Overexpression of fibronectin type III domain containing 3B is correlated with epithelial-mesenchymal transition and predicts poor prognosis in lung adenocarcinoma

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Abstract. Fibronectin (FN) type III domain containing 3B (FNDC3B), a member of the FN family, regulates the invasion and metastasis of cells in numerous tumor types. However, the mechanisms through which FNDC3B regulates carcinogenesis in lung adenocarcinoma (LADC) tissues have remained elusive. The present study revealed that the protein levels of FNDC3B and vimentin were significantly elevated in LADC tissues compared with those in normal lung tissues. By contrast, the expression of E-cadherin was decreased in LADC tissues compared with that in normal lung tissues. Furthermore, the aberrant expression of FNDC3B and epithelial-mesenchymal transition (EMT) markers was significantly associated with histological differentiation, lymph node metastasis and tumor-nodes-metastasis stage. Kaplan-Meier analysis indicated that a high expression of FNDC3B may be associated with poor overall survival of patients with LADC. In addition, overexpression of FNDC3B promoted the protein expression of EMT-associated genes in the A549 lung adenocarcinoma cell line. In conclusion, the present results support the notion that FNDC3B acts as an oncogene in LADC; it may serve a pivotal role in the development and progression of LADC and may participate in the regulation of the EMT.

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Introduction

Lung cancer is one of the most common cancer types with the highest incidence and mortality rate of all cancer types worldwide (1-5). Lung adenocarcinoma (LADC) accounts for ~75% of non-small cell lung cancer cases, which is the most common subtype of lung cancer (6,7). LADC has a marked clinical, molecular and histological heterogeneity. The five-year survival rate of LADC patients in 2011 was <15% (8). Therefore, it is essential to investigate the genes associated with tumor cell invasion and metastasis in patients with LADC. Furthermore, it is essential to identify the relevant molecular mechanisms of LADC. With this information, scientists may be able to develop better treatment methods for the diagnosis and prognosis of LADC.

Fibronectin type III (FNIII) domain containing 3B (FNDC3B), which is also known as factor for adipocyte differentiation 104, is a member of the FN family. It contains 9 FNIII domains and one transmembrane domain (9). Furthermore, FNDC3B regulates the differentiation of adipocytes and osteoblasts (10-12). FNIII domains have an important role in cell adhesion and in growth signaling pathways (13,14). The metastasis of hepatocellular carcinoma has been reported to be promoted by overexpression of FNDC3B, while knockdown of FNDC3B suppressed the proliferation and metastatic capacity of tumor cells (15). It is therefore indicated that FNDC3B is associated with the growth, invasion and metastasis of tumors. However, only few studies have reported on the involvement of FNDC3B in LADC.

Epithelial-mesenchymal transition (EMT) is a process in which epithelial cells gradually transform into mesenchymal-like cells, while losing their epithelial functionality and characteristics (16). After undergoing EMT, cells typically exhibit higher expression of the proteins vimentin, N-cadherin

and fibronectin; however, these cells exhibit downregulated expression of E-cadherin and cytokeratins (17). In patients with lung cancer, the invasiveness and metastatic capacity of tumor cells is characterized by the diagnostic marker vimentin (18). When a cell undergoes extensive EMT, it exhibits a lack E-cadherin expression and an induced expression of vimentin (19). Furthermore, EMT is also induced by overexpression of FNDC3B. In different cell types, including hepatocytes, mammary epithelial cells and renal epithelial cells, the induced expression of the mesenchymal marker vimentin and the suppression of E-cadherin occurs with the transformation of FNDC3B (20). However, it remains elusive whether the expression of FNDC3B is associated with the EMT in LADC patients.

In the present study, it was revealed that FNDC3B and EMT-associated markers were aberrantly expressed in LADC versus normal tissues. The expression of FNDC3B and EMT markers was demonstrated to be significantly associated with histological differentiation, lymph node metastasis and TNM stage. When FNDC3B was ectopically overexpressed, the protein expression of EMT-associated genes was increased in the A549 LADC cell line. Thus, FNDC3B acts as an oncogene in the development and progression of LADC. Furthermore, the present results may suggest that the EMT is regulated by FDNC3B.

Materials and methods

Patients and tissue samples. A total of 276 cases of LADC (Median age at diagnosis, 62 years; age range, 39-77 years) and 82 normal lung tissues adjacent to the LADC were collected from 276 patients that were treated at the Affiliated Hospital of Nantong University in China (Nantong, China). All of these patients were treated by surgical resection between 2007 and 2011. A total of 125 females were recruited, which accounted for 45.2% of the subjects included in the present study. The clinical data of all patients included were carefully recorded after the diagnosis of LADC was confirmed by two pathologists. The pathological stage was determined by according to the 8th Edition of the tumor-nodes-metastasis (TNM) Classification for Lung Cancer (21). The follow-up was completed by June 30, 2014 and the median follow-up duration was 52 months.

Tissue microarray (TMA) construction and immunohistochemistry (IHC). The TMA was generated using the Quick-Ray system (cat no. UT06; Unitma, Seoul, Korea). Core tissue biopsies (2 mm in diameter) were taken from individual formalin-fixed and paraffin-embedded tissue blocks and arranged in paraffin blocks. TMA specimens were cut into 4-μm sections and placed on super frost-charged glass microscope slides. Hematoxylin and eosin staining was performed for 15 min at room temperature for TMA analysis.

The EnVision method (22) was used for IHC staining. A total of 276 LADC samples and 82 normal lung tissues were included in the TMA. The samples were incubated for 40 min at 70˚C, dewaxed and dehydrated in a graded series of ethanols. Using TMA in antigen repairing 22 min at 99˚C, dripping with 3% hydrogen peroxide for 20 min at room temperature. Samples were then blocked using 10% normal goat serum (0060-01; Shanghai Haoran Biotechnology Co., Ltd., Shanghai, China) for 30 min at room temperature. Subsequently, the samples were washed with PBS containing Triton X-100 three times, and incubated with polyclonal rabbit anti human FNDC3B (1:150 dilution; cat. no. HPA007859; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), mouse anti-human E-cadherin (1:100 dilution; cat. no. MAB-0738) and rabbit anti-human vimentin (1:100 dilution; RMA-0547) (both from Maixin Biotech, Co. Ltd., Fuzhou, China) overnight at 4˚C. Subsequently, the samples were incubated with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (1:500 dilution; cat. no. sc-2004; SantaCruz Biotechnology, Inc., Dallas, TX, USA) for 2 h at room temperature. Antibodies were visualized using diaminobenzedene hydrochloride and PBS was used as a negative control instead of the primary antibody.

FNDC3B staining was quantified by evaluating at least 1,000 cells from at least five randomly selected fields of view. Prior to performing univariate and multivariate analyses, the intensity of immunostaining in each tumor section was classified as follows: 3, strong; 2, moderate; 1, weak; and 0, negative. Furthermore, the percentage of stained cells was determined using the following semi-quantitative scale: 0, <5% of cells; 1, 5-25%; 2, 26-50%; 3, 51-75%; and 4, >75% of cells. The two scores were multiplied and according to the final score obtained, the samples were classified into two groups: i) Score of 0-6, low expression and ii) score of 7-12, high expression (23,24). The cut-off for E-cadherin expression was set at 70%. In certain tumor samples, E-cadherin expression was >70%; a membranous staining developed in the entire tumor containing these cells, indicating a high expression of E-cadherin. Expression of vimentin was frequently observed in the cytoplasm of the tumor cells. According to cytoplasm staining, a high expression of vimentin was defined as >20% of tumor cells (24-27).

Western blot analysis. Cells were lysed in cell lysis solution containing 50 mM Tris-Cl, 1% (w/v) SDS, sodium pyrophosphate, β-glycerophosphate, sodium orthovanadate, sodium fluoride, EDTA and leupeptin (cat. no. P0013; Beyotime Institute of Biotechnology, Haimen, China). A protease inhibitor cocktail (Roche Applied Science, Penzburg, Germany) was supplemented prior to use. Protein (50 μg) was separated using 10% SDS-PAGE gel and transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Non-specific protein interactions were blocked by incubation with 3% fat-free milk in Tris-buffered saline (150 mm NaCl and 50 mM Tris-HCl, pH 7.6) at 4˚C for 1 h. Subsequently, the membrane was incubated at 4˚C for 2 h with polyclonal rabbit anti-human FNDC3B (1:300 dilution; cat. no. HPA007859; Sigma-Aldrich; Merck KGaA), mouse anti-human E-cadherin (1:1,000 dilution; cat. no. MAB-0738), rabbit anti-human vimentin (1:1,000 dilution; cat. no. RMA-0547) (both from Maixin Biotech, Co. Ltd.) and rabbit anti β-actin antibody (1:2,000 dilution; cat. no. 4970; Santa Cruz Biotechnology, Inc.). Following five washes with PBS with Tween-20 (5 min/wash), membranes were incubated with horseradish peroxidase conjugated-polyclonal goat anti-mouse secondary antibodies (1:10,000; cat. no. ab6789; Abcam, Cambridge, UK) for 1 h at room temperature. Signals were detected with an enhanced

Table I. FNDC3B immunohistochemical staining of protein in normal lung tissues and LADC tissues.

Group	Cases (n)	FNDC3B expression		P-value
		Low, n (%)	High, n (%)	
Normal lung tissue	82	69 (84.15)	13 (15.85)	<0.001
LADC	276	63 (22.83)	213 (77.17)	

FNDC3B, fibronectin type III domain containing 3B; LADC, lung adenocarcinoma.

chemiluminescence system (Sigma-Aldrich; Merck KGaA) according to the manufacturer's protocol. β-actin was used as the loading control.

Cell culture. The A549 human LADC cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). Cell culture of A549 cells was performed in RPMI-1640 medium (Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (Haoyang Biological Manufacture Co. Ltd., Tianjin, China) and 100 units penicillin-streptomycin in a humidified atmosphere with 5% CO_2 at 37˚C.

Construction of the pcDNA3.1-FNDC3B vector. Based on the Genbank sequence (NM_022763.3), upstream and downstream primers were synthesized for FNDC3B gene amplification. The restriction enzyme sites for *Bam*HI and *Kpn*I were added to the 5' end of each primer. Next, the full-length FNDC3B gene was amplified by polymerase chain reaction (PCR) from the complementary DNA template with the primers containing the *Bam*HI and *Kpn*I restriction sites. The PCR product was then ligated into the pGEM-T vector (Promega Corporation, Madison, WI, USA), which was transformed into A549 cells that were screened for positive clones. After sequence verification, the correct sequence was subcloned into the pcDNA3.1 expression vector (Shenzhen Zhonghong Boyuan Biological Technology Co., Ltd., Shenzhen, China) to construct the pcDNA3.1-FNDC3B recombinant expression vector.

Transfection of pcDNA3.1-FNDC3B or FNDC3B small hairpin (sh)RNA into A549 cells. The A549 cells were transfected with either the pcDNA3.1-FNDC3B eukaryotic expression vector or FNDC3B shRNA using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocols. After transfection, the cells were diluted at a 1:16 ratio and re-seeded in 12-well plates at a density of $2x10^5$ cells per well. The media was replaced with complete medium containing G418 (800 μg/ml). Following isolation, cells were re-suspended and cultured in medium containing G418 (cat. no. 10131035; Thermo Fisher Scientific, Inc.) for subsequent experiments. The stable positive clones were selected approximately 21 days later and protein expression was evaluated via western blotting.

RNA extraction and reverse transcription-quantitative (RTq) PCR. RTqPCR was performed according to a procedure described in previous study (28). The total RNA was extracted with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to manufacturer's protocol. The mRNA expression of FNDC3B and β-actin was determined with ABI7500 PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA).The data were obtained by normalizing the quantification cycle (Cq) values for FNDC3B to those of the corresponding β-actin, followed by calculation of $2^{-\Delta\Delta Cq}$ (29). The primer sequences were as follows: FNDC3B forward, 5'-GGGACAGACACCCGTTTTGA-3' and reverse, 5'-GTG TTGCCCACGGTAATGCT-3'; β-actin forward, 5'-GCACCAC ACCTTCTACAATGAG-3' and reverse, 5'-ACAGCCTGGATG GCTACGT-3'.

Invasion and migration assays. The invasion and migration assays were performed according to a procedure described in a previous study (28).

Colony formation assay. A total of ~200 cells were seeded into six-well plates in triplicate. The resultant cell culture was formed within 14 days in these plates. The cells were fixed with methanol for 15 min at room temperature and stained with 1% crystal violet for 30 min at room temperature. A colony was regarded as a cell aggregate derived from a single cell containing >50 cells.

Statistical analysis. Statistical analysis of the RT-qPCR and western blot data was performed using an unpaired Student's t-test when two groups were compared. The comparisons multile groups were made using a chi-square analysis or Fisher's exact test, if appropriate. The Chi-square and Phi coefficient were used to confirm the association between the expression of FNDC3B and EMT indicators. Kaplan-Meier analysis with the log-rank test was used for calculating and analyzing survival curves. Cox regression analysis was performed for univariate and multivariate analyses. $P<0.05$ was considered to indicate a statistically significant difference. The data were analyzed by using SPSS 22.0 software for Windows (IBM Corporation, Chicago, IL, USA).

Results

FNDC3B protein is increased in LADC tissues. IHC analysis indicated that FNDC3B protein was highly expressed in LADC tumors; the high expression of FDNC3B was determined to be 77.17% (213/276) in these tumors. By contrast, the overexpression of FDNC3B was only 15.85% (13/82) in the adjacent non-cancerous tissues. The expression of FNDC3B protein was significantly upregulated in LADC patients ($P<0.001$; Table I).

Table II. Association of FNDC3B expression with clinicopathological characteristics of lung adenocarcinoma patients.

	FNDC3B expression			
Characteristic	Low, n (%)	High, n (%)	χ^2 test	P-value
Sex				
Male	66 (54.55)	85 (54.55)	0.002	0.961
Female	55 (45.45)	70 (45.45)		
Age (years)				
≤60	56 (46.28)	69 (46.28)	0.085	0.77
>60	65 (53.72)	86 (53.72)		
Smoking history				
No	79 (65.29)	97 (65.29)	0.216	0.642
Yes	42 (34.71)	58 (34.71)		
Maximum tumor diameter (cm)				
≤3	57 (47.11)	86 (47.11)	1.91	0.167
>3	64 (52.89)	69 (52.89)		
Histological differentiation				
Well	32 (26.45)	4 (26.45)	54.176	<0.001
Moderate	65 (53.72)	64 (53.72)		
Poor	24 (19.83)	87 (19.83)		
Lymph node metastasis				
No	107 (88.43)	34 (88.43)	120.238	<0.001
Yes	14 (11.57)	121 (11.57)		
TNM stage				
I	105 (86.78)	30 (86.78)	127.668	<0.001
II	13 (10.74)	50 (10.74)		
III	3 (2.48)	66 (2.48)		
IV	0 (0.00)	9 (0.00)		

FNDC3B, fibronectin type III domain containing 3B; TNM, tumor-nodes-metastasis.

Representative IHC images for FNDC3B are presented in Fig. 1A. In normal lung tissues, the expression of FNDC3B protein was relatively low (Fig. 1A1). Furthermore, FNDC3B protein was predominantly localized in the cytoplasm of tumor cells, as well as in the membrane. The intensity of FNDC3B staining was increased with the deterioration of the degree of differentiation of the tumors (Fig. 1A2-4).

Association between the expression of FNDC3B in LADC tissues and clinicopathological characteristics. Table II provides the association of FNDC3B expression in LADC tissues and the clinicopathological characteristics. The expression of FNDC3B (high vs. low) was significantly associated with the histological differentiation, lymph node metastasis and TNM stage (P<0.001); however, it was not significantly associated with age, sex, smoking history and the tumor maximum diameter (P>0.05).

Overexpression of FNDC3B indicates poor prognosis. Kaplan-Meier survival curves indicated that the expression of FNDC3B may be used to predict the prognosis of LADC patients (χ^2=122.2695, P<0.0001). The survival rate of patients with high protein expression of FNDC3B in LADC tissues was significantly lower than that with a low expression (Fig. 2A). In these LADC patients, the expression of FNDC3B was an independent prognostic factor as indicated by logistic regression analysis using Cox's proportional hazards model (P=0.007; Table III). Kaplan-Meier survival analysis was also used to evaluate the impact of the histological degree of differentiation on patient survival (Fig. 2B). The histological differentiation was revealed to be an independent prognostic factor in LADC patients (P<0.001), which was also confirmed by Cox regression analysis (Table III).

EMT markers are aberrantly expressed in LADC tissues. The high expression rates of E-cadherin and vimentin were 20.29% (56/276) and 74.28% (205/276), respectively (Table IV). According to the IHC results, E-cadherin was mainly expressed in the cell membrane of LADC tissues. Furthermore, vimentin was mainly expressed in the cytoplasm. In normal lung tissues, vimentin expression was low, while that of E-cadherin was high (Fig. 1B1 and C1). In LADC tissues, the intensity of IHC staining for E-cadherin declined with the deterioration of the degree of differentiation (Fig. 1B2-4), while the opposite trend was observed for vimentin (Fig. 1C2-4).

Table III. Univariate and multivariate analyses of various prognosis parameters in 276 patients with lung adenocarcinoma using Cox regression model.

	Univariate analysis			Multivariate analysis		
Covariate	HR	95% CI	P-value	HR	95% CI	P-value
Age	0.929	(0.669-1.288)	0.657	0.952	(0.676-1.341)	0.779
Sex	1.002	(0.722-1.391)	0.991			
Smoking history	1.344	(0.964-1.875)	0.081			
Maximum tumor diameter	1.026	(0.739-1.424)	0.878			
Histological differentiation	4.597	(3.349-6.310)	<0.001	2.663	(1.899-3.736)	<0.001
Lymph node metastasis	7.009	(4.664-10.534)	<0.001	0.776	(0.36-1.672)	0.517
TNM stage	2.974	(2.434-3.633)	<0.001	1.278	(0.89-1.835)	0.184
FNDC3B	8.967	(5.616-14.317)	<0.001	2.311	(1.255-4.254)	0.007
E-cadherin	0.086	(0.054-0.135)	<0.001	0.428	(0.218-0.842)	0.014
Vimentin	11.707	(7.384-18.561)	<0.001	2.428	(1.247-4.728)	0.009

HR, hazard ratio; CI, Confidence interval; FNDC3B, fibronectin type III domain containing 3B; TNM, tumor-nodes-metastasis.

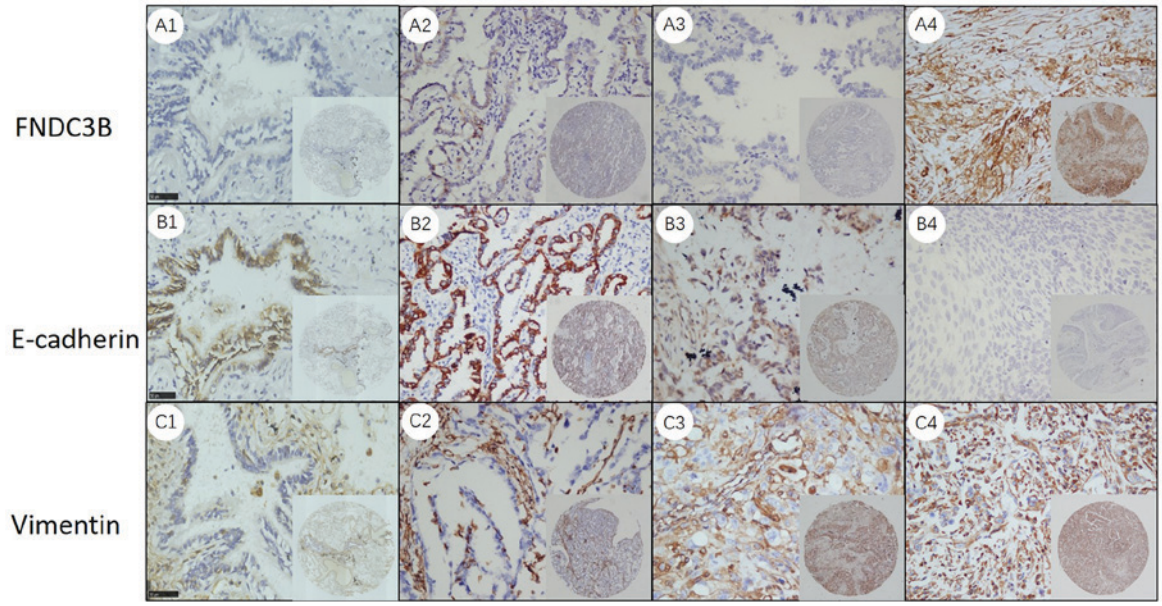

Figure 1. FNDC3B and markers of epithelial to mesenchymal transition in LADC tissues were determined by immunohistochemistry. (A) 1-4: Expression of FNDC3B (B) 1-4: Expression of E-cadherin and (C) 1-4: Expression of vimentin in normal lung tissue, as well as in well, moderately and poorly differentiated LADC tissues, respectively (original magnification, x40 or x400, respectively). FNDC3B, fibronectin type III domain containing 3B; LADC, lung adenocarcinoma.

Association between the expression of EMT markers in LADC tissues and clinicopathological characteristics. Table V presents the association of EMT marker expression in LADC tissues (high vs. low) with clinicopathological parameters. An aberrant expression of EMT markers was significantly associated with histological differentiation, metastasis and TNM stage (P<0.001); however, it was not significantly associated with age, sex, smoking history and the tumor maximum diameter (P>0.05).

Abnormally expressed EMT markers indicate poor prognosis. Kaplan-Meier survival analysis indicated that the expression of E-cadherin and vimentin was associated with the OS of LADC patients (χ^2=173.7963, P<0.0001; χ^2=170.3951, P<0.0001). In LADC patients, an increased expression of vimentin indicated a lower OS rate. By contrast, a high expression of E-cadherin indicated that the probability of survival for LADC patients at any given time-point was significantly higher compared with that for patients with low E-cadherin expression (Fig. 2C and D). Multivariate Cox regression analyses further revealed that E-cadherin and vimentin may be used as independent prognostic markers for determining the prognosis of LADC patients (P=0.014; 0.009; Table III).

Association between the expression of FNDC3B and markers of EMT. The association between the expression of FNDC3B and the two EMT markers determined by IHC was calculated and presented in Table VI. The results indicate that the expression of FNDC3B was positively associated with expression of

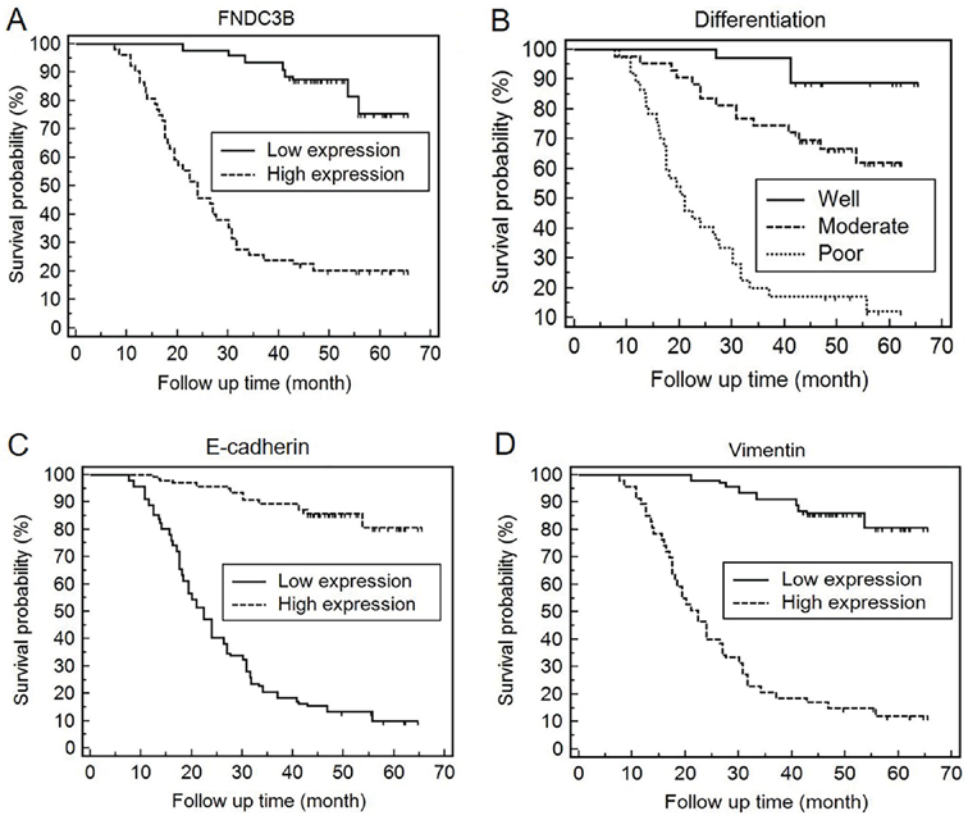

Figure 2. Survival curves of LADC were determined by Kaplan-Meier analysis and significant differences were determined using the log-rank test. (A) FNDC3B overexpression was negatively associated with the overall survival rate of patients (P<0.001). (B) In patients with well-differentiated cells in LADC tissues, the overall survival rate was significantly higher than that in patients with moderate and poor differentiation of cells in LADC tissues (P<0.001). The overall survival rate of moderate differentiation group was significantly higher than that in patients with poor differentiation of cells in LADC tissues (P<0.001). (C) Survival curves were drawn for LADC with high and low expression of E-cadherin. A low expression of E-cadherin was positively associated with the overall survival rate of patients (P<0.001). (D) Survival curves were used for assessing the effect of the expression of vimentin in LADC tissues on patient survival. A high expression of vimentin was negatively associated with the overall survival rate of patients (P<0.001). FNDC3B, fibronectin type III domain containing 3B; LADC, lung adenocarcinoma.

Table IV. Immunohistochemical analysis of epithelial-mesenchymal transition markers in normal lung tissues and LADC tissues.

		E-cadherin expression			Vimentin expression		
Tissue sample	Cases (n)	Low, n (%)	High, n (%)	P-value	Low, n (%)	High, n (%)	P-value
Normal lung tissue	82	12 (14.63)	70 (85.37)	<0.001	71 (86.59)	11 (13.41)	<0.001
LADC	276	220 (79.71)	56 (20.29)		71 (25.72)	205 (74.28)	

LADC, lung adenocarcinoma.

vimentin (P<0.001) but negatively associated with the levels of E-cadherin levels in LADC tissues (P<0.001).

FNDC3B has an oncogenic role in LADC. By performing colony formation assays using transfected A549 cells, it was revealed that LADC cells proliferated rapidly when the levels of FNDC3B were increased, while it was suppressed following knockdown of FNDC3B (Fig. 3A and B). In a Transwell system, an increased expression of FNDC3B significantly promoted the migration and invasion of A549 cells. By contrast, the migration and invasion of LADC cells was reduced following knockdown of FNDC3B (Fig. 3C and D). In the present study, the A549 cell line was transfected with pcDNA3.1-FNDC3B or shFNDC3B, and the efficacy of ectopic overexpression or knockdown of FNDC3B was determined by RT-qPCR (Fig. 3E). Overexpression of the FNDC3B protein resulted in downregulation of E-cadherin levels but upregulation of vimentin levels, while the opposite effect was obtained with FNDC3B knockdown (Fig. 3F and G). These results indicate that FNDC3B may have an oncogenic role in LADC.

Discussion

The present study suggested that FNDC3B may be used as a prognostic marker in patients with LADC. Compared with

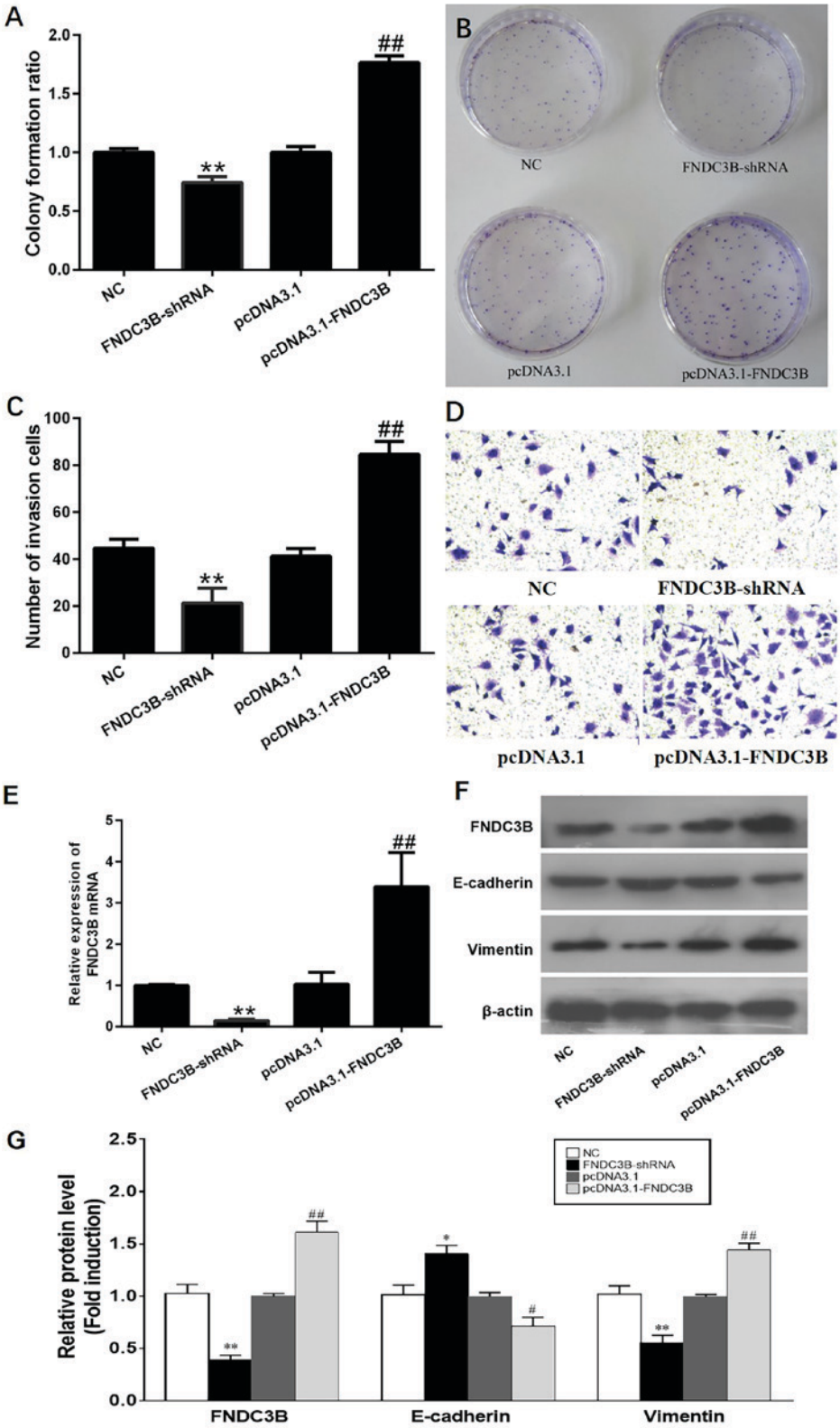

Figure 3. FNDC3B promotes the development of lung adenocarcinoma. The A549 cell line was transfected with shFNDC3B or NC, or with pcDNA3.1-FNDC3B or pcDNA3.1. (A and B) A clonogenic assay was performed to determine the colony formation ability of the cells after transfection. (Cand D) Transwell migration and invasion assays were used to detect the migration and invasion potential of A549 cells in the NC, FNDC3B-shRNA, pcDNA3.1 and pcDNA3.1-FNDC3B groups (original magnification, x400). (E) The efficacy of shFNDC3B overexpression/knockdown was assessed via reverse transcription-quantitative polymerase chain reaction analysis. (F and G) The expression of E-cadherin and vimentin in the different groups was examined by western blot analysis. *P<0.05 and **P<0.01 vs. NC group; $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. pcDNA3.1 group. NC, negative control; FNDC3B, fibronectin type III domain containing 3B; shRNA, small hairpin RNA; pcDNA3.1-FNDC3B, overexpression vector for FNDC3B.

that in normal lung tissues, the expression of FNDC3B was significantly higher in LADC tissues. A high expression of FNDC3B was detected in TMAs of LADC specimens by using IHC, and it was identified to be significantly associated

Table V. Association between markers of the epithelial-mesenchymal transition and clinicopathological characteristics.

	E-cadherin expression				Vimentin expression			
Characteristic	Low	High	χ^2 test	P-value	Low	High	χ^2 test	P-value
Sex								
Male	74	77	0.01	0.922	72	79	0.339	0.561
Female	62	63			64	61		
Age (years)								
≤60	65	60	0.679	0.41	62	63	0.01	0.922
>60	71	80			74	77		
Smoking history								
No	80	96	2.837	0.092	93	83	2.471	0.116
Yes	56	44			43	57		
Maximum tumor diameter (cm)								
≤3	70	73	0.012	0.911	70	73	0.012	0.911
>3	66	67			66	67		
Histological differentiation								
Well	4	32	59.444	<0.001	32	4	64.94	<0.001
Moderate	48	81			80	49		
Poor	84	27			24	87		
Lymph node metastasis								
No	23	118	125.316	<0.001	120	21	148.069	<0.001
Yes	113	22			16	119		
TNM stage								
I	22	113	120.939	<0.001	115	20	146.977	<0.001
II	44	19			19	44		
III	61	8			2	67		
IV	9	0			0	9		

TNM, tumor-nodes-metastasis.

Table VI. Association between the expression of FNDC3B and EMT marker proteins detected by immunohistochemistry.

EMT marker expression	FNDC3B expression		Phi coefficient	P-value
	Low	High		
E-cadherin				
Low	9	127	-0.739	<0.001
High	112	28		
Vimentin				
Low	110	26	0.736	<0.001
High	11	129		

EMT, epithelial-mesenchymal transition; FNDC3B, fibronectin type III domain containing 3B.

with histological differentiation, lymph node metastasis and TNM stage. Furthermore, a high expression of FNDC3B was associated with a poor prognosis in terms of OS. Several studies have reported that FNDC3B is an oncogene, which promotes cell proliferation in the following types of malignancies: Esophageal squamous cell carcinoma, glioblastoma and hepatocellular carcinoma (20,30). A previous study has also indicated that the metastatic potential of prostate cancer cells may be enhanced by modulating the expression of FNDC3B (31). This result further supports the notion that FNDC3B may be an important oncogenic driver gene (20). In line with this, the results of the present study indicate that FNDC3B protein was overexpressed in LADC tissues.

Metastasis is one of the most decisive factors that affect the prognosis of patients. The detection of EMT is widely used to determine tumor metastasis (32). A lack of E-cadherin expression, which also leads to an upregulation of vimentin expression, is regarded to be a hallmark of EMT (33). The EMT significantly increases the invasive potential of tumor cells (34). Tumor metastasis significantly impairs the long-term survival of patients; therefore, it is important to identify the molecular markers associated with metastasis. These molecular markers may be used to predict the prognosis

of patients with lung cancer (35). An abnormal expression of EMT markers indicates the presence of invasive and metastatic tumor cells in patients with lung cancer (18). Therefore, EMT markers may be considered as independent diagnostic markers of lung cancer. In the present study, an aberrant expression of molecular markers of the EMT was associated with the TNM stage, metastasis and prognosis of lung cancer patients. A high expression of vimentin in LADC tissues was associated with a significantly decreased probability of OS. However, a high expression of E-cadherin in LADC tissues was associated with a significantly increased probability of OS. By performing Cox regression analysis, E-cadherin and vimentin were identified as independent prognostic factors for LADC. In LADC tissues, FNDC3B expression was positively associated with vimentin levels but negatively associated with E-cadherin levels. The IHC results indicated that the intensity of FNDC3B and vimentin staining in LADC tissues was increased with deteriorating degree of differentiation; however, the opposite effect was observed for E-cadherin. In the present study, it was concluded that in LADC tissues, FNDC3B expression was positively associated with vimentin levels ($r=0.736$, $P<0.01$) but negatively associated with E-cadherin levels ($r=-0.739$, $P<0.001$).

Overexpression of FNDC3B protein may not only induces EMT but also activate several pathways associated with the development of cancer, including phosphoinositide-3 kinase/Akt, retinoblastoma 1 and transforming growth factor (TGF)-β signaling (20). A recent study demonstrated that FNDC3B is a novel suppressor of TGF-β signalling and represses the TGF-β-mediated EMT in cervical cancer cells (36). The present study indicated that FNDC3B is an oncogene that promotes the invasion and migration of A549 LADC cells. In the *in vitro* experiments of the present study, overexpression of FNDC3B protein not only enhanced the expression of vimentin but also reduced the expression of E-cadherin. These results allow for the following conclusion: In LADC tissues, FNDC3B may instigate the process of EMT to promote the invasion and metastasis of tumor cells.

Despite these significant results, the present study has several limitations. First, it was a retrospective research study, which may have led to selection bias and the availability of clinical data was limited. Consequently, it is required to confirm the present results in future studies including a larger number of LADC cases. Furthermore, the present study only included LADC patients; therefore, the results are restricted to lung cancer only. In addition, the present study only identified the function of FNDC3B in A549 cells. In future studies, the function of FNDC3B will be verified in another LADC cell line. Furthermore, EMT markers not only include the decreased expression of E-cadherin and increased expression of vimentin, but also other markers, e.g. N-cadherin, Snail and Slug. Therefore, the association between FNDC3B and those other EMT proteins will be examined in cancer specimens and *in vitro* in a subsequent study. Finally, the present study did not examine the mechanism through which FNDC3B regulates the EMT in LADC. A follow-up study will determine whether FNDC3B is regulated by the classical pathway of the EMT to promote the progression of LADC.

In conclusion, the present study indicated that FNDC3B is overexpressed in LADC tissues and is an independent prognostic factor of an inferior outcome. Furthermore, FNDC3B functions as an oncogene in LADC, and its high expression may promote the invasion and metastasis of tumor cells. Furthermore, the present results indicate that FNDC3B has an important role in the regulation of genes associated with the EMT in LADC tissues. FNDC3B may be utilized as a novel molecular marker in LADC and may serve as target for drug development.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

TB, LZ, YL and SY conceived and designed the present study. JL, JZ, JF, QZ, LQ and HQ collected the data and performed the experiments. DJ performed the data analysis and interpretation. TB and YL were involved in the preparation of manuscript. All the authors have read and approved the final manuscript.

Ethical approval and consent to participate

Written informed consent was obtained from all study participants, and the study protocol was approved by the Human Research Ethics Committee of the Affiliated Hospital of Nantong University in China (Nantong, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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