Expression of *KISS1* and MMP-9 in Non-small Cell Lung Cancer and Their Relations to Metastasis and Survival

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Abstract. *KISS1 and matrix metalloproteinase-9 (MMP-9) may play important roles as metastasis suppressor and metastasis promoter genes, respectively, in a variety of malignancies. However, there is little information about their possible roles in non-small cell lung cancer (NSCLC). The goals of this study were to determine the mRNA and protein expressions of KISS1 and MMP-9 in NSCLC and their relations to metastasis and prognosis. The mRNA and protein expressions of KISS1 and of MMP-9 protein were detected by in situ hybridization and immunohistochemistry respectively in 85 cases of NSCLC, and their matched lymph node metastases. Expressions of KISS1 mRNA and protein were significantly higher in low TNM stages of NSCLC (I-II) compared to more advanced stages (III-IV) ($p<0.05$). Moreover, in advanced TNM stages, cases without metastasis had higher KISS1 gene expression compared to those with lymph node metastasis ($p<0.05$). In contrast, MMP-9 expression was higher in stage III-IV NSCLC cases compared to stage I-II tumors ($p<0.05$) and higher in NSCLC cases with metastasis than those without metastasis ($p<0.05$). There was negative correction between KISS1 and MMP-9 protein expression ($p<0.01$). The 5-year survival rate in cases with higher KISS1 protein expression was significantly higher than in those with low expression (20.9 vs. 2.4%, $p<0.01$). However, the 5-year survival rate of patients with high MMP-9 protein expression were lower than those with low expression (19 vs. 4.7%, $p<0.05$). Our data suggest that KISS1 and MMP-9 may serve as potential prognostic and therapeutic markers in lung cancer.*

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Lung cancer is the most common cause of cancer-related deaths worldwide, accounting for 159,390 deaths in the United States in 2009 (1). Although recent advances in chemotherapy and radiation therapy have yielded modest improvements in patient outcomes, overall survival remains poor in advanced lung cancer patients (2, 3). Therefore, new therapeutic targets to block the metastatic process at its earliest stage are urgently needed (4).

Tumor metastasis is a large and complex process involving loss of cell–cell adhesion, invasion of the extracellular matrix, spread *via* lymphatics or vascular channels, and colonization at the metastatic site where secondary tumors must continue to grow and cause clinically significant disease (5-7). Numerous reports have shown that changes of oncogenes and tumor suppressor genes are involved in the process of metastasis (8). Recent findings indicate that the process of metastasis is subject to a specific class of proteins which play an important role in suppressing the metastatic phenotype (9). These proteins are encoded by suppressor genes of metastasis, defined as genes that suppress *in vivo* metastasis without inhibiting primary tumor growth. The *KISS1* gene, originally identified in melanoma by Lee *et al.* in 1996 (10), has been reported to function as a metastasis suppressor gene in a number of malignancies including those of the thyroid gland, liver, stomach, esophagus, kidney and urinary bladder (10-17). The *KISS1* gene is located on chromosome 1 near q32.1 with regulatory elements localized in chromosome 6 at 6q16.3-q23 (9). *KISS1* is primarily expressed in placenta and testis but is also found in the central nervous system, ovary, pancreas and intestine. The

KISS1 product is a 145 amino acid peptide, known as kisspeptin, which is cleaved to smaller peptides, including a 54 amino acid known as metastatin (9). The metastasis suppressor function is thought to act by inhibiting chemotaxis and local invasion of the malignant cells (18). Interestingly, *KISS1* product appears to be involved in regulation of collagenase activity that degrades extracellular matrix, which is important in metastasis. The expression and function of the *KISS1* appears to be cell/organ specific. Ikeguchi *et al*. reported that the expression of *KISS1* in esophageal cancer with lymph node metastasis was significantly lower than that without metastasis, suggesting deletion of *KISS1* was closely related to lymph node metastasis in esophageal cancer (15). Ringel *et al*. came to a similar conclusion in the study of thyroid cancer (19). In contrast, *KISS1* expression is increased in breast cancer patients with aggressive tumors and high mortality (12). Matrix metalloproteinase-9 (MMP-9) has been shown to be involved in degradation of the extracellular matrix and promotion of tumor growth and metastasis by its angiogenic properties (6). Little information is known about *KISS1* and MMP-9 expression in non-small cell lung cancer (NSCLC). In this study, we investigated the expression of *KISS1* and MMP-9 in 85 cases of NSCLC and their matched lymph node metastases to determine the relationship between their expression levels and metastasis, and survival.

Patients and Methods

Patients. This study included 85 patients with NSCLC who underwent tumor resection between 1999 and 2002 at the Affiliated Hospital of North China Coal Medical College Hospital. None of the patients had received radiotherapy, chemotherapy, or immunotherapy prior to tumor resection. The diagnosis and histological classification of all specimens were performed following the criteria for classification of lung cancer by the World Health Organization (WHO) (20). All available hematoxylin and eosin stained slides from each case were reviewed. Clinical information and follow-up were obtained by medical records. Patients were evaluated for age, sex, tumor size, histological type, tumor grade, TNM stage, presence of lymph node metastases and overall survival.

KISS1 in situ hybridization. *KISS1 in situ* hybridization was performed according to the manufacturer's instructions using a *KISS1 in situ* hybridization kit (Boshide Company, P.R. China). Briefly, sections were washed with phosphate buffered saline PBS) and treated with 1 μg/ml pepsin (diluted with 3% citric acid) at 37˚C to digest protein, and then incubated with pre-hybridization solution (0.25% acetic anhydride in 0.1 M triethanolamine) for 3 h at 38˚C. For hybridization, the sections were incubated with hybridization solution including 1 μg/μl digoxigenin (DIG)-labeled antisense cRNA probes at 38˚C overnight, and then washed with saline sodium citrate (SSC) several times. The sections were then incubated with mouse anti-digoxin biotinylated, drop-wise peroxidase biotinylated and visualized by 3, 3'-diaminobenzidine tetrahydrochloride (DAB). After hematoxylin staining, sections were coverslipped. Sections of normal placental tissue were used as positive controls and PBS replaced the probe in the negative controls.

Immunohistochemical staining for KISS1 and MMP-9. Formalin-fixed, paraffin-embedded tissue sections were cut into 4 μm-thick sequential sections. After deparaffinization and rehydration, sections were boiled in citrate buffer (0.01 M, pH 6.0) for antigen retrieval. Sections were then incubated with 3% H_2O_2 and 5% serum to block endogenous peroxidase activity and non-specific binding. For *KISS1* protein, sections were incubated with rabbit anti-human *KISS1* polyclonal antibody (phoenix Pharmaceutical Inc, USA). For the MMP-9 protein, sections were incubated with mouse anti-human MMP-9 monoclonal antibody (Mai Xin Biotechnology Co. P.R. China). The sections were then incubated with biotinylated secondary antibodies (Mai Xin Biotechnology) and visualized by DAB (Mai Xin Biotechnology). Counterstaining was carried out with hematoxylin. The sections were dehydrated in alcohol and coverslipped. For the negative controls, PBS replaced the primary antibody.

Evaluation of staining. For each case, five images at high magnification (×200) were obtained. The integral optical density (IOD) was determined by a BI2000 medical image analysis system (Chengdu Taimeng Science & Technology Inc., Chengdu, P.R. China) and the average value was calculated.

Statistical analysis. All data were expressed as the mean±SE. The differences of *KISS1* mRNA and protein expression, and MMP-9 protein expression between samples were analyzed by *t*-tests. The relationship was analyzed using linear correlation analysis. The probabilities of overall survival were calculated using the Kaplan-Meier method and were compared using the log-rank test. All statistical analyses were performed using SPSS 13.0 (SPSS Statistics, Chicago, USA) and *p*-values less than 0.05 were considered statistically significant.

Results

Clinical and pathologic features. The clinical and pathological features of the 85 cases of NSCLC are summarized in Table I. The patients ranged in age from 41 to 80 years, with an average age of 57 years at the time of surgery. There were 68 male and 17 female patients. Fifty-six out of the 85 cases (66%) had lymph node metastases. Sixty out of the 85 cases were classified as squamous cell carcinomas, 22 were adenocarcinomas, and the remaining 3 cases were adenosquamous carcinoma. For histological grade, 7 cases were well differentiated, 47 cases were moderately differentiated and 31 cases were poorly differentiated. There were 26 cases with stage I disease, 40 cases with stage II disease, 17 cases with stage III disease, and 2 cases with stage IV disease.

KISS1 protein expression relative to the clinical and pathological variables. The expression of *KISS1* mRNA and protein had no relation to patient age or sex, tumor size, histological tumor subtype, or histological grade ($p>0.05$)

Table I. *The expressions (integral optical density values) of KISS1 and MMP-9 in non-small cell lung cancer.*

Clinicopathological factors	n	*KISS1* mRNA		*KISS1* protein		MMP-9 protein	
		$\bar{X}$ ±SE	*p*-value	$\bar{X}$ ±SE	*p*-value	$\bar{X}$ ±SE	*p*-value
Gender							
Male	68	10.43±1.31	>0.05	11.60±1.26	>0.05	10.72±1.90	>0.05
Female	17	10.23±1.05		11.30±1.25		10.47±1.99	
Age (years)							
≤50	36	10.38±1.44	>0.05	11.47±1.37	>0.05	10.80±1.89	>0.05
>50	49	10.40±1.13		11.60±1.18		10.58±1.94	
Tumor size							
≤3 cm	48	10.50±1.05	>0.05	11.66±1.12	>0.05	10.12±1.66	<0.05
>3 cm	37	10.25±1.50		11.40±1.43		11.34±2.00	
Histological type							
Squamous cell	60	10.55±1.10	>0.05	11.66±1.20	>0.05	10.25±1.76	<0.05*
Adenocarcinoma	22	9.87±1.59		11.12±1.40		11.75±1.84	>0.05#
Adenosquamous	3	11.10±0.15		12.35±0.13		11.27±2.74	>0.05Δ
Differentiation							
Well or Moderate	54	10.54±1.22	>0.05	11.73±1.25	>0.05	10.38±1.79	>0.05
Poor	31	10.13±1.29		11.24±1.23		11.11±2.04	
TNM Stage							
I-II	66	10.68±1.04	<0.01	11.93±1.05	<0.05	10.02 ±1.54	<0.01
III-IV	19	9.35±1.43		10.24±1.01		12.92±1.24	
Lymph node metastasis							
–	54	10.80±1.02	<0.01	12.00±1.07	<0.01	9.55±1.28	<0.01
+	31	9.68±1.32		10.76±1.18		12.62±1.06	
Lesion site							
Primary	85	10.38±1.26	<0.05	11.55±1.26	<0.05	10.67±1.91	<0.01
Metastasis	56	7.28±1.52		8.30±1.31		15.80±2.59	

*Squamous *vs*. adenocarcinoma in histological type; #adenocarcinoma *vs*. adenosquamous in histological type; Δadenosquamous *vs*. squamous in histological type.

(Table I). However, *KISS1* expression was significantly higher in the low stages of NSCLC (I-II) compared to more advanced stages (III-IV) (mRNA: 10.68±1.04 *vs*. 9.35±1.43; protein: 11.93±1.05 *vs*. 10.24±1.01; $p<0.05$). In addition, *KISS1* expression was significantly higher in NSCLC without metastasis than cases with metastasis (mRNA: 10.80±1.02 *vs*. 9.68±1.32; protein: 12.00±1.07 *vs*. 10.76±1.18; $p<0.05$). *KISS1* expression was also significantly higher in the primary tumor compared to secondary sites mRNA: 10.38±1.26 *vs*. 7.28±1.52; protein: 11.55±1.26 *vs*. 8.30±1.3; $p<0.05$) (Table I, Figure 1). Linear correlation analysis of the IOD value of *KISS1* mRNA and protein showed a positive correlation between them (r=0.954, $p<0.01$).

MMP-9 protein expression relative to the clinical and pathological variables. Similar to *KISS1* expression, MMP-9 expression had no relation to the age, sex, pathological classification or grade ($p>0.05$) (Table I). However, MMP-9 protein expression was markedly higher in adenocarcinoma than in squamous carcinoma (11.75±1.84 *vs*. 10.25±1.76), and significantly higher in tumors with a diameter >3 cm compared to those with a diameter ≤3 cm (11.34±2.00 *vs*. 10.12±1.66). In addition, MMP-9 protein expression was significantly higher in stage III-IV NSCLC compared to stage I-II tumors (12.92±1.24 *vs*. 10.02 ±1.54, $p<0.01$). NSCLC MMP-9 expression in tumors with metastasis was significantly higher than in cases without metastasis (12.62±1.06 *vs*. 9.55±1.28, $p<0.01$), and significantly higher in the metastatic site compared to the corresponding primary tumor (15.80±2.59 *vs*. 10.67±1.91, $p<0.05$) (Table I, Figure 2) Furthermore, KISS1 and MMP-9 protein expression had a negative correlation (r=–0.523, $p<0.01$).

KISS1 and MMP-9 expression and survival rate of NSCLC patients. Comparison between *KISS1* expression and 5-year survival rates showed significant differences. The 5-year survival rate was the highest, at 20.9%, in patients with low stage (I-II) NSCLC with high *KISS1* expression, whereas the 5-year survival rate was 2.4% in patients with more advanced tumors (stage III-IV) and low *KISS1* expression level. The difference in 5-year survival rates between these two groups was statistically significant ($p<0.01$) (Figure 3, left panel).

Comparisons between MMP-9 expression and the 5-year survival rate also showed significant differences. A low level

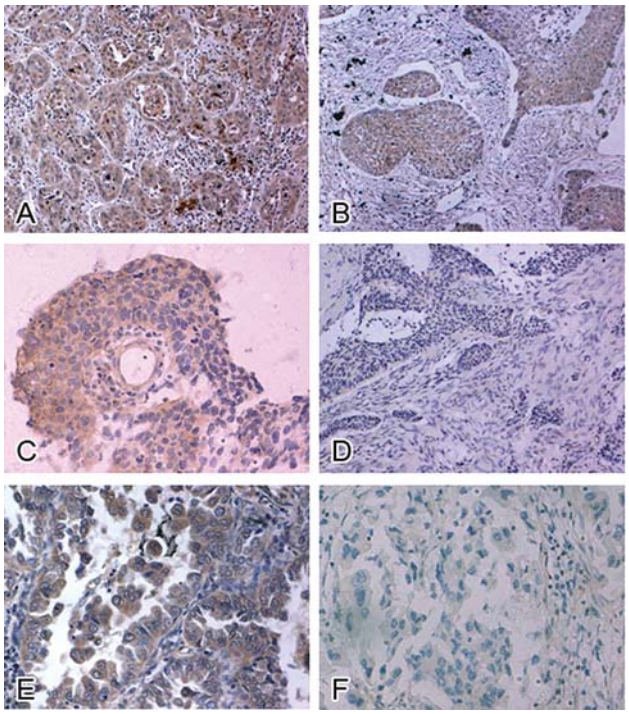

Figure 1. *Representative immunohistochemical staining and in situ hybridization histochemistry (A-F) showing KISS1 protein and mRNA expressions in different pathological types and TNM stages of non-small cell lung cancer (×200). KISS1 protein expression in squamous carcinoma, A: stage I and B: stage III; KISS1 mRNA expression in squamous carcinoma, C: stage I and D: stage III, and in adenocarcinoma, E: stage I and F: stage III.*

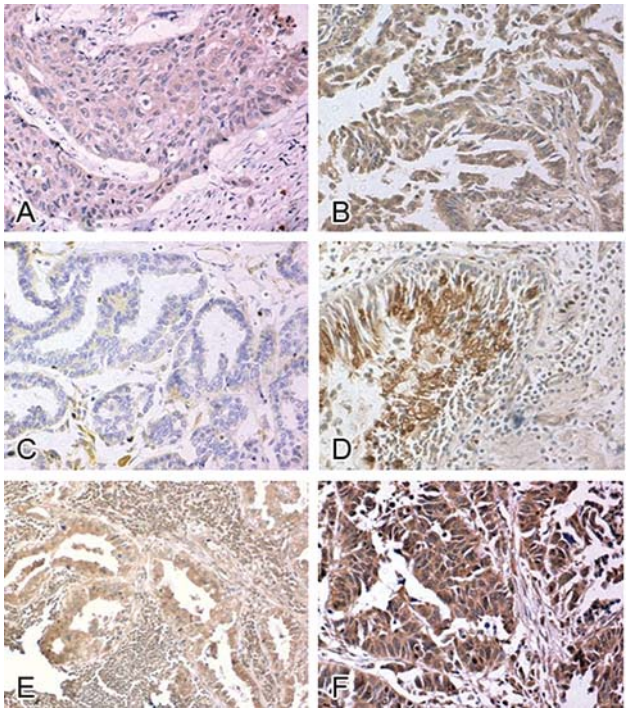

Figure 2. *Representative immunohistochemical staining and in situ hybridization histochemistry (A-F) showing MMP-9 protein expressions in different pathological types and TNM stages of non-small cell lung cancer (×200). A: Squamous carcinoma stage I; B: squamous carcinoma stage III; C: adenocarcinoma stage I; D: adenocarcinoma stage III; E: metastasized lymph node; F: primary lung carcinoma.*

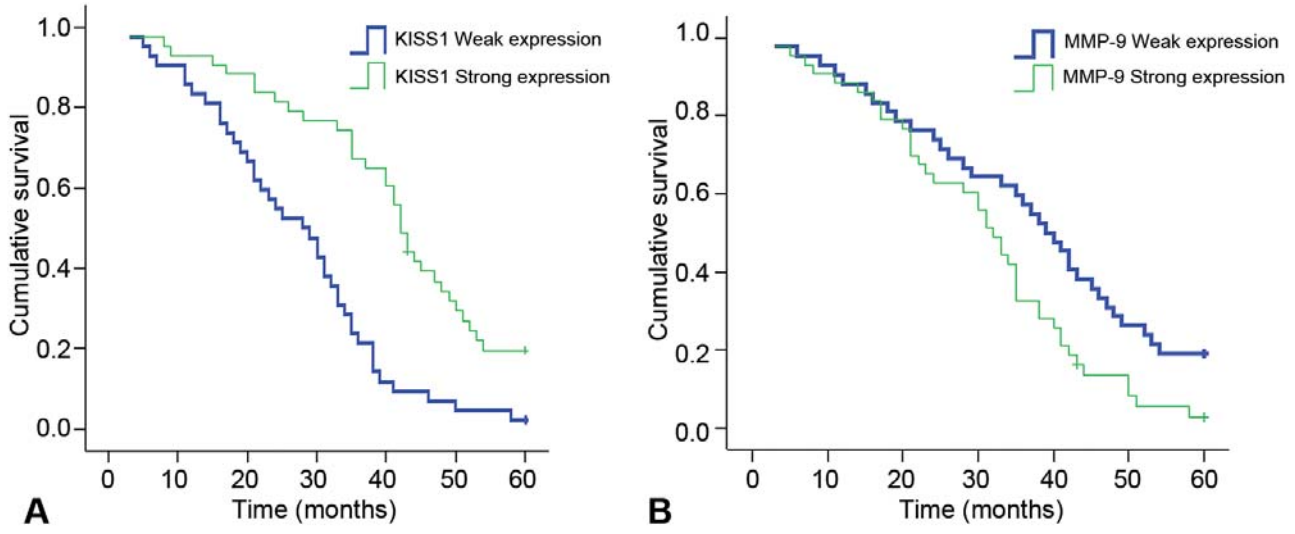

Figure 3. *Kaplan-Meier survival curves according to the levels of KISS1 (left) and MMP-9 expression (right).*

of MMP-9 expression was found in cases with low stages of NSCLC (I-II). These patients had a 5-year survival rate of 19.0%. In contrast, in patients with advanced stage disease (III-IV), MMP-9 had a high expression and the 5-year survival rate was 4.7%. The differences in the 5-year survival rates between the two groups were significant ($p<0.05$) (Figure 3, right panel).

Discussion

We analyzed the differential expression of *KISS1* and MMP-9 in 85 cases of NSCLC and their matched lymph node metastasis and found that the expression of these markers correlated with pathological stage, lymph node metastasis and survival. There was an inverse relationship between of KISS1 and MMP-9 expressions. *KISS1* expression was significantly higher in stage I-II disease compared to stage III-IV disease, indicating an inverse correlation between *KISS1* expression and NSCLC progression. Furthermore, *KISS1* expression was significantly higher in the primary tumors compared to the secondary metastatic site, supporting the notion that *KISS1* functions as a metastasis suppressor in NSCLC. Our findings suggest that *KISS1* and MMP-9 may serve as important biomarkers for assessing metastatic potential and prognosis in NSCLC patients.

The exact mechanism(s) of down-regulation of *KISS1* in the process of metastasis is not known. Loss of heterozygosity (LOH) in the chromosome region 6q16.3-q23 correlated significantly with down-regulation of *KISS1* expression, supporting a regulatory function of chromosome 6 on *KISS1* expression (15). Interestingly, LOH on chromosome 6q16.3 has been identified in some cases of NSCLC, suggesting a similar role in regulating *KISS1* expression in lung cancer. Down-regulation of *KISS1* was associated with cancer progression and metastasis. Dhar *et al*. found that gastric tumors with low *KISS1* expression frequently had distant metastasis and tumor recurrence (11). Furthermore, they found that patients with tumors expressing a low level of *KISS1* had a significantly worse overall survival. In another study, high *KISS1* expression was found to be an independent prognostic indicator for disease-free and overall survival in patients with hepatocellular carcinoma (14).

The role of MMP-9 in degradation of extracellular matrix and promoting angiogenesis and growth of metastatic tumor cells is well established (6). In this study, we found that MMP-9 expression increased with tumor size and that its expression was significantly higher in NSCLC cases with metastasis compared to those without metastasis. In addition, MMP-9 expression was significantly higher in lymph node metastasis than primary lesions. Taken together, these results support the notion that MMP-9 plays an important role in tumor growth and metastasis of NSCLC.

We also found an inverse relationship between *KISS1* and MMP-9 protein expression. These data suggest that *KISS1* has an inhibitory effect on the metastatic process of NSCLC, while MMP-9 has a metastasis-promoting role. An imbalance between expressions of these two proteins appears to be an important cause of NSCLC spread. The survival rate in patients with low *KISS1* expression was lower than in those patients with high *KISS1* expression, whereas low MMP-9 expression indicated a favorable prognosis. Therefore, assessing the *KISS1* and MMP-9 expressions adds important prognostic information for lung cancer patients.

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