Relationship between epithelial to mesenchymal transition and chemoresistance of lung cancer

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Abstract  Objective: The aim of this study was to explore the correlation between epithelial to mesenchymal transition (EMT) and chemoresistance of non-small-cell lung cancer (NSCLC).  Methods: In vitro, the drug resistance index of cisplatin resistant lung adenocarcinoma cell line (A549/DDP) was detected by CCK-8 assay; the morphological change between A549/DDP cells and lung adenocarcinoma cells (A549) was observed by phase contrast microscope; expression of EMT markers (including E-cadherin and vimentin) and resistance protein, excision repair cross-complementing 1 (ERCC1) was detected by immunocytochemistry. The expression of E-cadherin, vimentin and ERCC1 was investigated by immunohistochemistry in 120 cases of NSCLC, half of that were treated with pre-operative neoadjuvant chemotherapy (neoadjuvant chemotherapy group), and the other underwent surgery alone (simple surgery group).  Results: There was a significant difference between the IC50 (half maximal inhibitory concentration) of A549/DDP cells (5.20) and A549 cells (1.88) (P < 0.05), and the drug resistance index of A549/DDP cells was 2.77. Compared with A549 cells, A549/DDP cells increased expression of ERCC1 (P < 0.05). Moreover, A549/DDP cells showed morphological and phenotypic changes consistent with EMT: with spindle-shaped morphology, and decreased expression of E-cadherin and increased expression of vimentin. Immunohistochemistry showed significant positive correlation between the expression of ERCC1 and vimentin (r = 0.496, 0.332, P < 0.05), and significant negative correlation between the ERCC1 and E-cadherin (r = -0.403, -0.295, P < 0.05) in neoadjuvant chemotherapy group and simple surgery group. In addition, compared with simple surgery group, the expression of ERCC1 (P = 0.003) and vimentin (P = 0.004) was significantly increased, and the expression of E-cadherin was decreased in neoadjuvant chemotherapy group (P = 0.032).  Conclusion: A549/DDP cells acquired cisplatin-resistance and occurred EMT simultaneously; the phenomenon of chemoresistance and EMT was caused more easily in neoadjuvant chemotherapy group. As such, we further confirmed the close correlation between chemoresistance and EMT of NSCLC, and provided theoretical basis for the targeting therapy with EMT regulatory factor for chemoresistant NSCLC patients.

Key words  epithelial to mesenchymal transition; chemoresistance; lung cancer

Worldwide, lung cancer was the most commonly diagnosed and most fatal cancers in males, while having the fourth highest incidence rate and second highest mortality rate in females. The total 5-year survival rate is less than 15%[1]. Surgery is considered the best treatment for non-small-cell lung cancer (NSCLC). However, lung cancer is usually found at an advanced stage, and fewer than 25% of NSCLC patients are considered fit for surgery[2]. Chemotherapy is the main adjuvant therapeutic strategy for cancer treatment, but the chemoresistance often leads to the failure of chemotherapy[3].

Epithelial to mesenchymal transition (EMT) refers to the process that epithelial cells change to mesenchymal cells, during which epithelial cells lose their polarity and acquired increased motility and invasion[4-7]. EMT has been shown to be essential for embryonic development, neural nest and development of heart and other tissues and organs[4]. Recent studies have extended the knowledge that EMT is also involved in organ fibrosis, tissue repair, and cancer progression[5]. Accumulating studies demonstrated that EMT is involved in the metastasis, treatment resistance and associated with the progression of many type of tumors[6-7].

In order to explore the correlation between chemoresistance and EMT, we observed the changes of morphology and expression of EMT markers as well as resistance protein between A549/DDP cells and A549 cells, and the differential expression of resistance protein and EMT marker in neoadjuvant chemotherapy group and simple surgery group.
Materials and methods

Cell lines and cell culture
The A549/DDP cells and A549 cells were purchased from the cell bank of Xiangya Central Laboratory. The cell lines were cultured in RPMI-1640 (Hyclone, USA) supplemented with 10% FBS (Zhejiang Tianhang Biological Technology Co., Ltd, China) and 1% penicillin-streptomycin at 37 °C in 5% CO₂. Cisplatin (2 µg/mL) was regularly added to A549/DDP cells to maintain their drug resistance.

Drug cytotoxicity assay
The drug resistance index of A549/DDP cells was evaluated by CCK-8 assay (Boster, China). 5 × 10³ cells per well were seeded in 96-well plates in 100 µL medium with cisplatin of various concentration (0, 1, 2, 4, 8, 16 µg/mL) and left to incubate for 48 h at 37 °C and 5% CO₂. After 48 h, 10 µL CCK-8 was added to each well and cells were left for 2 h. The absorbance of each well was measured at 450 nm. Each experiment was performed using 5 replicate wells and 3 independent experiments. The dose-response curve could be plotted with the inhibition rate. The IC₅₀ (half maximal inhibitory concentration) was defined as the drug concentration required to produce a 50% reduction of optimal density in each test. Resistance index was calculated as IC₅₀ resistant/IC₅₀ sensitive cell line.

Primary tumor specimens
One hundred and twenty tumor specimens of NSCLC patients were taken from the Affiliated Hospital of Qingdao University Medical College, 60 of which underwent surgery following neoadjuvant chemotherapy in the last 5 years (neoadjuvant chemotherapy group). Each patient received 2 cycles of cisplatin-based chemotherapy. Another group of 60 patients underwent surgery alone without neoadjuvant chemotherapy (simple surgery group) was collected between May 2011 and June 2013. NSCLC was definitively identified based on P-vimentin in neoadjuvant chemotherapy group (31/60) and 31.67% (19/60) in simple surgery group (Table 2, Fig. 2B). Then we investigated the protein expression of EMT marker by immunocytochemistry to further explore whether A549/DDP cells occurred EMT.

Morphology changes were observed by phase contrast microscope that A549/DDP cells showed a spindle-shaped morphology, while the A549 cells showed polygon shape (Fig. 2A). Then we investigated the protein expression of EMT marker by immunocytochemistry to further explore whether A549/DDP cells occurred EMT. Immunocytochemical results showed that, the expression of E-cadherin was significantly decreased and the expression of vimentin was increased (P < 0.05) compared with A549 cells (Table 2, Fig. 2B).

Verification of drug resistance of A549/DDP cells
The IC₅₀ (half maximal inhibitory concentration) of A549/DDP cells and A549 cells was detected by CCK-8 assay to define the drug resistance index of A549/DDP cells. Fig. 1A showed that the cell inhibition rate increased with the increasing concentration of cisplatin. The IC₅₀ of A549/DDP cells was significantly higher than that of A549 cells (P < 0.05), and the drug resistance index of A549/DDP cells was 2.77 (Table 1). In addition, immunocytochemistry showed that the expression of ERCC1 of A549/DDP was significantly increased (P < 0.05) (Table 2, Fig. 1B).

Results

Effects of cisplatin resistance on EMT phenotype of A549 cells
Morphology changes were observed by phase contrast microscope that A549/DDP cells showed a spine-shaped morphology, while the A549 cells showed polygon shape (Fig. 2A). Then we investigated the protein expression of EMT marker by immunocytochemistry to further explore whether A549/DDP cells occurred EMT. Immunocytochemical results showed that, the expression of E-cadherin was significantly decreased and the expression of vimentin was increased (P < 0.05) compared with A549 cells (Table 2, Fig. 2B).

Correlation between the expression of drug resistance protein and EMT maker protein in specimens of NSCLC
Immunohistochemical findings showed that the expression of ERCC1, vimentin and E-cadherin were 76.67% (46/60), 66.67% (40/60) and 28.33% (17/60) in neoadjuvant chemotherapy group, and 55.0% (33/60), 51.67% (31/60) and 31.67% (19/60) in simple surgery group (Table 3). A significant positive correlation between ERCC1 and vimentin in neoadjuvant chemotherapy group (r = 0.496, P = 0.001) and simple surgery group (r = 0.332, P = 0.06),
and a significant negative correlation between ERCC1 and E-cadherin in neoadjuvant chemotherapy group ($r = -0.403, P = 0.004$) and simple surgery group ($r = -0.295, P = 0.037$) (Table 4) were found by Spearman analysis.

Meanwhile, the expression of ERCC1 ($P = 0.003$) and
vimentin ($P = 0.004$) was significantly increased, and the expression of E-cadherin was decreased ($P = 0.032$) compared with simple surgery group.

**Discussion**

Chemotherapy is the main adjuvant therapeutic strategy for NSCLC treatment. The combined chemotherapy based on cisplatin prolonged the survival time of patients with NSCLC effectively, but their 5-year survival rate was not significantly increased, the main reason is the drug resistance to cisplatin of NSCLC cells.

EMT is a fundamental biological process during which epithelial cells lose their polarity and change to a mesenchymal phenotype accompanied by increased motility and invasion. Recent studies found the process of EMT was widely involved in the acquisition of chemoresistance. Sarkar et al. found an upregulation of E-cadherin in pancreatic cancer cells sensitive to gefitinib (L3/6pl, Clol357, BxPC-3 and HPAC), while an upregulation of vimentin and ZEB-1 in the pancreatic cancer cells resistant to gefitinib (MiaPaCa-2, Panc-1 and Aspc-1). Foroni et al. established breast cancer cell lines resistant to taxol, doxorubicin and docetaxel, and found an increased expression of vimentin and a decreased expression of E-cadherin. In this study, we observed that A549/DDP cells acquired cisplatin-resistance and occurred EMT simultaneously, including a change of morphology that A549/DDP cells showed a spindle-shaped morphology, and phenotypic changes-downregulation of E-cadherin and upregulation of vimentin. We further supplemented the above results from the perspective of EMT and confirmed the correlation between chemoresistance and EMT.

We also showed the positive correlation between vimentin and ERCC1, and the negative correlation between E-cadherin and ERCC1 in neoadjuvant chemotherapy group and simple surgery group. In addition, we found the phenomena of EMT and chemoresistance were more apparent in neoadjuvant chemotherapy group, it indicated that the chemoresistance and EMT were caused more easily in neoadjuvant chemotherapy group, which contributed to the controversy about the curative effect of neoadjuvant chemotherapy treatment. The results above confirmed the correlation between chemoresistance and EMT of NSCLC from the histological level, and consisted with the results of our cell culture experiments.

Our study confirmed the relationship between chemoresistance and EMT from the cytological and histological level, but the underlying mechanism was not clear. Currently, most scholars accepted the mechanism that EMT and chemoresistance have focal points in their signal pathways. The activation and disorder of EMT-related signal transduction pathway regulate both EMT and chemoresistance progress. The EMT signal pathways, including PI3K/AKT/GSK-3β/Snail, NF-κB/Y1/RKIP/PTEN, PI3K/AKT/HIF-1α, Notch-2 etc, were confirmed to play a key role in the process of drug resistance to cisplatin, gemcitabine, gefitinib etc. Moreover, the production of multi-drug resistance (MDR) of tumor cells has also been shown to be accompanied with EMT. Han et al. found that the activation of PI3K/AKT, MAPK signal upregulated the expression of P-gp (the critical transcription regulator of MDR), while the PI3K/AKT and MAPK are important pathways of EMT. Furthermore, it was demonstrated that the P-gp protein and ATP-binding cassette (ABC) which were essential for MDR, were mediated by the EMT transcription factor-Snail and Twist. The above study results indicated that EMT and MDR exist meeting points in their signal pathways.

The interrelationship between chemoresistance and EMT was extremely complex, and there may be more common regulatory factors or signal pathways. This study provided theoretical basis for the further research about the correlation mechanisms between chemoresistance and EMT. Exploration on the correlation between EMT-related signal pathways and chemoresistance will be the focus of future research, and will provide new strategy for treatment on restoring chemotherapy sensitivity for tumor patients.

**References**