ORIGINAL RESEARCH

Low claudin-6 expression correlates with poor prognosis in patients with non-small cell lung cancer

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Objective: Claudins are found in junctional complexes mediating cell adhesion and are involved in the attachment of tight junctions to the underlying cytoskeleton. Abnormal claudin-6 expression has been observed for a variety of malignant solid tumors, but the expression of claudin-6 in non-small cell lung cancer (NSCLC) has not yet been characterized.

Methods: Immunohistochemistry, reverse transcription-polymerase chain reaction (RT-PCR), and western blot analysis were used to quantify claudin-6 expression in 123 cases of NSCLC and non-cancerous adjacent tissue. We analyzed the relationship between claudin-6 expression and clinicopathological features of NSCLC. The Kaplan–Meier method was used to analyze postoperative survival rates, and the log-rank test was used to assess differences in survival rates. The Cox regression model was used to perform multivariate analysis.

Results: Claudin-6 expression was low for 61 of 123 (49.6%) NSCLC tissue samples and for 33 of 123 (26.8%) normal adjacent tissue samples. RT-PCR and western blot analyses confirmed the immunohistochemistry results. Claudin-6 expression was associated with lymph node metastasis ($P<0.001$) and TNM stage ($P=0.007$). Kaplan–Meier analysis indicated that patients with low claudin-6 expression had significantly lower survival rates than those with high claudin-6 expression. Multivariate analysis suggested that low claudin-6 expression was an independent indicator of prognosis in NSCLC patients.

Conclusion: Low claudin-6 expression is an independent prognostic biomarker that indicates a worse prognosis in patients with NSCLC.

Keywords: NSCLC, claudin-6, immunohistochemistry, RT-PCR, western blot

Introduction

Non-small cell lung cancer (NSCLC) is the leading cause of cancer mortality in the world, and its incidence and mortality are increasing annually.[1] The 5-year survival rate for lung cancer patients in the United States is only 15%, and in the People's Republic of China, the survival rate is even lower.[2] The initiation and progression of NSCLC comprise a complicated process that depends on multiple factors and steps, including proto-oncogene activation, tumor-suppressor gene inactivation, and abnormal expression of various proteins.

Tight junctions (TJs) provide extracellular adhesive contacts between cells; destabilization of junctional complexes directly affects nutrient intake, cell proliferation, and migration.[3] Claudins are a large family of 24 proteins and critical components of TJs and help to attach junctional complexes to the underlying cell cytoskeleton.[4] As an important component of TJs, claudin-6 is closely related to a variety of biological habits of cells. For example, it is possible to regulate cell proliferation and gene transcription.

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Numerous studies have demonstrated an association between abnormal claudin expression and multiple forms of cancer.[5–8] However, the study of claudin-6 expression in NSCLC was rarely reported. Here, immunohistochemistry, reverse transcription-polymerase chain reaction (RT-PCR), and western blot analyses were together used to quantify claudin-6 expression in NSCLC and to better define the relationship between claudin-6 expression and prognosis in NSCLC patients.

Materials and methods
Patients and tumor specimens
NSCLC and adjacent non-cancerous tissue samples were surgically removed and collected from 123 patients of Xinjiang Medical University Affiliated Tumor Hospital from March 2005 to December 2010. All tissues were routinely fixed in 10% buffered formalin and embedded in paraffin blocks. Complete clinical data were available for all patients, and a pathologist confirmed the NSCLC diagnosis postoperatively. For adjacent non-cancerous tissue samples, tissue was obtained at a distance of more than 5 cm from the cancerous tissue; pathological analysis confirmed that the adjacent tissue samples were normal lung tissues. A total of 63 patients were male and 60 were female, with an age range of 32–75 years and an average age of 67.3±10.5 years. The histologic grade and clinical stage of the tumors were defined according to the seventh edition of the TNM classification of the International Union Against Cancer. None of the 123 NSCLC patients received chemotherapy or radiation therapy before surgery. All patients or their family members provided signed consent forms for the use of patient samples. The Ethics Committee of Subsidiary Tumor Hospital of Xinjiang Medical University approved the study.

Immunohistochemistry
Two-step method was used to stain the samples for immunohistochemical analysis. Paraffin-embedded samples were sectioned with a thickness of 4 µm, incubated overnight at 60°C, and then dewaxed. Sections were incubated at room temperature for 10 minutes in 3% hydrogen peroxide prepared fresh in deionized water in order to block endogenous peroxidase. After high-pressure retrieval in 0.01 mol/L citrate buffer solution (pH =6.0), the sections were incubated with rabbit antihuman claudin-6 monoclonal antibody (1:200; Santa Cruz Biotechnology Inc., Dallas, TX, USA) overnight at 4°C. Following three washes in phosphate-buffered saline (PBS), the sections were incubated with secondary antibody

at 37°C for 10 minutes. After the sections were washed three times in PBS, they were incubated with horseradish peroxidase-conjugated streptavidin at 37°C for 10 minutes. Following three washes in PBS, the sections were incubated with diaminobenzidine to initiate the colorimetric reaction, which was monitored using microscopy and then terminated with distilled water.

The pathologists were blinded with respect to clinical data associated with patients. At medium magnification (200×), five visual fields were randomly selected, with each visual field containing 200 tumor cells, for a total of 1,000 cells. Cells were rated according to staining intensity on the following scale: 0 point for no staining, 1 point for pale yellow staining, 2 points for yellowish brown staining, and 3 points for brown staining. Cells were also rated according to the proportion of positive cells, ranging from 0% to 100%. If the product of the staining intensity rating and the percentage of positive cell nuclei was greater than zero, we defined the sample as claudin-6 positive; otherwise, the sample was considered negative for claudin-6 expression. For positive samples, low claudin-6 expression was defined by staining intensities of 0−1 scores, whereas staining intensities of 2−3 scores defined high expression. In addition, we performed immunohistochemistry without the primary antibody as negative control.

Reverse transcription-polymerase chain reaction
RNA was extracted from samples in a sterile, RNAse-free environment by using TRIzol in strict accordance with manufacturer instructions. cDNA was produced from the purified mRNA transcripts by using reverse transcriptase. PCR was used to amplify and quantify claudin-6 cDNA using the following primer sequences: sense, 5′-CACTGCCACTTCTGGATGG-3′ and antisense, 5′-CAGTGCAGCTCCTTCAACCT-3′. The PCR program was as follows: initial denaturation at 94°C for 3 minutes; 35 cycles each of denaturation at 94°C for 30 seconds, annealing at 56.2°C for 30 seconds (annealing temperature of the internal reference GAPDH, 65°C), and extension at 72°C for 45 seconds and extension at 72°C for 2 minutes. Target gene and reference gene PCR products were visualized on a 1.5%–2.0% Sepharose gel using the JS-780 Automatic Gel Imaging Analysis System (Hangzhou Chincan Trading Co., Ltd., Zhejiang, People's Republic of China) and gray-scale analysis. The internal reference for each sample was set to one, and the claudin-6 mRNA level was defined as (claudin-6 mRNA gray value)/(internal reference mRNA gray value).

Western blotting

Cryopreserved tissue samples were weighed and then lysed in lysis buffer (Beyotime Institute of Biotechnology, Shanghai, People's Republic of China). In all, 50 μg of protein were resolved by 8%–12% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membranes (Bio-Rad Laboratories Inc., Hercules, CA, USA), followed by western blotting using standard procedures. The blot was incubated in Tris-buffered saline (TBST) containing 5% skimmed milk powder to block nonspecific binding, followed by incubation with claudin-6 rabbit antihuman monoclonal antibody (1:1,000) overnight at 4°C. The blot was washed three times with TBST (10 minutes per wash). Finally, the blot was incubated with goat antirabbit IgG (1:2,000; Santa Cruz Biotechnology Inc.) at 37°C for 1 hour and washed three times with TBST (10 minutes per wash). Electrogenerated chemiluminescence was used for autoradiography. The relative amount of Raf kinase inhibitor protein was represented by the claudin-6/GAPDH gray scale ratio, which was analyzed using the Quantity One software (Bio-Rad Laboratories Inc.).

Statistical analysis

The Mann–Whitney test was used to compare claudin-6 expression in NSCLC to that in normal tissues and lymph nodes. Categorical data comparisons were made using the χ^2 test, and quantitative comparisons were made using the Student's t-test. The primary outcomes were disease-free survival (DFS) and overall survival (OS). The Kaplan–Meier method was used to analyze postoperative survival rates, and the log-rank test was used to assess differences in survival rates. The Cox regression model was used to perform multivariate analysis. All statistical analyses were performed using SPSS version 13.0 (SPSS Inc., Chicago, IL, USA). Two-sided P-values were calculated, and $P<0.05$ was considered to be statistically significant.

Results

Low claudin-6 expression in primary NSCLC

Claudin-6 protein was mainly localized to the nucleus of NSCLC tissues and adjacent tissues, as detected by immunohistochemistry. Claudin-6 was expressed at low levels in 61 of 123 (49.6%) NSCLC tissue samples and in 33 of 123 (26.8%) normal adjacent tissue samples ($P<0.05$, Figure 1). RT-PCR
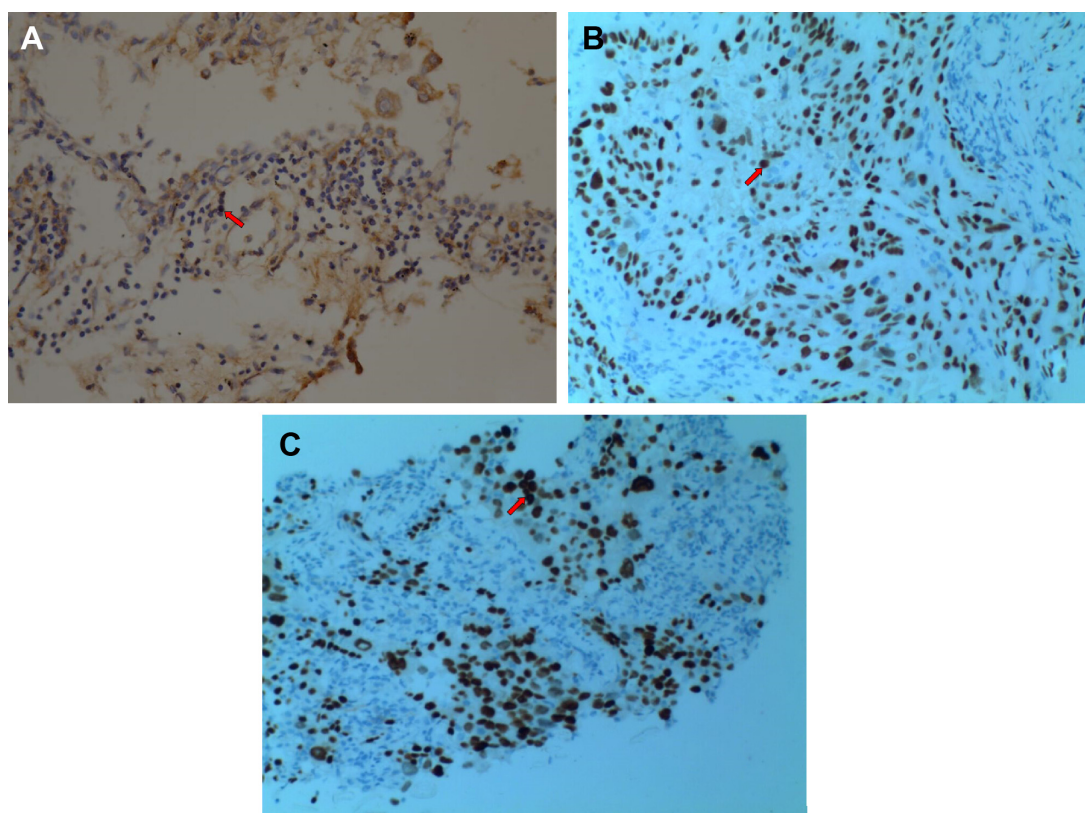
Figure 1 Claudin-6 expression in non-small cell lung cancer and adjacent tissues detected by immunohistochemistry (SP ×100).
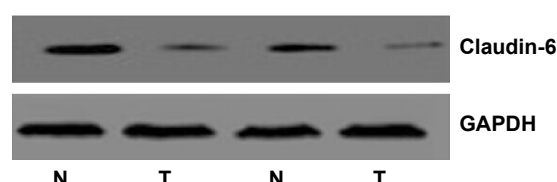**Notes:** The arrows indicate positive expression of claudin-6. (**A**) Normal adjacent tissue, (**B**) squamous cell carcinoma, and (**C**) adenocarcinoma.
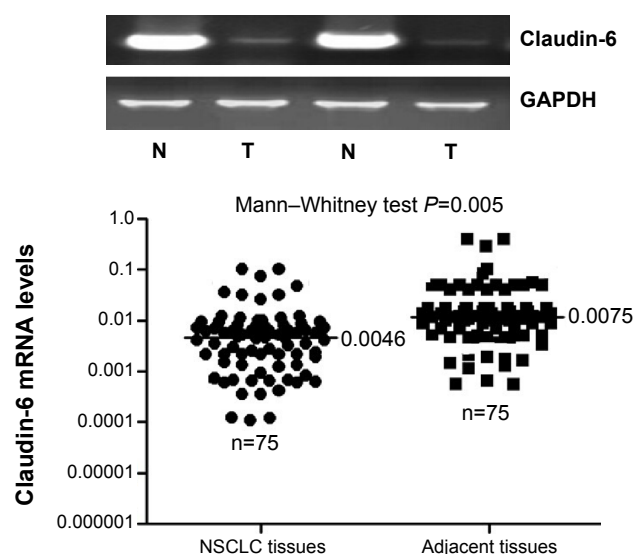
Figure 2 Claudin-6 mRNA was detected in NSCLC tissues (T) and adjacent tissues (N) by RT-PCR.
Abbreviations: NSCLC, non-small cell lung cancer; RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Figure 3 Claudin-6 protein was detected in NSCLC tissues (T) and adjacent tissues (N) by western blot analysis.
Abbreviations: NSCLC, non-small cell lung cancer; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

Expression of claudin-6 and clinicopathological factors of NSCLC

We analyzed the relationship between claudin-6 expression and clinicopathological factors of NSCLC (Table 1). Low claudin-6 expression was associated with lymph node metastasis ($P<0.001$) and TNM stage ($P=0.007$). Low claudin-6 expression had no significant correlation with other clinical parameters, including age, sex, smoking status, pathological type, differentiation, or tumor size ($P>0.05$).

Claudin-6 expression and prognosis in NSCLC patients

Tissue samples from 123 NSCLC patients were classified according to the claudin-6 expression level. The Kaplan–Meier

and western blot analyses were used to quantify mRNA and protein levels, respectively; we found that claudin-6 mRNA expression and protein levels were significantly higher in adjacent tissues than in NSCLC tissues ($P<0.05$, Figures 2 and 3). Thus, these data suggest that claudin-6 protein and mRNA levels are low in NSCLC tissues.

Table 1 Correlation between claudin-6 expression and clinicopathological factors of NSCLC

Variable	Cases	Claudin-6 expression			
		Low (n=61)	High (n=62)	χ^2	*P*-value
Age					
≤60 years	56	25	31	1.008	0.315
>60 years	67	36	31		
Sex					
Male	63	31	32	0.008	0.93
Female	60	30	30		
Smoking status					
No	65	32	33	0.007	0.932
Yes	58	29	29		
Pathological type					
SCC	58	27	21	0.250	0.617
AD	65	34	32		
Differentiation					
Well and moderate	86	41	45	0.421	0.516
Poor	37	20	17		
Tumor size					
T1–T2	55	28	27	0.069	0.793
T3–T4	68	33	35		
Lymph node metastasis					
Absent	63	21	42	13.659	<0.001
Present	60	40	20		
TNM stage					
I–II	102	45	57	7.166	0.007
III	21	16	5		

Abbreviations: NSCLC, non-small cell lung cancer; SCC, squamous cell carcinoma; AD, adenocarcinoma.

**Table 2** The survival of patients with non-small cell lung cancer

	Case number	Survival time (months)		P-value
		Mean ± SE	95% CI	
DFS				
Low expression	61	32.13±2.86	26.52–37.74	0.028
High expression	62	41.68±2.54	36.69–46.66	
OS				
Low expression	61	40.23±2.51	35.36–45.20	<0.001
High expression	62	53.08±1.63	49.88–56.28	

Abbreviations: DFS, disease-free survival; OS, overall survival; NSCLC, non-small cell lung cancer; SE, standard error; CI, confidence interval.

analysis suggested that patients with low claudin-6 expression levels had significantly shorter survival rates (DFS and OS) than patients with high claudin-6 expression levels (Table 2 and Figure 4). Univariate analysis showed that lymph node metastasis, TNM stage, and low claudin-6 expression levels were correlated with OS (Table 3). Multivariate analysis using the Cox regression model suggested that low claudin-6 expression was an independent indicator of poor patient prognosis (Table 4).

Discussion

TJs between epithelial and endothelial cells form a tight barrier that allows the passage of water and solute through the paracellular pathway.[9] Junctional complexes are critical in maintaining adhesive contacts between cells, establishing cell polarity, controlling permeability, and regulating cell proliferation.[10] Claudins are a large family of 24 proteins that are critical components of TJs.[11,12] Claudins range in molecular mass from 17 kDa to 27 kDa, but all contain
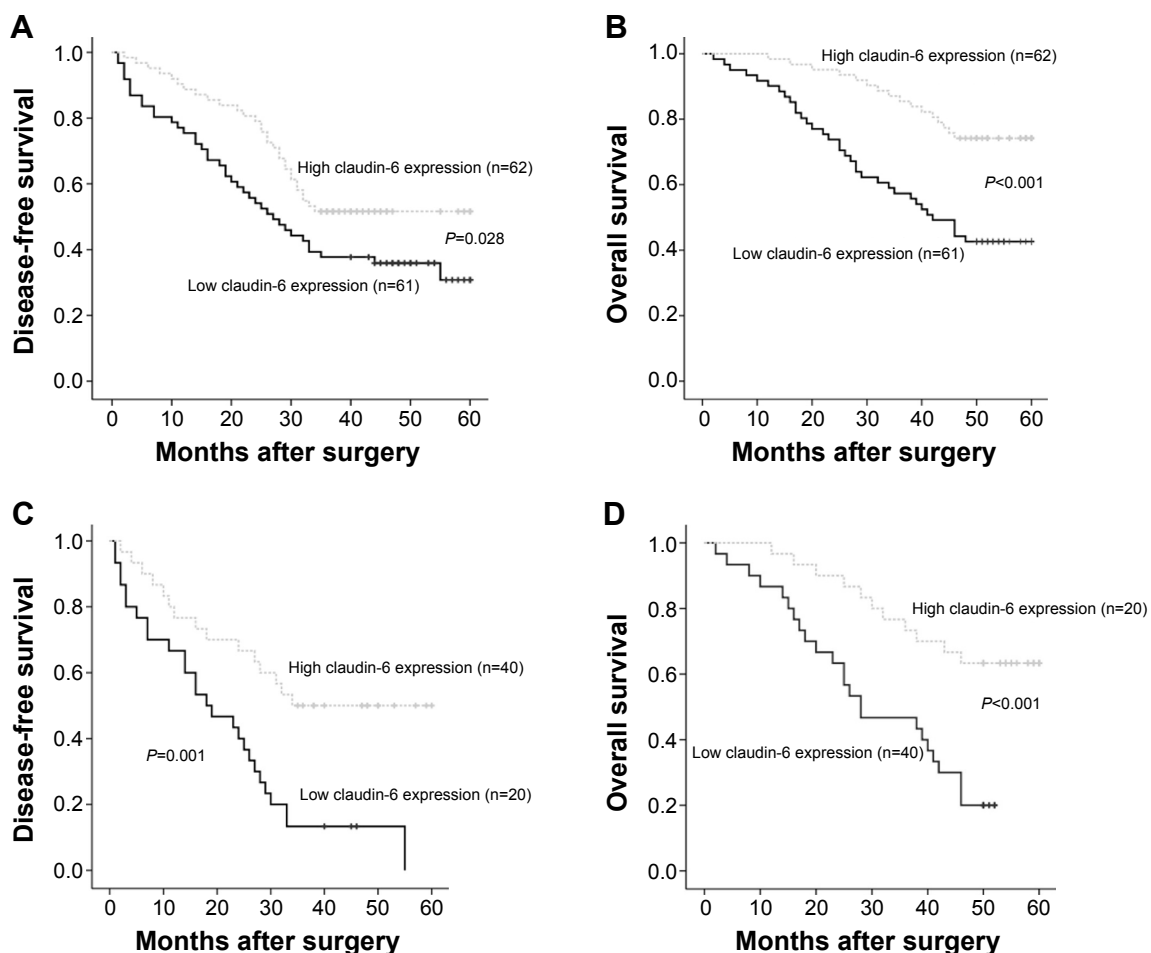
Figure 4 Kaplan–Meier survival curves of patients based on claudin-6 expression.
Notes: (**A** and **B**) all patients and (**C** and **D**) patients with lymph node metastasis.

Table 3 Univariate analysis of clinicopathological factors for overall survival

Variable	Cases	HR	95% CI	P-value
Age				
≤60 years	56	I	0.667–2.022	0.597
>60 years	67	1.161		
Sex				
Male	63	I	0.660–1.979	0.634
Female	60	1.143		
Smoking status				
No	65	I	0.554–1.670	0.891
Yes	58	0.962		
Pathological type				
SCC	58	I	0.597–1.213	0.637
AD	65	1.033		
Differentiation				
Well and moderate	86	I	0.483–1.653	0.72
Poor	37	0.894		
Tumor size				
T1–T2	55	I	0.412–1.239	0.232
T3–T4	68	0.715		
Lymph node metastasis				
Absent	63	I	1.126–4.568	0.016
Present	60	2.057		
TNM stage				
I–II	102	I	1.064–3.759	0.031
III	21	1.999		
Claudin-6 expression				
High	62	I	1.649–5.400	<0.001
Low	61	2.984		

Abbreviations: HR, hazard ratio; CI, confidence interval; SCC, squamous cell carcinoma; AD, adenocarcinoma.

four transmembrane domains and two extracellular annular areas.[13] Claudins are also involved in intercellular signal transduction.[11–13] Abnormal claudin expression plays an important role in the occurrence and development of multiple cancers; low claudin expression is associated with tumors.[14–16]

Claudin-6 is located at chromosome 16p13.3 and encodes a 219-amino-acid protein with a molecular mass of 23 kDa.[17] As an important component of TJs, claudin is not

Table 4 Multivariate analysis of clinicopathological factors for overall survival

Variable	Cases	HR	95% CI	P-value
Lymph node metastasis				
Absent	63	I	1.448–5.648	0.002
Present	60	2.86		
TNM stage				
I–II	102	I	1.039–5.384	0.04
III	21	2.366		
Claudin-6 expression				
High	62	I	1.056–4.067	0.034
Low	61	2.072		

Abbreviations: HR, hazard ratio; CI, confidence interval.

only capable of regulating intercellular material flow and maintaining epithelial cell polarity but also involved in cell proliferation, gene transcription, and tumor suppression.[18] Claudin downregulation can influence the initiation and progression of cancer by causing a decrease in cell adhesiveness and cohesion, variation in differentiation, and an increase in cell invasiveness.[19,20] The study by Micke et al was to assess which fraction of patients with NSCLC express claudin-6 by immunohistochemistry and gene expression microarray data. The results suggested that high claudin-6 protein expression was associated with worse prognosis in lung adenocarcinoma.[21] This finding encourages further clinical exploration of targeting ectopically activated claudin-6 expression as a valuable treatment concept in NSCLC. We do not know whether there is a similar phenomenon in Chinese patients and other pathological types with NSCLC. Here, we have quantified claudin-6 expression levels in NSCLC and have uncovered a correlation between low claudin-6 expression and decrease in survival rates of NSCLC patients.

In this study, we examined the expression levels of claudin-6 in NSCLC and corresponding adjacent tissues by immunohistochemistry. The results suggest that the rate of claudin-6 low expression was significantly higher in NSCLC tissues than in the surrounding non-tumor tissues. At the mRNA level, we have verified the above results using RT-PCR and western blot analysis, and we reached the same conclusion. In addition, we investigated the relationship between claudin-6 expression levels and clinicopathological features of NSCLC patients and found that claudin-6 expression was significantly correlated with lymph node metastasis and TNM stage, but not with age, sex, smoking status, pathological type, differentiation, and tumor size of patients with NSCLC. For patients with NSCLC, carrying out survival and prognostic analysis is necessary. The Kaplan–Meier analysis suggested that patients with low claudin-6 expression levels had significantly shorter survival rates (DFS and OS) than patients with high claudin-6 expression levels. Moreover, multivariate survival analysis demonstrated that low claudin-6 expression could be a significantly independent hazard factor for OS of patients with NSCLC, along with lymph node metastasis and TNM stage.

In conclusion, low claudin-6 expression is an independent prognostic biomarker that indicates a worse prognosis in patients with NSCLC.

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had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Disclosure

The authors report no conflicts of interest in this work.

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