**Abstract.** – **OBJECTIVE:** Lung cancer is the most common cause of death in cancer worldwide, and cisplatin plays an important role in its treatment. However, the response to chemotherapy is poorly attributable to drug resistance. Our present study aimed to investigate the relation of the exosomal miR-146a-5p level with the chemosensitivity of NSCLC to cisplatin and the molecular mechanism that miR-146a-5p mediated to effect on chemotherapy response.

**PATIENTS AND METHODS:** The exosomes were isolated by ExoQuick kit. The exosomal morphology and particle size distribution were evaluated by the transmission electron microscopy and nanoSight assay respectively. Cell proliferation was detected using the MTT assay. NSCLC cells were infected with mimics or inhibitor to overexpress or downregulate miR-146a level. Besides, Quantitative real-time PCR, Western blot analysis, and immunohistochemistry were applied to detect the relative miRNA and protein levels.

**RESULTS:** Advanced NSCLC patients with low serum exosomal miR-146a-5p levels had higher recurrence rates than those with high levels. A549/DDP cells and exosomes expressed higher miR-146a-5p than A549. In the process of cisplatin-induced drug resistance, the expression of miR-146a-5p decreased in either NSCLC cell lines or the exosomes gradually. What’s more, the overexpression of miR-146a-5p could reverse the resistance of A549/DDP. And the possible mechanism of miR-146a-5p increasing chemosensitivity of NSCLC to cisplatin could be targeting Atg12 to inhibit autophagy.

**CONCLUSIONS:** Serum exosomal miR-146a-5p may be a new biomarker predicting the efficacy of cisplatin for NSCLC patients and real-time monitoring drug resistance.

**Key Words:** Exosomes, miR-146a-5p, Prognosis biomarker, Cisplatin efficiency.

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**Introduction**

Lung cancer, with non-small cell lung cancer (NSCLC) accounting for about 85% of all cases, is the leading cause of cancer-related mortality worldwide. Systemic therapy for lung cancer consists mainly of platinum-based chemotherapy except for some patients in stage I. Unfortunately, the clinical effectiveness of such chemotherapy is limited by intrinsic and acquired resistance. It has been reported that mounting factors are involved in platinum-based drug resistance, but the underlying mechanisms remain largely obscure. These years, lots of efforts were paid to identify pharmacogenomics markers which could be used to predict platinum-based chemotherapy response in NSCLC patients. However, no robust pharmacogenomics marker was successfully used as a predictor in the clinical practice by now.

Exosomes are small, cell-secreted vesicles of about 30-200 nm that carry a wide range of active molecules, including proteins, RNAs, and lipids. In the clinical setting, exosomes are present in a variety of bodily fluids, including blood, serum, urine, amniotic fluid, and tumor malignant effusions. Given the relative ease and non-invasive nature of isolating exosomes from patient samples, and their distinctive protein and nucleotide contents, several studies have suggested using exosomal biomarkers for disease diagnostic purposes. Exosome-derived miRNAs circulate in body fluids in a highly stable and cell-free form, probably due to their incorporation in exosomes, allowing their use as novel diagnostic and prognostic markers. To date, 764 miRNAs have been identified in exosomes derived from different tissues. Each tumor is characterized by a specific miRNA profile. Exosomal miRNAs could be...
used as diagnostic and prognostic indicators for lung cancer, esophageal squamous cell carcinoma (ESCC), prostate cancer, ovarian cancer and other types of tumors.\(^{10,12,13}\)

miR-146a has been demonstrated to be up-regulated in various cancers, such as cervical cancer\(^ {24}\) and thyroid cancer\(^ {15,16}\). Moreover, miR-146a levels have therapeutic potential to suppress invasion and migration capacity in breast cancer\(^ {17}\) and pancreatic cancer\(^ {18}\). However, there are few investigations regarding the roles of miR-146a in drug resistance of NSCLC.

In this study, we aimed to investigate the relation of the exosomal miR-146a-5p level with the chemosensitivity of NSCLC to cisplatin by analyzing the clinical samples and cell lines. The molecular mechanism that miR-146a-5p mediated to effect on chemotherapy response was also explored. Our findings might provide a new biomarker predicting the efficacy of cisplatin for NSCLC patients.

**Patients and Methods**

**Ethical Committee Approval**

Our study was approved by the Ethics Committee on Human Research of the First Affiliated Hospital of Nanjing Medical University and Affiliated Hospital of Jiangsu University. Signed written informed consents were obtained from all participants before the study.

**Reagents**

Cisplatin was purchased from Sigma-Aldrich (St. Louis, MO, USA). Anti-LC3B, anti-p-AKT (S473), anti-p-mTOR (S2448), and anti-Beclin1 antibody were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-β-actin, anti-CD9, and anti-CD63 antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-SQSTM1/p62 and anti-Atg12 antibody were from Abcam (Cambridge, MA, USA). Anti-LC3B-I antibody was from Novus Biologicals (Littleton, CO, USA). 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) and dimethylsulfoxide were from Sunshine Biotechnology (Nanjing, China).

**Cell Culture**

The cisplatin-sensitive human NSCLC cell line A549 and its cisplatin-resistant derivate A549/DDP were obtained from the Institute of Hematology, Chinese Academy of Medical Sciences (Tianjin, China). The fold of resistance (A549/DDP vs. A549) is about 5 times as described in the previous studies\(^ {19}\). To monitor the change of cell properties during cisplatin treatment, we developed 3 cisplatin-resistant variants by exposure of A549/DDP cells to step-wise increase in cisplatin concentration. And we maintained them at a final concentration of 500 (A549/DDP-500), 1000 (A549/DDP-1000) and 2000 ng/mL (A549/DDP-2000), respectively. All of the cells were cultured in RPMI-1640 medium (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Life Technologies). For resistant variants, cisplatin was withdrawn from the culture medium 3 days before use.

**Tissue, Blood Samples**

The serum samples (about 1 mL each) were from 100 advanced NSCLC patients diagnosed at First Affiliated Hospital of Nanjing Medical University and Affiliated Hospital of Jiangsu University before the first treatment. All of the selected patients had accepted standard first-line platinum-based chemotherapy as the initial treatment. The paraffin sections of lung cancer were from 12 NSCLC patients diagnosed at the First Affiliated Hospital of Nanjing Medical University. Advanced NSCLC patients were divided into two groups according to the assessment of objective responses after 2 courses of cisplatin-based chemotherapy. 6 patients with progressive disease were defined as cisplatin-resistant NSCLC, the other 6 patients with partial response were defined as cisplatin-sensitive NSCLC, the other 6 patients with partial response were defined as cisplatin-sensitive NSCLC.

**Immunohistochemical (IHC) Analysis**

Immunostaining of LC3B-I, Atg12 protein was performed using a Real Envision Detection kit from the Gene Tech Company (Shanghai, China) according to the manufacturer’s instructions. To quantify protein expression, the integral optical density (IOD) as the product of mean density and area was evaluated with Image-pro plus software (Media Cybernetics, Bethesda, MD, USA). Each slice was taken 3 photos in random fields of view and analyzed on average IOD.

**Western Blot**

Western blot was performed as described previously\(^ {20}\). The densitometry of immunoblots was quantified with Image J software (NIH, Bethesda, MD, USA).
**MTT Assay**
Briefly, treated 3000 cells were seeded into the wells of 96-well plates and incubated with various concentrations of cisplatin for 72 h. The survival rate was detected as previously described.\(^{20}\)

**Isolation and Purification of Exosomes**
Exosomes were isolated and purified by ExoQuick exosome precipitation kit (SBI, Mountain View, CA, USA) according to manufacturer’s instructions.

**Transmission Electron Microscopy Assay**
Exosomes were added into an appropriate volume of fixative. Then samples were embedded and sectioned, and finally observed under transmission electron microscopy (Hitachi, Tokyo, Japan).

**NanoSight Assay**
Exosome pallets were resuspended in 1 mL PBS then the particle size distribution was analyzed under NanoSight NS300 (Malvern Instruments, Shanghai, China).

**Exosomal RNA Extraction and RT-qPCR of miRNAs**
Total RNA was extracted from exosome pellets using the miRNeasy Serum/Serum Kit (Qiagen, Hilden, Germany) according to manufacturer’s instructions. Quantitative real-time PCR analyses were performed using Qiagen miScript II RT Kit (Hilden, Germany) and Qiagen miScript SYBR Green PCR Kit (Hilden, Germany) according to manufacturer’s instructions. The absolute concentration of exosomal miRNA was the ratio of miRNA absolute expression level to the corresponding exosomal protein content. Absolute expression level was calculated by employing the standard curves. All data were collected and analyzed with a CFX96 Touch Real-Time PCR Detection System.

The primers sequences are the follow: has-miR-146a-5p; ACACACCAGCTGGGTGAGAACT-GAATTCCA.

**Cell Transfection with Mimics and Inhibitors**
NSCLC cells were transfected with the miR-146a-5p mimic, miR-146a-5p inhibitor, or their respective negative controls (NC) (GenePharma, Shanghai, China) according to the manufacturer’s instructions. After 24 h transfection to NSCLC cells, cells were verified the transfection efficiency by qRT-PCR.

**Target Prediction**
The conventional online programs, including Targetscan (http://www.targetscan.org) and Findtar3 (http://bio.sz.tsinghua.edu.cn) were used to predict the targets of hsa-miR-146a-5p.

**Statistical Analysis**
All statistical analyses were performed using GraphPad Prism5.0 (GraphPad Software, La Jolla, CA, USA). All data were expressed as the mean ± standard deviation of the mean (SD). The significance of differences between groups was estimated using the Student’s t-test, Mann-Whitney test or one-way analysis of variance (one-way ANOVA) with Tukey post hoc analysis, as appropriate. Progression-free survival (PFS) rates were calculated by the Kaplan-Meier method with the log-rank test applied for comparison. A probability level of 0.05 was chosen for statistical significance.

**Results**

**MiR-146a-5p Level in Serum Exosomes is Relative to the Cisplatin Responsiveness in NSCLC Patients**
We collected 100 serum samples from advanced NSCLC patients who received standard first-line treatment of cisplatin-based chemotherapy after diagnosis and the collection of tissues as described in Methods. Then we isolated exosomes from serum by Exoquick kit and identified exosomes as typical small vesicles with a diameter of 30 to 200 nm in transmission electron microscopy (Figure 1A). Moreover, we detected the particle size distribution under nanoSight and the marker proteins CD9 and CD63 in two clinical samples by Western blot (Figure 1B-C). After ensuring successful isolation of exosomes, we extracted exosomal total RNA and tested miR-146a-5p expression level by RT-qPCR. To evaluate the correlation between miR-146a-5p expression levels and prognosis in NSCLC patients, we used Kaplan-Meier survival analysis and log-rank test to assess miR-146a-5p expression levels and progression-free survival (PFS). The results showed that patients with low miR-146a-5p expression levels had a shorter PFS compared with patients with high miR-146a-5p expression (Figure 1D).
Exosomal miR-146a-5p predicts cisplatin response of NSCLC

MiR-146a-5p Level in NSCLC Cells and Exosomes

To further investigate the relation between exosomal miR-146a-5p level and cisplatin responsiveness, we developed three cisplatin-resistant variant cells as previously described. Compared with the parental sensitive NSCLC cell line A549, these variants showed gradually reduced sensitivity to cisplatin and A549/DDP-2000 was the strongest resistant. Either in NSCLC cells or in the tumor-derived exosomes, the miR-146a-5p level is decreased along with the increased resistance of NSCLC cells determined by RT-qPCR (Figure 2A-B).

Overexpression or Downregulation of miR-146a-5p Effected the Sensitivity of NSCLC Cells to Cisplatin

Our former data have shown that miR-146a-5p level was lower in the A549/DDP cells compared with A549 cells. To deeply investigate the role of miR-146a-5p in the cisplatin resistance in DDP-resistant NSCLC cells, we transfected miR-146a-5p mimics into A549/DDP cells. As shown in Figure 3A, miR-146a-5p expression was significantly increased. MTT assay was then performed to determine the effects of miR-146a-5p on cell viability of A549/DDP cells when exposed to cisplatin. As a result, A549/DDP cells after transfected were more sensitive to cisplatin than the controls (Figure 3A). Consistently, by transfecting miR-146a inhibitor, the sensitivity of A549 to cisplatin declined (Figure 3B), which indicated that miR-146a level could partially reverse the cisplatin resistant in NSCLC cells.

Atg12 was Identified as a Functional Target of miR-146a

As we know, miRNAs exert their function by affecting their target genes expression. Thus,
Figure 2. MiR-146a-5p level in A549 and A549/DDP cells and exosomes. (A) Absolute concentrations of miR-146a-5p in NSCLC cell derived exosomes were determined by RT-qPCR. (B) Relative miR-146a-5p levels in A549 and A549/DDP cells were determined by RT-qPCR. *p < 0.05 vs. A549 cells.

Figure 3. Responses of A549 and A549/DDP cells to cisplatin after transfection. (A) A549/DDP-2000 cells transfected with NC or miR-146a-5p-mimics (B) A549 cells transfected with NC or miR-146a-5p-inhibitor were incubated with various concentrations of cisplatin for 72 h. Cell viability was determined by MTT assay (*p < 0.05, **p < 0.01 vs. negative controls).
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the target genes of miR-146a were predicted through 2 publicly available web (TargetScan and Findtar3), and Atg12 was selected as a putative target (Figure 4A). We next determined whether overexpression of miR-146a could downregulate Atg12 expression. As shown in Figure 4B, the expression of Atg12 protein level was significantly lower in miR-146a overexpressed A549/DDP cells than that in NC group by Western blot. Correspondingly, we found the change of autophagy associated proteins (LC3B lipidation and p62) but upstream molecules (pAKT, pmTOR, and Beclin1) were not impacted. These results further suggested that Atg12 is a direct target of miR-146a-5p in NSCLC cells, and miR-146a may negatively regulate the expression of Atg12 to inhibit autophagy. A similar conclusion was got by inhibiting miR-146a expression in A549 cells (Figure 4B).

Atg12 was Involved in Sensitivity to Cisplatin in NSCLC

We collected 12 lung cancer tissue samples from advanced NSCLC patients who received standard first-line treatment of cisplatin-based chemotherapy. By evaluation of the efficacy after 2 courses of treatment, six of them were cisplatin-resistant (progressive disease) and the last were cisplatin-sensitive NSCLC (partial response). Immunohistochemical analysis showed that lung cancer tissues from cisplatin-resistant NSCLC displayed higher levels of basal autophagy as determined by the increase in both Atg12 and LC3 lipidation expression than those from cisplatin-sensitive NSCLC (Figure 5A and B).

Discussion

Cisplatin is the most widely used chemotherapy drugs for the treatment of lung cancer and other tumors21. Approximately 1,590,000 lung cancer patients succumb to the disease every year, 61% of which are primary drug resistance and 33% have an acquired drug resistance. Thus, a prognosis biomarker is urgent needed.

A most recent trend of pharmacogenomics study is to conduct a comprehensive molecular characterization of different response subtype patients, with the identification of DNA mutation, fusion gene, copy number variation, mRNA and noncoding RNA expression and epigenetic alteration in tumor tissues22,23. However, such data are still not available for sensitive or resistant NSCLC patients receiving platinum-based chemotherapy. Most of the NSCLC patients receiving platinum chemotherapy were at an advanced stage and not candidates for surgery. Therefore, it is very difficult to conduct such a research in a large number of patients. Liquid biopsy such as serum exosomes is ideal to overcome the limitation of invasive biopsy and represents one of the most important future directions of pharmacogenomics study. Existed studies aimed to investigate the association of specific genetic markers with drug response in retrospective analysis, but it was very difficult to demonstrate the real clinical value of these biomarkers. In the present work, we conducted our experiments based on clinical samples. The serum exosomal miR-146a-5p levels in 100 advanced NSCLC patients were evaluated. Our results suggested that the low-miR-146a-5p patients had higher recurrence rates than the high-146a-5p patients did. Further analysis indicated that in the process of cisplatin-induced drug resistance, the expression of miR-146a-5p decreased in either NSCLC cell lines or the tumor-derived exosomes gradually. What’s more, the overexpression of miR-146a-5p could

Figure 4. Atg12 was a novel target gene of miR-146a-5p. (A) The binding site of miR-146a-5p and the prediction target genes (Atg12) through Targetscan web. (B) The protein levels of p-AKT, p-mTOR, Beclin1, Atg12, LC3B, and p62 were analyzed in A549/DDP and A549 cells transfected with miR-146a-5p-mimics or miR-146a-5p-inhibitor respectively by Western blot. Western blots are representative of three similar experiments. Actin was used as a loading control.
reverse the resistance of A549/DDP determined by MTT assay. And the silico analysis showed that Atg12 was the target gene of miR-146a-5p. As we know, Atg12 is a member participating in autophagy process. The conjugation between Atg12 and Atg5 is essential for LC3 lipidation and therefore for autophagosome formation. Recent studies demonstrate that cisplatin induces autophagy in many kinds of cancer cells. Our previous study highlighted the involvement of autophagy in the cisplatin-resistance development. Inhibition of autophagy via tuning the Akt/mTOR signaling could be a promising strategy in the therapy for cisplatin-resistant non-small cell lung cancer. The Western blot results showed that miR-146a-5p inhibited the autophagy process by down regulating Atg12. Immunohistochemical analysis showed that lung cancer tissues from cisplatin-resistant NSCLC displayed higher levels of basal autophagy as determined by the increase in both LC3 lipidation and Atg12 expression than those from cisplatin-sensitive NSCLC. It is possible that miR-146a-5p increases the chemosensitivity of NSCLC to cisplatin by inhibiting autophagy. Thus, the expression levels of miR-146a-5p in NSCLC patients’ serum exosomes could be used as a potential new biomarker which could predict prognosis to cisplatin for NSCLC and realtime monitor drug resistance.

Conclusions

The present work indicates serum exosomal miR-146a-5p may be a new biomarker predicting the efficacy of cisplatin for NSCLC patients and
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real-time monitoring drug resistance. And the possible mechanism of miR-146a-5p increasing chemosensitivity of NSCLC to cisplatin could be targeting Atg12 to inhibit autophagy.

Conflict of Interest
The Authors declare that they have no conflict of interests.

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