

High expression of DLC family proteins predicts better prognosis and inhibits tumor progression in NSCLC

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Abstract. The incidence of primary lung cancer (PLC) is increasing and is becoming a leading cause of cancer-associated mortality worldwide. Non-small cell lung cancer (NSCLC) accounts for ~80% of PLC cases and has the worst prognosis among malignant tumors. Deleted in liver cancer (DLC) proteins belong to the RhoGTPase-activating protein family and are considered to be tumor suppressor genes. However, the role of the proteins, particularly DLC2 and DLC3, in NSCLC, has not been fully elucidated. The present study investigated the expression levels and prognostic values of DLCs in NSCLC using The Cancer Genome Atlas, the Genotype-Tissue Expression project and Kaplan-Meier plotter datasets. The current study demonstrated that the three DLCs were downregulated in NSCLC. High expression levels of DLC1 and DLC2 were associated with an improved survival in NSCLC. Additionally, the effects of DLCs on the proliferation and apoptosis of the lung cancer cell line A-549 were investigated *in vitro* using a Cell Counting Kit-8 assay and flow cytometry analysis. DLC2 and DLC3 overexpression inhibited proliferation and induced apoptosis in A549 cells. To the best of our knowledge, the current study was the first to investigate the expression level and prognostic values of DLC2 and DLC3 in NSCLC. The results indicated that DLC1 DLC2 and DLC3 serve specific roles in the occurrence and development of NSCLC, and may be considered as potential prognostic indicators in NSCLC.

Introduction

Primary lung cancer (PLC) has an increasing incidence and is a leading cause of cancer-associated mortality worldwide. Non-small cell lung cancer (NSCLC) accounts for ~80% of PLC cases and the prognosis of which is one of the worst among

all malignant tumors (1). The 5-year overall survival (OS) rate of patients with NSCLC is <15% in the United States, and lower in other countries (2). A good prognosis for NSCLC requires a timely diagnosis and appropriate treatment. Furthermore, a positive prognosis is associated with various pathological characteristics, including Tumor, Node and Metastasis staging (3) and pathological grading (4,5), and genetic backgrounds. For example, the CRP 1846T/T genotype has been identified to be associated with the prognosis of patients with NSCLC (6). Various tumor-associated molecules, signaling pathways, and proteases and their inhibitors are involved in the development of NSCLC. The genetic and molecular analysis of these factors may be important for the development of novel therapeutic agents and predicting the prognosis of patients with NSCLC (7,8).

Deleted in liver cancer (DLC) proteins are members of the RhoGTPase-activating protein (RhoGAP) family, and consist of DLC1-3. DLC2 and DLC3 are also known as StAR related lipid transfer domain containing 13 and 8, respectively (9). The RhoGAP family proteins are negative regulators of the Rho family of small GTPases, and promote the inactivation of RhoA by catalyzing the conversion of RhoA-GTP to RhoA-GDP (9,10). DLC1 and DLC2 are tumor suppressor genes in liver cancer and a number of other tumors, including breast cancer, gastric cancer and renal cell carcinoma, and the expression levels of DLC1 and 2 are downregulated in these tumors (11,12). DLC2 is similar to DLC1 in structure, and DLC3 is essential for maintaining the integrity of adherens junctions. The function and prognostic value of the DLC family in NSCLC remains largely unknown. Thus, the present study investigated whether there is abnormal DLC1-3 expression in patients with NSCLC, and whether the expression of DLC1-3 is associated with the prognosis of patients NSCLC. In addition, the online Kaplan-Meier (KM) plotter database (kmplot.com/analysis) was used to determine the prognostic roles of DLC mRNA expression in patients with NSCLC. A number of genes associated with gastric, breast, ovarian and lung cancer have been identified and validated by the KM plotter using patient samples measured by gene chips or RNA-sequencing (13-16). In the present study study, the KM plotter provided prognostic information and mRNA mapping of 1,926 patients with lung cancer patients. Additionally, the role of DLC2 and DLC3 overexpression in lung cancer cells was investigated *in vitro*.

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Materials and methods

Analysis of the datasets. The expression level of the DLC family was analyzed using Gene Expression Profiling Interactive Analysis (GEPIA; <http://gepia.cancer-pku.cn/>). GEPIA an interactive web server for analyzing the RNA sequencing expression data of 9,736 tumors and 8,587 normal samples from The Cancer Genome Atlas (TCGA; <https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga>) and Genotype-Tissue Expression project (GTEx; <http://commonfund.nih.gov/GTEx/>) (17).

The KM plotter database was used to analyze the prognostic values of the DLC family in NSCLC. In the KM plotter database, the data corresponding to four types of cancer (gastric, breast, ovarian and lung cancers) were downloaded from Gene Expression Omnibus (Affymetrix microarrays only), European Genome-Phenome Archive and TCGA. The data included gene expression data, and information on relapse, and OS information for patients. Clinical data from 1,926 patients with NSCLC including gender, histology, clinical stage, smoking history, pathological grades and chemotherapy agents used were collected from KM plotter database. The Affymetrix IDs of DLC1, DLC2 and DLC3 were entered into the KM plotter and data were compared using the KM survival plots. According to the median expression levels of DLC1, DLC2 and DLC3, the samples were divided into two groups: i) High expression group; and ii) low expression group. Subsequently, the 95% CI, the log-rank P-value and the hazard ratio were calculated. The Affymetrix ID corresponding to DLC1 used in the Kaplan Meier plotter was 210762_s_at, the Affymetrix ID corresponding to DLC2 was 213103_at and the Affymetrix ID corresponding to DLC3 was 206868_at.

Immunohistochemistry. Cancer and adjacent normal tissues were collected from 40 patients with NSCLC (male to female ratio, 27:13; age, 54.82±14.39) at the Zaozhuang Municipal Hospital (Zaozhuang, China) between June 2016 and June 2018. Patients who received preoperative radiotherapy, chemotherapy or hormone therapy were excluded from the present study. The current study was approved by The Ethics Committee of Zaozhuang Municipal Hospital (approval no. 2016ZMHE011) and all patients signed informed consent. These tissues were fixed in 4% paraformaldehyde solution overnight at room temperature, dehydrated and embedded in paraffin, and cut into 5- μ m-thick paraffin slices. Following dewaxing with xylene (2x5 min, at room temperature) and rehydration in descending ethanol series at room temperature, the sections were boiled in citrate buffer (pH=6.0) at 95°C for 15 min for antigen retrieval. The sections were blocked with 5% goat serum (cat. no. ab7481; Abcam) at room temperature for 60 min. Tissue sections were incubated with primary antibodies against DCL1 (cat. no. ab126257; 1:100; Abcam), DCL2 (cat. no. ab126489; 1:100; Abcam) and DCL3 (cat. no. 13899-1-AP; 1:100; ProteinTech Group, Inc.). Primary antibodies were incubated with the membranes overnight at 4°C and horseradish peroxidase-labeled secondary antibodies (cat. no. A00001-2; 1:500; ProteinTech Group, Inc.) were incubated at 37°C for 1 h. The slides were developed in diaminobenzidine at room temperature for 3 min and counterstained with hematoxylin at room temperature for 1 min.

Images were taken and processed by MetaMorph software (version 2.2; Molecular Devices, LLC).

The expression levels of DLC1, DLC2 and DLC3 were evaluated by the staining intensity and the percentage of positive stained cells, as previously described (18). Subsequently, the staining intensity was scored as follows: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining. The percentage of positive stained cells was scored as follows: 0, 0-10%; 1, 1-25%; 2, 26-50%; 3, 51-75%; and 4, >75%. A final score was assigned to each sample by multiplying these two scores, and samples presenting a score >6 were included in the high expression group, whereas samples presenting a score \leq 6 were included in the low expression group.

Cell culture and transfection. The human lung cancer cell line A-549 was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). DMEM (HyClone; GE Healthcare Life Sciences) supplemented with 10% FBS (Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 0.1 mg/ml streptomycin, was used for cell culture at 37°C with 5% CO₂. DLC1, DLC2 or DLC3 cDNAs cloned into pcDNA3.1 vectors were purchased from Oligobio Biotechnology Co., Ltd. (Beijing, China) and pcDNA3.1-DLC1, pcDNA3.1-DLC2 and pcDNA3.1-DLC3 vectors were used for subsequent experiments. Empty pcDNA3.1 vector was used as the control group. Cells were harvested 24 h after transfection prior to further experimentation.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The mRNA expression levels of DLC2 and DLC3 in A-549 cells were measured by RT-qPCR. Total RNA was extracted from A-549 cells using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.) followed by RT reaction to synthesize the cDNA using the HiFiScript cDNA Synthesis Kit (CW BIO) at 37°C for 15 min followed by an incubation at 85°C for 5 sec). qPCR was subsequently performed using SYBR[®] Premix Ex Taq[™] II (Thermo Fisher Scientific, Inc.). The thermocycling conditions were as follows: Initial denaturation at 95°C 30 sec, followed by 40 cycles of 95°C for 5 sec, 56°C for 20 sec and 72°C for 30 sec. The following primer pairs were used for the qPCR: DLC1, forward 5'-TGC GCCAGATGAATGAAGGT-3' and reverse 5'-GCATGATGG CAGCCTTGATG-3'; DLC2, forward 5'-GAACACGACCAG CAGTGAGA-3' and reverse 5'-CGGGTTGTCTGTACAGCA CT-3'; DLC3, forward 5'-CCTCCAGCGAACTTGACAGT-3' and reverse 5'-CCTGCAGGGTCTGGTAAGTG-3'; GAPDH, forward 5'-AAGGTGAAGGTCGGAGTCAA-3' and reverse 5'-AATGAAGGGGTCATTGATGG-3'. mRNA levels were quantified using the 2^{- $\Delta\Delta$ C_q} method (19) and normalized to the internal reference gene GAPDH.

Western blotting. A-549 cells transfected with the empty vector pcDNA3.1, pcDNA3.1-DLC1, pcDNA3.1-DLC2 or pcDNA3.1-DLC3 were lysed using radioimmunoprecipitation assay buffer (CW Biotech) for protein extraction. Total protein was quantified using a bicinchoninic acid assay and 20 μ g protein/lane was separated by a 10% SDS-PAGE. The separated proteins were subsequently transferred onto a PVDF membrane and blocked in 5% non-fat milk at 25°C for 1 h. The membranes were incubated with

primary antibodies against DLC1 (cat. no. ab126257; 1:500; Abcam), DLC2 (cat. no. ab126489; 1:500; Abcam), DLC3 (cat. no. 13899-1-AP; 1:400; ProteinTech Group, Inc.) and GAPDH (cat. no. 10494-1-AP; 1:5,000; ProteinTech Group, Inc.) overnight at 4°C. Following the primary incubation, membranes were incubated with horseradish peroxidase-labeled secondary antibodies (cat. no. A00001-2; 1:5,000; ProteinTech Group, Inc.) for 1 h at room temperature. Protein bands were visualized using the enhanced chemiluminescence system. Protein expression was density-quantified using ImageJ software (version 1.49; National Institutes of Health) with GAPDH as the loading control.

Cell Counting Kit-8 (CCK-8) assay. A-549 cells transfected with pcDNA3.1-DLC1, pcDNA3.1-DLC2 or pcDNA3.1-DLC3 were seeded into a 96-well plate at a density of 2×10^3 cells/well and cultured at 37°C. Fresh DMEM containing 10 μ l CCK-8 solution was added to the cells at 0, 24, 48 and 72 h post-transfection, and incubated for 2 h at 37°C. Cell proliferation was determined by measuring the optical density value at a wavelength of 450 nm. The CCK-8 assay was performed in triplicate.

Flow cytometry. Flow cytometry was used to investigate cell apoptosis and was performed using the Annexin V-FITC Apoptosis Detection Kit I (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. After transfection, cells were incubated for 48 h, and A-549 cells (2×10^5 cells) were stained with the Annexin V/FITC mix. A total of 10 ml propidium iodide and 400 μ l PBS were added prior to detecting cell apoptosis. Apoptotic cells were subsequently analyzed using a flow cytometer and FlowJo software (version 10; Tree Star, Inc.) was used for the data analysis.

Statistical analysis. Data are expressed as the mean \pm SD. Data were analyzed using GraphPad Prism software (version 7.0; GraphPad Software, Inc.). Analysis of the differential expression of DLCs between normal and tumor samples from The Cancer Genome Atlas and Genotype-Tissue Expression databases was performed by unpaired t-test. The KM method and the Log-rank test were performed to compare the survival rates among different groups, whose data were downloaded from the KM plotter database. The Cox proportional hazard model was used for the survival analysis. χ^2 test was used to compare the expression levels of DLCs in tumor tissues and normal lung tissues in the immunohistochemistry assays. One-way analysis of variance (ANOVA) was used for the analysis of multiple groups, followed by Newman-Keuls post-hoc test. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Differential expression of DLC between normal and tumor tissues in NSCLC. The expression level of the DLC family in NSCLC was analyzed by GEPIA database. GEPIA analyzed the mRNA expression levels of DLC in 483 lung adenocarcinoma (LUAD) and 347 normal lung tissues which were obtained from TCGA and GTEx databases, as well as 486 lung squamous cell carcinoma (LUSC) samples and 338 normal lung tissues. Analytical results of DLC expression are presented

in Fig. 1A. Decreased expression levels of DLC1, DLC2 and DLC3 were observed in LUAD and LUSC tumors compared with normal lung tissue ($P < 0.01$). To further investigate DLC expression, cancer and adjacent normal tissues were collected from 40 patients with NSCLC. As shown in Fig. 1B, immunohistochemical staining revealed higher expression of DLCs in normal lung tissues compared with lung cancer tissues. The percentage of low expression in lung cancer tissues reached to 87.5% (35/40), whereas in normal lung tissues was only 20% (8/40). However, no further clinical correlation analysis was performed due to the lack of pathological grade and metastasis data of these patients.

Prognostic value of DLC in NSCLC. The prognostic value of DLC1, DLC2 and DLC3 in NSCLC was assessed using the KM plotter database. Additionally, their potential association with clinicopathological parameters was investigated. The valid Affymetrix IDs in the KM plotter dataset of DLC1, DLC2 and DLC3 are 210762_s_at, 213103_at and 206868_at, respectively. Survival curves for the 20-year OS time were plotted for the patients with NSCLC ($n=1,926$), with LUAD ($n=720$) and with LUSC ($n=524$).

Effect of DLC1 expression on survival was assessed using the KM plotter. As shown in Fig. 2, a high expression level of DLC1 was positively associated with survival duration in all NSCLCs (HR, 0.64; 95% CI, 0.56-0.73; $P < 0.0001$). High expression level of DLC1 was also associated with prolonged survival in patients with LUAD (HR, 0.62; 95% CI, 0.49-0.78; $P < 0.0001$), but not in patients with LUSC. The prognostic value of the expression level of DLC2 expression in lung cancer was also investigated (Fig. 3). High mRNA expression of DLC2 was associated with improved survival time in all lung cancers (HR, 0.69; 95% CI, 0.6-0.78; $P < 0.0001$). High mRNA expression of DLC2 was also associated with improved OS in patients with LUAD (HR, 0.52; 95% CI, 0.41-0.67; $P < 0.0001$), but not in patients with LUSC. As shown in Fig. 4, a high expression level of DLC3 did not have a significant association with the survival rates in patients with any of the lung cancer types analyzed (all $P > 0.05$).

The KM plotter was used to further investigate the association of the expression levels of DLCs and several variables, including smoking habits (Table I), clinical stages (Table II) and pathological grades (Table III). As shown in Table I, the mRNA expression level of DLC1 was significantly association with OS in patients with NSCLC who smoked and those who never smoked (smoked, HR, 0.74; 95% CI, 0.6-0.91; $P=0.0041$; never smoked, HR, 0.23, 95% CI, 0.12-0.43, $P < 0.0001$). A high mRNA expression level of DLC2 was associated with improved survival in patients with NSCLC who never smoked (HR, 0.49; 95% CI, 0.27-0.89; $P=0.016$). However, the mRNA expression level of DLC3 was not associated with improved survival in patients with NSCLC with different smoking habits. As seen in Table II, a high mRNA expression level of DLC1 was associated with improved prognosis in patients with stage 1 NSCLC (HR, 0.44, 95% CI, 0.33-0.58; $P < 0.0001$). A similar result was observed for DLC2 (HR, 0.44; 95% CI, 0.33-0.59; $P < 0.0001$) and DLC3 (HR, 0.73; 95% CI, 0.55-0.97; $P=0.028$). However, the mRNA expression levels of DLC1, DLC2 and DLC3 were not associated with the OS in patients with stage 2 and NSCLC. As seen in Table III, only DLC1 was associated with an improved prognosis in patients with higher grade NSCLC

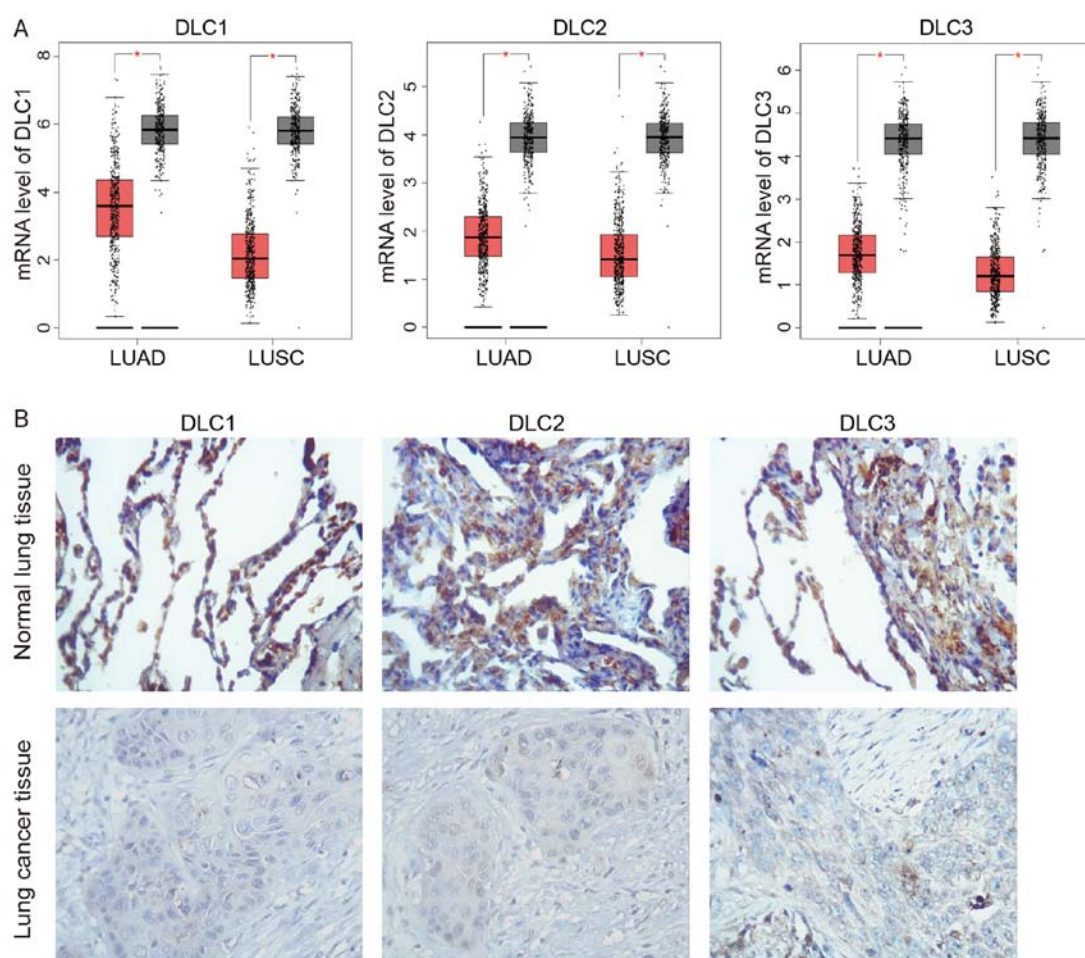


Figure 1. Analysis of DLC expression in NSCLC tissues. (A) Analysis of DLC expression in samples from The Cancer Genome Atlas and Genotype-Tissue Expression databases analyzed by GEPIA. The red and gray boxes represent tumor and normal tissues, respectively. DLC1, DLC2, and DLC3 expression was lower in LUAD and LUSC compared with normal tissues. LUAD, tumor n=483, normal n=347; LUSC, tumor n=486, normal n=338. *P<0.01, as indicated. (B) Immunohistochemical staining of DLC1, DLC2 and DLC3 in normal and lung cancer tissues (magnification, x100). DLC, deleted in liver cancer; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma.

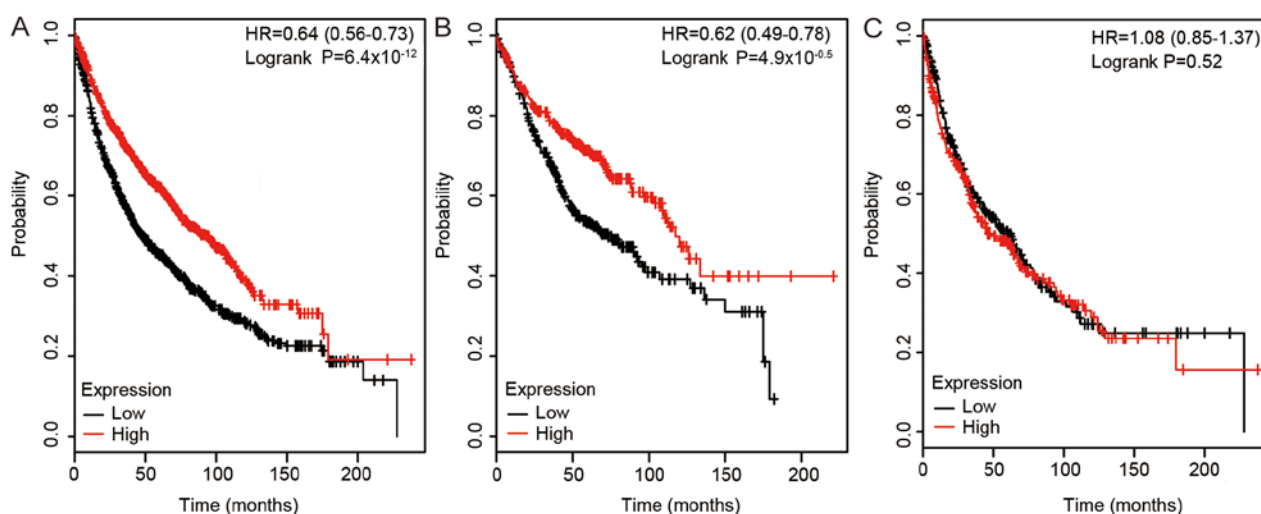


Figure 2. Prognostic value of DLC1 mRNA expression determined using the Kaplan Meier plotter. (A) Survival curves for all patients with non-small cell lung cancer (n=1,926). (B) Survival curves for patients with adenocarcinoma (n=720). (C) Survival curves for patients with squamous cell carcinoma (n=524). DLC1, deleted in liver cancer 1; HR, hazard ratio.

(grade II, HR=0.63; 95% CI, 0.46-0.87; P=0.0043; grade III, HR, 0.44; 95% CI, 0.22-0.87; P=0.016).

DLC1, DLC2, and DLC3 reduce the proliferation of A549 cells. As the expression levels of DLC1, DLC2, and DLC3

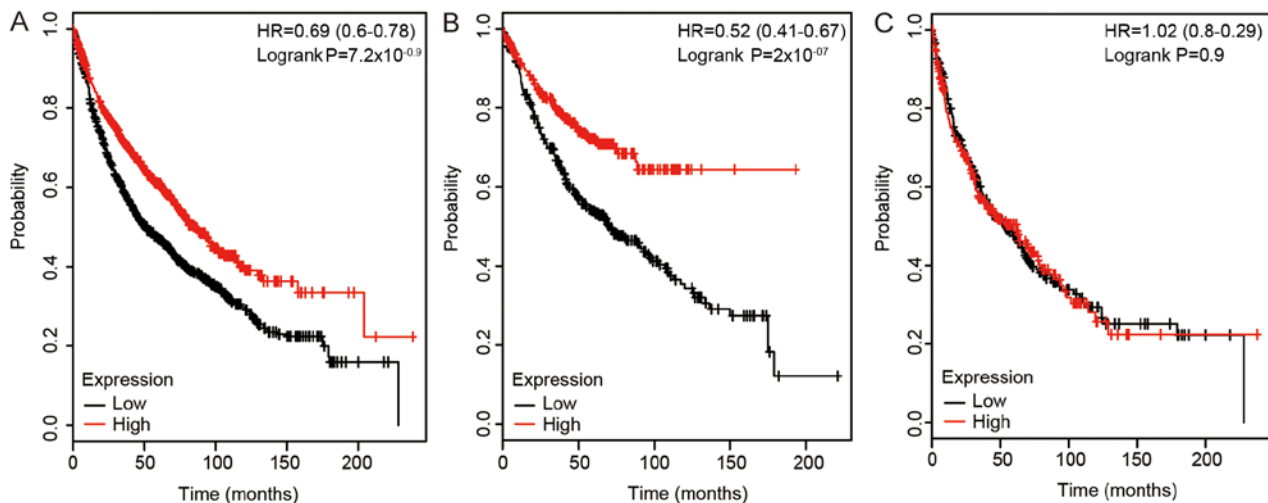


Figure 3. Prognostic value of DLC2 mRNA expression determined using the Kaplan Meier plotter. (A) Survival curves are plotted for all patients with non-small cell lung cancer (n=1,926). (B) Survival curves for patients with adenocarcinoma (n=720). (C) Survival curves are plotted for patients with squamous cell carcinoma (n=524). DLC2, deleted in liver cancer 2; HR, hazard ratio.

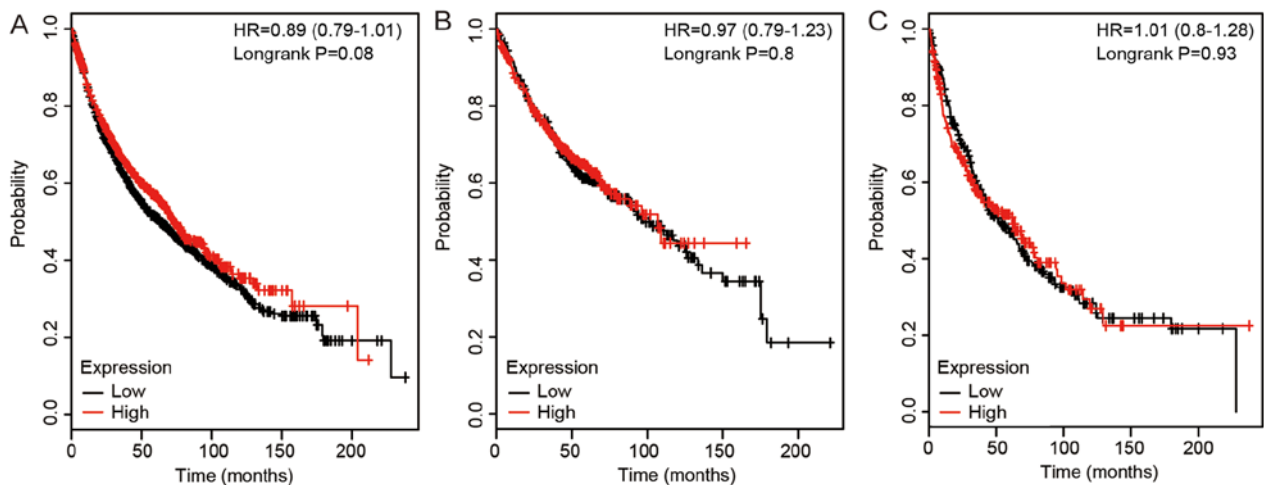


Figure 4. Prognostic value of DLC3 mRNA expression determined by using the Kaplan Meier plotter. (A) Survival curves are plotted for all patients with NSCLC (n=1,926). (B) Survival curves for patients with adenocarcinoma (n=720). (C) Survival curves are plotted for patients with squamous cell carcinoma (n=524). DLC3, deleted in liver cancer 3; HR, hazard ratio.

were lower in NSCLC tissues compared with healthy tissues, and their roles in NSCLC remained unclear, their effects on the proliferation of the A549 cell line were investigated. Plasmids expressing DLC1, DLC2 or DLC3 were used to transfect the cells, and RT-qPCR and western blotting were used to demonstrate the overexpression of DLCs in the cells (Fig. 5A-C). Following successful transfection, the proliferation of the transfected cells was assessed using the CCK8 assay. Cells transfected with empty plasmids served as the negative control. As shown in Fig. 5D, overexpression of DLC1, DLC2 and DLC3 decreased the proliferation of A549 cells compared with cells transfected with the empty plasmid.

DLC2 and DLC3 promote apoptosis of A549 cells. The upregulation of DLC1 was previously identified to induce apoptosis in NSCLC cells (20). To further explore the roles of DLC2 and DLC3 on apoptosis in A549 cells, flow cytometry was performed. The overexpression of DLC2 and DLC3 increased the percentage of

apoptotic cells compared with cells transfected with the plasmid control. The results indicated that DLC2 and DLC3 may induce the apoptosis of A549 cells *in vitro* (Fig. 5E and F).

Discussion

RhoGTPs are a family of small G-proteins that are important regulators of the actin cytoskeleton at the cell surface. Additionally, RhoGTPs regulate the cell cycle, cell migration, polarization, malignant transformation, invasion and metastasis (21). The DLC tumor suppressor proteins are members of the RhoGAP family that promote catalysis of GTP to GDP to inactivate RhoA (22).

DLC proteins are generally unstable in cells, and are expressed at low levels or deleted in various types of cancer (23-25). The DLC subfamily is composed of three members (DLC1, 2 and 3) with highly conserved amino acid sequences. The proteins contain three domains: i) RhoGAP

Table I. Association of DLC with different smoking history of patients with non-small cell lung cancer.

Gene	Smoking history	No. of cases	Hazard ratio	95% confidence interval	P-value
DLC1	Smoked	820	0.74	0.60-0.91	0.0041 ^a
	Never smoked	205	0.23	0.12-0.43	1.0000x10 ^{-6a}
DLC2	Smoked	820	0.98	0.80-1.20	0.8300
	Never smoked	205	0.49	0.27-0.89	0.0160 ^a
DLC3	Smoked	820	1.12	0.91-1.37	0.2900
	Never smoked	205	1.07	0.61-1.86	0.8100

Data presented in this table were obtained from Kaplan-Meier plotter database. DLC, deleted in liver cancer. ^aP<0.05.

Table II. Association of DLC with different clinical stages of patients with non-small cell lung cancer.

Gene	Clinical stage	No. of cases	Hazard ratio	95% confidence interval	P-value
DLC1	1	577	0.44	0.33-0.58	5.200x10 ^{-9a}
	2	244	1.03	0.71-1.48	0.880
	3	70	1.04	0.60-1.79	0.890
DLC2	1	577	0.44	0.33-0.59	2.200x10 ^{-8a}
	2	244	0.73	0.50-1.06	0.100
	3	70	1.02	0.60-1.75	0.940
DLC3	1	577	0.73	0.55-0.97	0.028 ^a
	2	244	1.28	0.89-1.85	0.180
	3	70	1.09	0.63-1.88	0.760

Data presented in this table were obtained from Kaplan-Meier plotter database. DLC, deleted in liver cancer. ^aP<0.05.

Table III. Association of DLC with different pathological grades of patients with non-small cell lung cancer.

Gene	Pathological grade	No. of cases	Hazard ratio	95% confidence interval	P-value
DLC1	I	201	0.86	0.60-1.23	0.4000
	II	310	0.63	0.46-0.87	0.0043 ^a
	III	77	0.44	0.22-0.87	0.0160 ^a
DLC2	I	201	0.89	0.62-1.27	0.5100
	II	310	0.94	0.69-1.28	0.6900
	III	77	0.89	0.46-1.71	0.7200
DLC3	I	201	1.20	0.84-1.72	0.3200
	II	310	0.75	0.54-1.03	0.0760
	III	77	0.93	0.48-1.79	0.8200

Data presented in this table were obtained from Kaplan-Meier plotter database. DLC, deleted in liver cancer. ^aP<0.05.

domain; ii) sterile α motif (SAM) domain; and iii) StAR-related lipid transfer (START) domains (26). Previous studies have revealed that the RhoGAP domain in DLC proteins serves an important role in the inhibition of tumors (27,28). The RhoGAP domain enhances GTP hydrolysis of the RhoGTP family members, RhoA, cell division cycle 42 (Cdc42), RhoB and RhoC, acting as a negative regulator of RhoGTPs (which are inactive when GDP-bound). The SAM domain may also serve as a negative intramolecular regulator in RhoGAPs (27,29).

The START domain binds to lipids, aiding the localization of DLC1 on cell adhesion plaques, which is required for DLC1 to exert its tumor suppressor role. However, the specific role of the START domain requires further investigation (20,30).

DLC1 is the most well investigated member of the DLC protein subfamily (31,32). Yuan *et al* (33) isolated the full-length cDNA of DLC1 by representational difference analysis in human primary hepatocellular carcinomas (HCCs) and HCC cell lines. DLC1, a well-known tumor suppressor gene located

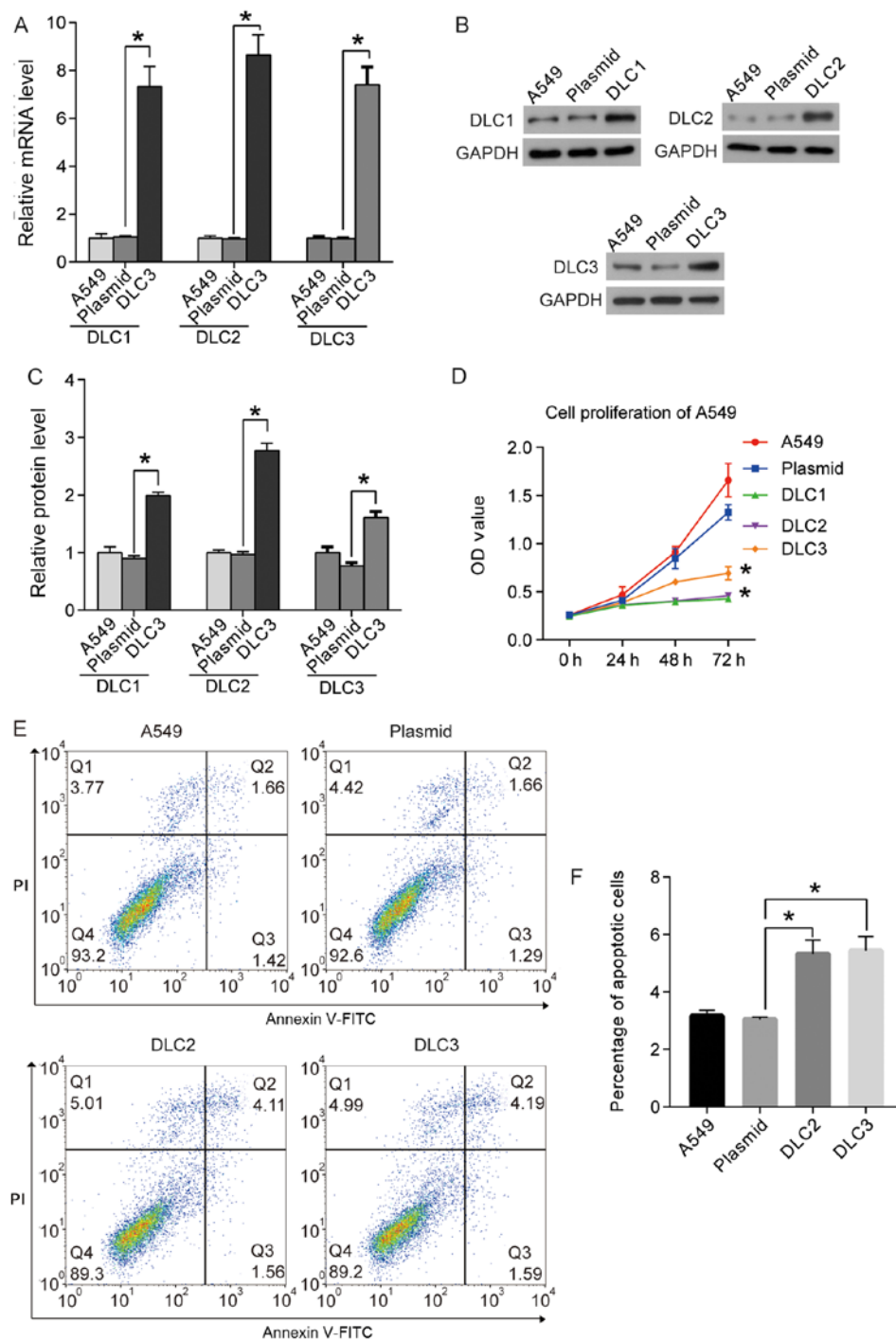


Figure 5. DLCs inhibited the proliferation and increased apoptosis of the human lung cancer cells line A549. (A) The expression levels of DLC1, DLC2 and DLC3 were measured by reverse-transcription quantitative polymerase chain reaction. The protein levels of DLC1, DLC2 and DLC3 were (B) detected and (C) semi-quantified by western blotting. (D) The Cell Counting Kit-8 assay was performed to detect the proliferation of A549 cells. (E) Flow cytometry was used to analyze the apoptosis of A549 cells. (F) Percentage of apoptotic cells as determined by flow cytometry. All the experiments in this figure were performed in triplicate. A549, untransfected cells. Plasmid, cells transfected with empty plasmid. *P<0.01, vs. Plasmid group. DLC, deleted in liver cancer; OD, optical density; PI, propidium iodide.

in the 8p21.3-p22 region of the human chromosome eight, has been previously reported to be associated with the occurrence of a number of malignant tumors, including gastric, colorectal and breast cancer as well as NSCLC (32,34). Through extensive genome screening, DLC1 has been suggested to be a significant susceptibility gene for NSCLC (35); however, clinical evidence for its application as a prognostic indicator is limited.

The present study investigated the prognostic value of DLC1 in NSCLC, and the results obtained demonstrated that a high expression level of DLC1 indicated improved survival. A previous study, using pull down analyses with the GTP-binding fragment of the RhoA effector, Rhotekin, revealed that reduced DLC1 transcript expression levels in NSCLC resulted in reduced RhoA-GTP levels *in vivo* (36). A previous study revealed that overexpression of DLC1 not only resulted in

morphological alterations that manifested as cytoplasmic extensions and membrane blebbing, but also inhibited tumor cell proliferation and migration, increasing the apoptosis of NSCLC cells *in vitro* (20). Furthermore, DLC1 suppresses tumor cell growth and invasion by RhoGAP-dependent and independent mechanisms in NSCLC (27).

Several genes are involved in the regulation of DLC1 anti-tumor activity in the RhoGAP-dependent mechanism (37,38). Caveolin-1 is one of these genes and it forms a complex with DLC1 by interacting with its START domain (37). The proinflammatory S100 calcium binding protein A10 also binds to DLC1 and inhibits cell migration, invasion and anchorage-independent growth in NSCLC (38). The present study demonstrated the prognostic role of DLC1 at the clinical sample level, suggesting that DLC1 is not only a tumor suppressor gene, but also a potential prognostic marker in NSCLC.

DLC2 was successfully cloned in 2003 and has a similar protein structure to DLC1 (25). Ullmannova and Popescu (39) reported low-expression of DLC2 in lung, renal, ovarian, breast, gastric, uterine, colon and rectal tumors for the first time using cancer-profiling arrays. Related research revealed that DLC2 was downregulated in a variety of tumors and that it inhibited the growth of tumor cells through its RhoGAP domain (40). The present study revealed that low expression of DLC2 was associated with survival in NSCLC.

In a number of related studies, DLC2 was reported to be associated with the development and metastasis of tumor cells. Tang *et al* (41) revealed that DLC2 mRNA is a direct target of microRNA (miR)-125b, and that the activation of DLC2 may be responsible for the metastasis induced by miR-125b in breast cancer cells. A previous study revealed that DLC2 is a central component of a signaling network that guides spindle positioning, cell-cell adhesion and mitotic fidelity (42). However, studies on the role of DLC2 in NSCLC remain limited. Thus the present study investigated the effect of DLC2 through its overexpression in A549 cells and revealed that DLC2 inhibits the proliferation of A549 cells, suggesting its potential role as a tumor suppressor in human NSCLC.

DLC3 is a tumor suppressor gene similar to DLC1 and DLC2 (43); however, the molecular function of DLC3 remains poorly understood. DLC3 is located on chromosome Xq13 and the protein localizes to focal adhesions. DLC3 acts as a GAP for RhoA and Cdc42 *in vitro*; its GAP activity is responsible for the morphological changes observed in Hela cells, which become round following disruption of the actin fibers (44). Hendrick and Olayioye (45) revealed that the interaction between DLC3 and Scribble planar cell polarity protein is essential for junctional DLC3 recruitment, and serves as a local regulator of RhoA-Rho-associated protein kinase signaling, which regulates adherens junction integrity and Scribble localization. Another study revealed that DLC3 is a Rho-specific GAP protein (46).

Braun *et al* (47), reported that loss of DLC3 inhibited the degradation of epidermal growth factor receptor (EGFR), increasing the activity of the EGFR signaling pathway. Additionally, Braun *et al* (47), showed that knockdown of DLC3 decreased the expression level of N-cadherin on the cell surface and decreased cell aggregation. In the current study, DLC3 was downregulated in patients with NSCLC, and a high expression level of DLC3 was associated with an improved prognosis in patients with stage 1 NSCLC.

Furthermore, DLC3 overexpression *in vitro* inhibited proliferation and promoted apoptosis of A549 cells, suggesting the role of DLC3 as a tumor suppressor gene in NSCLC. As DLC1, DLC2 and DLC3 are all downregulated in NSCLC and have similar domains, they may share the same signaling mechanisms involved in their tumor suppressor function. However, the specific molecular mechanism underlying their actions requires further elucidation.

The present study revealed that DLC1 and DLC2 are associated with prognosis in patients with NSCLC. Furthermore, the current study demonstrated the lower expression levels of DLC1, DLC2 and DLC3 in patients with NSCLC compared with normal controls, suggesting that DLC1-3 may serve negative roles in the progression of human NSCLC cells. The present results provided novel insights on the roles of the DLC members in NSCLC. Furthermore, in addition to DLC1, DLC2 and DLC3 may represent novel potential therapeutic targets for the treatment of NSCLC.

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Availability of data and materials

The data and materials are available from the corresponding author on reasonable request.

Authors' contributions

LS and JDS designed the study. LS and JS performed all the experiments and analyzed the data. JDS wrote the manuscript.

Ethics approval and consent to participate

The present study was approved by The Ethics Committee of Zaozhuang Municipal Hospital (approval no. 2016ZMHE011) and all patients signed informed consent.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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