EFFECT OF ITGA9-AS1ON PROLIFERATION, APOPTOSIS AND MIGRATION OF A549/DDP CELLS BY NEGATIVELY REGULATING MIR-629-5P

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ABSTRACT

Objective: *To investigate the effect of lncRNA ITGA9-AS1 on proliferation, apoptosis and migration of cisplatin (DDP) resistant lung cancer cells A549/DDP and its mechanism.*

Methods: *Using para-carcinoma tissue and lung cancer cell A549 as the control, RT-qPCR was used to detect the expression levels of ITGA9-AS1 and miR-629-5p in lung cancer tissues and A549/DDP cells. A549/DDP cells were divided into pcDNA group, pcDNA-ITGA9-AS1 group, pcDNA-ITGA9-AS1+miR-NC group, and pcDNA-ITGA9-AS1+miR-629-5p group. CCK-8 method and clone formation experiment were used to detect cell proliferation; flow cytometry was used to detect apoptosis; Transwell was used to detect cell migration; and Western Blot was used to detect Ki67, Cleaned-caspase3, Pro-caspase3, N-cadherin, and E-cadherin protein expressions. Dual-luciferase reporter gene assay verified the regulatory relationship between ITGA9-AS1 and miR-629-5p.*

Results: *Compared with para-carcinoma tissue, lung cancer tissue had significantly reduced ITGA9-AS1 expression (P<0.05). Compared with A549 cells, A549/DDP cells had significantly reduced ITGA9-AS1expression (P<0.05). Compared with pcDNA group, pcDNA-ITGA9-AS1 group had decreased OD value, clone formation number, migrating cell number and Ki67, N-cadherin and Pro-caspase3 protein expression (P<0.05), but increased apoptosis rate and E-cadherin and Cleaned-caspase3 protein expressions in A549/DDP cells (P<0.05). ITGA9-AS1 negatively regulated miR-629-5p expression in A549/DDP cells. Compared with pcDNA-ITGA9-AS1+miR-NC group, pcDNA-ITGA9-AS1+miR-629-5p group had increased OD value, clone formation number, migrating cell number and Ki67, N-cadherin and Pro-caspase3protein expressions (P<0.05), but decreased apoptosis rate, E-cadherin and Cleared-caspase3 protein expressions (P<0.05) in A549/DDP cells.*

Conclusion: *Overexpression of ITGA9-AS1 can negatively regulate miR-629-5p to lower proliferation and migration capability of A549/DDP cells and promote its apoptosis.*

Keywords: *Lung cancer, ITGA9-AS1, miR-629-5p, drug resistance.*

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Introduction

Lung cancer is a common malignant tumor and a cause of high rates of morbidity and mortality worldwide. Platinum-based combination chemotherapy is the first-line treatment for lung cancer[1]. Cisplatin (DDP) is a commonly used platinum drug, and cisplatin resistance is a major reason for lung cancer chemotherapy[2]. At present, the mechanism of lung cancer drug resistance remains unclear, and reversing chemotherapy resistance of lung cancer cells seems highly efficient in lung cancer chemotherapy. Long-chain non-coding RNA (lncRNA), a type of small-molecule non-coding RNA involved in regulating cell proliferation, differentiation, and apoptosis, has a close relation to the occurrence, development, and drug resistance of many human diseases, especially tumors[3-4]. Studies of Liu Zhengtai et al.[5] showed that down-regulation of lncRNA KCNQ1OT1 expression can enhance the

sensitivity of ovarian cancer resistant cells SKOV3/DDP to DDP. ITGA9-AS1 is a recently newly discovered lncRNA located on chromosome 3, which is 578 bp long and consists of 4 exons. Studies have shown that overexpression of ITGA9-AS1 can inhibit the proliferation and cloning capability of breast cancer T47D cells and enhance cell sensitivity to the chemotherapy drug cisplatin[6]. Nonetheless, at present, the influence and regulation mechanism of ITGA9-AS1 on drug resistance of lung cancer cells remains unknown. Studies have also shown that lncRNA can be used as a competitive endogenous RNA to combine with microRNA (miRNA), regulate the expression of target genes, and jointly affect the occurrence and development of tumors[7].

Bioinformatics software predicts that ITGA9-AS1 may have a targeted regulatory relationship with miR-629-5p. It has been reported that miR-629-5p has increased expression in lung adenocarcinoma tissues, and overexpression of miR-629-5p can promote tumor cell migration and invasion[8]. Therefore, this study took cisplatin-resistant lung cancer cells A549/DDP as the research object, observed the effect of ITGA9-AS1 on proliferation, apoptosis and migration of A549/DDP cells and aimed to find out whether it can target the regulation of miR-629-5p expression to provide a new molecular target for reversing cisplatin resistance of lung cancer cells.

Materials and methods

Clinical data

The cancer tissues and corresponding para-carcinoma tissue samples of 37 patients with non-small cell lung cancer who underwent surgical treatment in our hospital from January 2016 to March 2019 were collected and stored in liquid nitrogen. Among the 37 patients, 26 were male and 11 were female, with an average age of (59.21±8.35) years. For tissue differentiation, there were 5 cases with high differentiation, 19 cases with moderate differentiation, and 13 cases with low differentiation; for TNM staging, there were 6 cases in stage I, 18 cases in stage II, and 13 cases in stage III; there were 15 cases of lymph node metastasis, 22 cases of non-metastatic patients.

Inclusion criteria:

• Postoperatively pathologically confirmed as non-small cell lung cancer;

• No radiotherapy or chemotherapy before surgery;

• No other malignant tumors.

This study was approved by the ethics committee of our hospital, and the patients voluntarily signed an informed consent form.

Cells and reagents

Lung cancer cells A549 and cisplatin-resistant cells A549/DDP were purchased from Quanzhou Ruixin Biotechnology Co., Ltd.; fetal bovine serum (FBS) was purchased from Zhejiang Tianhang Biotechnology Co., Ltd., RPMI 1640 medium, cell counting kit- 8 (CCK-8), LipofectamineTM 2000 kit, Annexin V-FITC/PI cell apoptosis kit, bicinchoninic acid (BCA) protein detection kit and dual luciferase activity detection kit were purchased from Beijing SolarbioScience &Technology Co., Ltd., reverse transcription kit and PCR kit were purchased from Shenzhen Jingmei Bioengineering Co., Ltd., Trizol reagent was purchased from Invitrogen, USA, PCR primers, ITGA9-AS1 overexpression vector, empty vector, miR-629-5p mimics and simulated control sequences were purchased from Shanghai Sangon Biological Engineering Co., Ltd., rabbit anti-human Ki67, activated cleaved-caspase3, pro-caspase3, neuronal cadherin (N-cadherin), epithelial cadherin (E-cadherin) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibodies were purchased from Santa Cruz, USA.

Method

Cell culture and transfection

Both A549 and A549/DDP cells were cultured in RPMI 1640 medium containing 10% FBS. Incubator conditions: temperature 37°C, CO_2 volume fraction 5%, humidity 97%. A549/DDP cells in logarithmic growth phase were divided into pcDNA group, pcDNA-ITGA9-AS1 group, pcDNA-ITGA9-AS1+miR-NC group, and pcDNA-ITGA9-AS1+miR-629-5p group. pcDNA group cells were transfected with empty vector, pcDNA-ITGA9-AS1 group cells were transfected with ITGA9-AS1 overexpression carrier vector, pcDNA-ITGA9-AS1+miR-NC group cells were co-transfected with ITGA9-AS1 overexpression vector and simulation control sequence, pcDNA-ITGA9-AS1+miR-629-5p group cells were co-transfected with ITGA9-AS1 overexpression vector and miR-629-5p mimics. The specific transfection steps followed the instructions of LipofectamineTM 2000 kit. 6 h after transfection, the medium was changed. The culture continued for 24 h, and the cells were collected for subsequent experiments.

Detection of itga9-as1 and mir-629-5p expression by real-time fluorescence quantitative PCR (RT-qPCR)

Trizol reagent was used to extract total RNA from tissues or cells. After reverse transcription into cDNA, perform PCR amplification. Amplification conditions: 95 °C 5 min, 95 °C 10 s, 60 °C 30 s, 72 °C 30 s, a total of 35 cycles.

Primer sequence:

ITGA9-AS1upstream5'-CTCCATGCCAG-GTCTGTTCT-3', downstream 5'-TTGTCAG-CAAACCACCCTTG-3';

miR-629-5pupstream5'-TGAACGT-CAAGTCGATGA-3', downstream 5'-CCG-TAACCCCAATGCTGAT-3';

GAPDH upstream5'-GAGTCAT-CATCTCTGCCCCA-3', downstream 5'-TTCTGT-GTTGCTGTGATGGC-3';

U6upstream5'-TGCCGAACACTCACGGT-GTC-3', downstream 5'-CGTGTGGACTCCTCA-CAC-3'.

The relative expression levels of ITGA9-AS1 and miR-629-5p were calculated using $2^{-\triangle\triangle Ct}$ method.

CCK-8experiment

The transfected A549/DDP cells of each group were seeded in 96-well plates at 0.5×10^4 cells/well. After 24 hours of culture, 10 μLCCK-8 working solution was added.

After 2 h incubation, the optical density (OD) value was measured at 450 nm using a microplate reader. The experiment was repeated three times.

Clone formation experiment

The transfected A549/DDP cells in each group were seeded in 6-well plates at 0.5×104 cells/well.

After 14 d culture, the medium was discarded, fixed with 4% paraformaldehyde for 30 min, and stained with 0.4% crystal violet for 15 min. Observe with an inverted microscope and count clones involving more than 50 cells.

Apoptosis detection by flow cytometry

The transfected A549/DDP cells of each group were seeded in 24-well plates at 2.5×10^4 cells/well. After 24 h culture, the medium was discarded, and the cells were collected.

According to the Annexin V-FITC/PI kit instructions, flow cytometry was used to detect apoptosis.

Cell migration detection by Transwell

The concentration of A549/DDP cells after transfection in each group was adjusted to 5×104 cells/mL. Set Transwell in a 24-well plate, add 100 μL cell suspension to the upper chamber and 500 μL of medium containing 10% FBS to the lower chamber.

After incubation for 24 h, fix with 4% paraformaldehyde for 30 min and stain with 0.4% crystal violet for 15 min. Observe with an inverted microscope by randomly selecting 5 fields of vision, and count the migrating cells.

Protein expression detection by Western Blot

RIPA reagent was used to extract the total protein in the cells, and the protein concentration was detected by BCA method for quantification. SDS-PAGE electrophoresis was performed with 30 μg protein per well.

After electrophoresis, electrotransfer to PVDF membrane and block in 5% skimmed milk powder solution for 1 h. Add Ki67 (1:800), Cleaned-caspase3 (1:1000), Pro-caspase3 (1:1000), N-cadherin (1:800) and E-cadherin (1:800) primary antibodies, incubate at 4 °C overnight.

Horseradish peroxidase-labeled secondary antibody (1:3000) was added and incubated at 37°C for 1 h. Add chemiluminescent reagent, expose and photograph after dark development.

Dule luciferase reporter gene assay

A549/DDP was inoculated into a 24-well plate at 2.5×10^4/well.

According to the instructions of the LipofectamineTM 2000 kit, co-transfect WT-ITGA9-AS1 and miR-629-5p or miR-NC, MUT-ITGA9-AS1 and miR-629-5p or miR-NC. 6 h after transfection, the medium was replaced. Continue culture for 24 h, collect cells to detect luciferase activity.

Statistical analysis

SPSS.22.0 software was used to analyze the experimental data.

Measurement data conforming to normal distribution are expressed as mean ± standard deviation ($\bar{x}\pm s$).

Independent sample t-test was used for comparison between two groups; one-way analysis of variance was used for comparison between multiple groups, and SNK-q test was used for further pairwise comparison. $P<0.05$ indicates a statistically significant difference.

Results

Expression of ITGA9-AS1 in lung cancer tissues and A549/DDP cells

Compared with para-carcinoma tissues, lung cancer tissues have significantly reduced ITGA9-AS1expression (P<0.05). Compared with A549 cells, A549/DDP cells have significantly reduced ITGA9-AS1expression (P<0.05), as shown in Table 1 and Table 2.

Group	n	ITGA9-AS1
Para-carcinoma tissue	37	1.01±0.08
Lung cancer tissue	37	0.23±0.03*
t	--	55.531
P	--	0.000

Table 1: Expression of ITGA9-AS1 in lung cancer tissue ($\bar{x}$±s).
*Note: Compared with para-carcinoma tissue, *P<0.05.*

Group	ITGA9-AS1
A549	0.96±0.04
A549/DDP	0.38±0.02#
t	22.463
P	0.000

Table 2: Expression of ITGA9-AS1 in A549/DDP ($\bar{x}$±s, n=3).
Note: Compared with A549 cells, #P<0.05.

The effect of ITGA9-AS1 on proliferation, apoptosis and migration of A549/DDP cells

Compared with pcDNA group, pcDNA-ITGA9-AS1 group has increased ITGA9-AS1 expression (P<0.05), decreased miR-629-5p expression (P<0.05), decreased cell OD value, clone formation number and migrating cell number in A549/DDP cells (P<0.05), but increased apoptosis rate (P<0.05), as shown in Figure 1, Table 3.

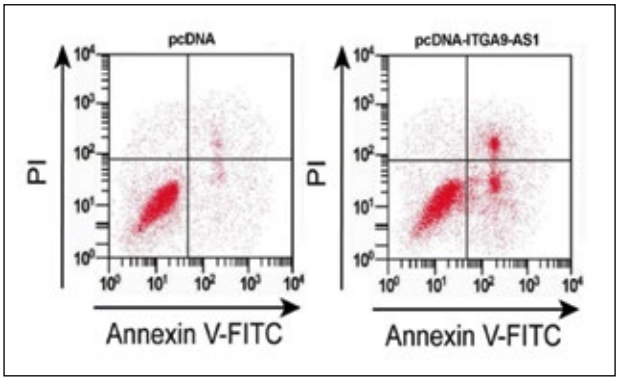

Figure 1: Effect of ITGA9-AS1 on apoptosis of A549/DDP cells.

Group	ITGA9-AS1	miR-629-5p	OD value	Clone formation number	Migrating cell number	Apoptosis rate(%)
pcDNA	0.97± 0.03	0.99± 0.04	0.89± 0.03	109.67± 1.70	189.33± 2.87	7.87± 0.36
pcDNA-ITGA9-AS1	3.27± 0.06*	0.26± 0.02*	0.34± 0.01*	52.00± 0.82*	89.33± 1.25*	23.80± 0.64*
t	59.386	28.273	30.125	5.869	55.330	37.575
P	0.000	0.000	0.000	0.004	0.000	0.000

Table 3: The effect of ITGA9-AS1 on proliferation, apoptosis and migration of A549/DDP cells ($\bar{x}$±s, n=3).
*Note: Compared with pcDNA group, *P<0.05.*

The effect of ITGA9-AS1 on proliferation, apoptosis and migration-related protein expressions in A549/DDP cells

Compared with pcDNA group, pcDNA-ITGA9-AS1 group has decreased Ki67, N-cadherin and Pro-caspase3 protein expression (P<0.05), and increased E-cadherin and Cleared-caspase3 protein expressions (P<0.05) in A549/DDP cells, as shown in Figure 2 and Table 4.

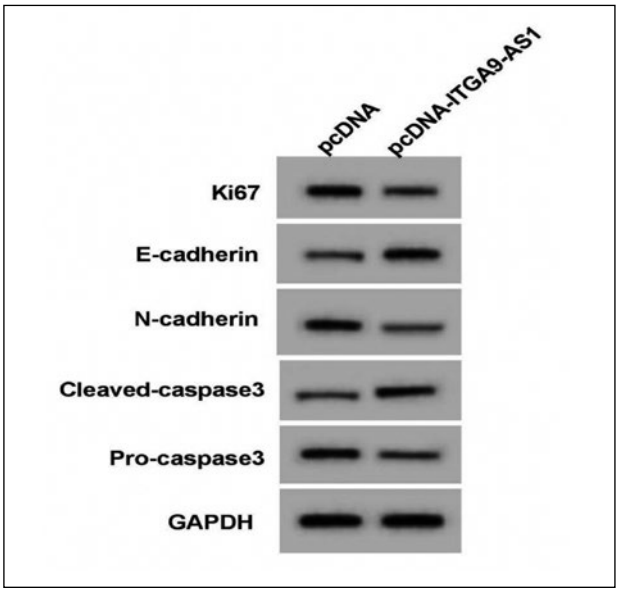

Figure 2: The effect of ITGA9-AS1 on proliferation, apoptosis and migration-related protein expressions in A549/DDP cells.

Group	Ki67	E-cadherin	N-cadherin	Cleaved-caspase3	Pro-caspase3
pcDNA	0.76±0.03	0.15±0.01	0.59±0.03	0.23±0.01	0.56±0.02
pcDNA-ITGA9-AS1	0.24±0.01*	0.65±0.03*	0.12±0.01*	0.66±0.03*	0.20±0.01*
t	28.482	27.386	25.743	23.552	27.885
P	0.000	0.000	0.000	0.000	0.000

Table 4: The effect of ITGA9-AS1 on proliferation, apoptosis and migration-related protein expressions in A549/DDP cells ($\bar{x}$±s, n=3).
*Note: Compared with pcDNA group, *P<0.05.*

ITGA9-AS1 targets regulation of miR-629-5p

See Figure 3 for the binding sites of ITGA9-AS1 and miR-629-5p nucleotide sequence. After WT-ITGA9-AS1 is co-transfected with miR-629-5p mimics, the cell has decreased luciferase activity (P<0.05); but after WT-ITGA9-AS1 is co-transfected with miR-629-5p mimics, no significant change is shown in cell luciferase activity (P>0.05), as shown in Table 5.

Figure 3: Binding sites of ITGA9-AS1 and miR-629-5p nucleotide sequence.

Group	WT-ITGA9-AS1	MUT-ITGA9-AS1
miR-NC	1.00±0.03	0.99±0.04
miR-629-5p	0.24±0.02*	1.00±0.03
t	36.509	0.346
P	0.000	0.746

Table 5: Dual-luciferase reporter assay ($\bar{x}$±s, n=3).
*Note: Compared with the miR-NC group, *P<0.05.*

Overexpression of miR-629-5p reverses the effect of ITGA9-AS1 on proliferation, apoptosis and migration of A549/DDP

Compared with the pcDNA group, pcDNA-ITGA9-AS1 group has increased OD value, clone formation number and migrating cell number (P<0.05), but decreased apoptosis rate (P<0.05) in A549/DDP cells, as shown in Figure 4 and Table 6.

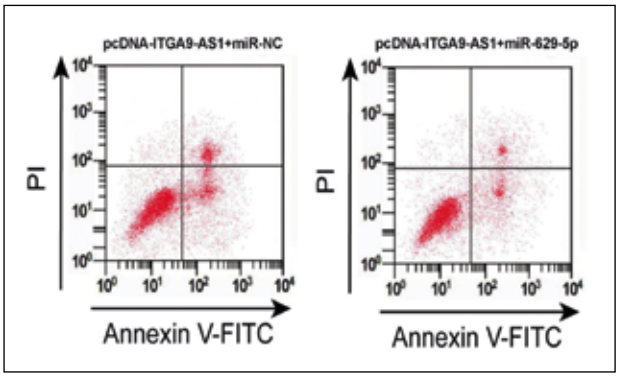

Figure 4: MiR-629-5p reverses the effect of ITGA9-AS1 on apoptosis of A549/DDP cells.

Group	OD value	Clone formation number	Migrating cell number	Apoptosis rate (%)
pcDNA-ITGA9-AS1+miR-NC	0.34±0.02	52.67±0.94	89.00±1.41	23.85±0.55
pcDNA-ITGA9-AS1+miR-629-5p	0.72±0.02*	98.67±1.25*	159.67±1.70*	11.18±0.38*
t	23.270	50.943	55.420	32.827
P	0.000	0.000	0.000	0.000

Table 6: MiR-629-5p reverses the effect of ITGA9-AS1 on proliferation, apoptosis and migration of A549/DDP cells ($\bar{x}$±s, n=3).
*Note: Compared withpcDNA-ITGA9-AS1+miR-NC group, *P<0.05.*

MiR-629-5p reverses the effect of ITGA9-AS1 on A549/DDP proliferation, apoptosis and migration-related proteins

Compared with pcDNA-ITGA9-AS1+miR-NC group, pcDNA-ITGA9-AS1+miR-629-5p group has increased Ki67, N-cadherin and Pro-caspase3 protein expression (P<0.05), decreased E -Cadherin and Cleaved-caspase3 protein expression (P<0.05) in A549/DDP cells, as shown in Figure 5 and Table 7.

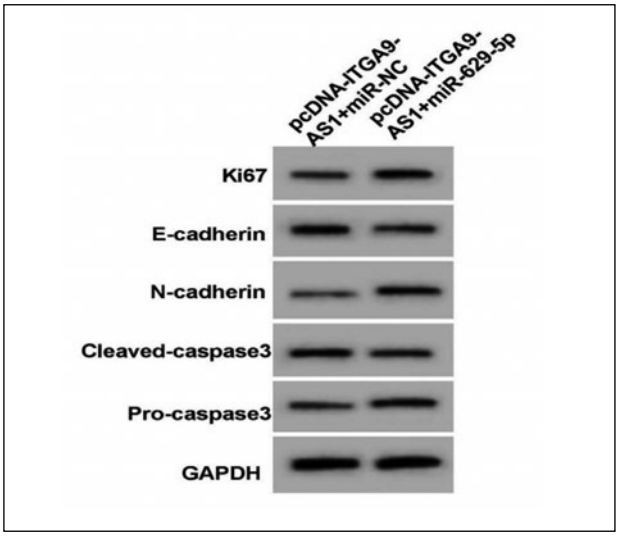

Figure 5: MiR-629-5p reverses the effect of ITGA9-AS1 on Ki67, E-cadherin, N-cadherin, Cleared-caspase3, Pro-caspase3 protein expression in A549/DDP cells.

Group	Ki67	E-cadherin	N-cadherin	Cleaved-caspase3	Pro-caspase3
pcDNA-ITGA9-AS1+miR-NC	0.24±0.01	0.65±0.03	0.11±0.01	0.67±0.03	0.21±0.01
pcDNA-ITGA9-AS1+miR-629-5p	0.60±0.02*	0.27±0.02*	0.44±0.02*	0.34±0.02*	0.46±0.02*
t	27.885	18.255	25.562	15.853	19.365
P	0.000	0.000	0.000	0.000	0.000

Table 7: MiR-629-5p reverses the effect of ITGA9-AS1 on A549/DDP proliferation, apoptosis and migration-related proteins ($\bar{x}$±s, n=3).
*Note: Compared with pcDNA-ITGA9-AS1+miR-NCgroup, *P<0.05.*

Discussion

Tumor cell drug resistance is the main cause of tumor chemotherapy failure, so reversing tumor cell drug resistance is a hot issue in current research. lncRNA widespread in eukaryotes participates in the regulation of life activities such as cell proliferation and apoptosis as well as drug resistance of tumor cells. Studies have shown that a variety of lncRNAs are abnormally expressed in lung cancer patients resistant to chemotherapy drugs and can be used as molecular targets to reverse lung cancer resistance. Studies of Wang Liwen[9] have shown that lncRNA MVIH is significantly elevated in cancer tissues of lung cancer patients resistant to gemcitabine, which can be used as a marker for predicting gemcitabine resistance. Wang Qi et al.[10] showed that lncRNA uc002ktr.3 is highly expressed in cancer tissues of patients with cisplatin resistance in lung squamous cell carcinoma, and interference with its expression can enhance sensitivity of lung squamous cell carcinoma cells to cisplatin. Xiao et al.[11] showed that lncRNA CASC2 had reduced expression in cisplatin-resistant lung cancer tissues, and overexpression of lncRNA CASC2 could inhibit the proliferation, migration and invasion of cisplatin-resistant lung cancer cells. Its mechanism of action is possibly to down-regulate miR-18a and then promote IRF-2 expression. ITGA9-AS1 is a recently discovered lncRNA. Its expression in lung cancer and its effect on drug resistance of lung cancer cells still remain unknown.

This study indicated that lung cancer tissues and A549/DDP cells significantly increased ITGA9-AS1 expression, suggesting that ITGA9-AS1 may also act as a tumor suppressor gene in lung cancer and participate in regulating the sensitivity of cisplatin-resistant lung cancer cells to cisplatin. This study showed that after overexpression of ITGA9-AS1, the OD value, clone formation number and Ki67 protein expression in A549/DDP cells were significantly reduced, which was consistent with related reports[12], suggesting that overexpression of ITGA9-AS1 can inhibit proliferation of A549/DDP cells. At the same time, overexpression of ITGA9-AS1 increased the apoptosis rate of A549/DDP cells and Cleaved-caspase3 protein expression in the cells, but decreased Pro-caspase3 protein expression, which was consistent with related reports[13], indicating that overexpression of ITGA9-AS1 can promote A549/DDP cell apoptosis. This study also showed that after overexpression of ITGA9-AS1, the A549/DDP cell migration number and N-cadherin protein expression in cells were decreased, while E-cadherin protein expression was increased, which was consistent with related reports[14], indicating that overexpression of ITGA9 -AS1 can weaken the migration capability of A549/DDP cells.

In this study, dual-luciferase reporter gene assay confirmed that ITGA9-AS1 can be bound to miR-629-5p, and after overexpression of ITGA9-AS1, miR-629-5p expression was reduced in A549/DDP cells, indicating that ITGA9-AS1 targets the negative regulation of miR-629-5p expression. MiR-629-5p has increased expression in a variety of tumors, promoting tumorigenesis and development.

Studies have shown that miR-629-5p expression is up-regulated in tissue samples of hepatocellular carcinoma (HCC), and the overexpression of miR-629-5p promotes the proliferation, migration and invasiveness of human HCC cells in vitro and tumor growth and metastasis in vivo through targeted inhibition of secreted frizzled-related protein 2 (SFRP2)[15]. MiR-629-5p has up-regulated expression in colorectal cancer tissues and cell lines.

Overexpression of miR-629-5p reduces the sensitivity of colon cancer cells to 5FU by negatively regulating the expression of CXXC finger protein 4 (CXXC4)[16]. This study showed that overexpression of miR-629-5p reversed the effect of overexpression of ITGA9-AS1 on proliferation, apoptosis and migration of A549/DDP cells, i.e. overexpression of miR-629-5p reversed the promotion effect of ITGA9-AS1overexpression on A549/DDP cell drug resistance[17].

Conclusion

Lung cancer tissues and cisplatin-resistant lung cancer cells have low expression of ITGA9-AS1. Overexpression of ITGA9-AS1 can effectively inhibit the proliferation and migration of cisplatin-resistant lung cancer cells and promote apoptosis, possibly by targeting negative regulation of miR-629-5p, while the ITGA9-AS1/miR-629-5p axis may provide a molecular target for reversing cisplatin resistance in lung cancer[18].

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