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

H. ZHENG[1], J.-F. LIU[2]

[1]Department of Ultrasonography, Hebei Chest Hospital, Shijiazhuang, China
[2]Department of Thoracic Surgery, the First Hospital of Hebei Medical University, Shijiazhuang, China

**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 health, as shown in the statistical results of American Cancer Society[1] in 2014. Moreover, the statistical results of another recent report[2] showed that lung cancer became one of the common malignant tumors and cancers all over the world. Moreover, it has been ranked at the first place of the mortality and fatality due to lack of specific diagnosis and treatment drugs[3]. Furthermore, deteriorating living environment, also contributing towards its continuously elevating morbidity year by year. Therefore, accelerating the study on the pathogenesis of lung cancer to provide theoretical basis for the diagnosis and treatment of lung cancer has become an important subject of studies on lung cancer these days. Moreover, many studies[4,5] 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 body[6]. 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 body[7]. 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 inhibition[8]. 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 cells[9]. It could also act as a type IV collagenase, so as to promote the migration of some tumor cells.



*Corresponding Author:* JinFeng Liu, MM; e-mail: liu_jinfeng111@163.com

However, there are fewer reports on the correlation between P13K/AKT signal pathway-mediated MMP-9 and lung cancer. In the present study, we tried to explore the correlation between P13K/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.[illegible] $t$=0.243, $p$=0.915). Experiment specimens obtained through operation should be stored in liquid nitrogen for the subsequent exp[illegible]t.

### Methods

The molecular reagents, RT[illegible]R kits, [illegible]ELISA determination kits were p[illegible]sed [illegible]KaRa (Dalian, China). An[illegible]al pr[illegible]xtraction kits were purchased fr[illegible]AXYGE[illegible]zhou, China). SP kits were [illegible]rchased from [illegible]en Biotech Co., Ltd. (B[illegible]China). The rest molecular reagents w[illegible]pu[illegible]d from Shanghai Biotech Co., Ltd. ([illegible]anghai,

### RT-PCR

RNA ext[illegible]ion: (1) About 0.2 g experimental sample w[illegible]taken f[illegible]n -80°C refrigerator and immedi[illegible]lace[illegible]nto the [illegible]pared ice box for melting. A[illegible]of it wa[illegible]elts, 0.45 ml RNA Plus was adde[illegible]equ[illegible]y, the experimental sa[illegible]rapidly [illegible]d back and forth with [illegible]pette[illegible]) After be[illegible]g centrifuged at 12,000 [illegible]m and 4[illegible] for 15 min, supernatant was discard-[illegible]200 [illegible]n was added into sediment, [illegible]was sharply absorbed back and forth with pip[illegible]shaken with hand for 15s and then conducted by still standing on ice for 15[illegible] (3) trifugation was conducted at 12,00[illegible]pm and 4 for 15 min. (4) Supernatant was [illegible]orbed slightly with pipette and transferred i[illegible]EP tube without RNase. After commensura[illegible]opropanol had been added, it was bo[illegible]omed up[illegible]ix wel and then conducted by st[illegible]tanding on [illegible] min. (5) Centrifugation[illegible]as conducted at [illegible]0 rpm and 4°C for 5 m[illegible](6) Sup[illegible]atant was discarded, and 750 ml[illegible]ethyl [illegible]ohol w[illegible]added along tube wall, mix[illegible]y. Cent[illegible]gation was conducted [illegible]2,000 [illegible]d 4°C [illegible]10 min. (7) Supernata[illegible]was discard[illegible]e residual ethyl alcoho[illegible]s removed as [illegible]as possible (or placed [illegible]mperature for 15-20 min). (8) Appropr[illegible]wate[illegible]ut RNase was added to measure the quality [illegible]racted RNA, and the rest [illegible]used for reverse[illegible]scription.

[illegible]orescent quantization. Operation was con-d[illegible]d according to the instructions of TaKaRa f[illegible]ogenic quantitat[illegible]e PCR, with slight improve-m[illegible]. The fluoroge[illegible]quantitative PCR primers w[illegible]ompounde[illegible]y Shanghai Bioengineering Tec[illegible]d. See Table I for sequences.

### ELISA

[illegible]rement of P13K, AKT, and MMP-[illegible]protei[illegible]n different samples in control group [illegible]nd observation group was conducted according to instructions of ELISA kit (TaKaRa Corporation, Otsu, Shiga, Japan), with modifications[10]. [illegible]he standard protein sample used in this study [illegible]s diluted with dilution buffer according to the [illegible]atio 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 I.** Fluorogenic quantitative PCR primers.

| Primer name | Sequences |
|---|---|
| P13K-F | ATGAGCAGAAGCAAGCCGTGAC |
| P13K-R | CTGGGCTTTAAGTCCCGATG |
| AKT-F | GTGCTAGCTAGTCGATCGAT |
| AKT-R | TGCTAGCTAGTCGATAGCTAC |
| MMP-9-F | GCTAGCTAGCTAGTAGCTAC |
| MMP-9-R | TGCTAGCTAGCTAGAGTCGATCG |
| GAPDH-F | GTCGATGGCTAGTCGTAGCATCGAT |
| GAPDH-R | TGCTAGCTGGCATGCCCGATCGATC |

absorption value was tested at 495 nm. Then, the content and concentration of P13K, 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 proteinase 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-P13K, AKT, 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, membrane for 3 times, coloration was conducted with diaminobenzidine and imaging was taken by Fluorchem 9900 imaging system. The integral optical densities of all protein colloration strips were determined to calculate the relative content of protein of P13K, AKT, and MMP-9.

### Immunohistochemistry

Steps of immunohistochemistry: Detection was conducted by applying immunohistochemistry SP-connection, and the specific experimental steps were as follows: (1) Lung specimens of the control group and experiment group were immobilized with 10% formaldehyde and embedded in paraffin. During the test, the thickness of paraffin sections is about 4 μm; the prepared experiment sections were attached on a glass slide to be baked at 60°C for about 1h. (2) De-waxing processes conducted through the prepared xylene and elution was conducted with anhydrous alcohol. After elution, the residual ethyl alcohol was washed with ultrapure water. Finally, washing was conducted with phosphate buffered saline (PBS, 7.2 pH) (for 5 times, 5 min for each). After being heated in the steaming pot at 121°C for 2 min and cooled, it was placed in PBS solution at room temperature for 5 min. (3) After PBS solution had dried up, 3% peroxidase blocker was added to the above sections. After being placed at 37°C for about 10 min, washing was conducted with PBS for 5 times, 3 min each). After PBS solution had been removed, 5 ml non-immune serum was added to incubate at room temperature for 10 min. (4) Primary antibody was added to the sections which were placed to incubate from room temperature for about 2h (or at 4°C overnight) and washed with PBS (for 5 times, 5 min for each time). About 50 ml streptomycin peroxidase was added into the above sections, which were incubated at 37°C for 2h and washed with PBS for 5 times, 5 min for each time). 100 ml coloring agent A was added and observation was conducted under a microscope. (7) 10 min later, flushing was conducted with distilled water. Then counterstaining was conducted with hematoxylin for 5 min and flushing conducted again. Dewatering and drying with anhydrous alcohol and sections sealed with neutral balsam.

Identification of results: The positive results of pulmonal 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[11].

### Experiment on Blocking P13K/AKT Signal Pathway

In this study, we detected the expression quantities of P13K, AKT, and MMP-9 genes before and after adding P13K/AKT signal pathway blocking agent LY294002 into cultured lung cancer tissue cells (specific methods refer to[12]).

### 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.

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

| Gene | Adjacent lesion tissues (μg/l) | Adjacent normal lung tissues (μg/l) | *p*-value |
|---|---|---|---|
| P13K | 0.15 | 0.67 | <0.05 |
| AKT | 0.23 | 0.92 | <0.05 |
| MMP-9 | 0.47 | 4.32 | <0.05 |

Note: $p<0.05$ 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 [illegible] there were significant differences between [illegible] ($p<0.05$) in comparison adjacent normal [illegible] tissues. Moreover, there was a certain correlation between P13K, AKT, and MMP-9 genes and lung cancer.

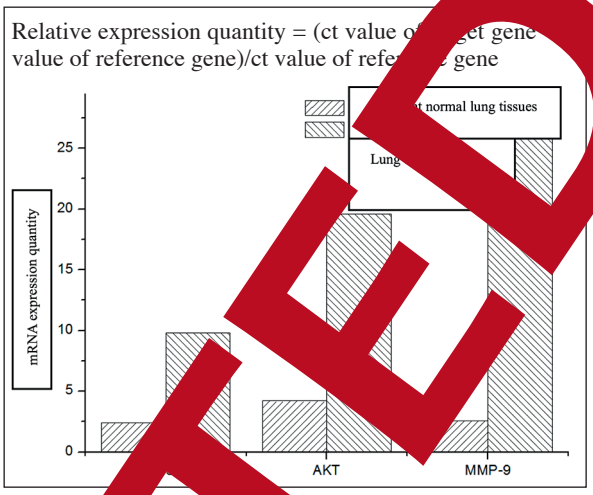



**Figure 1.** The mRNA expression quantities of P13K, AKT, and MMP-9 in control group and observation group.

### 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 expressions 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-

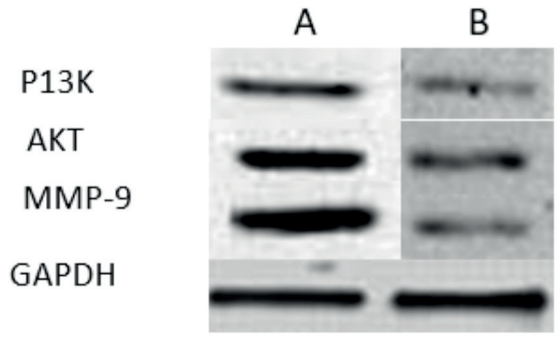



**Figure 2.** Protein expression quantities of P13K, AKT, and MMP-9 in the control group and the observation group. ***A,*** Lung cancer tissues sample; ***B,*** Adjacent normal tissue sample.

tities 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 n- ber of MMP-9 positive cells in adjacent le tissues and adjacent normal lung ti s sh that most of the MMP-9 positive s were lun cancer cells (87.2%). The resu of Spearman correlation detection showed t re was a positive correlation between MMP- ng cancer (r=0.232, $p$<0.05), which c nfirmed MMP- gene took part in the o rence and processes of lung canc

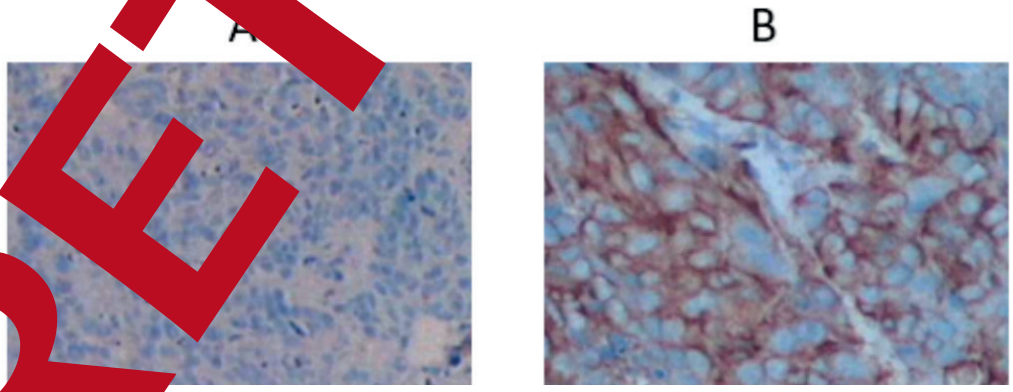
## Di

Among ma eatures of t ant tumor, invasion an tastasis are con ed to be the main facto ible for the recurrence after treatmen The e study results showed that as a kin of impor tercellular substance prot In the human b he family of matrix m oproteinases (MMPs) played a very im- p nt role in the process of body inflammation, w nd healing, reg eration of injured vessels a ell as the repl ion and transfer of tumor ce [5]. Accordi the differences of structure and MMPs metabolic substrates, most of MPs in the human body can be di- ided into four main types, namely gelatinases, stromatolysis, and model MMPs[16]. recen udy[17] also showed that MMPs usually

**Table III.** Statistical results of positive cell count of CD4 n normal ovarian tissues, ovarian benign tumor and ovarian cancer tissues.

| Group | Total ular sc (n) | Number o -9 positive lls | Number of MMP-9 negative cells | KI value (%) | *p*-value |
|---|---|---|---|---|---|
| Adjacent lesion tissues | 0 | 55 | 547 | 8.83 | <0.05 |
| Adjacent normal lung tissues | 600 | 523 | 77 | 87.2 | |

Note: $p$<0.05 indicated that t was a significan ce.




**F 3.** Immunohistochemical staining of MMP-9 in adjacent lesion tissues and adjacent normal lung tissues. ***A,*** Adjacent normal l es. ***B,*** Adjacent lesion tissues (magnification: × 400). Note: $p$<0.05 indicated that there was significant difference.

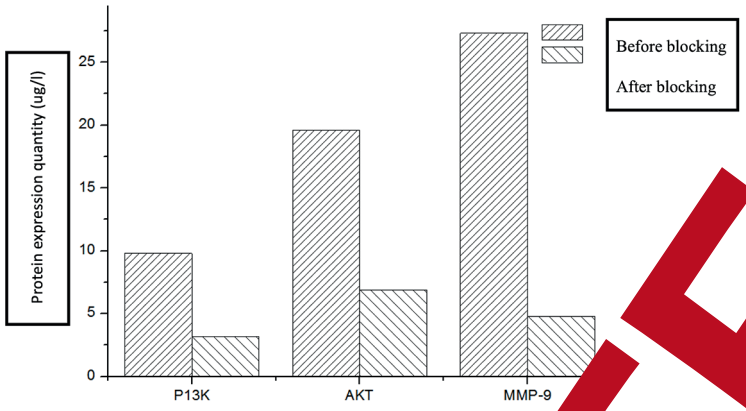



**Figure 4.** P13K/AKT specific inhibitor LY294002 bl[illegible]experiment.

existed in inactive status in the human body and it could be activated to degrade the intercellular special substances only under certain conditions[18]. 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[19].

With the deepening investigation on si[illegible] transduction pathway in recent years, i[illegible]as found that P13K/AKT signal pathway could [illegible]ticipate in many signal transduction proces[illegible] in the human body[20,21]. It was also proved tha[illegible] the activation of P13K/AKT sig[illegible]athway could participate in the process[illegible]ptosis and migration of tissue cells [illegible] tum[illegible]cells. For instance, the utilization [illegible]pecific i[illegible]ibitor LY294002 and Wortmannin[illegible]3K/A[illegible] nal pathway could reduce[illegible]he in[illegible]ular SKT content to promote the [illegible]ptosis of [illegible]cells. Study results[20] show[illegible]hat P13K/AK[illegible]al pathway could regu[illegible] migration of t[illegible]nor cells through many[illegible]nes[illegible] human body and there is a paucity [illegible]informa[illegible] the correlation between P[illegible]K/AKT signal p[illegible]y-mediated MMP-9 a[illegible]ung cancer.

## C[illegible]clusio[illegible]

In the prese[illegible]y, t[illegible]gh the detection of ex[illegible]quantity[illegible]K/AKT signal pathway [illegible]e and[illegible]MP-9 gene[illegible] lung cancer tissues and [illegible]jacent [illegible]rmal lung tissues, we found that the [illegible]pressi[illegible]P13K/AKT signal pathway [illegible]MP-9 gene[illegible] lung cancer tissues increased sig[illegible]tly by comparing with adjacent normal lung tissues. Howeve[illegible]xpression quantity of MM[illegible] protein decrease[illegible]nificantly after adding[illegible]3K/AKT signal pathway inhibitor, which i[illegible]ated that the MMP-9 gene could participate i[illegible]e occurrence a[illegible] deterioration processes of l[illegible] cancer of org[illegible]sm through P13K/AKT to s[illegible]xtent.

## Conflict of interest

[illegible]declare no conflicts of interest.

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