

The mitotic serine threonine kinase, *Aurora-2*, is a potential target for drug development in human pancreatic cancer

Sangeeta Rojanala,^{1,2} Haiyong Han,^{1,2}
Rubén M. Muñoz,¹ Walden Browne,¹
Raymond Nagle,^{1,3} Daniel D. Von Hoff,¹
and David J. Bearss¹

¹Arizona Cancer Center and Departments of ²Molecular and Cellular Biology and ³Pathology, University of Arizona, Tucson, AZ

Abstract

***Aurora-2* is a serine threonine kinase that associates with the centrosome. Overexpression or ectopic expression of *Aurora-2* appears to alter centrosome number and function and has been implicated in a variety of human cancers. In this work, we demonstrate that *Aurora-2* is both amplified and overexpressed in human pancreatic cancer cell lines, with a 2–5-fold increase in gene copy number and a 3–4-fold increase in protein levels compared with controls. *Aurora-2* is also amplified and overexpressed in pancreatic cancers taken directly from patients. An immunohistochemistry of tissues taken directly from patients demonstrated an overexpression of *Aurora-2* in 26 of 28 pancreatic cancers compared with 18 normal pancreas samples. Antisense nucleotides specifically targeted at *Aurora-2* arrest the cell cycle in pancreatic cancer cells, indicating the potential of *Aurora-2* as a therapeutic target in pancreatic cancer. [Mol Cancer Ther. 2004;3(4):451–457]**

Introduction

Pancreatic cancer is the fourth leading cause of cancer death among both adult men and women in the United States. Adenocarcinoma of the pancreas has one of the highest mortality rates of all human tumor types and accounts to ~5–6% of all cancer deaths in the United States (1). Close to 90% of patients diagnosed with pancreas cancer will die within the first year following diagnosis. The high mortality rate of pancreatic cancers is largely due to difficulty of early detection and diagnosis and lack of effective therapies for this disease. Chemotherapy in management of patients with advanced pancreatic cancers

has been disappointing. The only recent approved therapy, gemcitabine, provides only a moderate increase in survival in patients with advanced pancreatic cancer (2). New therapeutics that specifically target pancreatic cancers are obviously needed.

We have reported previously the use of cDNA microarray analysis to discover new potential targets that are significantly up-regulated in pancreatic cancer cell lines compared with normal pancreas cells (3). One of the genes that we identified from that study is the serine threonine kinase *Aurora-2*. Of the 5289 different genes examined by the arrays, 30 genes showed an expression ratio greater than 2 SD from the mean in at least three of the nine pancreatic cell lines studied. Of those 30 genes, the *Aurora-2* gene was one of the most consistently up-regulated genes, showing an average of 4-fold increase in pancreatic cells versus normal pancreas cells. The overexpression of *Aurora-2* at the mRNA level in pancreatic cancer cell lines was also confirmed by reverse transcription (RT)-PCR and Northern blot.

The *Aurora-2* (*STK15*, *BTAK*, *Aurora-A*, *Aik*, and *ARK1*) gene encodes a protein that is part of a family of three human mitotic serine threonine kinases called Aurora/Ipl1-related kinases (AIRKs; Refs. 4). AIRKs are a conserved group of proteins that play a role in regulation of centrosome maturation and chromosome segregation, with homologues in *Saccharomyces cerevisiae* (5), *Caenorhabditis elegans* (6), *Drosophila* (7), and humans (8). Of the three human AIRKs (*Aurora-1*, *-2*, and *-3*) that have been identified, *Aurora-2* has been shown to possibly play a role in human cancers. *Aurora-2* is overexpressed in many tumor types and maps to chromosome 20q13, a region that is frequently amplified in human tumors (9–12). The mechanism(s) responsible for mediating the oncogenic activity of *Aurora-2* are still unclear but are likely due to the role of *Aurora-2* in the regulation of centrosome function.

Centrosomes are the microtubule organizing centers in eukaryotic cells, which promote the recruitment of mitotic proteins (13, 14). Centrosomes establish bipolar spindles during cell division, which in turn ensure equal segregation of replicated chromosomes to the two daughter cells. Abnormalities in centrosome duplication or ectopic assembly of microtubule nucleating proteins can lead to multipolar spindles, which may result in unequal segregation of chromosomes to the two daughter cells (15). In contrast, centrosomes that fail to duplicate form monopolar spindles, leading to inability to segregate chromosomes and mitotic failure (7). In addition, cells with abnormal centrosome numbers result in aberrant chromosome segregation and aneuploidy, a common feature present in many malignancies including pancreatic cancers (9, 16, 17).

Several studies have shown that *Aurora-2* is a centrosome-associated kinase, which has a functional role in centrosome maturation and spindle assembly. Ectopic

Received 4/10/03; revised 1/20/04; accepted 1/27/04.

Grant support: NIH/National Cancer Institute (CA95031 and CA88310) and Arizona Cancer Center tissue culture shared service (grant P30CA023074).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for Reprints: Daniel D. Von Hoff, Arizona Cancer Center, University of Arizona, 1515 North Campbell Avenue, Tucson, AZ 85724. Phone: (520) 626-7925; Fax: (520) 626-6898. E-mail: dvonhoff@azcc.arizona.edu

expression of *Aurora-2* in mouse NIH3T3 cells leads to transformation *in vitro* and to the appearance of abnormal centrosome numbers (11). Overexpression of *Aurora-2* in near-diploid human breast epithelial cells resulted in centrosome abnormalities with induction of aneuploidy (10, 11). In addition, the gene encoding *Aurora-2* is amplified in many of these tumor types, suggesting a mechanism associated with its overexpression (10, 11).

In this article, we present evidence that the *Aurora-2* gene is amplified in human pancreatic cell lines and that *Aurora-2* is overexpressed at the mRNA and protein levels in both human pancreatic cancer cell lines and pancreatic cancers taken directly from patients compared with normal pancreas cells. Furthermore, we demonstrate that inhibition of *Aurora-2* expression in pancreatic cancer cell lines leads to cell cycle arrest, suggesting that *Aurora-2* may be of considerable interest as a potential target for drug development.

Materials and Methods

Cell Culture

AsPC-1, BxPC-3, Capan-1, CFPAC-1, HPAF II, Mia PaCa-2, Panc-1, and SU.86.86 (human pancreatic tumor cell lines) and NIH3T3 (mouse fibroblast cell line) were obtained from American Type Culture Collection (Manassas, VA). Forf (human foreskin fibroblast cell line) and Mutj (UACC-462; human pancreatic tumor cell line) were established from fresh tissues at Arizona Cancer Center (courtesy of the tissue culture shared service at Arizona Cancer Center). Human pancreatic tumor cell lines were cultured at 37°C in RPMI 1640 (Mediatech, Herndon, VA) with 10% fetal bovine serum (FBS), 50 units/ml penicillin G sodium, and 50 units/ml streptomycin sulfate (Invitrogen, Carlsbad, CA). NIH3T3 cells were cultured in DMEM (Mediatech) with 10% FBS, 50 units/ml penicillin G sodium, and 50 units/ml streptomycin sulfate. Cells were grown to 80% confluency and passaged as required. The growth medium was aspirated by vacuum and the cells were washed with 1× PBS (Mediatech); sufficient trypsin (Invitrogen) was added to cover the cells and incubated at 37°C for 2–3 min or until cells detached from the flask surface; trypsin was neutralized with an equal volume of culture medium and the cells were counted using a hemocytometer.

Isolation of Total RNA

Total RNA from different cell lines was isolated using RNeasy Mini Kit following the manufacturer's protocol (Qiagen, Inc., Valencia, CA). Briefly, cells were trypsinized and collected as a cell pellet by centrifugation at 3000 × *g* for 5 min. Buffer RLT (50 µl; RNeasy lysis buffer) was added to the cell pellet and gently vortexed to lyse the cells. The samples were homogenized by passing through the QIAshredder column. After mixing 500 µl of 70% ethanol, the lysates were added onto the RNeasy mini spin columns and centrifuged for 15 s at 8000 × *g*. The spin columns, now bound with RNA, were then washed once with 700 µl Buffer RWI and twice with 500 µl Buffer RPE. RNA was eluted with 25 µl RNase-free water from the columns and stored at –80°C for later use in RT-PCR.

RT-PCR

The RT reactions were carried out using the Omniscript RT Kit (Qiagen) according to the manufacturer's protocol. Each 20 µl reaction contained 1× Omniscript RT Buffer (Qiagen); 500 µM each of dCTP, dATP, dGTP, and dTTP; 1 µM Oligo dT primer; 1 M random decamer primers; 1 unit Omniscript RT; and 2 µg total RNA. The reaction was incubated at 37°C for 60 min and 92°C for 10 min. Both incubations were carried out in a DNA Engine Peltier Thermal Cycler (MJ Research, South San Francisco, CA). PCR was performed using the following protocol: Each 50 µl reaction contained 1× PCR buffer; 50 µM each of dCTP, dATP, dGTP, and dTTP; 0.5 µl β-actin primer pair (Ambion, Austin, TX); 2.5 units Taq polymerase (Promega, Madison, WI); 0.5 µM of each *Aurora-2* primer (see below); and 2 µl of the RT reaction. Primer sequences were as follows: *Aurora-2* (upstream) 5'-ATTACAGCTAGAGG-CATCATG-3' and *Aurora-2* (downstream) 5'-GGCGACA-GATTGAAGGGC-3'. The reactions were carried out in a DNA Engine Peltier Thermal Cycler using the following program: 94°C, 4 min; 94°C, 1 min; 60°C, 1 min; 72°C, 1 min for 25 cycles and 72°C, 5 min. PCR products were resolved on 1% agarose gels and visualized with ethidium bromide.

Real-Time PCR

Real-time PCR was performed using the following protocol: Each 25 µl reaction contained 2× SYBR Green Master Mix, 0.25 µl β-actin primer pair (Ambion), 0.5 µM of each *Aurora-2* primer (see below), and 0.5 µl of the RT reaction. Primer sequences were as follows: *Aurora-2* (upstream) 5'-ATTACAGCTAGAGGCATCATG-3' and *Aurora-2* (downstream) 5'-GGCGACAGATTGAAGGGC-3'. The reactions were carried out in a DNA Engine Opticon (MJ Research) using the following program: 95°C, 14 min; 94°C, 30 s; 58°C, 45 s; 72°C, 45 s for 40 cycles. The samples were quantified and normalized to β-actin that served as an internal control.

Isolation of Genomic DNA

Genomic DNA was isolated from nine human pancreatic cancer cell lines. The cells were trypsinized and collected as a cell pellet by centrifugation at 3000 × *g* for 5 min. The supernatant was aspirated, and 600 µl tail blot solution [50 mM Tris (pH 8.0), 100 mM EDTA, 100 mM NaCl, and 1% SDS], RNase (1 µg/ml), and proteinase K solution (10 mg/ml) were added followed by incubation at 55°C for 1 h. DNA was extracted with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) by centrifugation for 10 min and isopropanol was added to allow DNA precipitation. Using a pipette, DNA was removed and dipped into a beaker of 70% and 100% ethanol and air dried and resuspended in 200 µl TE buffer [10 mM Tris-HCl (pH 7.5) and 1 mM EDTA].

Probe Preparation

The *Aurora-2* and β-actin probes used in the Southern blot analysis were prepared using the RTS RadPrime DNA Labeling System (Invitrogen). The *Aurora-2* PCR fragment subcloned into pcDNA 3.1/V5-His TOPO TA cloning vector and a plasmid containing the β-actin were used

as a template. Template DNA (25 ng) was denatured in 45 μ l TE buffer by heating to 100°C for 5 min and immediate cooling on ice. The denatured template and 5 μ l [³²P]dCTP (5 μ Ci/ μ l) were added to RTS RadPrime DNA Labeling System and gently mixed. The reaction mix was centrifuged for 30 s and incubated at 37°C for 10 min followed by stopping the reaction with 0.2 M EDTA (5 μ l). The labeled probes were purified by Micro BioSpin 30 columns (Bio-Rad Laboratories, Hercules, CA).

Southern Blot Analysis

Genomic DNA was digested with *Pst*I restriction endonuclease at 37°C overnight. Digested genomic DNA (10 μ g) was subjected to electrophoresis on agarose gel, stained with ethidium bromide, and visualized by UV light to check DNA loading and migration. The agarose gel was destained in distilled water and treated with 0.25 M HCl for 30 min, 1.5 M NaCl/0.5 M NaOH for 20 min twice, 1.5 M NaCl/0.5 M Tris-HCl (pH 7.0) for 20 min twice, and distilled water for 20 min twice while shaking at room temperature followed by a capillary transfer onto a Zeta-probe nylon membrane (Bio-Rad Laboratories). The membrane was rinsed in 2 \times SSC and irradiated using a UV transilluminator at 254 nm wavelength (Stratagene, La Jolla, CA). Hybridization with the radiolabeled *Aurora-2* and -actin probes was performed in a solution containing 5 \times Denhardt's reagent, 6 \times saline-sodium phosphate-EDTA (SSPE; pH 7.4), 0.5% SDS, 100 μ g/ml denatured salmon sperm DNA, and 50% formamide at 42°C for overnight. The membrane was then washed once with 1 \times SSPE and 0.1% SDS at 42°C for 45 min and twice with 0.1 \times SSPE and 0.1% SDS at 65°C for 1 h. The radioactive signals on the membrane were visualized using PhosphorImager and analyzed with the ImageQuant software (Amersham Biosciences, Piscataway, NJ).

Western Blotting

Protein extracts from nine different pancreatic cancer cell lines were prepared and separated by SDS-PAGE. The proteins from the gels were transferred to a nitrocellulose membrane by tank transfer. Membrane was blocked in 5% nonfat dry milk in Tris-buffered saline and 0.1% Tween 20 (TBST; 100 mM Tris, 0.9% NaCl) on an orbital shaker for 1 h at room temperature. The membrane was probed with 1:250 dilution of a monoclonal antibody against *Aurora-2* (BD Transduction Laboratories, San Diego, CA) in TBST/0.5% milk and incubated while shaking at room temperature for 1 h followed by 1:1000 dilution of rabbit anti-mouse horseradish peroxidase secondary antibody (Bio-Rad Laboratories) in TBST/0.5% milk for 1 h. Membranes were washed thrice for 5 min each in TBST, in between each incubation, and antibody-bound proteins were detected with enhanced chemiluminescence detection system (Amersham Biosciences) using Kodak Biomax MR X-ray film (Kodak, New Haven, CT).

Cloning *Aurora-2* cDNA and Sequencing

Homo sapiens coding sequence for *Aurora-2* was obtained from Genbank (accession no. NM_00360). RT first-strand synthesis was carried out from pooled RNA isolated from

several pancreatic cancer cell lines and PCR was performed with the RT cDNA as a template. The PCR reaction conditions were as follows: 95°C for 5 min; 94°C, 1 min; 59°C, 1 min and 10 s; 72°C, 1 min and 30 s, 34 times; 72°C, 1 min and 30 s. The resulting 1233-bp product was gel purified and subcloned into the pcDNA 3.1/V5-His TOPO TA cloning vector following the manufacturer's protocol (Invitrogen). The insertion direction and sequence of *Aurora-2* were verified by sequencing.

Tissue Microarray-Based Immunohistochemistry

Core tissue biopsies (diameter 0.6 mm, height 3–4 mm) were taken from individual "donor" blocks and arrayed into a new "recipient" paraffin block (45 \times 20 mm) using a tissue microarraying instrument (Beecher Instruments, Sun Prairie, WI). On average, 100 sections were cut from one tumor tissue microarray block. H&E staining for histology verification was performed every 50th section cut from the block to ensure that the histology was consistent from section to section. The 5 m thick sections were deparaffinized in 0.1 M citrate buffer (pH 6.6) and 1 mM EDTA followed by microwaving. The sections were then stained using an automated immunostainer (VMS ES; Ventana Medical Systems, Tucson, AZ) with a rabbit anti-human *Aurora-2* polyclonal antibody followed with biotinylated goat anti-rabbit antibody (DAKO, Carpinteria, CA) and then with avidin-biotin-peroxidase complex (DAKO), each for 30 min at 42°C. After staining, the slide was evaluated and photographed using a light microscope equipped with a digital camera.

Antisense Experiments

Aurora-2 antisense oligonucleotides (Isis Pharmaceuticals, Carlsbad, CA) were screened against sequences in the Genbank database to ensure that they only matched *Aurora-2* kinase sequence. *Aurora-2* antisense oligonucleotide sequence is 5'-CTAGATTGAGGGCAGCA and the scrambled oligonucleotide sequence is 5'-GTACAGTTATGCGCGGTAGA. Mia PaCa-2 cells (0.5×10^6) were seeded in T-25 flasks with 5 ml RPMI 1640 supplemented with 10% FBS. Cells were grown to 50% confluency, the medium was aspirated, cells were washed with PBS, and 6 μ l LipofectAMINE reagent (Invitrogen)/1 ml Opti-MEM medium (Invitrogen) was added to T-25 culture flask. *Aurora-2* antisense oligonucleotides were added directly to T-25 flask at 200 nM concentrations. Cells were incubated at 37°C with 5% CO₂ and harvested for isolation of RNA and protein extraction after 24 and 48 h to detect the *Aurora-2* kinase expression levels by RT-PCR and Western blotting.

Flow Cytometry

Mia PaCa-2 cells (0.5×10^6) were seeded in T-25 flasks with 5 ml RPMI 1640 supplemented with 10% heat-inactivated FBS to 50% confluency. The cells were treated with *Aurora-2* antisense oligonucleotides as described in antisense experiments and harvested after 24 and 48 h. The cells were then washed with PBS and resuspended in 1 ml Krishan buffer (0.1% sodium citrate, 0.2 mg/ml RNase, and 0.3% NP40) containing 0.05 μ g/ml propidium iodide for incubation at 4°C for 30 min. The cells were subjected to

flow analysis using FACScan (Becton Dickinson, San Jose, CA). Data were acquired using Soft CellQuest software and analyzed using ModFitLT 2.0 (Verity Software House, Topsham, ME).

Caspase-3 Assay

Mia PaCa-2 cells (0.5×10^6) were seeded and cultured to 50% confluency. The medium was aspirated, cells were washed with PBS, and 5 ml Opti-MEM medium (Life Technologies, Inc., Carlsbad, CA) containing 6 μ l lipofectin reagent (Life Technologies)/1 ml Opti-MEM medium was added to each T-25 culture flask. *Aurora-2* antisense oligonucleotides were added directly to T-25 flask at 200 nM concentration and staurosporine was added directly to flasks at 1 μ M concentration for incubation at 37°C for 6 h. The media was then replaced with 5 ml RPMI 1640 supplemented with 10% FBS and harvested at 24 and 48 h. Cells (1×10^6) were collected for each sample and suspend in cell lysis buffer for 10 min on ice. The cell lysates were centrifuged in a microcentrifuge at 14,000 rpm for 10 min at 4°C and the supernatant was transferred to Eppendorf tubes to detect caspase-3 activity using BD ApoAlert Caspase-3 Fluorescent Assay Kit (BD Biosciences Clontech, Palo Alto, CA). The samples were read using Wallac Victor2 multilabel fluorometer model 1420-011 (Perkin-Elmer, Boston, MA) with 405 nm excitation filter and 500 nm emission filter.

Results

The *Aurora-2* Gene Is Amplified in Pancreatic Cancer Cell Lines

Aurora-2 gene copy number increases have been reported in colorectal, breast, and gastric cancers (10, 12). The *Aurora-2* gene has been mapped to chromosome 20q, a region that is frequently amplified in pancreatic cancer (10, 18). To examine whether the *Aurora-2* gene is amplified in pancreatic cancers, Southern blot analysis was performed in nine human pancreatic cell lines. These cell lines showed 2–5-fold amplification of *Aurora-2* compared with normal diploid foreskin fibroblast cells (data not shown).

Aurora-2 Is Overexpressed in *Aurora-2* Pancreatic Cancer Cell Lines

We demonstrated previously that *Aurora-2* mRNA was overexpressed in pancreatic cancer cell lines, with an average of a 4-fold increase compared with normal pan-

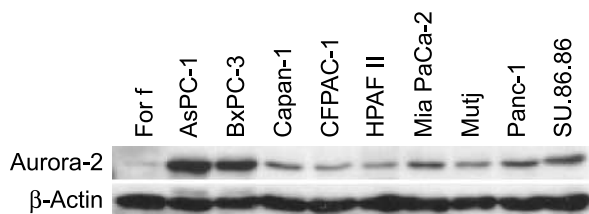


Figure 1. Protein expression of *Aurora-2* kinase in pancreatic cancer cell lines. Western blot analysis indicates an up-regulation of 3–4-fold of *Aurora-2* in nine different pancreatic cancer cell lines (*AsPC-1*, *BxPC-3*, *Capan-1*, *CFPAC-1*, *HPAF II*, *Mia PaCa-2*, *Mutj*, *Panc-1*, and *SU.86.86*) in comparison with normal diploid foreskin fibroblast cells (*Forf*). β -actin served as an internal control.

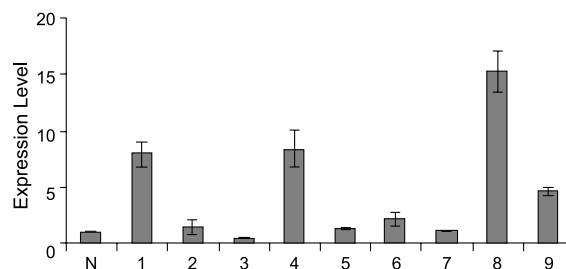


Figure 2. *Aurora-2* kinase expression in pancreatic patient tumors. Real-time PCR of pancreatic patient tumor samples indicates an up-regulation of *Aurora-2* mRNA expression in samples 1, 4, 6, 8, and 9 taken directly from patients compared with normal pancreas tissue (N). Normalized ratio is obtained from pancreatic tumor tissue versus normal tissue.

creas (19). To determine if this mRNA increase was matched by overexpression of protein, we carried out a Western blot analysis on nine human pancreatic cancer cell lines using a polyclonal anti-*Aurora-2* antibody (Cell Signaling Technology, Beverly, MA). Western blot demonstrated a 3–4-fold increase level of *Aurora-2* protein in all nine pancreatic cancer lines compared with the normal foreskin fibroblast cells (Fig. 1).

Aurora-2 Is Overexpressed in Patients with Pancreatic Cancer

To determine if *Aurora-2* is overexpressed in pancreatic cancer patient samples, we examined *Aurora-2* mRNA and protein levels in tumor tissues using real-time PCR and immunohistochemistry, respectively. As demonstrated in Fig. 2, of the nine patient samples studied by real-time PCR, five patients (56%) showed increased mRNA level compared with the normal pancreas tissue (N). To investigate *Aurora-2* protein levels in multiple tissue samples, we established a tissue microarray that contains core sections from 28 pancreatic adenocarcinomas and 18 sections from normal pancreas. The sections were stained with a polyclonal rabbit anti-*Aurora-2* antibody (Cell Signaling Technology) using an automated immunostaining station. The staining intensities were scored from 0 (no staining) to 4+ (the strongest staining; Fig. 3). Twenty-six of 28 adenocarcinomas (93%) had 2+ or higher staining of *Aurora-2* protein whereas all of the 18 normal cases were graded as negative or 1+ above background. The stain was

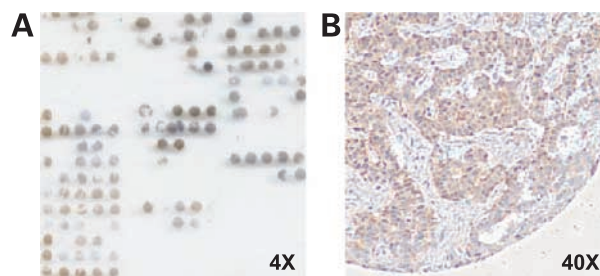


Figure 3. Immunohistochemistry of pancreatic tissue array. **A**, an array of paraffin-embedded pancreatic tumor samples. **B**, a specific pancreatic tumor sample at higher magnification from the array indicates a high level of *Aurora-2* protein expression.

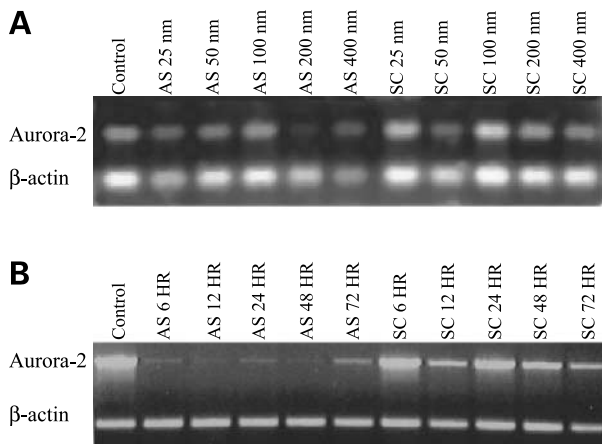


Figure 4. Inhibition of *Aurora-2* using antisense oligonucleotides. **A**, RT-PCR of a human pancreatic tumor cell line (Mia PaCa-2) treated with antisense *Aurora-2* at 25, 50, 100, 200, and 400 nm concentrations. Antisense-treated cells indicate a significant inhibition of *Aurora-2* expression at 200 nm concentration compared with the scrambled oligonucleotides. **B**, RT-PCR of time course study of Mia PaCa-2 cells treated with *Aurora-2* antisense indicates inhibition of *Aurora-2* expression as early as 6 h.

diffused throughout the cytoplasm. These results confirmed that *Aurora-2* is highly up-regulated in pancreatic cancer and presents a potential molecular target for drug development.

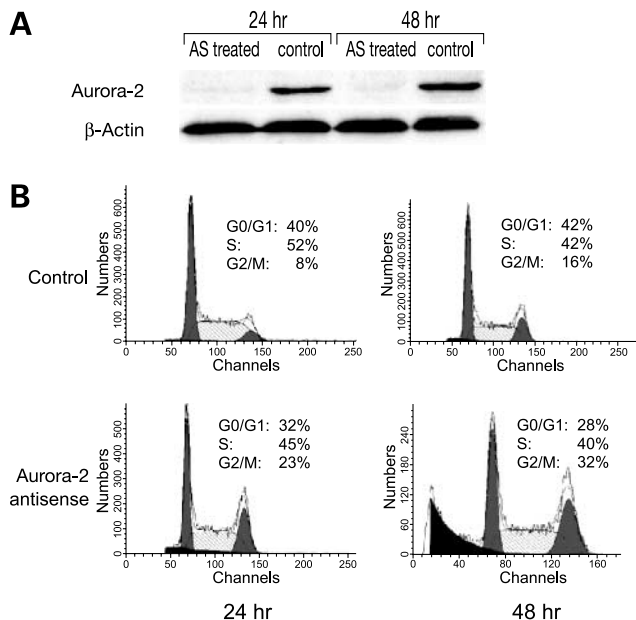


Figure 5. Inhibiting *Aurora-2* arrests cell cycle. **A**, time-dependent study showing Western blot analysis of protein extracts from pancreatic cell line (Mia PaCa-2) treated with antisense oligonucleotides shows inhibition of *Aurora-2* expression at 24 and 48 h. **B**, following inhibition of *Aurora-2* expression using antisense oligonucleotides, the flow analysis indicates a fraction of cells in sub-G₁ phase of the cell cycle in the antisense-treated cells compared with the untreated control, indication of cells undergoing apoptosis.

Validation of *Aurora-2* as a Potential Target for Drug Development

Time and Dose Effects of *Aurora-2* Antisense Oligonucleotides. To study the effects of *Aurora-2* inhibition on pancreatic cancer cell growth, we used an antisense approach to inhibit the expression of *Aurora-2*. *Aurora-2* antisense oligonucleotides (obtained from Isis Pharmaceuticals) were screened against sequences in the Genbank database to ensure a unique match with *Aurora-2* sequence. A scrambled oligonucleotide was also synthesized to serve as a control. The ability of the antisense to inhibit the expression of *Aurora-2* was verified by RT-PCR. A human pancreatic tumor cell line (Mia PaCa-2) was treated with antisense *Aurora-2* to determine the optimal dose at which *Aurora-2* expression was inhibited. Antisense-treated cells showed a significant inhibition of *Aurora-2* expression at 200 nm concentration when compared with the scrambled oligonucleotide (Fig. 4A). A time course study was also performed to determine the effect of *Aurora-2* antisense. Mia PaCa-2 cells treated with antisense and scrambled oligonucleotides for 6, 24, 48, and 72 h indicated inhibition of *Aurora-2* expression 6 h post-transfection (Fig. 4B).

Inhibition of *Aurora-2* Induces Cell Cycle Arrest and Apoptosis. We predicted that inhibition of *Aurora-2* expression with antisense oligonucleotides would result in cell cycle arrest in the G₂-M phase and may lead to cell death. Treatment with *Aurora-2* antisense resulted in complete suppression of *Aurora-2* protein expression (Fig. 5A). Following the treatment with *Aurora-2* antisense oligonucleotide, the cells were analyzed by flow cytometry to determine the cell cycle distribution (Fig. 5B). In controls, the percentage of cells in G₂-M was 8% at 24 h and 16% at 48 h, while in antisense-treated cells, it was 23% and 32%, respectively. Furthermore, we performed caspase-3 assay

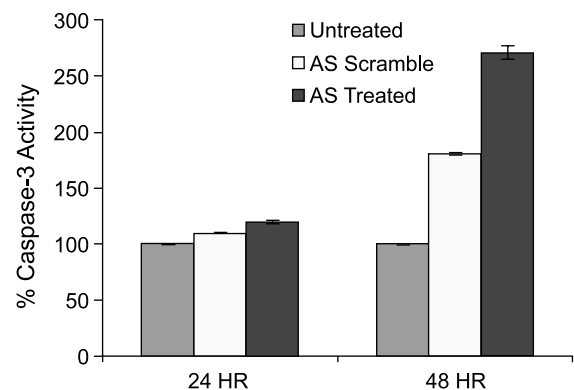


Figure 6. Inhibiting *Aurora-2* expression induces caspase-3 activation. Mia PaCa-2 cells treated with *Aurora-2* antisense show an induction of caspase-3 activity at 24 and 48 h. The untreated cells indicate a baseline of caspase-3 activity at 100% at 24 and 48 h. The antisense scrambled cells (AS Scramble) indicate an increase in caspase-3 activity at 24 h (9%) and 48 h (80%) compared with the untreated control cells. However, the antisense-treated (AS Treated) cells show a significant increase in apoptosis compared with the untreated and the antisense scrambled controls. The antisense-treated cells indicate a 20% increase in caspase-3 activity at 24 h and 90% increase at 48 h compared with the scrambled antisense controls.

to see if the inhibition of *Aurora-2* expression caused cells to undergo apoptosis (Fig. 6). Treatment with *Aurora-2* antisense resulted in an induction of caspase-3 activity. If untreated, the cells undergo apoptosis at normal levels inducing caspase-3 activity at 24 and 48 h. In the scrambled antisense-treated controls, there was a 9% induction in caspase-3 activity at 24 h and a 80% induction in caspase-3 activity at 48 h compared with the untreated cells. However, in the antisense-treated cells, there was 20% increase in caspase-3 activity at 24 h and 90% at 48 h compared with the scrambled antisense controls. These results indicate a significant increase in caspase-3 activity in the antisense-treated pancreatic cancer cell line compared with both the scrambled antisense and the untreated control cells. The control cells treated with lipofectin only did not result in an increase of caspase-3 activity compared with the untreated cells (data not shown). However, we failed to detect a significant increase in apoptosis in antisense *Aurora-2*-treated cells using Annexin V and terminal deoxynucleotidyl transferase-mediated nick end labeling assays.

Discussion

In this study, we investigated whether *Aurora-2* is up-regulated in pancreatic cancer cell lines and pancreatic cancers taken directly from patients. Using Western blotting, we demonstrated a 3–4-fold increase in *Aurora-2* expression in nine human pancreatic cell lines. Real-time PCR and immunohistochemistry showed that there are significant increases in *Aurora-2* in human pancreatic cancer tissues as well. We also showed, using Southern blot analysis, that the *Aurora-2* gene is amplified 2–5-fold in pancreatic cancer cell lines. The data with overexpression of *Aurora-2* along with amplification of the gene are reminiscent of the situation with *HER-2/neu* where both amplification and increased expression were noted in some breast cancers taken directly from patients (20). Of course, a very successful drug development program to develop a therapeutic agent against breast cancer followed that finding (21, 22).

Our data in pancreatic cancers are consistent with reports showing overexpression of *Aurora-2* in human colon, breast, ovarian, cervical, prostate, neuroblastoma, and pancreas cancer cell lines as well as overexpression and amplification of *Aurora-2* in colon, breast, gastric, bladder, and pancreatic cancers taken directly from patients (9, 11, 12, 19, 23–25). Previous immunofluorescence studies in colon and breast cancers revealed centrosomal localization of *Aurora-2* during mitosis, while we observed diffuse cytoplasmic staining in tumors taken directly from patients. Thus, *Aurora-2* overexpression apparently is indicative of the pathological states of cancer cells.

We have demonstrated that antisense suppression of *Aurora-2* arrests progression of the cell cycle and the cells may undergo apoptosis, indicating that *Aurora-2* may be a valuable target for treatment of pancreatic cancer and that *Aurora-2* inhibitors may be useful therapeutics for pancreatic cancer. We are currently in the process of further

evaluating these possibilities. Whether *Aurora-2* suppression proves to be a fruitful avenue for treatment of pancreatic cancer, further understanding of its normal function and role in carcinogenesis is likely to yield important insights into regulation of mitotic processes and how these are changed in the process of carcinogenesis.

Acknowledgments

We thank Richard Robinson for his excellent input into the preparation of this manuscript and Yu Zhao for his excellent scientific input to the Aurora Kinase Project.

References

- Jemal A, Murray T, Samuels A, Ghafoor A, Ward E, Thun MJ. Cancer statistics, 2003. *CA Cancer J Clin*, 2003;53:5–26.
- Burris HI, Moore M, Andersen J, et al. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. *J Clin Oncol*, 1997;15:2403–13.
- Han HY, Bearss DJ, Browne LW, Calaluca R, Nagle RB, Von Hoff DD. Identification of differentially expressed genes in pancreatic cancer cells using cDNA microarray. *Cancer Res*, 2002;62:2890–6.
- Giet R, Prigent C. Aurora/Ipl1p-related kinases, a new oncogenic family of mitotic serine-threonine kinases. *J Cell Sci*, 1999;112:3591–601.
- Chan CS, Botstein D. Isolation and characterization of chromosome-gain and increase-in-ploidy mutants in yeast. *Genetics*, 1993;135:677–91.
- Schumacher JM, Ashcroft N, Donovan PJ, Golden A. A highly conserved centrosomal kinase, AIR-1, is required for accurate cell cycle progression and segregation of developmental factors in *Caenorhabditis elegans* embryos. *Development*, 1998;125:4391–402.
- Glover DM, Leibowitz MH, McLean DA, Parry H. Mutations in aurora prevent centrosome separation leading to the formation of monopolar spindles. *Cell*, 1995;81:95–105.
- Bischoff JR, Plowman GD. The Aurora/Ipl1p kinase family: Regulators of chromosome segregation and cytokinesis. *Trends Cell Biol*, 1999;9:454–9.
- Bischoff JR, Anderson L, Zhu Y, et al. A homologue of *Drosophila* aurora kinase is oncogenic and amplified in human colorectal cancers. *EMBO J*, 1998;17:3052–65.
- Sen S, Zhou H, White RA. A putative serine/threonine kinase encoding gene BTAK on chromosome 20q13 is amplified and overexpressed in human breast cancer cell lines. *Oncogene*, 1997;14:2195–200.
- Zhou H, Kuang J, Zhong L, et al. Tumor amplified kinase STK15/BTAK induces centrosome amplification, aneuploidy and transformation [see comments]. *Nat Genet*, 1998;20:189–93.
- Sakakura C, Hagiwara A, Yasuoka R, et al. Tumor-amplified kinase BTAK is amplified and overexpressed in gastric cancers with possible involvement in aneuploid formation. *Br J Cancer*, 2001;84:824–31.
- Kimura M, Kotani S, Hattori T, et al. Cell cycle-dependent expression and spindle pole localization of a novel human protein kinase, Aik, related to Aurora of *Drosophila* and yeast Ipl1. *J Biol Chem*, 1997;272:13766–71.
- Gopalan G, Chan CS, Donovan PJ. A novel mammalian, mitotic spindle-associated kinase is related to yeast and fly chromosome segregation regulators. *J Cell Biol*, 1997;138:643–56.
- Doxsey S. The centrosome—a tiny organelle with big potential [letter; comment]. *Nat Genet*, 1998;20:104–6.
- Sato N, Mizumoto K, Nakamura M, et al. Centrosome abnormalities in pancreatic ductal carcinoma. *Clin Cancer Res*, 1999;5:963–70.
- Pihan GA, Purohit A, Wallace J, et al. Centrosome defects and genetic instability in malignant tumors. *Cancer Res*, 1998;58:3974–85.
- Tanner MM, Grenman S, Koul A, et al. Frequent amplification of chromosomal region 20q12-q13 in ovarian cancer. *Clin Cancer Res*, 2000;6:1833–9.
- Han H, Bearss DJ, Browne LW, Calaluca R, Nagle RB, Von Hoff DD. Identification of differentially expressed genes in pancreatic cancer cells using cDNA microarray. *Cancer Res*, 2002;62:2890–6.

20. Slamon DJ, Clark GM. Amplification of c-erbB-2 and aggressive human breast tumors? *Science*, 1988;240:1795–8.
21. McNeil C. Herceptin raises its sights beyond advanced breast cancer. *J Natl Cancer Inst*, 1998;90:882–3.
22. Baselga J, Norton L, Albanell J, Kim YM, Mendelsohn J. Recombinant humanized anti-HER2 antibody (Herceptin) enhances the antitumor activity of paclitaxel and doxorubicin against HER2/neu overexpressing human breast cancer xenografts. *Cancer Res* 1998;58:2825–31.
23. Tanaka T, Kimura M, Matsunaga K, Fukada D, Mori H, Okano Y. Centrosomal kinase AIK1 is overexpressed in invasive ductal carcinoma of the breast. *Cancer Res*, 1999;59:2041–4.
24. Sen S, Zhou H, Zhang RD, et al. Amplification/overexpression of a mitotic kinase gene in human bladder cancer. *J Natl Cancer Inst*, 2002;94:1320–9.
25. Gritsko TM, Coppola D, Paciga JE, et al. Activation and overexpression of centrosome kinase BTAK/Aurora-A in human ovarian cancer. *Clin Cancer Res*, 2003;9:1420–6.

Molecular Cancer Therapeutics

The mitotic serine threonine kinase, *Aurora-2*, is a potential target for drug development in human pancreatic cancer

Sangeeta Rojanala, Haiyong Han, Rubén M. Muñoz, et al.

Mol Cancer Ther 2004;3:451-457.

Updated version Access the most recent version of this article at:
<http://mct.aacrjournals.org/content/3/4/451>

Cited articles This article cites 24 articles, 15 of which you can access for free at:
<http://mct.aacrjournals.org/content/3/4/451.full#ref-list-1>

Citing articles This article has been cited by 11 HighWire-hosted articles. Access the articles at:
<http://mct.aacrjournals.org/content/3/4/451.full#related-urls>

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link
<http://mct.aacrjournals.org/content/3/4/451>.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.