Silencing of decoy receptor 3 (DcR3) expression by siRNA in pancreatic carcinoma cells induces Fas ligand-mediated apoptosis in vitro and in vivo

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Abstract. Decoy receptor 3 (DcR3) is abundantly expressed in human tumors and protects cells from a wide range of apoptotic stimuli. In this study, we demonstrate that DcR3 is overexpressed in pancreatic carcinoma cells, and that the pancreatic carcinoma cell lines, Panc-1 and SW1990, are resistant to Fas ligand (FasL)-mediated apoptosis. To further define the function of DcR3 in cell growth and apoptosis, we used small interfering RNA (siRNA) to knockdown the expression of the DcR3 gene in Panc-1 and SW1990 cells. Our results revealed that the silencing of DcR3 expression enhanced the inhibitory effects of FasL and reduced the capability of the cells for proliferation and colony formation in vitro. In addition, the downregulation of DcR3 modulated the cell apoptotic regulators, Fas-associated death domain (FADD), caspase-3 and caspase-8, thus triggering cell apoptosis. Furthermore, the knockdown of DcR3 inhibited the growth of Panc-1 tumor xenografts. Taken together, our findings indicate that DcR3 is important in cancer progression and may be a used as a potential therapeutic target for the gene therapy of pancreatic carcinoma.

Introduction

Human pancreatic carcinoma, a highly malignant cancer with a poor prognosis, is the sixth leading cause of mortality due to malignant disease in China and the fourth leading cause of cancer-related mortality in the United States (1,2). The current literature indicates that the 5-year survival rate of pancreatic carcinoma patients remains <5% and has not increased significantly over the past 20 years, partly due to the fact that pancreatic carcinoma cells are relatively resistant to chemotherapy and radiotherapy (3,4). It has been suggested that resistance to Fas-Fas ligand (FasL)-mediated apoptosis may play an important role in the pathogenesis of pancreatic carcinoma; although human pancreatic adenocarcinoma cells express Fas and FasL, they are still resistant to Fas-mediated apoptosis (5).

As previously reported, a soluble decoy receptor 3 (DcR3) binds to FasL and inhibits FasL-mediated apoptosis. DcR3, also known as Tr6 or M68, is a member of the tumor necrosis factor receptor (TNFR) superfamily and maps to chromosome position 20q13, which is associated with gene amplification in various types of cancer. It shares sequence homology with osteoprotegerin (31%), TNFR2 (29%) and has relatively less homology with Fas (17%) (6). It has been reported that DcR3 has 3 ligands: FasL, TNF-like molecule 1A (TL1A) and homologous to lymphotoxins, exhibits inducible expression, competes with herpes simplex virus glycoprotein D for HVEM, expressed by T lymphocytes (LIGHT) (7-9). DcR3 contributes to tumor growth by blocking apoptosis, impeding the immune response and inducing angiogenesis (10,11). There is strong evidence indicating that DcR3 is overexpressed in a variety of human tumors, including cancers of lungs (6), colon (12) and liver (13), as well as gastric carcinoma (14) and malignant gliomas (15).

In this study, we examined the expression of DcR3 in pancreatic carcinoma tissues, serum and cell lines. Moreover, using small interfering RNA (siRNA) to silence the expression of DcR3, we investigated the effects of FasL-mediated apoptosis. Our findings indicated that DcR3 may be a potential target for gene therapy of pancreatic carcinoma.

Materials and methods

Clinical samples. Tissue samples from 50 pancreatic carcinoma patients were collected during surgical resections performed at the First Affiliated Hospital of Soochow University, Suzhou, China, from January 2008 to June 2012. Tumor tissues and
adjacent non-tumor tissues were frozen immediately after surgical removal in liquid nitrogen and stored at -80°C. Serum for ELISA was obtained from cancer patients prior to surgery and serum from healthy individuals was used as the control. The patients had not received any pre-operative chemotherapy, radiotherapy or immunotherapy. All samples were obtained with patient consent and local ethics committee approval.

**Lentiviral vectors for DcR3 siRNA.** Three different siRNAs targeting DcR3 were designed using the DcR3 gene sequence (GenBank, NM-003823) as a template. The sequence with the most effective silencing effect was selected for subsequent experiments (data not shown). The recombinant lentivirus was synthesized and purified by Genechem Co., Ltd. (Shanghai, China).

**Cell culture and transfection.** The human pancreatic carcinoma cells, Panc-1 and SW1990, from the Shanghai Institute of Cell Biology (Shanghai, China) were maintained in Dulbecco's modified Eagle's medium (DMEM) or RPMI-1640 (Gibco, Carlsbad, CA, USA) respectively, supplemented with 10% fetal bovine serum (Gibco) and 100 µg/ml each of penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA) in 5% CO₂, at 37°C.

In 6-well plates, 5x10⁴ cells/well were cultured overnight, then transfected with 10 µl recombinant lentivirus of DcR3 siRNA (LV-RNAi) or mock lentivirus (LV-NC) using Lipofectamine 2000 (Invitrogen). Single-stranded cDNA for a PCR template was synthesized from 10 µg of total RNA using random primers and M-MLV reverse transcriptase (Takara, Dalian, China). Total RNA from the tissues and cells was extracted using TRIzol reagent (Invitrogen). Single-stranded cDNA for a PCR template was synthesized from 10 µg of total RNA using random primers and M-MLV reverse transcriptase (Takara, Dalian, China). The relative levels of mRNA transcripts to the control (actin) were determined by qRT-PCR. The primers used for PCR were as follows (forward and reverse):

- **Cas-8 (192 bp):** 5'-AGCGAGCAAGGGAAGGCTGG-3' and 5'-TGAACCCAAGAGGTCAAG-3'
- **Cas-3 (278 bp):** 5'-ACAAAGCGACTGGATGAA-3' and 5'-ACAAAGTC-3'
- **DcR3 (112 bp):** 5'-AAGGAGTACACAGATTGAA-3' and 5'-CTCTTCCTCCCATGACAC-3'
- **Fasl (684 bp):** 5'-GGAAATGGGACAAA-3' and 5'-GGTGATATTTACTCAAGTG-3'
- **FasL (192 bp):** 5'-TGAGGTTGAGGAACTGGGATT-3' and 5'-CCAA  
- **Hoechst (1% crystal violet). The colonies containing >50 cells were counted using a microscope.

**Cell proliferation assay.** After 72 h of transfection, the cells were cultured at 500 cells/well in 6-well plates at 37°C for 20 days. The cells were then treated with sFasL (25 ng/ml) for 24 h and fixed with methanol and stained with 1% crystal violet. The cell proliferation rate was calculated as follows: (EdU⁺ cell number/Hoechst⁺ cell number) x100%.

**Colony formation assay.** After 72 h of transfection, 2x10⁴ Panc-1 cells in 200 µl saline were inoculated subcutaneously into the flanks of nude mice. When the tumors reached approximately 5-7 mm in diameter, the mice were randomly divided into groups and injected intratumorally with PBS, LV-NC or LV-RNAi/DcR3 (1x10⁷ pfu/50 µl) once per week for 4 weeks. The tumor size was measured every 5 days with calipers, and the tumor volumes were calculated according to the formula: V = (L x W²) x0.5

**Cell cycle and apoptosis analysis.** After 72 h of transfection, the cells were treated with sFasL (25 ng/ml) for 24 h. For cell cycle analysis, the cells were collected and fixed in 70% ethanol at 4°C overnight, followed by staining with propidium iodide (PI) (BD Biosciences, Franklin Lakes, NJ, USA) and were kept in the dark at 4°C for 30 min. The cell cycle was analyzed by FACSCalibur with CellQuest software. For apoptosis analysis, a volume of 100 µl of cell suspension (1x10⁶ cells/ml) was labeled with 10 µl of PI and 5 µl of Annexin V/FITC (BD Biosciences). The cells were incubated in the dark for 15 min at room temperature and early apoptotic cells were assessed by FACSCalibur.

**ELISA.** Cell culture supernatants and serum from cancer patients were collected and DcR3 levels were measured using ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

**In vivo tumor model.** Male BALB/C nude mice (4-6 weeks old and weighing 16-20 g) were purchased from the Shanghai Experimental Animal Center (Shanghai, China) and housed in a specific pathogen-free environment. Animal experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals of Soochow University. To establish a tumor xenograft model, 2x10⁴ Panc-1 cells in 200 µl saline were inoculated subcutaneously into the flanks of nude mice. When the tumors reached approximately 5-7 mm in diameter, the mice were randomly divided into groups and injected intratumorally with PBS, LV-NC or LV-RNAi/DcR3 (1x10⁷ pfu/50 µl) once per week for 4 weeks. The tumor size was measured every 5 days with calipers, and the tumor volumes were calculated according to the formula: V = (L x W²) x0.5
by measuring tumor length (L) and width (W). At the end of experiment, the tumors were dissected and weighed.

Statistical analysis. SPSS software version 16.0 was used for statistical analysis. Data are expressed as the means ± SD. One-way analysis of variance (one-way ANOVA) or the t-test was performed for inter-group comparisons. A P-value <0.05 was considered to indicate a statistically significant difference.

Results

DcR3 is overexpressed in pancreatic carcinoma. In our cohort of 50 patients recently operated for pancreatic carcinoma, paired samples of tumor and non-tumor tissues were subjected to qRT-PCR (Fig. 1A). DcR3 mRNA was overexpressed in the pancreatic carcinoma tissues compared with the non-tumor tissues (P<0.05). As DcR3 lacks a transmembrane sequence and is a soluble protein, we used ELISA assay to determine the serum levels of DcR3. As shown in Fig. 1B, the serum from the cancer patients had significantly higher levels of DcR3 than the serum from the controls (P<0.01). These results demonstrate that DcR3 is overexpressed in pancreatic carcinoma and that the protein expression of DcR3 is compatible with its mRNA expression.

Expression of DcR3 in pancreatic carcinoma cells and inhibitory effect of sFasL. The expression of FasL and that of its receptor, Fas, as well as that of DcR3, was assessed by RT-PCR (Fig. 2A). The Panc-1 cells and SW1990 cells expressed Fas and DcR3. The mRNA ratio of DcR3 to actin was 2.48±0.72 for Panc-1 and 2.57±0.81 for SW1990 cells. HT29 cells was used as the negative controls for DcR3 expression. We could not detect FasL expression by RT-PCR in the Panc-1 and SW1990 cells. To investigate whether DcR3 plays a role in the sensitivity to apoptosis mediated by FasL, we examined the effects of 24 h of exposure to sFasL (25 ng/ml) by CCK-8 assay (Fig. 2B). The results indicated that sFasL did not inhibit the cell growth of Panc-1 and SW1990 cells, but only that of HT29 cells, which do not express DcR3.

Silencing DcR3 expression by lentivirus-mediated RNA interference (RNAi). To investigate the role of DcR3 in the cell apoptosis mediated by FasL, the Panc-1 and SW1990 cells were transfected with recombinant lentivirus-mediated
RNAi targeting DcR3 and further used for functional analysis. As shown in Fig. 3A and B, the significant decrease in DcR3 expression was verified by RT-PCR and ELISA in the cells transfected with LV-RNAi.

Knockdown of DcR3 expression by RNAi enhances the effects of FasL. To explore the function of DcR3, cell proliferation was determined by CCK-8 assays, colony formation and EdU assays. As shown in Fig. 4, there was no significant effect on cell viability without exposure to sFasL, whereas when the cells were transfected with DcR3 siRNA and treated with sFasL for 24 h, cell growth inhibition was observed. An analysis of clonogenicity indicated that the LV-RNAi-transfected cells displayed much fewer and smaller colonies than the LV-NC-transfected cells (Fig. 5A). The relative colony number of LV-RNAi-transfected cells was reduced by almost 50 and 40% in the Panc-1 and SW1990 cells, respectively (Fig. 5B). Similarly, we found that the number of EdU+ LV-RNAi-transfected cells was significantly reduced compared with the LV-NC-transfected cells (P<0.05). After silencing DcR3...
expression, the relative cell proliferation rate (LV-RNAi to LV-NC-transfected cells) was 41.46±4.25% for Panc-1 and 48.29±5.16% for SW1990 cells (Fig. 6).

The cell cycle and apoptosis are associated with tumor cell growth and proliferation. Thus, we examined the effects of silencing DcR3 expression on the cell cycle and apoptosis by flow cytometry. As shown in Fig. 7, the percentage of G0/G1 phase LV-RNAi-transfected cells was 78.03±1.06% for Panc-1 and 71.9±0.93% for SW1990 cells, which was significantly higher than that of the LV-NC-transfected cells (55.78±0.76% for Panc-1 and 56.98±0.71% for SW1990 cells; P<0.05, respectively). An analysis of apoptosis revealed that the number of apoptotic cells was significantly increased in the LV-RNAi-transfected cells compared with the LV-NC-transfected cells (P<0.05) (Fig. 8). These results suggest that the knockdown of DcR3 expression by RNAi enhances the apoptotic effects of FasL on pancreatic carcinoma cells.

Silencing DcR3 expression modulates the expression of cell apoptotic regulators. FADD, caspase-3 and caspase-8 are the regulators of the FasL-mediated apoptotic pathway. Following treatment with sFasL at 25 ng/ml for 24 h, the relative mRNA...
levels of FADD, caspase-3 and caspase-8 to the control, actin, were determined by RT-PCR (Fig. 9). The results revealed that the cells transfected with LV-RNAi had an upregulated expression of FADD, caspase-3 and caspase-8. These results further support the hypothesis that silencing the expression of DcR3 modulates cell apoptotic regulators, thus triggering cell apoptosis.

**Discussion**

In this study, we demonstrate that DcR3 is overexpressed in pancreatic carcinoma tissues, serum and cell lines. In our previous study, we demonstrated that the expression of DcR3 was associated with clinicopathological features, such as lymph node metastasis, tumor size and clinical stage (16). In this study, we also found that silencing DcR3 expression...
enhanced the apoptotic effects mediated by FasL in vitro and inhibited the tumor growth in vivo. DcR3 is a member of the TNFR superfamily and is regarded as a secreted molecule, as it lacks a transmembrane sequence. Wu et al. reported that 55% of patients with liver, gastric and colon carcinoma were serum DcR3-positive (17). The detection of DcR3 in serum offers an easy-to-access method for tumor diagnosis.

Apoptosis is a cell suicide mechanism which maintains a stable internal environment. The imbalance between cell proliferation and apoptosis plays an important role in the occurrence and progression of malignant tumors. FasL is mainly expressed in activated T cells and natural killer (NK) cells, and it induces apoptosis in target cells through the death receptor, Fas. The most common function of the FasL/Fas system is to mediate the killing of tumor cells by cytotoxic T cells. However, studies have indicated that many tumors, including pancreatic carcinoma, are resistant to FasL/Fas-mediated growth inhibition signals, despite expressing Fas (5,18). Similar results were obtained in this study using Panc-1 and SW1990 cells. Several mechanisms have been suggested to play a role in this phenomena, such as the downregulation of Fas (19), the upregulation of FasL (20) and the expression of soluble Fas (21). DcR3, which binds to FasL and inhibits FasL-induced apoptosis, may also play a role in the resistance to FasL/Fas-mediated growth inhibition signals.

DcR3 is a soluble decoy receptor belonging to the TNFR superfamily, which binds to FasL, LIGHT and TL1A. DcR3 can block the effects of FasL, TL1A and LIGHT by inhibiting the FasL-Fas, TL1A-death receptor 3 (DR3) and LIGHT-HVEM interaction (22-24). Evidence has shown that DcR3 not only protects tumor cells from apoptosis induced by FasL, LIGHT and TL1A, but also suppresses immune surveillance by blocking T cell costimulation mediated by TL1A and LIGHT (25,26). Previous studies have demonstrated that DcR3 neutralizes the FasL-mediated apoptotic signal; therefore, we wished to determine whether the silencing of DcR3 expression enhances the apoptotic effects mediated by FasL.

In this study, we succeeded in silencing DcR3 using lentivirus-mediated RNAi targeting DcR3. We found that the knockdown of DcR3 expression in Panc-1 and SW1990 cells treated with sFasL inhibited cell viability, proliferation and clonogenicity. Flow cytometric analysis revealed that silencing DcR3 expression induced G0/G1 phase arrest in the cells transfected with LV-RNAi and induced apoptosis, compared with the cells transfected with LV-NC. These results suggest that the downregulation of DcR3 expression enhances the effects of FasL in Panc-1 and SW1990 cells. FADD, caspase-3 and caspase-8 are the major regulators of the death receptor apoptotic pathway. FADD is a central adaptor molecule for Fas and forms a complex with Fas and pro-caspase-8 (27). Once caspase-8 is released from the complex in an active form to transmit death signals to downstream caspase family members, such as caspase-3, apoptosis is induced. We found that the knockdown of DcR3 expression upregulated the expression of FADD, caspase-3 and caspase-8. These results further demonstrate that silencing DcR3 expression modulates cell apoptotic regulators, thus triggering cell apoptosis.

Pancreatic carcinoma is insensitive to radiotherapy and chemotherapy and anticancer therapy remains a major clinical challenge. Gene therapy has provided us with an alternative approach for the treatment of human cancer. The data presented in this study suggest the potential use of DcR3 as a therapeutic target. To the best of our knowledge, the anticancer effects of silencing DcR3 expression in vivo have not been reported to date. Our experiments on animals indicated that LV-RNAi/DcR3 suppressed the growth of pancreatic carcinoma xenografts in nude mice. The blocking of DcR3 may prove to be an effective therapeutic strategy, by supporting T lymphocytes and NK cells, which express FasL, to kill tumor cells.

In conclusion, this study demonstrates that DcR3 is over-expressed in pancreatic carcinoma tissues and serum. Silencing DcR3 by lentivirus-mediated DcR3 RNAi enhances the effects of FasL and inhibits tumor growth in vitro and in vivo. These findings indicate that DcR3 may be a potential therapeutic for the gene therapy of pancreatic carcinoma.

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