Expression of p53, p16 and Ki67 proteins in ductal adenocarcinoma of the pancreatic head and their relation with survival and cell differentiation

Expressão das proteínas p53, p16 e ki67 no adenocarcinoma da cabeça do pâncreas e sua relação com a sobrevida e diferenciação celular

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ABSTRACT
Objective: To determine the expression of p53, p16 and Ki-67 and its relevance in survival and cell differentiation. Methods: Fifteen duodenopancreatectomized patients were included. Immunohistochemical expression of p53, p16 and Ki-67 was determined in paraffin embedded tumor blocks. The relation of these expressions with different variables was studied. Results: Ninety-three per cent of tumors showed expression of p53 and p16. Ki-67 was expressed in 86.66% of tumors (labeling index – LI 11.91 ± 9.47). The presence of combined alterations was not related to significant differences in tumor type, stage or survival; similar results were obtained analyzing isolated expressions. When groups of p16 and Ki-67 expressions where created, the median survival was not significant. However, there was a slightly better survival in patients with focal expression of p16 (median survival 20.75 versus 14.34), when compared to patients with diffuse expression. Conclusion: The overexpression of p53, p16 and Ki-67 was not related to survival or tumor grade, when comparing isolated or combined expressions.

Keywords: Tumor suppressor proteins/analysis; Cell cycle proteins/analysis; Ki-67 antigen/analysis; Tumor suppressor protein p53; Pancreatic neoplasms; Survivorship

INTRODUCTION
Pancreatic cancer (PC) is one of the deadliest cancers. Its incidence and mortality are similar and it represents a Public Health problem in Western countries(1-3). Most of the time late diagnosis contributes to poor prognosis and precludes adequate surgical treatment(4). Understanding carcinogenesis and its relation with...
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OBJECTIVE
To determine the expression of p53, p16 and Ki-67 and its relevance in survival and cell differentiation and compare the results of combined and isolated immunostaining for each marker.

METHODS
Patients and samples
Fifteen cases of PC – all ductal adenocarcinomas of pancreatic head – were studied. All patients were seen and operated on at Surgical Gastroenterology Department of the Universidade Federal de São Paulo (UNIFESP-EPM), in São Paulo, Brazil, from 1993 to 2003. The sample consisted of 45 routinely neutral-formalin fixed and paraffin-embedded blocks strictly containing tumor tissue, and there were three for each patient. The subjects were 11 males (73.33%) and 4 females (26.66%) with a median age of 56 years (range of 45-66 years). According to the Union for International Cancer Control (UICC), 40% (6 patients) had IIB stage, 33.33% (5 patients) had IIA stage, and 13.33% (2 patients) had IB and III stages each. Tumors were graded and 6 were classified as well differentiated while 9 as moderately differentiated. By the end of the study, 2 patients were still alive with 34 and 119 months of survival each.

Antibodies and immunohistochemical staining
The primary antibodies were anti-p53 DO-7 (DakoCytomation, Glostrup, Denmark) against human p53, anti-p16 clone Ab7 16PO7 (Neomarkers, Fremont, CA, USA) against p16, and anti-Ki67 clone MIB-1 (Immunotech, Marseille, France) against Ki-67 antigen. Immunohistochemistry was performed according to Hsu et al. Briefly, 4-μm thick sections were deparaffined and incubated with primary antibodies against human proteins and antigen was diluted to 1:20 for p53, 1:100 for p16, and 1:80 for Ki-67, at 4°C, overnight. A microwave irradiation procedure was applied for antigen retrieval. Immunolocalization was performed using the Streptavidin Biotin Complex IHRP; Duet, Mouse/Rabbit (DakoCytomation, Glostrup, Denmark) kit. The color of the reactions was developed using 3-3'diaminobenzidine (DAB) (SIGMA Chemical Co., St. Louis, MO, USA) and counterstaining was carried out with Harris hematoxylin. For positive controls sections of colon adenocarcinoma for p53, sections of cervical cancer for p16, and sections of oligodendrogloma for Ki-67 were included. For negative controls sections of normal pancreatic tissue were included. Positive staining included nuclear and cytoplasmic staining for p53 of more than 10% of target cells; for p16, more than 5% and any immunostained nuclei, regardless of intensity, for Ki-67 and expressed for labeling index (LI).

Statistical analysis
The data were analyzed by Fisher’s exact test ($\chi^2$) to compare proportions of isolated and combined staining. A cutoff was used in immunostaining of p16 and Ki-67 of 25 and 15% of the stained cells, respectively. Overall survival estimates were obtained according to the actuarial method of Kaplan-Meier. Survival curves below and above the chosen cutoff were plotted monthly and compared by log rank. Finally, to determine significance of the risk factor, Cox proportional univariate analysis was used. Statistical tests were paired and a p value > 0.05 was determined. Statistical software SAS version 8.02 (SAS Institute, Cary, NC, USA) was used.

RESULTS
Overall results of protein immunostaining are summarized in table 1, and typical examples of the positive and negative groups for p53 and p16 are shown in figure 1.
According to TNM stage and cutoff point, expression data are shown in Table 2.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Total (%)</th>
<th>p53</th>
<th>p16</th>
<th>Ki-67</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>&lt; 25</td>
<td>&gt; 25</td>
<td>&lt; 15</td>
</tr>
<tr>
<td>IB</td>
<td>2 (13.3)</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>IIA</td>
<td>5 (33.3)</td>
<td>5</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>IIB</td>
<td>6 (40.0)</td>
<td>5</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>III</td>
<td>2 (13.3)</td>
<td>2</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>15 (100)</td>
<td>14 (93.3)</td>
<td>6 (42.9)</td>
<td>8 (57.1)</td>
</tr>
</tbody>
</table>

The association of p53 immunostaining with mortality did not show significance (p = 0.667) even considering staining with cell differentiation. Similar findings were obtained comparing p16 (p ≥ 0.05) and Ki-67 (p > 0.05). Median global survival was 17.71 months (95%CI: 9.07-20.47) (Figure 2). Survival of p53 positive patients was 15.7 months. Using the cutoff point, survival of patients with expression of p16 above 25% of cells was 14.34 (95%CI: 5.39-18.46) compared to expression below 25% that was 20.75 (95%CI: 9.07-24.45), log rank p = 0.088 and Hazard Ratio (HR) 2.821 (95%CI: 0.817-9.740) (Table 3).

Patients with staining for MIB-1 above 15% of cells showed survival of 17.46 months (95%CI: 5.39-27.56), compared to those with below 15% who survived 17.71 months (95%CI: 9.07-19.94), log rank p = 1.955 and HR 0.419 (95%CI: 0.108-1.618) (Table 3).

Finally, association of immunoexpressions was calculated and 53% of the patients shared p53 and p16 (> 25%); 33.3% of the patients presented p53 and Ki-67 (> 15%), 26.7% showed association between p16 (> 25%) and Ki-67 (> 15%), and those accumulating p53, p16 (> 25%), and Ki-67(>15%) expression represented 26.7%. None of the combined expressions appear to be related to survival (p ≥ 0.05).

**DISCUSSION**

Identification of mutated genes in PC led to better understanding of this condition. Early in literature, researchers accepted that DNA content in tumoral cells would influence prognosis. However, little effect was observed in clinical practice; most of the time, because some mutations were relatively specific and rare, and
technology involved in detection and identification of such alterations was expensive and not always available. PC carcinogenesis is a multiple step process and would certainly be better understood by the study of more than one gene or marker involved at a time. The possibility of immunohistochemical detection of protein expressions is an important tool in genetic research. This is specially considered in gene p53, since mutation or alteration in its function confers stability and increases half-life of the nuclear protein. Similarly, p16 expression would be easily determined by IH(19,27,29). Since its first description, Ki-67 has been employed to accurately measure proliferation of cell fraction, which would be a marker of cell differentiation. This would create a great deal of possible uses as an excellent marker for prognosis and not only for prediction(23).

In this study, all samples showed positive reaction for DO-7 for more than 50% of cells, except for patient 14 who was negative, and was the only one who survived more than 5 years (119 months). Ninety-three percent of the samples were positive for Ab7 16PO7. These proportions were not significant and the results influenced the decision to establish groups using a cutoff point of positive expressions. Two publications showed interesting findings. One published that lack of expression of p16 was related to better survival(14), and the other reported that mutation of p16 was related to clearly longer survival rates(20). LI was quite heterogeneous (11.9 ± 9.47) and not significant. Some authors(16,30) published similar findings in PC and other gastrointestinal tumors due to heterogeneous expression of Ki-67. On the other hand, this was not observed in some neurologic tumors, in which LI would be easily used as a predictor and a prognostic factor as well(23,31).

Correlation of TNM with protein expression was also described(32,33), but this association was not clear enough and requires further studies. Our group could not find this association, neither a p value > 0.05. In this study, using the Kaplan-Meyer method overall survival estimates were carried out on samples with expressions below and above cutoff point. Although it was not possible to use a cutoff point of p53 expressions, interesting observations in p16 groups were found. Survival of patients with expressions focally positive for Ab7 16PO7 (< 25%) was better than diffuse positive (> 25%) ones. It was not possible to find statistically significant differences between these groups, and the Ki-67/LI groups as well; besides, it is possible that the findings reflected the small number of subjects studied.

There are several publications analyzing genetic mutations and altered expressions of various genes as responsible for differences in survival(10,23). However, likewise in our study, the negative results could be due to the small sample(10,16). That bias could explain our findings of association of expressions and the lack of differences in survival.

CONCLUSION
In this study, overexpression of p53, p16 and Ki-67 was not related to survival or tumor grade, when comparing isolated or combined expressions.

Taken together, the present results encourage us to create a cooperative group to study not only these mutations, but also a pool of them in an expressive series using other specific technologies, such as microarrays and gene sequencing.

REFERENCES