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ORIGINAL ARTICLE

Overexpression of ANLN in lung adenocarcinoma is associated with metastasis

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Keywords
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Abstract

Background: ANLN has been identified as an actin-binding protein and previous studies have suggested that ANLN is associated with actin cytoskeletal dynamics. Lung adenocarcinoma is a poor prognosis tumor. Metastasis is a very common complication and is regarded as the main cause for an unsatisfactory treatment outcome. Whether ANLN is involved in the metastasis of lung adenocarcinoma is unknown.

Methods: We performed immunohistochemical staining and analyzed the correlation between the expression level and pathological parameters. We tested the migration and invasion of A549 and PC9 cell after interference with ANLN expression by Transwell and scratch wound healing assays. Protein expression of E-cadherin, vimentin and N-cadherin were detected using Western blotting and immunofluorescence staining. The same experiment was also tested after overexpression of ANLN.

Results: The metastasis of patients with high expression of ANLN was significantly more than that of patients with low expression of ANLN. In vitro, after interfering with ANLN expression, E-cadherin and vimentin expression were increased and N-cadherin expression was decreased in A549 and PC9 cells. Migration and invasion ability of A549 and PC9 cells were decreased,vice versa.

Conclusion: Our study suggests that the expression of ANLN in lung adenocarcinoma is associated with metastasis of cancer cells. ANLN may be involved in the metastasis of lung adenocarcinoma by promoting epithelial mesenchymal transformation of tumor cells.

Introduction

Lung cancer is one of the most common tumors in China and also the world.[1,2] More than 80% of lung cancers are non-small cell lung cancer.[3]The most common pathological type of NSCLC is adenocarcinoma.[4]Despite significant advances in early diagnosis and treatment, the five-year survival rate for non-small cell lung cancer is still less than 15%.[1,5] One of the most important reasons is that non-cell lung cancer is prone to metastasis,[6,7] and it is therefore necessary to study the mechanism of invasion and metastasis of lung adenocarcinoma.

Anillin actin-binding protein (ANLN) encodes an actin-binding protein that plays a role in cell growth and migration.[8] Previous studies have found that ANLN can regulate actin cytoskeletal dynamics in podocytes.[9] Previous studies have also confirmed that ANLN expression is associated with prognosis in patients with breast,[10] bladder,[11] and colorectal cancers.[12] Pandi *et al.*[13] confirmed that ANLN is a Wnt/β-catenin responsive gene in gastric cancer and can regulate the proliferation of gastric cancer cells. So far, there have been few studies on the role of ANLN in tumorigenesis and development of lung adenocarcinoma. Whether ANLN is involved in the metastasis of lung adenocarcinoma has not previously been well studied.

Methods

Patients

This study included 102 patients with lung adenocarcinoma treated in Tianjin Medical University Cancer Institute and Hospital between 2011 to 2016. The use of samples and information for these patients was approved by the Ethics Committee of Tianjin Medical University Cancer Institute and Hospital, and both of them had written informed consent.

Cell culture and treatments

Human lung adenocarcinoma tumor cells A549 and PC9 cells were stored in our Institute of Oncology. A549 and PC9 cells were cultured in a RPMI 1640 medium supplemented with 1% penicillin/streptomycin mixture and 10% fetal bovine serum (Biological Industries, Israel). The cells were maintained in a humidified incubator at 37°C containing 5% CO_2. SiRNA for ANLN was purchased from Ribobio (Guangzhou, China).

Transwell invasion assay

The invasive and migration ability were determined by Transwell assay (Corning, NY, USA). To determine the invasion ability, the matrigel matrix (Corning, NY, USA) was diluted in a ratio of 1:5 with an empty medium and added to the upper chamber of the transwell chamber. Tumor cells were resuspended in empty medium and added to the upper chamber at a density of 500/μL, and 500 μL complete RPMI 1640 medium was added to the bottom side of the Transwell assay. After incubating in a cell culture incubator for 28 hours, the cells in the upper chamber were wiped off, and the cells that migrated to the lower side of the chamber were stained. Lower cells were stained with a Thermo Scientific Three-step stain kit (ThermoFisher Scientific, USA) according to the instruction manual. To determine the migration ability, cells were added to the upper chamber without matrigel and incubated for eight hours. The dyeing procedure is as described above. Cells were counted under a 200× microscope field and statistically analyzed.

Immunohistochemical staining

The tissue sections were hydrated with xylene and gradient alcohol after two hours at 60°C. The antigen was repaired with EDTA antigen repair solution, horse serum was used as blocking solution, and the antibody was incubated at 4°C overnight. After rewarming for two hours at room temperature, the secondary antibody was incubated at 37°C for two hours. A DAB color development kit (ZLI-9018) was used to develop, it was counterstained with hematoxylin, and then dehydrated with gradient ethanol and xylene to seal. Images were taken at 100× and 400× by Universal upright fluorescence microscope and imaging system (OLYMPUS BX61, Japan).

Scratch wound healing assay

The migration ability of A549 and PC9 cells was determined by scratch wound healing assay. A549 and PC9 cells were harvested and 8×10^5 cells plated in six-well plates. When the plates yielded cells at about 80% confluence, the monolayer was scraped in a straight line to create a scratch using a 10 μl pipette tip. Debris was then removed using phosphate buffered saline and serum-free medium was added. Photographs were taken under a microscope at 0 and 24 hours.

Table 1 Primers used

CDH1	**Forward primer** TGCCCAGAAAATGAAAAAGG
	Reverse primer RGTGTATGTGGCAATGCGTTC
CDH2	**Forward primer** GACAATGCCCCTCAAGTGTT
	Reverse primer CCATTAAGCCGAGTGATGGT
Vimentin	**Forward primer** GAGAACTTTGCCGTTGAAGC
	Reverse primer TCCAGCAGCTTCCTGTAGGT
Snail	**Forward primer** ACCCCACATCCTTCTCACTG
	Reverse primer TACAAAAACCCACGCAGACA
Slug	**Forward primer** CTTTTTCTTGCCCTCACTGC
	Reverse primer GCTTCGGAGTGAAGAAATGC

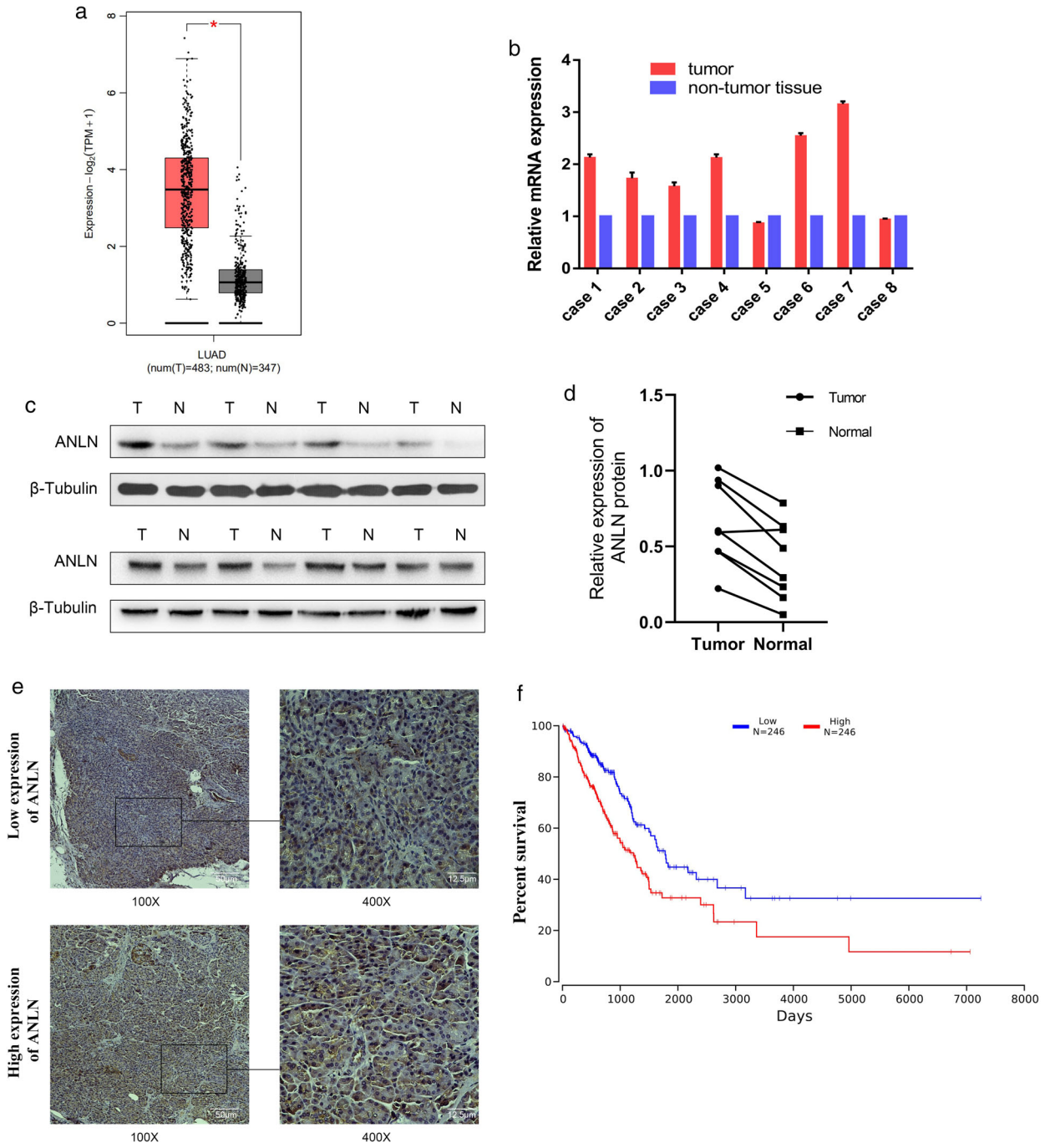

Figure 1 (**a**) Expression of ANLN in LUAD and normal lung tissues was analyzed in the TCGA database. (**b**–**d**) ANLN expression of RNA and protein in eight pairs of LUAD and matched paracancerous tissues (**e**). High and low expression of ANLN in LUAD tissues (**f**). Overall survival is associated with tumor ANLN level in 492 LUAD patients.

Western blotting

Total proteins from cultured cells were lysed with SDS lysis buffer. Protein concentration was assayed using a BCA assay kit (Thermo Fisher Scientific, USA). Equal quantities of protein were separated on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto polyvinylidene fluoride membranes (Merk, Germany), and then reacted with primary antibodies against ANLN, CDH1, CDH2, Snail, Vimentin, Slug, and β-Tubulin (Proteintec, China) at 4°C overnight. The membranes were then

Table 2 Association between ANLN mRNA expression and clinical characteristics in LUAD patients

Characteristics	ANLN low expression	ANLN high expression	*P*
Gender			
Male	32	28	0.8875
Female	23	19	
Age (year)			
≥60	35	25	0.2854
<60	20	22	
pT			
T1/T2	29	31	0.1760
T3/T4	26	16	
pN			
N0	38	22	0.0226*
N1/N2	17	25	
pTNM			
I	31	29	0.5851
II-III	24	18	

*$P < 0.05$ indicates a significant association between the variables. LUAD, lung adenocarcinoma.

washed with TBST Buffer and incubated for one hour with Goat anti-Mouse/Rabbit IgG(H+L)-HRP (Ray antibody Biotech, Beijing, China). The bands were visualized using electrochemiluminescent luminous fluid (Merk, Germany). Photographs were taken using by a Tanon 6600 Luminescence imaging workstation (Tanon, China).

RNA extraction and real-time polymerase chain reaction

Total RNA was extracted from A549 and PC9 cells using TRIzol reagent (TaKaRa, China). Primer sequences are shown in Table 1. Reverse transcription Polymerase Chain Reaction (RT-PCR) was performed using an all-in-one kit (Bimake, China) according to the manufacturer's instructions and the T100 Thermal Cycler (Bio-Rad, USA). Real-time PCR amplification was performed using a 2X SYBR Green qPCR Master Mix (Bimake, China). Quantitative PCR (qPCR) was performed by the System (Bio-Rad, USA). PCR products were verified by melting curve analysis. The relative messenger RNA (mRNA) levels of target genes were calculated using the $2-\Delta\Delta Ct$ method.

Statistical analysis

All statistical analyses were performed using SPSS version 19.0 (SPSS Inc., Chicago, IL, USA), GraphPad Prism software (La Jolla, CA, USA), Adobe Photoshop CS6 (San Jose, CA, USA), and Image J software (NIH). Data are expressed as mean standard deviation. Comparisons were analyzed using a Chi-square test and P value < 0.05 was considered statistically significant.

Results

Expression of ANLN elevated in lung adenocarcinoma

Expression of ANLN in lung adenocarcinoma and matched paracancerous tissues was analyzed in the TCGA database by GEPIA and oncolnc. The expression of ANLN in 483 samples of cancer tissues was significantly higher than that in 347 samples of normal tissues (Fig 1a $P = 0.01$). We examined the expression of RNA and protein in ANLN in eight pairs of fresh lung adenocarcinoma and matched paracancerous tissues by q-PCR and Western Blot (Fig 1b–d). The expression level of ANLN mRNA in lung adenocarcinoma was significantly higher than that in adjacent tissues. The same was true for protein levels.

Poor prognosis in patients with high expression of ANLN

Immunohistochemical staining of tissue sections showed that ANLN expression varies in different patients (Fig 1e). The expression of ANLN in lung adenocarcinoma patients in the TCGA database was divided into high and low groups, and the relationship analyzed between ANLN expression and overall survival (OS). The OS in patients with high expression of ANLN was significantly shorter than those in low expression group (Fig 1f median survival time: 689.5 days vs. 601 days, $P < 0.0001$).

ANLN associated with metastasis of lung adenocarcinoma

The relationship between ANLN expression and TNM staging was analyzed (Table 2) and indicated that there was significant association between ANLN and pN.

Lung cancer cell migration and invasion ability decrease after expression of ANLN

To further confirm the effect of ANLN on the migration and invasion of lung adenocarcinoma cells, we performed Transwell and scratch wound healing assay after interfering the expression of ANLN by using SiRNA. As shown in Fig 2, 24 hours passed after the cells were wounded, and the migratory activity was inhibited when ANLN was knocked down in both A549 ($P = 0.0054$) and PC9 ($P = 0.0076$) cell lines (Fig 2a–f). Both the migratory activity and invasiveness of siANLN cells were obviously attenuated (Fig 2e–h).

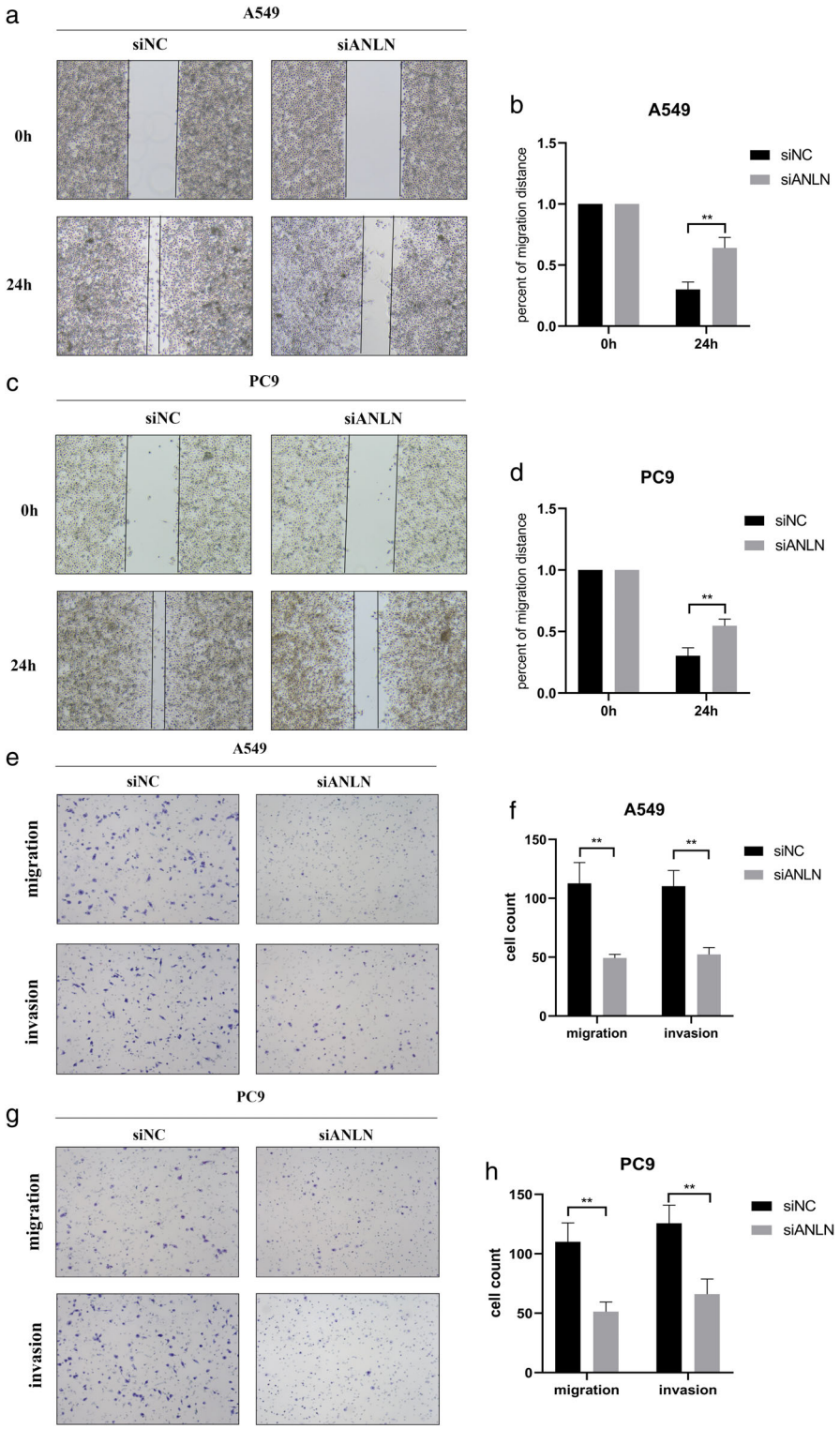

Figure 2 (**a**–**d**) A549 and PC-9 cells were transfected with siRNA of ANLN, the motility of the cells was evaluated 24 hours after transfection by wound healing assay. (**e**–**h**). Same cells described in (**a**–**d**) were used in Transwell migration and invasion assay.

ANLN may affect migration and invasion via EMT

In order to explore the mechanism of ANLN affecting migration and invasion, we conducted bioinformatics analysis. Epithelial-mesenchymal transition (EMT) is one of the most popular research mechanisms related to migration and invasion. The relationship between ANLN and EMT was analyzed by GSEA and TCGA database (Fig 3).

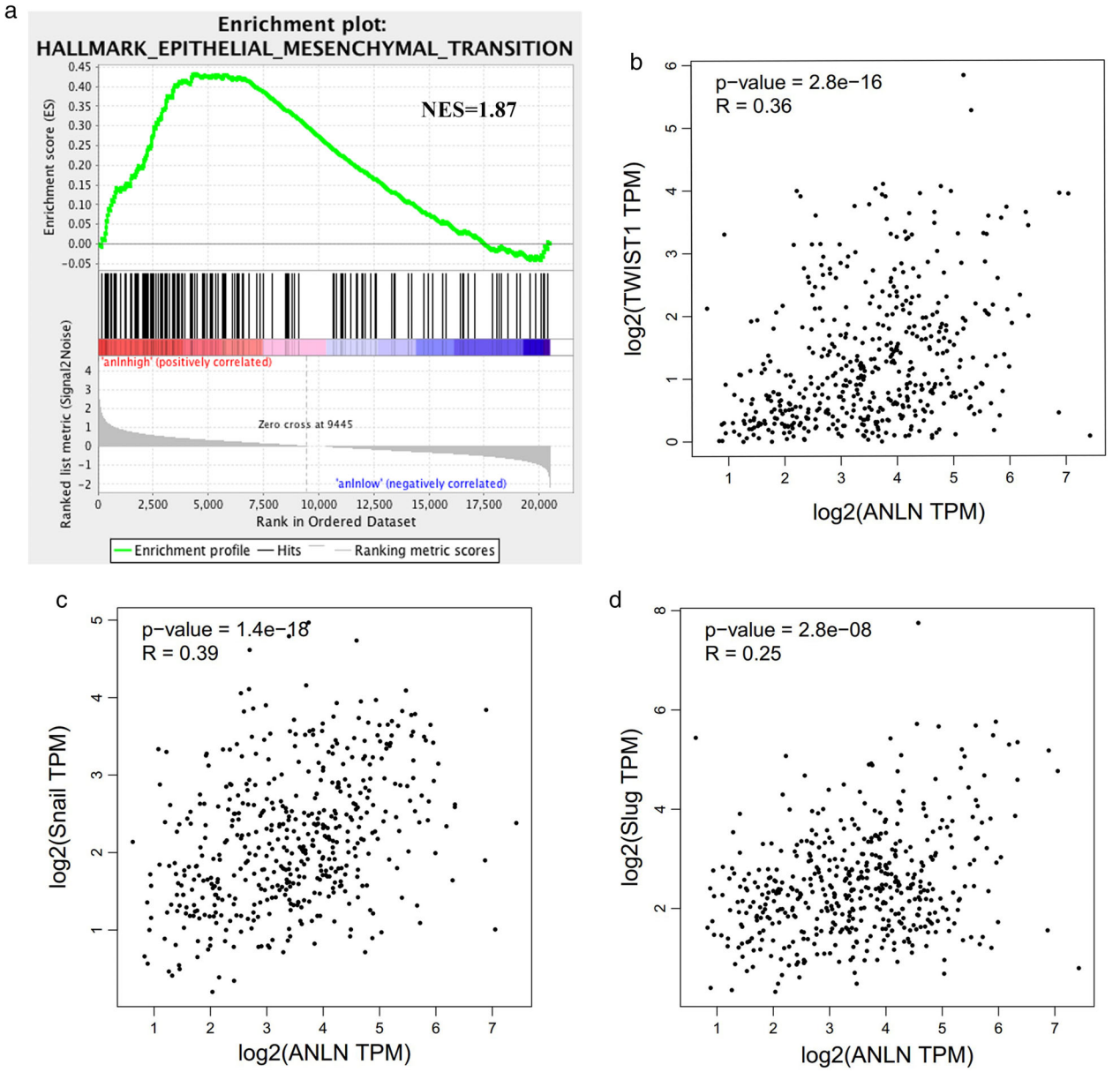

Figure 3 (**a**) Gene-set enrichment analysis (GSEA) of ANLN. (**b**,**c**) The correlation between ANLN and TWIST1, Snail and Slug was analyzed in the TCGA database.

We examined the correlation between mRNA levels of ANLN and EMT biomarkers TWIST1, Snail and Slug in LUAD patients from The Cancer Genome Atlas (TCGA) database. There was exact correlation between the mRNA level of TWIST1 and ANLN (R = 0.36 $P < 0.0001$), as well as those of Snail and ANLN (R = 0.39 $P < 0.0001$) or Slug and ANLN (R = 0.25 $P < 0.0001$). As indicated in Fig 4, in both A549 and PC9 cell lines, we found that the expression of mesenchymal markers snail, slug and vimentin were decreased when ANLN was knocked down, while epithelial marker CDH1 (E-Cadherin) was upregulated (Fig 4a,b). The tendency of relative mRNA levels was quite similar to proteins, CDH1 was upregulated ($P < 0.0001$) by knocking down ANLN, and CDH2 (N-Cadherin), Vimentin, Snail and Slug were both decreased in A549(CDH2: $P < 0.0001$, Vimentin: $P < 0.0001$, Snail: $P = 0.0002$, Slug: $P < 0.0001$) and PC9(CDH2: $P < 0.0001$, Vimentin: $P = 0.0002$, Snail: $P < 0.0001$, Slug: $P = 0.0002$) cell lines (Fig 4c,d).

Discussion

The prognosis of patients with lung adenocarcinoma has improved significantly with early diagnosis, early treatment, and the widespread use of targeted drugs.[14]Metastasis

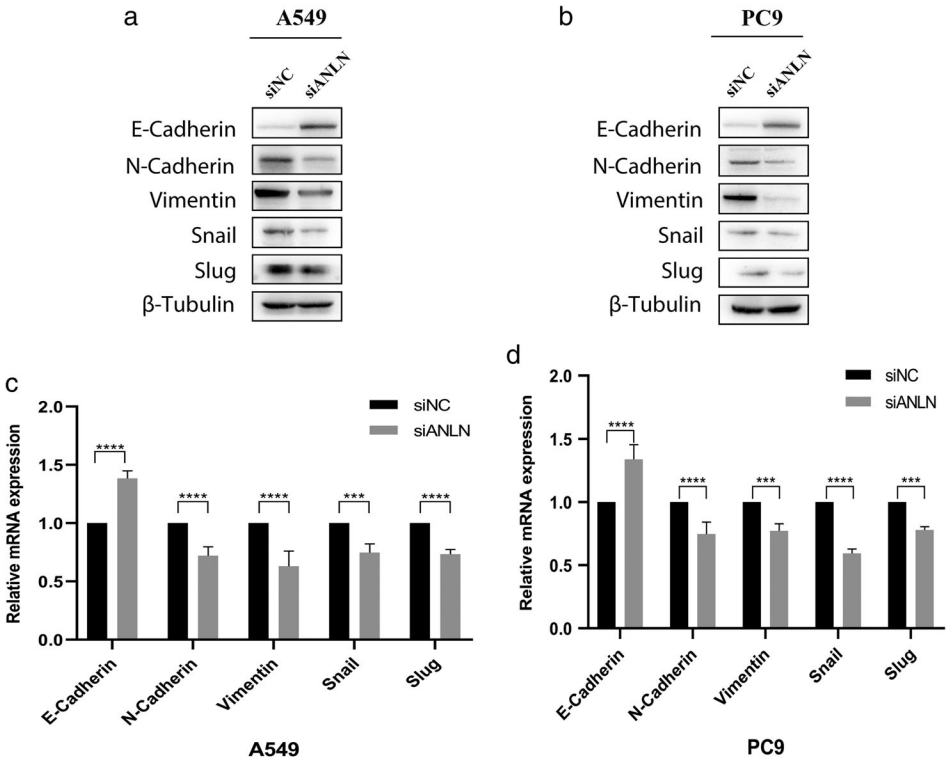

Figure 4 (**a**,**b**) Western blot of protein from A549 and PC9 which were transfected with control and ANLN siRNA. (**c**,**d**) q-PCR of cDNA from the same cells described in (**a**,**b**).

becomes an increasingly important factor affecting the prognostic factors of patients with lung adenocarcinoma. Therefore, there is an urgent need to study the molecular mechanism of lung adenocarcinoma metastasis. In our study, we found a significant positive correlation between ANLN expression and lung adenocarcinoma metastasis. The migration and invasion of lung adenocarcinoma cells A549 and PC9 are greatly reduced after interference of the expression of ANLN. However, due to the limitations of the conditions, we did not conduct an experimental study related to overexpression of ANLN. However, since the expression of ANLN in lung adenocarcinoma is significantly higher than that in adjacent tissues, we believe that it is more important to reduce the expression of ANLN by experimental means to avoid a "ceiling effect". The analysis results of existing third-party public database data and our experimental data also explain the influence of ANLN on lung adenocarcinoma metastasis (Fig S1).

The impact of ANLN on the biological behavior of cells is multifaceted, although existing research has confirmed the impact of ANLN on tumor cell cycle and other aspects.[15] In this article, we focus on the migration and invasion of lung adenocarcinoma. With the advancement of surgical resection techniques and the use of a variety of new drugs, the treatment of primary tumors is becoming more and more effective. The metastasis of lung adenocarcinoma in clinical work is the main cause of unsatisfactory treatment. In the advanced stages of lung adenocarcinoma, the transfer to various organs, such as bone, brain, liver and other organs, can cause corresponding symptoms, which often cause great pain in patients. Therefore, we first explored the relationship between ANLN and migration and invasion of lung adenocarcinoma.

The existing data explores the specific mechanism of ANLN affecting EMT and this study does not report mechanisms other than EMT. However, due to the numerous mechanisms of tumor cell metastasis it would be difficult to cover all aspects in one article. ANLN, as a cytoskeletal binding protein, may affect actin dynamics and cytoskeletal signaling after altering the expression of ANLN. From another perspective, different mechanisms may possibly play a leading role under different factors. In conclusion, in this study, we demonstrated that increased expression of ANLN in LUAD is associated with clinicopathological parameters, especially N stage and M stage, and patients with high expression of ANLN have shorter survival rates. ANLN is therefore a promising biomarker and potential therapeutic target for LUAD.

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Disclosure

No authors report any conflict of interest.

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Supporting Information

Additional Supporting Informationmay be found in the online version of this article at the publisher's website:

Figure S1 The expression of ANLN in the absence of serum-induced apoptosis did not affect the apoptosis of A549 and PC9.

