Differences of inflammatory microenvironment and sensitive correlation to cisplatin-chemotherapy in lung adenocarcinoma and squamous cell carcinoma

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Abstract

Introduction: Non-small cell lung cancer (NSCLC) is the most prevalent type of cancer worldwide and associated with high mortality rate. An effective treatment for NSCLC is urgently needed. This study aimed to investigate differences of inflammatory microenvironment and sensitive correlation to cisplatin-chemotherapy in lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC), thereby providing insights into the mechanisms underlying inflammation in NSCLC.

Materials and methods: The resistance of A549 and SK-MES-1 cells to cisplatin treatment was determined by IC50. The migration and invasion capacity of A549 and SK-MES-1 cells was determined using migration and invasion assay kits. The apoptosis rate was evaluated. The protein expression levels of caspase-3 and Ki67 were measured by western blot. ELISA assay was used to determine the levels of inflammatory cytokines, including IL-4, IL-6, IL-8, and TNF-α.

Results: Patients with LUAD showed shorter survival than LUSC patients after the chemotherapy treatment. LUAD was more resistant to cisplatin treatment, corresponding with a higher IC50 observed in A549 cells than SK-MES-1 cells. Moreover, A549 cells showed higher migration and invasion capacity and lower apoptosis rate than SK-MES-1 cells. In addition, SK-MES-1 LUAD samples showed stronger inflammation response than LUAD samples and A549 cells. Accordingly, inflammation cytokines promoted the migration and invasion of SK-MES-1 cells, whereas inhibited cell apoptosis.

Conclusions: The present study suggests that LUAD is more resistant to chemotherapy and shows a stronger inflammation response than LUSC. Inflammatory cytokines could enhance the resistance of LUAD to chemotherapy and further promote tumor cell proliferation, migration, and invasion.

Keywords: non-small cell lung cancer; lung adenocarcinoma; squamous cell carcinoma; inflammation; cisplatin

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Lung cancer is the most common cancer worldwide and associated with high mortality (1). There are approximately 2 million new cases of lung cancer each year. Currently, chemotherapy is the major strategy to treat lung cancer. Platinum-based chemotherapy has been considered as an optimal option for the management of lung cancer (2). Mechanistically, platinum-DNA adducts inhibit DNA repair, inhibiting tumor proliferation and promoting tumor cell apoptosis (3).

It has been commonly recognized that tumor tissues are associated with inflammatory response in clinic (4, 5). Inflammation tumor microenvironment plays a crucial role in promoting tumor cell proliferation, progression, and invasion. Disregulated inflammation leads to the development of tumor microenvironment and releasing of inflammatory factors, which alters gene expression and post-translational modification (6). For example, IL-4 secretion is reported to associate with anti-inflammation...
and immunosuppression in lung cancer, breast cancer, etc. (7, 8). IL-4 has been demonstrated to inhibit proliferation and progression of cancer cells through inhibiting angiogenesis (9). IL-6, a pro-inflammatory cytokine, is a key factor stimulating the downstream STAT3 signaling, which further induces the anti-apoptosis pathway. As a result, release of IL-6 enhances tumor cell metastasis and invasion (10, 11). IL-8 has been considered as an important tumor-promoting factor by potentially promoting angiogenesis and release of growth factors, thereby causing tumor proliferation and progression (12). In addition, TNF-α plays an important role in tumor proliferation and metastasis. Study has shown that deficient TNF-α induces tumor apoptosis in patients with lung cancer (13). Overall, IL-4, IL-6, IL-8, and TNF-α could serve as indicators to characterize the tumor microenvironment in lung cancer.

Non-small cell lung cancer (NSCLC) mainly includes lung adenocarcinoma (LUAD) and lung squamous cell carcinoma (LUSC). Currently, with regard to the response of LUAD and LUSC to chemotherapy such as cisplatin, most studies are focusing on pathophysiology. Recently, a very nice review article has explained in details the inflammatory effect on the lung cancer progression and therapy (14). However, the differences between different types of lung carcinoma require more study. However, the knowledge on the inflammatory tumor microenvironment of malignant tumor remains unclear (15–17). Herein, the present study mainly focused on investigating the differences in inflammatory responses between LUAD and LUSC, as well as the correlation between inflammatory response and cisplatin treatment.

**Methods**

**Lung cancer specimens**

Lung cancer specimens were obtained from resections of 19 and 21 brain-dead patients with LUAD and LUSC, respectively. This study was approved by the ethics committee of XinHua Hospital Affiliated to Shanghai Jiao Tong University School of Medicine.

**Cell culture**

Human LUAD cell line A549 and human lung squamous carcinoma cell line SK-MES-1 were purchased from ATCC. Cells were thawed quickly in 37°C water bath after taking out of −150°C freezer. Cells were transferred to a sterilized centrifuge tube mixed with fresh complete media. Subsequently, cells were subcultured after achieving 80% confluency. Cells were seeded in plate for 24 h before stimulation, and the cells were stimulated when the cells grown into 70%, with 0.1 μg/mL lipopolysaccharides for 24 h.

**Cisplatin treatment**

A549 and SK-MES-1 cells were seeded at 5,000 cells per well in 96-well plates. After achieving 80% confluence, cells were treated with vehicle or different concentrations of cisplatin (2, 4, 8, 12, and 16 μmol/L) for 48 h. Subsequently, the old media were removed completely, and 100 μL fresh RPMI1640 media and 10 μL CCK-8 reagent were added to each well. After 1-h incubation, plate was read at 450 nm, and IC50 was assessed using GraphPad Prism5.

**Cell migration assay**

100 μL of A540 and SK-MES-1 cell suspension (2 × 10^4 cells) were added in the upper chamber of the Transwell chamber (BD Biosciences, NJ, USA). Cells were incubated with media supplemented with 10% FBS for 36 h. The migrated cells on the lower chamber surface were fixed and stained with crystal violet for 20 min. The migrated cells were imaged under a light microscope (magnification × 200).

**Flow cytometry**

A549 and SK-MES-1 cells were treated with cisplatin and harvested. A 500 μL binding buffer was added to resuspend cells. Cell suspension was mixed gently and incubated at room temperature for 10 min. Apoptotic cells (TMRM-/Annexin V(FITC)+/DAPI-) were evaluated using flow cytometry.

**Enzyme-linked immunosorbtent assay**

100 mg of tumor tissue and tumor-adjacent tissue were homogenized with 5 mL PBS for 10 min on ice. Homogenates were centrifuged, and the supernatants were collected for analysis of IL-4, IL-6, IL-8, and TNF-α using ELISA according to manufacturer’s instruction (BD Bioscience Pharmingen).

**Statistical analysis**

All experiments were repeated at least three times. All data presented were analyzed using SPSS22.0 and were expressed as mean ± standard deviation (SD). Paired-t tests, independent t tests, and one-way ANOVA analysis were used to analyze the significance of differences between groups. P < 0.05 was considered statistically significant.

**Results**

**Patients with LUAD survived longer than patients with LUSC**

The specimens from 19 patients with LUAD and 21 patients with LUSC were obtained after patients were pronounced brain-dead. There was no statistical significance in terms of age, gender, or the patient number administrated with gemcitabine or combination of gemcitabine and cisplatin between LUAD and LUSC patients (Table 1). We found that patients with LUSC survived longer than patients with LUAD (P < 0.01, Fig. 1A). Importantly, the survival of LUSC patients received chemotherapy was markedly longer than LUAD patients received...
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Chemotherapy (P = 0.04, Fig. 1). To further evaluate LUAD and LUSC in cisplatin resistance, we measured the resistance of A549 and SK-MES-1 cell lines to different concentrations of cisplatin. Our results showed that IC50 of A549 cells was significantly higher than SK-MES-1 cells (Fig. 1B), suggesting that LUAD was more resistant to cisplatin than LUSC.

The migration and invasion capacity of A549 was higher than SK-MES-1

In order to evaluate the migration capacity of A549 and SK-MES-1 cells, scratch test and invasion test were performed with the treatment of cisplatin at concentrations of 2, 4, 8, 12, and 16 μmol/L. The effect of each treatment should be first relatively estimated with corresponding control, and after that the results between two different cell lines were compared. A549 cells showed higher migration capacity than SK-MES-1 cells in both the control group and the cisplatin group (P < 0.01, Fig. 2B). Taken together, these findings indicated that LUAD showed higher migration and invasion capacity than LUSC.

Cell apoptosis plays a crucial role in cancer treatment. There is an unbalanced cell apoptosis and proliferation in cancer cells. Defective cell apoptosis was observed in cancer cells, resulting in excessive proliferation. To evaluate the cell apoptosis rate with the treatment of cisplatin, we measured apoptosis capacity of A549 and SK-MES-1 cells using a flow cytometer. The effect of each treatment should be first relatively estimated with corresponding control, and after that the results between two different cell lines were compared. Our results showed that the apoptosis rate of A549 was significantly lower than SK-MES-1 cells after the treatment with cisplatin (P < 0.01, Fig. 3A). Consistently, the relative protein level of apoptosis executor caspase-3 in A549 cells was significantly lower than SK-MES-1 cells. In contrast, the relative protein level of proliferation indicator Ki67 in A549 cells was markedly higher than SK-MES-1 cells after the treatment with cisplatin (Fig. 3B). These findings indicated higher apoptosis capacity and lower proliferation rate in LUSC than with LUAD.
A549 showed stronger proinflammatory response than SK-MES-1 with chemotherapy treatment

Proinflammation associates with tumor cell progression, which requires cytokines such as IL-6, IL-8, and TNF-α released from proinflammatory cells (18). Here, we found that the reduction of IL-4 level relative to that of control in patients with LUAD was smaller than LUSC. On the other hand, the relative levels of IL-6, IL-8, and TNF-α in LUAD patients were significantly lower than LUSC patients \((P < 0.01, \text{Fig. 4A})\). In line with the results observed in patient specimens, in both intracellular and supernatant cell samples, the relative level of IL-4 in A549 cells was higher than SK-MES-1 cells. Correspondingly, the relative levels of IL-6, IL-8, and TNF-α a in A549 cells were significantly lower than SK-MES-1 cells in both intracellular and supernatant cell samples \((P < 0.01, \text{Fig. 4B})\). These results suggested that proinflammation mediators could play an important role in prompting tumor proliferation and reducing apoptosis.

Proinflammatory factors promoted SK-MES-1 cell invasion and proliferation and reduced the apoptosis rate of SK-MES-1

To further evaluate the inflammation effects, SK-MES-1 cells were cultured with the supernatants of A549 cells

![Fig. 3.](image)

The apoptosis rate of A549 was lower than that of SK-MES-1. A, the A549 and SK-MES-1 cell lines with the treatment of cisplatin \((2, 4, 8, 12, \text{and } 16 \mu\text{mol/L})\) were placed into 24-well plates and incubated with fresh medium. The apoptosis capacity of cells was determined by Flow cytometry. Data represent mean ± SD of three independent experiments \((P < 0.01)\). B, upper panel, the A549 and SK-MES-1 cell lines with the treatment of cisplatin \((16 \mu\text{mol/L})\) were placed on 6-well plates and incubated with fresh medium. The protein expression levels of caspase-3 and KI67, and the internal control \(\beta\)-actin of A549 and SK-MES-1 cell lines were assessed by Western blotting. Protein expression from three independent experiments. Quantitation by densitometry was shown on below \((P < 0.01)\). Lower panel, the extended expression of caspase-3 and KI67 of A549 and SK-MES-1 cell lines is also detected.

![Fig. 4.](image)

The proinflammatory factors of A549 were more than that of SK-MES-1. A, the LUAD and LUSC patient specimens with the treatment of gemcitabine and cisplatin were dissociated. The levels of IL-4, IL-6, IL-8, and TNF-α were determined by ELISA. Data represent mean ± SD of three independent experiments \((P < 0.01)\). B, the A549 and SK-MES-1 cell lines with the treatment of cisplatin \((16 \mu\text{mol/L})\) were placed into 96-well plates and incubated with fresh medium. The levels of IL-4, IL-6, IL-8, and TNF-α of A549 and SK-MES-1 cells (upper panel) and their supernatant (lower panel) were detected by ELISA, and range lines represent mean ± SD of three independent experiments \((P < 0.01)\). LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma.
for 48 h, along with the treatment with different concentrations of cisplatin. The effect of each treatment should be first relatively estimated with corresponding control, and after that the results between two different cell lines were compared. Next, we measured SK-MES-1 cell viability using the CCK-8 assay. Results showed that SK-MES-1 cell viability was significantly increased when compared to control (P < 0.01, Fig. 5A). In addition, we found that the invasion ability of SK-MES-1 cells was also notably increased after being cultured with the supernatants of A549 cells (P < 0.01, Fig. 5B). Furthermore, apoptosis and proliferation of SK-MES-1 cells were measured after treatment with the highest concentration of cisplatin (16 μmol/L). The apoptosis rate of SK-MES-1 cells was significantly reduced after being cultured with the supernatants of A549 cells (P < 0.01, Fig. 5C). The protein level of caspase-3 was decreased compared to control, whereas Ki67 was markedly increased relative to control (Fig. 5C). Taken together, these observations indicated that proinflammation could promote SK-MES-1 cell invasion and proliferation and reduce the apoptosis rate.

Discussion

In the present study, we demonstrated that in comparison with LUSC, LUAD was more resistant to cisplatin treatment and showed higher inflammatory response after the treatment, which, in turn, promoted tumor cell proliferation and migration. Importantly, we found that proinflammatory mediators could promote tumor cell proliferation and inhibit apoptosis.

Lung cancer is the most prevalent type of cancer worldwide and associated with high mortality rate. Currently, the most commonly used approach for the treatment of NSCLC is chemotherapy. There are two major subtypes of NSCLC: LUAD and LUSC, each contributing to 60 and 25% of NSCLC, respectively (19). NSCLC mainly includes squamous cell carcinoma and adenocarcinoma. It was focuses on the differences in the expression of different inflammatory factors and the correlation between the expression of inflammatory factors and cisplatin sensitivity in these two types of cancers in this study. Cell line A549 of human squamous cell carcinoma and cell line SK-MES-1 of human LUAD are the most common and stable cell lines that can be purchased commercially, such as ATCC. Together above, we chosen them as the cell objects in this study.

In light of cytotoxicity of cisplatin and many other chemotherapeutic agents, there is a large number of patients with NSCLC who do not effectively respond to the treatment. To improve the outcome of chemotherapy, inhibiting chemoresistance and promoting apoptosis are crucial for the treatment of NSCLC. Ning et al. suppressed cisplatin resistance in LUAD tissue by stabilizing p53 through inhibiting MDM2-mediated p53 ubiquitination, resulting in promoted tumor apoptosis (20). With regard to LUSC, a study reported that lncRNA SFTA1P was downregulated when treated with cisplatin, and the overexpression of SFTA1P notably increased tumor cell apoptosis (21). However, differences in the resistance of LUAD and LUSC to chemotherapy remain unclear. In this study, we used two cell lines A549 and SK-MES-1 to represent LUAD and LUSC cells, respectively. We showed that patients with LUAD were more resistant to cisplatin than LUSC. Consistently, our in vitro results showed higher capability of invasion and migration in LUAD cells than LUSC cells after the treatment with cisplatin. As a result, we found that LUAD cells showed significantly lower apoptosis rate and higher proliferation ability than LUSC cells. Herein, our findings support that both LUAD and LUSC are resistant to chemotherapy, while LUAD is more resistant to cisplatin treatment than LUSC.

Fig. 5. Proinflammatory factors promoted the invasion and proliferation and reduced the apoptosis rate of A549. The A549 cells were cultured the supernatant of SK-MES-1 for 48 h and also with the treatment of cisplatin (2, 4, 8, 12, and 16 μmol/L) and were placed on plates. The viability of cells (A), invasion capacity (B), and the apoptosis rate (C) were determined (P < 0.01).
Inflammation has been well-documented to associate with cancer development (22, 23). In our study, we found that the anti-inflammatory cytokine IL-4 was less significantly reduced in LUAD than LUSC in the control group. However, the elevated levels of pro-inflammatory cytokines such as IL-6, IL-8, and TNF-α in LUAD were less than LUSC in the control group after the treatment with cisplatin. These results indicated that LUAD showed higher inflammation response after cisplatin treatment than LUSC. As shown before, less apoptosis rate and higher invasion and proliferation capability were observed in LUAD than LUSC. These findings suggested that inflammation could play an essential role in chemotherapy resistance of LUAD. Currently, the role of inflammation in chemotherapy resistance in cancer remains unclear. Consistent with our results, a recent study revealed a novel mechanism where Wnt/β-catenin and NF-kB pathways were activated by inflammatory cytokines released from breast cancer cells after chemotherapy, thereby facilitating drug-resistance in breast cancer (24). Furthermore, we found that incubating LUSC cells with inflammatory cytokines released by LUAD cells reduced their sensitivity and increased their resistance to cisplatin, leading to reduced apoptosis and increased proliferation and invasion of LUSC. Therefore, these results suggested that LUAD was more resistant to chemotherapy, causing release of inflammatory cytokines, which subsequently increased drug-resistance of LUSC. Ultimately, patients with LUAD and LUSC responded poorly to chemotherapy.

However, the mechanisms of inflammation in chemotherapy for the treatment of cancer are still unclear (25). Studies have demonstrated that drugs that target inflammatory cytokine receptors, such as IL-6 receptor antagonist, successfully inhibit myeloma cell growth by blocking TNF-α expression (26, 27). Correspondingly, a clinical study has shown that treatment with anti-IL-6 receptor antibody could improve treatment outcome of patients with cancer cachexia (28, 29). These reports indicate that anti-inflammatory could be an approach to prevent and treat cancer (30, 31). Moreover, the differences between LUAD and LUSC in responding to chemotherapy physiologically and biologically require further research. One of the limitations of this study is the lack of mechanisms responsible for chemotherapy resistance of LUAD and LUSC. We aim to gain better insight into the differences between LUAD and LUSC in chemotherapy resistance, as well as microenvironmental communications between drug-resistant tumor cells. Insights into the intracellular signaling pathways involved in drug resistance would also be beneficial to develop approaches for prevention and treatment of NSCLC.

In summary, we demonstrated that both LUAD and LUSC were resistant to cisplatin administration, and LUAD was more resistant to cisplatin than LUSC. Consistent with longer survival in patients with LUSC than those with LUAD after the treatment with cisplatin, A549 cells showed stronger proliferation and invasion capability, as well as reduced apoptosis rate, than SK-MES-1 cells. Moreover, levels of pro-inflammatory cytokines, such as IL-6, IL-8, and TNF-α, were reduced by a lesser extent in A549 and LUAD specimens than SK-MES-1 and LUSC specimens after the treatment with cisplatin. In addition, our findings showed that incubating SK-MES-1 cells with inflammatory cytokines further increased their drug resistance. Our data have provided new evidence to treat and manage NSCLC in the clinical setting.

This study suggests that LUAD is more resistant to chemotherapy and shows a stronger inflammation response compared to LUSC. Inflammatory mediators could elevate the resistance of LUSC to chemotherapy and promote tumor cell proliferation, migration, and invasion. Targeting the inflammation tumor microenvironment could serve as a novel approach for the treatment and management of NSCLC.

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Conflict of Interest and funding
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Authors’ contributions
Yange Gong, Hongyan Pang, Zhiqiang Yu, Xue Wang, Ping Li, and Qiyun Zhang performed the experiments, and analyzed and interpreted the data. Yange Gong and Qiyun Zhang were the major contributors in writing the manuscript. All authors read and approved the final manuscript.

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