Elevated FUS/TLS expression is negatively associated with E-cadherin expression and prognosis of patients with non-small cell lung cancer

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Abstract. Fused in sarcoma/translocated in liposarcoma (FUS/TLS), a ubiquitous and multifunctional DNA and RNA-binding protein, contributes an important function in cancer and neurodegenerative disease; however, its role in lung cancer remains unclear. In the present study, the expression of FUS/TLS in non-small cell lung cancer (NSCLC) and the significance of FUS/TLS for predicting the clinical outcome of patients with NSCLC, was examined. FUS/TLS expression was investigated in NSCLC tissues and their matched adjacent non-tumorous tissues by reverse transcription-quantitative polymerase chain reaction, western blotting, and immunohistochemistry. Tissue microarrays representing 208 patients with NSCLC were used to determine the expression pattern and associations with FUS/TLS using immunohistochemistry. Prognostic significance was assessed by Kaplan-Meier survival estimates and log-rank tests. Data revealed that FUS/TLS expression was elevated in NSCLC tissues compared with corresponding normal tissue mRNA (9.27±0.73 vs. 6.15±0.60) and protein (3.32±0.75 vs. 0.30±0.07) levels. In tissue microarrays, FUS/TLS was highly expressed in 103 (49.5%, 103/208) NSCLC tissues compared with adjacent normal lung tissues (28.4%, 59/208). Overexpression of FUS/TLS was associated with higher tumor node metastasis stage (P=0.016), poorer differentiation (P=0.008), large tumor size (P=0.019) and predicted poor prognosis (P=0.005) in patients with NSCLC. Notably, correlation analysis revealed a significant inverse association between the expression of FUS/TLS and E-cadherin (r^2=0.51; P=0.036). Furthermore, patients with NSCLC with high FUS/TLS and impaired E-cadherin expression had a notably poor prognosis (P=4.01x10^{-4}). Thus, the results from the present study indicate that elevated FUS/TLS expression promotes NSCLC progression. FUS/TLS, alone or in combination with E-cadherin, is a novel prognostic predictor for patients with NSCLC.

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Introduction

Lung cancer is one of the very few cancer types in which a continuous increase in incidence has emerged over recent years. Currently, ~1.6 million new cases of lung cancer are diagnosed annually, worldwide (1). Of these, >85% of lung cancer cases are classified as non-small cell lung cancer (NSCLC) (2). Despite advancements and improvements in surgical, and medical treatments of this patient population, the 5-year survival rate of patients with lung cancer remains at ~17.4% (2). This is largely due to the late stage at which the majority of patients are diagnosed, and the lack of effective treatments for this disease. In addition, local control for early-stage NSCLC has dramatically improved over previous decades for operable and inoperable patients; however, ~20% of early-stage patients still develop distant metastasis (2,3). Thus, understanding the molecular markers that regulate invasion and disease spread is crucial in identifying novel and reliable prognostic markers for NSCLC, and may be exploited to refine patient selection for already existing therapies.

The FET (previously TET) gene family comprise fused in sarcoma/translocated in liposarcoma (FUS/TLS), Ewing's sarcoma and TATA-binding protein-associated factor 15. These proteins, encoded by the FET gene family, are similar in structure and function (4). FET proteins contain an N-terminal domain, a G-rich domain, an RRM (RNA-binding

domain), a zinc finger motif and a C-terminal RGG-rich (arginine-glycine-rich) domain (4,5). Multifunctional characteristics of the FET protein family have been authenticated. The N-terminal domain, rich in Gln, Gly, Ser and Tyr, has a transcriptional activation function (6,7). The RRM, zinc-finger and RGG-rich domains contribute to the RNA-binding ability of the FET-proteins (8-10). In addition, FET proteins also bind single-stranded DNA and possibly double-stranded DNA. The unique functions also include pre-mRNA splicing and DNA repair and recombination (11). Notably, chromosomal rearrangements result in the 5' regions of FET genes being fused with different transcription factor genes in sarcomas or other types of cancer (12-14). FUS/TLS was identified as a fusion gene with DNA damage inducible transcript 3 (*CHOP*) (also known as *GADD153, DDIT3*) in human myxoid liposarcoma with chromosomal translocation t(12; 16)(q13; p11) (15). In myxoid liposarcoma, >85% of cases were associated with the translocation of FUS/TLS (4,12). Subsequently, TLS/FUS-ERG ETS transcription factor (ERG) chimeric transcripts were observed to contribute an important role in the development of acute myeloid leukemia (16,17). In addition, overexpression of FUS/TLS protein or mRNA has been reported in liposarcoma cell lines (18), breast cancer cells (19) and sporadic colorectal cancer cells (20). Thus, FUS/TLS serves an important function in the development of cancer.

Epithelial-mesenchymal transition (EMT) is a crucial event in cancer development and metastasis (21). One of the key processes of EMT is the loss of E-cadherin. E-cadherin has been reported to be a hallmark in the progression of NSCLC (22). A previous study indicated that FUS/TLS protein was located in the spreading initiation centers of adhering cells and was involved in cell spreading (23), while E-cadherin primarily expressed on the membrane of epithelial cells, contributes an important role in cell-cell adhesion (24). This suggested that FUS may be involved in regulating E-cadherin expression. The aim of the present study was to examine FUS/TLS levels in NSCLC and matched paratumor tissues, and evaluate the association between FUS/TLS expression and the clinicopathological characteristics of patients with NSCLC. In addition, the prognostic effect of FUS/TLS and E-cadherin expression was determined by multivariate analysis, either as FUS/TLS as an independent parameter or combined with E-cadherin.

Materials and methods

Patients and specimens. From January 2005 to December 2005, archival specimens were consecutively collected from 208 patients (male 148, female 60; median age 65 years; age range 40-83 years) with NSCLC who received curative resection at Zhongshan Hospital of Fudan University (Shanghai, China). Patient characteristics: All the patients' clinicopathological information were in agreement with the description of the authors previous study (25). The collection and conservation of patient samples, and details were permitted with the evidence of written informed consent. The follow-up was terminated in July 2010. The median follow-up duration was 43 months (range, 1-66 months). The overall survival (OS) was calculated from the day of surgery to the date of either mortality as a result of lung cancer or the last follow-up. Ethical approval was obtained from the Zhongshan Hospital Research Ethics Committee (Shanghai, China).

Cell culture. The 16HBE cell line was obtained from Xiangbio (Shanghai, China). H460, 95-C, A549 and 95-D cell lines were purchased from the Institute of the Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). 16HBE, A549 and H460 cell lines were maintained in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS, Gibco; Thermo Fisher Scientific, Inc.) and antibiotics (100 IU/ml penicillin, and 100 μg/ml streptomycin sulfate). 95-C and 95-D cells were cultured in RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS and antibiotics. All cell lines were incubated in a humidified atmosphere of 5% CO_2 at 37˚C.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA of tissues was extracted using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Total RNA was reverse-transcribed to cDNA using PrimeScript RT reagent kit (Takara Biotechnology Co., Ltd., Dalian, China), according to the manufacturer's protocol. Amplification and detection were performed using the ABI PRISM 7900 Sequence Detection system (Applied Biosystems; Thermo Fisher Scientific, Inc.) starting with 1 μl cDNA and SYBR Green Real-Time PCR Master mix (Takara Biotechnology Co., Ltd.). Primers were designed as follows: FUS/TLS forward, 5'-AGCTGAAGGGAGAGGCAAC-3' and reverse, 5'-GGCGAGTAGCAAATGAGACC-3'; GADPH forward, 5'-GGTATGACAACGAATTTGGC-3' and reverse, 5'-GAGCACAGGGTACTTTATTG-3'. Relative expression levels of the gene of interest were calculated using the $2^{-\Delta\Delta Cq}$ method (26). GADPH was used as an internal standard and triplicate RT-qPCR samples were performed in each assay. GraphPad Prism 5.0 software was applied to the quantification of relative mRNA expression (GraphPad Software, Inc., La Jolla, CA, USA).

Western blot analysis. Total protein was prepared using radioimmunoprecipitation assay lysis buffer (Beyotime; Shanghai, China) protein concentration was determined using an Enhanced BCA Protein Assay Kit (Beyotime Institute of Biotechnology, Haimen, China). Equal amounts of protein (30 μg) were separated via 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Merck KGaA, Darmstadt, Germany). Membranes were blocked in 5% fat-free milk at room temperature for 1 h, and subsequently incubated with primary antibodies rabbit anti-FUS/TLS (dilution 1:4,000; cat. no. 11570-1-ap; ProteinTech Group, Inc., Chicago, IL, USA) and mouse anti-β-actin (dilution 1:1,000; cat. no. AA128; Beyotime Institute of Biotechnology), which was used as an internal control. The primary antibodies were incubated at 4˚C overnight. Subsequent to washing with Tris-buffered saline containing 0.1% Tween-20 (TBST), the membranes were incubated with a horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibody (catalog no. A0208 or A0216; dilution, 1:1,000; Beyotime Institute of Biotechnology) at room temperature for 1 h. Finally, the membranes were washed with TBST and visualized using

ECL solution (EMD Millipore, Billerica, MA, USA) using an automatic chemiluminescence Image analysis system (Tanon Technology Co., Ltd., Shanghai, China), Image-Pro Plus software version 6.0 (Media Cybernetics, Inc., Rockville, USA) was used to analyze relative protein expression and exhibited as the density ratio vs. β-actin. All experiments were performed in triplicate.

Immunofluorescence. The localization of FUS/TLS protein in 16HBE, A549, H460, 95C and 95D cells was examined by immunofluorescence. Cells were fixed with 4% paraformaldehyde (Yesen, Shanghai, China) for 30 min at room temperature and then permeated with 0.3% Triton X (Beyotime Institute of Biotechnology) for 10 min, washed three times with PBS. Then the cells were blocked with 5% BSA/PBS for 30 min at room temperature. Subsequently, cells were incubated at 4˚C overnight with rabbit anti-FUS/TLS. (1:400 dilution; catalog no. 11570-1-ap; ProteinTech Group, Inc.; Chicago, IL, USA). Following washing, the cells were incubated with secondary anti-rabbit IgG (1:400 dilution; catalog no. A0468; Beyotime; Shanghai, China) at room temperature for 2 h. Nuclei were counterstained with DAPI. (catalog no. C1006; Beyotime; Shanghai, China) for 5 min at room temperature and washed with PBS. The locations of FUS/TLS were detected by fluorescence microscopy (Olympus Corporation, Tokyo, Japan) used a magnification of x200.

Tissue microarray (TMA) and immunohistochemistry (IHC). TMAs were constructed by Shanghai Biochip Co., Ltd. (Shanghai, China). Pathological types of all samples were reviewed by hematoxylin and eosin staining, in accordance with protocols from a previous study (25). IHC of paraffin sections was performed according to a protocol from a previous study (27). In brief, paraffin sections were deparaffinized by heating at 65˚C for 2 h, subsequently washed with xylene and rehydrated in ethanol. After antigen retrieval was performed by incubating in 10 mmol/l Citrate Sodium Buffer (pH 6.0; Yesen) and the slides boiled in a microwave, slides were incubated in 0.3% H_2O_2 for 15 min to block endogenous peroxidase activity at room temperature. Slides were rinsed in PBS and immersed in 5% BSA for 1 h at room temperature to block nonspecific binding sites. Subsequently, the sections were incubated with primary antibody, rabbit anti-human FUS/TLS (dilution, 1:5,000; catalog no. 11570-1-ap; ProteinTech Group, Inc.; Chicago, IL, USA), overnight at 4˚C. The following day, samples were washed three times with PBS. After 30 min of incubating slides in horseradish peroxidase labeled secondary antibody (GTVision III immunohistochemical kit; cat. no. GK500705; Gene Tech, Shanghai, China) at room temperature and washing in PBS buffer, the slides were stained with 3,3'-diaminobenzidine (DAB)-H_2O_2 under a microscope for 2 min at room temperature, and hematoxylin was used to counterstain the nuclei at room temperature for 40 sec. Finally, the sections were dehydrated and covered with glass microscope glass using neutral resins.

Evaluation of immunostaining intensity of TMAs. The staining results scored were as follows. The proportion of immunoreactive cells in total cell number of every point: 0 (0%), 1 (>0% to 25%), 2 (>25% to 50%), 3 (>50% to 75%) and 4 (>75%). The cellular staining intensity was determined by the degree of color: 0 (none), 1 (weak), 2 (moderate), 3 (strong) (28). The final score for FUS/TLS expression was the summation of both scores. The combined scores were divided into negative (-, 0-1), weak positive (+, 2-3), moderate positive (++, 4-5) and strong positive (+++, 6-7), respectively. For statistical analysis, the negative and weak positive staining were considered the FUS/TLS low level group, and the moderate and strong positive staining were deemed the FUS high level group.

Statistical analysis. SPSS 21.0 software package (SPSS, Chicago, IL, USA) was utilized for statistical analyses. OS was plotted using the Kaplan-Meier method (log-rank test). Spearman's rank correlation analysis identified a correlation between FUS/TLS and E-cadherin expression. Significant factors in univariate analysis were further incorporated into multivariate Cox proportional hazards regression model to selection of independent prognostic factors. The differences between categorical variables were analyzed by χ^2 test. $P<0.05$ was considered to indicate a statistically significant difference (two-tailed).

Results

FUS/TLS is highly expressed in NSCLC tissues. RT-qPCR, western blotting and immunohistochemistry were performed to detect mRNA, and protein levels of FUS/TLS in NSCLC tissues and matched adjacent nontumorous tissues. FUS/TLS expression was significantly increased in NSCLC compared with that in corresponding adjacent normal lung tissues at the mRNA (9.27±0.73 vs. 6.15±0.60; Fig. 1A) and protein (3.32±0.75 vs. 0.30±0.07; Fig. 1B) levels. The pathological type of NSCLC and normal lung tissues were confirmed by hematoxylin and eosin staining (Fig. 1C). Immunohistochemical analysis demonstrated that strong intensity FUS/TLS staining in NSCLC tissues was markedly higher than that in paratumor tissues (Fig. 1C). Overall, FUS/TLS-positive immunostaining was more frequent in tumor tissues (49.5%, 103/208) compared with matched nontumor tissues (28.4%, 59/208; Fig. 1D). Immunohistochemically, FUS/TLS demonstrated primarily nuclear and to a lesser degree, cytoplasmic localization in NSCLC tissues. The expression of FUS/TLS in normal lung endothelial cells (16HBE) and NSCLCs cell lines (A549, H460, 95C and 95D) was further examined by immunofluorescence. The results presented in Fig. 1E demonstrate a predominant nuclear localization of FUS/TLS expression and high level of FUS/TLS was observed in NSCLC cells. The aforementioned results indicate that FUS/TLS contributes to the onset and progression of NSCLC.

Association between FUS/TLS and clinicopathological features of NSCLCs. The association between the clinicopathological features of the patient cohort and FUS/TLS was analyzed. Detailed clinical and pathological information was presented in a previous study (29). Briefly, analysis included a total of 208 cases of primary NSCLC, and the cohort consisted of 60 (28.8%) women and 148 (71.2%) men. The number of patients with squamous cell carcinoma, adenocarcinoma and other pathologic subtypes of NSCLC was 85, 110 and 13, respectively. There were 144 (69.2%) tumors in tumor node

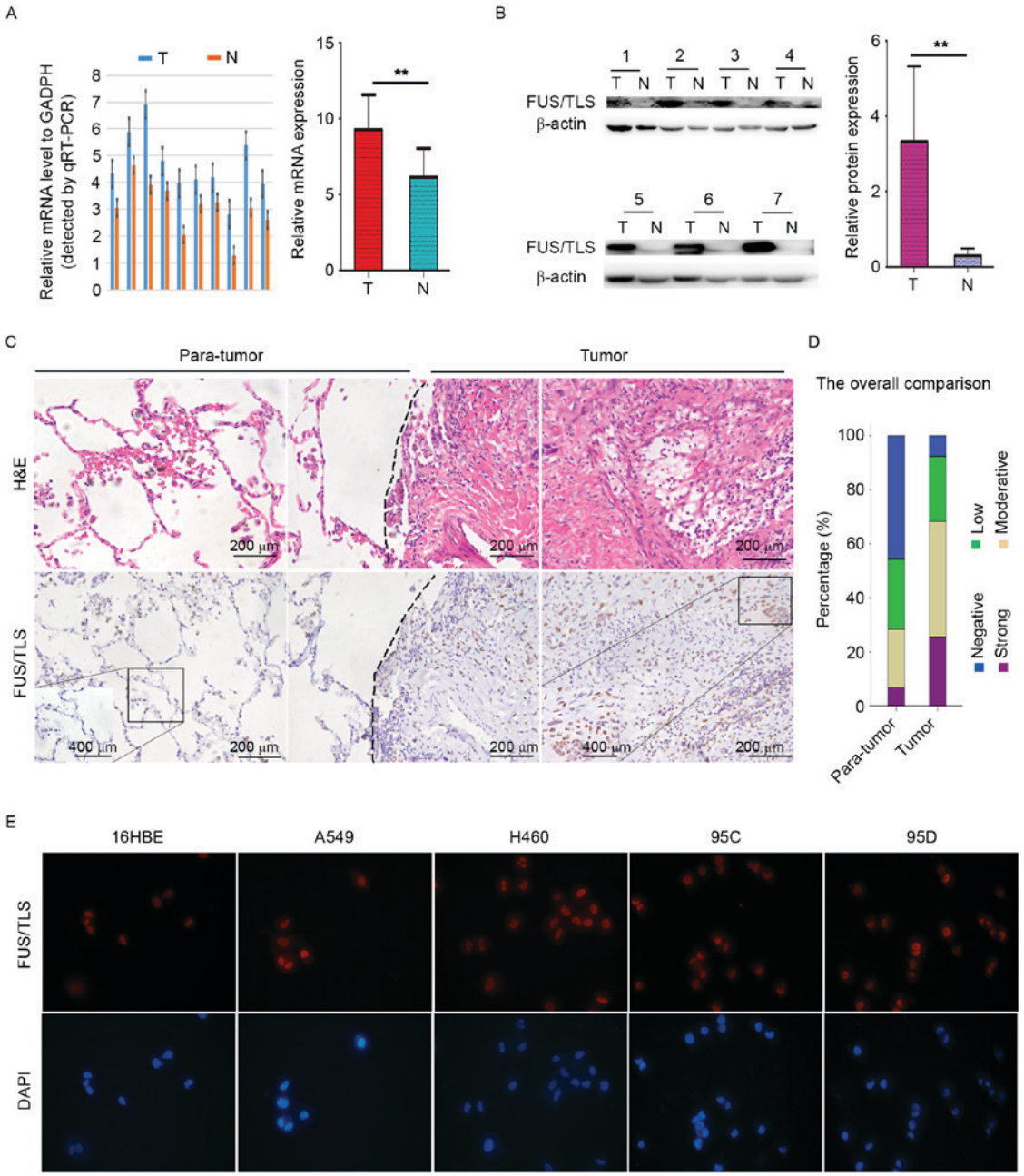

Figure 1. Expression of FUS/TLS in NSCLC tissues and cell lines. (A) FUS/TLS gene and (B) protein expression in 7 pairs of NSCLC and matched normal lung tissue, by RT-qPCR and western blotting, respectively. (C) The proportion of different FUS/TLS expression level in NSCLC tissues and corresponding adjacent normal lung tissues. (D) FUS/TLS mRNA expression levels in 10 NSCLC tissues and matched adjacent lung tissues as detected by RT-qPCR. (E) Immunofluorescent detection of FUS/TLS in cell lines. FUS/TLS, Fused in sarcoma/translocated in liposarcoma; NSCLC, non-small cell lung cancer; T, tumor tissue; N, matched normal lung tissue; RT-qPCR, reverse transcription-quantitative polymerase chain reaction. Original magnification, x200. *P<0.05, **P<0.01.

metastasis (TNM) stages I-II and 64 (30.8%) tumors in stages III-IV. In addition, tumors with low and high differentiation were 93 and 115 respectively. Lymph node metastasis was identified in 90 (43.3%) of samples.

The association between FUS/TLS expression with clinicopathological parameters was demonstrated in Table I and Fig. 2. FUS/TLS expression was significantly associated with histological type (P=0.009), high TNM stage (P=0.016), poor tumor differentiation (P=0.008) and larger tumor size (P=0.019). The proportion of patients with high FUS/TLS levels in TNM stage III-IV (62.5%, 40/64) was greater than patients in TNM stage I-II (43.75%, 63/144; Fig. 2D). Compared with patients with adenocarcinoma (40.91%, 45/110), high levels of FUS/TLS were more common in patients with squamous cell carcinoma (62.35%, 53/85; Fig. 2E). Notably, high FUS level patients with larger tumor size (≥3 cm, 55.4%, 77/139) were more frequent than patients with smaller tumor size (<3 cm, 37.68%, 26/69; Fig. 2F). Furthermore, a high proportion of elevated FUS/TLS level was also present in tissues with poorer differentiation (60.22%, 56/93) than well/moderate differentiation (47/115; Fig. 2G). Different subtype of NSCLCs revealed different OS

Table I. Association of expression of FUS/TLS and E-cadherin with clinicopathological features of NSCLC tissues.

		FUS/TLS			E-cadherin		
Variables	No.	Low	High	P-value[a]	Low	High	P-value[a]
Age, years				0.074			0.092
<60	102	58	44		66	36	
≥60	106	47	59		56	50	
Sex				0.022			0.877
Male	148	67	81		86	62	
Female	60	38	21		36	24	
Smoking status				0.397			0.567
Smokers	84	39	45		47	37	
Non-smokers	124	66	58		75	49	
Histologic type				0.009[a]			0.585
Squamous carcinoma	85	32	53		52	33	
Adenocarcinoma	110	65	45		64	46	
Other[b]	13	8	5		6	7	
Differentiation				0.008[a]			0.571
Well/moderate	115	68	47		65	50	
Poor	93	37	56		57	36	
Tumor stage				0.016[a]			0.222
I+II	144	81	63		80	64	
III+IV	64	24	40		42	22	
Lymph node metastasis				0.263			0.011[a]
Yes	90	41	49		62	28	
No	118	64	54		60	58	
Tumor size, cm				0.019[a]			0.072
<3	69	43	26		34	35	
≥3	139	62	77		88	51	

[a]P<0.05 difference was statistically significant. [b]Other including adenosquamous carcinoma, large-cell carcinoma, mucoepidermoid carcinoma and carcinosarcoma. FUS/TLS, fused in sarcoma/translocated in liposarcoma; NSCLC, non-small cell lung cancer.

rates. The patients with higher TNM stage, larger tumor size, and poorer differentiation exhibited a more unfavorable OS rate. These clinicopathological features were all significantly associated with high FUS/TLS expression. However, other clinicopathological features, including age and smoking status, were not directly associated with the level of FUS/TLS.

High level of FUS was associated with poor prognosis of patients with NSCLC. The 5-year OS rate after surgery was 47.6% for the entire cohort. Patients with high FUS/TLS levels exhibited a worse prognosis compared with those with low FUS/TLS expression (P=0.005; Fig. 2A). The relationship between FUS/TLS expression and prognosis by various subset analyses was examined. High FUS/TLS level was associated with a poorer outcome in patients with adenocarcinoma (P=0.005; Fig. 2A), whereas no distinct statistical difference was observed in patients with squamous cell carcinoma (P=0.314). It was of interest that smokers with high FUS/TLS levels underwent a worse prognosis compared with smokers with low FUS/TLS levels (P=0.011; Fig. 2C). In addition, the level of FUS/TLS had an indistinctive effect on non-smokers within this cohort (P=0.150). These data further revealed that high FUS levels may predict a poor prognosis for patients with NSCLC.

The univariate analysis indicated that FUS/TLS expression, tumor size, TNM stage, lymph node metastasis and tumor differentiation demonstrated a distinct impact on OS. In multivariate analysis, tumor size and lymph node metastasis remained associated with OS; however, FUS/TLS levels lost value as an independent predictor of OS (P=0.093). Owing to the association observed with TNM stage (P=0.016) and tumor size (P=0.019), FUS/TLS level significantly influences the OS rate of patients (Table II).

High level of FUS/TLS impaired E-cadherin expression and the combination of the two markers defined a subset of NSCLC patients with worse prognosis. The association between E-cadherin expression with clinicopathological characteristics are exhibited in Table I. No significant associations were observed between impaired E-cadherin expression

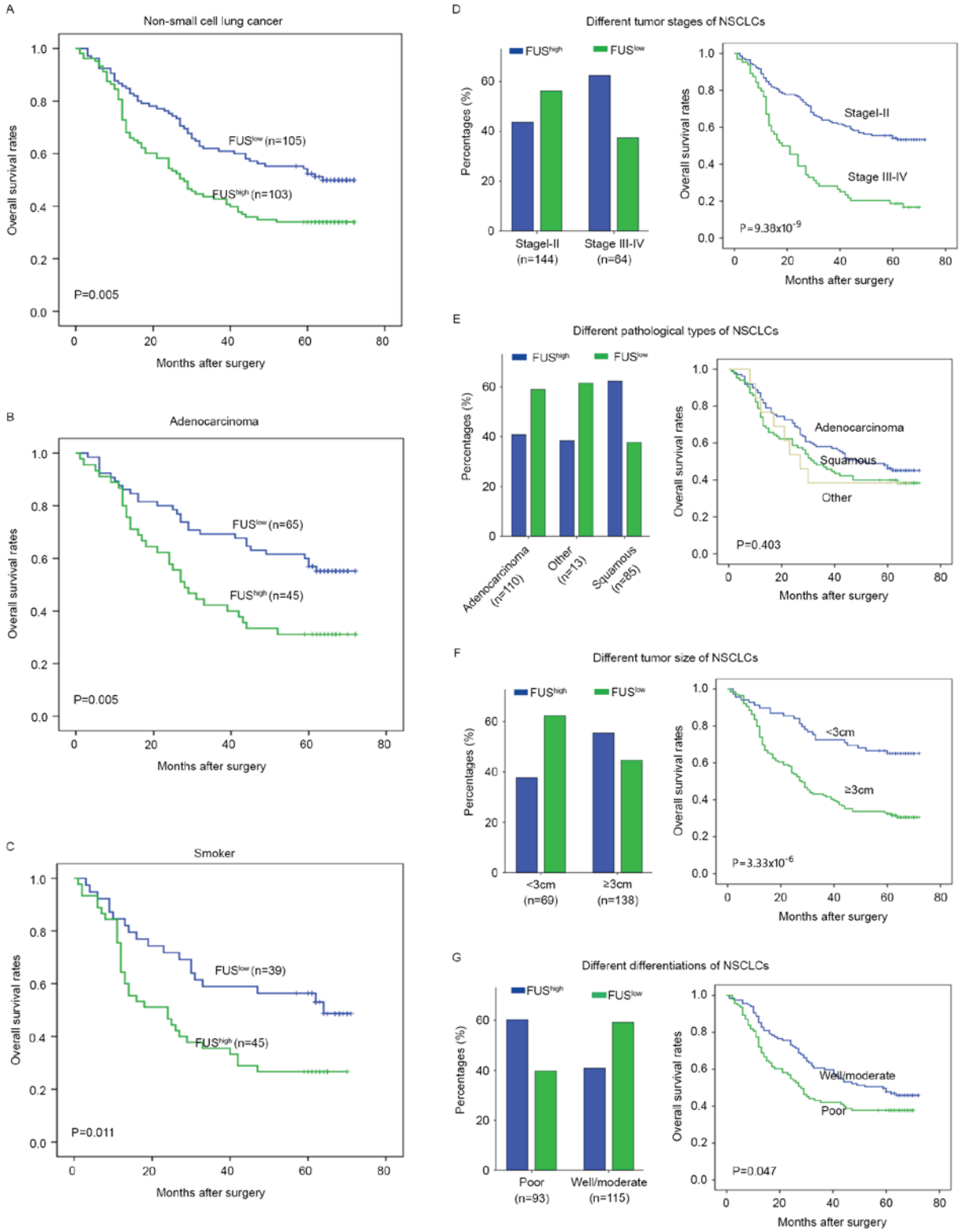

Figure 2. High FUS/TLS expression is associated with poor prognosis of patients with NSCLC. (A) Kaplan-Meier analysis of overall survival in patients with NSCLC according to FUS/TLS expression level. Overall survival in subsets of patients divided by (B) adenocarcinoma and (C) smoking status. FUS/TLS levels associated with different clinicopathological features of patients with NSCLC, including the evaluation of their corresponding overall survival: (D) Tumor stage, (E) pathological type, (F) tumor size and (G) differentiation status. FUS^{high}: high expression of FUS/TLS; FUS^{low}: low expression of FUS/TLS. FUS/TLS, Fused in sarcoma/translocated in liposarcoma; NSCLC, non-small cell lung cancer.

and any of the clinicopathological variables, except lymph nodal involvement (P=0.011). Patients with low E-cadherin expression exhibited a shorter OS compared to those with high E-cadherin expression (P=0.025; Fig. 3A). In univariate analysis, E-cadherin expression demonstrated a significant influence on OS (P=0.027). However, in the multivariate analysis, E-cadherin was not significant as an independent predictor of survival (P=0.211; Table II).

Table II. Univariate and multivariate analysis of factors associated with OS.

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	P-value	HR	95% CI	P-value
Gender						
(Male vs. female)	0.789	0.526-1.183	0.251			
Smoking status						
(Non-smokers vs. smokers)	1.284	0.895-1.843	0.175			
Tumor size						
(≥3 cm vs. <3 cm)	2.755	1.758-4.318	$9.76x10^{-6}$	2.059	1.296-3.271	0.002[a]
Tumor stage						
(III-IV vs. I-II)	2.771	1.922-3.993	$4.65x10^{-8}$	1.430	0.904-2.262	0.127
Lymph node metastasis						
(Yes vs. no)	3.042	2.103-4.399	$3.47x10^{-9}$	2.172	1.371-3.443	0.001[a]
Differentiation						
(Well/moderate vs. poor)	1.431	1.000-2.049	0.050[a]	1.122	0.772-1.629	0.546
FUS/TLS level						
(Low vs. high)	1.667	1.160-2.394	0.006[a]	1.387	0.947-2.030	0.093
E-cadherin expression						
(Low vs. high)	0.655	0.450-0.954	0.027[a]	0.785	0.538-1.147	0.211
FUS(TLS)/E-cadherin expression			0.001[a]			0.044[a]
1 vs. 3	1.461	1.144-1.866	0.002[a]	1.255	0.968-1.628	0.086
2 vs. 3	1.959	1.310-2.930	0.001[a]	1.683	1.115-2.539	0.013[a]
1+2 vs. 3	2.037	1.414-2.934	$2.03x10^{-4}$	1.634	1.122-2.381	0.010[a]

Variables were adopted for their prognostic significance by univariate analysis with forward stepwise selection (forward, likelihood ratio). Variables were adopted for their prognostic significance by univariate analysis ([a]P<0.05). OS, overall survival; 95% CI, 95% confidence interval; multivariate analysis, Cox proportional hazards regression model FUS/TLS, fused in sarcoma/translocated in liposarcoma; NSCLC, non-small cell lung cancer.

The association between FUS/TLS and E-cadherin expression was also examined (Fig. 3). A significant negative association was observed between FUS/TLS and E-cadherin expression (r^2=0.51 and P=0.036; Fig. 3B and Table III). High FUS/TLS expression was associated with impaired E-cadherin expression and low FUS/TLS expression was associated with increased E-cadherin expression, representative images are shown in Fig. 3D. To investigate the combined influence of FUS/TLS and E-cadherin expression on the prognosis of patients with NSCLC, patients were divided into three groups: Group 1, low FUS/TLS and preserved E-cadherin (n=51); Group 2, FUS/TLS and E-cadherin high or low (n=89); Group 3, high FUS/TLS and impaired E-Cadherin (n=68). Significant differences of 5-year OS rates were observed among the three groups (Fig. 3C). Group 1 and 2 exhibited a more favorable 5-year OS rates (52.9 and 48.3%, respectively) as compared with Group 3 (26.5%). In addition, the multivariate analysis demonstrated the index that combined the two markers (FUS/TLS and E-cadherin) was an independent prognostic factor in patients' overall survival (P=0.044; Table II).

Table III. Association between FUS/TLS and E-cad expression in NSCLC tissues.

E-cadherin	FUS/TLS		Total	P-value
	Low	High		
Low	54	68	122	0.036[a]
High	51	35	86	
Total	105	103	208	

[a]P<0.05 difference was statistically significant. FUS/TLS, fused in sarcoma/translocated in liposarcoma; NSCLC, non-small cell lung cancer.

Discussion

In the present study, it was demonstrated that the mRNA and protein levels of FUS/TLS were upregulated in NSCLC tissues compared with corresponding normal tissues. Increased expression of FUS/TLS was associated with higher TNM stage, poor tumor differentiation and large tumor size, and may be beneficial in predicting poor prognosis in patients with NSCLC. Furthermore, it was revealed that there was a significant negative association between FUS/TLS and E-cadherin expression. Combined evaluation of FUS/TLS and E-cadherin

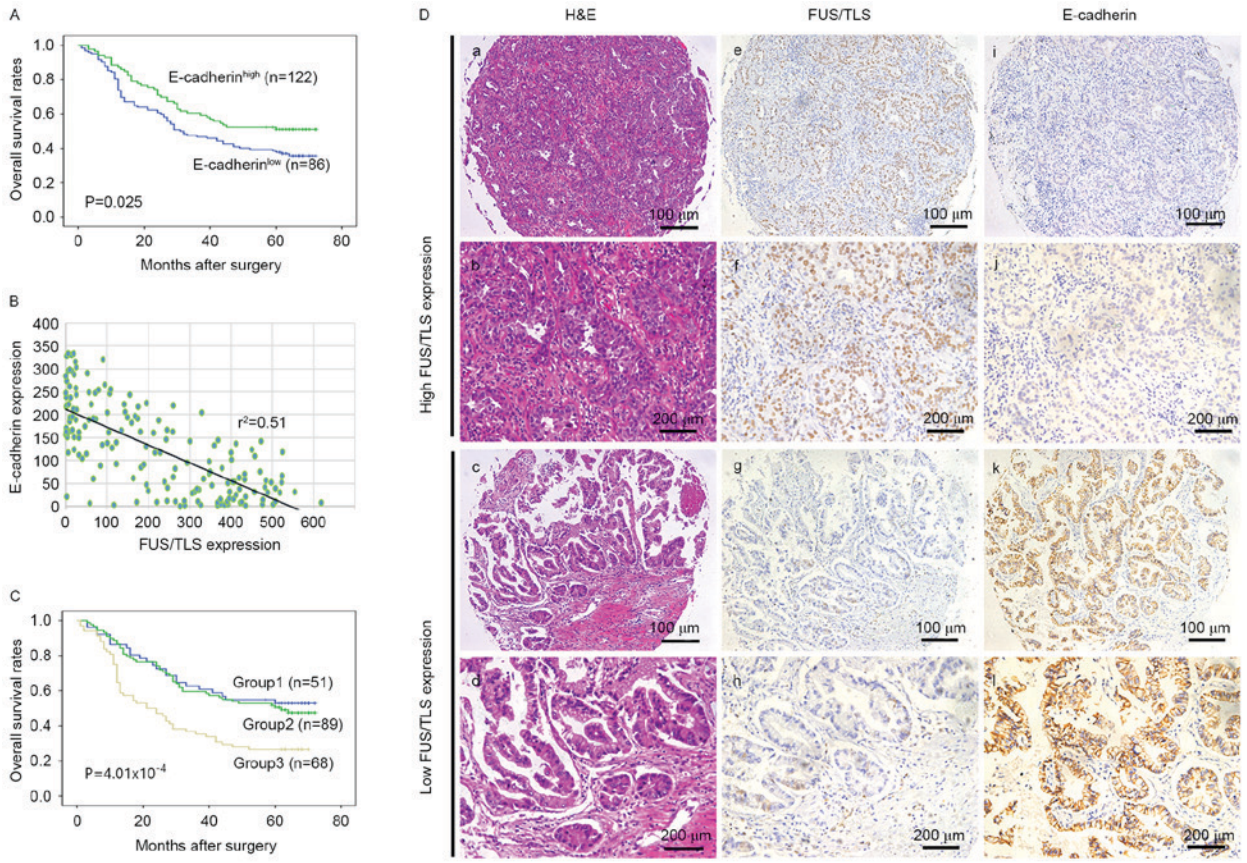

Figure 3. Correlation between FUS/TLS and E-cadherin expression, and the significance of the combination of the two markers. (A) Kaplan-Meier survival analysis for overall survival based on E-cadherin expression in NSCLC cohort. (B) The correction between FUS/TLS and E-cadherin expression according to the integrated optical density of the immunostaining. (C) Kaplan-Meier curves for patients with NSCLC according to the combination of FUS/TLS and E-cadherin expression. (D) Representative images of FUS/TLS and E-cadherin expression in NSCLC samples. (a-d) NSCLC in tissue microarray was identified by H&E staining; (e and f) represent high FUS/TLS expression, (i and j) represent impaired E-cadherin expression, (g and h) represent low FUS/TLS expression, (k and l) represent high E-cadherin expression. FUS/TLS, Fused in sarcoma/translocated in liposarcoma; NSCLC, non-small cell lung cancer; E-cadherin^{high}, high expression of E-cadherin; E-cadherin^{low}, low expression of E-cadherin; H&E, hematoxylin and eosin.

expression was an independent prognostic factor that defined a new subgroup of patients with NSCLC with shortened survival. The results from the present study indicated that high FUS/TLS levels contributed to the progression of NSCLC.

FUS/TLS, a ubiquitous and multifunctional DNA and RNA-binding protein, participates in a wide range of cellular processes, including RNA transcription, microRNA biogenesis, splicing, and nucleo-cytoplasmic shuttling (30-32). In addition, loss of FUS/TLS function significantly restricts cell proliferation (33). The functions of the fusion gene FUS-CHOP and TLS/FUS-ERG in solid tumor, and acute myeloid leukemia have been extensively studied (16,17,34). For example, FUS/TLS combined with the androgen receptor was reported to promote prostate cancer cell proliferation (35). Furthermore, FUS/TLS physically binds with nuclear paraspeckle assembly transcript 1 and contributes an important role in the survival of breast cancer cells (19). FUS/TLS has been demonstrated to participate in various types of cancer and neurodegeneration. However, little attention has been given to the investigation of the association between FUS/TLS and lung cancer. The results from the present study indicated that elevated FUS/TLS expression significantly associated with shortened survival in patients with NSCLC. Clinically, FUS/TLS expression in lung adenocarcinoma and squamous cell carcinoma was present heterogeneously. Upregulated FUS/TLS expression was observed in a higher proportion in squamous cell carcinoma tissues (62.35%) than in adenocarcinoma tissues (40.91%). Although the gene expression profiles of the two histological subtypes vary, the biological reason remains unclear. Furthermore, higher tumor TNM stage, larger tumor size and poor differentiation, the three factors that predict a poor survival, were all significantly associated with high FUS/TLS expression. Subgroup analyses revealed that the proportion of elevated FUS/TLS level in patients with NSCLC with TNM stage III-IV was significantly higher than patients with TNM stage I-II. The same phenomenon was demonstrated in patients with tumor size ≥3 cm and poor differentiation compared with tumor size <3 cm and well or moderate differentiation, respectively.

E-cadherin, an important cell-to-cell adhesion molecule serves a critical role in cancer development and metastasis. Loss of E-cadherin expression in epithelial cancer cells lead to loss of cell polarity and epithelial markers, and increased motility due to acquired mesenchymal markers (3). E-cadherin is also considered to be a hallmark of NSCLC, associated with invasiveness, metastasis and prognosis (4). The results from the present study indicated that FUS/TLS and E-cadherin had a significant influence on the prognosis of patients with

NSCLCs. However, in multivariate analysis, the significance value was lost as an independent predictor of OS. It was also demonstrated in the present study that there were potential associations between FUS/TLS and E-cadherin expression in NSCLC. An important finding is the statistically significant inverse association between FUS/TLS and E-cadherin expression in NSCLC. Furthermore, in the multivariate analysis, high FUS/TLS expression in combination with low E-cadherin expression was an independent prognostic factor for shortened OS.

In summary, FUS/TLS may represent a potential prognostic biomarker of NSCLC. This combination may provide a novel effective risk stratification scheme for NSCLC.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

DX and YBW designed the experiment and wrote the paper. CJ and JJL performed the assays. JG, HBW and SQZ participated in clinical data collection. YFL and XL supervised the research program and performed the data analysis. JYD and JJX had significant roles in the study design and manuscript review. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Ethical approval was obtained from the Zhongshan Hospital Research Ethics Committee (Shanghai, China). The collection and conservation of patient samples, and details were permitted with the evidence of written informed consent.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Siegel RL, Miller KD and Jemal A: Cancer statistics, 2016. CA Cancer J Clin 66: 7-30, 2016.
2. Ettinger DS, Wood DE, Akerley W, Bazhenova LA, Borghaei H, Camidge DR, Cheney RT, Chirieac LR, D'Amico TA, Dilling TJ, *et al*: NCCN guidelines insights: non-small cell lung cancer, version 4.2016. J Natl Compr Canc Netw 14: 255-264, 2016.
3. Zappa C and Mousa SA: Non-small cell lung cancer: Current treatment and future advances. Transl Lung Cancer Res 5: 288-300, 2016.
4. Trautmann M, Menzel J, Bertling C, Cyra M, Isfort I, Steinestel K, Elges S, Grünewald I, Altvater B, Rossig C, *et al*: FUS-DDIT3 fusion protein driven IGF-IR signaling is a therapeutic target in myxoid liposarcoma. Clin Cancer Res 23: 6227-6238, 2017.
5. Morohoshi F, Ootsuka Y, Arai K, Ichikawa H, Mitani S, Munakata N and Ohki M: Genomic structure of the human RBP56/hTAFII68 and FUS/TLS genes. Gene 221: 191-198, 1998.
6. Bertolotti A, Bell B and Tora L: The N-terminal domain of human TAFII68 displays transactivation and oncogenic properties. Oncogene 18: 8000-8010, 1999.
7. Zinszner H, Albalat R and Ron D: A novel effector domain from the RNA-binding protein TLS or EWS is required for oncogenic transformation by CHOP. Genes Dev 8: 2513-2526, 1994.
8. Lerga A, Hallier M, Delva L, Orvain C, Gallais I, Marie J and Moreau-Gachelin F: Identification of an RNA binding specificity for the potential splicing factor TLS. J Biol Chem 276: 6807-6816, 2001.
9. Nguyen CD, Mansfield RE, Leung W, Vaz PM, Loughlin FE, Grant RP and Mackay JP: Characterization of a family of RanBP2-type zinc fingers that can recognize single-stranded RNA. J Mol Biol 407: 273-283, 2011.
10. Ohno T, Ouchida M, Lee L, Gatalica Z, Rao VN and Reddy ES: The EWS gene, involved in Ewing family of tumors, malignant melanoma of soft parts and desmoplastic small round cell tumors, codes for an RNA binding protein with novel regulatory domains. Oncogene 9: 3087-3097, 1994.
11. Cassiday LA and Maher LJ III: Having it both ways: Transcription factors that bind DNA and RNA. Nucleic Acids Res 30: 4118-4126, 2002.
12. Panagopoulos I, Aman P, Mertens F, Mandahl N, Rydholm A, Bauer HF and Mitelman F: Genomic PCR detects tumor cells in peripheral blood from patients with myxoid liposarcoma. Genes Chromosomes Cancer 17: 102, 1996.
13. Campos-Melo D, Droppelmann CA, Volkening K and Strong MJ: RNA-binding proteins as molecular links between cancer and neurodegeneration. Biogerontology 15: 587-610, 2014.
14. Riggi N, Cironi L, Suva ML and Stamenkovic I: Sarcomas: Genetics, signalling, and cellular origins. Part 1: The fellowship of TET. J Pathol 213: 4-20, 2007.
15. Crozat A, Aman P, Mandahl N and Ron D: Fusion of CHOP to a novel RNA-binding protein in human myxoid liposarcoma. Nature 363: 640-644, 1993.
16. Ichikawa H, Shimizu K, Hayashi Y and Ohki M: An RNA-binding protein gene, TLS/FUS, is fused to ERG in human myeloid leukemia with t(16;21) chromosomal translocation. Cancer Res 54: 2865-2868, 1994.
17. Kong XT, Ida K, Ichikawa H, Shimizu K, Ohki M, Maseki N, Kaneko Y, Sako M, Kobayashi Y, Tojou A, *et al*: Consistent detection of TLS/FUS-ERG chimeric transcripts in acute myeloid leukemia with t(16;21)(p11;q22) and identification of a novel transcript. Blood 90: 1192-1199, 1997.
18. Spitzer JI, Ugras S, Runge S, Decarolis P, Antonescu C, Tuschl T and Singer S: mRNA and protein levels of FUS, EWSR1, and TAF15 are upregulated in liposarcoma. Genes Chromosomes Cancer 50: 338-347, 2011.
19. Ke H, Zhao L, Feng X, Xu H, Zou L, Yang Q, Su X, Peng L and Jiao B: NEAT1 is required for survival of breast cancer cells through FUS and miR-548. Gene Regul Syst Bio 10: 11-17, 2016.
20. Lepourcelet M, Tou L, Cai L, Sawada J, Lazar AJ, Glickman JN, Williamson JA, Everett AD, Redston M, Fox EA, *et al*: Insights into developmental mechanisms and cancers in the mammalian intestine derived from serial analysis of gene expression and study of the hepatoma-derived growth factor (HDGF). Development 132: 415-427, 2005.
21. Kalluri R and Weinberg RA: The basics of epithelial-mesenchymal transition. J Clin Invest 119: 1420-1428, 2009.
22. Bremnes RM, Veve R, Gabrielson E, Hirsch FR, Baron A, Bemis L, Gemmill RM, Drabkin HA and Franklin WA: High-throughput tissue microarray analysis used to evaluate biology and prognostic significance of the E-cadherin pathway in non-small-cell lung cancer. J Clin Oncol 20: 2417-2428, 2002.
23. Andersson MK, Stahlberg A, Arvidsson Y, Olofsson A, Semb H, Stenman G, Nilsson O and Aman P: The multifunctional FUS, EWS and TAF15 proto-oncoproteins show cell type-specific expression patterns and involvement in cell spreading and stress response. BMC Cell Biol 9: 37, 2008.
24. Hirohashi S: Inactivation of the E-cadherin-mediated cell adhesion system in human cancers. Am J Pathol 153: 333-339, 1998.
25. Gu J, Ding JY, Lu CL, Lin ZW, Chu YW, Zhao GY, Guo J and Ge D: Overexpression of CD88 predicts poor prognosis in non-small-cell lung cancer. Lung Cancer 81: 259-265, 2013.

26. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
27. Liao YF, Wu YB, Long X, Zhu SQ, Jin C, Xu JJ and Ding JY: High level of BRD4 promotes non-small cell lung cancer progression. Oncotarget 7: 9491-9500, 2016.
28. Liao Y, Gu J, Wu Y, Long X, Ge DI, Xu J and Ding J: Low level of 5-Hydroxymethylcytosine predicts poor prognosis in non-small cell lung cancer. Oncol Lett 11: 3753-3760, 2016.
29. Zhao GY, Ding JY, Lu CL, Lin ZW and Guo J: The overexpression of 14-3-3ζ and Hsp27 promotes non-small cell lung cancer progression. Cancer 120: 652-663, 2014.
30. Uranishi H, Tetsuka T, Yamashita M, Asamitsu K, Shimizu M, Itoh M and Okamoto T: Involvement of the pro-oncoprotein TLS (translocated in liposarcoma) in nuclear factor-kappa B p65-mediated transcription as a coactivator. J Biol Chem 276: 13395-13401, 2001.
31. Zinszner H, Sok J, Immanuel D, Yin Y and Ron D: TLS (FUS) binds RNA in vivo and engages in nucleo-cytoplasmic shuttling. J Cell Sci 110: 1741-1750, 1997.
32. Mastrocola AS, Kim SH, Trinh AT, Rodenkirch LA and Tibbetts RS: The RNA-binding protein fused in sarcoma (FUS) functions downstream of poly(ADP-ribose) polymerase (PARP) in response to DNA damage. J Biol Chem 288: 24731-24741, 2013.
33. Ward CL, Boggio KJ, Johnson BN, Boyd JB, Douthwright S, Shaffer SA, Landers JE, Glicksman MA and Bosco DA: A loss of FUS/TLS function leads to impaired cellular proliferation. Cell Death Dis 5: e1572, 2014.
34. Panagopoulos I, Aman P, Fioretos T, Höglund M, Johansson B, Mandahl N, Heim S, Behrendtz M and Mitelman F: Fusion of the FUS gene with ERG in acute myeloid leukemia with t(16;21)(p11;q22). Genes Chromosomes Cancer 11: 256-262, 1994.
35. Haile S, Lal A, Myung JK and Sadar MD: FUS/TLS is a co-activator of androgen receptor in prostate cancer cells. PLoS One 6: e24197, 2011.