Frequent Loss of p16 Expression and Its Correlation with Clinicopathological Parameters in Pancreatic Carcinoma

Yu-Xin Hu, Hiroyuki Watanabe, Koushiro Ohtsubo, Yasushi Yamaguchi, Aiguli Ha, Takashi Okai, and Norio Sawabu

Department of Internal Medicine, Cancer Research Institute, Kanazawa University, 4-86 Yoneizumi, Kanazawa 921, Japan

ABSTRACT

Expression of the p16 gene product in human primary pancreatic carcinoma (PC) was investigated in paraffin-embedded tissue using a monoclonal antibody against p16 protein, clone G175-405, by means of immunohistochemistry, and the correlation of results with various clinicopathological parameters was evaluated. All six cases of normal pancreas and all but 1 of 20 cases of chronic pancreatitis expressed p16 protein, whereas 37.5% (3 of 8) of cystadenomas and 41.9% (26 of 62) of PCs lost p16 expression. There was a significant difference between chronic pancreatitis and PC for frequency of the loss of p16 expression ($P < 0.01$). Moreover, loss of p16 expression in pancreatic malignancy was significantly associated with histological grade (G1 versus G2 and G3, $P < 0.01$) but not with sex, age, clinical stage, tumor location, or resectability. The survival period was shorter and metastasis is more likely in those cases that did not show p16 expression than those that did.

INTRODUCTION

The incidence of PC has increased in recent decades in both China and Japan. Moreover, the aggressive nature of this malignancy, its refractory response to treatment, and the lack of an early diagnostic method have contributed to a poor prognosis. However, understanding the molecular pathogenesis of PC may well be the foundation upon which to develop novel strategies for identifying genetic markers useful for early diagnosis. An association has been demonstrated between PC and expression of the K-ras oncogene (1) as well as $p53$ tumor suppressor gene mutation (2). We reported previously K-ras gene mutations in stimulated samples of pancreatic juice in 81% of patients with PC (3). Recently, a new tumor suppressor gene, $p16$ (also called MST1, CDKN2, and p16INK4), located at chromosome 9p21 has received special attention. This gene encodes p16 protein, which regulates proliferation through the $G_1$ phase of the cell cycle. Because the p16 protein is a cyclin-dependent kinase 4 inhibitor, deletion or mutation of the $p16$ gene may increase the activity of cyclin-dependent kinase 4, resulting in hyperphosphorylation of the Rb protein, progression of the cell cycle from $G_1$ to $S$, and hence, enhancement of cell proliferation (4). Many studies have examined genetic changes of the $p16$ gene, but little attention has been paid to the expression of its product in primary cancers. Although mutations and deletions of the $p16$ gene have been found to be relatively common in PC by genetic analysis (5–8), subsequent studies also have reported discrepancies between primary PC and cultured cell line (9), which raises the question of whether genetic alterations of $p16$ are a consequence of cell culture or selective conditions during the establishment of a cell line. Furthermore, there are some difficulties associated with assessing the role of the $p16$ gene in tumorigenesis using genetic analysis due to the relatively high GC content of this gene, methodological limitations, and infiltration of nonmalignant cells in primary resected tumor sample. In recent years, some investigators have developed an immunohistochemical assay using polyclonal antibodies to detect altered p16 expression using sections from paraffin-embedded cell lines and archival tissues. This method may be a suitable modality with which to screen for p16 expression abnormalities, especially in a large number of samples (10–14). In the present study, we examined p16 expression in 62 cases of human primary PC using a monoclonal antibody to human p16 protein by means of IHC and correlated the results with various clinicopathological parameters to elucidate whether altered p16 expression play a role in the development and progression of PC.

MATERIALS AND METHODS

Patients and Samples. Sixty-two cases of PC, consisting of 52 pancreatic ductal adenocarcinomas, 8 cystadenocarcinomas, and 2 adenosquamous carcinomas were used. All samples were collected at surgery, except for one, which was obtained at autopsy, at Shanghai Hospital, Second Military Medical University in Shanghai, China, and the Cancer Research Institute, Kanazawa University in Kanazawa, Japan, from 1977–1996. The subjects were 37 males and 25 females with a median age of 56 years (range, 29–74 years). Tumors were predominantly primary pancreatic lesions ($n = 37$) and some metastatic tumors to the liver and lymph nodes ($n = 25$; liver involvement, 5 cases, and regional or distant lymph node involvement, 20 cases) also included. No patient received preoperative radiation or chemotherapy. Tissues were routinely fixed in neutral formalin and embedded in paraffin wax. Histopathological grade and clinical staging were evaluated according to the criteria...
established by Klöppel (15) and Hermreck (16), respectively, with 21 cases graded as well differentiated (G1), 24 as moderately differentiated (G2), and 17 as poorly differentiated (G3); and eight patients scored as stage I, 16 as stage II, 20 as stage III, and 18 as stage IV. Based on the surgical results, tumors of 35 patients were either radically resected or partially resected. The remaining 27 cases were nonresectable due to their advanced stage. Follow-up data were available for 32 patients: 15 who survived less than 6 months and 17 who survived more than 6 months. Eight cystadenomas obtained at surgery (mucinous cystadenoma, four cases; serous cystadenoma, solid and cystic tumor, two cases each, respectively) were also available. In addition, 6 cases of normal pancreas and 20 CPs were used as controls. Control samples were obtained at operation or autopsy from deceased patients and accident victims.

**Antibody and Immunohistochemical Staining.** The primary antibody, clone G175-405 (PharMingen, Inc., San Diego, CA), is a mouse monoclonal antibody against purified human p16 protein. The specificity of this antibody has been confirmed previously by Western blot analysis and is known to be suitable for IHC of paraffin-embedded tissue sections (see information on the technical data sheet of this antibody). IHC was performed according to the method of Hsu et al. (17). Briefly, sections (4-μm thick) were deparaffinized and incubated with primary antibody against human p16 protein diluted to 1:100 (5 μg/ml) at 4°C overnight. Immunolocalization was performed using the streptavidin-biotin complex method (SLAB kit; Dako Corp., Carpinteria, CA). The color of the reactions was developed using diaminobenzidine (Sigma Chemical Co., Louis, MO) and by counterstaining with hematoxylin. Cell lines, H2009 and H2373 (kindly provided by Dr. J. Geradts, Department of Pathology and Laboratory Medicine, University of North Carolina, Chapel Hill, NC) established from a small cell lung cancer and a mesothelioma, were used as positive and negative controls, respectively. Staining specificity and distribution in these cell lines have been confirmed by Western blot and IHC (10). Only nuclear staining of more than 5% of target cells, regardless of cytoplasmic staining, was regarded as positive immunoreactivity. Positive staining of the surrounding stromal cells was used as internal control. Absence of any cell staining, including stromal cells, was not interpretable, and such sample was excluded from the study to avoid technical artifact.

**Statistical Analysis.** The data were analyzed with the χ² and log rank tests to compare differences between the two subgroups of patients classified based on the results of p16 staining.

**RESULTS**

The overall results of p16 protein IHC are summarized in Table 1, and typical examples of the positive and negative groups are shown in Fig. 1. In the p16-positive group, immunoreactivity for p16 protein always simultaneously occurred in both the cytoplasm and nucleus, and nuclear staining alone was not seen in this series. In the p16-negative case, less than 5% of target cells had stained nuclei.

All 6 cases of normal pancreas and all but 1 of 20 cases of CP showed expression of the p16 gene product. Absent or aberrant p16 expression was seen in 3 of 8 (37.5%) cystadenomas and more frequently in pancreatic carcinomas (26 of 62, 41.9%). The frequency of aberrant p16 expression significantly differed between CP and PC (P < 0.01).

Results of immunostaining of p16 expression in 62 cases of PC and the correlation with various clinicopathological parameters are shown in Table 2. The distribution of 26 cases with negative staining in pancreatic malignancy was as following: pancreatic ductal adenocarcinomas (22 of 52, 42.3%); cystadenocarcinomas (3 of 8, 37.5%), and adenosquamous carcinomas (1 of 2, 50.0%). Among clinicopathological parameters, only histological grade (G1 versus G2 and G3) revealed a significant difference in the frequency of loss of p16 expression (P < 0.01). The Kaplan-Meier survival analysis and log rank test demonstrated a longer survival period in positive than negative p16 patients: 1-year survival rate and the median survival period was 20.0% and 7 months for p16-positive, and 17.6% and 5 months for p16-negative patients, respectively. The difference between the two groups was not statistically significant (P > 0.05). Moreover, the frequency of loss of p16 expression in those with a shorter survival period (≤6 months) was higher (66.7%, 10 of 15) than those with a longer survival period (>6 months; 41.2%, 7 of 17), although the difference between the two groups remained insignificant.

**DISCUSSION**

There is now substantial evidence that p16 is a tumor suppressor gene, at least in specific types of human tumors. The best evidence for genetic alterations of p16 is found in gliomas, melanomas, leukemia, and carcinoma of the esophagus (18–21). In contrast, structural abnormalities of this gene are relatively rare in primary breast (22) and ovarian cancers (23). Although deletions and mutations of p16 gene have been reported to be common in cell lines and xenografts of human PC (5, 6, 8), subsequent studies using human primary resected PC demonstrated that this is not the case. Huang et al. (9) examined 30 cases of resected human PCs and found deletions and mutations in only 8 (26.7%). Bartsch et al. (7) found somatic p16 mutations in 11 of 32 (34.4%) primary PCs. These studies showed that the exact frequency of abnormalities of the p16 gene in primary PCs is not as high as in cancer cell lines or xenografts of human PCs. In the present study, we evaluated the status of altered expression of p16 in human primary PCs by IHC, a technique confirmed to be suitable for examination of archival tissues (10–14). About 42% of PCs did not display p16 expression. This figure is high compared to that of genetic abnormalities in human primary PCs reported by previous reports (7, 9). We could not exclude the possibility that the frequency of loss of p16 expression was underestimated in the present study, as not all possible p16 expressions could be identified by our assay. p16 protein seems to be unstable and much more susceptible to
damage, especially when its immunoreactivity is too weak to be detected (10). However, it is unlikely that deletions and mutations might be the only primary modes of p16 inactivation in human cancer, including PC. Hypermethylation of the p16 gene associated with transcriptional silencing has been frequently found in lung, head and neck cancer, glioma cell lines, and fresh tumors without deletion and mutations (24). Although hypermethylation of p16 in PC has not been elucidated, codeletion of p16 and p15 in PC has been reported (8). The relatively high frequency of aberrant p16 expression in PCs obtained in the present study suggests that epigenetic factors, as mentioned above, play a role in the functional inactivation of p16. Direct genetic analysis of the p16 gene in PCs is currently under way in our department to further determine the relationship between altered p16 expression and genetic changes of this gene.

The significant difference in p16 expression between CP and PCs might reflect that loss of p16 expression plays a role in tumorigenesis and progression of PC. Also, it is noteworthy that the rate of loss of p16 expression was consistently higher in groups G2 and G3 than in G1, and this implies that p16 protein, as a product of tumor suppressor gene, might maintain phenotypic characteristics under normal conditions and inhibit cells from undergoing malignant transformation. Alternatively, if normal p16 expression can be maintained during the cell cycle, loss of p16 expression, perhaps at a point during cellular phenotypic differentiation, may interfere with terminal differentiation, and even in established malignancies may contribute to the evolution of malignant clones during the clinical course. A similar finding was observed in melanocytic lesions (13). This conclusion is supported by the trend toward a positive correlation between loss of p16 expression and poor prognosis of PC, although the relationship did not reach significance. We consider aberrant expression of p16 to be an additional factor, at least in the cascade of molecular alterations seen in PC, because frequently aberrant expression of p53 protein and Ki-ras mutation at codon 12 were found in this series.4

Only three cystadenomas were negative for p16 in the present study, and two of which, the mucinous, and solid and

\[4\text{ Data not published.}\]
Table 2  Correlation between loss of p16 and clinicopathological parameters in PC

<table>
<thead>
<tr>
<th>Parameters</th>
<th>No.</th>
<th>p16 loss (%)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥60</td>
<td>29</td>
<td>14 (48.3)</td>
<td></td>
</tr>
<tr>
<td>&lt;60</td>
<td>33</td>
<td>12 (36.5)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>37</td>
<td>17 (45.9)</td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>25</td>
<td>9 (36.0)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Pathological grade</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G1</td>
<td>21</td>
<td>4 (19.0)</td>
<td></td>
</tr>
<tr>
<td>G2 and G3</td>
<td>41</td>
<td>22 (53.7)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Clinical stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I and II</td>
<td>24</td>
<td>7 (29.2)</td>
<td></td>
</tr>
<tr>
<td>III and IV</td>
<td>38</td>
<td>19 (50.0)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Tumor location</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primary site</td>
<td>37</td>
<td>13 (35.1)</td>
<td></td>
</tr>
<tr>
<td>Metastatic site</td>
<td>25</td>
<td>13 (52.0)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Resectability</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RR and PR</td>
<td>35</td>
<td>14 (40.0)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>NR</td>
<td>27</td>
<td>12 (44.4)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>Survival (mo)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤6</td>
<td>15</td>
<td>10 (66.7)</td>
<td>&gt;0.05</td>
</tr>
<tr>
<td>&gt;6</td>
<td>17</td>
<td>7 (41.2)</td>
<td></td>
</tr>
</tbody>
</table>

* RR, radically resected; PR, partially resected; NR, not resected.

p16-positive classification. We agree with this because p16 staining could be easily detected in the cytoplasm in normal pancreas and benign pancreatic diseases in our series, and it may be a nonspecific background staining. Criteria of lost p16 expression used the in previous studies with IHC are ambiguous (10, 11–14). Taking what are mentioned above into consideration, we selected 5% as a cutoff value to differentiate positive from negative staining, which was mostly used in IHC. Furthermore, positive staining of the surrounding stromal cells was always an internal control. Absence of any cell staining, including stromal cells, was not interpretable, and such sample was excluded from this study to avoid technical artifact. Thus, the methods used in this study are supposed to be able to discriminate between negative immunoreactivity and absence due to technical failure of p16 staining.

Taken together, the present results suggest that loss of p16 expression occurs frequently in PC and plays a role in the tumorigenesis and cellular phenotypic differentiation of this malignant disease.

ACKNOWLEDGMENTS

We thank J. Geradts for help in providing control cell block sections and for critical suggestions regarding this work.

REFERENCES

Frequent loss of p16 expression and its correlation with clinicopathological parameters in pancreatic carcinoma.

Y X Hu, H Watanabe, K Ohtsubo, et al.