Overexpression of Cyclin D1 in Human Pancreatic Carcinoma Is Associated with Poor Prognosis

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Abstract

We have investigated the expression of cyclin D1 in adenocarcinoma of the pancreas and the relevance of cyclin D1 expression to clinical outcome. In comparison to normal pancreas, Southern blot analyses revealed amplification of the cyclin D1 coding gene in 25% of the cases, whereas with reverse transcription-PCR, overexpression of mRNA was observed in 82% of the examined tissues. Immunohistochemically, we could demonstrate nuclear overexpression in tumor cells in 68.4%, and this protein accumulation correlated significantly with poor prognosis [median survival, 18.1 versus 10.5 months; \( P < 0.01 \) (\( \chi^2 \) test)].

Introduction

Cyclins are involved in the regulation of the cell cycle progression. They are necessary for the activity of the cdks and are one of the subunits of the active kinases (1, 2). As a G1 cyclin, cyclin D1 was identified originally as a putative proto-oncogene, BCLI/PRADI, located at chromosome 11q13, as a suppressor of yeast G1 cyclin mutations and as a delayed early response gene induced by colony-stimulating factor 1 (3). Overexpression of cyclin D1 has been reported in different human cancers, including esophageal cancer, breast cancer, and hepatocellular carcinoma (4–6). Overexpression of cyclin D1 is known to shorten the G1-S transition and thus it promotes cell progression and differentiation (7). A further hint that cyclin D1 could be associated with tumorigenesis is that cyclin D1 is inducible by activated myc and ras oncogenes (8, 9). Recent evidence suggests that cyclin D1 is a mediator of apoptotic neuronal cell death (10). In accordance with these findings, Pagano et al. (11) showed that transient overexpression of cyclin D1 in fibroblasts arrests cells in the G1-phase of the cell cycle. In senescent fibroblasts, an increased level of cyclin D1 has been found (12, 13), also indicative of two opposing aspects of the impact of overexpressed cyclin D1 in cell cycle control.

To investigate the role of cyclin D1 in pancreatic cancer, we immunohistochemically analyzed 53 pancreatic cancer tissues and 7 specimens of normal pancreas on the expression of cyclin D1. From 28 of the carcinoma tissues and 7 of the normal tissues, we assessed the amount of mRNA by RT-PCR and tested for DNA amplification via Southern blot. Chen et al. (1) recently reported that cyclin D1 is inducible by the p21WAF1/CIP1 inhibitor of cdks (14), which is known to regulate the cell cycle directly. For that reason, we examined the expression of p21WAF1/CIP1 in our series of tissues and were able to demonstrate a significant correlation between p21WAF1/CIP1 expression and cyclin D1 overexpression.

Materials and Methods

Tissue Samples. Pancreatic carcinoma tissues were obtained from patients who underwent surgical operations for pancreatic cancer at the Department of General Surgery, University of Ulm. The group of patients included 26 female and 27 male patients. The median age was 63 years in a range from 39–85. Tissues were collected after surgical removal, snap-frozen immediately in liquid nitrogen, and stored at —80°C, or were fixed in 4% formalin for 1 day at room temperature, processed, and embedded in paraffin. Fifty-three adenocarcinoma tissues, 3 normal tissues from organ donors, and 4 normal pancreatic tissues from patients undergoing surgery for pancreatic cancer were used for immunohistochemistry, and with 28 of the pancreatic carcinoma tissues and 7 normal pancreas tissues, we performed RT-PCR and Southern blot analysis.

Immunohistochemistry. Five-μm paraffin sections were adhered to silanized slides, deparaffinized, and hydrated by passing through xylene (three times for 5 min each time), a graded series of ethanol (1 X 100, 80, 70, and 50%) for 5 min, and distilled water for 10 min. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol (30 min) between the first two steps of washing with ethanol. After each following step, sections were washed with 0.5 M Tris buffer (pH 7.6) three times for 10 min. Permeabilization of the sections was achieved by incubation with 0.05% trypsin for 30 min. The tissue sections were then covered with 2% rabbit normal serum in Tris-buffered saline for 20 min and were incubated overnight at 4°C with the anti-cyclin D1 monoclonal antibody (clone DCS-6; Ref. 15), which was purchased from Oncogene Science (Cambridge, MA). For each case, a corresponding section was incubated in Tris-buffered saline without the primary antibody as a control for nonspecific staining. As positive control, we used a pancreatic carcinoma tissue with a clear overexpression of cyclin D1. Biotinylated rabbit antimouse secondary antibody was added for 40 min followed by the avidin-biotinylated peroxidase complex for an additional 40 min. After washing with distilled water for 10 min, staining was achieved by using 3,3′-diaminobenzidine. The sections were then counterstained with Mayer’s hemalaun and mounted. Staining of the p21WAF1/CIP1 protein was achieved by using the monoclonal antibody WAF1 clone EA10 (Oncogene Science) according to a protocol described elsewhere (16).

RT-PCR. Frozen pancreatic tissue was cut in small blocks, which were examined by hematoxylin staining on the amount of tumor cells and tissue of chronic pancreatitis. From 100 mg of 28 tissues, which showed less than 10% chronic pancreatitis and more than 30% tumor cells, we isolated mRNA using a guanidinium thiocyanate method and oligodeoxythymidylate-cellulose column chromatography (QuickPrep Micro mRNA Purification kit; Pharmacia Biotech). The mRNA was dissolved in the elution buffer provided in the kit in a final volume of 30 μl. cDNA was prepared by RT of mRNA using SuperScript RT RNase H-Reverse Transcriptase (Life Technologies, Inc.) and diluted with distilled water to a final volume of 40 μl. A 434-bp cyclin D1 fragment was amplified using the following primers: 5′-CTGGAGCCCGT-GAAAAGAGC-3′ and 5′-CTGGAGAGAG-AGCGTGAGG-3′. A 5-μl aliquot of cDNA was amplified under the following cycling conditions: 94°C for 60 s, 55°C for 90 s, and 72°C for 120 s for 30 cycles. The PCR products were electrophoresed through a 1% agarose gel containing ethidium bromide and visualized by UV rays. β-Actin was used as an internal standard to confirm equal loading in each experiment.

Probe Preparation. RT was carried out on mRNA extracted from the human pancreatic cell line AsPC-1, and PCR amplification was performed with following primers: 5′-CCAGCCATGGAACACCAGCTCCGT-3′ and 5′-GGAATGAGAAGCTTCCCTGGA-3′. The cycling conditions were: 40 cycles, 94°C for 45 s, 55°C for 30 s, and 72°C for 50 s.
were as follows: 94°C for 3 min, 65°C for 1 min, 72°C for 1 min for 30 cycles, and a last step of 10 min at 72°C. The amplified 1015-bp cyclin D1 fragment was used as the probe for Southern hybridization.

**Southern Blot Analysis.** Genomic DNA was isolated from homogenized human pancreatic cancer tissue as described previously (17). Ten μg of DNA were digested with EcoRI (Boehringer Mannheim, Mannheim, Germany) and electrophoresed through a 1% agarose gel. The DNA was then transferred to a Nytran membrane (NY 12N; Schleicher und Schüll, Dassel, Germany) with the Turboblotter (Schleicher und Schüll), a downward transfer system, according to the manufacturer's instructions. After prehybridization for 3 h at 65°C in 6× SSC [0.15 M NaCl; 0.015 M Na-citrate], 5× Denhardt’s solution (0.1% BSA, 0.1% polyvinylpyrrolidone, and 0.1% Ficoll), 0.5% SDS, and 100 μg/ml denatured salmon sperm, DNA hybridization was carried out for 15 h at 65°C in prehybridization solution containing 5 × 10⁶ cpm ³²P-labeled probe. For autoradiography, membranes were exposed to Kodak X-OMAT XAR-5 film (Siemens, Stuttgart, Germany) at −70°C with intensifying screens for 72 h.

**Results**

**Cyclin D1 Is Overexpressed in Pancreatic Carcinoma.** In normal pancreatic tissue, no staining of cyclin D1 was observed (Fig. 1). Using the anti-cyclin D1 antibody immunohistochemistry revealed a nuclear staining of the tumor cells in 68.4% of the carcinoma tissues investigated; in 31.6% of the pancreatic carcinoma tissues, no staining was achieved. Staining of the tumor cells was mostly moderate, with only a few cells exhibiting a strong nuclear staining reactivity (Fig. 1). One case showed a strong staining of both the nuclei and the cytoplasm of the carcinoma cells. Staining with an antibody against p21WAIF1/CIP1 also revealed a clear nuclear staining of cancer cells in 58.5% of the pancreatic carcinoma tissue, as described elsewhere (16).

In our series of tissues from pancreatic adenocarcinomas, we were able to demonstrate a close correlation between p21WAIF1/CIP1 expression and cyclin D1 overexpression (P < 0.001; Table 1). In the only cancer tissue judged to be strongly positive for cyclin D1 expression, a very strong p21WAIF1/CIP1 expression occurred as well. In chronic pancreatitis tissue that often surrounds pancreatic carcinoma, some areas exhibited strong immunoreactivity with the anti-cyclin D1 antibody, and staining of these cells was not limited to the nucleus (Fig. 1).

**Amplification of Cyclin D1 in Pancreatic Carcinoma.** Because it is well known that cyclin D1 is amplified at DNA levels in different carcinomas, such as breast or esophageal carcinoma, we performed Southern blot analysis of DNA isolated from 28 cases of pancreatic carcinomas. Amplification of the cyclin D1 gene was observed in 25% of the cases investigated (Fig. 2), whereas all cases of normal pancreatic tissue showed no gene amplification (Fig. 2). All of these carcinomas with cyclin D1 gene amplification were positive in immunohistochemistry. In the pancreatic carcinoma cases that showed no gene amplification, 62% stained positively and 38% stained negatively in immunohistochemistry using an anti-cyclin D1 antibody.

**Cyclin D1 mRNA Overexpression Correlates Significantly with Immunoreactivity in Pancreatic Cancer.** Cyclin D1 mRNA expression was determined by RT-PCR, amplifying a 434-bp product (Fig. 3). In comparison to normal pancreatic tissue, an overexpression of cyclin D1 mRNA was found in 82% (23 of 28) of the cases. In 75% (21 of 28) of the cases investigated, cyclin D1 mRNA expression correlated with cyclin D1 protein expression. At any rate, 17.8% (5 of 28) of the tissues revealed high levels of cyclin D1 mRNA and showed no cyclin D1 antigen overexpression, and 7.1% (2 of 28) with protein staining exhibited no elevated cyclin D1 mRNA signal in RT-PCR.

**Correlation of Cyclin D1 Antigen with Clinicopathological Features.** To assess the clinical importance of cyclin D1, we correlated the overexpression of cyclin D1 protein with age, sex, differentiation, stage (UICC), tumor size, tumor involvement, and tumor localization, and we were not able to show a significant correlation among them (Table 1). Interestingly, there was a significant difference between cyclin D1-positive and -negative cases. Patients whose tumors were cyclin D1 negative in immunohistochemistry lived significantly longer as compared to patients whose pancreatic tumors stained positively with the anti-cyclin D1 antibody in immunohistochemistry, as follows: (a) median survival, 18.1 versus 10.5 months; P < 0.01 (χ² test); (b) at 18 months, cyclin D1 negative, 56% alive; cyclin D1 positive, 28% alive; P < 0.01 (Fisher’s exact test; Fig. 4).
Table 1: Comparison of cyclin D1 expression and clinicopathological findings

The correlation of immunohistochemical staining for cyclin D1 in pancreatic carcinoma with clinicopathological features revealed no significant correlation between cyclin D1 antigen expression and age, sex, grading, stage (UICC), tumor size, tumor localization, and lymph node metastasization. The comparison of cyclin D1 and p21\(^{WAF1/CIP1}\) expression yielded a significant correlation between them (P < 0.001).

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<tr>
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<td>12</td>
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<tr>
<td>Poor</td>
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*NS, not significant.

Discussion

In many types of human tumor cells, cyclin D1 is deregulated, and overexpression of cyclin D1 contributes to oncogenic transformation of cells in vitro and in vivo (3, 5, 6, 18). To examine to what extent amplification and overexpression of cyclin D1 contributes to tumorigenesis and clinical outcome, we investigated the expression of cyclin D1 in human pancreatic carcinoma. In immunohistochemistry, we never achieved staining of healthy pancreatic tissue, but 68.4% of the pancreatic cancer tissues showed a clear moderate nuclear staining of the tumor cells. Only one case was judged to be strongly positive with further staining of the cytoplasm. Overexpression of cyclin D1 antigen was associated with mRNA overexpression in most cancer tissue, and these findings are consistent with other reports in esophageal tumor (18).

In other tumors, such as breast, liver, and esophageal carcinomas, other groups have found cyclin D1 gene amplification in 16–42% (4, 5, 15, 18). In our study, 25% of the tumors investigated showed cyclin D1 gene amplification. Immunohistochemically, we revealed positive staining for cyclin D1 in 68.4% of the pancreatic cancer tissues, implying that there exist other mechanisms leading to cyclin D1 overexpression than gene amplification. This finding, that cyclin D1 antigen overexpression is not always absolutely due to cyclin D1 gene amplification, has also been described by Gillett et al. in breast cancer tissue (5). Amplification of other genes located at 11q13, such as int-2 or hst-1, has been reported to be associated with prognosis in esophageal and breast cancer (4, 18–22). But the fact that not only gene amplification contributes to cyclin D1 overexpression in pancreatic tissue may be an important argument that cyclin D1 overexpression is not only determined by the amplification of the 11q13 region.

Comparative studies of cyclin D1 expression and clinical features revealed a significant correlation between poor prognosis and over-
expression of the cyclin D1 antigen in tumor cells in our series of 53 pancreatic carcinomas, in agreement with other reports in breast and esophageal carcinoma (15, 18), and we were not able to show a significant correlation between overexpression of cyclin D1 and sex, age, grading, resectability, stage (UICC), tumor size, and lymph node metastasis. This may suggest that cyclin D1 overexpression is involved in abnormal cell proliferation and, because there was no statistical difference in expression and no obvious difference in staining intensity between early and advanced tumor stages, cyclin D1 deregulation seems to be an early event in tumorigenesis. In pancreatic carcinoma, most of the tumor tissue is surrounded by areas of chronic pancreatitis, and this tissue, with many dying cells, often showed cyclin D1 expression more strongly than the tumor cells and not limited to the nucleus. On the one hand, the expression of cyclin D1 in chronic pancreatitis tissue could be interpreted as an early link to tumorigenesis, but on the other hand, in chronic pancreatitis, the areas that stained positive for cyclin D1 showed strong fibrosis, pointing to dying pancreatic cells. Also, the staining intensity of these cells was clearly stronger than that of most of the tumor cells. With regard to the attempt to transfect and to overexpress cyclin D1 in cell lines, which then showed a reduced viability, high levels of cyclin D1 seem to be toxic (23). This paradox is also reflected in our results concerning pancreatic tumor cells: cyclin D1 overexpression correlated significantly with poor prognosis and, on the other hand, with the expression of the cdk inhibitor p21WAF1/CIP1 (P < 0.001), which mediates G1 arrest and apoptosis. Chen et al. (14) recently reported that p21WAF1/CIP1 induces cyclin D1 synthesis, supporting the hypothesis that cyclin D1 not only contributes to oncogenic transformation but also to growth arrest. These interrelations between p21WAF1/CIP1-mediated growth arrest and cyclin D1 are also discussed by Del Sal et al., who were able to show that both cyclin D1 and p21WAF1/CIP1 are involved in p53-mediated growth suppression in murine cell lines expressing temperature-sensitive p53 protein (24). But how can these findings be explained in view of the fact that overexpression of cyclin D1 contributes at least in some carcinomas to tumorigenesis? A speculative explanation could be that the decision for growth or apoptotic cell death in a given cell type is dependent on the concentration of cyclin D1. In conclusion, our findings suggest that, besides gene amplification, cyclin D1 accumulation is mainly due to induction by other factors participating in cell cycle control, and one possible candidate could be p21WAF1/CIP1. Along with the observation that cyclin D1 overexpression in tumor cells was associated with shortened survival, it can be suggested that cyclin D1 overexpression may reflect the aggressiveness of pancreatic cancer.

Acknowledgements

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References


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