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Original Article

Association of ZEB1 and FOXO3a protein with invasion/metastasis of non-small cell lung cancer

Liang Tian[1], Nan Guo[2], Nan Zhang[2], Zhigang Miao[1], Xiaozhong Guo[1], Ningning Peng[3], Rongju Zhang[1], Yu Miao[1]

Departments of [1]Pathology, [2]Cardiothoracic Surgery, [3]Orthopedics, Hebei Medical University Affiliated Cangzhou Central Hospital, Cangzhou 061001, Hebei Province, China

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Abstract: Objective: The research was aimed to study the expression of ZEB1 and FOXO3a in NSCLC tissue, and to explore the effect of its expression on infiltration/metastasis of NSCLC. Methods: Total of 130 pairs of NSCLC tumor tissue and adjacent normal tissue were collected from June 2013 to June 2015 in Cangzhou Central Hospital. The expression of ZEB1 and FOXO3a protein was detected by immunohistochemistry and Western blot, and mRNA by qPCR. We analyzed the relationship between clinical data of NSCLC and gene expression of ZEB1 and FOXO3a. Results: The expression of ZEB1 protein in NSCLC tissues was significantly higher than that in adjacent normal tissue ($P<0.05$), but the FOXO3a was just opposite ($P<0.05$). The expression of ZEB1 and FOXO3a protein was significantly correlated with tumor size, differentiation degree, lymph node metastasis, distal metastasis and TNM staging ($P<0.05$). The expression of ZEB1 protein was significantly increased and the expression of FOXO3a protein was significantly decreased in NSCLC patients with lymph node metastasis or distant metastasis ($P<0.05$). The 2-year survival rate of patients with high expression of ZEB1 or low expression of FOXO3a was significantly lower than that of NSCLC patients with low expression of FOXO3a ($P<0.05$). Pearson analysis showed that the expression of ZEB1 mRNA in NSCLC was negatively correlated with FOXO3a mRNA expression ($r = -0.705$, $P<0.05$). Conclusion: ZEB1 is highly expressed in NSCLC tissues and FOXO3a is lowly, and they collaborate to promote NSCLC infiltration and metastasis.

Keywords: FOXO3a, ZEB1, non-small cell lung cancer, infiltration

Introduction

Non-small cell lung cancer (NSCLC) is a primary bronchogenic carcinoma of the lung, which accounts for about 80% of the total lung cancer patients, and is the most common and lethal tumor in the world [1, 2]. Over 90% NSCLC patients die from cancer cell metastasis, but the molecular mechanism of NSCLC cell metastasis remains unknown [3]. Other studies [4-6] have pointed out that, the epithelial-mesenchymal transition (EMT) process, a process during which closely connected and polarized epithelial cells gradually transform into loosely organized mesenchymal cells that are devoid of cell junctions and cell polarity, enables cancer cells to acquire invasion and migration capability, and is a prerequisite for the metastasis of cancer cells. Therefore, the study of the expression of EMT related proteins in NSCLC tissues will help to reveal the molecular mechanism of the initiation of NSCLC cell metastasis.

E box binding zinc finger protein 1 (ZEB1) can inhibit the expression of epithelial markers like E-cadherin, and act as a key inducer gene in EMT [7, 8]. The forkhead box family protein FOXO3a is a tumor suppressor that has been intensively studied, and it can regulate the EMT process by inhibition of the related pathways [9]. Current studies have also demonstrated that aberrant expression of ZEB1 and FOXO3a proteins is associated with tumor invasion and migration [7-9]. However, there is still little research on the expressions of ZEB1 and FOXO3a in NSCLC tissues. In this study, we examined the expression of ZEB1 and FOXO3a proteins by immunohistochemistry and western blotting, as well as the relative expression of ZEB1 and FOXO3a mRNAs in different tissues

by real-time quantitative PCR. We also analyzed the relationship between their expressions and the clinical pathological data of NSCLC, and discussed the correlation of the expression of ZEB1 and FOXO3a in NSCLC tissues with its influence on the invasion and metastasis of NSCLC, thus providing theoretical basis for revealing the molecular mechanism underlying the intonation of NSCLC cell metastasis.

Materials and methods

Clinical data and specimens

A total of 130 pairs of tumor tissues and the adjacent normal tissues (distance >5 cm) of NSCLC patients diagnosed by clinical pathology were collected from June 2013 to June 2015 at the Cangzhou Central Hospital. Among them, there were 79 male patients and 51 female patients. Other general data were shown in **Table 1**; each tissue specimen was separated into 3 parts, 2 of which were soybean-sized and direct frozen into liquid nitrogen, while the other part was made into paraffin sections. In addition, this study was approved by the e Ethical Commission of Cangzhou Central Hospital.

Reagents and instruments

VECTASTAIN® Elite® ABC Kit (PK-7200, Vector Laboratories, USA); FOXO3a rabbit monoclonal antibody (ab53287, Abcam, UK), ZEB1 rabbit monoclonal antibody (ab203829, Abcam, UK) and goat anti-rabbit IgG monoclonal antibody (ab190492, Abcam, UK); 10×PBS (Hyclone, USA); tissue total protein extraction kit, BCA protein concentration detection kit and tissue total RNA extraction kit (Beyotime Biotechnology, China); PrimeScript™ RT reagent kit (#RR037A, Takara, Japan); fluorescence real-time quantitative PCR instrument (aglient, USA); two color infrared laser imaging system (LICOR, USA); NANODROP 1000 (Thermo fisher, USA).

Experimental method

Immunohistochemical detection of the expression of ZEB1 and FOXO3a proteins

The prepared paraffin sections of the tumor tissues and the adjacent normal tissues were subjected for immunochemistry according to the instructions of the purchased VECTASTAIN® Elite® ABC Kit as well as ZEB1 and FOXO3a antibodies (1×PBS was used in replacement of the primary antibody in the negative control group). Fox3a was located in the nucleus, while ZEB1 was located in the nucleus and cytoplasm. Immunohistochemical results were assessed by reference to Yueming Zhang [10]: positive cell rate <25% = 0 point, 25%-75% = 1 point, >75% = 2 points; For staining intensity, none (colorless) = 0 point, weak (light yellow) = 1 point, medium (pale brown) = 2 points, strong (dark brown) = 3 points; the product of positive cell rate and staining intensity score A ≥4 was identified as high expression.

Western blot detection of ZEB1 and FOXO3a proteins

The total tissue proteins were extracted from the tissue samples frozen in liquid nitrogen according to the instructions of the purchased tissue total protein extraction kit. After detecting the protein concentrations, SDS-PAGE was performed, and the membrane was blocked with 5% no-fat milk, incubated with primary antibodies of FOXO3a, ZEB1 or GAPDH and goat anti-rabbit secondary antibodies before image development. The band density was analyzed by Image-J software and normalized against GAPDH levels to be presented as the final results.

qPCR detection of the expression of ZEB1 and FOXO3a mRNAs

The total tissue RNA was extracted from the tissue samples frozen in liquid nitrogen according to the instructions of the purchased tissue total RNA extraction kit. NANODROP 1000 was used to detect the RNA concentrations, and 1000 ng total RNA was revers-transcribed into cDNA. About 25 ng cDNA was subjected for real-time PCR to detect the relative expressions of FOXO3a (Forward: 5'-AATTTTTCCTGGGCACACGC-3'; Reverse: 5'-CAACAGTTCATCCTGGCAGC-3'), ZEB1 (Forward: 5'-TCCAAAGAGCATTTGGATGTCTGAA-3'; Reverse: 5'-TCCAAAGCACGTTACCCAGG-3') and ZEB1 (Forward: 5'-GAACTCTCACGGAAACGGGT-3'; Reverse: 5'-AGGCCTGTGTACCTAGGAGG-3'). The results were expressed as $2^{-\Delta\Delta Ct}$.

Statistical analysis methods

Statistical analysis was performed using the SPSS20.0 statistical program. Chi-square test

Table 1. Baseline data of the 130 patients with NSCLC

Item	Case (n)	Ratio (%)
Gender		
Male	79	60.76
Female	51	35.38
Age (years)		
≥60	55	42.31
<60	75	57.69
Clinical typing		
Squamous cell carcinoma	38	29.23
Adenocarcinoma	92	70.77
Tumor size (cm)		
≤5	38	29.23
>5	92	70.77
Differentiation degree		
Poorly differentiated	24	18.46
Moderately differentiated	38	29.23
Well differentiated	68	52.31
Lymph node metastasis		
Transfer	80	61.54
Not transferred	50	38.46
Distant metastasis		
Transfer	76	58.46
Not transferred	54	41.54
TNM staging		
I+II	42	32.21
III+IV	88	67.69

was used to compare the differences of the enumeration data between groups (percentage), while *t* test was used to compare the differences of the measurement data between groups (mean ± standard deviation). Pearson method was used to analyze the correlation between two indexes, and $P<0.05$ was considered statistically significant.

Results

Baseline data of the 130 patients with NSCLC

As shown in **Table 1**, among the 130 NSCLC patients enrolled in the study, 79 were males and 51 were females, while 55 were at or over the age of 60 years old and 75 were at the age less than 60 years old. 38 cases were squamous cell carcinomas and 92 cases were adenocarcinomas. There were 24 cases with low differentiation, 38 cases with middle differentiation, and 68 cases with high differentiation, while 76 cases were with distant metastasis and 54 cases were without distant metastasis. Moreover, the TNM stage was stage I/II in 42 cases and stage III/IV in 88 cases.

Immunohistochemical detection of the expression of ZEB1 and FOXO3a proteins

Immunohistochemical staining and scoring were performed for the 130 pairs of NSCLC tumor tissues and the adjacent normal tissues. The results showed that: (1) ZEB1 protein was over expressed in 83 cases of tumor tissues while lower expressed in 47 cases, and the immunohistochemical score was (4.02±1.56); ZEB1 protein was lower expressed in all the adjacent normal tissues, and the immunohistochemical score was (0.94±0.78); (2) FOXO3a protein was over expressed in 55 cases of tumor tissues while lower expressed in 75 cases, and the immunohistochemical score was (2.69±1.79); FOXO3a protein was over expressed in all the adjacent normal tissues, and the immunohistochemical score was (4.97±1.00). See the detail in **Figure 1**.

Relationship of the expression of ZEB1 and FOXO3a proteins with the clinical data of NSCLC patients

In the 130 patients with NSCLC, over expression of ZEB1 protein was found in 83 cases while lower expression in 47 cases. The relationship of the expression of ZEB1 and FOXO3a proteins with the clinical data of NSCLC patients was analyzed, and the results showed that: the expression of EB1 and FOXO3a proteins was independent of sex, age or the clinical classification of the NSCLC patients ($P>0.05$), while it was significantly related to tumor size, degree of differentiation, lymph node metastasis, distant metastasis, as well as TNM staging ($P<0.05$), as shown in **Table 2**.

Relationship of the expression of ZEB1 and FOXO3a proteins with NSCLC cell metastasis

In the 130 patients with NSCLC, 80 cases were found with lymph node metastasis while 50 cases were without lymph node metastasis. The immunohistochemistry results showed that: the immunohistochemical score of ZEB1 protein in the NSCLC patients with lymph node metastasis was (4.39±1.35), which was signifi-

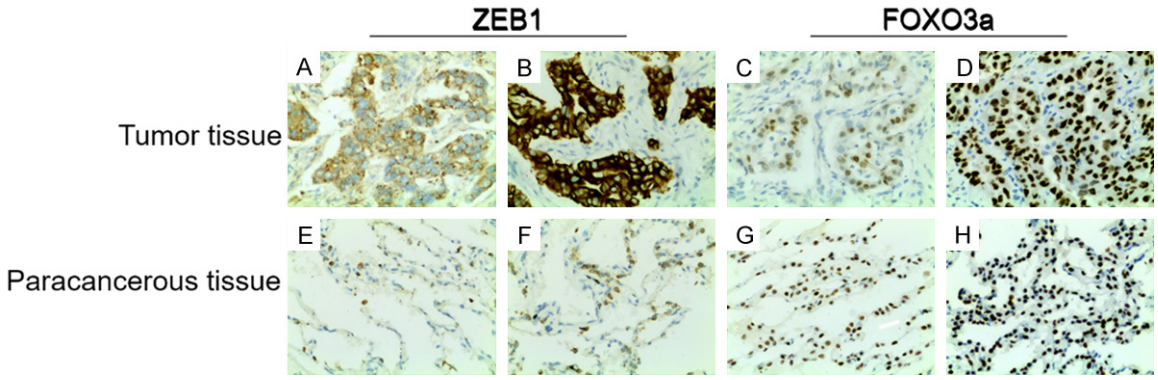

Figure 1. Immunohistochemical staining of ZEB1 and FOXO3a proteins. (1) For the immunohistochemistry results, Fox3a localized in the nucleus while ZEB1 was located in the nucleus and cytoplasm; (2) ZEB1 protein was lower expressed (A) or over expressed (B) in NSCLC tumor tissues, while lower expressed (E) or over expressed (F) in the adjacent normal tissues; FOXO3a protein was lower expressed (C) or over expressed (D) in NSCLC tumor tissues, while lower expressed (G) or over expressed (H) in the adjacent normal tissues.

Table 2. Relationship of the expression of ZEB1 and FOXO3a proteins with the clinical data of NSCLC patients

Item	ZEB1		χ^2	*P*	FOXO3a		χ^2	*P*
	High (case)	Low (case)			High (case)	Low (case)		
Gender								
Male	53	26	0.917	0.338	30	49	1.549	0.213
Female	30	21			25	26		
Age (years)								
≥60	39	16	2.060	0.151	26	29	0.963	0.326
<60	44	31			29	46		
Tumor size (cm)								
≤5	18	20	6.313	0.012	22	16	5.345	0.021
>5	65	27			33	59		
Clinical typing								
SC	31	25	3.071	0.080	19	37	2.830	0.093
AC	52	22			36	38		
Differentiation degree								
Poor	12	12	12.285	0.002	14	10	7.821	0.020
Moderate	20	18			20	18		
Well	51	17			21	47		
Lymph node metastasis								
Transfer	59	21	8.839	0.003	27	53	6.241	0.012
Non	24	26			28	22		
Distant metastasis								
Transfer	64	12	32.871	0.000	21	55	16.145	0.000
Non	19	35			34	20		
TNM staging								
I+II	20	22	7.078	0.008	23	19	8.215	0.004
III+IV	63	25			22	56		

cantly higher than that in the patients without lymph node metastasis (3.42±1.70, $P<0.05$); the immunohistochemical score of FOXO3a protein in the NSCLC patients with lymph node

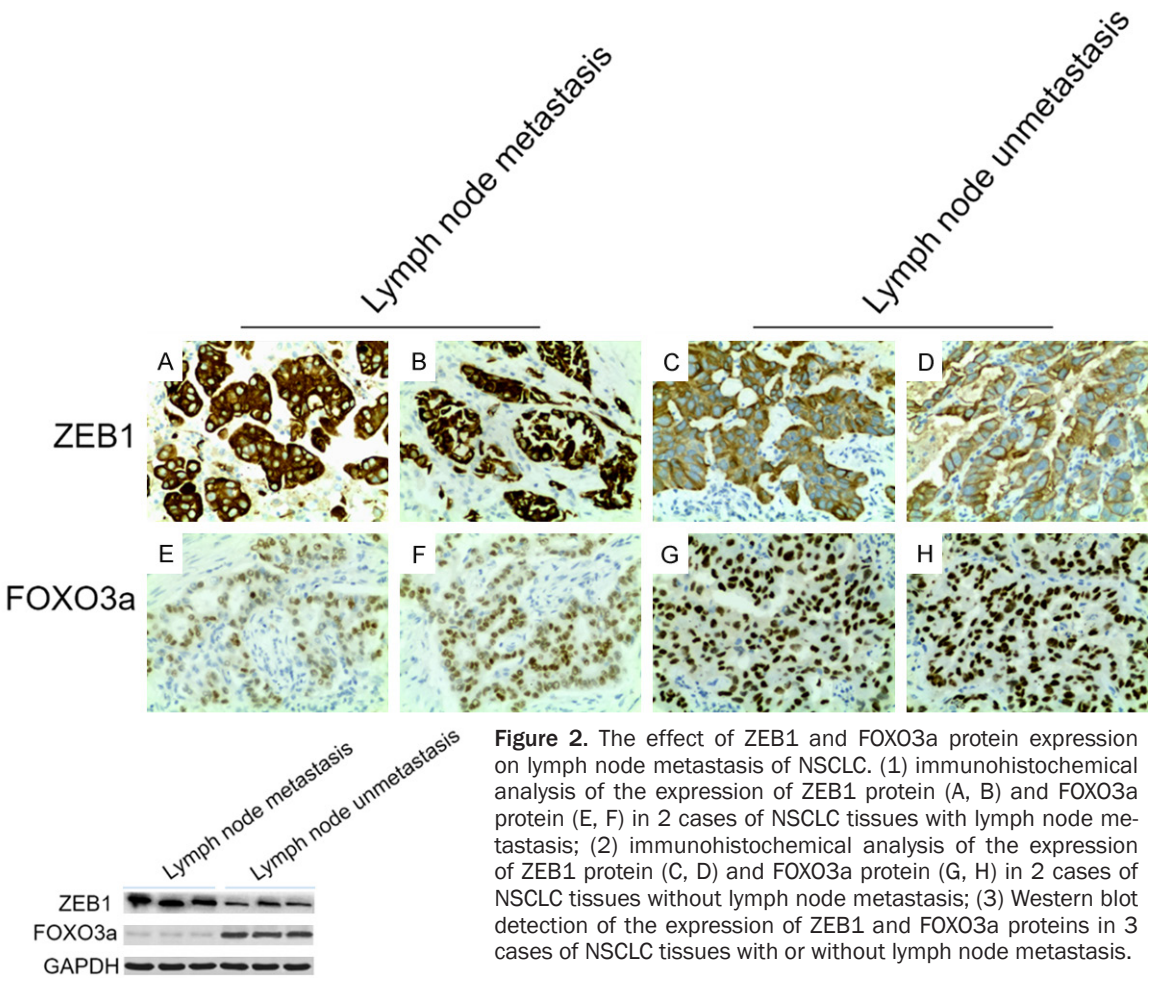

Figure 2. The effect of ZEB1 and FOXO3a protein expression on lymph node metastasis of NSCLC. (1) immunohistochemical analysis of the expression of ZEB1 protein (A, B) and FOXO3a protein (E, F) in 2 cases of NSCLC tissues with lymph node metastasis; (2) immunohistochemical analysis of the expression of ZEB1 protein (C, D) and FOXO3a protein (G, H) in 2 cases of NSCLC tissues without lymph node metastasis; (3) Western blot detection of the expression of ZEB1 and FOXO3a proteins in 3 cases of NSCLC tissues with or without lymph node metastasis.

metastasis was (2.44±1.57), which was significantly lower than that in the patients without lymph node metastasis (3.10±2.04, $P<0.05$). The results of Western bolt showed that: the relative expression of ZEB1 protein in the NSCLC patients with lymph node metastasis was (1.47±0.32), which was significantly higher than that in the patients without lymph node metastasis (0.62±0.13, $P<0.05$); the relative expression of FOXO3a protein in the NSCLC patients with lymph node metastasis was (0.10±0.02), which was significantly lower than that in the patients without lymph node metastasis (0.81±0.14, $P<0.05$), as shown in **Figure 2**.

In the 130 patients with NSCLC, 76 cases were found with distant metastasis of tumor cells while 54 cases were without distant metastasis. Immunohistochemistry results showed that: the immunohistochemical score of ZEB1 protein in the NSCLC patients with distant metastasis was (4.55±1.78), which was significantly higher than that in the patients without distant metastasis (3.16±1.72, $P<0.05$); the immunohistochemical score of FOXO3a protein in the NSCLC patients with distant metastasis was (2.29±1.58), which was significantly lower than that in the patients without distant metastasis (3.34±1.92, $P<0.05$). The results of Western bolt showed that: the relative expression of ZEB1 protein in the NSCLC patients with distant metastasis was (1.67±0.29), which was significantly higher than that in the patients without distant metastasis (0.71±0.18, $P<0.05$); the relative expression of FOXO3a protein in the NSCLC patients with distant metastasis was (0.15±0.04), which was significantly lower than that in the patients without distant metastasis (0.93±0.19, $P<0.05$), as shown in **Figure 3**.

Relationship of the expression of ZEB1 and FOXO3a with the clinical prognosis of NSCLC

The 130 patients with NSCLC were followed up for two years after the operation, and the

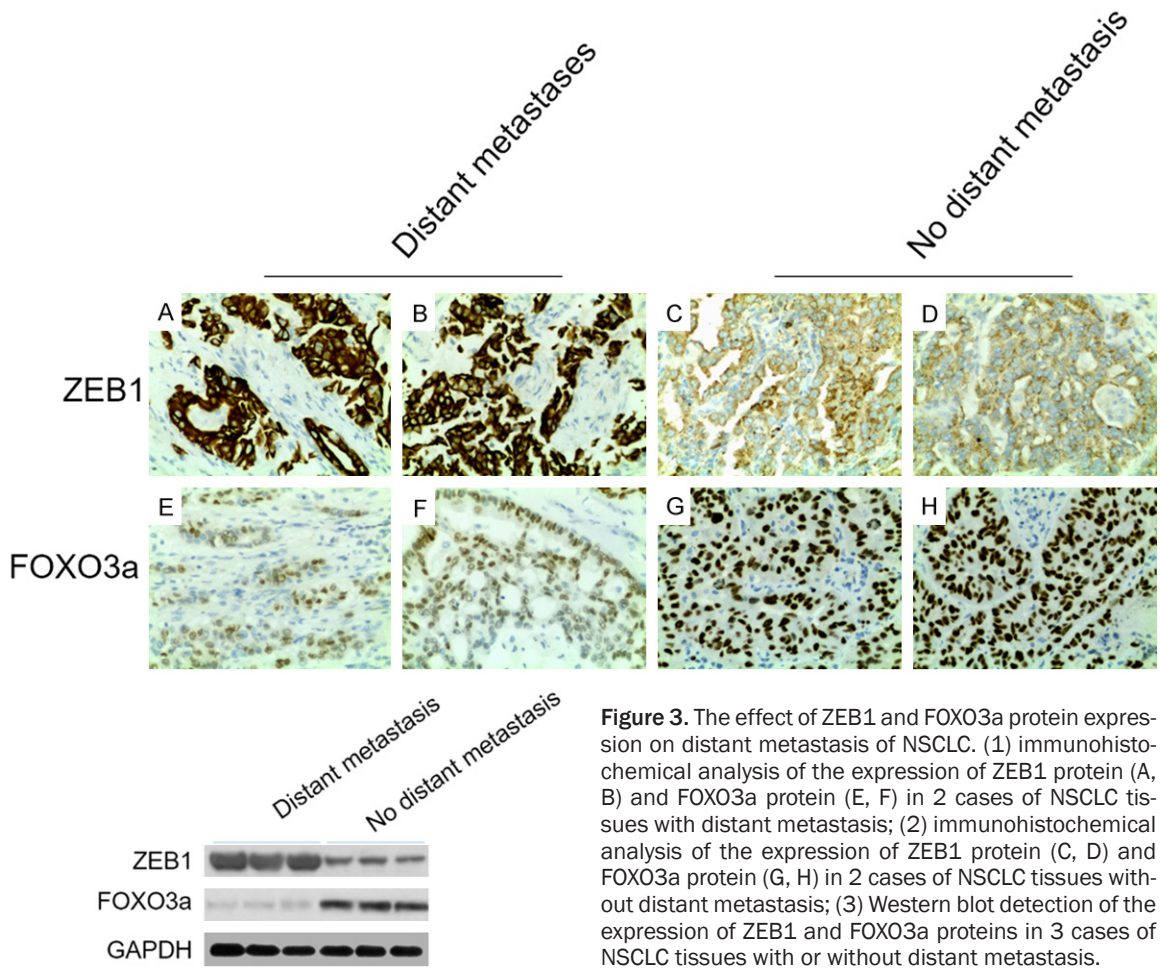

Figure 3. The effect of ZEB1 and FOXO3a protein expression on distant metastasis of NSCLC. (1) immunohistochemical analysis of the expression of ZEB1 protein (A, B) and FOXO3a protein (E, F) in 2 cases of NSCLC tissues with distant metastasis; (2) immunohistochemical analysis of the expression of ZEB1 protein (C, D) and FOXO3a protein (G, H) in 2 cases of NSCLC tissues without distant metastasis; (3) Western blot detection of the expression of ZEB1 and FOXO3a proteins in 3 cases of NSCLC tissues with or without distant metastasis.

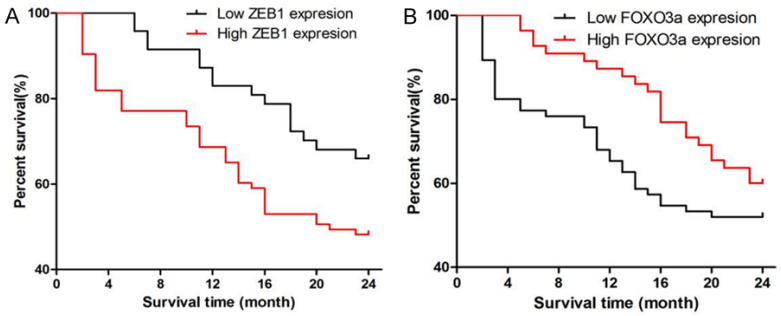

Figure 4. Effect of ZEB1 and FOXO3a protein expressions on the prognosis of patients with NSCLC.

results showed that: The 2-year survival rate of the NSCLC patients with over expressed ZEB1 was 48.19%, which was significantly lower than that of the patients with low ZEB1 expression (65.96%, $P<0.05$), as shown in **Figure 4A**; the 2-year survival rate of the NSCLC patients with lower expressed FOXO3a was 52.00%, which was significantly lower than that of the patients with high FOXO3a expression (60.00%, $P<0.05$), as shown in **Figure 4B**.

Correlation between ZEB1 and FOXO3a protein expressions in NSCLC tissues

The expression of ZEB1 and FOXO3a proteins in the 130 cases of NSCLC tumor tissues were detected by immunohistochemistry and Western blot: the higher the expression of ZEB1 protein, the lower the expression of FOXO3a protein in the tumor tissues of the same NSCLC patients, as shown in **Figure 5**.

The expression of ZEB1 and FOXO3a mRNAs in the 130 cases of NSCLC tumor tissues were detected by qPCR, and Pearson method was used to analyze the correlation between the two proteins: the expression of ZEB1 mRNA

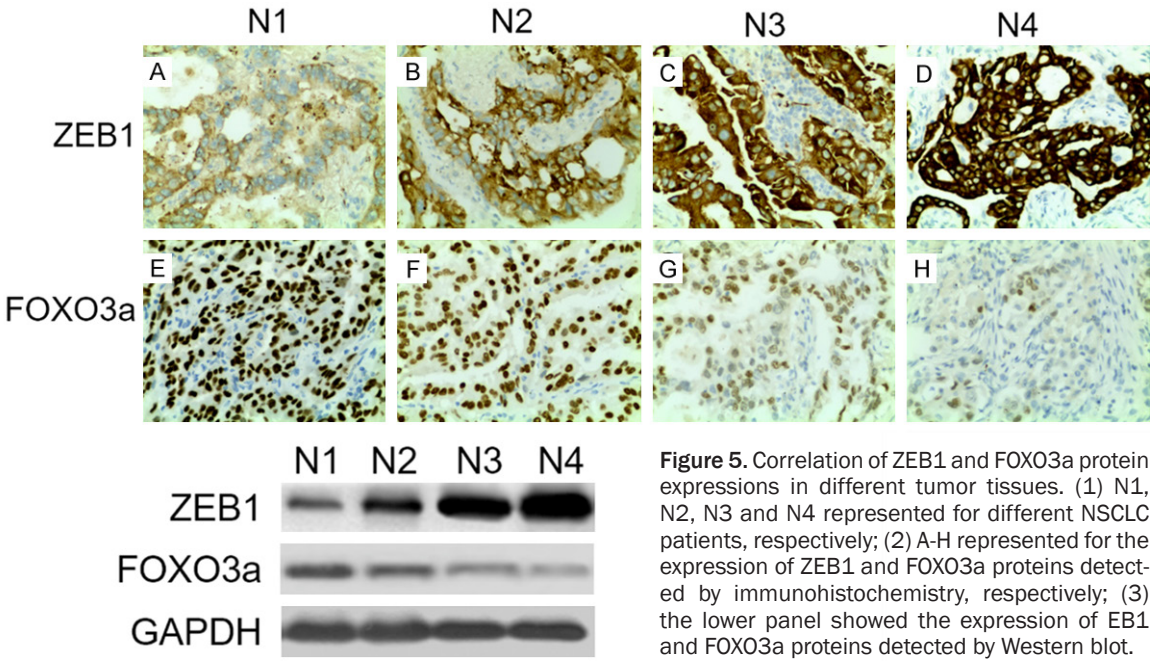

Figure 5. Correlation of ZEB1 and FOXO3a protein expressions in different tumor tissues. (1) N1, N2, N3 and N4 represented for different NSCLC patients, respectively; (2) A-H represented for the expression of ZEB1 and FOXO3a proteins detected by immunohistochemistry, respectively; (3) the lower panel showed the expression of EB1 and FOXO3a proteins detected by Western blot.

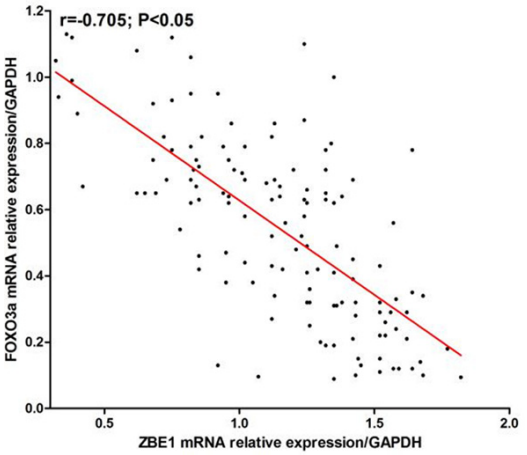

Figure 6. The scatter diagram of the correlation between ZEB1 and FOXO3a.

was negatively correlated with the expression of FOXO3a mRNA in NSCLC tissues ($r = -0.705$, $P<0.05$), as shown in **Figure 6**.

Discussion

The genesis and development of tumor is a multi-step process involving internal and external factors. Among them, EMT process is a crucial step for the invasion and migration of tumor cells, and is also one of the major causes of death in patients with malignant tumors [4-6]. For patients with non-small cell lung cancer, most patients have cancer cell infiltration and metastasis after clinical diagnosis, which is one of the main causes of death after resection of primary lesions in NSCLC patients [3]. Therefore, studying the molecular mechanism of NSCLC cell invasion and migration not only provides a theoretical basis for the development of targeted drugs for NSCLC, but also helps to determine the prognosis of patients accurately.

ZEB1, also known as TCF8 or δEF1, is located on the short arm of human chromosome 10, which is a transcription factor required for early embryonic development [11]. However, recent studies have indicated that ZEB1 protein is over expressed in breast cancer [7], thyroid cancer [12], ovarian cancer [13] as well as lung cancer [14], and that over expression of ZEB1 can confer or enhance the invasion/migration ability of cancer cells by driving EMT. In this study, we found that ZEB1 protein is over expressed in NSCLC tissues. Further analysis of the relationship between ZEB1 protein expression and the clinical data of NSCLC patients showed that the expression of ZEB1 protein in NSCLC tumor tissues was significantly correlated with tumor size, degree of differentiation, lymph node metastasis, distant metastasis as well as TNM staging, and that it was even higher expressed in the NSCLC patients with the cancer cell metastasis.

E-cadherin protein is involved in the mutual adsorption between cells as well as between cell and the basal membrane like materials. As an epithelial marker in the EMT process, E-cadherin protein inhibits tumor cell metastasis, and the decrease of its expression indicates promoted metastasis/invasion abilities of tumor cells [15, 16]. Several studies [7, 8, 17, 18] have confirmed that ZEB1 protein inhibits the expression of E-cadherin protein not only by binding with the transcription factor Slug, but also through its up-regulation by oncogenes or micro-RNAs, thus promoting tumor cell EMT.

Meanwhile, our results also suggested that FOXO3a protein showed low expression in NSCLC tissues FOXO3a, and that its expression was significantly related to NSCLC tumor size, degree of differentiation, lymph node metastasis, distant metastasis and TNM staging. FOXO3a is one of the important proteins that have been thoroughly studied recently. It is an essential transcription factor that regulates many cellular functions. As a tumor suppressor gene, its loss-of-expression has been found in multiple types of tumor tissues [19, 20]. In recent years, the study of FOXO3a and tumor cell EMT has been gradually appreciated: Ni D et al [19] found that in clear-cell carcinoma cell lines, the loss-of-expression of FOXO3a could up regulate the expression of SNAIL1 protein and induce renal cancer cell EMT, thus promoting renal cancer cell metastasis both *in vitro* and *in vivo*; Belguise K et al [20] found that FOXO3a could suppress breast cancer cell invasion by activating ERα signaling. As for lung cancer, Cheng CW et al [21] found that FOXO3a could block tumor cell metastasis by inhibiting the EMT process activated by HIF-1α. Therefore, the low expression of FOXO3a in NSCLC may be a contributing factor for NSCLC invasion and migration.

In conclusion, over expressed ZEB1 protein and lower expressed FOXO3a protein both promote NSCLC progression by enhancing the invasion and migration ability of NSCLC cells. Meanwhile, our results suggested that the expression of ZEB1 mRNA and FOXO3a mRNA was negatively correlated in NSCLC tissues. Although the mechanisms of the interaction between FOXO3a and ZEB1 in tumor tissues have not been revealed, related studies have shown that PI3K/AKT can not only bind with SHP-2 protein to drive the ZEB1-miR-200 feedback loop mediated by PDGFRα in gliomas [22], but also control the transcription of ZEB1 gene through targeted regulation of GSK3β/β-catenin signaling transduction, thus regulating the expression of cytokeratin, vimentin and MMP2 proteins [23]. In addition, miR-200c enhances the sensitivity of NSCLC cells to gefitinib and induces NSCLC cell apoptosis via PI3K/Akt signaling pathway as well as by targeting ZEB1 [24]. As a key effector protein downstream of the PI3K/AKT signaling pathway, FOXO3a may also be involved in the regulation of ZEB1 gene expression in tumor cells.

In summary, lower expression of FOXO3a and over expression of ZEB1 may synergistically contribute to NSCLC invasion/metastasis by enhancing the invasion/migration abilities of NSCLC cells mediated by the PI3K/AKT signaling pathway in non-small cell lung cancer tissues.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Liang Tian, Department of Pathology, Hebei Medical University Affiliated Cangzhou Central Hospital, 201 Xinhuazhong Road, Cangzhou 061001, Hebei Province, China. Tel: +86-18730777668; Fax: +86-317-2075552; E-mail: liang123ab@aliyun.com

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