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Potential key roles of tumor budding: a representative malignant pathological feature of non-small cell lung cancer and a sensitive indicator of prognosis

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Authors' contributions

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Conflict of interest

The authors have disclosed that they have no significant relationships with, or financial interest in, any commercial companies pertaining to this article.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

NO. 2018-L068.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Abstract

Aims: The Union for International Cancer Control has officially recognized tumor budding as an independent prognostic factor for patients with colorectal cancer. However, only a few studies discuss its significance in lung cancer. Airway spread and peritumoral space are important events in the metastasis of lung cancer cells. But few studies have examined the relationship between tumor budding and spread through airspaces (STAS) or whether there is a correlation between tumor budding and the peritumoral space.

Methods: In this study, we selected 532 patients with non-small cell lung cancer from China, including 380 patients with adenocarcinoma and 152 with squamous cell carcinoma, to explore the correlation between tumor budding, the clinicopathological characteristics of these patients, and prognosis. Pan-cytokeratin staining showed tumor budding more clearly than hematoxylin and eosin staining.

Results: In patients with lung adenocarcinoma, there was a correlation between tumor budding and airway spread ($P < 0.001$), and in patients with squamous cell carcinoma, tumor budding state was closely related to the peritumoral space ($P < 0.001$). On Cox regression analysis, multivariate analysis showed that tumor budding, pleural and vascular invasion, airway spread, tumor size, lymph node metastasis, and Tumor Node Metastasis stage were independent risk factors of prognosis for non-small cell lung cancer patients.

Conclusions: As an effective and simple pathological diagnostic index, it is necessary to establish an effective grading system in the clinical diagnosis of lung cancer to verify

the value of tumor budding as a prognostic indicator. We hope that this analysis of Chinese patients with non-small cell lung cancer can provide useful reference material for the continued study of tumor budding.

Key words: lung cancer, prognosis, tumor budding

Strengths and limitations of this study

We selected 532 patients with non-small cell lung cancer from China, including 380 patients with adenocarcinoma and 152 with squamous cell carcinoma, to explore the correlation between tumor budding, the clinicopathological characteristics of these patients, and prognosis.

Through the evaluation of tumor budding in lung cancer specimens of Chinese patients, we hope to provide reference for the establishment of tumor budding criteria in the diagnosis of lung cancer.

Our research was limited to the tumor budding analysis of NSCLC patients in China, and the results of different ethnicities may differ.

This study only included surgical resection specimens, no biopsy specimens.

Introduction

Lung cancer is among the most common malignant tumors in China and the world. According to global cancer data from 2020, lung cancer is the most common type of cancer (11.4% of the total) and cancer-related death (18% of total cancer deaths)[1]. Early lung cancer has few clinical manifestations and is easily ignored or even missed. With

the spread and infiltration of tumor cells, most patients lose the opportunity for radical surgery. Invasion and metastasis are among the main causes of lung cancer death and play a decisive role in lung cancer staging and management.

As a pathological phenomenon, tumor budding has been attracting increased attention. Some studies have shown that tumor budding is a factor that reflects the malignant invasion and poor prognosis of digestive tract tumors[2]. The Union for International Cancer Control (UICC) has officially recognized that tumor budding is an independent prognostic factor for colorectal cancer (CRC) patients. However, only a few studies have explored its significance in lung cancer.

In recent years, with the increasing research on cancer prognosis, some scholars have reported that the morphological characteristics of the peritumoral space are related to patient prognosis. Peritumoral spaces have been noted in breast, lung, bladder, and prostate cancers as well as other malignant tumors. Tumor cells generally spread to the corresponding lymph nodes through the lymphatic system, a phenomenon that is considered an important early event of tumor metastasis[3 4]. However, the presence of a correlation between tumor budding and the peritumoral space has been rarely reported.

In this study, we selected 532 cases of NSCLC patients from China, including 380 cases of adenocarcinoma and 152 cases of squamous cell carcinoma, to explore the correlation between tumor budding, patients' clinicopathological characteristics, and prognosis with the aim of determining a reference value for evaluating patient prognosis and clinical treatment.

Material and methods

Patients' general information

We retrieved the pathological reports of patients who met the inclusion criteria from the files of the pathology system and obtained other clinical pathological information from the electronic medical record system. All 532 cases included in this study were radical surgical specimens. The data of 380 patients with primary lung adenocarcinoma and 152 patients with primary lung squamous cell carcinoma treated in the Cardiothoracic Disease Department of the Affiliated Hospital of Nantong University between June 2009 and July 2015. We excluded patients for whom follow-up information was lacking; thus, and a total of 532 patients (302 males, 230 females; 202 patients were ≤65 years old, while 328 patients were >65 years old). None of the patients received chemotherapy or radiotherapy preoperatively. The clinical and pathological information and medical records were complete for each patient.

We took the corresponding paraffin blocks of each patient from the pathological diagnosis center and sliced them into 3-μm-thick slices. Each slice was floated in 45°C warm water on a spreader to flatten the tissue, which was then picked up with a slide and baked in an oven at 65°C. Cytokeratin immunohistochemical staining (CK) and hematoxylin and eosin (HE) staining were performed. Rabbit polyclonal anti-human pan-cytokeratin (CKpan) antibody was used (dilution 1:50; ab215838, Abcam, USA). The evaluations were independently performed by three experienced pathologists using a multi-head microscope (Precise Instrument Co., Ltd., Beijing, China) to reach consensus.

Patient and Public Involvement

All patients signed an informed consent form, and the study was approved by the Ethics Committee of Affiliated Hospital of Nantong University (2018-L068). The patients were followed up by telephone and outpatient service. The starting point of follow-up was the operation time for each patient, while the end point was the time of death. If the patient was still alive, we selected the last follow-up appointment as the termination point.

Histological type assessment

We observed the histopathological structure of each tissue sample under the microscope and classified the tumor tissues according to the diagnostic criteria formulated by the WHO in 2015. The Tumor Node Metastasis (TNM) staging was based on UICC/American Joint Committee on Cancer (AJCC) 8th edition.

Evaluation of tumor budding

The slides stained with HE were placed under a 10 × 20 light microscope to observe the densest portion of the budding. The clarity of HE and CKpan staining on tumor budding were compared. The areas of budding were then counted in high-power fields (HPFs).

It remains controversial whether HE or CK staining should be used for budding markers. CK staining can reportedly more clearly show the bud focus covered by the significant peritumoral inflammatory reaction[5]. CK staining also aides in the observation of a large number of germinal foci mixed with stromal fibroblasts[6]. CK

staining can produce three to four times more buds than HE staining[7]. In many studies, many scholars chose CK staining for sprouting evaluations[6 8-14]. Therefore, here we used both HE staining and CKpan staining and observed the budding state of each level between methods. The budding site was more easily observed and the scope of the bud focus was clearer using CKpan staining.

The judgment of tumor budding refers to the standard of Ueno et al.[15], that is, an isolated single tumor cell or small clusters of tumor cells composed of no more than four tumor cells in the stroma at the start of the tumor invasion were considered tumor budding.

To employ a semiquantitative method to analyze tumor budding, we counted the mean number of tumor buds under 10 HPFs. The tumor budding was divided into non-budding, low budding (≤10 buds/10 HPFs) and high budding (>10 buds/10 HPFs).

Tumor cell clusters surrounded by tumor stroma were defined as tumor cell nests. Based on Moritz's research method[16] and according to the histomorphology characteristics of lung cancer, we divided the cell nests in tumor stroma into two to four tumor cell nests and a single invasive cancer cell in the matrix of the tumor invasion edge. We also divided tumor interstitial fibrosis into negative, very low (10% of the total tumor area), low (10–25%), medium (25–50%), and high (>50%).

Statistical analysis

The data were analyzed using SPSS 26.0 software (IBM Corporation, Armonk, NY, USA). The χ^2 test and t-test were used to compare the count data and measurement

data, respectively. The follow-up result was the overall survival (OS) rate. The Kaplan-Meier method was used to draw the survival curves, while the log rank method was used to analyze the differences among groups. A multivariate Cox proportional hazard model was used to determine the independent prognostic factors of the lung cancer patients. The difference was statistically significant ($P < 0.05$).

Results

Tumor budding in NSCLC patients

In cases of lung cancer with tumor budding, the front edge was not smooth and the budding tumor cells were heteromorphic, irregularly shaped, rich in cytoplasm, often fused, and eosinophilic. The nucleus was irregularly shaped and the staining was deeper than that of stromal cells. However, the tumor budding foci were sometimes easily confused with poorly differentiated stromal cells. However, compared with HE staining, CK staining can more clearly show tumor budding spores (Figure 1).

Relationship between tumor budding and clinicopathological features of patients with NSCLC

Tumor interstitial fibrosis was defined as fibrosis observed under 100× magnification. According to the area of fibrosis, it was classified as negative, ≤10%, 10–25%, 25–50% and >50%. The peritumoral space, that between the tumor cells and the stroma, was the morphological manifestation of the interaction between them that clearly divided the tumor components and the stroma[3]. Shah et al.[17] reported that the

peritumoral space was very common in tumors and related to invasive cancer cell nests.

Among the 380 cases of lung adenocarcinoma, 46 showed no tumor budding and 334 showed tumor budding. Tumor budding status was closely related to the 5-year OS status of patients with lung adenocarcinoma. In addition, it was closely related to tumor histological subtype ($P < 0.001$), tumor size ($P < 0.001$), lymph node metastasis ($P < 0.001$), vascular invasion ($P < 0.001$), pleural invasion ($P < 0.001$), STAS ($P < 0.001$), tumor necrosis ($P = 0.005$), tumor interstitial fibrosis ($P < 0.001$), and TNM stage ($P < 0.001$). However, tumor budding was not related to the patient gender patient sex ($P = 0.875$) or age ($P = 0.898$). The proportion of tumor budding in patients with vascular tumor thrombus was significantly higher than that in patients without vascular tumor thrombus. The greater the degree of lymph node metastasis, the higher the proportion of tumor budding (Table 1). In the 152 patients with primary squamous cell carcinoma of the lung (Table 2), tumor budding status was significantly correlated with the 5-year OS status ($P < 0.001$), peritumoral space ($P < 0.001$), vascular invasion ($P = 0.001$), tumor size ($P < 0.001$), lymph node metastasis ($P < 0.001$), airway spread ($P = 0.001$), tumor necrosis ($P = 0.030$), TNM stage ($P < 0.001$), and tumor interstitial fibrosis ($P < 0.001$).

Survival analysis of patients

All 532 patients were included in the survival analysis study by July 2020. The follow-up time was 3–82 months. At the end of the study, 261 patients were still alive. Among the dead patients, the proportion of high-grade budding was significantly higher

than those of the low-grade budding and non-budding groups. The Kaplan-Meier method was used to analyze the postoperative survival rate, while the log rank method was used to test the intergroup differences.

In patients with lung adenocarcinoma, univariate analysis showed that tumor budding, tumor budding nucleus size, pleural and vascular invasion, airway spread, histological subtype, necrosis area, and TNM stage were significantly associated with 5-year survival (Table 3). We then used the Cox proportional hazard regression model to analyze the statistically significant indicators of the univariate analysis. For the budding model, we took the above factors as variables, and the tumor budding (hazard ratio [HR], 1.298; 95% confidence interval [CI], 1.033–1.630; P = 0.025), nuclear size (HR, 1.477; 95% CI, 1.070–2.039; P = 0.018), pleural invasion (HR, 1.527; 95% CI, 1.052–2.217; P = 0.026), vascular invasion (HR, 2.144; 95% CI, 1.285–3.578; P = 0.004), airway spread (HR, 2.695; 95% CI, 1.597–4.548; P < 0.001), necrosis (HR, 1.328; 95% CI, 1.016–1.734; P = 0.038), histological subtype (HR, 0.855; 95% CI, 0.758–0.965; P = 0.011), pT (HR, 2.011; 95% CI, 1.645–2.458; P < 0.001), pN (HR, 2.038; 95% CI, 1.413–2.940; P < 0.001), and TNM stage (HR, 0.481; 95% CI, 0.299–0.773; P = 0.002) also showed a statistically significant correlation with the 5-year survival rate based on the univariate Cox regression univariate analysis (Figure 2).

The Kaplan-Meier survival curve showed that the higher the budding grade, the lower the 5-year OS rate (P < 0.001) (Figure 3). In the histological subtypes of lung adenocarcinoma, the higher the level of tumor budding, the worse the prognosis in cases with micropapillary subtypes and solid subtypes (Figure 4). In the adherent subtype (P

= 0.356), papillary subtype (P = 0.567), and acinar subtype (P = 0.353), there was no statistical correlation between tumor budding degree and survival status. Compared with tumor budding cell nucleus containing fewer than three lymphocytes (small size), when the tumor budding nucleus had four or more lymphocytes (large size), the 5-year OS rate of lung adenocarcinoma patients was significantly reduced (Figure 5A).

In cases of lung squamous cell carcinoma, tumor budding size, budding tumor nest, pleural and vascular invasion, airway spread, tumor interstitial fibrosis area, peritumoral space, tumor size and lymph node metastasis, and TNM stage influenced patient 5-year survival rate (Table 4). To eliminate the interactions between variables, multivariate Cox regression analysis was used to analyze the data. The above factors independently affected the prognosis of patients with squamous cell carcinoma (Figure 2). The Kaplan-Meier survival curve showed that the 5-year OS rate of patients with lung squamous cell carcinoma in TNM stage II was significantly higher than that of patients with high-grade tumor budding (Figure 6B), while the 5-year OS rate of lung squamous cell carcinoma patients with single cell tumor budding was significantly lower (Figure 5B).

Discussion

Cancer is an issue of great concern worldwide, and its prognosis mainly depends on the pathological type, TNM stage, tumor differentiation degree, and microvascular invasion, and patients with the same TNM stage but quite different prognoses are often seen in the clinical setting. In recent years, as a pathological phenomenon, tumor

budding has attracted increasing attention. Tumor budding, also known as focal dedifferentiation is the first step in the process of a malignant tumor's invasion and metastasis. Therefore, tumor budding is considered a key step in a tumor's invasive growth process[18]. Tumor budding spores are considered cancer stem cells, which are defined as isolated single tumor cells or clusters of fewer than five tumor cells at the start of tumor invasion[5]. Some studies stated that tumor budding is not a static histological feature; rather, it involves a small focal tumor cell complex separated from the main body of the tumor that enters the surrounding tissue in a "budding" manner, which represents a dynamic process[19]. Gabbert et al.[18] also supported this conclusion. Shinto et al.[8] reported that there were interconnected cytoplasmic pseudo fragments similar to pseudopodia processes between budding tumor cells, which may be related to the increase in cell invasion ability. In addition, some studies have speculated that tumor budding is a step in the progression of malignant tumors from focal lesions to systemic diseases[20]. Tumor budding is now considered of great significance in tumor invasion and metastasis[21-24]. Some studies have shown that tumor budding reflected the invasiveness and poor prognosis of digestive tract tumors[2]. The presence of tumor budding may be related to the late stage of a tumor, frequent lymphatic vascular invasion, and lymph node and distant metastasis. The UICC officially recognizes tumor budding as an independent prognostic factor for CRC. It was recently used as a significant prognostic indicator for the treatment of esophageal squamous cell carcinoma, gastroesophageal junction adenocarcinoma, and gastric adenocarcinoma[25]. In the current study of 380 cases of primary lung adenocarcinoma and 152 cases of

primary lung squamous cell carcinoma, we found that tumor budding was closely related to the 5-year OS, tumor size, lymph node metastasis, vascular invasion, airway spread, tumor necrosis, tumor interstitial fibrosis, and TNM stage. This suggests that tumor budding may be an important indicator of malignant invasion and metastasis. Compared with NSCLC patients without tumor budding, those with the morphological characteristics of tumor budding have a worse 5-year OS prognosis.

The detection accuracy of abdominal B-ultrasound and abdominal computed tomography for lymph node metastasis is reportedly 12.2–80.0%[26] and 50–80%, respectively[27-30]. Guluoglu et al.[31] evaluated 126 patients with gastric cancer and found that lymph node metastasis was the only parameter associated with tumor budding. Masaki *et., al*[32] established a model formula for predicting the probability of lymph node metastasis in 76 patients with T1 stage CRC as follows: $z = 0.070 \times$ (budding count) - 3.726, probability = $1/1 + e^{-z}$. Furthermore, the tumor budding count was included in the clinical decision-making analysis of patients to determine whether patients require additional surgery after endoscopic treatment. Some studies have shown that the presence of tumor budding in biopsy specimens before CRC surgery increases the possibility of lymph node and distant metastasis. Therefore, neoadjuvant therapy and surgical treatment can be considered for these patients[33]. The Japanese Society for Cancer of the Colon and Rectum has incorporated the index of tumor budding into the guidelines for patients with pT1 disease who require further surgery[34]. In our study, 244 of 253 patients with lymph node metastasis had tumor budding. The sensitivity of budding for predicting lymph node metastasis was 96.44%, indicating that

tumor budding is an effective pathological index with high sensitivity for predicting lymph node metastasis. Therefore, we believe that for patients with NSCLC, we can refine the significance of tumor budding through a larger sample study to contribute to clinical decision-making.

The peritumoral space is the space between the tumor cells and the stroma that divides the tumor components from the stroma and is morphological manifestation of the interaction between the tumor cells and the stromal cells. The peritumoral space is commonly seen in paraffin-embedded tissue sections fixed with formalin. The peritumoral space is one of the pathomorphological manifestations of tumor biological behavior that is considered a prognostic factor by some scholars. Peritumoral spaces have been noted in breast, lung, bladder, and prostate cancers and other malignant tumors. Tumor cells usually spread to the corresponding lymph nodes through the lymphatic system, this phenomenon is considered an important early event of tumor metastasis[3 4]. In prostate cancer, an extensive peritumoral space indicates a higher tumor grade, shorter disease-free survival, and poor prognosis[35 36]. At the same time, the peritumoral space in breast cancer is closely related to histological grade, lymphatic invasion, lymph node metastasis, and prognosis and can be used as an important marker to judge the prognosis of breast cancer patients[37 38]. Acs et al.[39] observed the relationship between a large peritumoral space and lymph angiogenesis, and the results confirmed a poor prognosis of patients with large peritumoral spaces, which was consistent with this hypothesis. In our study, we found that in patients with lung squamous cell carcinoma, the peritumoral space is closely related to tumor budding, which is also an

independent risk factor for patient 5-year OS. A joint evaluation of the peritumoral space and tumor budding can effectively evaluate the prognosis of patients with lung squamous cell carcinoma.

Lung adenocarcinoma spreads through the bronchus, known as lung metastasis, and the airways, known as airway metastasis. A small number of lung adenocarcinoma cancer cells enter the bronchial cavity, and with the respiratory movement through the bronchial discontinuous, they diffuse into other lung segments or lobes on the same or opposite side, forming new lung metastases[40]. Our study revealed that tumor budding was closely related to airway spread. Tumor budding can be combined with spread through airspaces (STAS) to evaluate the malignant aggressive behavior of NSCLC.

Che et al.[2] found that the OS rate of patients with high budding gastric adenocarcinoma was significantly lower than that of patients with low budding gastric adenocarcinoma. Some studies reported that the presence of tumor budding in surgical specimens of patients with gastric cancer may indicate a poor prognosis and early recurrence[25]. We also found that the 5-year OS rate of lung adenocarcinoma or squamous cell carcinoma patients with high-grade budding was significantly lower than that of patients with low-grade or no budding. However, Hass et al.[41] emphasized that tumor budding and cancer classification based on cell differentiation were neither the same nor related. Some researchers believed that tumor budding and tumor growth pattern were independent prognostic parameters[15]. However, in our study of lung adenocarcinoma, tumor budding was closely related to histological subtype. In patients with papillary and solid subtypes of lung adenocarcinoma, the 5-year survival rate of

patients with high-grade budding was significantly lower than that of patients with low-grade budding. In patients with TNM stage I, the 5-year OS rate of patients with high-grade tumor budding was lower than that of patients with low-grade or no budding (Figure 6A). The results are consistent with those of Kyuichi et al.[42].

In our study, Cox regression analysis showed a significant correlation between tumor budding and 5-year OS rate. Tumor budding, pleural and vascular invasion, airway spread, tumor size, lymph node metastasis, and TNM stage were independent risk factors for the prognosis of NSCLC patients. In addition, tumor budding nucleus size, tumor necrosis area, and histological subtype were independent prognostic factors of lung adenocarcinoma. The area of interstitial fibrosis, presence of a peritumoral space, and small tumor cell nest were independent prognostic factors in patients with squamous cell carcinoma. Therefore, we speculate that tumor budding may be a representative malignant pathological feature of NSCLC and a sensitive indicator reflective of its prognosis.

The research results of Wang et al. suggested that tumor budding should be included in the routine histopathological report to better stratify the risk of CRC patients[43]. The AJCC and College of American Pathologists guidelines on CRC proposed that tumor budding should be considered an optional reporting indicator and should be evaluated in all cases of stage I and II CRC. This provides us with a standardized reporting tool for tumor budding[44]. However, there is no unified scoring standard for lung cancer.

The current study had several limitations. First, our research is limited to the tumor

budding analysis of NSCLC patients in China, and the results of different ethnicities may differ. In addition, because the number of surgical specimens selected for this operation before 2015 was limited, the sample size was insufficient, which might result in sample bias. However, as an effective and simple pathological diagnosis index, it is necessary to establish an effective grading system to verify its value as a standard prognostic indicator. In addition, prospective clinical trials including multicenter samples are needed to evaluate the role of tumor budding in predicting the prognosis of lung cancer and produce reference values for the pathological diagnosis and clinical treatment of lung cancer.

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Table1 The correlation of tumor budding with clinicopathological characteristics of lung adenocarcinoma patients

Characteristic	All cases	Tumor budding		χ^2	P
		Negative	Positive		
Total	380				
Age (year)				0.016	0.898
≤65	150	18(11.84%)	134(88.16%)		
> 65	228	28(12.28%)	200(87.72%)		
Gender				0.067	0.875
Male	208	26(12.50%)	182(87.50%)		
Female	172	20(11.63%)	152(88.37%)		
Histological subtype				128.953	**< 0.001***
Adherent type	63	34(53.97%)	29(46.03%)		
Acinar type	140	1(0.71%)	139(99.29%)		
Papillary type	49	2(4.08%)	47(95.92%)		
Micro- papillary type	62	7(11.29%)	55(88.71%)		
Solid type	66	2(3.03%)	64(96.97%)		
Pleural invasion				48.730	**< 0.001***
Absent	151	40(26.49%)	111(73.51%)		
Present	229	6(2.62%)	223(97.38%)		
Vascular invasion				15.095	**< 0.001***
Absent	179	34(18.99%)	145(81.01%)		
Present	201	12(5.97%)	189(94.03%)		
Airway spread				103.402	**< 0.001***
Absent	102	41(40.20%)	61(59.80%)		
Present	278	5(1.80%)	273(98.20%)		
Interstitial fibrosis				141.608	**< 0.001***
Negative	11	7(63.64%)	4(36.63%)		
≤10%	94	39(41.49%)	55(58.51%)		
10-25%	99	0(0.00%)	99(100.00%)		
25-50%	113	0(0.00%)	113(100.00%)		
> 50%	63	0(0.00%)	63(100.00%)		
Necrosis				10.737	**0.005***
Absent	114	22(19.30%)	92(80.70%)		
Focal area	216	16(7.41%)	200(92.59%)		

A large area	50	8(16.00%)	42(84.00%)		
pT				115.713	**< 0.001***
pT1a	18	14(77.80%)	4(22.22%)		
pT1b	64	6(9.38%)	58(90.63%)		
pT1c	65	20(30.77%)	45(69.23%)		
pT2a	64	4(6.25%)	60(93.75%)		
pT2b	84	1(1.19%)	83(98.81%)		
pT3	77	1(1.3%)	76(98.70%)		
pT4	8	0(0.00%)	8(100.00%)		
pN				27.761	**< 0.001***
pN0	195	40(20.51%)	155(79.49%)		
pN1	82	1(1.22%)	81(98.78%)		
pN2	82	5(6.10%)	77(93.90%)		
pN3	21	0(0.00%)	21(100.00%)		
TNM stage				41.194	**< 0.001***
Ⅰa1	6	4(66.67%)	2(33.33%)		
Ⅰa2	76	12(15.79%)	64(84.21%)		
Ⅰa3	48	11(22.92%)	37(77.08%)		
Ⅰb	41	9(21.95%)	32(78.05%)		
Ⅱa	15	3(20.00%)	12(80.00%)		
Ⅱb	87	2(2.30%)	85(97.70%)		
Ⅲa	76	4(5.26%)	72(94.74%)		
Ⅲb	27	1(3.70%)	26(96.30%)		
Ⅲc	3	0(0.00%)	3(100.00%)		
Ⅳ	1	0(0.00%)	1(100.00%)		
5-year survival				32.644	**< 0.001***
No	183	4(2.19%)	179(97.81%)		
Yes	197	42(21.32%)	155(78.68%)		

Table2 The correlation of tumor budding with clinicopathological characteristics of lung squamous cell carcinoma patients

Characteristic	All cases	Tumor budding		χ^2	P
		Negative	Positive		
Total	152				
Age (year)				3.776	0.075
≤65	52	3(5.77%)	49(94.23%)		
> 65	100	17(17.00%)	83(83.00%)		
Gender				0.457	0.622

Male	94	11(11.70%)	83(88.30%)		
Female	58	9(15.52%)	49(84.48%)		
Peritumoral space				27.333	**< 0.001***
Absent	36	14(38.89%)	22(61.11%)		
Present	116	6(5.17%)	110(94.83%)		
Pleural invasion				1.341	0.475
Absent	132	19(14.39%)	113(85.61%)		
Present	20	1(5.00%)	19(95.00%)		
Vascular invasion				11.160	**< 0.001***
Absent	62	15(24.19%)	47(75.81%)		
Present	90	5(5.56%)	85(94.44%)		
spread through airspaces (STAS)				11.715	**0.001***
Absent	75	17(22.67%)	58(77.33%)		
Present	77	3(3.90%)	74(96.10%)		
Interstitial fibrosis				51.047	**< 0.001***
Negative	6	6(100.00%)	0(0.00%)		
≤10%	32	8(25.00%)	24(75.00%)		
10-25%	49	4(8.16%)	45(91.84%)		
25-50%	36	0(0.00%)	36(100.00%)		
> 50%	29	2(6.90%)	27(93.10%)		
Necrosis				6.983	**0.030***
Absent	7	2(28.57%)	5(71.43%)		
Focal area	92	16(17.39%)	76(82.61%)		
A large area	53	2(3.77%)	51(96.23%)		
pT				31.561	**< 0.001***
pT1a	1	1(100.00%)	0(0.00%)		
pT1b	20	6(30.00%)	14(70.00%)		
pT1c	31	10(32.26%)	21(67.74%)		
pT2a	33	2(6.06%)	31(93.94%)		
pT2b	34	0(0.00%)	34(100.00%)		
pT3	22	0(0.00%)	22(100.00%)		
pT4	11	1 (9.09%)	10(90.91%)		
pN				8.284	**0.040***
pN0	84	17(20.24%)	67(79.76%)		
pN1	47	2(4.26%)	45(95.74%)		
pN2	19	1(5.26%)	18 (94.74%)		
pN3	2	0 (0.00%)	2(100.00%)		

TNM stage				32.131	**< 0.001***
Ⅰa1	4	1(25.00%)	3(75.00%)		
Ⅰa2	18	5(27.78%)	13(72.22%)		
Ⅰa3	23	10(43.48%)	13(56.52%)		
Ⅰb	16	2(12.50%)	14(87.50%)		
Ⅱa	19	0(0.00%)	19(100.00%)		
Ⅱb	38	1(2.63%)	37(97.37%)		
Ⅲa	25	1(4.00%)	24(96.00%)		
Ⅲb	5	0(0.00%)	5(100.00%)		
Ⅲc	3	0(0.00%)	3(100.00%)		
Ⅳ	1	0(0.00%)	1(100.00%)		
5-year survival				17.383	**< 0.001***
No	88	3(3.41%)	85(96.59%)		
Yes	64	17(26.56%)	47(73.44%)		

Table 3 The univariate analysis of 5-year survival prognostic factors in lung adenocarcinoma patients.

Variable	Univariate analysis	
	P > |z|	HR(95%CI)
Tumor Budding (10HPF) Low(n=141) vs. high(n=193)	**0.011***	1.374(1.077-1.753)
Nuclear size Small(n=145) vs. Large(n=189)	**0.023***	1.467(1.054-2.042)
Smallest tumor cell nest Single cell(n=166) vs. 2-4cells(n=168)	0.699	0.943(0.702-1.267)
Gender Male(n=208) vs. female(n=172)	0.252	0.835(0.614-1.136)
Age(years) ≤65 (n=150) vs. > 65 (n=228)	0.050	1.362(1.00-1.854)
Pleural invasion Absent (n=151) vs. present (n=229)	**0.021***	1.560(1.071-2.272)
Vascular invasion Absent (n=179) vs. present (n=201)	**0.001***	2.357(1.401-3.965)
spread through airspaces		

(STAS)		
Absent (n=102) vs. present (n=278)	**< 0.001***	2.874(1.690-4.887)
Necrosis		
Absent (n=114) vs. present (n=266)	**0.047***	1.315(1.004-1.722)
Histological subtype		
Adherent type(n=63) vs. Acinar type(n=140) vs. Papillary type (n=49) vs. Micro-papillary type(n=62) vs Solid type (n=66)	**0.014***	0.858(0.759-0.969)
Interstitial fibrosis		
Absent(n=11) vs. present(n=369)	0.200	0.900(0.766-1.057)
pT		
pT1+pT2(n=295) vs pT3+pT4 (n= 85)	**< 0.001***	2.069(1.687-2.538)
pN		
pN0(n=195) vs pN1+pN2+pN3 (n=185)	**< 0.001***	1.974(1.363-2.858)
TNM stage		
Ⅰ + Ⅱ (n= 273)vs Ⅲ+Ⅳ(n=107)	**0.003***	0.484(0.301-0.780)

Table 4 The univariate analysis of 5-year survival prognostic factors in lung squamous cell carcinoma patients

Variable	Univariate analysis	
	P > |z|	HR(95%CI)
Tumor Budding (10HPF)		
Low(n=83) vs. high(n=49)	**0.002***	0.589(0.423-0.820)
Nuclear size		
Small(n=129) vs. Large(n=3)	0.159	0.390(0.880-2.196)
Smallest tumor cell nest		
Single cell(n=49) vs. 2-4cells(n=77)	**0.002***	0.485(0.307-0.769)
Gender		
Male(n=94) vs. female(n=58)	0.964	1.014(0.552-1.863)
Age(years)		
≤65 (n=52) vs. > 65 (n=100)	0.908	0.972(0.600-1.575)
Pleural invasion		

Absent (n=132) vs. present (n=20)	**0.001***	0.302(0.149-0.613)
Vascular invasion		
Absent (n=62) vs. present (n=90)	**0.005***	2.397(1.307-4.396)
spread through airspaces (STAS)		
Absent (n=75) vs. present (n=77)	**0.004***	2.426(1.327-4.435)
Necrosis		
Absent (n=7) vs. present (n=145)	0.287	1.252(0.828-1.896)
Peritumoral space		
Absent(n=36) vs. Present (n=116)	**< 0.001***	4.389(1.920-10.035)
Interstitial fibrosis		
Absent(n=6) vs. present(n=146)	**0.009***	1.315(1.071-1.614)
pT		
pT1+pT2(n=119) vs pT3+pT4 (n= 33)	**< 0.001***	2.398(1.584-3.629)
pN		
pN0(n=84) vs pN1+pN2+pN3 (n=68)	**0.029***	1.440(1.038-1.999)
TNM stage		
Ⅰ+ Ⅱ(n= 118)vs Ⅲ+ Ⅳ(n=34)	**0.016***	1.954(1.133-3.372)

Figure legends

Figure 1: The tumor budding with HE staining and immunohistochemical staining.
A-D: the budding of the tumor in lung squamous cell carcinoma.
E-H: the tumor budding in lung adenocarcinoma.
A, C, E and G were ×20 magnification.
B, D, F and H were×40 magnification (bar = 500 μm).

Figure 2: The forest map of multivariate survival analysis.
A: the results of multivariate analysis of lung adenocarcinoma.
B: the results of multivariate analysis of lung squamous cell carcinoma.

Figure 3: Kaplan – Meier analysis of the relationship between tumor budding and 5-year overall survival rate in patients with NSCLC.
A: in patients with lung adenocarcinoma, the 5-year survival rate of patients with high-grade budding group was significantly lower than that of patients without tumor

budding and low-grade tumor budding.
B: in patients with lung squamous cell carcinoma, the higher the level of tumor budding, the worse the prognosis of patients was.

Figure 4: Kaplan – Meier analysis showed that the 5-year survival rate of patients with different histological subtypes in adenocarcinoma.
A: the survival rates of patients with different histological subtypes were different. Among them, the 5-year prognosis of patients with micropapillary subtype and solid subtype was significantly lower than that of adherent subtypes.
B: in patients with solid subtypes, the 5-year survival rate of patients with high-grade budding was significantly lower than that of patients with low-grade budding and non-budding.
C: in patients with micropapillary subtypes, the higher the grade of tumor budding, the worse the prognosis.

Figure 5: The relationship between the size of tumor budding nests and the nuclear size of tumor budding, as well as the 5-year survival rate of patients with NSCLC.
A: in patients with lung adenocarcinoma, the larger the nucleus of tumor budding, the lower the 5-year overall survival rate was.
B: in patients with lung squamous cell carcinoma, single cell invasion showed a worse prognosis.

Figure 6: The relationship between tumor budding level and patients at different TNM stages.
A: in patients with TNM stage I lung adenocarcinoma, the higher the tumor budding level, the lower the 5-year overall survival rate.
B: in patients with TNM stage II squamous cell carcinoma, the prognosis of patients without tumor budding and low-grade tumor budding was significantly higher than that of patients with high-grade tumor budding.

Figure 1

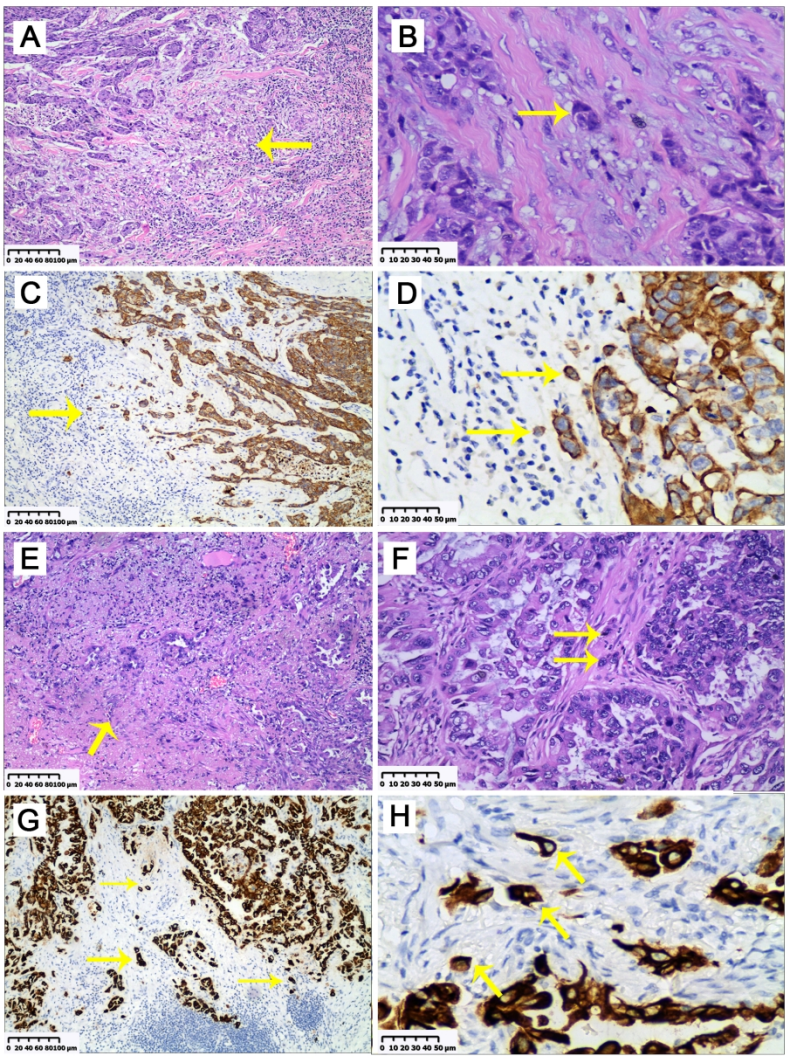


Figure 2

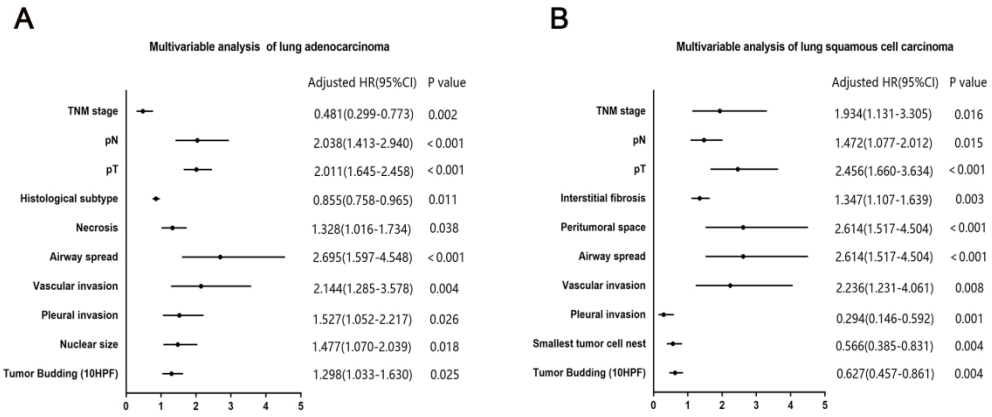


Figure 3

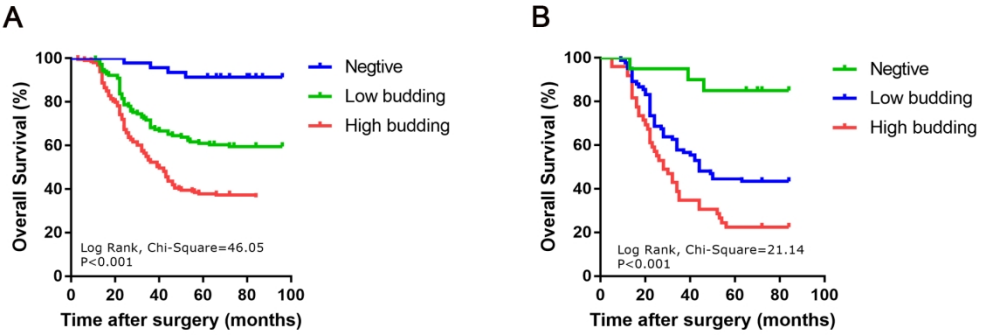


Figure 4

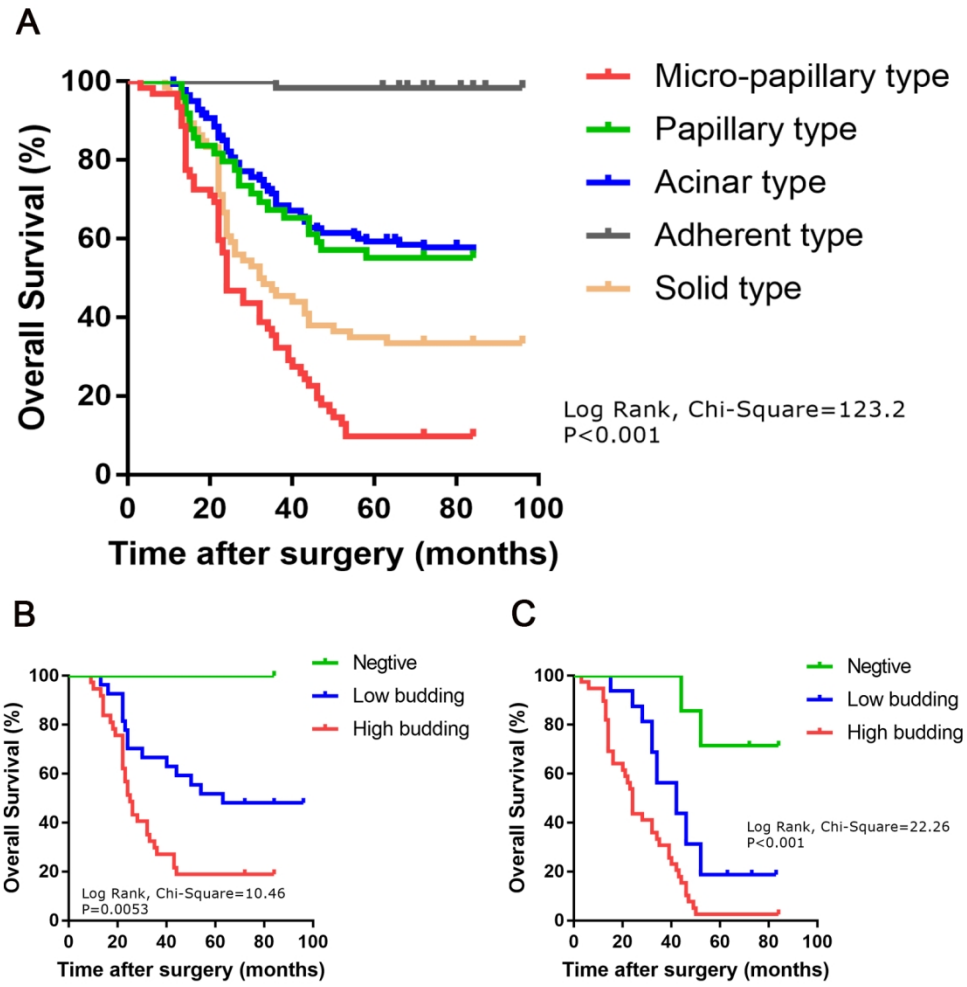


Figure 5

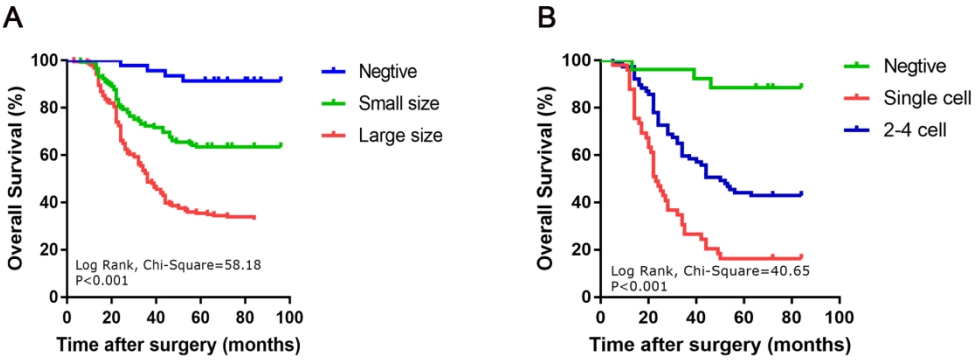
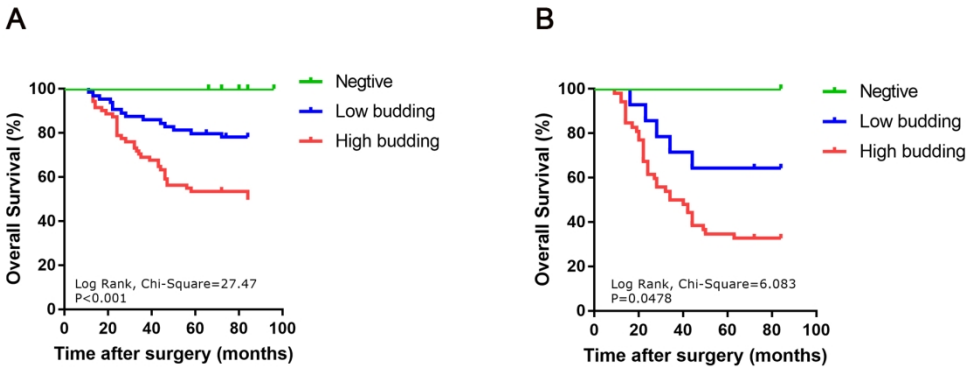


Figure 6



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Potential key roles of tumor budding: a representative malignant pathological feature of non-small cell lung cancer and a sensitive indicator of prognosis

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Abstract

Objectives: To investigate the relationship between tumor budding, clinicopathological characteristics of patients, and prognosis in non-small cell lung cancer.

Study design: A retrospective study was used.

Participants: We selected 532 patients with non-small cell lung cancer from China, including 380 patients with adenocarcinoma and 152 with squamous cell carcinoma

Primary and secondary outcome measures: Tumor budding was visible using hematoxylin and eosin staining as well as pan-cytokeratin staining. The count data and measurement data were compared using the $\chi 2$ test and the t-test, respectively. The overall survival (OS) rate was the follow-up result. The survival curves were drawn using the Kaplan-Meier method, and the differences between groups were analyzed using the log-rank method. The independent prognostic factor of lung cancer patients was determined using a multivariate Cox proportional hazard model.

Results: In patients with lung adenocarcinoma, there was a correlation between tumor budding and airway spread (OR: 36.698; 95% CI: 13.925–96.715; $P < 0.001$), and in patients with squamous cell carcinoma, tumor budding state was closely related to the peritumoral space (OR: 11.667; 95% CI: 4.041–33.683; $P < 0.001$). On Cox regression analysis, multivariate analysis showed that tumor budding, pleural and vascular invasion, airway spread, tumor size, lymph node metastasis, and Tumor Node Metastasis stage were independent risk factors of prognosis for non-small cell lung

cancer patients.

Conclusions: As an effective and simple pathological diagnostic index, it is necessary to establish an effective grading system in the clinical diagnosis of lung cancer to verify the value of tumor budding as a prognostic indicator. We hope that this analysis of Chinese patients with non-small cell lung cancer can provide useful reference material for the continued study of tumor budding.

Key words: lung cancer, prognosis, tumor budding

Strengths and limitations of this study

- We selected 532 patients with non-small cell lung cancer from China, including 380 patients with adenocarcinoma and 152 with squamous cell carcinoma, to explore the correlation between tumor budding, the clinicopathological characteristics of these patients, and prognosis.
- Through the evaluation of tumor budding in lung cancer specimens of Chinese patients, we hope to provide reference for the establishment of tumor budding criteria in the diagnosis of lung cancer.
- Our research was limited to the tumor budding analysis of NSCLC patients in China, and the results of different ethnicities may differ.
- This study only included surgical resection specimens, no biopsy specimens.

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Conflict of interest

The authors have disclosed that they have no significant relationships with, or financial interest in, any commercial companies pertaining to this article.

Authors' contributions

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

NO. 2018-L068.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Introduction

Lung cancer is among the most common malignant tumors in China and the world. According to global cancer data from 2020, lung cancer is the most common type of cancer (11.4% of the total) and cancer-related death (18% of total cancer deaths)[1]. Early lung cancer has few clinical manifestations and is easily ignored or even missed. With the spread and infiltration of tumor cells, most patients lose the opportunity for radical surgery. In recent years, with the rapid development of medical technology, immunotherapy has become a hot spot in the treatment of lung cancer. In a Meta-analysis study by Alfredo tartarone et al., the results showed that in pretreated NSCLC patients, three immune checkpoint inhibitors (ICIs) such as nivolumab, pembrolizumab, and atezolizumab, as well as two anti-PD-1 (nivolumab and pembrolizumab) and one

anti-PD-L1 (atezolizumab) can be administered. The findings support the superiority of ICIs over docetaxel in pretreated NSCLC patients, and suggest that anti-PD-1 inhibitors may have a minor advantage over anti-PD-L1 inhibitors[2]. Fausto Petrelli et al. confirmed in their meta-analysis that there is moderate evidence that adding immune checkpoint inhibitors to chemotherapy improves overall survival when compared to chemotherapy alone[3]. However, in a review of Jianwei Zhu et al put forward different opinions. Their research results show that immunotherapy for patients with non-small cell lung cancer after surgery or radiotherapy cannot prolong their survival time. At the same time, they noted that an interim analysis for one of these trials revealed that treated participants with stage III NSCLC had a better PFS[4]. Most current studies are combined therapies, such as dendritic cells (DCs) or DCs/cytokine induced killer (CIK) therapy in combination with chemotherapy in advanced lung cancer, according to a review by Monireh Mohsenzadegan et al[5]. However, these medications have only had little success in the treatment of advanced NSCLC[5]. Invasion and metastasis are among the main causes of lung cancer death and play a decisive role in lung cancer staging and management.

As a pathological phenomenon, tumor budding has been attracting increased attention. Some studies have shown that tumor budding is a factor that reflects the malignant invasion and poor prognosis of digestive tract tumors[6]. The Union for International Cancer Control (UICC) has officially recognized that tumor budding is an independent prognostic factor for colorectal cancer (CRC) patients. However, only a

few studies have explored its significance in lung cancer.

In recent years, with the increasing research on cancer prognosis, some scholars have reported that the morphological characteristics of the peritumoral space are related to patient prognosis. Peritumoral spaces have been noted in breast, lung, bladder, and prostate cancers as well as other malignant tumors. Tumor cells generally spread to the corresponding lymph nodes through the lymphatic system, a phenomenon that is considered an important early event of tumor metastasis[7 8]. However, the presence of a correlation between tumor budding and the peritumoral space has been rarely reported.

In this study, we selected 532 cases of NSCLC patients from China, including 380 cases of adenocarcinoma and 152 cases of squamous cell carcinoma, to explore the correlation between tumor budding, patients' clinicopathological characteristics, and prognosis with the aim of determining a reference value for evaluating patient prognosis and clinical treatment.

Material and methods

Patients' general information

We retrieved the pathological reports of patients who met the inclusion criteria from the files of the pathology system and obtained other clinical pathological information from the electronic medical record system. All 532 cases included in this study were radical surgical specimens. The data of 380 patients with primary lung adenocarcinoma and 152 patients with primary lung squamous cell carcinoma treated in the Cardiothoracic Disease Department of the Affiliated Hospital of Nantong

University between June 2009 and July 2015. We excluded patients for whom follow-up information was lacking; thus, and a total of 532 patients (302 males, 230 females; 202 patients were ≤65 years old, while 328 patients were >65 years old). None of the patients received chemotherapy or radiotherapy preoperatively. The clinical and pathological information and medical records were complete for each patient.

We took the corresponding paraffin blocks of each patient from the pathological diagnosis center and sliced them into 3-μm-thick slices. Each slice was floated in 45°C warm water on a spreader to flatten the tissue, which was then picked up with a slide and baked in an oven at 65°C. Cytokeratin immunohistochemical staining (CK) and hematoxylin and eosin (HE) staining were performed. Rabbit polyclonal anti-human pan-cytokeratin (CKpan) antibody was used (dilution 1:50; ab215838, Abcam, USA). The evaluations were independently performed by three experienced pathologists using a multi-head microscope (Precise Instrument Co., Ltd., Beijing, China) to reach consensus.

Patient and Public Involvement

All patients signed an informed consent form, and the study was approved by the Ethics Committee of Affiliated Hospital of Nantong University (2018-L068). The patients were followed up by telephone and outpatient service. The starting point of follow-up was the operation time for each patient, while the end point was the time of death. If the patient was still alive, we selected the last follow-up appointment as the termination point.

Histological type assessment

We observed the histopathological structure of each tissue sample under the microscope and classified the tumor tissues according to the diagnostic criteria formulated by the WHO in 2015. The Tumor Node Metastasis (TNM) staging was based on UICC/American Joint Committee on Cancer (AJCC) 8th edition.

Evaluation of tumor budding with HE

The slides stained with HE were placed under a 10 × 20 light microscope to observe the densest portion of the budding. The areas of budding were then counted in high-power fields (HPFs).

The judgment of tumor budding refers to the standard of Ueno et al.[9], that is, an isolated single tumor cell or small clusters of tumor cells composed of no more than four tumor cells in the stroma at the start of the tumor invasion were considered tumor budding.

To employ a semiquantitative method to analyze tumor budding, we counted the mean number of tumor buds under 10 HPFs. The tumor budding was divided into non-budding, low budding (≤10 buds/10 HPFs) and high budding (>10 buds/10 HPFs).

Tumor cell clusters surrounded by tumor stroma were defined as tumor cell nests. Based on Moritz's research method[10] and according to the histomorphology characteristics of lung cancer, we divided the cell nests in tumor stroma into two to four

tumor cell nests and a single invasive cancer cell in the matrix of the tumor invasion edge. We also divided tumor interstitial fibrosis into negative, very low (10% of the total tumor area), low (10–25%), medium (25–50%), and high (>50%).

Evaluation of tumor budding assisted with Cytokeratin

The clarity of HE and pan-cytokeratin staining on tumor budding were compared.

It remains controversial whether HE or Cytokeratin (CK) staining should be used for budding markers. CK staining can reportedly more clearly show the bud focus covered by the significant peritumoral inflammatory reaction[11]. CK staining also aides in the observation of a large number of germinal foci mixed with stromal fibroblasts[12]. CK staining can produce three to four times more buds than HE staining[13]. In many studies, many scholars chose CK staining for sprouting evaluations[12 14-20]. Therefore, here we used both HE staining and pan-cytokeratin staining and observed the budding state of each level between methods. The budding site was more easily observed and the scope of the bud focus was clearer using pan-cytokeratin staining.

Statistical analysis

The data were analyzed using SPSS 26.0 software (IBM Corporation, Armonk, NY, USA). The χ^2 test and t-test were used to compare the count data and measurement data, respectively. The follow-up result was the overall survival (OS) rate. The Kaplan-Meier method was used to draw the survival curves, while the log rank method was

used to analyze the differences among groups. A multivariate Cox proportional hazard model was used to determine the independent prognostic factors of the lung cancer patients. The difference was statistically significant ($P < 0.05$).

Results

Tumor budding in NSCLC patients

In cases of lung cancer with tumor budding, the front edge was not smooth and the budding tumor cells were heteromorphic, irregularly shaped, rich in cytoplasm, often fused, and eosinophilic. The nucleus was irregularly shaped and the staining was deeper than that of stromal cells. However, the tumor budding foci were sometimes easily confused with poorly differentiated stromal cells. However, compared with HE staining, CK staining can more clearly show tumor budding spores (Figure 1).

Relationship between tumor budding and clinicopathological features of patients with NSCLC

Tumor interstitial fibrosis was defined as fibrosis observed under 100× magnification. According to the area of fibrosis, it was classified as negative, ≤10%, 10–25%, 25–50% and >50%. The peritumoral space, that between the tumor cells and the stroma, was the morphological manifestation of the interaction between them that clearly divided the tumor components and the stroma[7]. Shah et al.[21] reported that the peritumoral space was very common in tumors and related to invasive cancer cell nests.

Among the 380 cases of lung adenocarcinoma, 46 showed no tumor budding and 334 showed tumor budding. Tumor budding status was closely related to the 5-year OS status of patients with lung adenocarcinoma. In addition, it was closely related to tumor histological subtype ($P < 0.001$), tumor size ($P < 0.001$), lymph node metastasis ($P < 0.001$), vascular invasion (OR, 3.693; 95% CI, 1.847–7.383; $P < 0.001$), pleural invasion (OR, 13.393; 95% CI, 5.512–32.542; $P < 0.001$), STAS (OR, 36.698; 95% CI, 13.925–96.715; $P < 0.001$), tumor necrosis ($P = 0.005$), tumor interstitial fibrosis ($P < 0.001$), and TNM stage ($P < 0.001$). However, tumor budding was not related to the patient gender patient sex (OR, 1.086; 95% CI, 0.583–2.021; $P = 0.875$) or age (OR, 0.959; 95% CI, 0.510–1.804; $P = 0.898$). The proportion of tumor budding in patients with vascular tumor thrombus was significantly higher than that in patients without vascular tumor thrombus. The greater the degree of lymph node metastasis, the higher the proportion of tumor budding (Table 1). In the 152 patients with primary squamous cell carcinoma of the lung (Table 2), tumor budding status was significantly correlated with the 5-year OS status (OR, 0.098; 95% CI, 0.027–0.350; $P < 0.001$), peritumoral space (OR, 11.667; 95% CI, 4.041–33.683; $P < 0.001$), vascular invasion (OR, 5.426; 95% CI, 1.855–15.865; $P = 0.001$), tumor size ($P < 0.001$), lymph node metastasis ($P < 0.001$), airway spread (OR, 7.230; 95% CI, 2.021–25.863; $P = 0.001$), tumor necrosis ($P = 0.030$), TNM stage ($P < 0.001$), and tumor interstitial fibrosis ($P < 0.001$).

Survival analysis of patients

All 532 patients were included in the survival analysis study by July 2020. The follow-up time was 3–82 months. At the end of the study, 261 patients were still alive. Among the dead patients, the proportion of high-grade budding was significantly higher than those of the low-grade budding and non-budding groups. The Kaplan-Meier method was used to analyze the postoperative survival rate, while the log rank method was used to test the intergroup differences.

In patients with lung adenocarcinoma, univariate analysis showed that tumor budding, tumor budding nucleus size, pleural and vascular invasion, airway spread, histological subtype, necrosis area, and TNM stage were significantly associated with 5-year survival (Table 3). We then used the Cox proportional hazard regression model to analyze the statistically significant indicators of the univariate analysis. For the budding model, we took the above factors as variables, and the tumor budding (hazard ratio [HR], 1.298; 95% confidence interval [CI], 1.033–1.630; P = 0.025), nuclear size (HR, 1.477; 95% CI, 1.070–2.039; P = 0.018), pleural invasion (HR, 1.527; 95% CI, 1.052–2.217; P = 0.026), vascular invasion (HR, 2.144; 95% CI, 1.285–3.578; P = 0.004), airway spread (HR, 2.695; 95% CI, 1.597–4.548; P < 0.001), necrosis (HR, 1.328; 95% CI, 1.016–1.734; P = 0.038), histological subtype (HR, 0.855; 95% CI, 0.758–0.965; P = 0.011), pT (HR, 2.011; 95% CI, 1.645–2.458; P < 0.001), pN (HR, 2.038; 95% CI, 1.413–2.940; P < 0.001), and TNM stage (HR, 0.481; 95% CI, 0.299–0.773; P = 0.002) also showed a statistically significant correlation with the 5-year survival rate based on the Cox regression univariate analysis (Figure 2).

The Kaplan-Meier survival curve showed that the higher the budding grade, the lower the 5-year OS rate ($P < 0.001$) (Figure 3). In the histological subtypes of lung adenocarcinoma, the higher the level of tumor budding, the worse the prognosis in cases with micropapillary subtypes and solid subtypes (Figure 4). In the adherent subtype ($P = 0.356$), papillary subtype ($P = 0.567$), and acinar subtype ($P = 0.353$), there was no statistical correlation between tumor budding degree and survival status. Compared with tumor budding cell nucleus containing fewer than three lymphocytes (small size), when the tumor budding nucleus had four or more lymphocytes (large size), the 5-year OS rate of lung adenocarcinoma patients was significantly reduced (Figure 5A).

In cases of lung squamous cell carcinoma, tumor budding size, budding tumor nest, pleural and vascular invasion, airway spread, tumor interstitial fibrosis area, peritumoral space, tumor size and lymph node metastasis, and TNM stage influenced patient 5-year survival rate (Table 4). To eliminate the interactions between variables, multivariate Cox regression analysis was used to analyze the data. The above factors independently affected the prognosis of patients with squamous cell carcinoma (Figure 2). The Kaplan-Meier survival curve showed that the 5-year OS rate of patients with lung squamous cell carcinoma in TNM stage II was significantly higher than that of patients with high-grade tumor budding (Figure 6B), while the 5-year OS rate of lung squamous cell carcinoma patients with single cell tumor budding was significantly lower (Figure 5B).

Discussion

Cancer is an issue of great concern worldwide, and its prognosis mainly depends on the pathological type, TNM stage, tumor differentiation degree, and microvascular invasion, and patients with the same TNM stage but quite different prognoses are often seen in the clinical setting. In recent years, as a pathological phenomenon, tumor budding has attracted increasing attention. Tumor budding, also known as focal dedifferentiation is the first step in the process of a malignant tumor's invasion and metastasis. Therefore, tumor budding is considered a key step in a tumor's invasive growth process[22]. Tumor budding spores are considered cancer stem cells, which are defined as isolated single tumor cells or clusters of fewer than five tumor cells at the start of tumor invasion[11]. Some studies stated that tumor budding is not a static histological feature; rather, it involves a small focal tumor cell complex separated from the main body of the tumor that enters the surrounding tissue in a "budding" manner, which represents a dynamic process[23]. Gabbert et al.[22] also supported this conclusion. Shinto et al.[14] reported that there were interconnected cytoplasmic pseudo fragments similar to pseudopodia processes between budding tumor cells, which may be related to the increase in cell invasion ability. In addition, some studies have speculated that tumor budding is a step in the progression of malignant tumors from focal lesions to systemic diseases[24]. Tumor budding is now considered of great significance in tumor invasion and metastasis[25-28]. Some studies have shown that tumor budding reflected the invasiveness and poor prognosis of digestive tract tumors[6]. The presence of tumor

budding may be related to the late stage of a tumor, frequent lymphatic vascular invasion, and lymph node and distant metastasis. The UICC officially recognizes tumor budding as an independent prognostic factor for CRC. It was recently used as a significant prognostic indicator for the treatment of esophageal squamous cell carcinoma, gastroesophageal junction adenocarcinoma, and gastric adenocarcinoma[29]. In the current study of 380 cases of primary lung adenocarcinoma and 152 cases of primary lung squamous cell carcinoma, we found that tumor budding was closely related to the 5-year OS, tumor size, lymph node metastasis, vascular invasion, airway spread, tumor necrosis, tumor interstitial fibrosis, and TNM stage. This suggests that tumor budding may be an important indicator of malignant invasion and metastasis. Compared with NSCLC patients without tumor budding, those with the morphological characteristics of tumor budding have a worse 5-year OS prognosis.

The detection accuracy of abdominal B-ultrasound and abdominal computed tomography for lymph node metastasis is reportedly 12.2–80.0%[30] and 50–80%, respectively[31-34]. Guluoglu et al.[35] evaluated 126 patients with gastric cancer and found that lymph node metastasis was the only parameter associated with tumor budding. Masaki *et., al*[36] established a model formula for predicting the probability of lymph node metastasis in 76 patients with T1 stage CRC as follows: $z = 0.070 \times$ (budding count) - 3.726, probability = $1/1 + e^{-z}$. Furthermore, the tumor budding count was included in the clinical decision-making analysis of patients to determine whether patients require additional surgery after endoscopic treatment. Some studies have

shown that the presence of tumor budding in biopsy specimens before CRC surgery increases the possibility of lymph node and distant metastasis. Therefore, neoadjuvant therapy and surgical treatment can be considered for these patients[37]. The Japanese Society for Cancer of the Colon and Rectum has incorporated the index of tumor budding into the guidelines for patients with pT1 disease who require further surgery[38]. In our study, 244 of 253 patients with lymph node metastasis had tumor budding. The sensitivity of budding for predicting lymph node metastasis was 96.44%, indicating that tumor budding is an effective pathological index with high sensitivity for predicting lymph node metastasis. Therefore, we believe that for patients with NSCLC, we can refine the significance of tumor budding through a larger sample study to contribute to clinical decision-making.

The peritumoral space is the space between the tumor cells and the stroma that divides the tumor components from the stroma and is morphological manifestation of the interaction between the tumor cells and the stromal cells. The peritumoral space is commonly seen in paraffin-embedded tissue sections fixed with formalin. The peritumoral space is one of the pathomorphological manifestations of tumor biological behavior that is considered a prognostic factor by some scholars. Peritumoral spaces have been noted in breast, lung, bladder, and prostate cancers and other malignant tumors. Tumor cells usually spread to the corresponding lymph nodes through the lymphatic system, this phenomenon is considered an important early event of tumor metastasis[7 8]. In prostate cancer, an extensive peritumoral space indicates a higher

tumor grade, shorter disease-free survival, and poor prognosis[39 40]. At the same time, the peritumoral space in breast cancer is closely related to histological grade, lymphatic invasion, lymph node metastasis, and prognosis and can be used as an important marker to judge the prognosis of breast cancer patients[41 42]. Acs et al.[43] observed the relationship between a large peritumoral space and lymph angiogenesis, and the results confirmed a poor prognosis of patients with large peritumoral spaces, which was consistent with this hypothesis. In our study, we found that in patients with lung squamous cell carcinoma, the peritumoral space is closely related to tumor budding, which is also an independent risk factor for patient 5-year OS. A joint evaluation of the peritumoral space and tumor budding can effectively evaluate the prognosis of patients with lung squamous cell carcinoma.

Lung adenocarcinoma spreads through the bronchus, known as lung metastasis, and the airways, known as airway metastasis. A small number of lung adenocarcinoma cancer cells enter the bronchial cavity, and with the respiratory movement through the bronchial discontinuous, they diffuse into other lung segments or lobes on the same or opposite side, forming new lung metastases[44]. Our study revealed that tumor budding was closely related to airway spread. Tumor budding can be combined with spread through airspaces (STAS) to evaluate the malignant aggressive behavior of NSCLC.

Che et al.[6] found that the OS rate of patients with high budding gastric adenocarcinoma was significantly lower than that of patients with low budding gastric adenocarcinoma. Some studies reported that the presence of tumor budding in surgical

specimens of patients with gastric cancer may indicate a poor prognosis and early recurrence[29]. We also found that the 5-year OS rate of lung adenocarcinoma or squamous cell carcinoma patients with high-grade budding was significantly lower than that of patients with low-grade or no budding. However, Hass et al.[45] emphasized that tumor budding and cancer classification based on cell differentiation were neither the same nor related. Some researchers believed that tumor budding and tumor growth pattern were independent prognostic parameters[9]. However, in our study of lung adenocarcinoma, tumor budding was closely related to histological subtype. In patients with papillary and solid subtypes of lung adenocarcinoma, the 5-year survival rate of patients with high-grade budding was significantly lower than that of patients with low-grade budding. In patients with TNM stage I, the 5-year OS rate of patients with high-grade tumor budding was lower than that of patients with low-grade or no budding (Figure 6A). The results are consistent with those of Kyuichi et al.[46].

In our study, Cox regression analysis showed a significant correlation between tumor budding and 5-year OS rate. Tumor budding, pleural and vascular invasion, airway spread, tumor size, lymph node metastasis, and TNM stage were independent risk factors for the prognosis of NSCLC patients. In addition, tumor budding nucleus size, tumor necrosis area, and histological subtype were independent prognostic factors of lung adenocarcinoma. The area of interstitial fibrosis, presence of a peritumoral space, and small tumor cell nest were independent prognostic factors in patients with squamous cell carcinoma. Therefore, we speculate that tumor budding may be a

representative malignant pathological feature of NSCLC and a sensitive indicator reflective of its prognosis.

The research results of Wang et al. suggested that tumor budding should be included in the routine histopathological report to better stratify the risk of CRC patients[47]. The AJCC and College of American Pathologists guidelines on CRC proposed that tumor budding should be considered an optional reporting indicator and should be evaluated in all cases of stage I and II CRC. This provides us with a standardized reporting tool for tumor budding[48]. However, there is no unified scoring standard for lung cancer.

The current study had several limitations. First, our research is limited to the tumor budding analysis of NSCLC patients in China, and the results of different ethnicities may differ. For example, demographic heterogeneity in the frequency of genetic susceptibility alleles was addressed in Zahra Fathi, et al's review of lung cancer in the Iranian population[49]. They focused on germline and somatic gene variation, putative operable drivers of these genes, their impact on tumor immune monitoring, and the drug resistance mechanism of cancer treatment in which they engage in this work. In addition, because the number of surgical specimens selected for this operation before 2015 was limited, the sample size was insufficient, which might result in sample bias. However, as an effective and simple pathological diagnosis index, it is necessary to establish an effective grading system to verify its value as a standard prognostic indicator. In addition, prospective clinical trials including multicenter samples are needed to

evaluate the role of tumor budding in predicting the prognosis of lung cancer and produce reference values for the pathological diagnosis and clinical treatment of lung cancer.

Conclusion

To validate the utility of tumor budding as a prognostic indicator, an effective and straightforward pathological diagnostic index should be established in the clinical diagnosis of lung cancer. We selected 532 Chinese patients with non-small cell lung cancer for this investigation, including 380 with adenocarcinoma and 152 with squamous cell carcinoma. Our findings reveal a link between tumor budding and airway spread in patients with lung adenocarcinoma, and a connection between tumor budding and the peritumoral space in patients with squamous cell carcinoma. Multivariate analysis revealed that tumor budding, pleural and vascular invasion, airway spread, tumor size, lymph node metastasis, and Tumor Node Metastasis stage were independent risk variables of prognosis for non-small cell lung cancer patients by Cox regression analysis. We think that this study of Chinese patients with non-small cell lung cancer will be relevant for future research into tumor budding.

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Table1 The correlation of tumor budding with clinicopathological characteristics of lung adenocarcinoma patients

Characteristic	All cases	Tumor budding		χ^2	P
		Negative	Positive		
Total	380				
Age (year)				0.016	0.898
≤65	150	18(11.84%)	134(88.16%)		
> 65	228	28(12.28%)	200(87.72%)		
Gender				0.067	0.875
Male	208	26(12.50%)	182(87.50%)		
Female	172	20(11.63%)	152(88.37%)		
Histological subtype				128.953	**< 0.001***
Adherent type	63	34(53.97%)	29(46.03%)		
Acinar type	140	1(0.71%)	139(99.29%)		
Papillary type	49	2(4.08%)	47(95.92%)		
Micro- papillary type	62	7(11.29%)	55(88.71%)		
Solid type	66	2(3.03%)	64(96.97%)		
Pleural invasion				48.730	**< 0.001***
Absent	151	40(26.49%)	111(73.51%)		
Present	229	6(2.62%)	223(97.38%)		
Vascular invasion				15.095	**< 0.001***
Absent	179	34(18.99%)	145(81.01%)		
Present	201	12(5.97%)	189(94.03%)		
Airway spread				103.402	**< 0.001***
Absent	102	41(40.20%)	61(59.80%)		
Present	278	5(1.80%)	273(98.20%)		
Interstitial fibrosis				141.608	**< 0.001***
Negative	11	7(63.64%)	4(36.63%)		
≤10%	94	39(41.49%)	55(58.51%)		
10-25%	99	0(0.00%)	99(100.00%)		
25-50%	113	0(0.00%)	113(100.00%)		
> 50%	63	0(0.00%)	63(100.00%)		
Necrosis				10.737	**0.005***

Absent	114	22(19.30%)	92(80.70%)		
Focal area	216	16(7.41%)	200(92.59%)		
A large area	50	8(16.00%)	42(84.00%)		
pT				115.713	**< 0.001***
pT1a	18	14(77.80%)	4(22.22%)		
pT1b	64	6(9.38%)	58(90.63%)		
pT1c	65	20(30.77%)	45(69.23%)		
pT2a	64	4(6.25%)	60(93.75%)		
pT2b	84	1(1.19%)	83(98.81%)		
pT3	77	1(1.3%)	76(98.70%)		
pT4	8	0(0.00%)	8(100.00%)		
pN				27.761	**< 0.001***
pN0	195	40(20.51%)	155(79.49%)		
pN1	82	1(1.22%)	81(98.78%)		
pN2	82	5(6.10%)	77(93.90%)		
pN3	21	0(0.00%)	21(100.00%)		
TNM stage				41.194	**< 0.001***
Ⅰa1	6	4(66.67%)	2(33.33%)		
Ⅰa2	76	12(15.79%)	64(84.21%)		
Ⅰa3	48	11(22.92%)	37(77.08%)		
Ⅰb	41	9(21.95%)	32(78.05%)		
Ⅱa	15	3(20.00%)	12(80.00%)		
Ⅱb	87	2(2.30%)	85(97.70%)		
Ⅲa	76	4(5.26%)	72(94.74%)		
Ⅲb	27	1(3.70%)	26(96.30%)		
Ⅲc	3	0(0.00%)	3(100.00%)		
Ⅳ	1	0(0.00%)	1(100.00%)		
5-year survival				32.644	**< 0.001***
No	183	4(2.19%)	179(97.81%)		
Yes	197	42(21.32%)	155(78.68%)		

Table2 The correlation of tumor budding with clinicopathological characteristics of lung squamous cell carcinoma patients

Characteristic	All cases	Tumor budding		χ^2	P
		Negative	Positive		
Total	152				
Age (year)				3.776	0.075

≤65	52	3(5.77%)	49(94.23%)		
> 65	100	17(17.00%)	83(83.00%)		
Gender				0.457	0.622
Male	94	11(11.70%)	83(88.30%)		
Female	58	9(15.52%)	49(84.48%)		
Peritumoral space				27.333	**< 0.001***
Absent	36	14(38.89%)	22(61.11%)		
Present	116	6(5.17%)	110(94.83%)		
Pleural invasion				1.341	0.475
Absent	132	19(14.39%)	113(85.61%)		
Present	20	1(5.00%)	19(95.00%)		
Vascular invasion				11.160	**< 0.001***
Absent	62	15(24.19%)	47(75.81%)		
Present	90	5(5.56%)	85(94.44%)		
spread through airspaces (STAS)				11.715	**0.001***
Absent	75	17(22.67%)	58(77.33%)		
Present	77	3(3.90%)	74(96.10%)		
Interstitial fibrosis				51.047	**< 0.001***
Negative	6	6(100.00%)	0(0.00%)		
≤10%	32	8(25.00%)	24(75.00%)		
10-25%	49	4(8.16%)	45(91.84%)		
25-50%	36	0(0.00%)	36(100.00%)		
> 50%	29	2(6.90%)	27(93.10%)		
Necrosis				6.983	**0.030***
Absent	7	2(28.57%)	5(71.43%)		
Focal area	92	16(17.39%)	76(82.61%)		
A large area	53	2(3.77%)	51(96.23%)		
pT				31.561	**< 0.001***
pT1a	1	1(100.00%)	0(0.00%)		
pT1b	20	6(30.00%)	14(70.00%)		
pT1c	31	10(32.26%)	21(67.74%)		
pT2a	33	2(6.06%)	31(93.94%)		
pT2b	34	0(0.00%)	34(100.00%)		
pT3	22	0(0.00%)	22(100.00%)		

pT4	11	1 (9.09%)	10(90.91%)		
pN				8.284	**0.040***
pN0	84	17(20.24%)	67(79.76%)		
pN1	47	2(4.26%)	45(95.74%)		
pN2	19	1(5.26%)	18 (94.74%)		
pN3	2	0 (0.00%)	2(100.00%)		
TNM stage				32.131	**< 0.001***
Ⅰa1	4	1(25.00%)	3(75.00%)		
Ⅰa2	18	5(27.78%)	13(72.22%)		
Ⅰa3	23	10(43.48%)	13(56.52%)		
Ⅰb	16	2(12.50%)	14(87.50%)		
Ⅱa	19	0(0.00%)	19(100.00%)		
Ⅱb	38	1(2.63%)	37(97.37%)		
Ⅲa	25	1(4.00%)	24(96.00%)		
Ⅲb	5	0(0.00%)	5(100.00%)		
Ⅲc	3	0(0.00%)	3(100.00%)		
Ⅳ	1	0(0.00%)	1(100.00%)		
5-year survival				17.383	**< 0.001***
No	88	3(3.41%)	85(96.59%)		
Yes	64	17(26.56%)	47(73.44%)		

Table 3 The univariate analysis of 5-year survival prognostic factors in lung adenocarcinoma patients.

Variable	Univariate analysis	
	P > |z|	HR(95%CI)
Tumor Budding (10HPF) Low(n=141) vs. high(n=193)	**0.011***	1.374(1.077-1.753)
Nuclear size Small(n=145) vs. Large(n=189)	**0.023***	1.467(1.054-2.042)
Smallest tumor cell nest Single cell(n=166) vs. 2-4cells(n=168)	0.699	0.943(0.702-1.267)
Gender Male(n=208) vs. female(n=172)	0.252	0.835(0.614-1.136)
Age(years)		

≤65 (n=150) vs. > 65 (n=228)	0.050	1.362(1.00-1.854)
Pleural invasion Absent (n=151) vs. present (n=229)	**0.021***	1.560(1.071-2.272)
Vascular invasion Absent (n=179) vs. present (n=201)	**0.001***	2.357(1.401-3.965)
spread through airspaces (STAS) Absent (n=102) vs. present (n=278)	**< 0.001***	2.874(1.690-4.887)
Necrosis Absent (n=114) vs. present (n=266)	**0.047***	1.315(1.004-1.722)
Histological subtype Adherent type(n=63) vs. Acinar type(n=140) vs. Papillary type (n=49) vs. Micro-papillary type(n=62) vs Solid type (n=66)	**0.014***	0.858(0.759-0.969)
Interstitial fibrosis Absent(n=11) vs. present(n=369)	0.200	0.900(0.766-1.057)
pT pT1+pT2(n=295) vs pT3+pT4 (n= 85)	**< 0.001***	2.069(1.687-2.538)
pN pN0(n=195) vs pN1+pN2+pN3 (n=185)	**< 0.001***	1.974(1.363-2.858)
TNM stage Ⅰ+Ⅱ(n= 273)vs Ⅲ+Ⅳ(n=107)	**0.003***	0.484(0.301-0.780)

Table 4 The univariate analysis of 5-year survival prognostic factors in lung squamous cell carcinoma patients

Variable	Univariate analysis	
	P > |z|	HR(95%CI)
Tumor Budding (10HPF) Low(n=83) vs. high(n=49)	**0.002***	0.589(0.423-0.820)

Nuclear size Small(n=129) vs. Large(n=3)	0.159	0.390(0.880-2.196)
Smallest tumor cell nest Single cell(n=49) vs. 2-4cells(n=77)	**0.002***	0.485(0.307-0.769)
Gender Male(n=94) vs. female(n=58)	0.964	1.014(0.552-1.863)
Age(years) ≤65 (n=52) vs. > 65 (n=100)	0.908	0.972(0.600-1.575)
Pleural invasion Absent (n=132) vs. present (n=20)	**0.001***	0.302(0.149-0.613)
Vascular invasion Absent (n=62) vs. present (n=90)	**0.005***	2.397(1.307-4.396)
spread through airspaces (STAS) Absent (n=75) vs. present (n=77)	**0.004***	2.426(1.327-4.435)
Necrosis Absent (n=7) vs. present (n=145)	0.287	1.252(0.828-1.896)
Peritumoral space Absent(n=36) vs. Present (n=116)	**< 0.001***	4.389(1.920-10.035)
Interstitial fibrosis Absent(n=6) vs. present(n=146)	**0.009***	1.315(1.071-1.614)
pT pT1+pT2(n=119) vs pT3+pT4 (n= 33)	**< 0.001***	2.398(1.584-3.629)
pN pN0(n=84) vs pN1+pN2+pN3 (n=68)	**0.029***	1.440(1.038-1.999)
TNM stage Ⅰ+ Ⅱ(n= 118)vs Ⅲ+ Ⅳ(n=34)	**0.016***	1.954(1.133-3.372)

Figure legends

Figure 1: The tumor budding with HE staining and immunohistochemical staining.

A-D: the budding of the tumor in lung squamous cell carcinoma.
E-H: the tumor budding in lung adenocarcinoma.
A, C, E and G were ×20 magnification.
B, D, F and H were×40 magnification (bar = 500 μm).

Figure 2: The forest map of multivariate survival analysis.
A: the results of multivariate analysis of lung adenocarcinoma.
B: the results of multivariate analysis of lung squamous cell carcinoma.

Figure 3: Kaplan – Meier analysis of the relationship between tumor budding and 5-year overall survival rate in patients with NSCLC.
A: in patients with lung adenocarcinoma, the 5-year survival rate of patients with high-grade budding group was significantly lower than that of patients without tumor budding and low-grade tumor budding.
B: in patients with lung squamous cell carcinoma, the higher the level of tumor budding, the worse the prognosis of patients was.

Figure 4: Kaplan – Meier analysis showed that the 5-year survival rate of patients with different histological subtypes in adenocarcinoma.
A: the survival rates of patients with different histological subtypes were different. Among them, the 5-year prognosis of patients with micropapillary subtype and solid subtype was significantly lower than that of adherent subtypes.
B: in patients with solid subtypes, the 5-year survival rate of patients with high-grade budding was significantly lower than that of patients with low-grade budding and non-budding.
C: in patients with micropapillary subtypes, the higher the grade of tumor budding, the worse the prognosis.

Figure 5: The relationship between the size of tumor budding nests and the nuclear size of tumor budding, as well as the 5-year survival rate of patients with NSCLC.
A: in patients with lung adenocarcinoma, the larger the nucleus of tumor budding, the lower the 5-year overall survival rate was.
B: in patients with lung squamous cell carcinoma, single cell invasion showed a worse prognosis.

Figure 6: The relationship between tumor budding level and patients at different TNM stages.
A: in patients with TNM stage I lung adenocarcinoma, the higher the tumor budding level, the lower the 5-year overall survival rate.
B: in patients with TNM stage II squamous cell carcinoma, the prognosis of patients without tumor budding and low-grade tumor budding was significantly higher than that of patients with high-grade tumor budding.

Figure 1

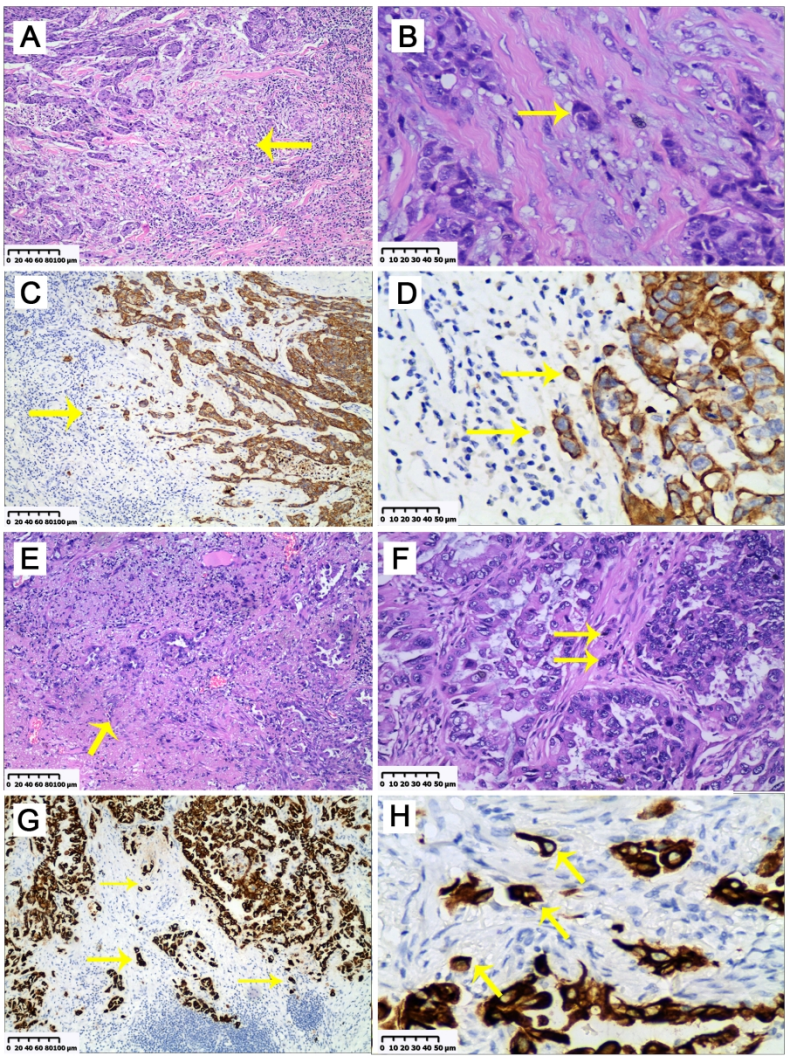

Figure 2

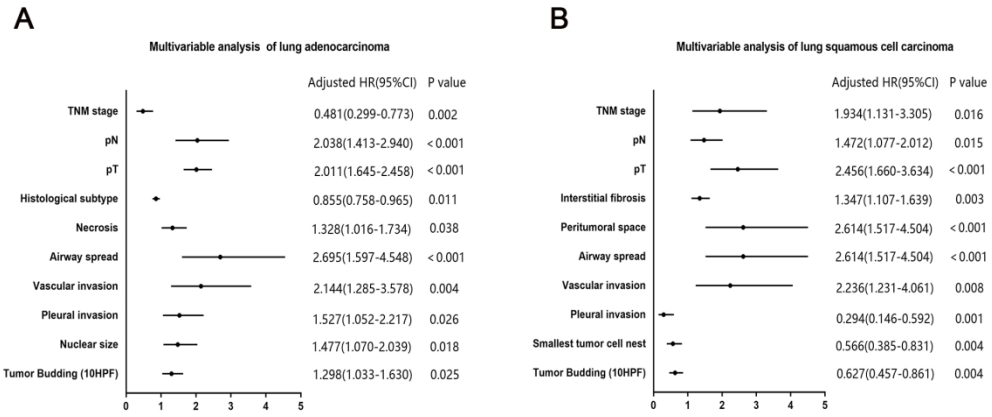

Figure 3

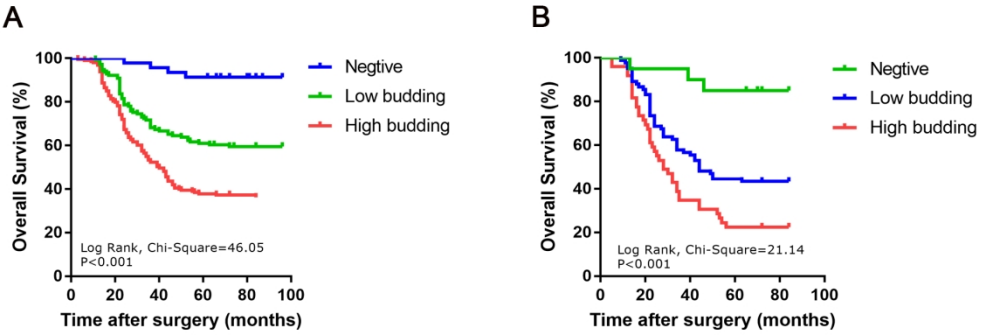

Figure 4

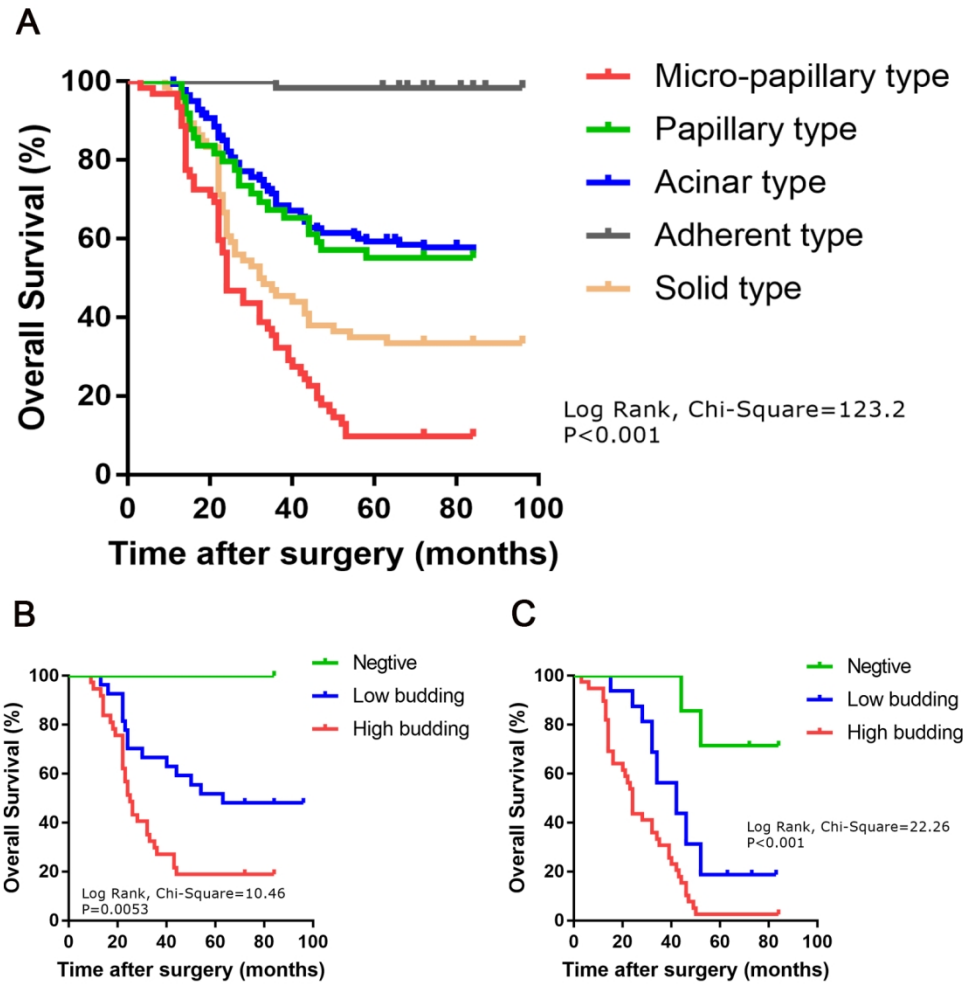

Figure 5

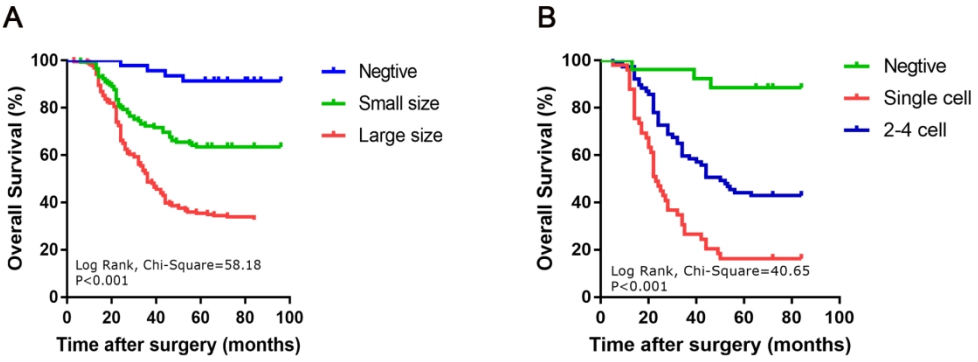

Figure 6