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SNCG gene silencing in gallbladder cancer cells inhibits key tumorigenic activities

Shenghua Han[1,2], Feifei She[2,3], Dong Wang[1],Xiangqing Yao[1], Lei Jiang[1],Yanling Chen[1,2]

[1]Department of Hepatobiliary Surgery, Union Hospital, Fujian Medical University, Fuzhou 350001, China, [2]Key Laboratory of Ministry of Education for Gastrointestinal Cancer, Fujian Medical University, Fuzhou 350001, China, [3]Research Center for Molecular Medicine, The Key Laboratory of Infection and Oncology of Universities in Fujian Province, Fujian Medical University, Fuzhou 350004, China

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1. ABSTRACT

We recently determined that synuclein-gamma (SNCG) is highly expressed in human gallbladder cancer (GBC), and its abnormal expression is associated with tumor aggressiveness. To investigate the effects of SNCG gene silencing on the tumorigenic profiles of the GBC cell line, NOZ, short-hairpin RNA (shRNA) interference was employed. Specifically, the SNCG transcript was targeted by SNCG-shRNA lentiviral particles designed to silence SNCG gene expression. Following selection of NOZ cells stably expressing SNCG-shRNA, SNCG expression was examined by western blot and semi-quantitative RT-PCR analyses. Phenotypic hallmarks of gallbladder carcinogenesis were assayed by CCK-8, soft agar (colony formation), modified Boyden-Chamber (invasion), and flow cytometry (cell-cycle and apoptosis) assays. Our results showed that SNCG gene silencing in NOZ cells inhibited cell growth, colony formation, and invasion. In addition, it directly increased the effectiveness of paclitaxel in inducing G2/M cell-cycle arrest and cell apoptosis. Data from our *in vivo* study showed a decrease in tumor growth and weight in mice injected with SNCG-silenced NOZ cells. Together, these findings suggest that SNCG plays an important role in the progression of GBC.

2. INTRODUCTION

Primary gallbladder cancer (GBC) is highly invasive, as it can metastasize in its early stages to distant organs such as the lung and the liver, by either lymphatic or hematogenous spread. Early GBC has no specific symptoms or clinical manifestations, thus making it a highly malignant carcinoma. In addition, this disease has a poor prognosis and high mortality rate. Thus, it is imperative that new therapeutic targets for GBC are identified.

Synuclein-gamma (SNCG) is a member of the synuclein family of proteins, which consisting of alpha-, beta-, and gamma- synuclein. These proteins are highly conserved and are strongly expressed in nervous tissues (1). Synuclein-alpha and synuclein-beta, are predominantly expressed in the central nervous system, and are localized to presynaptic terminals. They participate in the development and function of the central nervous system, and may be important in the etiology and pathogenesis of neurodegenerative disorders, such as Alzheimer's disease and Parkinson's disease (2–6) . SNCG is a cytoplasmic protein that is localized to the perinuclear area and centrosomes in several types of human interphase cells. It is

expressed at low levels in the brain and peripheral nervous system, including the dorsal root ganglia and trigeminal ganglia (7-10). During murine embryonic development, SNCG is expressed only in primary sensory neurons and motor neurons. SNCG mRNA expression increases in the aging mouse cerebral cortex, and the corresponding protein also accumulates with age (11).

The SNCG gene (also referred to as breast cancer-specific gene 1 [BCSG1]) was initially cloned from advanced infiltrating breast cancer cells using the expressed sequence tag-based differential cDNA sequencing approach (12). SNCG maps to chromosome region 10q23. It is composed of five coding exons, and is transcribed into an mRNA of about 1 kb that encodes a 127 amino acids polypeptide (1). SNCG expression is usually tissue-specific and restricted to brain tissue and presynaptic terminals (13). However, this tissue specificity is lost during breast and ovarian cancer disease progression. SNCG is abnormally expressed in a high percentage of advanced and metastatic breast and ovarian cancers, but not in normal or benign tissues (14). Furthermore, over-expression of SNCG can stimulate proliferation, and induce invasion and metastasis of breast cancer cells (15). SNCG has also been shown to compromise normal mitotic checkpoint controls, resulting in multinucleation as well as faster breast cancer cell growth (16, 17). In addition to tumors of the breast and ovary, Liu found that the SNCG protein is also abnormally expressed in a high percentage of other carcinomas as well, including tumor tissues of liver, gastric, lung, prostate and cervical cancer; it is rarely expressed in tumor-matched non-neoplastic adjacent tissues (NNAT) (18). Interestingly, the opposite phenotype was found in esophagus cancer. Zhou found low expression levels of SNCG in human esophageal squamous cell carcinoma (ESCC), and the biological effects of SNCG over-expression on ESCC cells suggested that SNCG might play a role as a negative regulator in the development of human ESCC (19). Therefore, further study in cancer tissues and cultured cell lines is needed to understand the roles of SNCG in the development of other human neoplastic diseases.

There is currently little known about the functional and mechanistic roles of SNCG in GBC development and progression. In our present study, we were thus interested in determining if there is a functional correlation between SNCG expression and the biologic behavior of GBC. To address this, we used lentivirus-mediated short hairpin RNA (shRNA) interference to silence SNCG in the NOZ human GBC cell line, and investigated the subsequent tumorigenic effects.

3. MATERIALS AND METHODS

3.1. Cell lines

The following three human GBC cell lines were obtained and cultured according to the supplier's instructions (20): 1) GBC-SD (Shanghai cellbank of CAS, Shanghai, China), 2) SGC-996 (Tumor Cytology Research Unit, Medical College, Tongji University, Shanghai, China), 3) NOZ (Health Science Research Resources Bank, Japan). Briefly, GBC-SD and SGC-996 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% calf bovine serum. NOZ/shSNCG cells stably expressing SNCG-shRNA, and the corresponding control cell lines NOZ/shCTRL, will be described later. NOZ cells (NOZ/parental) were cultured in Williams E medium supplemented with 10% fetal bovine (FBS) and 2 mmol/L L-glutamine (Invitrogen, Carlsbad, CA). All cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2.

3.2. Establishment of NOZ cells stably expressing SNCG-shRNA

The target-specific 59-bp sequence designed to silence SNCG gene expression, was inserted into the lentiviral vector (Plvx-shRNA) through BamH1 and EcoRI restriction sites. The resulting vector, SNCG-shRNA, was confirmed by sequencing. The empty lentiviral vector (shCTRL) was used as negative control, and did not cause degradation of any known cellular mRNA. A day prior to transfection, 1.5×10^4 logarithmically growing and healthy NOZ cells were split into three equal groups, each plated in a 6-well plate to achieve 70–80% confluency by the next day. Both the empty lentiviral vector, and the lentiviral vector encoding SNCG-shRNA were transfected into NOZ cells. Stable cell lines expressing SNCG-shRNA were isolated via selection with puromycin (2μg/ml). Stable cells were expanded, harvested, and prepared for western blot and semi-quantitative RT-PCR analyzes.

3.3. Semi-quantitative RT-PCR

Cultured cells were washed twice with phosphate buffered saline (PBS) and harvested. Total cellular RNA was isolated from GBC-SD, SGC-996, NOZ cell lines, and selected shRNA clones by Trizol Reagent (Invitrogen, Carlsbad, CA) The oligo (dT)-primed first-strand cDNA was synthesized from 1 μg total RNA using the PrimeScript RT-PCR Kit (Takara Co., Ltd. Japan). Primers are as follows: upstream primer: 5'-TGTGGTGAGCAGCGTCAACACT-3'; downstream primer: 5'-TTGGATGCCACACCCTCCTGTT-3' for SNCG (132 bp). A 500 bp beta-actin fragment was amplified as a control: upstream primer: 5'-GGC ATG GGT CAG AAG GAT TCC-3'; downstream primer: 5'-ATG TCA CGC ACG ATT TCC CGC-3'. PCR involved 40 cycles of pre-denaturation at 94 °C for 3 min, denaturation at 94 °C for 30 sec., annealing at 60 °C for 40 sec., and extension at 72 °C for 30 sec., with a final extension at 72 °C for 10 min. The final PCR products were analyzed by agarose gel electrophoresis.

3.4. Western blot analysis

The expression of SNCG in GBC-SD, SGC-996, NOZ (NOZ/parental), NOZ/shCTRL and NOZ/shSNCG cells was analyzed by western blotting. Briefly, cells were harvested on ice with RIPA buffer (50mM Tris, pH7.2, 150mM NaCl, 0.1% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 1% NP-40, 2mM EDTA, 50mM NaF, 1mM PMSF, complete protease inhibitor cocktail [Roche]). Protein concentration was determined using the BCA protein assay kit (Beyotime, Jiangsu, China). Proteins (35μg) were solubilized with sample buffer and heated at 95 °C for 3 min. Samples were then run on a 12%

acrylamide gel (Biorad) and electrophoretically transferred to a PVDF membrane (Millipore). The membrane was blocked for 1 h in TBST (TBS and 0.1% Tween 20) containing 10% milk, and then incubated for 16 h with a 1:250 dilution of rabbit anti-SNCG antibody (Sigma) or rabbit anti-cleaved PAPR antibody (cell signaling). The membrane was then washed 3× with TBST (0.4% Tween 20) and incubated with alkaline phosphatase-conjugated goat anti-rabbit IgG (Santa Cruz) at a 1:2000 dilution for 2 h. Following 3 more washes, the membrane was incubated with the CDP-star detection reagent (Roche) for 5 min at room temperature, and then exposed to X-ray film. Film images were scanned using an Image Scanner (Amersham Biosciences). The membrane was re-probed with a 1:2000 dilution of beta-actin monoclonal antibody (Sigma), followed by incubation with AP-conjugated goat anti-mouse IgG (Santa Cruz) at a 1:2000 dilution. The rest of the procedure was the same as noted above. All Western blots are representative of 3 independent experiments.

3.5. Cell proliferation analysis

Cell proliferation of NOZ/shCTRL, NOZ/shSNCG and NOZ/parental was determined using the Cell Counting Kit-8 (CCK-8) assay (Dojindo, Japan). Briefly, cells were seeded in 96-well plates at a density of 5×10^3 cells/ well in 100 μl medium supplemented with 10% FBS, and incubated at 37 ˚C and 5% CO_2. Cells were cultured for 24 h, 48 h, 72 h, 96 h or 120 h. Following each time point, 10 μL of the CCK-8 solution was added to each well of the microplate, and the absorbance at 450 nm was measured using a microplate reader (Quant, Bio-Tek, USA) after a 1 h incubation. Assays were done in triplicate and 3 separate assays were conducted.

3.6. Colony formation assay

Cells (1000/well) were suspended in medium containing 0.35% agar, and seeded into 6-well plates that were overlaid with 0.5% agar. The agar-containing cells were allowed to solidify at room temperature. Cells then grew undisturbed for 15 days at 37˚C in 5% CO_2. Visible colonies (more than 10 cells) were counted with the aid of a dissecting microscope. 3 separate assays were conducted.

3.7. Transwell cell invasion assay

Boyden chambers with 8 μm polycarbonate membranes in 24-well dishes (BD, USA) were used for the invasion assay. The chambers were coated with serum-free Williams E medium reduced Matrigel (21.5μg; BD) in facies medialis, and serum-free Williams E medium reduced Fibronectin (5μg; BD) in facies lateralis. Cells (3×10^4) were resuspended in Williams E medium with 2.5% FBS and added to the upper chamber in triplicate. Consecutively, Williams E medium with 10% FBS was added to the lower chamber. Chambers were incubated at 37˚C in the 5% CO_2 incubator for 48 h. After incubation, NOZ/shCTRL, NOZ/shSNCG and NOZ/parental cells on the surface of the upper chamber were removed by swiping with cotton swabs. The chambers were fixed with 95% ethanol and 5% acetic acid for 30 min, and then stained with H&E. The number of invasion cells in the lower chamber was determined by light microscopy at a 400× magnification. Five random fields of vision were counted. All experiments were performed in triplicate and values were analyzed and presented as means ± standard deviation (SD).

3.8. Flow cytometry in analysis of the cell cycle and apoptosis

NOZ/shCTRL, NOZ/shSNCG and NOZ/parental cells were grown in serum-free Williams E medium for 24h to block cells at the G0 phase of the cell cycle. A 100 nM concentration of paclitaxel (Cell Signaling) was added to the cells. After 24 h, cells were harvested and fixed in 70% ethanol at 4˚C for 2 h and then washed with PBS. Cells were then stained with 5 μg/ml propidium iodide (PI) along with 50μg/ml RNaseA, and subjected to flow cytometry. The histogram of DNA distribution was modeled as a sum of G1, G2-M, and S phase using ModFitLT software. For apoptosis analyses, harvested cells were analyzed by flow cytometry using the Annexin V-FITC assay (Abcam). The cells were washed twice with cold PBS and then incubated in binding buffer (10 nM HEPES, pH 7.4, 5 nM $CaCl_2$, and 140 nM NaCl). Following incubation, 0.1 ml of the solution was transferred to a new tube, and 5 μl of Annexin V-FITC and 10 μl of propidium iodide were added. After 15 min at room temperature in the dark, the cells were analyzed immediately by flow cytometry. Cells were simultaneously stained with FITC-Annexin V (green fluorescence, X-axis) and the non-vital dye propidium iodide (red fluorescence, Y-axis) to facilitate the discrimination of intact cells (FITC-PI-), early apoptotic cells (FITC+PI-) and late apoptotic or necrotic cells (FITC+PI+)

3.9. Antitumor effects of SNCG gene silencing in vivo

Homozygous BALB/c nu/nu athymic female mice (4 weeks of age) were purchased from Slac Labroatory Animal Co. Ltd., Shanghai, China and maintained at 22–24˚C under pathogen-limiting conditions as required. All animal manipulations were carried out in a laminar airflow biological safety cabinet. Cages, bedding, and food were autoclaved before use. Sterile water was provided ad lib. 3.0×10^6 NOZ/shSNCG cells and their control counterparts were subcutaneously injected into the right front legs of 5 mice. Tumor weight and size were monitored and measured every 4 days for 24 days. Tumor volume was calculated using the formula $(1/2)\times A\times B\times B$, where ‘‘A’’ is tumor length and ‘‘B’’ is tumor breadth.

3.10. Statistical analysis

The results were presented as means ± standard deviation (SD) of three or more independent determinations. Statistical analysis was performed by SPSS16.0 (SPSS Inc., Chicago, IL), using two-tailed Student t-test for unpaired data and ANOVA for multiple comparisons. A value of $P < 0.05$ was considered statistically significant.

4. RESULTS

4.1. Generation of NOZ cells stably expressing SNCG-shRNA

To examine endogenous expression levels of SNCG protein and mRNA in GBC-SD, SGC-996 and NOZ cells, western blot and semi-quantitative RT-PCR analyses

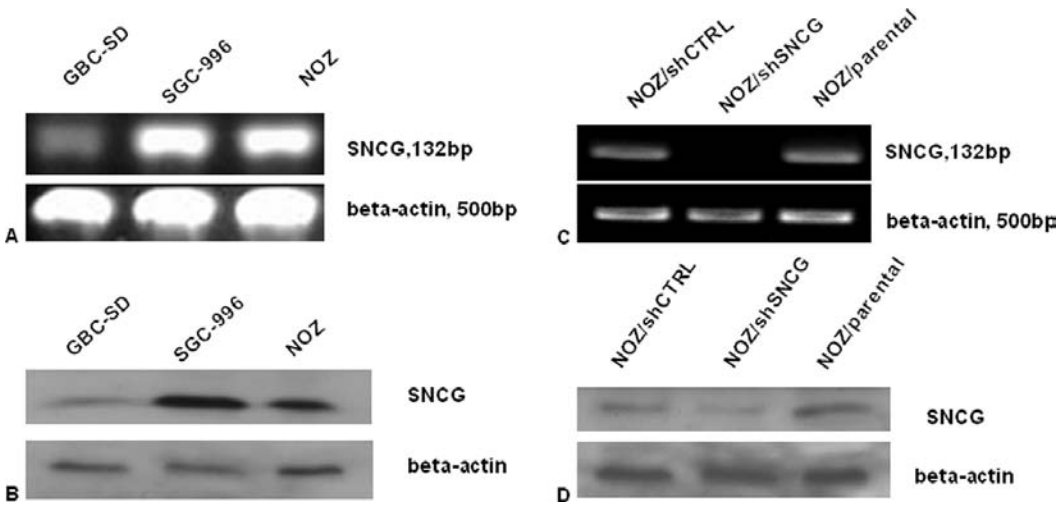

Figure 1. Establishment of NOZ cells that stably express SNCG-shRNA. A: SNCG mRNA levels in NOZ, SGC-996, and GBC-SD cells. B: SNCG protein levels in NOZ, SGC-996, and GBC-SD cells. C: SNCG mRNA levels in NOZ/parental, NOZ/shCTRL, and NOZ/shSNCG cells. D: SNCG protein levels in NOZ/parental, NOZ/shCTRL, and NOZ/shSNCG cells. RT-PCR and western blots used beta-actin as a control.

were carried out on whole-cell lysates and total RNA extracts, respectively. SNCG was more highly expressed in NOZ and SGC-996 cells compared to GBC-SD cells at both the mRNA (figure 1A) and protein (figure 1B) levels. NOZ cells were further used to investigate the effect of SNCG for its higher tumorgenic ability and high expression of SNCG.

The effectiveness of the SNCG-shRNA lentiviral construct in stably silencing SNCG in NOZ cells was verified by western blot and semi-quantitative RT-PCR analyses as shown in figure 1C,D. The results show significant reduction of SNCG mRNA and protein levels compared to NOZ/shCTRL cells and NOZ/parental cells. This shRNA-mediated effect was specific, as beta-actin levels did not significantly differ between SNCG-shRNA transfected cells and control cells.

4.2. SNCG gene silencing leads to a decrease in cell proliferation

In order to determine whether SNCG gene silencing affects the proliferation of NOZ cells, NOZ/shSNCG, NOZ/shCTRL and NOZ/parental cells were cultured for 24 h, 48 h, 72 h, 96 h or 120 h. After each time point, cells were harvested and CCK-8 assays were performed. Compared to NOZ/shCTRL and NOZ/parental cell lines, the OD450 value of NOZ/shSNCG cells decreased slightly between 24 h and 96 h, and decreased significantly at the 120 h time point, with an accompanying reduction in cell proliferation (figure 2; $^{**}P<0.01$; n =3). These results suggest that shRNA-mediated SNCG silencing decreases the proliferation of NOZ cells.

4.3. SNCG gene silencing inhibits cell colony formation in NOZ cells

With respect to the ability to form colonies, NOZ/shSNCG cells formed colonies in soft agar that were considerably smaller and fewer in number than those from both NOZ/shCTRL cells and NOZ/parental cells (figure 3A). The number of colonies formed in NOZ/shSNCG cells (113±26 colonies) was significantly reduced (305±21 of NOZ/parental cells and 283±31 of NOZ/shCTRL cells, $^{**}P<0.01$) as shown in figure 3B.

4.4. SNCG gene silencing decreases invasion of NOZ cells

As described in the Material and Methods section, we performed invasion assays using modified Boyden Chamber experiments in order to determine whether SNCG-silencing affects invasion of NOZ cells. As shown in figure 4A, the number of invasive NOZ/shSNCG cells (27.2±3.6) was significantly decreased compared to NOZ/shCTRL cells (51.7±1.6) and NOZ/parental cells (52.2±3.2) ($P<0.001$ for each comparison) (figure 4B). Based on these findings we hypothesize that SNCG plays a significant role in invasiveness within the NOZ microenvironment to at least aid the local spread of tumors. Furthermore, the results from these *in vitro* studies indicate that SNCG may play a role in the metastasis of primary NOZ cells.

4.5. SNCG-silencing in NOZ cells treated with paclitaxel induces G2/M arrest

To determine the effects of SNCG-silencing on the cell cycle, cells were treated with paclitaxel for 24 h followed by flow cytometric analysis. The proportions of NOZ/shSNCG cells in the G0/G1 and S phases of cells were 31.42% and 16.37%, respectively, compared to 37.73% (G0/G1) and 17.47% (S) for NOZ/parental cells, and 34.57% (G0/G1) and 19.78% (S) for NOZ/shCTRL cells. The proportion of NOZ/shSNCG cells in the G2/M phase was significantly increased compared to that of NOZ/shCTRL and that of NOZ/parental cells (figure 5). As shown in figure 5A, 52.22% of NOZ/shSNCG cells accumulated in the G2/M phase, in contrast to 44.80% and 46.55% of NOZ/parental and NOZ/shCTRL cells, respectively.

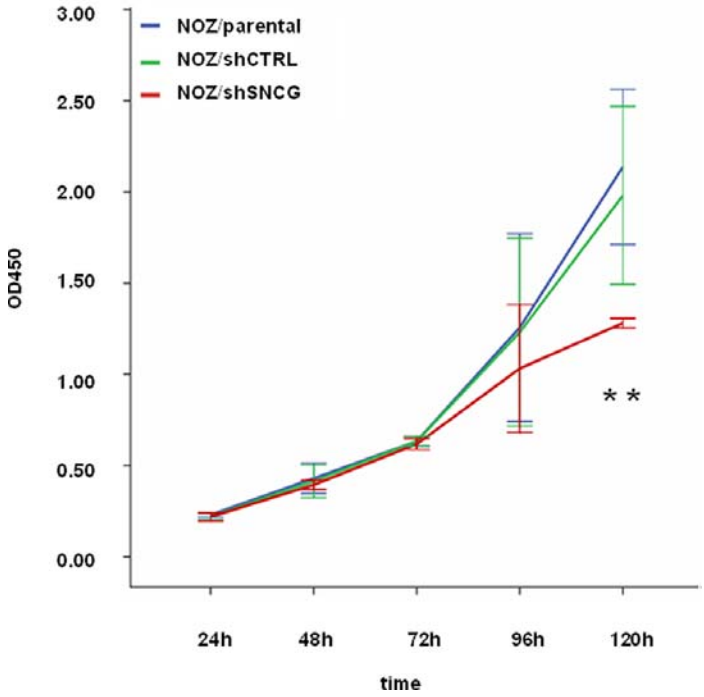

Figure 2. The effect of SNCG gene silencing on cell proliferation. NOZ/parental, NOZ/shCTRL, and NOZ/shSNCG cells were cultured for 24 h, 48 h, 72 h, 96h, or 120 h and proliferation was measured by CCK-8 assays. **P < 0.01 vs. control.

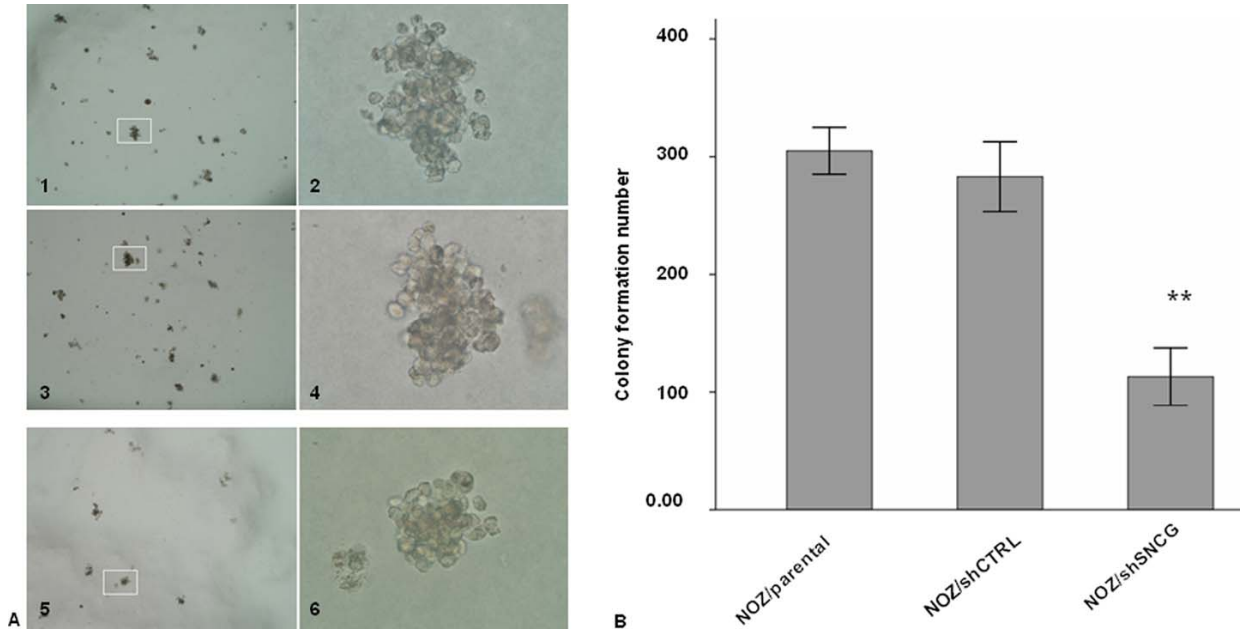

Figure 3. The effects of SNCG gene silencing on colony formation. A: NOZ/shSNCG, NOZ/parental, and NOZ/shCTRL cells formed colonies in agar. (1/2 NOZ/parental; 3/4 NOZ/shCTRL; 5/6 NOZ/shSNCG; 1/3/5, 40×; 2/4/6 , 400×) B: The colony formation number was calculated in each group. **P < 0.01 vs. control.

4.6. SNCG-silencing in NOZ cells increases paclitaxel-induced apoptosis

To investigate the effects of paclitaxel-induced apoptosis in SNCG-silenced NOZ cells, flow cytometry was employed to analyze apoptosis in NOZ/shSNCG, NOZ/shCTRL and NOZ/parental cells treated with 100 nM paclitaxel. As shown in figure 6A, the proportion of apoptotic NOZ cells was 5.1%, but increased to 12.5% in the SNCG-silenced cells. Statistic analyses showed that there were more apoptotic cells in the NOZ/shSNCG

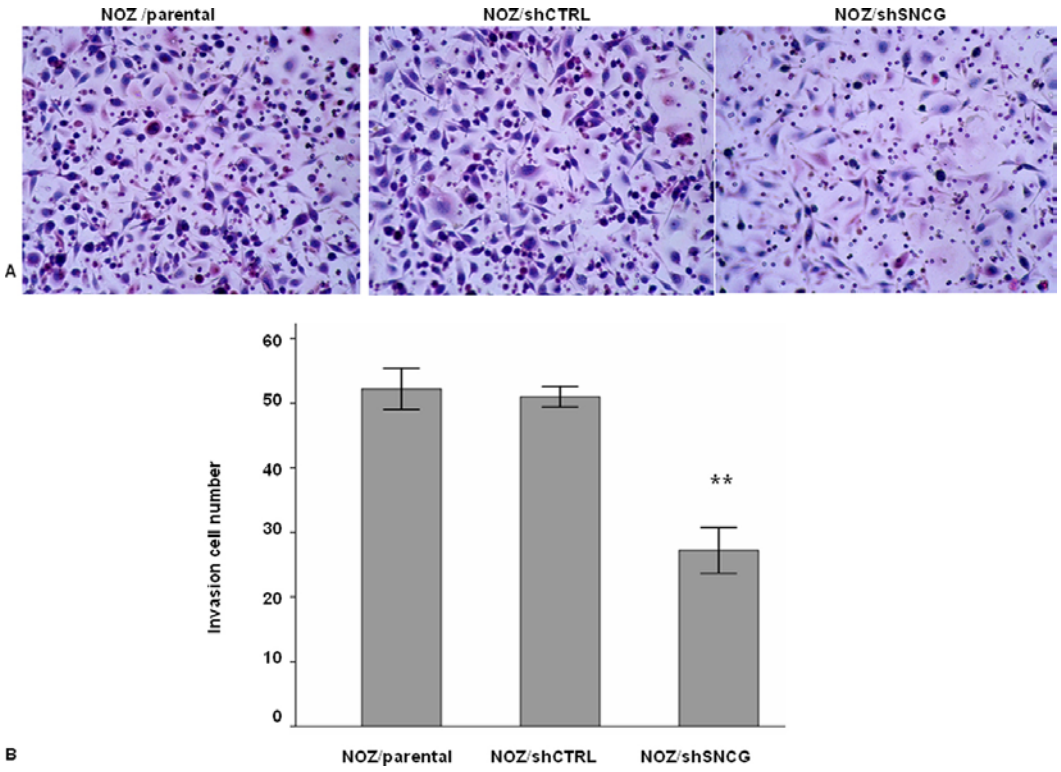

Figure 4. The invasive ability of SNCG-silenced NOZ cells. A: Invasive cells in the lower chamber were viewed under light microscopy at a 200× magnification. B: The number of invasive cells was calculated in each group. **P < 0.01 vs. control.

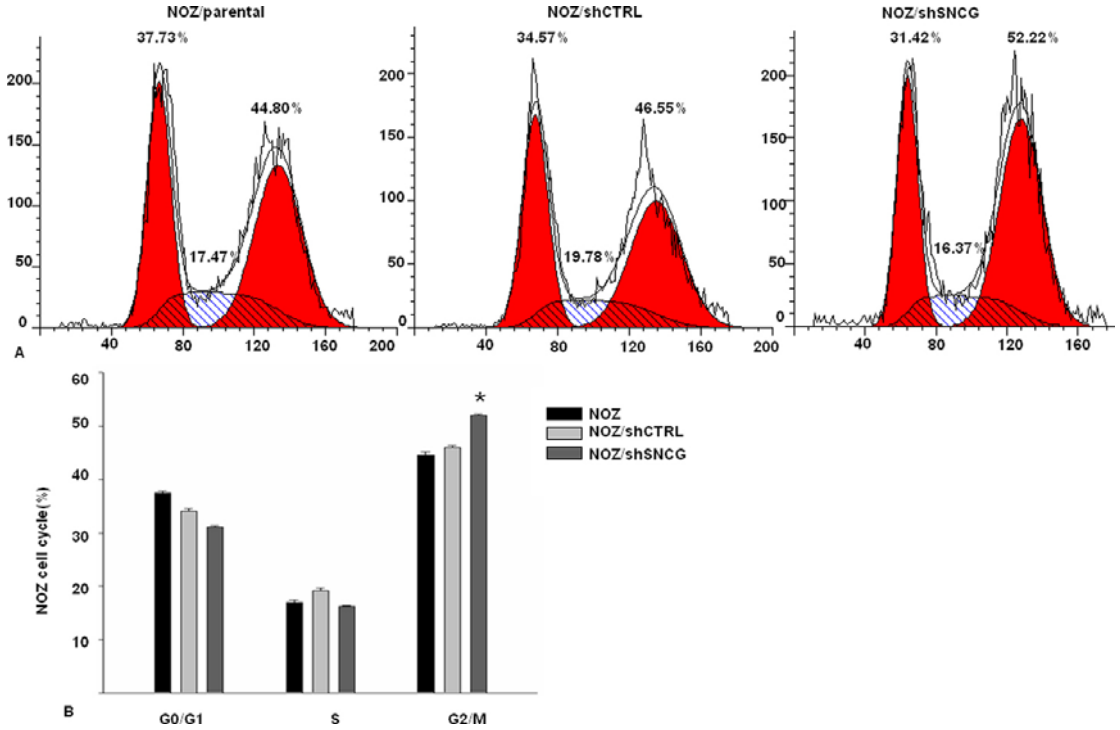

Figure 5. The effects of SNCG-silencing on the cell cycle in NOZ cells treated with paclitaxel. A: NOZ/parental, NOZ/shCTRL, and NOZ/shSNCG cells were treated with paclitaxel (100 nM) and the cell cycle was examined by flow cytometry. B: The proportion of cells in the G1/G0,S, and G2/M phase was calculated. All experiments were performed in triplicate; values were analyzed and presented as means ± standard deviation (SD). *P < 0.05 vs. Control.

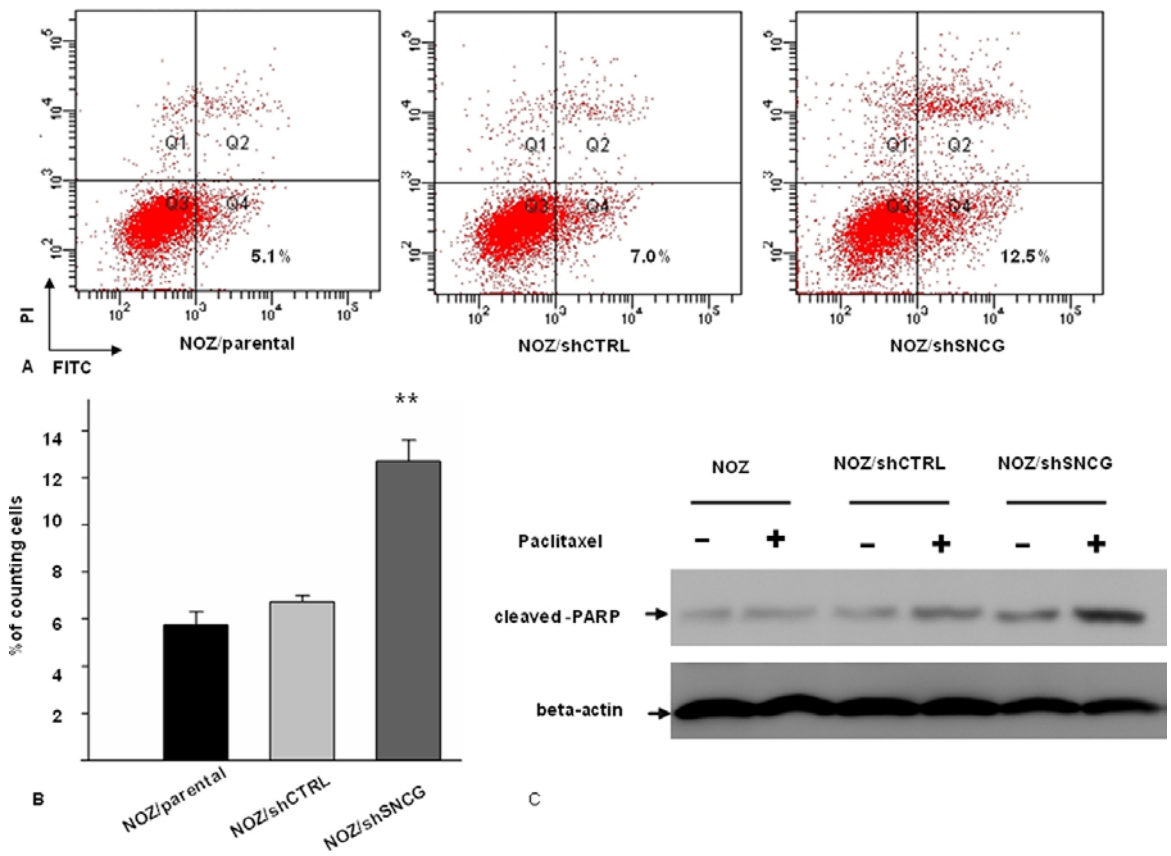

Figure 6. The effects of SNCG-silencing on paclitaxel-induced cell apoptosis. A: NOZ/parental, NOZ/shCTRL, and NOZ/shSNCG cells were treated with paclitaxel (100 nM) and apotosis was examined by flow cytometry. B: The apoptotic rate of NOZ cells was calculated in each group. C: Western blot was used to detect cleaved PARP expression in each group; All experiments were performed in triplicate, and values were analyzed and presented as means ± standard deviation (SD). **P < 0.01 vs. control.

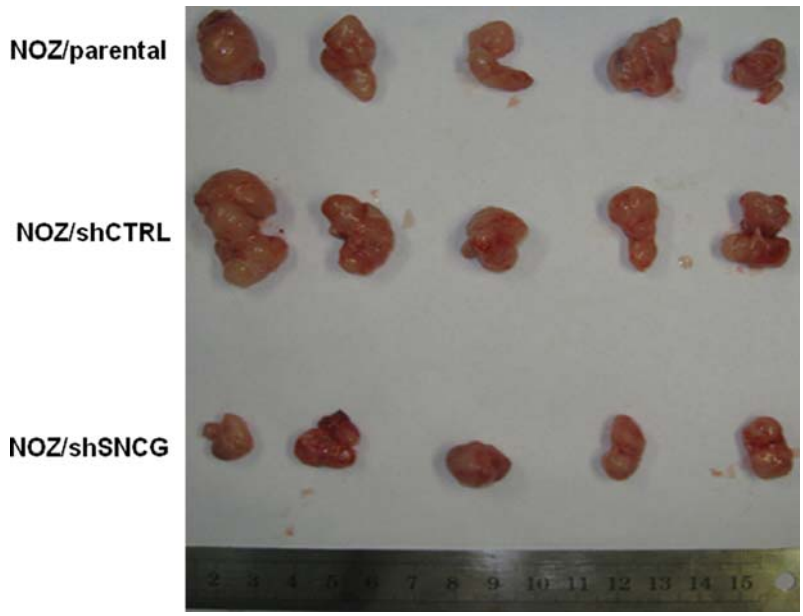

Figure 7. The antitumor effects of SNCG gene silencing in mice. NOZ/shSNCG tumors developed slower, and had a lighter weight and smaller volume compared to NOZ/shCTRL or NOZ/parental tumors over a period of 24 days. *P < 0.05 vs. control.

Table 1. Weight and volume of tumors in mice injected with NOZ/shSNCG, NOZ/shCTRL, and NOZ/parental cells

	Group	Average	SD
Weight (g)	NOZ/parental	2.244	0.848
	NOZ/shCTRL	2.496	1.166
	NOZ/shSNCG	0.976	0.101
Volume (cm^3)	NOZ/parental	2.278	0.507
	NOZ/shCTRL	2.491	1.491
	NOZ/shSNCG	0.925	0.435

group, compared to the NOZ/shCTRL or NOZ/parental cells (figure 6B; **P<0.01; n =3). To determine whether apoptosis-related factors were also upregulated, western blot was used to detect the expression of cleaved PARP. As shown in figure 6C, compared to the control cells, the expression of cleaved PARP was highest in the NOZ/shSNCG cells. Together, these results indicated that SNCG-silencing increases the apoptotic rate of NOZ cells treated with paclitaxel.

4.7. *In vivo* antitumor effects of SNCG-silencing on NOZ cells

The antitumor effects of SNCG-silencing in NOZ cells was tested in 15 BALB/c nude mice that had been subcutaneously injected with NOZ/shSNCG cells, NOZ/shCTRL, and NOZ/parental cells in their front right legs. As shown in the figure 7 and table 1, NOZ/shSNCG tumors developed slower and had a lighter weight and smaller volume compared to NOZ/shCTRL and NOZ/parental tumors over a period of 24 days. These results suggest that the reduced tumorigenic hallmarks of SNCG that were observed in our *in vitro* experiments are reproducible *in vivo* as well.

5. DISCUSSION

In the current study, we were interested in investigating the functional role of SNCG in GBC. To this end, we developed SNCG-shRNA lentiviral particles designed to silence SNCG gene expression, and generated NOZ cells stably expressing SNCG-shRNA. Our results showed that SNCG gene silencing inhibited GBC cell growth (NOZ cells) and invasion. It also facilitated paclitaxel-induced apoptosis thereby increasing the effectiveness of this antimicrotubule drug.

The present study showed that cell proliferation decreased in NOZ/shSNCG cells, compared to NOZ/parental cells and NOZ/shCTRL cells, thus indicating that SNCG indeed has the ability to promote cell growth. Previous studies have shown that SNCG constitutively activates ERK1/2, and increases estrogen receptor alpha (ER-alpha) transcriptional activity through an HSP-based multiprotein chaperone complex, resulting in an increase in breast or ovarian cancer cell survival and proliferation (21-25). In addition to the noted effects on cell proliferation, SNCG is also associated with cell invasion and metastasis. Previous *in vitro* studies showed that retinoblastoma cell lines overexpressing SNCG had higher MMP9 protein levels and activity, which led to enhanced cell motility and invasion (26). This was confirmed by *in vivo* studies which demonstrated that SNCG caused metastasis in nude mice upon implantation of SNCG-expressing MDA-MB 435 breast cancer cells in the fat pads of these mice. Specifically, mice that were implanted with SNCG-upregulated cells, displayed an increase in tumor growth and metastasis in the axillary lymph nodes and lungs, compared to mice implanted with control cells (15). In the current study, we presented data indicating that SNCG plays a key role in GBC invasion, as reduced SNCG expression led to decreased cell invasion of NOZ cells. Together, our results suggest that SNCG may function as a key mediator of cancer cell growth and invasion, thus making it a promising target for GBC treatment.

In our study, we explored the clinical implications of SNCG expression as a biomarker in predicting the effectiveness of paclitaxel in the treatment of GBC, using NOZ cells as *in vitro* models. We provided strong evidence of a clear inverse relationship between SNCG expression and lower sensitivity to antimicrotubule chemotherapeutic agents. Biological treatments targeting SNCG has been studied in breast cancer. In fact, Singh designed and characterized a peptide inhibitor of SNCG that enhances sensitivity of breast cancer cells to antimicrotubule drugs (27). Our present study demonstrated a similar effect; we observed that SNCG gene silencing enhanced sensitivity of GBC cells to the antimicrotubule drug, paclitaxel. Specifically, we showed that NOZ/shSNCG cells had an increased sensitivity to paclitaxel comparing to NOZ/parental and NOZ/shCTRL cells. Moreover, paclitaxel also induced an increase in apoptosis and the proportion of cells in the G2/M phase. These results provide evidence that supports the notion that SNCG expression inhibits mitotic checkpoint function, thereby leading to cell resistance to antimicrotubule drugs. Therefore, the expression status of SNCG on GBC may be useful as a biomarker in predicting the effectiveness of paclitaxel treatment.

Results from our *in vivo* study showed that SNCG gene silencing caused decreased tumor growth rates and decreased weight in nude mice, indicating that SNCG has the ability to promote gallbladder tumor growth. Previous studies have demonstrated that ectopic expression of SNCG increased breast cancer cell growth in anchorage dependent and independent conditions through interaction with BubR1, a mitotic checkpoint kinase. This led to inhibition of the mitotic checkpoint control (16,17, 28). The *in vivo* antitumor effects of SNCG–silencing, while so far only demonstrated in 15 mice, nevertheless, suggest a trend towards reduced tumor growth.

In summary, we show that the downregulation of SNCG expression inhibits GBC cell growth and invasion, facilitates paclitaxel-mediated apoptosis, and increases the effectiveness of antimicrotubule drugs. Therefore, SNCG is likely to play an important role in the progression of GBC. Further studies are needed to determine if SNCG will indeed make an effective biomarker or molecular target for GBC diagnosis, prognosis evaluation and therapy.

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Abbreviations: SNCG: Synuclein-gamma; GBC: gallbladder cancer; shRNA: short-hairpin RNA; NOZ/shSNCG: SNCG-silenced NOZ cells; BCSG1: breast cancer-specific gene 1; NNAT: non-neoplastic adjacent tissues; ESCC: esophageal squamous cell carcinoma; PBS: phosphate buffered saline; CCK-8: the Cell Counting Kit-8

Key Words: SNCG, Tumorigenic Activities, Gallbladder Cancer, NOZ

Send correspondence to: Yanling Chen, Department of Hepatobiliary Surgery, Union Hospital, Fujian Medical University, Key Laboratory of Ministry of Education for Gastrointestinal Cancer, Fujian Medical University, 29 Xinquan Road, Fuzhou, Fujian 350001, China, Tel: 86-591-83357896-8012, Fax: 86-591-83325046, E-mail: ylchenfjmu@163.com