients often have CE due to esophageal stasis that has extended the esophagus, increasing the risk of bacterial proliferation and damage to the mucosa (21, 29, 30). These bacteria belong to the normal microflora of the mouth and oropharynx and create an environment for anaerobic microorganisms capable of reducing nitrate into nitrite, producing carcinogens which may trigger esophageal carcinogenesis (30).

In the present study, 70% CM patients also had CE, of whom 5/6 had altered p53 expression and soft to severe CE. Therefore, the altered expression of p53 in the CM (6/23) and in CE (12/23) patients may have occurred in response to the inflammatory process. In addition, a moderately increased risk of developing ESCC was reported in CE patients, suggesting that CE is the precursor lesion for squamous cell carcinoma (3).

Genetic alterations in dysplasia of esophageal carcinoma has shown p53 immunoreactivity ranging from 40% in moderate dysplasia to 48% in carcinoma, indicating an increase in p53 expression in dysplasia compared with both normal epithelia and esophagitis. The increase of p53 expression was greater in dysplasia associated with carcinoma than in non-carcinoma-associated dysplasia, suggesting that the expression of p53 shown by immunohistochemistry is an important biomarker in esophageal carcinogenesis in pre-cancerous lesions (12).
In an Iranian study of familial and sporadic esophageal carcinoma, it was noted that 64.3% of family members studied had the CDKN2A/P16 gene hypermethylated in the promoter region. In sporadic esophageal carcinoma, P16 hypermethylation was observed in 73.3% of tumor cases; in samples of serum and blood, of the same patients, hypermethylation was found in 26.6% and 43.3%, respectively. These results indicate that P16 could be used as a biomarker in the early identification of esophageal cancer in high-risk populations and in cases of family history (31). Therefore, both the occurrence of mutation and methylation of DNA, as the allelic loss (LOH) at P16, appear to play an important role in esophageal carcinoma development (31, 32). However, in the present work, analysis of the expression of p16 in the patients with benign esophageal lesions and carcinoma was not consistent, since no changes were found between these groups.

The abnormal expression of Fhit has been described as a common event in the early stage of esophageal carcinoma development, with complete loss or reduction of the protein expression, increasing along with histopathological severity, such as dysplasia to carcinoma in situ and invasive carcinoma (18, 19). However, no difference was found for Fhit expression in the esophageal lesions in the present study.

Smokers and alcoholic patients exhibit altered p53 expression, as shown by immunohistochemistry (7, 11). Excessive consumption of alcohol and tobacco are related to higher rates of loss of Fhit expression than do those of low consumption, confirming the finding that the FHIT/FRA3B locus is susceptible to damage caused by environmental carcinogens (18, 20). However, analysis of all the groups using the Fisher’s exact test showed no association between the changes in the expression of the analyzed proteins and the demographic and clinicopathological parameters (sex, age, smoking and alcohol consumption).

According to the literature, the regulator proteins of the cell cycle are controlled by tumor suppressor genes and oncogenes; this interaction works in the regulation of cell cycle progression (33). The overexpression of p53 protein and loss of expression of Fhit and p16 proteins were reported by Chang et al. (34) in 64%, 75% and 81% respectively, of cases of invasive esophageal carcinoma, indicating that expression changes in these proteins are related to neoplasia development. However, in our study, Kappa test analysis showed no agreement in the expression levels of these proteins, even though Fisher’s exact test indicated an association between p53 and p16 (CD, CM and CE), p53 and Fhit (ESCC), p16 and Fhit (CD, CM, CE and ESCC) protein expression. In cells, thousands of molecules act together, but we cannot expect a perfect correlation among specific ones, which may explain the lack of agreement among the three proteins when examined simultaneously. It should also be considered that benign lesions such as CE and megaesophagus should not present multiple genetic alterations similar to carcinomas which show an accumulation of different alterations, involving both oncogenes and tumor suppressor genes.

### Table II. Kappa test to describe the intensity of agreement among the expression of three proteins simultaneously and association between protein expression, two by two, by Fisher’s exact test on esophageal mucosa, in each evaluated group.

<table>
<thead>
<tr>
<th>Group</th>
<th>p53</th>
<th>p16</th>
<th>Fhit</th>
<th>Kappa</th>
<th>Fisher exact test (P-value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Altered</td>
<td>Normal</td>
<td>Altered</td>
<td>Normal</td>
<td>Altered</td>
</tr>
<tr>
<td>NM</td>
<td>(0%)</td>
<td>(100%)</td>
<td>(60%)</td>
<td>(40%)</td>
<td>(10%)</td>
</tr>
<tr>
<td>CD</td>
<td>(7.7%)</td>
<td>(92.4%)</td>
<td>(76.9%)</td>
<td>(23.1%)</td>
<td>(7.7%)</td>
</tr>
<tr>
<td>CM</td>
<td>(26.1%)</td>
<td>(73.9%)</td>
<td>(26.1%)</td>
<td>(73.9%)</td>
<td>(17)</td>
</tr>
<tr>
<td>CE</td>
<td>(52.2%)</td>
<td>(47.8%)</td>
<td>(95.7%)</td>
<td>(4.3%)</td>
<td>(8)</td>
</tr>
<tr>
<td>ESCC</td>
<td>(100%)</td>
<td>(0%)</td>
<td>(90.9%)</td>
<td>(9.1%)</td>
<td>(2)</td>
</tr>
</tbody>
</table>

NM, normal mucosa; CD, Chagas disease patients without megaesophagus; CM, chagasic megaesophagus; CE, chronic esophagitis; ESCC, esophageal squamous cell carcinoma. Immunostain: Altered, p53+ > 3%, p16 and Fhit (absent/weak); Normal, p53+ ≤3%, p16 and Fhit (moderate/strong). Kappa test: *ns Kappa value <0, no agreement between protein expressions (p53 vs. p16 vs. Fhit). Fisher exact test: *ns no significant association; *p<0.05, statistical association between protein expression.
Figure 1. Immunohistochemical stain in esophageal mucosa. A, p53 protein: a, NM, negative; b, CD, negative; c, CM, p53+; d, CE, p53+ and e, ESCC, p53+. B, p16 protein: a, NM, weak; b, CD, absent; c, CM, cytoplasm moderate and nuclear strong; d, CE, moderate and e, ESCC, absent. C, Fhit protein: a, NM, weak; b, CD weak; c, CM, moderate; d, CE, strong and e, ESCC, strong. NM, normal mucosa; CD, Chagas disease patients without megaesophagus; CM, chagasic megaesophagus; CE, chronic esophagitis; ESCC, esophageal squamous cell carcinoma; Magnifications ×200.
The present study of p53, p16 and Fhit protein expression in esophagitis and megaesophagus patients without cancer is unprecedented. The results support the involvement of p53 protein in the progression of esophageal carcinoma from precursor lesions, indicating that the cell cycle may be delayed due to persistent inflammation (esophagitis and the accumulation of food caused by megaesophagus) and so increases the risk of DNA damage. This association may gradually emerge as a sequence of severity of injuries (CM, CE and ESCC) and may be related to increased risk of tumor development. However, no significant alterations in the expressions of p16 and Fhit proteins were observed that could indicate a major role in the early steps of esophageal carcinogenesis.

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References


