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CITED4 enhances the metastatic potential of lung adenocarcinoma

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Abstract

Background: CITED4 belongs to the CBP/p300-interacting transactivator with glutamic acid and aspartic acid-rich tail (CITED) family which is induced by various cytokines and participates in cytokine-induced proliferation and differentiation. CITED4 is induced by HB-EGF in lung cancer cells. However, it is unclear whether and how CITED4 contributes to the invasion and metastasis of lung adenocarcinoma (ADC).

Methods: CITED4 expression in lung adenocarcinoma and its association with disease-free survival (DFS) and overall survival were analyzed based on a cohort of 261 patients. The roles of CITED4 were validated via loss-of-function and gain-of-function experiments. The relationship between CITED4 and CLDN3 was validated by immunohistochemistry, Western blotting, and luciferase reporter assays. The function of the CITED4-CTNNB1-CLDN3 complex was fully validated and described.

Results: CITED4 expression was significantly upregulated in ADC tissues and cells and a predictor for DFS. Downregulation of CITED4 attenuated the proliferation and invasion, whereas CITED4 overexpression enhanced these effects. Overexpression and knockdown of CITED4 resulted in the upregulation and downregulation of CLDN3, respectively. Moreover, CITED4 downregulation suppressed CLDN3-mediated ADC cell metastasis in vivo. CITED4 was highly expressed and positively correlated with CLDN3. Mechanistically, CITED4 interacted with CTNNB1 and functioned synergistically to enhance CLDN3 transcription. Importantly, CITED4 induced ADC invasion via a CLDN3-dependent pathway. CITED4 determined the level of CLDN3, which in turn affected the sensitivity of tumors to Clostridium perfringens enterotoxin treatment.

Conclusions: The CITED4-CTNNB1-CLDN3 axis plays a key role in the invasion and metastasis of ADC and provides a novel therapeutic target for lung cancer treatment.

KEYWORDS
CITED4, CLDN3, lung adenocarcinoma, metastasis, Wnt

INTRODUCTION

Lung cancer remains the major cause of cancer-related death worldwide.[1] Lung adenocarcinoma (ADC) is the most common histological subtype and is increasing in prevalence.[2] Recurrence and metastasis are the most important reasons for the failure of postoperative treatment in lung cancer, but the molecular mechanism of ADC metastasis has not yet been clarified. Claudin-3 (CLDN3) is the major structural molecule that forms tight junctions (TJs) between epithelial cells, and its expression levels vary in different subtypes of lung carcinomas.[3,4] A previous study

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found that CLDN3 affects the proliferation and metastasis of ADC.[5]

CITED4 belongs to the CBP/p300-interacting transactivators with glutamic acid and aspartic acid-rich tail (CITED) family, the members of which have critical roles during development.[6–8] CITED family proteins are induced by various cytokines and participate in cytokine-induced proliferation and differentiation.[9–12] CITED4 interacts with CBP/p300, as well as the transcription factor TFAP2, mediating TFAP2-dependent transcription.[7] Analysis of CITED4 in a large series of human breast tumors assessed by immunohistochemistry (IHC) showed both cytoplasmic and nuclear staining, and the nuclear expression of CITED4 was negatively correlated with HIF-1 alpha expression, tumor size and tumor grade.[13] CITED4 knockdown led to decreased cellular proliferation and modulation of the expression of a large number of genes, including c-MET tyrosine kinase and several actin-associated adherens junction (AJ)/TJ genes.[14] CITED4 also interacted with Myc and potentiated Myc-mediated transactivation of the CCND1 promoter, leading to cell cycle progression.[15] However, the underlying mechanisms that regulate the function and expression of CITED4, particularly in ADC, are poorly understood.

In this study, we observed that CITED4 was highly expressed in most ADC tissues and cells, the invasion and proliferation of which were dependent on CLDN3 signaling. Moreover, we found that CITED4 is involved in the expression of CLDN3 induced by the β-catenin pathway. Thus, we further examined the biological and clinical significance of the CITED4/CLDN3 signaling axis in ADC progression.

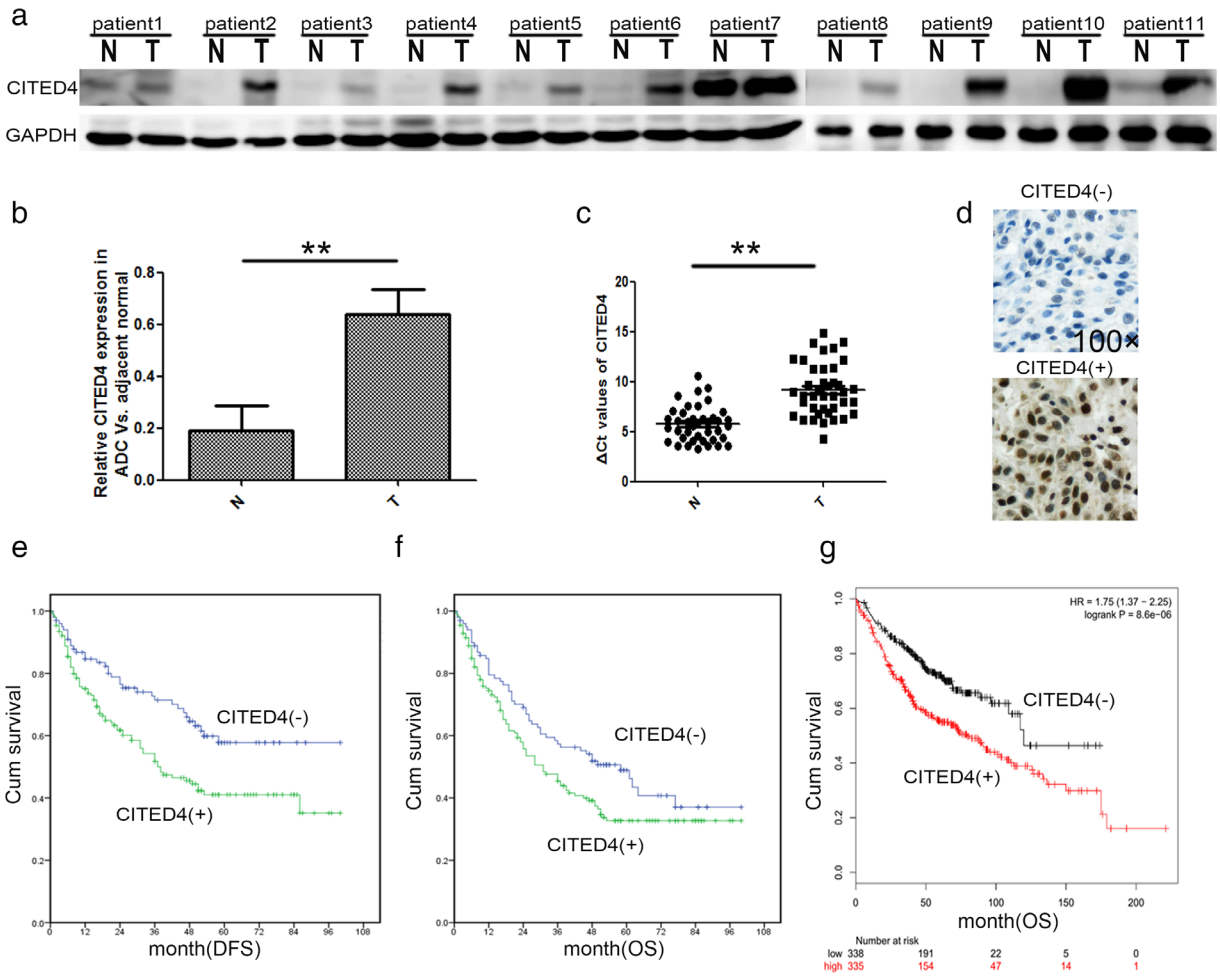


FIGURE 1 CITED4 expression and its association with poor outcomes in patients with lung adenocarcinoma (ADC). (a) CITED4 expression in ADC and adjacent normal lung samples. Total tissue lysates were prepared using frozen matched normal and lung cancer tissues from the same patient. GAPDH was used as a loading control (N, adjacent normal tissue; T, tumor tissue). (b&c) CITED4 was significantly increased in the ADC group compared with the normal group (**$p < 0.01$). Dot plots represent the ΔCt values of CITED4 (higher ΔCT values correspond to lower expression; mean ± SEM; Mann–Whitney U test). (d) Representative images of protein expression in an ADC determined by immunohistochemistry (IHC) with anti-CITED4 (brown). The slide was counterstained with hematoxylin. Original magnification ×100. (e&f) Kaplan–Meier curves for the disease-free survival (DFS) and overall survival (OS) rates of patients with ADC according to the expression level of CITED4. Blue, patients with low CITED4 expression ($n = 106$); green, patients with high expression of CITED4 ($n = 155$, median DFS was 37 months vs. NR (not reached) and median OS was 31 vs. 58 months; pDFS = 0.004, pOS = 0.046). (g) Data from public databases also show the association of CITED4 expression with OS in lung cancer patients (http://kmplot.com/)

METHODS

Cell culture and clinical specimens

ADC cell lines, including NCI-H1650, NCI-H1299, CaLu-3, A549, PC9, NCI-H358, H460, NCI-H1299 and HEY-293T, were obtained from the American Type Tissue Culture Collection (ATCC). The cell lines were maintained in Roswell Park Memorial Institute (RPMI)-1640 containing 10% fetal bovine serum (FBS; Invitrogen).

The acquisition of ADC tissues and clinical data from our institute was approved by the Institutional Review Board of China (approval ID 81702277). We also obtained cell lysates from the clinical specimens and subjected them to Western blot analysis.

Western blot analysis

Total protein was extracted from the sample, and the protein concentrations were determined using the Bradford Protein Assay (Bio-Rad, Hercules, CA). A total of 50 μg of protein from each sample was separated by 8%–12% SDS-PAGE and transferred onto a polyvinylidene fluoride membrane (Bio-Rad). The proteins were detected by incubation of the membranes with antibodies against CLDN3, CITED4 (Abcam), β-catenin, MMP-2 and GAPDH (Cell Signaling). WNT- and control-conditioned medium (Wnt-CM [ATCC number: CRL-2647] and L-CM) were collected according to the directions from ATCC and incubated with the cells for 24 h during the experiments. Wnt signaling activity was then determined by performing various assays, such as Western blotting.

Real-time quantitative PCR (qPCR)

Total RNA was extracted by Direct-zol RNA MiniPrep kit (Zymo Research) from cell pellets or tissues. cDNA was synthesized by MultiScribe Reverse Transcriptase (ABI). Q-PCR was performed with Power SYBR Green PCR Master Mix reagent (ABI). A total of 10-ng cDNA template and 3 pmol of primer pairs were applied in each 10-μL PCR reaction. All qPCR primers used in this study were acquired from online primer database (medgen.ugent.be) and purchased commercially (Invitrogen).

Immunohistochemical analysis

Patient tumors were removed, weighed, fixed in 5% formalin, and prepared for histological analysis. Immunohistochemical staining was carried out using the ABC Staining Kit (Santa Cruz Biotechnology) and a secondary biotinylated rabbit IgG antibody (Invitrogen). CLDN3, CITED4, and Ki-67 immunoreactivity was assessed based on a combined score of the extent and intensity of staining. Scores of 0–3 were assigned according to the percentage of positive tumor cells (0 = 0%; 1 = <33%; 3 = 33%–66%; 3= > 66%) and the intensity of tumor staining (0 = 0; 1 = 1+; 3 = 2+; 3 = 3+). The two scores were multiplied to give an overall score of 0–9, for which 0 was considered negative, 1–2 was considered weak, 3–6 was considered moderate, and 9 was considered strong staining. Negative and weak expression was considered to be low, whereas moderate and strong expression was considered to be high. The scorers reviewed any discordant scores together to obtain a consensus score.

Lentivirus-based short hairpin RNA (shRNA) transduction

shRNA lentiviral transduction particles for the CLDN3 and CITED4 knockdown experiments were obtained from

TABLE 1 Association between CITED4 expression and clinicopathological characteristics of patients with ADC

Characteristics	No	CITED4 (+)	CITED4 (−)	$\chi^2 p$
Age (years)				.801
≤60	143	84 (58.5%)	59 (41.5%)	
>60	118	71 (60.2%)	47 (39.8%)	
Gender				.900
Male	124	73 (58.5%)	51 (41.5%)	
Female	137	82 (59.9%)	55 (40.1%)	
Smoking history				.900
Never	140	84 (59.7%)	56 (40.3%)	
Ever	121	71 (58.7%)	50 (41.3%)	
Tumor size (cm)				.528
≤3	134	76 (56.8%)	58 (43.2%)	
>3	127	78 (61.2%)	49 (38.8%)	
T stage				.503
T1	118	66 (55.6%)	52 (44.4%)	
T2	88	56 (63.6%)	32 (36.4%)	
T3	55	33 (60.0%)	22 (40.0%)	
N stage				.255
N0	140	82 (58.6%)	58 (41.4%)	
N1	31	23 (74.2%)	8 (25.8%)	
N2	90	49 (54.7%)	41 (45.3%)	
TNM stage				.642
I (IA, IB)	121	73 (60.2%)	48 (39.8%)	
II (IIA, IIB)	52	33 (63.5%)	19 (36.5%)	
IIIA	88	49 (55.7%)	39 (44.3%)	
Histology				
Micropapillary predominant	33	19 (57.6%)	14 (42.4%)	.423
Acinar predominant	29	19 (65.5%)	10 (34.5%)	.513
Lepidic predominant	99	50 (50.5%)	49 (49.5%)	.141
Solid predominant	78	45 (57.7%)	33 (42.3%)	.736
Papillary predominant	22	17 (77.3%)	5 (22.7%)	.590
Recurrence or metastasis				.003
Absent	146	74 (50.7%)	72 (49.3%)	
Present	115	80 (69.6%)	35 (30.4%)	

Sigma. Two of three CLDN3-specific and CITED4-specific shRNA constructs (shRNA sequence targeting CLDN3: CCGGCGACCGCAAGGACTACGTCTACTCGAGTAGA-CGTAGTCCTTGCGGTCGTTTTTG; CCGGGACTACG TCTAAGGGACAGACCTCGAGGTCTGTCCCTTAGAC-GTAGTCTTTTTTG; shRNA sequence targeting CITED4: CCGGCGCCGAACTC ATCGACGAGGACTCGAGTCC TCGTCGATGAGTTCGGCGTTTTT; CCGGCGGCATGG ACGCCGAACTCATCTCGAGATGAGTTCGGCGTCCA-TGCCGTTTTT))[14] and one nontargeting construct were transduced separately into 293T cells. The nontargeting construct contained an shRNA sequence that did not target any known human gene and served as a scrambled negative control. Briefly, 293T cells were transduced with CLDN3- or CITED4-specific shRNA lentiviral particles for 24 h in the presence of hexadimethrine bromide to improve the transduction efficiency. Afterward, the medium containing viral particles was removed and replaced with fresh medium containing 10 μg/mL puromycin. Before lentiviral transduction, a puromycin titration was performed that identified 10 μg/mL as the minimum puromycin concentration to cause the complete death of the 293T cells after a 5-day incubation. CITED4 knockdown was confirmed using a Western blot analysis to compare the protein levels in cells with targeting versus nontargeting shRNA.

Generation of NCI-H358 CITED4 cells

NCI-H358 CITED4 or empty-vector (NCI-H358 pCDH) cells were generated by transducing wild-type NCI-H358 cells with pCDH-CITED4 or empty vector and then selecting successfully transduced cells with 7.5 mg/mL puromycin for at least 5 days. The clones were isolated, and the overexpression of CITED4 was confirmed by Western blotting.

Fluorescence confocal microscopy

A549 cells were cultured with WNT- and control-conditioned medium (Wnt-CM and L-CM) for 72 h. Cells were fixed in 4% formaldehyde and subjected to indirect immunofluorescence microscopy with anti-CLDN3 and anti-CITED4 antibodies. Confocal immunofluorescence microscopy (Olympus) was performed using an Olympus confocal microscope according to the manufacturer's protocol. The magnification used was 40×.

Cell proliferation assay

After the cells were seeded in 24-well plates, 5-ethynyl-2′-deoxyuridine (EdU) was added to the culture medium at a concentration of 50 μM/mL for 8 h to chase the DNA template according to the instructions in the Cell Light EdU DNA Cell Kit (Apollo 488/567, RiboBio). Briefly, after being fixed in 4% paraformaldehyde and treated with 0.5% Triton X for 15 min, the cells were incubated with Apollo in the dark, and the nuclei were stained with Hoechst 33342. EdU-labeled cells were counted manually in five fields of view randomly selected from each well, and the percentage of positively stained cells was calculated.

Anchorage-dependent colony formation assay

For the anchorage-dependent colony formation assays, A549 (shCITED4), PC9 (shCITED4) and the relevant control cells were seeded in a 6-well plate at a low density (5 × 102 cells/well) for 7 days. After 7 days, the cells were fixed with ethanol for 6 min and stained with a crystal violet solution (0.05% crystal violet and 20% methanol). The cells were then washed twice with water and permeabilized with methanol. All experiments were performed in triplicate.

TABLE 2 Association of various factors with DFS and OS in ADCs determined by COX regression model

	Multivariate analysis for DFS		Multivariate analysis for OS	
Variable	**HR (95% CI)**	***p*-value****	**HR (95% CI)**	***p*-value**
Gender Male vs. female	0.699 (0.468–1.045)	.081	0.897 (0.624–1.289)	.557
Age >60 year vs. ≤60 yr	0.576 (0.407–0.815)	.002	1.004 (0.742–1.359)	.979
Smoking history Never vs. ever	0.768 (0.513–1.150)	.200	1.319 (0.992–1.886)	.129
TNM stage Early vs. advanced	1.443 (1.198–2.584)	.001	1.685 (1.421–1.997)	.001
Chemotherapy Yes vs. no	1.738 (1.169–2.584)	.006	1.245 (0.882–1.757)	.213
Radiotherapy Yes vs. no	1.521 (0.998–2.317)	.051	1.199 (0.798–1.801)	.382
CITED4 expression Low vs. high	1.494 (1.054–2.116)	.024	1.354 (0.989–1.855)	.059

Abbreviations: CI, confidence interval; HR, hazard ratio for death.
*$p < 0.05$ was considered statistically signifiant.

Wound scratch assay

Each group of A549 (shCITED4), PC9 (shCITED4) and relevant control cells was seeded into a six-well plate. On the following day, when the cells were approximately 90% or more confluent, each well was scraped with a 20-μL pipette tip to create three linear regions devoid of cells. Then, the cells in each well were cultured with RPMI-1640 medium (Gibco) containing 2% FBS (Gibco) in a humidified incubator. Photographs of the wounded area were taken immediately after making the scratch (0 h time point) and after 24 h to monitor the invasion of cells into the wounded area.

Transwell tumor cell migration assay

A 24-well Boyden chamber with an 8-μm pore size polycarbonate membrane (Corning, NY) was used to evaluate cell motility. A total of 105 cells were seeded in the upper chamber with 200-μL serum-free medium. Then, 600-μL medium with 10% serum was added into the lower chamber as a chemoattractant. A total of 16 h after incubation, the membranes were fixed with methanol and stained with a three-step staining set (Thermo, UK). Five visual fields were randomly selected from each membrane for analysis. All experiments were performed in triplicate.

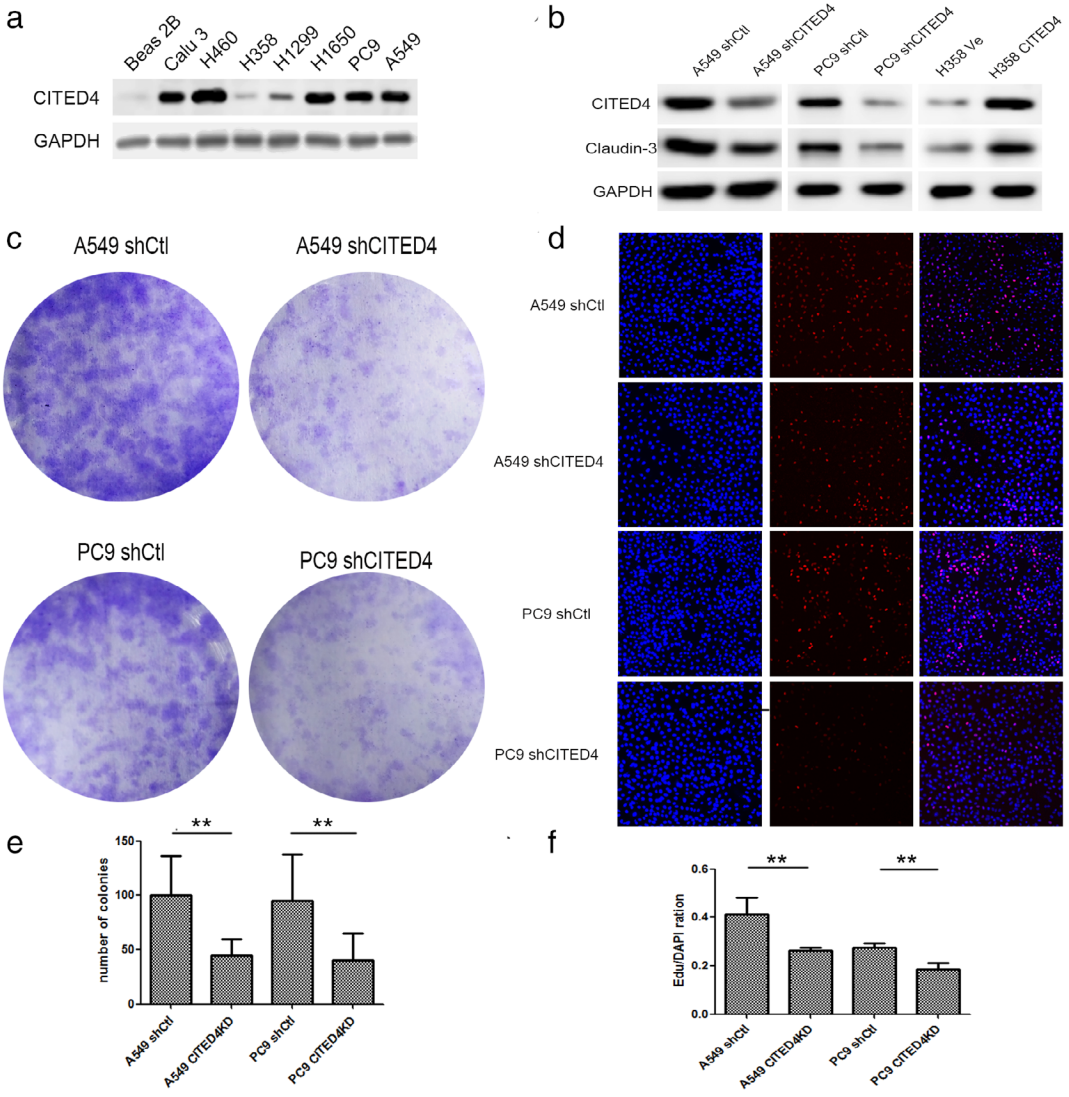


FIGURE 2 CITED4 expression alterations affect the clonality and proliferation of ADC cells. (a) CITED4 expression was detected in ADC cell lines: A549, PC9, H1650, H460 and CaLu-3 cells express very high levels of CITED4; NCI-H1299 cells express moderate levels of CITED4; and H358 cells and alveolar epithelial Beas-2B cells most do not express CITED4 at all. (b) Western blot of CLDN3 protein expression in CITED4- transfected and CITED4-knockdown cells. (c&d) Representative photographs of anchorage-dependent colonies that were stained with crystal violet. The bar graphs show that the number of colonies increased in cells with ectopic expression of CITED4 compared to control cells (**$p < 0.01$, *t*-test). (e&f) The proliferation of A549 (shCITED4) and PC9 (shCITED4) cells and the respective control cells was assessed using the 5-ethynyl-2′-deoxyuridine (EdU) assay (**$p < 0.01$, *t*-test)

Luciferase reporter assay

Luciferase reporter assays were used to measure the effect of CITED4 expression on promoter activity in HEK293T cells transfected with the pGL3-CLDN3 promoter reporter plus or minus CITED4 cDNA. PGL3-basic was used as a negative control. The results are representative of three independent experiments and are expressed as the mean ± SD. Chromatin immunoprecipitation (ChIP) was used to assess CITED4 binding to the CLDN3 promoter. A549 cells treated with or without Wnt-CM for 2 h were subjected to ChIP with antibodies against CITED4 and β-catenin, followed by Q-PCR analysis to assess the binding of CITED4 to the CLDN3 promoter. The 5′ upstream region of human CLDN3 (−833 to +189, relative to the transcriptional start site) was amplified by PCR and inserted into the pGL3-Basic vector (Promega).

In vivo tumorigenicity

A total of 1 x 107 stable A549 (shCITED4) cells and control cells were injected subcutaneously into the left groin of 6-week-old male BALB/c nude mice ($n = 7$ per group). The tumor diameter was measured every 3 days from the 28th day after inoculation for 25 days. The tumor volume was calculated by the formula $V = 0.5 \times L \times W2$. All animal experiments were approved by the Animal Experimentation Ethics Committee of the Tianjin Medical University Cancer Hospital and Institute. The *C. perfringens* carboxy-terminal fragment peptide was synthesized by Invitrogen (Grand Island, NY; final concentration 5 μg/mL).

Statistical analysis

Associations between CITED4 expression and clinical and biological characteristics were analyzed by χ^2 or Fisher's exact test. Survival curves were drawn using the Kaplan–Meier method. Cox proportional hazards regression (forward likelihood ratio model) was used for multivariate survival analyses. All two-group comparisons utilized Student's *t*-test with the assumption of unequal variance. Data are presented as the mean ± SEM determined from a minimum of three independent experiments. All data were analyzed using the Statistical Package for the Social Sciences Version 21.0 Software (SPSS Inc). The two-sided significance level was set at $p < 0.05$.

RESULTS

CITED4 expression is significantly upregulated in ADC and is an independent predictor of survival in postoperative patients

We detected the expression levels of CITED4 protein in paired adjacent normal tissues and ADC specimens

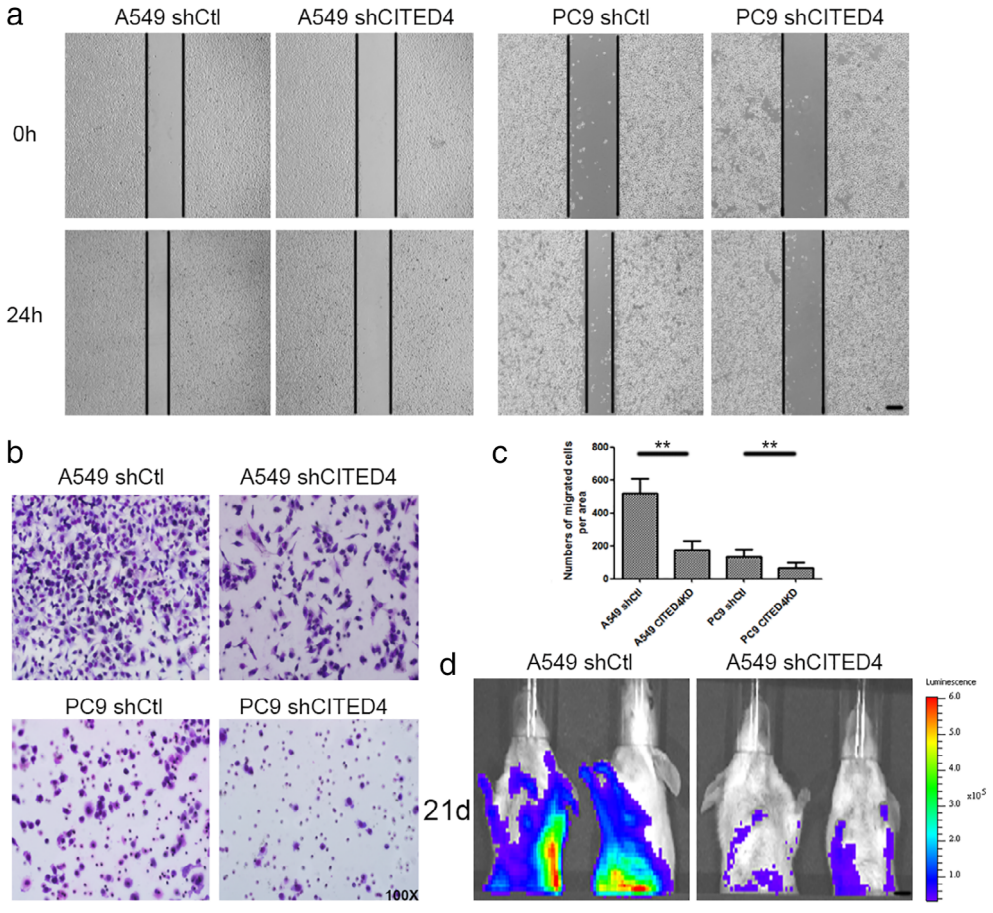


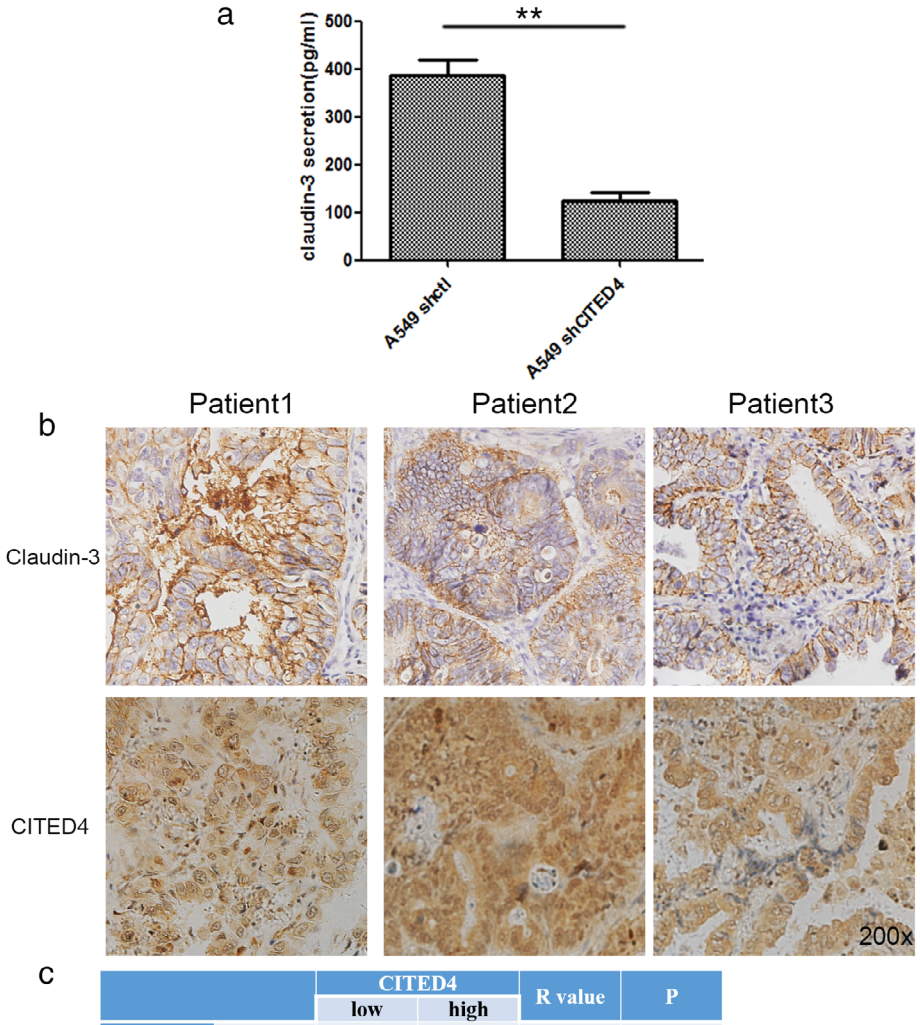
FIGURE 3 CITED4 expression alterations affect the migration and invasion of ADC cells. (a) The effect of CITED4 on cell migration was determined by a wound healing assay. During a period of 24 h, the spreading speed of A549 (shCITED4) and PC9 (shCITED4) cells along the wound edge was slower than that of control cells. (migration rate was quantified, Bar: 100 μm. [$n = 3$]; *$p < 0.01$ as determined by an ANOVA). (b&c) Similarly, the downregulation of CITED4 significantly attenuated the migration ability of A549 and PC9 cells, and the number of migrated tumor cells is quantified in the panel below (**$p < 0.01$, *t*-test). (d) shCITED4 knockdown reduced the metastatic capacity in a mouse metastasis model. Representative images of serial noninvasive bioluminescence monitoring after tail vein injection of the same number (1 × 106) of luciferase transfected A549-shCITED4 and control cells. Mice were imaged dorsally for 3 min and then ventrally for another 3 min

(n = 11) and found that the CITED4 protein expression level was significantly higher in ADC specimens than in normal tissues (Figure 1(a)&(b), $^{**}p < 0.01$). The mRNA assessment also confirmed the above results (Figure 1(c), $^{**}p < 0.01$). We next performed IHC analysis of 261 ADC patients using an anti-CITED4 antibody. The intensity of CITED4 staining was independently scored by two pathologists and classified as low or high (see Methods). CITED4 expression was low in most of the normal lung tissues but increased in the ADC tissues (overexpression rate 19.5% vs. 59.4%, $p < 0.01$).

The correlation between the CITED4 expression status and the clinicopathological features of 261 ADCs was further evaluated, and the findings are summarized in Table 1. A positive correlation was observed between CITED4 upregulation and recurrence and/or metastasis (Table 1). Furthermore, Kaplan–Meier survival analysis demonstrated that the 5-year survival rate was significantly lower in patients with CITED4 upregulation (n = 155) than in patients with low CITED4 expression (n = 106, the median disease-free survival (DFS) was 37 months vs. NR [not reached]), and the median overall survival (OS) was 31 vs. 58 months (p_{DFS} = 0.004; p_{OS} = 0.046, Figure 1(e)& (f)). Moreover, multivariate analysis showed that the upregulation of CITED4 (p_{DFS} = 0.024) was an independent metastatic predictor for ADC (Table 2). It has also been confirmed that the expression of CITED4 affects the recurrence and metastasis of ADC. Data from public databases also support this result (Figure 1(g), http://kmplot.com/).

Alterations in CITED4 expression affect proliferation, clonality, and migration

Similarly, CITED4 was significantly upregulated in 6/7 (85.7%) of the ADC cell lines assessed (CaLu-3, H460, H358, H1299, H1650, A549 and PC9, Figure 2(a)) but was not detected in Beas-2B cells. Taken together, our data demonstrate significant CITED4 overexpression in ADC tissues and cells and predict a potential correlation between CITED4 expression and cancer progression.



		CITED4		R value	P
		low	high		
Cldudin-3	low	106	12	0.91	<0.001
	high	9	134		

FIGURE 4 Co-expression of CITED4 and CLDN3 in ADC. (a) Downregulation of CITED4 also reduced the level of secreted CLDN3 in the culture medium ($^{**}p < 0.01$, t-test). (b&c) Co-expression of CITED4 and CLDN3 in ADC tissues and further verification by tissue microarray (χ^2 test, $p < 0.001$)

It is well known that cell transformation plays an important role in the evaluation of malignant potential.[16] In this context, we evaluated the cell-transforming potential of CITED4 in A549 and PC9 cells. As observed in Figure 2(b), the CITED4 downregulation group displayed reduced anchorage-dependent growth compared with that in the control group (Figure 2(d), $^{**}p < 0.01$). As shown in Figure 2(d), under CITED4 downregulation conditions (Figure 2(b)), we observed decreases in proliferation in both A549 (shCITED4) and PC9 (shCITED4) cells (Figure 2(e), $^{**}p < 0.01$). Furthermore, the wound healing assay and transwell system were used to examine the migration ability of the cells and 24 h after seeding, wound closure monitoring and the transwell assay showed that the downregulation of CITED4 significantly restrained cell migration (Figure 3 (a)–(c), $^{**}p < 0.01$). This phenomenon was also confirmed in vivo: downregulation of CITED4 reduced the number of metastatic lesions transferred by A549 cells (Figure 3(d)). Taken together, the results above suggest that CITED4 acts as an oncogene in ADC, and that CITED4 overexpression significantly increases the tumorigenicity and malignant behavior of these two cell types.

CITED4 induces ADC invasion via a CLDN3-dependent pathway

CLDN3, a member of the cellular adhesion molecule family, was identified in previous work as a key mediator of the oncogenic effects caused by hyperactivated EGFR signaling in ADC.[5]CLDN3, as a secreted protein, can also affect cell migration and metastasis. Although CITED4 and CLDN3 are located in different parts of the cells, we found by serial sectioning that CITED4 and CLDN3 are consistently highly expressed in ADC (Figure 4(c), $^{**}p < 0.01$); downregulation of CITED4 significantly reduced the level of CLDN3 in vitro, while overexpression of CITED4 increased the level of CLDN3 (Figure 4(a)). Further studies found that downregulation of CITED4 also reduced the level of secreted CLDN3 in the culture medium (Figure 4(a), $^{**}p < 0.01$).

We therefore examined whether CITED4 induces malignancy through a CLDN3-dependent pathway. We further confirmed that cultivation of ADC cells with CLDN3-rich medium promoted their migration (Figure 5(a), $^{**}p < 0.01$). CITED4 knockdown cells were cultured in CLDN3-rich medium, and compared with the control medium, the CLDN3-rich medium promoted cell invasion (Figure 5(a), $^{**}p < 0.01$). We also cultured H358 cells overexpressing

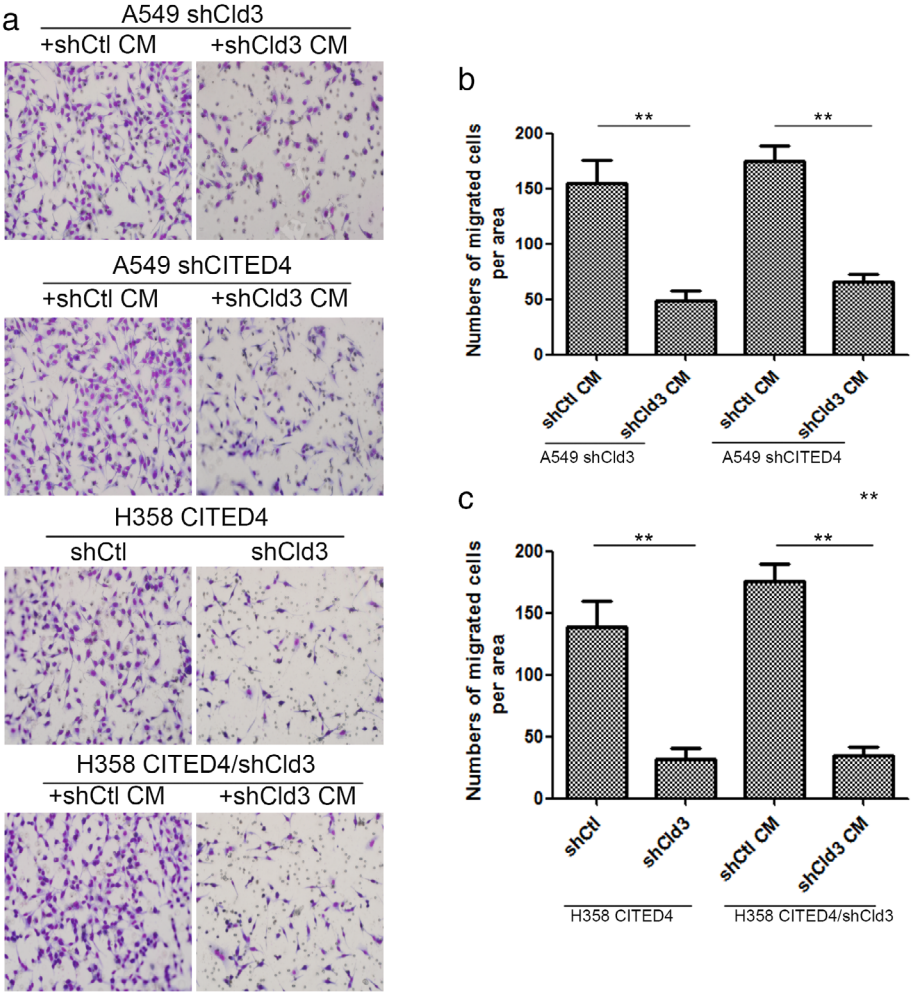


FIGURE 5 CITED4 induces the migration of ADC cells via CLDN3-dependent modalities. (a–c) Migration assay upon the addition of conditioned medium (CM) from CLDN3 shRNA (shCLDN3) and control shRNA (shCtrl)-transfected cells to A549-shCLDN3 or A549-shCITED4 cells. Overexpressing CITED4 in H358 cells and downregulating CLDN3 in the medium attenuated cell invasion and migration. Recovery experiments indicated that CITED4 induces the migration of ADC cells via CLDN3. Student's *t*-test was performed. Data are presented as mean ± SEM of triplicate experiments

CITED4 in medium lacking CLDN3. Culture in medium lacking CLDN3 attenuated cell invasion and migration. To further reduce the effect of CITED4 overexpression on the secretion level of CLDN3, CLDN3 was knocked down in H358 cells overexpressing CITED4; then, cells were cultured with culture media with different CLDN3 protein levels. The culture medium lacking CLDN3 attenuated ADC cell progression. Therefore, depending on the CLDN3 level, CITED4 may affect the movement of ADC cells and then promote the invasion of ADC.

CITED4 interacts with CTNNB1 and binds to the CLDN3 promoter

To further confirm the interaction between CITED4 and CLDN3, we investigated the WNT signaling pathway and its key molecule CTNNB1 (β-catenin). Previous studies have shown that CTNNB1 can bind to the promoter region of CLDN3, affecting its protein level.[17,18] We next examined whether CITED4 binds to the CLDN3 promoter together with CTNNB1. Our study found that in 293 T cells cotransfected with CITED4 and CLDN3, CLDN3 was mainly located in the cytoplasm, while CITED4 was mainly located in the nucleus and perinuclear region (Figure 6(a)). Culture of the cells in WNT agonist-containing medium revealed that CLDN3 appeared in the nucleus and perinuclear region and colocalized with CITED4 (Figure 6(a)). Further study by immunoprecipitation (IP) found that CTNNB1 interacts with CITED4 in 293 T cells. Moreover, we constructed a luciferin reporter plasmid containing the CLDN3 promoter region. The promoter reporter assay confirmed that CTNNB1 stimulated CLDN3 promoter reporter activity and that CITED4 enhanced CTNNB1-mediated CLDN3 transcription (Figure 6(c)). We further demonstrated that activation of WNT signals can induce CLDN3 expression and that CITED4 levels play a key role in this process (Figure 6(d)–(g)). Taken together, these findings support the notion that CITED4 interacts with CTNNB1 and functions synergistically to enhance CLDN3 transcription.

CITED4/CLDN3 signaling mediates lung tumor growth in vivo

To further confirm whether CITED4 signaling is crucial for tumor development, we injected CITED4-silenced A549 cells subcutaneously into nude mice. We monitored tumor volume over time and found that knockdown of CITED4 significantly attenuated tumor growth and reduced tumor size (Figure 7(a)&(b)). These results showed that CITED4 signaling has an important role in lung cancer proliferation. Furthermore, the downregulation of CITED4 also had an impact on the expression levels of CLDN3 and MMP-2 in vivo (Figure 7(c)), and in addition, A549 (shCITED4) cells showed a lower proliferation rate than the control cells (Figure 7(d)), consistent with the in vitro findings.To further study whether CITED4/CLDN3 signaling can act as a potential therapeutic target for lung cancer treatment, we treated lung cancer cell lines with *Clostridium perfringens* enterotoxin (CPE), an inhibitor of and natural ligand for CLDN3.[19] CPE significantly inhibited the proliferation of cells with high CLDN3 expression (A549) (Figure 5(e)), but

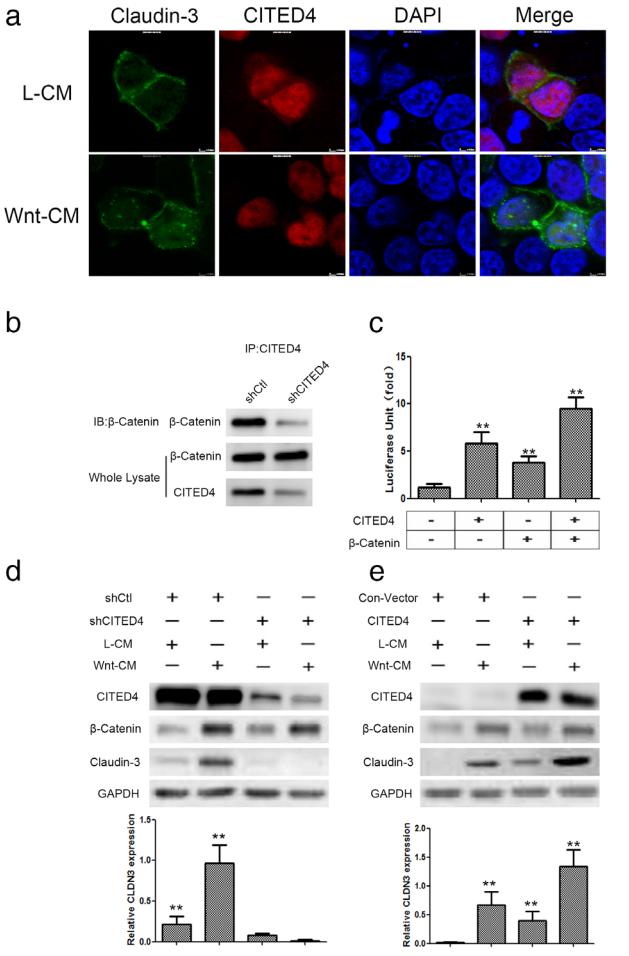


FIGURE 6 CITED4 interacts with CTNNB1 and binds to the CLDN3 promoter&#13. (a) Coculture of cells with Wnt-CM revealed that CLDN3 appeared in the nucleus and perinuclear region and colocalized with CITED4. (b) Coimmunoprecipitation (co-IP) analysis to assess the interaction between endogenous CITED4 and β-catenin in A549 cells infected with lentiviral vectors encoding shCITED4 or shCtrl, followed by IP with antibodies against CITED4. The immunoprecipitated complexes were subjected to Western blotting with antibodies against β-catenin and CITED4. (c) Luciferase reporter assay to measure CLDN3 promoter activity in 293T cells transfected with the pGL3-CLDN3 promoter reporter plus cDNA of CITED4 and/or β-catenin. The results are representative of three independent experiments and are expressed as the mean ± SD (**$p < 0.01$, ANOVA). (d) Knockdown of CITED4 inactivates CLDN3 and attenuates Wnt-induced CLDN3 in A549 cells. Cells were cotransfected with the control or shCITED4 and then treated with L- or Wnt-CM. Relative luciferase analysis was performed as described above. Asterisks indicate a statistically significant difference in cells transfected with control shRNA versus shCITED4 (**$p < 0.01$, ANOVA). (e) In contrast, restoring CITED4 expression in H358 cells (CITED4-negative cells) promoted Wnt-induced CLDN3 expression. Asterisks indicate statistically significant differences in cells transfected with the control vector versus CITED4 (**$p < 0.01$, ANOVA)

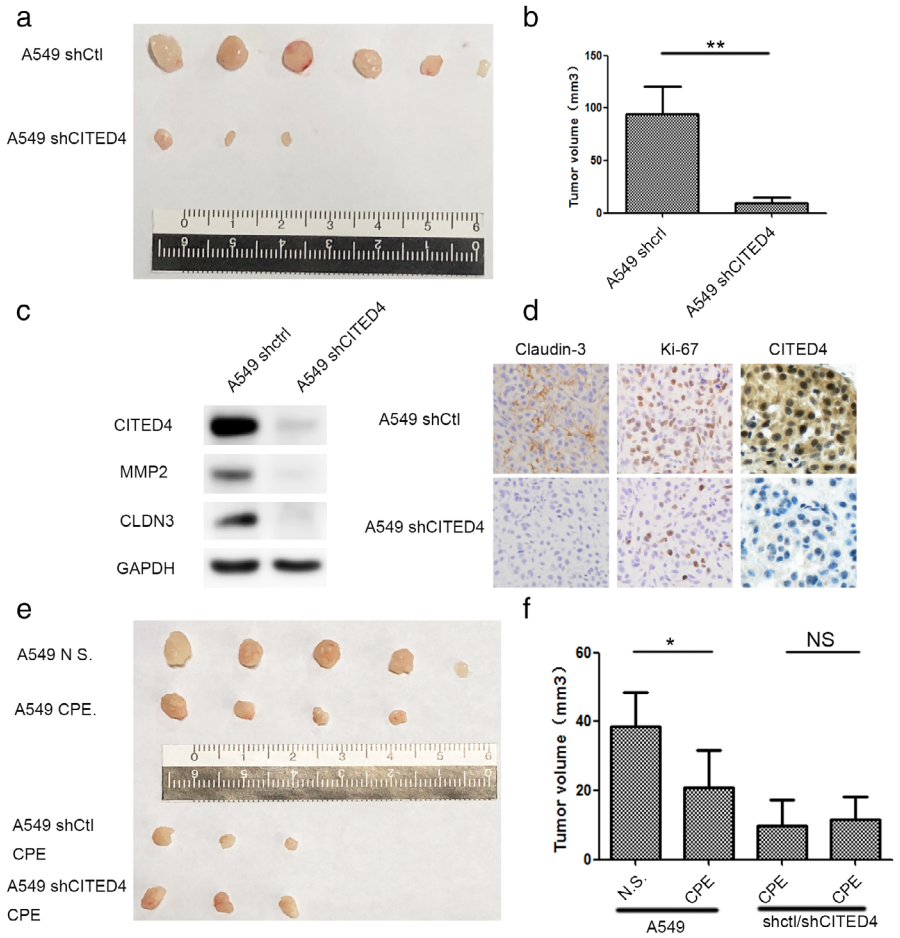


FIGURE 7 CITED4/CLDN3 signaling mediates lung tumor growth in vivo. (a) Representative examples of tumors formed in nude mice following the injection of shCITED4-expressing A549 cells (lower panel) and shCtrl-transduced A549 cells (upper panel). (b) The tumor volume of the shCITED4 group is much smaller than that of the control group (**$p < 0.01$, *t*-test). (c&d) Knockdown of CITED4 in A549 cells led to a reduction in CLDN3 and MMP-2 expression and the Ki-67 proliferation index. (e&f) *Clostridium perfringens* enterotoxin (CPE) significantly inhibited the proliferation of cells with high expression of CLDN3 (A549), but the antitumor effect of CPE was significantly attenuated in cells with CITED4 downregulation (*$p < 0.05$; NS, not significant)

in the cells with CITED4 downregulation, the antitumor effect of CPE was significantly attenuated (Figure 5(e)). Furthermore, it was also confirmed by in vivo experiments that CITED4 determined the level of CLDN3, which in turn affected the sensitivity of tumors to CPE treatment. These findings support the notion that CITED4/CLDN3 signaling could exert potent anticancer activity against human ADC.

DISCUSSION

In recent years, the spectrum of non-small cell lung cancer (NSCLC) has continuously changed, and the proportion of ADC cases among lung cancer cases has increased year by year. Radical resection is still the most important treatment for stage I–IIIA patients, but metastasis is the most important cause of postoperative treatment failure in lung cancer. Many factors affect metastasis, such as postoperative pathological staging and pathological subtypes; however, the molecular mechanisms are poorly understood.

CITED4 is one of the three members of the CITED family in humans (CITED1, CITED2, CITED4). The CITED family members show not only fairly high sequence conservation but also share binding partners, such as HIF-1alpha, CBP/P300 and TFAP2. All three members of the CITED4 family have been shown to be involved in tumorigenesis to some degree.[14]

Here, we found that the expression of the transcriptional cofactor CITED4 was significantly increased in ADC tissues compared with paired normal lung tissues and that its expression could predict the risk of recurrence and metastasis in patients, which affects the DFS and OS of cancer patients. In addition, we found that CITED4 can promote the invasion and migration of ADC cells. We also confirmed this phenomenon in vivo, but the mechanism is not clear.

CLDN3 belongs to a family of proteins important in TJ formation and function. A previous study demonstrated that CLDN3 was frequently upregulated in human ADC, and its upregulation was significantly associated with poor DFS and OS in ADC patients. We also observed that forced expression of CLDN3 increased the malignant potential of ADC.[5] Moreover, we found that increased CLDN3 expression was accompanied by increased CITED4 expression. In colorectal cancer cells, phenotypic analysis of CITED4 shRNA knockdown cells demonstrated decreased cell proliferation and G2 cell cycle blockade. Microarray analysis identified many deregulated genes, and pathway analysis revealed genes linked to activated adherens junctions/TJs (including claudin-4).[14] The results also showed that CITED4 affects the level of secreted CLDN3, which in turn affects the invasion and migration of ADC cells. CITED4 functions as a molecular switch for CLDN3-mediated cell proliferation, migration and invasion. This explains how CITED4 affects the cell

cycle and hence lung cancer proliferation. Our study also found that how CITED4 affects the invasion and migration of ADC depends on the level of CLDN3. Culturing cells in medium lacking CLDN3 attenuated the effect of CITED4 on the invasion and migration of ADC cells.

CITED4 and CLDN3 are located in the nucleus and membrane, respectively, and thus, it is difficult for them to physically interact. To further explore the interaction between CITED4 and CLDN3, we introduced the key molecule CTNNB1 (β-catenin) of the WNT signaling pathway. In general, β-catenin not only enhances cell–cell adhesion by associating with cadherin complexes in the adherens junctions of the cell membrane but also functions as a transcriptional coactivator after interacting with TCF transcription factor complexes in the nucleus.[20] Our previous study demonstrated β-catenin involvement in epithelial mesenchymal transition.[21] We also predicted the interaction between CITED4 and CTNNB1 through bioinformatics and verified the interaction between them through co-IP. Knockdown of CITED4 in SW480 cells resulted in the deregulation of a large number of genes, several of which encode proteins involved in cell proliferation (β-catenin).[14] The activation of the WNT pathway caused the intracellular migration of CLDN3 and CITED4 cells. The observation of nuclear and perinuclear colocalization provides a spatial basis for the interaction between them. Both CITED4 and CTNNB1 can activate the promoter region of CLDN3, and the presence of both results in a greater level of activation. In the case of downregulation of CITED4, even if the WNT pathway is activated, the expression of CLDN3 cannot be induced. Protein studies further confirmed that although both CITED4 and CTNNB1 can activate the promoter activity of CLDN3, CLDN3 expression may be more dependent on CITED4. Therefore, the CITED4-CTNNB1-CLDN3 axis forms a complex that affects the invasion and metastasis of ADC through the key effector protein CITED4.

Furthermore, we confirmed the above phenomenon in vivo. Knockdown of CITED4 significantly inhibited tumor growth, and knockdown of CITED4 triggered the downregulation of CLDN3 and MMP-2 levels and also affected cell proliferation, as indicated by the Ki-67 index, which further confirmed the influence of CITED4 on the proliferation and invasion of ADC. Moreover, CLDN3 is the epithelial receptor for CPE, a potent cytolytic toxin.[19,22] CPE triggers the lysis of mammalian epithelial cells through interactions with claudin-3 and claudin-4 receptors, while cells that do not express CPE receptors (i.e., mesothelial cells and most healthy human tissues) are protected from the lethal effects of CPE because they fail to bind the toxin. Because claudin-3 and claudin-4 have been recognized as receptors for CPE).

At present, the application of CPE and its modified fraction is mainly focused on the treatment of ovarian cancer and drug-resistant ovarian cancer. Our previous experiments confirmed that CLDN3 exhibits similar expression trends (high expression) in ADC and ovarian cancer, indicating that CPE and its modified fraction could inhibit the growth of ADC cells in vivo. However, the antitumor effect of CPE on ADC cells with CITED4 downregulation was obviously attenuated. It was further confirmed that CITED4 could affect the level of CLDN3 and then affect the sensitivity of ADC to CPE in vivo.

Taken together, our findings indicate that CITED4 protein expression may enhance the invasion and metastasis of ADC and further affect the short- and long-term survival of ADC patients. The direct correlation between CITED4 and CLDN3 expression in ADC tissues further suggests that the level of CITED4 may affect the level of CLDN3. The key transcription factor of the classical Wnt signaling pathway CTNNB1, which acts as a bridge between them, also participates in this process. Thus, the CITED4-CTNNB1-CLDN3 axis plays a key role in the invasion and metastasis of ADC and can be used as a new target for future treatment.

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REFERENCES

1. Lee W, Jiang Z, Liu J, Haverty PM, Guan Y, Stinson J, et al. The mutation spectrum revealed by paired genome sequences from a lung cancer patient. Nature. 2010;465(7297):473–7.
2. Feldser DM, Kostova KK, Winslow MM, Taylor SE, Cashman C, Whittaker CA, et al. Stage-specific sensitivity to p53 restoration during lung cancer progression. Nature. 2010;468(7323):572–5.
3. Moldvay J, Jackel M, Paska C, Soltész I, Schaff Z, Kiss A. Distinct claudin expression profile in histologic subtypes of lung cancer. Lung Cancer. 2007;57(2):159–67.
4. Gao F, Duan X, Lu X, Liu Y, Zheng L, Ding Z, et al. Novel binding between pre-membrane protein and claudin-1 is required for efficient dengue virus entry. Biochem Biophys Res Commun. 2010;391(1):952–7.
5. Zhang L, Wang Y, Zhang B, Zhang H, Zhou M, Wei M, et al. Claudin-3 expression increases the malignant potential of lung adenocarcinoma cells: role of epidermal growth factor receptor activation. Oncotarget. 2017;8(14):23033–47.
6. Boyle S, Shioda T, Perantoni AO, de Caestecker M. Cited1 and Cited2 are differentially expressed in the developing kidney but are not required for nephrogenesis. Dev Dyn. 2007;236(8):2321–30.
7. Braganca J, Swingler T, Marques FI, Jones T, Eloranta JJ, Hurst HC, et al. Human CREB-binding protein/p300-interacting transactivator with ED-rich tail (CITED) 4, a new member of the CITED family, functions as a co-activator for transcription factor AP-2. J Biol Chem. 2002;277(10):8559–65.
8. Xu B, Qu X, Gu S, Doughman YQ, Watanabe M, Dunwoodie SL, et al. Cited2 is required for fetal lung maturation. Dev Biol. 2008;317(1):95–105.
9. Huang T, González YR, Qu D, Huang E, Safarpour F, Wang E, et al. The pro-death role of Cited2 in stroke is regulated by E2F1/4 transcription factors. J Biol Chem. 2019;294(21):8617–29.
10. Qu X, Lam E, Doughman YQ, Chen Y, Chou YT, Lam M, et al. Cited2, a coactivator of HNF4alpha, is essential for liver development. EMBO J. 2007;26(21):4445–56.
11. Jayaraman S, Doucet M, Lau WM, Kominsky SL. CITED2 modulates breast cancer metastatic ability through effects on IKKalpha. Mol Cancer Res. 2016;14(8):730–9.

12. Chou YT, Wang H, Chen Y, Danielpour D, Yang YC. Cited2 modulates TGF-beta-mediated upregulation of MMP9. Oncogene. 2006;25 (40):5547–60.
13. Fox SB, Braganca J, Turley H, Campo L, Han C, Gatter KC, et al. CITED4 inhibits hypoxia-activated transcription in cancer cells, and its cytoplasmic location in breast cancer is associated with elevated expression of tumor cell hypoxia-inducible factor 1alpha. Cancer Res. 2004;64(17):6075–81.
14. Rogers MA, Kalter V, Marcias G, Zapatka M, Barbus S, Lichter P. CITED4 gene silencing in colorectal cancer cells modulates adherens/tight junction gene expression and reduces cell proliferation. J Cancer Res Clin Oncol. 2016;142(1):225–37.
15. Hsieh CH, Chou YT, Kuo MH, Tsai HP, Chang JL, Wu CW. A targetable HB-EGF-CITED4 axis controls oncogenesis in lung cancer. Oncogene. 2017;36:2946–56.
16. Robbs BK, Cruz AL, Werneck MB, Mognol GP, Viola JP. Dual roles for NFAT transcription factor genes as oncogenes and tumor suppressors. Mol Cell Biol. 2008;28(23):7168–81.
17. Jiang L, Yang YD, Fu L, Xu W, Liu D, Liang Q, et al. CLDN3 inhibits cancer aggressiveness via Wnt-EMT signaling and is a potential prognostic biomarker for hepatocellular carcinoma. Oncotarget. 2014;5 (17):7663–76.
18. Ahmad R, Kumar B, Chen Z, Chen X, Müller D, Lele SM, et al. Loss of claudin-3 expression induces IL6/gp130/Stat3 signaling to promote colon cancer malignancy by hyperactivating Wnt/beta-catenin signaling. Oncogene. 2017;36(47):6592–604.
19. Walther W, Petkov S, Kuvardina ON, Aumann J, Kobelt D, Fichtner I, et al. Novel *Clostridium perfringens* enterotoxin suicide gene therapy for selective treatment of claudin-3- and -4-overexpressing tumors. Gene Ther. 2012;19(5):494–503.
20. Li J, Zhou BP. Activation of ß-catenin and Akt pathways by Twist are critical for the maintenance of EMT associated cancer stem cell-like characters. BMC Cancer. 2011;11:49.
21. Wang C, Su Y, Zhang L, Wang M, You J, Zhao X, et al. The function of SARI in modulating epithelial-mesenchymal transition and lung adenocarcinoma metastasis. PLoS One. 2012;7(9):e38046.
22. English DP, Santin AD. Claudins overexpression in ovarian cancer: potential targets for *Clostridium perfringens* enterotoxin (CPE) based diagnosis and therapy. Int J Mol Sci. 2013;14(5):10412–37.

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