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Introduction of a Survivin Gene-specific Small Inhibitory RNA Inhibits Growth of Pancreatic Cancer Cells

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Abstract. *Background: The anti-apoptotic molecule survivin is expressed in human cancers of various origins. Since this molecule possesses multiple functions, including apoptosis inhibition, cell cycle promotion and enhancement of Fas ligand expression, survivin has attracted growing attention as a target in cancer treatment. A survivin-specific small inhibitory RNA (siRNA) was introduced into pancreatic cancer cells to investigate its effect on cancer cell growth. Materials and Methods: Survivin mRNA and protein expression were examined by RT-PCR and Western blotting, respectively. DNA histogram analysis was performed using a flow cytometer. Results: The introduction of survivin-specific siRNA reduced survivin mRNA and protein expression in PANC-1 cells by over 90% and to an undetected amount, respectively, and induced growth inhibition. The siRNA transfectants showed pronounced morphological changes including enlargement of cells and multinucleation. siRNA transfectants did not show cell cycle arrest, but underwent apoptosis. Conclusion: Our data suggest that the use of survivin-specific siRNA deserves further investigation as a novel approach to cancer therapy.*

Suppression of apoptosis is thought to contribute to tumor initiation, progression and drug resistance (1). Several proteins involved in inhibition of apoptotic signaling have been identified, including the *bcl-2* family and the inhibitor of apoptosis protein (IAP) family (2, 3). Certain members of the latter family directly inhibit terminal effector caspases engaged in the execution of programmed cell death. Survivin, a member of the IAP family, is expressed in human cancers of various origins such as breast (4), esophagus (5), stomach (6, 7), colon (8, 9) and pancreas (10), and is associated with poor patient survival (5, 7-9). Since overexpression of survivin desensitizes cancer cells to several anticancer drugs (7, 11) and irradiation (12), which otherwise would induce apoptosis, survivin acts as a resistance factor against these cancer treatment modalities.

Li *et al.* demonstrated that survivin was expressed in the G2/M-phase of the cell cycle in a cycle-regulated manner (13). Bolton *et al.* and Chen *et al.* showed that survivin interacted directly with a mitotic regulator, aurora-B kinase, and enhanced its activity (14, 15). These results suggested that survivin promotes mitosis through activation of aurora-B kinase and contributes to the aberrant growth of cancer cells.

In addition, we recently reported that survivin enhanced the expression of the Fas ligand (FasL) in cancer cells through up-regulation of specific protein-1-mediated gene transcription, and also that survivin enables cancer cells to counter-attack immune cells by inducing FasL-triggered apoptosis in cells of the immune surveillance system (16).

These findings depict survivin as a multifunctional protein important for cancer cell proliferation *in vivo*. Therefore, survivin has attracted growing attention as a possible point of therapeutic attack in cancer treatment.

One recent technique, used to rapidly and efficiently identify the function of individual genes, is RNA interference (RNAi, 17-19). This process has been found to be evolutionarily conserved in plants, worms and flies, and has been demonstrated recently to function in mammalian cells (20). Use of RNAi in mammalian cells involves transfection of an annealed 21-mer of sense and antisense RNA oligonucleotides (small inhibitory RNA, siRNAs) corresponding to a portion of a gene of interest. These RNAs then bind specifically to cellular RNA and activate a process that leads to degradation of the mRNA and a subsequent 80% to 90% decrease in expression of the corresponding protein. Thus, RNAi represents an important technique for specifically down-regulating the expression of cellular genes.

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In the present study, siRNA targeted to the survivin gene was introduced into pancreatic cancer cells and its effect on cancer cell growth was investigated.

Materials and Methods

Cell culture. The human pancreatic cancer cell line, PANC-1 was obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in DMEM medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) and grown at 37˚C in a humidified atmosphere of 5% CO_2.

Transfection of siRNA for survivin into pancreatic cancer cells. An siRNA was designed to target the coding region of the survivin gene (nucleotides 366 to 385, relative to the start codon), and prepared by Japan Bio Service (Saitama, Japan). Single-strand RNAs were annealed by incubating a 20 μM concentration of each strand in annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH buffer at pH 7.4, and 2 mM magnesium acetate) for 1 min at 90˚C, followed by 1-h incubation at 37˚C. As a transfection control, scramble RNA was prepared to contain numbers of each nucleotide equal to those in the siRNA targeted to the survivin gene. The siRNA duplexes used in this study were as follows:

Survivin	5'-GAA UUU GAG GAA ACU GCG A TT-3'
	3'-TT CUU AAA CUC CUU UGA CGC U-5'
Scramble RNA	5'-GCA UUG GAU AAG ACG UAG A TT-3'
	3'-TT CGU AAC CUA UUC UGC AUC U-5'

Transfections were performed using OligofectAmine reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's protocol. Briefly, $5x10^4$ of PANC-1 cells were cultured in 6-well culture plates (Costar, New Bedford, MA, USA) in 2 ml of DMEM supplemented with 10% FBS, and incubated for 24 h. After the cells had been washed twice with OPTI-MEM medium (Invitrogen), the cells were incubated in 800 μL of OPTI-MEM medium and 200 μl of OligofectAmine with the addition of 1 μM of either siRNA. After 4 h of incubation, 300 μl of FBS were added, and the cells were incubated for an additional 120 h.

Quantification of survivin mRNA. The expression of survivin mRNA was determined by a quantitative RT-PCR using an ABI PRISM 7700 sequence detector system (Applied Biosystems, Foster City, CA, USA). The isolation of total RNA and synthesis of cDNA were performed using ISOGEN reagent (Nippon Gene, Toyama, Japan) and TaqMan Reverse Transcription Reagents (Applied Biosystems), according to the manufacturers' protocols. The gene-specific primers and fluorescent hybridization probes used in the quantitative PCR were as follows: forward primer, 5'-AAG AAC TGG CCC TTC TTG GA-3'; reverse primer, 5'-CAA CCG GAC GAA TGC TTT T-3'; and probe, 5'-(FAM) CCA GAT GAC GAC CCC ATA GAG GAA CA (TAMRA)-3'. Two splice variants of survivin, survivin-2B (retaining a part of intron 2 as a cryptic exon) and survivin-ΔEx3 (lacking exon 3), were not detected by this set of primers and probe (21). Quantitative RT-PCR was performed using a TaqMan Universal PCR Master Mix (Applied Biosystems) as described previously (16). To compare the amounts of mRNA encoding survivin in different samples, the quantity of specific mRNA was normalized as a ratio to the amount of 18S ribosomal RNA (22), which was determined using TaqMan Ribosomal RNA Control Reagents (Applied Biosystems), according to the manufacturer's protocol.

Western blot analysis. Cytoplasmic proteins were extracted from survivin-specific siRNA transfectants using NE-PER nuclear and cytoplasmic extraction reagents (Pierce, Rockford, IL, USA) with a protease inhibitor cocktail (Sigma-Aldrich). Equal amounts of cytoplasmic proteins (10 μg / lane) were separated on a 4 to 20% gradient Tris-glycine gel (Invitrogen) under denaturing conditions using Tris-glycine sodium dodecyl sulfate (SDS) running buffer (Invitrogen). The proteins were electroblotted to a nitrocellulose membrane (Invitrogen) detected using a WesternBreeze chromogenic Western blot immunodetection kit (Invitrogen), according to the manufacturer's protocol. Briefly, after incubation in blocking solution for 30 min at room temperature, the membrane was incubated with mouse anti-human survivin IgG mAb (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or mouse anti-human actin IgG mAb (Santa Cruz Biotechnology) at room temperature for 1 h. The membrane was washed and incubated with secondary antibody conjugated to alkaline phosphatase at room temperature for 30 min. Alkaline phosphatase labelling was detected using a chromogenic substrate containing 5-bromo-4-chloro-3-indolyl- 1-phosphate (BCIP) and nitroblue tetrazolium (NBT).

Cell staining. Survivin-specific siRNA transfectants were grown for 72 h on 2-well culture slides (Becton Dickinson). The cells were stained using Pappenheim stain by Sp-100 slide preparation unit with May-Grunwald's stain solution and Giemsa's stain solution (Sysmex, Kobe, Japan).

Cell proliferation assay. The viability of survivin-specific siRNA transfectants was determined with an 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2- (4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay, CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). Survivin-specific siRNA transfectants were harvested at 24 h after transfection and $2x10^3$ cells were cultured in 96-well culture plates (Costar) in 100 μl of DMEM supplemented with 10% FBS, and incubated for 24 to 120 h. At the end of incubation, 20 μl of MTS solution were added to be incubated for 2 h at 37˚C. The amount of soluble formazan from reduction of MTS by viable Jurkat cells was assessed by measurement of absorbance at 490 nm using a microculture plate reader (Spectla Fluor, TECAN, Maennedorf, Switzerland).

DNA histogram analysis. Survivin-specific siRNA transfectants were harvested at 48 or 72 h after transfection. After centrifugation, the cell pellets were resuspended in 500 μl of hypotonic fluorochrome solution (propidium iodide at 50 μg/ml in 0.1% sodium citrate and 0.1% Triton X-100) and incubated at 4˚C for 4 h in the darkness (23, 24); 20000 events were measured per sample using an EPICS XL-MCL cytometer (Beckman Coulter, Tokyo, Japan). The propidium iodide was excited with argon laser light at 488 nm, and emission was collected as red fluorescence using a 560 nm dichroic mirror and a 600 nm band pass filter.

Results

Introduction of survivin-specific siRNA completely abrogated survivin expression in pancreas cancer cells. We designed an siRNA to target the coding region of the survivin gene (nucleotides 366 to 385, relative to the start codon) and introduced it into PANC-1 cells, which abundantly express

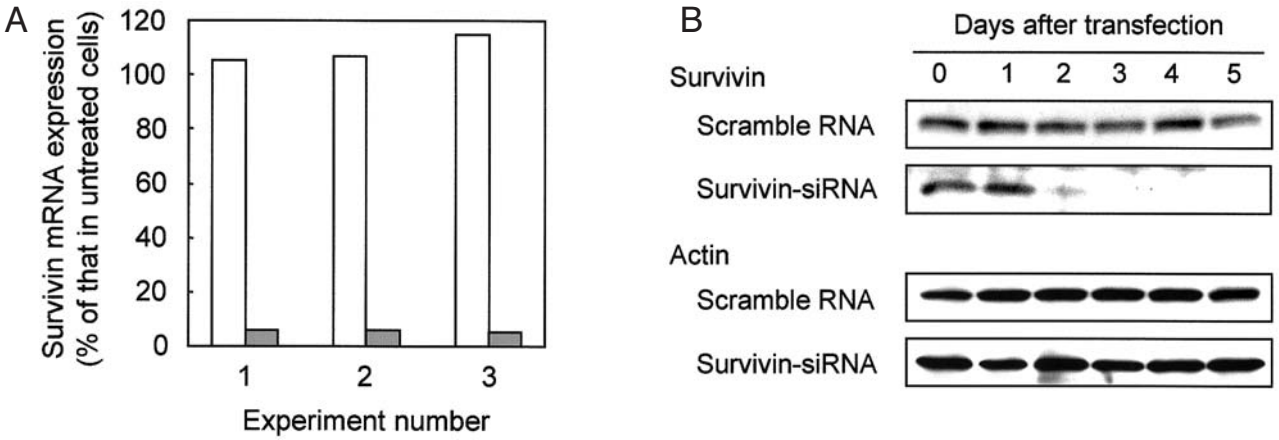

Figure 1. *Introduction of survivin-specific siRNA completely abolished survivin expression in pancreatic cancer. A. Survivin mRNA expression in scramble RNA transfectants (open bar) and survivin-specific siRNA transfectants (shaded bar). Total RNA was extracted from both transfectants 3 days after transfection. Survivin mRNA expression was quantified using real-time PCR. Each data-point indicates the mean value obtained from each experiment. B. Survivin protein expression in scramble RNA transfectants and survivin-specific siRNA transfectants. Cytoplasmic protein was extracted from both transfectants at the indicated number of days after transfection. Survivin protein expression was determined by Western blotting.*

survivin transcripts. As a transfection control, scramble RNA was prepared to contain numbers of each nucleotide equal to those in the siRNA targeted to the survivin gene. Survivin mRNA expression, quantified using real-time PCR in survivin-specific siRNA transfectants 3 days after transfection, is shown in Figure 1A. While the introduction of scramble RNA did not affect the expression levels of survivin mRNA, the introduction of survivin-specific siRNA decreased survivin mRNA expression to less than 10% of that in untreated cells. Western analysis indicated that survivin protein expression began to decrease 2 days after transfection and was completely abrogated at 3 days after transfection (Figure 1B). This expression-reducing effect of survivin-specific siRNA was still present at 5 days after transfection.

Introduction of survivin-specific siRNA showed a growth inhibitory effect in pancreatic cancer cells. To examine the effect of survivin-specific siRNA on the growth of PANC-1 cells, the viable cell numbers from 1 to 5 days after transfection were evaluated using an MTS assay (Figure 2). The growth inhibitory effect of survivin-specific siRNA began to be significant 4 days after transfection, resulting in 50% fewer viable cells than in scramble RNA transfectants 5 days after transfection.

Morphological changes were evident in survivin-specific siRNA transfectants. Representative examples of the morphology of survivin-specific siRNA transfectants 3 days after transfection are presented in Figure 3. Although no clear difference in morphology could be seen between untreated

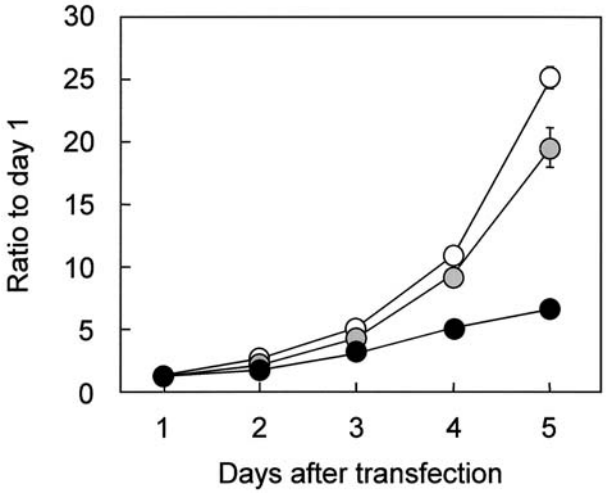

Figure 2. *Introduction of survivin-specific siRNA inhibited growth of pancreatic cancer. The viability of untreated cells (open circle), scramble RNA transfectants (gray circle) and survivin-specific siRNA transfectants (closed circle) were determined by an MTS assay. The data represent mean±S.D. of three independent experiments.*

cells and scramble RNA transfectants, the survivin-specific siRNA transfectants showed enlargement of cells and multinucleation.

Introduction of survivin-specific siRNA did not arrest the cell cycle, but induced apoptosis in pancreatic cancer cells. Survivin has been reported to be expressed in the

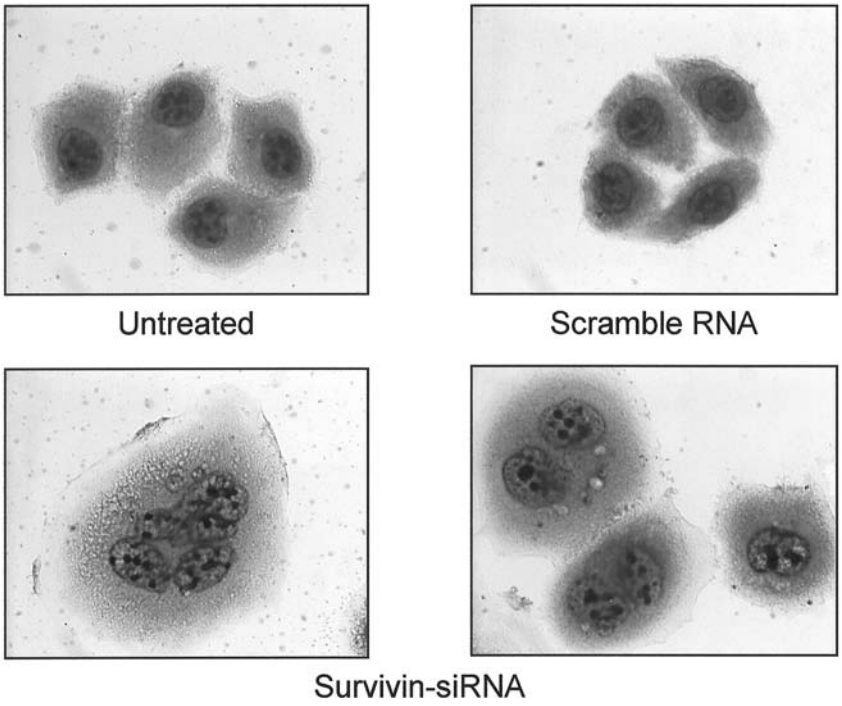

Figure 3. *Survivin-specific siRNA transfectants showed severe morphological changes Cells were stained using the Pappenheim stain 3 days after transfection (original magnification X 400).*

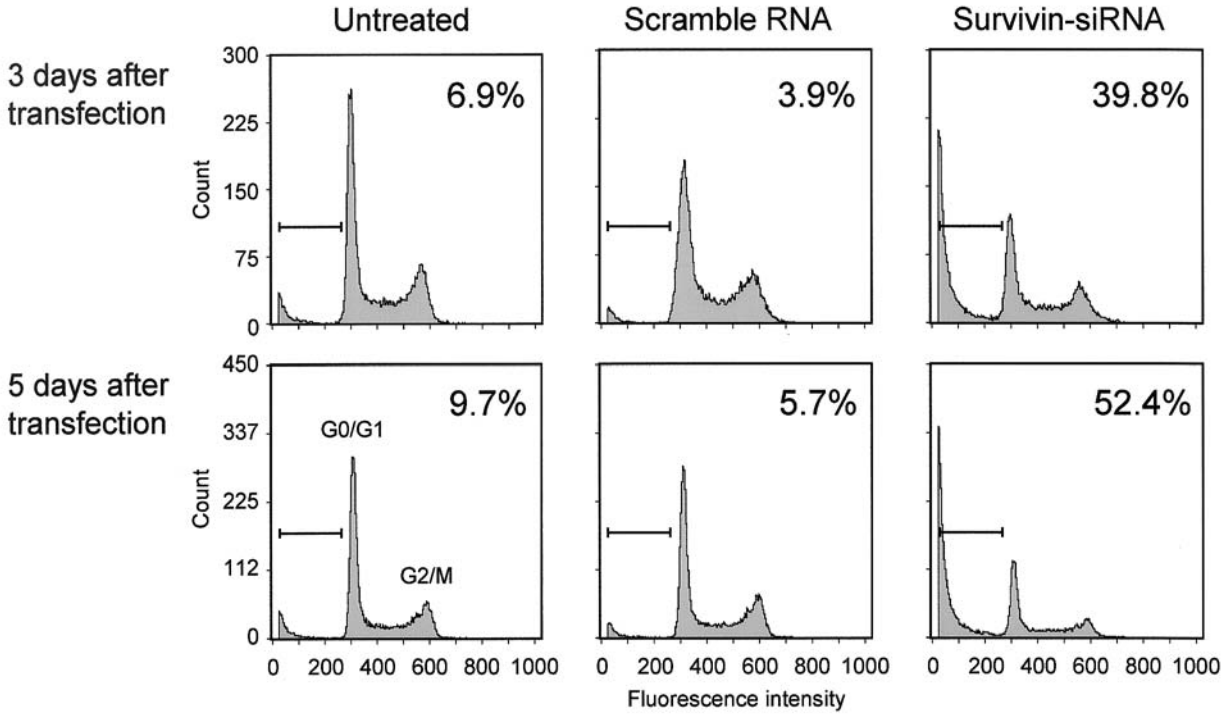

Figure 4. *Survivin-specific siRNA transfectants showed increased apoptosis. The DNA content was analyzed using a flow cytometer after propidium iodide staining of the nucleus on days 3 and 5 after transfection. The percentage of apoptotic cells with hypodiploid DNA content is indicated for each condition tested.*

G2/M- phase of the cell cycle and to contribute to the aberrant progression of cancer cells through mitosis (13). We hypothesized that the growth inhibition observed in survivin-specific siRNA transfectants could result from cell cycle arrest at the G2/M-phase and, therefore, performed DNA histogram analyses on days 3 and 5 after transfection (Figure 4). However, survivin- specific siRNA transfectants did not show accumulation in the G2/M-phase, but instead demonstrated increased hypodiploid DNA content, indicating apoptosis of cells, resulting in 46.7% more apoptotic cells than in the scramble RNA transfectants 5 days after transfection.

Discussion

In the present study, we introduced siRNA, targeting the survivin gene, into pancreatic cancer cells and successfully reduced survivin mRNA and protein expressions by over 90% and to an undetectable amount, respectively. Olie *et al.* reported that the introduction of antisense oligonucleotides targeting survivin mRNA into lung cancer cells down-regulated survivin mRNA expression by 70% (25). However, when we introduced their antisense oligonucleotides into PANC-1 cells and SW480 human colon cancer cells, the reduction of survivin mRNA expression was less than 20% (data not shown). Therefore, the efficacy of antisense oligonucleotides may vary depending on the cell type. We introduced our survivin-specific siRNA into SW480 and T-47D human breast cancer cells, and observed that this siRNA reduced survivin mRNA and protein expression to an extent similar to that in PANC-1 cells (data not shown). These results represent a remarkable effect against survivin expression of our survivin-specific siRNA in cancer cells of various origins.

We also demonstrated that the introduction of survivin-specific siRNA caused growth inhibition associated with severe morphological changes in the PANC-1 cells. DNA histogram analysis demonstrated that growth inhibition by survivin-specific siRNA was not a result of cell cycle arrest at the G2/M-phase, but reflected induction of apoptosis indicated by increased hypodiploid DNA content. In contrast, Kappler *et al.* demonstrated that 5 human sarcoma cell lines, transfected with a survivin-specific siRNA different from the construct used in our study, showed G2/M arrest, with fewer than 10% of these cells undergoing apoptosis (26). Williams *et al.* showed that the introduction of a survivin-specific siRNA into colon cancer cells induced both apoptosis and cell cycle arrest (27). The reason for these differences remains unclear. The blockade of apoptotic signaling by survivin involves inhibition of the activation of caspase-9, which subsequently activates effector caspases, including caspase-3 and -7 (28, 29). Janicke *et al.* reported that procaspase-3 was absent in MCF-7 human breast cancer cells because of partial deletion of the *CASP-3* gene (30). Therefore, one possible explanation might be that cancer cells with low caspase expression undergo cell cycle arrest after treatment with survivin-specific siRNA.

The mechanism by which the introduction of survivin-specific siRNA induces apoptosis in the absence of additional inducers of apoptosis is unclear. However, down-regulation of survivin expression may trigger a pro-apoptotic signal to be identified in subsequent investigations.

In conclusion, our data suggest that the use of survivin-specific siRNA deserves further investigation as a novel approach to cancer therapy.

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