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# Correlation between matrix metalloproteinase-9 and vascular endothelial growth factor expression in lung adenocarcinoma

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**ABSTRACT.** The aim of this study was to investigate the correlation between the expression of matrix metalloproteinase-9 (MMP-9) and vascular endothelial growth factor (VEGF) and clinicopathological features of lung adenocarcinoma. The expression of MMP-9 and VEGF was evaluated by immunohistochemistry of 30 samples from lung adenocarcinoma patients and 12 paratumoral (normal) tissue samples. In addition, the change in VEGF or MMP-9 expression after MMP-9 or VEGF blockade, respectively, was measured using western blot in lung adenocarcinoma A549 cells. High expression of MMP-9 was found in 63.3% of adenocarcinoma tissues versus 16.7% in normal tissues ($P < 0.05$). High expression of VEGF was found in 70% of adenocarcinoma tissues versus 25% in normal tissues ($P < 0.05$). A positive correlation was identified between MMP-9 and VEGF expression (correlation coefficient = 0.7094, $P < 0.001$), and their mutual overexpression was associated with clinical staging and lymph node status ($P < 0.05$). In addition, an decrease in VEGF protein expression was observed after MMP-9 blockade by an MMP-9-specific monoclonal antibody. Similarly, a decrease in MMP-9 protein expression was found after VEGF blockade by

a VEGF-specific monoclonal antibody. In conclusion, VEGF and MMP-9 are overexpressed in lung adenocarcinoma tissues, and they have a synergistic effect on the invasion and metastasis of adenocarcinoma.

**Key words:** MMP-9; VEGF; Lung adenocarcinoma

## INTRODUCTION

Vascular endothelial growth factor (VEGF) is one of the most important factors promoting angiogenesis, an important process in tumor growth and metastasis. Expression of matrix metalloproteinase-9 (MMP-9), a proteinase involved in basement membrane degradation during angiogenesis, is also reported to be involved in tumor invasion and metastasis. Previous studies (Nagakawa et al. 2002; Li et al. 2009) have demonstrated overexpression of these two markers in various malignancies, and found a possible correlation between their overexpression and lymph node status and clinical staging in cancer. However, there are few studies on MMP-9 and VEGF expression in lung adenocarcinoma and their involvement in tumor invasion and metastasis. Our study evaluated VEGF and MMP-9 expression in 30 lung adenocarcinoma tissue samples and 12 paratumoral normal tissue samples using immunohistochemistry. We further explored the involvement of VEGF and MMP-9 in lung cancer invasion and metastasis through the measurement of changes in VEGF and MMP-9 expression after blockade with the corresponding MMP-9 and VEGF antibody in cultured A549 cells *in vitro*.

## MATERIAL AND METHODS

### Patient demographics

Between March 2009 and March 2012, surgical samples from 30 patients with lung adenocarcinoma were obtained from the Jiangjin Central Hospital (Chongqing, China). Twelve paratumoral normal tissue samples were also collected as controls. All samples were fixed in 10% formaldehyde, embedded in paraffin, and serially sectioned (5 µm thickness). Among the 30 patients (20 males and 10 females; aged 40-82 years), 14 patients were older than 60 years, 18 patients had lesions greater than 3 cm, and 16 patients had lymph node metastasis. No patients had prior adjunct chemotherapy or radiotherapy before surgery, and medical records of all patients were complete. Thirteen patients were classified as stage I-II with the remaining 17 patients as stage III-IV according to American Joint Committee on Cancer (AJCC) and Union for International Cancer Control (UICC) criteria (Wang, 2011).

### Cell lines

Human lung adenocarcinoma cell line A549 was provided by the Institute of Basic Medical Sciences, Chongqing Medical University (Chongqing, China). Following recovery, the A549 cells were cultured in RPMI 1640 (Gibco, USA) media supplemented with 10% fetal bovine serum (Senrun Biotech Co., Ltd., Tianjin, China) at 37°C in a 5% $CO_2$ humidified incubator. Trypsin (0.1%) was used for cell passaging.

### Immunohistochemistry

Immunohistochemical staining of MMP-9 and VEGF was performed according to the

manufacturer protocol of the immunohistochemistry kit (S-P Hypersensitivity Immunohistochemistry Kit; ZSGB Biotech Co., Ltd., Beijing, China) and mouse anti-human MMP-9 and VEGF (mouse anti-human) monoclonal antibodies (mAb) were purchased from Santa Cruz Biotechnology, Inc. (USA). Normal lung tissues were used as controls. Negative controls for non-specific staining were done on tissue sections using pre-immune serum IgG in place of primary antibodies while positive controls were done on specimens known to express specific antigens. The results were evaluated using the methods of Li et al. (2009). Tissues scored between 0 and 1 were classified as low expression and tissues with a score ≥2 were classified as high expression.

### Western blot

A549 cells were divided into three subgroups after several passages: A549, A549 + MMP-9 mAb (Santa Cruz; 5 µg/mL) and A549 + VEGF mAb (Santa Cruz; 5 µg/mL). When the cultured cells reached 90% confluence, cells were washed twice with PBS at 4°C and 200 µL cell lysis buffer was added. After 30 min of oscillation on ice, cells were removed by scraping and centrifuged to retrieve total protein in the supernatant. Loading buffer was added to the supernatant at a ratio of 1:4. After boiling for 3-5 min, the retrieved protein was frozen. The Bradford method was adopted for measurements of protein concentration. 8% SDS-PAGE was performed with 40 µg total protein in each well. The isolated protein was then transferred to PVDF membrane. The blot was blocked with 5% fat-free milk for 1 h and then incubated with antibodies against MMP-9 or VEGF (same as above) diluted 1:500 in 1X TBS-T. Following overnight incubation at 4°C, the corresponding secondary antibodies were added and incubated at 37°C for 1.5 h. The resulting bands were evaluated by enhanced chemiluminescence (ECL). In this study, β-actin was used as an internal control and all experiments were repeated in triplicate. Horseradish peroxidase (HRP)-labeled rabbit anti-mouse and mouse anti-human β-actin polyclonal antibodies and diaminobenzidine (DAB) horseradish peroxidase color development kit were obtained from ZSGB Biotech Co., Ltd.

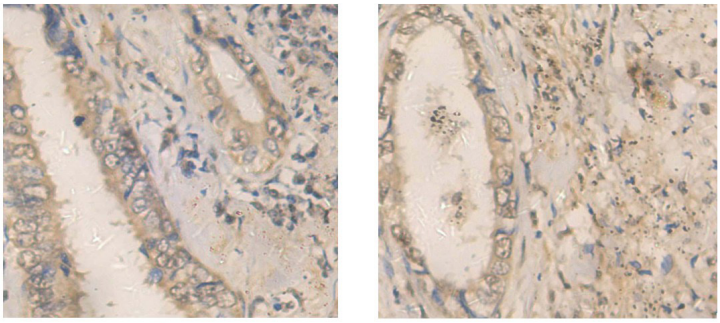
### Statistical analysis

The SAS 8.2 statistical software (Chicago, IL, USA) was used for analysis and the Fisher exact probability test and Spearman correlation analysis (r value) were performed. P value <0.05 was considered to be statistically significant.

## RESULTS

### Expression of MMP-9 and VEGF in lung adenocarcinoma

Immunohistochemical staining showed that both MMP-9 and VEGF were exclusively located in the cytoplasm in the form of brownish-yellow granules. As seen in Figure 1, the expression of both proteins was higher in cancerous tissue compared to normal tissue (MMP-9: 63.3 *vs* 16.7%; VEGF: 70 *vs* 25%, cancerous *vs* normal tissue). There was overexpression of VEGF in 21 adenocarcinoma cases and overexpression of MMP-9 in 18 adenocarcinoma cases compared to the controls. MMP-9 overexpression only occurred in one adenocarcinoma case with low VEGF expression. This finding indicates a positive correlation between MMP-9 and VEGF expression ($r = 0.7094$, $P < 0.001$).



A B

**Figure 1.** MMP-9 (**A**) and VEGF (**B**) expression in lung adenocarcinoma tissue (200X magnification).

## Relationship between MMP-9 and VEGF expression and clinicopathological features

Statistical analysis revealed increased expression of MMP-9 and VEGF in patients with lymph node metastasis compared to patients without lymph node involvement ($P < 0.05$) and in patients in stage III-IV compared to stage I-II ($P < 0.05$). There was no association between biomarker expression level and patient age or size of tumor ($P > 0.05$; Table 1).

**Table 1.** Relationship between MMP-9 and VEGF expression and clinicopathological features.

| Features | MMP-9 | | P value | VEGF | | P value |
|---|---|---|---|---|---|---|
| | Low expression | High expression | | Low expression | High expression | |
| Clinical stage | | | | | | |
| I + II | 10 | 3 | <0.01 | 9 | 4 | <0.01 |
| III + IV | 1 | 16 | | 0 | 17 | |
| Size of tumor | | | | | | |
| ≤3 | 6 | 6 | 0.2663 | 5 | 7 | 0.4181 |
| >3 | 5 | 13 | | 4 | 14 | |
| Age (years) | | | | | | |
| ≤60 | 6 | 10 | 0.9193 | 6 | 10 | 0.4397 |
| >60 | 5 | 9 | | 3 | 11 | |
| Lymphatic metastasis | | | | | | |
| No | 9 | 5 | | 9 | 5 | <.01 |
| Yes | 2 | 14 | 0.0068 | 0 | 16 | |

## Change in VEGF protein expression in lung adenocarcinoma A549 cells after MMP-9 blockade with monoclonal antibody

A549 cells were treated with MMP-9 mAb (final concentration = 5 µg/mL) for 24 h. The western blot showed a significant subsequent decrease in VEGF protein expression ($P < 0.05$; Figure 2).

## Change in MMP-9 protein expression in lung adenocarcinoma A549 cells after VEGF blockade with monoclonal antibody

VEGF mAb (final concentration = 5 µg/mL) was added to A549 cells. After a 24-h incubation, a significant decrease in MMP-9 expression was detected by western blot ($P < 0.05$; Figure 3).


**Figure 2.** Western blot showing VEGF protein expression in A549 cells after a 24-h treatment with MMP-9 mAb. *Lane 1* = 0 µg/mL and *lane 2* = 5 µg/mL. β-actin was used as an internal control.

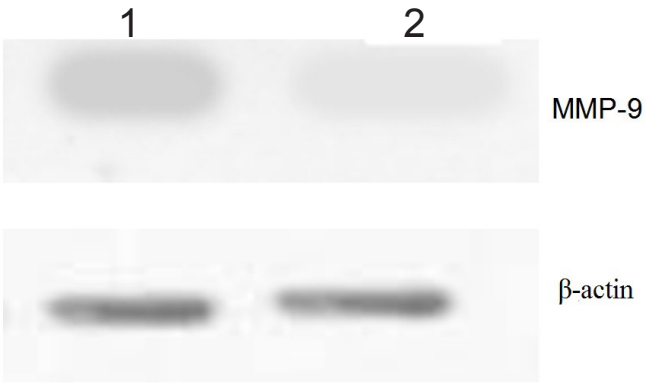



**Figure 3.** Western blot showing MMP-9 protein expression in A549 cells after a 24-h treatment with VEGF mAb. *Lane 1* = 0 µg/mL and *lane 2* = 5 µg/ml. β-actin was used as an internal control.

## DISCUSSION

It is well established that VEGF plays a central role in the angiogenesis of tumors through its pro-angiogenic effect on vascular endothelial cells and its potent anti-apoptotic actions on newly formed vascular endothelium. Studies on pancreatic cancer (Tang et al., 2001; Bogoevski et al., 2004) have demonstrated a marked overexpression of VEGF in cancerous tissue, which is associated with lymphatic metastasis and prognosis. Similar studies (McDonnell et al., 2000; Ye, 2001) on lung cancer have also revealed increased VEGF expression and a parallel increase in microvessel density in lung cancer tissues compared with benign and normal tissue. Moreover, adenocarcinoma tissue is reported to have higher VEGF expression levels than squamous carcinoma tissue. This could be partially responsible for the early metastasis and rapid progression observed in adenocarcinoma.

MMP-9 is also involved in the invasion and metastasis of multiple malignancies. The underlying mechanism is that MMP-9 degrades and disrupts types IV and V collagen and gelatin, which are crucial components of the extracellular matrix. The disruption of intact matrix facilitates invasion of tumor vasculature into mesenchyme. With the increased microvessel density, the tumor

grows and migrates to distant sites. Studies on pancreatic cancer (Nagakawa et al., 2002; Li et al., 2008a) have shown higher expression levels of VEGF and MMP-9 in cancerous tissue compared to normal tissue, indicating that these two markers may be involved in the pathogenesis of pancreatic cancer. Moreover, their expression has been found to be associated with lymph node status, suggesting that they may play vital roles in cancer metastasis. It is also notable that their changes in expression exhibit rough synchronization. In our study, we found MMP-9 and VEGF overexpression in lung adenocarcinoma, and their overexpression was correlated with lymph node status and clinical staging. A positive correlation between MMP-9 and VEGF expression was also identified.

The synchronized overexpression of MMP-9 and VEGF in tumor pathogenesis led scientists to explore their relationship. Meadows et al. (2001) found that VEGF can activate the Ras-activated extracellular signal-regulated kinase (ERK) pathway. Activation of the ERK signaling pathway is reported to promote MMP-9 gene transcription in breast cancer and hepatocellular carcinoma cells. In breast cancer cells, ERK activation leads to MMP-9 activation, while the ERK inhibitor PD 98059 suppresses MMP-9 expression in a dose-dependent manner. These findings suggest MMP-9 may be a downstream target of the ERK signaling pathway. Moreover, our study showed decreased MMP-9 expression following VEGF blockade, indicating that VEGF may regulate MMP-9 expression through the ERK signaling pathway. Li et al. (2008b) found that the MMP inhibitor GM 6001 could significantly inhibit retinal neovascularization and downregulate VEGF expression in rats. Decreased VEGF release may be due to decreased extracellular matrix degradation owing to inhibited MMP-9. To further investigate the interaction between VEGF and MMP-9, lung cancer A549 cells were cultured *in vitro*, and VEGF and MMP-9 expression was evaluated by western blot. We observed a decrease in VEGF expression with MMP-9 blockade and a similar decrease in MMP-9 expression with VEGF blockade. These findings suggested a mutual promotion between VEGF and MMP-9 expression.

In this study, we observed a positive feedback loop involving VEGF and MMP-9. Intermediate signaling pathways exist between these two biomarkers, contributing to mutual stimulation and promotion. This loop promotes angiogenesis, invasion and metastasis in lung cancer; however, the exact mechanism remains unclear. Deeper knowledge of the regulatory mechanism between VEGF and MMP-9 will allow for a better understanding of mechanisms underlying tumor growth, invasion and metastasis, which is valuable in the diagnosis, treatment and prognosis of lung adenocarcinoma.

## Conflicts of interest

The authors declare no conflict of interest.

## ACKNOWLEDGMENTS

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