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Original Article

The down-regulation of SNCG inhibits the proliferation and invasiveness of human bladder cancer cell line 5637 and suppresses the expression of MMP-2/9

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Abstract: γ-synuclein (SNCG) is highly expressed in bladder cancer tissues and associated with tumor recurrence. However, the functional effect of SNCG on the development of bladder cancer remains unknown. In the present study, the effects of SNCG down-regulation by RNA interference (RNAi) on the proliferation and invasiveness of human bladder cancer cell line 5637 were explored. Three pairs of SNCG-specific small interference RNA (siRNA) were designed and transfected into the 5637 cell lines, and then the SNCG expressions in the three siRNA were assessed using reverse transcription-polymerase chain reactions (RT-PCR) and Western blot, while the cell proliferation and invasiveness of the 5637 cells were evaluated using cell counting kit-8 (CCK-8) and transwell assays, respectively. In addition, the expressions of matrix metalloproteinase-2 and -9 (MMP-2/9) were analyzed using enzyme-linked immunosorbent assays after the down-regulation of SNCG. The results showed that compared with the negative and empty vector controls, all three SNCG siRNAs were observed to significantly inhibit the SNCG expressions at the mRNA and protein levels ($P < 0.05$), among which the lowest SNCG expression was measured in SNCG-siRNA-244. And the SNCG suppression mediated by RNAi successfully inhibited the proliferation and invasiveness of the 5637 cell lines ($P < 0.05$), as well as the down-regulation of MMP-2/9. In conclusion, the proliferation and invasiveness of bladder cancer cells can be decreased by specifically interfering with the SNCG expression. And SNCG siRNA deserves further study as a novel target for biomedical therapy in bladder cancer.

Keywords: Synuclein-γ, bladder cancer, 5637 cell line, RNA interference

Introduction

Bladder cancer (BC) is one of the most common urinary tract malignancies worldwide [1, 2]. More than 90% of BC is urothelial carcinoma, which can be divided into non-muscle-invasive (NMIBC) and muscle-invasive (MIBC). At their initial presentation, about 30% of patients have MIBC and 70% have NMIBC [3]. Of the patients with MIBC, 50% have distant metastasis within 2 years, and 60% die within 5 years despite treatment [4-6]. Among the patients with NMIBC, the recurrence rate ranges from 50-70%, and 10-15% will progress to MIBC over a 5-year period [7]. Therefore, it is urgent to explore the pathogenesis of BC so as to develop effective methods for the early diagnosis and treatment of BC.

Synuclein-γ (SNCG) belongs to the family of synuclein proteins, and it is a soluble, small, and highly conserved neuronal protein. Since it was initially observed to be over-expressed in breast cancer tissues and related to the stage, SNCG was previously named breast cancer specific gene 1 (BCSG1) [8]. Currently, SNCG is widely studied in breast cancer as a tumor marker, and the associations between SNCG and a variety of other kinds of tumors such as ovarian cancer, liver cancer, lung cancer, gastric cancer, pancreatic cancer, prostate cancer and so on, have received increased attention [9-13]. As to BC, our previous study demonstrated that SNCG is highly expressed in BC tissues and is associated with tumor recurrence [14]. However, the functional effect of SNCG on the development of BC remains unknown.

Table 1. Sequences of the siRNA vectors

oligo	Sequences
oligo-178	5'-UCAAGAAGGGCUUCUCCAUTT-3' (sense) 5'-AUGGAGAAGCCCUUCUUGATT-3' (antisense)
oligo-244	5'-UGACGGAAGCAGCUGAGAATT-3' (sense) 5'-UUCUCAGCUGCUUCCGUCATT-3' (antisense)
oligo-388	5'-UCAACACUGUGGCCACCAATT-3' (sense) 5'-UUGGUGGCCACAGUGUUGATT-3' (antisense)
negative control	5'-UUCUCCGAACGUGUCACGUTT-3' (sense) 5'-ACGUGACACGUUCGGAGAATT-3' (antisense)

In the present study, the effects of SNCG down-regulation via RNA interference (RNAi) on the proliferation and invasion of the BC cell line 5637 was evaluated in vitro. It was observed that the specific down-regulation of SNCG was achieved at the mRNA and protein levels by this approach, and the proliferation and invasive capabilities of the cancer cells were significantly diminished. This result suggests that SNCG may be associated with the progression of BC, and SNCG suppression mediated by RNAi may become an effective therapeutic strategy for the control of BC.

Materials and methods

Cell lines, cell culture, and reagents

Human BC cell line 5637 was obtained from the GenePharma Biotech Co., Ltd. (Shanghai, China). All the cells were cultured in an RPMI-1640 medium (Gibco, Carlsbad, USA), and the media were supplemented with 10% fetal bovine serum (FBS; Invitrogen, Shanghai, China) at 37°C in a humidified atmosphere of 5% CO_2. This study was approved by the Institutional Review Board of Peking University Third Hospital.

siRNA for 5637

Based on the full SNCG cDNA sequence, three pairs of siRNA vectors targeting the SNCG gene and a negative control vector were designed and synthesized by GenePharma Biotech Co., Ltd. (Shanghai, China) as follows (**Table 1**). All the sequences were then constructed into 5637 cell lines by the transfection reagent siRNA-mate (GenePharma Biotech Co., Ltd., Shanghai, China).

RT-PCR assays

The total RNA of each of the SNCG RNAi-stable 5637 cell lines and the negative controls were extracted using Trizol reagent (Invitrogen Life Technologies). After extraction, the total RNA was reverse-transcribed into cDNA using a reverse transcription kit (Dingguo Changsheng Biotech Co., Ltd., Beijing, China) complying with the manufacturer's protocol. The primer sequences used in the RT-PCR were as follows: upstream primer, 5'-TGACCTCAGTGGCCGAGAA-3' and downstream primer, 5'-GCCTCACCCTCCTGTTGG-3' (178 bp). ACTB was used as the internal control and the primer sequences were upstream primer, 5'-CGTGGACATCCGCAAAGA-3' and downstream primer, 5'-GAAGGTGGACAGCGAGGC-3' (199 bp). The PCR amplification reaction was as follows: initial denaturation at 95°C for 3 min, then 40 cycles of (95°C for 30 sec, 62°C for 40 sec). The PCR products were analyzed using electrophoresis on a 2% agarose gel and were visualized under ultraviolet light. The SNCG mRNA expressions relative to the ACTB expressions were calculated using the 2-ΔΔCt method.

Western blot analyses

The total proteins of each of the SNCG RNAi-stable 5637 cell lines and the negative controls were extracted 48 h after the transfection, followed by the addition of a loading buffer. After being heated at 100°C for 10 min, the proteins were separated using 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Next, the proteins were transferred to polyvinylidene fluoride (PVDF) membranes for 30 min at 100 V and 70 min at 120 V. The membranes were then incubated with primary antibodies: rabbit anti-SNCG (1:200, #ab52633; Abcam, England), followed by the addition of the secondary antibody, horseradish peroxidase-conjugated goat anti-rabbit IgG (1:100000, #JIR 111-035-003; Jackson, USA). Then, the protein bands were revealed using an enhanced chemiluminescence (ECL) detection reagent, and the images were captured using Kodak X-Omat BT film. Finally, the absorbance was analyzed using the Gel-Pro analyzer software.

CCK-8 assay for cell proliferation

The cells were seeded in 96-well plates with a volume of 100 μl and incubated for 0, 24, 48, and 72 h. At the end of the incubation, 10 μl of CCK-8 reagent (Beyotime, Jiangsu, China) was

added to each well according to the manufacturer's instructions. The plates were then incubated at 37°C, under 5% CO_2 for 1 h. Finally, the absorbance was measured at the 490 nm wavelength.

Matrigel transwell assay for cell invasion

A transwell chamber (#3422, Corning, USA) was used to detect the cells' invasion abilities. The Matrigel (#356234, BD, USA) was put into a 4°C refrigerator overnight for melting. The next day, the Matrigel was diluted by 1:4 with serum-free medium, and 30 μl Matrigel was added to the upper chamber. Then, more than 100 μl cells were seeded in the upper chamber of each transwell insert; meanwhile, the RPMI-1640 medium containing 10% FBS was added to the lower chamber. The cells were then cultured at 37°C for 24 h to allow cell migration. In the end, the noninvasive cells in the upper chamber were gently removed with a cotton swab, and the invasive cells attached to the lower chamber were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. The cells were counted and photographed under an inverted microscope at 200× magnification.

Enzyme-linked immunosorbent assay

The cells were treated as described above. The concentrations of MMP-2 and MMP-9 in the cell culture supernatants were quantified using MMP-2 and MMP-9 ELISA kits (R&D Systems, USA). The testing of each sample was repeated 3 times. Briefly, the cellular supernatants were collected and centrifuged, then a total of 100 μl supernatants or standard samples or positive control samples were added into the 96 well plates and incubated for 1 h, followed by 100 μl enzyme-linked antibody incubation for 0.5 h at 4°C. After being washed 9 times with a washing buffer, the template was added and incubated for 0.5 h, followed by a H_2SO_4 termination reaction. The 450 nm absorbance was determined using a microplate reader.

Statistical analysis

All the experiments were independently repeated in triplicate. The data were expressed as the mean ± standard deviation (mean ± SD), and the differences among the means of the multiple groups were compared based on ANOVA, and T tests were used to compare the differences between the two groups. SPSS 18.0 was used for the statistical analysis.

Results

SNCG RNAi significantly inhibits SNCG mRNA expression

5637 cells transfected with four SNCG siRNA-expressing sequences (SNCG-siRNA-178, -244, -388 and negative control sequence) were constructed. After the corresponding stable cell lines were established, the SNCG mRNA expression was measured using RT-PCR and compared among the groups. The results demonstrated that the SNCG mRNA expressions in the 3 siRNA groups were lower than they were in the control groups, and the strongest effect was observed in the SNCG-siRNA-244 group ($P < 0.05$).

SNCG RNAi significantly inhibits SNCG protein expression

The SNCG protein expression levels were evaluated using Western blot. In accordance with the results for the SNCG expressions at the mRNA level, the SNCG protein expressions were also significantly reduced in the three SNCG siRNA groups, and the lowest expressions of the SNCG protein were observed in the SNCG-siRNA-244 group (**Figure 1**).

Down-regulation of SNCG via RNAi suppresses the proliferation of the BC cell lines

A CCK-8 assay was used to investigate the effects of the SNCG down-regulation via RNAi on the proliferation of the BC cell line 5637. As shown in **Figure 2**, the cell proliferation decreased significantly in the groups treated with siRNA-244 ($P < 0.05$), compared with the negative and blank control groups. The results demonstrate that SNCG siRNA can inhibit the proliferation of BC cells.

Down-regulation of SNCG via RNAi inhibits the invasiveness of the BC cell lines

The effect of SNCG down-regulation via RNAi on the invasiveness of BC cell line 5637 was evaluated using a chamber assay. As shown in **Figure 3**, the number of the migrated cells was determined to be lower in the SNCG siRNA-244

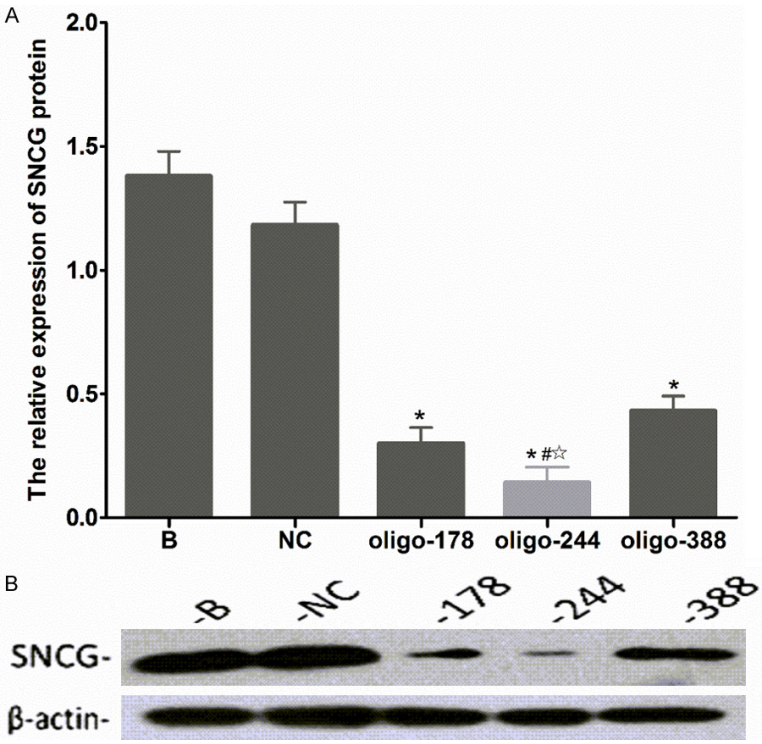

Figure 1. The expression of the SNCG protein in the siRNA-5637 cells. A. Gray quantitative; B. Protein expression. (*P < 0.05 vs. B and NC; #P < 0.05 vs. oligo-178; ☆P < 0.05 vs. oligo-388; B, blank control; NC, negative control).

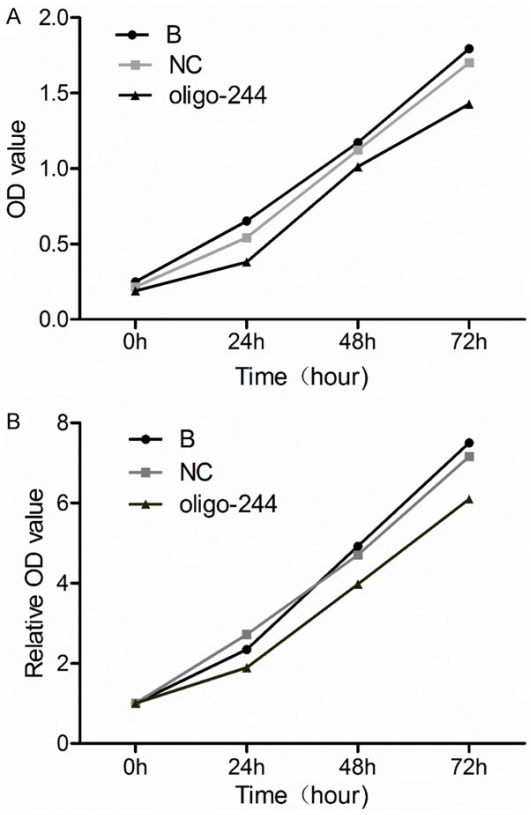

Figure 2. Cell growth analyzed using CCK-8 assays for the indicated time. A: The proliferation rate of cell line 5637 at a variety of times; B: The relative proliferation rate of cell line 5637 at a variety of times benchmarked against the rate of 0 hours. (*P < 0.05 vs. the control groups; B, blank control; NC, negative control).

group (P < 0.05), compared with the control groups. The result indicates that SNCG siRNA can suppress the migration of BC cells.

The effects of SNCG down-regulation on MMP-2 and MMP-9

We also assessed whether the SNCG knockdown in the 5637 cells had an effect on the protein expressions of MMP-2 and MMP-9. The results showed that the MMP-2 and MMP-9 proteins in the RANi group were significantly reduced compared to the blank control and negative control groups (P < 0.05).

Discussion

As the third member of the neuronal protein synuclein family, SNCG has been reported to be highly expressed in a variety of cancer types, including BC, but not in normal or benign cancer tissues [14, 15]. When over-expressed, SNCG may be associated with increased cancer cell proliferation and adverse outcomes in various solid tumors [16]. And several studies suggest that the suppression of SNCG expression may be an effective therapy in diversified cancer types [17, 18]. In the current study, we were interested in investigating the functional role of SNCG in BC.

RNA interference (RNAi) is a powerful genetic technology that can lead to gene silencing [19]. And as an ancient and highly conserved gene regulatory mechanism, RNAi has been extensively used in genetic research and therapy [20]. Several studies showed that RNAi can effectively reduce the expression of the gene of

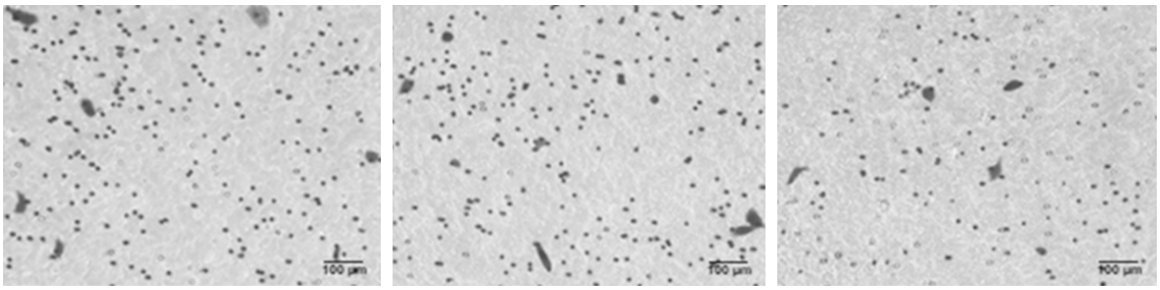

Figure 3. The effects of SNCG-siRNA suppression on the invasiveness of the 5637 cells. The number of migrated cells in the 5637 RNAi-244 group was much lower than it was in the blank control and negative controls.

interest [21, 22]. Therefore, RNAi technology was utilized in the current study. A series of SNCG siRNA sequences were designed and constructed into siRNA expression vectors and then introduced into 5637 cells. The results showed that all these small molecules were able to specifically and significantly suppress the SNCG expressions at the mRNA and protein levels.

Although advanced techniques for the early detection and treatment of BC are available, the mortality of BC has not changed markedly. Therefore, increased attention has been given to illuminating the molecular mechanisms involved in the development of BC. Recently, Zhang investigated the role of CCAT1 in the development of BC using the cell functional test method [23], which is consistent with the method used in our study. In our study, the CCK-8 and transwell chamber models were used to investigate the effect of the SNCG RNAi sequences on the proliferation and invasiveness of the BC cell lines. The results showed that the proliferation capability and the migration and invasion ability of the SNCG RNAi-transfected 5637 cells were significantly reduced, with the percentage as much as 30.88% in proliferation and 75.86% in invasiveness, respectively. This result suggests that the down-regulation of SNCG may be helpful in attenuating BC progression.

The mechanism by which SNCG regulates tumor cell growth was not further investigated in the current study, but it has been determined that SNCG takes part in several signaling pathways, including estrogen-receptor alpha36-mediated estrogen signaling [24], AKT/mTOR signaling [25], mitogen-activated protein kinases (MAPK) pathways [26], and microtubule regulation [27]. Based on these studies, we think that SNCG may play a role in the proliferation of bladder cancer cells via one of these known signaling pathways; however, this needs further investigation.

This study also showed that the secretions of MMP-2 and MMP-9 in the cell culture supernatants were relatively reduced in the group treated by RNAi. Two members of the MMPs family, MMP-2 and MMP-9 have been proved to be highly related to tumor progression and metastasis [28, 29]. It is well known that the members of the MMP family secreted by the invading tumor cells can degrade all the essential components of the extracellular matrix (ECM). ECM degradation is pivotal for tumor cell invasion. Thus, MMPs levels may effectively reflect the invasiveness of tumor cells in multiple cancers. Indeed, in the current study, we found that the inhibition of the MMP-2 and MMP-9 expressions is associated with the suppression of BC cell proliferation and invasion in vitro after the knockdown of SNCG. Nevertheless, the underlying mechanism by which SNCG regulates the MMP-2 and MMP-9 expressions in BC cells is unknown and warrants further study.

Our study has some limitations that should be noted. First, only one BC cell line - 5637 - was used due to the low expression of SNCG in the other three BC cell lines. Moreover, the effect of the over-expression of SNCG on the proliferation and invasion of bladder cancer cell line 5637 was not examined. Second, the study was done only in vitro, and if it were confirmed in vivo, the result would be more persuasive. Last but not least, the mechanism of how SNCG affects the proliferation, migration, and inva-

sion of bladder cancer was not further explored in our study. Despite these drawbacks, our present study preliminarily presents the functional role of SNCG in BC, which lays the foundation for our future work.

Disclosure of conflict of interest

None.

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