RNA interference-mediated inhibition of survivin and VEGF in pancreatic cancer cells *in vitro*

JIANLIN SONG, LIPIN CAO and YIXIONG LI

Department of General Surgery, Xiangya Hospital, Central South University, Changsha, Hunan 410008, P.R. China

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Abstract. The aim of the present study was to investigate the effects of simultaneous short hairpin RNA (shRNA)-targeted survivin and vascular endothelial growth factor (VEGF) inhibition on the proliferation, apoptosis and angiogenesis of human pancreatic cancer cells (Panc-1). Targeted small interfering RNA (siRNA) expression vectors of survivin and VEGF were constructed and transfected into Panc-1 cells. The downregulation of survivin and VEGF expression was evaluated by real-time PCR and western blot analysis. The effects of targeted shRNA on the proliferation and apoptosis of Panc-1 cells were analyzed by MTT assay and flow cytometry (FCM). The culture medium from Panc-1 cells transfected with siRNA was collected and human umbilical vein endothelial cells (HUVECs) were seeded in this media. The proliferation and apoptosis of the HUVECs were also investigated by MTT assay and FCM. A transfected cell line (Panc-1/survivin-shRNA and Panc-1/VEGF-shRNA) was established in which the expression of survivin and VEGF was downregulated. The cell viabilities of Panc-1 cells and HUVECs in the combined inhibition groups were markedly decreased compared with the controls. The cell apoptosis rates of Panc-1 cells and HUVECs in the combined inhibition groups were observed to be significantly increased compared with the controls. The simultaneous RNA interference-mediated downregulation of survivin and VEGF expression inhibited proliferation and induced the apoptosis of Panc-1 cells and HUVECs, indicating that combined therapy with survivin and VEGF inhibition may serve as a potential strategy for the treatment of pancreatic cancer.

Introduction

Pancreatic cancer is one of the most common types of cancer with 300,000 mortalities every year worldwide. Morbidity and mortality are gradually increasing (1). At present, the success of tumor resection and the efficacy of chemotherapy and radiotherapy are extremely low (2). In addition to conventional cancer therapies, several alternative approaches for limiting tumor progression are currently under investigation. These strategies aim to reduce the expression of tumor-related genes, for example, by the use of small interfering RNAs (siRNAs). The main targets of these strategies are central regulatory genes which control cell proliferation, cell death and angiogenesis, including the apoptosis inhibitor survivin and vascular endothelial growth factor (VEGF). Survivin, a member of the inhibitor of apoptosis protein (IAP) family, has been demonstrated to be involved in the regulation of apoptosis, cellular proliferation and angiogenesis in cancer and has attracted growing attention as a potential target for cancer therapy (3). VEGF is the most effective and specific factor for the promotion of tumor angiogenesis and is vital for tumor growth and metastasis (4). VEGF and survivin are overexpressed in the majority of cancer types, including human pancreatic cancer (5-8). It has been reported that antisense oligodeoxynucleotides (AS-ODNs) or siRNAs, specifically directed at survivin or VEGF, induced apoptosis and inhibited the proliferation of tumor cells (9,10). However, the effects of combined target gene silencing of survivin and VEGF on the proliferation, apoptosis and angiogenesis of human pancreatic cancer cells have not yet been reported.

The aim of the present study was to investigate the effects of simultaneously targeting survivin and VEGF with short hairpin RNA (shRNA) on the proliferation, apoptosis and angiogenesis of human pancreatic cancer cells (Panc-1). Gene therapy simultaneously targeting survivin and VEGF may be a potent and attractive strategy for the treatment of pancreatic cancer.

Materials and methods

shRNA design and plasmid construction. siRNA target design tools from oligo designer 3.0 were used to design survivin-, VEGF- and non-specific-shRNA sequences. Four vectors were designed, which included 4 survivin- and VEGF-specific siRNAs designated as S1, S2, S3 and S4 and V1, V2, V3 and V4, respectively. The vectors including nonsense sequences were designated Snc and Vnc. Sequences of siRNAs targeted at survivin and VEGF and the nonsense control constructs are presented in Table I. The oligonucleotides were annealed and inserted into the pGPU6/GFP/Neo expression vector according

Correspondence to: Professor Yixiong Li, Department of General Surgery, Xiangya Hospital, Central South University, 87 Xiangya Rd, Changsha, Hunan 410008, P.R. China
E-mail: liyixiong2011@hotmail.com

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Table I. Sequences of shRNA against human survivin and VEGF.

Vector	Target sequences	Sequences cloned into the vector (5'-3')
S1	GCGCTTTCCTTTCTGTCAAGA	S:CACCGCGCTTTCCTTTCTGTCAAGATTCAAGAGATCTTGACAGAAAGGAAAGCGCTTTTTTG A:GATCCAAAAAAGCGCTTTCCTTTCTGTCAAGATCTCTTGAATCTTGACAGAAAGGAAAGCGC
S2	GACAGAGAAAGAGCCAAGAAC	S:CACCGACAGAGAAAGAGCCAAGAACTTCAAGAGAGTTCTTGGCTCTTTCTCTGTCTTTTTTG A:GATCCAAAAAAGACAGAGAAAGAGCCAAGAACTCTCTTGAAGTTCTTGGCTCTTTCTCTGTC
S3	GCACCACTTCCAGGGTTTATT	S: CACCGCACCACTTCCAGGGTTTATTTCAAGAGAATAAACCCTGGAAGTGGTGCTTTTTTG A: GATCCAAAAAAGCACCACTTCCAGGGTTTATTCTCTTGAAATAAACCCTGGAAGTGGTGC
S4	GCACTTCAGACCCACTTATTT	S: CACCGCACTTCAGACCCACTTATTTCAAGAGAATAAGTGGGTCTGAAGTGCTTTTTTG A: GATCCAAAAAAGCACTTCAGACCCACTTATTCTCTTGAAATAAGTGGGTCTGAAGTGC
Snc	GTTCTCCGAACGTGTCACGTC	S: CACCGTTCTCCGAACGTGTCACGTCAAGAGATTACGTGACACGTTCGGAGAATTTTTTG A: GATCCAAAAAATTCTCCGAACGTGTCACGTAATCTCTTGACGTGACACGTTCGGAGAAC
V1	GCAGATTATGCGGATCAAACC	S: CACCGCAGATTATGCGGATCAAACCTTCAAGAGAGGTTTGATCCGCATAATCTGCTTTTTTG A: GATCCAAAAAAGCAGATTATGCGGATCAAACCTCTCTTGAAGGTTTGATCCGCATAATCTGC
V2	GCGCAAGAAATCCCGGTATAA	S: CACCGCGCAAGAAATCCCGGTATAATTCAAGAGATTATACCGGGATTTCTTGCGCTTTTTTG A: GATCCAAAAAAGCGCAAGAAATCCCGGTATAATCTCTTGAATTATACCGGGATTTCTTGCGC
V3	GCGAGGCAGCTTGAGTTAAAC	S: CACCGCGAGGCAGCTTGAGTTAAACTTCAAGAGAGTTTAACTCAAGCTGCCTCGCTTTTTTG A:GATCCAAAAAAGCGAGGCAGCTTGAGTTAAACTCTCTTGAAGTTTAACTCAAGCTGCCTCGC
V4	GCCAGCACATAGGAGAGATGA	S: CACCGCCAGCACATAGGAGAGATGATTCAAGAGATCATCTCTCCTATGTGCTGGCTTTTTTG A: GATCCAAAAAAGCCAGCACATAGGAGAGATGATCTCTTGAATCATCTCTCCTATGTGCTGGC
Vnc	GTTCTCCGAACGTGTCACGTC	S: CACCGTTCTCCGAACGTGTCACGTCAAGAGATTACGTGACACGTTCGGAGAATTTTTT G A: GATCCAAAAAATTCTCCGAACGTGTCACGTAATCTCTTGACGTGACACGTTCGGAGAAC

shRNA, short hairpin RNA; VEGF, vascular endothelial growth factor; S, sense strand; A, antisense strand.

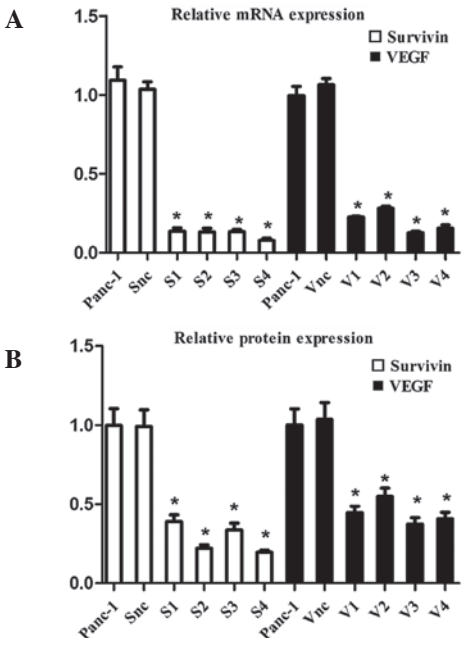

Figure 1. Relative expression of survivin and VEGF (A) mRNA and (B) protein following transfection with shRNA. *P<0.05, vs. control. VEGF, vascular endothelial growth factor; shRNA, short hairpin RNA.

to the manufacturer's instructions (Genepharma, Shanghai, China). The recombinant vectors were confirmed by digestion analysis using restriction endonucleases and all inserted sequences were verified by DNA sequencing.

Cell culture and transfection. The human pancreatic cancer cell line, Panc-1 (American Type Culture Collection, Manassas, VA, USA), was cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen Life Technologies, Carlsbad, CA, USA) containing 10% fetal calf serum in a 37˚C incubator with a 5% CO_2-humidified atmosphere. Panc-1 cells were seeded in 6-well plates at 4-5x10^4 cells/well and cultured overnight to 70% confluence prior to transfection. Transfection was performed using Lipofectamine™ 2000 and the cells were transfected with the vectors according to the manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA, USA). Following this, assays were performed using transfectants.

Real-time PCR analysis of mRNA expression. Total cellular RNA was isolated using TRIzol reagent according to the manufacturer's instructions (Invitrogen Life Technologies). Real-time PCR was performed using total RNA (2 mg) using oligo$(dT)_{18}$ primers at 42˚C for 60 min and 70˚C for 10 min. The primer sequences used were as follows: survivin (136 bp), 5'-accgcatctctacattcaag-3' (forward) and 5'- ttgaagcagaagaaacactg-3' (reverse); VEGF (136 bp), 5'-actgaggagtccaacatcac-3' (forward) and 5'-gtctgcattcacatttgttg-3' (reverse); β-actin (208 bp), 5'-cattaaggagaagctgtgct-3' (forward) and 5'-gttgaaggtagtttcgtgga-3' (reverse). The relative quantification of the target gene expression was performed using the $2^{-\Delta\Delta Ct}$ method. Each experiment was performed at least three times.

Western blot analysis of target protein expression. Untransfected or stably transfected Panc-1 cells were lysed in lysis buffer and the lysates were cleared by centrifuging. Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gele electrophoresis (SDS-PAGE), electroblotted onto a nitrocellulose membrane, blocked by 5% skimmed milk and probed with anti-survivin, -VEGF and -GAPDH antibodies (Sigma-Aldrich, St. Louis, MO, USA). Following incubation with secondary antibody, immunoblots were visualized by chemiluminescence using a chemiluminescence kit and the specific bands were recorded on X-ray film. GAPDH protein levels were used as a control to verify equal protein loading.

Cell proliferation assay. Panc-1 cells and HUVECs (American Type Culture Collection) were seeded in the collected culture medium of each group and cell viability was measured by 3-(4,5-dimethylthazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich). Panc-1 cells or HUVECs (1x10^4 cells/well) were seeded into seven 96-well culture plates and each group consisted of 3 parallel wells. MTT was added to each well and the cells were incubated at 37˚C. The reaction was then stopped by lysing the cells with 150 ml DMSO for 5 min. Optical densities were determined using a Versamax microplate reader (Molecular Devices, Sunnyvale, CA, USA) at 570 nm.

Apoptosis detection. The apoptosis of Panc-1 cells and HUVECs seeded in the collected culture medium of each group was assessed 72 h following transfection by staining cells with Annexin V/propidium iodide (PI) and analyzed using flow cytometry (FCM).

Statistical analysis. All statistical analyses were performed using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA). Comparisons among all groups were performed using the one-way analysis of variance (ANOVA) test and Student Newman Keuls method. P<0.05 was considered to indicate a statistically significant difference.

Results

Inhibitive effect of specific shRNA vectors on survivin and VEGF expression in Panc-1 cells and selection of the most effective specific shRNA vector. mRNA and protein expression levels of survivin and VEGF, inhibited by specific-shRNAs in the Panc-1 cells, were analyzed by real-time PCR and western blot analysis. As demonstrated in Fig. 1, real-time PCR revealed that the expression of survivin was inhibited in the S1, S2, S3 and S4 groups (13.63, 13.14, 13.27 and 7.81%, respectively, compared with the normal and positive controls; P<0.05) and the expression of VEGF was inhibited in the V1, V2, V3 and V4 groups (22.51, 28.27, 12.69 and 15.46% respectively, compared with the normal and positive controls; P<0.05). No significant difference was identified between survivin and the VEGF positive and normal controls (P>0.05). Western blot analysis revealed that survivin and VEGF protein expression was significantly inhibited, consistent with the real-time PCR results. S4 and V3, directed at survivin and VEGF, respectively, were selected as the most effective inhibitors for investigation in the latter experiments.

Individual and combined inhibitive effect of siRNA on survivin and VEGF expression in Panc-1 cells. Real-time PCR and

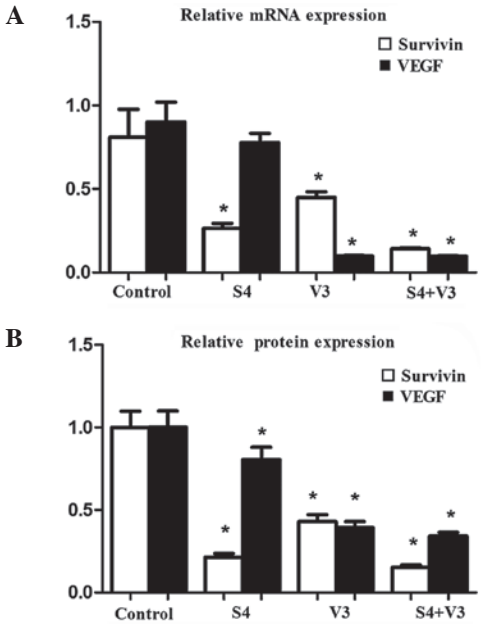

Figure 2. Individual and simultaneous suppression of survivin and VEGF in Panc-1 cells by shRNA (S4 and V3). Relative expression of survivin and VEGF (A) mRNA and (B) protein levels determined by real-time PCR and western blot analysis, respectively. *P<0.05, vs. control. VEGF, vascular endothelial growth factor; shRNA, short hairpin RNA.

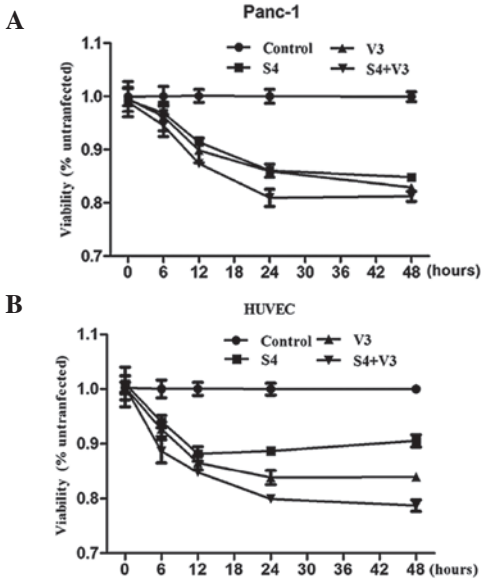

Figure 3. Survivin and VEGF gene knockdown inhibited cell growth. Cell growth curves of (A) Panc-1 cells and (B) HUVECs. Cell viability was analyzed by MTT for 48 h and the highest inhibitory rate of Panc-1 cells and HUVECs in the S4+V3 group was 81.2 ±0.95 and 78.7±1.06%, respectively, at 48 h. Viability of Panc-1 cells and HUVECs in the S4 or V3 group was decreased. This decrease was higher in the S4+V3 group. *P<0.05, vs. control. VEGF, vascular endothelial growth factor; HUVECs, human umbilical vein endothelial cells.

western blot analysis (Fig. 2) demonstrated that the expression of survivin and VEGF by Panc-1 cells was inhibited in the S4 and V3 groups, respectively, at the mRNA and protein levels, and the expression levels of survivin and VEGF mRNA and protein were significantly reduced simultaneously in the S4+V3 group compared with the control (P<0.05).

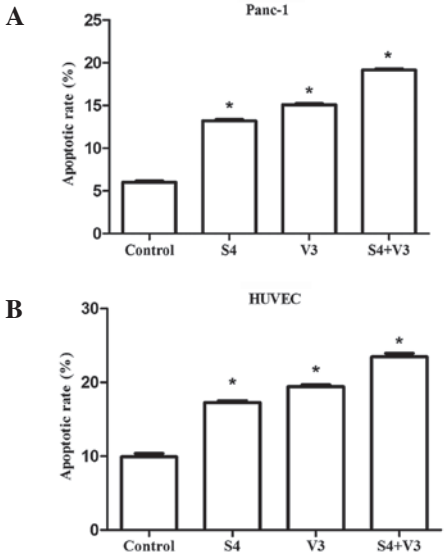

Figure 4. Survivin and VEGF gene knockdown induced cell apoptosis. Cell apoptosis rate of (A) Panc-1 cells and (B) HUVECs. The strongest apoptotic signals appeared in Panc-1 cells and HUVECs of the S4+V3 group and the percentage of apoptotic cells was 19.17±0.09 and 23.45 ±0.49%, respectively. The apoptosis rate of Panc-1 cells and HUVECs in the S4 or V3 group was higher than the control and this decrease was higher in the S4+V3 group. *P<0.05, vs. control. VEGF, vascular endothelial growth factor; HUVECS, human umbilical vein endothelial cells.

Proliferation assay. Cell growth curves of Panc-1 cells and HUVECs determined by MTT for 48 h are presented in Fig. 3 and revealed that the viability of the Panc-1 cells and HUVECs was inhibited in a time-dependent manner and the highest inhibitory rates were 81.2 ±0.95 and 78.7±1.06%, respectively, at 48 h. Compared with control cells, the viability of Panc-1 cells and HUVECs in the S4 or V3 groups was reduced and the reduction was greater in the S4+V3 group (P<0.05).

Apoptosis of Panc-1 cells and HUVECs detected by Annexin V-FITC and PI staining. Apoptosis was assessed following transfection by staining cells with Annexin V/PI and analyzed using FCM (Fig. 4). The strongest apoptotic signals were identified in the Panc-1 cells and HUVECs of the S4+V3 group and the percentages of apoptotic cells were 19.17±0.09 and 23.45±0.49%, respectively. The results indicate that the apoptosis rates of Panc-1 cells and HUVECs in the S4 and V3 groups were higher than those in the control. This increase was higher in the S4+V3 group (P<0.05).

Discussion

Abnormal proliferation and angiogenesis and resistance to apoptosis are hallmarks of various forms of cancer and commonly lead to the failure of cancer therapy. Survivin is a novel human IAP family member containing a single baculoviral IAP repeat domain. Survivin inhibits caspases and blocks the apoptotic pathway and its α-helix structure interacts with microtubules and interfers with mitosis (11). In addition, survivin is involved in tumor angiogenesis by inhibition of vascular endothelial cell apoptosis (12). VEGF, a vascular permeability factor, is a highly specific endothelial cell mitogen which inhibits apoptosis and promotes the

survival of vascular endothelial cells (13,14). VEGF is secreted by malignant tumor cells and plays a critical role in angiogenesis by binding receptors on vascular endothelial cells (15,16). Therefore, simultaneous inhibition of expression of survivin and VEGF in pancreatic cancer cells may inhibit proliferation and angiogenesis and induce apoptosis more effectively than individual inhibition.

Survivin and VEGF are upregulated in various malignancies, including pancreatic cancer, and are associated with aggressive tumor behavior and recurrence (5-8). Previous studies have reported that inhibition of survivin in pancreatic cancer cells by AS-ODNs or siRNA reduces tumor cell growth and induces apoptosis (17). It has also been reported that inhibition of survivin in the endothelial cells may induce the apoptosis of endothelial cells and reduce tumor-associated angiogenesis (18,19). In addition, inhibition of VEGF in tumor cells by AS-ODNs or siRNA has been identified to reduce tumor cell growth, induce apoptosis and affect tumor angiogenesis (20,21). However, studies concerning the effect of simultaneous targeting of survivin and VEGF on the proliferation, apoptosis and angiogenesis of human pancreatic cancer cells have not been performed to date.

RNA interference (RNAi) is a powerful post-transcriptional gene silencing technique and is characterized by high efficiency and specificity and low toxicity. At present, the technique is widely utilized in gene therapy and has became a powerful tool for studies on gene function (22). To explore the potential of survivin and VEGF as effective therapeutic targets, RNAi was performed to silence endogenous survivin and VEGF expression in Panc-1 cells.

In the present study, mRNA and protein expression levels of survivin and VEGF in Panc-1 cells were markedly downregulated. Consistent with this downregulation, MTT assay and FCM revealed increased levels of cell apoptosis and inhibition of cell growth. This effect on proliferation and apoptosis was higher in the combined survivin and VEGF inhibition group. In addition, due to the downregulation of survivin and VEGF in the culture medium of the Panc-1 cells transfected by siRNA, cell apoptosis rate was also observed to be increased and cell growth was inhibited in HUVECs. Again, this effect was more apparent in the combined survivin and VEGF inhibition group. However, the molecular mechanism by which these effects are mediated in HUVECs remain unknown. Further studies are required to validate survivin and VEGF as pharmaceutical targets for anti-tumorigenesis in pancreatic cancer *in vivo*.

In summary, the results of the current study indicate that survivin and VEGF are associated with the development of pancreatic cancer and the anti-tumorigenic effects of simultaneous shRNA-targeted survivin and VEGF are considerably greater than those of a single inhibitor. Through investigation of the anti-tumorigenic mechanisms of simultaneous inhibition of survivin and VEGF in Panc-1 cells and HUVECs, we hypothesize that combined therapy with survivin and VEGF inhibition should be analyzed further as a potential therapeutic strategy for human pancreatic cancer.

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