Abstract. – OBJECTIVE: To investigate the relative expression of long non-coding RNA (IncRNA) ASAP1-IT1 (hereafter called ASAP1-IT1) in tissues and cells of non-small cell lung cancer (NSCLC) patients, so as to explore the effect of ASAP1-IT1 on the biological effect of NSCLC cells.

PATIENTS AND METHODS: Real-time quantitative polymerase chain reaction (qRT-PCR) was performed to detect the relative expressions of ASAP1-IT1 on tissues of 68 NSCLC patients and 5 cell lines. Besides, the interference sequence of ASAP1-IT1 was designed to detect the transfection efficiency through qRT-PCR experiment. Cell count kit 8 (CCK-8) and clone formation experiment were also carried out to determine the effect of ASAP1-IT1 expression under interference on the proliferation ability of NSCLC cells. In addition, transwell experiment was also performed to investigate the effects of ASAP1-IT1 expression under interference on the invasion and metastasis of NSCLC cells. Furthermore, the Western blotting assay was also conducted to detect the downstream signal pathways through which ASAP1-IT1 regulated the biological behaviors of NSCLC.

RESULTS: The results of qRT-PCR experiment showed that in 68 NSCLC samples, upregulation of ASAP1-IT1 expression was identified in 51 samples (82.4%) in comparison with the expression in tumor-adjacent tissues, and a similar upregulation was also observed in 5 NSCLC cells. CCK-8 and clone formation experiment were also carried out to determine the effect of ASAP1-IT1 expression under interference on the proliferation ability of NSCLC cells. In addition, transwell experiment was also performed to investigate the effects of ASAP1-IT1 expression under interference on the invasion and metastasis of NSCLC cells. Furthermore, the Western blotting assay was also conducted to detect the downstream signal pathways through which ASAP1-IT1 regulated the biological behaviors of NSCLC.

CONCLUSIONS: In this study, it was found that the expression of ASAP1-IT1 is relatively upregulated in NSCLC cells and tissues, which can promote the proliferation, invasion and metastasis of NSCLC cells through regulating the PTEN/AKT signal pathway. Thus, the therapeutic target of ASAP1-IT1 is expected to provide important ideas for reversing the malignant phenotype of NSCLC in clinical practice.

Key Words: LncRNA ASAP1-IT1, NSCLC, Proliferation, Invasion and metastasis, PTEN/AKT signal pathway.

Introduction

Occupying 21.7% in cancers that lead to death, lung cancer has become the most frequent malignant tumor, and also the major cause accounting for the cancer-related death in the world. Currently, lung cancer has been divided into two kinds, i.e. non-small cell lung cancer (NSCLC, about 80%) and small-cell lung cancer (about 20%). Based on the multiple mutations in target genes that have been discovered, such as epidermal growth factor receptor (EGFR), Kirsten rat sarcoma viral oncogene (KRAS), phosphoinositide 3-kinase α (PI3KA), human epidermal growth factor receptor 2 (HER2), cellular mesenchymal to epithelial transition factor (MET), anaplastic lymphoma kinase (ALK) and c-ros oncogene 1, receptor tyrosine kinase (ROS1) fusion gene, solid foundation has been laid for the individualized precise treatment of lung cancer. However, prognoses of lung cancer patients in Stage IIIIB and IV remain poor with 5-year survival rate of only 5% and 1%, respectively. Thus, it is an ur-
gent task to discover new molecular markers and therapeutic targets for improvement of prognoses of NSCLC patients.

The encyclopedia of deoxyribonucleic acid (DNA) elements (ENCODE) project revealed that transcription product of only a few genes in human beings can encode proteins, while the remaining product without the protein-encoding function is named as non-coding ribonucleic acid (ncRNA), in which long non-coding RNA (lncRNA) is a kind of ncRNA in length of over 200 bp. According to some latest research, it is reported that lncRNA is involved in the cell-biological functions through the roles of messenger, primer, guide or stent, and the abnormal expression of lncRNA correlates with the variations in biological behaviors of NSCLC closely, like proliferation, invasion, metastasis and chemoresistance.

LncRNA ASAP1-IT1 is located in chromosome 8q24.21 in whole length of 1179 bp. It was initially found in ovarian cancer for its abnormal expression by Fu et al, which is correlated with the overall prognosis of ovarian cancer patients. Yang et al reported that ASAP1-IT1 is highly expressed in bladder cancer tissues, and ASAP1-IT1 in high expression can promote the development stem cell-like functions in bladder cancer cells. However, there remain no studies reporting the expression, biological functions or potential molecular mechanisms of ASAP1-IT1 in NSCLC tissues and cells.

In this study, it was firstly found the relatively high expression of ASAP1-IT1 in NSCLC tissues and cells, and interference on ASAP1-IT1 expression can inhibit the proliferation, migration and metastasis of tumor cells, while the regulatory role of PTEN/AKT signal pathway is the potential molecular mechanism. The result of this study is expected to provide a new idea for the development of more efficient therapeutics for NSCLC.

**Patients and Methods**

**Tissues and Cells**

The tissue samples were collected from a total of 68 lung cancer patients who were admitted to the Thoracic Surgery of Suzhou Wujiang District First People’s Hospital between January 2014 and December 2016. All these patients were diagnosed as NSCLC and had not received any chemotherapy, radiotherapy or targeted therapy before the study. After section, samples were preserved in liquid nitrogen, and all preservation and transfer procedures were carried out in accordance with the bacterial- and enzyme-free principle. Collection and operation of samples were also conformed to the ethic regulation and operation guidelines of clinical experiment. This study had been approved by the Ethic Committee of Suzhou Wujiang District First People’s Hospital, and patients or their authorized delegates signed the agreement to participate in this study.

16 Human bronchial epithelial (HBE) cell lines (Cell Bank, CAS, Shanghai, China) and 5 NSCLC cell lines (A549, H1299, H460, SPCA-1 and SK-MES-1) were cultured in Dulbecco’s modified eagle Medium (DMEM) Hyclone (South Logan, UT, USA) or Roswell Park Memorial Institute-1640 (RPMI-1640); Invitrogen (Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin-penicillin mixture (100 U/mL penicillin and 100 μg/mL streptomycin) in a thermostat incubator (37°C, 5% (v/v) CO2 and saturated humidity). Before study, all cell lines should undergo morphological test, growth curve analysis and karyotype analysis.

**Interference Sequence and Primer Synthesis**

Interference sequences of ASAP1-IT1 synthesized by Invitrogen (Carlsbad, CA, USA) were shown as follows: 1# 5’-GC UGCGAACAAUAGACUAGGAGUUU-3’, 2# 5’-CAGCACCCGAUGUAUCCCUGGA-3’, 3# 5’-UGAAGGCGAGAGUGGUAGGCUCUGAA-3’. Primers were designed using oligo and Primer 5.0 software (Invitrogen, Carlsbad, CA, USA): GAPDH (F) 5’-GGGAGCCAAGGTCAT-3’, GAPDH (R): 5’-GAGTCTTCCAC-GATACCA-3’.

**Detection of ASAP-AS1 Expression**

TRIzol method was used to extract the total RNA from tissues and cells; with an ultraviolet/visible spectrophotometer (NanoDrop 1000, Thermo Fisher Scientific, Waltham, MA, USA), optical density (OD) 260/280 ratio was determined for calculation of RNA concentration. Perfect Real-time kit (PrimeScript™ RT Master Mix, TaKaRa, Otsu, Shiga, Japan) was also used in synthesis of cDNA with the reverse-transcription reaction system (20 μL) prepared in strict accordance with the instructions. Under the instruction of SYBR® Premix Ex Taq™ II (TaKaRa, Otsu, Shiga, Japan), the reaction system for PCR was
prepared, and the RT-PCR was carried out with ABI7500RTC-PCR using two-step method in following conditions: initial denaturation at 95°C for 30 s; 40 cycles of 95°C for 5 s and 60°C for 34 s. Expression of ASAP1-IT1 was calculated with the method of $2^{-\Delta\Delta Ct}$.

**Cells Count Kit 8 (CCK-8) and Clone Formation Experiment**

Transient transfection for NSCLC cells with si-ASAP1-IT1 and si-NC was carried out. Next, the cells in each group were collected and seeded on a 96-well plate at density of $3\times10^4$/well. With 0, 24, 48, 72 and 96 h as time points of observation, 110 mL mixture of CCK-8 and serum-free medium (1:10) were added into cells after they were cultured for different time, followed by measurement of OD value at wavelength of 450 nm with a microplate reader for preparation of growth curve. Transient transfection for NSCLC cells with si-ASAP1-IT1 and si-NC was carried out, and cells in each group were collected and seeded on a 6-well plate at density of $10^5$/well. Culture medium was replaced every 3 days; 12 days later, cells were mounted with formaldehyde, followed by staining with crystal violet and cell count.

**Transwell Experiment**

Transwell chambers without any matrix gel were placed in a 24-well plate (Millipore, Billerica, MA, USA). Transient transfection was carried out for NSCLC cells with si-ASAP1-IT1 and si-NC, and transfected cells in each group were collected for preparation of cell suspension ($3.0\times10^5$/mL). Thereafter, cells were transferred into the upper transwell chambers in 0.2 mL/well (0.4$\times10^5$). After 24 h of culture in serum-free medium, cells that migrated through the membrane to the lower chambers were observed and counted for analysis of the migration ability of cells in each group.

Transwell chambers with matrix gel (BD Sciences, Franklin Lakes, NJ, USA) were placed in a 24-well plate (Millipore, Billerica, MA, USA). Transient transfection was carried out for NSCLC cells with si-ASAP1-IT1 and si-NC, and transfected cells in each group were collected for preparation of cell suspension ($3.0\times10^5$/mL). Thereafter, cells were transferred into the upper transwell chambers in 0.2 mL/well (0.8$\times10^5$); after 48 h of culture in serum-free medium, cells that migrated through the membrane to the lower chambers were observed and counted for analysis of the invasion ability of cells in each group.

**Western Blotting Assay**

Total protein was extracted in the experiment and control groups after cell lysis was added, and samples were loaded (30 μg/well) for electrophoresis for 40 min in stacking gel at 80 V, and 2 h in separation gel at 100 V. Proteins on gel were then transferred onto the membrane for 2 h using the regular method followed by blocking with 5% skimmed milk. Then, the membrane was incubated with primary anti-glycereraldehyde-phosphate dehydrogenase (GAPDH) antibody (1:1000) overnight and then with secondary antibodies produced in rabbits (1:10000) for 2 h. The membrane was washed with Tris-buffered saline and Tween 20 (TBST) for 3 times (10 min/time), and enhanced chemiluminescence (ECL) reagent was added for color development in gel imaging system.

**Statistical Analysis**

Statistical Product and Service Solutions (SPSS) 16.0 software (SPSS Inc., Chicago, IL, USA) was applied in statistical analysis. $t$-test was carried out for comparison between either two groups, and one-way analysis of variance (ANOVA) for comparison among groups: as for multiple comparison between groups, Bonferroni method was applied only with equal variances; otherwise, Welch test would be performed, and Dunnett’s T3 method would be adopted for intergroup multiple comparison. $p < 0.05$ suggested that the difference had statistical significance.

**Results**

**ASAP1-IT1 Expression**

Among the tissue samples collected from 68 NSCLC patients previously, the RNA was extracted from the tissues using TRizol method for reverse transcription of cDNA, and the relative expression of ASAP1-IT1 in tissue samples were detected in qRT-PCR experiment. The results suggested that, compared with the tumor-adjacent tissues, upregulation in ASAP1-IT1 expression was identified in 51 patients (Figure 1A). Similar methods were also adopted for detecting the expression of ASAP1-IT1 in NSCLC cells, and the results showed that in comparison with that in the 16HBE cell line, the expression of ASAP1-IT1 was upregulated in 4 NSCLC cell lines, while the comparison between 16HBE cell line and the remaining 1 cell line showed that the difference had no statistical significance (Figure 1B). To further investigate the biological role of ASAP1-IT1 in
NSCLC cells, ASAP1-IT1-specific interference sequence was designed and synthesized, and after 48 h, the interference efficiency in qRT-PCR experiment was measured (Figure 1C-D).

**Interference on ASAP1-IT1 Expression Inhibits Cell Proliferation**

Transient transfection for NSCLC cells with si-ASAP1-IT1, and si-NC as control was carried out, and, after cells adhered to the wall, they were seeded in each group on a 96-well plate at density of $3 \times 10^4$/well. With 0, 24, 48, 72 and 96 h as time points of observation, CCK-8 solution was added into cells for detection of OD value to prepare the growth curve. The results showed that the proliferation ability of cells in the experiment group was significantly lower than that in the control group (Figure 2A and B); after cells were treated using the same methods, they were seeded on a 6-well plate at density of $10^4$/well, followed

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**Figure 1.** Detecting the expression of ASAP1-IT1 in NSCLC. **A**, Results of qRT-PCR experiment for relative expression of ASAP1-IT1 in tissues samples collected from 68 NSCLC patients show that there are 51 with upregulation, and 17 with downregulation, in which GAPDH expression serves as internal reference. **B**, Results of qRT-PCR experiment for relative expression of ASAP1-IT1 in 5 NSCLC cell lines show that in comparison with the 16HBE cell line, upregulation was identified in 4 cell lines, and the comparison between the remaining 1 cell line and 16HBE cell line shows that the difference has no statistical significance. **C**, and **D**, A549 and SPCA-1 cells are transfected with si-ASAP1-IT1, and si-NC as control, and 48 h later, qRT-PCR experiment is conducted to detect the transfection efficiency ($**p < 0.01; *p < 0.05$).
by mounting, fixing and counting after 12 days, and the results coincided with the results in CCK-8 experiment (Figure 2C and D).

**Interference on ASAP1-IT1 Expression Suppresses the Migration and Invasion of Cells**

After NSCLC cells were transiently transfected with the si-ASAP1-IT1 and si-NC as control for 24 h, 3 × 10^4 cells were collected from two groups and seeded into the chambers without matrix gel. 24 h later, cells that migrated through the membrane were mounted, stained and photographed under microscope (Figure 3A-B). Similar treatment methods were also performed for cells, and 6×10^4 cells were added into the chambers with matrix gel. After 48 h, cells that migrated through the membrane were mounted and stained (Figure 3C-D).
Role of ASAP1-IT1 in NSCLC

ASAP1-IT1 Regulates the PTEN/AKT Signal Axis

Literature has reported that the activation and suppression of PTEN/AKT signaling pathway are correlated closely with the abilities of proliferation, migration and invasion of tumor cells. However, lncRNAs, as the major epigenetic regulatory factors, are involved in many signaling pathways, including PTEN/AKT, thereby regulating the variations of oncobiological behaviors\textsuperscript{12,13}. In this study, the results of Western blotting assay showed that after interference on expression of ASAP1-IT1 in NSCLC cells, variations occurred in the expressions of key molecules in its downstream signaling pathway, PTEN/AKT (Figure 4A-B).

Discussion

Lung cancer is a kind of malignant tumors with the highest incidence rate and mortality rate
in the world. Many researchers are engaged in exploring the pathogenesis of NSCLC, which, nevertheless, remains elusive, so the treatment of NSCLC is still unsatisfied. It has been confirmed that lncRNAs are crucial to the pathophysiological processes of multiple tumors, and, currently, lncRNAs have become new focuses in research of pathogenesis and development of therapeutic strategies of NSCLC.

LncRNAs are divided into two kinds, onco-lncRNAs and tumor-suppressor lncRNAs. Zhai et al. found that the high expression of lncRNA hox transcript antisense intergenic RNA (HOXATIR) can inhibit the expression of p53 through binding with polycomb repressive complex 2 (PRC2), thereby suppressing the apoptosis and increasing the proliferation of tumor cells. LncRNA AK126698 in high expression can also increase the resistance of NSCLC cells to cisplatin. Besides, Sun et al. reported that BRAF activated non-coding RNA (BANCR) is relatively downregulated in NSCLC cells. Upregulating the expression of BANCR can inhibit the migration and invasion of cells, which may be realized through regulating the epithelial-mesenchymal transition (EMT). In this study, it was firstly found that the interference on relatively high expression of ASAP1-IT1 in NSCLC tissues and cells can inhibit the proliferation, migration and invasion of tumor cells.

PTEN/Akt signal transduction pathway is a kind of classic intracellular transduction pathway, and its roles in repair, migration, proliferation of cells, and angiogenesis, have been confirmed by many studies. However, the abnormal activation of this pathway is also correlated with the development of diseases, such as diabetes mellitus, autoimmune disease and tumors. In recent years, more and more studies have indicated that lncRNAs are involved in the proliferation, invasion and metastasis of tumor cells through regulating the PTEN/AKT signaling pathway. For example, Guo et al. reported that lncRNA AFAP1-AS1 can promote the proliferation and inhibit the apoptosis of gastric cancer cells through PTEN/AKT signaling pathway; Li et al. found that in hepatocellular carcinoma, lncRNA ARSP could enhance the chemoresistance of hepatocellular carcinoma cells to adriamycin through regulating the PTEN/AKT signal pathway. In this study, we confirmed for the first time that ASAP1-IT1 can mediate the expression of key molecules in PTEN/Akt signaling pathway to regulate the proliferation, invasion, and metastasis of NSCLC cells.

**Conclusions**

Studies above are an important theoretical foundation for in-depth exploration of potential molecular mechanisms accounting for the development and progression of NSCLC, while the ASAP1-IT1/PTEN/Akt axis, as a therapeutic target, serves as reference for reversing the formation of NSCLC malignant phenotype.

**Conflict of Interest**

The Authors declare that they have no conflict of interests.

**References**

Role of ASAP1-IT1 in NSCLC


