Studies on the relationship between P13K/AKT signal pathway-mediated MMP-9 gene and lung cancer

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Abstract. – OBJECTIVE: The present study has been planned to explore the relationship between P13K/AKT signal pathway-mediated matrix metalloproteinase-9 (MMP-9) gene and lung cancer.

PATIENTS AND METHODS: The adjacent lesion tissues were removed from 68 cases of patients with lung cancer admitted to our hospital from February 2013 to February 2015. They were selected as observation group, and the pulmonary adjacent normal section specimens from 18 cases were taken as control group. The differences in expression quantities of P13K, AKT and MMP-9 genes in adjacent lesion tissues and adjacent normal tissues were detected by fluorescence quantitative PCR technology, enzyme-linked immunosorbent assay (ELISA), Western-blotting technology and immunohistochemical technique and SP staining technology.

RESULTS: The mRNA expression quantities of P13K, AKT and MMP-9 genes in adjacent lesion tissues increased significantly in comparison to adjacent normal tissues, and the differences were statistically significant (p<0.05). The results of Spearman correlation detection showed that the expression quantity of MMP-9 in adjacent lesion tissues had a significantly positive correlation with that of P13K and AKT (r=0.232, p<0.05).

CONCLUSIONS: MMP-9 gene could promote the generation of lung cancer through P13K/AKT signal pathway, which provides certain theoretical and experimental basis for subsequent diagnosis and treatment of lung cancer.

Key Words:
P13K/AKT signal pathway, MMP-9, Lung cancer.

Introduction

Cancer has exceeded cardiovascular and cerebrovascular diseases and is the major killer of human lives at present, as shown in the statistical results of American Cancer Society¹ in 2014. Moreover, the statistical results of another recent report² showed that lung cancer became one of the common malignant tumors and cancers all over the world. Moreover, cancer has been ranked at the first place of the morbidity and fatality due to lack of specific diagnosis and treatment drugs³. Further, deteriorating living environment, also contributing towards its continuously elevating morbidity year by year. Therefore, accelerating the studies on the pathogenesis of lung cancer to provide a good theoretical basis for the diagnosis and treatment of lung cancer has become an important subject of studies on lung cancer these days. In recent years, many studies⁴,⁵ showed that P13K/AKT (phosphoinositide 3-kinases/ serine-threonine kinase) signal pathway could take part in many signal transduction pathways, among which P13K could phosphorylate phosphatidylinositol to generate inositol lipids with the function of the second messenger in the human body⁶. On the other hand, AKT could take part in many physiological processes of the organism, including cell cycle control and cell apoptosis process initiation, through the interaction with P13K signal pathway in the human body⁷. In recent years, as the studies on P13K/AKT signal pathway strengthened, it was noticed that there was a certain correlation between the changes of P13K/AKT signal transduction pathway leading to the occurrence and deterioration of some malignant tumors in the human body. For instance, migration of human tracheal epithelial cell activated by AKT caused the loss of its contact inhibition function. However, AKT activity could be inhibited through P13K specific inhibition⁸. Moreover, matrix metalloproteinases (MMPs) as a zinc ion-dependent extracellular proteinase in the human body, could take part in the physiological processes, including the migration of tumor cells⁹. It could also act as a type IV collagenase, so as to promote the migration of some tumor cells.

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However, there are fewer reports on the correlation between PI3K/AKT signal pathway-mediated MMP-9 and lung cancer. In the present study, we tried to explore the correlation between PI3K/AKT signal pathway-mediated MMP-9 and lung cancer, hoping to provide certain theoretical and experimental basis for subsequent in-depth studies.

Patients and Methods

Materials

The lung cancer tissues excised from 68 cases of patients with lung cancer admitted to our hospital from February 2013 to February 2015 were selected as observation group. It included 38 males and 30 females, with the average age of (37.5±8.6) years old. The pulmonary adjacent normal section specimens from 18 cases were taken as the control group, including 12 males and 6 females, with the average age of (36.3±9.2) years old. All above research objects were primary lung cancer cases and passed pathological diagnosis after operation (WHO/UICC special diagnosis criteria for lung cancer in 2011). There were no significant differences between the control group and the observation group in gender and age ($X^2=1.763$, $p=0.328$; $t=0.243$, $p=0.915$). Experiment specimens obtained through operation should be stored in liquid nitrogen for the subsequent experiment.

Methods

The molecular reagents, RT-PCR kits, and ELISA determination kits were purchased from TaKaRa (Dalian, China). Animal protein extraction kits were purchased from AXYGEN (Suzhou, China). SP kits were purchased from Tiangen Biotech Co., Ltd. (Beijing, China). The rest molecular reagents were purchased from Shanghai Biotech Co., Ltd. (Shanghai, China).

RT-PCR

RNA extraction: (1) About 0.2 g experimental sample was taken from -80°C refrigerator and immediately placed into the prepared ice box for melting. After most of it was melted, 0.45 ml RNA Plus was added. Subsequently, the experimental sample was rapidly absorbed back and forth with a pipette. (2) After being centrifuged at 12,000 rpm and 4°C for 15 min, supernatant was discarded. 200 ml chloroform was added into sediment, which was sharply absorbed back and forth with pipette or shaken with hand for 15s and then conducted by still standing on ice for 15 min. (3) Centrifugation was conducted at 12,000 rpm and 4°C for 15 min. (4) Supernatant was absorbed slightly with pipette and transferred into EP tube without RNase. After commensurable isopropanol had been added, it was bottomed up to mix well and then conducted by still standing on ice for 10 min. (5) Centrifugation was conducted at 12,000 rpm and 4°C for 5 min. (6) Supernatant was discarded, and 750 ml 75% ethyl alcohol was added along tube wall, mixing slightly. Centrifugation was conducted at 12,000 rpm and 4°C for 10 min. (7) Supernatant was discarded, and the residual ethyl alcohol was removed as much as possible (or placed at room temperature for 15-20 min). (8) Appropriate water without RNase was added to measure the quality of extracted RNA, and the rest was used for reverse transcription.

Fluorescent quantization: Operation was conducted according to the instructions of TaKaRa fluorogenic quantitative PCR, with slight improvement. The fluorogenic quantitative PCR primers were compounded by Shanghai Bioengineering Technology Co., Ltd. See Table I for sequences.

ELISA

The measurement of PI3K, AKT, and MMP-9 proteins in different samples in control group and observation group was conducted according to instructions of ELISA kit (TaKaRa Corporation, Otsu, Shiga, Japan), with modifications. The standard protein sample used in this study was diluted with dilution buffer according to the ratio of 1:50, based on which standard curve was designed. After samples to be tested were diluted with phosphate buffered saline (PBS, 7.2 pH) according to the ratio of 1:1000, 100 ml samples to be tested were added to each well of 96-well plates. Subsequently, 50 ml test liquid A was added. After the 96-well plates were placed at room temperature for 2h, tetramethylbenzidine (TMB) chromogenic substrate was added, and the light

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>PI3K-F</td>
<td>ATGAGCAGAAGCAAGGCAGCTGAC</td>
</tr>
<tr>
<td>PI3K-R</td>
<td>CTGGGCTTTAAGTCCCGGATG</td>
</tr>
<tr>
<td>AKT-F</td>
<td>GTGCTAGTCTAGTCGATCATGAT</td>
</tr>
<tr>
<td>AKT-R</td>
<td>TGCTAGTCTAGTGCTGATGCTAC</td>
</tr>
<tr>
<td>MMP-9-F</td>
<td>GCTAAGCTAGTCTAGTCTAGTAC</td>
</tr>
<tr>
<td>MMP-9-R</td>
<td>TGCTAGTCTAGTCTAGTCTAGTAC</td>
</tr>
<tr>
<td>GAPDH-F</td>
<td>GTGCTAGTGTGCACTCATGATGCTAC</td>
</tr>
<tr>
<td>GAPDH-R</td>
<td>TGCTAGTGGCATGGCGCATGATC</td>
</tr>
</tbody>
</table>
absorption value was tested at 495 nm. Then, the content and concentration of PI3K, AKT and MMP-9 proteins in all samples to be tested were calculated according to the standard curve.

**Western-Blotting**

Experimental samples stored in -80°C refrigerator was taken out and melt on ice for the extraction of total protein. About 150 mg experimental tissue sample was taken from -80°C refrigerator and then grinded rapidly in the pre-cooling mortar on the ice. After the completion of grinding, it was transferred into 1.5 mL Eppendorf (EP) tube rapidly, added with 300 ml protein extraction solution A and 5 ml protease inhibitor B. After being kept warm at 37°C for 30 min, centrifugation was conducted at 12,000 rpm for 10 min, supernatant was slightly absorbed with pipette and put into new 1.5 ml EP tube. Target protein was determined through Western-blot. 10 ml supernatant and loading buffer A were absorbed and mixed well to conduct sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) electrophoresis and transmembrane by semi-dry turn method. After being sealed at room temperature for 1h, 5% skim milk powder was incubated with anti-PI3K, AKT and MMP-9 antibodies (1:1 000, 4°C overnight) and second antibody marked with horseradish peroxidase (1:1 000, shaking at room temperature for 1h). After Tris buffered saline-tween (TBS-T) washing were conducted with anhydrous alcohol and elution was conducted with hematoxylin for 5 min and flush with distilled water. Then counterstaining was conducted with hematoxylin for 5 min and flushing was conducted again. Dewatering and drying were conducted with anhydrous alcohol and sections were sealed with neutral balsam.

Identification of results: The positive results of experimental sections passed through immunohistochemistry process were as follows: in case yellow granules occurred in cytomembrane or cytoplasm, namely the negative results were membrane staining <10% or tumor cells after staining were negative, it could be judged as negative. In the study, the quantitative decision was conducted for the results through LI index. KI index referred to the number of positive cells in each field[1].

**Experiment on Blocking PI3K/AKT Signal Pathway**

In this study, we detected the expression quantities of PI3K, AKT, and MMP-9 genes before and after adding PI3K/AKT signal pathway blocking agent LY294002 into cultured lung cancer tissue cells (specific methods refer to[2]).

**Statistical Analysis**

The obtained experimental data were processed by SPSS 20.0 statistical software (SPSS Inc., Chicago, IL, USA). In this study, the experimental data were all expressed by mean ± standard deviation (x±s). Single factor analysis method was applied to implement data analysis among different groups. p<0.05 indicated that there was a significant difference.
Results

**The mRNA Expression Quantities of P13K, AKT, and MMP-9 in Control Group and Observation Group**

The mRNA was extracted from adjacent lesion tissues and adjacent normal lung tissues. On this basis, the relative mRNA expression quantities of P13K, AKT, and MMP-9 were determined by fluorogenic quantitative PCR method (Figure 1). The results showed that the mRNA contents of P13K, AKT, and MMP-9 genes in the specimens of adjacent lesion tissues were higher and there were significant differences between them ($p < 0.05$) in comparison adjacent normal lung tissues. Moreover, there was a certain correlation between P13K, AKT, and MMP-9 genes and lung cancer.

**Protein Expression Quantities of P13K, AKT, and MMP-9 in Control Group and Observation Group Detected by ELISA Method**

Protein expression quantities of P13K, AKT, and MMP-9 in adjacent lesion tissues and adjacent normal lung tissues were determined by ELISA method, and the results were shown in Table II. The data in Table II showed that the protein contents of P13K, AKT and MMP-9 in adjacent lesion tissues were higher than those in adjacent normal lung tissues ($p<0.05$), which indicated that there was a certain correlation between the protein contents of P13K, AKT and MMP-9 and lung cancer.

**Protein Expression Quantities of P13K, AKT, and MMP-9 in Control Group and Observation Group Detected by Western-blotting Method**

The protein expression quantities of P13K, AKT, and MMP-9 genes in adjacent normal lung tissues and adjacent lesion tissues were determined by Western-blotting method, and the results were shown in Figure 2. Figure 2 showed that there were significant differences in the protein contents of P13K, AKT, and MMP-9 between adjacent normal lung tissues and adjacent lesion tissues, namely the protein contents of P13K, AKT and MMP-9 in adjacent lesion tissues were significantly higher than those in adjacent normal lung tissues. This result was consistent with that of ELISA.

**Immunohistochemical Results of MMP-9 Protein in Control Group and Observation Group**

The immunohistochemistry detection for MMP-9 protein in adjacent lesion tissues and adjacent normal lung tissues showed that adjacent normal lung tissues had less MMP-9 positive cells (namely, there were less dark brown cells after cell staining), while the adjacent lesion tissues had more MMP-9 positive cells. There were significant differences between them ($p<0.01$). Moreover, the counting results of MMP-9 positive cells in adjacent lesion tissues and adjacent normal lung tissues showed that the number of MMP-9 positive cells in adjacent normal lung tissues was more than that in adjacent lesion tissues ($p<0.01$) (Table III). This result was consistent with that of ELISA and Western-blotting.

**P13K/AKT Specific Inhibitor LY294002 Blocking Experiment**

The determination of expression quantities of P13K, AKT and MMP-9 genes before and after adding blocking agent LY294002 into lung cancer cells showed that (Figure 4) the expression quan-

![Relative expression quantity](image)

**Table II.** Protein expression quantities of P13K, AKT and MMP-9 in adjacent lesion tissues and adjacent normal lung tissues.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Adjacent lesion tissues (μg/l)</th>
<th>Adjacent normal lung tissues (μg/l)</th>
<th>$p$-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>P13K</td>
<td>0.15</td>
<td>0.67</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>AKT</td>
<td>0.23</td>
<td>0.92</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>MMP-9</td>
<td>0.47</td>
<td>4.32</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

Note: $p<0.05$ indicated that there was a significant difference.
tivities of P13K, AKT and MMP-9 genes in lung cancer cells after adding blocking agent were significantly decreased by comparing with those before adding a blocking agent. It indicated that the blocking of P13K/AKT signal pathway could lower the content of MMP-9 protein in lung cancer cells.

**Correlation Between MMP-9 and Lung Cancer**

The detection of correlation between the number of MMP-9 positive cells in adjacent lesion tissues and adjacent normal lung tissues showed that most of the MMP-9 positive cells were lung cancer cells (87.2%). The results of Spearman correlation detection showed that there was a positive correlation between MMP-9 and lung cancer ($r=0.232$, $p<0.05$), which confirmed that MMP-9 gene took part in the occurrence and evolution processes of lung cancer.

**Discussion**

Among many features of the malignant tumor, invasion and metastasis are considered to be the main factors responsible for the recurrence after treatment\(^1\). The earlier study results showed that as a kind of important intercellular substance protease in the human body, the family of matrix metalloproteinases (MMPs) played a very important role in the process of body inflammation, wound healing, regeneration of injured vessels as well as the replication and transfer of tumor cells\(^14,15\). According to the differences of structure and properties of MMPs metabolic substrates, most of the MMPs in the human body can be divided into four main types, namely gelatinases, collagenase, stromatolysis, and model MMPs\(^16\).

A recent study\(^17\) also showed that MMPs usually

<table>
<thead>
<tr>
<th>Group</th>
<th>Total cellular score (n)</th>
<th>Number of MMP-9 positive cells</th>
<th>Number of MMP-9 negative cells</th>
<th>KI value (%)</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adjacent lesion tissues</td>
<td>600</td>
<td>53</td>
<td>547</td>
<td>8.83</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Adjacent normal lung tissues</td>
<td>600</td>
<td>523</td>
<td>77</td>
<td>87.2</td>
<td></td>
</tr>
</tbody>
</table>

Note: $p<0.05$ indicated that there was a significant difference.

**Figure 2.** Protein expression quantities of P13K, AKT, and MMP-9 in the control group and the observation group. A, Lung cancer tissues sample; B, Adjacent normal tissue sample.
existed in inactive status in the human body and it could be activated to degrade the intercellular special substances only under certain conditions. Since MMPs could degrade the intercellular adhesive substances, it could reduce the adhesive characters between cells to a large extent so as to promote the transfer of cells in human body, which was characterized by invasion and metastasis of tumor cells in the tumor cells.

With the deepening investigation on signal transduction pathway in recent years, it was found that P13K/AKT signal pathway could participate in many signal transduction processes in the human body. It was also proved that the activation of P13K/AKT signal pathway could participate in the processes of apoptosis and migration of tissue cells and tumor cells. For instance, the utilization of specific inhibitor LY294002 and Wortmannin of P13K/AKT signal pathway could reduce the intracellular SKT content to promote the apoptosis of tumor cells. Study results showed that P13K/AKT signal pathway could regulate the migration of tumor cells through many genes in the human body and there is a paucity of information on the correlation between P13K/AKT signal pathway-medi- ed MMP-9 and lung cancer.

Conclusions

In the present study, through the detection of expression quantity of P13K/AKT signal pathway gene and MMP-9 gene in lung cancer tissues and adjacent normal lung tissues, we found that the expression quantity of P13K/AKT signal pathway and MMP-9 gene in lung cancer tissues increased significantly by comparing with adjacent normal lung tissues. However, the expression quantity of MMP-9 protein decreased significantly after adding P13K/AKT signal pathway inhibitor, which indicated that the MMP-9 gene could participate in the occurrence and deterioration processes of lung cancer of organism through P13K/AKT to some extent.

Conflict of interest

The authors declare no conflicts of interest.

References


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