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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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4 **Potential key roles of tumor budding: a representative malignant pathological**
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6 **feature of non-small cell lung cancer and a sensitive indicator of prognosis**
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34 interest in, any commercial companies pertaining to this article.
35
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39

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44

45 **Availability of data and materials**

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47 The datasets used and/or analyzed during the current study are available from
48
49 the corresponding author upon reasonable request.
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56 Ethics approval and consent to participate

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58 NO. 2018-L068.
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7 **Consent for publication**
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9 Not applicable.
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14 **Competing interests**
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16
17 The authors declare that they have no competing interests.
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For peer review only

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

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4 the value of tumor budding as a prognostic indicator. We hope that this analysis of
5
6 Chinese patients with non-small cell lung cancer can provide useful reference material
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8
9 for the continued study of tumor budding.
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11 **Key words:** lung cancer, prognosis, tumor budding
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17 **Strengths and limitations of this study**

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19 We selected 532 patients with non-small cell lung cancer from China, including 380
20
21 patients with adenocarcinoma and 152 with squamous cell carcinoma, to explore the
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23 correlation between tumor budding, the clinicopathological characteristics of these
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25 patients, and prognosis.
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30 Through the evaluation of tumor budding in lung cancer specimens of Chinese patients,
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32 we hope to provide reference for the establishment of tumor budding criteria in the
33
34 diagnosis of lung cancer.
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38 Our research was limited to the tumor budding analysis of NSCLC patients in China,
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40 and the results of different ethnicities may differ.
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43 This study only included surgical resection specimens, no biopsy specimens.
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48 **Introduction**

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50 Lung cancer is among the most common malignant tumors in China and the world.
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52 According to global cancer data from 2020, lung cancer is the most common type of
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54 cancer (11.4% of the total) and cancer-related death (18% of total cancer deaths)¹. Early
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56 lung cancer has few clinical manifestations and is easily ignored or even missed. With
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4 the spread and infiltration of tumor cells, most patients lose the opportunity for radical
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6 surgery. Invasion and metastasis are among the main causes of lung cancer death and
7
8 play a decisive role in lung cancer staging and management.
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11 As a pathological phenomenon, tumor budding has been attracting increased
12 attention. Some studies have shown that tumor budding is a factor that reflects the
13 malignant invasion and poor prognosis of digestive tract tumors². The Union for
14 International Cancer Control (UICC) has officially recognized that tumor budding is an
15 independent prognostic factor for colorectal cancer (CRC) patients. However, only a
16 few studies have explored its significance in lung cancer.
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27 In recent years, with the increasing research on cancer prognosis, some scholars
28 have reported that the morphological characteristics of the peritumoral space are related
29 to patient prognosis. Peritumoral spaces have been noted in breast, lung, bladder, and
30 prostate cancers as well as other malignant tumors. Tumor cells generally spread to the
31 corresponding lymph nodes through the lymphatic system, a phenomenon that is
32 considered an important early event of tumor metastasis^{3 4}. However, the presence of a
33 correlation between tumor budding and the peritumoral space has been rarely reported.
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45 In this study, we selected 532 cases of NSCLC patients from China, including 380
46 cases of adenocarcinoma and 152 cases of squamous cell carcinoma, to explore the
47 correlation between tumor budding, patients' clinicopathological characteristics, and
48 prognosis with the aim of determining a reference value for evaluating patient prognosis
49 and clinical treatment.
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57 **Material and methods**

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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

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

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

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40 The slides stained with HE were placed under a 10 × 20 light microscope to
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42 observe the densest portion of the budding. The clarity of HE and CKpan staining on
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44 tumor budding were compared. The areas of budding were then counted in high-power
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46 fields (HPFs).
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51 It remains controversial whether HE or CK staining should be used for budding
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53 markers. CK staining can reportedly more clearly show the bud focus covered by the
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55 significant peritumoral inflammatory reaction⁵. CK staining also aides in the
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57 observation of a large number of germinal foci mixed with stromal fibroblasts⁶. CK
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4 staining can produce three to four times more buds than HE staining⁷. In many studies,
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6 many scholars chose CK staining for sprouting evaluations^{6 8-14}. Therefore, here we
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8 used both HE staining and CKpan staining and observed the budding state of each level
9
10 between methods. The budding site was more easily observed and the scope of the bud
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12 focus was clearer using CKpan staining.
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17 The judgment of tumor budding refers to the standard of Ueno et al.¹⁵, that is, an
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19 isolated single tumor cell or small clusters of tumor cells composed of no more than
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21 four tumor cells in the stroma at the start of the tumor invasion were considered tumor
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23 budding.
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27 To employ a semiquantitative method to analyze tumor budding, we counted the
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29 mean number of tumor buds under 10 HPFs. The tumor budding was divided into non-
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31 budding, low budding (≤ 10 buds/10 HPFs) and high budding (> 10 buds/10 HPFs).
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36 Tumor cell clusters surrounded by tumor stroma were defined as tumor cell nests.
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38 Based on Moritz's research method¹⁶ and according to the histomorphology
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40 characteristics of lung cancer, we divided the cell nests in tumor stroma into two to four
41
42 tumor cell nests and a single invasive cancer cell in the matrix of the tumor invasion
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44 edge. We also divided tumor interstitial fibrosis into negative, very low (10% of the
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46 total tumor area), low (10–25%), medium (25–50%), and high ($> 50\%$).
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53 *Statistical analysis*

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56 The data were analyzed using SPSS 26.0 software (IBM Corporation, Armonk,
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58 NY, USA). The χ^2 test and t-test were used to compare the count data and measurement
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4 data, respectively. The follow-up result was the overall survival (OS) rate. The Kaplan-
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6
7 Meier method was used to draw the survival curves, while the log rank method was
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10 used to analyze the differences among groups. A multivariate Cox proportional hazard
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12 model was used to determine the independent prognostic factors of the lung cancer
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14 patients. The difference was statistically significant ($P < 0.05$).
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18 19 20 **Results**

21 22 *Tumor budding in NSCLC patients*

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25 In cases of lung cancer with tumor budding, the front edge was not smooth and the
26
27 budding tumor cells were heteromorphic, irregularly shaped, rich in cytoplasm, often
28
29 fused, and eosinophilic. The nucleus was irregularly shaped and the staining was deeper
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31 than that of stromal cells. However, the tumor budding foci were sometimes easily
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33 confused with poorly differentiated stromal cells. However, compared with HE staining,
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35 CK staining can more clearly show tumor budding spores (Figure 1).
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43 44 *Relationship between tumor budding and clinicopathological features of patients with* 45 46 *NSCLC*

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48 Tumor interstitial fibrosis was defined as fibrosis observed under 100×
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50 magnification. According to the area of fibrosis, it was classified as negative, ≤10%,
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52 10–25%, 25–50% and >50%. The peritumoral space, that between the tumor cells and
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54 the stroma, was the morphological manifestation of the interaction between them that
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56 clearly divided the tumor components and the stroma³. Shah et al.¹⁷ reported that the
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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

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4 than those of the low-grade budding and non-budding groups. The Kaplan-Meier
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6 method was used to analyze the postoperative survival rate, while the log rank method
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8 was used to test the intergroup differences.
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11 In patients with lung adenocarcinoma, univariate analysis showed that tumor
12 budding, tumor budding nucleus size, pleural and vascular invasion, airway spread,
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14 histological subtype, necrosis area, and TNM stage were significantly associated with
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16 5-year survival (Table 3). We then used the Cox proportional hazard regression model
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18 to analyze the statistically significant indicators of the univariate analysis. For the
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20 budding model, we took the above factors as variables, and the tumor budding (hazard
21
22 ratio [HR], 1.298; 95% confidence interval [CI], 1.033–1.630; $P = 0.025$), nuclear size
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24 (HR, 1.477; 95% CI, 1.070–2.039; $P = 0.018$), pleural invasion (HR, 1.527; 95% CI,
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26 1.052–2.217; $P = 0.026$), vascular invasion (HR, 2.144; 95% CI, 1.285–3.578; $P =$
27
28 0.004), airway spread (HR, 2.695; 95% CI, 1.597–4.548; $P < 0.001$), necrosis (HR,
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30 1.328; 95% CI, 1.016–1.734; $P = 0.038$), histological subtype (HR, 0.855; 95% CI,
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32 0.758–0.965; $P = 0.011$), pT (HR, 2.011; 95% CI, 1.645–2.458; $P < 0.001$), pN (HR,
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34 2.038; 95% CI, 1.413–2.940; $P < 0.001$), and TNM stage (HR, 0.481; 95% CI, 0.299–
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36 0.773; $P = 0.002$) also showed a statistically significant correlation with the 5-year
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38 survival rate based on the univariate Cox regression univariate analysis (Figure 2).
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50 The Kaplan-Meier survival curve showed that the higher the budding grade, the
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52 lower the 5-year OS rate ($P < 0.001$) (Figure 3). In the histological subtypes of lung
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54 adenocarcinoma, the higher the level of tumor budding, the worse the prognosis in cases
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56 with micropapillary subtypes and solid subtypes (Figure 4). In the adherent subtype (P
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4 = 0.356), papillary subtype (P = 0.567), and acinar subtype (P = 0.353), there was no
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6 statistical correlation between tumor budding degree and survival status. Compared
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8 with tumor budding cell nucleus containing fewer than three lymphocytes (small size),
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10 when the tumor budding nucleus had four or more lymphocytes (large size), the 5-year
11
12 OS rate of lung adenocarcinoma patients was significantly reduced (Figure 5A).
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16
17 In cases of lung squamous cell carcinoma, tumor budding size, budding tumor nest,
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19 pleural and vascular invasion, airway spread, tumor interstitial fibrosis area,
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21 peritumoral space, tumor size and lymph node metastasis, and TNM stage influenced
22
23 patient 5-year survival rate (Table 4). To eliminate the interactions between variables,
24
25 multivariate Cox regression analysis was used to analyze the data. The above factors
26
27 independently affected the prognosis of patients with squamous cell carcinoma (Figure
28
29 2). The Kaplan-Meier survival curve showed that the 5-year OS rate of patients with
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31 lung squamous cell carcinoma in TNM stage II was significantly higher than that of
32
33 patients with high-grade tumor budding (Figure 6B), while the 5-year OS rate of lung
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35 squamous cell carcinoma patients with single cell tumor budding was significantly
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37 lower (Figure 5B).
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48 Discussion

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50 Cancer is an issue of great concern worldwide, and its prognosis mainly depends
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52 on the pathological type, TNM stage, tumor differentiation degree, and microvascular
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54 invasion, and patients with the same TNM stage but quite different prognoses are often
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56 seen in the clinical setting. In recent years, as a pathological phenomenon, tumor
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4 budding has attracted increasing attention. Tumor budding, also known as focal
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6 dedifferentiation is the first step in the process of a malignant tumor's invasion and
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8 metastasis. Therefore, tumor budding is considered a key step in a tumor's invasive
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10 growth process¹⁸. Tumor budding spores are considered cancer stem cells, which are
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12 defined as isolated single tumor cells or clusters of fewer than five tumor cells at the
13
14 start of tumor invasion⁵. Some studies stated that tumor budding is not a static
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16 histological feature; rather, it involves a small focal tumor cell complex separated from
17
18 the main body of the tumor that enters the surrounding tissue in a "budding" manner,
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20 which represents a dynamic process¹⁹. Gabbert et al.¹⁸ also supported this conclusion.
21
22 Shinto et al.⁸ reported that there were interconnected cytoplasmic pseudo fragments
23
24 similar to pseudopodia processes between budding tumor cells, which may be related
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26 to the increase in cell invasion ability. In addition, some studies have speculated that
27
28 tumor budding is a step in the progression of malignant tumors from focal lesions to
29
30 systemic diseases²⁰. Tumor budding is now considered of great significance in tumor
31
32 invasion and metastasis²¹⁻²⁴. Some studies have shown that tumor budding reflected the
33
34 invasiveness and poor prognosis of digestive tract tumors². The presence of tumor
35
36 budding may be related to the late stage of a tumor, frequent lymphatic vascular
37
38 invasion, and lymph node and distant metastasis. The UICC officially recognizes tumor
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40 budding as an independent prognostic factor for CRC. It was recently used as a
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42 significant prognostic indicator for the treatment of esophageal squamous cell
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44 carcinoma, gastroesophageal junction adenocarcinoma, and gastric adenocarcinoma²⁵.
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60 In the current study of 380 cases of primary lung adenocarcinoma and 152 cases of

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4 primary lung squamous cell carcinoma, we found that tumor budding was closely
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6 related to the 5-year OS, tumor size, lymph node metastasis, vascular invasion, airway
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8 spread, tumor necrosis, tumor interstitial fibrosis, and TNM stage. This suggests that
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10 tumor budding may be an important indicator of malignant invasion and metastasis.
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12 Compared with NSCLC patients without tumor budding, those with the morphological
13
14 characteristics of tumor budding have a worse 5-year OS prognosis.
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19 The detection accuracy of abdominal B-ultrasound and abdominal computed
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21 tomography for lymph node metastasis is reportedly 12.2–80.0%²⁶ and 50–80%,
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23 respectively²⁷⁻³⁰. Guluoglu et al.³¹ evaluated 126 patients with gastric cancer and found
24
25 that lymph node metastasis was the only parameter associated with tumor budding.
26
27 Masaki *et. al*³² established a model formula for predicting the probability of lymph
28
29 node metastasis in 76 patients with T1 stage CRC as follows: $z = 0.070 \times (\text{budding}$
30
31 $\text{count}) - 3.726$, probability = $1/1 + e^{-z}$. Furthermore, the tumor budding count was
32
33 included in the clinical decision-making analysis of patients to determine whether
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35 patients require additional surgery after endoscopic treatment. Some studies have
36
37 shown that the presence of tumor budding in biopsy specimens before CRC surgery
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39 increases the possibility of lymph node and distant metastasis. Therefore, neoadjuvant
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41 therapy and surgical treatment can be considered for these patients³³. The Japanese
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43 Society for Cancer of the Colon and Rectum has incorporated the index of tumor
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45 budding into the guidelines for patients with pT1 disease who require further surgery³⁴.
46
47 In our study, 244 of 253 patients with lymph node metastasis had tumor budding. The
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49 sensitivity of budding for predicting lymph node metastasis was 96.44%, indicating that
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4 tumor budding is an effective pathological index with high sensitivity for predicting
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6 lymph node metastasis. Therefore, we believe that for patients with NSCLC, we can
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8 refine the significance of tumor budding through a larger sample study to contribute to
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10 clinical decision-making.
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13
14 The peritumoral space is the space between the tumor cells and the stroma that
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16 divides the tumor components from the stroma and is morphological manifestation of
17
18 the interaction between the tumor cells and the stromal cells. The peritumoral space is
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20 commonly seen in paraffin-embedded tissue sections fixed with formalin. The
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22 peritumoral space is one of the pathomorphological manifestations of tumor biological
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24 behavior that is considered a prognostic factor by some scholars. Peritumoral spaces
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26 have been noted in breast, lung, bladder, and prostate cancers and other malignant
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28 tumors. Tumor cells usually spread to the corresponding lymph nodes through the
29
30 lymphatic system, this phenomenon is considered an important early event of tumor
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32 metastasis^{3 4}. In prostate cancer, an extensive peritumoral space indicates a higher
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34 tumor grade, shorter disease-free survival, and poor prognosis^{35 36}. At the same time,
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36 the peritumoral space in breast cancer is closely related to histological grade, lymphatic
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38 invasion, lymph node metastasis, and prognosis and can be used as an important marker
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40 to judge the prognosis of breast cancer patients^{37 38}. Acs et al.³⁹ observed the relationship
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42 between a large peritumoral space and lymph angiogenesis, and the results confirmed
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44 a poor prognosis of patients with large peritumoral spaces, which was consistent with
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46 this hypothesis. In our study, we found that in patients with lung squamous cell
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48 carcinoma, the peritumoral space is closely related to tumor budding, which is also an
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4 independent risk factor for patient 5-year OS. A joint evaluation of the peritumoral
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6 space and tumor budding can effectively evaluate the prognosis of patients with lung
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8 squamous cell carcinoma.
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11 Lung adenocarcinoma spreads through the bronchus, known as lung metastasis,
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13 and the airways, known as airway metastasis. A small number of lung adenocarcinoma
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15 cancer cells enter the bronchial cavity, and with the respiratory movement through the
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17 bronchial discontinuous, they diffuse into other lung segments or lobes on the same or
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19 opposite side, forming new lung metastases⁴⁰. Our study revealed that tumor budding
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21 was closely related to airway spread. Tumor budding can be combined with spread
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23 through airspaces (STAS) to evaluate the malignant aggressive behavior of NSCLC.
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30 Che et al.² found that the OS rate of patients with high budding gastric
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32 adenocarcinoma was significantly lower than that of patients with low budding gastric
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34 adenocarcinoma. Some studies reported that the presence of tumor budding in surgical
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36 specimens of patients with gastric cancer may indicate a poor prognosis and early
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38 recurrence²⁵. We also found that the 5-year OS rate of lung adenocarcinoma or
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40 squamous cell carcinoma patients with high-grade budding was significantly lower than
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42 that of patients with low-grade or no budding. However, Hass et al.⁴¹ emphasized that
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44 tumor budding and cancer classification based on cell differentiation were neither the
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46 same nor related. Some researchers believed that tumor budding and tumor growth
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48 pattern were independent prognostic parameters¹⁵. However, in our study of lung
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50 adenocarcinoma, tumor budding was closely related to histological subtype. In patients
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52 with papillary and solid subtypes of lung adenocarcinoma, the 5-year survival rate of
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4 patients with high-grade budding was significantly lower than that of patients with low-
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6 grade budding. In patients with TNM stage I, the 5-year OS rate of patients with high-
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8 grade tumor budding was lower than that of patients with low-grade or no budding
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10 (Figure 6A). The results are consistent with those of Kyuichi et al.⁴².
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14 In our study, Cox regression analysis showed a significant correlation between
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16 tumor budding and 5-year OS rate. Tumor budding, pleural and vascular invasion,
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18 airway spread, tumor size, lymph node metastasis, and TNM stage were independent
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20 risk factors for the prognosis of NSCLC patients. In addition, tumor budding nucleus
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22 size, tumor necrosis area, and histological subtype were independent prognostic factors
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24 of lung adenocarcinoma. The area of interstitial fibrosis, presence of a peritumoral
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26 space, and small tumor cell nest were independent prognostic factors in patients with
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28 squamous cell carcinoma. Therefore, we speculate that tumor budding may be a
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30 representative malignant pathological feature of NSCLC and a sensitive indicator
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32 reflective of its prognosis.
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40 The research results of Wang et al. suggested that tumor budding should be
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42 included in the routine histopathological report to better stratify the risk of CRC
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44 patients⁴³. The AJCC and College of American Pathologists guidelines on CRC
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46 proposed that tumor budding should be considered an optional reporting indicator and
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48 should be evaluated in all cases of stage I and II CRC. This provides us with a
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50 standardized reporting tool for tumor budding⁴⁴. However, there is no unified scoring
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52 standard for lung cancer.
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58 The current study had several limitations. First, our research is limited to the tumor
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4 budding analysis of NSCLC patients in China, and the results of different ethnicities
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6 may differ. In addition, because the number of surgical specimens selected for this
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8 operation before 2015 was limited, the sample size was insufficient, which might result
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10 in sample bias. However, as an effective and simple pathological diagnosis index, it is
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12 necessary to establish an effective grading system to verify its value as a standard
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14 prognostic indicator. In addition, prospective clinical trials including multicenter
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16 samples are needed to evaluate the role of tumor budding in predicting the prognosis of
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18 lung cancer and produce reference values for the pathological diagnosis and clinical
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20 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*
I a1	6	4(66.67%)	2(33.33%)		
I a2	76	12(15.79%)	64(84.21%)		
I a3	48	11(22.92%)	37(77.08%)		
I b	41	9(21.95%)	32(78.05%)		
II a	15	3(20.00%)	12(80.00%)		
II b	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 airspace (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*
I a1	4	1(25.00%)	3(75.00%)		
I a2	18	5(27.78%)	13(72.22%)		
I a3	23	10(43.48%)	13(56.52%)		
I b	16	2(12.50%)	14(87.50%)		
II a	19	0(0.00%)	19(100.00%)		
II b	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 $\times 20$ magnification.

B, D, F and H were $\times 40$ 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

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3 budding and low-grade tumor budding.

4 B: in patients with lung squamous cell carcinoma, the higher the level of tumor budding,
5 the worse the prognosis of patients was.
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7

8 Figure 4: Kaplan – Meier analysis showed that the 5-year survival rate of patients with
9 different histological subtypes in adenocarcinoma.

10 A: the survival rates of patients with different histological subtypes were different.
11 Among them, the 5-year prognosis of patients with micropapillary subtype and solid
12 subtype was significantly lower than that of adherent subtypes.
13

14 B: in patients with solid subtypes, the 5-year survival rate of patients with high-grade
15 budding was significantly lower than that of patients with low-grade budding and non-
16 budding.
17

18 C: in patients with micropapillary subtypes, the higher the grade of tumor budding, the
19 worse the prognosis.
20
21

22 Figure 5: The relationship between the size of tumor budding nests and the nuclear size
23 of tumor budding, as well as the 5-year survival rate of patients with NSCLC.

24 A: in patients with lung adenocarcinoma, the larger the nucleus of tumor budding, the
25 lower the 5-year overall survival rate was.
26

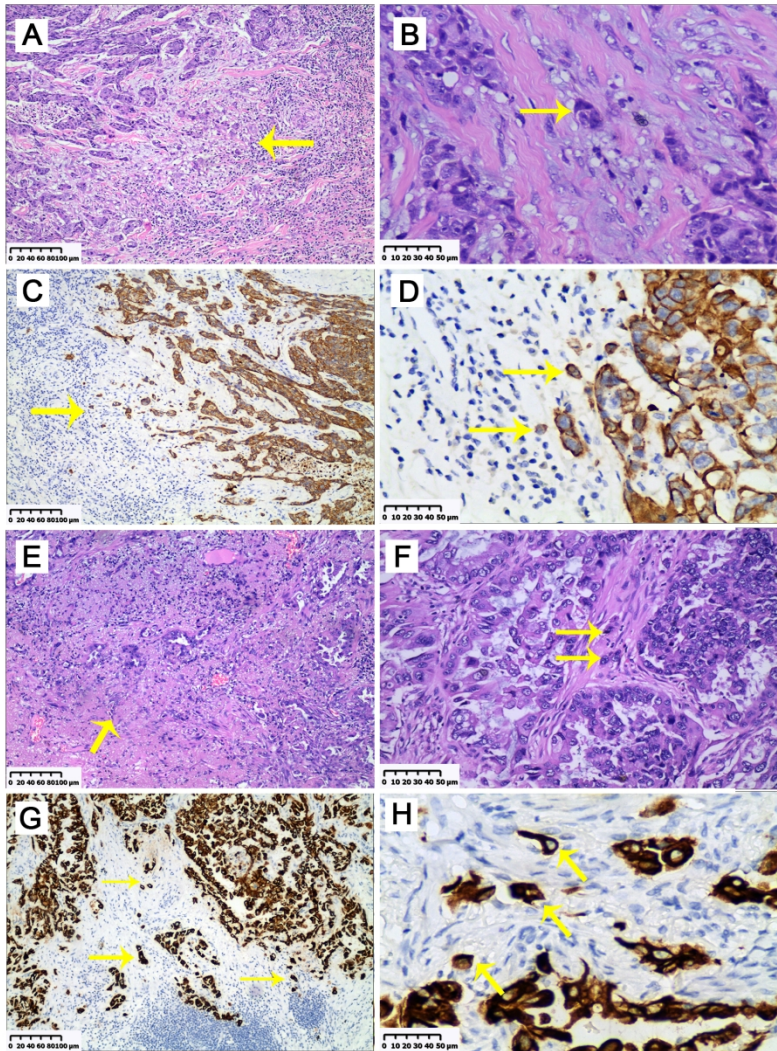
27 B: in patients with lung squamous cell carcinoma, single cell invasion showed a worse
28 prognosis.
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30

31 Figure 6: The relationship between tumor budding level and patients at different TNM
32 stages.
33

34 A: in patients with TNM stage I lung adenocarcinoma, the higher the tumor budding
35 level, the lower the 5-year overall survival rate.
36

37 B: in patients with TNM stage II squamous cell carcinoma, the prognosis of patients
38 without tumor budding and low-grade tumor budding was significantly higher than that
39 of patients with high-grade tumor budding.
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Figure 1



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Figure 2

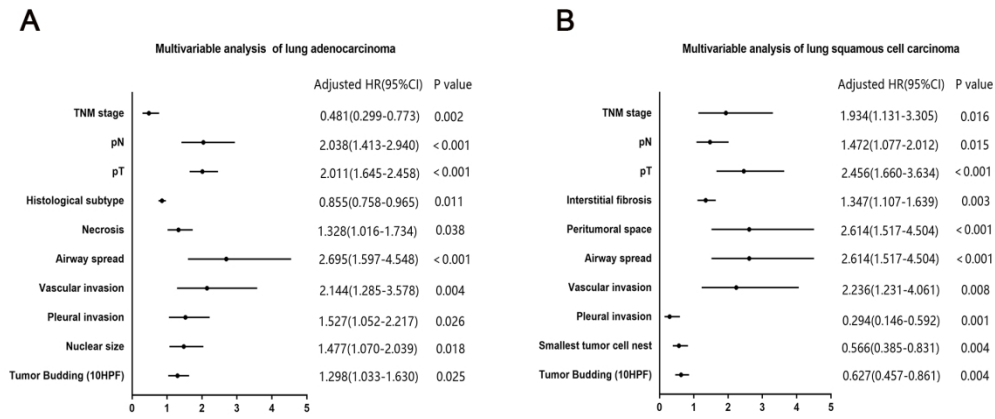


Figure 3

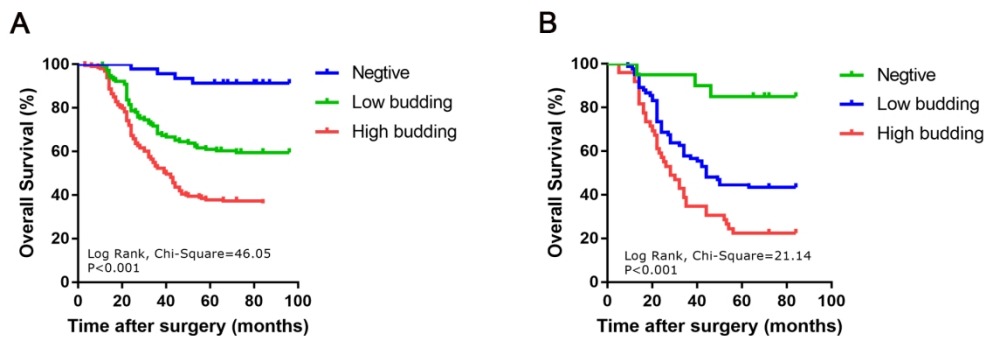
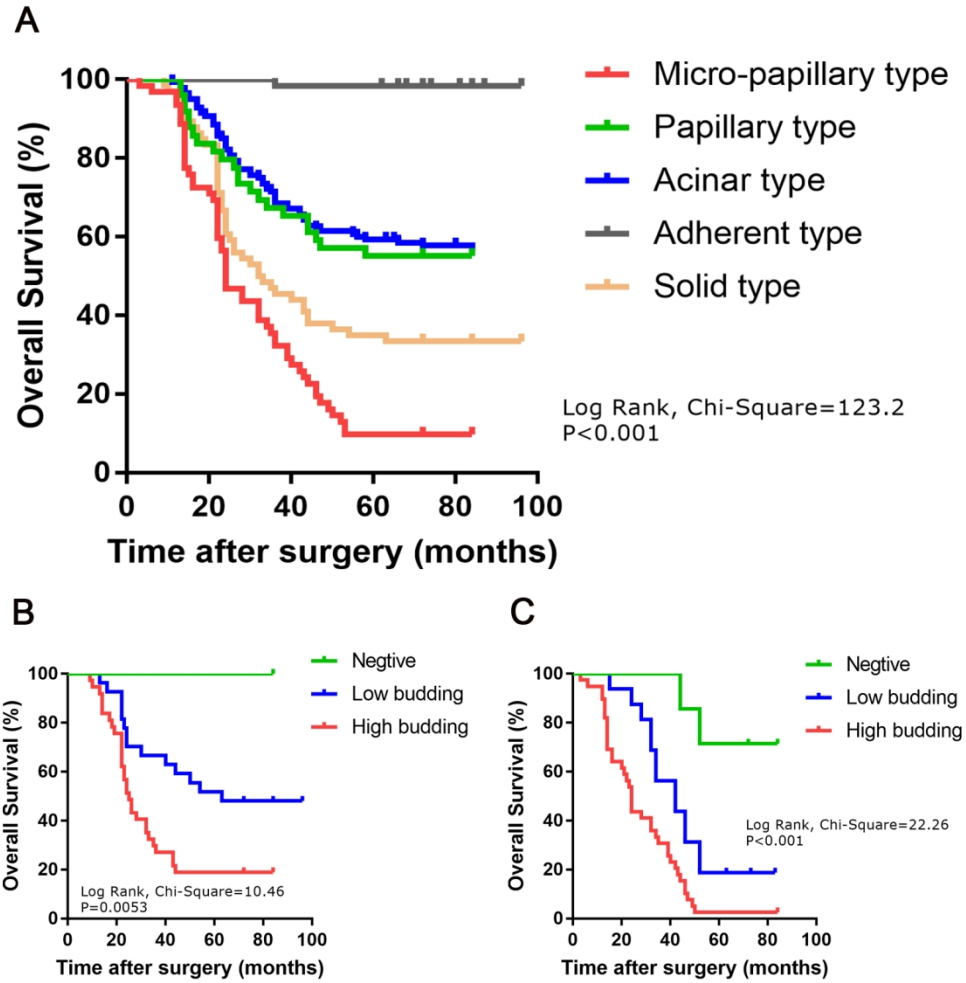
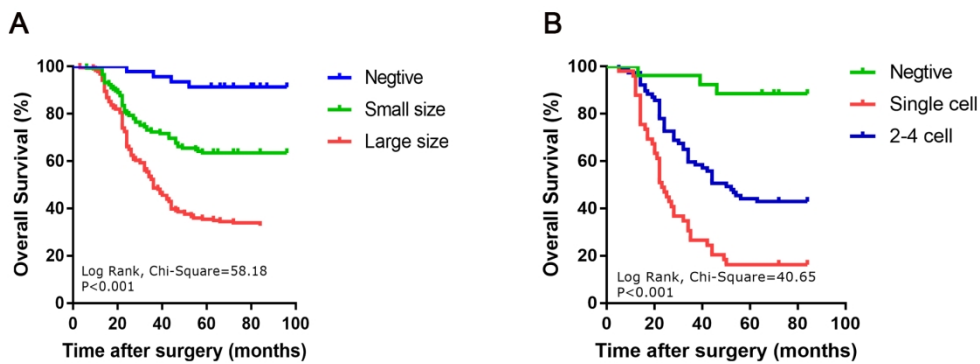


Figure 4



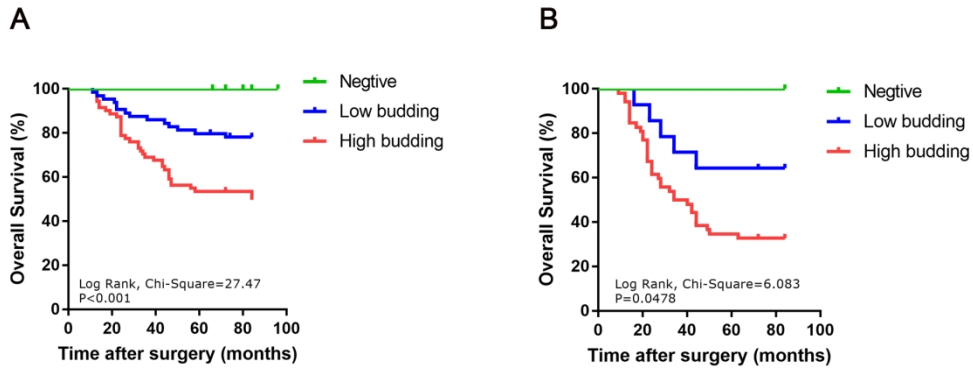
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Figure 5



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Figure 6



BMJ Open

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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4 **Potential key roles of tumor budding: a representative malignant pathological**
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6 **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 χ^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

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4 cancer patients.
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7 **Conclusions:** As an effective and simple pathological diagnostic index, it is necessary
8
9 to establish an effective grading system in the clinical diagnosis of lung cancer to verify
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11 the value of tumor budding as a prognostic indicator. We hope that this analysis of
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13 Chinese patients with non-small cell lung cancer can provide useful reference material
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16
17 for the continued study of tumor budding.
18

19
20 **Key words:** lung cancer, prognosis, tumor budding
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23 24 25 **Strengths and limitations of this study** 26

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28 ➤ We selected 532 patients with non-small cell lung cancer from China, including
29
30 380 patients with adenocarcinoma and 152 with squamous cell carcinoma, to
31
32 explore the correlation between tumor budding, the clinicopathological
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34 characteristics of these patients, and prognosis.
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39 ➤ Through the evaluation of tumor budding in lung cancer specimens of Chinese
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41 patients, we hope to provide reference for the establishment of tumor budding
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43 criteria in the diagnosis of lung cancer.
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47 ➤ Our research was limited to the tumor budding analysis of NSCLC patients in
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49 China, and the results of different ethnicities may differ.
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53 ➤ This study only included surgical resection specimens, no biopsy specimens.
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17 **Conflict of interest**

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20 The authors have disclosed that they have no significant relationships with, or financial
21
22 interest in, any commercial companies pertaining to this article.
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28 **Authors' contributions**

29
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35
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46
47 Not applicable.
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53 **Availability of data and materials**

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

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4 anti-PD-L1 (atezolizumab) can be administered. The findings support the superiority
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6 of ICIs over docetaxel in pretreated NSCLC patients, and suggest that anti-PD-1
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8 inhibitors may have a minor advantage over anti-PD-L1 inhibitors². Fausto Petrelli et
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10 al. confirmed in their meta-analysis that there is moderate evidence that adding immune
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12 checkpoint inhibitors to chemotherapy improves overall survival when compared to
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14 chemotherapy alone³. However, in a review of Jianwei Zhu et al put forward different
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16 opinions. Their research results show that immunotherapy for patients with non-small
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18 cell lung cancer after surgery or radiotherapy cannot prolong their survival time. At the
19
20 same time, they noted that an interim analysis for one of these trials revealed that treated
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22 participants with stage III NSCLC had a better PFS⁴. Most current studies are combined
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24 therapies, such as dendritic cells (DCs) or DCs/cytokine induced killer (CIK) therapy
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26 in combination with chemotherapy in advanced lung cancer, according to a review by
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28 Monireh Mohsenzadegan et al⁵. However, these medications have only had little
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30 success in the treatment of advanced NSCLC⁵. Invasion and metastasis are among the
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32 main causes of lung cancer death and play a decisive role in lung cancer staging and
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34 management.
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47 As a pathological phenomenon, tumor budding has been attracting increased
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49 attention. Some studies have shown that tumor budding is a factor that reflects the
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51 malignant invasion and poor prognosis of digestive tract tumors⁶. The Union for
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53 International Cancer Control (UICC) has officially recognized that tumor budding is an
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55 independent prognostic factor for colorectal cancer (CRC) patients. However, only a
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4 few studies have explored its significance in lung cancer.
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7 In recent years, with the increasing research on cancer prognosis, some scholars
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9 have reported that the morphological characteristics of the peritumoral space are related
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11 to patient prognosis. Peritumoral spaces have been noted in breast, lung, bladder, and
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13 prostate cancers as well as other malignant tumors. Tumor cells generally spread to the
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15 corresponding lymph nodes through the lymphatic system, a phenomenon that is
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17 considered an important early event of tumor metastasis^{7,8}. However, the presence of a
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19 correlation between tumor budding and the peritumoral space has been rarely reported.
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26 In this study, we selected 532 cases of NSCLC patients from China, including 380
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28 cases of adenocarcinoma and 152 cases of squamous cell carcinoma, to explore the
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30 correlation between tumor budding, patients' clinicopathological characteristics, and
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32 prognosis with the aim of determining a reference value for evaluating patient prognosis
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34 and clinical treatment.
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38 39 **Material and methods**

40 41 42 *Patients' general information*

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45 We retrieved the pathological reports of patients who met the inclusion criteria
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47 from the files of the pathology system and obtained other clinical pathological
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49 information from the electronic medical record system. All 532 cases included in this
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51 study were radical surgical specimens. The data of 380 patients with primary lung
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53 adenocarcinoma and 152 patients with primary lung squamous cell carcinoma treated
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55 in the Cardiothoracic Disease Department of the Affiliated Hospital of Nantong
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4 University between June 2009 and July 2015. We excluded patients for whom follow-
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6 up information was lacking; thus, and a total of 532 patients (302 males, 230 females;
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8 202 patients were ≤ 65 years old, while 328 patients were >65 years old). None of the
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10 patients received chemotherapy or radiotherapy preoperatively. The clinical and
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12 pathological information and medical records were complete for each patient.
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17 We took the corresponding paraffin blocks of each patient from the pathological
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19 diagnosis center and sliced them into 3- μm -thick slices. Each slice was floated in 45°C
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21 warm water on a spreader to flatten the tissue, which was then picked up with a slide
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23 and baked in an oven at 65°C. Cytokeratin immunohistochemical staining (CK) and
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25 hematoxylin and eosin (HE) staining were performed. Rabbit polyclonal anti-human
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27 pan-cytokeratin (CKpan) antibody was used (dilution 1:50; ab215838, Abcam, USA).
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29 The evaluations were independently performed by three experienced pathologists using
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31 a multi-head microscope (Precise Instrument Co., Ltd., Beijing, China) to reach
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33 consensus.
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42 *Patient and Public Involvement*

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45 All patients signed an informed consent form, and the study was approved by the
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47 Ethics Committee of Affiliated Hospital of Nantong University (2018-L068). The
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49 patients were followed up by telephone and outpatient service. The starting point of
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51 follow-up was the operation time for each patient, while the end point was the time of
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53 death. If the patient was still alive, we selected the last follow-up appointment as the
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55 termination point.
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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 method¹⁰ and according to the histomorphology characteristics of lung cancer, we divided the cell nests in tumor stroma into two to four

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

11 12 13 14 15 *Evaluation of tumor budding assisted with Cytokeratin*

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

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20 It remains controversial whether HE or Cytokeratin (CK) staining should be used
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23 for budding markers. CK staining can reportedly more clearly show the bud focus
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25 covered by the significant peritumoral inflammatory reaction¹¹. CK staining also aides
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27 in the observation of a large number of germinal foci mixed with stromal fibroblasts¹².
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30 CK staining can produce three to four times more buds than HE staining¹³. In many
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33 studies, many scholars chose CK staining for sprouting evaluations^{12 14-20}. Therefore,
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36 here we used both HE staining and pan-cytokeratin staining and observed the budding
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39 state of each level between methods. The budding site was more easily observed and
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42 the scope of the bud focus was clearer using pan-cytokeratin staining.
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48 *Statistical analysis*

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50 The data were analyzed using SPSS 26.0 software (IBM Corporation, Armonk,
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53 NY, USA). The χ^2 test and t-test were used to compare the count data and measurement
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56 data, respectively. The follow-up result was the overall survival (OS) rate. The Kaplan-
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59 Meier method was used to draw the survival curves, while the log rank method was
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4 used to analyze the differences among groups. A multivariate Cox proportional hazard
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6 model was used to determine the independent prognostic factors of the lung cancer
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8 patients. The difference was statistically significant ($P < 0.05$).
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14 15 **Results**

16 17 *Tumor budding in NSCLC patients*

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20 In cases of lung cancer with tumor budding, the front edge was not smooth and the
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22 budding tumor cells were heteromorphic, irregularly shaped, rich in cytoplasm, often
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24 fused, and eosinophilic. The nucleus was irregularly shaped and the staining was deeper
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26 than that of stromal cells. However, the tumor budding foci were sometimes easily
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28 confused with poorly differentiated stromal cells. However, compared with HE staining,
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30 CK staining can more clearly show tumor budding spores (Figure 1).
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45 46 47 48 49 50 51 52 53 54 55 56 57 58 59 60 *Relationship between tumor budding and clinicopathological features of patients with NSCLC*

45 Tumor interstitial fibrosis was defined as fibrosis observed under 100×
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47 magnification. According to the area of fibrosis, it was classified as negative, ≤10%,
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49 10–25%, 25–50% and >50%. The peritumoral space, that between the tumor cells and
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51 the stroma, was the morphological manifestation of the interaction between them that
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53 clearly divided the tumor components and the stroma⁷. Shah et al.²¹ reported that the
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55 peritumoral space was very common in tumors and related to invasive cancer cell nests.
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4 Among the 380 cases of lung adenocarcinoma, 46 showed no tumor budding and
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6 334 showed tumor budding. Tumor budding status was closely related to the 5-year OS
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8 status of patients with lung adenocarcinoma. In addition, it was closely related to tumor
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10 histological subtype ($P < 0.001$), tumor size ($P < 0.001$), lymph node metastasis ($P <$
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12 0.001), vascular invasion (OR, 3.693; 95% CI, 1.847–7.383; $P < 0.001$), pleural
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14 invasion (OR, 13.393; 95% CI, 5.512–32.542; $P < 0.001$), STAS (OR, 36.698; 95% CI,
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16 13.925–96.715; $P < 0.001$), tumor necrosis ($P = 0.005$), tumor interstitial fibrosis ($P <$
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18 0.001), and TNM stage ($P < 0.001$). However, tumor budding was not related to the
19
20 patient gender patient sex (OR, 1.086; 95% CI, 0.583–2.021; $P = 0.875$) or age (OR,
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22 0.959; 95% CI, 0.510–1.804; $P = 0.898$). The proportion of tumor budding in patients
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24 with vascular tumor thrombus was significantly higher than that in patients without
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26 vascular tumor thrombus. The greater the degree of lymph node metastasis, the higher
27
28 the proportion of tumor budding (Table 1). In the 152 patients with primary squamous
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30 cell carcinoma of the lung (Table 2), tumor budding status was significantly correlated
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32 with the 5-year OS status (OR, 0.098; 95% CI, 0.027–0.350; $P < 0.001$), peritumoral
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34 space (OR, 11.667; 95% CI, 4.041–33.683; $P < 0.001$), vascular invasion (OR, 5.426;
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36 95% CI, 1.855–15.865; $P = 0.001$), tumor size ($P < 0.001$), lymph node metastasis ($P <$
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38 0.001), airway spread (OR, 7.230; 95% CI, 2.021–25.863; $P = 0.001$), tumor necrosis
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40 ($P = 0.030$), TNM stage ($P < 0.001$), and tumor interstitial fibrosis ($P < 0.001$).
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58 *Survival analysis of patients*

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4 All 532 patients were included in the survival analysis study by July 2020. The
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7 follow-up time was 3–82 months. At the end of the study, 261 patients were still alive.
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10 Among the dead patients, the proportion of high-grade budding was significantly higher
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12 than those of the low-grade budding and non-budding groups. The Kaplan-Meier
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14 method was used to analyze the postoperative survival rate, while the log rank method
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16 was used to test the intergroup differences.
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21 In patients with lung adenocarcinoma, univariate analysis showed that tumor
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23 budding, tumor budding nucleus size, pleural and vascular invasion, airway spread,
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25 histological subtype, necrosis area, and TNM stage were significantly associated with
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27 5-year survival (Table 3). We then used the Cox proportional hazard regression model
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29 to analyze the statistically significant indicators of the univariate analysis. For the
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31 budding model, we took the above factors as variables, and the tumor budding (hazard
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33 ratio [HR], 1.298; 95% confidence interval [CI], 1.033–1.630; $P = 0.025$), nuclear size
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35 (HR, 1.477; 95% CI, 1.070–2.039; $P = 0.018$), pleural invasion (HR, 1.527; 95% CI,
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37 1.052–2.217; $P = 0.026$), vascular invasion (HR, 2.144; 95% CI, 1.285–3.578; $P =$
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39 0.004), airway spread (HR, 2.695; 95% CI, 1.597–4.548; $P < 0.001$), necrosis (HR,
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41 1.328; 95% CI, 1.016–1.734; $P = 0.038$), histological subtype (HR, 0.855; 95% CI,
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43 0.758–0.965; $P = 0.011$), pT (HR, 2.011; 95% CI, 1.645–2.458; $P < 0.001$), pN (HR,
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45 2.038; 95% CI, 1.413–2.940; $P < 0.001$), and TNM stage (HR, 0.481; 95% CI, 0.299–
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47 0.773; $P = 0.002$) also showed a statistically significant correlation with the 5-year
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49 survival rate based on the Cox regression univariate analysis (Figure 2).
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4 The Kaplan-Meier survival curve showed that the higher the budding grade, the
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6 lower the 5-year OS rate ($P < 0.001$) (Figure 3). In the histological subtypes of lung
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8 adenocarcinoma, the higher the level of tumor budding, the worse the prognosis in cases
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10 with micropapillary subtypes and solid subtypes (Figure 4). In the adherent subtype (P
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12 = 0.356), papillary subtype ($P = 0.567$), and acinar subtype ($P = 0.353$), there was no
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14 statistical correlation between tumor budding degree and survival status. Compared
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16 with tumor budding cell nucleus containing fewer than three lymphocytes (small size),
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18 when the tumor budding nucleus had four or more lymphocytes (large size), the 5-year
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20 OS rate of lung adenocarcinoma patients was significantly reduced (Figure 5A).
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28 In cases of lung squamous cell carcinoma, tumor budding size, budding tumor nest,
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30 pleural and vascular invasion, airway spread, tumor interstitial fibrosis area,
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32 peritumoral space, tumor size and lymph node metastasis, and TNM stage influenced
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34 patient 5-year survival rate (Table 4). To eliminate the interactions between variables,
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36 multivariate Cox regression analysis was used to analyze the data. The above factors
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38 independently affected the prognosis of patients with squamous cell carcinoma (Figure
39
40 2). The Kaplan-Meier survival curve showed that the 5-year OS rate of patients with
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42 lung squamous cell carcinoma in TNM stage II was significantly higher than that of
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44 patients with high-grade tumor budding (Figure 6B), while the 5-year OS rate of lung
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46 squamous cell carcinoma patients with single cell tumor budding was significantly
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48 lower (Figure 5B).
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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 process²². 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 invasion¹¹. 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 process²³. 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 diseases²⁴. Tumor budding is now considered of great significance in tumor invasion and metastasis²⁵⁻²⁸. Some studies have shown that tumor budding reflected the invasiveness and poor prognosis of digestive tract tumors⁶. The presence of tumor

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4 budding may be related to the late stage of a tumor, frequent lymphatic vascular
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6 invasion, and lymph node and distant metastasis. The UICC officially recognizes tumor
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8 budding as an independent prognostic factor for CRC. It was recently used as a
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10 significant prognostic indicator for the treatment of esophageal squamous cell
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12 carcinoma, gastroesophageal junction adenocarcinoma, and gastric adenocarcinoma²⁹.
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15 In the current study of 380 cases of primary lung adenocarcinoma and 152 cases of
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17 primary lung squamous cell carcinoma, we found that tumor budding was closely
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19 related to the 5-year OS, tumor size, lymph node metastasis, vascular invasion, airway
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21 spread, tumor necrosis, tumor interstitial fibrosis, and TNM stage. This suggests that
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23 tumor budding may be an important indicator of malignant invasion and metastasis.
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26 Compared with NSCLC patients without tumor budding, those with the morphological
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28 characteristics of tumor budding have a worse 5-year OS prognosis.
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37 The detection accuracy of abdominal B-ultrasound and abdominal computed
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39 tomography for lymph node metastasis is reportedly 12.2–80.0%³⁰ and 50–80%,
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41 respectively³¹⁻³⁴. Guluoglu et al.³⁵ evaluated 126 patients with gastric cancer and found
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43 that lymph node metastasis was the only parameter associated with tumor budding.
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46 Masaki *et. al*³⁶ established a model formula for predicting the probability of lymph
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48 node metastasis in 76 patients with T1 stage CRC as follows: $z = 0.070 \times (\text{budding}$
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50 $\text{count}) - 3.726$, probability = $1/1 + e^{-z}$. Furthermore, the tumor budding count was
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52 included in the clinical decision-making analysis of patients to determine whether
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54 patients require additional surgery after endoscopic treatment. Some studies have
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4 shown that the presence of tumor budding in biopsy specimens before CRC surgery
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6 increases the possibility of lymph node and distant metastasis. Therefore, neoadjuvant
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8 therapy and surgical treatment can be considered for these patients³⁷. The Japanese
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10 Society for Cancer of the Colon and Rectum has incorporated the index of tumor
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12 budding into the guidelines for patients with pT1 disease who require further surgery³⁸.
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15 In our study, 244 of 253 patients with lymph node metastasis had tumor budding. The
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17 sensitivity of budding for predicting lymph node metastasis was 96.44%, indicating that
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19 tumor budding is an effective pathological index with high sensitivity for predicting
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21 lymph node metastasis. Therefore, we believe that for patients with NSCLC, we can
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23 refine the significance of tumor budding through a larger sample study to contribute to
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25 clinical decision-making.
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34 The peritumoral space is the space between the tumor cells and the stroma that
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36 divides the tumor components from the stroma and is morphological manifestation of
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38 the interaction between the tumor cells and the stromal cells. The peritumoral space is
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40 commonly seen in paraffin-embedded tissue sections fixed with formalin. The
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42 peritumoral space is one of the pathomorphological manifestations of tumor biological
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44 behavior that is considered a prognostic factor by some scholars. Peritumoral spaces
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46 have been noted in breast, lung, bladder, and prostate cancers and other malignant
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48 tumors. Tumor cells usually spread to the corresponding lymph nodes through the
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50 lymphatic system, this phenomenon is considered an important early event of tumor
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52 metastasis^{7 8}. In prostate cancer, an extensive peritumoral space indicates a higher
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4 tumor grade, shorter disease-free survival, and poor prognosis^{39 40}. At the same time,
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6 the peritumoral space in breast cancer is closely related to histological grade, lymphatic
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8 invasion, lymph node metastasis, and prognosis and can be used as an important marker
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10 to judge the prognosis of breast cancer patients^{41 42}. ACS et al.⁴³ observed the relationship
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12 between a large peritumoral space and lymph angiogenesis, and the results confirmed
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14 a poor prognosis of patients with large peritumoral spaces, which was consistent with
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16 this hypothesis. In our study, we found that in patients with lung squamous cell
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18 carcinoma, the peritumoral space is closely related to tumor budding, which is also an
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20 independent risk factor for patient 5-year OS. A joint evaluation of the peritumoral
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22 space and tumor budding can effectively evaluate the prognosis of patients with lung
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24 squamous cell carcinoma.
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34 Lung adenocarcinoma spreads through the bronchus, known as lung metastasis,
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36 and the airways, known as airway metastasis. A small number of lung adenocarcinoma
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38 cancer cells enter the bronchial cavity, and with the respiratory movement through the
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40 bronchial discontinuous, they diffuse into other lung segments or lobes on the same or
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42 opposite side, forming new lung metastases⁴⁴. Our study revealed that tumor budding
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44 was closely related to airway spread. Tumor budding can be combined with spread
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46 through airspaces (STAS) to evaluate the malignant aggressive behavior of NSCLC.
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53 Che et al.⁶ found that the OS rate of patients with high budding gastric
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55 adenocarcinoma was significantly lower than that of patients with low budding gastric
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57 adenocarcinoma. Some studies reported that the presence of tumor budding in surgical
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4 specimens of patients with gastric cancer may indicate a poor prognosis and early
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6 recurrence²⁹. We also found that the 5-year OS rate of lung adenocarcinoma or
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8 squamous cell carcinoma patients with high-grade budding was significantly lower than
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10 that of patients with low-grade or no budding. However, Hass et al.⁴⁵ emphasized that
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12 tumor budding and cancer classification based on cell differentiation were neither the
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14 same nor related. Some researchers believed that tumor budding and tumor growth
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16 pattern were independent prognostic parameters⁹. However, in our study of lung
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18 adenocarcinoma, tumor budding was closely related to histological subtype. In patients
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20 with papillary and solid subtypes of lung adenocarcinoma, the 5-year survival rate of
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22 patients with high-grade budding was significantly lower than that of patients with low-
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24 grade budding. In patients with TNM stage I, the 5-year OS rate of patients with high-
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26 grade tumor budding was lower than that of patients with low-grade or no budding
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28 (Figure 6A). The results are consistent with those of Kyuichi et al.⁴⁶.

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

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9 The research results of Wang et al. suggested that tumor budding should be
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11 included in the routine histopathological report to better stratify the risk of CRC
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13 patients⁴⁷. The AJCC and College of American Pathologists guidelines on CRC
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15 proposed that tumor budding should be considered an optional reporting indicator and
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17 should be evaluated in all cases of stage I and II CRC. This provides us with a
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19 standardized reporting tool for tumor budding⁴⁸. However, there is no unified scoring
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26 standard for lung cancer.

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28 The current study had several limitations. First, our research is limited to the tumor
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30 budding analysis of NSCLC patients in China, and the results of different ethnicities
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32 may differ. For example, demographic heterogeneity in the frequency of genetic
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34 susceptibility alleles was addressed in Zahra Fathi, et al's review of lung cancer in the
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36 Iranian population⁴⁹. They focused on germline and somatic gene variation, putative
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38 operable drivers of these genes, their impact on tumor immune monitoring, and the drug
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40 resistance mechanism of cancer treatment in which they engage in this work. In addition,
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42 because the number of surgical specimens selected for this operation before 2015 was
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44 limited, the sample size was insufficient, which might result in sample bias. However,
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46 as an effective and simple pathological diagnosis index, it is necessary to establish an
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48 effective grading system to verify its value as a standard prognostic indicator. In
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60 addition, prospective clinical trials including multicenter samples are needed to

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

11 **Conclusion**

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15 To validate the utility of tumor budding as a prognostic indicator, an effective and
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17 straightforward pathological diagnostic index should be established in the clinical
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19 diagnosis of lung cancer. We selected 532 Chinese patients with non-small cell lung
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21 cancer for this investigation, including 380 with adenocarcinoma and 152 with
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23 squamous cell carcinoma. Our findings reveal a link between tumor budding and airway
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25 spread in patients with lung adenocarcinoma, and a connection between tumor budding
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27 and the peritumoral space in patients with squamous cell carcinoma. Multivariate
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29 analysis revealed that tumor budding, pleural and vascular invasion, airway spread,
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31 tumor size, lymph node metastasis, and Tumor Node Metastasis stage were independent
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33 risk variables of prognosis for non-small cell lung cancer patients by Cox regression
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35 analysis. We think that this study of Chinese patients with non-small cell lung cancer
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37 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*
I a1	6	4(66.67%)	2(33.33%)		
I a2	76	12(15.79%)	64(84.21%)		
I a3	48	11(22.92%)	37(77.08%)		
I b	41	9(21.95%)	32(78.05%)		
II a	15	3(20.00%)	12(80.00%)		
II b	87	2(2.30%)	85(97.70%)		
III a	76	4(5.26%)	72(94.74%)		
III b	27	1(3.70%)	26(96.30%)		
III c	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*
	I a1	4	1(25.00%)	3(75.00%)		
	I a2	18	5(27.78%)	13(72.22%)		
	I a3	23	10(43.48%)	13(56.52%)		
	I b	16	2(12.50%)	14(87.50%)		
	II a	19	0(0.00%)	19(100.00%)		
	II b	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.

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3 A-D: the budding of the tumor in lung squamous cell carcinoma.

4 E-H: the tumor budding in lung adenocarcinoma.

5 A, C, E and G were $\times 20$ magnification.

6 B, D, F and H were $\times 40$ magnification (bar = 500 μm).
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10 Figure 2: The forest map of multivariate survival analysis.

11 A: the results of multivariate analysis of lung adenocarcinoma.

12 B: the results of multivariate analysis of lung squamous cell carcinoma.
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15 Figure 3: Kaplan – Meier analysis of the relationship between tumor budding and 5-
16 year overall survival rate in patients with NSCLC.

17 A: in patients with lung adenocarcinoma, the 5-year survival rate of patients with high-
18 grade budding group was significantly lower than that of patients without tumor
19 budding and low-grade tumor budding.
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21 B: in patients with lung squamous cell carcinoma, the higher the level of tumor budding,
22 the worse the prognosis of patients was.
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26 Figure 4: Kaplan – Meier analysis showed that the 5-year survival rate of patients with
27 different histological subtypes in adenocarcinoma.

28 A: the survival rates of patients with different histological subtypes were different.
29 Among them, the 5-year prognosis of patients with micropapillary subtype and solid
30 subtype was significantly lower than that of adherent subtypes.
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32 B: in patients with solid subtypes, the 5-year survival rate of patients with high-grade
33 budding was significantly lower than that of patients with low-grade budding and non-
34 budding.
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36 C: in patients with micropapillary subtypes, the higher the grade of tumor budding, the
37 worse the prognosis.
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41 Figure 5: The relationship between the size of tumor budding nests and the nuclear size
42 of tumor budding, as well as the 5-year survival rate of patients with NSCLC.

43 A: in patients with lung adenocarcinoma, the larger the nucleus of tumor budding, the
44 lower the 5-year overall survival rate was.
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46 B: in patients with lung squamous cell carcinoma, single cell invasion showed a worse
47 prognosis.
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51 Figure 6: The relationship between tumor budding level and patients at different TNM
52 stages.

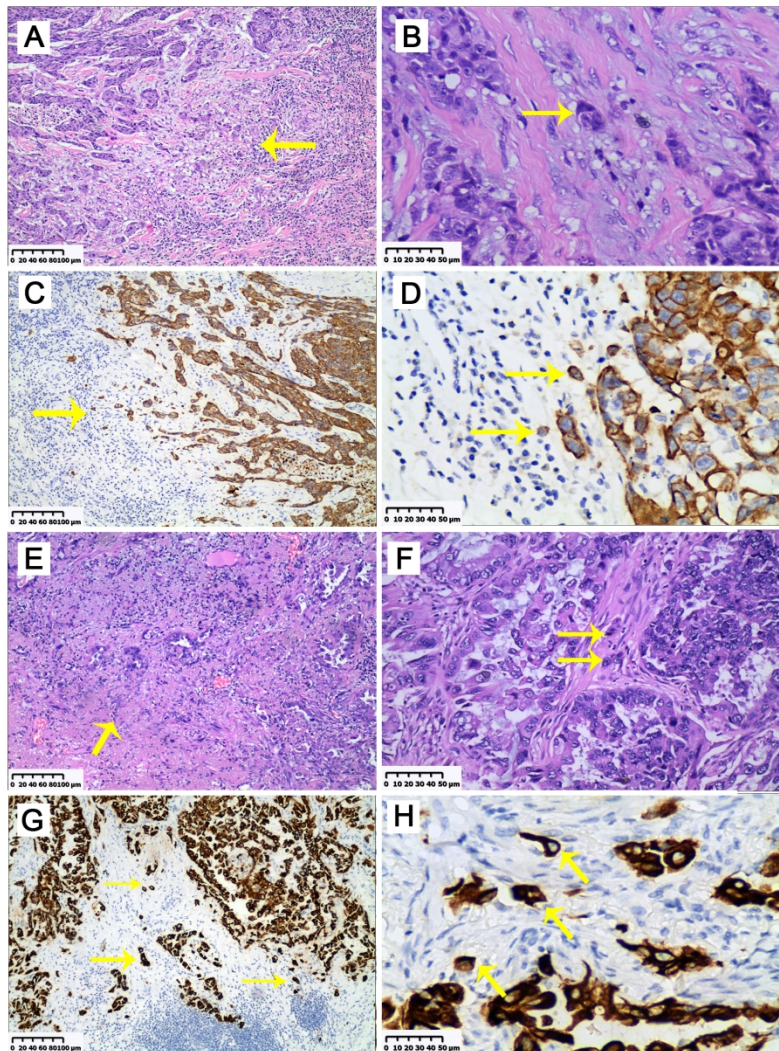
53 A: in patients with TNM stage I lung adenocarcinoma, the higher the tumor budding
54 level, the lower the 5-year overall survival rate.
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56 B: in patients with TNM stage II squamous cell carcinoma, the prognosis of patients
57 without tumor budding and low-grade tumor budding was significantly higher than that
58 of patients with high-grade tumor budding.
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Figure 1



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Figure 2

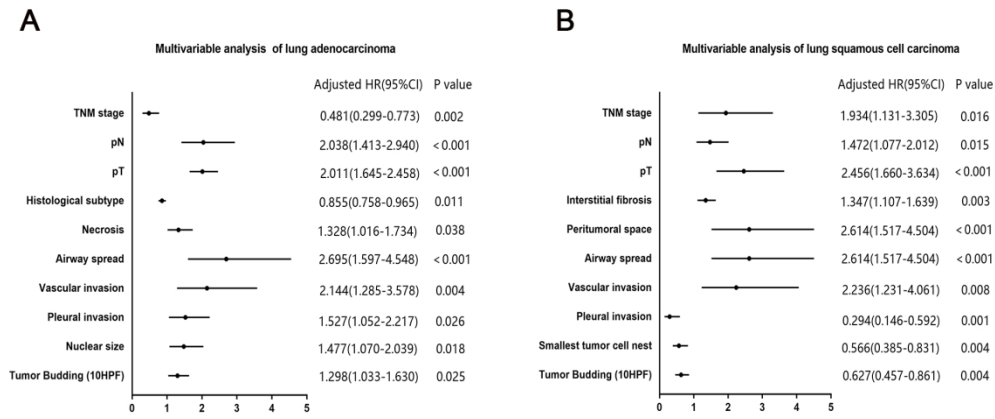


Figure 3

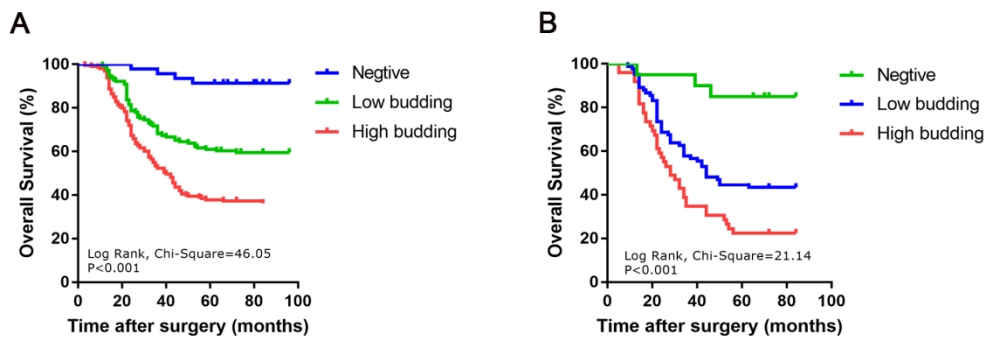
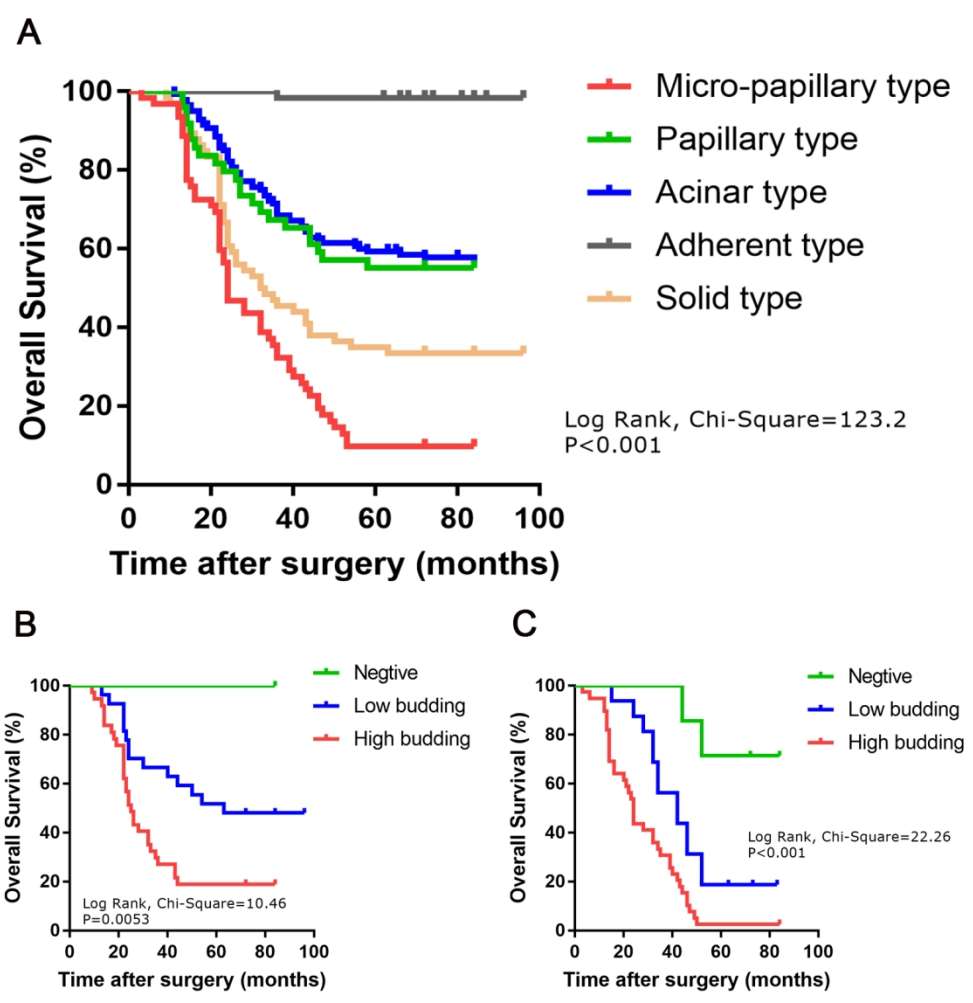
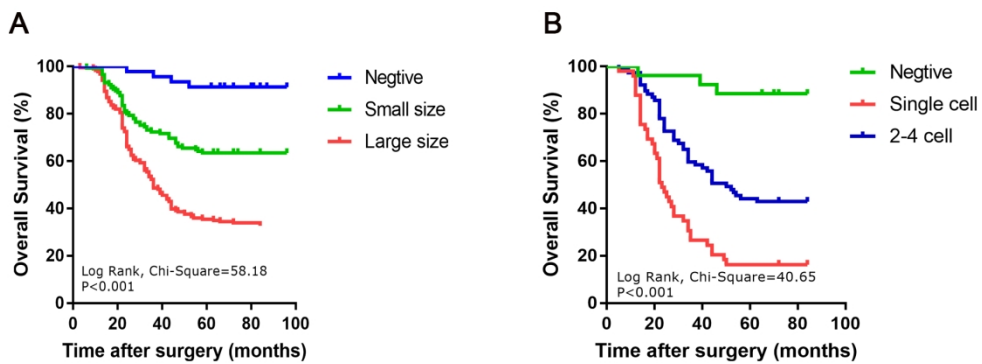


Figure 4



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Figure 5



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Figure 6

