Original Article

Knock-down of B7-H3 by small interfering RNA promotes gemcitabine-induced apoptosis in pancreatic cell line Patu8988t

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Abstract: Objective: To investigate the effect of B7-H3 on the growth of Patu8988t. Methods: According to expression of B7-H3 detected by RT-PCR and Western blot, the optimal B7-H3 siRNA was been used in the following experimental procedures. Patu8988t were divided into blank group, negative control siRNA group, B7-H3 siRNA group, gemcitabine group, gemcitabine + negative control siRNA group and gemcitabine + B7-H3 siRNA group. CCK-8 kits were used to detecte the survival and growth inhibition of Patu8988t in different groups. Hoechst 33342 and Annexin V/PI assay were used to investigate the effect of B7-H3 on apoptosis of Patu8988t. Expression of caspase-3, caspase-8, caspase-9, Bcl-2, and Bax mRNA were detected by RT-PCR. Results: the B7-H3 siRNA 4# sequence has a highest inhibition rate and were used in the following experimental procedures. A more inhibitory effect of gemcitabine on B7-H3 siRNA treated Patu8988t cells was found in the CCK-8 kit assay. Significant higher expressions and activities of caspase-3, caspase-8 and caspase-9 were observed in B7-H3 siRNA treated cells, when incubated with gemcitabine. Conclusions: B7-H3 RNAi-mediated gene silencing enhance the sensitivity of gemcitabine in pancreatic cancer Patu8988t cells markedly, and promotion of apoptosis in Patu8988t cells might be the major mechanism.

Keywords: B7-H3, RNA interference, gemcitabine, pancreatic cancer, apoptosis

Introduction

As a highly fatal disease, pancreatic cancer is commonly seen in the department of gastroenterology, among the worst malignancies whose average 5-year survival is a dismal 4% [1]. Pancreatic cancer is often diagnosed in an advanced state, and is limitedly respond to current treatments, which result in an exceptionally poor prognosis, though medical and surgical therapy has been being improved for decades [2]. Now gemcitabine is used as the standard chemotherapy for pancreatic cancer, but the resistance to gemcitabine result in unsatisfactory response rate. Therefore, an efficient therapy of this disease is of great importance. New approaches, including gene therapy, are required to improve treatment results. B7-H3, a recently discovered B7 family member, is well-documented as a regulator in the T cell-mediated immune responses [3]. Many studies have been found that B7-H3 express in many types of human cancer, while high expression of B7-H3 is correlated with a poor outcome in patients. In the study, we aimed to investigate the effect of silencing B7-H3 on pancreatic carcinoma cell line Patu8988t with or without gemcitabine, and the underlying mechanisms.

Materials and methods

Cell culture and transfection

Human pancreatic cancer cell line Patu8988t was kindly gifted by Laboratory of Cellular and Molecular Tumor Immunology of Soochow University, and were cultured in RPMI-1640 (Gibco, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified incubator at 37°C in 5% CO₂. The when the coverage rate
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reached 50% to 70%, cells were cultured with serum-free medium for transfection. The sequences of siRNA applied in the experiment are shown in Figure 1. siRNA and RNAi-mate transfection reagents (Shanghai GenePharma Co., China) diluted in serum-free medium (μM). 8 μl RNAi-mate transfection reagents (Shanghai GenePharma Co., China) was added, and oscillated. The solution was placed at room temperature for the formation of siRNA/RNAi-Mate complexes. The obtained solution was added into the pores for 6-hour culture. The medium was then replaced with serum medium.

Cell viability assay

Patu8988t cells were plated in 100 μl medium per well in 96-well plates, blank and zero wells were set. One day after seeding, cell viability was measured with Cell Counting Kit-8 (Peptide Institute Inc., Osaka, Japan) at 24 h, 48 h and 72 h after transfection for 2-hour culture at 37°C, and the survival rate and inhibition rate were calculated. The OD value at the wavelength of 490 nm was detected using an enzyme-labeled analyzer. The cell survival rate was calculated based on the formula: the survival rate = (the OD value of the experimental group/the OD value of the blank group) × 100%.

Hoechst staining

At 48 h after the transfection of siRNA, Patu8988t cells (treated with or without gemcitabine (20 μmol/L)) were fixed for 10 min in 4% (v/v) paraformaldehyde, and then incubated with Hoechst 33342 dye (Sigma Aldrich, St. Louis, MO, USA) (10 μg/ml) for 10 min. After washed with PBS, cells were observed using an inverted fluorescence microscope (IX70; Olympus, Tokyo, Japan).

Annexin V/PI assay

Patu8988t cells were plated in six-well plate. Cells of blank group, negative control siRNA group, B7-H3 siRNA group, gemcitabine group (10 μmol/L), gemcitabine + negative control siRNA group and gemcitabine + B7-H3 siRNA group, after 48 h incubation were collected, washed in cold PBS twice and then the cells (the cell density was adjusted to 1 × 10⁶/ml) were mixed in 100 μl of 1 × binding buffer and incubated with an Annexin V/PI double staining solution (5 μl FITC Annexin V and 5 μl PI) (Sigma-Aldrich, USA) at room temperature for 15 min. The stained cells were analyzed by flow cytometry and the percentage of apoptotic cells were calculated with Mod Fit LT software (Verity Software House, Topsham, ME, USA). The percentage of apoptotic cells were calculated as mentioned before.

RT-PCR assay

Total RNA of Patu8988t cells was extracted, using Trizol reagent (Invitrogen, Carlsbad, CA, USA), and the first strand cDNA was synthesized with the Ex Script RT reagent kit (Takara Bio Inc., Shiga, Japan), using 2000 ng of RNA per 20 μl reaction and oligo (dT) primer. cDNA was then utilized in RT-PCR reactions for caspase-3, caspase-8, caspase-9, Bcl-2, and Bax. GAPDH was utilized as internal standard. The product was amplified in a reaction volume of 10 μl containing 1 μl RT product, 5 μl SYBR Premix Ex Taq II (5 ×), 0.2 μl ROX Reference Dye II (50 ×), and 20 pmol of each primer. PCR reactions were then amplified for 40 cycles in a 7900 HT Fast Real-Time PCR System (ABI, Palo Alto, CA, USA). Each cycle consisted of denatured for 1 min at 94°C, annealing for 1 min at 60°C, and polymerization of 2 min at 72°C. GAPDH was utilized as internal standard. Relative mRNA levels were normalized to GAPDH mRNA, and the fold change for each mRNA was calculated using the delta Ct method. Primers used in the PCR reaction are presented as below: B7-H3: forward: 5′-ATGAACAGACCACCAAGAUA-3′, backward: 5′-CCACGCACTGGAAA-3′; Caspase-3: forward: 5′-GCCGTTGGTACAGAAGCTG-3′, backward: 5′-GCAAAACGACACTGGATGAA-3′; Caspase-8: forward:
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5'-ATGTTGGAGGAAAGCAATCTG-3', backward: 5'-TGAGCCCTGCCTGGTGTCT-3'; Caspase-9: 5'-CCCCTCAACCCTCCTATC-3', backward: 5'-TCATCTGCTCCCTTCCA-3'; Bcl-2: forward: 5'-CAGCTGCACCTGACGCCCTT-3', backward: 5'-TCCTCCGTTATCCTGGATCC-3'; Bax: forward: 5'-GCCTCCGTATCTGACCGCCCT-3', Bcl-2: forward: 5'-GCCTCCGTTATCCTGGATCC-3', backward: 5'-ACCACCTGGTCTTGGATCC-3'; GAPDH: forward: 5'-CATGAGAAGTATGACAACCAGCCT-3', backward: 5'-AGTCCTTCCACGATCCAAAGT-3'.

Western blot analysis

Cells were collected after trypsinization and centrifugation, and then lysed for protein extraction. The protein concentration was measured by the enzyme-labeled BCA method. Total proteins were electrophoresed on a 12% SDS-PAGE gel, and were then transferred to a PVDF membrane. Membranes were blocked in 5% non-fat milk and probed with anti-B7-H3 monoclonal antibody (1:200; Santa Cruz, CA, USA). The blots were incubated with 1:1000 diluted anti-mouse HRP-conjugated secondary antibody (Santa Cruz, CA, USA). Immunecomplex was detected using an ECL detection kit (Beyotime Institute of Biotechnology, Jiangsu, China). β-actin (Santa Cruz, CA, USA) was used as an internal standard.

Evaluation of caspase-3, caspase-8 and caspase-9 activity

Caspase-3, caspase-8 and caspase-9 activities were detected in cell extracts using caspase-3, caspase-8 and caspase-9 colorimetric assay kits, respectively. Enzymatic activities of the three caspases in cell extracts from mice treated only by NS were also detected as controls. The kits were used as recommended by the manufacturer (Jiancheng, Bioengineering Institute, Nanjing, China).

Statistical analysis

Data were expressed as mean ± standard error (SE) and were analyzed using SPSS PC version 18.0 (SPSS Inc, Chicago, Ill, USA). Statistical analysis was performed using one-analysis of variance (ANOVA) followed by SNK tests as post hoc test. Kruskal-Wallis test was used to evaluate the differences of categorical values followed by Mann-Whitney U tests as post hoc test. The criterion of significance was a P value of less than 0.05.

Results

Transfection of B7-H3 siRNA

In our pilot study, expression of B7-H3 was analysed by flow cytometry (FCM). FCM assessment showed that B7-H3 was expressed in the pancreatic carcinoma cell line Patu8988t (Figure 2). Patu8988t cells were transfected with the negative control siRNA and RNAi-mate transfection reagents at a final concentration of 10 nmol/L, 20 nmol/L and 40 nmol/L. 6 h after transfection, transfected cells were observed under the microscope. The highest transfection efficiency rate was found in the 40 nmol/L group, and such concentration was utilized in the following procedures. Compared with the blank group, the negative control siRNA treated group did not show influences on the B7-H3 mRNA and protein expression in Patu8988t after 24 h or 48 h transfection. In siRNA 4# groups, B7-H3 mRNA expression were not affected 24 h after transfection, but decreased 48 h after transfection, compared with that in the negative control group. Western blot analysis showed that B7-H3 protein expression reduced in siRNA 4# groups, but siRNA 3# group had the lowest inhibition rate, while the...
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The highest inhibition rate was observed in siRNA 4# group. Thus the B7-H3 siRNA 4# sequence were used in the following experimental procedures and the results are shown in Figure 3.

Figure 3. The transfection of B7-H3 siRNA into Patu8988t cells. A. The transfection efficiency of B7-H3 siRNA at the concentration of 40 nmol/L into Patu8988t cells: a, c. Observation of the same visual field under a regular microscope; b, d. Observation under a fluorescence microscope (a, b × 200; c, d × 400). B. The influence of B7-H3 siRNA on the B7-H3 mRNA expression in Patu8988t cells. C, D. Downregulation of the B7-H3 protein expression by B7-H3 siRNA in Patu8988t cells. Data were shown as mean ± SE.

Figure 4. B7-H3 siRNA promotes the antitumor effect of gemcitabine in Patu8988t. A. B7-H3 siRNA transfection alone did not affect the growth of Patu8988t. B. B7-H3 siRNA transfection could promote the growth inhibitory effect of gemcitabine against Patu8988t. Data were shown as mean ± SE. *P < 0.05, compared with gemcitabine group and gemcitabine + negative control siRNA group.
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B7-H3 siRNA promotes the antitumor effect of gemcitabine in vitro (Figure 4).

B7-H3 siRNA promotes gemcitabine-induced apoptosis in vitro

The apoptotic frequency of Patu8988t cells transfected with B7-H3 siRNA was similar to that of cells treated with equivalent dose of negative control siRNA and blank group, and no obvious apoptotic cells were detected in these three groups without gemcitabine incubation. After the incubation with gemcitabine (10 μmol/L) for 48 h, typical apoptotic morphological changes were found in the Patu8988t cells, including shrinkage, deformation, and detachment after the incubation with gemcitabine (10 μmol/L) for 48 h, and more apoptotic cells were found after B7-H3 siRNA transfection, compared with gemcitabine + negative control siRNA group and gemcitabine group. B. Apoptotic cell percentage was calculated. Data were shown as mean ± SE. *P > 0.05, compared with gemcitabine group; #P < 0.05, compared with gemcitabine group and gemcitabine + negative control siRNA group.

**Figure 5.** Apoptosis of Patu8988t after B7-H3 siRNA transfection. A. Hoechst 33342 staining of Patu8988t. No obvious apoptotic cells were detected in these three groups without gemcitabine incubation. Typical apoptotic morphological changes were found in the Patu8988t cells, including shrinkage, deformation, and detachment after the incubation with gemcitabine (10 μmol/L) for 48 h, and more apoptotic cells were found after B7-H3 siRNA transfection, compared with gemcitabine + negative control siRNA group and gemcitabine group. B. Apoptotic cell percentage was calculated. Data were shown as mean ± SE. *P > 0.05, compared with gemcitabine group; #P < 0.05, compared with gemcitabine group and gemcitabine + negative control siRNA group.

**B7-H3 siRNA promotes the antitumor effect of gemcitabine in vitro**

We measured the survival and growth inhibition of Patu8988t cells with or without B7-H3 siRNA transfection, using the CCK-8 kit assay. The results showed that the proliferation and survival was not affected by B7-H3 siRNA during the observation period. The proliferation of Patu8988t cells was significantly inhibited 48 h after gemcitabine treatment, and the negative control siRNA did not impact on the inhibitory effect of gemcitabine. However, a more inhibitory effect of gemcitabine on Patu8988t cells was found after B7-H3 siRNA transfection, indicating that B7-H3 siRNA could promote the antitumor effect of gemcitabine in vitro (Figure 4).
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did not show such an effect (Figure 6; Table 1). RT-PCR analysis showed the mRNA of caspase-3, caspase-8 and caspase-9 increased after gemcitabine treatment, and B7-H3 siRNA could promote the upregulation. But B7-H3 siRNA did not affect the downregulation of Bcl-2 mRNA and the upregulation of Bax mRNA induced by gemcitabine (Figure 7). Similarly, the activities of caspase-3, caspase-8 and caspase-9 were not obviously affected after the transfection of B7-H3 siRNA. However, significant higher activities of caspase-3, caspase-8 and caspase-9 were observed in B7-H3 siRNA treated cells, when incubated with gemcitabine, compared with the cells treated with gemcitabine alone (Figure 8). Taken together, these data indicated that silencing of B7-H3 could increase the antitumor activity of gemcitabine by promoting apoptosis in the pancreatic cancer cell line.

Table 1. Impact of B7-H3 siRNA transfection on viability of Patu8988t cells (Annexin V/PI assay)

<table>
<thead>
<tr>
<th></th>
<th>Viable cells</th>
<th>Early apoptosis</th>
<th>Late apoptosis</th>
<th>Necrosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>94.67%±0.51%</td>
<td>3.53%±0.21%</td>
<td>0.63%±0.15%</td>
<td>1.16%±0.21%</td>
</tr>
<tr>
<td>Negative control siRNA</td>
<td>94.60%±0.36%</td>
<td>3.20%±0.26%</td>
<td>0.70%±0.10%</td>
<td>1.50%±0.17%</td>
</tr>
<tr>
<td>B7-H3 siRNA</td>
<td>94.13%±0.21%</td>
<td>3.13%±0.40%</td>
<td>0.77%±0.06%</td>
<td>1.96%±0.67%</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>76.40%±1.04%</td>
<td>18.6%±0.76%</td>
<td>3.33%±0.21%</td>
<td>1.63%±1.53%</td>
</tr>
<tr>
<td>Gemcitabine + Negative control siRNA</td>
<td>74.60%±0.80%*</td>
<td>20.30%±1.08%*</td>
<td>3.73%±0.40%*</td>
<td>1.37%±0.15%</td>
</tr>
<tr>
<td>Gemcitabine + B7-H3 siRNA</td>
<td>67.67%±1.02%#</td>
<td>28.83%±0.95%#</td>
<td>4.03%±0.21%#</td>
<td>1.47%±0.15%#</td>
</tr>
</tbody>
</table>

Data were shown as mean ± SE. *P > 0.05, compared with gemcitabine group; #P < 0.05, compared with gemcitabine group and gemcitabine + negative control siRNA group.

Figure 6. Apoptosis of Patu8988t were measured by Annexin V/PI assessment. Annexin V/PI assay showed that no apoptosis existed in the three groups treated without gemcitabine. Obvious apoptosis of Patu8988t cells was observed in gemcitabine-treated cells, and B7-H3 siRNA transfection could significantly sensitize the apoptosis (especially the early apoptosis) after 48 h of gemcitabine incubation.

Figure 7. RT-PCR analysis showed the mRNA of caspase-3, caspase-8 and caspase-9 increased after gemcitabine treatment, and B7-H3 siRNA could promote the upregulation. But B7-H3 siRNA did not affect the downregulation of Bcl-2 mRNA and the upregulation of Bax mRNA induced by gemcitabine.
B7-H3 silencing promotes gemcitabine-induced apoptosis in pancreatic cell line P20881

Discussion

The co-stimulatory molecule B7-H3 is a novel member of the B7 ligand family, which could be induced to express in activated dendritic cells, monocytes and T cells and might play an important role in regulating T-cell responses in various conditions [4]. B7-H3 is often expressed in many types of human cancer, including kidney cancer, prostate cancer, lung cancer and pancreatic cancer as well [5-8]. Many studies have reported that the high expression of B7-H3 is positively correlated with a poor outcome in patients with these types of cancer mentioned above. But the role of B7-H3 in pancreatic cancer still remains unclear, as well as its underlying mechanisms. Therefore, we attempted to test the function of B7-H3 in the pancreatic cells in vitro. In our pilot study, we found that B7-H3 was highly expressed in the pancreatic carcinoma cell line Patu8988t. That was why we chose this cell line in our following experiment. As a phenomenon of posttranscriptional gene silencing, RNA interference has now been extensively applied in researching gene function identification and post-transcriptional control of gene expression, with the virtues of high performance and specificity [9-11]. Thus, RNA interference technique has provided a new way of gene therapies for various diseases, particularly for malignant tumors. Here, the cell growth and death of Patu8988t was detected after B7-H3 gene silencing using siRNA. In the current study, the mRNA and protein expression of B7-H3 was obviously inhibited 48 h after the designed B7-H3 siRNA transfection in Patu8988t cells. But, the B7-H3 siRNA treatment alone did not show direct effect on the growth inhibition of Patu8988t cells, while it promoted the antitumor effect of gemcitabine.

Gemcitabine is now used as the standard chemotherapy for pancreatic cancer, especially the rescetable ones. However, the median survival duration was less than 6 months following gemcitabine monotherapy, and the overall response rate was only 5.0%-11.0% [12]. Combination therapies including gemcitabine with other cytotoxic agents (such as irinotecan and oxaliplatin), could induced improved response rates over gemcitabine monotherapy [13, 14]. However, some trials have reported that these combinations had shown no significant survival benefits and no demonstration of improved efficacy for combination of gemcitabine with the cytotoxic agents, like exatecan and pemetrexed, and the targeted agent tipifarnib [15]. These poor outcomes indicate that more effective treatment strategies meet the urgent necessity for advanced pancreatic cancer. The development of resistance to gemcitabine may be a significant barrier to the effective therapy, and many cases resistant to gemcitabine have been reported. Thus, a better understanding of the molecular mechanisms of resistance to gemcitabine is essential to allow it to be used more effectively. Our present results showed that silencing of the co-stimulatory molecule B7-H3 by small interfering RNA technique could increases the sensitivity of gemcitabine in pancreatic cancer cell line Patu8988t.

Proapoptosis is thought to be a pivotal mechanism for a large number of chemotherapeutic agents [16, 17]. Due to numerous defects of the apoptosis machinery, pancreatic cancer shows, to a certain extent, resistance towards conventional oncological therapies. There are Figure 7. RT-PCR analysis. mRNA of caspase-3, caspase-8 and caspase-9 increased after 48 h of gemcitabine treatment, and B7-H3 siRNA could promote the upregulation. But the downregulation of Bcl-2 mRNA and the upregulation of Bax mRNA induced by gemcitabine were not affected by B7-H3 siRNA transfection. Data were shown as mean ± SE. *P < 0.05, compared with blank group; #P < 0.05, compared with gemcitabine group.
B7-H3 silencing promotes gemcitabine-induced apoptosis in pancreatic cell line P20882


two alternative pathways to initiate apoptosis and both finally activate the executioner caspases-3, -6 and -7. Intrinsic pathway (also called mitochondrial pathway) takes a key position by initiating apoptosis, and an imbalance of pro- and anti-apoptotic members of the BCL-2 protein family is involved in this process, which finally leads to the activation of the pro-apoptotic BCL-2 family members BAX and/or BAK and the perturbation of the integrity of the outer mitochondrial membrane [18, 19]. This induces the release of cytochrome c and other apoptotic regulators and, finally, the cleavage of the executioner caspases, like caspase-3. The other pathway is the extrinsic pathway (also called death receptor pathway) and is mediated by different death receptors on the cell surface, such as TNF-, FAS-(APO-1, CD95) and TRAIL-(TNF-related apoptosis inducing ligand) receptors. Activation of the receptors after extracellular binding of the specific ligands (TNF-α, FAS-L and TRAIL) initiates the recruitment of FADD (FAS-associated death domain protein), pro-caspase-8 and -10 to the death domain, which are forming the DISC (death inducing signaling complex), and then the initiator caspase-8 is activated. The extrinsic pathway in pancreatic carcinoma cells needs the signal enhancing-effect of mitochondria to induce apoptosis. Then, caspase-8 cleaves the pro-apoptotic BH3-only BCL-2 family member BID, which translocates to the mitochondrial membrane and induces the release of apoptogenic factors from the mitochondria. Our result showed that knock-down of B7-H3 by siRNA transfection could promote gemcitabine through increasing the activity of caspase-3, -8 and -9, but it did not affect the balance of Bcl-2/Bax, indicating that B7-H3 may not function on the mitochondrial pathway, but death receptor pathway, and the related mechanisms need further investigation.

In conclusion, B7-H3 maybe closely correlated with the apoptosis of pancreatic cancer cell line Ptu8988t. It could promote the antitumor effect of gemcitabine by facilitating gemcitabine-induced apoptosis and may serve as an effective target for gene therapy.

Disclosure of conflict of interest

None.

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Figure 8. Evaluation of Caspase-3, Caspase-8 and Caspase-9 activity. The activities of caspase-3, caspase-8 and caspase-9 were not obviously affected in those three groups without gemcitabine. While, higher activities of caspase-3, caspase-8 and caspase-9 were observed in B7-H3 siRNA treated cells after 48 h of gemcitabine treatment. Data were shown as mean ± SE. *P > 0.05, compared with gemcitabine group; **P < 0.05, compared with gemcitabine group and gemcitabine + negative control siRNA group.
References


