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The expression of Elf-1 and Ki-67 and their correlations in non-small-cell lung cancer*

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Abstract ***Objective:*** Elf-1 is a member of the proto-oncogenes Ets-related transcription factor family and over-expressed in many human tumors, Ki-67 is an important nuclear antigen associated with cell proliferation. This study investigated the expression of Elf-1 and Ki-67 in non-small-cell lung cancer (NSCLC) and studied their correlation with the clinicopathological features. ***Methods:*** Tissue microarray from 64 cases lung cancer tissue and 10 cases normal lung tissue was constructed, immunohistochemical method was used to evaluate the protein expressions of Elf-1 and Ki-67, correlations of the expression of Elf-1 and Ki-67 to clinicopathological features of NSCLC were analyzed. ***Results:*** Expression of Elf-1 and Ki-67 in NSCLC tissues were significantly higher than in normal lung tissues ($P < 0.05$), the positive rate of Elf-1 and Ki-67 was 73.44% and 64.06% in NSCLC group, Overexpression of Elf-1 in NSCLC was significantly related to histopathological grading, different clinical staging and the intensity of ELF-1 expression was significantly higher in the group with lymph node metastasis than that without ($P < 0.05$). Overexpression of Ki-67 was also closely related to tumor differentiation, clinical stages and lymph node metastasis ($P < 0.05$). In addition positive correlation was found between the expressive intensity of Elf-1 and Ki-67 ($\tau = 0.295$, $P = 0.018$). ***Conclusion:*** The high expression and positive correlation of Elf-1 and Ki-67 in NSCLC suggest that they probably play a role in onset and progression of lung cancer, united detecting their expression could be used as an valuable molecular biological index for predicting the malignant behavior and early diagnosis of NSCLC.

Key words Elf-1; Ki-67; immunohistochemistry; correlation; non-small-cell lung cancer (NSCLC)

Primary lung cancer is the most common malignant tumor which presents a major threat to human's health in worldwide and its incidence keeps increasing. Accumulating researches have demonstrated that abnormal proliferation and apoptosis inhibition of cells are the molecular and biological basis of malignancy. E74-like factor 1 (Elf-1), one of the E Twenty Six (Ets) transcription factor family members, regulates the genes involved in cellular growth and differentiation; the proliferating antigen Ki-67 is an effective biological marker in cell proliferation and widely used to assess the proliferative activity of cells in the tumor tissue. Therefore, we used tissue microarray technique and immunohistochemistry PowerVision™-9000 to detect the protein expression levels of Elf-1 and Ki-67 in non-small-cell lung cancer (NSCLC) and study their correlations with clinical pathologic characteristics.

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Materials and methods

Materials

Formalin-fixed by 40 g/L and paraffin-embedded tumor and control samples were obtained from a total of 64 cases with NSCLC and 10 normal lung tissues, then they were prepared into 4 μm serial sections and stained with hematoxylin and eosin (HE). The pathologic histology of each sample was examined by two pathologists to verify the presence of NSCLC tissue (patients did not receive preoperative chemotherapy and preoperative radiotherapy), all specimens of the NSCLC group were obtained between December 2006 and October 2009 at the Department of Thoracic Surgery, Yantai Affiliated Hospital of Binzhou Medical University. Among these specimens, 52 were collected from male patients and 22 from female patients. The age of the patients was from 37 to 79 years, with a median age of 56.5. Among these cancer samples, 27 were squamous cell carcinoma (4 cases were classified as grade I, 11 cases as grade II and 12 cases as grade III) and 37 were adenocarcinoma (10 cases were classified as grade I, 12 cases as grade II and 15 cases as grade III). As for

tumor volume, tumor diameter was < 5 cm in 44 cases and > 5 cm in 20 cases. Lymph node metastasis for all patients were recorded, among which 35 cases showed lymph node metastases. The samples were classified according to a staging system of the 1997 international Union Against Cancer (UICC) for lung cancer , 17 patients were at stage I, 33 at stage II and 14 at stage III.

Main reagents

Reagents were obtained from the following sources: rabbit anti-human Elf-1 and mouse anti-human Ki-67 were purchased from Santa Cruz Biotechnology, inc . (USA), the PowerVision™-9000 reagent Kit was obtained from Beijing Zhongshan Biotechnology Co., Ltd. (China).

Detection methods

The design and preparation of tissue microarray (TMA)

TMA with 2.0 mm in diameter tissue core (typical area of cancer) was constructed from sixty-four cases of NSCLC tissue and ten normal lung tissues. Two arrays were designed as N1 and N2. N1 array contained one Marker and forty-two cases of NSCLC tissue, N2 array contained one Marker and twenty-two cases of NSCLC tissue and ten normal lung tissues. One human Kidney tissue as a marker (Marker) was placed on the starting position of each wax block so as to determine the chips order and the spacing between two dots was 1 mm on TMAs. The tissue micro-arrays were built as standard method and could better represent the pathological feature of the original wax block.

Immunohistochemistry

This experiment applied immune histochemical method (SP), the paraffin-embedded tissue was dewaxed and hydrated through the graded series of ethanol to distilled water, then endogenous peroxidases was inactivated in 3% (v/v) H_2O_2. After microwave retrieval, the mouse anti-human Ki-67 (1:50) and rabbit anti-human Elf-1 antibodies (1:100) were processed according to reagents instructions. The chromogenic reagent diaminobenzidine (DAB) was added to and observed the sections under microscope. Afterward, the chromogenic reaction was terminated with tap water and the tissue sections were re-stained with Mayer hematoxylin and were mounted by neutral gum.

Determining results

A total of 10 high power fields (HPF) were observed randomly in each section, with 100 cancer cells counted per HPF. The extent of staining was observed and the percent of positively stained cells was recorded, Elf-1 was located mainly in the nucleus, presenting yellow to brown and the positive staining of Ki-67 was brown to tan, mainly localized in the nucleus, both of them was counted only the nuclear staining. According to Lu *et al* [1], the extent of staining was scored as follows: positive scores were 0 = no staining; 1 = light yellow; 2 = yellow; 3 = brown or dark brown. According to the percent of positively stained cells, positive scores were: 0 < 5%; 1 = 6%–30%; 2 = 31%–70% and 3 > 71%. The total score equals the sum of the positive range score and the positive extent score: 0 was defined as negative (–), 1–2 was defined as weak positive (+), 3–4 was defined as moderate positive (++) and 5–6 was defined as strong positive (+++).

Statistical analysis

The statistical software SPSS16.0 was used for the statistical analysis. The relationship between each indicator and the clinicopathologic features were analyzed by Kruskal-Wallis tests and the interrelationships between indicators used the Kendell rank correlation for analysis. Statistical significance was assumed when $P < 0.05$.

Results

Tissue dots of TMAs were orderly arranged and integral and uniformed in size with good HE staining, Pathological information of TMA basically represents traditional slides. Expression of Elf-1 and Ki-67 in NSCLC tissues were significantly higher than in normal lung tissues(the weakly expressed of Elf-1 and Ki-67 was individually detected and no positive expression in normal tissues ($P < 0.05$; Table 1).

Correlation between the expression of Elf-1 and the clinicopathologic features in NSCLC (Table 2)

Positive staining for Elf-1 was yellow-brown granules and mainly located in the nucleus of cancer cells (Fig. 1) and also was discovered in the cytoplasm of some cancer cells . The positive expression of Elf-1 was 47 cases among 64 specimens and the positive rates was 73.44% in NSCLC cells, the expressive intensity of Elf-1 was related to histopathological grading of the cancer ($P = 0.023$), the significant differences were observed between different clinical stages ($P = 0.020$) and the intensity of Elf-1 expression was significantly higher in the group with lymph node metastasis than that without ($P = 0.002$). The expression of Elf-1 was independent of pathological type sex, age and tumor size of the patients ($P > 0.05$).

Correlation between the expression of Ki-67 and the clinicopathologic features in NSCLC (Table 2)

The positive staining for Ki-67 was yellow-brown to tan granules and mainly localized in the nucleus of cancer

Table 1 Comparison of expressions of Elf-1 and Ki-67 in lung normal and NSCLC tissues

Biological markers	*n*	Normal		NSCLC			χ^2	*P*
		–	+	–	+	Positive rate (%)		
Elf-1	64	63	1	17	47	73.44	10.299	0.002
Ki-67	64	63	1	23	41	64.06		

Table 2 Correlation among the positive expressions of Elf-1 and Ki-67 and various clinicopathological features of NSCLC

Characteristics	*n*	Elf-1				*Z*	*P*	Ki-67				*Z*	*P*
		–	+	++	+++			–	+	++	+++		
Pathological types													
Squamous carcinoma	27	8	8	8	3	–0.170	0.865	10	8	7	2	–0.036	0.972
Adenocarcinoma	37	9	14	9	5			13	13	8	3		
Gender													
Male	52	13	19	14	6	–0.063	0.950	18	18	12	4	–0.181	0.857
Female	12	4	3	3	2			5	5	3	1		
Age (years)													
≤ 55	24	6	9	7	2	–0.181	0.857	8	9	6	1	–0.058	0.954
> 55	40	11	13	10	6			15	12	9	4		
Tumor diameter (cm)													
≤ 5	44	12	15	11	6	–0.053	0.958	16	15	10	3	–0.335	0.738
> 5	20	5	7	6	2			7	6	5	2		
Histological grade													
I	14	5	5	4	0			11	2	1	0		
II	23	9	7	6	1	7.560	0.023	8	9	5	1	15.513	0.000
III	27	3	10	7	7			4	10	9	4		
Lymph node metastasis													
Yes	35	4	13	11	7	–3.100	0.002	7	13	11	4	–2.956	0.003
No	29	13	9	6	1			16	8	4	1		
Clinical stage													
I	17	8	6	1	2			10	5	2	0		
II	33	7	14	9	3	7.875	0.020	7	14	9	3	6.709	0.035
III	14	2	2	7	3			6	2	4	2		

Table 3 The correlation between the expression of Elf-1 and Ki-67 in NSCLC (*n*)

The expression of Ki-67	The expression of Elf-1			
	Negative	Weak positive	Positive	Strong positive
Negative	14	6	2	4
Weak positive	2	7	5	3
Positive	0	6	10	1
Strong positive	1	2	0	1

τ = 0.295, *P* = 0.018

cells (Fig. 2), among 64 specimens, the positive expression of Ki-67 was 41 cases and the positive rates was 64.06% in NSCLC cells. The expression of Ki-67 was independent of pathological type , tumor size, age and sex of the patients ($P > 0.05$) while it was correlated with tumor differentiation histopathological grading of the cancer ($P = 0.000$), clinical stages ($P = 0.035$) and lymph node metastasis ($P = 0.003$).

Correlation between the expression of Elf-1 and Ki-67 (Table 3)

Elf-1 was positively correlated with Ki-67 in NSCLC ($\tau = 0.295$, $P = 0.018$) with the Kendell rank correlation for analysis.

Discussion

The TMA put lots of tissues from different sources on one solid carrier such as glass slab and it can represent the pathological information of traditional slides, it is developed rapidly since TMA have been applied. TMA is a novel array-based high-throughput technique to facilitate analysis of multi-specimens with high-efficiency simultaneously only in one microarray, it significantly saves time and costs in situ analysis of gene and protein expression, the advantages of TMA technology

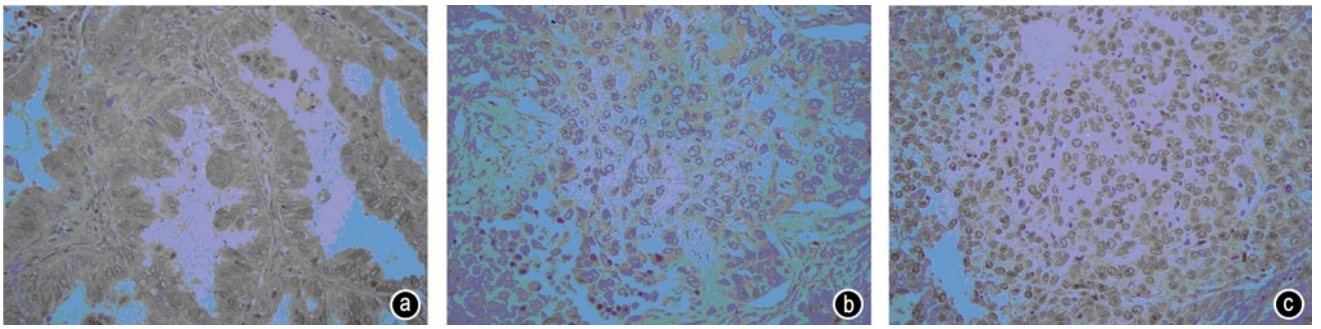

Fig. 1 Expression of Elf-1 protein in NSCLC tissue (SP stain × 400). (a) Expression of Elf-1 in the nucleus of lung adenocarcinoma; (b) Weakly expression of Elf-1 in well differentiated lung squamous carcinoma; (c) Strongly expression of Elf-1 in poorly differentiated lung squamous carcinoma

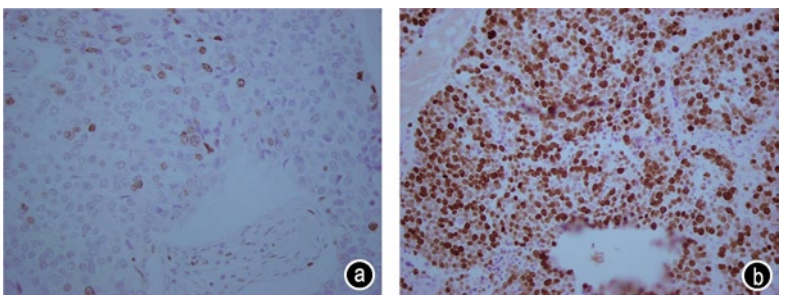

Fig. 2 Ki-67 expression in NSCLC (SP stain × 400). (a) Weakly expressed of Ki-67 in well differentiated lung squamous carcinoma; (b) Intensely expressed of Ki-67 in poorly differentiated lung squamous carcinoma

also include small bulk, efficiency and high resolution. Nowadays, combined with histochemical techniques, immunohistochemical techniques and conventional pathology techniques, TMA is considered as a reliable and high-throughout tool which is playing a key role in the researches of large sample, retrospective clinipathological and post-genomics of human cancers, it is said to have wide and valuable application prospects.

Lung cancer is one of the most common malignancies with poor prognosis that has seriously threatened the human life, in recent decades, researchers found that cancer is a genetic-disease, the occurrence and development of malignancies are extremely complex multi-gene regulation and multi-step process, it is important clinical significance that the distribution and expression of tumor markers are detected and studied timely. Ets is transcription factors family, the Ets transcription factors regulate a wide variety of biologic processes, they regulate the genes involved in cellular proliferation and differentiation, migration, apoptosis and cell-cell interactions [2]. Many of the Ets family members are proto-oncogenes, one such factor, Elf-1, an Ets-related transcriptional factor that is a less investigated among the Ets family members. Elf-1 contains a LXCXE retinoblastoma protein-binding motif near its amino terminus, its activity of Elf-1 is regulated by interactions with the retinoblastoma protein and these interactions are in turn regulated in a cell-cycle-dependent fashion [3], Elf-1 is expressed in specific cell cycles and correlated with developmental processes, it has been characterized an important role in tumor angiogenesis, invasion and metastasis. The current studies have confirmed that Elf-1 is overexpressed in many malignant tumors, while the weakly expression in the normal tissue or benign lesions, overexpression of Elf-1 has been reported in prostate cancer [4], breast cancer [5], epithelial ovarian carcinoma [6], osteosarcoma [7] and NSCLC [8]. Protein expression of Elf-1 in 64 lung cancer and 10 normal lung tissues are detected using immunohistochemistry, respectively, the results show that the positive rate of Elf-1 is 73.44% in NSCLC group and the positive expression is only in NSCLC tissues, the expression of Elf-1is significantly higher in NSCLC than in normal lung tissues ($P < 0.05$). In this study, chi-square test reveals that Elf-1 expression intensity in NSCLC is related to the differentiation degree and different clinical stages, the intensity of Elf-1 expression is significantly higher in the group with lymph node metastasis than that without, on the basis of above results, Elf-1 protein is expressed in a cell restricted pattern might display oncogenic potential, this study is consistent with the conclusions from other studies that ELF-1 may serve as an important regulator of cell cycle-specific gene expression [9].

Excessive proliferation and invasion of cells are a sign of malignancy and an important factor in the proliferation of tumor formation, development and prognosis. The Ki-67 production of a mouse monoclonal antibody was described by Gerdes J *et al* in 1983 [10], it is a nuclear antigen expressed in proliferating cells during the G_1, S, G_2 and M-phase of the cell cycle and its expression is widely used as a marker of cell proliferation [11]. it also appears to

label proliferating cells in some tumors, many studies have confirmed that there is a correlation between a high Ki-67 labeling index and a malignant biological behavior and a poor prognosis [11]. Currently, it is a commonly applied diagnostic and prognostic assessment tool of malignancy. Numerous studies have showed that Ki-67 were overexpressed in gastric cancer, non-small-cell lung cancer and colorectal cancer [12–14]. The results of this study showed that Ki-67 protein positive expression rate is 64.06% (41/64) in NSCLC and it is significantly higher than in normal lung tissues ($P < 0.05$), in addition, overexpression of Ki-67 is closely related to the biological behavior of lung cancer, but unrelated to patient sex, age, pathological types and tumor size, these findings are consistent with the conclusions from Soomro [15]. Ki-67 expression was highly associated with Elf-1, positive correlation is found between the expressive intensity of Elf-1 and Ki-67 ($\tau = 0.295$, $P = 0.018$) which reveals higher proliferative activity of cells with overexpression Elf-1. Elf-1is the cell cycle regulatory protein, which plays a positive regulatory role during the cell cycle, the overexpression of Elf-1 is correlate with the proliferative states, while, Ki-67 is a specific marker of cell proliferation [16], the simultaneous overexpression of Elf-1 and Ki-67 are both associated significantly with the malignant potential of tumor cells, which could be used as an important indicator for preliminary identification of tumor grade and prognosis judgment [16] and could be used to evaluate biological behaviors of NACLC. The development and progression of lung cancer is caused by interaction of multiple factors, on the basis of above observations, we will further explore the roles and the molecular mechanisms of Elf-1 and Ki-67 underlying the carcinogenesis of cancer, a multivariate analysis should be performed to compare Elf-1 and Ki-67 with outcome of NSCLC in order to indicate a new tumor gene therapy.

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