Identification of human polo-like kinase 1 as a potential therapeutic target in pancreatic cancer

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Abstract
Pancreas cancer is the fourth leading cause of cancer-related death in adults in the United States. New molecular targets for diagnosis and therapy of this disease are desperately needed. In this study, we report on the mitotic serine-threonine kinase polo-like kinase 1 (Plk1) in pancreatic cancer. Plk1 mRNA was found to be overexpressed in 9 of 10 tested pancreatic cancer cell lines and in 4 of 4 tested human tumors. Immunohistochemical staining of a pancreatic tissue microarray showed that 26 of the 35 tumors taken directly from patients overexpressed Plk1. We also examined the effects of depleting Plk1 in pancreatic cancer cells by the use of antisense oligonucleotides. Antisense-treated pancreatic cancer cells showed cell cycle arrest in G2-M as well as a drastic reduction in proliferation rates. These data suggest that Plk1 is a potential therapeutic target in devising a treatment for patients with pancreatic cancer. [Mol Cancer Ther 2004;3(5):641–6]

Introduction
Adenocarcinoma of the pancreas is among the most aggressive type of human tumors. While accounting for only 2–3% of cancer diagnoses each year, it is the fourth leading cause of cancer-related death in adults in the United States (1). Current therapeutic strategies have shown only moderate success in increasing patient survival (2). New treatments for pancreatic cancer are greatly needed and our increased understanding of the events that drive the onset and progression of this disease will aid in the development of these therapies. In the present study, we report on the identification and validation of the polo-like kinase 1 (Plk1) as a potential new target for drug development for treatment of patients with pancreatic cancer.

Plk1, a mitotic cyclin-independent serine-threonine kinase, is a member of a family of polo-like kinases involved in a wide variety of cell cycle processes. Polo kinase was first identified in Drosophila melanogaster, where mutants display abnormal mitotic and meiotic divisions caused by failure to properly organize the mitotic spindle (3). In mammalian cells, Plk1 is primarily localized at the centrosome where it is responsible for centrosome separation and maturation (4). Plk1 specific antibodies introduced into HeLa cells by microinjection prevent centrosome separation as well as lower γ-tubulin accumulation (4). In addition to its role in regulating centrosome function, Plk1 plays a role in the timing of mitotic entry and exit (5). It has been implicated in targeting cyclin B1 to the nucleus during prophase (6), the activation of Cdc25c phosphatase (7), and the inactivation of the Cdk1-cyclin B complex required for mitotic exit (5). Plk1 is also a target of the G2 DNA damage checkpoint, where it undergoes ubiquitin-dependent proteolysis mediated by the checkpoint protein Chfr, implicating the loss of Plk1 function as an important response to DNA damage during the G2 phase of the cell cycle (8).

Elevated Plk1 protein levels have been correlated with poor prognosis in a number of human cancers including: non-small cell lung cancer (9), squamous cell carcinoma (10), esophageal carcinoma (11), melanoma (12, 13), oropharyngeal carcinoma (14), breast cancer (15), colorectal cancer (16), and endometrial carcinoma (17). Elevated expression of Plk1 has also been shown to be associated with a higher grade and a more advanced stage of ovarian cancer (18).

Overexpression of the Plk1 gene has also been found in multiple cancer cell lines which include: U87MG (human glioma), MDA-MB-435 (breast cancer), HeLa S3 (cervical carcinoma), and A549 (non-small cell lung cancer) (19, 20). Plk1 has also been described as a diagnostic marker for cellular proliferation (21). Furthermore, overexpression of Plk1 in NIH-3T3 murine fibroblasts results in oncogenic transformation and causes tumor growth in nude mice (22).

The above studies implicate Plk1 as playing a role in a variety of human cancers. In the present study, we report the overexpression of Plk1 in pancreatic adenocarcinomas as detected in both pancreatic cancer cell lines as well as in pancreatic tumors taken directly from patients. We also report on initial validation of Plk1 as a potential therapeutic target by inhibiting Plk1 expression in pancreatic cancer cells by the use of antisense oligonucleotides. Our data indicate that suppression of Plk1 expression in pancreatic cancer cells results in cell cycle arrest as well as a drastic reduction in cell proliferation and viability.

Materials and Methods
Cell Culture
Pancreatic cancer cell lines AsPC-1, BxPC-3, Capan-1, Capan-2, CFPAC-1, HS766T, MiaPaCa-2, PANC-1, and SU.86.86 were...
purchased from the American Type Tissue Culture Collection (Manassas, VA). Pancreatic cell line Mutj (UACC-462) was established at the Arizona Cancer Center (23). Cell lines were maintained in RPMI 1640 supplemented with fetal bovine serum (10% for AsPC-1, BxPC-3, MiaPaCa-2, PANC-1, and Mutj; 15% for CFPAC-1 and SU.86.86; 20% for Capan-1), penicillin, and streptomycin. Cell line HPDE6c7 (an immortalized but not transformed pancreatic epithelial cell line) was maintained in keratinocyte serum-free medium supplemented by epidermal growth factor and bovine pituitary extract (Invitrogen, Carlsbad, CA). All cells were grown in a humidified incubator at 37°C and 5% CO2. Cells were harvested with trypsin at 80–90% confluency. Cell counting was done using trypan blue stain on a hemocytometer.

RT-PCR
Total RNA from cell pellets was isolated using the SNAP RNA isolation kit (Invitrogen). Two micrograms of total RNA were used for reverse transcriptase (RT) reactions (20 µl total volume), which was carried out using the Omniscript RT kit (Qiagen, Valencia, CA). PCRs were then carried out by mixing 2 µl of the reverse transcription reaction mixture, 5 µl of 10× PCR buffer containing 15 mM Mg²⁺, 1 µl of 10 mM deoxynucleotide triphosphate mixture, 1 µl of a 100 µM Plk1 specific primer, 1 µl of β-actin primer pair, 1 µl of β-actin competitors (Ambion, Austin, TX), 37 µl of dH₂O, and 0.5 µl of 5 units/µl Taq polymerase (Promega, Madison, WI). The Amplification cycle (94°C for 30 s, 59°C for 45 s, and 72°C for 1 min) was repeated 29 times. PCR primers for Plk1 were designed to produce a DNA fragment ~450 bp in length. The sequences are 5'-AAGAGATCCCGGAGGTCCTA-3' and 5'-TCATTCAGGGAAAGGTTGCC-3'.

Antisense Transfection
MiaPaCa-2 cells were grown to 40–50% confluency the day of the transfection and washed with Dulbecco’s PBS (Cellgro, Herdon, VA). OPTI-MEM transfection medium (Invitrogen) containing 3 µl Lipofectin reagent (Invitrogen)/ml of medium for each 100 nM of oligo used was added to the cell culture plates. Plk1 specific or scrambled oligo provided by Isis Pharmaceuticals (Carlsbad, CA) was then added dropwise to the final concentrations (200 nM for most of the experiments). Cells were incubated in transfection media for 6 h, then washed once with Dulbecco’s PBS, and given normal growth media. Cells were harvested by the described method at the time points indicated in the text.

Western Blotting
Protein extracts were prepared in NP40 lysis buffer (10 mM Tris-Cl (pH 7.4), 10 mM NaCl, 3 mM MgCl2, 0.5% NP40) and concentration was determined by using the BCA protein assay kit (Pierce, Rockford, IL). Twenty micrograms of protein/lane were run on a 10% SDS-PAGE gel which was then transferred to a nitrocellulose membrane by tank transfer. The membrane was then blocked in 5% milk in Tris-buffered saline with Tween 20 [TBS-T: 20 mM Tris-HCl, 150 mM NaCl (pH 7.5), 0.05% Tween 20] overnight at 4°C. The membrane was then probed with a mouse monoclonal antibody against Plk1 (Zymed, San Francisco, CA) at a 1:1000 dilution or a mouse monoclonal antibody against β-actin (Sigma, St. Louis, MO) at a 1:3000 dilution. Following probing, the membrane was washed in TBS-T and probed with a 1:1000 dilution of antimouse horse radish peroxidase-linked antibody (Bio-Rad, Hercules, CA) and visualized with a chemiluminescence kit (Cell SIGNaling, Beverly, MA) and X-ray film.

Flow Cytometry
Cells were treated and harvested as described and stored at ~80°C until day of assay. Cell pellets were resuspended in 1 ml of Krishan’s buffer (0.1% sodium citrate, 0.02 mg/ml RNase A, 0.3% NP40) containing 0.05 mg/ml propidium iodide and incubated for 4 h. DNA content analysis was then performed using a Becton Dickinson FacScan, modeling 20,000 events/sample.

Real-Time PCR
An Opticon DNA Engine (MJ Research, Reno, NV) was used to perform real-time fluorescence detection PCR. One microliter of cDNA produced from reverse transcription reactions with tumor sample or cell line-derived RNA was added to 12.5 µl of SYBR green PCR Master Mix (Qiagen), 0.5 µl of Plk1 or β-actin specific primer pair, and 12 µl of dH₂O (final volume of 25 µl). Amplification (94°C for 1:00 min, 59°C for 1:10 min, and 72°C for 1:30 min) was repeated for 39 cycles. Following the PCR reaction, a melting curve assay was performed to determine the purity of the amplified product. Data were provided as a threshold cycle value (CT) for each sample which indicated the cycle at which a statistically significant increase in fluorescence was first detected. These data were then normalized to β-actin, which served as an unaffected control gene, for each data point and compared to a normal pancreas control [total pancreas RNA (Ambion) for patient tumors and HPDE6c7 RNA for cell lines] to determine a relative expression ratio.

Southern Blotting
Genomic DNA was isolated from cultured cells by use of the DNeasy Tissue Extraction Kit (Qiagen). Fifteen micrograms of BamH1-digested total cellular DNA was blotted onto a Zeta probe GT membrane (Bio-Rad) according to established laboratory methods (24). cDNA probes were produced for Plk1 or glyceraldehyde-3-phosphate dehydrogenase by using the Random Primers DNA Labeling System (Invitrogen). Signal intensity was normalized by measuring glyceraldehyde-3-phosphate dehydrogenase expression and relative expression ratios for Plk1 were then determined.

Pancreatic Tumor Tissue Array and Immunohistochemistry
Morphologically representative areas of 42 archival cases of pancreatic tumors, 35 of which are documented ductal adenocarcinomas, from the University of Arizona Health Sciences Center and the Tucson Veterans Administration Medical Center, were selected from formalin-fixed tissue samples embedded in paraffin blocks. Two 1.5-mm-diameter cores/case (84 discs total) were re-embedded in a tissue microarray using a tissue arrayer (Beecher Instruments, Silver Spring, MD) according to a method described previously (25). Serial sections of the paraffin-embedded pancreatic tissue array were deparaffinized and reacted.
with primary antibodies specific for Plk1 (Zymed) for 1 h. Biotinylated anti-mouse/anti-rabbit secondary antibodies were applied, followed by streptavidin-peroxidase complex (DAKO, Carpinteria, CA). Colored products were produced using the diaminobenzidine substrate. Staining reactions were scored as diffuse or focal and were graded (from 0, negative to +, intensely positive) for both neoplasm and background stroma.

Results

Plk1 Expression and Copy Number in Pancreatic Cancer Cell Lines

Real-time quantitative PCR was used to analyze the mRNA signal levels present in 10 pancreatic cancer cell lines. These results were then compared to HPDE6c7, an immortalized non-transformed pancreatic epithelial cell line (26). Because Plk1 is involved in cell cycle progression and is generally present in proliferating cells, HPDE6c7 served as a useful control for analyzing the Plk1 expression level in these cells. HPDE6c7 and all of the pancreatic cell lines tested were rapidly proliferating with similar population doubling times (24–36 h). Plk1 mRNA signal showed substantial up-regulation in 9 of the 10 tested cell lines (Fig. 1). Cell line MiaPaCa-2 showed the highest elevation of Plk1 mRNA levels relative to HPDE6c7 with almost 58-fold overexpression of the gene. The lowest level of overexpression, 8-fold, was seen in the Panc-1 cell line. The rest of the cell lines tested fell within this range except cell line Mutj, the expression level of which was near that of HPDE6c7.

Southern blotting was used to analyze gene copy number in these same cell lines. There was no sizeable amplification (defined as a greater than 2-fold increase in signal strength) of the Plk1 gene in any of the tested cell lines relative to the normal pancreas line HPDE6c7 (data not shown). These data taken together seem to indicate that Plk1 overexpression in pancreatic cancer cells is a result of transcriptional up-regulation. While up-regulation can occur as a result of increased proliferation rate, the levels found in the tested pancreatic cancer lines were markedly higher than the non-transformed line (HPDE6c7), which was proliferating at a similar rate.

Plk1 Is Overexpressed in Pancreatic Adenocarcinoma

RNA isolated from four human pancreatic tumor samples, all confirmed ductal adenocarcinomas, was subjected to real-time quantitative PCR analysis to determine relative expression ratios compared to RNA isolated from normal human pancreatic cells (Ambion). mRNA signal showed up-regulation of Plk1 mRNA in all four tumor samples tested (Fig. 2). One of the tumor samples in particular showed more than a 20-fold increase over normal, suggesting that Plk1 expression level may be highly variable from tumor to tumor. The overall level of Plk1 overexpression was slightly lower than that for pancreatic cancer cell lines, but still considerably higher than the normal pancreas control. To determine whether or not detectable RNA overexpression was accompanied by an increase in protein levels, we carried out immunohistochemical staining using a monoclonal Plk1 antibody on a paraffin-embedded pancreatic tumor tissue array. The tissue microarray contained 35 different pancreatic adenocarcinoma samples archived and collected at our facility (Fig. 3). The overall staining for Plk1 showed that 26 of 35 (74%) tumors had positive staining (tumor score is at least 1+ over background stroma score; see Materials and Methods), while 11 of 35 (31%) had high staining (3+ or over). As two cores are present for each sample, these data refer to samples by origin and not by core as seen in Fig. 3. Taken together, these data demonstrate that Plk1 protein level is frequently increased in pancreatic adenocarcinomas taken directly from patients.

Inhibition of Plk1 Expression by Antisense Oligonucleotides

The high frequency of Plk1 overexpression in pancreatic adenocarcinomas suggests that Plk1 may be playing an important role in maintaining the tumor phenotype of pancreatic cancer cells. To assess the consequences of
inhibition of Plk1 expression, we treated pancreatic cancer cells with antisense oligonucleotides directed against the Plk1 mRNA. The pancreatic cancer cell lines MiaPaca-2 and Panc-1 were used in all of the antisense experiments. Three antisense oligonucleotides were tested along with their scrambled controls. One of these, Isis 121969, was found to completely abolish the Plk1 mRNA signal when transfected into MiaPaca-2 and Panc-1 cells. Optimal transfection concentration for both cell lines was determined to be 200 nM (data not shown). Two hundred nanomolar concentrations of Isis 121969 or scrambled control (Isis 124485) antisense were transfected into MiaPaCa-2 (Fig. 4A) and Panc-1 cells (Fig. 4B), which were then harvested and assayed at 12, 24, and 48 h post-transfection. Cell lysates were assayed by Western blot and probed with a monoclonal Plk1 antibody and a monoclonal β-actin antibody. Western blotting indicated that levels of Plk1 in cells transfected with Plk1 antisense were drastically reduced by 12 h post-transfection and remained low up through 48 h post-transfection. Levels of Plk1 in cells transfected with control oligo were unaffected.

**Plk1 Antisense Treatment Causes Cell Cycle Arrest and Decreased Proliferation**

As Plk1 is an important cell cycle regulator, we studied the cell cycle distribution of antisense-treated cells using flow cytometry. Analysis of Plk1 antisense-treated MiaPaCa-2 (Fig. 5A) and Panc-1 (Fig. 5B) cells showed that by 24 h post-transfection, a large percentage (57% for MiaPaCa-2 and 58% for Panc-1) of the cell populations had moved from G0-G1 to S phase. By 48 h post-transfection 74% of antisense-treated MiaPaCa-2 cells and 42% of antisense-treated Panc-1 cells were blocked in G2-M, indicating the inability of cells to complete mitosis without active Plk1. Cells treated with scrambled oligo showed no apparent changes in their cell cycle profiles throughout the experiments. There is a noticeable sub-G1 peak that develops 48 h post-transfection in antisense-treated Panc-1 cells. There is a similar sub-G1 accumulation in MiaPaCa-2 cells, although this is not readily visible in the data shown. This sub-G1 peak may indicate cell death via apoptosis, although this proved difficult to detect by standard methods (see Discussion).

MiaPaCa-2 (Fig. 6A) and Panc-1 (Fig. 6B) cells transfected with Plk1 antisense showed a marked decrease in cell proliferation rate compared to those transfected with control oligo. This decrease in proliferation was also associated with a loss of viability as the overall number of attached cells dropped in both treated cell lines. Both treated Panc-1 and MiaPaCa-2 cells had a large number of cells detached from the flask bottom 48 h post-transfection. MiaPaCa-2 cells again demonstrated a greater response to Plk1 depletion with loss of viability beginning 24 h post-transfection.

The cell cycle and antiproliferative effects of Plk1 antisense oligonucleotides seem to be selective for pancreatic cancer cells. When the normal pancreas cell line HPDE6c7 was transfected with both Plk1 specific and control oligos,
there was no distinguishable difference between the cell cycle profiles or proliferation rates of the two groups (data not shown). This may indicate that pancreatic cancer cells are either more dependent on Plk1 function to carry out mitosis, or are missing important checkpoint proteins that may allow normal cells to recover from a transient loss of Plk1.

Discussion
As described earlier, the high mortality rate of pancreatic cancer has created a great need to search for factors affecting its onset as well as a need to better understand the molecular events involved in pancreatic tumor progression. Understanding the effects of specific genetic abnormalities that are common in the development of pancreas cancer could lead to new and more effective ways to diagnose, treat, and prevent this devastating disease. In the present study, we have investigated the overexpression of the gene Plk1 in pancreatic adenocarcinomas. Our data have shown that Plk1 expression is up-regulated in the majority of pancreatic cancer cell lines and patient tumors, although gene amplification was not evident. We have also shown that antisense oligonucleotides directed against the Plk1 mRNA message induce cell cycle arrest and cause a decrease in proliferation and loss of viability in pancreatic cancer cells.

The cytotoxicity of the antisense oligonucleotides in treated pancreatic cancer cells is likely due to the induction of mitotic failure. Similar effects have been seen by the use of a dominant-negative Plk1 construct transfected into the SAOS-2 (osteosarcoma) cell line (27). In that experiment, treated SAOS-2 cells underwent a number of abnormal mitoses before the onset of apoptosis. In addition, other studies have reported similar cell cycle and apoptosis effects on cancer cells treated with small interference RNAs (siRNAs) directed against Plk1 (28, 29). The results from many of these studies suggest that the cytotoxic effects of Plk1 depletion are selective for tumor cells, confirming our own observations that Plk1 antisense affects the cell cycle and proliferation of pancreatic cancer cells but not the untransformed pancreas line HPDE6c7.

Cell cycle analysis performed for this study indicated that at 48 h post-transfection, there was an accumulation of cells containing sub-G1 levels of DNA. While this accumulation may indicate that Plk1 depletion results in an induction of apoptosis, we found this difficult to detect by common methods (analysis of pro-caspase-3 and cleaved caspase-3 levels by Western blot). Another study on Plk1 depletion in HeLa cells seemed to show that detection of apoptosis by this means did not become useful until Plk1 had been depleted for more than 3 days (28). That study also found that apoptosis was more evident in Plk1 depleted cells treated with caffeine or wortmannin. These findings may indicate that apoptosis in our experiments is simply not occurring at levels we can detect this early after antisense transfection. Drawing Plk1 inhibition out for
longer than 48 h in our experiments required multiple transfections which resulted in non-specific liposome-related toxicity. For this reason, our data report only on experiments involving a single transfection.

In each of our antisense transfection experiments, we found that the MiaPaCa-2 cell line seemed to be more sensitive to Plk1 depletion than was the Panc-1 cell line. This is not unexpected in light of the relative Plk1 expression levels as determined in our initial experiments. MiaPaCa-2 expresses Plk1 at a level 7 times greater than that of Panc-1. As such, MiaPaCa-2 may be more dependent on Plk1 overexpression to maintain its tumor phenotype. This conclusion is again supported by the apparent lack of effect seen in antisense-treated HPDE6c7 cells when compared with the drastic effects of antisense on MiaPaCa-2 and Panc-1.

There has been an ongoing interest in the use of antisense drugs in cancer patients. There are several antisense therapies that have undergone or are currently undergoing clinical trials (30). These include antisense targeted against bcl-2, protein kinase C-α, c-ras, and ras, and protein kinase A-type 1 (31). To date, none of the completed randomized trials have demonstrated efficacy of the antisense approach. However, there are still many ongoing clinical trials using the antisense approach. We are currently planning experiments to study the efficacy of Plk1 antisense against mouse tumor xenografts as well as other in vivo applications of this technology.

The extremely high level of overexpression in pancreatic cancer also suggests the usefulness of developing potent small molecule inhibitors of Plk1. While cells often possess alternate pathways that can be activated to cope with the loss of function of a particular protein in the cell, the results of our experiments suggest that the majority of pancreatic cancers may lack such pathways to overcome Plk1 depletion. The above data taken together demonstrate that plk 1 is an attractive drug target in pancreatic cancer. Ongoing studies will define the potential effectiveness of Plk1 inhibitors as treatment for patients with pancreatic adenocarcinoma as well as a wide variety of other human cancers that also overexpress Plk1.

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References

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