Effects of a human plasma membrane-associated sialidase siRNA on prostate cancer

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Abstract: Human plasma membrane-associated sialidase (Neu3) mainly localized in plasma membranes and plays crucial roles in the regulation of cell surface functions. We investigated the effects and molecular mechanisms of Neu3 on cell growth and apoptosis in vivo and in vitro in this study. Initially, we found the levels of Neu3 expression were higher in prostate cancer tissues and cell lines than in normal prostate tissues. We then applied a Neu3 siRNA approach to block Neu3 signaling using PC-3M cells as model cells. PC-3M cells transfected with Neu3-specific short hairpin RNA plasmid inhibited cell proliferation and induced apoptosis significantly. Knocking down neu3 decreased the expression of Bcl-2 and increased the expression of Bax, Caspase-3 in PC-3M cells. The experiments suggest that Neu3 is a promising molecular target for prostate cancer therapy.

Keywords: Neu3, siRNA, tunnel, apoptosis, Attenuated Salmonella

Introduction

Cancer, or uncontrolled cellular proliferation, can result either from mutations that “turn on” and over express the oncogenes that stimulate growth, or from mutations that result in loss of tumor suppressor genes and their products that inhibit growth [1, 2]. Prostate cancer is the second leading cause of cancer-related deaths among men in Western countries [3], and its incidence is increasing year by year in China [4]. The humanity urgently needs to explore effective therapeutic measures for prostate cancer. Human plasma-membrane-associated sialidase (Neu3) NEU3), specifically hydrolyzing gangliosides, plays crucial roles in the regulation of cell surface functions. is markedly up regulated in human cancers, leading to apoptosis suppression [5]. In these tumors, the expression level of Neu3 mRNA and protein in the tumor tissue significantly increased compared with the adjacent non-tumor tissue [6]. The level of Neu3 expression is higher; the tumor malignancy is higher [7]. It had been demonstrated that siRNA mediated reduction in sialidase NEU3 induces apoptosis in human cancer cells, but not normal cells [9]. Suppression of Neu3 expression could be a new and effective therapeutic approach for prostate cancer.

Materials and methods

Cell culture and tissue samples

PC-3M prostate cancer cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The cells were maintained at 37°C under 5% CO₂ in IMDM (Hyclone, Utah, USA) containing 10% (v/v) FBS (Hyclone, Utah, USA). Lipofectamine 2000 (Invitrogen, Carlsbad, CA) was used for transfection cells, which were harvested for plasma membrane purification or total RNA extraction. Human tissue samples were obtained from archival pathology specimens and all were made anonymous. Patient consents for use of tissue samples for research were obtained according to policies of the ethics committees of Jilin University and the China-Japan Union Hospital of Jilin University.
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Total RNA was isolated with Trizol (Invitrogen) according to the protocol provided by the manufacturer. Total RNA (0.6 μg) was transcribed in cDNA with the High Capacity cDNA reverse Transcription Kit (Applied Biosystems, Foster City, CA) in a reaction volume of 20 μl. Primer oligo-dT, a component of the Superscript III First-Strand Synthesis System (Invitrogen), was used to reverse-transcribe cDNA. After cDNA synthesis, 1 μl from the reaction volume was utilized for RT-PCR measurements. Relative Neu3 mRNA levels were calculated and compared between tumors and normal tissues or between siRNA and control-transfected cells respectively. All measurements were done in duplicate and the experiments were repeated in triplicate. PCR products were analyzed by electrophoresis on a 2% (w/v) agarose gel and visualized with ethidium bromide. Primers are shown in Table 1.

Western blot

For Western blot analyses, cells were harvested 48 hours after transfection. The cells were homogenized in RIPA lysis buffer (Reference TAKARA manufacture). After centrifugation at 7000 g for 25 minutes, the supernatant was analyzed for protein content using the Bradford reagent (Bio-Rad). After boiling at 100°C for 10 min, 50 μg of total protein was added to each lane. The proteins were separated by 10-12% SDS-PAGE and transferred onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The sheet was incubated in 5% nonfat milk for 1 h at room temperature and then exposed to the primary antibodies against Neu3 (kindly provided from Professor Taeko Miyagi, Miyagi cancer center, Japan) MMPs and β-actin (DAKO Biotech, Inc. Glostrup, Denmark) (diluted 1:1000) at 4°C overnight. The membrane were washed with TBST three times. The sheet was then incubated with secondary antibodies for 1 h at room temperature. The bands were visualized with either Horseradish peroxidase reaction or alkaline phosphatase reaction. The absorbance of each band in these Western blots was measured using densitometry using a Tannon Gis analysis System (Tannon, China). The Western blot assays were repeated in triplicate.

MTT assay

The viability of the transfected PC-3M cells was measured with the colorimetric MTT [3-(4, 5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide] assay as described before [8]. Briefly, PC-3M cells were inoculated in 96 well plates at 1×10^5 cells/well in IMDM containing 10% (v/v) FBS. After transfection, for each time point, 20 μl MTT (5.0 mg/ml) was added into each well and the cells were incubated at 37°C for 4 h. After incubation, 100 μl DMSO was added into each well, and must clean MTT solution and the samples were mixed well. The purplish-blue crystals were dissolved at room temperature. The absorbance (A) at λ = 570 nm was read on a Spectra Max plus 550 (BIORAD, USA).

FACS apoptosis assay

For quantification of apoptosis, cells were digested with 0.25% trypsin and 0.02% EDTA (1:1) at room temperature for 2-3 min. Cells were collected by centrifugation at 900 rpm for 6 min and then washed twice with cold PBS (pH 7.4). Subsequently they were resuspended in 400 μl binding buffer at a concentration of 1×10^6 cell/ml. 5 μl Annexin V-FITC was added and incubated for 15 min at 4°C in the dark. 10 μl PI was added to the tube and incubated at 4°C in the dark for 30 min. The cells were analyzed by flow cytometry (FACS alibur, Becton Dickinson, Franklin Lakes, NJ USA). Data acquired by CELL Quest (Becton Dickinson, NJ USA) was analyzed using the software ModFit LT that came with the machine.

Histochemistry and TUNEL assay

Blocks of tumor tissue were fixed in formalin, cut into 5-Am-thick slices, stained with hema-

| Table 1. The sequence of the primers |
|-----|-----|
| Primer | Sequence |
| GAPDH | 5'-GGGTGATGCTGGTGCTGATGT-3' |
| | 5'-AAGAATGGGAGTTGCTGTAAGTC-3' |
| Neu3 | 5'-CTGGTTCTTGGCTTTCCAGC-3' |
| | 5'-CTGGCACTATGGGGATCT-3' |
| Bcl-2 | 5'-GAGGATTGGGCGCTTT-3' |
| | 5'-CCACCGCTCGTTATCT-3' |
| BAX | 5'-TTTCACAGGCAACTCAGC-3' |
| | 5'-GGAGGAAGTCCAATGCAG-3' |
| Caspase-3 | 5'-TAACCAGGGCTCTGAGGTA-3' |
| | 5'-GTGGAAATTGATGCGATGT-3' |
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Figure 1. RT-PCR and Western blot analysis the expression of Neu3 and the effect of siRNA-mediated Neu3 silencing in PC-3M cells. A. Semi-quantitative RT-PCR analysis the expression of Neu3 in PC-3M cells, normal tissue and primary cancer tissue with 15 ug of total mRNA for each sample. GAPDH was amplified as a control. B. Quantified Neu3 mRNA levels from three separate experiments. C. Western blotting analyses. β-actin was amplified as a control. D. Quantified Neu3 protein levels from three separate experiments. E. Semi-quantitative RT-PCR analysis of the effect of siRNA-mediated NEU3 silencing in PC-3M cells. GAPDH was amplified as a control. F. Quantified Neu3 mRNA levels from three separate experiments. G. Western blotting analyses. β-actin was amplified as a control. H. Quantified Neu3 protein levels from three separate experiments.

As a negative control, rabbit immunoglobulins (Vector Laboratories, Burlingame, CA) were used to replace the primary antibody. Goat anti-rabbit IgG conjugated with horseradish peroxidase was used as a second antibody. Immunohistochemical staining was done manually at room temperature, using an avidin-biotin-peroxidase complex method (Vectastain Elite ABC kit; Vector Laboratories). The criteria for immunohistochemical assay results are as follows: positive cells contained brown particle staining in the nucleus or cytoplasm. Samples with <5% positive cells were designated as negative (−); samples stained slightly between 5% and 25% positive were designated as (+); samples stained moderately (between 25% and 50% positive) as (++), and stained deeply (>50% strongly positive) as (+++). TUNEL assay was performed with the in situ Cell Death Detection kit (Roche, Inc., Indianapolis, IN) instruction book. Results were recorded by confocal microscopic system (OLYMPUS, Japan).

Mouse models for human prostate cancer

Male BALB/c nudes, aging 6 weeks, were purchased from the Beijing Institute for Experimental Animals. All animals were housed and experiments were performed according to the guidelines established by Jilin University for the ethical use of animals in research. To generate tumor xenografts, PC-3M prostate cancer cells ($2\times10^6/100 \mu l$) were injected subcutaneously in the back of nude mice. The tumor mass was visible after 5 days. And it was stripped and cut into blocks of $1\times1\times1\ mm^3$ after 15 days. For analyzing the effect Neu3 to tumor, a sizing...
1×1×1 mm³ of tumor was implanted in lateral lobes of prostate gland of nude mice, respectively. After 5 days, the mice were randomized into four experimental groups (five mice per group). Animals were treated by intranasal as follows: (a) mock (PBS buffer alone), (b) AS (Attenuated Salmonella 1×10⁷ cfu/mouse), (c) pGCsi-Scramble (Attenuated Salmonella carrying pGCsi-Scramble vector 1×10⁷ cfu/mouse), (d) pGCsi-Neu3 (Attenuated Salmonella carry-
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Table 2. The weights of the tumors (Unit: gram)

<table>
<thead>
<tr>
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<th>Mock</th>
<th>AS</th>
<th>pGCsi-Scramble</th>
<th>pGCsi-Neu3</th>
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<tr>
<td>Tumor weight</td>
<td>0.71±0.15</td>
<td>0.65±0.10</td>
<td>0.65±0.16</td>
<td>0.35±0.08</td>
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Discussion

The application of gene transfer technologies has led to the development of new experimental approaches, such as inhibition of oncogene function and restoration of activity of tumor suppressor genes. Human plasma membrane-associated sialidase (NEU3) as a key enzyme for ganglioside degradation has been the focus of much research. In this study, we investigated the effects of Neu3 knockdown on the growth and apoptosis of prostate cancer cells.

Antitumor effects of Neu3 knockdown in vivo

To determine whether the transfected PGCsi-Neu3 recombinant plasmid could inhibit tumor growth using a xenograft tumor model, a sizing 1×1×1 mm³ of tumor was implanted in lateral lobes of prostate gland of nude mice. The animals were treated by intranasal as follows: (a) mock, (b) AS, (c) pGCsi-Scramble, (d) pGCsi-Neu3. After 45 days, mice were killed and final tumor weights and volumes were determined. In the pGCsi-Neu3 group, mice treated with siRNA-Neu3 had smaller tumor weight compared with mice treated with AS, pGCsi-Scramble and mock group (P<0.01, Table 2). Besides, the tumors of pGCsi-Neu3 group were paler and had fewer tumor vessels compared with the other groups (Figure 3A). In addition, we determined the effects of si-Neu3 on apoptosis by FACS and TUNEL assay and on cell proliferation by PCNA immunohistochemistry. FACS and TUNEL showed that tumors in the pGCsi-Neu3 group had undergone massive apoptosis and necrosis (Figure 3B, 3C). The apoptosis index was 42.3±2.8% in the pGCsi-Neu3 group, significantly higher than pGCsi-Scramble group (6.3±1.9%), AS group (6.3±1.9%) and mock group (5.9±1.7%), (P<0.01). Cells with cell nuclei brown staining were positive cells; the number of positive cells in pGCsi-Neu3 group was remarkable decreased compared with other groups (Figure 3D). Immunohistochemistry showed that the expression of Bcl-2 decreased in the pGCsi-Neu3 group, while the expression of Bax, and Caspase-3 increased in the pGCsi-Neu3 group (Figure 4A-C).

Statistical analysis

The significance of the differences between various samples was determined using Student’s two-tailed t-test. The significance of the differences between the median values of the data was determined using the two-tailed Mann-Whitney test. For all analyses, the level of significance was set at P<0.05. All statistical calculations were carried out using the SigmaStat statistical software package 10.0 (SPSS, Chicago, IL). Data are presented as the mean ± SE and represent three independent experiments.

Results

Overexpression of Neu3 in PC-3M cells and primary cancer tissue and the effect of siRNA-mediated NEU3 silencing in PC-3M cells

To determine whether Neu3 is overexpressed in primary cancer, we compared the levels of Neu3 expression in PC-3M cells, normal tissue and primary cancer tissue by RT-PCR and Western blot. The levels were measured by densitometric analysis. Quantitative evaluation of the relative expression of Neu3 revealed that mRNA and protein were overexpressed by an average of 2.7-fold in the 28 primary prostate tumors, PC-3M compared with normal prostate tissue (Figure 1B, 1D). Both methods revealed that Neu3 was overexpressed in cancer cell line and tissue compared with normal tissue (Figure 1A, 1C). RT-PCR and Western blot assay showed a similar and statistically significant decrease of the level of Neu3 after transfected PGCsi-Neu3 recombinant plasmids (P<0.01) (Figure 1E-G).

PGCsi-Neu3 recombinant plasmid inhibited cell growth, promoted cell apoptosis

We used a MTT assay to monitor the growth of transfected PGCsi-Neu3 recombinant plasmid cells. Transfected PGCsi-Neu3 recombinant plasmid cells showed significantly inhibited cell proliferation after 2 days in vitro (Figure 2A). (P<0.01). A further analysis of the flow cytometric data showed that PGCsi-Neu3 recombinant plasmid significantly promoted cell apoptosis compared with the Mock and PGCsi-scramble (Figure 2B).

Antitumor effects of Neu3 knockdown in vivo

To determine whether the transfected PGCsi-Neu3 recombinant plasmid could inhibit tumor growth using a xenograft tumor model, a sizing 1×1×1 mm³ of tumor was implanted in lateral lobes of prostate gland of nude mice. The animals were treated by intranasal as follows: (a) mock, (b) AS, (c) pGCsi-Scramble, (d) pGCsi-Neu3. After 45 days, mice were killed and final tumor weights and volumes were determined. In the pGCsi-Neu3 group, mice treated with siRNA-Neu3 had smaller tumor weight compared with mice treated with AS, pGCsi-Scramble and mock group (P<0.01, Table 2). Besides, the tumors of pGCsi-Neu3 group were paler and had fewer tumor vessels compared with the other groups (Figure 3A). In addition, we determined the effects of si-Neu3 on apoptosis by FACS and TUNEL assay and on cell proliferation by PCNA immunohistochemistry. FACS and TUNEL showed that tumors in the pGCsi-Neu3 group had undergone massive apoptosis and necrosis (Figure 3B, 3C). The apoptosis index was 42.3±2.8% in the pGCsi-Neu3 group, significantly higher than pGCsi-Scramble group (6.3±1.9%), AS group (6.3±1.9%) and mock group (5.9±1.7%), (P<0.01). Cells with cell nuclei brown staining were positive cells; the number of positive cells in pGCsi-Neu3 group was remarkable decreased compared with other groups (Figure 3D). Immunohistochemistry showed that the expression of Bcl-2 decreased in the pGCsi-Neu3 group, while the expression of Bax, and Caspase-3 increased in the pGCsi-Neu3 group (Figure 4A-C).
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of intensive study. It is markedly upregulated in colon cancers, leading to apoptosis suppression. Besides, it had been demonstrated that siRNA mediated reduction in sialidase NEU3 induces apoptosis in human cancer cells, but not normal cells [9]. We found Neu3 mRNA and protein overexpressed in prostate cancer compared with normal prostate tissues. To confirm further whether NEU3 directly affects prostate cancer, we employed siRNA-mediated silencing of NEU3 gene. The siRNA targeting NEU3 reduced mRNA level by 70% and the scrambled control showed no significant change of level, as estimated by reverse transcription-PCR and western blotting.

PC-3M cells were transfected with pGCsi-Neu3 expressing plasmid or the control vector by lipofectamine transfection. Cell proliferation was determined by MTT assay. We found the cell growth after transfection was inhibited obviously. According to the test results of MTT, we found After cell was transfected with pGCsi-neu3 plasmid for 48 h, the apoptosis rate were 37.2% by FCM measurement, whereas it were 0.5% and 0.8% in groups of single transfected

Figure 3. Antitumor effects of Neu3 knockdown in vivo. Tumor-bearing mice were treated with various plasmids. After 45 days, mice were killed. A. Inset images show the sizes of representative tumors. AS: Attenuated Salmonella typhimurium. B. A part of every tumor tissue was ground into single cell for FACS. C. TUNEL assay were analyzed by means of fluorescence microscopy. D. Histochemistry-PCNA, reduced tumor proliferation (reduced PCNA levels).
lipofectamine and control in respectively. Therefore, knockdown Neu3 induced growth inhibition and apoptosis in PC-3M cell line.

Apoptosis is an ordered process, numerous molecules and approaches are involved in its initiation. Bcl-2 family protein plays an important role in turning on or off of the mitochondrial permeability transition pore (MPTP). Turning on of MPTP is an important step of mitochondrial apoptosis. When the cell-stimulating factor stimulation received, pro-apoptotic gene Bax protein localized, targeted to the mitochondrial outer membrane from the cytoplasm, and directly with the voltage-dependent anion channel, and to improve channel activity.

Figure 4. Expression of Bcl-2, Bax, and Caspase-3 in vivo. A. Immunohistochemical analysis of Bcl-2 expression; B. Immunohistochemical analysis of Bax expression; C. Immunohistochemical analysis of Caspase-3 expression.
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the mitochondrial membrane permeability, release of kinds of pro-apoptotic factors such as cytochrome C etc, induce apoptosis. Casepase-3 is the direct executors of apoptosis. Therefore, we examined if the expression of these genes was altered by Si-Neu3. The expression of Bcl-2 was significantly knocked down in the presence of Si-Neu3 but not Si-Scramble (Figure 2C-F). Thus, the Neu3 siRNA decreases tumor cell survival.

As the complexity of the environment in vivo, differences in test results often appear between in vivo and vitro. To develop a convenient, we used a xenograft tumor model.

The discovery that genes vectored by bacteria can be functionally transferred to mammalian cells has suggested the possible use of bacterial vectors as vehicles for gene therapy. Bioengineered attenuated strains of Salmonella enterica serovar typhimurium (S. typhimurium) have been shown to accumulate preferentially >1,000-fold greater in tumors than in normal tissues and to disperse homogeneously in tumor tissues [10, 11]. These attenuated bacteria have been found to be safe in mice, pigs, and monkeys when administered i.v. [12-15], and certain live attenuated Salmonella strains have been shown to be well tolerated after oral administration in human clinical trials [16-18]. We choose attenuated Salmonella as vehicles. We next examined the tumorigenic properties of Si-Neu3 cells in vivo. BALB/c nudes (n = 5) were implanted with a sizing 1×1×1 mm³ of tumor into the upper flank. After 45 days, mice were killed and the tumor sizes and weights were measure. The weights of tumors from Mice treated with mock, AS, pGCSI-Scramble was 0.71±0.15 g, 0.65±0.10 g, 0.65±0.16 g, respectively (Table 2). Thus, the bacteria carrying pGCSI-Scramble did not significantly affect tumor growth any differently compared with the Salmonella vector alone. However, mice treated with Salmonella-pGCSI-Neu3 developed tumors with a median reduced weights of 0.35±0.08 g. The differences in tumor weights between Mock, AS, Salmonella-pGCSI-scamble versus Salmonella-pGCSI-Neu3 (P<0.01) were statistically very significant. In summary, tumor suppressive effect can be achieved with a single dose of bacteria transformed with a siRNA expression vector than those treated with Salmonella alone or Salmonella carrying Si-Scramble control.

The data shown in Figure 3B suggest that inhibition of tumor growth is due to activation of apoptosis. Tumors from mice treated with Mock, AS, pGCSI-Scramble or pGCSI-Neu3 were analyzed with TUNEL assays (Figure 3C). PGCsi-Neu3 treated tumors show massive apoptosis with sparsely dispersed chromatin, several TUNEL-positive cells, and some necrotic regions compared with the Mock, AS, PGCsi-Scramble control, which showed a finely granular cytoplasm with evenly dispersed chromatin and very fewer TUNEL-positive cells. Proliferating cell nuclear antigen is closely related to cell DNA synthesis and a good tumor marker. PCNA and tumor grade, tumor stage and chemotherapy sensitivity are closely related. Mock, AS, PGCsi-Scramble control treated tumors show PCNA labelling index was higher compared with PGCsi-Neu3 treated tumors. These data show that the Neu3 siRNA carried by Salmonella exerts a strong apoptotic antitumor effect in vivo. Ultimately, the Neu3 siRNA was found to be effective in suppressing prostate tumor growth in an in vivo xenograft mouse model via both growth inhibition and increased apoptosis.

In summary, we have presented evidence for the potential use of Neu3-specific siRNAs to block persistent Neu3 signaling, weaken tumor growth and promote tumor apoptosis in vivo and in vitro. Suppression of Neu3 expression could be a new and effective therapeutic approach for prostate cancer.

Disclosure of conflict of interest

None.

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