Downregulation of DLL4 predicts poor survival in non-small cell lung cancer patients due to promotion of lymph node metastasis

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Abstract. Delta-like 4 (DLL4) is a membrane-bound ligand, which belongs to the Notch signaling pathway and plays important roles in angiogenesis and vascular development. The expression of DLL4 in non-small cell lung cancer (NSCLC) remains unclear. Therefore, DLL4 expression was detected in clinical specimens using quantum dots (QDs)-immunohistochemistry (IHC) and lung cancer cell lines by quantitative real-time polymerase chain reaction. The protein levels of DLL4 were decreased in the tumor tissues of NSCLC patients and lung cancer cell lines. Kaplan-Meier analysis indicated that low expression of DLL4 predicted poor survival rate of NSCLC patients. A549 and A427 cells transfected with pCMV-DLL4 exhibited reduced cell proliferation, migration and invasion using MTT assay, wound healing assay and Transwell assay. These data indicate that DLL4 represents a new prognostic biomarker for NSCLC, and DLL4 overexpression inhibits cell proliferation and metastasis *in vitro*.

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Introduction

Non-small cell lung cancer (NSCLC) is one of the most common cancers and the leading cause of cancer-related deaths in China (1). Although several targeted therapies (EGFR and ALK) have been developed, NSCLCs still have a tendency for recurrence and metastasis (2,3). Furthermore, our understanding of lung cancer is very limited, which has resulted in poor patient outcomes. Discovery of new targeted biomarkers for prognosis is important in cancer research (4-6). Recently, accumulating studies have revealed that angiogenesis-related genes, including DLL4 are dysregulated in lung cancer and they act as oncogenes or tumor suppressors.

DLL4 is a member of the Notch signaling family and plays an important role in angiogenesis (7-9). Various studies have found that DLL4 regulates vessel sprouting via angiogenic stimuli (10-13). Promotion of new vessel sprouting is a very fundamental factor in tumor growth and metastasis (12). Therefore, DLL4 may function as an oncogene in bladder cancer and breast cancer (12,13). However, other reports have indicated that DLL4 acts as a tumor suppressor in other cancer cell types due to deregulated vascular development (14-16). Based on these reports, our knowledge of the roles of DLL4 in NSCLC is conflicting and limited.

In the present study, expression levels of DLL4 in NSCLC patients and lung cancer cell lines were determined, and its clinical significance of prognosis was analyzed. The effects of DLL4 on cell proliferation and invasion of lung cancer cell

lines were also determined. Understanding the DLL4 functions will hopefully provide a new prognostic biomarker for lung cancer.

Materials and methods

Patients and Ethics statement. One hundred and two formalin-fixed, paraffin-embedded lung tissues and non-cancerous lung tissues were collected before any patient treatment from NSCLC patients, who were enrolled in the study between January 2007 and January 2012 at the Central Hospital (Table I). This study was approved by the Ethics and Scientific Committees of the Central Hospital (Wuhan, China) and complied with the Declaration of Helsinki. Written informed consent was obtained from all patients.

A total of 63 men and 39 women with a mean age of 52 (range, 24-76 years) years were included. All patients were followed up from the date of surgery to December, 2014. Pathological features, such as age and sex are shown in Table I. All of the NSCLC lung tissue samples were classified according to the 7th edition of the TNM classification by the International Association for the Study of Lung Cancer (IASLC) (17,18). Overall survival (OS) was calculated, which was the period from the date of initial diagnosis to death or the last follow-up. At the end of the study, 53 patients (52.0%) were still alive and 49 patients (48.0%) died of NSCLCs.

Data involving gene mutations were not obtained. No patients received new adjuvant therapy before or after surgery. The data of patients who received chemotherapy and radiotherapy were not fully collected; 61 patients received chemotherapy prior to or after surgery and 14 patients received radiotherapy before surgery. The clinical data of other patients were not collected. Interactions of these clinical data were not evaluated.

Table I. Characteristics of the NSCLC patients (N=102).

Characteristics	Data
Mean age (range) in years	52 (24-76)
Sex, n (%)	
Male	63 (61.8)
Female	39 (38.2)
Survival status, n (%)	
Dead	53 (52.0)
Surviving	49 (48.0)
Depth of invasion (T), n (%)	
T1	21 (20.6)
T2	71 (69.6)
T3	8 (7.8)
T4	2 (2.0)
Lymph node metastasis (N), n (%)	
N0	66 (64.7)
N1	23 (22.6)
N2	4 (3.9)
NX	9 (8.8)
Distant metastasis (M), n (%)	
M0	99 (97.1)
M1	3 (2.9)
TNM stage, n (%)	
Ia/Ib	19/12 (30.4)
IIa/IIb	38/18 (54.9)
IIIa/IIIb	11/1 (11.8)
IV	3 (2.9)
Total	102 (100)

NSCLC, non-small cell lung cancer.

Tissue microarray construction and QDs-IHC. Initially, hematoxylin and eosin-staining was performed and screened for tumor tissues and matched non-cancerous tissues. Two tissue microarray (TMA) slides, which consisted of 102 NSCLC tissues and adjacent non-cancerous lung tissues, were constructed with a diameter of 1.5 mm and technological support was provided from Beijing Do Biotech Co., Ltd. (19,20).

The expression of Atg4C and DLL4 was assessed by QDs-IHC staining according to the manufacturer's instructions and Wuhan Jiayang Quantum Dots Co., Ltd. (Wuhan, China) provided the technological support. In brief, the TMAs were prepared in xylene and in graded alcohol. Antigen retrieval of Atg4C was performed in EDTA buffer (1 mM, pH 8.0) at microwave oven for 20 min, while DLL4 was in EDTA buffer (1 mM, pH 8.0) using autoclave for 4 min. Tris-buffered saline (TBS) was used for dilution (antibodies and QDs), containing 2% bovine serum albumin (BSA; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). At first, TMAs were incubated in 2% BSA buffer, and then, TMAs were incubated with primary antibodies, which included rabbit anti-Atg4C (diluted 1:200; cat. no. ab191705; Abcam, Cambridge, MA, USA) and rabbit anti-DLL4 (diluted 1:200; cat. no. ab7280; Abcam). Then, TBS-T (0.5% Tween in TBS) was used for washing the TMAs. Goat anti-rabbit IgG was used as a secondary antibody (1:400; cat. no. 7074; Cell Signaling Technology, Inc., Danvers, MA, USA). Finally, the TMAs were incubated in QDs (605 nm) conjugated to streptavidin (1:300; Wuhan Jiayang Quantum Dots Co., Ltd.), and TMAs were sealed in 90% glycerin (Sigma-Aldrich; Merck KGaA). TBS instead of two primary antibodies was used for negative control, which showed auto-fluorescence signal.

Scoring of QDs-IHC staining. The signals of QDs-IHC staining were detected using Olympus BX53 fluorescence microscopy (Olympus Corp., Tokyo, Japan) at 605 nm and the results were evaluated by two independent researchers. They were also blinded to the clinical parameters of the patients. The scoring was calculated using the positive area and the staining intensity. The area of positivity (AD) was calculated as 0 (no positive area or positive area <5%), 1 (5-25%), 2 (26-50%), 3 (51-75%) and 4 (>75%), while the intensity of staining (IS) was scored as 1 (weak), 2 (moderate) and 3 (strong) (2). Intensity distribution (ID) = AP x IS, with the ID score being the final expression level of protein, which ranged from 0 to 12. The cutoff point of high or low expression

of DLL4 protein was determined on the receiver operating characteristic (ROC) curve analysis with respect to OS.

Cell culture and transfection. A549, H1299 and A427 cell lines were obtained from the Cell Bank of Shanghai Institutes for Biological Sciences (Shanghai, China). They were cultured in RPMI-1640 media with 10% fetal bovine serum (FBS) (Life Technologies, Beijing, China) in 5% CO_2. A549 and A427 cell lines were seeded in 6-well plates at 10^6 cells/wells. The pCMV-myc vector, and pCMV-myc-DLL4 (Life Technologies, Shanghai, China) were used for overexpression. Total proteins were isolated for Western blot analysis at 48 h after transfection using RIPA lysis buffer (Beyotime, Shanghai, China). The pSILENCE vector, pSILENCE-A and pSILENCE-B (Life Technologies, Shanghai, China) were used for knockdown with Invitrogen™ Lipofectamine 2000 (Thermo Fisher Scientific, Inc., Waltham, MA, USA).

Quantitative real-time PCR (qPCR). cDNA was obtained using the RevertAid First Strand cDNA Synthesis kit (Fermentas, Burlington, ON, Canada). The relative expression of *DLL4* (reference gene transcript ID: NM_019074.3) mRNA was measured using qRT-PCR in a CFX 96 Real-Time PCR system (Bio-Rad Laboratories, Shanghai, China) using a SYBR-Green kit (Takara Bio Co., Ltd., Japan), and the relative changes were quantified. Forward primer of *DLL4* was, 5-CTAGCTGTGGGTCAGAACTGGTTATT-3 and the reverse primer was, 5-ATGACAGCCCGAAAGACAGAT-3. *GAPDH* was used as a control. The primers were as follows: Forward primer, 5-GGAGTCAACGGATTTGGTCGTA-3 and reverse primer, 5-GGCAACAATATCCACTTTACCAGAGT-3. Relative gene expression levels were determined by the $2^{-\Delta\Delta Cq}$ [2 - (testCq {DLL4} - testCq {GAPDH}) - (controlCq {DLL4} - control Cq{GAPDH})] method (2). qPCR conditions: SYBR Green (2x) 10 μl, forward primer and reverse primer (10 pmol) 1 μl, cDNA 2 μl, ddH_2O 6 μl; Initial denaturation 94˚C for 5 min; denaturation 94˚C for 30 sec, annealing 64˚C for 30 sec, extension 72˚C for 45 sec, cycle 35; extension 72˚C for 5 min.

Cell proliferation. Cell proliferation was assessed using an MTT assay. Cells were plated in 24-well plates at $3x10^5$ cells/well. Then cells were incubated with 100 μl MTT dye (0.5 mg/ml; Sigma-Aldrich; Merck KGaA) for 4 h and 150 μl DMSO (Sigma-Aldrich; Merck KGaA) was added after the supernatant was removed. The absorbance was detected at 570 and 655 nm was used as the reference wavelength. The absorbance was determined at 12, 24, 36, 48, 60 and 72 h after transfection and the MTT assay was performed in triplicate.

Cell migration and invasion. Cell migration and invasion abilities were detected using wound healing a and Transwell chamber assays (Corning, Beijing, China) with or without Matrigel (Invitrogen; Thermo Fisher Scientific, Inc., Beijing, China). For the determination of cell migration, a wound was produced using a plastic pipette tip when 90-100% cell confluence was reached. Then the migrated cells were washed and cultured in low serum (2.5%) media for 48 h. Wound closure (%) was defined as the area of migrated cells at 48 h divided by the area at 0 h.

Transwell chambers (Corning, Beijing, China) with Matrigel were used for detection of cell invasion. Transwell chambers were placed into 6-well plates, and coated with Matrigel. A total of $4x10^4$ cells were seeded in the upper chambers into serum-free media at 24 h after transfection. Meanwhile media of 10% FBS/DMEM (Gibco; Thermo Fisher Scientific) was added to the lower chambers. After 48 h, the cells which had invaded through the membrane were fixed in 20% methanol and stained with 0.1% crystal violet. The non-migrated A549 and A427 cells were removed by cotton swabs. Other cells on the upper surface of the membrane were removed by cotton swabs. Images were captured using microscope (Olympus Corp., Tokyo, Japan) for calculating the number of migrated cells at x200 magnification.

Western blot analysis. Protein samples were isolated from A549 and A427 cells using RIPA lysis buffer and protein concentrations were detected using the BCA kit (Beyotime Institute of Biotehnology, Shanghai, China). The cellular extracts were separated on 10% SDS-PAGE gel and transferred onto PVDF membranes (Bio-Rad Laboratories). Membranes were blocked using 1% non-fat milk and incubated with the primary DLL4 antibody (cat. no. ab7280; Abcam) or GAPDH antibody (cat. no. ab9485; Abcam) overnight. Next, the secondary antibody (cat. no. 7074; Cell Signaling Technology) was added and incubation was carried out. Finally, protein bands were visualized using the enhanced chemiluminescence (ECL) assay.

Statistical analysis. Statistical analyses were performed using SPSS19.0 software (IBM Corp., Armonk, NY, USA). Data are expressed as means ± SD and the differences between groups were assessed with the Student's t-test. Comparisons of multiple groups were performed using ANOVA and the S-N-K test as a post hoc test was used. The association between protein levels and clinical parameters were estimated using the Chi-square test. Kaplan-Meier test and log-rank test were performed for survival analysis. K-M plotter database was used for NSCLC survival analysis. Statistically significant differences were considered when two-tailed P-values <0.05.

Results

Expression of DLL4 in clinical specimens and lung cancer cell lines. *DLL4* mRNA levels were determined using qPCR in lung tissues of 22 NSCLC patients and 20 healthy controls. As shown in Fig. 1A, the DLL4 mRNA levels were downregulated in the lung tissues of NSCLC patients compared with these levels in the non-cancerous tissues and healthy controls. DLL4 mRNA levels were also detected in lung cancer cell lines. The expression of DLL4 was decreased (0.25-fold) in three lung cancer cell lines compared with that noted in primary human alveolar epithelial cells (Fig. 1B). The cutoff value of DLL4 expression levels was determined using ROC curve analysis (Fig. 1C); 4.2 was defined as the cutoff point of DLL4 in NSCLC patients (an ID score ≥4.2 defined high expression and ID <4.2 indicated low expression). The cutoff value of DLL4 expression had optimal sensitivity and specificity. The area under the curve was 0.691 and the 95% confidence interval (CI) was 0.621-0.769.

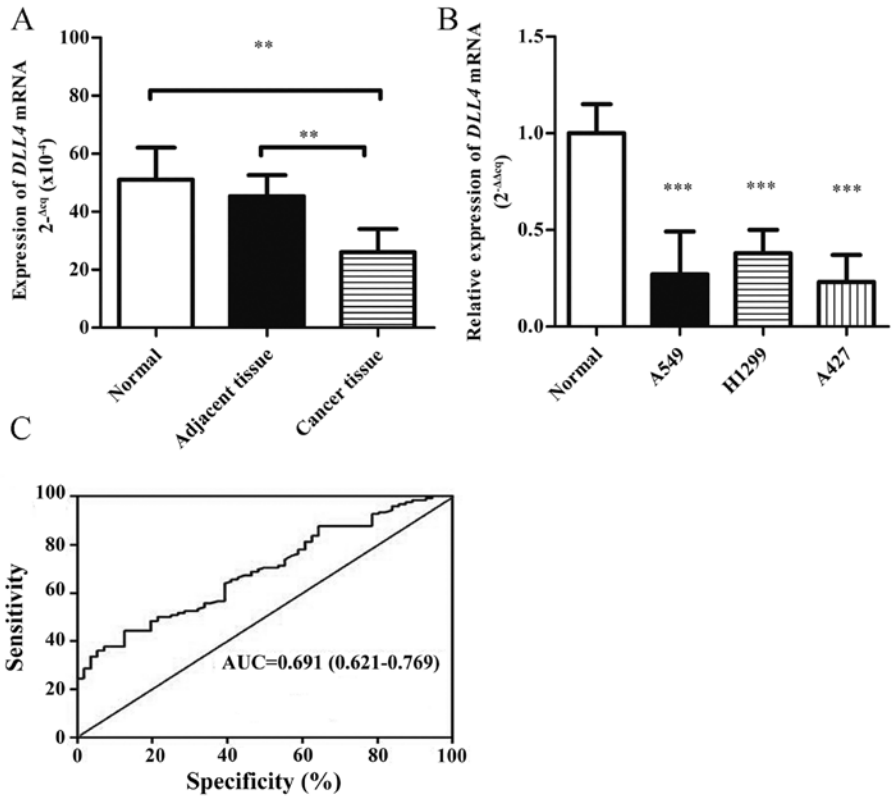

Figure 1. Expression of DLL4 in clinical specimens and lung cancer cell lines. (A) qPCR assay was performed and DLL4 levels of lung tissues in patients (n=22) and controls (n=20) were detected and the levels were normalized to GAPDH. (B) DLL4 levels in lung cancer cell lines and normal primary human alveolar epithelial cells, normalized to GAPDH. (C) ROC analysis of DLL4 scores using the overall survival (OS) status of lung NSCLC patients. **P<0.01, ***P<0.001. DLL4, Delta-like 4; NSCLC, non-small cell lung cancer.

Table II. Association between DLL4 expression and clinicopathological parameters of the NSCLC patients.

		DLL4 expression		
Characteristics	n	Low n (%)	High n (%)	P-value
Age (years)				>0.05
<60	72	42 (41.2)	30 (29.4)	
≥60	30	19 (18.6)	11 (10.8)	
Sex				>0.05
Male	63	35 (34.3)	28 (27.5)	
Female	39	26 (25.5)	13 (12.7)	
Depth of invasion (T)				>0.05
T1-T2	92	53 (52.0)	39 (38.2)	
T3-T4	10	8 (7.8)	2 (2.0)	
Lymph node metastasis (N)				<0.05
N0	66	31 (30.4)	35 (34.3)	
N1, N2, N_X	36	30 (29.4)	6 (5.9)	
Distant metastasis (M)				>0.05
M0	99	59 (57.8)	40 (39.3)	
M1	3	2 (2.0)	1 (0.9)	
TNM stage				>0.05
I-II	87	50 (49.0)	37 (36.3)	
III-IV	15	11 (10.8)	4 (3.9)	
Total		61 (59.8)	41 (40.2)	

NSCLC, non-small cell lung cancer.

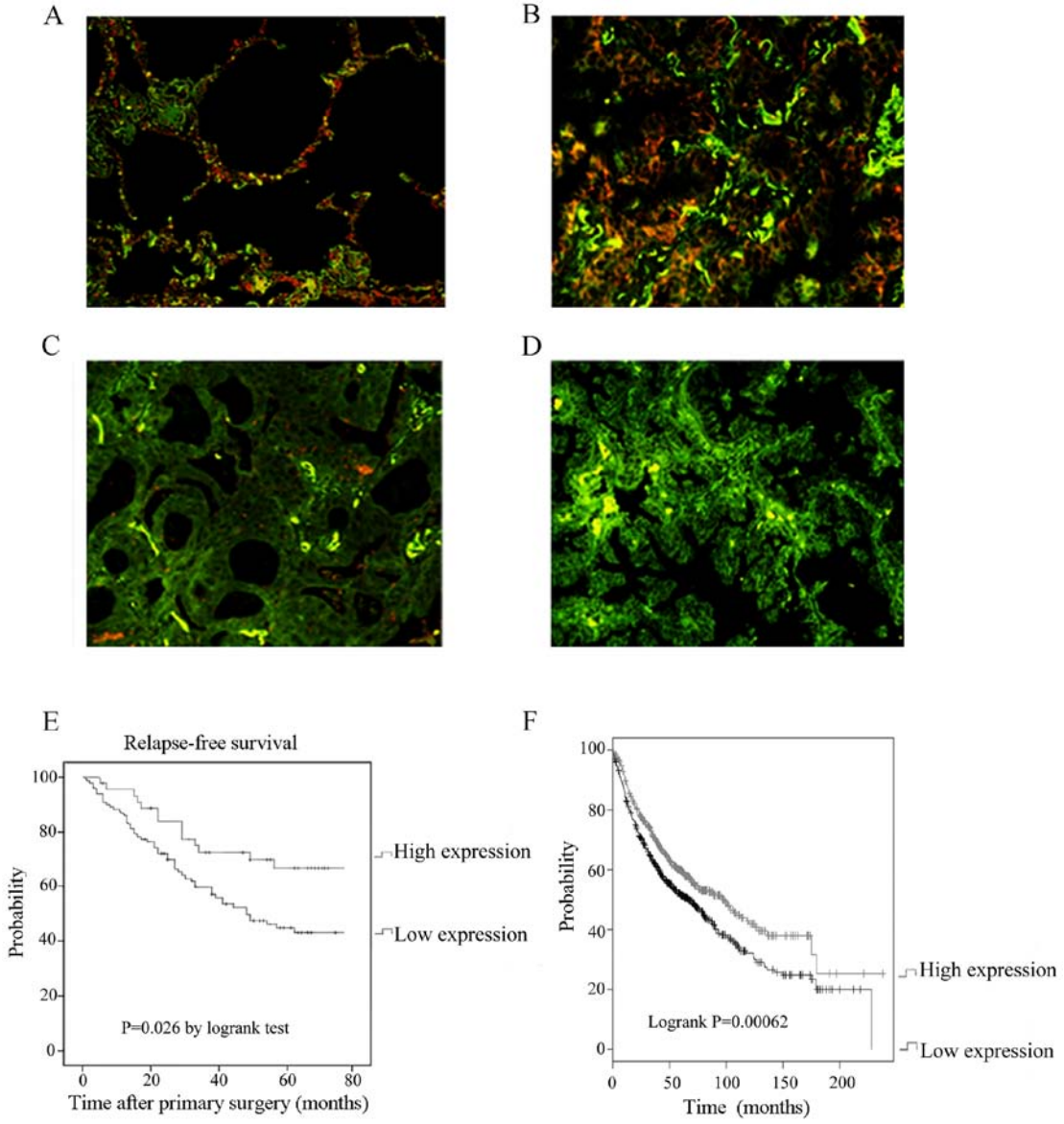

Figure 2. QD-based IHC staining of DLL4 in NSCLC tissues and survival curves of lung NSCLC patients according to DLL4 expression. (A) Positive DLL4 expression in non-cancerous tissue (magnification, x100). (B) Positive DLL4 expression in NSCLC tissue (magnification, x200). (C) Weak DLL4 expression in NSCLC tissue (magnification, x40). (D) Negative expression of DLL4 in NSCLC tissue (magnification, x40). (E) NSCLC patients with a low level of DLL4 protein showed poor relapse-free survival when compared with patients with a high level of DLL4 protein (P=0.026). (F) K-M plotter software was used and NSCLC patients with low expression of *DLL4* mRNA had a poor survival rate (n=1,422). DLL4, Delta-like 4; NSCLC, non-small cell lung cancer; QD, quantum dot; IHC, immunohistochemistry.

Expression of DLL4 protein in TMA and overall survival analysis. To validated the results that levels of DLL4 mRNA were downregulated in lung cancer tissues and lung cancer cell lines, we performed ODs-IHC staining in a larger cohort of NSCLC patients (n=102). DLL4 was expressed in both cancer tissues and adjacent non-cancerous lung tissues including vascular endothelial cells (Fig. 2). DLL4 was located in the cell membrane and cytoplasm (Fig. 2A). In tumor tissues, expression of DLL4 was significantly decreased (Fig. 2B-D). Forty-one (40.2%) patients showed high DLL4 expression and 61 (59.8%) patients showed low DLL4 expression in tumor tissues. Subsequently, the prognostic value of DLL4 expression was investigated in the NSCLC patients. The results demonstrated that high DLL4 protein expression predicted a prolonged survival rate (Fig. 2E, P=0.026) using Kaplan-Meier analysis and log-rank test. K-M plotter database of lung cancer patients (n=1422) was used and the result of survival analysis supported our conclusion (Fig. 2F, P<0.001). This was in contrast to the results in breast cancer and this finding warrants further research. Atg4C expression was also determined. However, no significant difference was observed in this study (data not shown).

Clinical significance of DLL4 expression. As shown in Table II, the association between the level of DLL4 protein and clinicopathological variables was analyzed. The expression level of DLL4 was not significantly associated with sex, age, T, M, or TNM stage of the NSCLC patients. Notably, a significant association between lymph node metastasis (N) status and DLL4 expression was observed.

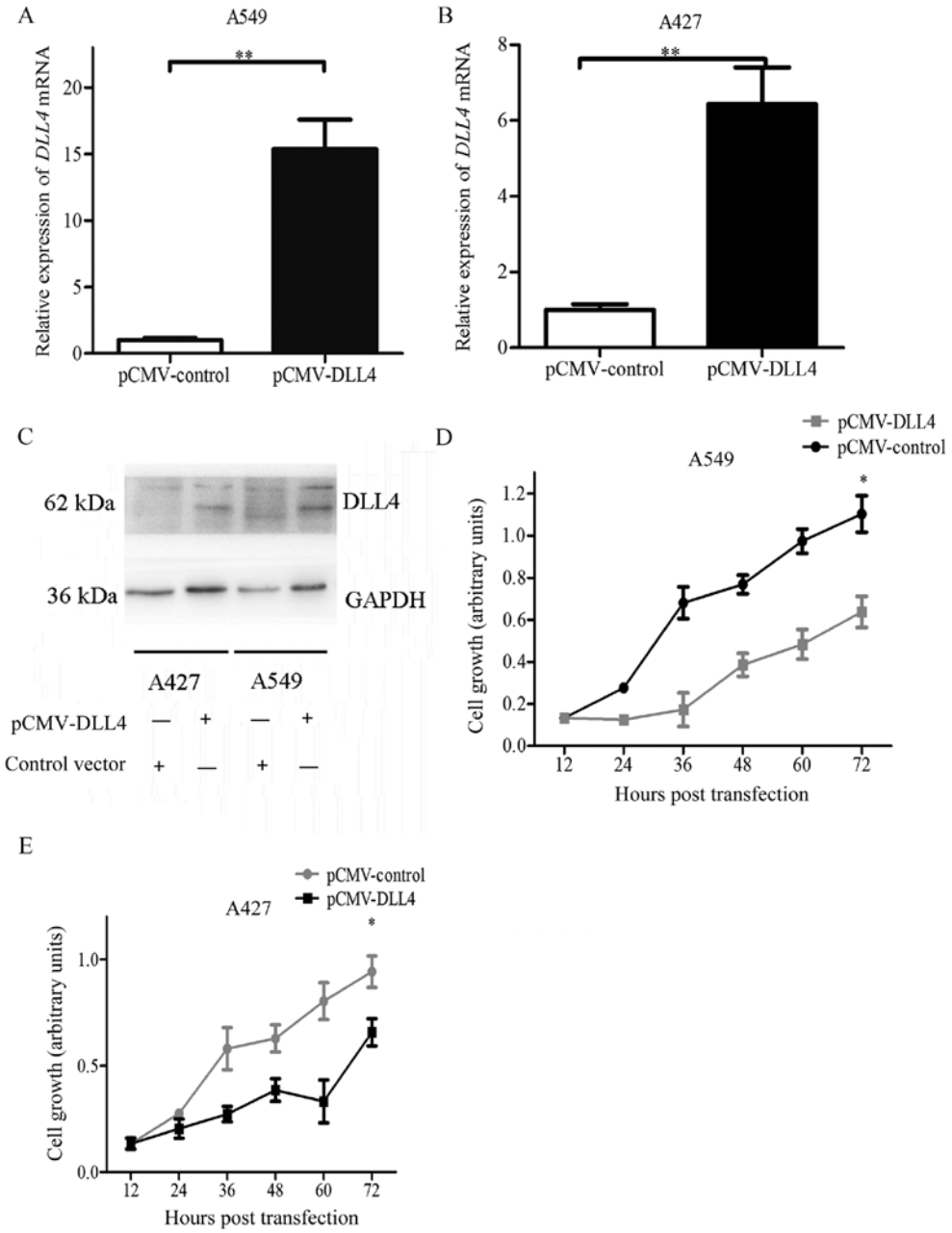

Figure 3. Overexpression of DLL4 reduces cell proliferation in A549 and A427 cell lines. (A and B) The A549 and A427 cells were transfected with pCMV-DLL4 and *DLL4* mRNA expression was detected using qPCR. (C) Upper panel in the blot shows DLL4 expression and the lower panel shows GAPDH. The DLL4 protein levels were upregulated after transfection. (D and E) Cell viability was determined using MTT assay and cell viability and proliferation were decreased in the A549 and A427 cells transfected with pCMV-DLL4. *P<0.05, **P<0.01. DLL4, Delta-like 4.

Overexpression of DLL4 reduces cell proliferation in A549 and A427 cell lines. Cell viability was detected using the MTT assay. Transfection with pCMV-myc-DLL4 significantly increased *DLL4* mRNA and protein levels in the A549 and A427 cell lines (Fig. 3A-C). Transfection efficiency was ~30-40% (data not shown). Compared with the control vector, cell viability and proliferation were significantly decreased in the A549 cells transfected with the pCMV-DLL4 vector (Fig. 3D). Identical results were also observed in the A427 cells (Fig. 3E). These results demonstrated that DLL4 overexpression inhibited cell viability and proliferation in lung cancer cell lines. They also indicated that DLL4 acted as a tumor suppressor in NSCLC cell lines.

Overexpression of DLL4 inhibits cell migration and invasion. Wound healing assay and Transwell invasion assay were used to detect the effects of DLL4 overexpression on the migration and invasion of NSCLC cell lines. As shown in Fig. 4A, the closure rate of cells transfected with pCMV-DLL4 was less than the rate of cells transfected with pCMV-control in the A549 cell line. The rate of cells transfected with the control vector was 0.64-fold, and the rate with pCMV-DLL4 was 0.37-fold (Fig. 4B). Similarly, identical results were also observed in the A427 cell line (Fig. 4C). These results demonstrated that cell migration was inhibited in cancer cells with DLL4 overexpression.

As shown in Fig. 4D, the number of invaded cells were decreased in the A549 cells transfecting with pCMV-DLL4.

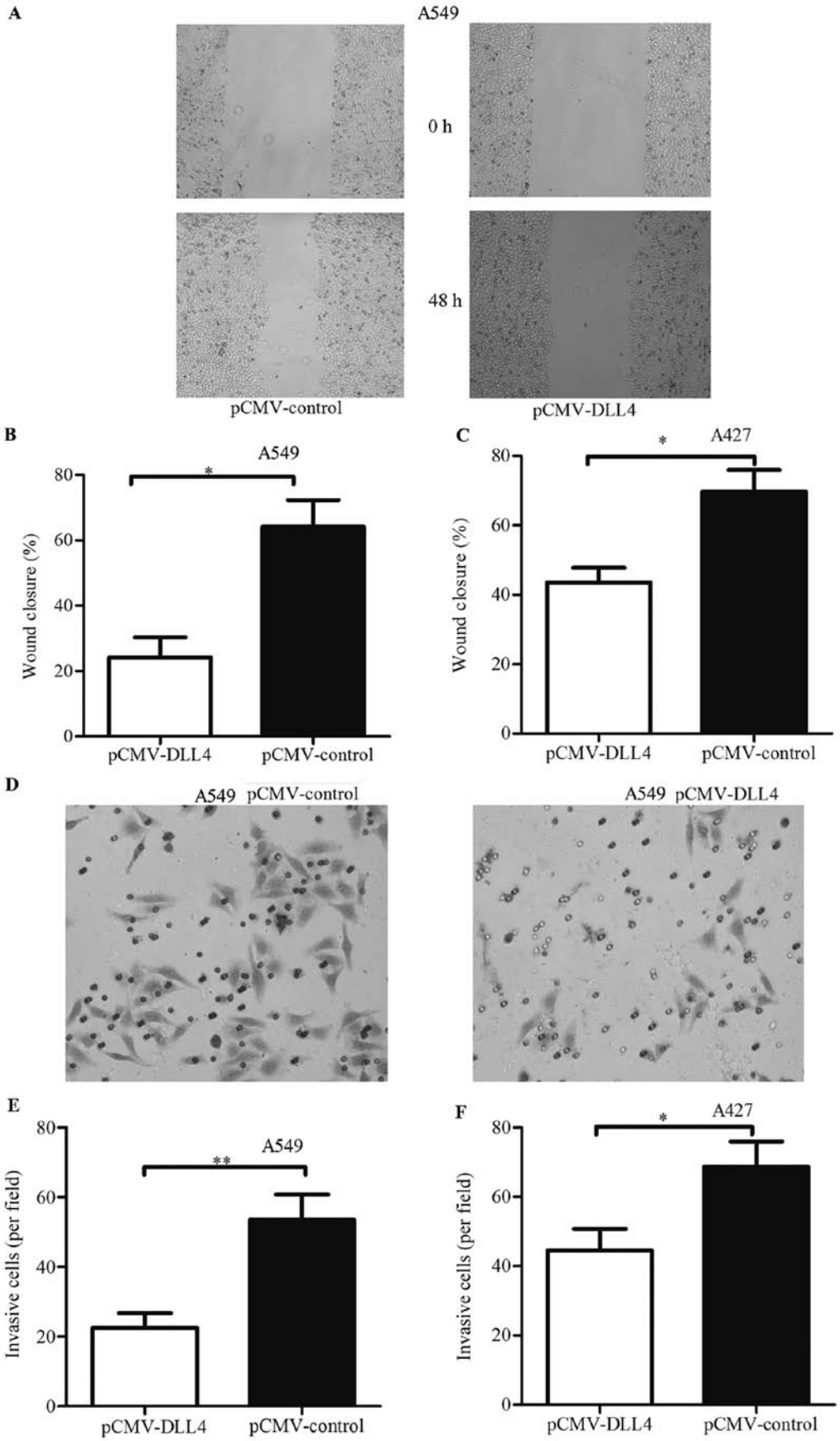

Figure 4. Effects of DLL4 on cell migration and invasion in A549 and A427 cell lines. (A) Wound healing assay was performed in A549 cells transfected with pCMV-DLL4 and control. Cell migration was detected at 0 and 48 h (magnification, x40). (B) Wound closure rates were calculated using ImageJ software (National Institutes of Health, Bethesda, MD, USA) and the rate was decreased compared with the control in the A549 cells. (C) Wound closure rate was also measured in A427 cells and it was less than that noted in the control. (D) Transwell assays were performed in A549 cells transfected with pCMV-DLL4 and invaded cells were stained with crystal violet (magnification, x200). (E) The number of invaded cells was decreased compared with the control. (F) Invasion of A427 cells was also detected and it was less than that noted in the control. $^{*}P<0.05$, $^{**}P<0.01$. DLL4, Delta-like 4.

Compared with the control vector, the ability of invasion was decreased to 0.22-fold (Fig. 4E). These results were also observed in the A427 cells (Fig. 4F). These results indicated that DLL4 functioned as a tumor suppressor that inhibited cell viability, migration and invasion.

Discussion

Many studies have reported that DLL4 expression in lung cancer may be associated with tumor metastasis and prognosis (3,20-25). In the present study, DLL4 expression was assessed using tissue microarray and the prognostic value was examined in NSCLC patients. We found that DLL4 expression was significantly decreased in NSCLC patients compared with that noted in normal subjects, and low DLL4 expression predicted a poor survival rate and was significantly correlated with lymph node metastasis. DLL4 expression was downregulated in A549, H1299 and A427 cells. Furthermore, overexpression of DLL4 reduced cell proliferation and invasion in both A549 and A427 cells. Our results suggest that DLL4 is an independent prognostic biomarker for lung cancer.

There are many preclinical models which have focused on dll4 allele deletion and systemic application of DLL4/Notch inhibitors, which have been found to result in significant suppression of tumor growth (14,26). Our findings were completely contrasting to the results in other tumor types where upregulation of DLL4 correlates with tumor promotion (16-29). In an attempt to determine the role of DLL4 in lung cancer cells, DLL4 was overexpressed in two NSCLC cell lines. The results revealed that DLL4 overexpression had a negative effect on the growth, migration and invasion of lung cancer cells. Although bioinformatics analysis using K-M plotter supported our conclusion, these conflicting results need further research and should be explained carefully.

On the one hand, as a member of Notch signaling, DLL4 plays an important role in vessel sprouting (30). Expression of DLL4 was found to stimulate Notch signaling and regulate the ratio of tip cells to stalk cells (30,31). When DLL4 was inhibited, tip-cell specification was not able to be controlled and excessive sprouting occurred, leading to tumor migration. DLL4 was considered as a tumor suppressor due to reducing endothelial sensitivity to VEGF and increased DLL4 could reduce tumor growth and VEGF-induced overall tumor blood supply (16,32). DLL4 overexpression was found to prevent metastasis formation and allow for increased delivery to the tumor of concomitant chemotherapy and improve its efficacy (32).

On the other hand, *DLL4* expression was found to be downregulated in NSCLC patients due to posttranscriptional mechanisms, due to upregulation of the miR-30 family. microRNAs (miRNAs) are small non-coding RNAs, which regulate target gene expression by mRNA degradation and translational inhibition (1). Numerous miRNAs are found to play roles in carcinogenesis of NSCLC, such as the miR-30 family (21-23). Furthermore, the miR-30 family and miR-27b are implicated in *DLL4* regulation (24,25). It is unclear whether or not these miRNAs believed to suppress DLL4 specifically lead to tumor growth and invasion in NSCLC patients.

There are some limitations to this study. All the patients were diagnosed and treated between 2007 and 2012 according to the 7th edition of the TNM classification by IASLC. However, it was difficult to reappraise according to the 8th edition of the TNM classification by UICC/AJCC. *In vivo* xenograft study should be conducted for further research. The data of the patients' pulmonary function test, histological classification, and gene mutations could not obtain and interactions of these clinical data were not evaluated. An experiment using knockdown was not performed, as the expression of DLL4 was difficult to silence. pSILENCE-A and pSILENCE-B failed to knock down DLL4. Thus, we did not discuss it in the results. We will perform this again in further research. The use of the MTT assay in the growth studies should be explained carefully. Overexpression for DLL4 could partly support the conclusions. However, it may result in loss of viability (ie. cell death) which could explain the apparent effects on growth, migration and invasion. Therefore, further research is needed.

In conclusion, we identified low expression of DLL4 in NSCLC patients. Downregulation of DLL4 was found to be associated with poor OS and overexpression of DLL4 inhibited proliferation, migration and invasion of cancer cells.

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Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

HL, ZL and SL conceived and designed the study. HL, JP, MZ, PM and JZ performed the experiments. HL, ZL and MZ wrote the paper. SL, JP, MZ, ZL and PM reviewed and edited the manuscript. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

The present study was approved by the Ethics and Scientific Committees of the Central Hospital (Wuhan, China) and complied with the Declaration of Helsinki. Written informed consent was obtained from all patients.

Patient consent for publication

Not applicable.

Competing interests

All authors declare that they have no competing interests.

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