Expressions of CD44, PCNA and MRP1 in lung cancer tissues and their effects on proliferation and invasion abilities of lung cancer cell line 95D

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Summary

Purpose: To investigate the expressions of CD44 non-small cell lung cancer cells, proliferating cell nuclear antigen (PCNA) and multidrug resistance-associated protein 1 (MRP1) in the lung cancer tissues and their effects on the proliferation and invasion abilities in vitro of lung cancer cell line 95D.

Methods: 138 lung cancer tissues and 127 adjacent normal tissues were collected from lung cancer patients after operation in Shandong Provincial Third Hospital from January 2015 to December 2017. CD44 siRNA (experimental CD44 group), PCNA siRNA (experimental PCNA group) and MRP1 siRNA (experimental MRP1 group) were transfected into human lung cancer 95D cells, and a negative control group (cells transfected with miR-Native Control) and a blank group (untransfected cells) were established. MTT assay was used for detecting the proliferation of cells, and Transwell chamber was used for detecting their invasion ability.

Results: The relative expressions of CD44, PCNA and MRP1 in the lung cancer tissues were higher than those in the adjacent tissues (p<0.050). At 24th h, the cell survival rate in the experimental MRP1 group was lower than that in the experimental PCNA group (p<0.050); At 48th the cell survival rate in the experimental MRP1 group was higher than that in the experimental CD44 group (p<0.050). At 72th h, the cell survival rate in the experimental PCNA group was significantly higher than that in the experimental CD44 group and the experimental MRP1 group (p<0.05). The cell invasion number in the experimental CD44 group, the experimental PCNA group and the experimental MRP1 group were significantly lower than cells in the negative control group and blank group (p<0.05).

Conclusion: CD44, PCNA and MRP1, which may be involved in the regulation of the proliferation and invasion abilities of lung cancer cells, may serve as new molecular targeting markers for the diagnosis and treatment of lung cancer.

Key words: CD44, PCNA, MPP1, lung cancer cell line 95D, proliferation, invasion

Introduction

Lung cancer, a malignant tumor with high incidence, poses a serious threat to human life and health [1,2]. At present, its incidence and mortality rank first among malignant tumors [3,4]. Besides, its incidence and detection rate are increasing rapidly in the world with the change of modern lifestyle and the influence of the environment [5]. Currently, lung cancer is mainly treated by operation, but many patients with early stage still have tumor recurrence or metastasis within 5 years after operation [6,7]. It is difficult to rely on the pathological section and classification of the lung cancer tissues or the TNM staging for the diagnosis, treatment and prognosis of patients due to its complex pathogenesis [8]. In recent years, more and more molecules have been found to play an important role in the regulation of the biological properties of lung cancer cells, such as CD44, PCNA and MRP1.
role in the occurrence and development of lung cancer with the continuous development of molecular biology, which becomes a research hotspot in the medical field [9].

CD44 is a transmembrane glycoprotein mainly expressed in epithelial-derived and tumor cells, which has been reported in the literature to be involved in cell adhesion events, including lymphocyte migration and metastasis, proliferation and invasion of tumor cells [10,11]. Proliferating cell nuclear antigen (PCNA) is a nucleoprotein in cells, which is closely related to the proliferation of tumor cells and can be an indicator for judging diseases according to reports in the literature [12,13]. Multidrug resistance-associated protein (MRP1), a transmembrane glycoprotein that is mainly expressed in cells, in the tissues and the peripheral blood of the human body, is associated with cellular oxidative stress and inflammatory responses. It has been reported in the literature that MRP1 protects the body when carcinogens invade, which leads to drug resistance [14,15]. With the deepening of research, more and more data prove that CD44, PCNA and MRP1 are related to the diagnosis, treatment and prognosis of tumors, and further discussion is expected to provide new ideas and targets for the diagnosis and treatment of lung cancer.

Methods

General information

A retrospective analysis was performed on 138 patients with lung cancer undergoing operation in Shandong Provincial Third Hospital from January 2015 to December 2017, with an average age of 63.14±6.28 years, who agreed with the removal and collection of 138 lung cancer tissues and 127 adjacent normal tissues during the operation. Inclusion criteria: All patients were diagnosed with lung cancer by pathological diagnosis and signed an informed consent form. Exclusion criteria: Patients complicated with other severe organ diseases and tumors were excluded; patients with communication disorders and mental illness; patients who did not cooperate with the study staff. All specimens were stored in a liquid nitrogen container immediately after their excision, and all sample collections were approved by the Ethics Committee of Shandong Provincial Third Hospital.

Experimental reagents and materials

Human lung cancer 95D cells were purchased from the cell bank of Shanghai Institutes for Biological Sciences; real-time quantitative PCR instrument from Bio-Rad, USA; fetal bovine serum (FBS) and 0.25% pancreatin from HyClone, USA; Trizol reagent and qRT-PCR kit from Applied Invitrogen, USA; DMEM medium from Gibco, USA; MTT solution from Sigma, USA; Transwell chamber from Corning, USA; cDNA reverse transcription kit from Invitrogen, USA; Lipofectamine 2000 transfection reagent from Invitrogen, USA. All primers and transfected plasmids were designed and synthesized by Sangon Biotech (Shanghai) Co., Ltd.

Experimental methods

RT-PCR detection of mRNA expressions of CD44, PCNA and MRP1 in the lung cancer tissues and the adjacent tissues

The lung cancer tissues and the adjacent tissues were taken out from the liquid nitrogen container. The total RNA was extracted from CD44 mRNA, PCNA mRNA and MRP1 mRNA using the Trizol reagent, and its purity and concentration were detected by UV spectrophotometer. Each of 5 μg of total RNA was taken to reversely transcribe cDNA according to the kit instructions, with the reaction parameters as follows: at 37°C for 15 min, at 42°C for 42 min and at 70°C for 5 min. The transcribed cDNA was used for PCR amplification with β-actin as an internal reference, and the reaction system was as follows: 5 μl of 2×qPCR Mix, each of 1 μl of upstream and downstream primers, 2 μl of template cDNA, and 1 μl of dH₂O (sterilized distilled water) added to the 10 μl system. The reaction conditions were as follows: pre-denaturation at 94°C for 2 min and then for 40 cycles (denaturation at 95°C for 10 s, annealing at 60°C for 40 s and extension at 72°C for 30 s). The relative expressions of the genes were expressed by 2^-ΔΔCT, and RT-PCR detection was carried out using the PCR instrument, with the experiment repeated 3 times. Primer sequences are shown in Table 1.

Cell culture and transfection

Human lung cancer 95D cells were placed in a DMEM medium containing 10% PBS, and cultured at 37°C, 5%CO₂. CD44 siRNA, PCNA siRNA and MRP1 siRNA were respectively transfected into cells in the logarithmic growth phase, and then the cells were divided into the experimental CD44 group, the experimental PCNA group and the experimental MRP1 group. In addition, cells untransfected were set as the blank group, and cells transfected with miRNA-Negative Control

### Table 1. Related primer sequences

<table>
<thead>
<tr>
<th>Factor</th>
<th>Upstream primer</th>
<th>Downstream primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD44 mRNA</td>
<td>5’-TCCACACCTCCTCCCCAGTATGCA-3’</td>
<td>5’-GGGAGGTCTGTGATCTGTACA-3’</td>
</tr>
<tr>
<td>PCNA mRNA</td>
<td>5’-TGATGAGGTTCTTTAGTG-3’</td>
<td>5’-GAGTGGCTGTTCTCTTCC-3’</td>
</tr>
<tr>
<td>MRP1 mRNA</td>
<td>5’-ATGGACTACACAAGGGTGAT-3’</td>
<td>5’-TTCCACCTCCTTCTCC-3’</td>
</tr>
<tr>
<td>β-actin</td>
<td>5’-CGGAAATCTCGTGATGAC-3’</td>
<td>5’-CAGGAAGGAAGGCTGGAAG-3’</td>
</tr>
</tbody>
</table>
Expression of CD44, PCNA and MRP1 in lung cancer

Transfection was performed in strict accordance with the instructions of the Lipofectamine 2000 transfection kit. Cells in the logarithmic growth phase were inoculated into a 6-well plate at a density of $3 \times 10^5$ cells/well, and Lipofectamine 2000 was mixed with CD44 siRNA, PCNA siRNA, MRP1 siRNA and miR-NC, and then transfected at $37^\circ C$, 5%CO$_2$. After transfection for 24 h, the relative expressions of CD44 mRNA, PCNA mRNA and MRP1 mRNA in 95D cells transfected in the groups were detected using RT-PCR, and the cells were collected for subsequent experiments.

**MTT assay for cell proliferation**

After 48 h of transfection the cells in the groups were inoculated into a 96-well plate, and each well was inoculated with approximately 100 μl of solution with a density of $2 \times 10^4$ cells/ml and a cell number of 200 cells. At 24th, 48th and 72th h, 20 μl of MTT solution was added to each well of cells, respectively. Then, the cells were incubated in the incubator for 4 h, added with 150 μl of dimethyl sulfoxide and shaken for 10 min. The optical density (OD) at 490 nm was measured using a microplate reader to detect cell proliferation, and the experiment was repeated 3 times. The cell survival rate (%) = (OD in the experimental group - OD in the blank group) / (OD in the control group - OD in the blank group).

**Transwell chamber detection of the invasion ability of cells in vitro**

The transfected 95D cells in the groups were added to the Transwell upper chamber at a density of $3 \times 10^4$ cells/well, and then 600 μl of DMEM medium containing 10% FBS was added to the lower chamber and cultured at $37^\circ C$, 5% CO$_2$ for 24 h. The cells in the lower chamber were fixed with a 95% ethanol solution for 15 min, taken out, rinsed with PBS, stained with 0.1% crystal violet, and then rinsed again with PBS until clarification. Finally, the cell invasion numbers of 5 visual fields were randomly selected under the microscope to calculate the average value. The experiment was repeated 3 times.

**Statistics**

SPSS19.1 (Bizinsight (Beijing) Information Technology Co., Ltd.) software package was used for statistical analyses. Measurement data were expressed as mean ± standard deviation, and t-test was used for difference comparison between the two groups, while repeat measurement analysis of variance (ANOVA) was used between the three groups. When p<0.05, the difference was statistically significant.

**Results**

**mRNA expressions of CD44, PCNA and MRP1 in the lung cancer tissues and the adjacent tissues**

The relative expression of CD44 in the lung cancer tissues was higher than that in the adjacent tissues, with a statistically significant difference (t=3.317, p=0.001); that of PCNA in the lung cancer tissues was significantly higher than that in the adjacent tissues, with a statistically significant difference (t=43.410, p<0.001); that of MRP1 in the lung cancer tissues was significantly higher than that in the adjacent tissues, with a statistically significant difference (t=21.700, p<0.001) (Table 2).

**Relative expressions of CD44, PCNA and MRP1 in the groups of cells after transfection**

The expression of CD44 in the experimental CD44 group was significantly lower than that in the negative control group and the blank group, with a statistically significant difference (p<0.05), while there was no significant difference between the negative control group and the blank group (p>0.05). The expression of PCNA in the experimental PCNA group was significantly lower than that in the negative control group and the blank group, with a statistically significant difference.

- **Table 2.** mRNA expressions of CD44, PCNA and MRP1 in the lung cancer tissues and the adjacent tissues

<table>
<thead>
<tr>
<th></th>
<th>Cancer tissues (n=138)</th>
<th>Adjacent tissues (n=127)</th>
<th>t</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD44</td>
<td>3.46±1.53</td>
<td>2.87±1.35</td>
<td>3.317</td>
<td>0.001</td>
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<tr>
<td>PCNA</td>
<td>1.34±0.23</td>
<td>0.58±0.10</td>
<td>43.410</td>
<td>&lt;0.001</td>
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<tr>
<td>MRP1</td>
<td>0.91±0.18</td>
<td>0.54±0.07</td>
<td>21.700</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

- **Table 3.** Relative expressions of CD44, PCNA and MRP1 in the groups of cells after transfection

<table>
<thead>
<tr>
<th></th>
<th>Experimental group</th>
<th>Negative control group</th>
<th>Blank group</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD44</td>
<td>2.08±1.29*</td>
<td>3.46±1.57</td>
<td>3.51±1.68</td>
<td>59.230</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>PCNA</td>
<td>0.47±0.16*</td>
<td>1.36±0.51</td>
<td>1.58±0.27</td>
<td>574.600</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>MRP1</td>
<td>0.53±0.26*</td>
<td>0.93±0.62</td>
<td>0.91±0.68</td>
<td>23.000</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* indicates that compared to the blank group and the negative control group, p<0.05.
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(p<0.050), while there was no significant difference between the negative control group and the blank group (p>0.05). The expression of MRP1 in the experimental MRP1 group was significantly lower than that in the negative control group and the blank group, with a statistically significant difference (p<0.05), while there was no significant difference between the negative control group and the blank group (p>0.05) (Table 3).

Comparison of the proliferation ability of 95D cells in the groups after transfection

Comparison between the groups was performed. The cell survival rate was compared between the experimental CD44 group, the experimental PCNA group, the experimental MRP1 group, the negative control group and the blank group. At the 24th h, the cell survival rate in the experimental MRP1 group was lower than that in the experimental PCNA group, while that in the experimental PCNA group was higher than that in the negative control group and the blank group, with statistically significant differences (p<0.05); there was no difference in the cell survival rate between the experimental CD44 group and the experimental MRP1 group, and between the experimental CD44 group and the negative control group, the blank group (p>0.05). At the 48th h, the cell survival rate in the experimental MRP1 group was higher than that in the experimental CD44 group, while that in the three experimental groups was lower than that in the negative control group and the blank group, with statistically significant differences (p<0.05); there was no difference in the cell survival rate between the experimental CD44 group and the experimental PCNA group, and between the experimental PCNA group and the experimental MRP1 group (p>0.05). At the 72th h, the cell survival rate in the experimental PCNA group was higher than that in the experimental CD44 group and the experimental MRP1 group, while that in the three experimental groups was lower than that in the negative control group and the blank group, with statistically significant differences (p<0.05); there was no difference in the cell survival rate between

Table 4. Comparison of cell survival rate (%) in the groups

<table>
<thead>
<tr>
<th>Group</th>
<th>F</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental CD44 group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experimental PCNA group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Experimental MRP1 group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative control group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blank group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24h</td>
<td>3.191</td>
<td>0.013</td>
</tr>
<tr>
<td>48h</td>
<td>1481.000</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>72h</td>
<td>3568.000</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>F</td>
<td>2063.000</td>
<td>0.007</td>
</tr>
<tr>
<td>p</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

* indicates that compared to the blank group and the negative control group, p<0.05; * indicates that compared to the experimental CD44 group, p<0.05; ^ indicates that compared to the experimental PCNA group, p<0.05; & indicates that compared to the 24th hour, p<0.05; @ indicates that compared to the 48th hour, p<0.05.

Figure 1. Comparison of the proliferation ability of 95D cells in the groups after transfection. * indicates that compared to the blank group and the negative control group, p<0.05; * indicates that compared to the experimental CD44 group, p<0.05; * indicates that compared to the experimental PCNA group, p<0.05; * indicates that compared to the 24th hour, p<0.05; * indicates that compared to the 48th hour, p<0.05.
the experimental CD44 group and the experimental MRP1 group (p>0.05).

Comparison within the groups was performed. From the 24th to 72th h, the cell survival rate in the experimental CD44 group, the experimental PCNA group and the experimental MRP1 group showed a gradual decline, and the differences were statistically significant within the three groups at different time points (p<0.001); that in the negative control group and the blank group also showed a gradual decline, but the differences were not statistically significant (p>0.05) (Table 4, Figure 1).

Comparison of the invasion ability of 95D cells in the groups after transfection

The cell invasion number in the experimental CD44 group, the experimental PCNA group and the experimental MRP1 group were 197.42±22.05 cells, 200.04±21.51 cells and 198.19±22.26 cells, respectively, which were significantly lower than 398.84±46.32 cells in the negative control group and 400.21±45.97 cells in the blank group (p<0.05); but there was no significant difference between the experimental CD44 group, the experimental PCNA group, the experimental MRP1 group, the blank group and the negative control group (p>0.05) (Table 5, Figure 2).

Discussion

Lung cancer, a common malignant tumor, has less obvious symptoms in the early stage and rapid progression, which leads to the current lack of corresponding means for its diagnosis and treatment [16]. Lung cancer has a complex pathogenesis related to the environment, the activation of oncogenes and the inactivation of tumor suppressor genes, but there is no detailed report on its specific mechanism [17]. Gene therapy is a key research issue for the treatment of tumors in recent years, while no effective correlation factor for the treatment and diagnosis of lung cancer has been found currently. Therefore, finding an effective factor for the treatment and diagnosis of lung cancer is also a problem that needs to be solved urgently in clinical practice [18]. In this study, 138 lung cancer patients undergoing operation in Shandong Provincial Third Hospital were analyzed. The mRNA expression levels of CD44, PCNA and MRP1 in 138 lung cancer tissues and 127 adjacent normal tissues were detected using RT-PCR, and the effects of CD44, PCNA and MRP1 on the proliferation and invasion abilities of 95D NSCLC cells were detected, in order to provide a reference for the clinical treatment of lung cancer.

In this study, the relative expressions of CD44, PCNA, and MRP1 in the lung cancer tissues were higher than those in the adjacent tissues, with sta-
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Statistically significant differences. Then, the effects of CD44, PCNA and MRP1 on the proliferation and invasion abilities of lung cancer 95D cells were analyzed. The results suggest that the interference with the expressions of CD44 mRNA, PCNA mRNA and MRP1 mRNA will reduce the survival rate and cell invasion number of lung cancer 95D cells, and inhibit the proliferation and their invasion abilities to some extent. CD44 interacts with a variety of transcription factors to specifically regulate the multiple differentiation and developmental functions of cells or tissues. A study also shows that it plays a key role in promoting tumor cell differentiation and morphogenesis in lung cancer [19]. According to a study by Yasuda et al [20], CD44 is highly expressed in lung cancer and speculated to play an important role in lung cancer cells. Studies have explored the mechanism of action of CD44 on tumor cells, and found that CD44 inhibits cell proliferation and metastasis by regulating Rho A and c-Jun signaling pathways, which is consistent with our conclusions [21,22]. PCNA is a nucleoprotein abundantly expressed in the S phase, the positive detection rate of which is 86% in lung cancer according to Chen et al [23]. There are a large number of reports in the literature that PCNA is involved in the occurrence and development of gastric cancer, lung cancer and other malignant tumors, the level changes of which are consistent with the cell proliferation, as an indicator for measuring the grade of tumor differentiation, and cell proliferation and invasion abilities [24-26]. MRP1 causes multidrug resistance by promoting the excretion of glutathione-binding drugs from cells. Advanced lung cancer is mainly treated by chemotherapy, and the overexpression of membrane transport protein is one of the main factors of lung cancer cell resistance [27,28]. Therefore, the effect of the MRP1 expression on lung cancer was explored in this study. There is a report in the literature that the expression level of MRP1 mRNA in the lung cancer tissues is significantly higher than that in the normal lung tissues [29]. However, there are few related literature reports on the effect of MRP1 on the proliferation and invasion abilities of lung cancer cells, so this study is more innovative and research-worthy, and more in-depth research can be carried out in the follow-up. According to Huang et al [30], MRP1 plays an important role in the invasion and proliferation of human fibrosarcoma cell line HT-1080.

There are still certain limitations in this study. For example, the number of patients included is not enough to be a big data statistics. In addition, the correlation of CD44, PCNA and MRP1 is not analyzed. The subject number and research directions will continue to be increased, and the results will be tested and analyzed in order to improve the research.

Conclusion

In summary, CD44, PCNA and MRP1 are highly expressed in the lung cancer, and the interference with their mRNA overexpression inhibits the proliferation and invasion of lung cancer 95D cells. This experiment believes that CD44, PCNA and MRP1, which are involved in the biological process of lung cancer cells, can serve as diagnostic markers and treatment targets for lung cancer.

Conflict of interests

The authors declare no conflict of interests.

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