Expression of KISS1 and KISS1R (GPR54) may be used as favorable prognostic markers for patients with non-small cell lung cancer

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Abstract. Lung cancer is the most commonly diagnosed cancer worldwide. Loss of KISS1 expression has been associated with progression and poor prognosis of various cancers, however, the precise role of KISS1 expression in non-small cell lung cancer (NSCLC) is not well defined. KISS1 receptor (KISS1R, also named GPR54) coupled to KISS1, has been shown to play a pivotal role in suppressing cancer metastasis. In this study, 56 NSCLC specimens were divided into stage IIIB (locally advanced) and stage IV (metastatic). The mRNA and protein levels of KISS1 and KISS1R in cancer tissues were found to be lower compared to that in normal tissues using RT-PCR and western blot analysis, respectively. In addition, the expression of both KISS1 and KISS1R in stage IV NSCLC was lower compared to that in stage IIIB stage NSCLC. The cumulative survival rate of the patients with KISS1 or KISS1R expression was significantly higher compared to that without expression. KISS1 or KISS1R expression in NSCLC can be used to indicate favorable prognosis for disease outcome. Metastin, the product of the *KISS1* gene, was lower in the serum of patients with stage IV NSCLC compared to that in stage IIIB NSCLC.

Introduction

Lung cancer was the most commonly diagnosed cancer as well as the leading cause of cancer death in males in 2008 globally (1). Male lung cancer death rates are decreasing in most Western countries, including many European countries, North America and Australia (2). In contrast, lung cancer rates are increasing in countries such as China and several other countries in Asia and Africa (3,4). Although chemotherapy and radiation therapy have yielded modest improvements in patient outcomes, overall survival of lung cancer patients remains poor (5,6). Therefore, new therapeutic targets are urgently needed.

KISS1 was originally identified in melanoma by Lee *et al* (7) in experiments designed to identify the molecules responsible for the anti-metastatic effect of human chromosome 6. The *KISS1* gene is located on chromosome 1 near q32.1 with regulatory elements localized in chromosome 6 at 6q16.3-q23 (8). 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 (8). Associations between loss of KISS1 expression and increased tumor progression and poor prognosis were found in various solid tumors, such as pancreatic, breast, bladder, brain, epithelial ovarian and gastric cancer (9-14). KISS1 receptor (KISS1R, also named GPR54) coupled to kisspeptins, has been revealed to play a pivotal role for the onset of puberty and to suppress cancer metastasis (15-17). Kisspeptins regulate cell proliferation, migration, and invasion in different cell lines via KISS1R/GPR54 (18-20). The role of GPR54 in cancer has been difficult to discern. A recent study has shown that the expression of KISS1 and GPR54 correlates with breast tumor progression and poor patient prognosis (10). Ikeguchi *et al* (21) reported that overexpression of KISS1 and GPR54 was correlated with the progression of HCC. Zhang *et al* (22) and Hata *et al* (13) surveyed RNA expression of the KISS1 and GPR54 in ovarian cancer and observed a trend towards favorable prognosis where KISS1/GPR54 RNA expression is elevated.

To our knowledge, little information is known about KISS1 and KISSR1 expression in the Chinese Han people with non-small cell lung cancer (NSCLC). In this study, we investigated the expression of KISS1 and KISSR1 in 56 cases of NSCLC to determine the relationship between their expression levels and survival of lung cancer patients.

Materials and methods

Subjects. A total of 56 patients with NSCLC were obtained from Department of Thoracic Surgery, the First Affiliated Hospital of China Medical University (January 2006 to December 2010). Twenty-eight patients had stage IIIB NSCLC (locally advanced) and other 28 patients had stage IV NSCLC (metastatic) according to the International Association for the

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Study of Lung Cancer (IASLC) staging committee (23). All patients underwent standard laboratory tests (cytology and histology), confirmed computerized tomography of the thorax. None of the patients underwent radiotherapy or chemotherapy before operation. The study was approved by our University Ethics Committee and was conducted in accordance with the Helsinki Declaration. All patients gave their written informed consent to participate in the study.

RNA isolation and Reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was isolated using an RNeasy mini kit (Biomed; Beijing, China). First strand cDNA was reverse transcribed with 1 μg of total RNA, using Takara Reverse Transcription kit (Takara; Dalian, China) and oligo (dT) 15 primers (Takara). The *KISS1* primers were: 5'-TGAACTC ACTGGTTTCTTGGC-3' (forward primer) and 5'-CAGCCT GGCAGTAGCAGCT-3' (reverse primer). The *KISS1R* primers were: 5'-ATCTACGTCATCTGCCGCCAC-3' (forward primer) and 5'-TCACGTACCAGCGGTCCACAC-3' (reverse primer). The house-keeping gene, *GAPDH* was used as an internal control for normalization of the results. The *GAPDH* primers were: 5'-AGAAGGCTGGGGCTCATTTG-3' (forward primer) and 5'-AGG GGCCATCCACAGTCTTC-3' (reverse primer). PCR amplification of cDNA was performed in 20 μl mixtures. Finally, amplicons were electrophoresed in 2% agarose gel with ethidium bromide and visualized under UV illumination.

Methylation-specific PCR (MSP). Genomic DNA was extracted from lung cancer specimens using a TissueGen DNA kit (CWbiotech; Beijing, China). Genomic DNA (2 μg) was denatured with 0.2 M NaOH. Then, 10 mM hydroquinone (Sigma-Aldrich, St. Louis, MO, USA) and 3 M sodium-bisulfite (Sigma-Aldrich) were added. The solution was incubated at 55˚C for 16 h. DNA samples were then purified using a WizardDNA purification resin (Promega; Madison, WI, USA). In this procedure unmethylated (but not methylated) cytosines can convert to uracil, which is then converted to thymidine during subsequent PCR to give sequence differences between methylated and unmethylated DNA. The modified DNA was used as a template both for MSP and USP. The primer sequences for the methylated *KISS1* gene were: 5'-CGGGTTGGAAGT TTTAGC-3' (forward primer) and 5'-GCTTCGACAAACGA AAAAC-3' (reverse primer), and for the unmethylated allele were: 5'-TTTTGGGTTGGAAGTTTTAG-3' (forward primer) and 5'-ACTTCAACAAACAAAAAACAAC-3' (reverse primer). The PCR products were separated in 2% agarose gel with ethidium bromide and visualized under UV illumination.

Western blot analysis. Tissues were lysed in lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100) containing a protease inhibitor cocktail (Sigma-Aldrich). Extract protein amounts were quantified using the BCA protein assay kit (CWbiotech). Equivalent amounts of protein (40 μg) were separated using 10% SDS-PAGE and transferred to a PVDF membrane (Millipore Corporation; Billerica, MA, USA). Western blot analysis was performed using primary antibodies: KISS1 (sc-15400), KISS1R (sc-134499) and β-actin (sc-130657, Santa Cruz Biotechnology; Santa Cruz, CA, USA). Each specific antibody binding was detected with horseradish peroxidase (HRP)-conjugated respective secondary antibodies (Amersham Biosciences; Amersham, UK) and ECL solutions (Amersham Biosciences).

Immunohistochemical staining for KISS1 and KISS1R. 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 (sc-101246). For the KISS1R protein, sections were incubated with mouse anti-human KISS1R monoclonal antibody (H-048-61, Phoenix Pharmaceuticals; Burlingame, CA, USA). The sections were then incubated with biotinylated secondary antibodies and visualized by DAB. Counterstaining was carried out with hematoxylin. The sections were dehydrated in alcohol and coverslipped. For the negative controls, PBS replaced the primary antibody.

Blood samples and ELISA for plasma metastin. Plasma levels of metastin were measured by ELISA, by the methods of Katagiri *et al* (24). Baseline metastin serum levels were measured in all patients. All blood samples were collected between 08.00 and 10.00 in the morning. Blood samples for metastin were collected before surgery, placed in a chilled tube containing aprotinin (500 KIU/ml) and EDTA (1.2 mg/ml), and immediately centrifuged at 3,000 rpm for 20 min. Circulating serum metastin was determined using a sandwich enzyme immunoassay (CSB-EL012373HU, Cusabio Life Science, Wuhan, China) with a sensitivity of 0.078 ng/ml, while the variations of intra-assay precision (precision within an assay) and inter-assay precision (precision between assays) were less than 8 and 10%, respectively, according to the manufacturer.

Cell cycle and apoptosis analysis. Cancer samples were subjected to chemical digestion by incubating with 0.5% pepsin at 37˚C in water bath for 30 min with intermittent stirring. Disaggregated tissues were filtered through a 50 μM nylon mesh. Cells were collected in PBS and fixed on ice with 1% paraformaldehyde, followed by 70% cold ethanol containing 10 μg/ml RNase. Then the cells were stained with 50 μg/ml propidium iodide (PI, KeyGen; Nanjing, China) for 15 min at room temperature for cell cycle analysis. The apoptotic cells were detected with Annexin V-FITC/PI double staining. Following the manufacturer's instructions for the Apoptosis Assay kit (KeyGen), the stained cells were analyzed by flow cytometry. Data analysis was performed with CellQuest software (BD Biosciences; Rockville, MD, USA).

Statistics and survival analysis. Association between KISS1 or KISS1R expression and clinical covariates was assessed univariately. Overall survival rates were determined using the Kaplan-Meier estimator. Kaplan-Meier survival plots were generated and comparisons were made with log-rank statistics. Cox proportional hazard model was used to identify significant factors correlated with prognosis in multivariate analysis. For all analyses, only $P<0.05$ were considered significant. All the statistical analyses and graphics were performed with GraphPad Prism 5.

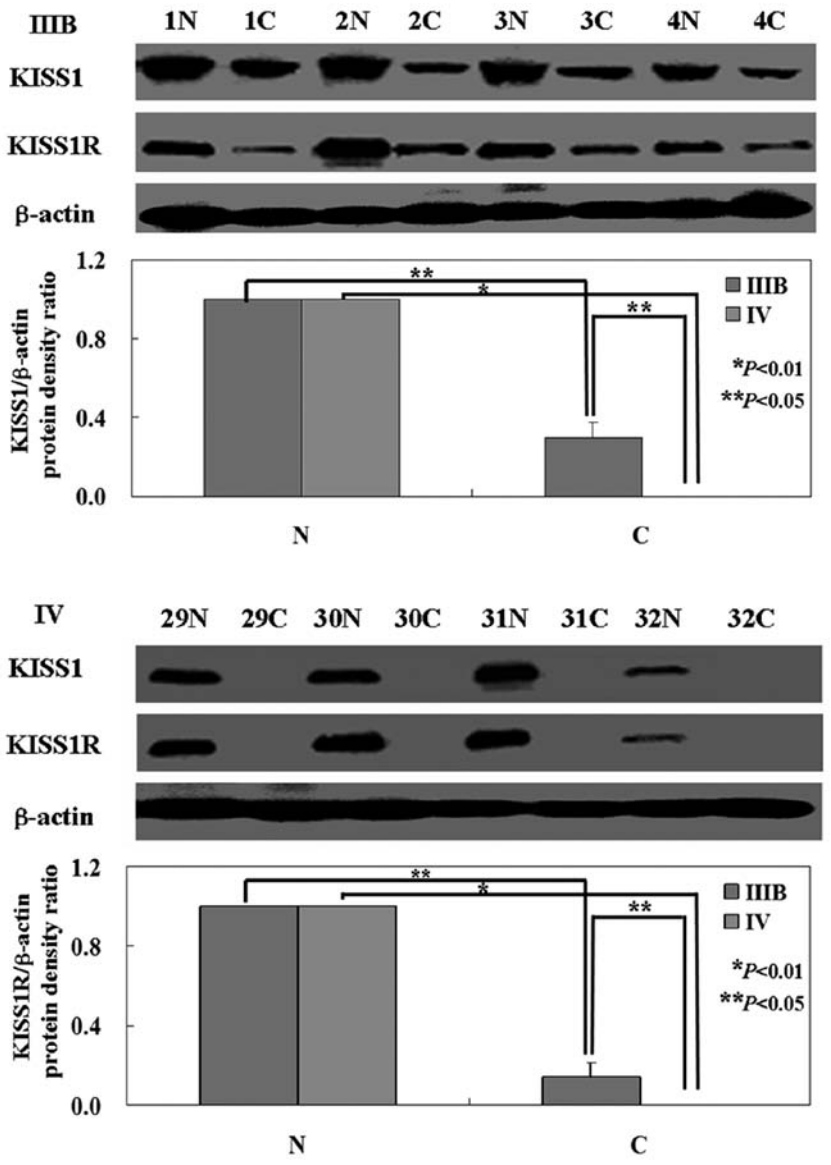

Figure 1. Representative results of two paired lung cancer and corresponding normal tissue by western blot analysis with anti-KISS1 antibody and anti-KISS1R antibody, respectively. KISS1 and KISS1R protein expression was lower in cancer tissues than matched normal tissues (P<0.05). β-actin was used as an internal control. N, normal; C, cancer.

Results

KISS1 protein expression relative to the clinical and pathological variables. The levels of KISS1 mRNA and protein in tumor tissue were both lower than that in normal tissue (P<0.05; Figs. 1 and 2) by using western blot analysis and RT-PCR. Interesting, KISS1 expression was higher in the low stage of NSCLC (IIIB) compared to advanced stage (IV) (P<0.05; Figs. 1 and 2). Fifty-six tumor samples were examined for KISS1 methylation by MSP. Representative examples are illustrated in Fig. 3. We found a correlation between CpG island KISS1 promoter methylation and downregulated KISS1 mRNA levels in tumor samples. The immunostaining results showed that KISS1 expression was distributed in the cytoplasm of normal lung cells and lung cancer cells (Fig. 4). Consistent with the results of western blot analysis, the results of immunostaining also showed that KISS1 protein was weakly expressed in lung cancer specimens, but highly in normal parts of specimens. There was a significant difference in KISS1 expression between the low stage of NSCLC (IIIB) compared to advanced stage (IV). We then analyzed the potential relationship between the expression of KISS1 and the clinicopathological characteristics of these patients. The levels of KISS1 protein had no relation to patient age or sex, tumor size, lymphatic invasion, pN category, venous invasion or histological type (P>0.05; Table I). However, in addition, KISS1 expression was associated with the differentiation of the patients with stage IV NSCLC (P<0.05; Table I).

KISS1R protein expression relative to the clinical and pathological variables. RT-PCR and western blot analysis were carried out to investigate the levels of KISS1R mRNA and protein in NSCLC specimens. As shown in the results, the levels of KISS1R mRNA and protein were lower in tumor

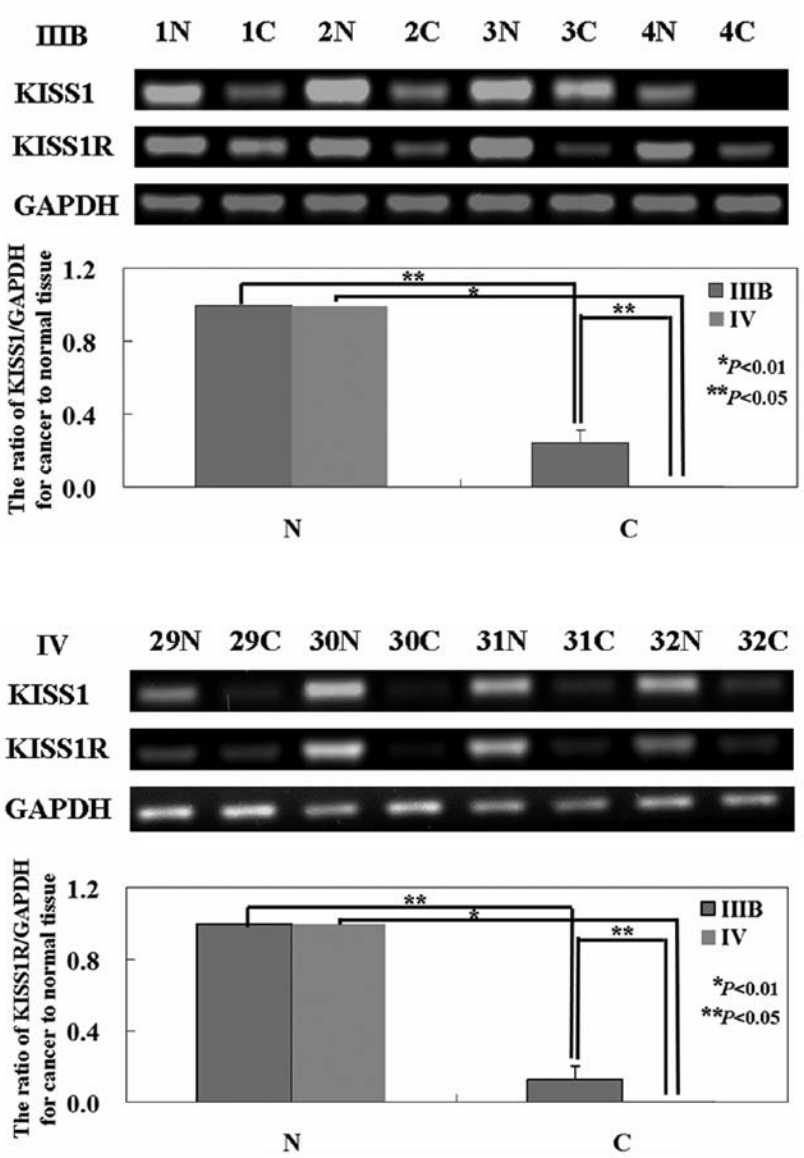

Figure 2. The levels of *KISS1* and *KISS1R* mRNA were measured in specimens using RT-PCR. The levels of *KISS1* and *KISS1R* mRNA were lower in cancer tissues than matched normal tissues (P<0.05). *GAPDH* was used as an internal control. N, normal; C, cancer.

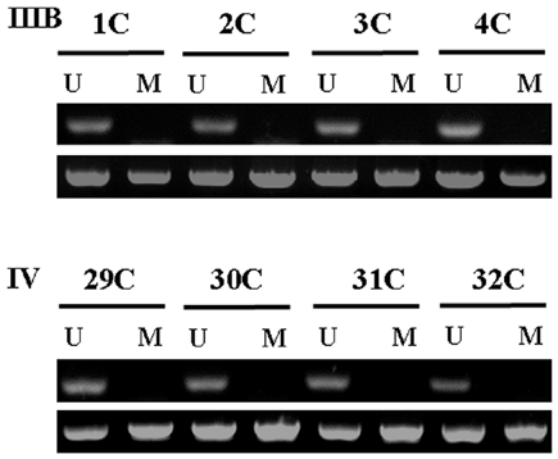

Figure 3. Methylation-specific PCR (MSP) analysis of the CpG island methylation status of the KISS1 promoter region in specimens. PCR products specific for unmethylated (U) and methylated (M) CpG sites were analyzed in 2% agarose gel.

tissue than that in normal tissue (P<0.05; Figs. 1 and 2). The levels of KISS1R mRNA and protein were higher in cancer tissues from stage IIIB NSCLC than that from stage IV NSCLC (P<0.05; Figs. 1 and 2). The immunostaining results showed that KISS1R expression was distributed to the cytomembrane (Fig. 4). However, KISS1R expression showed no correlation with the clinical and pathological variables of the patients (P>0.05; Table II).

Kaplan-Meier survival analysis and Cox proportional hazard analysis. To investigate the association of KISS1 expression or KISS1R expression with patient survival, the survival data from 50 patients with NSCLC (6 missing follow-up) were assessed. Comparison between KISS1 expression and 5-year survival rates showed significant differences. In patients with stage IIIB and IV NSCLC, comparison by the Kaplan-Meier method for low versus high KISS1 expression showed a significant difference in the 5-year survival rate (P<0.05;

Table I. Relationship between KISS1 expression and clinicopathological parameters of patients with stage IIIB and IV NSCLC.

Clinicopathological features	KISS1 expression in stage IIIB						KISS1 expression in stage IV					
	n	-	+	PR (%)	χ^2	P-value	n	-	+	PR (%)	χ^2	P-value
Sex					1.04	0.058					1.56	0.325
Female	8	5	3	37.5			10	7	3	30.0		
Male	20	17	3	15.0			18	16	2	11.1		
Age (years)					0.11	0.251					0.22	0.264
<50	11	9	2	18.2			8	7	1	12.5		
≥50	17	13	4	23.5			20	16	4	20.0		
Differentiation					0.84	0.162					5.38	0.027
Well or moderate	9	8	1	11.1			6	3	3	50.0		
Poor	19	14	5	26.3			22	20	2	9.1		
Lymphatic invasion					0.85	0.146					0.17	0.435
-	14	10	4	28.6			19	16	3	15.8		
+	14	12	2	14.3			9	7	2	22.2		
Venous invasion					1.77	0.235					0.65	0.237
-	12	8	4	33.3			18	14	4	22.2		
+	16	14	2	12.5			10	9	1	10.0		
Histological type					1.87	0.215					2.17	0.073
Squamous cell	9	8	1	11.1			9	8	1	11.1		
Adenocarcinoma	8	5	3	37.5			9	6	3	33.3		
Small cell	11	9	2	18.2			10	9	1	10.0		
Tumor size					0.04	0.487					1.10	0.081
<3 cm	15	12	3	20.0			17	15	2	11.8		
≥3 cm	13	10	3	23.1			11	8	3	27.3		
pN category					0.57	0.279					1.33	0.067
pN0	6	5	1	16.7			6	5	1	16.7		
pN1	7	6	1	14.3			7	6	1	14.3		
pN2	8	6	2	25.0			6	4	2	33.3		
pN3	7	5	2	28.6			9	8	1	11.1		

NSCLC, non-small cell lung cancer; PR, positive rate; χ^2, chi-square distribution.

Fig. 5A). Comparisons between KISS1R expression and the 5-year survival rate also showed significant differences. Kaplan-Meier analysis showed that KISS1R expression was closely correlated with the favorable prognosis of patients with NSCLC (P<0.05; Fig. 5B). Cox proportional hazard analysis indicated that KISS1 and KISS1R were independent prognostic factors for stage IIIB and IV NSCLC (P<0.05; Tables III and IV).

Correlations of metastin serum levels at diagnosis. There was significant correlation between serum metastin levels in patients and the stages of NSCLC. When we examined separately the patients with NSCLC stage IIIB and the patients with stage IV NSCLC, a statistically significant difference was observed between circulating metastin levels (P<0.05; Fig. 6). The plasma level of metastin in patients with stage IIIB NSCLC ranged from 0.53 to 2.1 ng/ml (mean, 1.07±0.08 ng/ml) and the mean plasma level of metastin in patients with stage IV NSCLC was 0.24±0.03 ng/ml.

The assessment of tumors by flow cytometry. We compared the apoptotic percentage of cells collected from stage IIIB and stage IV NSCLC tissues by using Annexin V-FITC and propidium iodide (PI) double staining. As shown in Fig. 7A, the percentage of apoptotic cells in stage IIIB was 2.39±0.42%, whereas 0.32±0.03% of cells in stage IV were undergoing apoptosis (P<0.05). Cells from stage IIIB cancer tissue had a higher ratio in the G_1 phase than the ones from stage IV cancer tissue. The mean value of G_1 phase fraction of stage IIIB and stage IV cells was 55.3±5.7 and 34.3±5.8%, respectively (P<0.05, Fig. 7B).

Discussion

Previous reports showed that the KISS1/KISS1R system plays an important role in tumor progression in a wide variety of tumor types (8). However, to date, the presence and potential role of the KISS1/KISS1R system in NSCLC has not been reported yet. In this work, we analyzed the differential expres-

Table II. Relationship between KISS1R expression and clinicopathological parameters of patients with stage IIIB and IV NSCLC.

Clinicopathological features	KISS1R expression in stage IIIB						KISS1R expression in stage IV					
	n	-	+	PR (%)	χ^2	P-value	n	-	+	PR (%)	χ^2	P-value
Sex					0.39	0.657					0.41	0.425
Female	8	6	2	25.0			10	8	2	20.0		
Male	20	17	3	15.0			18	16	2	11.1		
Age (years)					0.95	0.386					0.03	0.827
<50	11	10	1	9.1			8	7	1	12.5		
≥50	17	13	4	23.5			20	17	3	15.0		
Differentiation					0.41	0.648					0.04	0.732
Well or moderate	9	8	1	11.1			6	5	1	16.7		
Poor	19	15	4	21.1			22	19	3	13.6		
Lymphatic invasion					0.24	0.835					0.11	0.242
-	14	11	3	21.4			19	16	3	15.8		
+	14	12	2	14.3			9	8	1	11.1		
Venous invasion					3.43	0.175					0.23	0.185
-	12	8	4	33.3			18	15	3	16.7		
+	16	15	1	6.25			10	9	1	10.0		
Histological type					0.56	0.464					0.69	0.095
Squamous cell	9	8	1	11.1			9	8	1	11.1		
Adenocarcinoma	8	6	2	25.0			9	7	2	22.2		
Small cell	11	9	2	18.2			10	9	1	10.0		
Tumor size					0.45	0.341					0.22	0.641
<3 cm	15	13	2	13.3			17	15	2	11.8		
≥3 cm	13	10	3	23.1			11	9	2	18.2		
pN category					0.41	0.379					0.13	0.879
pN0	6	5	1	16.7			6	5	1	16.7		
pN1	7	6	1	14.3			7	6	1	14.3		
pN2	8	6	2	25.0			6	5	1	16.7		
pN3	7	6	1	14.3			9	8	1	11.1		

NSCLC, non-small cell lung cancer; PR, positive rate; χ^2, chi-square distribution.

sion of KISS1 and KISS1R in 28 patients with stage IIIB NSCLC and 28 patients with stage IV NSCLC and found that KISS1 and KISS1R expression was higher in stage III disease compared to stage IV disease. The results indicated an inverse correlation between KISS1 and KISS1R expression and NSCLC progression. KISS1 was originally identified as a metastasis suppressor by microcell-mediated transfer in melanoma lines, responsible for tumor cell invasive and migratory properties without affecting their tumourigenicity (25). Loss of KISS1 expression was found to be a significant predictor and a potential biomarker of lymph node metastasis in esophageal squamous cell carcinoma (26). Dhar *et al* (27) found that gastric cancers with low KISS1 had a frequent venous invasion, distant metastasis and tumor recurrence. Schmid *et al* (28) studied the expression of *KISS1* gene in HCC and its role in invasion, metastasis and prognosis of human HCC by immunohistochemistry. Another study showed that KISS1 expression in NSCLC was significantly higher in the primary tumors compared to the secondary metastatic site (29). However, in our study, we did not find the relationship between KISS1 and metastasis of NSCLC. Consistent with our study, Karapanagiotou *et al* (30) found that KISS1 is not involved in metastatic potential of non-small cell lung cancer. Data from our study suggest that KISS1 is less likely to serve as a diagnostic marker for NSCLC or metastatic disease. Possible explanations for our findings could be the role of KISS1 seems to be different in different types of cancer. Another explanation could be either the lack of KISS1 expression in lung tissue or the lack of KISS1R expression. In an attempt to uncover the mechanisms by which KISS1 is lost in NSCLC, we tested the promoter hypermethylation of *KISS1*. Consistent with our results, Cebrian *et al* (31) found that *KISS1* hypermethylation was frequent in bladder cancer cells analyzed by methylation-specific PCR and bisulfite sequencing and was associated with low gene expression. Another study showed that *KISS1* hypermethylation correlated with transcript and protein expression loss, being increased *in vitro* by azacytidine (32).

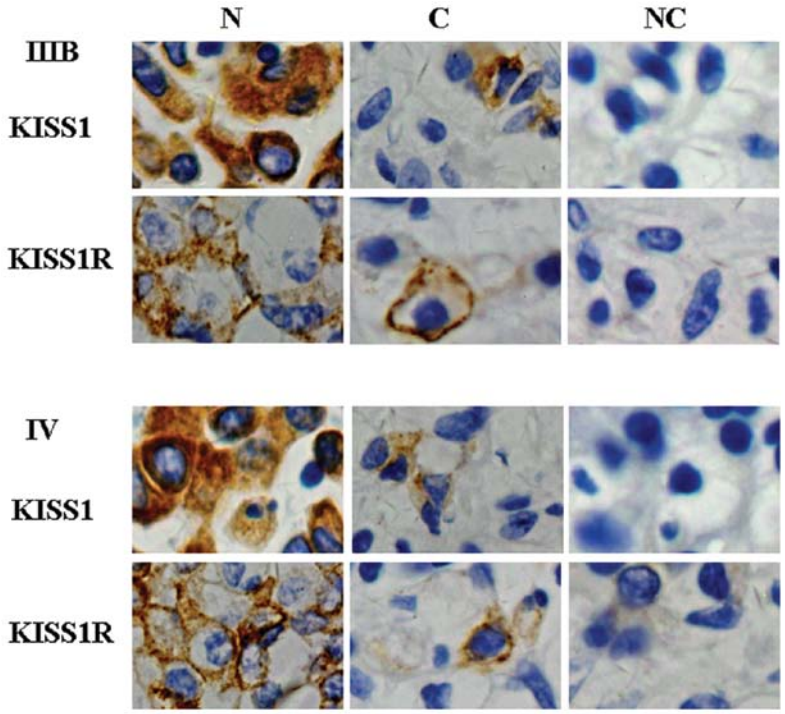

Figure 4. Immunohistochemical staining for KISS1 and KISS1R protein in specimens. The nuclei were counterstained with hematoxylin. N, normal; C, cancer; NC, negative control.

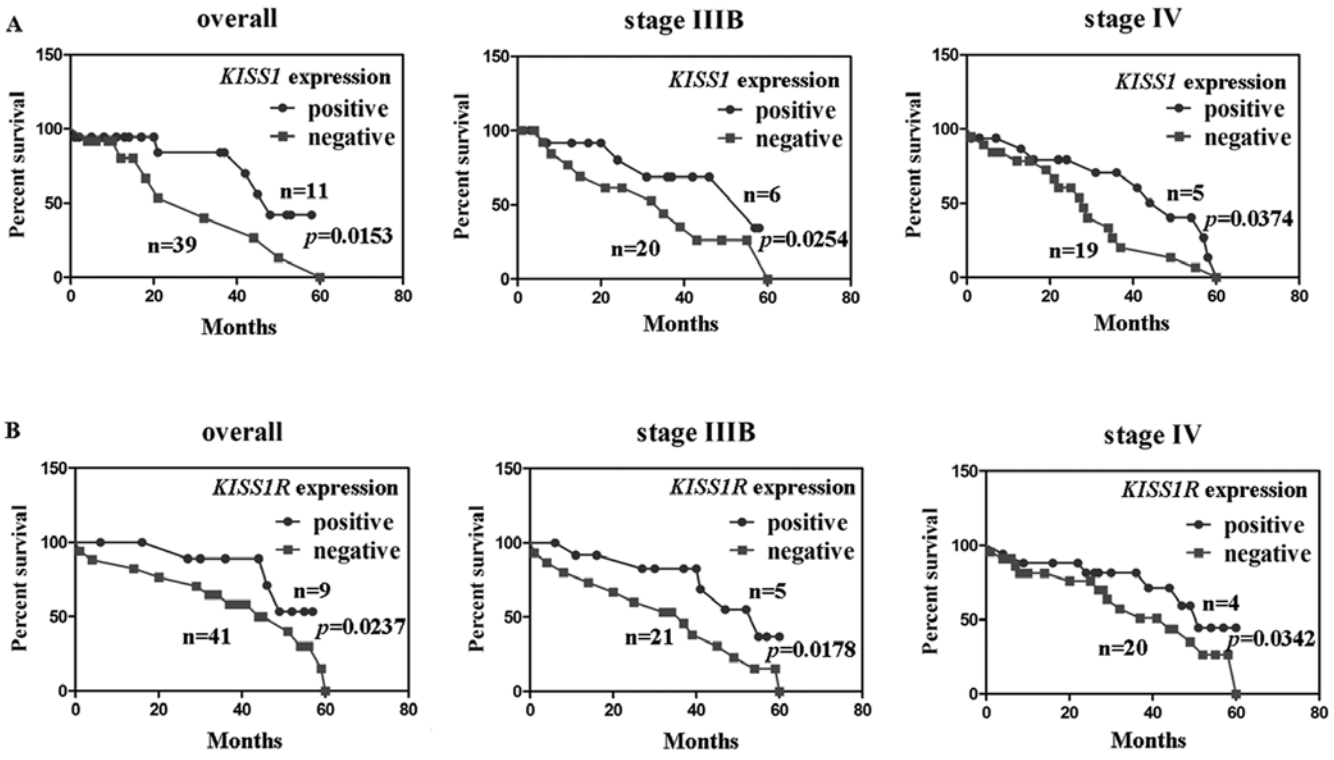

Figure 5. KISS1 or KISS1R protein and prognosis of the patients with NSCLC. Kaplan-Meier curve survival analysis indicating that tumors without (A) KISS1 or (B) KISS1R expression had poorer disease specific survival than those with KISS1 or KISS1R expression.

KISS1R is a G protein-coupled receptor with a common structure of seven transmembrane α-helices (33). The binding of KISS1 results in conformation of KISS1R, which leads to signalling via G-proteins and downstream effectors to prevent invasion and metastasis (17,34). Martin *et al* (10) found that GPR54 expression increased in the invasive

Table III. Multivariate analysis of clinical variables for stage IIIB and IV NSCLC.

Clinicopathological parameters	KISS1 expression in stage IIIB		KISS1 expression in stage IV	
	Relative risk (95% CI)	P-value	Relative risk (95% CI)	P-value
Sex (male)	0.683 (0.45-1.05)	0.224	0.498 (0.33-0.77)	0.407
Age (>50 years)	0.727 (0.48-1.12)	0.231	0.712 (0.47-1.10)	0.236
Differentiation	0.654 (0.43-1.01)	0.342	0.622 (0.41-0.96)	0.325
Lymphatic invasion	0.715 (0.47-1.10)	0.267	0.643 (0.42-0.99)	0.281
Venous invasion	0.592 (0.39-0.91)	0.453	0.518 (0.34-0.80)	0.463
Lymph node metastasis	0.788 (0.52-1.21)	0.231	0.678 (0.44-1.05)	0.354
Tumor size (≥3 cm)	0.573 (0.37-0.88)	0.463	0.624 (0.41-0.96)	0.442
KISS1 expression (+ to +++)	0.894 (0.58-0.38)	0.045	1.122 (0.73-1.73)	0.038
KISS1R expression (+ to +++)	0.921 (0.60-1.42)	0.037	1.352 (0.88-2.09)	0.023

CI, confidence interval.

Table IV. Comparison of the NSCLC patients with positive for metastin, and those negative, by using ELISA.

Clinicopathological features	Positive for metastin (n=11)	Negative for metastin (n=45)	P-value
Age (years)	60.3±6.7	58.2±6.2	0.467
Sex			0.432
Female	4	14	
Male	7	31	
Differentiation			0.162
Well or moderate	5	10	
Poor	6	35	
Lymphatic invasion			0.321
-	6	27	
+	5	18	
Venous invasion			0.154
-	2	28	
+	9	17	
Histological type			0.752
Squamous cell	3	15	
Adenocarcinoma	4	13	
Small cell	4	17	
Tumor size			0.534
<3 cm	4	28	
≥3 cm	7	17	
pN category			0.576
pN0	2	10	
pN1	1	13	
pN2	4	10	
pN3	4	12	
Stage			0.034
IIIB	9	19	
IV	2	26	

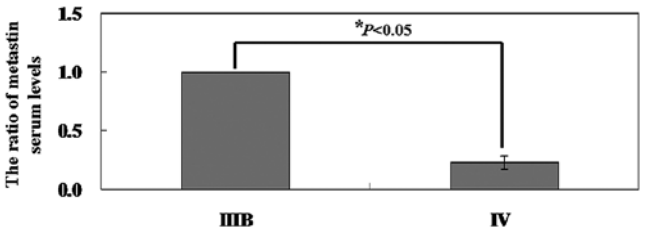

Figure 6. Plasma metastin levels were detected by using ELISA. There was a significant difference between the patients with stage IIIB NSCLC and the patients with stage IV NSCLC (P<0.05).

ductal tumor. Higher levels of GPR54 mRNA were observed in the moderately differentiated tumors compared to the poorly differentiated high-grade cancers (35).Consistent with previous studies, we confirmed that the KISS1R was lower in low stage of NSCLC (IIIB) compared to advanced stage (IV). Another study conducted by Zajac *et al* (36) suggested that KISS1/GPR54 signaling is pro-migratory and invasive in breast cancer cells. However, in our study, we did not find the relationship between KISS1R expression and metastasis of NSCLC. Most studies have shown that the KISS1/GPR54 system is negatively correlated with tumor progression. The prognostic relevance of KISS1 and GPR54 has been investigated in some solid tumors (9-17). We also confirmed the KISS1 or KISS1R expression was closely correlated with the favorable prognosis of patients with NSCLC.

The bioactive sequence of the KISS-1 gene product metastin is the C-terminal 10 amino acids (37). Metastin was initially purified from human placenta (17). An enormous increase in circulating metastin levels has been detected during pregnancy (38). No statistically significant difference in circulating metastin levels was observed between healthy volunteers and patients with resectable pancreatic cancer (39). Data from Karapanagiotou *et al* study suggested that metastin is less likely

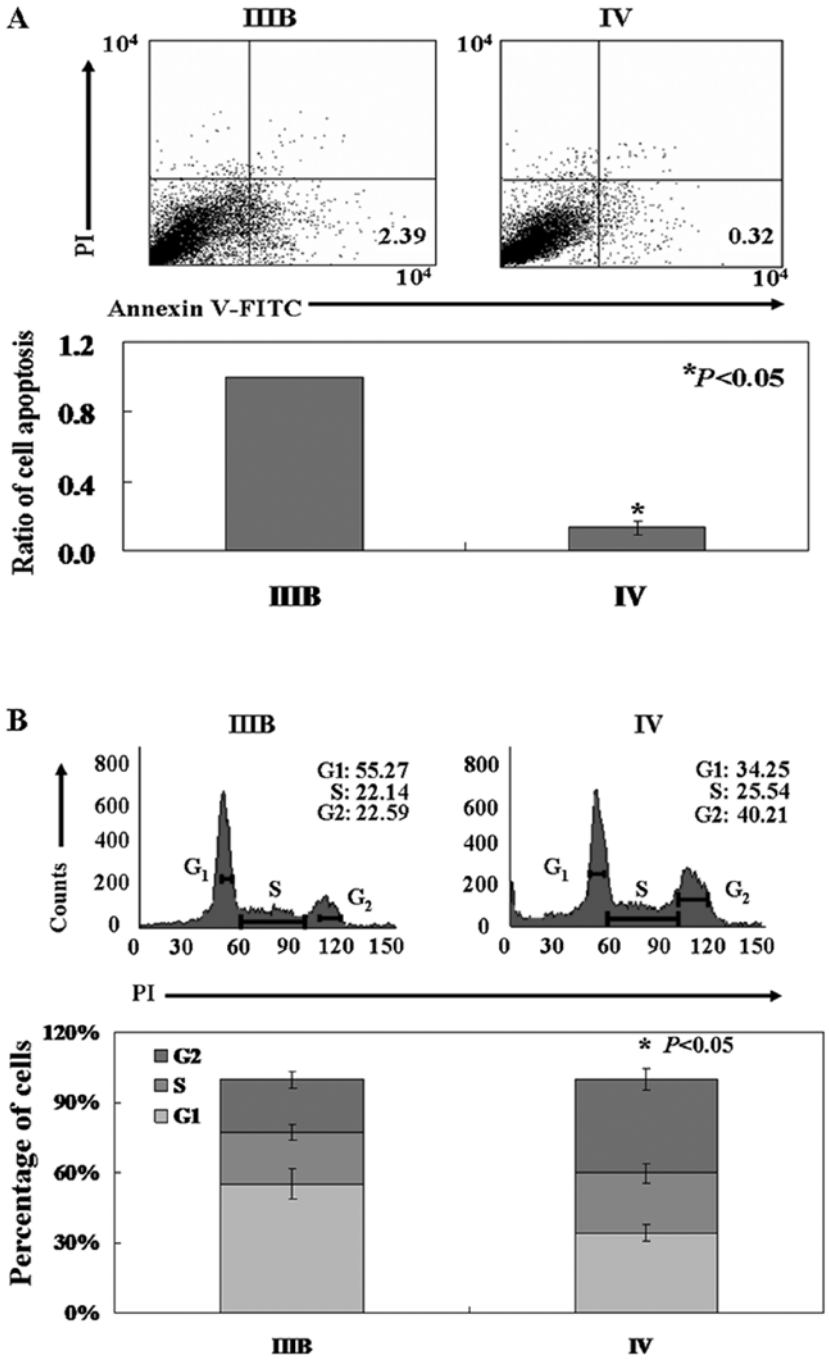

Figure 7. The assessment of NSCLC stage IIIB and IV tissue by flowcytometry. (A) Apoptotic ratio of NSCLC stage IIIB and IV cells was determined from Annexin V/PI double-staining assays. (B) Propidium iodide staining showed changes in the cell cycle. The histogram shows the results have statistical significance (P<0.05).

to serve as a diagnostic marker for NSCLC or metastatic disease (30). However, in our study, we confirmed the plasma metastin levels of the patients with stage IIIB NSCLC were significantly higher than that of patients with stage IV NSCLC. The possible explanation for the difference between Karapanagiotou *et al* results (30) and ours could be that the subjects derived from different ethnic groups. In our future studies, we will further investigate this problem.

We confirmed cells collected from stage IIIB tumor had higher apoptotic ratio than the ones from stage IV tumor specimens. In addition, we also found the KISS1/GPR54 system could induce G_1 arrest in lung cancer cells. These results indicated that the KISS1/GPR54 system may not only inhibit invasion and migration of cancer cells but also induce apoptosis and cell cycle arrest in the cells.

In conclusion, the expression of KISS1 or KISS1R was associated with better survival of patients with NSCLC. KISS1 hypermethylation was identified in cancer tissues, providing a potential mechanistic explanation for the observed loss of KISS1. Furthermore, the serum metastin level could become an independent prognostic tool for the Chinese Han people with NSCLC.

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