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Review Article

High expression of ADPGK increases the sensitivity of gastric cancer to chemotherapy

Wenhua Hu[1], Ye Hua[2]

[1]*Department of Gastroenterology, Tonglu First People's Hospital, No. 338, Xuesheng Road, Chengnan Street, Tonglu County, Hangzhou 311500, Zhejiang Province, China;* [2]*Department of General Surgery, Chongqing Emergency Medical Center, No. 1 Jiankang Road, Yuzhong District, Chongqing 400014, China*

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Abstract: Objective: To verify that high expression of LNCRNA ADPGK-AS1 can increase the sensitivity of gastric cancer (GC) to chemotherapy. Methods: The expression of ADPGK-AS1 in MKN-45, MKN-28, MGC-803, AZ521 and HFE-145 cell lines was detected by qRT-PCR. In addition, si-LncRNA ADPGK-AS1, sh-LncRNA ADPGK-AS1 and Si-NC sequences were transfected into MKN-45 and AZ521 cells. QRT-PCR was employed to detect the ADPGK-AS1 expression in si-LncRNA ADPGK-AS1 and sh-LncRNA groups. MTT, Transwell and flow cytometry (FC) were adopted to test the proliferation, invasion and apoptosis of transfected cells, while Western blot (WB) was utilized to examine the expression of Bax and Bcl-2 in MKN-45 and AZ521 cells. What's more, the cells were respectively subjected to cisplatin (4, 8, 16 and 32 μmol/L) treatment at different concentrations, and the cell survival was measured 24 hours later. Results: The ADPGK-AS1 level in HFE-145 was significantly higher than that in MKN-45, MKN-28, MGC-803 and AZ521 ($P<0.05$). After transfection, the ADPGK-AS1 level in the MKN-45 and AZ521 cells in the Si-RNA group was significantly lower than that in the Sh-RNA and Si-NC groups ($P<0.05$). Compared with the Sh-RNA group, the proliferation of MKN-45 and AZ521 in the Si-RNA group was remarkably higher ($P<0.05$), the invasion capacity was evidently higher ($P<0.05$) and the apoptosis rate was lower ($P<0.05$). The Bax protein was reduced statistically in MKN-45 and AZ521 cells in the Si-RNA group ($P<0.05$), while Bcl-2 protein was increased significantly ($P<0.05$). At the concentration of 16 μmol/L and 32 μmol/L, the survival rate of cells in the Si-RNA group was dramatically higher than that in the other two groups ($P<0.05$). Conclusion: Highly expressed LncRNA ADPGK-AS1 can inhibit the proliferation and invasion of GC cells and promote apoptosis, and can increase the sensitivity of GC to chemotherapy.

Keywords: ADPGK, gastric cancer, chemotherapy sensitivity

Introduction

GC is one of the most common clinical malignancies and one of the three leading causes of cancer-related death in the world [1]. In 2018, there were approximately 1.03 million cases newly diagnosed as GC and approximately 780,000 deaths worldwide, ranking the fifth and the third in the incidence and mortality rate of malignancies. In China, it is the second prevalent malignant tumors that ranks the third in the mortality rate, accounting for about half of the morbidity and mortality globally [2, 3], which seriously threatens the life and health of patients. Studies have shown that most patients with early GC do not have typical clinical symptoms, so most patients are already in the advances stage when diagnosed [4]. Currently, surgery is the first choice for the treatment of early GC, but most patients will have recurrence [5]. In the case that patients develop postoperative recurrence and/or metastasis, reoperation or chemoradiotherapy will be necessary [6]. For those with advanced GC, they are often treated with chemotherapy to prolong the survival [7]. However, chemotherapy is often accompanied by a variety of adverse reactions. According to statistics, platinum(Pt)-based chemotherapy is the most extensively used chemotherapy regimen for GC, but it is prone to Pt resistance, resulting in poor chemotherapy and low overall effective rate. As a result, most patients cannot benefit from chemotherapy [8]. Therefore, studying the mecha-

Table 1. Primer sequences

Primer sequences	R (5'-3')	F (5'-3')
ADPGK-AS1	AATTTCACGGAGAAGGGCGT	ACGCGTGATGTCTGCCTTTA
GAPDH	AACAGGAGGTCCCTACTCCC	GCCATTTTGCGGTGGAAATG

nism and development of GC and reversing Pt resistance is the hotspot and challenge in clinical research, and overcoming it will be of great significance to carry out individualized chemotherapy for GC.

Long non-coding RNAs (lncRNAs), which are increasingly defined as non-coding RNAs over 200 nt, have a finite or no protein-coding capacity [9]. The function of lncRNA has been characterized in a variety of cancers [10], and it mainly plays a role at three levels: transcription, post-transcription, and epigenetic modification [11]. Previous studies have reported that lncRNA can play the role of competing endogenous RNA (ceRNA) and interact with coding genes or other types of non-coding RNA (including miRNA) and regulate their expression [12]. According to some research, numerous lncRNAs in GC are associated with the tumor onset and progression in GC [13]. Among them, ADPGK-AS1 is a novel one that has been found to be involved in the occurrence of GC [14], but its specific mechanism of action remains a subject of investigation. Therefore, for the purpose of further elucidating the role of ADPGK-AS1 in GC, this experiment explored its effect on the sensitivity of GC to chemotherapy, providing a reliable theoretical basis for the future clinical diagnosis and treatment of GC.

Methods

Main instruments and reagents

RIPA reagent, protein extraction reagent, ECL luminescence kit, trypsin, Lipofectamine™ 2000, DMEM, PBS and fetal bovine serum were all purchased from Thermo Fisher. TransScript II Green Two-Step qRT-PCR SuperMix was purchased from Beijing Transgen Biotechnology Co., Ltd., China. CCK-8 kit and Annexin V/PI apoptosis detection kit were purchased from Shanghai Yeasen Biotechnology Co., Ltd., China. Transwell kit and Penicillin-Streptomycin were purchased from Gibco, United States. Dual luciferase reporter gene detection kit was purchased from Promega Corporation, United States. EZMagna RIP kit was puchased from Millipore, Billerica, MA, United States.

Cell purchasing and culture

Human GC cells MKN-45, MKN-28, MGC-803, AZ521 and human gastric mucosa normal epithelial cells HFE-145 were all purchased from Bena Biotech, with the cell numbers of BNCC337682, BNCC102156, BNCC100665, BNCC338084, and BNCC342464 respectively. The purchased MKN-45, AZ521, MGC-803, AZ521 and HFE-145 were cultured in RPMI 1640 medium containing 10% fetal bovine serum (FBS) (Gibco), and incubated in a moist atmosphere at 37°C with 5% CO_2. Si-LncRNA ADPGK-AS1 (si-RNA), sh-LncRNA ADPGK-AS1 (sh-RNA), and LncRNA negative control group (si-NC) were transfected into the cells using Lipofectamine Lipofectamine 2000 kit (Invitrogen, Carlsbad, USA). The procedure was carried out strictly according to the kit instructions.

QRT-PCR detection

The total serum RNA was isolated as instructed by serum RNA extraction kit (Bioteke, China, BC1710213), and qRT-PCR was performed after reversely transcripting the total RNA to cDNA using genomic reverse transcription kit (Takara, China, 16072315). The operation process was carried out according to the instructions of SYBR GREEN (CoWin Biosciences Co., Ltd., China, MIC091465). The circulation conditions were: denatured at 95°C, 5 min; 95°C, 30 s; 61°C, 30 s; 72°C, 30 s, cycling 45 times. Each group was equipped with 3 multiple holes. In this experiment, GAPDH was used as the internal reference and $2^{-\Delta\Delta ct}$ was utilized for data analysis. See **Table 1** for primer sequences.

MTT detection

The transfected cells were inoculated into 96-well plates, each containing 5×10^3 cells, and then grew in a 37°C and 5% CO_2 incubator. The plates were removed at 24 h, 48 h, 72 h and 96 h, respectively, and MTT solution (5 mg/mL, Sigma-Aldrich) was added at each time

point for a 4-hour culture. After discarding the MTT solution, 150 μL DMSO (Sigma-Aldrich) was added to each well, and the optical density (OD) at 570 nm wavelength was measured by enzyme scale instrument after shaking for 10 min. The experiment was repeated three times and the results were averaged.

FC

The transfected cells were collected and treated according to the instructions of the apoptosis detection kit (FITC) before they were re-collected, centrifuged, and incubated at a density of 1×10^6/mL. Then 100 μL cell suspension was incubated in the dark with 10 μL Annexin-V-FITC for 15 min, and re-stained with PI for 30 min, followed by the detection with a flow cytometer. In the double-parameter diagram, those with Annexin-V and PI negative were normal cells, those with Annexin-V PI negative were early apoptotic cells, and those with Annexin-V and PI positive were late apoptotic cells. Apoptosis rate of cells = percentage of early apoptotic cells + percentage of late apoptotic cells.

Cell invasion test (Transwell)

After transfection for 24 h, cells were collected (~3×10^4), and seeded in the upper chamber of the Transwell. Then it was rinsed with PBS twice and inoculated in the upper chamber where 200 μL DMEM was added, while the lower chamber was filled with 500 mL DMEM containing 20% FBS. After culturing at 37°C for 48 h, the substrates and cells that did not pass through the membrane surface of the upper chamber were wiped, and rinsed 3 times with PBS, fixed with paraformaldehyde for 10 min, and washed with double steam water for 3 times. After drying, the cells were stained with 0.5% crystal violet, and under the microscope (200×), 10 fields were randomly selected for cell counting, and the results were averaged.

WB detection

The protein was extracted from the cultured cells of each group with RIPA buffer as instructed, added with lysate and centrifuged at 10,000×g for 20 min to collect the supernatant. The protein concentration was determined by the BCA method (using bovine serum albumin as the standard). Then the same amount of protein was isolated by SDS-PAGE before it was transferred to a membrane and sealed with 5% skim milk powder at room temperature for 1 h. Subsequently, Bax (1: 1,000), Bcl-2 (1: 2,000) and GAPDH (1: 5,000) primary antibodies were added and incubated at 4°C overnight. Then the goat anti-mouse IgG secondary antibody labeled with HRP was added and incubated for 2 h, and the chemiluminescence solution was added to develop.

Finally, the test results were photographed and quantity-one software was used to analyze the gray value of each band.

Cisplatin treatment

The cells were treated with cisplatin of 4 μmol/L, 8 μmol/L, 16 μmol/L and 32 μmol/L respectively 24 h after transfection. After that, the cell survival was detected, and the average value was obtained after 3 times of experiment.

Statistical methods

In this study, the data was statistically analyzed using SPSS20.0, and the relevant pictures were plotted by GraphPad 7. The measurement data were expressed in the form of mean ± standard deviation (Mean ± SD) and verified by the t test. Independent sample t-test was adopted for inter-group comparisons (expressed as t), and one-way ANOVA was employed for multi-group comparisons. The post hoc pairwise comparison was performed by LSD-t test, the multi-time expressions were analyzed by repeated measurement analysis of variance, and multi-point expression was analyzed by repeated measures analysis of variance and Bonferroni post-hoc test. A statistically significant difference was considered with $P<0.05$.

Results

ADPGK-AS1 was expressed in gastric cancer cells

ADPGK-AS1 was found to be significantly increased in HFE-145 and was markedly higher than that in other four cell lines MKN-45, MKN-28, MGC-803, and AZ521 ($P<0.05$), of which MKN-45, AZ521 showed the greatest difference with HFE-145 (**Figure 1**).

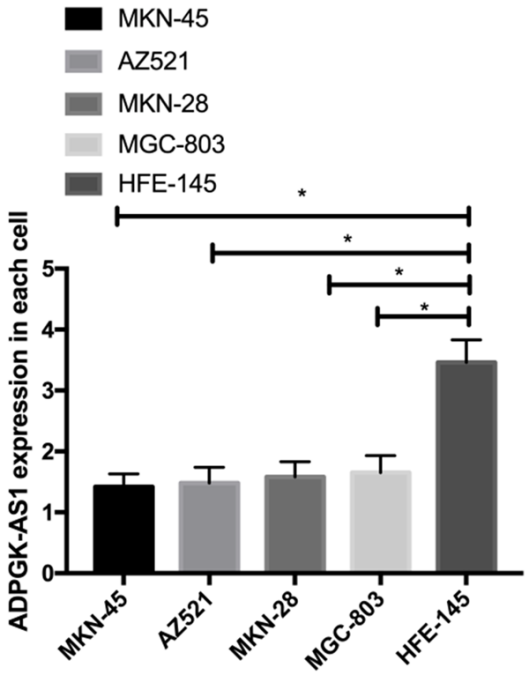

Figure 1. ADPGK-AS1 expression in each cell line. Note: * indicated P<0.05 between two groups.

Detection of transfection success rate

As mentioned in the preceding paragraph, the difference of ADPGK-AS1 expression between MKN-45, AZ521 and HFE-145 was the biggest, so the former two were selected for transfection. It was found that the ADPGK-AS1 in the Si-RNA group of MKN-45 and AZ521 was noticeably lower than that in other two groups (P<0.05), and the ADPGK-AS1 in the Sh-RNA group of MKN-45 and AZ521 was evidently higher compared with the Si-NC and Si-RNA groups (P<0.05) (**Figure 2**).

Overexpression of ADPGK-AS1 inhibited the proliferation and invasion of gastric cancer cells and promoted apoptosis

As shown by the MTT experiments, the proliferation capacity of MKN-45 and AZ521 cells in the Si-RNA group was the highest among the three groups (P<0.05), and compared between the Si-NC group and the Sh-RNA group, the proliferation capacity of the former was notably higher than the latter (P<0.05). Transwell experiments exhibited that, the cell invasion rate of MKN-45 and AZ521 cells in the Si-RNA group was dramatically higher (P<0.05), and that in the Sh-RNA group was noticeably lower when

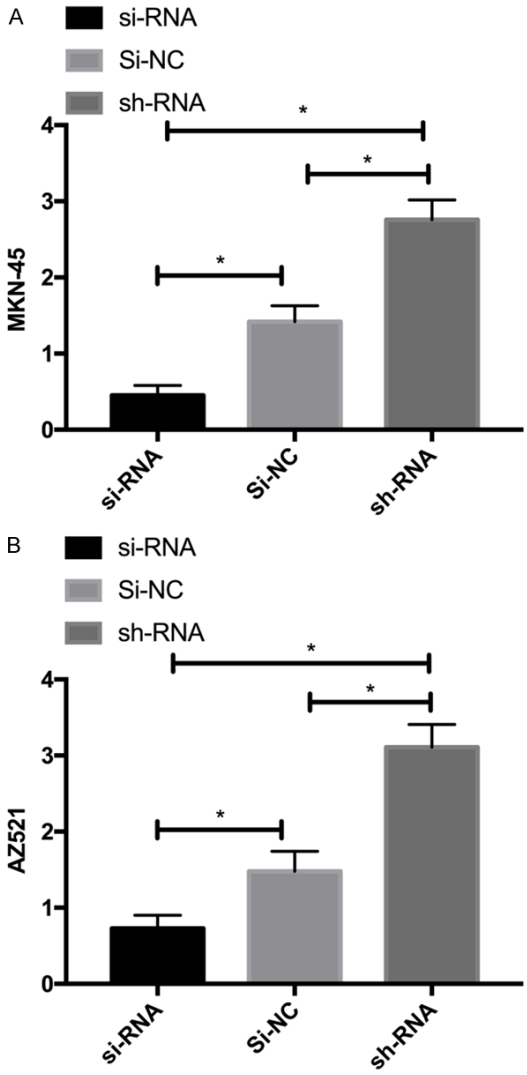

Figure 2. ADPGK-AS1 expression in transfected cells. A. MKN-45 expression. B. AZ521 expression. Note: * indicated P<0.05 between two groups.

compared with the Si-NC group (P<0.05). FC experiments demonstrated that the apoptosis rate of MKN-45 and AZ521 in the Sh-RNA group was noticeably higher than the other two groups (P<0.05) (**Figures 3**-**5**).

Overexpression of ADPGK-AS1 inhibited Bax expression and promoted Bcl-2 expression

The Bax and Bcl-2 protein expression was detected by WB. The results revealed that Bax protein was decreased greatly in the Si-RNA group of MKN-45 and AZ521 cells (P<0.05), and increased markedly in the Sh-RNA group (P<0.05). Bcl-2 protein was elevated dramatically in the Si-RNA group of MKN-45 and AZ521

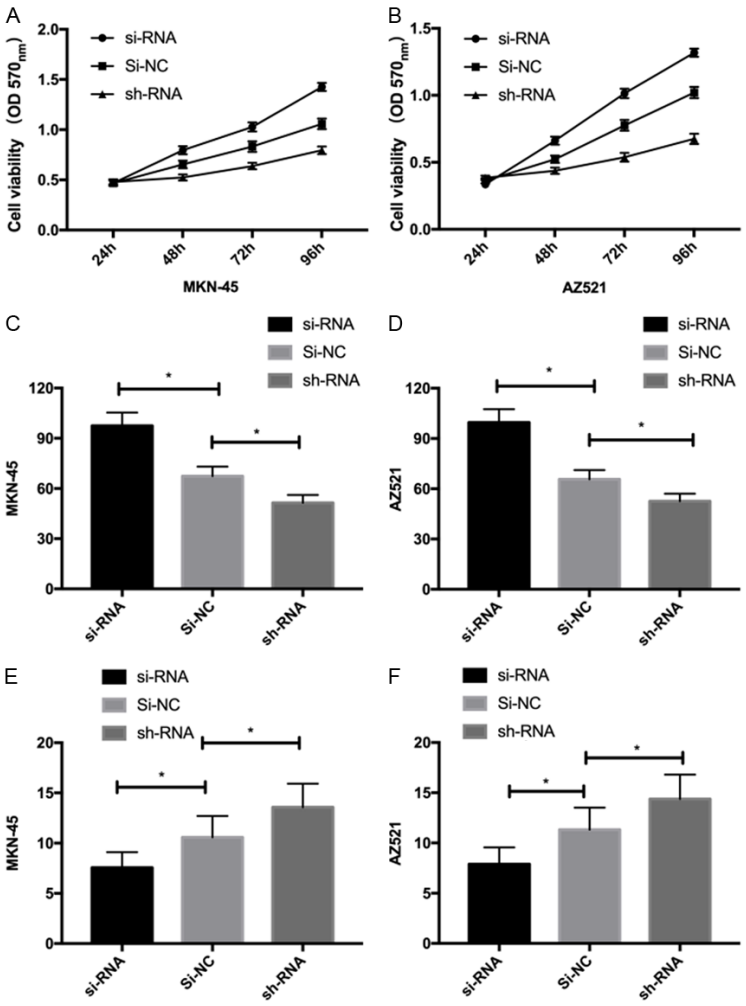

Figure 3. Effects of transfection on biological function of cells. A. Proliferation capacity of MKN-45. B. Proliferation capacity of AZ521. C. Invasion capacity of MKN-45. D. Invasion capacity of AZ521. E. Apoptosis rate of MKN-45. F. Apoptosis rate of AZ521. Note: * indicated P<0.05 between two groups.

cells (P<0.05), and decreased notably in the Sh-RNA group (P<0.05) (**Figure 6**).

Inhibition of ADPGK-AS1 improved the survival rate of gastric cancer cells after cisplatin treatment

Cells were processed for cisplatin treatment for 24 h with different concentrations (4, 8, 16 and 32 μmol/L), and the survival rates of cells were observed. At 4 μmol/L and 8 μmol/L, no marked difference was noticed in the survival rate of MKN-45 and AZ521 cells among the three groups (P>0.05), while at 16 μmol/L and 32 μmol/L, the survival rate of cells in the Si-RNA group were remarkably higher than that in the Si-NC group and Sh-RNA group (P<0.05) (**Figure 7**).

Discussion

The incidence and mortality of GC are relatively high, and most patients are already in the advanced stage [15] when they seek medical treatment, which deprive them of the optimal surgical period, resulting in poor prognosis and low survival rate [16]. While chemotherapy can provide surgical opportunities for patients who cannot undergo surgical treatment by reducing the preoperative surgical stage [17]. Research has shown that oxaliplatin combined with tegafur gimeracil oteracil potassium capsule or capecitabine are often used for neoadjuvant chemotherapy in patients with advanced GC, but some patients are not sensitive to chemotherapy drugs and even develop drug resistance, which affects the efficacy of chemotherapy [18]. Other evidence has indicated that chemotherapy sensitivity can be improved by inhibiting and promoting some related genes [19]. Referring to the previous literature, we found that LncRNA ADPGK-AS1 regulated a variety of cancers and was related to GC [20]. Therefore, we speculated that the chemosensitivity of GC may be affected by regulating the level of LncRNA ADPGK-AS1. In order to verify our speculation, we conducted research with the following results.

In this study, the expression of ADPGK-AS1 increased significantly in human normal gastric mucosal cells HFE-145, which was dramatically higher than that in the four GC cell lines MKN-45, MKN-28, MGC-803 and AZ521. Among the four GC cell lines, the ADPGK-AS1 expression in MKN-45 and AZ521 was dramatically different from that of HFE-145, so we selected MKN-45 and AZ521 for transfection, and established the Si-RNA group, Sh-RNA group and Si-NC

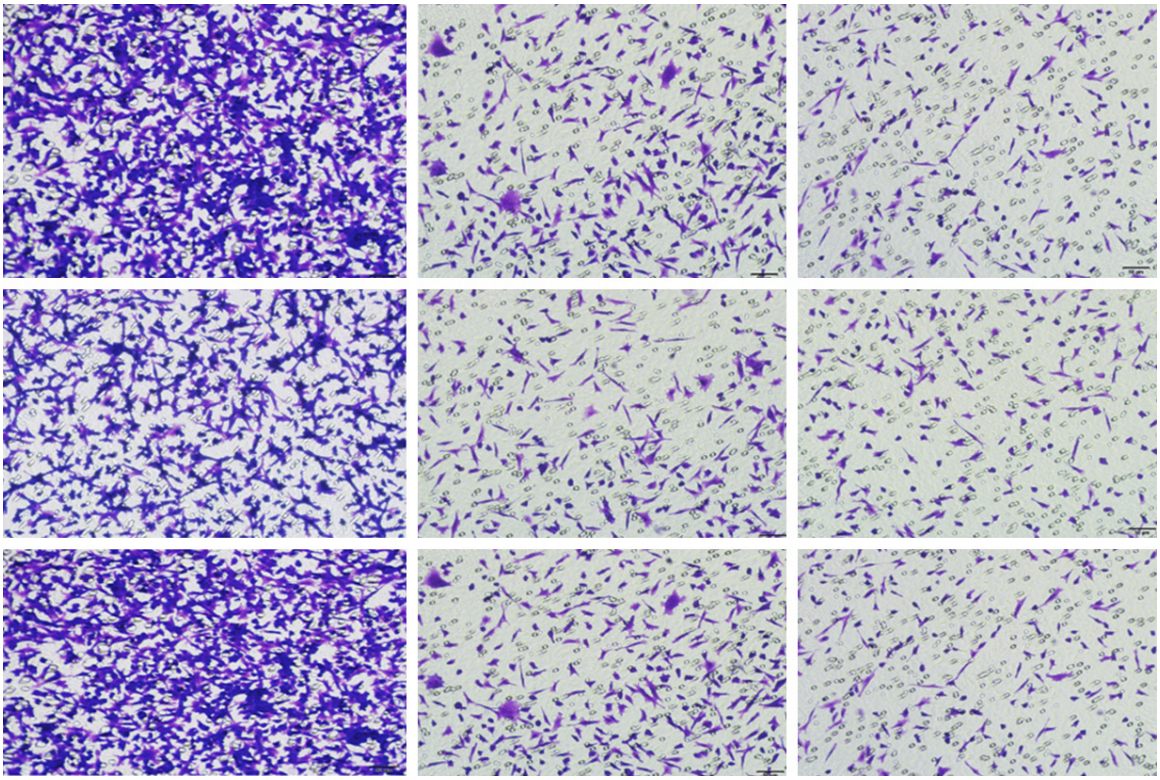

Figure 4. Invasion of MKN-45 cells.

group to test the ADPGK-AS1 level in them after transfection. The results showed that the ADPGK-AS1 in the Si-RNA group of MKN-45 and AZ521 was notably lower than that in the Sh-RNA and Si-NC groups, and it was greatly elevated in the Sh-RNA group of MKN-45 and AZ521 compared with the Si-NC and Si-RNA groups, indicating that our transfection was successful. Next, we observed the biological function of the transfected cells. MTT experiments displayed that the proliferation capacity of MKN-45 and AZ521 cells in the Si-RNA group was the highest, followed by the Si-NC group, with that of the Sh-RNA group being the lowest, and the differences were statistically significant. Transwell experiments exhibited that, the cell invasion rate of MKN-45 and AZ521 cells in the Si-RNA group was dramatically higher, and that in the Sh-RNA group was noticeably lower when compared with the Si-NC group. FC experiments demonstrated that the apoptosis rate of MKN-45 and AZ521 in the Sh-RNA group was noticeably higher than the other two groups. These results exhibited that overexpression of LncRNA ADPGK-AS1 reduced GC cell activity, confirming the role of LncRNA ADPGK-AS1 in GC, that is, high expression of LncRNA ADPGK-AS1 inhibited the proliferation and invasion of GC cells, promoted apoptosis, and reduced the occurrence and development of GC. This also suggests that LncRNA ADPGK-AS1 may be a promising therapeutic target for GC. In the future, the expression of LncRNA ADPGK-AS1 may be regulated clinically to affect the progression of cancer cells. The study by Gao et al. [21] showed that lncRNA NBAT-1 was underexpressed in GC, and its low expression promoted the occurrence and development of GC and was associated with poor prognosis, which is similar to our findings. Yang et al. [22] revealed that lncRNA acted on the progression of various tumors including breast cancer (BC). In the study of Qin et al. [23], LncRNA MDC1-AS was found to present low expression in GC, and its inhibition would increase the proliferation and invasion and reduce the apoptosis rate of cancer cells, while on the contrary, its overexpression could inhibit the development and proliferation and increase the apoptosis rate of cancer cells. Therefore, we speculated that LncRNA ADPGK-AS1 might become a new potential therapeutic target for GC. Then we

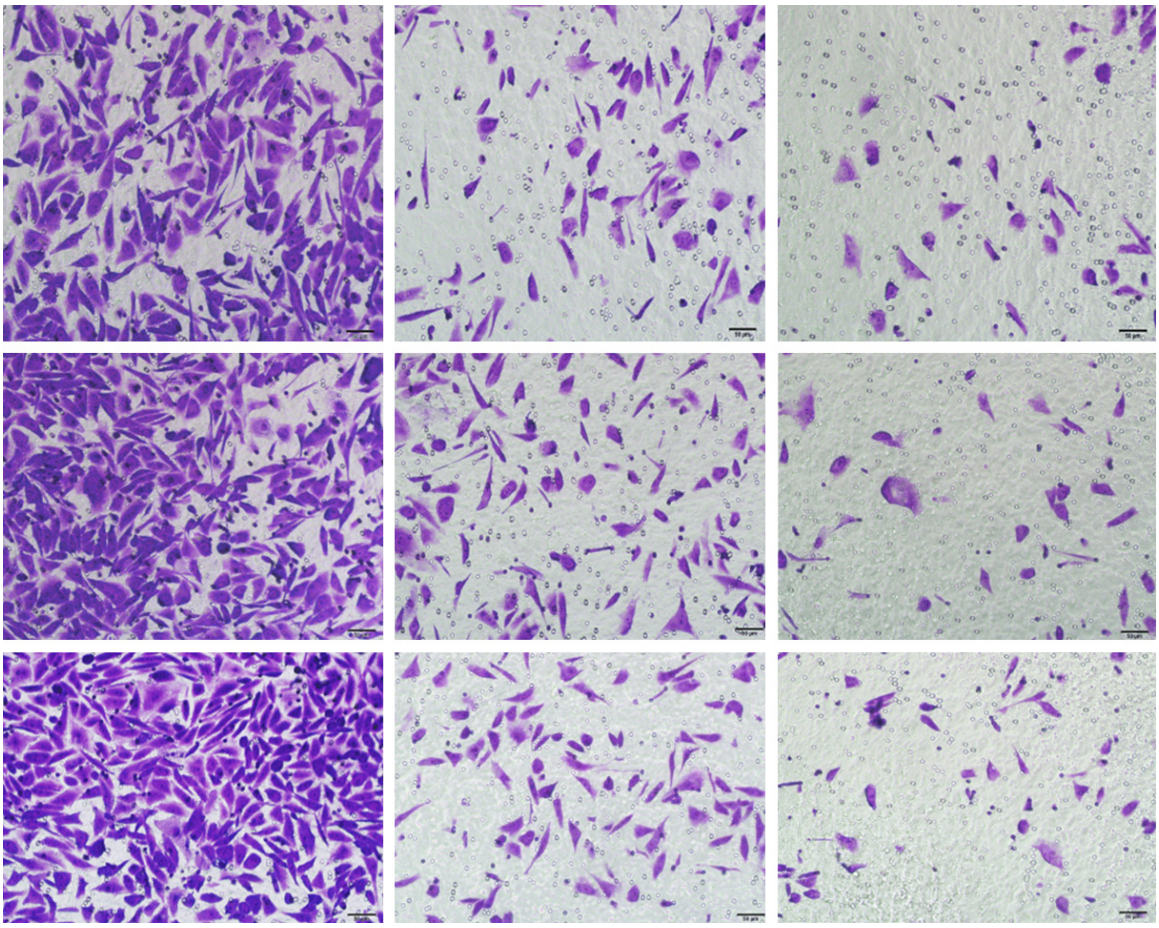

Figure 5. Invasion of AZ521 cells.

measured Bax and Bcl-2 protein expression with WB. The results showed that Bax reduced statistically in MKN-45 and AZ521 cells in the Si-RNA, and elevated markedly in the Sh-RNA group, while Bcl-2 increased dramatically in the Si-RNA group, and declined notably in the Sh-RNA group. Bax is a pro-apoptotic protein and Bcl-2 is an anti-apoptotic protein, which further supports our above experimental results. Finally, in order to test whether the highly expressed LncRNA ADPGK-AS1 can increase the chemotherapy sensitivity of GC, we treated the cells with cisplatin at concentrations of 4, 8, 16, and 32 μmol/L for 24 h. The survival rate of the MKN-45 and AZ521 turned out to be differed insignificantly among the three groups at concentrations of 4 μmol/L and 8 μmol/L, while at the concentration of 16 μmol/L and 32 μmol/L, the survival rate in the Si-RNA group was remarkably higher than that in the Si-NC group and Sh-RNA group. Cisplatin is an orange-yellow or yellow crystalline powder, which is slightly soluble in water and easily soluble in dimethylformamide, and can be gradually converted into trans and hydrolyzed in aqueous solution. It can bind to DNA and trigger cross-binding, thereby damaging DNA function and inhibiting cell mitosis. Apart from that, it is a non-specific cell drug that can block the proliferation of tumor cells and induce apoptosis [24]. The results suggested that overexpressed LncRNA ADPGK-AS1 could enhance the sensitivity of GC cells to cisplatin at the concentrations of 16 μmol/L and 32 μmol/L, indicating that it can be used as an auxiliary index of chemotherapy for GC in the future to enhance the effect of chemotherapy and improve the prognosis.

However, there are still some inadequacies in this experiment. To begin with, due to limited experimental conditions, tumor formation experiments in nude mice has not been carried out in this experiment, so it is not clear whether

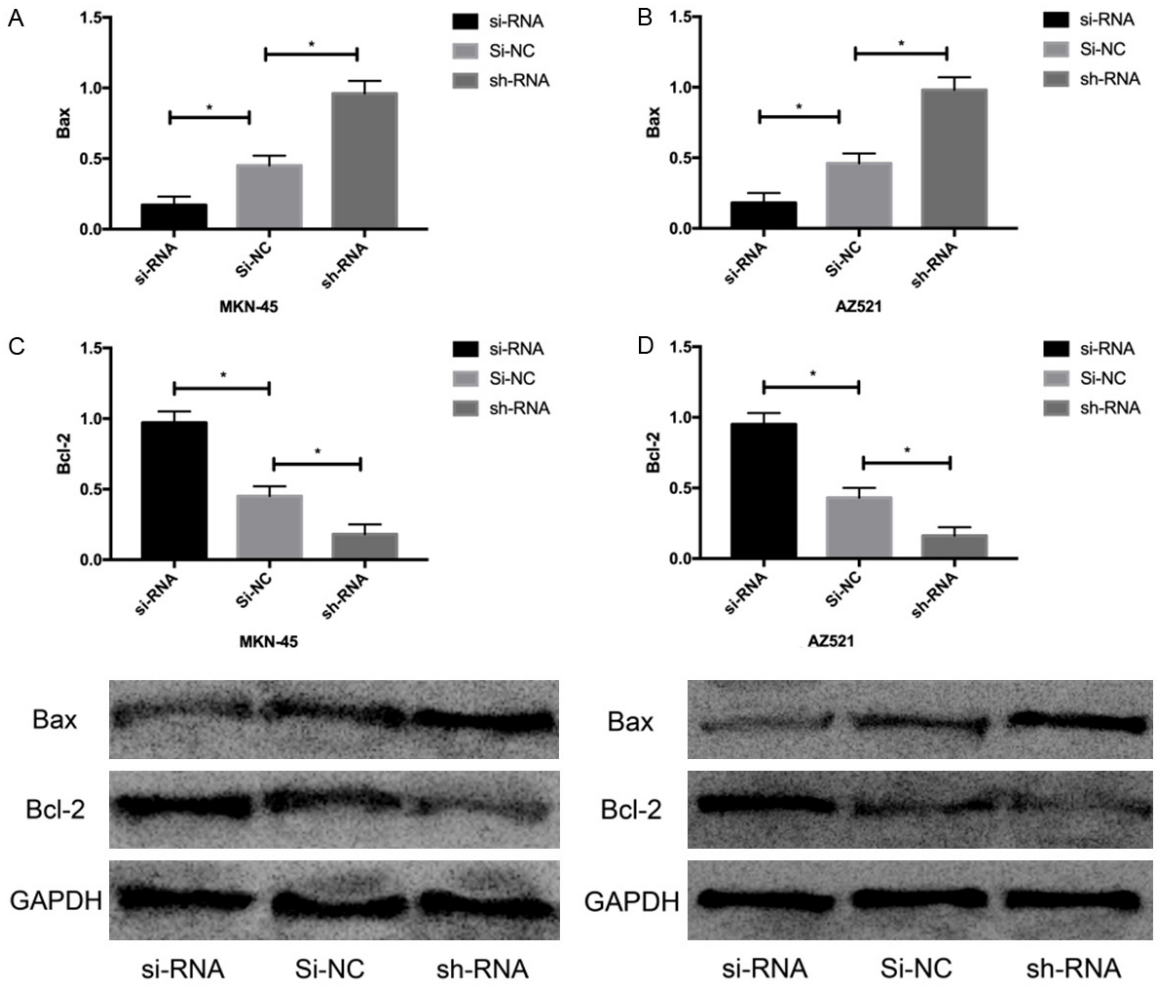

Figure 6. Detection of Bax and Bcl-2 expression. A. Bax expression in MKN-45. B. Bax expression in AZ521. C. Bcl-2 expression in MKN-45. D. Bcl-2 expression in AZ521. Note: * indicated P<0.05 between two groups.

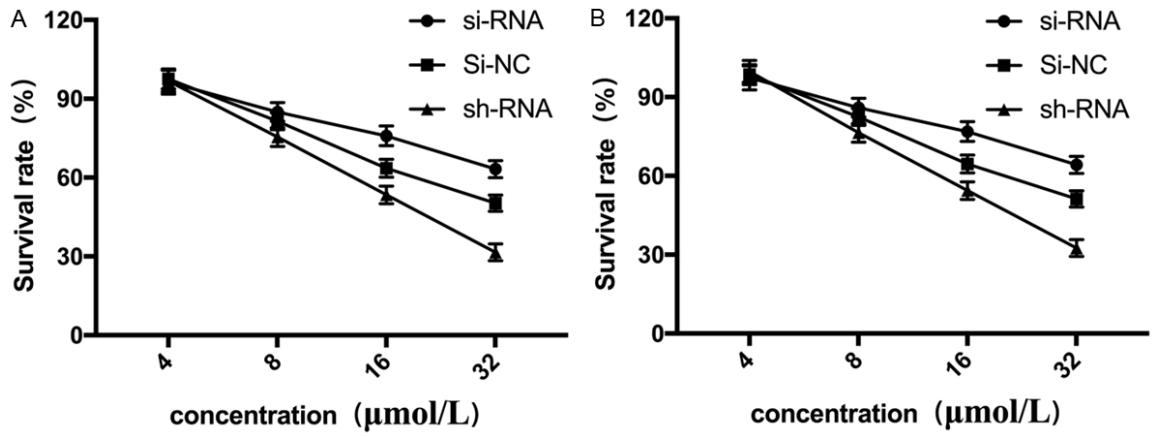

Figure 7. Cell viability after cisplatin treatment. A. Survival rate of MKN-45. B. Survival rate of AZ521.

LncRNA ADPGK-AS1 can be used as a therapeutic target for GC. In addition, clinical trials are absent in this study, so whether the serum expression of patients with GC is similar to that

of cells remains to be further explored. Moreover, we are unable to evaluate the prognosis of LncRNA ADPGK-AS1 in patients with GC due to the short experimental period. We will carry out experimental analysis on the above shortcomings as soon as possible to obtain more complete experimental results.

Conclusion

Highly expressed LncRNA ADPGK-AS1 can inhibit the proliferation and invasion of GC cells and promote apoptosis, and can increase the sensitivity of GC to chemotherapy.

Disclosure of conflict of interest

None.

Address correspondence to: Ye Hua, Department of General Surgery, Chongqing Emergency Medical Center, No. 1 Jiankang Road, Yuzhong District, Chongqing 400014, China. E-mail: huayyzbvy86993@163.com

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