

# Vinculin expression in non-small cell lung cancer

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## Abstract

**Objective:** This study aimed to explore the expression of vinculin in non-small cell lung cancer (NSCLC) and to analyze its correlation with clinical features and prognosis of NSCLC.

**Methods:** The expression of vinculin in cancer tissues and paracancer tissues was detected by real-time PCR, western blotting, and immunohistochemistry. Correlations between vinculin-positive expression and clinical features were analyzed by Pearson correlation analysis, and those between vinculin expression and prognosis were analyzed by Cox multivariate analysis.

**Results:** Vinculin expression was significantly lower in cancer tissues than in paracancer tissues. Pearson correlation analysis showed that vinculin expression was significantly correlated with tumor–node–metastasis (TNM) stage and lymph node metastasis in NSCLC. Cox multivariate analysis showed that vinculin-negative expression and TNM stage were independent risk factors for NSCLC prognosis. Kaplan–Meier analysis showed that the 5-year overall survival (OS) rate was 20.51% for all NSCLC patients, and was significantly higher for vinculin-positive patients with NSCLC than vinculin-negative patients.

**Conclusions:** Vinculin gene transcription is inhibited in NSCLC, and low vinculin expression promotes malignancy in NSCLC. Therefore, vinculin could be used as a prognostic marker for NSCLC and a potential target for its treatment.

## Keywords

Non-small cell lung cancer, Vinculin, prognosis, overall survival, tumor–node–metastasis, lymph node metastasis

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## Introduction

Lung cancer is one of the most malignant tumors with the highest mortality rate in the world.<sup>1,2</sup> According to histopathological features, it can be divided into small cell lung cancer and non-small cell lung cancer (NSCLC).<sup>3</sup> NSCLC is a clinically common type of lung cancer. Although NSCLC treatment has improved in recent years, the 5-year overall survival (OS) rate for NSCLC patients is still less than 20%.<sup>4</sup> Poor prognosis seriously affects the quality of life of patients and brings a heavy financial burden to families and society. Many advances have been made in molecular biology, but the pathogenesis of NSCLC is still poorly understood.<sup>5</sup> Moreover, many NSCLC patients are diagnosed at an advanced stage, often with associated tumor invasion and metastasis, which is the main cause of treatment failure and death in these patients.

Recent studies have shown that the invasion and metastasis of malignant tumors are related to the destruction of extracellular matrix and cell migration. In this process, tumor cell adhesion, cytoskeletal protein reconstitution, and recombination play key roles.<sup>6</sup> Cell-matrix adhesion and cell-cell adhesion are critical for cell metabolism, protein synthesis, cell survival, and cancer metastasis. Integrins and cadherins are transmembrane receptors predominantly involved in these functions; the former are responsible for cell-matrix adhesion, while the latter play an important role in cell-cell adhesion.<sup>7</sup> Integrin-mediated cell-matrix adhesion occurs in all tumor stages, and vinculin is involved in this process.

Vinculin acts as a tumor suppressor gene that affects tumorigenesis, metastasis, and invasion.<sup>8</sup> Tumor metastasis requires cells to invade connective tissue, which is essentially a mechanical event involving adhesion, shape changes, movement, and force generation.<sup>6</sup> Vinculin has no enzymatic

activity and regulates adhesion by directly binding actin, stimulating actin polymerization, and remodeling. Several studies<sup>9-12</sup> have shown that cells with low vinculin expression have increased metastatic potential and are closely related to the degree of tumor malignancy. However, the expression and clinical importance of vinculin in NSCLC have rarely been reported.

Therefore, this study aimed to explore the correlation of vinculin expression with clinical features and prognosis in NSCLC. Patients with TNM I-III NSCLC who underwent surgical treatment were enrolled in this study. Real-time PCR and western blotting were used to detect the mRNA and protein expression of vinculin in cancer tissues and paracancer tissues, while immunohistochemistry detected vinculin expression in tissues. The relationship between vinculin expression, clinical characteristics, and the 5-year OS rate of NSCLC patients was analyzed.

## Patients and methods

### *Patients and tissue samples*

Seventy-eight patients with primary NSCLC who underwent surgery at the People's Hospital of Rugao from January 2011 to December 2014 were enrolled in the study. None of the 78 patients had received radiotherapy, chemotherapy, or other anti-cancer treatments before surgery. Patients received conventional radiotherapy and chemotherapy after surgery. Clinical data are summarized in Table 1.

NSCLC tissues and corresponding paracancer tissues were collected from surgical resection specimens and snap-frozen in liquid nitrogen. All patients provided written informed consent. This study was approved by the Institutional Review Board of the People's Hospital of Rugao (IRB20110132).

**Table 1.** The relationship between vinculin expression and clinicopathological characteristics.

	N	Vinculin		$\chi^2$	P
		(-)	(+)		
Sex				0.212	0.645
Male	62	47	15		
Female	16	13	3		
Smoking history				0.148	0.700
Yes	49	37	12		
No	29	23	6		
Age (years)				0.325	0.569
≤60岁	26	21	5		
>60岁	52	39	13		
Histological type				0.210	0.647
Squamous cell carcinoma	34	27	7		
Adenocarcinomas	44	33	11		
Differentiation				0.575	0.750
Well	6	4	2		
Moderate	37	28	9		
Poor	35	28	7		
Tumor size				0.216	0.642
<3 cm	47	37	10		
≥3 cm	31	23	8		
Lymphatic metastasis				<b>8.187</b>	<b>0.004</b>
No	42	27	15		
Yes	36	33	3		
TNM stage				<b>9.339</b>	<b>0.009</b>
I	25	14	11		
II	32	27	5		
III	21	19	2		

TNM, tumor–node–metastasis

Note: Boldface P<0.01.

### Real-time PCR

Total RNA of cancer and paracancer samples from six NSCLC patients was isolated using the UNIQ-10 Spin Column RNA Purification Kit (Sangon, Shanghai, China) according to the manufacturer's instructions. cDNA was prepared from 2–6 µg of total RNA using the cDNA Synthesis Kit (Fermentas, Burlington, Canada) following the manufacturer's instructions. Real-time PCR was performed to detect Vinculin mRNA using the SYBR Green Master Mix (Roche, Basel,

Switzerland) according to the manufacturer's instructions in the Corbett RG-6000 PCR system (QIAGEN, Dusseldorf, Germany). Reactions contained 1 µl of template cDNA, 10 µl master mix, 12 µl double-distilled H<sub>2</sub>O, and 1 µl (10 µM/L) primers. The primers were synthesized as follows: GAPDH: sense 5'-GCAAGTTCAACGGCACAG-3', antisense 5'-GCCAGTAGACTCCACGACAT-3'; and vinculin: sense 5'-ATGGCCACCATGCCAGTGTTCATACGCGC-3', antisense 5'-ATGCCTACTGGTACCAGGAGTCTT-3'. PCR conditions were:

initial denaturation at 95°C for 5 minutes, followed by 40 cycles of 94°C for 15 seconds, 60°C for 20 seconds, and 72°C for 20 seconds. Fold-changes in gene expression were analyzed by the  $2^{-\Delta\Delta C_t}$  method.

### Western blotting

Western blotting was performed as described previously.<sup>13</sup> Briefly, proteins were extracted from previously frozen cancer and paracancer tissues by lysing them in radioimmunoprecipitation assay buffer containing complete protease inhibitor cocktail (Pierce Chemical Company, Rockford, IL, USA). The protein concentration was determined with the Bradford assay (Bio-Rad, Hercules, CA, USA). Denatured protein (200 µg) was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to a polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). The membrane was blocked with 5% nonfat milk in Tris-buffered saline–Tween 20 for 2 hours, then incubated with rabbit anti-vinculin (1:800; Abcam, Cambridge, MA, USA) and rabbit anti-β-actin antibody (1:1000; Abcam) at room temperature for 12 hours. The membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG secondary antibody (1:2000; Abcam) for 2 hours after washing with rinse buffer at room temperature. Membranes were developed with enhanced chemiluminescence (Pierce Protein Research Products), and optical densities were analyzed by ImageMasterTM2D Platinum (Version 5.0; Amersham Pharmacia Biotech GE, Shanghai, China).

### Immunohistochemistry

Immunohistochemistry was performed as described previously.<sup>14</sup> Briefly, tissue slides were sequentially incubated with rabbit anti-vinculin (1:500; Abcam) and HRP-

conjugated goat anti-rabbit IgG antibodies (1:1000; Abcam). Counterstaining was performed with hematoxylin. Slides were observed under a DMR fluorescence microscope (Leica, Solms, Germany).

Results were analyzed independently by two expert pathologists who were blinded to the clinical data. A semi-quantitative histopathology (H) score was obtained from the staining intensity score (0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining) × percentage score (0–300). An H-score lower than the median was considered to be negative.

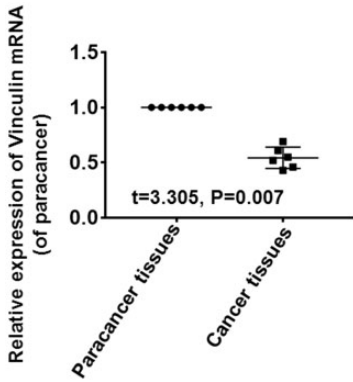
### Statistical methods

Data are shown as means ± SD or % prevalence. Data were analyzed by SPSS 21.0 software (SPSS Inc., Chicago, IL, USA). The independent t test was used to analyze vinculin mRNA and protein expression. The Pearson chi-square test or Fisher's exact test were used to analyze the correlation between vinculin expression and clinicopathological characteristics. The 5-year OS rate was estimated using the Kaplan–Meier method. Multivariate analysis was performed using the Cox regression model. *P*-values less than 0.05 were considered statistically significant.

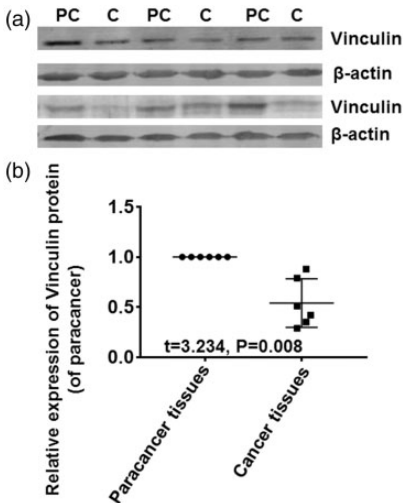
## Results

### Vinculin mRNA and protein expression decreased in NSCLC

Vinculin mRNA expression was around 1.8-fold higher in paracancer samples than in cancer samples, representing a significant difference (*P*=0.007; Figure 1). Similarly, semi-quantitative western blotting analysis revealed significantly higher vinculin protein levels in paracancer samples than cancer samples (*P*=0.008; Figure 2).



**Figure 1.** Vinculin mRNA expression was detected by RT-PCR and shown to be 1.8-fold higher in paracancer samples than in cancer samples, which is a significant difference ( $P = 0.007$ ).



**Figure 2.** Vinculin protein expression was detected by semi-quantitative western blotting, which showed significantly higher levels in paracancer samples than in cancer samples ( $P = 0.008$ ). PC = paracancer tissues, C = cancer tissues.

**The vinculin-positive rate decreased with TNM stage in NSCLC**

Immunohistochemical analysis revealed a large number of vinculin-positive cells in paracancer tissues, with vinculin protein

mainly located in the cell membrane and cytoplasm (Figure 3a). The positive rate of vinculin in paracancer tissues was 100.00%. However, only a small number of lightly stained vinculin-positive cells were observed in cancer tissues, and the positive rates of vinculin were 44.00% (TNM I), 15.63% (TNM II), and 9.52% (TNM III) (Figure 3b).

**Correlations between vinculin expression and clinicopathologic features**

An overview of vinculin expression and clinicopathological features is summarized in Table 1. Vinculin expression was significantly correlated with TNM stage and lymph node metastasis ( $P < 0.05$ ). However, no significant associations were observed between vinculin expression and other clinicopathologic features such as histological type, patient age, sex, tumor size, smoking history, and differentiation (Table 1).

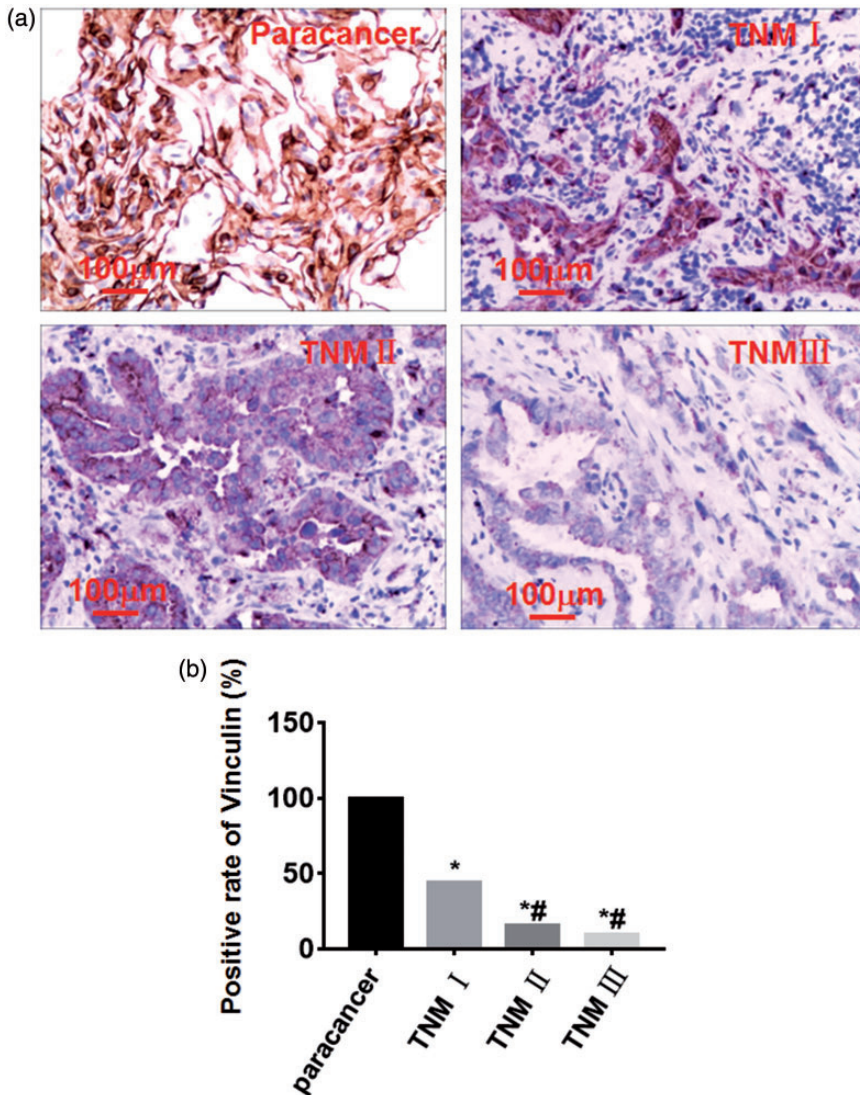
**Vinculin positive rate and 5-year OS**

The median OS of vinculin-positive patients was 54 months (95% confidence interval (CI): 46.52–61.48), compared with 42 months (95% CI: 38.32–45.68) for vinculin-negative patients. Therefore, vinculin-negative patients had a decreased survival time compared with vinculin-positive patients, and this difference was statistically significant  $P = 0.006$ ; Figure 4).

**Low vinculin expression is an independent prognostic factor for NSCLC**

The Cox multivariate regression model showed that vinculin-negative expression and TNM stage were independent prognostic indicators for NSCLC patients ( $P < 0.05$ ). Low expression of vinculin (hazard ratio (HR): 0.237; 95% CI: 0.107–0.527) and high TNM stage (HR: 0.377; 95% CI: 0.194–0.735) were associated with





**Figure 3.** The vinculin positive rate was detected by immunohistochemistry. (a) Immunohistochemistry revealed a large number of vinculin-positive cells in paracancer tissues, mainly in the cell membrane and cytoplasm. Only a small number of lightly stained vinculin-positive cells were observed in cancer tissues. (b) The positive rate of vinculin in paracancer tissues was 100.00% compared with 44.00% (TNM I), 15.63% (TNM II), and 9.52% (TNM III). \* vs. paracancer specimens,  $P < 0.05$ ; # vs. TNM I specimens,  $P < 0.05$ .

an increased risk of death. Other clinicopathologic features such as histological type, patient age, sex, tumor size, smoking history, lymphatic metastasis, and differentiation were not associated with poor survival of NSCLC patients (Table 2).

## Discussion

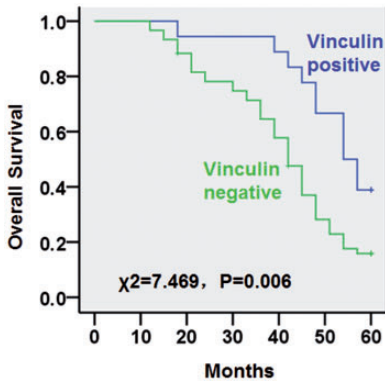
Current research suggests that vinculin protein is involved in the invasion and metastasis of a variety of tumors.<sup>8</sup> Vinculin is a 117-kDa protein consisting of 1066 amino

acids that was shown by animal developmental models to have a key role in cell–cell and cell–matrix adhesion. Previous studies indicated that vinculin affects adhesion protein turnover and contractility,<sup>15,16</sup> controls cell signaling processes,<sup>17–19</sup> and provides a mechanical link between the extracellular matrix and the actin cytoskeleton.<sup>20–23</sup> Consequently, knockout of vinculin protein affects cell adhesion and cell

migration, which are both critical processes for embryonic development. Moreover, the abnormal expression of vinculin is associated with a variety of diseases, such as cancer and cardiomyopathy.<sup>6,7,24</sup> In this study, we explored the correlation of vinculin expression with clinical features and prognosis in NSCLC patients.

Previous studies showed that the loss of vinculin was associated with the metastasis of a variety of tumors.<sup>8</sup> Metastasis is the main reason for poor prognosis and high recurrence, which is the distinguished phenotype of malignant tumors. The adhesion ability of fibroblasts isolated from vinculin-deficient mice was previously shown to be decreased and their migration ability increased compared with control cells, while restoring vinculin expression reversed this;<sup>25</sup> vinculin-null carcinoma cells showed similar findings. Therefore, loss of vinculin protein expression may induce changes in cell signal transduction, thereby directly promoting cell tumorigenicity.

The present study revealed significantly lower vinculin mRNA and protein expression in cancer tissues than paracancer tissues, with the lowest expression seen in TNM III cancer tissues. Paracancer tissues showed a 100% positive rate for vinculin, which compared with 44.00%, 15.63%, and 9.52% for NSCLC TNM I, TNM II, and



**Figure 4.** The relationship between the vinculin positive rate and 5-year OS. The median OS of vinculin-positive patients was 54 months (95% CI, 46.52–61.48) compared with 42 months for vinculin-negative patients (95% CI, 38.32–45.68). Vinculin-negative patients had a significantly decreased survival time compared with vinculin-positive patients ( $P = 0.006$ ).

**Table 2.** Cox multivariate regression analysis.

	B	SE	Hazard ratio	95% CI	P
Age	-6.607	0.315	0.545	0.294–1.012	0.054
Sex	-0.633	0.382	0.531	0.251–1.121	0.097
Smoking history	0.003	0.302	1.003	0.555–1.811	0.993
Histological type	0.208	0.329	1.231	0.647–2.345	0.526
Differentiation	0.104	0.331	1.109	0.579–2.124	0.754
Tumor size	0.182	0.332	1.200	0.638–2.256	0.572
Lymphatic metastasis	-0.161	0.333	0.851	0.443–1.636	0.629
TNM stage	-0.975	0.340	0.377	0.194–0.735	<b>0.004</b>
Vinculin	-1.439	0.407	0.237	0.107–0.527	<b>0.000</b>

CI, confidence interval.  
 Note: Boldface  $P < 0.01$ .

TNM III, respectively. Thus, vinculin expression was significantly decreased in NSCLC compared with non-cancerous tissues. Pearson's correlation analysis showed that the positive rate of vinculin was significantly correlated with TNM stage and lymph node metastasis in NSCLC patients, and the 5-year OS rate of vinculin-positive NSCLC patients was significantly higher than that of vinculin-negative patients ( $P < 0.05$ ). Moreover, Cox multivariate analysis identified vinculin-negative expression and TNM stage as independent risk factors for the prognosis of NSCLC patients.

This study is limited by the small number of samples, so further studies with a larger number of patients are warranted to confirm these findings. Other studies have observed similar phenomena in different types of cancer, such as breast cancer,<sup>26</sup> colon cancer,<sup>27</sup> and prostate cancer.<sup>28</sup> Together, these findings indicate that vinculin is a tumor suppressor gene. Some studies showed that vinculin suppresses tumors by supporting anchorage-dependent cell growth and reducing cell motility.<sup>10–12,29</sup> However, the mechanism by which vinculin functions as a tumor suppressor is unclear so additional studies are required.<sup>7</sup>

In conclusion, vinculin transcription is inhibited in NSCLC, and its low expression promotes malignancy. Vinculin could be used as a prognostic marker for NSCLC and is a potential target for its treatment.

### Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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