

Cisplatin suppresses tumor proliferation by inhibiting autophagy in ovarian cancer via long non-coding RNA RP11-135L22.1

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Abstract. – **OBJECTIVE:** To observe the effect of cisplatin-induced autophagy in human ovarian cancer cell lines and explore the correlation between RP11-135L22.1 with cisplatin-induced autophagy.

MATERIALS AND METHODS: Genome-wide expression profile and chemotherapy sensitivity data of ovarian cancer were downloaded from TCGA database. It was found that the expression level of lncRNA RP11-135L22.1 differed between chemotherapy-sensitive group and insensitive group. Besides, RP11-135L22.1 expression levels were detected in 64 ovarian cancer tissues and 30 normal tissues by qRT-PCR. Relationship between RP11-135L22.1 expression levels in 64 ovarian cancer tissues and their clinicopathological characteristics were analyzed by χ^2 -test. Cell viability was detected by CCK8 assay. Cell apoptosis and cell cycle were accessed by flow cytometry. HO8910 cells were selected for transfection of pcDNA-RP11-135L22.1, and qRT-PCR was used to evaluate RP11-135L22.1 expression in cisplatin-treated HO8910 cells. Western blot was performed to analyze the expression changes of autophagy-related proteins.

RESULTS: Genome-wide expression profile of chemotherapy-sensitive and -insensitive patients with ovarian cancer from TCGA database was analyzed by edgeR package. It was found that RP11-135L22.1 level in chemotherapy-sensitive group was significantly lower than that of insensitive group. QRT-PCR results confirmed that RP11-135L22.1 was lowly expressed in ovarian cancer. The overall survival of patients was positively correlated with the expression of RP11-135L22.1. Furthermore, RP11-135L22.1 was associated with FIGO stage and tumor size. Flow cytometry showed that cisplatin could induce apoptosis and arrest cell cycle in ovarian cancer cells lines. CCK8 assay showed that cisplatin decreased viability of ovarian cancer cells. For *in vitro* study, HO8910 cells were cultured with medium containing different concentrations of cisplatin or treated with cisplatin for different times. The results revealed that RP11-135L22.1 expression was negatively correlated with the treating time and dose of cisplatin. Western blot showed that cisplatin induced autophagy in ovarian

cancer cells in a time- and dose-dependent manner. Cisplatin combined with RP11-135L22.1 can reduce autophagy, increase the apoptosis and inhibit its activity of ovarian cancer cells to a certain extent.

CONCLUSIONS: Cisplatin can induce autophagy in HO8910 ovarian cancer cells. After overexpression of RP11-135L22.1, it inhibited cisplatin-induced autophagy, thus enhancing the effect of cisplatin on ovarian cancer cells.

Key Words

Ovarian cancer, Cisplatin, lncRNA, RP11-135L22.1, Autophagy.

Introduction

Ovarian cancer is a common malignant tumor in reproductive truck. The incidence of ovarian cancer ranks after lung cancer, breast cancer, rectal cancer and pancreatic cancer in women¹. In the last 20 years, the incidence of the disease increases at a rate of 0.1% per year, with 238 700 new cases each year². Ovarian cancer is easy to metastasize and relapse, and it is prone to be resistant to chemotherapy. Its curative effect and long-term prognosis were poor. Specifically, the 5-year survival rate was only about 30%³. At present, there are still no effective methods of early diagnosis; consequently, 75% of patients were already in an advanced stage when diagnosed⁴. Therefore, it is important to explore and clarify the drug resistance mechanism of ovarian cancer and enhance the chemosensitivity of ovarian cancer.

Cisplatin is widely-used for cancer chemotherapy. It induces apoptosis of tumor cells through irreversible inserting into DNA bases. Therefore, it can further induce DNA damage response, which prevents cell division and activates apoptosis⁵. Because of its broad spectrum of anti-cancer effects, in recent years, cisplatin received wide-

spread attention in treating ovarian cancer^{6,7}, but the mechanism is still unclassified. Thus, it is necessary to further exploring the mechanism of cisplatin in treating ovarian cancer.

Long non-coding RNAs (lncRNAs) are a class of RNAs whose transcripts are over 200 nucleotides in length and do not encode a protein⁸. With the in-depth study of lncRNA, its expression has been found to vary significantly in tumor cells. Therefore, the relationship between lncRNA and tumor has drawn attention⁹. Further research of lncRNA and ovarian cancer may provide a new strategy for diagnosis and treatment. Currently, there are no reports of lncRNA RP11-135L22.1; then, its effects in ovarian cancer need to be further explored.

Autophagy is a basic life process, in which cells are highly conserved to maintain homeostasis¹⁰. Autophagy can degrade organelles and macromolecular proteins. Under normal conditions, intracellular autophagy remains a relatively low rate. However, when cells are attacked by starvation or lack of growth factors, autophagy would be immediately activated. As a result, autophagy is commonly considered to be involved in cell survival, but excessive activation of autophagy can also cause cell death^{11,12}. Similarly, studies on tumors also found that autophagy was not only a protective factor for tumor escape¹³, but also an inhibitor of tumor development in other cases. In addition, studies^{14,15} have found that inhibition of autophagy indirectly inhibited drug-induced cell death. Therefore, autophagy has become a hot research topic in cancer drug therapy. In ovarian cancer, there is little research on the role of autophagy.

In this study, we first examined the effects of lncRNA RP11-135L22.1 on the cisplatin-mediated autophagy in treating ovarian cancer.

Materials and Methods

Expression Analysis of lncRNA RP11-135L22.1

Tumor genome-wide expression profile and drug sensitivity data were downloaded from TCGA database. Differential levels of lncRNAs in normal and tumor tissues, as well as in the chemosensitive and insensitive groups, were analyzed by edgeR package. RP11-135L22.1 expression in ovarian cancer patients was analyzed by qRT-PCR. This study was approved by the Ethical Committee of Yantai Yuhuangding Hospital.

Cell Culture

The ovarian cancer cell line HO8910 was cultured in Roswell Park Memorial Institute 1640 (RPMI-1640) medium (Gibco, Rockville, MD, USA) supplemented with 10% fetal bovine serum (FBS) (HyClone, South Logan, UT, USA) and penicillin/streptomycin, and placed in a thermostatic incubator at 37°C with 5% CO₂. After 4-5 generations of cell passage, cells with good condition were selected for research. When cell density reached 80% confluency, cells were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Specifically, 8 μL Lipofectamine 2000 and 4 μg pcDNA-RP11-135L22.1 dissolved in 500 μL of 1640 medium (Gibco, Rockville, MD, USA) were added to experimental group. In addition, the same amount of Lipofectamine 2000 and pcDNA-NC were added to the control group. Full growth medium was supplemented 6 h after transfection.

CCK-8 Assay

After 24 h of transfection, cells were seeded into 96-well plates at a density of $2 \times 10^3/100 \mu\text{L}$ and cell counting kit-8 (CCK-8) assay was performed at 24, 48, 72 and 96 h after inoculation. For CCK-8 assay, the serum-free medium was replaced at the time and 10 μL of CCK8 were added to each well. After incubation at 37°C and 5% CO₂ for 1 h, the OD value was measured at 450 nm. Each measurement was performed in quintuplicates.

Flow Cytometry Detection of Apoptosis Rate

Treated cells were trypsinized and transferred to the appropriate centrifuge tube for centrifugation. After centrifugation, the supernatant was removed, and cell pellet was washed twice with cold phosphate-buffered saline (PBS) and resuspended with Annexin V binding solution. 5 μL of Annexin V-fluorescein isothiocyanate (FITC) and 5 μL of PI were used for staining in dark for 15 min. Stained cells were diluted with 400 μL of $1 \times$ binding buffer. Measurement was performed by the flow cytometry and results were analyzed by software. Each experiment was performed in triplicates.

Flow Cytometry Detection of Cell Cycle

Cells were inoculated into 60 mm culture plates at a density of $1 \times 10^6/\text{ml}$. Transfection was performed when cell confluency was up to

80%. After 24 h, appropriate antibiotics (resistant markers on eukaryotic expression vector) were added to fresh medium for culturing. After 48–72 h, cells were digested by trypsin and washed twice with PBS. The supernatant was discarded and 1 mL of 70% ethanol was added. Cells were homogenized and fixed at 4°C for over 12 h. Cells were washed to remove ethanol, centrifuged at 1 000 r/min for 5 min, and washed again. The cells were then resuspended with 0.5 mL PBS. 1 mg/mL PI and 10 mg/mL RNase A were added to a final concentration of 50 g/ml for incubation at 37°C for 30 min. Cell cycle was measured by flow cytometry.

Western Blot

Total protein was extracted from the treated HO8910 cells, and bicinchoninic acid (BCA) kit was used for determining protein concentration. 40 µg protein were used for gel electrophoresis, which was transferred to polyvinylidene difluoride (PVDF) membrane, blocked with 5% skim milk for 1 h. Next, the membrane was incubated with primary antibodies overnight at 4°C and was followed by secondary antibodies incubation for 1 h. Enhanced chemiluminescence (ECL) was used for imaging exposure.

Statistical Analysis

SPSS 22.0 software (IBM, Armonk, NY, USA) was used for statistical analysis. Kaplan-Meier survival curve was used for survival analysis. Flow cytometry results were analyzed using WinMDI software. Clinicopathological characteristics were analyzed by χ^2 -test. Measurement data was analyzed by *t*-test. $p < 0.05$ was considered statistically significant.

Results

Relationship Between lncRNA RP11-135L22.1 Expression and Clinical Data in Ovarian Cancer

Genome-wide expression profiles of patients with chemotherapy-sensitive and non-responsive data were downloaded from TCGA. Our analysis revealed that lncRNA RP11-135L22.1 level was significantly decreased in the chemotherapy-insensitive group (Figure 1A and 1B). QRT-PCR results showed that there was a significantly lower expression of lncRNA RP11-135L22.1 in 64 ovarian cancer tissues compared with that in the 30 normal tissues (Figure 2A). It was also significantly lower in the chemotherapy-insensitive group than that of chemotherapy-sensitive group (Figure 2B). In addition, the overall survival was positively correlated with the expression of RP11-135L22.1 (Figure 2C, $p = 0.0485$, HR = 0.4570). Statistical results showed that the lower RP11-135L22.1 coupled with the later tumor stage and larger tumor size (Table I). These results indicated that highly expressed RP11-135L22.1 inhibited tumorigenesis.

Cisplatin Inhibits the Proliferation While Promoting Apoptosis of Ovarian Cancer Cells

To determine the effect of cisplatin on ovarian cancer cells, flow cytometry and CCK8 assays were performed in this paper. Apoptosis was measured by flow cytometry, which indicated that cisplatin induced apoptosis in a dose-dependent manner (Figure 2D). Cell cycle analysis suggested that the percentage of cells arrested in G1 phase was positively correlated with the cisplatin concentration on HO8910 cells (Figure 2E).

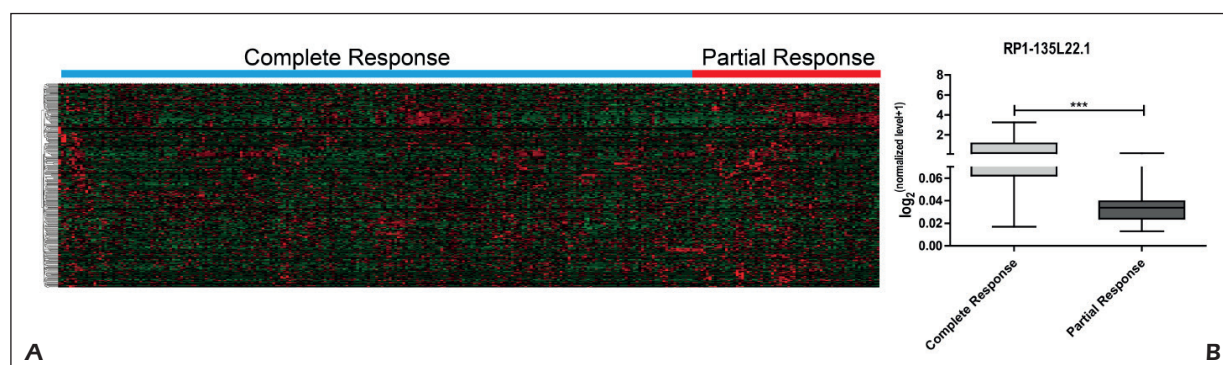


Figure 1. RP11-135L22.1 is overexpressed in chemosensitivity patients with ovarian cancer. **A**, Heat map of differential expression between chemotherapy-sensitive and chemotherapy-insensitive ovarian cancer. **B**, RP11-135L22.1 expression in the chemotherapy-insensitive group was lower than those in the chemotherapy-sensitive group.

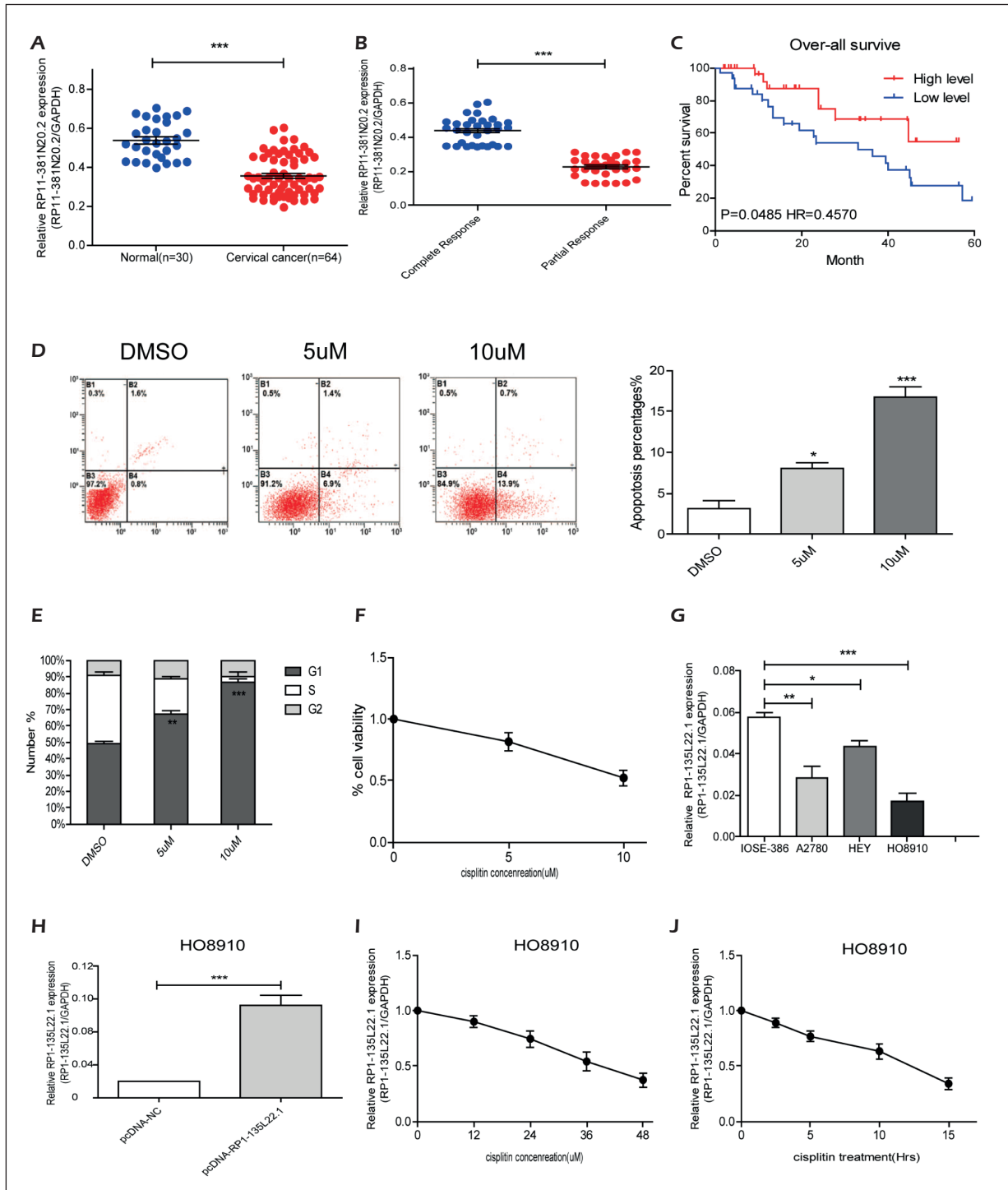


Figure 2. RP1-135L22.1 is lowly expressed in ovarian cancer. **A**, RP1-135L22.1 expression in 64 ovarian cancer tissues was significantly lower than that of 30 normal ovarian tissues. **B**, RP1-135L22.1 expression in chemosensitive patients with ovarian cancer was higher than those chemotherapy-insensitive patients. **C**, The overall survival in RP1-135L22.1 high expression group was higher than that of RP1-135L22.1 low expression group. **D**, Cell apoptosis under cisplatin treatment with different concentrations. **E**, Cell cycle alteration under cisplatin treatment with different concentrations. **F**, CCK8 results showed the proliferation after treatment with different concentrations of cisplatin. **G**, RP1-135L22.1 expression in ovarian normal cell line IOSE-386 and ovarian cancer cell lines (A2780, HEY, HO8910). **H**, Transfection of pcDNA-RP1-135L22.1 in HO8910. **I**, RP1-135L22.1 expression in HO8910 cell line after treatment of different concentrations of cisplatin. **J**, RP1-135L22.1 expression in the HO8910 cell line after treatment of cisplatin at different times.

Table 1. Correlation between the RP1-135L22.1 expression and clinicopathological characteristics in ovarian cancer patients (n = 64).

Clinicopathologic features	Number of cases	RP1-135L22.1 expression		p-value
		Low (n=32)	High (n=32)	
Age (years)				
< 50	27	15	12	0.4477
≥ 50	37	17	20	
Histological subtype				
Serous	25	14	11	0.4421
Others	39	18	21	
Tumor size				
< 8 CM	33	10	23	0.0011*
≥ 8 CM	31	22	9	
FIGO stage				
I-II	35	13	22	0.0238*
III-IV	29	19	10	
Histological grade				
G1-G2	27	16	11	0.2056
G3	37	16	21	
Lymph node metastasis				
Absent	34	18	16	0.6164
Present	30	14	16	

CCK8 assay showed that the viability of HO8910 cells decreased with the increased cisplatin concentration (Figure 2F). The above results showed that cisplatin could induce apoptosis, inhibit cell viability and proliferation of ovarian cancer cells.

RP11-135L22.1 Expression in HO8910 Cells Decreases after Cisplatin Treatment

It was found that HO8910 cell line had the lowest RP11-135L22.1 expression by qRT-PCR (Figure 2G). Therefore, HO8910 cells were selected for overexpression experiments. Effective transfection of pcDNA-NC and pcDNA-RP11-135L22.1 into HO8910 cell line resulted in a significant increase in the RP11-135L22.1 expression (Figure 2H). To explore the relationship between cisplatin and RP11-135L22.1, we cultured HO8910 cells in medium containing different concentrations of cisplatin. It was found that cisplatin concentration was negatively correlated with RP11-135L22.1 expression (Figure 2I). Similarly, long time treatment of HO8910 cells in the same cisplatin concentration medium led to lower expression of RP11-135L22.1 (Figure 2J). These data implied that cisplatin inhibited RP11-135L22.1 expression in cells, thus inhibiting tumor growth.

RP11-135L22.1 Promotes the Suppressor Effect of Cisplatin

After RP11-135L22.1 transfected into cells cultured in medium containing cisplatin, we found a significant increase in apoptosis level of

ovarian cancer cells (Figure 3A) and a significant decrease in cell viability (Figure 3B). These results showed that cisplatin combined with RP11-135L22.1 could promote apoptosis and reduce the vitality of ovarian cancer cells, thereby inhibiting tumor progression.

RP11-135L22.1 Inhibits Cisplatin-induced Autophagy

Expressions of autophagy related proteins in HO8910 cells, including LC3A/BI, LC3A/B-II and Atg7, were increased after cisplatin treatment at a time- and dose-dependent manners (Figure 3C, D). In contrast, expressions of Atg7 and LC3A/B-II were downregulated after treatment of cisplatin combined with RP11-135L22.1 (Figure 3E), indicating that RP11-135L22.1 inhibited cisplatin-induced autophagy in ovarian cancer. Thus, cisplatin can induce autophagy in ovarian cancer cells in order to inhibit tumor progression, while RP11-135L22.1 can inhibit cisplatin-induced autophagy.

Discussion

Ovarian cancer is a malignant tumor that seriously threatens women's health. It is the most malignant tumor of gynecologic cancer¹⁶. Previously, chemotherapy drugs eliminated tumor cells mainly through the induction of apoptosis,

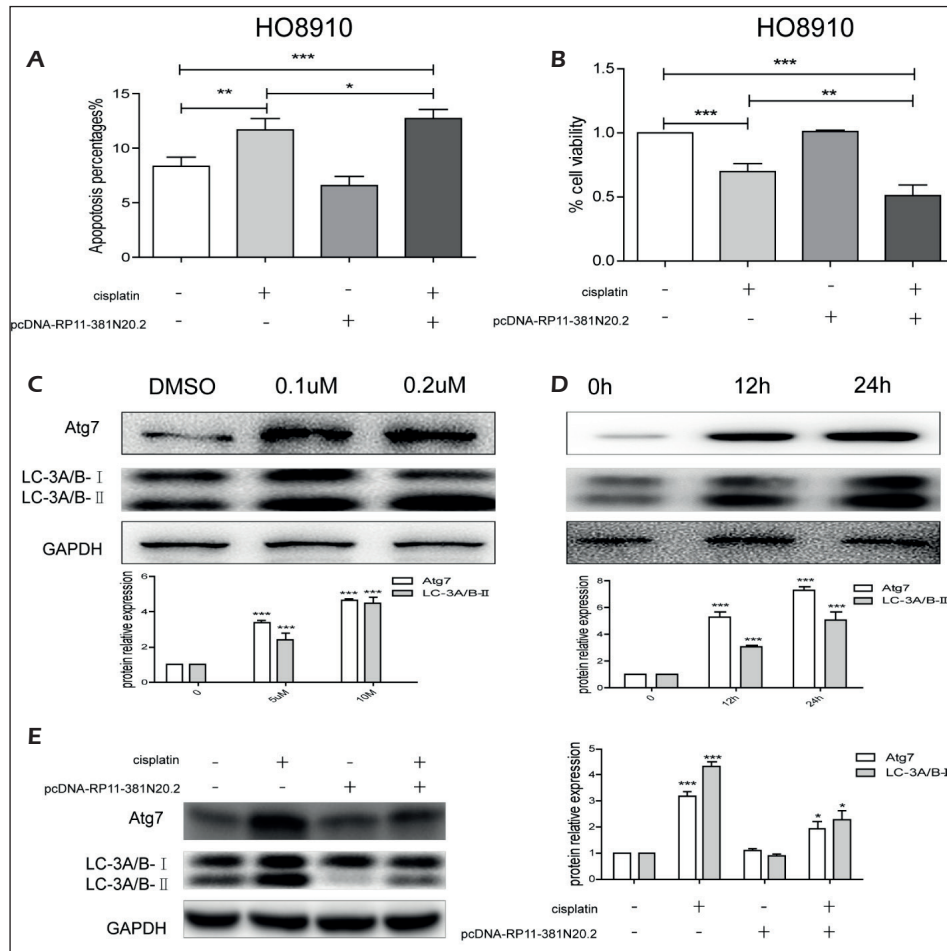


Figure 3. Cisplatin induces autophagy in ovarian cancer cells. High expression of RP11-135L22.1 inhibits autophagy in ovarian cancer cells. **A**, Apoptosis of ovarian cancer cells after transfection into RP11-135L22.1. **B**, Proliferation of ovarian cancer cells after transfection into RP11-135L22.1. **C-D**, Western blot showed that the protein expressions of Atg7, LC3A/B-II and GAPDH in ovarian cancer cells treated with different concentrations of Cisplatin (left) and treated with 10 μ M cisplatin for different times (right). **E**, Protein expressions of Atg7, LC3A/B-II and GAPDH in HO8910 cells under cisplatin treatment (0.1 μ M) and highly expressed RP11-135L22.1 during cell culture.

whereas autophagy has been considered as a mechanism of cell survival. In recent years, cell autophagy has gradually become a research focus. Studies^{17,18} found that changes in autophagy played a dual role in tumor development, which may either promote the death of cancer cells, or improve the resistance of cancer cells to chemotherapy. Therefore, understanding the specific effect of autophagy on the development and treatment of ovarian cancer is expected to become a potential strategy for treating ovarian cancer. The Cancer Genome Atlas (TCGA), is a cancer research project of similar importance to the Human Genome Project conducted by the International Association for Cancer Genomics. Its main purpose is to observe genes that cause cancer mutations and to map out the complete-

ness of these gene maps. Therefore, in this study, we downloaded genome-wide expression profile and its drug-related sensitivity information from TCGA. Next, we analyzed dysregulated lncRNAs in chemotherapy-sensitive and insensitive patients with ovarian cancer by edgeR package. LncRNA RP11-135L22.1, downregulated in chemotherapy insensitive patients, was selected for further verification. In 64 ovarian cancer tissues and 30 normal ovarian tissues, it was found that RP11-135L22.1 was lowly expressed in ovarian cancer tissues. Additionally, the overall survival rate was positively correlated with the expression. Statistical analysis showed that the expression of RP11-135L22.1 was correlated with tumor size and stage. These suggested that RP11-135L22.1 had an inhibito-

ry effect on ovarian cancer. Cisplatin, as the first-line chemotherapy drug in clinical ovarian cancer, exerts its anticancer mechanism by interfering with DNA replication and transcription, thereby inhibiting normal self-replication of tumor cells, inducing apoptosis, and finally inhibiting tumor cell growth¹⁹. In this report, cisplatin was found to be capable of promoting autophagy in ovarian cancer cells. Similarly to other studies, cisplatin was also found to induce different cellular autophagic functions in other tissues and cells. For example, a study showed that autophagy-deficient mice exhibited more acute kidney injury with severe DNA damage and more apoptotic cells induced by cisplatin than the control group²⁰. In other lung cancer related studies, it was also proposed that cisplatin and immunomodulatory protein GMI cooperatively induced autophagy and promoted apoptosis²¹. Yang et al²² found that cisplatin in tumor chemotherapy could kill tumor cells by inducing autophagic cell death. Nevertheless, the biological effect of autophagy on treating cancer was very complex, which may exert different mechanisms in different tumor types²³. Therefore, it is important to understand the role of cisplatin as well as autophagy in ovarian cancer. In this work, we performed CCK8 and flow cytometry to investigate the biological functions of cisplatin. Our findings showed that cisplatin inhibited proliferation and increased apoptosis of ovarian cancer cells. Western blot was used to study the correlation between cisplatin and autophagy, suggesting that cisplatin had the ability to induce autophagy in ovarian cancer cells. Furthermore, after cisplatin treatment combined with RP11-135L22.1, the autophagy was significantly reduced. This confirmed that exerted a significant anti-cancer potential in ovarian cancer, combination of which with RP11-135L22.1 could inhibit autophagy and promote apoptosis of ovarian cancer cells.

Conclusions

We observed that cisplatin had a profound antitumor effect on ovarian cancer by inducing autophagy. RP11-135L22.1 inhibited cisplatin-induced autophagy in ovarian cancer, thus inhibiting its progression.

Conflict of Interest

The authors declared no conflict of interest.

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