·Clinical Research·

Expression and significance of Elf-1 and vascular endothelial growth factor in non-small cell lung cancer

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[Abstract] **Background and Objective:** The roles of vascular endothelial growth factor (VEGF) in tumor angiogenesis is related with Ets family. Elf-1, a member of Ets family, has seldom been studied. This study aimed to investigate the expression of Elf-1 and VEGF in non-small cell lung cancer (NSCLC), and explore their correlations to clinicopathologic features of NSCLC. **Methods:** Tissue microarray containing 69 specimens of NSCLC and six specimens of normal lung tissues was constructed. The expression of Elf-1 and VEGF was detected by PowerVision™-9000 immunohistochemistry. **Results:** Elf-1 and VEGF were not detected in all normal tissues; the positive rates of Elf-1 and VEGF were 72.46% and 63.77% in NSCLC, respectively. The expression levels of both Elf-1 and VEGF were significantly related with tumor differentiation, lymphatic metastasis, clinical stage, and postoperative survival time (all $P < 0.01$). Overexpression of them was related with poor prognosis: the survival rates were significantly lower in positive patients than in negative patients (both $P < 0.01$). Elf-1 expression was positively correlated to VEGF expression ($r = 0.702$, $P < 0.01$). **Conclusions:** The expression of Elf-1 and VEGF in NSCLC is related to differentiation, lymphatic metastasis, clinical stage and prognosis. Detecting their expression in combination can help to predict the malignant behavior of NSCLC.

Key words: Elf-1, vascular endothelial growth factor, lung neoplasm, non-small cell, clinical stage, biological behavior, predictor

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Accumulating evidence has demonstrated that vascularization of tumor lesion can accelerate the growth and metastasis of solid tumor.[1, 2] Hitherto, VEGF is the most potent vascularization promoter that has been identified. In recent years, investigations have revealed that its effect is in close correlation with Ets family.[3-6] For the time being, Ets-1 and Ets-2 are the most intensively studied members of Ets family,[7-9] while Elf-1 is hardly investigated. Nevertheless, the effect of Elf-1 in vascularization is often more important than that of Ets-1 and Ets-2.[4-6] To explore the roles of Elf-1 in the vascularization of NSCLC, we used tissue microarray technique and immunohistochemistry PowerVision™-9000 to detect the protein expression levels of Elf-1 and VEGF in NSCLC, and their correlations with clinicopathologic characteristics.

Materials and Methods

Materials. A total of 69 surgically resected and pathologically

confirmed NSCLC specimens (no radiotherapy or chemotherapy was given before surgery), along with six specimens of normal lung tissue, were collected in the Affiliated Hospital of Binzhou Medical College between March 2001 and March 2003. These specimens were fixed by 10% formaldehyde (v/v) and embedded by paraffin as routine. Then they were prepared into 4-μm sections, which thereafter underwent hematoxylin and eosin (HE) staining. Among these specimens, 44 were collected from male patients and 25 from female patients. The patients aged from 36 to 73 years, with a median age of 54.5 years. Among these cancer samples, 36 were squamous cell carcinoma (11 cases were classified as grade I, 18 cases as grade II and seven cases as grade III) and 33 were adenocarcinoma (8 cases as grade I, 19 cases as grade II and 6 cases as grade III). As for tumor volume, tumor diameter was ≤ 3 cm in 24 cases, 3-5 cm in 18 cases and ≥ 5 cm in 27 cases. As for ages, 31 patients were ≤ 55 years old and another 38 patients were >55 years old. Lymph node examination was recorded in all patients, among which 32 patients showed lymph node metastases. According to the lung cancer staging criteria by UICC (1997), 12 patients were at stage T1N0M0, nine at stage T1N1M0, three at stage T1N2M0, 12 at stage T2N0M0, five at stage T2N1M0, one at stage T2N2M0, 13 at stage T3N0M0, three at stage T3N1M0, three at stage T3N1M1, one at stage T3N2M0, one at stage T3N2M1, two at stage T3N3M1, one at stage T4N1M0, one at stage T4N1M1, one at stage T4N2M0 and one at stage T4N2M1. All these patients were followed for more than five years (up to September 2008) with 25 patients (included into X ≤ 1 year group) died within one year and another 21 patients (included into X ≥ 5 year group) achieved five-year survival.

Main reagents. Both rabbit anti-human Elf-1 and rabbit anti-human VEGF antibodies were purchased from Santa Cruz Co. (US). PowerVision™-9000 reagent kit was purchased from Beijing Zhongshan Biotechnology Co., Ltd.

Detection method. Tissue microarray was prepared using perforating needle in a tissue microarray apparatus, with a needle diameter of 2 mm. Immunohistochemical staining PowerVision™ 9000 was used. Control groups were prepared for each batch of staining, with PBS in replacement of primary antibody as negative controls and known positive section as positive controls. Forimmunohistochemical staining, the paraffin sections were dewaxed and hydrated by gradient ethanol. Then intrinsic peroxidase was deactivated by 3% (v/v) hydrogen peroxide. The sections were then recovered by microwave and was added with rabbit anti-human Elf-1 antibody (1:100) or rabbit anti-human VEGF antibody (1:200). Reagent II and III were added in turn, followed by incubation. Chromogenic reagent DAB was added. The sections were then observed under microscope and chromogenic process was terminated with tap water. The sections were then re-stained by Mayer hematoxylin and were sealed by neutral gum.

Determination of results. To determine VEGF expression levels, 5 high-power visual fields were randomly selected (necrotic field was excluded) and 1000 lung cancer cells were counted for the calculation of percentage of positive cells. For varied percentages of positive cells: 0% was recorded as 0 point; ≤ 25% was recorded as 1; 26%-50% was recorded as 2; >50% was recorded as 3. For staining intensity, 0 point indicated negative staining, 1 indicated weak positive, 2 indicated positive, and 3 indicated strong positive. Total score was calculated by adding percentage points and intensity points. Maximum total score was 6 points, while a total score >2 was regarded as positive immunohistochemical staining. Classifications of VEGF score: a total score of 0-2 was regarded negative; a score of 3-4 was regarded as low level and a score of 5-6 as high level.[10]

To determine Elf-1 expression levels, 10 high-power visual fields were randomly selected from each section and 100 cancer cells were counted in each visual field; percentages of positive cells were calculated and stain intensity in positive cell was observed. Scores based on staining intensity: negative staining was recorded

as 0 point; light-yellowish color was 1 ; brown-yellowish color was 2; dark-brown or tan-brown was 3. Scores based on percentage of positive cells: ≤ 5% was recorded as 0 point, 6-30% as 1, 31-70% as 2, and ≥ 71% as 3. Total score was calculated by adding intensity points and percentage points. Total score of 0 indicated negative stain; 1-2 points indicated weak positive; 3-4 points indicated moderate positive and 5-6 points indicated strong positive.[8]

Statistical processing. SPSS for windows 11.0 software was used. The correlation between each parameter and clinical pathological features was analyzed by rank sum test (Kruskal-Wallis H and Mann-Whitney U methods); correlation between parameters was analyzed by Kendall rank correlation method. Survival was analyzed by Kaplan-Meier method and the difference was tested for significance by Log-rank test. $P<0.05$ indicated significant difference.

Results

Expression of Elf-1 and VEGF. Positive rates of Elf-1 and VEGF expression in NSCLC were 72.46% and 63.77%, respectively. Neither Elf-1 nor VEGF was expressed in normal lung tissue. Positively stained Elf-1 particles were mainly located in the nucleus of cancer cells (Figure 1) and also in some parts of the cytoplasm of cancer cells. Since Elf-1 was a transcriptional factor mainly located in the nucleus,[11] only those cells with stained nuclei were taken into account when calculating positive cells. On the other hand, positively stained VEGF particles were seen mainly in the cytoplasm of cancer cells but rarely in plasma membrane of cancer cells. Positive staining was also found in some vascular endothelial cells (Figure 2).

Correlations between Elf-1 expression in NSCLC and clinicopathologic features, and survival. Elf-1 expression was not correlated with tumor volume, age or gender of the patient ($P>0.05$), but was correlated with differentiation level of tumor cells ($P=0.001$), lymph node metastasis

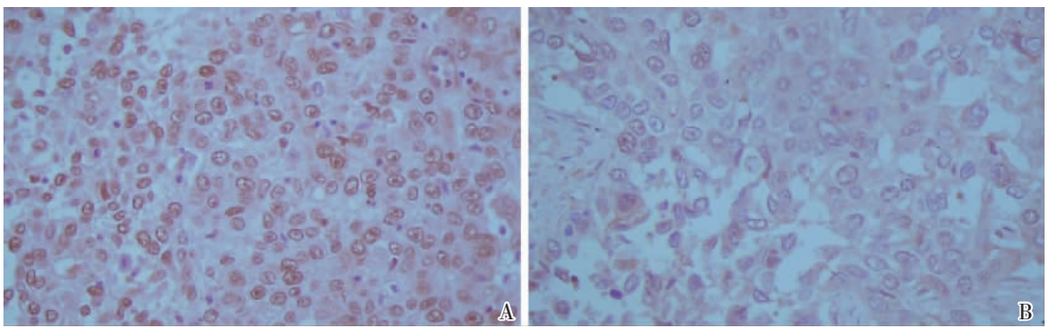

Figure 1 Elf-1 expression in lung carcinoma (PowerVision-9000 ×400)

A: Elf-1 is intensely expressed in the nuclei of cancer cells in poorly differentiated lung squamous cell carcinoma.

B: Elf-1 is weakly expressed in the nuclei of cancer cells in well differentiated lung squamous cell carcinoma.

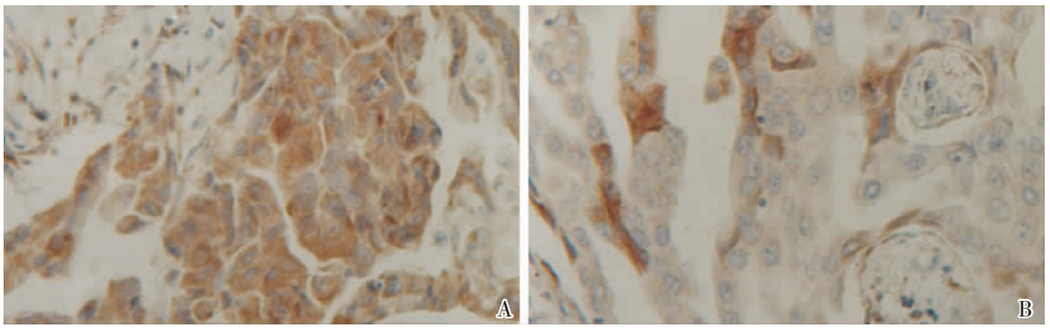

Figure 2 Vascular endothelial growth factor (VEGF) expression in lung carcinoma (PowerVision-9000 ×400)

A: VEGF is intensely expressed in the cytoplasm of cancer cells in lung adenocarcinoma with lymphatic metastasis.

B: Elf-1 is weakly expressed in the cytoplasm of cancer cells in lung adenocarcinoma without lymphatic metastasis.

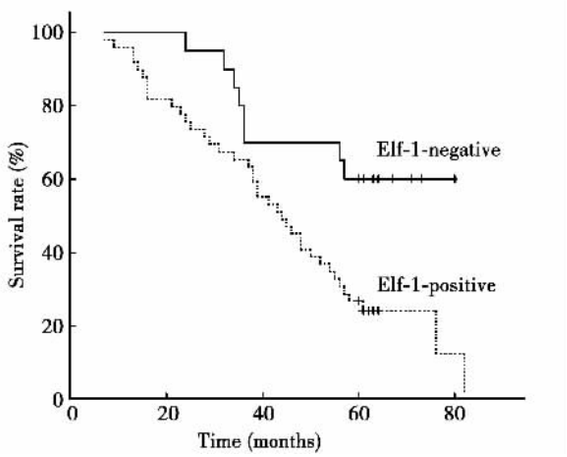

Figure 3 Relationship between Elf-1 expression and prognosis of non-small cell lung cancer (NSCLC)

(P<0.001), clinical staging (P=0.001) and post-surgical survival duration (P<0.001) (Table 1). Kaplan-Meier survival analyses showed that the mean survival duration in the NSCLC patients with positive Elf-1 expression was 45.46 months, while it was 63.50 months in the NSCLC patients with negative Elf-1 expression. Test with Log-rank method revealed χ^2 =7.193, P = 0.007, indicating that the survival rate in the patients with positive Elf-1 expression was significantly lower than that in the patients with negative Elf-1 expression.

Correlations between VEGF expression in NSCLC and clinicopathologic features, and survival. VEGF expression was not correlated with tumor volume, age or gender of the patient (P>0.05), but was correlated with differentiation level of tumor cells (P=0.006), lymph node metastasis (P<0.001), clinical staging (P=0.001) and post-surgical survival duration (P<0.001) (Table 2). Kaplan-Meier survival analyses showed that the mean survival duration in the NSCLC

Table 1 Correlation of Elf-1 expression to clinicopathologic features of non-small cell lung cancer

Item	Cases	Elf-1 expression [cases (%)]				Statistic (χ^2 or Z)	*P* value
		−	+	++	+++		
Sex						−1.331	0.183
Man	44	14(20.29)	15(21.74)	10(14.19)	5 (7.25)		
Woman	25	5 (7.25)	8(11.59)	7(10.14)	5 (7.25)		
Age						−0.816	0.415
≤55	31	8(11.59)	12(17.40)	8(11.59)	3 (4.35)		
>55	38	11(15.94)	11(15.94)	9(13.04)	7(11.59)		
Histological type						−0.356	0.722
Squamous cell carcinoma	36	8(11.59)	13(18.84)	12(17.39)	3 (4.35)		
Adenocarcinoma	33	11(15.94)	10(14.19)	5 (7.25)	7(10.14)		
Histological grade						13.496	0.001[a]
Ⅰ	19	8(11.59)	9(13.04)	1 (1.45)	1 (1.45)		
Ⅱ	37	10(14.19)	13(18.84)	9(13.04)	5 (7.25)		
Ⅲ	13	1 (1.45)	1(1.45)	7(10.14)	4 (5.80)		
Lymph node metastasis						−3.386	<0.001
Positive	32	0(0.00)	15(21.74)	10(14.19)	7(10.14)		
Negative	37	19(27.54)	8(11.59)	7(10.14)	3 (4.35)		
Tumor size (cm)						−0.108	0.914
≤3	24	7(10.14)	7(10.14)	8(11.59)	2 (2.90)		
>3, <5	18	5 (7.25)	6 (8.70)	3 (4.35)	4 (5.80)		
≥5	27	7(10.14)	10(14.19)	6 (8.70)	4 (5.80)		
Clinical stage						−3.411	0.001
Ⅰ−Ⅱ	51	17(24.64)	22(31.88)	6 (8.70)	6 (8.70)		
Ⅲ−Ⅳ	18	2 (2.90)	1 (1.45)	11(15.94)	4 (5.80)		
Postoperative survival (years)						19.296	<0.001[b]
≥5	21	12(17.40)	7(10.14)	1 (1.45)	1 (1.45)		
>1, <5	23	3 (4.35)	13(18.84)	6 (8.70)	1 (1.45)		
≤1	25	4 (5.80)	3 (4.35)	10(14.19)	8(11.59)		

[a]Between grade Ⅱ and grade Ⅲ, *P*=0.011; between grade Ⅰ and grade Ⅲ, *P*<0.001. [b]Between every two subgroups, *P*<0.05.

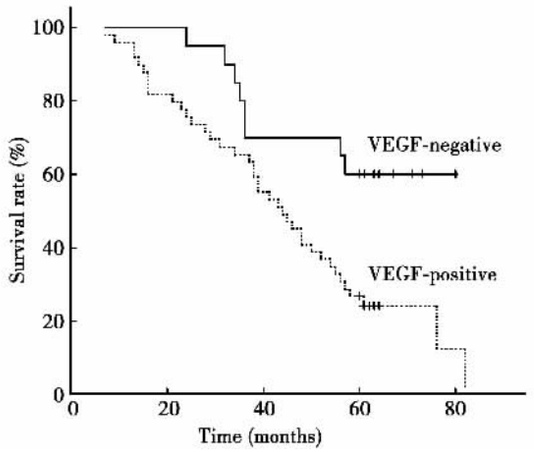

Figure 4 Relationship between VEGF expression and prognosis of NSCLC

patients with positive VEGF expression was 44.8 months, while it was 61.2 months in the NSCLC patients with negative VEGF expression. Test with Log rank method revealed χ^2=7.062, P = 0.008, indicating that the survival rate in the patients with positive VEGF expression was significantly lower than that in the patients with negative VEGF expression.

Correlation between Elf-1 and VEGF expressions. In NSCLC, expression of Elf-1 was positively correlated with that of VEGF (r = 0.702, P<0.001).

Discussion

Nowadays, lung cancer is the most frequent malignant tumor with high mortality rate, and its incidence keeps increasing. Modern molecular

Table 2 Correlation of vascular endothelial growth factor (VEGF) expression to clinicopathologic features of non-small cell lung cancer

Item	Cases	VEGF expression [cases (%)]			Statistic	*P* value
		Negative(0–2 scores)	Low(3–4 scores)	High(5–6 scores)	(χ^2 or Z)	
Sex						
Man	44	17(24.64)	17(24.64)	10(14.19)	−1.084	0.278
Woman	25	8(11.59)	9(13.04)	8(11.59)		
Age						
≤55	31	10(14.19)	14(20.29)	7(10.14)	−0.096	0.923
>55	38	15(21.74)	12(17.40)	11(15.94)		
Histological type						
Squamous cell carcinoma	36	11(15.94)	14(20.29)	11(15.94)	−1.120	0.263
Adenocarcinoma	33	14(20.29)	12(17.40)	7(10.14)		
Histological grade						
Ⅰ	19	12(17.40)	6 (8.70)	1 (1.45)		
Ⅱ	37	11(15.94)	18(26.09)	8(11.59)	10.160	0.006[a]
Ⅲ	13	2 (2.90)	2 (2.90)	9(13.04)		
Lymph node metastasis						
Positive	32	4 (5.80)	16(23.19)	12(17.40)	−3.512	<0.001
Negative	37	21(30.43)	10(14.19)	6 (8.70)		
Tumor size(cm)						
≤3	24	8(11.59)	9(13.04)	7(10.14)		
>3, <5	18	6 (8.70)	8(11.59)	4 (5.80)	0.243	0.885
≥5	27	11(15.94)	9(13.04)	7(10.14)		
Clinical stage						
Ⅰ–Ⅱ	51	21(30.43)	24(34.78)	6 (8.70)	−3.725	0.001
Ⅲ–Ⅳ	18	4 (5.80)	2 (2.90)	12(17.40)		
Postoperative survival (years)						
≥5	21	13(18.84)	7(10.14)	1 (1.45)		
>1, <5	23	6 (8.70)	14(20.29)	3 (4.35)	18.532	<0.001[b]
≤1	25	6 (8.70)	5 (7.25)	14(13.04)		

[a]Between grade Ⅱ and grade Ⅲ, P=0.026; between grade Ⅰ and grade Ⅲ, P=0.005. [b]Between every two subgroups, P<0.05.

and biological researches have demonstrated that before neo-vascularization, tumor lesion merely exists as small and asymptomatic lesion; when new vasculature is developed, the tumor can grow rapidly and its ability of developing distant metastasis is enhanced.[1, 2, 12] Therefore, it is possible to control the growth and metastasis of tumor if we can identify the pathways regulating vascularization.

VEGF, also known as vascular permeability factor (VPF), is an important vascularization promoter. With a molecular weight of 46 kD, it is a highly conservative glycoprotein dimer connected by disulfide bond. It can be released by many types of tumor cells and is also expressed in in-situ tumors. VEGF specifically acts on vascular endothelial cells and triggers proliferation and vascularization; it can increase the permeability of primary capillary and veinule to macromolecules and also stimulate elongation and duplication in certain endothelial cells of different originations. The results of our study showed that VEGF expression was mainly seen in the cytoplasm of lung cancer cells, with marked heterogeneity. Its expression was significantly increased in the cancer tissue around blood vessels [I cannot find the images or description in the "Results" section], suggesting that VEGF was closely related to the vascularization of tumor. Expression of VEGF showed statistically significant difference between lymph node metastatic group and non-metastatic group, among histological grade I, II and III, between different post-surgical survival groups, and among varied clinical stages, which was in consistent with the perspective that VEGF, as the most important vascularization promoter, was correlated with the tendency toward lymph node metastasis, more invasiveness and poor prognosis of tumors.[13-15] The lower level the differentiation of lung cancer cells is, the rapider the tumor grows and the more it is prone to hypoxia, which induces increased production of VEGF and thereby increases tumor vascularization to provide more oxygen and nutrition for cancer cells and to promote growth of lung cancer. When tumor volume increases, the marginal cancer cells are more frequently exposed to lymphatic ducts, or they may enter bloodstream via anastomosis of vein to lymphatic duct and then join the lymphatic fluid, which precipitates lymph node metastasis of lung cancer and subsequent poor prognosis. Results of our study also showed that expression of VEGF was correlated withthe survival rates in the patients, and that the patients with negative VEGF expression enjoyed significantly longer survival duration than those with positive expression.

Studies revealed the effects of VEGF mentioned above were closely related to Elf-1.[3-6] Elf-1 was first composed by hybridizing the probes of DNA-binding domain in human Ets-1 cDNA as obtained from human T cell gene pool. It was a less investigated transcriptional factor among Ets family. Ets family is group of transcriptional factors containing a DNA-binding domain (ETS domain) constituted by 85 amino acids, with a winged helix-turn-helix structure; they can recognize and bind to the purine-abundant core DNA sequence GGAA/T, which is located in 5-flanking regulation domain of many genes related to the degeneration of extracellular matrix and vascularization, such as MMMP-3 and urokinase plasminogen activator (uPA). Thereby, Ets family regulates the transcription of these genes. We noticed that Elf-1 was highly expressed in non-small cell lung cancer; patients with lymph node metastasis showed noticeably higher positive rate of Elf-1 expression than patients without lymph node metastasis; expression of Elf-1 significantly increased in invasive margin of tumor [I cannot find this finding the "Results" section]; in addition, expression of Elf-1 remarkably increased with the malignancy of tumor. These findings are consistent with the conclusions from other studies that Elf-1 was correlated with the occurrence and metastasis of endometrial carcinoma, prostate carcinoma and osteosarcoma.[11, 16-18] Knatun and colleagues[3] suggested that Elf-1 up-regulated the expression of MMP-3, integrin β and uPA and brought about the transformation from endothelial cells to vascular phenotype, and thus induced vascularization in cancer tissue and promoted the invasiveness and metastasis of cancer, which resulted in poor

prognosis. Our study also demonstrated that the expression level of Elf-1 was significantly increased along with the progression of tumors (as indicated by TNM staging) and the shortening of post-surgical survival in the patients. Kaplan-Meier survival analysis also showed that Elf-1 expression was correlated withpatient survival, and the patients with negative Elf-1 expression achieved significantly longer survival duration than those with positive expression.

Dube and colleagues[4] performed Northern blot and in-situ hybridization in cultured cells and found that Elf-1 played important roles in vascularization and growth process by regulating the promoter genes of Tie1 and Tie2. Our study indicated that VEGF expression was positively correlated with that of Elf-1, supporting the perspective that VEGF can induce the expression of Elf-1. Therefore, it raises the hypothesis that VEGF and Elf-1 might act in synergy during vascularization. Hahne and colleagues[5] report that VEGF induces Ets-1 expression in endothelial cells, and Ets-1 up-regulates the expression of proteolytic enzymes, such as MMP-1, -3, -9 and uPA, which further promote the degeneration of basement membrane and participate in the vascularization process. In this pathway, Ets-1 is an intermediate link. It is therefore possible to interrupt vascularization of tumors by blocking the expression of Ets-1.[19] Expression of VEGF in human endothelial cells and vascular smooth muscle cells could be effectively inhibited by using antisense oligonucleotides of Ets-1.[20] While Huang and colleagues[6] believe that Elf-1 has decisive role in vascularization, because two members of Ets family, ETS-1 and ETS-2, regulate promoter genes of Tie1 and Tie2 via Elf-1, and thereby exerted important effects in vascularization and tumor growth process. Hence, to block the expression of Elf-1 might be an effective way to inhibit tumor growth.

In conclusion, our study demonstrated high level expression of Elf-1 in NSCLC was significantly correlated with differentiation level of tumor cell, lymph node metastasis, clinical staging and prognosis, and had important role in tumor vascularization, invasiveness and metastasis. To block the expression of Elf-1 might be an effective way to inhibit tumor growth. Both Elf-1 and VEGF were involved in tumor infiltration and lymph node metastasis. Combined detection on their expressions might serve as a useful biomarker to monitor the biological behaviors of NSCLC, and might have prognostic and predictive values for NSCLC patients.

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