Infrequent Expression of p21 Is Related to Altered p53 Protein in Pancreatic Carcinoma

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ABSTRACT

This study is designed to investigate the expression of p21 and its relation to altered p53 protein in pancreatic carcinoma (PC). Immunohistochemical staining of formalin-fixed, paraffin-embedded tissue sections of PC was performed using a monoclonal antibody against p21 (187), with a parallel examination of altered p53 protein. The expression of p21 was only found in 12 of 58 (20.7%) PCs and, moreover, was mainly restricted to the well-differentiated ductal epithelium. Sixty-four% (37 of 58) of PCs showed positive p53 staining, and this change was significantly related to the absence of p21 expression (P < 0.01). In a subgroup, the proportion of the undetectable p21 expression and the expression of p53 were increased with increasing tumor grade but decreased with advancing clinical stage. The results of the present study suggest that the absence of p21 expression is very common in PCs and appears to relate to altered p53 protein. Moreover, the abnormalities involving the expression of p21 and p53 may play a more important role in the development than in the progression of this malignancy.

INTRODUCTION

Extensive studies on the abnormalities of certain oncogenes, tumor suppressor genes, and their products involved in the cell cycle have revealed that the control of cell proliferation is managed by a series of checkpoints that regulate cell cycle progression, and disruption of any component during this process deprives cells of critical antiproliferative drives and promotes cellular carcinogenesis (1). p21 (also known as waf1, cip1, and sel) and p53 protein are two important components that regulate G1-to-S-phase transition through the cell cycle. Furthermore, previous studies have suggested that p53 can induce transcription of p21, the product of which can inhibit cyclin-dependent kinases in response to DNA-damaging agents that trigger Gi arrest and apoptosis (2). Hence, an alternative approach to investigating p53 functional status is to evaluate its downstream effector, p21. Some studies on human tumors, however, did not show such a relationship, demonstrating that the expression of p21 can also be induced by p53-independent pathways (3–5). An important role for altered p53 has been confirmed in the development and progression of PCs (6–10), whereas tumor-specific alterations in the p21 gene, similar to a variety of other human malignancies, are either rare or not associated with PC (6, 11, 12). On the other hand, very little is known about the expression of p21 in PC. A study by DiGiuseppe et al. (13) on a group of 21 patients with PC showed p21-positive immunostaining in all except two cases and no correlation of the expression of p21 with either p53 mutational status or p53 overexpression. These results, however, were obtained from a small series of PCs. Again, we also noted a recent report in which an inverse relationship with respect to the expression of p21 and p53 was found in PC (14). Therefore, further information will obviously be necessary before the role of p21 protein in PC can be exactly assessed. In the present study, we investigated the functional status of p21 and p53 protein in a large series of PCs to further evaluate the correlation between the expression of these two proteins and some clinicopathological parameters.

MATERIALS AND METHODS

Patients and Specimens. Fifty-eight primary PC tissues, all but 1 of which were obtained from operation of surgical resection or biopsy at Shanghai Hospital, Second Military Medical University in Shanghai, China, and Cancer Research Institute, Kanazawa University in Kanazawa, Japan, were available for this study. The age of patients ranged from 29 to 79 (median, 55) years, with a male:female ratio of 2.0. Only those specimens that were diagnosed with primary pancreatic ductal adenocarcinoma histologically and had adequate clinicopathological data were selected. No patient was treated with radio- or chemotherapy prior to operation. In addition, six tissues of normal pancreas were also studied as controls.

Immunostaining. Immunohistochemical staining was performed using a labeled streptavidin-biotin technique (SLAB kit; DAKO, Carpinteria CA) on 4-μm-thick, formalin-fixed, and paraffin-embedded sections. In brief, after deparaffinization, the

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The abbreviations used are: PC, pancreatic carcinoma; TGF, transforming growth factor.
sections were microwaved at 95°C for 20 min in hot 10 mM sodium citrate buffer for p21 staining and target retrieval solution (DAKO Corp.) for p53 staining for a total of 20 min at about 3-min intervals. Nonspecific binding activity was inhibited with blocking serum at room temperature for 20 min. Sections then were incubated with primary monoclonal antibody against p21 (187; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) diluted at 1:100 and antibody against p53 (DO-7; DAKO, Glostrup, Denmark) at 1:50 at 4°C overnight, respectively, and followed with biotinylated link antibody and peroxidase-labeled streptavidin at room temperature for 20 min. Staining was detected using 3-amino-9-ethylcarbazol (DAKO Corp.) for p21 and 3',3'-diaminobenzidine (Sigma Chemical Co., Louis, MO) for p53 and then counterstained with hematoxylin. Human PC cell lines with known expression of p21 and p53, PANC-1 and BxPC-3, were used as positive controls for p21 and p53 staining, respectively (6, 9), both of which were purchased from Dainippon Pharmaceutical Company (Osaka, Japan). Negative controls were obtained by omitting primary antibody. Only nuclear staining and/or nuclear staining above any cytoplasmic background were considered to be specific immunoreactivity. Staining of more than at least 20% of target cells was defined as positive staining for p53, in consideration of what was shown to be closely related with p53 mutation (15). The score for positive staining of p21 was more than 5% of specific staining cells.

Statistical analysis was performed using the χ² or Fisher’s exact method to compare differences and relationships between two subgroups of patients classified by p21 and/or p53 staining. Differences were considered statistically significant when the probability value was less than 0.05.

RESULTS

Tumor Grading and Clinical Staging. Among 58 cases of PC, 37 tissues were primary, and the remaining 21 were metastatic (5 from liver and 16 from local or distant regional lymph nodes). Tumors were graded according to Klöppel’s criteria (16): well-differentiated adenocarcinoma (G1) in 20 cases, moderately differentiated (G2) in 21 cases, and poorly differentiated (G3) in 17 cases. Clinical staging was performed in accordance with the标准 put forward by Hermreck et al. (17): stage I, 8 cases; stage II, 16 cases; stage III, 17 cases; and stage IV, 17 cases.

p21 and p53 Expression. Ductal epithelium of all normal pancreas showed negative nuclear staining for p21, but immunoreactivity was readily observed in some nuclei of vessel endothelial cells, the cytoplasm of scattered cells with acinar structure, and histologically normal cells adjacent to cancerous tissues in metastatic sites. Twelve cases (20.7%) of PC were found with positive nuclear staining for p21 protein, but indetectable nuclear staining was as high as 79.3% (46 of 58). The incidence of p21 expression was not significantly distributed among histological grades: 4 of 20 (20.0%) in G1; 6 of 21 (28.6%) in G2; 2 of 17 (11.8%) in G3, nor among clinical stages: 2 of 8 (25.0%) in stage I; 3 of 16 (18.8%) in stage II; 4 of 17 (23.5%) in stage III; and 3 of 17 (17.6%) in stage IV (P > 0.05, respectively). Of note was that positive staining for p21 was almost specially localized to the well-differentiated ductal epithelium in most PCs, regardless of tumor grade (Fig. 1).

No nuclear staining for p53 was found in six normal pancreas. Thirty-seven of 58 tumors (63.8%) showed positive immunoreactivity for p53 protein. Unlike p21, p53 staining was more diffuse and heterogeneous. Tumors with more than 50% of p53-positive cells were found in 23 cases and were distributed more frequently in the of group G3 or advanced clinical stages (Fig. 2).

Relationship of p21 to p53 Expression. Thirty-two (86.5%) of 37 cases with the expression of p53 did not express
p21 (Fig. 3), and an additional seven cases with the expression of p21 were negative for the expression of p53. Hence, in the whole series, the expression of p21 was significantly related to altered p53 protein ($P < 0.01$; Table 1). We divided tumors examined into three subgroups based on the presence (+) and absence (−) of p21 and p53 expression: I, p21+/p53− (no abnormality in either of the two tumor suppressor gene products); II, p21−/p53− or p53+/p21− (an altered expression in one of the two proteins); and III, p21+/p53+ (altered expression involved in both products) and compared them with some clinicopathological features. No correlation was found between any one of three subgroups and any one of clinicopathological features ($P > 0.05$, Table 2). However, a trend that the proportion of p21−/p53+ was increased with increasing tumor grade but decreased with advancing clinical stage was found in subgroup III.

**DISCUSSION**

To elucidate the role of p21 expression and its relation to altered p53 protein in the development and progression of PC, we immunohistochemically analyzed the expression of these two proteins in 58 archival tissues of this malignancy. The results showed that the undetectable expression of p21 in PC was very common (46 of 58; 79.3%). Further analysis of the relationship between the expression of these two proteins demonstrated that the absence of p21 expression in most PCs was accompanied by altered p53 expression (32 of 46; 69.6%); i.e., altered expression of p21 and p53 was closely related ($P < 0.01$). Based on these results, we emphasize here that the undetectable expression of p21 related to altered p53 may play an important role in the development of PC.

Positive-p21 staining was found in only about 21% of PCs in our study but in 90 and 57% in the studies of DiGiuseppe et al. (13) and Dergham et al. (14), respectively. We do not consider the difference in antibody used in these studies to be the reason for the highly variable results. The monoclonal antibody p21 (187) used in the present study has been characterized, showing it only reacts with p21 protein of human origin (from the datasheet of this reagent). Moreover, we additionally stained two well-defined PC cell lines using this antibody to confirm its reliability, and the results examined were consistent with those established by Western blotting using Ab-1 (6), another monoclonal antibody used in the studies of DiGiuseppe et al. (13) and Dergham et al. (14), showing that these two antibodies have a similar sensitivity and identical specificity (data not shown). Furthermore, the staining results with p21 (187) in some human tumors have been variable, suggesting that the specificity of this antibody is dependent on the tissue type (5, 18). Therefore, the positive immunostaining detected in our series undoubtedly reflects a genuine abundance of p21 protein. Possible explanations for the difference in positive rate of staining may lie in differences in sampling and the scoring standard for staining. We used a low percentage as the cutoff for separating p21-positive from negative cases; however, it was necessary to select such a value because not many cells could be stained for p21 in most samples of this series of PC. Again, we consider that it is very difficult to exactly define staining pattern and cutoff value for p21 protein at the present time because of varying tissue specificity and the distribution of p21 protein. Recently, we also examined the expression of p27kip1, which has 42% homology with p21 in its NH2-terminal region. Interestingly, we found that this protein has some similar features to p21 in staining pattern and distribution. Based on our experiences, we felt that PC tissue perhaps is different from some
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other organs in the degree of intracellular proteolytic activity or ability to produce, at least for these two proteins, in response to extracellular signals.

In agreement with the studies of Jian et al. (19) and Mælandsmo et al. (20) on melanoma, our results suggest that the absence of p21 expression plays a role in the development of PC and support that p21 is a tumor suppressor. Similar observations in human gastric, breast, and ovarian cancers were published recently (5, 21, 22). We postulate that expression of altered p53 mediated by mutations or other abnormalities results in failure to induce the expression of p21 during pancreatic carcinogenesis. We clearly observed a lack of p21 in some areas of PC in which p53 was expressed, exhibiting that the expression of p21 might be regulated by p53. Moreover, it is possible that PC with mutant p53 protein, coupled with abnormal p21 expression, obtains a survival

Table 1  Relationship between expression of p21 and altered p53 protein in PCs

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Fig. 3 p21 staining is completely negative in tumor nests of metastatic PC (A), whereas p53 protein is diffusely stained in the same area (B). Histologically normal hepatic cells adjacent to cancer tissue show non-specific cytoplasmic staining. Both A and B, ×50.
advantage and opportunity for genetic instability or another genetic event.

Although this study shows no significant difference between the expression of p21 and PC tumor grade, there is a relative tendency showing a higher rate of the expression of p21 in group G1 or G2 compared with group G3. p21 has been known to have a role in regulating cellular differentiation in many different cell lines and tissues (23, 24). Induction of cellular differentiation seems to be consistent with suppression of tumor growth mediated by p21. In some PCs with heterogeneously differentiated tissues, some areas showing pronounced positive staining of p21 were found with well-differentiated histological features, whereas other areas showing weak immunoreactivity of p21 had moderately or poorly differentiated features. This further supports the opinion of Stincomb (25) that the expression of p21 in the tumor not only inhibits cell proliferation but simultaneously induces cell differentiation. A recent study of Gorospe et al. (26) suggests that p21 affects human melanoma cell survival in a dependent-p53 pathway. Caffo et al. (21) reported that patients with breast cancer with p21<sup>-/p53</sup> had a worse prognosis. In the present study, among 46 PCs with the absent expression of p21, 32 cases had altered expression of p53. Furthermore, the rate of the aberrant expression involved in both p21 and p53 was increased with increasing tumor grade, but no such tendency was found when a singular abnormal parameter, either p21 or p53, was analyzed. Therefore, it is possible that these two altered proteins might act cooperatively to promote malignant progression.

On the other hand, the relationship between the expression of p21 and altered p53 is not absolute in PC. Actually, some factors other than p53 could influence the expression of p21. Recently, the expression of p21 has been confirmed to be up-regulated by TGF-β1 (27). Moreover, loss of a functional TGF-β pathway may contribute to the tumorigenetic behavior of human PC cells (28). Furthermore, p21 expression in PC cell lines has been reported to be transcriptionally regulated by TGF-β, which requires the expression of DPC4 (29). Moreover, cyclin D1 was reported to be induced by p53 through p21 (30), and its expression was reported as being correlated significantly with poor prognosis of PC (31). Recently, our group found that the lack of p16 expression in PC is a common event (32). Other investigations stressed the role of p27<sup>kip1</sup> as a more important regulator of the cell cycle among cyclin-dependent kinase inhibitors during carcinogenesis (33, 34). Our study also confirmed a frequent lack of this protein in PCs examined. Therefore, it is possible that numerous products of oncogenes and tumor suppressor genes ultimately have a substantial effect on the cell cycle regulatory system by direct or indirect pathways through complicated mechanisms. We consider that identification of the mechanisms of the cell cycle regulatory system involved in tumor development is necessary for understanding tumor biological behavior and being able to seek reliable diagnostic markers as well as new therapeutic strategies.

### REFERENCES

Expression of p21/WAF-1 in Pancreatic Carcinoma


Infrequent expression of p21 is related to altered p53 protein in pancreatic carcinoma.

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