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Prognostic impact of matrix metalloproteinases (MMP-9 and MMP-2) and vascular endothelial growth factor expression in non-small cell lung cancer

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Aim: To investigate the role of matrix metalloproteinases (MMPs), MMP-9 and MMP-2, and vascular endothelial growth factor (VEGF) in metastasis and their relationship with survival. Early metastasis is the most common cause of lung cancer death. Angiogenesis and basement membrane damage are necessary for growth and invasion of the tumoral cells. VEGF, involved in angiogenic activity, and MMPs, involved in matrix destruction, are important for metastasis in lung cancer.

Materials and methods: Using immunohistochemistry analysis, we evaluated MMP-9, MMP-2, and VEGF expressions in paraffin specimens from 91 patients with histopathologically confirmed non-small cell lung cancer and the relationship between MMP-9, MMP-2, and VEGF expressions and the prognosis.

Results: For MMP-9 expression, 52 (57.1%) patients were positive and 39 (42.9%) were negative. Patients whose tumors had MMP-9-positive staining had a shorter duration of survival than patients whose tumors had no expression, but it was not statistically significant (14.1 ± 1.7 (median: 12) vs. 16.7 ± 2.2 (median: 15), $P > 0.05$). For MMP-2 expression, 13 (14.3%) patients were positive and 78 (85.7%) were negative. Patients whose tumors had MMP-2-positive staining had a shorter duration of survival than patients whose tumors had no expression, but it was not statistically significant (12.6 ± 3.6 (median: 8) vs. 16.1 ± 1.5 (median: 14), $P > 0.05$). For VEGF expression, 34 (37.4%) patients were positive and 57 (62.6%) were negative. There was no significant relationship between the duration of survival for patients whose tumors had VEGF-positive and VEGF-negative staining (15.3 ± 1.7 (median: 18) vs. 15.6 ± 2.1 (median: 13), $P > 0.05$).

Conclusion: Angiogenesis and basement membrane damage are the main components for metastasis, but there was no significant relationship between MMP and VEGF expressions and prognosis. This indicates that the metastasis biology of lung cancer is a complex and multistep pathogenetic mechanism, although a small number of patients were included in our study.

Key words: Lung cancer, metastasis, matrix metalloproteinases, vascular endothelial growth factor

Küçük hücreli dışı akciğer kanserli hastalarda matriks metalloproteinaz (MMP-9 ve MMP-2) ve vasküler epitelyal büyüme faktörü ekspresyonunun prognoz üzerine olan etkisi

Amaç: Akciğer kanserli hastalarda ölüm nedeni çoğunlukla hastalığın kısa sürede metastaz yapmasıdır. Tümör hücrelerinin büyümesi ve invazyon yapabilmesi için anjiogenezin olması ve bazal membranın bozulması gerekmektedir. Akciğer kanserlerinde anjiogenik aktivite gösteren vasküler endotelyal büyüme faktörü (VEGF) ve matriksin harabiyetinde yer alan matriks metalloproteinazlar (MMPs) metastaz oluşumunda önemli bir yer tutarlar. Bu çalışmanın amacı, MMP-9, MMP-2 ve VEGF'nin metastaz oluşumundaki yeri ve prognoz ile olan ilişkisini araştırmaktır.

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Yöntem ve gereç: Histopatolojik olarak küçük hücreli dışı akciğer kanseri tanısı konulan 91 hastanın parafin blokları immunohistokimyasal yöntem kullanılarak, MMP-9, MMP-2 ve VEGF ekspresyonu açısından incelendi ve hastalığın prognozu ile olan ilişkisi değerlendirildi.

Bulgular: 52 (% 57,1) hasta MMP-9 pozitif, 39 (% 42,9) hastada ise MMP-9 negatifti. MMP-9 pozitif hastaların sağ kalım süreleri, MMP-9 negatif olanlara göre daha kısa olmasına rağmen istatistiksel anlamlılık yoktu (sırasıyla 14,1 ± 1,7 (ortanca: 12), 16,7 ± 2,2 (ortanca: 15), P > 0,05). 13 (% 14,3) hasta MMP-2 pozitif, 78 (% 85,7) hastada ise MMP-2 negatifti. MMP-2 pozitif hastaların sağ kalım süreleri, MMP-2 negatif olanlara göre daha kısa olmasına rağmen istatistiksel anlamlılık yoktu (sırasıyla 12,6 ± 3,6 (ortanca: 8), 16,1 ± 1,5 (ortanca: 14), P > 0,05). 34 (% 37,4) hasta VEGF pozitif, 57 (% 62,6) hastada ise VEGF negatifti. VEGF pozitif ve negatif hastaların sağ kalım süreleri arasında istatistiksel olarak anlamlılık yoktu (sırasıyla 15,3 ± 1,7 (ortanca: 18), 15,6 ± 2,1 (ortanca: 13), P > 0,05).

Sonuç: Tümör metastazında anjiogenez ve bazal membran devamlılığı önemli bir yer tutmakla birlikte, MMPs ve VEGF ekspresyonu ile prognoz arasında anlamlı bir ilişki bulunamamıştır. Çalışmaya alınan hasta sayısı az olmasına rağmen bu durum akciğer kanserinde metastaz biyolojisinin çok basamaklı ve kompleks bir olay olduğuna işaret etmektedir.

Anahtar sözcükler: Akciğer kanseri, metastaz, matriks metalloproteinazlar, vasküler endotelyal büyüme faktörü

Introduction

Lung cancer is the foremost malignancy in the world in terms of incidence and mortality, representing 12.4% of all of the newly detected cancers with an estimated 1.35 million new cases each year. At the same time, it is the most common cause of death from cancer, with the number of deaths totaling 1.18 million each year (1). The lung cancer incidence in Turkey is 52.7/100,000 in men and 7.2/100,000 in women (2). The prognosis of lung cancer is worse than those of all other cancers. With 5-year survival of 5%-15% in stage III and less than 2% in stage IV (3), non-small cell lung cancer (NSCLC) accounts for approximately 85% of all cases of lung cancer (4).

Early metastasis is the most common cause of lung cancer death. Tumor metastasis is a multistep process consisting of several sequential events including detachment of malignant cells from the primary tumor, invasion to surrounding tissue, intravasation into the circulatory system, adhesion to vascular endothelial cells at distant sites, and extravasation through the endothelial basement membrane to colonize new tissues. Among these steps, angiogenesis and degradation or remodeling of the basement membranes are the most critical steps in tumor invasion (5). The extracellular matrix (ECM) is a network of proteins and proteoglycans that determine the architecture of tissues and plays an essential role in cell adhesion, migration, proliferation, and differentiation. Degradation of the ECM requires the participation of different proteolytic enzymes, including matrix metalloproteinases (MMPs), cysteine proteinases, and serine proteinases (6). MMPs are a 21-member family of zinc-dependent endopeptidases, which are capable of degrading most ECM components, including collagen, elastin, fibronectin, and gelatin (7). Among the MMPs, 72-kDa and 92-kDa type IV collagenases (gelatinase A and B or MMP-2 and MMP-9, respectively) are especially active in the degradation of type IV collagen, the main constituent of the basement membranes. Both type IV collagenases are expressed in many human cancers (8-10).

Vascular endothelial growth factor (VEGF), involved in angiogenic activity, is also important for metastasis in lung cancer. It was shown that tumors can reach 1-2 mm in size without angiogenesis and cannot grow more than this size (11,12). Although many factors affect angiogenesis (FGF, PD-ECGF, etc.), VEGF is the most powerful endothelial cell-specific mitogen associated with tumor neovascularization (13).

The aim of this study was to investigate the role of MMP-9, MMP-2, and VEGF in metastasis and their relationship with survival in lung cancer patients.

Materials and methods

Enrolled in this study were 91 patients with histopathologically confirmed NSCLC (Table).

Using immunohistochemistry analysis, MMP-9, MMP-2, and VEGF expressions were evaluated in paraffin specimens from 91 patients with histopathologically confirmed NSCLC, and the

Table. Demographic characteristics of the patients.

Age, mean (years)		59.9 ± 10.5
Sex, female/male		38/53
Smoking (packs/year)		50.3 ± 26.6
Histological type (NSCLC)	Epidermoid carcinoma	60 (65.9%)
	Adenocarcinoma	18 (19.8%)
	Adenosquamous carcinoma	1 (1.1%)
	Unclassified carcinoma	12 (13.2%)
Stage	Stage 4	39 (42.9%)
	Stage 3	37 (40.7%)
	Stage 2	7 (7.6%)
	Stage 1	8 (8.8%)

relationship between MMP-9, MMP-2, and VEGF expressions and the prognosis was analyzed.

Survival times were measured from the diagnosis of the disease to the time of the last follow-up or death. Stage III patients received chemotherapy and radiotherapy with the best supportive care and stage IV patients received chemotherapy with the best supportive care. The tumors were classified according to the Union for International Cancer Control TNM classification (3).

Immunohistochemistry for MMP-9, MMP-2, and VEGF

Tissue sections of 4 μm were cut from representative formalin-fixed and paraffin-embedded blocks. Sections were deparaffinized in xylene and rehydrated. Immunoperoxidase staining was performed using the streptavidin-biotin peroxidase method. The sections were treated with 0.3% H_2O_2 in order to suppress endogenous peroxidase activity. Antigen retrieval was performed using 0.01 M citrate buffer, pH 6.0, through microwave processing for MMP-9 and MMP-2 antibodies. Sections were pretreated with EDTA to retrieve antigen expression for the VEGF antibody. The sections were incubated with anti-MMP-2 (72-kDa collagenase IV) monoclonal antibody (1:50 diluted, clone A-Gel VC2, NeoMarkers, CA, USA), anti-MMP-9 (92-kDa collagenase IV) polyclonal antibody (1:50 diluted, NeoMarkers), and anti-VEGF monoclonal antibody (1:50 diluted, clone VG1, NeoMarkers). As a chromogen for color development, 3-amino-9-ethylcarbazole substrate (AEC; LabVision, NeoMarkers) was used. The slides were counterstained with hematoxylin, dehydrated, and mounted. Sections of placenta tissue for MMP-9 and MMP-2, and angiosarcoma for VEGF, were used as positive controls. In the negative control, phosphate buffered saline replaced the primary antibody. Stained slides were examined to identify the cellular localization of MMP-9, MMP-2, and VEGF immunoreactivity and were scored for both intensity (1+ = weak, 2+ = moderate, and 3+ = intense) and proportion (1 = less than 5%, 2 = 5%-25%, 3 = 25%-50%, 4 = 50%-75%, and 5 = >75%) of cells stained. These values were added to provide a single score for each case. When the score was higher than 1, it was considered positive.

Statistical analyses

SPSS for Windows was used for statistical calculations. For analysis of survival times, the Kaplan-Meier method was used, and survival distributions were compared with log-rank statistics. Significant correlations between the variables were clarified by Cox's multivariate hazards model.

Results

The demographic characteristics of the patients are summarized in the Table. For MMP-2 expression, 13 (14.3%) patients were positive and 78 (85.7%) were negative. Patients whose tumors had MMP-2-positive staining had a shorter duration of survival than patients whose tumors had no expression, but it was not significant statistically (12.6 ± 3.6 (median: 8) vs. 16.1 ± 1.5 (median: 14), P > 0.05, respectively) (Figures 1 and 2). There was no significant relationship between the MMP-2 expression and patient age, sex, tumor histologic type, or tumor stage (P > 0.05).

For MMP-9 expression, 52 (57.1%) patients were positive and 39 (42.9%) were negative. Patients whose tumors had MMP-9-positive staining had a shorter duration of survival than patients whose tumors had no expression, but it was not significant statistically (14.1 ± 1.7 (median: 12) vs. 16.7 ± 2.2 (median: 15), P > 0.05, respectively) (Figures 3 and 4). There was no significant relationship between the MMP-9 expression and patient age, sex, tumor histologic type, or tumor stage (P > 0.05).

For VEGF expression, 34 (37.4%) patients were positive and 57 (62.6%) were negative. There was

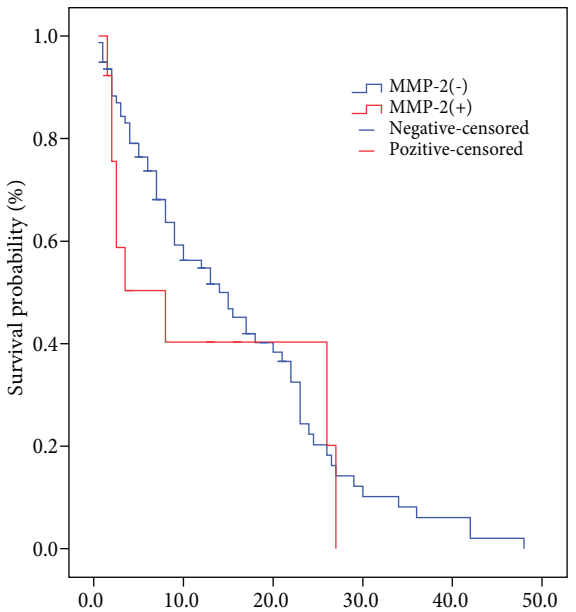

Figure 1. Overall survival of patients with MMP-2-positive and MMP-2-negative staining in non-small cell carcinoma.

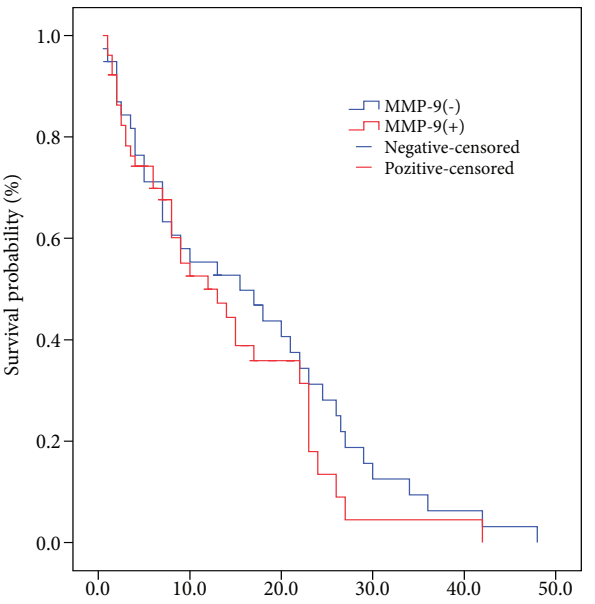

Figure 3. Overall survival of patients with MMP-9-positive and MMP-9-negative staining in non-small cell carcinoma.

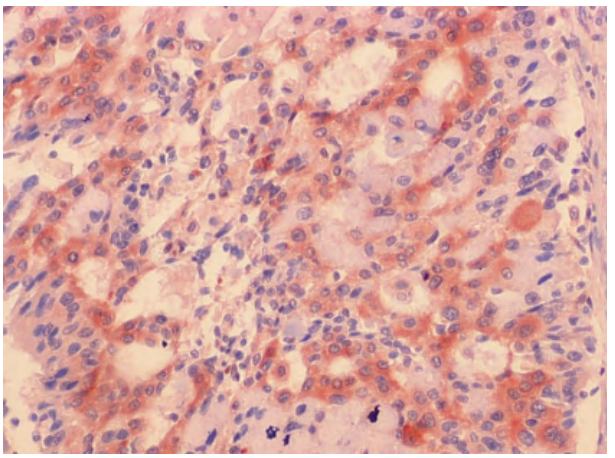

Figure 2. Expression of MMP-2 in adenocarcinoma cells (streptavidin-biotin-peroxidase, AEC, 200×).

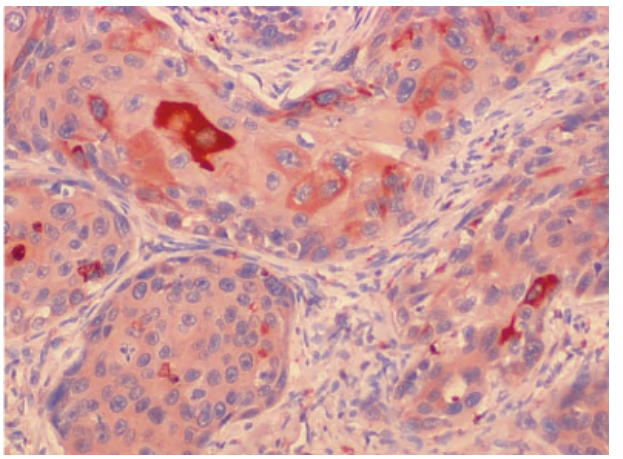

Figure 4. Expression of MMP-9 in squamous cell carcinoma (streptavidin-biotin-peroxidase, AEC, 200×).

no significant relationship between the duration of survival for patients whose tumors had VEGF-positive staining and patients whose tumors had no VEGF expression (15.3 ± 1.7 (median: 18) vs. 15.6 ± 2.1 (median: 13), P > 0.05, respectively) (Figures 5 and 6). There was no significant relationship between the VEGF expression and patient age, sex, tumor histologic type, or tumor stage (P > 0.05).

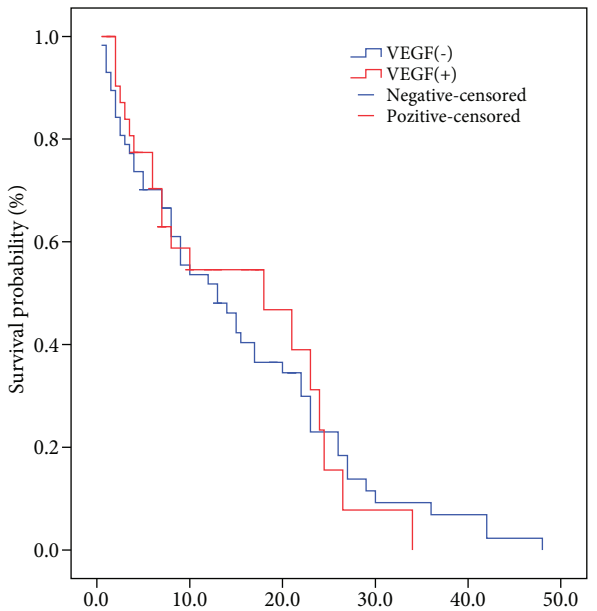

Figure 5. Overall survival of patients with VEGF-positive and VEGF-negative staining in non-small cell carcinoma.

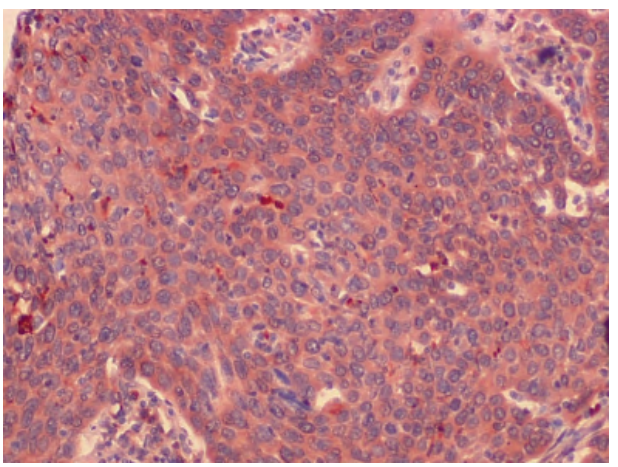

Figure 6. Expression of VEGF in squamous cell carcinoma (streptavidin-biotin-peroxidase, AEC, 200×).

Discussion

The most important cause of death in lung cancer patients is tumor metastasis. Angiogenesis and basement membrane degradation are necessary for growth and invasion by tumor colonies. Many studies have endeavored to establish a relation between tumor invasion, metastasis, and expression of MMPs and VEGF. Folkman described the association between growing solid malignant tumors and angiogenesis (14). Many of the proteolytic enzymes worked on paraffin-embedded material, and the development of antibodies against angiogenic factors allowed for the study of the prognostic role of molecules involved in these transactions (15). In our study, paraffin blocks of VEGF and MMP expression were investigated immunohistochemically.

In previous studies of patients with NSCLC, 29%-84.6% were found to be VEGF-positive (13,16-19). In our study, 37.4% of the patients with NSCLC were VEGF-positive. In cancer patients, the source of VEGF associated with tumor neovascularization is tumor cells. The serum VEGF levels in patients with colorectal cancer were found to be higher in the tumor-draining vein than in the peripheral vein (20).

In previous studies, there was no significant relation between the histologic type of NSCLC and VEGF positivity (17,21). Volm et al. could not determine a significant relationship between age and VEGF in patients with NSCLC (21). In our study, we could not determine a significant relationship between histologic type or age and VEGF positivity.

Aikawa et al. found a VEGF positivity of 22% in stages I and II, and 43% in stage III (17); however, Volm et al. could not detect a significant association between stage and VEGF positivity (21). In our study, we could not detect a significant association between tumor stage and VEGF positivity. There may be many reasons for these different results. Although VEGF is an effective factor in angiogenesis, the fact that angiogenesis is multistep and includes many growth factors and cytokines may lead to different study outcomes. Liao et al. could not detect a significant relationship between VEGF positivity and survival, yet they found a statistically significant relationship with survival when VEGF, p53, and the proliferation index were evaluated together (19). In addition, tumor cells secrete angiogenic and antiangiogenic

factors at the same time. In a previous study, the survival time was shorter in patients who were VEGF-positive and were not secreting angiostatin, which is an angiogenesis inhibitor, than in patients who were VEGF-positive and secreting angiostatin (22). However, the small number of patients, along with the study of only one angiogenic factor and no VEGF isoforms, could have impacted the results significantly.

Molecular studies continue intensively for the use of VEGF and VEGF receptor antibodies as potential treatments for lung cancer (16). In a previous study, VEGF-121, a neutralizing antibody, was shown to inhibit lung metastasis of sarcomas (16). For this reason, molecular predictors that display the risk of metastasis must be investigated.

It is well documented that hypoxia, a potent stimulator of angiogenesis, induces the expression of VEGF, having a mitogenic effect on vascular endothelial cells (23). The migration of proliferating endothelial cells requires the degradation of the ECM by MMPs. It has been shown that VEGF can stimulate MMP release from endothelial cells and, indirectly, their activation (23). A positive correlation between the expression of type IV collagenases and VEGF in human NSCLC has been found (24). The invasion of surrounding tissues by neoplastic cells is one of the most important steps in tumor progression. Proteolytic enzymes such as MMPs contribute to tumor expansion by degrading components of the ECM (7).

Two studies on lung cancer using northern analysis, immunohistochemistry, and in situ hybridization showed that MMP was absent from normal lung parenchyma, although the total MMP activity was 2-fold higher in lung cancer preparations (9,25).

Many studies have shown that MMPs are expressed in squamous cell carcinoma (26,27), with particular involvement by MMP-2 and MMP-9 (26,28,29). As MMPs appear to be essential for tumor invasion and metastatic spread, characterizing their expression may help to determine a patient's treatment or prognosis. In our study, patients whose tumors had positive staining for MMPs had a shorter duration of survival than patients whose tumors had no expression, but it was not statistically significant.

In NSCLC, MMP-2 has been reported to increase in tumor cells and in the peritumoral tissues (30,31). In addition, MMP-2 expression has been reported to be an indicator of poor prognosis (13,32,33), whereas some studies did not yield the same results (31,34,35). In the metaanalysis of MMP-2 studies about the relation between MMP-2 and survival in NSCLC, it was indicated that MMP-2 overexpression was a poor prognostic factor for survival in NSCLC (36).

Leinonen et al. demonstrated that high MMP-9 expression indicated aggressive tumor behavior and high MMP-7 indicated less aggressive tumor behavior in NSCLC. However, MMP-9 and MMP-7 expressions had no prognostic value in NSCLC patients (37).

Sienel et al. showed that MMP-9 expression in cancer cells is of independent prognostic impact in NSCLC patients (38), whereas some studies did not demonstrate the prognostic impact of MMP-9. Shou et al. showed that MMP-9 expression correlated with the T-status and the N-status, suggesting that the observed prognostic value of MMP-9 could result from possible joint effects with the T- or N-status (13). However, in spite of studies performed with lung carcinomas, the relationship of MMP-9 with clinicopathological variables is unclear (39,40).

Hrabec et al. found no significant differences between MMP levels and tumor stage. They explained that the expression of these enzymes by tumor or stromal cells reached a high level (optimal for malignant growth) even in patients with stage I tumors (6). In our study, there was no significant relationship between MMP expression and tumor stage.

In addition, in the extracellular space, the activity of MMPs is regulated by a specific class of natural inhibitors and it cannot be excluded that metastatic capacity depends on the balance between MMPs and their inhibitors (6). In the future, MMPs might be considered as a potential target for studies investigating the benefit of adjuvant MMP inhibitor therapy.

As a result, although angiogenesis and basement membrane damage are the main components of metastasis, there was no significant relationship between MMP and VEGF expressions and prognosis. The metastasis biology of lung cancer is a complex and multistep pathogenetic mechanism.

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