VEGF-C ShRNA Inhibits Pancreatic Cancer Growth and Lymphangiogenesis in an Orthotopic Fluorescent Nude Mouse Model

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Abstract. The aim of this study was to assess the inhibitory efficacy of short hairpin RNA (ShRNA) targeting vascular endothelial growth factor C (VEGF-C) in an orthotopic pancreatic cancer mouse model. BxPC-3 human pancreatic cancer cells expressing green fluorescent protein (GFP) were orthotopically implanted onto the pancreas of nude mice. All mice were randomly divided into four groups when the average tumor size had reached 100 mm³ and were treated with either vehicle or gemcitabine at 150 mg/kg; or intravenous VEGF-C ShRNA at 150 mg/kg; or intratumoral VEGF-C ShRNA at 150 μg/kg. In vivo fluorescence imaging was performed to monitor tumor growth and metastasis during the study. Real-time quantitative polymerase chain reaction (RT-qPCR) and an enzyme-linked immunosorbent assay (ELISA) were performed to determine the mRNA and protein level of VEGF-C in tumor tissues. Lymphatic vessel marker D2-40, blood vessel marker CD31 and proliferation marker Ki67 expression of the tumor tissues were analyzed by immunohistochemistry staining. Intravenous and intratumoral VEGF-C ShRNA treatment significantly inhibited tumor growth, downregulated the expression of VEGF-C mRNA, reduced tumor microlymphatic vessel density (MLVD), and inhibited cancer cell proliferation. Gemcitabine, as the standard treatment for pancreatic cancer, demonstrated a stronger inhibitory effect on tumor growth, with less inhibition of MLVD and more inhibition of microvessel density (MVD) and proliferation than VEGF-C ShRNA. These results indicate that different mechanisms are associated with the efficacy of gemcitabine and VEGF-C ShRNA.

Pancreatic cancer is a highly treatment-resistant disease. Realistic orthotopic mouse models of pancreatic cancer have been developed (1, 2) for this disease. These models are enhanced by fluorescent protein expression to image the disease in real time and to visualize the efficacy of therapeutics, including gemcitabine, first-line therapy for this disease (3, 4).

Treatment of the orthotopic model of pancreatic cancer with metronomic gemcitabine and sunitinib significantly prolonged median overall survival compared with control and with either regimen alone (5). However, improved therapeutics for pancreatic cancer are still urgently needed.

Lymph node metastasis is the most common metastatic pathway of pancreatic cancer and often occurs earlier than hematogenous metastasis (6, 7). Vascular endothelial growth factor C (VEGF-C) expression stimulates lymph node metastasis in pancreatic cancer (8-10). Interfering with the VEGF-C/VEGFR-3 signaling pathway suppresses lymphangiogenesis and lymph node metastases (11-13). Blocking VEGF-C inhibits tumor growth and metastasis (14, 15).
Short hairpin RNA (ShRNA) expression vector systems (16-18) induce targeted gene silencing in mammalian cells. Lentivirus vectors encoding ShRNAs are capable of efficient transfection into human cells, including non-dividing cells, resulting in long-term and stable expression of the ShRNAs (19).

In this study, we examined the antitumor and anti-metastasis effects of a lentivirus vector encoding VEGF-C ShRNA in an orthotopic mouse model of pancreatic cancer expressing green fluorescent protein (GFP). *In vivo* fluorescence imaging was used to visualize and quantify tumor growth and metastasis development, thereby allowing real-time efficacy evaluation.

**Materials and Methods**

**Cell culture.** The human pancreatic cancer cell line BxPC-3 GFP was obtained from AntiCancer, Inc. (San Diego, CA, USA). The cells were cultured in RPMI-1640 (GIBCO Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Hyclone, Logan, UT, USA) at 37°C in 5% CO₂ in saturated air. All media were supplemented with penicillin/streptomycin (GIBCO/BRL, Grand Island, NY, USA).

*Animal care.* BALB/C male nu/nu nude mice, aged 4-6 weeks and weighing 20-25 g, were purchased from the Beijing Kelihua laboratory animal center (Beijing, PRC). The mice were maintained in a HEPA-filtered environment at 24-25°C and with 50-60% humidity. The animals were fed an autoclaved laboratory rodent diet. Animal experiments were approved by the Animal Committee of Origin Biosciences Inc. (Nanjing, PRC).

*Surgical orthotopic implantation (SOI).* BxPC-3-GFP tumor stock was established by subcutaneously (s.c.) by injecting BxPC-3-GFP cells (5x10⁶) in the flank of nude mice. Pancreatic tumors grown s.c. in nude mice were harvested at the exponential growth phase and resected under aseptic conditions. Strong GFP expression of the BxPC-3-GFP tumor tissue was confirmed by fluorescence microscopy. Necrotic tissues were removed and viable tissues were cut with scissors and minced into 1-mm³ pieces. For SOI, the animals were anesthetized by injection of 0.02 ml of solution of 50% ketamine, 38% xylazine, and 12% acepromazine maleate. The abdomen was sterilized using iodine solution and alcohol and an incision was made through the left upper abdominal pararectal line and peritoneum. The pancreas was exposed, and the capsule of the pancreatic body and tail was stripped. Then one piece of tumor fragment was transplanted to the pancreatic tail with 8-0 surgical sutures and this segment of the pancreas was fixed on the anterior peritoneum. The pancreas was then returned to the peritoneal cavity and the abdomen was closed with sterile 5-0 surgical sutures (1-3). All surgical procedures and animal manipulations were conducted under HEPA-filtered laminar-flow hoods with a x8 surgical microscope (YZ20P5; Alltion (Wuzhou) Co. Ltd., Wuzhou, Guangxi, PRC).

**VEGF-C ShRNA.** Lentiviral vector plasmids encoding VEGF-C ShRNA were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). VEGF-C ShRNA plasmid is a pool of three target-specific lentiviral vector plasmids each encoding 19-25 nt (plus hairpin) ShRNAs designed to knock-down gene expression. Each plasmid contains a puromycin resistance gene for the selection of cells stably expressing ShRNA.

**Drug treatment.** Gemcitabine (Jiangsu Hansoh Pharmaceutical Co., Ltd., Jiangsu, PRC) was reconstituted in saline and administered intraperitoneally. VEGF-C ShRNA plasmid was diluted in saline and administered by intravenous or intratumoral injection. Mice were randomly divided into four groups of eight mice per group for treatment when the average tumor size had reached 100 mm³ at day 16 after orthotopic implantation. Group 1 served as the vehicle control and received saline in the same volume of drug as the treated animals. Group 2 was treated weekly i.p. with gemcitabine at 150 mg/kg. Group 3 received intravenous (i.v.) VEGF-C ShRNA treatment every three days at 150 μg/kg. Group 4 received intratumoral (i.t.) VEGF-C ShRNA treatment every three days at 150 μg/kg. The treatment duration was two weeks. Clinical signs and body weights for each tumor-bearing mouse were observed daily or measured twice per week, respectively, during the course of the treatment.

**In vivo fluorescence imaging.** Real-time, quantitative measurements of tumor volume were carried out twice per week by whole-body fluorescence imaging during the study. Tumor volume was calculated using the formula (LxW²) ×0.5, where W and L represent the perpendicular minor and major dimensions of the tumor, respectively. At the end of the study, all mice were sacrificed and open fluorescence imaging was performed (3). Primary tumors and all metastases to distant organs, including the mesentery lymph nodes, lung, liver, abdominal cavity and thoracic cavity were imaged. The primary tumor was removed and weighed after open-body imaging. A fluorescence stereo microscope model (MZ650; Nanjing Optic Instrument Inc., Nanjing, PRC) equipped with D510 long-pass and HQ600/50 band-pass emission filters (Chroma Technology, Brattleboro, VT) and a cooled color charge-coupled device camera (Qimaging, Surrey, BC, Canada) was used. Selective excitation of GFP was produced through an illuminator equipped with HQ470/40 and HQ540/40 excitation band-pass filters (Chroma Technology, Brattleboro, VT). Images were processed and analyzed with the use of IMAGE PRO PLUS 6.0 software (Media Cybernetics, Silver Spring, MD).

**Real-time quantitative polymerase chain reaction (RT-qPCR).** Frozen tumor tissues were homogenized in a pre-cooled tissue homogenizer. Total RNA was extracted from the homogenate using a Trizol kit (Invitrogen, Carlsbad, CA, USA), and reverse-transcribed to cDNA using a First-Strand cDNA synthesis kit (Fermentas, Vilnius, Lithuania) according to the manufacturer’s instructions. RT-qPCR was performed using SYBR Green Real-Time PCR Master Mix (Toyobo, Osaka, Japan). The PCR primers were as follows: VEGF-C, sense: 5’-AGGCAAGCTACTCTGAAAGA-3’, antisense: 5’-GGAGACGTCTAATAATGGAAATG-3’, β-action, sense: 5’-GGCAAGGAGATCAGCTGCCCT-3’, antisense: 5’-GCTGTAGCCACATCTGCTGGAA-3’. Reactions were analyzed on a DA7600 real-time PCR machine (Zhongshan Bio-Tech Co., Ltd., Zhongshan, PRC) using the following cycle conditions: 5 minutes pre-denaturation at 95°C, followed by 40 cycles of 15 s denaturation at 95°C, 20 s annealing at 60°C and 40 s extension at 72°C. Results were normalized against β-action mRNA expression.
Enzyme-linked immunosorbent assay (ELISA). VEGF-C production in the tumors was measured in tumor homogenates by ELISA using a human VEGF-C ELISA kit (Invitrogen). ELISA was performed according to the manufacturer’s instruction. Briefly, the flat-bottom 96-well microtiter plates were coated with antibodies to VEGF-C and incubated overnight at 4˚C, then blocked with bovine serum albumin buffer at 37˚C for 3 h. The blank wells were then loaded with diluted sample (supernatant of tumor homogenates) or standard, and the remaining wells were loaded with sample or standard at different concentrations (100 μl/well) and incubated at 37˚C for 2 h. The plates were washed five times and incubated with biotinylated antibody to VEGF-C at 37˚C for 1 h. After washing five times, the plates were incubated with horseradish peroxidase-labeled avidin at 37˚C for 30 min. After washing again five times, the plates were incubated with substrate tetramethyl benzidine (100 μl/well) at 37˚C for 30 min. The color reaction was stopped by addition of H2SO4 (2 mol/l, 50 μl/well), and the optical density was measured at 450 nm using a microplate reader (ELx800uv; Bio-Tec Instruments, Winooski, VT, USA). A standard curve was constructed and the protein level of VEGF-C was calculated on the basis of the standard curve.

Immunohistochemistry. Formalin-fixed paraffin-embedded tumor tissue sections (4 μm) were deparaffinized in xylene, rehydrated in graded ethanol solutions, and rehydrated in phosphate-buffered saline (PBS; pH 7.2). To enhance antigen retrieval, sections were microwaved for 5 min. Each slice was treated with two drops of 3% H2O2-methanol solution for 15 min at 37˚C and washed with PBS. Monoclonal antibodies to D2-40, CD31 and Ki67 (Biocare Medical Co., Concord, CA, USA) were used as a lymphatic vessel marker, a blood vessel marker, and a proliferating cell marker, respectively. Tissue sections were incubated overnight at 4˚C with diluted primary antibodies (1:100 D2-40; 1:100 CD31; and 1:50 Ki67). After washing in PBS, the slices were incubated with horseradish peroxidase-labeled secondary antibody (1:200, Maixin Bio-Tech Co., Ltd, Fuzhou, PRC) for 30 min at room temperature. After color development using diaminobenzidine (Maixin...
Bio-Tech Co., Ltd), the slices were counterstained in hematoxylin and mounted with a neutral resin medium. The microlymphatic vessel density (MLVD) and microvessel density (MVD) were quantified as described previously (20, 21). Briefly, a scanning slice was first examined at ×100 magnification for a ‘hot spot’ representing the area of the highest vessel density. Then the field was switched to ×400 magnification for clear depiction and better counting. For each slice, the MLVD or MVD was calculated as the average of vessels in four fields. Proliferation was calculated as the average ratio of Ki67-positive cells to 100 total cells in four fields (at ×400 magnification).

Figure 2. Tumor growth curves after treatment with vascular endothelial growth factor C (VEGF-C) short hairpin RNA (ShRNA) or gemcitabine in the orthotopic BxPC-3-GFP pancreatic cancer mouse model. Whole-body fluorescence imaging was performed and tumor volumes were calculated as described in Materials and Methods. Data are the means ± SD from eight mice in each group at each time point.

Figure 3. Efficacy of vascular endothelial growth factor C (VEGF-C) short hairpin RNA (ShRNA) and gemcitabine on tumor weights in the orthotopic BxPC-3-GFP pancreatic cancer mouse model. Primary tumors were excised and tumor weights were determined for each animal at autopsy. Data are the means ± SD from eight mice in each group.
Statistical analysis. Data are expressed as means ± SD, and were analyzed by one-way analysis of variance (ANOVA), using SPSS software version 16.0 (SPSS Inc., Chicago, USA), where \( p<0.05 \) was considered to be statistically significant.

Results

Effect of VEGF-C ShRNA on the growth of orthotopic pancreatic tumors. Tumor growth was monitored and measured by whole-body fluorescence imaging during the course of the treatment (Figure 1). Gemcitabine \((i.p.)\), VEGF-C ShRNA \((i.v.)\), and VEGF-C ShRNA \((i.t.)\)-treated mice started showing tumor-growth inhibition beginning from day 20 after orthotopic implantation. The tumor size was significantly reduced in all three treated groups compared to control group \((p<0.01)\) at the end of the study (Figure 2).

At autopsy, tumors were excised and tumor weights were determined for each animal. The mean tumor weights for each group are presented in Figure 3. Tumor weights were significantly reduced by gemcitabine \((i.p.)\), VEGF-C ShRNA \((i.v.)\), and VEGF-C ShRNA \((i.t.)\) as compared to the control \((p<0.01)\). The tumor-bearing animals treated with gemcitabine \((i.p.)\), VEGF-C ShRNA \((i.v.)\) and VEGF-C ShRNA \((i.t.)\) demonstrated a 67.1% (1.7 ± 0.3; \( p<0.01 \)); a 35.1% (3.3 ± 0.8; \( p<0.01 \)); and a 40.7% (3.0 ± 0.5; \( p<0.01 \)) decrease, respectively, in tumor weight as compared with control animals.

Efficacy of VEGF-C ShRNA on lymph node metastasis. At the end of the study, all animals were sacrificed and whole-body imaging was conducted for metastasis evaluation. The metastasis incidence, especially for lymph node metastasis, was determined and analyzed. Gemcitabine \((i.p.)\), VEGF-C ShRNA \((i.v.)\), and VEGF-C ShRNA \((i.t.)\) groups showed a reduced incidence of metastasis compared with the control group. However, no statistically-significant difference was found between three treated groups and the control group \((p>0.05)\) (Figure 4).
Effect of VEGF-C ShRNA on VEGF-C production in tumors.

RT-qPCR and ELISA were performed to determine the mRNA and protein level of VEGF-C in tumor tissues from all three treated and control groups. VEGF-C ShRNA and gemcitabine treatment significantly down-regulated the expression of VEGF-C mRNA compared with the control (p<0.01). Both VEGF-C ShRNA (i.v.) and VEGF-C ShRNA (i.t.) groups had significantly lower levels of VEGF-C mRNA expression than the gemcitabine group (p<0.01) (Figure 5A). A lower level of VEGF-C protein was found in VEGF-C ShRNA and gemcitabine-treated animals, although statistical difference was not achieved (Figure 5B).

Effects of VEGF-C ShRNA on expression of lymphangiogenesis, angiogenesis and a proliferation marker.

Expression of lymphatic vessel (MLVD) marker D2-40, blood vessel (MVD) marker CD31, and a proliferation marker Ki67 in the tumor tissues was analyzed by immunohistochemistry. Significant reduction in the MLVD was found in the gemcitabine- and VEGF-C ShRNA-treated groups compared with the control group (p<0.01). MLVD was significantly more reduced by VEGF-C ShRNA than by gemcitabine (p<0.01) (Figure 6).

No significant difference of MVD was found between the VEGF-C ShRNA-treated and untreated control groups (p>0.05), although a significant reduction in MVD was
Figure 6. Efficacy of vascular endothelial growth factor C (VEGF-C) short hairpin RNA (ShRNA) and gemcitabine on lymphangiogenesis, angiogenesis and proliferation in the orthotopic BxPC-3-GFP pancreatic cancer mouse model. A: Representative immunostaining for D2-40, CD31, and Ki-67 in BxPC-3 tumors that were treated with saline control, gemcitabine, i.v. VEGF-C ShRNA or i.t. VEGF-C ShRNA, respectively. Original magnification, ×400. B: Quantitative morphometry for D2-40, CD31 and Ki-67 immunoreactivity from the tumors shown in (A). Data are the means ± SD from four tumors per group, with four fields analyzed for each tumor. *p<0.05 when compared with controls; †p<0.05 when compared with gemcitabine; ‡p>0.05 when compared with control.
observed in the gemcitabine group as compared with the control group (p<0.01) (Figure 6).

Ki67 expression was significantly inhibited by VEGF-C ShRNA and gemcitabine as compared with the untreated control (p<0.01). Gemcitabine treatment resulted in a more significant reduction of Ki67 expression level than did VEGF-C ShRNA treatment (p<0.01) (Figure 6).

Body weight and toxicity. The body weights of mice were measured to evaluate the toxic side-effects of all treatments. The body weights of VEGF-C ShRNA-treated groups were not significantly different from the untreated control (p>0.05). However, a significant decline on body weight was noticed in the gemcitabine-treated group as compared with the untreated control (p<0.05) and the VEGF-C ShRNA-treated groups (p<0.05), beginning from day 20 after orthotopic implantation (Figure 7). No other physical or behavioral signs that indicated adverse effects due to the treatments were observed in any group.

Discussion

In the present study, we demonstrated that i.v. and i.t. VEGF-C ShRNA treatment significantly inhibited growth of the human pancreatic BxPC-3 tumor in an orthotopic mouse tumor model. VEGF-C ShRNA treatment significantly down-regulated the expression of VEGF-C mRNA, reduced tumor MLVD and inhibited tumor cell proliferation. However, tumor MVD was not significantly affected by VEGF-C ShRNA. The result indicates that inhibition of VEGF-C expression and lymphangiogenesis contributes to the anticancer efficacy of VEGF-C ShRNA.

To investigate if the inhibitory effects of VEGF-C ShRNA on VEGF-C expression and lymphangiogenesis may result in reduction of regional lymph node metastases, metastasis incidence was evaluated in this study with the orthotopic pancreatic cancer mouse model. The VEGF-C ShRNA-treated groups showed a decreased incidence of metastasis compared with the control group, however, the difference was not significant. This may be attributable to the fact that lymph node metastasis is regulated by factors beside VEGF-C (13, 22).

Gemcitabine, the treatment standard for pancreatic cancer, was selected for comparison and showed more inhibitory efficacy on tumor growth than did VEGF-C ShRNA treatment. However, gemcitabine induced less inhibition of MLVD and more inhibition of MVD and proliferation than did VEGF-C ShRNA, indicating a different mechanism was associated with efficacy of gemcitabine and VEGF-C ShRNA.

GFP- and red fluorescent protein-expressing orthotopic pancreatic tumor models have been shown to be useful for the evaluation of novel treatment strategies for pancreatic cancer (3-5, 23-25). Such strategies are needed to combat this highly lethal tumor. RNA interference as a new emerging therapy is still under development. Treatment conditions need to be further investigated and optimized to increase the anticancer therapeutic potential of this modality.
Conflicts of Interest

None of the Authors have a conflict of interest regarding this study.

Acknowledgements

This study was supported by research grants from the National Natural Science Foundation in China (numbers 81071195, 30870689 and 81271630).

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