

Increased Midkine and Estrogen Receptor- β Expression in Human Non-Small Cell Lung Cancer

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ABSTRACT

Objective: Midkine (MK), a new member of the heparin-binding growth factor family, has been found recently to have a high expression level in many tumor specimens including lung carcinoma. Estrogens may be involved in lung carcinogenesis, and estrogen receptors, mainly estrogen receptor- β (ER- β), are present and functional in normal lung and tumor cell lines and tissues. In addition, estrogens and growth factors may promote the progression of human non-small cell lung cancer (NSCLC). Previously, we have immunohistochemically demonstrated that MK and ER- β proteins were overexpressed in NSCLC and their expression levels were both significantly negatively correlated with the pathological classification. The purpose of this study was to further verify their expression and its correlation with NSCLC.

Methods: Taking NSCLC tissues and their corresponding paraneoplastic and normal lung as research objects, we further examined the expression of MK and ER- β by means of RT-PCR, *in situ* hybridization and Western blot analyses at the levels of messenger RNA (mRNA) and protein, respectively.

Results: The increased MK and ER- β mRNA expression was found in NSCLC by RT-PCR and *in situ* hybridization analyses. Furthermore, Western blot analysis also displayed increased expression of MK and ER- β proteins in NSCLC. Finally, their correlation analysis at the levels of mRNA and protein expression in NSCLC demonstrated that MK protein level was significantly correlated to estrogen receptor- β ($P < 0.01$, $r_s = 0.535$); in spite of their correlation at the mRNA level, there was no remarkable difference between MK and ER- β ($P > 0.05$, $r_s = 0.178$).

Conclusion: All these results in the present study confirmed that MK and ER- β were overexpressed in human NSCLC.

Key words: Midkine (MK); Estrogen receptor- β (ER- β); Non-small cell lung cancer (NSCLC)

INTRODUCTION

Lung cancer is the most common malignancy and the leading cause of cancer death worldwide^[1,2]. At present, there are few therapeutic options available for lung cancer patients.

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Midkine (MK) gene was found in the cDNA library constructed from HM-1 embryonal carcinoma cell line of mouse induced by retinoic acid^[3]. Researches have showed that MK protein is a multifunctional cytokine closely related with carcinogenesis and growth. Since earlier studies proposed that the main biological activities of MK were neurotrophic effects, MK was thought to be a neurotrophic factor to embryonic spinal cord and dorsal root ganglion neurons and to play a significant role in embryogenesis of the nervous system^[4]. However, further researches showed that MK had several cancer-related activities^[5], mainly including mitogenic, anti-apoptotic, transforming, fibrinolytic, chemotactic, and angiogenic ones. In 2006, Krzystek-

Korpicka et al^[6] presented up-to-date views on the biological activity of MK at the cell and tissue levels. In recent years, MK was found to express at high levels in a variety of human tumors^[7], and some studies indicated that MK was overexpressed in NSCLC and its expression level was much higher than that of normal lung tissue^[8-10].

Estrogens are pivotal in the growth and development of both normal and neoplastic mammary tissues^[11] and can act as tumor promoters through a receptor-mediated mechanism which increases cell proliferation^[12]. The role of estrogen in promoting tumors has been proven during breast and endometrial cancer development^[13]. At present, it is well known that the actions of estrogens are mainly mediated via estrogen receptors (ERs) and ERs are ligand-activated transcription factors that belong to the nuclear hormone receptor superfamily. Since 1996 two different ERs have been identified, which are now known as ER- α and ER- β ^[14]. Although the etiology of NSCLC is not fully defined, earlier work shows that estrogens as well as growth factors may promote its progression^[15]. Furthermore, Li et al^[16] found that ERs expression may be closely associated with the biological behaviors of NSCLC, and some studies demonstrated expression of only ER- β in human NSCLC^[17-19].

Nevertheless, the studies on the expression of MK and ER- β in NSCLC have been limited and the results are somewhat inconsistent. To our knowledge, no document has reported their correlation in NSCLC. To verify our previous immunohistochemical analysis, we further examined their expression in NSCLC using RT-PCR, *in situ* hybridization and Western blot analyses. Also, in this present study, their correlation analysis at the levels of mRNA and protein expression in NSCLC was performed.

MATERIALS AND METHODS

Patients and Tissue Specimens

The present research is retrospective and based on the histological materials collected from a cohort of 28 patients who underwent operation for NSCLC at the People's Hospital of Jiangsu Province (Nanjing, China). Some suitable histological materials were available from 24 patients with NSCLC who had not received chemotherapy, a range of age from 32 to 76 years (median, 62.5 years). The specimens were 72 lung tissues, including 24 cases of NSCLC tissues, and their corresponding paraneoplastic and normal lung tissues. Interview and biospecimen collection was initiated before the law involved was

implemented and each subject provided informed consent. These lung tissues were immediately frozen in liquid nitrogen for RNA isolation or freezing microtomy (Leica CM 1900). The clinicopathological data of these 24 patients with NSCLC are summarized in Table 1.

RNA Isolation

Total cellular RNA was extracted from about 100mg frozen tissues using TRIzol Reagent (BioFlux Corp, USA) according to the manufacturer's instructions. The concentration and purity of the isolated total RNA were determined with the spectrophotometer: $OD_{260}/OD_{280}=1.7-2.0$ was taken as the range of pure RNA.

First Strand cDNA Synthesis

With 1 μ g total RNA as template, the first strand of cDNA of each species was synthesized using RT Kit Reagent (TOYOBO Bio-Technology Co., Ltd., China) according to the manufacturer's instructions.

Polymerase Chain Reaction (PCR)

Human β -actin gene was chosen as a reference gene. The oligonucleotides used for amplification were summarized in Table 2. For MK, the conditions for PCR were as follows: pre-denaturation at 94°C for 30 s, 30 cycles of denaturation (94°C for 30 s), re-annealing (53°C for 30 s), and extension (72°C for 30 s), then kept at 72°C for 5 min. Correspondingly, the conditions for ER- β PCR were as follows: pre-denaturation at 94°C for 30 s, 31 cycles of denaturation (94°C for 30 s), re-annealing (46.5°C for 30 s), and extension (72°C for 30 s), then kept at 72°C for 5 min. Finally, 5 μ l PCR products were detected by 1% agarose gel electrophoresis with ethidium bromide (EB) staining. All assays were repeated at least three times and found to be reproducible.

In situ Hybridization Analysis of MK and ER- β

MK and ER- β antisense oligonucleotide probes were also summarized in Table 2, and synthesized respectively by Wuhan Boster Biological Technology, LTD. and Tianjin HaoYang Biological Manufacture CO., LTD, China. *In situ* hybridization was performed for 72 lung tissues, which was conducted according to the manufacturer's instructions with some changes. Briefly, for each specimen, 8- μ m-thick fresh frozen sections were prepared under sterile conditions. The sections were then fixed by 4% paraformaldehyde with 1/1000 DEPC for 10 min at room temperature and

digested by 1% pepsin for 20 min at 4°C, followed by pre-hybridization for 2 h for MK and for 1 h for ER- β at 37°C. After pre-hybridization, tissues sections were incubated with antisense oligonucleotide probes of MK overnight at 37°C and ER- β for 2 h at 37°C in an incubation chamber. After washed fully in standard saline citrate (SSC) solution, biotinylated mouse anti-digoxin primary antibody, alkaline phosphatase

(AP)-conjugated SABC solution and color substrate solution with 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/ NBT) were applied subsequently, followed by nuclear fast red for nuclear counterstaining for MK but not for ER- β . Negative control slides were incubated with respective pre-hybridization solution without MK or ER- β antisense oligonucleotide probe.

Table 1. Relationships between MK and ER- β mRNA expression levels by *in situ* hybridization analysis in NSCLC and clinicopathological data

Characteristic	Cases (n)	MK mRNA expression			ER- β mRNA expression		
		-	+	Positive (%)	-	+	Positive (%)
Sex							
Male	19	3	16	84.2	4	15	78.9
Female	5	3	2	40.0	1	4	80.0
Age (years)							
<65	11	4	7	63.6	2	9	81.8
\geq 65	13	2	11	84.6	3	10	76.9
Megascopic type							
Central	14	5	9	64.3	3	11	78.6
Peripheral	10	1	9	90.0	2	8	80.0
Tumor size (cm)							
<5	11	3	8	72.7	1	10	90.9
\geq 5	13	3	10	76.9	4	9	69.2
Histological type							
Squamous cell carcinoma	7	0	7	100	1	6	85.7
Adenocarcinoma	17	6	11	64.7	4	13	76.5
Pathological classification							
I/II	15	3	12	80.0	3	12	80.0
III	9	3	6	66.7	2	7	77.8
TNM stage							
I/II	19	6	13	68.4	5	14	73.7
III/IV	5	0	5	100	0	5	100
Regional lymph node metastasis							
Yes	13	5	8	61.5	2	11	84.6
No	11	1	10	90.9	3	8	72.7

Table 2. Sequence of primers/hybridization probes of β -actin, MK and ER- β

Target gene	Sequence	PCR product size
β -actin	F:5'-CCACGAAACTACCTTCAACTCC-3' R:5'-TCATACTCCTGCTTGCTGATCC-3'	270bp
MK	F:5'-AAAGAAAGATAAGGTGAAGAAGGGCGG-3' R:5'-GGTCCTTTCCCTTCCCTTTCTTG-3' A:5'-CCCTGCAACTGGAAGAAGGAGTTTGGAGCC-3' 5'-GCACCAAAGTCCGCCAAGGCACCCCTGAAGA-3' 5'-GGAGACCATCCGCGTCACCAAGCCCTGCAC-3'	362bp
ER- β	F:5'-TCCAGCCATGACATTCTA-3' R:5'-GAGGTTCCGCATACAGAT-3' A:5'-GCATTGAGCATCTCCAGCAGCAGGTCATACAC-3'	179bp

F: forward primer; R: reverse primer; A: antisense oligonucleotide probe

Western Blot Analysis of MK and ER- β

Fresh lung tissues were rinsed twice with ice-cold TBS, and solubilized in lysis buffer containing 20 mmol/L Tris (pH 7.5), 135 mmol/L NaCl, 2 mmol/L EDTA, 2 mmol/L DTT, 25 mmol/L β -glycerophosphate, 2 mmol/L sodium pyrophosphate, 10% glycerol, 1% Triton X-100, 1 mmol/L sodium orthovanadate, 10 mmol/L NaF, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride (PMSF) for 30 min and also treated with Ultrasonic Cell Disruptor (Sanyo, Japan). Lysates were centrifuged by 15,000g at 4°C for 15 min. Equal amounts of the soluble protein were denatured in SDS, electrophoresed and then transferred to PVDF membranes. After that the horseradish peroxidase (HRP)-conjugated goat anti-mouse and anti-rabbit IgG antibodies were used against β -actin mouse monoclonal antibody (Enogene Biotech Co. Ltd., China), MK (C-term) rabbit monoclonal antibody (Epitomics Corp., USA) and rabbit anti-human ER- β polyclonal antibody (Bioworld Technology Corp., USA), respectively. Eventually, the proteins were visualized using BeyoECL Plus (Beyotime Institute of Biotechnology, China).

Statistical Analysis

With the Quantity One 4.5.0 Software attached in Gel Doc 2000 (BIO-RAD, USA), the PCR products and western blot analytical results of MK and ER- β were semi-quantified by densitometric measurement and the relative quantity of MK, ER- β to β -actin was obtained by $OD_{MK}/OD_{\beta\text{-actin}}$ and $OD_{ER\text{-}\beta}/OD_{\beta\text{-actin}}$. Each slide was microscopically examined at $\times 400$ magnification and assessed semi-quantitatively

according to both percentage of MK and ER- β mRNA-immunoreactive cells (Score-P:1, 0-10%; 2, 10-30%; 3, 30-70%; 4, 70-100%) and staining intensity (Score-I:1, faint; 2, moderate; 3, strong). Score-P \times Score-I was estimated in each microscopic field, of which Score-1~3 was defined as negative but Score- ≥ 4 as positive. Using SPSS 13.0 for Windows, independent-samples T test, chi-square (χ^2) tests and Fisher's exact probability were performed for statistical analysis. In addition, correlation analysis of MK and ER- β expression at the mRNA and protein levels in NSCLC was conducted by the Bivariate (Spearman's correlation) analysis. Differences were considered significant when a two-tailed *P* value less than 5% was obtained.

RESULTS

Increased MK and ER- β Expression at the Gene Level in NSCLC

In the present study, RT-PCR analysis was performed to test whether MK or ER- β expression was upregulated at the transcription level in NSCLC. The results were showed in Table 3. Evidently, MK mRNA was expressed in NSCLC and corresponding paraneoplastic and normal lung; its expression level in NSCLC was stronger compared to normal lung ($P < 0.001$, Figure 1A). Similarly, compared to normal lung, ER- β mRNA expression level in NSCLC was stronger ($P < 0.01$, Figure 1B) and it seemed that there was no significant difference in its expression level in paraneoplastic and normal lung. These results indicated that the expression of MK and ER- β at the mRNA level increased in NSCLC and suggested that MK and ER- β may play an important role in lung tumorigenesis.

Table 3. MK and ER- β mRNA expression level by RT-PCR analysis in NSCLC and corresponding paraneoplastic and normal lung ($\bar{x} \pm s$)

Tissue type	Cases (n)	$OD_{MK}/OD_{\beta\text{-actin}}$ ($\bar{x} \pm s$)	$OD_{ER\text{-}\beta}/OD_{\beta\text{-actin}}$ ($\bar{x} \pm s$)
NSCLC	24	0.63 \pm 0.26	0.54 \pm 0.27
Paraneoplastic lung	24	0.54 \pm 0.24	0.45 \pm 0.20
Normal lung	24	0.40 \pm 0.13 ^{***#}	0.36 \pm 0.13 ^{**}

^{***} $P < 0.001$, ^{**} $P < 0.01$ compared to NSCLC, respectively; [#] $P < 0.05$ compared to paraneoplastic lung

To observe cellular localization of MK and ER- β mRNA in those above lung tissues mentioned, *in situ* hybridization analysis was further conducted. Accordingly, the positive expression rates of MK mRNA and ER- β mRNA by *in situ* hybridization analysis in NSCLC and corresponding paraneoplastic and normal lung were 75%, 20.8%, 0 and 79.2%,

41.7%, 20.8%, respectively. For MK mRNA, royal purple or purple black particles were observed in the cytoplasm of lung cancer cells. The histological characteristics of lung cancer cells with MK mRNA positive in NSCLC were diverse, including the focal pattern, flake pattern, tubular pattern and diffuse pattern. Among these characteristics, the most

prominent and common one was the diffuse pattern. However, the MK mRNA positive cells were single, scattered, or focal-form distributed over the detected paraneoplastic lung tissues (Figure 2.). Likewise, in contrast to negative cells with colorless nuclei, the expression of ER- β mRNA in NSCLC was also mainly localized to the cytoplasm of lung cancer cells with royal purple or purple black in color and diffuse

in distribution, and the extent of the staining was different as well. In addition, the expression of ER- β mRNA was visible in the epicyte of fewer lung cancer cells which were not counted. In comparison, ER- β mRNA was mainly expressed in the cytoplasm of bronchial epithelia in the paraneoplastic and normal lung tissues (Figure 3). There was no significant signals in negative controls.

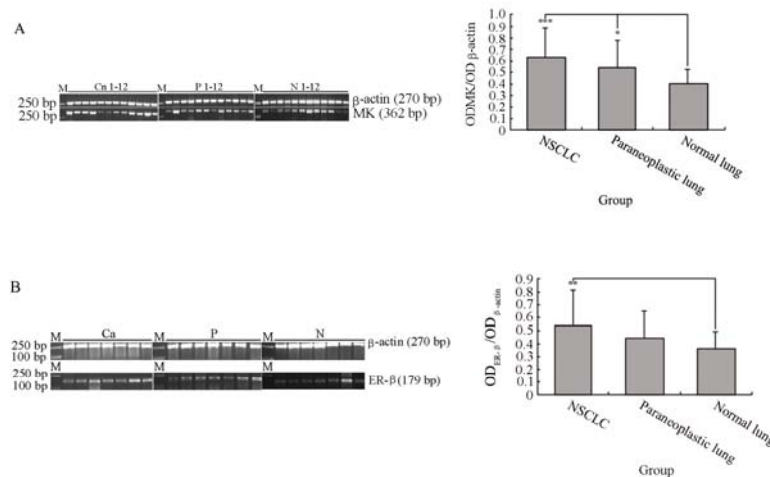


Figure 1. Expression and statistical analysis of MK and ER- β by RT-PCR at the transcription level in NSCLC and corresponding paraneoplastic and normal lung. RT-PCR products were detected by Ethidiumbromide staining. The amount of mRNA loaded in each lane was confirmed by β -actin. (A) M: marker 2000, Ca 1~12: NSCLC samples, P 1~12: paraneoplastic lung samples, N 1~12: normal lung samples. (B) M: marker 2000, Ca: NSCLC samples, P: paraneoplastic lung samples, N: normal lung samples.

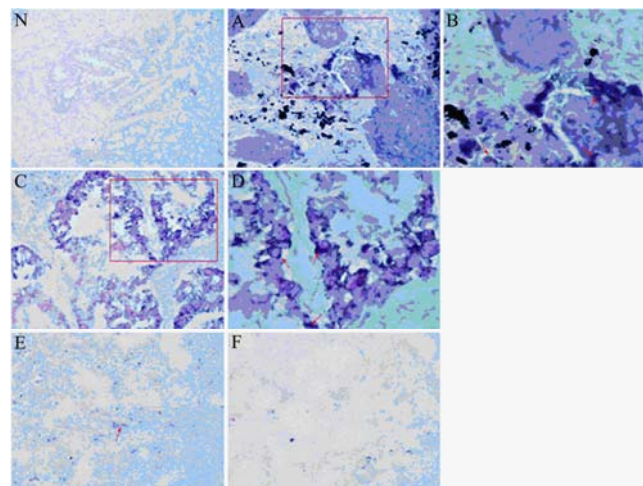


Figure 2. *In situ* hybridization analysis of MK transcripts in NSCLC as well as corresponding paraneoplastic and normal lung. N (Negative control): There was no significant signals in cytoplasm of lung cancer cells in well differentiated adenocarcinoma of the lung (original magnification, 100 \times); A, B: MK mRNA diffuse expression in cytoplasm of lung cancer cells (arrows) in moderately differentiated squamous cell carcinoma of the lung (original magnification, 200 \times and 400 \times , respectively); C, D: MK mRNA diffuse expression in cytoplasm of lung cancer cells (arrows) in well differentiated adenocarcinoma of the lung (original magnification, 200 \times and 400 \times , respectively); E: MK mRNA positive weakly expression in cytoplasm of lung cancer cells (arrow) infiltrating paraneoplastic lung (original magnification, 100 \times); F: There was no significant signals in normal lung (original magnification, 100 \times). All tissue sections were stained by alkaline phosphatase (AP) with nuclear fast red counterstaining.

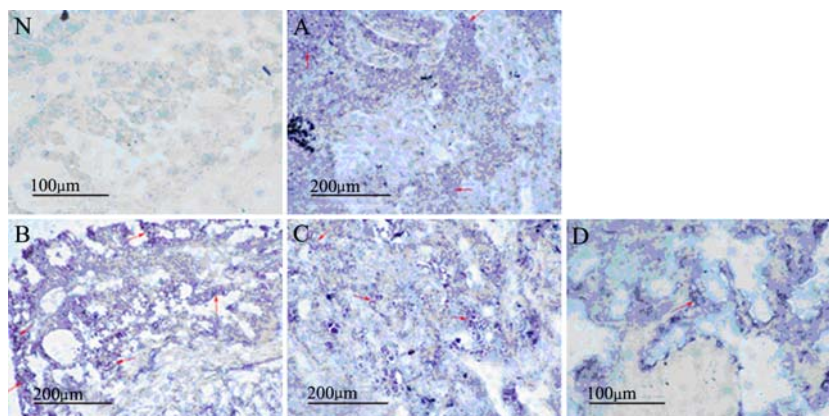


Figure 3. The expression of ER- β transcripts detected by *in situ* hybridization in NSCLC as well as corresponding paraneoplastic and normal lung. N (Negative control): There was no significant signals in cytoplasm of lung cancer cells in well differentiated adenocarcinoma of the lung (original magnification, 200 \times); A: ER- β mRNA diffuse expression in cytoplasm of lung cancer cells (arrows) in moderately differentiated squamous cell carcinoma of the lung (original magnification, 100 \times); B: ER- β mRNA diffuse expression in cytoplasm of lung cancer cells (arrows) in moderately differentiated adenocarcinoma of the lung (original magnification, 100 \times); C: ER- β mRNA positive expression in cytoplasm of lung cancer cells (arrows) in poor differentiated adenocarcinoma of the lung (original magnification, 100 \times); D: ER- β mRNA positive weakly expression in cytoplasm of bronchial epithelial cells (arrow) in paraneoplastic lung (original magnification, 200 \times). All tissue sections were stained by alkaline phosphatase (AP) without nuclear fast red counterstaining.

In addition, with the aid of Fisher's exact probability, the relationships of the positive expression rates of MK and ER- β mRNA with sex, age, megascopic type, tumor size, histological type, TNM stage or regional lymph node metastasis were analyzed. As shown in Table 1, the data suggested that the expression levels of MK and ER- β mRNA in NSCLC were not closely related with all above clinicopathologic data.

Increased Expressions of MK and ER- β Proteins in NSCLC

In order to examine whether the expression of MK and ER- β mRNA did always correspond with the expression of their respective functional proteins, we further investigated the expression of MK and ER- β at the protein level in NSCLC and corresponding paraneoplastic and normal lung. Tricine-SDS-PAGE is commonly used to separate proteins in the mass range 1-100 kDa. It is the preferred electrophoretic system for the resolution of proteins smaller than 30 kDa^[20]. In view of the molecular weight of MK (13.4 kD), the tricine-SDS-PAGE was used to western blot analysis of MK. Accordingly, the routine tris-SDS-PAGE was for ER- β . The results were showed in Table 4. Statistically, it was clear that there was weak MK protein expression in NSCLC (Figure 4A) but no MK

protein expression in corresponding paraneoplastic and normal lung (Figure not shown). Analogously, there was stronger ER- β protein expression in NSCLC but weak or no ER- β protein expression in corresponding paraneoplastic ($P < 0.001$) and normal ($P < 0.001$) lung (Figure 4B). On one hand, these results suggested that the expression of MK and ER- β mRNA did basically correspond with the expression of their respective functional protein, and on the other hand they made it clear again that upregulated MK and ER- β proteins in NSCLC may be involved in lung tumorigenesis.

Correlation Analysis of MK and ER- β Expression at the mRNA and Protein Level in NSCLC.

Since both *in situ* hybridization and immunohistochemistry analyzed MK and ER- β expression semi-quantitatively and localizatonally, we investigated the correlation of *in situ* hybridization analysis of MK and ER- β with our previous immunohistochemical analysis. Table 5 demonstrated that the expressions of MK and ER- β mRNA did correspond with the expressions of their respective functional proteins. At the same time, the correlation of them at the protein level was stronger ($P < 0.01$, $r_s = 0.535$); in spite of their correlation, there was no difference at the mRNA level ($P > 0.05$, $r_s = 0.178$).

Table 4. Expression levels of MK and ER-β proteins by Western blot analysis in NSCLC and corresponding paraneoplastic and normal lung($\bar{x}\pm s$)

Tissue type	Cases (n)	OD _{MK} /OD _{β-actin} ($\bar{x}\pm s$)	OD _{ER-β} /OD _{β-actin} ($\bar{x}\pm s$)
NSCLC	24	0.41±0.34	0.84±0.30
Paraneoplastic lung	24	0.00±0.00	0.40±0.27***
Normal lung	24	0.00±0.00	0.35±0.28***

***P<0.001 compared to NSCLC

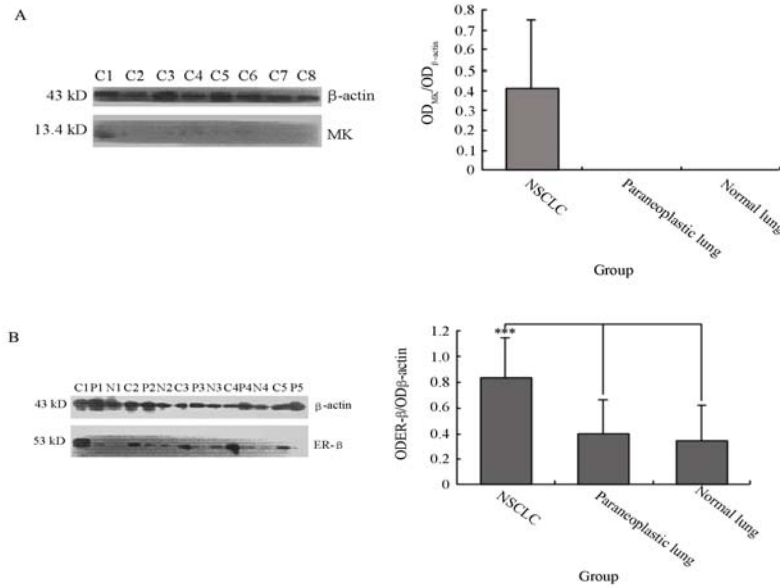


Figure 4. Detection and statistical analysis of MK and ER-β protein by Western blot analysis in NSCLC and corresponding paraneoplastic and normal lung. β-actin expression was used as control. (A) C1~C8: NSCLC samples. (B) C1~C5: NSCLC samples; P1~P5: paraneoplastic lung samples; N1~N4: normal lung samples.

Table 5. Correlation analysis of MK and ER-β at the mRNA and protein levels in NSCLC

Parameter 1	Parameter 2	P	r _s
MK mRNA	MK protein		
	+		
+	18	0.001**	0.655
-	3		
ER-β mRNA	ER-β protein		
	+		
+	16	0.000***	0.725
-	0		
MK protein	ER-β protein		
	+		
+	16	0.007**	0.535
-	0		
MK mRNA	ER-β mRNA		
	+		
+	18	0.406	0.178
-	1		

P<0.01, *P<0.001

DISCUSSION

Aberrant expression of growth factors is often associated with tumorigenesis, and its effects on the signal transduction system are considered to lead to tumorigenesis^[21]. Upregulation of MK mRNA has been found in various human carcinomas such as Wilms' tumor; lung, breast, gastric, and colon carcinomas; and brain tumors^[21]. In the present study, our results also showed that expression of MK mRNA by RT-PCR analysis was upregulated in NSCLC. Additionally, it seemed that its expression level in paraneoplastic lung was also higher than that in normal lung, which in part was due to the lung cancer cells infiltrating the former. Estrogens may be involved in lung carcinogenesis, and estrogen receptors (ERs), mainly ER- β , are present and functional in normal lung and tumor cell lines and tissues^[22]. In 2002, Mollerup et al^[23] proved that ER genes were abundantly expressed in both histologically normal human lung and lung tumor cell lines. Similarly, compared to normal lung, ER- β mRNA expression level in NSCLC was stronger. These results suggested that increased expressions of MK and ER- β at the mRNA level in NSCLC may play an important role in lung tumorigenesis.

Concerning the cellular localization of MK protein, the previous conclusions are diverse: some researchers report that MK is located in the nucleus and nucleolus, whereas others think that it localizes in the cytoplasm^[24]. Nevertheless, it is reported that MK transcripts always localize in the cytoplasm of hepatocellular carcinoma cells^[25], intrahepatic cholangiocarcinoma cells^[26] and thyroid papillary carcinoma cells^[21]. In our study, *in situ* hybridization analysis showed that MK transcripts were found to be in the cytoplasm of lung cancer cells and the histological characteristics of lung cancer cells with MK mRNA positive in NSCLC were different from those of MK mRNA-positive lung cancer cells in corresponding paraneoplastic lung. Besides, we failed to detect significant signals of MK transcripts in normal lung. In the same way, for ER- β mRNA, we detected its increased expression in the cytoplasm of lung cancer cells in NSCLC but low expression in the cytoplasm of bronchial epithelia in paraneoplastic and normal lung. Furthermore, although well differentiated and moderately differentiated NSCLC tended to express more MK and ER- β transcripts than the poorly differentiated, our statistical analysis showed that the expression levels of their transcripts in NSCLC were not closely related with all clinicopathologic data.

Next, in order to examine whether the expression of MK and ER- β mRNA did always correspond with

the expression of their respective functional proteins, we further investigated the expression of MK and ER- β at the protein level by Western blot analysis. Analogously, at the protein level, Western blot analysis demonstrated that MK was expressed in NSCLC but not in paraneoplastic and normal lung, and ER- β was overexpressed in NSCLC, the level being much stronger than that of bronchial epithelia in paraneoplastic and normal lung.

From our previous study^[27], it was indicated that MK and ER- β proteins were overexpressed in NSCLC and their expression levels were both significantly negatively correlated with the pathological classification, i.e. well differentiated and moderately differentiated NSCLC more significantly expressed MK and ER- β proteins than the poorly differentiated type. Based on the fact that in normal developing mouse embryos MK gene and protein play a role in cell differentiation and organogenesis^[25], these results suggested that MK mRNA and protein along with ER- β mRNA and protein may have some relationship to the lung cancer cell differentiation in NSCLC. Another earlier study^[28] in our lab indicated that small interfering RNA (siRNA) targeting MK gene can inhibit gastric cancer cell growth and induce apoptosis via mitochondrial depolarization and caspase-3 activation, which suggested that MK siRNA may be a promising novel and potential therapeutic strategy for the treatment of gastric cancers. Therefore, MK siRNA should also be a good candidate for treating NSCLC. In addition, treatment of NSCLC cells with siRNA directed to ER- β effectively reduced mRNA expression of ER- β and specific inhibitors of ER and epidermal growth factor receptor (EGFR) signaling elicited marked blockade of the growth of human NSCLC xenografts *in vivo*^[15]. Therefore, it may be feasible to achieve inhibition of NSCLC growth by the use ER- β siRNA and effective antagonists blocking ER and EGFR signaling as well.

Since *in situ* hybridization and immunohistochemistry can analyze MK and ER- β expressions semi-quantitatively and localizationally, the correlation of *in situ* hybridization analysis of MK and ER- β with our previous immunohistochemical analysis was also examined. Spearman's correlation analysis showed that in spite of their correlation, there was no difference between MK and ER- β expressions at the mRNA level ($P > 0.05$, $r_s = 0.178$). In addition, it was stated clearly that their transcript levels in NSCLC coincided with their respective protein levels, and especially the distribution and localization of MK protein previously determined by immunohistochemistry were similar to those obtained by *in situ* hybridization analysis.

In conclusion, the results of MK and ER- β

expression in NSCLC using RT-PCR, *in situ* hybridization and Western blot analyses at the mRNA and protein levels indicate their increased expressions in human NSCLC, suggesting that they have some important roles in its genesis. And together with our previous immunohistochemical analysis, we uphold that the combined detection of MK with ER- β at the protein level may be of great value in early diagnosis and treatment of patients with NSCLC as well as determining their prognosis.

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