Knockdown of long non-coding RNA HOTAIR increases cisplatin sensitivity in ovarian cancer by inhibiting cisplatin-induced autophagy

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Summary

Purpose: Ovarian cancer is one of the most malignant tumors in the female reproductive system. With the widespread application of chemotherapeutic drugs, many ovarian cancer patients develop drug resistance. The aim of this study was to explore the function of HOTAIR in the treatment of ovarian cancer with cisplatin and its underlying mechanism.

Methods: Quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect HOTAIR expressions in tissues and cells of ovarian cancer. Cell counting kit-8 (CCK-8) assay was used to examine the viability of ovarian cancer cells. Besides, small interfering (si) RNA was transfected to knockdown HOTAIR so as to explore its biological function. Western blot was performed to detect the expression levels of autophagy-related proteins. Flow cytometry was applied to detect apoptosis of ovarian cancer cells.

Results: HOTAIR was upregulated in ovarian cancer. Meanwhile, expression levels of autophagy-related proteins Atg7 and LC3 II/I in ovarian cancer cells increased with the increase of cisplatin concentration. Transfection of si-Atg7 could improve the therapeutic effect of cisplatin on ovarian cancer via inhibiting autophagy. Additionally, HOTAIR knockdown could increase the sensitivity of cisplatin in ovarian cancer treatment by inhibiting cisplatin-induced autophagy.

Conclusions: Knockdown of long non-coding (lnc) RNA HOTAIR could increase the sensitivity of cisplatin in ovarian cancer by inhibiting cisplatin-induced autophagy. Our research attempts to find a more effective treatment and provides new ideas for the clinical treatment of ovarian cancer.

Key words: apoptosis, autophagy, cisplatin, HOTAIR, ovarian cancer

Introduction

Ovarian cancer is one of the most malignant tumors in the female reproductive system, with a 5-year survival rate of only 30% even after active treatment [1,2]. Current treatments of ovarian cancer mainly include surgical resection, postoperative chemotherapy and radiotherapy. Therefore, it is of great importance to explore more effective treatments for ovarian cancer.

The long non-coding RNA HOTAIR (lncRNA HOTAIR) exists between the HOXC11 and HOXC12 locus, which regulates HOXD expressions in various tissues. In addition, HOTAIR has a regulatory effect on histone modification and gene correct expression [3]. A large number of studies has shown that HOTAIR is aberrantly expressed in many tumors, including liver cancer, colorectal cancer, pancreatic cancer, and gastric cancer [4-7].

Cisplatin is one of the most classic chemotherapeutic drugs for ovarian cancer, and plays an important role in clinical treatment. With the

widespread application of chemotherapeutic drugs, however, many ovarian cancer treatments have developed drug resistance. Therefore, reversal of drug resistance in ovarian cancer has been well recognized in clinical and basic researches [8]. Recent investigations have shown that autophagy is closely related to the occurrence and development of cancer [9,10]. Autophagy regulation could reverse the resistance to chemotherapy, including cisplatin resistance. It has been confirmed that the activated autophagy could lead to cisplatin resistance in lung cancer [11]. HOTAIR has also been reported to participate in drug resistance in ovarian cancer [12]. The relationship between autophagy and tumorigenesis has been explored in a previous study [13].

Stress factors are capable of upregulating autophagy in cancer cells, including cell injury caused by anticancer drug treatment. Besides, some cancer cells may develop drug resistance or promote tumor growth through autophagy enhancement [14]. Although it has been confirmed that HOTAIR could induce tumor autophagy [15], whether or not HOTAIR could affect cell growth by regulating cisplatin-induced autophagy has not been proved in ovarian cancer yet.

In our study, we explored the relationship among HOTAIR, ovarian cancer and autophagy and found that cisplatin could induce activation of protective autophagy in ovarian cancer. Interfering with HOTAIR increased the cisplatin sensitivity in ovarian cancer through reducing cisplatin-induced autophagy. Our research may provide new ideas and a basis for the treatment of ovarian cancer.

Methods

Patient samples

All the experimental tissue specimens were obtained from the Department of Reproduction, Children and Women's Healthcare of Laiwu City. A total of 20 ovarian cancer tissues and 10 adjacent normal tissues were collected from July 2013 to February 2017. Patients were preoperatively diagnosed with ovarian cancer and signed informed consent. This study was approved by the ethics committee of the Children and Women's Healthcare of Laiwu City.

Cell culture

The human ovarian cancer cell lines SKOV3 and A2780 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). These cells were cultured in high glucose Dulbecco's modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and placed at 37°C in a 5% CO_2 incubator. Cisplatin was purchased from Selleck Chemicals (Houston, TX, USA).

Transfection of small interfering RNAs

24 hrs before transfection, cells were digested and seeded into 6-well plates. Briefly, 5 µL of siRNA and 5 µL of Lipofectamine2000 were diluted with 250 µL of Opti-MEM for 5 min, respectively. Then, the two reagents were mixed into a transfection complex and placed at room temperature for 20 min. Then, 100 µL of transfection complex were added into each well and gently mixed.

Cell counting kit-8 (CCK-8) assay

The SKOV3 and A2780 cells were digested, centrifuged and resuspended. After cell counting, 2000 cells per well were seeded into a 96-well plate and 10 µL of CCK-8 reagent were added 24 hrs later. Cells were then incubated at 37°C for another 2 hrs. The absorbance at 450 nm wavelength was measured by a microplate reader.

Western blot analysis

Total proteins were extracted with radioimmunoprecipitation assay (RIPA) lysate, and the protein concentration was detected by Bio-Rad Protein assay (Bio-Rad Laboratories, Hercules, CA, USA). Samples were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS/PAGE) and then transfected to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After blocking with 5% nonfat milk at room temperature, the immunoblots were incubated with the following primary antibodies overnight at 4°C, including Atg7 (Cell Signaling Technology, # 8558, Danvers, MA, USA), LC3A / B (CST, # 12741, Danvers, MA, USA), Bcl-2 (CST, # 4223, Danvers, MA, USA), Bax (CST, # 5023, Danvers, MA, USA) and GAPDH (Beyotime, #AF0009, Shanghai, China). Membranes were then incubated with HRP-labeled secondary antibody for 1 hr. The protein bands were analyzed by Find-do × 6 Tanon (Tanon, China) after washing with TBST (Phosphate Buffer Solution with 0.05% Tween20).

Quantitative real-time PCR

Total RNA was extracted by TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The RNA concentration was measured by a Nanodrop Spectrophotometer (IMPLEN GmbH, Munich, Germany). The cDNA was synthesized by reverse transcription reaction with a PrimeScript RT reagent kit (TaKaRa, Tokyo, Japan). QRT-PCR was performed using the SYBR Primix kit (TaKaRa, Tokyo, Japan). The Ct values were obtained and statistically analyzed by $2^{-\Delta\Delta Ct}$ quantification method. GAPDH and HOTAIR primers were synthesized by Bioengineering Co., Ltd, (Shanghai, China). Primer sequences used in this study were as follows: GAPDH forward 5'-CCGGGAAACTGTGGCGGT-GATGGT-3', reverse 5'-AGGTGGAGGTATGGGTGTCGCT-GTT-3'; HOTAIR forward 5'-GGTAGAAAAAGCAACCAC-GAAGC-3', reverse 5'-ACATAAACCTCTGTGAGTGCC-3'.

Flow cytometry

Cells were washed with phosphate buffered saline (PBS), digested with EDTA-free trypsin and centrifuged. Then cell pellets were resuspended, washed with PBS

and centrifuged at 1000 rpm for 3 min. Propidium iodide (PI) and Annexin V-FITC were added into cells. After incubation for 20 min, cell suspension (1×10^5 cells/mL) was prepared and analyzed by flow cytometry.

Statistics

Data analyses were performed with Prism GraphPad 6.0 software (La Jolla, CA, USA). Data were presented as mean ± SD. Student's t-test was applied to assess the statistical significance between different groups and p<0.05 was considered statistically significant.

Results

Knockdown of HOTAIR inhibits autophagy in ovarian cancer cells

Twenty ovarian cancer samples and 10 ovarian cancer-adjacent normal tissues were collected. HOTAIR expressions in these tissues were detected by qRT-PCR and found that its expressions were decreased in adjacent normal samples compared with cancer samples (p<0.01) (Figure 1A), suggesting that HOTAIR was overexpressed in ovarian cancer. Transfection of si-HOTAIR in SKOV3 and A2780 cells significantly decreased its expression (p<0.01) (Figure 1B). Besides, CCK-8 assay showed that knockdown of HOTAIR significantly inhibited

the viability of ovarian cancer cells (p<0.05) (Figure 1C). In addition, interfering with HOTAIR inhibited the expressions of autophagy-related proteins Atg7 and LC3II/I (p<0.05) (Figure 1D). These results indicated that HOTAIR knockdown could inhibit autophagy.

Cisplatin induces autophagy in ovarian cancer

SKOV3 and A2780 cell lines were treated with different doses of cisplatin for 24hrs, respectively. Then cell viability was detected by CCK-8 assay. The results showed that cisplatin inhibited the proliferation of ovarian cancer cells in a dose-dependent manner (Figure 2A). To further explore the pharmacological effects of cisplatin on ovarian cancer, we detected the protein expressions of autophagy-related genes by Western blot and found that protein levels of Atg7 and LC3II/I were elevated with the increase of cisplatin dose (Figure 2B). These results suggested that cisplatin could lead to autophagy in ovarian cancer cells.

Inhibition of autophagy increases the cisplatin sensitivity of ovarian cancer cells

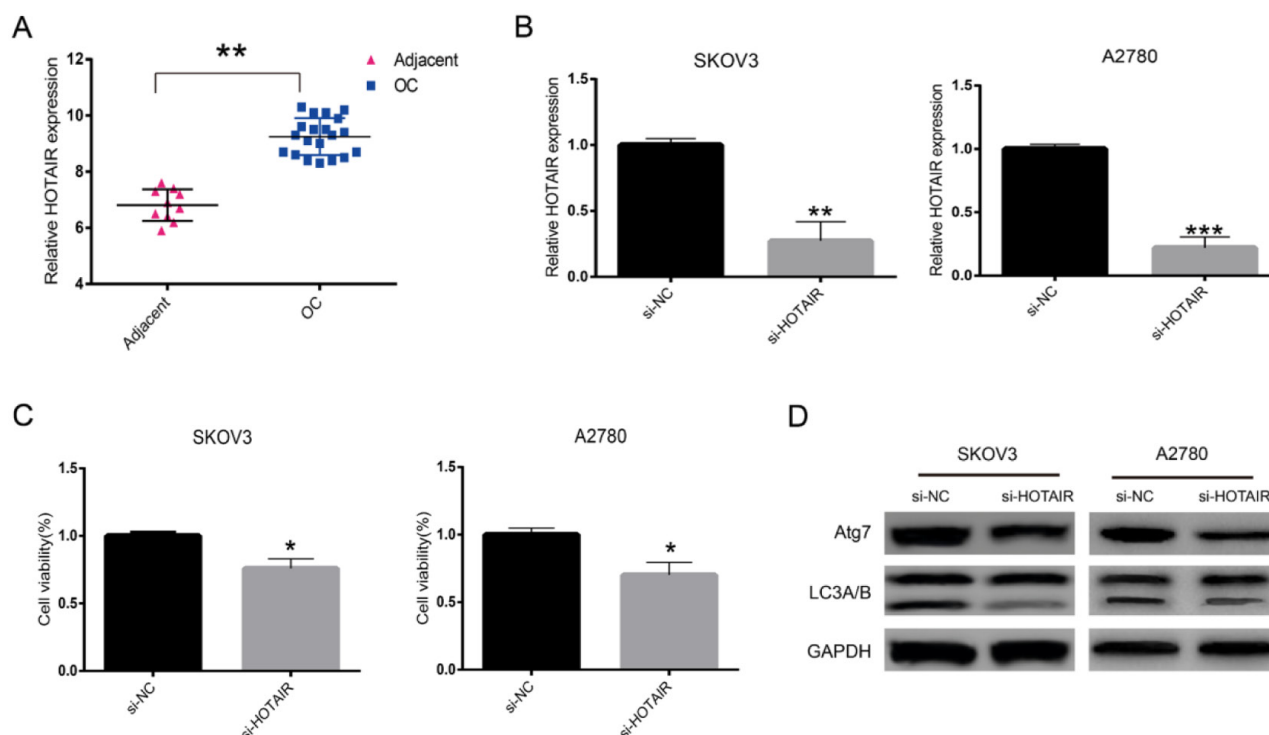To further investigate the role of cisplatin-induced autophagy, we suppressed autophagy in

Figure 1. Knockdown of HOTAIR inhibits autophagy in ovarian cancer cells. **(A)** HOTAIR expression was detected by qRT-PCR in ovarian cancer tissues (n=20) and adjacent normal tissues (n=10, **p<0.01); **(B)** HOTAIR expression in SKOV3 and A2780 cells after transfection with si-HOTAIR was detected by qPCR (**p<0.01; ***p<0.001); **(C)** Cell viability of SKOV3 and A2780 cells after transfection with si-HOTAIR was evaluated by CCK-8 assay (*p<0.05); **(D)** Protein levels of Atg7, LC3A / B and GAPDH protein in SKOV3 and A2780 cells were detected by Western blot after transfection of si-HOTAIR. Interfering with HOTAIR reduced the expressions of Atg7 and LC3A/B.

ovarian cancer by transfection of si-Atg7. As shown in Figure 3A, the expressions of Atg7 and LC3II/I in ovarian cancer cells were significantly reduced (p<0.05) after transfection of si-Atg7. In addition, cisplatin-induced autophagy was also reduced after transfection of si-Atg7. These findings indicated that knockout of Atg7 could significantly inhibit cisplatin-induced autophagy in ovarian cancer cells. Besides, CCK-8 assay revealed that knockout of Atg7 could increase the inhibition of cisplatin on ovarian cancer (p<0.01) (Figure 3B). Taken together, these results showed that autophagy could lead to cisplatin resistance in ovarian cancer cells, while inhibiting autophagy could increase the sensitivity of ovarian cancer cells to cisplatin.

Knockdown of HOTAIR reduces cisplatin-induced autophagy and increases the therapeutic effect of cisplatin on ovarian cancer cells

Since knockdown of Atg7 could inhibit autophagy and increase the toxicity of cisplatin to ovarian cancer cells, we explored whether knockdown of HOTAIR could increase the effect of cisplatin on ovarian cancer cells via inhibiting autophagy. To validate the above hypothesis, ovarian cancer A2780 cells were transfected with si-HOTAIR or si-NC, respectively, followed by adding10 µM cisplatin or not. As shown in Figure 4A, HOTAIR knockdown combined with cisplatin stimulation significantly inhibited cell growth (p<0.05) compared to those with individual HOTAIR knockdown or cisplatin treatment. Besides, cisplatin-induced autophagy was significantly inhibited after knockdown of HOTAIR (p<0.05) (Figure 4B). Meanwhile, the expression level of anti-apoptotic protein Bcl-2 was decreased, whereas the level of pro-apoptotic protein Bax was elevated (Figure 4B). Flow cytometry also indicated an increase of apoptosis in A2780 cells treated with both HOTAIR knockdown and cisplatin compared to those with HOTAIR knockdown or cisplatin treatment alone (Figure 4C). These results suggested that knockdown of HOTAIR could
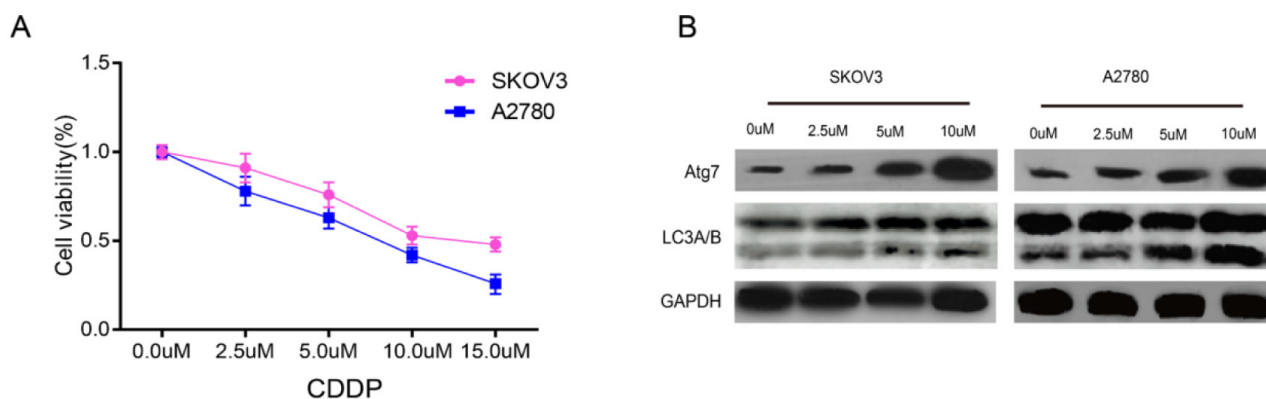

Figure 2. Cisplatin (CDDP) induces autophagy in ovarian cancer. **(A)** Cell viability was detected by CCK-8 assay after ovarian cancer cells were treated with different concentrations of cisplatin (p<0.05); **(B)** In Western blot, the protein levels of Atg7, LC3A/B were significantly increased with the increase of cisplatin dose in SKOV3 and A2780 cells (p<0.05).
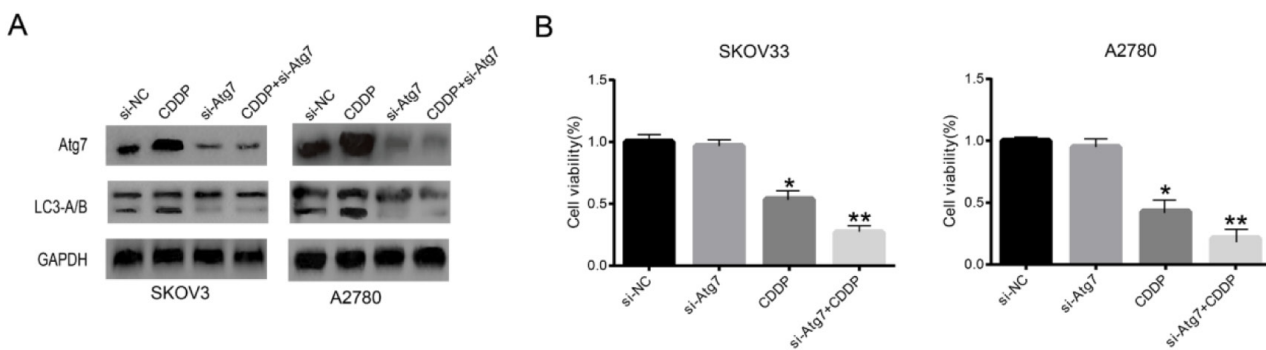

Figure 3. Inhibition of autophagy increases the sensitivity of ovarian cancer cells to cisplatin. **(A)** Protein levels of Atg7 and LC3A / B were detected by Western blot after transfection of si-NC, siRNA-siAtg7 (si-Atg7) and 10 µM cisplatin treatment. Tranfection of si-Atg7 significantly reduced the expressions of Atg7 and LC3A/B in ovarian cancer cells (p<0.05). **(B)** Cell viability was measured by CCK-8 assay after transfection of si-NC, siRNA-siAtg7 (si-Atg7) and 10 µM cisplatin treatment. Knockout of Atg7 significantly decreased cell viability of SKOV3 and A2780 cells (*p<0.05, **p<0.01).
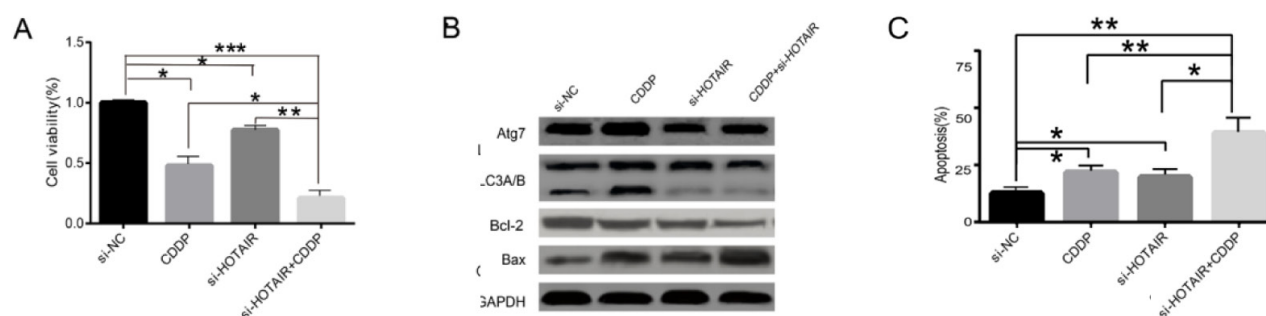
Figure 4. Knockdown of HOTAIR increases cisplatin-induced apoptosis in ovarian cancer cells. **(A)** Viability of A2780 cells was detected by CCK-8 assay. Cells were pretreated with 10 μM cisplatin, followed by transfection with si-HOTAIR. HOTAIR combined with cisplatin stimulation significantly decreased cell viability (*p<0.05, **p<0.01, ***p<0.001). **(B)** Protein levels of Atg7, LC3A/B, Bcl-2, Bax and GAPDH in ovarian cancer cells after cisplatin treatment and si-HOTAIR transfection were detected by Western blot. Knockdown of HOTAIR significantly inhibited the expressions of autophagy-related proteins. **(C)** Cell apoptosis was detected by flow cytometry after si-HOTAIR transfection or the combination of cisplatin treatment and si-HOTAIR transfection in ovarian cancer cells (*p<0.05, **p<0.01). HOTAIR knockdown combined with cisplatin stimulation significantly increased cell apoptosis in A2780 cells.

reduce cisplatin-induced autophagy and increase the therapeutic effect of cisplatin on ovarian cancer.

Discussion

Cisplatin and the platinum analogues are used in the first-line chemotherapy of ovarian cancer. However, most patients eventually develop cisplatin resistance and experience tumor recurrence. Therefore, it is of great importance to avoid the cisplatin resistance in the clinical treatment of ovarian cancer. Currently, the role of autophagy in ovarian cancer has attracted much attention. Autophagy not only plays a key role in tumorigenesis and development, but also is involved in the drug therapy of tumors [18,19].

Lnc RNA HOTAIR is overexpressed in about 25% of patients with breast cancer which is an independent marker of death and metastasis in this disease [20,21]. Yang et al. [22] reported that HOTAIR is upregulated in hepatocellular carcinoma compared to adjacent normal tissue. Moreover, patients with higher expression of HOTAIR had a significantly shorter recurrence-free survival. Studies on ovarian cancer cell lines A2780 and OVCA429 have shown that silencing HOTAIR could induce apoptosis by altering cell cycle and expressions of apoptosis-related proteins [23]. In this study, we found that HOTAIR was significantly increased in ovarian cancer tissues through analysis of 20 ovarian cancer tissues and 10 adjacent normal tissues. Besides, knockdown of HOTAIR inhibited the proliferation of ovarian cancer cells. Ozes et al. [12] found that HOTAIR leads to cisplatin resistance after cisplatin-induced DNA damage via activating the NF-κB pathway. In our study, we found that HOTAIR knockdown could inhibit Atg7, thus inhibiting autophagy in ovarian cancer. Liu et al. [24] showed that autophagy activator (rapamycin along with arsenic trioxide) could inhibit the proliferation of ovarian cancer cells. Meanwhile, it has been found that inhibiting autophagy could increase the cisplatin sensitivity of ovarian cancer [25,26]. In our study, we found that cisplatin induced autophagy in a concentration-dependent manner in ovarian cancer cells. Knockdown of Atg7 inhibited cell autophagy and proliferation, indicating that cisplatin-induced autophagy exerted a protective effect on ovarian cancer cells. Therefore, HOTAIR could inhibit the cisplatin-induced autophagy, which ultimately improved the cisplatin sensitivity of ovarian cancer cells. Besides, HOTAIR knockdown combined with cisplatin stimulation reduced autophagy and increased apoptosis in ovarian cancer cells.

Conclusions

This study has shown that knockdown of lncRNA HOTAIR inhibits autophagy via decreasing Atg7 expression. More importantly, knockdown of lncRNA HOTAIR leads to inhibited cisplatin-induced autophagy and elevated cisplatin sensitivity to ovarian cancer, which could provide a more effective treatment of ovarian cancer.

Conflict of interests

The authors declare no conflict of interests.

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