

Overexpression of G₁-S Cyclins and Cyclin-Dependent Kinases during Multistage Human Pancreatic Duct Cell Carcinogenesis

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ABSTRACT

Purpose: Molecular analysis of pancreatic intraepithelial neoplasia lesions and ductal adenocarcinoma suggested a multistage paradigm for pancreatic duct cell carcinogenesis. This study investigated the molecular basis for the neoplastic duct cells in this pancreatic intraepithelial neoplasia–carcinoma sequence to acquire progressive enhancement of their proliferative potential.

Experimental Design: Using tissue microarray blocks containing 15 to 40 pancreatic intraepithelial neoplasia lesions and ductal adenocarcinoma of pancreas, we studied by immunohistochemistry the expression profiles of cyclins and cyclin dependent kinases (CDKs) that regulate the G₁-S cell cycle checkpoints. The role of cyclins D3 and D1 in three pancreatic cancer cell lines was investigated using specific short interfering RNA technique.

Results: Cyclin D3 overexpression was noted the earliest in pancreatic intraepithelial neoplasia-1A and was prevalent in 90% to 100% of high-grade pancreatic intraepithelial neoplasias and ductal cancer. Cyclin A overexpression was also noted early and reached 50% to 100% of high-grade pancreatic intraepithelial neoplasias and cancer, but the percentage of abnormal duct cells showing overexpression of cyclin A was significantly lower than cyclin D3. Cyclin E overexpression occurred in 20% to 25% of high-grade pancreatic intraepithelial neoplasias and in 75% of ductal carcinoma. Cyclin D1 demonstrated the lowest frequency of overexpression that occurred late. CDK2 and

CDK4 overexpression was also noted in early pancreatic intraepithelial neoplasias and progressively increased to reach 60% to 75% in carcinoma. The down-regulation of cyclin D3 mRNA and protein levels using specific short interfering RNA resulted in growth inhibition of pancreatic cancer cell lines.

Conclusion: The results provide additional insight into the mechanism of G₁-S cell cycle checkpoints deregulation during stepwise pancreatic duct cell carcinogenesis, and suggest a p16-independent role for cyclin D3 in deregulating the G₁ cell cycle checkpoints during early stages of pancreatic duct cell carcinogenesis.

INTRODUCTION

The entry and progression of a cell through the cell cycle is controlled by changes in the levels and activities of several cyclins, cyclin-dependent kinases (CDKs) and their inhibitors. Disruption of the G₁-S checkpoints leads to uncontrolled cell growth, resulting in the development of cancers. Important players at the G₁-S checkpoints are cyclin D-CDK4/6 and cyclin E-CDK2 complexes, which phosphorylate the retinoblastoma proteins, thus committing the cell to progress into DNA replication and mitosis. Cyclin A associates with CDK2 and is involved at both the G₁-S and G₂-M checkpoints of the cell cycle (1).

Pancreatic carcinoma is a highly malignant tumor with >90% mortality. There were previous reports on altered expression of D- and A-type cyclins in pancreatic cancer, but these results require additional confirmation (2–8). Pancreatic ductal carcinogenesis is a multistage process, of which the putative preinvasive intermediate lesions have been defined as pancreatic intraepithelial neoplasia (9). Although an increasing number of progressive genetic aberrations are being demonstrated to occur in these pancreatic ductal cancer precursor lesions (10, 11), genetic or expression changes of the cyclins in these lesions have not been extensively studied (11–13). In this study, we report the results of a systematic immunohistochemical investigation on the G₁-S cyclins and their associated CDKs using tissue microarray in pancreatic intraepithelial neoplasia and pancreatic cancer lesions.

MATERIALS AND METHODS

Tissue Materials and Cell Lines. After the approval of this study design by the University Health Network Research Ethics Board, we searched the surgical pathology files at the Toronto General Hospital Department of Pathology for specimens of Whipple resection and partial pancreatectomy during 1999 to 2002. Autopsy cases or cases of familial pancreatic carcinoma were not included. Ninety-four cases were identified for retrieval of their formalin-fixed, paraffin-embedded tissue

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Note: M. M. Al-Aynati and N. Radulovich contributed equally to this work.

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blocks. Among these, 38 cases were from pancreatic ductal adenocarcinoma patients, 28 cases from miscellaneous neoplastic conditions of pancreas other than ductal cancer, and 30 cases were associated with benign or reactive conditions.

The human pancreatic cancer cell lines were purchased from the American Type Culture Collection (Rockville, MD). The PK1 cell line was a gift of Dr. Masao Kobari (Tohoku University, Sendai, Japan). The lines were routinely propagated and maintained in DMEM containing 10% fetal bovine serum, 1% penicillin-streptomycin, and 2 mmol/L L-glutamine at a humidified atmosphere of 5% CO₂ at 37°C.

Tissue Microarray. The H&E slides of these cases were reviewed for the presence of normal ducts, pancreatic intraepithelial neoplasia lesions, and adenocarcinoma. The pancreatic intraepithelial neoplasia lesions were graded according to criteria reported previously (10). Using the manual tissue arrayer (Beecher Instruments, Silver Spring, MD), we constructed six tissue microarray blocks containing 1.0- and 1.5-mm cores of 38 pancreatic adenocarcinomas, 18 pancreatic intraepithelial neoplasia-1A lesions, 40 pancreatic intraepithelial neoplasia-1B lesions, 30 pancreatic intraepithelial neoplasia-2 lesions, 19 pancreatic intraepithelial neoplasia-3 lesions, and 29 normal ducts. The final number of lesions assessed was slightly variable among different markers as a result of losses during block trimming or immunostaining procedures.

Immunohistochemistry and Scoring. Serial 4- μ m-thick sections from the tissue microarray blocks were cut and dried overnight in a 60°C oven. Sections were dewaxed in xylene and rehydrated through graded alcohol to water. Initial sections were stained in H&E for histologic verification. Endogenous peroxidase was blocked in 3% hydrogen peroxide. After performing microwave antigen retrieval in 10 mmol/L Citrate buffer (pH 6.0) in a pressure cooker, slides were blocked for endogenous biotin with a biotin blocking kit (Vector Laboratories, Burlingame, Ontario, Canada). After additional blocking with normal serum, sections were covered in primary antibody for 16 hours at room temperature in a moist chamber. The following concentrations of primary antibodies were used: cyclin D1 (clone DCS-6, DAKO Laboratories, Mississauga, Ontario, Canada) at 1:50 dilution; cyclin D3 (clone DCS-22, NovoCastra Laboratories, Newcastle-upon-Tyne, United Kingdom) at 1:20 dilution; cyclin E (clone 13A3 from NovoCastra Laboratories) at 1:50 dilution, cyclin A (rabbit polyclonal, Santa Cruz Biotechnology Laboratories, Santa Cruz, CA) at 1:500 dilution; CDK4 (mouse monoclonal, Transduction Laboratory) at 1:100 dilution; CDK2 (mouse monoclonal, Transduction Laboratory) at 1:500 dilution; and p130-Rb2 (BD Bioscience-Transduction laboratories, San Diego, CA) at 1:500 dilution. After washing in PBS, secondary incubations were carried out with Ultra-streptavidin Detection Pathology System (Signet Pathology System, Dedham, MA), followed with streptavidin-horse-radish peroxidase for 20 minutes each. Immunoreactivities were revealed by incubation in Nova Red substrate (Vector Laboratories) for 5 minutes. Slides were counterstained in Mayer's hematoxylin and mounted with Permount. Lesions were semi-quantitatively scored as a percentage of nuclei staining positive within each duct lesion or tumor, and the intensity of staining was qualitatively graded from weak to strong. Lesions with no or only very occasional (<5% but almost always <2%) nuclei

demonstrating positive staining were considered negative. Data were statistically analyzed using the Cochran-Armitage test for trend and nonparametric Mann Whitney *t* test, with two-tailed *P*s < 0.05 being considered as statistically significant.

Short Interfering RNA. The short interfering RNAs directed against cyclin D3 and cyclin D1 were obtained as SMARTpool duplexes from Dharmacon Research Inc. (Lafayette, CO). Each SMARTpool duplex contains four pooled SMARTselected short interfering RNA duplexes with "UU" overhangs and a 5' phosphate on the antisense strand directed against either cyclin D3 or cyclin D1 mRNA. Two short interfering RNA controls were used in our experiments, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) short interfering RNA (Ambion Inc., Austin, Texas) and SMARTpool nonsilencing (NS short interfering RNA) control (Dharmacon Research Inc.). All of the short interfering RNAs were aliquoted and stored at -20°C.

Short Interfering RNA Transfection. To deliver the short interfering RNA, cells were plated in six-well plate in medium containing 10% fetal bovine serum to give 30% to 50% confluence, and the short interfering RNA was transfected using Oligofectamine (Invitrogen, Carlsbad, CA), as per the protocol provided by the manufacturer. For each six-well plate, 10 μ L of 20 μ mol/L short interfering RNA stock was added. Cells were lysed in either RNA or protein lysis buffer at different points after transfection or were plated into 96-well plates 24 hours after transfection for proliferation assay.

Western Blot Analysis. Cells were prepared in Protein Lysis buffer [50 mmol/L HEPES (pH 8.0), 10% glycerol, 1% Triton X-100, 150 mmol/L NaCl, 1 mmol/L EDTA, 1.5 mmol/L MgCl₂, 100 mmol/L NaF, and 10 mmol/L Na₂P₂O₇·H₂O supplemented with 5 μ g/mL leupeptin, 5 μ g/mL aprotinin, and 100 μ g/mL phenylmethylsulfonylfluoride]. The antibodies and dilutions used included anticyclin D3 (610279; 1:500; BD Transduction Laboratories, Mississauga, Ontario, Canada), anticyclin D1 (554180; 1:250; Becton Dickinson, San Diego, CA) and anti-GAPDH (4300; 1:500; Ambion). After washing with 1 \times Tris-buffered saline Tween 20, the membranes were incubated with antimouse or antirabbit IgG-horse-radish peroxidase conjugate antibody (Cell Signaling, Beverly, MA) at a 1:5,000 dilution for 1 hour at room temperature and developed using BM Chemiluminescence Western Blotting kit (Roche Diagnostics, Mannheim, Germany).

RNA Isolation and Assay. RNA was isolated using Qiagen RNeasy kit (Qiagen, Canada, Mississauga, Ontario, Canada) as recommended by the manufacturer. RNA was quantified by spectrophotometry, and the RNA quality was verified by agarose gel electrophoresis. First-strand cDNA was prepared from 2 μ g of total RNA using the Taqman Reverse Transcription reagents and Random Hexamer primer (Perkin-Elmer Applied Biosystems, Foster City, CA). Primer sets for PCR amplifications were designed using the Primer Express software (Perkin-Elmer Applied Biosystems). The sequences (5' to 3') of the primers were as follows: cyclin D3 (CCND3, GenBank accession no. NM 001760), forward GCAGCGCCTTCCAACT, reverse TCAAAGGAATGCTGGTGTATGTATC; Cyclin D1 (CCND1, GenBank accession no. NM 053056), forward AGGTCTGCGAGGAACAGAAGTG, reverse TGCA-GCGGCTCTTTTC; GAPDH (GenBank accession no.

NM 002046), forward GAAGGTGAAGGTCGGAGTC, reverse GAAGATGGTGATGGGATTTTC. Real-time PCR amplification was performed in the SYBR Green PCR Master Mix (Perkin-Elmer Applied Biosystems) and using an ABI PRISM 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems). Assay for each sample and primer set was performed in duplicate, with each reaction using reverse transcription product equivalent to 10 ng RNA and 0.4 μ mol/L primers. Thermal cycle condition was 50°C for 2 minutes, 95°C for 10 minutes, 40 cycles of 95°C 15 seconds, and 60°C for 1 minute. The data were analyzed by the Comparative CT Method as per the manufacturer's instructions, using the values of ribosomal 18S as calibrator for RNA amount.

Cell Proliferation Assay. Cells were transfected with the indicated short interfering RNAs as outlined above. At 24 hours after transfection, cells were replated in a number of 96-well plates at a density of 3,000 cells/well. The cells were then grown in a tissue culture medium (100 μ L per well) in a humidified atmosphere of 5% CO₂ at 37°C. At 1, 3, 4, 5, and 7 days after transfection, 20 μ L of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfonyl)-2H-tetrazolium reagent

(Promega, Madison, WI) was added to each well and incubated for 1 hour at 37°C. The absorbance was then measured at 492 nm directly from 96-well plate using Tecan Plate Reader. The quantity of formazan product produced by cells upon addition of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxy-phenyl)-2-(4-sulfonyl)-2H-tetrazolium reagent as measured by the amount of 492 nm absorbance is directly proportional to the number of living cells in culture.

RESULTS

Expression of Cyclins. In normal duct epithelium or acinar cells, none of the protein studied were expressed (Fig. 1A). However, islet cell nuclei showed strong positive staining for cyclin D1 and D3 (Fig. 1B). Table 1 summarizes the frequency of various duct lesions and cancers that demonstrated nuclear staining for cyclins D1, D3, A, and E, as well as CDK 2 and 4.

Nuclear immunoreactivity for cyclin D1 was not seen in pancreatic intraepithelial neoplasia-1 lesions but was found in 11–27% of pancreatic intraepithelial neoplasia-2 or -3 and ductal cancer. In overexpressing lesions that demonstrated 5% or

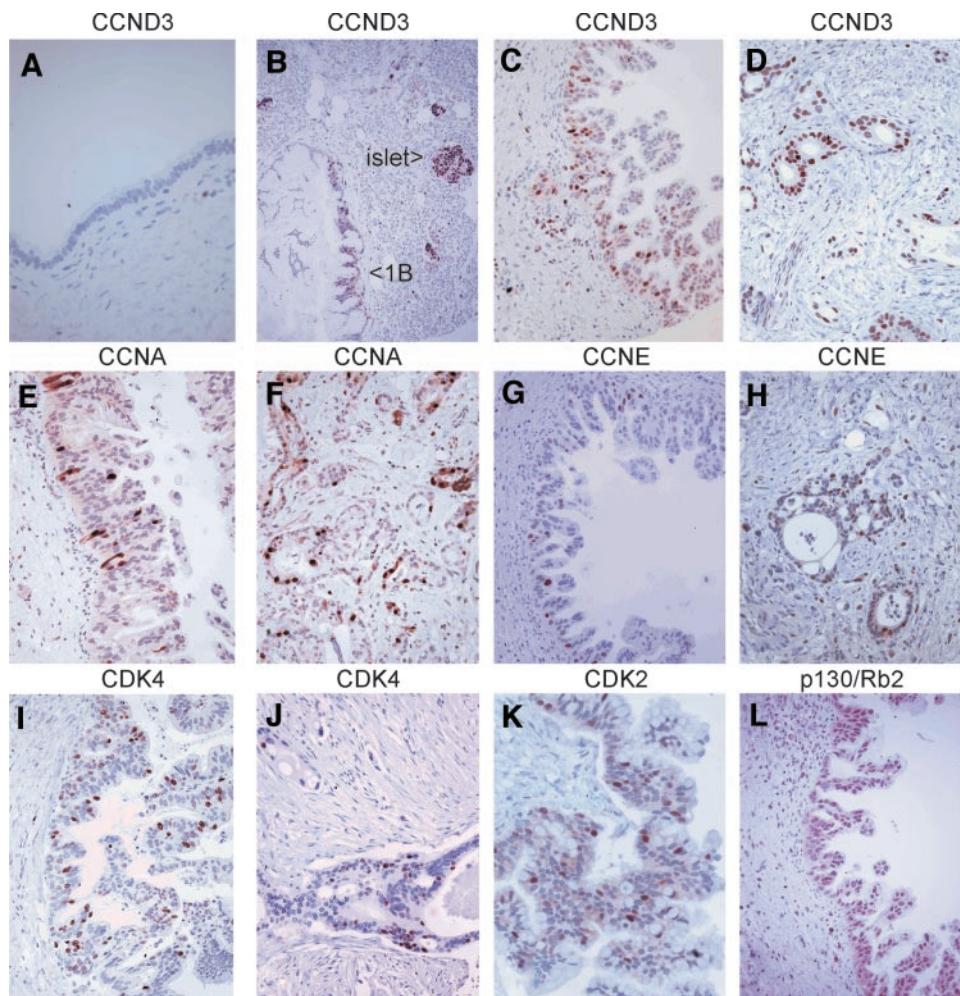


Fig. 1 Representative microphotographs of the duct lesions that demonstrated positive staining for cyclin D3 (A–D), cyclin A (E and F), cyclin E (G and H), CDK4 (I and J), CDK2 (K), and p130RB2 (L). A, normal duct; B, PanIN-1B; C, E, G, I, and L, PanIN-3; K, PanIN-2; D, F, H, and J, adenocarcinoma. (Original magnifications: A, C–J, L, \times 100; B, \times 50; K, \times 200).

Table 1 Frequency of overexpression of cyclins and CDKs in pancreatic intraepithelial neoplasia—duct cell carcinoma sequence

	No. of lesions studied	CCND1	CCND3	CCNA	CCNE	CDK4/6	CDK2
Normal	27–29	0	0	0	0	0	0
PanIN-1A	15–18	0	31%	0	0	0	0
PanIN-1B	36–40	0	55%	15%	0	11%	7%
PanIN-2	23–30	22%	93%	56%	22%	48%	33%
PanIN-3	15–19	11%	100%	100%	24%	53%	53%
ADC	37–38	27%	92%	87%	71%	76%	61%

Abbreviations: CCN, cyclin; PanIN, intraepithelial neoplasia; ADC, adenocarcinoma.

more stained nuclei, the median of approximated percentages of positively stained nuclei were 8% to 15%, which was not correlated with neoplastic progression (Fig. 2). In contrast to cyclin D1, the immunoreactivity for cyclin D3 was detected in 31% and 55% of pancreatic intraepithelial neoplasia-1A and -1B lesions, respectively (Fig. 1B). The intensity of immunoreactivity dramatically increased, and it was noted in 90% to 100% of high-grade pancreatic intraepithelial neoplasia and ductal cancer (Fig. 1, C and D). The median percentage of positively stained nuclei in overexpressing lesions was also significantly

increased from 10% in pancreatic intraepithelial neoplasia-1A and 20% in pancreatic intraepithelial neoplasia-1B lesions to 80% in high-grade pancreatic intraepithelial neoplasia (2 and 3) epithelia and cancer cells ($P < 0.0001$).

Six of 40 (15%) pancreatic intraepithelial neoplasia-1B lesions showed overexpression of cyclin A. This frequency progressively increased to 56% in pancreatic intraepithelial neoplasia-2 and 87% to 100% in pancreatic intraepithelial neoplasia-3 or cancer (Fig. 1, E and F). However, the medians percentage of positively stained nuclei in overexpressing lesions

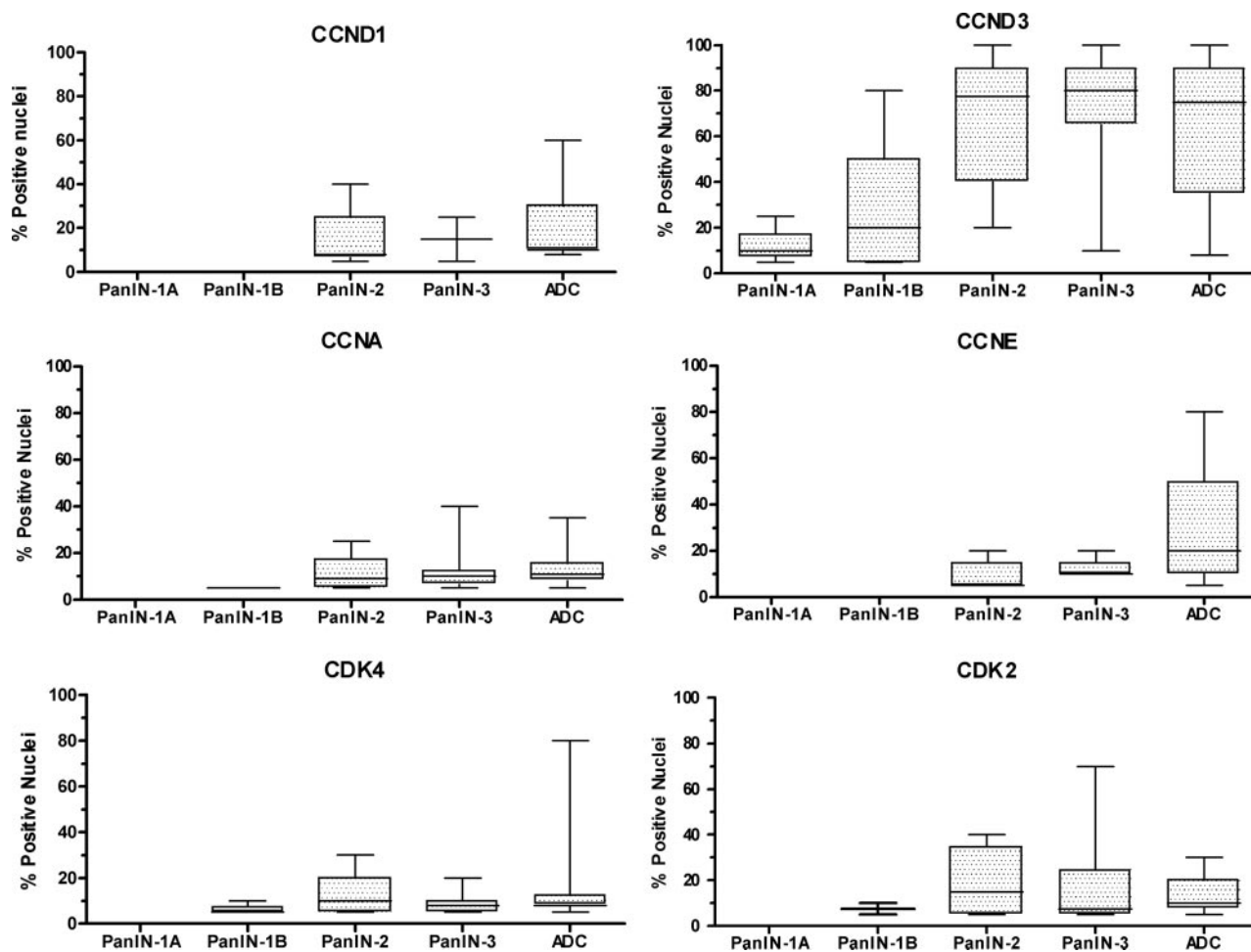


Fig. 2 The distribution of percent positively stained nuclei in lesions that overexpressed the various cyclins and cyclin dependent kinases. Negative cases with occasional (<5%) stained nuclei were not included in this analysis. CCN, cyclin.

ranged from 5% to 10%, and this failed to show an increasing trend that correlated with neoplastic progression. Similar to cyclin D1, cyclin E overexpression was not seen before high-grade pancreatic intraepithelial neoplasia epithelia, but 70% of cancer showed overexpression (Fig. 1, *G* and *H*). The median percentage of positively stained nuclei in overexpressing lesions showed a progressive increase and reached 20% in cancer cells.

Expression of Cyclin-Dependent Kinases. Overexpression of CDK4 and CDK2 were noted early in ~10% of pancreatic intraepithelial neoplasia-1B lesions, but they demonstrated progressive increases in higher-grade pancreatic intraepithelial neoplasia lesion and carcinoma (Fig. 1, *I-K*). Variation of the percentage of positively stained nuclei in overexpressing lesions was noted, but the medians remained between 5% and 15% without showing significant trend that correlated with neoplastic progression.

p130/Rb2 Expression. *Rb2* is a third member of the Rb family genes besides *p110/Rb* and *p107*, and it was reported as

the preferred target for cyclin D3 (CCND3) and cyclin E (CCNE) -associated CDK activities (14, 15). The p130/Rb2 immunoreactivity was ubiquitously detected in the normal and aberrant pancreatic duct epithelial cells, as well as in adenocarcinoma (Fig. 1*L*).

Role of D-Type Cyclins in Pancreatic Cancer Cell Lines. Western blot analysis demonstrates that both cyclins D1 and D3 were expressed in all seven of the pancreatic cancer cell lines that we studied (Fig. 3*A*). Interestingly, there appears to be an apparent inverse relationship between the expression levels of these two D-type cyclins. BxPC3, Capan-2, and HPAC expressed relatively higher cyclin D1 levels, whereas MiaPaca-2, Panc-1, and PK1 cell lines demonstrated higher cyclin D3 levels (Fig. 3*A*). HPAF appears to demonstrate relatively equal abundance of cyclins D1 and D3. We then investigated the ability of short interfering RNA technique to down-regulate the expression of these two D-type cyclins. The short interfering RNA designed against cyclins D1 and D3 was highly effective

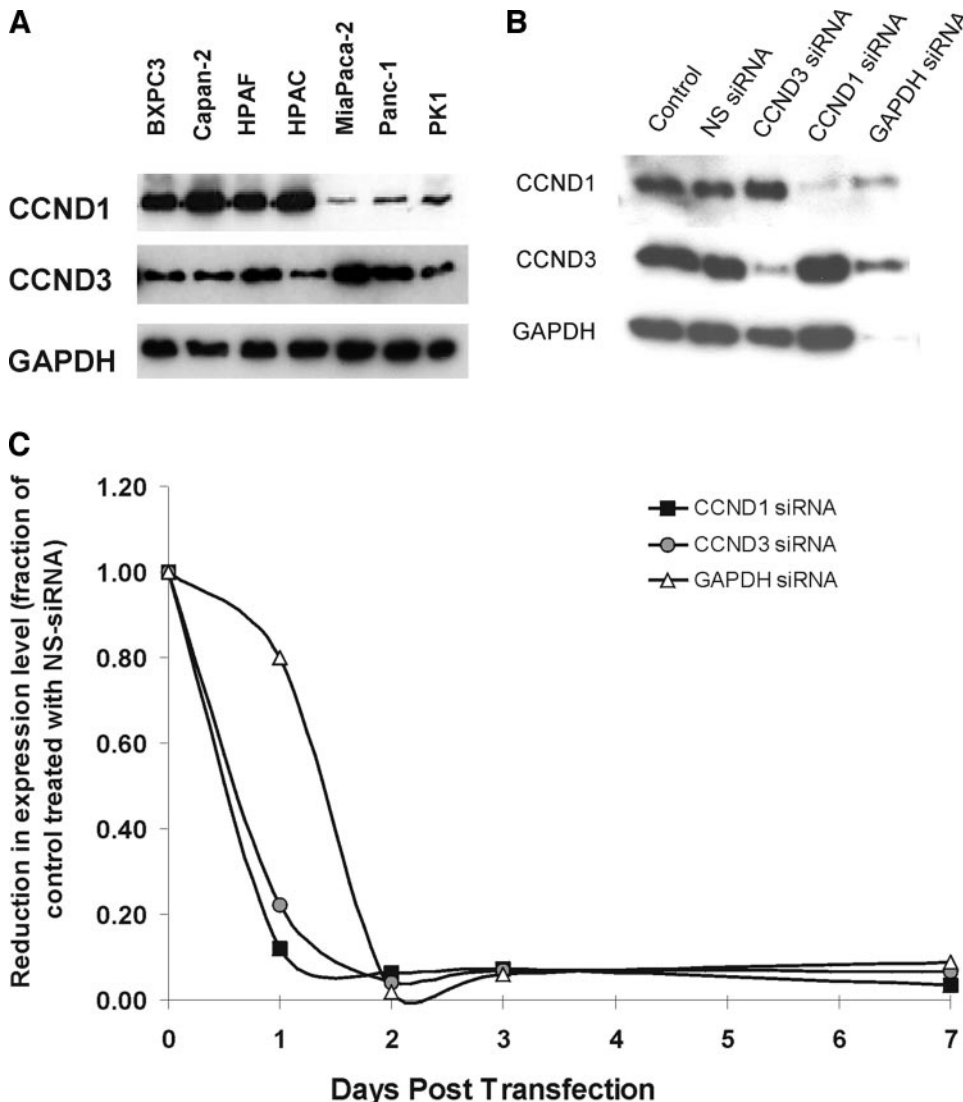


Fig. 3 Down-regulation of cyclin D1 and cyclin D3 expression by short interfering RNA treatment. **A**, Western blot demonstrates the cyclin D1 and cyclin D3 expression in seven pancreatic cancer cell lines studies. **B**, Western blot shows marked suppression of cyclin D1, cyclin D3, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) protein levels 48 hours after transfection of respective short interfering RNA in Panc-1 cells. **C**, real-time reverse transcription-PCR assay demonstrates rapid down-regulation of mRNA transcripts at 1 to 7 days after treatment of PANC-1 cells with short interfering RNA against cyclin D1, cyclin D, and GAPDH. The relative transcript levels (*Y*-axis) are represented as fraction of level in cells treated with non-suppressing siRNA. *CCND1*, cyclin D1; *CCND3*, cyclin D3.

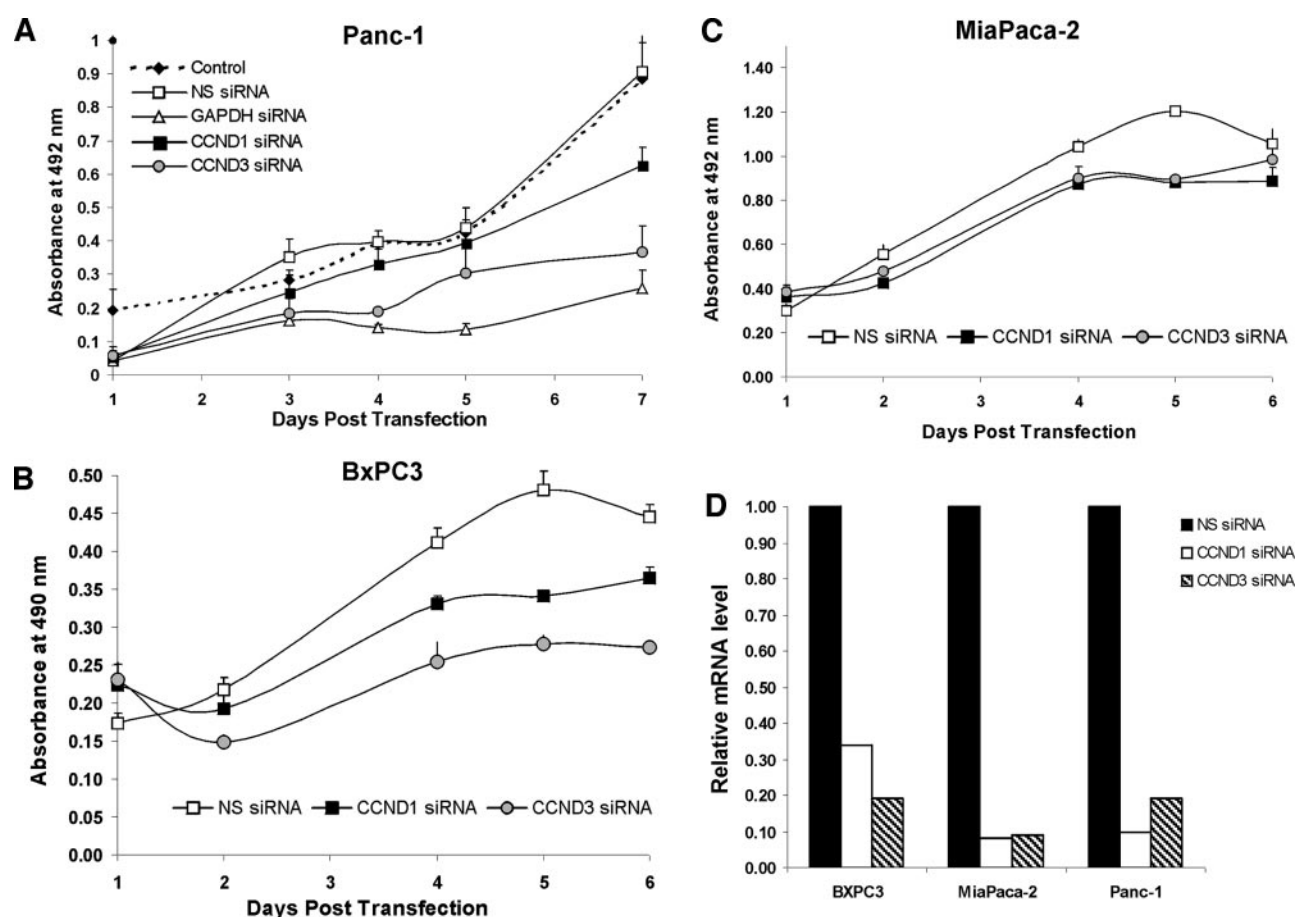


Fig. 4 Suppression of growth after cyclin D1 and cyclin D3 siRNA treatments in: (A) Panc-1, (B) BxPC-3, (B) and (C) MiaPaca-2 cell lines. D, the comparative cyclin D1 and cyclin D3 mRNA transcript levels in these cells 48 hours after small interfering RNA treatment with the respective small interfering RNA. The level measured in cells treated by nonsuppressing small interfering RNA was taken as 100%. NS, nonsuppressing; siRNA, small interfering RNA.

in suppressing the protein expression of these cyclins in the Panc-1 cells (Fig. 3B). The down-regulation at the mRNA level was dramatically observed at 24 hours after transfection, reached the maximum level at 48 hours, and persisted for at least 7 days (Fig. 3C). The short interfering RNA against GAPDH was used as a positive control in these studies.

Effect of Short Interfering RNA on Pancreatic Cancer Cell Growth. The treatment of Panc-1, MiaPaca-2, and BxPC3 cell lines by the short interfering RNAs against cyclin D1 and cyclin D3 resulted in significant but variable levels of growth suppression. In Panc-1 and BxPC-3 cells (Fig. 4, A and B), the suppressive effect of cyclin D3 short interfering RNA was more prominent than cyclin D1 short interfering RNA. In contrast, both short interfering RNAs were equally effective in the MiaPaca-2 cells (Fig. 4C). The treatment with nonsuppressing RNA was used as controls, and Fig. 4D demonstrates the marked suppression of cyclin D1 and cyclin D3 mRNA levels in these cells after treatment with their respective short interfering RNA. In PANC-1 cell line, the GAPDH short interfering RNA was also used as a control, and its marked antiproliferative effect was consistent with the result reported previously (16).

DISCUSSION

We have exploited the tissue microarray technology to perform a systematic analysis of the expression of G₁-S cyclins in pancreatic intraepithelial neoplasia lesions. Previous studies that addressed the role of cell cycle regulators in pancreas carcinogenesis have focused mainly on p16^{INK4A} and cyclin D1. To the best of our knowledge, this is the first report on the overexpression patterns of other members of G₁-S cyclins (D3, A, and E) and their associated CDKs (2 and 4). The results demonstrate that progressive intraepithelial pancreatic duct cell neoplasia is associated with increasing overexpression of multiple cyclins as well as their respective CDK partners, thus providing a more comprehensive insight into the mechanism of deregulation of G₁-S cell cycle checkpoint in the pathogenesis of pancreatic cancer.

Cyclin D1 associates with CDK4/6 and plays an important role during the early G₁ phase of cell cycle. The cyclin D1-CDK4/6 complex is inhibited by p16^{INK4A} (1), the cell cycle regulator gene that has been most exhaustively studied in pancreatic ductal cancer. The inactivation of *p16* gene occurs in a

progressive manner with increasing grades of pancreatic intraepithelial neoplasia lesions (11, 17–19), and it occurs in practically all pancreatic ductal cancers. In contrast, the role of cyclin D1 remains unclear. Some reports have demonstrated higher expression of *cyclin D1* mRNA in pancreatic cancer cell lines and tissue compared with normal pancreas (2, 3), as well as *cyclin D1* gene amplification in pancreatic ductal carcinoma (2). However, Huang *et al.* (4) failed to document *cyclin D1* gene amplification in 80 pancreatic cancers studied, but detected weak to moderate nuclear and cytoplasmic staining by immunohistochemistry. We detected at best a moderate immunoreactivity of cyclin D1 in 15% to 25% of pancreatic intraepithelial neoplasias and ductal cancers but did not observe a significant trend for progressive increase with higher grades of dysplasia leading to malignancy. This is in contrast to other reports (11, 12) that showed the lack of cyclin D1 immunoreactivity in low-stage pancreatic intraepithelial neoplasia but an overexpression in 15% to 29% of pancreatic intraepithelial neoplasia-2, 41% to 57% of pancreatic intraepithelial neoplasia-3, and 47% of ductal cancers. Nevertheless, the data suggest that whereas cyclin D1 expression may play an important role in pancreatic cancer biology (2, 3, 20), its role during the early stages of neoplastic progression may be less significant. This should not be surprising when *p16* inactivation already occurs in 27% to 44% of low-grade and 50% to 85% of high-grade pancreatic intraepithelial neoplasias, as well as in practically 100% of ductal cancers. Because cyclin D1 partners only with CDK4/6, the inactivation of both *p16* and overexpression of cyclin D1 could be redundant.

Probably the most important finding of this study is the demonstration of cyclin D3 overexpression, which starts early and at very high frequency during pancreatic duct cell carcinogenesis. Ito *et al.* (6) reported previously the expression of cyclin D3 by immunohistochemistry in 33% of 60 pancreatic cancers, and this was correlated with high Ki-67 labeling index of the tumor cells. In our series, we detected an even higher rate of cyclin D3 overexpression in 92% of adenocarcinoma. Cyclin D3 overexpression has also been reported in several other human malignancies including breast cancer, malignant gliomas, renal cell carcinoma, and T-cell leukemia (21–24).

The importance of cyclin D3 overexpression becomes more apparent when we consider the *in vitro* evidence for its mechanism of action in regulating cell cycle. In contrast to cyclin D1, cyclin D3 associates predominantly with CDK6, and its major phosphorylation target is the p130/Rb2, which associates with the E2F4–5 (14, 15). In contrast to the activating E2F1–3, an abundance of repressive E2F4–5 has been detected in the nuclei of quiescent (G₀) cells, where it associates with histone deacetylases and suppresses the transcription of E2F responsive genes (25, 26). Importantly, Faast *et al.* (27) reported recently that cyclin D3 activity in ES cells is not inhibited by p16^{INK4A}. Thus, we postulate that the main function of cyclin D3 activation during the early stages of pancreatic duct cell carcinogenesis would be to inactivate the repressive E2F4–5, which would additionally complement and enhance the proliferative potential of E2F1–3 activation mediated by *p16* inactivation. The results of our short interfering RNA studies confirm the important role of cyclin D3 in the proliferation of pancreatic ductal cancer cells, even when *p16* is inactivated in all of these

cell lines. It is worth noting that p130/Rb2 is also a target of cyclin E-cdk2, but activation of cyclin E does not occur until late in high-grade pancreatic intraepithelial neoplasias. Consistent with this hypothesis on the role of cyclin D3 in inactivating p130-Rb2, we have also demonstrated that the latter is ubiquitously expressed in pancreatic intraepithelial neoplasia and pancreatic ductal cancer cells. We have not demonstrated increased phosphorylation of p130/Rb2, because its phosphospecific antibody is not yet available. Interestingly, Ma *et al.* (28) reported that the cyclin D3 promoter is regulated by E2F1, which is activated by *p16* inactivation. In contrast to cyclins D1 and D3, cyclin D2 expression was reported to undergo age-related inactivation in pancreatic cancer (13).

Overriding the role of cyclin D, the critical determinant for entry of cells into the S phase is the cyclin E-cdk2 interaction (1, 25). As the cell commences DNA synthesis and replication, cyclin E is degraded by proteolysis and replaced in its protein complex with cdk2 by the cyclin A, which eventually redistributes to CDK1. The progressive enhancement of proliferative activity in pancreatic intraepithelial neoplasia lesions as demonstrated by increased Ki-67 labeling indices (29) could possibly be related to the overexpression of these S-phase cyclins E and A. Consistent with our finding, previous reports (8, 9) have indicated that cyclin A overexpression was found in 56% to 77% of pancreatic ductal adenocarcinoma.

Last but not least, we have demonstrated for the first time that the CDK4 and CDK2 are also progressively overexpressed in pancreatic intraepithelial neoplasia–carcinoma sequence. These presumably lead to additional enhancement of the proliferative activities during the step-wise progression of duct cell carcinogenesis. Previous reports have demonstrated that CDK4 can result in a bypassing of the telomere-dependent replicative senescence in normal human epithelial cells (30), and CDK4 can cooperate with the *Ras* oncogene to induce malignant transformation of human epidermal cells (31). It is important to note that aside from the cyclins and CDKs, other cyclin-dependent kinase inhibitors, p21^{cip1} and p27^{kip1} also control the G₁-S cell cycle regulation. Biankin *et al.* (12) reported previously that p21^{cip1} is overexpressed early in pancreatic intraepithelial neoplasia lesions during pancreatic duct cell carcinogenesis. Because *Ki-ras* oncogene has been reported to induce the expression of p21, the progressive increases of the p21 levels in pancreatic intraepithelial neoplasia lesions and ductal cancer could possibly be attributed to *Ki-ras* oncogene activation.

In summary, we have presented a more complete paradigm on the expression and role of G₁-S cyclins during various stages of pancreatic ductal carcinogenesis, with cumulative increases in the expression of D-, A-, and E-type cyclins and their partners CDK4 and CDK2 during the pancreatic intraepithelial neoplasia–carcinoma progression. We have also demonstrated that parallel to the inactivation of p16^{INK4A}, cyclin D3 overexpression is likely to play a crucial role in completing the inactivation of G₁ cell cycle checkpoint during duct cell carcinogenesis, thus could represent a potential therapeutic target in the prevention and treatment of this lethal disease.

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