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LncRNA SNHG5 promotes cisplatin resistance in gastric cancer via inhibiting cell apoptosis

M. LI[1], Y.-Y. ZHANG[2], J. SHANG[3], Y.-D. XU[3]

[1]Department of Pharmacy, Yantai Yuhuangding Hospital, Yantai, China
[2]Physical Examination Center, Yantai Yuhuangding Hospital, Yantai, China
[3]Department of Central Sterile Supply, Yantai Yuhuangding Hospital, Yantai, China

Min Li and Yanyun Zhang contributed equally to this work

Abstract. – OBJECTIVE: To elucidate the function of long non-coding RNA (lncRNA) SNHG5 in cisplatin-resistant gastric cancer (GC), and its potential mechanism.

PATIENTS AND METHODS: We detected the expressions of SNHG5, apoptosis-specific genes (Bax and Bcl-2) and drug resistance-specific genes (MDR1 and MRP1) in cisplatin-sensitive and cisplatin-resistant GC patients. The expression levels were also detected in cisplatin-resistant GC cell lines (BGC823/DDP, SGC7901/DDP) and GC cell lines (BGC823 and SGC7901). Through the liposome transfection, the regulatory effects of SNHG5 on proliferative potential and apoptosis were examined by cytotoxicity assay and flow cytometry assay, respectively. The protein levels of apoptosis-related genes and drug resistance-related genes influenced by SNHG5 were detected by Western blot.

RESULTS: Compared with cisplatin-sensitive GC patients, SNHG5 expression was remarkably higher in cisplatin-resistant GC patients. Besides, higher SNHG5 expression was observed in BGC823/DDP and SGC7901/DDP cells relative to that of their parental cells. Proliferative rate (OD450) and IC50 decreased, but the apoptotic rate increased in BGC823/DDP and SGC7901/DDP cells with SNHG5 knockdown. It is found that SNHG5 overexpression reduced cisplatin sensitivity in BGC823 and SGC7901 cells. Decreased cisplatin cytotoxicity, elevated IC50 and inhibited apoptotic rate were observed in GC cells overexpressing SNHG5. Moreover, the expression levels of Bax, MDR1 and MRP1 were upregulated, while Bcl-2 downregulated in BGC823 and SGC7901 cells overexpressing SNHG5.

CONCLUSIONS: SNHG5 is highly expressed in cisplatin-resistant GC. SNHG5 promotes cisplatin resistance in GC by regulating apoptosis-related genes and drug resistance-related genes.

Key Words:
Gastric cancer, Cisplatin resistance, LncRNA SNHG5, Proliferation, Apoptosis.

Introduction

Gastric cancer (GC) is common in the digestive system. Currently, radiotherapy and chemotherapy are still the main treatment approaches for GC. Cisplatin (DDP) is the first-line platinum antitumor drug, directly acting on DNA to induce tumor cell apoptosis. It is widely applied in the treatment of GC[1]. However, with the widespread application of chemotherapeutic drugs, the emergence of drug resistance in tumor cells has become a major factor affecting chemotherapy efficacy. Hence, it is of clinical significance to clarify the mechanism of drug resistance. It is believed that several aspects are involved in the mechanism of chemoresistance[2-7], including (1) Enhancement of active efflux of antineoplastic drugs mediated by ABC-type membrane carriers such as P-glycoprotein (P-gp), (2) abnormal expressions of multidrug resistance genes, (3) dysregulation of DNA damage repair system and (4) inhibition of apoptosis pathway.

Long non-coding RNAs (lncRNAs) contain over 200 nucleotides in length and are transcribed by RNA polymerase II. They do not have an open reading frame. LncRNAs are produced through the transmutation of protein-coding gene sequences into non-coding genes, or generated with the insertion of transposon factor sequences[8]. A large number of lncRNAs are abnormally expressed in tumors, and regulate tumor cell proliferation, differentiation and apoptosis through different

Corresponding Authors: Yingdi Xu, MM; e-mail: ysxyd@21cn.com
Ju Shang, MM; e-mail: yssj0382@163.com

signaling pathways[9]. The intracellular apoptotic pathway and the anti-apoptotic pathway simultaneously act to maintain the normal cell cycle progression. LncRNA is capable of mediating tumor cell apoptosis by influencing apoptotic factors, further leading to drug resistance. The vital functions of lncRNAs have been identified in drug-resistant gastrointestinal tumors. They regulate nucleic acids or proteins in a sequence-specific and structure-specific manner at multiple levels, thus affecting the efficacy of chemotherapeutic drugs[8].

Zhao et al[10] showed downregulated SNHG5 in GC, serving as a tumor-suppressor gene. Moreover, SNHG5 is involved in imatinib-resistant chronic myeloid leukemia[11] and gefitinib-resistant lung adenocarcinoma[12]. Whether SNHG5 could regulate cisplatin resistance in GC remains unclear, which is specifically elucidated in this work.

Patients and Methods

Sample Collection

Fresh cisplatin-sensitive GC tissues (n=13) and cisplatin-resistant GC tissues (n=13) were harvested from patients who were pathologically diagnosed as GC and underwent surgical resection at the Yantai Yuhuangding Hospital from September 2012 to September 2016. All obtained surgical specimens were placed in cryogenic vials containing inactivated RNase within 15 min of *ex vivo*, and preserved in liquid nitrogen. The newly diagnosed patients were not treated with chemotherapy, radiotherapy or targeted therapy before surgery. Cisplatin-resistant GC patients only received cisplatin treatment and developed drug resistance. Patients did not have other diseases. The study was approved by the Ethics Committee of the Yantai Yuhuangding Hospital, and patients signed informed consent.

Cell Culture and Transfection

GC cell lines (BGC823 and SGC7901) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA), and cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), 100 U/mL L penicillin and 100 μg/mL streptomycin. Cisplatin-resistant GC cell lines (BGC823/DDP and SGC7901/DDP) were induced in our laboratory and cultured in RPMI-1640 medium with 16 μg/mL and 10 μg/mL cisplatin, respectively.

Cells that were logarithmically grown and in good condition were inoculated into 6-well plates one day before transfection with 1×10^5 cells/well. Cell abundance during transfection was 60-70%. Transfection was performed for 48 h using Lipofectamine™ 2000, and transfected vectors were provided by GenePharma (Shanghai, China).

RNA Extraction and Real Time-Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from tissues or cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and quantified using UV spectrophotometer. D260 nm/D280 nm value was calculated for selecting qualified RNA samples, which were preserved at -20°C for use. Extracted RNA was reversely transcribed into cDNA and amplified by Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) using SYBR Premix Ex Taq II kit (TaKaRa, Otsu, Shiga, Japan). PCR reaction conditions were: pre-denaturation at 95°C for 30 s, 95°C for 5 s, and 60°C for 31 s, for a total of 40 cycles. The relative levels were quantitatively analyzed using the $2^{-\Delta\Delta Ct}$ method. GAPDH was used as an internal reference. The experiment was repeated three times. Primer sequences were as follows: SNHG5: F: TACTGGCTGCGCACTTCG, R: TACCCTGCACAAACCCGAAA; Bax: F: GAGCTGCAGAGGATGATTGC, R: CCAATGTCCAGCCCATGATG; Bcl-2: F: CCTCGCTGCACAAATACTCC, R: TGGAGAGAATGTTGGCGTCT; MDR1: F: CTGAAATCCAGCGGCAGA, R: TGTATCGGAGTCGCTTGGTGAG; MRP1: F: TTGCCGTCTACGTGACCATT, R: AGGCGTTTGAGGGAGACACT; GAPDH: F: CGCTCTCTGCTCCTCCTGTTC, R: ATCCGTTGACTCCGACCTTCAC.

Cytotoxicity Assay

BGC823/DDP and SGC7901/DDP cells were transfected with si-SNHG5 or si-NC for 48 h, followed by cisplatin induction for different time points (16 μg/mL and 10 μg/mL). BGC823 and SGC7901 cells were transfected with pcDNA-SNHG5 or pcDNA-NC for 48 h, followed by cisplatin induction for different time points (7 μg/mL and 6 μg/mL). Cell density was adjusted to 2×10^4 /mL, and cells were inoculated in 96-well plates with 200 μL of suspension per well. At 24 h, 36 h and 48 h, 10 μL of Cell Counting Kit-8 (CCK-8; Dojindo, Kumamoto, Japan) was applied per well and optical density $(OD)_{450}$ value was recorded 2 h later.

For calculating IC_{50}, transfected cells were incubated with 0, 5, 10, 15, 20 or 25 μg/mL cisplatin

for 48 h. Each group had 6 replicate wells. OD_{450} value was recorded as abovementioned.

Apoptosis Determination

Cells were collected, washed twice with phosphate-buffered saline (PBS), and stained using the Annexin V/fluorescein isothiocyanate (FITC) Apoptosis Detection Kit (Beyotime, Shanghai, China). The single cell suspension was first incubated with 500 μL of 1×buffer, 5 μL of Annexin V and 5 μL of Propidium Iodide (PI) at 37°C for 20 min in the dark. Stained cells were examined by FACSAriaTM flow cytometry and analyzed by Win MDI. 2.9 software. Each experiment was repeated in triplicate.

Western Blot

Total protein from cells was extracted using radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China), quantified by bicinchoninic acid (BCA) method (Pierce, Waltham, MA, USA) and loaded for electrophoresis. After transferring on a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA), it was blocked in 5% skim milk for 2 hours, incubated with primary antibodies at 4°C overnight and secondary antibodies for 2 h. Bands were exposed by enhanced chemiluminescence (ECL) and analyzed by Image J Software (NIH, Bethesda, MD, USA).

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 17.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. Data were expressed as mean ± SD (Standard Deviation). The *t*-test was used for analyzing the differences between the two groups. $p<0.05$ indicated the significant difference.

Results

High Expression of SNHG5 in Cisplatin-Resistant GC Patients

SNHG5 expression in cisplatin-sensitive and cisplatin-resistant GC patients was determined. Patients with cisplatin-resistant GC had a higher level of SNHG5 relative to cisplatin-sensitive ones (Figure 1A). Identically, the cellular expression of SNHG5 was higher in cisplatin-resistant GC cells (BGC823/DDP and SGC7901/DDP) compared with that of parental GC cells (BGC823 and SGC7901) (Figure 1B).

Low Expression of SNHG5 Increased Cisplatin Sensitivity in GC Cells

To clarify the role of SNHG5 in cisplatin-resistant GC, we examined cisplatin sensitivity in BGC823/DDP and SGC7901/DDP cells with SNHG5 knockdown. First of all, the transfection efficacy of si-SNHG5 was determined (Figure 2A). OD_{450} and IC_{50} markedly decreased in cisplatin-resistant GC cells transfected with si-SNHG5 (Figure 2B, C). Higher apoptotic rate was observed in BGC823/DDP and SGC7901/DDP cells with SNHG5 knockdown (Figure 2D). It is indicated that SNHG5 silence enhanced cisplatin sensitivity in drug-resistant GC.

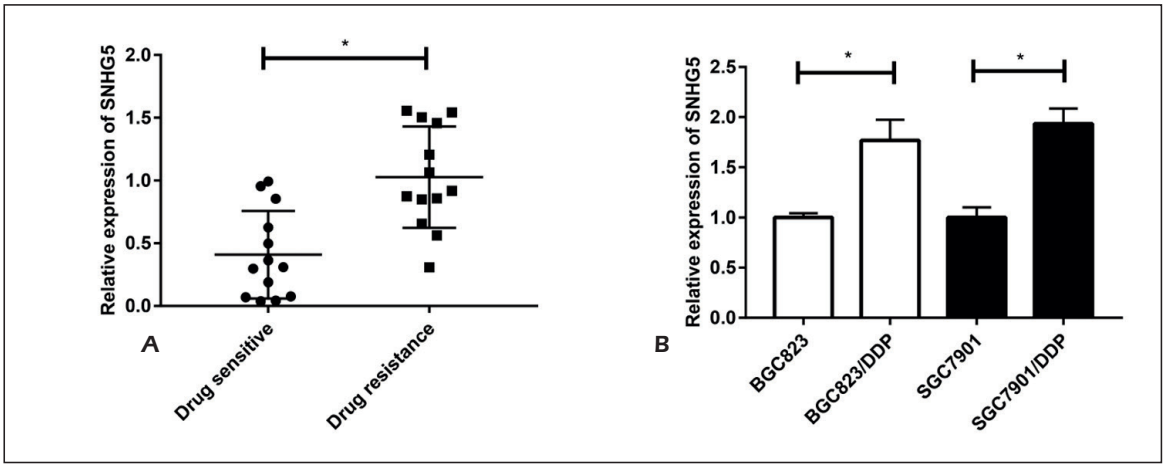

Figure 1. High expression of SNHG5 in cisplatin-resistant GC patients. **A**, SNHG5 expression was higher in cisplatin-resistant GC patients (n=13) than cisplatin-sensitive GC patients (n=13). **B**, Cellular expression of SNHG5 was higher in cisplatin-resistant GC cells (BGC823/DDP and SGC7901/DDP) compared with that of ordinary GC cells (BGC823 and SGC7901).

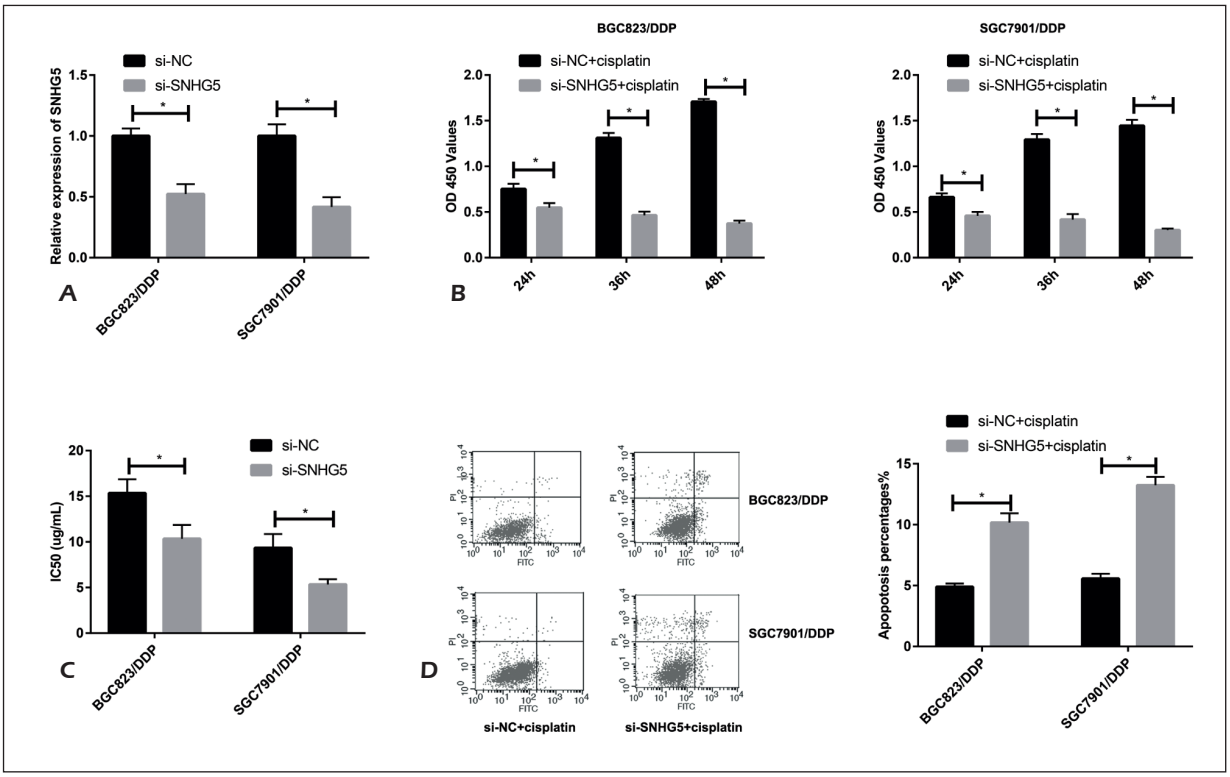

Figure 2. Low expression of SNHG5 increased cisplatin sensitivity in GC cells. **A**, Transfection efficacy of si-SNHG5 in BGC823/DDP and SGC7901/DDP cells. **B**, OD450 in BGC823/DDP and SGC7901/DDP cells transfected with si-SNHG5 or si-NC. **C**, IC50 in BGC823/DDP and SGC7901/DDP cells transfected with si-SNHG5 or si-NC. **D**, Apoptotic rate in BGC823/DDP and SGC7901/DDP cells transfected with si-SNHG5 or si-NC.

High Expression of SNHG5 Promoted Cisplatin Resistance in GC Cells

Subsequently, we detected the regulatory effect of SNHG5 on parental GC cells. Transfection of pcDNA-SNHG5 sufficiently upregulated SNHG5 expression in GC cells (Figure 3A). Higher OD_{450} and IC_{50} were observed in cisplatin-induced GC cells overexpressing SNHG5 (Figure 3B, 3C). Moreover, SNHG5 overexpression greatly decreased cisplatin-induced GC cell apoptosis (Figure 3D). It is suggested that SNHG5 overexpression attenuated cisplatin sensitivity of GC cells.

Overexpression of SNHG5 Inhibited Cell Apoptosis

Western blot and RT-PCR were conducted to observe changes in apoptosis-specific and drug resistance-specific genes in GC cells. SNHG5 overexpression downregulated the mRNA level of Bax (Figure 4A) and upregulated Bcl-2 (Figure 4B) in GC cells. The mRNA levels of MDR1 and MRP1 increased by SNHG5 overexpression (Figure 4C, 4D). Identically, expression changes were similar at their protein levels (Figure 4E).

Discussion

With the in-depth researches on lncRNAs, we have realized their vital function in tumor resistance. LncRNA UCA1 regulates the adriamycin chemosensitivity in GC by affecting the apoptotic pathway. UCA1 knockdown accelerates doxorubicin-induced GC cell apoptosis by upregulating RARP and downregulating Bcl-2[13]. LncRNA ANRIL is upregulated in cisplatin-resistant and 5-fluorouracil-resistant GC tissues and cells. Silence of ANRIL can reverse the multidrug resistance (MDR) by downregulating MDR1 and MRP1. Moreover, regression analysis found that the expression of ANRIL was positively correlated with the expression of MDR-related proteins[14].

Cisplatin induces tumor cell apoptosis through both the mitochondrial apoptotic pathway and endoplasmic reticulum stress pathway, thus achieving its anti-tumor effects[15,16]. However, drug insensitivity due to prolonged application time and increased blood drug concentration seriously influences the clinical

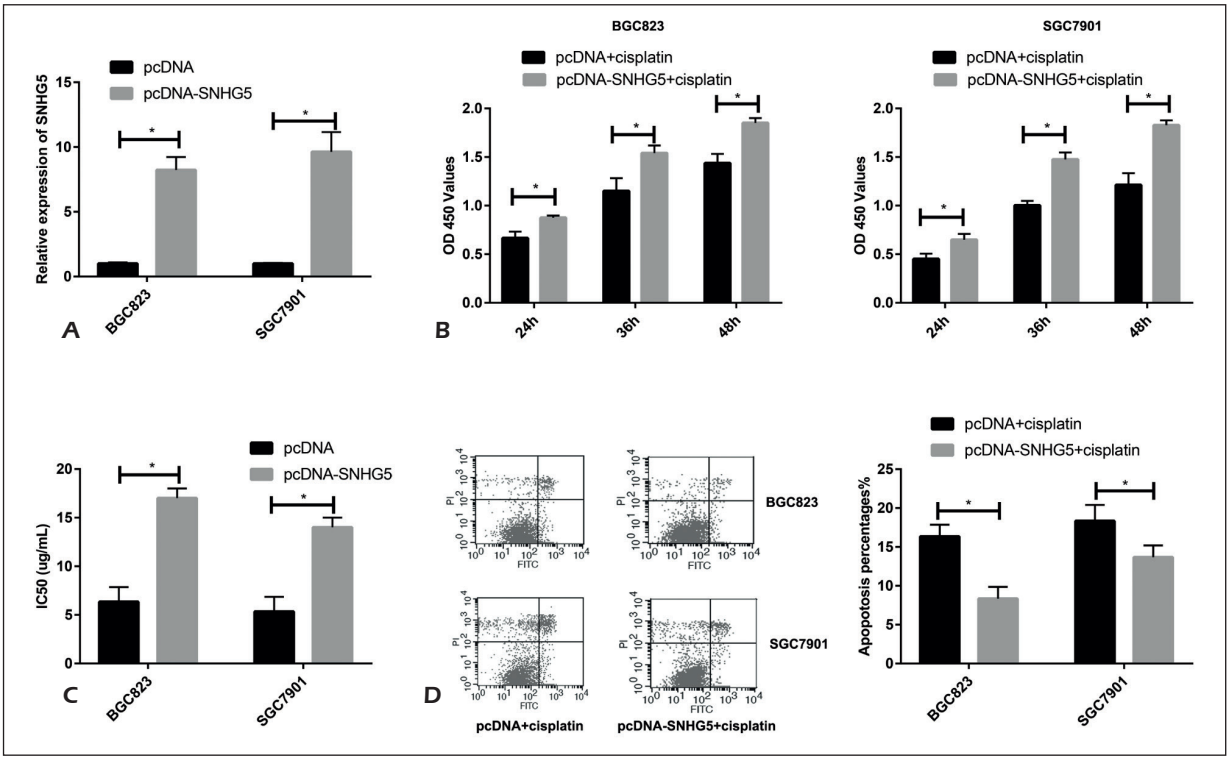

Figure 3. High expression of SNHG5 promoted cisplatin resistance in GC cells. **A**, Transfection efficacy of pcDNA-SNHG5 in BGC823 and SGC7901 cells. **B**, OD450 in BGC823 and SGC7901 cells transfected with pcDNA-SNHG5 or pcDNA-NC. **C**, IC50 in BGC823 and SGC7901 cells transfected with pcDNA-SNHG5 or pcDNA-NC. **D**, Apoptotic rate in BGC823 and SGC7901 cells transfected with pcDNA-SNHG5 or pcDNA-NC.

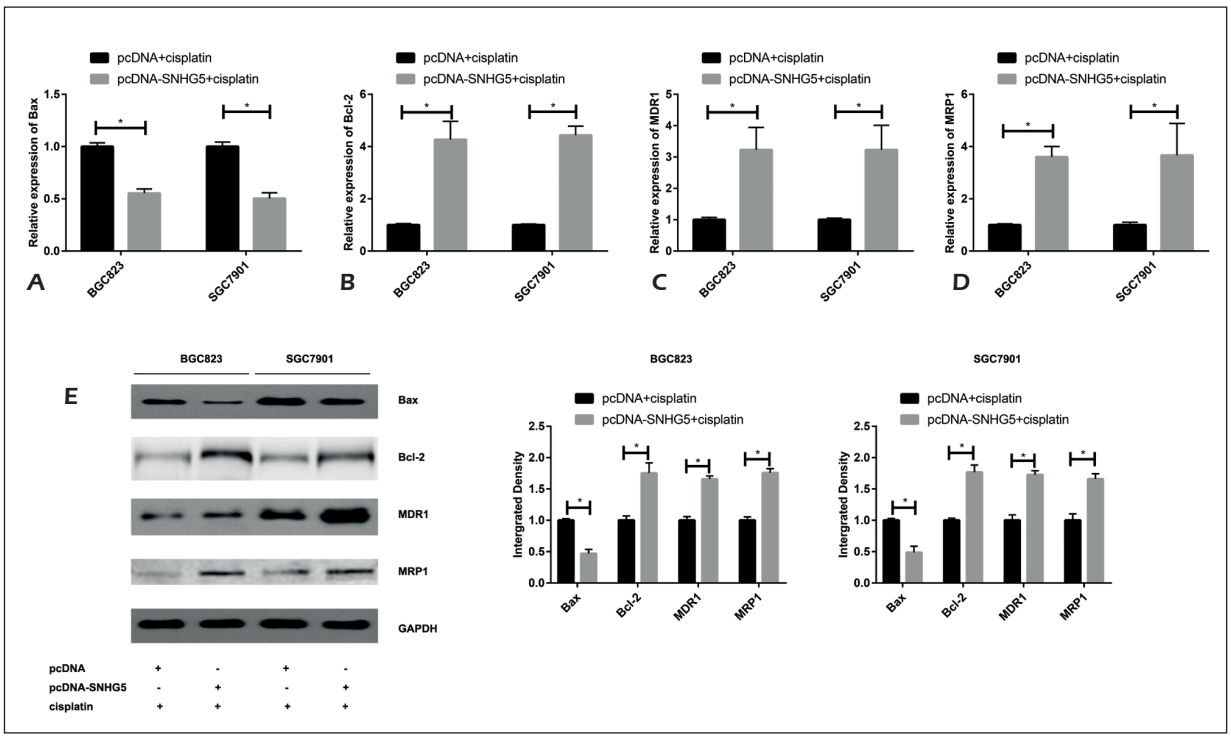

Figure 4. Overexpression of SNHG5 inhibited cell apoptosis. **A-D**, The mRNA levels of Bax, Bcl-2, MDR1 and MRP1 in cisplatin-induced BGC823 and SGC7901 cells transfected with pcDNA-SNHG5 or pcDNA-NC. **E**, The protein levels of Bax, Bcl-2, MDR1 and MRP1 in cisplatin-induced BGC823 and SGC7901 cells transfected with pcDNA-SNHG5 or pcDNA-NC.

outcomes of tumor patients. Apoptosis is precisely regulated by a series of specific genes, among which Bcl-2 family is important[17]. Bcl-2 family includes apoptosis-inhibiting genes represented by Bcl-2 and pro-apoptotic genes represented by Bax[18,19]. Studies have shown that Bcl-2 inhibits apoptosis by forming a heterodimer with Bax and thus releasing Bax in histiocytes[20]. The overexpression of Bcl-2 decreases apoptotic rate, while the overexpression of Bax accelerates apoptosis. Hence, the ratio of Bcl-2/Bax is an important molecular switch initiating apoptosis. Bcl-2 upregulation inhibits apoptosis through efficient tolerance to cisplatin, further influencing the therapeutic efficacy of platinum drugs. Qiu et al[21] have showed the overexpressed Bcl-2 in platinum-resistant cells, serving as an important intermediary for platinum resistance.

The mechanism of MDR in tumors is very complex. ATP-binding cassette (ABC) proteins located on the cell membrane exert a crucial function, including MDR-associated proteins (MRP1/ABCC1) and MDR proteins (MDR1/Pgp/ABCB1)[22]. In 1976, Juliano et al[23] first proposed that the drug pumping effect of transmembrane transporters markedly decreases the cellular concentration of antitumor drugs. They further found a presence of 170 KDa molecule, Pgp (also known as MDR1) in MDR cells. MDR1-transport drugs are usually lipophilic compounds with a large molecular weight, such as vincristine, taxanes, doxorubicin, etc[24]. MRP1 is an ABCC subfamily transporter discovered in MDR1$^{-/-}$ drug-resistant human-derived small cell lung cancer cell line H69AR. MRP1 contains 1531 amino acids, mainly resistant to anthracyclines, vincristine, antifolate drugs, etc., while it does not seem resistant to taxanes[25].

In this work, SNHG5 was upregulated in both cisplatin-resistant GC tissues and cells, suggesting that SNHG5 may be involved in cisplatin-resistant GC. The knockdown of SNHG5 elevated cisplatin sensitivity in drug-resistant GC cells. Conversely, SNHG5 overexpression could attenuate cisplatin sensitivity in ordinary GC cells, manifesting increased drug resistance. Furthermore, SNHG5 overexpression upregulated Bax, MDR1 and MRP1, downregulated Bcl-2 and increased Bcl-2/Bax ratio in GC cells. Our results demonstrated that SNHG5 regulated cisplatin resistance in GC by mediating apoptosis-specific and drug resistance-specific genes.

Conclusions

We demonstrated that SNHG5 is highly expressed in cisplatin-resistant GC. SNHG5 promotes cisplatin resistance in GC by regulating apoptosis-related genes and drug resistance-related genes.

Conflict of Interests

The authors declare that they have no conflict of interest.

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