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Correlation and Significance of Midkine and Estrogen Receptor Beta Protein Expression in Non-Small Cell Lung Cancer

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OBJECTIVE Midkine (MK), a new member of the heparin-binding growth factor family, was found recently to have a high expression level in many carcinoma specimens, including those of the esophagus, gall bladder, pancreas, colorectum, breast and lung. Estrogen receptor beta (ER-β), a recently cloned estrogen receptor subtype, was also found to be highly expressed in lung tumor tissue, in contrast to a lower level of expression in normal lung tissue. However, few relevant studies on these proteins have been published. The aims of our study were to investigate the expression of midkine and ER-β proteins in non-small cell lung cancer (NSCLC) and to examine the relationship between their expression and the clinicopathologic data as well as to analyse the correlation of their expression in NSCLC.
METHODS By immunohistochemistry, MK and ER-β were examined in 24 surgically resected cases of NSCLC with their corresponding paraneoplastic and normal lung tissues.
RESULTS MK and ER-β were overexpressed in NSCLC. The levels of MK and ER-β expression in NSCLC were found to be significantly negatively correlated with the pathological classification (P = 0.042 and 0.021, respectively), and their expression decreased with a raise in the classification. Spearman's correlation analysis showed that the correlation of their expression in NSCLC was strong (correlation coefficient [r_s] = 0.535, $P = 0.007 < 0.01$).
CONCLUSION The expression levels of MK and ER-β to some extent reflect the malignant degree of NSCLC, and their combined detection may be of great value in early diagnosis, treatments of patients with NSCLC and can predict the prognoses.

KEY WORDS: midkine (MK), estrogen receptor beta (ER-β), non-small cell lung cancer (NSCLC), immunohistochemistry.

Introduction

Lung cancer is the leading cause of cancer mortality in the Western world. In the developing world, a lung cancer epidemic is still spreading, and is suspected to have a major impact on public health throughout many of the next decades[1]. At present, there are few therapeutic options available for lung cancer patients.

The MK gene was found in the cDNA library constructed from the HM-1 embryonal carcinoma cell line of the mouse induced by retinoic acid[2]. Researchers have showed that the MK protein is a multifunctional cytokine closely related to carcinogenesis and growth. The early researches proposed that the main biological activities of MK were neurotrophic effects, so MK was thought to be a neurotrophic factor for the embryonic spinal cord and dorsal root ganglion neurons, and that it play a significant role in embryogenesis of the nervous system[3]. However, further research showed that MK had some other biological activities[4], mainly including mitogenic, anti-apoptotic, transforming, fibrinolytic, chemotactic, and angiogenic factors. In 2006, Krzystek-Korpacka et al.[5] presented up-to-date views on the biological activity of MK both at the cellular (mitogenic properties, participation in apoptosis, and cellular migration, adhesion, morphological differentiation, and chemokine synthesis stimulation) and tissue levels (involvement organogenesis, tissue regeneration and protection and in the formation and degradation of the extracellular matrix). Researchers found that MK was expressed at high levels in a variety of human tumors[6], and some studies also showed that MK was overexpressed in NSCLC and its expression level was much higher compared to normal lung tissue[7-9].

Estrogens are pivotal in the growth and development of both normal and neoplastic mammary tissues[10] and may function as tumor promoters through a receptor-mediated mechanism which increases cell proliferation[11]. The role of estrogen in promoting tumors has been demonstrated during breast and endometrial cancer development[12]. It is well known that the actions of estrogens are mainly mediated via estrogen receptors (ERs). ERs are ligand-activated transcription factors that belong to the nuclear hormone receptor superfamily. Since 1996, two kind of different ERs have been studied, which are now known as ER-α and ER-β[13]. The etiology of non-small cell lung cancer (NSCLC) is not completely clear, but earlier work shown that estrogens as well as other growth factors may promote the progression of human NSCLC[14]. Furthermore, Li et al.[15] found that ERs expression was closely associated with the biological behavior of NSCLC, and some other studies demonstrated that there was only the expression of ER-β in human NSCLC[16-18].

Fresh frozen sections have been widely used for immunohistochemical studies, and also as a standard to evaluate the efficiency of heat-induced antigen retrieval (HIAR) in formalin-fixed and paraffin-embedded specimens[19]. Up to now, the studies on the expression of MK and ER-β in NSCLC have been limited, and the results shown somewhat inconsistent. And to our knowledge, no reports have been published regarding their correlation in NSCLC. Thus, in our present research, the expression of MK and ER-β proteins as well as the relationship between their expression and the clinicopathologic data were investigated by immunohistochemistry in fresh frozen sections of 24 cases of NSCLC and their corresponding paraneoplastic and normal lung tissues. Moreover, the first time, correlation of the expression of MK and ER-β proteins in NSCLC has also been analyzed.

Materials and Methods

Patients and tissue specimens

The present research is retrospective and based on the histological materials collected from a cohort of 24 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. The clinicopathological data of these 24 patients with NSCLC are summarized in Table 1.

Immunohistochemistry of MK and ER-β

The affinity purified rabbit antihuman MK polyclonal antibody was produced in our laboratory. The prediluted rabbit antihuman ER-β polyclonal antibody (E14021) was purchased from the Spring Bioscience Corp. The ready-to-use DAKO En Vision™ (GK400305/15) and Liquid DAB+Substrate Chromogen System were purchased from the Gene Co. Ltd. The known sections with MK strong expression and ER-β-positive breast cancer tissues were used as positive controls; a negative control was created by substituting the primary antibody with TBS.

Statistical analysis

The expression of both MK and ER-β was assessed semi-quantitatively by estimating the percentage of cells with stained cytoplasm for MK and stained nuclei for ER-β on whole tissue slides. For MK, the immunohistochemical staining was scored by determining the percentage of positive cells (< 5%-, 5%~25%+, 25%~50%++, ≥ 50%+++) ; for ER-β, the immunohistochemical staining was scored by determining positive staining in the nuclei with at least weak staining in more than 10% of the tissue cells, this being the definition of a ER-β-positive tissue[20]. SPSS 13.0 for Windows, the chi-square (χ^2) test and Fisher's exact probability were ulilized for statistical analysis. In addition, the analysis of the correlation of MK and ER-β protein expression in NSCLC was conducted by the bivariate(Spearman's correlation) analysis. Differences were considered significant when a two-tailed P value was < 0.05.

Results

The specificity of MK and ER-β immunologic reactions in lung tissue

For MK, yellowish or brown or black particles were observed in the cytoplasm of lung cancer cells. The histological characteristics of lung cancer cells with positive MK in NSCLC were diverse, including the focal pattern,

Table 1. The relationships between MK, ER-β expression in NSCLC and the clinicopathological data.

Characteristic	Cases (n)	MK expression			ER-β expression		
		-	+	Positive (%)	-	+	Positive (%)
Sex							
Male	19	2	17	89.5	5	14	73.7
Female	5	1	4	80.0	3	2	40.0
Age (years)							
< 65	11	2	9	81.8	3	8	72.7
≥ 65	13	1	12	92.3	5	8	61.5
Megascopic type							
Central	14	2	12	85.7	4	10	71.4
Peripheral	10	1	9	90.0	4	6	60.0
Tumor size (cm)							
< 5	11	1	10	90.9	3	8	72.7
≥ 5	13	2	11	84.6	5	8	61.5
Histological type							
Squamous cell carcinoma	7	0	7	100	1	6	85.7
Adenocarcinoma	17	3	14	82.4	7	10	58.8
Pathological classification							
I/II	15	0	15	100	2	13	86.7
III	9	3	6	66.7*	6	3	33.3*
TNM stage							
I/II	19	3	16	84.2	8	11	57.9
III/IV	5	0	5	100	0	5	100
Regional lymph node metastasis							
Yes	13	3	10	76.9	4	9	69.2
No	11	0	11	100	4	7	63.6

*$P < 0.05$ compared with I/II pathological classification.

flake pattern, tubular pattern and diffuse pattern. Among these characteristics, the most prominent common one was the diffuse pattern. However, the MK positive cells were single, scattered, or distributed in a focal-form over the detected paraneoplastic and normal lung tissues. In contrast to negative cells with blue nuclei, the expression of ER-β in NSCLC was mainly localized to the nuclei of lung cancer cells demonstrated maroon color and diffuse distribution, and the extent of the staining was also different. In addition, the expression of ER-β was also visible in the cytoplasm of fewer lung cancer cells which were not counted. In comparison, ER-β was mainly expressed in the nuclei of bronchial epithelia in the paraneoplastic and normal lung tissues. There was no staining in negative controls (Fig.1).

The relationships between MK, ER-β expression in NSCLC and the clinicopathologic data

With the aid of Fisher's exact probability, the relationships of the positive expression rates of MK and ERβ proteins 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 ERβ proteins in NSCLC were closely related with the pathological classification ($P = 0.042$ and 0.021, respectively) and the expression decreased with a raise in the classification.

The expression levels of MK and ER-β in lung tissue

The positive expression rates of MK in 24 cases of NSCLC, and their corresponding paraneoplastic and normal lung tissues were 87.5%, 41.7% and 33.3%, respectively, while the rates of ER-β were 66.7%, 33.3% and 25%, respectively. The comparative analyses of MK and ER-β expression levels in the above tissues were performed using χ^2 test. The results showed that the expression level of MK protein in NSCLC tissues was remarkably higher than that in their paraneoplastic ($P = 0.001 < 0.01$) and normal lung tissues ($P = 0.000 < 0.001$), whereas no significant difference in its expression level was observed between the paraneoplastic and normal lung tissues ($P = 0.551 > 0.05$). Similar results were produced for ER-β (Table 2).

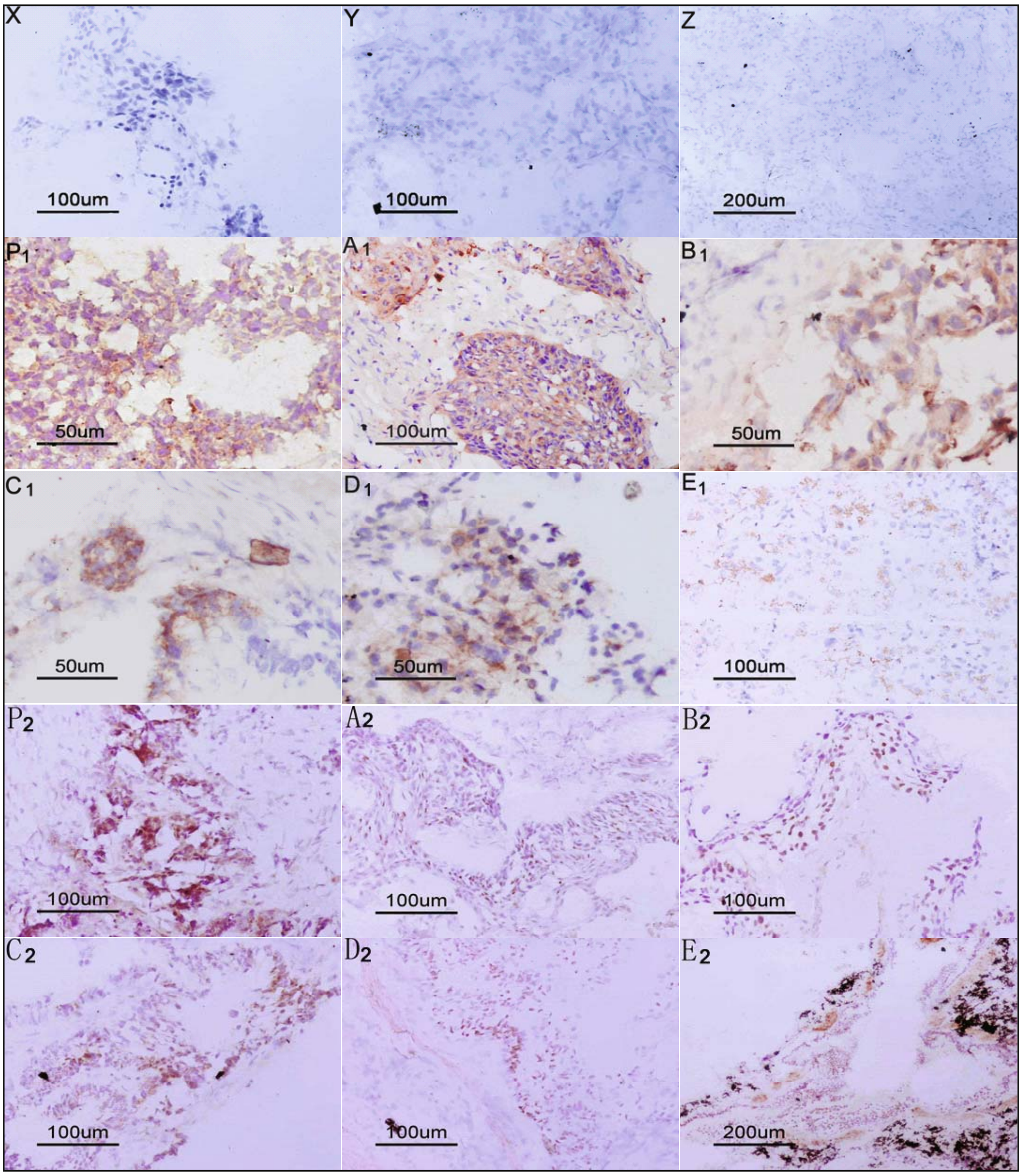

Fig.1. Expression of MK and ER-β proteins in NSCLC, and the corresponding paraneoplastic and normal lung tissue (En Vision Method). X, Y, Z (negative controls): No staining in NSCLC tissue (×200), paraneoplastic (×200) and normal lung tissue (×100), respectively; P_1 (MK positive control): The section of MK strong expression (×400), P_2 (ER-β positive control): ER-β positive expression in nuclei of breast cancer cells in breast cancer tissue (×200); A_1: MK diffuse expression in cytoplasm of lung cancer cells in moderately differentiated squamous cell carcinoma of the lung (×200), A_2: ER-β positive expression in nuclei of lung cancer cells in moderately differentiated squamous cell carcinoma of the lung (×200); B_1: MK positive expression in the cytoplasm of lung cancer cells in poorly differentiated squamous cell carcinoma of the lung (×400), B_2: ER-β positive expression in nuclei of lung cancer cells in poorly differentiated squamous cell carcinoma of the lung (×200); C_1: MK strong expression in cytoplasm of lung cancer cells in moderately differentiated adenocarcinoma of the lung (×400), C_2: ER-β positive expression in nuclei of lung cancer cells in moderately differentiated adenocarcinoma of the lung (×200); D_1: MK positive expression in cytoplasm of lung cancer cells in poorly differentiated adenocarcinoma of the lung (×400), E_1: MK weak expression in paraneoplastic lung tissue (×200); D_2, E_2: ER-β positive expression in nuclei of bronchial epithelial cells in paraneoplastic (×200) and normal lung tissue (×100)

Table 2. The statistical analysis of expression levels of MK and ER-β in NSCLC, and the corresponding paraneoplastic and normal lung tissue.

Group	MK		ER-β	
	χ^2	*P*	χ^2	*P*
NSCLC tissue *vs.* Paraneoplastic tissue	11.021	0.001**	5.333	0.021*
NSCLC tissue *vs.* Normal tissue	14.722	0.000***	8.392	0.004**
Paraneoplastic tissue *vs.* Normal tissue	0.356	0.551	0.403	0.525

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

The analysis of the correlation between MK and ER-β expression in NSCLC

As shown in Table 3, the correlation between MK and ER-β expression in NSCLC was strong (Spearman's correlation coefficient [r_s] = 0.535, $P = 0.007 < 0.01$).

Discussion

Although a minority of researchers claim that MK and ER-β proteins are only expressed in NSCLC tissue, the majority of them indicate there is expression of MK and ER-β proteins are not only in NSCLC tissues but also in normal lungs as well. Additionally, it is note worthy that Mollerup et al.[1] showed that ER genes were abundantly expressed in both histologically normal human lung and lung tumor cell lines. In the present research, we found that MK and ER-β proteins were expressed in NSCLC tissues as well as in their corresponding paraneoplastic and normal lung tissues. Especially for ER-β, in our results, it shown ER-β expression in the paraneoplastic and normal lung tissue tests. Therefore, we conclude that ER-β may have important physiological functions in normal lungs, which is consistent with the report of Omoto et al.[16]

From previous studies, we find data on MK and ER-β expression levels have been conflicting, which in part may be ascribed to different experimental approaches[1] and different standards judging a positive cell. For MK, in the present research, a positive rate of 87.5% was obtained in NSCLC tissues, while its positive expression rate in their corresponding paraneoplastic and normal lung tissues was much lower, 41.7% and 33.3%, respectively. The results are somewhat inconsistent with those of Garver et al.[7], Li et al,[8] and Xu et al.[9] For ER-β, similar results were obtained: the percentage of ER-β in the above tissues was 66.7%, 33.3%, and 25%, respectively, which is not consistent with those of Omoto et al.[16] but in agreement with those of Schwartz et al.[17] By the bivariate (Spearman's correlation) analysis, the analysis of the correlation between MK and ER-β expression in NSCLC was conducted (Table 3). An apparent strong correlation was obtained.

Table 3. The correlation between MK and ER-β expression in NSCLC.

MK	ER-β +	ER-β -	P	r_s
+	16	5	0.007	0.535
-	0	3		

Concerning the cellular localization of these two proteins are diverse, which may be due to problems with antibody specificity or other reasons. Up to now, consistent conclusions on the localization of MK have not been obtained. Some researchers reported that MK was located in the nucleus and nucleolus, whereas others considered that it localized in the cytoplasm[21]. In the present research, we found that MK protein was mainly expressed in the cytoplasm of lung cancer cells in NSCLC tissues with a brown color and diffuse distribution. In the paraneoplastic and normal lung tissues tests, weak staining for MK was also visible in the cytoplasm, which may be ascribed to the paracrine activity of lung cancer cells expressing MK protein.

With respect to localization of ER in lung tissue, the viewpoints are also inconsistent. We found that ER-β staining was localized to the nuclei of lung cancer cells in NSCLC tissues, the intensity was much stronger than that of bronchial epithelia in their corresponding paraneoplastic and normal lung tissues. These results are the same as those of Omoto et al.[16] In addition, in contrast to the nuclear staining of the smooth muscle cells reported by Taylor and Al-Azzawi[22], weak cytoplasmic staining of the smooth muscle cells was also observed in the paraneoplastic and normal lung tissues, which was considered to be nonspecific.

Kato et al.[23] pointed out that the increase of MK protein expression level was related to the pathological tumor classification. In the present research, the positive expression rate of the MK protein in NSCLC tissues was found to be significantly related with the pathological classification ($P = 0.042 < 0.05$) and expression decreased with its raise, but there was no notable relationship between its positive expression rate and the other clinicopathological data ($P > 0.05$). However, Li et al.[8] and Xu et al.[9] maintained that MK expression was notably related with regional lymph node metastasis ($P < 0.05$) rather than pathological classification. Besides, Xu et al.[9] also reported that MK expression was remarkably related with the TNM stage and tumor size ($P < 0.05$).

For ER, the past studies showed that its positive rates were related to the histological classification of lung cancer and increased along with its elevaton[1]. Nevertheless, like MK expression, our results showed that the positive expression rate of ER-β protein in NSCLC was exclusively related with the pathological classification ($P = 0.021 < 0.05$) and also decreased with the rise in histologically determined pathological stage. All of the above results demonstrate that MK and ER-β expression depends on the tumor tissue differentiation, and is inversely correlated with cell proliferation. In addition, Peng et al.[24] also suggested that the positive rate of ER decreased with the raise in the histological classification, and the protein contnet may reflect differentiation of lung tumor tissue. In 2005, Wu et al.[18] reported that ER-β overexpression could be used as a significantly favorable prognostic factor in patients with surgically resected Stage II and III non–small cell lung cancers. During the same year, Kawai et al.[25] concluded that the absence of ER-β could serve as a marker to identify patients at high risk even at an early clinical stage. Moreover, Skov et al.[20] reported the presence of ER-β in a tumor as an apparent positive prognostic factor for men with non-small cell lung cancer.

In view of the strong correlation between MK and ER-β expression in NSCLC, it is predictable that MK and ER-β may serve as the potential reference indexes in assessing the degree of malignancy in NSCLC; also blocking their expression may be a means to treat patients with NSCLC. Our former study[26] indicated that small interfering RNA (siRNA) targeting the MK gene can inhibit growth of gastric cancer cells and induce apoptosis via mitochondrial depolarization and caspase-3 activation, suggesting that MK siRNA may be a promising novel and potential therapeutic strategy for the treatment of gastric cancers. Therefore, MK siRNA may also be a new choice to treat NSCLC patients. In addition, it may be feasible to block NSCLC growth by using effective antagonists against ER-β and epidermal growth factor receptor (EGFR) signaling as well as ER-β siRNA[14].

In conclusion, first, MK and ER-β proteins are overexpressed in NSCLC, suggesting these have that they have important roles in its genesis. Second, the positive expression rates of MK and ER-β proteins decrease with the histological class elevated, which implies that their expression levels to some extent may reflect the malignant degree of NSCLC. Third, there is strong correlation in the expression of MK and ER-β proteins in NSCLC. All these results indicate that the combined detection of MK with ER-β protein may be of great value in early diagnosis, treatment and predicted prognosis for patients with NSCLC.

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