High-throughput RNAi Screening Identifies a Role for TNK1 in Growth and Survival of Pancreatic Cancer Cells

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Abstract

To identify novel targets in pancreatic cancer cells, we used high-throughput RNAi (HT-RNAi) to select genes that, when silenced, would decrease viability of pancreatic cancer cells. The HT-RNAi screen involved reverse transfecting the pancreatic cancer cell line BxPC3 with a siRNA library targeting 572 kinases. From replicate screens, approximately 32 kinases were designated as hits, of which 22 kinase targets were selected for confirmation and validation. One kinase identified as a hit from this screen was tyrosine kinase nonreceptor 1 (TNK1), a kinase previously identified as having tumor suppressor-like properties in embryonic stem cells. Silencing of TNK1 with siRNA showed reduced proliferation in a panel of pancreatic cancer cell lines. Furthermore, we showed that silencing of TNK1 led to increased apoptosis through a caspase-dependent pathway and that targeting TNK1 with siRNA can synergize with gemcitabine treatment. Despite previous reports that TNK1 affects Ras and NF-κB signaling, we did not find similar correlations with these pathways in pancreatic cancer cells. Our results suggest that TNK1 in pancreatic cancer cells does not possess the same tumor suppressor properties seen in embryonic cells but seems to be involved in growth and survival. The application of functional genomics by using HT-RNAi screens has allowed us to identify TNK1 as a growth-associated kinase in pancreatic cancer cells. Mol Cancer Res; 9(6); 724–32. ©2011 AACR.

Introduction

Pancreatic cancer is one of the most aggressive and lethal cancers known today, with a 5-year survival of only 4%. In 2009, pancreatic cancer was the fourth leading cause of cancer-related deaths (1). Patients diagnosed with pancreatic cancer typically have a poor prognosis because of a lack of early symptoms, leading to metastatic disease at the time of diagnosis. The treatment options for pancreatic cancer include chemotherapy, surgery, and radiation. The most preferred current therapeutic drug to treat pancreatic cancer is gemcitabine, yet the 1-year survival of pancreatic cancer patients treated with gemcitabine is about 18%, representing a significant but modest advancement in the quality of life (2). Clearly, novel targets and therapeutic combinations are needed to more effectively treat patients afflicted with this deadly disease.

Previously, our laboratory had reported on the use of a high-throughput RNA interference (HT-RNAi) platform to identify potential sensitizing targets to gemcitabine in pancreatic cancer cells (3) and to cisplatin in ovarian cancer (4). This procedure used a library of siRNA to identify functional mediators of the cytotoxic response to chemotherapeutic agents. This HT-RNAi screen is also capable of identifying targets which, when silenced, would cause a decrease in cancer cell viability in the absence of additional therapeutics as previously reported (5). The targets identified by these types of HT-RNAi screens are referred to as Achilles’ heel (AH) targets. In this study, we have used the HT-RNAi platform to identify novel AH targets in pancreatic cancer, using BxPC3 cells. One novel AH target identified from this screen was tyrosine kinase, nonreceptor, 1 (TNK1).

TNK1 was first identified in human umbilical cells (6) and murine embryonic stem cells as a tumor suppressor that downregulates Ras activity (7). It was further determined that Tnk1 knockout mice form spontaneous tumors (8) and TNK1 blocks NF-κB activation to facilitate TNFα-induced apoptosis (9). These data collectively suggest that TNK1 functions as a tumor suppressor, at least in embryonic and stem cells. In contrast, a recently published study identified TNK1 as having oncogenic potential based upon a retroviral insertion mutagenesis screen (10). A separate study has also identified an activated version of TNK1 in...
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Hodgkin’s lymphoma (11). Here we identify TNK1 as a novel AH target in pancreatic cancer and present evidence that TNK1 functions to promote growth and survival in pancreatic cancer cells.

Materials and Methods

Cell culture and reagents

The human pancreatic cancer cell lines MiaPaCa-2, BxPC3, Panc-1, AsPC-1, Su.86.86, CAPAN-1, and CAPAN-2 were obtained from the American Type Culture Collection (ATCC). Cells were grown in RPMI-1640 supplemented with 10% FBS, 2 mmol/L L-glutamine, 100 IU/mL penicillin G, and 100 μg/mL streptomycin. The immortalized human pancreatic ductal epithelial cell line HPDE6 was kindly provided by Dr. Ming-Sound Tsao (University of Toronto; Toronto, Ontario, Canada; refs. 12, 13). HPDE6 cells were maintained in keratinocyte serum-free medium supplemented with epidermal growth factor (0.2 ng/mL) and bovine pituitary extract (30 μg/mL). All media reagents were obtained from Invitrogen. The cell lines were routinely maintained at 37°C in a humidified 5% CO₂ atmosphere and periodically screened for mycoplasma.

Cell line identities were verified by STR profiling (14) by using the AmpFISTR Identifier PCR amplification kit (Applied Biosystems). This method simultaneously amplifies 15 STR loci and amelogenin in a single tube, using 5 dyes, 6-FAM, JOE, NED, PET, and LIZ which are then separated on a 3100 Genetic Analyzer (Applied Biosystems). GeneMapper ID v3.2. AmpFISTR control DNA mixtures 15 STR loci and amelogenin in a single tube, using 5 dyes, 6-FAM, JOE, NED, PET, and LIZ which are then separated on a 3100 Genetic Analyzer (Applied Biosystems). GeneMapper ID v3.2. AmpFISTR control DNA, including the AmpFISTR allelic ladder were run concurrently. Results were compared with published STR sequences from the ATCC. The STR profiling is repeated once a cell line has been passaged more than 6 months after previous STR profiling.

Gemcitabine hydrochloride (Eli Lilly) was obtained from the Mayo Clinic Pharmacy and stock solutions of 100 mmol/L were prepared by dissolving gemcitabine in PBS. Aliquots of gemcitabine were stored at −20°C until use. siRNA targeting TNK1 and nonsilencing control were obtained from Qiagen.

RNAi screening

High-throughput RNAi (HT-RNAi) was performed using the validated kinase siRNA library version 1 obtained from Qiagen. Stock siRNA was diluted in siRNA buffer (Qiagen) and 9.3 ng of siRNA was printed onto white Corning 384-well plates (Fisher Scientific). Nonsilencing and lethal siRNA (Qiagen) were included as negative and positive controls, respectively. Before actual RNAi screening, a transfection reagent test was done by testing commercially available transfection reagents for optimal transfection efficiency of a panel of pancreatic cancer cell lines. SiLentFect reagent (BioRad) was selected for siRNA transfection in all assays (Supplementary Table S1). HT-RNAi was done by using siRNA reverse transfection of cells as previously described (3). Briefly, diluted SiLentFect reagent (BioRad) in OptiMEM (Invitrogen) was added to the wells and allowed to complex with the siRNA. BxPC3 cells were resuspended in growth media without antibiotics at a final concentration of 1,000 cells per well. Cell viability was determined by the addition of CellTiter-Glo (Promega) and relative luminescence units (RLU) were measured by using an EnVision plate reader (Perkin-Elmer). Raw RLU data was used to calculate viability relative to control wells. Decreased viability in the lethal positive control wells served as an indicator of successful transfection for each plate. The screening data was normalized by using the standard z-score method by correcting the raw data for plate row variation and then normalizing and pooling data from all assay plates. The assumption is that the majority of the siRNAs are nonhits and the null distribution is normal (15). The criteria for identification of potential hits were z-score values of less than −1.65, which corresponded to a P value of 0.05, in at least 3 of the 4 screens of pancreatic cancer cells. This cutoff was chosen because of the relatively small size and focused nature of the screen. Validation of screening results with a panel of pancreatic cancer cell lines was done in a similar assay format.

Dose response assays

Cells were reverse transfected as described above in 384-well plates and incubated with siRNA (Qiagen) for 24 hours. Gemcitabine was added at a range of concentrations and cells were incubated for a further 72 hours. Cell viability was measured as described above. Drug–dose response curves were generated and IC₅₀ calculated by using Prism 5.0 (GraphPad Software).

Apoptotic activity assay

Analysis of apoptotic activity was completed by using a Caspase-Glo 3/7 Assay System (Promega). All reagents were added according to manufacturer’s instructions. Briefly, BxPC3 cells were reverse transfected with siRNA (Qiagen) on a 384-well plate at a density of 1,000 cells per well. Caspase-Glo reagent was added at 24, 48, and 72 hours to lyse cells and permit caspase-induced cleavage of the substrate. Activity was determined by measuring luminescence output as described above.

Western blot analysis

Cells were transfected with 16 nmol/L of TNK1 siRNA or nonsilencing siRNAs in 6 well plates by reverse transfection. Cells were treated with siRNA for 96 hours and whole cell lysates were prepared by using Complete Lysis-M reagent (Roche). Protein concentration was determined by BCA assay and lysates were resolved by SDS-PAGE on a 4% to 12% resolving gel. Proteins were transferred onto polyvinylidene difluoride membranes. Antibodies for TNK1, PARP, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), p-MEK 1/2, and MAP/ERK kinase (MEK) 1/2 were purchased from Cell Signaling Technology. Mouse anti-β-tubulin was purchased from Sigma Aldrich. Secondary horseradish peroxidase–conjugated anti-rabbit and anti-mouse antibodies were purchased...
from Jackson ImmunoResearch Laboratories, Inc. Bound antibodies were detected by using SuperSignal West Femto (Pierce) and imaged by using an AlphaInnotech Imager.

**Immunoprecipitation**

Whole cell lysates were immunoprecipitated by using bead-bound p-Tyr monoclonal antibody (Cell Signaling) according to manufacturer’s instructions. Protein was eluted from immunobeads, heat denatured, and loaded onto an SDS-PAGE gel. Protein levels were analyzed by Western blotting as described above. The anti-TNK1 antibody was purchased from Abgent and the anti–phospho-TNK1 (Y277) and anti-EGFR antibodies were purchased from Cell Signaling.

**Quantitative real-time PCR**

Cells were reverse-transfected with siRNA in 6-well plates and incubated for 24 to 72 hours. Total RNA was collected by using an RNaseasy MiniPrep Kit (Qiagen) and concentration was measured by using a Nano Drop (Thermo Scientific). cDNA was generated by using an iScript cDNA synthesis Kit (Bio-Rad). Primers for TNK1 were purchased from Qiagen. Samples were run in triplicate on a 96-well PCR plate by using an Opticon 2 (MJ Research). All samples were normalized to levels of GAPDH.

**Results**

**HT-RNAi screening for kinases important in growth of pancreatic cancer cells**

To identify genes that modulate viability of BxPC3 pancreatic cancer cells, we carried out loss-of-function screening by using high-throughput RNAi. A robust HT-RNAi assay was developed that allowed for high efficiency siRNA transfection of cells by cationic lipids in 384-well plates. The HT-RNAi screen involved reverse-transfecting BxPC3 pancreatic cancer cells with validated library siRNA targeting 572 kinases with 2 siRNA sequences/kinase. Cell viability was assessed by using a luminescence-based cell number assay and the data were normalized and analyzed as described in Materials and Methods. Two independent HT-RNAi screens were conducted to produce a biological replicate of the results. Data was normalized by z-score analysis and results are shown as the z-score for each kinase siRNA in each screen of the BxPC3 cells as well as previous screens by using MiaPaCa-2 cells (Fig. 1A). Hits were designated as having z-scores lower than −1.65 or at least 3 of the 4 assays on the pancreatic cell lines. These criteria identified 32 kinases as significant in growth of both BxPC3 and MiaPaCa-2 cells (Fig. 1B and Supplementary Table S2). Furthermore, comparison of these hits to z-scores from a HT-RNAi kinase screen done on the normal fibroblasts cell line GM05659 showed 22 kinase targets that did not overlap and thus seemed to be specific for the pancreatic cancer cells (Table 1). Several of these kinases have previously been associated with growth and survival of pancreatic cancer cells. One kinase identified as a hit in both cell lines was TNK1, which was the focus of subsequent studies due to reports that it had both tumor suppressor and oncogenic properties.

**Confirmation of gene silencing by TNK1 siRNA**

To show the silencing efficiency of the siRNA targeting TNK1, MiaPaCa-2 and BxPC3 cells were transfected with TNK1 or nonsilencing siRNA and incubated at 37°C for 72 hours. For these validation experiments, we used 3 different siRNA sequences of which 2 of these sequences were identical to the ones used in the initial screen. Total RNA was collected and cDNA was generated to determine levels of TNK1 mRNA by quantitative real-time PCR (qRT-PCR). In addition, cell lysates were analyzed by Western blot by using an anti-TNK1 antibody. Results using qRT-PCR show that all 3 TNK1 siRNA sequences tested were able to reduce the TNK1 message levels compared with nonsilencing siRNA (Fig. 2A). Furthermore, silencing of TNK1 resulted in decreased TNK1 protein level as shown by the Western blot results (Fig. 2B). Silencing of TNK1 by these sequences were further shown by qRT-PCR analysis in MiaPaCa-2, AsPC-1, and Su.86.86 (Supplementary Fig. S1).

**TNK1 expression in pancreatic cancer cells**

Because little is known about TNK1, we first sought to characterize its expression in pancreatic cancer cells. We selected 9 common pancreatic cancer cell lines and examined the protein expression levels of TNK1 along with SMAD4, vimentin, and E-cadherin by Western blotting (Fig. 3A). We further quantified TNK1 protein expression levels by using densitometry analysis (Fig. 3B). Only slight differences in expression exist across the panel of cell lines with MiaPaCa-2 exhibiting the lowest expression of TNK1, whereas HPAC exhibits the highest expression of TNK1. Of note, higher expression levels of TNK1 seem to correlate with mutation or low expression of the SMAD4 gene in this panel of cell lines (i.e., BxPC3, CAPAN-1, and HPAC). Conversely, the cell lines in this panel that express lower levels of TNK1 have all been previously characterized as having a wild-type SMAD4 gene (i.e., MiaPaCa-2, Panc-1; refs. 27, 28). Furthermore, cell lines that exhibit high vimentin expression (indicative of mesenchymal phenotype) tended to have lower expression of TNK1 whereas cell lines that have high E-cadherin expression (indicative of epithelial phenotype) appeared to have higher expression of TNK1.

To determine whether TNK1 knockdown affected SMAD4 expression, we treated Panc-1 cells with TNK1 or nonsilencing siRNA and analyzed SMAD4 expression by Western blot (Fig. 3C). Conversely, we also treated Panc-1 cells with siRNA against SMAD4 and analyzed for TNK1 expression. A densitometry graph correlating to the Western blot is shown for quantitative comparison (Fig. 3D). These results show that even though there seems to be a correlation between SMAD4 and TNK1 expressions based on the results of Figure 3A, TNK1 knockdown by siRNA does not directly affect
SMAD4 expression nor does SMAD4 knockdown directly affect TNK1 expression.

Silencing of TNK1 inhibits growth and induces apoptosis in pancreatic cancer cells

To determine how TNK1 affects pancreatic cancer cell viability, 5 pancreatic cell lines (BxPC3, MiaPaCa-2, AsPC-1, Panc-1, and Su.86.86) were treated with 3 different siRNA sequences targeting TNK1. As shown in Figure 2A and B, all sequences show reliable knockdown of the TNK1 message. In BxPC3, MiaPaCa-2, and Su.86.86 cells (Fig. 4A), TNK1 knockdown resulted in a significant decrease in cell viability. This effect was less notable in Panc-1 and AsPC-1 cells, with TNK1 inhibition resulting in an approximately 20% decrease in viability. Treatment of BxPC3 cells with the 3 TNK1 siRNA resulted in increased apoptosis as shown by using an assay for caspase 3/7 activity (Fig. 4B). These results suggest an important role for TNK1 in pancreatic cancer cell growth.

To test for expression of activated TNK1, we first immunoprecipitated whole cell lysates by using a bead-bound p-Tyr antibody. Blotting with a TNK1 antibody revealed high levels of phosphorylated TNK1 in BxPC3 and CAPAN-1 cells (Fig. 4C). These samples were also blotted with a phospho-TNK1 antibody specific for the Tyr 277 residue. We note that CAPAN-1, CAPAN-2, and HPAC cells all show high levels of phosphorylation at this particular residue. In contrast, we did not observe any phosphorylation of TNK1 in the normal pancreatic epithelial cell line HPDE6.

A role for TNK1 in cell response to gemcitabine

To determine the effect of TNK1 knockdown on gemcitabine response in pancreatic cancer cells, we treated...
BxPC3 cells with TNK1 siRNA or nonsilencing siRNA and dosed the transfected cells with gemcitabine (Fig. 5A). The results showed a shift in the IC50 values of the TNK1 siRNA treated cells compared with control siRNA-treated cells suggesting potentiation of gemcitabine activity. By using the active concentration of 8 nmol/L, the effect of TNK1 silencing with gemcitabine was examined in a panel of 5 pancreatic cell lines (Fig. 5B). Results show varying effects of TNK1 silencing on gemcitabine response. Of note, the combination of gemcitabine with TNK1 siRNA resulted in lower cell viability when compared with gemcitabine or TNK1 siRNA alone in all of the cell lines tested.

The role of TNK1 in KRAS and NF-κB pathways

Because previously published studies found a correlation between TNK1 expression and suppression of KRAS activity (7), we sought to determine whether this relationship holds true in pancreatic cancer cells. KRAS gain-of-function mutations are often present in pancreatic cancer cells (29), so we examined the ratio of phospho-MEK 1/2 (Ser217/221) to total MEK 1/2 (a downstream phosphorylation target of KRAS). Following incubation with TNK1 siRNA, we noticed a slight increase in the ratio of p-MEK to total MEK in BxPC3 cells (Supplementary Fig. S2). However, this trend was determined not to be statistically significant, suggesting that TNK1 knockdown does not affect KRAS activity in pancreatic cancer cells.

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Azoitei and colleagues noted that overexpression of TNK1 resulted in a dramatic increase in PARP cleavage following treatment with TNFα (9). Therefore, we sought to determine whether TNK1 knockdown results in a decrease in TNFα-induced cleavage of PARP. Our results indicated that TNK1 knockdown actually resulted in an increased cleavage of PARP following TNFα induction (Fig. 5C). In addition, we noted that treatment with TNK1 siRNA alone does not result in increased PARP cleavage (Supplementary Fig. S3). These data suggest that TNK1 levels are not closely tied to NF-κB activation in pancreatic cancer cells.

Discussion

In this study, we used HT-RNAi screening to identify kinases whose silencing decreased growth of pancreatic cancer cells. Furthermore, we validated the kinase TNK1 as a novel pancreatic cancer target important in cell growth and survival. HT-RNAi screens with a kinase siRNA library on the BxPC3 and MiaPaCa-2 pancreatic cancer cell lines (Fig. 1) identified 32 kinases as being important in cell growth of pancreatic cancer cells (Table 1). Of these, 22 kinases seemed to be specific for the pancreatic cancer cells compared with a previous HT-RNAi kinase dataset from normal fibroblasts. Although several of the kinases have been previously associated with cancer (i.e., p38, CALM1, HK1, and STK10), we focused on TNK1 in this study because of previous studies suggesting both tumor suppressor and oncogenic activity (8, 11).

TNK1 was identified as a hit in both the MiaPaCa-2 and BxPC3 siRNA screens and has not been previously associated with pancreatic cancer. Expression analysis of TNK1 in a panel of pancreatic cells showed only slight variations in levels of TNK1 expression with the highest

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Figure 3. Characterization of TNK1 expression in a panel of pancreatic cancer cell lines. A, whole cell lysates from 9 different pancreatic cancer cell lines were separated by SDS-PAGE and analyzed by Western blot analysis. Membranes were probed for expression of TNK1, β-actin (loading control), SMAD4, vimentin, and E-cadherin. Final images were cropped to highlight relevant bands. B, densitometry analysis was completed to compare levels of TNK1 normalized against the loading control. C, whole cell lysates were prepared from TNK1 and SMAD4 siRNA-treated Panc-1 cells, which were then resolved by SDS-PAGE and analyzed for TNK1, SMAD4, and β-tubulin expression relative to untreated (UT) and nonsilencing (NS) siRNA controls. D, densitometry analysis was completed to compare protein levels normalized against the loading control.
expression seen in HPAC cells (Fig. 2C). Although protein expression levels are relatively low overall, we did note the presence of phosphorylated TNK1 in several pancreatic cell lines (Fig. 2A and B). Silencing of TNK1 by siRNA reduced the cell viability of several pancreatic cell lines and induced apoptosis (Fig. 4A and B). Furthermore, TNK1 knockdown affected cell survival by potentiating gemcitabine-induced cytotoxicity (Fig. 5A and B).

Although all cell lines show a statistically significant decrease in viability when treated with TNK1 siRNA, Panc-1 and AsPC-1 were noticeably less affected than the BxPC3, MiaPaCa-2, and Su.86.86 cell lines. Although the exact nature of this observation is unclear, we do note that cell lines exhibiting a mesenchymal phenotype tend to show lower expression of TNK1 than those with epithelial characteristics (Fig. 3A). These data combined with the conflicting studies about the role of TNK1 as a tumor suppressor/oncogene (8, 10) provide support to the hypothesis that TNK1 exerts context-dependent roles within the cell. Further investigation is needed to determine whether TNK1 acts as an oncogene or a tumor suppressor.

Previously published results have shown that TNK1 in embryonic cells potentiates KRAS activity (7). However, this effect was not noted in BxPC3 cells as silencing of TNK1 did not affect MEK 1/2 phosphorylation (Supplementary Fig. S2). Also, despite reports that TNK1 overexpression enhances PARP cleavage following TNFα induction (9), our data indicates that silencing of TNK1 in BxPC3 cells with siRNA increases PARP cleavage when compared with untreated and nonsilencing siRNA controls (Fig. 5C), suggesting a role for TNK1 in apoptosis.

These data collectively suggest an alternative role for TNK1 in pancreatic cancer cells. Knowing that TNK1 knockdown results in a decrease in cell viability, it is unlikely that TNK1 acts as a tumor suppressor, as has been shown in embryonic cells (7, 8). In addition, this heretofore unknown role for TNK1 in growth and survival in pancreatic cancer makes it a promising target in the ongoing quest to find novel cancer targets and treatments. Further experimentation is needed to determine whether TNK1 plays an oncogenic role in other cancer types and data on expression levels in different tissue types is needed. Very few genes have been characterized as being a tumor suppressor under certain conditions and an oncogene in others (i.e., TGFβ). The addition of TNK1 to this very short list both complicates the view of how cancer develops and presents additional avenues for treating cancer more effectively.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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