Survivin depletion preferentially reduces the survival of activated K-Ras-transformed cells

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Abstract

To identify cancer-specific targets, we have conducted a synthetic lethal screen using a small interfering RNA (siRNA) library targeting ~4,000 individual genes for enhanced killing in the DLD-1 colon carcinoma cell line that expresses an activated copy of the K-Ras oncogene. We found that siRNAs targeting baculoviral inhibitor of apoptosis repeat-containing 5 (survivin) significantly reduced the survival of activated K-Ras-transformed cells compared with its normal isogenic counterpart in which the mutant K-Ras gene had been disrupted (DKS-8). In addition, survivin siRNA induced a transient G2-M arrest and marked polyploidy that was associated with increased caspase-3 activation in the activated K-Ras cells. These results indicate that tumors expressing the activated K-Ras oncogene may be particularly sensitive to inhibitors of the survivin protein. [Mol Cancer Ther 2007;6(1):269–76]

Introduction

In a series of classic yeast genetic studies, Hartwell et al. (1) showed the concept of identifying synthetic-lethal interactions between genes whose functional modifications are required for cellular phenotypes. With the advent of large-scale gene knockdown using small interfering RNA (siRNA) libraries, it has become possible to do similar screens in mammalian cells. By using isogenic, paired cell lines that differ by a single oncogenic lesion, targeted gene knockdown can be used to identify drug targets for selectively killing tumor cells. Such a screen could be used to identify cancer targets for drugs that could have a favorable therapeutic window. For example, tumors that have inactivating mutations of the von Hippel-Lindau tumor suppressor gene can become sensitive to changes in the levels or activity of hypoxia-inducible factor-1α (2). Similarly, tumors that carry mutations in the tumor suppressor gene PTEN can be sensitized to drugs that inhibit the mammalian target of rapamycin kinase (3).

We selected the K-Ras oncogene for our screen because Ras-activating mutations are one of the more prevalent genetic changes in cancer occurring in ~30% of patients (4). The Ras superfamily of proteins consists of several low molecular weight GTPases that serve to change the cellular balance between GTP and GDP (5). Activation of these GTPases by extracellular ligands mediates signaling events that are directed from the cell surface receptors to signaling molecules in the nucleus. Once Ras is in the active, GTP-bound conformation, three main effector pathways are modulated, including the RAF/mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) kinase/ERK signaling pathway, the Ras guanine-nucleotide exchange factor signaling pathway, and the phosphatidylinositol 3-kinase signaling pathway. Ras can thus affect many different cellular processes, including cell growth, differentiation, and cytoskeletal reorganization, all of which can contribute to tumorigenesis (5, 6).

In this study, we focused our screen using paired colon carcinoma cells that differ in the expression of the K-Ras protein (DLD-1/DKS-8 pair). DLD-1 is dependent on the activated K-Ras gene for tumor growth and contains one wild-type (wt) K-Ras allele and one mutated activated K-Ras allele. Its isogenic counterpart, DKS-8, was created through genetic disruption of the activated K-Ras allele using targeted homologous recombination and is impaired in both anchorage-independent growth and the ability to form tumors in mice (7). Using this isogenic pair, we identified proteins that cooperate with activated K-Ras for tumor cell survival.

Materials and Methods

Cell Lines

Human colon cancer cell lines DLD-1 (parental line containing both mutant and wt K-Ras alleles), DKO-1 (containing only mutant K-Ras allele), and DKS-8, DKO-3, and DKO-4 (all containing wt K-Ras allele) were obtained from the laboratory of Dr. Shirasawa (International Medical Center of Japan, Tokyo, Japan). Cells were cultured in DMEM with 10% fetal bovine serum (Life Technologies, Gaithersburg, MD) in a 37°C humidified chamber (5% CO2).
siRNAs and siRNA Library Design

Our siRNA library was selected to target ~4,000 genes representing a wide variety of protein families that include protein kinases, G protein–coupled receptors, ubiquitin E3 ligases, transporters, ion channels, and peptidases. The siRNAs were designed by Dharmacon Research, Inc. (Lafayette, CO) using their proprietary algorithm whereby each mRNA is targeted by a pool of siRNAs consisting of a combination of four siRNA duplexes directed at different regions. siRNA to the *Polo-like kinase 1* gene (Genbank accession no. NM_001168): survivin-1, 5′-CAAGGAAACCAACAAUA-3′; survivin-2, 5′-GCA-AAGGAAACCAACAAUA-3′; survivin-3, 5′-GAAACUGGACGAGGAAAGA-3′; survivin-4, 5′-UGAGGAAACUGC-GAAGAAA-3′; survivin-5, 5′-CAGCAGCAUCUCUCAAUCA-3′; and survivin-6, 5′-GGCCUUCUUCCCUUGUCA-3′. siRNA Library Screen

DLD-1 cells were seeded the day before at approximately 1 × 10^4 per well in 96-well plates to obtain 50% to 60% confluency at the time of transfection. The library was screened using 100 nmol/L SMARTpool siRNA (25 nmol/L/siRNA; four siRNAs per pool). In brief, for a single well of a 96-well plate, 6 μL of siRNA and 3 μL of Lipofectamine 2000 (Invitrogen Corp., Carlsbad, CA) were each incubated separately with 100 μL Opti-MEM (Invitrogen) for 10 min and mixed together for 20 min at room temperature and then 20 μL were applied to the cells plated in 100 μL of medium. The cells were incubated in the siRNA transfection reagent mixture for 4 to 5 h before receiving fresh medium (100 μL). Three days later, cell death was measured using the ToxiLight assay (Cambrex Corp.; Rockland, ME) according to the manufacturer’s instructions. In this cell-based assay, the release of adenylate kinase from damaged cells is measured using a bioluminescent readout that allows for a differential in DLD-1 versus the DKS-8 cell line. Western Blotting

Cell lysates were electrophoresed in Novex SDS-PAGE gels (Invitrogen), and the proteins were transferred onto a nitrocellulose membrane. Immunoblotting was done using anti survivin antibody from Sigma-Aldrich Corp. (St. Louis, MO). Blots were developed using the enhanced chemiluminescence reagent from Amersham Biosciences (Piscataway, NJ).

Quantitative Reverse Transcription-PCR

RNA was isolated using Trizol (Invitrogen), chloroform extraction, and Qiagen RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. All primers and Taqman probes were purchased from Integrated DNA Technologies (Coralville, IA). Primers and probes are as follows: 5′-GGCTGTAGCGTCCATGCC-3′ (forward), 5′-TGCGTGTACCTCCGCTTA-3′ (reverse), and 5′-ATGCCATGTGCTGGCCTTCC-3′ (probe) for the *RPL19* gene and 5′-GCGAGGCTGCTGCTTA-3′ (forward), 5′-GAAGAAAAACTGGGCCAAGTCT-3′ (reverse), and 5′-CCACTGCCCACCTGAAACGC-3′ (probe). For each sample, quantitative PCR was in a final volume of 25 μL containing 100 ng RNA, 0.4 μmol/L each of the forward and reverse PCR primers, and 100 nmol/L of the Taqman probe. Temperature conditions consisted of 50°C for 30 min, 95°C for 5 min followed by 40 cycles of 60°C for 1 min and 95°C for 15 s on a Prism 7700 and 7900HT sequence detector (Applied Biosystems, Inc., Foster City, CA). Data analysis was done with the ABI SDS software package, standard curves were generated in parallel, and the relative concentration of RNA in each sample was determined by comparison using methods described in the ABI Prism User Bulletin Number 2. Survivin expression values were normalized to the *RPL19* control gene, and the relative expression for each sample was divided by the average relative expression value of the scrambled control to simplify the display of the data.

Caspase-3 Assay

siRNA-transfected cells in 96-well plates were lysed in 120 μL of 1× lysis buffer [1.67 mmol/L HEPES (pH 7.4), 7 mmol/L KCl, 0.83 mmol/L MgCl₂, 0.11 mmol/L EDTA, 0.11 mmol/L EGTA, 0.57% CHAPS, 1 mmol/L DTT, 1× protease inhibitor cocktail tablet; EDTA-free; Roche Pharmaceuticals, Nutley, NJ] at room temperature with shaking for 20 min. After cell lysis, 80 μL of a caspase-3 reaction buffer [48 mmol/L HEPES (pH 7.5), 252 mmol/L sucrose, 0.1% CHAPS, 4 mmol/L DTT, 20 μmol/L Ac-DEVD-AMC substrate; Biomol Research Labs, Inc., Plymouth Meeting, PA] were added and the plates were incubated for 2 h at 37°C. The plates were read on a 1420 Victor Multilabel Counter using the following setting: excitation, 360/40; emission, 460/40. The units of fluorescence relative to the control were defined as fold increase in caspase-3 activity.

Flow Cytometry Analysis

Cell cycle distribution and DNA synthesis were assessed by propidium iodide labeling and bromodeoxyuridine (BrdUrd) incorporation, respectively. Approximately 1 × 10⁶ cells were pulse labeled with 30 μmol/L BrdUrd (Sigma-Aldrich) in six-well plates for 1 h at the selected times after siRNA transfection, harvested, and then fixed in 70% methanol. Methanol-fixed cells were resuspended in 1 mL of 2 N HCl, 0.5% Triton X-100 for 30 min at 25°C, after which the suspension was neutralized with the addition of 1 mL of 0.1 mol/L sodium tetraborate (pH 8.5). Cells were washed once in 1× washing buffer (0.5% Tween 20, 1% bovine serum albumin in 1× PBS). Replicative DNA
synthesis was detected by staining the BrdUrd-containing cells with 0.1 mL of FITC-conjugated anti-BrdUrd antibody (Becton Dickinson, San Jose, CA) for 30 min at 25°C. Cells were washed once in 1/2 washing buffer and resuspended in 800 μL of PBS, 200 μL of propidium iodide (0.1 mg/mL), and 5 μL of RNase A (10 mg/mL; Sigma-Aldrich), incubated in the dark (25°C, 30 min), and analyzed using a Becton Dickinson ExCalibur Flow Cytometer.

**Results**

**Identification of Genes Required for Cell Survival in K-Ras-Activated Cells**

To identify siRNAs that selectively kill cells with an activated K-Ras mutation, we conducted a synthetic lethal screen using a pair of human isogenic cancer cell lines that contained an activated K-Ras allele (DLD-1 parental) or a wt K-Ras allele (DKS-8 isogenic counterpart; ref. 7). From our initial screening of our siRNA library in the K-Ras-activated DLD-1 cells using a cell death assay (see Materials and Methods), we identified 75 siRNA hits. Figure 1 depicts an example of the results obtained from screening 60 siRNAs in duplicate from our library. Hits were scored as significant if cell killing was induced at or above one SD from the population mean. As shown in Fig. 1, survivin and GDF10 siRNAs induced a significant increase in cell killing relative to the population.

To determine which siRNA hits may preferentially induce cell killing in the context of an activated mutant K-Ras background, the 75 candidate hits were retested in the isogenic K-Ras paired cell lines (DLD-1/DKS-8) to identify hits that induced cell death specifically in the DLD-1 cells. As shown in Fig. 2, survivin, CDC2, and C20ORF18 induced differential cell killing in the DLD-1 versus DKS-8 cells. Of these screening hits, we chose to conduct further experiments on survivin due to its known importance in cancer. Survivin is a member of the inhibitor of apoptosis family of proteins but, unlike the other family members, it contains only a single baculoviral inhibitor of apoptosis repeat domain. It controls cell cycle progression and the apoptotic checkpoint, both of which are crucial variables of tumorigenesis. Survivin has been shown to bind to the inner centromeric protein and to bind/enhance the activity of the Aurora B kinase, molecules that are essential for both chromosome segregation and normal cytokinesis (9–11). In addition, survivin is minimally expressed in differentiated normal cells, although highly expressed in many cancers and is negatively correlated with survival (10). These features make survivin an attractive target to follow up in the context of synthetic-lethal interactions with activated K-Ras.

**Cell Killing of K-Ras-Activated Cells Is Specific to Survivin siRNA**

To confirm siRNA specificity, we evaluated multiple siRNAs against survivin to establish a correlation between target knockdown and cell death. Multiple siRNAs against survivin induced RNA and protein knockdown in both the DLD-1 and DKS-8 cell lines (Fig. 3A and B). The level of knockdown of endogenous survivin protein was equivalent in both the cell lines using the same concentration of survivin siRNA (25 nmol/L), although RNA knockdown was more efficient as detected by quantitative PCR in the DLD-1 cells. This could be due to differences in synthesis or stability of cleaved, inactive RNAs between the two cell lines. However, the survivin siRNA-induced cell death was at least 2-fold greater in the DLD-1 cell line containing the activated copy of the K-Ras oncogene (Fig. 3C). To confirm that cell death is mediated through an apoptotic mechanism by survivin-specific siRNAs, we tested the potency of these siRNAs in the DLD-1/DKS-8 isogenic pair using a caspase-3 assay. These studies revealed that low nanomolar concentrations of survivin siRNA were indeed active in the DLD-1 cells relative to the wt K-Ras DKS-8 cells as assessed by enhanced caspase-3 activation (Fig. 4A). These results correlated with the ToxiLight assay (Fig. 4B). Furthermore, microscopic analysis of cell death showed approximately 60% to 80% in DLD-1 cells, whereas marginal cell death (~10–20%) was observed in DKS-8 cells on reduction of survivin protein (data not shown).
Survivin Depletion Leads to Cell Death in Additional K-Ras-Disrupted Isogenic Clones of DLD-1

To rule out the possibility that the DLD-1/DKS-8 K-Ras isogenic cell line pair may have accumulated additional genetic alterations that are independent of the K-Ras pathway and that could affect cell survival, we extended our analysis to include additional K-Ras-disrupted clones. As shown in Fig. 5, multiple siRNAs against survivin at a low dose (5 nmol/L) induced 2- to 3-fold increases in cell death when transfected into DLD-1 cells or a cell line that contained only the activated mutant K-Ras allele (DKO-1) compared with cells containing only the wt K-Ras allele (DKO-3, DKO-4, and DKS-8). Results were similarly seen using the caspase-3 activity assay (data not shown). Importantly, this preferential cytotoxicity was not due to poor transfection efficiency of the wt K-Ras clones, as siRNA-mediated survivin protein knockdown was equally evident in all the clonal lines when assessed by Western blot analysis (data not shown). These data show that additional clones behave similarly to DLD-1 cells.

Survivin Knockdown in Activated Mutant K-Ras Cells Preferentially Induces Apoptosis and Correlates with a Transient G₂-M Arrest and Marked Polyploidy

Survivin is a cell cycle–regulated protein that serves a dual function as an endogenous inhibitor of caspases and as a regulator of mitotic progression. We showed that survivin siRNA-induced cell death is mediated through an apoptotic mechanism (Fig. 4A). The minimal amount of cell death seen in DKS-8 K-Ras wt cells was not due to an intrinsic cell death resistance mechanism, as transfection of a positive control Polo-like kinase 1 siRNA resulted in a similar cell death phenotype in both DLD-1 and DKS-8 cells.

Figure 2. Retest screening of preliminary siRNA hits in the K-Ras isogenic paired cell lines (DLD-1/DKS-8). siRNAs (20 nmol/L SMART-pool) were transfected into the K-Ras paired cell lines, and after 72 h, cell death was determined by the ToxiLight assay. Circles, siRNAs inducing differential kill in the DLD-1 cells compared with the DKS-8 cells. Points, mean of triplicates; bars, SE. Similar results were obtained in two additional experiments. Control siRNA included Polo-like kinase 1.

Figure 3. Additional siRNAs against survivin knock down RNA and protein target levels in DLD-1/DKS-8 cells but induce cell death phenotypes only in the DLD-1 cells. A, quantitative PCR analysis to determine survivin siRNA-mediated RNA knockdown in K-Ras isogenic pair. DLD-1 mutant and DKS-8 wt cells were transfected with four different survivin siRNAs (25 nmol/L) comprising the original SMART-pool, and at 48 h after transfection, RNA was extracted and analyzed. The experiments were done two independent times. B, Western blot analysis to determine protein knockdown. Parallel cultures were transfected as in A, and cell extracts were prepared and analyzed by Western blot using survivin antibody from R&D Systems (Minneapolis, MN). C, additional survivin siRNAs were transfected into the K-Ras mutant and K-Ras wt isogenic cell lines at 25 nmol/L and assayed 72 h after transfection by the ToxiLight assay. Columns, mean of triplicates; bars, SE. The experiment was done three independent times.
Furthermore, both cell lines have similar growth rates as measured by a cell proliferation assay (data not shown), consistent with previously published observations. Using fluorescence-activated cell sorting, we next examined whether survivin knockdown differentially affects the cell cycle profiles in the context of a mutant activated K-Ras background. Importantly, the distribution of logarithmically growing cells in G1, S, and G2-M was similar for untreated DLD-1 and DKS-8 cells alike. However, marked differences were seen in cell cycle distribution between the isogenic pair after treatment with survivin siRNAs. Specifically, treatment of DKS-8 cells with 25 nmol/L survivin siRNA resulted in an accumulation of cells at the G2-M phase by 48 h, which was stably maintained beyond 72 h (up to a 56-h time point shown in Fig. 6A). There was also ~20% polyploidy (8N) population relative to the scrambled siRNA control-treated cells at 48 h, a phenotype that is consistent with previously published observations using a survivin antisense oligonucleotide (12). In these studies, knockdown of survivin induces a typical rounded cell morphology and polyploidy, indicating that survivin is essential for cells to progress through mitosis (12). To better understand the effects of survivin siRNA on cell cycle progression, S-phase synthesis profiles were also assessed by measuring BrdUrd incorporation. As seen in Fig. 6B and C, DKS-8 cells harvested at various times after siRNA treatment exhibited a marked decrease in the number of cells entering S phase by 32 h, with a corresponding increase in the percentage of cells entering the G2-M phase. A small percentage of these G2-M cells escaped the block to re-replicate their DNA and form an 8N population. These results are consistent with the flow cytometry data shown in Fig. 6A. In contrast to the DKS-8 cell line, however, the DLD-1 cells treated with survivin siRNA rapidly accumulated in G2-M and exhibited polyploidy (8N DNA content) as early as 24 h. Interestingly, this G2-M checkpoint was transient as the majority of the blocked cells began a second round of DNA replication without dividing, resulting in a rapid decrease in G2-M with a concomitant increase in a polyploidy cells by 48 h (Fig. 6A–C). The observed apoptotic cell death is consistent with polyploidy triggering the cells to pass...
from a transient G2-M block into mitosis without undergoing cytokinesis because of damaged spindles and abnormalities in chromosome segregation (9).

**Discussion**

Large-scale siRNA libraries are ideally suited for knocking down the expression of individual genes in cancer cell lines to identify target genes that cancer cells require for cell survival. The addictive nature of both oncogenic and tumor suppressor mutations can be exploited for the discovery of drugs that specifically target tumors. Although small molecules have been used previously to screen isogenic cancer cell lines, such an approach has the disadvantage of not knowing the specific identity of the targets involved (13, 14). In this study, we have used a synthetic-lethal approach using a high-throughput RNA interference–based screen to identify genes that work cooperatively with K-Ras for tumor cell survival.

This study shows that siRNA-mediated reduction of endogenous survivin in activated K-Ras DLD-1 cells results in preferential cell death as evaluated in multiple cell-based human cancer cell lines. These findings suggest that survivin is a critical target gene that is required for K-Ras-driven tumor cell proliferation.

**Figure 6.** Survivin siRNA-induced cell cycle changes in DLD-1/DKS-8 cell lines. DLD-1 mutant and DKS-8 wt cells were transfected with scrambled siRNA (25 nmol/L) or survivin siRNA-6 (25 nmol/L), and cells were harvested at several time points and analyzed by flow cytometry as described in Materials and Methods. A, histogram plots display analysis of total DNA content as determined by propidium iodide staining. Cell cycle populations are characterized as G1 (2N DNA content), S phase (BrdUrd incorporation), G2-M (4N DNA content), and polyploidy (8N DNA content). B, the flow cytometric dot plots display simultaneous analysis of S-phase DNA synthesis (X axis), as determined by a 1-h BrdUrd pulse labeling of cells at different time points after siRNA transfection, and total DNA content (Y axis), as determined by propidium iodide staining. C, quantitation of cell cycle populations (% G1, S, G2-M, or polyploidy cells). Similar results were obtained in four independent experiments. 

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assays. At a cellular level, we have shown that survivin depletion in activated K-Ras cells induces a G2-M arrest that is subsequently bypassed followed by multiple rounds of DNA endoreduplication and a concomitant increase in polyploidy and cell death. In contrast, survivin depletion in the wt K-Ras DKS-8 cells results in a stable arrest in the G2-M phase of the cell cycle, correlating with little or no effects on cell survival. A possible explanation for our results may be that both Ras and survivin interact in a common cellular pathway. Ras-dependent cell cycle progression is controlled by downstream effectors, which include the RAF/MAPK/ERK kinase/ERK protein kinases. Cellular studies using dominant-negative MAPK/ERK kinase kinase or inhibitors of MAPK kinases have shown an association of MAPKs with microtubules and have suggested that this pathway is also used by cells to progress through the G2-M phases of the cell cycle (15). Using N-Ras-activated HeLa cells synchronized at G1-S, it was also shown that mitotic phosphorylation of MAPK/ERK kinase 1 kinase is required to prime cells for undergoing mitosis (16). Depletion of the chromosomal passenger protein survivin, which is required for chromosomal segregation and cytokinesis, may then selectively kill tumor cells expressing mutant activated K-Ras through a mechanism that involves synergy between K-Ras-mediated G2-M progression and survivin depletion-mediated chromosomal instability.

Our observations are consistent with previous data that have indicated a strong relationship between activated Ras oncogene and survivin. In one study, H-Ras activation was shown to increase the levels of survivin protein, thereby regulating the survival of hematopoietic cells (17). Recent studies involving dominant-negative mutants and antisense oligonucleotides have shown that survivin expression may depend on the activation of H-Ras in human cancer cells (18). H-Ras-induced survivin protein overexpression was found to be associated with survivin localization to the mitotic spindle, suggesting that the Ras pathway is required for survivin localization. Recent reports have also described an augmentation of Ras-mediated cell growth by two other members of the chromosomal passenger protein complex: Aurora kinases A and B and inner centromeric protein (10, 19). In a separate study using HeLa cells, it was shown that only N-Ras was significantly activated during mitosis despite the high expression of K-Ras protein (15). However, in our study, we have specifically shown that survivin depletion differentially reduces the survival of DLD-1 cancer cells in the context of activated K-Ras expression. Agents that disrupt this synergistic association between K-Ras and survivin would then potentiate cell death in K-Ras-activated cancer cells.

Due to the importance of survivin as a cell cycle regulator and a modulator of apoptosis, as well as its strong expression in cancers and minimal expression in normal cells, several attempts have been made to target survivin for anticancer therapy. These approaches include the use of small-molecule inhibitors against cyclin-dependent kinase proteins that regulate survivin activity, antisense oligonucleotides, and ribozymes directed against survivin or genetically engineered dominant-negative survivin mutants. In addition, reduction of survivin protein using siRNA or short hairpin RNA has been shown to increase sensitivity to a variety of chemotherapeutic or radiosensitizing agents (20–24). For example, hepatoma cells exhibit an increased trail-mediated sensitivity when treated with survivin siRNA in combination with chemotherapeutic agents (20). Similarly, siRNA-mediated suppression of survivin protein in Taxol-treated HeLa cells caused a checkpoint arrest (23). Using a single vector-derived short hairpin RNA against survivin transfected into Burkitt’s lymphoma Raji cell line, Gu et al. (24) have shown an increased apoptotic response to reduced survivin levels. Our studies show that multiple siRNAs against the survivin gene alone selectively kill K-Ras-activated cell line. Encouraging results from such studies have led to clinical trials targeting survivin in cancer patients both with antisense oligonucleotides and peptide-based immunotherapy (11). Peptide vaccines against restricted survivin epitopes are currently being evaluated in clinical trials in patients with melanomas and advanced stages of pancreas, colon, and cervical carcinomas (25).

Our studies indicate a codependence between survivin and activated K-Ras expression for cancer cell survival and suggest that therapies directed against survivin activity may prove to be especially efficacious for patients with cancers containing K-Ras-activated mutations. The low expression of survivin in normal cells may also allow such inhibitors to exhibit an excellent therapeutic window.

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