Int J Clin Exp Med 2018;11(2):840-847
www.ijcem.com /ISSN:1940-5901/IJCEM0066839

Original Article

Increased tumor ZEB1 protein expression is correlated with poor prognosis in patients with non-small cell lung cancers

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Received October 9, 2017; Accepted November 18, 2017; Epub February 15, 2018; Published February 28, 2018

Abstract: Background: Zinc finger E-box binding homeobox 1 (ZEB1), a protein related with epithelial-to-mesenchymal transition (EMT) might play a role in lung cancer progression. The current study investigated the expression patterns of ZEB1 in non-small cell lung cancer (NSCLC) and its relationship to survival time. Methods: ZEB1 protein expression was detected by immunohistochemistry in tumor tissues and adjacent normal tissues got from 103 patients with NSCLC. The degrees of tumor ZEB1 protein expression across patient characteristics (age, gender, TNM stage, histological type and etc.) were investigated. We further examined the relationship between ZEB1 expression degree and patient survival using survival analysis. Results: ZEB1 protein expression was significantly increased in the NSCLC tissues compared with that in the normal lung tissues (P<0.001). Increased ZEB1 expression in tumor tissues was associated with poor histological differentiation (P=0.013) and advanced TNM stage (P=0.027). Furthermore, high ZEB1 protein expression in the NSCLC tissues was inversely related with progression free survival (PFS, P=0.001) and 5-year overall survival (OS, P<0.001). Conclusion: Increased tumor ZEB1 protein expression is associated with poor prognosis in patients with NSCLC and could be used as a predictor of poor prognosis.

Keywords: Non-small cell lung cancer, immunohistochemistry, ZEB1, prognosis

Introduction

Although significant advances in diagnosis and management have taken place during the past few decades, patients with non-small cell lung cancer (NSCLC) still face the risk of recurrence and metastasis [1, 2]. NSCLC is thought to develop through a multistep process leading to oncogenic mutations in lung epithelial cells [3]. EMT, epithelial-to-mesenchymal transition, is critical in this process and remains an important contributor to tumor progression and metastatic spread [4, 5]. EMT is regulated by several transcription factors, including zinc-finger E-box-binding homeobox (ZEB), Snail, and Twist families [6, 7].

ZEB1, identified in early 1990s, can act as both transcriptional activators and repressors [8]. Through down-regulating or up-regulating expression of target proteins related with cycle progression, apoptosis, and senescence, ZEB1 plays an essential role in developmental and tumor-related EMT [9]. Specifically, ZEB1 is deemed as a major contributor to EMT process in lung cancer [3]. *In vitro* studies has found that ZEB1 promotes lung tumor cell proliferation, inhibits tumor suppressor genes expression and activated CD44 splicing pathways related with carcinogenesis and metastatic potential [3, 9, 10]. ZEB1 protein expression is up-regulated in NSCLC cells [11] and in primary lung cancer tissues [10, 12]. However, few studies have examined the relationship between ZEB1 expression in primary lung cancer tissues and clinical prognosis.

To further understand the prognostic role of ZEB1 in NSCLC, we enrolled 103 cases of NSCLCs and investigated the expression pat-

terns of ZEB1 in tumor tissues by immunohistochemistry (IHC), and investigated its association with clinicopathological manifestations and clinical outcomes.

Methods

Patients and samples

This prospective observational study was approved by the Ethic Committee of the First Affiliated Hospital of Zhengzhou University, China. Written informed consent was obtained from every enrolled patient. NSCLC tissues and adjacent normal tissues were obtained from 103 patients undergoing curative surgery at the Department of Thoracic Surgery, The First Affiliated Hospital of Zhengzhou University between January 2010 and December 2011. The eligible patients should met all the following criteria: 1) NSCLC confirmed based in pathological examination; 2) surgery should be potentially cured and not R1/R2 resection; 3) TNM staging from I to III based on the 7th edition of the TNM classification of malignant tumors [13]; 4) no other concomitant malignancies or lung diseases; 5) no chemotherapy or radiotherapy before surgery and 6) standard postoperative therapy and regular follow-up. Patients with any one of the following criteria were excluded: 1) lung metastatic cancer or primary lung cancer concomitant with other tumors; 2) chemotherapy, radiotherapy or surgical treatment before current surgery; 3) irregular follow-up or unstandardized therapy after surgery or 4) specimens contaminated or dissolved before immunohistochemistry. Specimens were fixed in 10% formalin, cut at an interval of 5 mm and embedded in paraffin for immunohistochemistry.

The patients' baseline clinical data were collected. After operation, all the patients received at least two-round docetaxel and cisplatin chemotherapy after operation with dosage similar to reports by Ma et al. [14]. The patients were followed up until death or December 2016 at least for five years. Chest CT or whole-body PET/CT were performed every 3 to 6 months to check the disease status and adjust therapy plans. Survival time was calculated from the first day of diagnosis of NSCLC to death or the last time of contact.

Immunohistochemistry

Three-micrometer (3 μm) sections of paraffin-embedded tissues were used for immunohistochemistry. Briefly, after deparaffinizing, antigen retrieval, and endogenous peroxidase blocking, the sections were subjected to 12-hour immunostaining using a rabbit anti-human ZEB1 polyclonal antibody (diluted 1:300, Sigma, USA) at 4°C. After washing, the sections were incubated with biotinylated secondary antibody, followed by rinsing and diaminobenzidine (DAB) exposure for 10 min. The slides were further counter-stained with hematoxylin for microscopic examination. The efficacy of primary antibody was confirmed using negative control.

Immunohistochemical expression of ZEB1 protein was evaluated using a semi-quantitative scoring system reported previously [15]. Percentage scores were defined as follows: 0 for 0-5%; 1 for 6-25%; 2 for 26-50%, or 3 for more than 50%. Four were differentiated for staining intensity: 0 for no staining; 1 for weak staining; 2 for moderate staining, and 3 for strong staining. The staining intensity score and the percentage score were summed as the tissue ZEB1 expression score with a score of 0 as negative; 1 to 3 as low; and 4 to 6 as high. Examination of immunostaining was performed by two independent observers and further verified by another pathologist.

Statistical analyses

SPSS software (version 17.0, Chicago, Illinois, USA) was used for data analysis. For categorical variables, absolute frequencies and incidences (rates) were reported. Continuous variables were expressed as mean and standard deviation (SD). The correlations between ZEB1 expression patterns and clinicopathologic variables were analyzed using the χ^2 test and linear-by-linear association for trend was further used to detect a possible linear association between ZEB1 positivity and TNM stage or between ZEB1 positivity and degrees of histological differentiation. Logistic regression models were also used to detect whether a clinicopathologic variable was an independent risk factor for ZEB1 expression. Kaplan-Meier survival curves and the log-rank test were used for univariate overall survival analysis. Multivariate analysis was performed using the Cox proportional-hazard regression model with a forward step-wise (Wald) variable selection method to determine the contribution of ZEB1 expression on OS and PFS. $P<0.05$ was considered to be statistically significant.

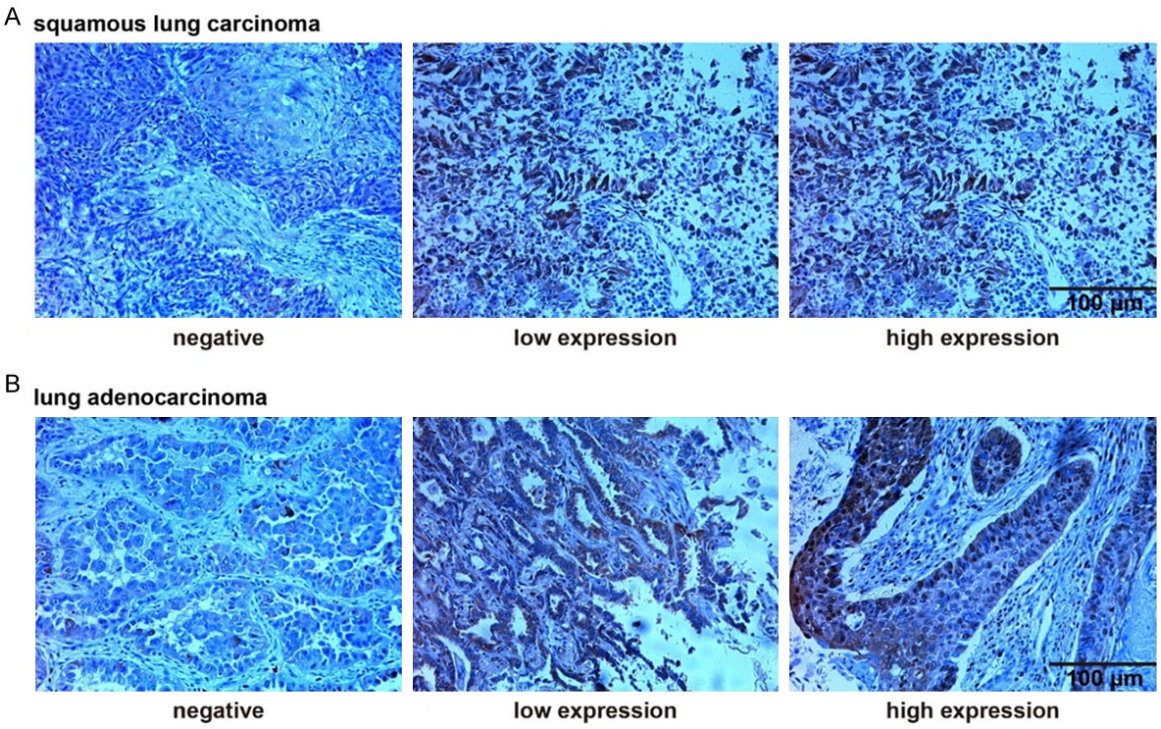

Figure 1. ZEB1 staining in non-small cell lung carcinoma tissues. A. Representative negative (left), low (middle) and high (right) immunoreactivity of ZEB1 protein in squamous lung carcinomas; B. Representative negative (left), low (middle) and high (right) immunoreactivity of ZEB1 protein in lung adenocarcinomas. ZEB1 positivity was mainly confined to the cell nuclei.

Results

Patient characteristics

A total of 103 cases were eligible including 67 males and 36 females. The ages ranged from 33 to 77 years with a median age of 59 years old. There were 45 cases with TNM stage I, 25 with stage II and 33 with stage III. Thirty-three cases were diagnosed with well differentiated carcinomas according to the WHO histologic classification recommendations [16]. Forty-one cases were diagnosed with moderate differentiated carcinomas, and 27 cases were with poor differentiated carcinomas. Among these tumors, 64 samples were adenocarcinomas and 39 were squamous cell carcinomas. The median progression free survival (PFS) was 11 months. The mean PFS was 16 months for patients at stage I, 16 months for cases at stage II and 13 months for patients at stage III. The median OS was 51 months with a range of 12 to 60 months. And 58 patients died during the follow-up.

ZEB1 expression patterns in NSCLC tissues

Among the 103 cases, ZEB1 protein positivity was detected in 76 (73.79%) tumor tissues and 20 (19.42%) adjacent normal tissues. There was a significantly higher proportion of ZEB1 protein expression in NSCLS tumor tissues compared to normal tissues (χ^2=61.18, P< 0.001). In the 76 cases with ZEB1 protein positivity, 32 cases had a low expression level and 44 cases had a high expression level of ZEB1. As shown in **Figure 1**, ZEB1 staining was found predominately in the cell nuclei of tumor cells, but for a few tumors, the ZEB1 signals were also located in either cytoplasm/nucleus or cytoplasm.

ZEB1 expression in relation to clinicopathological characteristics

ZEB1-immunoreactive cancer cells across age, sex, smoking habit, tumor size, histological subtype, histological grade, TNM stage and lymph node metastasis are shown in **Table 1**. Of those, older ages (≥60 years old), sex, smoking,

Table 1. ZEB1 expression in non-small cell lung cancer tissues across clinicopathological featuress

Characteristics	ZEB1 expression		χ^2	P
	Negative (%)	Positive (%)		
Age (year)			0.269	0.604
<60	14 (28.57)	35 (71.43)		
≥60	13 (24.07)	41 (75.93)		
Gender			1.311	0.252
Female	7 (22.22)	29 (77.78)		
Male	20 (28.36)	47 (71.64)		
Smoking			1.063	0.303
No	13 (31.71)	28 (68.29)		
Yes	14 (22.58)	48 (77.42)		
Tumor size			0.029	0.864
<5 cm	20 (26.67)	55 e		
≥5 cm	7 (25.00)	21 (75.00)		
Histology			1.055	0.304
Adenocarcinoma	19 (29.69)	45 (70.31)		
Squamous carcinoma	8 (20.51)	31 (79.49)		
Histological grade			9.126	0.010*
Well	15 (41.67)	21 (58.33)		
Moderate	10 (24.39)	31 (75.61)		
Poor	2 (7.69)	24 (92.31)		
TNM stage			8.155	0.017*
I	17 (37.78)	28 (62.22)		
II	7 (28.00)	18 e		
III	3 (9.09)	30 (90.91)		
Lymph node metastasis			3.788	0.052
No	15 (36.59)	26 (63.41)		
Yes	12 (19.35)	50 (80.65)		

Note: *P<0.05.

Table 2. Logistic regression model analysis of risk factors for ZEB1 positivity

Identified risk factors	Adjusted OR	95% CI	P
Histological differentiation grade	2.364	1.201-4.654	0.013
TNM stage	2.020	1.085-3.759	0.027

tumor size, lymph node metastasis and histological subtype had no influence on the expression of ZEB1 protein (all P>0.05). Tumor cells from well-differentiated carcinomas had a lower rate of ZEB1 positivity compared to moderately and poor differentiated cells (P=0.010). The ratio of expression of ZEB1 in well, moderate and poor grade tumor tissues were 58.33%, 75.61%, and 92.31%, respectively. Patients with advanced TNM stage were more likely to have ZEB1 protein detected in the tumor cells (P=0.017) with a significant linear-by-linear association (P=0.005). Logistic regression revealed that poor histological differentiation (P=0.013, adjusted OR (odds ratio)=2.36 per grade; 95% CI=1.20-4.65) and advanced TNM stage (P=0.027, adjusted OR=2.02 per grade; 95% CI=1.09-3.76) were independent risk factors for ZEB1 expression (**Table 2**).

Among the 76 cases with ZEB1 immunostaining positivity, 36 cases (47.37) were identified with ZEB1 immunoreactivity only in the nucleus and 8 cases only in the cytoplasm. **Table 3** depicts the ZEB1 intracellular locus in relation to clinicopathological characteristics. There was no difference of ZEB1 intracellular locus distribution across age, sex, smoking habit, tumor size, histological subtype, histological grade, or lymph node metastasis. Patients with advanced TNM stage had more ZEB1 immunoreactivity detected in the nucleus and cytoplasm (P=0.032).

High ZEB1 expression in NSCLC tissues was associated with shorter progression free survival (PFS)

As shown in **Figure 2A**, patients with different degrees of ZEB1 immunoreactivity had different PFSs (P<0.001). The median PFSs for patient with high, low and no expression of ZEB1 were 10, 18 and 18 months respectively. Patients with high ZEB1 expression had shorter PFS compared to those with low ZEB1 (χ^2=21.187, P<0.001) and no ZEB1 (χ^2=15.120, P<0.001) expression. There was no significant difference on PFSs between patients with low ZEB1 expression and those without ZEB1 immunoreactivity (χ^2=0.368, P=0.544). Further multivariate analyses found that increased ZEB1 expression (P=0.001) and poor cell differentiation (P=0.005) were independently associated with shorter PFS (**Table 4**).

Table 3. Cell locus of ZEB1 immunostaining across clinicopathological features

Characteristics	ZEB1 intracellular locus			χ^2	P
	Nucleus (%)	Cytoplasm (%)	Nucleus and Cytoplasm		
Age (year)				0.474	0.789
<60	16 (45.71)	3 (8.57)	16 (45.71)		
≥60	20 (48.78)	5 (12.20)	16 (39.02)		
Gender				0.148	0.942
Female	13 (44.83)	3 (10.34)	13 (44.83)		
Male	23 (48.94)	5 (10.64)	19 (40.43)		
Smoking				0.016	0.992
No	13 (46.43)	3 (10.71)	12 (42.86)		
Yes	23 (47.92)	5 (10.42)	20 (41.67)		
Tumor size				1.126	0.570
<5 cm	26 (47.27)	7 (12.73)	22 (40.00)		
≥5 cm	10 (47.62)	1 (4.76)	10 (47.62)		
Histology				0.378	0.828
Adenocarcinoma	20 (44.44)	5 (11.11)	20 (44.44)		
Squamous carcinoma	16 (51.61)	3 (9.68)	12 (38.71)		
Histological grade				1.698	0.791
Well	12 (57.14)	1 (4.76)	8 (38.10)		
Moderate	14 (45.16)	4 (12.90)	13 (45.16)		
Poor	10 (41.67)	3 (12.50)	11 (45.83)		
TNM stage				10.533	0.032*
I	18 (64.29)	4 (14.29)	6 (64.29)		
II	9 (50.00)	2 (11.11)	7 (38.89)		
III	9 (30.00)	2 (6.67)	19 (63.33)		
Lymph node metastasis				3.877	0.144
No	16 (61.54)	1 (3.85)	9 (34.62)		
Yes	20 (40.00)	7 (14.00)	23 (46.00)		

Note: *P<0.05.

High ZEB1 expression in NSCLC tissues was associated with shorter 5-year overall survival (OS)

Figure 2B depicts the effect of different ZEB1 expression levels in patients with NSCLC on 5-year overall survival (OS). There was significantly different OSs among patient with high (median 39 months), low (median 58 months) and negative (median 60 months) ZEB1 expression (P<0.001). Patients with high ZEB1 expression had shorter OS compared to those with negative ZEB1 expression (χ^2=11.450, P=0.001). There seems to be a difference on OSs between patients with high and low ZEB1 expression (χ^2=4.526, P=0.033>0.017) and the difference is nearly significant. A difference was also found for OS for patients with low ZEB1 expression and patients without ZEB1 expression (χ^2=10.295, P=0.001). Further multivariate analyses found that female patients were associated with longer OS (P=0.001) and increased ZEB1 expression (P<0.001) and older age (≥60, P=0.008) predicted short OS (**Table 5**).

Discussion

In this study, we found that ZEB1 protein was increased in NSCLC tissues and increased ZEB1 expression was associated with early postsurgical progression and short overall survival. These results suggested that increased ZEB1 expression was associated poor prognosis for NSCLC patients undergoing surgical resection.

We found that increased ZEB1 expression was correlated with poor cancer cell differentiation, advanced TNM stage and lymph node metasta-

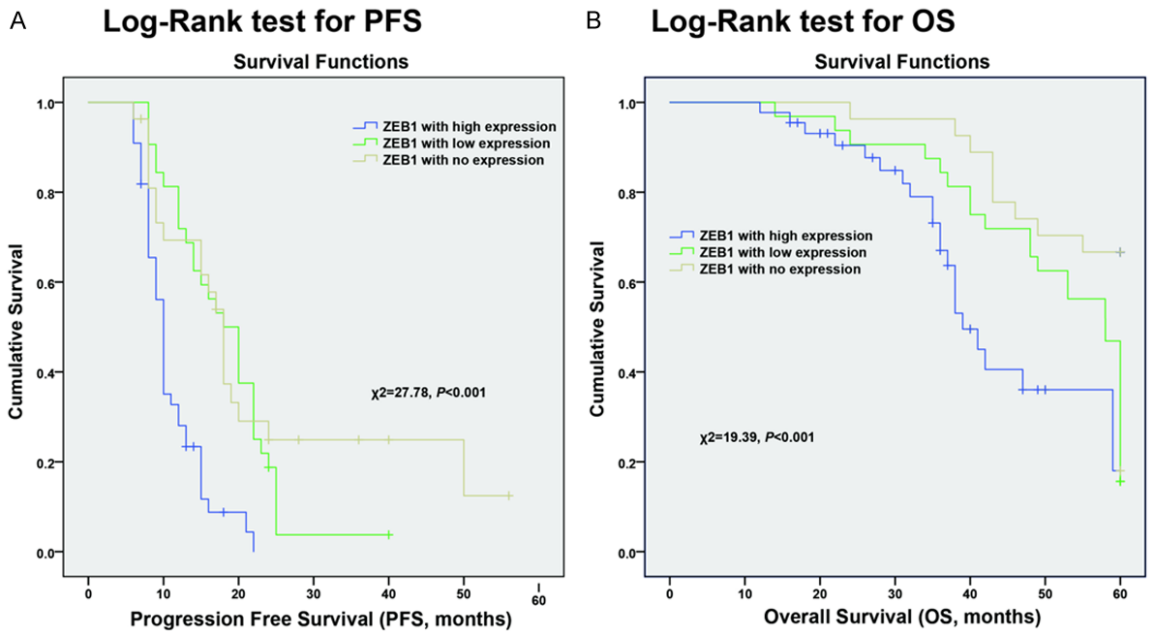

Figure 2. Kaplan Meier plots of the effect of ZEB1 expression on PFS (A) and OS (B) for patients with non-small cell lung carcinoma.

Table 4. Cox proportional hazard model analysis of risk factors for PFS

Identified risk factors	Adjusted HR	95% CI	P
ZEB1 expression	1.725	1.246-2.388	0.001
Differentiation grade	1.561	1.143-2.130	0.005

Table 5. Cox proportional hazard model analysis of risk factors for 5-year OS

Identified risk factors	Adjusted HR	95% CI	P
ZEB1 expression	2.365	1.631-3.431	<0.001
Age ≥60	2.117	1.218-3.677	0.008
Female	0.354	0.192-0.652	0.001

sis. Similarly, Aigner et al. reported that in invasive human cancer cells, ZEB1 could promote tumor cell dedifferentiation by repressing expression of proteins related with epithelial differentiation [17].

A meta-analysis stated that increased ZEB1 expression was associated with lymph node metastasis and TNM stage in patients with digestive cancer [18]. Larsen et al. also found that ZEB1 was positively associated with TNM stage even in early stage IB primary NSCLC [3]. Furthermore, ZEB1 was significantly overexpressed in bone-metastatic small cell lung cancer (SCLC) cell lines and ZEB1 could promote bone metastasis of SCLC through the EMT pathway while knockdown of ZEB1 expression significantly inhibited the progress of bone metastasis in SCLC [19, 20]. Miyahara at al also report that ZEB1 protein expression in pleomorphic lung carcinomas was associated with lymph node metastasis and pleural invasion, and diffuse ZEB1 expression in this tumor predicts poorer disease-specific survival [12]. Taken together, these results suggested that ZEB1 played a vital role in lung cancer progression.

The mechanism of ZEB1-increased tumor progression and poor prognosis might be related with E-cadherin and following EMT. NSCLC cell study has found ZEB1 expression showed close inverse correlation with E-cadherin, whose down-regulation has been thought as a marker of EMT in primary lung cancers [21]. ZEB1 knockdown with in NSCLC cell lines increased E-cadherin expression and induced cell apoptosis [22]. Moreover, ZEB1 could inhibit the expression of Semaphorin 3F, a lung tumor suppressor *in vitro* [23]. On the signaling mechanisms, studies had found that ZEB1 directly repressed epithelial splicing regulatory protein 1 (ESRP1) which further increased expression of a mesenchymal splice variant of CD44 leading to EMT and enhanced invasion [3].

Our univariate and multivariate analysis found that increased ZEB1 expression in tumor cells was significantly associated with short PFS and five years OS which was in accordance with the previous reports from Shuai Xiang et al. [24] and Zhang et al. [25]. They found that ZEB1 expression was correlated with metastasis and poor prognosis in patients with breast cancer or colorectal cancer. A recent meta-analysis also summarized that ZEB1 was positively associated with poor overall survival for patients with pancreatic cancer, gastric cancer and colorectal cancer [18]. Yang et al. also reported that ZEB1 activated PI3K signaling thereby promoting lung adenocarcinoma metastasis and antagonism of PI3K signaling could inhibit metastasis, suggesting promoting metastasis might be a mechanism of ZEB1 related short survival time. Interestingly, the role of ZEB1 on lung cancer development and progression might rely on KRAS mutations [26]. Zhang et al. reported that an opposite role of ZEB1 in EGFR-mutated lung cancer cells, in which ZEB1 was found to inhibit cancer cell growth [27]. Our study did not confirm the mutants of EGFR and KRAS mutations in our study which added uncertainty to our studies. Furthermore, individualized gene-based therapy was not applied or rejected in our study which might also confounded our results.

In conclusion, our results imply that ZEB1 expression is increased in patients with NSCLC, and the level of ZEB1 protein expression is associated with poor histological differentiation, advanced TNM stage and lymph node metastasis. Furthermore, positive ZEB1 protein expression is significantly associated with poor PFS and 5-year OS. However, the small number of patients enrolled and no further detection of the expression of ZEB1 related proteins should be considered when interpreting our findings. More studies were also needed to aim to target ZEB1 signaling to improve prognosis of patients with NSCLC.

Acknowledgements

The work was supported by Medical Science and Technology Project of Henan Province, China (201602045) and Overseas Study of Health and Family Planning Commission of Henan Province, China (5451 Program).

Disclosure of conflict of interest

None.

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