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Research Article

# Multicenter Study on Tumor Budding in Lung Squamous Cell Carcinoma: Comparison Between Biopsy and Resection With Interobserver Variability Assessment

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## ABSTRACT

Grading lung squamous cell carcinoma (LUSC) is controversial and not universally accepted. The histomorphologic feature of tumor budding (TB) is an established independent prognostic factor in colorectal cancer, and its importance is growing in other solid cancers, making it a candidate for inclusion in tumor grading schemes. We aimed to compare TB between preoperative biopsies and resection specimens in pulmonary squamous cell carcinoma and assess interobserver variability. A retrospective cohort of 249 consecutive patients primarily resected with LUSC in Bern (2000-2013, n = 136) and Lausanne (2005-2020, n = 113) with available preoperative biopsies was analyzed for TB and additional histomorphologic parameters, such as spread through airspaces and desmoplasia, by 2 expert pathologists (M.M., C.N.). Results were correlated with clinicopathologic parameters and survival. In resection specimens, peritumoral budding (PTB) score was low (0-4 buds/0.785 $mm^2$) in 47.6%, intermediate (5-9 buds/0.785 $mm^2$) in 27.4%, and high ($\geq$10 buds/0.785 $mm^2$) in 25% of cases (median bud count, 5; IQR, 0-26). Both the absolute number of buds and TB score were similar when comparing tumor edge and intratumoral zone ($P = .192$) but significantly different from the score obtained in the biopsy ($P < .001$). Interobserver variability was moderate, regardless of score location (Cohen kappa, 0.59). The discrepant cases were reassessed, and consensus was reached in all cases with identification of causes of discordance. TB score was significantly associated with stage ($P = .002$), presence of lymph node ($P = .033$), and distant metastases ($P = .020$), without significant correlation with overall survival, tumor size, or pleural invasion. Desmoplasia was significantly associated with higher PTB ($P < .001$). Spread through airspaces was present in 34% and associated with lower PTB ($P < .001$). To conclude, despite confirming TB as a reproducible factor in LUSC, we disclose areas of scoring ambiguity. Preoperative biopsy evaluation was insufficient in establishing the final TB score of the resected tumor.



These authors contributed equally: Marie Maillard and Christina Neppl.

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## Introduction

Lung cancer is responsible for most of cancer-associated deaths, despite the advancements made in the understanding of the disease and the emerging availability of personalized treatment options in subsets of non–small cell lung carcinoma.[1] Lung squamous cell carcinoma (LUSC) accounts for 12% to 25% of all lung cancers and approximately one-third of non–small cell lung carcinoma.[2,3] Grading LUSC remains controversial.[4] Recently, new histomorphologic parameters, such as tumor budding (TB), have been described that allow prognostic stratification.[5-7] TB is a particular pattern of tumor infiltration, with a tumor bud being defined as a single tumor cell or a small group of 2 to 4 cells that are visualized as detached from the main tumor mass on 2-dimensional sections, infiltrating the surrounding tissue. TB can be evaluated at the tumor infiltration front (peritumoral budding [PTB]) and in the tumor center (intratumoral budding [ITB]).

TB was first described in colorectal cancer and has been well established as a strong independent prognostic feature.[8-11] It has then been studied in different entities, such as head and neck squamous cell carcinoma (SCC),[12,13] pancreatic cancer,[14,15] cervical cancer,[16,17] and lung cancer.[5-7,18] Over the years, several studies have been published evaluating different methods of TB counting.[19] A consensus has been reached in 2016 during the International Tumor Budding Consensus Conference (ITBCC),[20] providing a clear methodology to evaluate TB in colorectal cancer, which is now included as an additional adverse prognostic parameter in universally recognized grading systems, the eighth edition of the Union for International Cancer Control (UICC) TNM classification[21] and the College of American Pathologists guidelines.[22] Moreover, TB was shown to have a prognostic value in preoperative biopsies in many carcinomas,[23] including head and neck SCC,[24] esophageal carcinoma,[25] and colorectal adenocarcinoma.[26-28] In those cancers, TB in biopsies could thus be used for clinical decision-making regarding administration of neoadjuvant therapy or choice of surgical procedure. Given that a large proportion of lung cancers are inoperable, there is a major interest in clarifying whether it is possible to transpose the prognostic value of TB to the preoperative setting.

To the best of our knowledge, this study is the first to compare TB in preoperative biopsies in LUSC with PTB and ITB in the paired resection specimens using the ITBCC guidelines, including interobserver variability and in-depth evaluation of specific pitfalls in the lung. We have also evaluated the association of TB with desmoplasia and spread through airspaces (STAS).

## Materials and Methods

### *Patient Cohort*

This multicentric retrospective study was conducted according to the REporting recommendations for tumour MARKer prognostic studies (REMARK) guidelines and approved by the local Ethics Commissions (projects-ID CER-VD: 2020-02354; KEK 200/14). We included 249 consecutive patients diagnosed with LUSC in resection specimens at the Institute of Tissue Medicine and Pathology, University of Bern and Institute of Pathology, University Hospital Lausanne, where both the resection specimens and the preoperative biopsies were available, and the resection was performed without prior neoadjuvant treatment. Patients with SCC in other anatomical sites diagnosed previously or simultaneously were excluded, in an effort to avoid potential metastatic lung lesions. The Lausanne cohort included 113 patients (2005-2020). The Bern cohort included 136 patients (2000-2013) and was a subcohort of a previously published cohort (n = 354),[6] defined by the availability of preresection biopsies. Most biopsies were transbronchial and bronchial forceps biopsies, with a minority being transthoracic needle biopsies (21/113, 18% for the Lausanne cohort). Neither cryobiopsies nor surgical lung biopsies (wedge resections) were included.

Clinical information was extracted from patient files. Histopathologic data were collected from the pathologic reports, and the histology was reevaluated for the present study. Tumor stages were revised according to the current UICC TNM eighth edition.[29] Overall survival (OS) was established as the time from surgical resection to death. The time measured from surgical resection to development of locoregional or metastatic recurrence (or death) was defined as disease-free survival (DFS). Disease-specific survival (DSS) was defined as the time between diagnosis until death because of LUSC. The characteristics regarding the patient cohort are provided in the Table, as well as their relation to TB.

### *Assessment of Tumor Budding*

TB was independently established by 2 board-certified pathologists (M.M. and C.N.) according to the recommendations for reporting TB in colorectal cancer based on the ITBCC (2016).[20] All hematoxylin and eosin (H&E)–stained slides of each case were evaluated using the microscope (Nikon Eclipse Ci microscope; Nikon AG Instruments), and the ones with the highest amount of TB were selected, digitized at high power (×40) (NanoZoomer S60 Digital slide scanner C13210-01, Hamamatsu Photonics), and visualized using TM-Microscopy (Telemis SA). We chose 2 slides per resection specimen: one from the tumor invasion front to assess PTB and one from the tumor center to evaluate ITB. We have defined the intratumoral zone as beginning at least 1 field distance (at ×20 magnification) from the tumor edge. Tumor buds were identified and counted at ×20 magnification (adjusted to a field measuring 0.785 $mm^2$). The absolute number of TB was recorded in 3 categories as suggested by the ITBCC: 0 to 4 buds (Bd1, low budding), 5 to 9 buds (Bd2, intermediate budding), and ≥10 buds (Bd3, high budding). Two additional measurements of the biopsies were assessed in square millimeters; the first was the whole tissue area and the second the area of interest, meaning tumoral tissue. For all slides, areas with necrosis or fragmentation were excluded from the evaluation. When a case was classified in a different budding category by the pathologists, it was considered discrepant and therefore rediscussed to reach consensus. We used Cohen kappa to measure interobserver variability.[30]

### *Immunohistochemical Staining*

Immunohistochemical evaluation with pancytokeratin staining was restricted to difficult cases. The slides were stained on automated immunostainers (Bern: Leica BOND RX; Leica Biosystems; Lausanne: Benchmark ULTRA; Ventana). We used the mouse monoclonal anti-cytokeratin AE1/AE3 antibody (clone M3515; Dako-Agilent) (Bern: dilution 1:200, pretreatment with citrate buffer, 20 minutes at 100 °C; Lausanne: dilution 1:100,

**Table**
Clinical and pathologic characteristics of the cohort in relation to PTB scores

| | Budding scores (PTB) | | | |
|---|---|---|---|---|
| | Low (Bd1), n = 118 | Moderate (Bd2), n = 68 | High (Bd3), n = 62 | *P* |
| Sex, n (%) | | | | .090[a] |
| Male | 98 (83.05) | 63 (92.64) | 50 (80.65) | |
| Female | 20 (16.95) | 5 (7.36) | 12 (19.35) | |
| Age (y) (median, IQR) | 68 (63-75.75) | 68 (63.5-75) | 68 (63-75) | .928[b] |
| Smoking status, n (%) | | | | .362[a] |
| Never | 3 (2.63) | | 1 (1.75) | |
| Former | 58 (50.88) | 30 (52.63) | 22 (38.60) | |
| Active | 53 (46.49) | 27 (47.37) | 34 (59.65) | |
| Size (mm) (median, IQR) | 45 (30.25-60) | 45 (31.5-64) | 50 (35.75-73.75) | .094[b] |
| pT, n (%) | | | | .296[a] |
| pT1 | 28 (23.73) | 11 (16.18) | 8 (12.90) | |
| pT2 | 42 (35.59) | 25 (36.76) | 22 (35.48) | |
| pT3 | 27 (22.88) | 13 (19.12) | 12 (19.35) | |
| pT4 | 21 (17.80) | 19 (27.94) | 20 (32.26) | |
| pN, n (%) | | | | .033[a] |
| pN0 | 72 (61.02) | 27 (39.71) | 26 (41.94) | |
| pN1 | 34 (28.81) | 30 (44.12) | 27 (43.55) | |
| pN2 | 12 (10.17) | 11 (16.18) | 9 (14.52) | |
| M, n (%) | | | | .020[a] |
| M0 | 77 (100.00) | 16 (88.89) | 11 (91.67) | |
| M1 | | 2 (11.11) | 1 (8.33) | |
| Stage, n (%) | | | | .002[a] |
| I | 35 (29.66) | 13 (19.12) | 6 (9.68) | |
| II | 46 (38.98) | 18 (26.47) | 24 (38.71) | |
| III | 37 (31.36) | 35 (51.47) | 30 (48.39) | |
| IV | | 2 (2.94) | 2 (3.23) | |
| PL, n (%) | | | | .105[a] |
| PL0 | 81 (68.64) | 49 (72.06) | 37 (59.68) | |
| PL1 | 25 (21.19) | 9 (13.24) | 8 (12.90) | |
| PL2 | 7 (5.93) | 6 (8.82) | 10 (16.13) | |
| PL3 | 5 (4.24) | 4 (5.88) | 7 (11.29) | |

Age is given at diagnosis. TNM stage values are informed according to the Union for International Cancer Control TNM eighth edition. *P* values were generated using the Fisher exact test (categorical variables) or Kruskal-Wallis test (continuous variables).
PL, pleural invasion; PTB, peritumoral budding.
[a] Fisher exact test.
[b] Kruskal-Wallis test.

pretreatment with CC1, 32 minutes at 100 °C, detection with Ultraview DAB Detection Kit). Final scoring was performed on H&E-stained slides.

### *Assessment of Desmoplasia and Spread Through Airspaces*

Desmoplasia was assessed as present or absent in the hotspot field. STAS, defined as tumor cell nests spreading in air spaces beyond the tumor border,[31] was reported on surgical specimen as present or absent.

### *Statistical Analysis*

Statistical analysis was performed using the R environment version 4.1.3. For comparison of the budding categories with clinicopathological characteristics, we used the Fisher exact test for categorical data and Kruskal-Wallis test for ordinal or continuous data. Interrater reliability regarding budding scores was assessed using Cohen kappa with quadratic weights. For univariable survival analysis, we used the log-rank test and Kaplan-Meier plots for visualization. A 2-sided $P < .05$ was considered statistically significant.

## Results

### *Tumor Budding Score Is Similar at the Infiltration Front and in the Tumor Center but Differs From That in the Biopsy*

TB was evaluated in 249 specimens and corresponding preoperative biopsies, for a total number of 714 slides, because 30 slides carried both the intratumoral and peritumoral hotspots. Three biopsies were not representative and have been disregarded. The entire biopsy surface between 1 and 280 $mm^2$ (median, 9 $mm^2$) and the area of interest between >1 and 240 $mm^2$ (median, 4 $mm^2$) in the Lausanne cohort (n = 113) were measured.

The median ITB were 5 buds (range, 0-45); the median PTB were 5 buds (range, 0-26), and the median TB in biopsies were 2 buds (range, 0-35). PTB showed low budding score (Bd1) in 47.4% (n = 118/249), intermediate score (Bd2) in 27.3% (n = 68/249), and high budding score (Bd3) in 25.3% of cases (n = 63/249). ITB showed Bd1 in 48.0% (n = 117/249), Bd2 in 28.9% (n = 72/249), and Bd3 in 24.1% (n = 60/249). Biopsies showed Bd1 in 76.8% (n = 189/246), Bd2 in 15.4% (n = 38/246), and Bd3 in 8.1% of cases (n = 20/246) (Fig. 1). When comparing the scores between PTB and ITB in resection specimens, we found a concordant budding category in 62.9% of cases (n = 156/249).

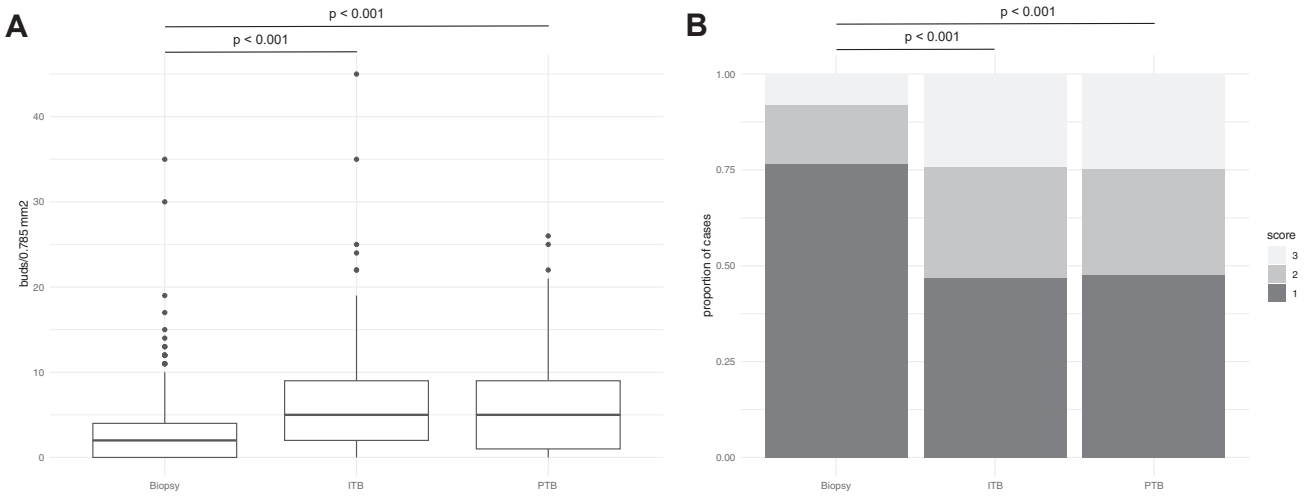


**Figure 1.**
Comparison of budding between the different regions of assessment. Budding score distribution is depicted according to (A) absolute budding counts (box plot) and (B) budding category scores (bar plot). Scores 1, 2, and 3 correspond to budding categories low (Bd1), moderate (Bd2), and high (Bd3), respectively. ITB, intratumoral budding; PTB, peritumoral budding.

The budding counts are not normally distributed in each region (Supplementary Fig. S1), and therefore, we used the Friedman test to assess the overall comparability and the Wilcoxon signed-rank test to perform a pairwise comparison. In general, there was a significant difference ($P < .001$). A pairwise Wilcoxon signed-rank test confirmed the significant differences in the absolute number of buds comparing biopsies with the infiltration front ($P < .001$) or tumor center ($P < .001$). The absolute bud counts were comparable between the infiltration front and tumor center in resection specimen ($P = .192$).

### *Interobserver Variability Between Tumor Budding Scores and Causes for Discordance*

A total of 744 hotspots were independently assessed for TB, and 240 slides showed discordant budding scores: 70 PTB (28.5%), 122 ITB (49.6%), and 48 biopsies (19.5%). Considering all hotspots independent of localization, a moderate Cohen kappa score of 0.59 was reached. Moderate Cohen kappa scores were also reached when considering individually the TB scores of different locations (infiltration front, 0.651; biopsies, 0.680; and tumor center, 0.416). Following the independent evaluation, a consensus of discordant cases could be reached in 100% of the biopsies and in 90.6% of the infiltration front or tumor center (174/192). Additional pan-cytokeratin staining was used in 18 slides corresponding to 9 patients, allowing to reach consensus in all cases. Discordant cases had significantly more often intermediate or high TB scores ($P < .001$).

Six causes of discordant TB scoring were identified (Fig. 2): morphologic interpretation (44%, n = 106), choice of hotspot (41%, n = 97), extensive inflammation (10%, n = 24), interpretation of hotspot localization (2.5%, n = 6), and extensive necrosis (1%, n = 1). For 6 cases, discrepancy was attributed to a transcription error.

### *Tumor Budding Correlates With Higher Stage and Is Significantly Associated With Lymph Node and Distant Metastases*

Higher TB score showed a significant association with higher UICC/The American Joint Committee on Cancer stage ($P = .002$), particularly regarding presence of infiltrated mediastinal lymph nodes (pN category according to UICC, $P = .033$) and distant metastases ($P = .020$). There was no correlation with pleural invasion and pT category.

### *Budding Relation to Desmoplasia and Spread Through Airspaces*

Desmoplastic reaction was evaluated in the tumor infiltration front of all cases (189/249) and in the tumor center (105/113) and biopsies of the Lausanne cases only (93/113). Desmoplastic reaction was statistically significantly associated with higher PTB ($P < .001$).

STAS was demonstrated in 34.15% of the resection cases (n = 84/246). STAS was statistically significantly associated with lower PTB ($P < .001$) but not with ITB ($P = .958$) or budding in the biopsies ($P = .292$).

### *Survival Analysis*

For the survival analysis, we examined the Lausanne cohort because the survival data for patients in the Bern cohort were already available and published.[6] OS was defined as the time from surgery (resection of the lung carcinoma) until death of any cause. DFS was defined as the time from surgery until death of any cause or relapse whatever occurred first. DSS relates to the time between diagnosis until death specifically because of LUSC, with other cause of death (n = 14) or unknown cause (n = 24) being censored.

Patients were included in the survival analysis if the follow-up could be assessed at least 30 days after the resection of the lung carcinoma. One patient was excluded because of missing follow-up information, and 3 patients were excluded because of follow-up information of <30 days after surgery.

Median OS was 51 months (95% CI, 36-not available), and 47 events were counted for 109 patients. Neither PTB ($P = .70$), ITB ($P = .76$) nor the budding score of the biopsy ($P = .92$) was associated with OS (Supplementary Fig. S2).

Median DFS was 31 months (95% CI, 18-59 months), and 57 events were counted for 109 patients. Likewise, neither PTB ($P = .27$), ITB ($P = .71$) nor the budding score of the biopsy ($P = .75$) was associated with DFS (Supplementary Fig. S3).

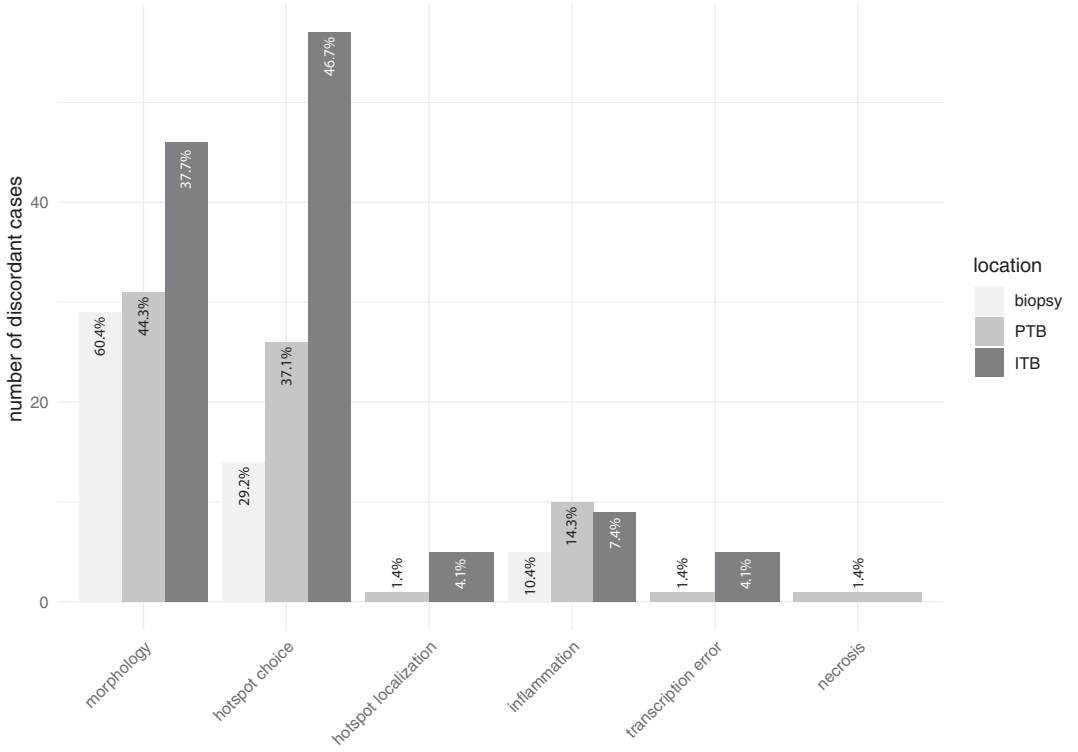


**Figure 2.**
Categories of discrepancy in interobserver variability. The reasons for discordance are given according to the region of assessment (ITB, PTB, or biopsy). The percentages indicate the proportion of discordant cases in that specific region. ITB, intratumoral budding; PTB, peritumoral budding.

Median DSS was not reached. Here as well, neither PTB ($P = .23$), ITB ($P = .59$) nor the budding score of the biopsy ($P = .73$) was associated with DSS (Supplementary Fig. S4).

## Discussion

Despite the constantly evolving knowledge of histologic and molecular characteristics of LUSC, there is currently no universally validated grading system.[21,29,32,33] Potential factors for grading LUSC include TB, degree of keratinization, nuclear size, stromal desmoplasia, and STAS.[4,5,34] TB is particularly interesting as it has demonstrated its reliable value as an independent prognostic factor in several other cancers, most prominently among them in colorectal carcinoma.[8-11,20,35] In LUSC, evaluation of TB differs among studies, rendering comparisons difficult.[6,34,36-38]

In our study, we used the ITBCC scoring methodology established for colorectal cancer,[20] which has also shown prognostic value in LUSC.[6] Moreover, it has been successfully assessed in preoperative biopsies of colorectal adenocarcinoma,[26-28] SCC of the oral cavity,[24] and esophageal carcinoma,[25] where the prognostic value correlated with that of the surgical specimen. Most tumors in the lung are diagnosed on fine-needle or forceps biopsies from within the mass,[39] and therefore, it would be of value to gather prognostic information prior to treatment.

We assessed the ITBCC approach on a well-characterized multicenter cohort of patients with LUSC with the aim to compare the TB scoring established in the preoperative biopsies with the corresponding surgical specimens. First, using the resection specimens, we could show a strong association of the TB score at the infiltration front (PTB) and in the center of the tumor (ITB), as previously described by others in colorectal and gastric carcinomas.[20,40,41] This facilitates scoring of TB in LUSC in daily practice.

However, we found no correlation of TB category between resection specimen and preoperative biopsy. This is contrary to previous studies on colorectal and head and neck cancers[24-26,28,42,43] and can be explained in several ways, some of them illustrated in Figure 3. First, LUSC typically shows significant tumor heterogeneity, and the hotspots are often isolated, representing a small percentage of the total tumor volume. Although a large area is screened in the resection specimen, the biopsy is taken randomly, not necessarily targeting areas with a high budding score. In addition, the biopsy samples only a very little part of the tumor. Although the diagnostic criteria of SCC can be determined on a few cells with certainty, the determination of the budding score requires a minimal tumor volume, including stroma. The biopsy often has a limited size and the area of interest even more. Furthermore, it is subject to crushing and dissociation artifacts, making the interpretation of budding more difficult. The biopsy itself, although small, has a certain thickness, and TB might be missed because of insufficient step sectioning. Therefore, it must be deeply cut with the microtome to guarantee a representative analysis and avoid an inappropriately superficial evaluation of the sample. In the same way as in the resection specimen, extensive inflammation or necrosis hinders interpretation. Finally, superficial sampling of an endobronchial mass may be sufficient for diagnosis but not always for TB scoring.

Given that several studies demonstrated a significant correlation between biopsy and surgical specimen as well as its prognostic value, notably in head and neck SCC and colorectal adenocarcinoma,[24-26,28,42,43] it seems legitimate to question the size of the

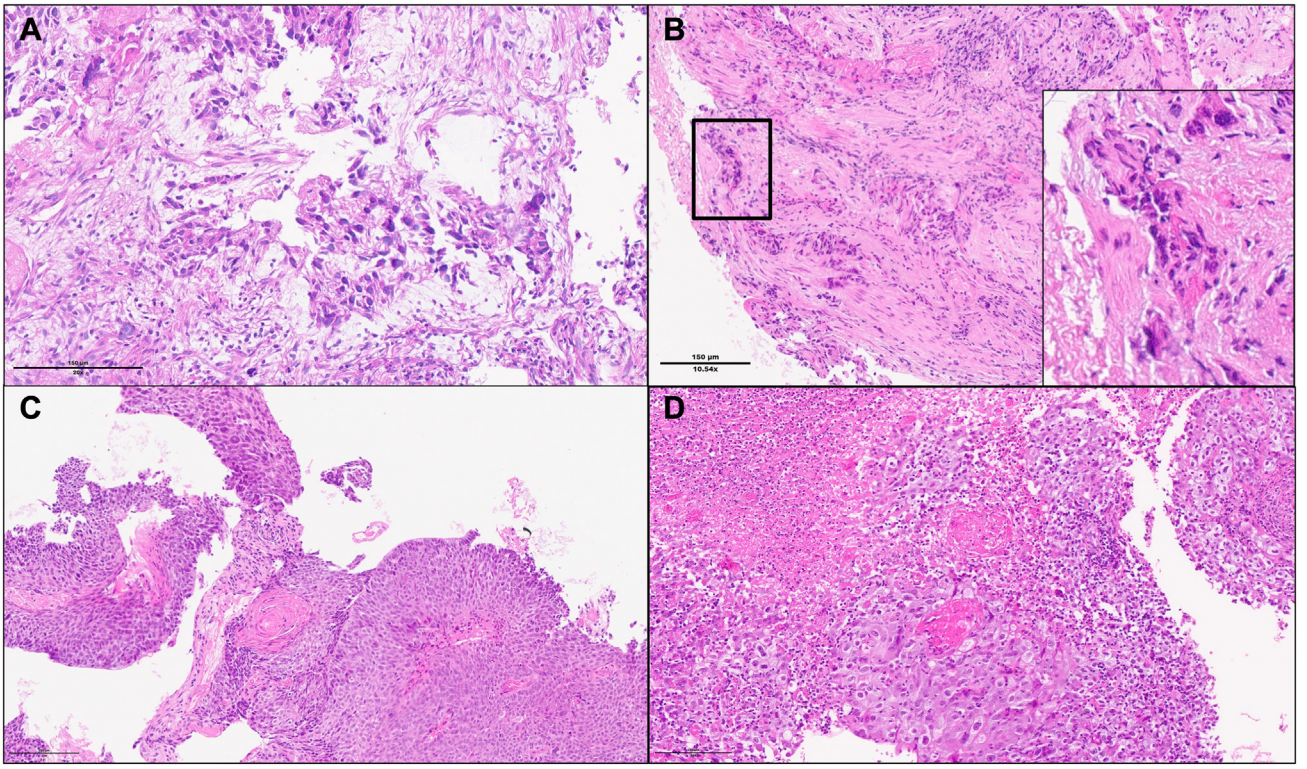


**Figure 3.**
Potential difficulties encountered when scoring biopsies using hematoxylin and eosin staining. (A) Tissular dissociation, separating stroma from tumor. (B) Crushing artifacts, making it challenging to identify separated small groups of tumor cells and assess the number of tumor cells they consist of (area of the inset is marked by a rectangle and highlights crushed tumor cells). (C) Superficial specimen of an endobronchial mass, allowing diagnosis but not assessment of the budding score. (D) Extensive necrosis, hindering distinction between stroma and tumor buds.

biopsy in order to guarantee a sufficient amount of material and establish a reliable TB score. Nevertheless, sample size was not documented in the previous studies, making it difficult to establish a minimal biopsy size to be defined as representative. This element warrants future studies to optimize the process.

Owing to the aerial nature of the lung, the presence of desmoplastic reaction is almost mandatory for TB to be present, apart from the rare invasion of pleura, vascular, or bronchial walls. This is not the case in other organs, such as the colon, cervix, or esophagus, which can present TB without the necessity of desmoplastic stroma. In lung tissue, this facilitates the analysis in tumors with minimal or no stromal reaction because there will be no tumor buds to count. STAS is a recently studied histologic factor with a controversial prognostic value.[6,44-46] We found an inverse correlation between STAS and desmoplastic stroma, suggesting that the desmoplastic process could prevent STAS. Given that budding is considered a manifestation of epithelial-mesenchymal transition,[19,38,47] evaluation of the tumor microenvironment and the role of fibroblasts and myofibroblasts in the development of TB is a promising topic.

In our cohort, we could not show the prognostic value of TB, as previously demonstrated.[5-7,18,34,36,38] The reason might be not only the relatively small number of patients with examinable biopsy ($n = 109$) but also a selection bias for patients with low budding scores (Bd1). Only few tumors were in the Bd2 and Bd3 categories, weakening the statistical power. Indeed, we had an underrepresentation of advanced tumor stages (more often inoperable), resulting from the inclusion criteria of primary resection without neoadjuvant therapy. However, we confirmed the statistically significant association of TB with higher stage, demonstrating TB correlation with lymph node and distant metastases, in agreement with previous results.[18,36]

The interobserver variability for TB was satisfactory, as previously shown in other studies on lung and oral SCC.[48,49] Nevertheless, we identified areas of uncertainty that need to be standardized in order to reach unambiguity in evaluating TB. For the first time to our knowledge, we report on the reasons for interobserver variability in assessing TB in LUSC. Diverging morphologic interpretation was the most important cause of mismatch. Indeed, tumoral stroma may present a highly cellular and reactive appearance, and the difference between activated fibroblasts and tumor cells can sometimes be subtle (Fig. 4). At low TB scores, a single bud could change the budding category. Precise evaluation of tumor cell nuclei and cytoplasm, as well as conservative counting (ie, only TB identified with certainty), enabled consensus to be reached in all cases. The choice of the hotspot, leading to discordant TB scores, can be explained by tumor heterogeneity and the large size of the tumors, making it more difficult to rapidly identify TB hotspot areas.

Areas with extensive inflammation should be excluded from the analysis.[20] Nevertheless, some tumors lack areas devoid of inflammation, and in our experience, immunohistochemical staining may be of great help in identifying budding hotspots and counting tumor buds. Pancytokeratin helped with hotspot identification and certainty level of budding count (Figs. 4 and 5). In those difficult cases, TB score would have been underestimated on H&E slides only (Fig. 6). Interpreting the location of the hotspot raises the question of a clear definition of tumor infiltration front. In the lung, cancer often involves large-caliber vessels or bronchi, embedded in the mass. At reaching consensus, those were

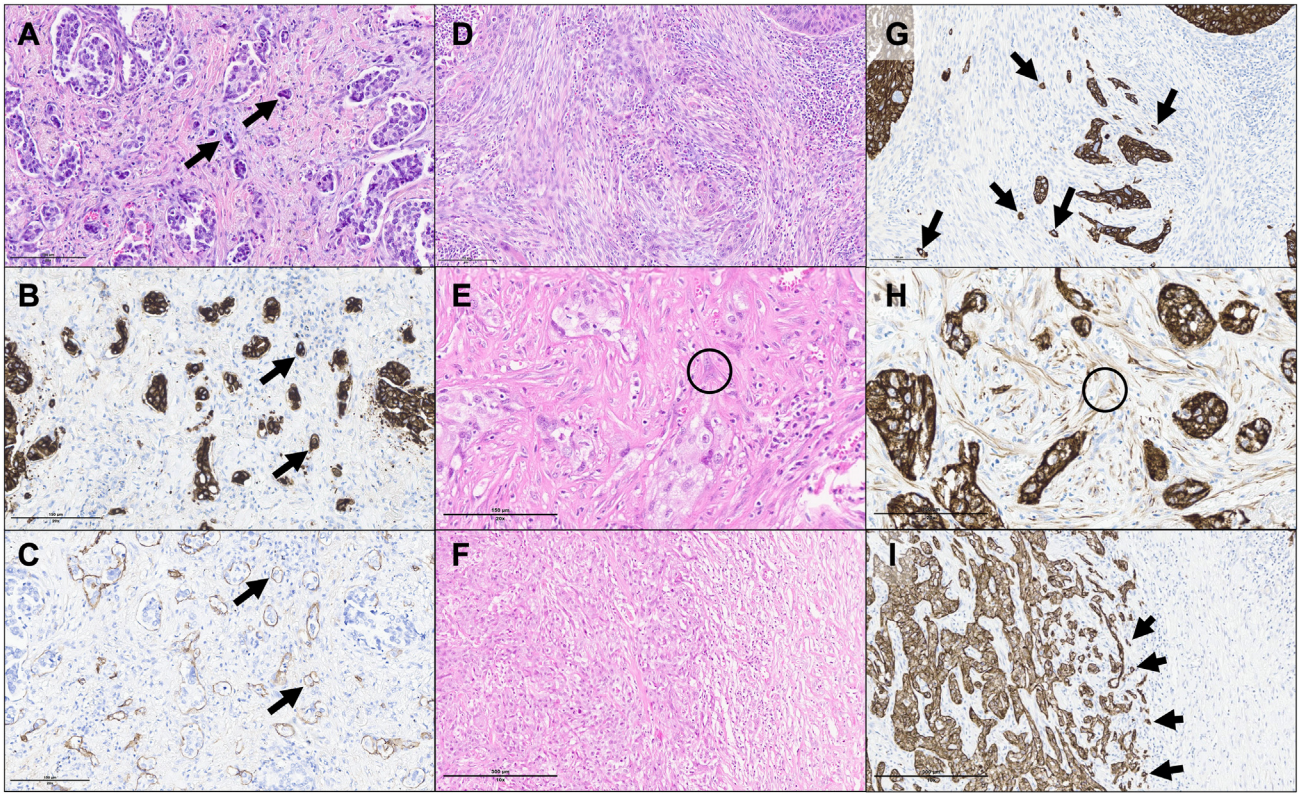


**Figure 4.**
Use of immunohistochemistry to help identify tumor buds in difficult cases of squamous cell carcinoma of the lung. (A-C) Lymphatic vascular invasion mimicking tumor buds with retraction artifacts (some examples highlighted by arrows). (B) Pancytokeratin stains the tumor cells (arrows: single tumor cell "buds"). (C) D2-40 highlights lymphatic vessel walls, confirming lymph vessel invasion (arrows: same "buds" surrounded by lymphatic wall). (D, G) Highly inflammatory stroma, masking single tumor cells that are readily identifiable in the pancytokeratin staining (G, arrows). (E, H) Reactive stroma containing activated myofibroblasts that morphologically mimic tumor cells but lack strong pancytokeratin staining (example circled in E and H). (F, I) Stromal reaction combining prominent inflammation and marked fibroblastic reaction, complicating the distinction of the tumor border. Pancytokeratin immunostaining greatly facilitates the hotspot identification and counting of tumor buds (arrows). (A, D, E, and F, hematoxylin and eosin; B, G, H, and I, CK AE1/AE3; and C, D2-40).

interpreted as tumor infiltration front, but a general guideline is lacking. More problematic were invaginations formed by the tumor stroma (Fig. 5). At reaching consensus, those were interpreted as tumor edge for the present study. To the best of our knowledge, we have for the first time described the potential sources of divergence. Although some have mentioned certain difficulties of interpretation, they have not further evaluated the topic.[9] Using a systematic approach, we have grouped reasons for interobserver variability in assessing TB, providing guidance for routine diagnostics and a base for refining the scoring criteria. The development of digital pathology and artificial intelligence algorithms will soon make it possible to use specific tools and facilitate our analysis, and therefore, it is even more imperative to set excessively unambiguous criteria and identify any possible sources of confusion to make the process reliable.

To conclude, we report the reproducibility of TB score in LUSC, highlighting its association with higher TNM stage and showing that preoperative biopsies are not sufficient to assess TB score of the resected tumor with its following prognostic value. We revealed specific scoring challenges, raising the necessity that future guidelines encompass recommendations for those difficult situations highlighted in this study. If TB scoring is to become a standard part of the pathology report, integrated in the World Health Organization and crucial to prognostication, it should be easily performable, clearly defined, and as reproducible as possible.

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*Author Contributions*

M.M. and S.B. designed the study concept. M.M., C.N., and J.A. performed the acquisition of data. P.Z. performed the statistical analysis. M.M., S.B., and P.Z. performed the interpretation of data and the writing. C.N., S.P., and T.K. revised the manuscript, and all the authors read and approved the final version.

*Data Availability*

All the data will be provided by the corresponding author on reasonable request.

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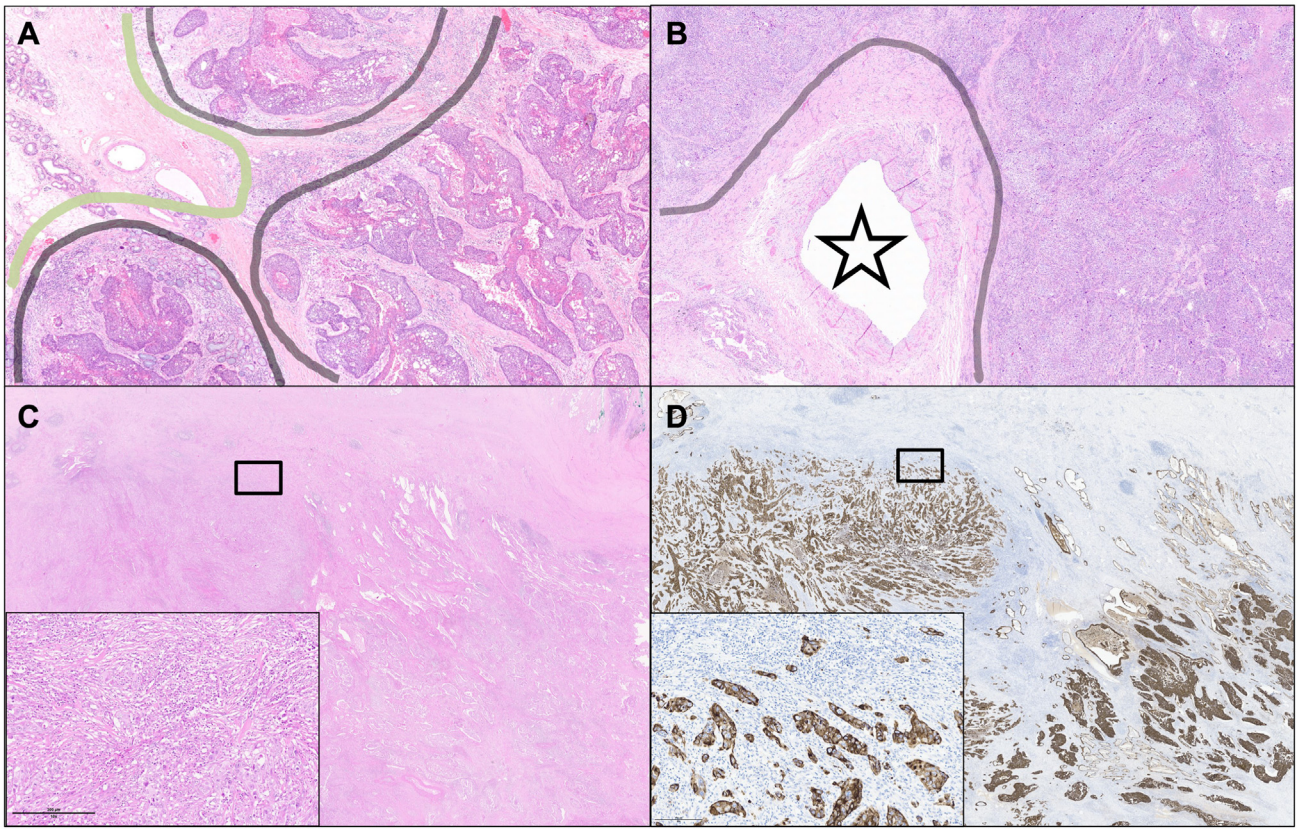


**Figure 5.**
Interpretation challenges in TB scoring of squamous cell carcinoma of the lung. (A) Squamous cell carcinoma demonstrating a nodular architecture, with numerous interpositions of fibrous septa, leading to questionable interpretation of tumor center vs infiltration front (green line vs black lines). (B) Tumor proliferation surrounding a large vascular structure (star). The black line should be considered tumor edge. The same situation occurs with tumor surrounding bronchial structures. (C) Illustration of tumor heterogeneity and the difficulty of identifying the TB hotspot and counting tumor buds using standard H&E staining. The area of the inset is marked by a rectangle, showing the infiltration front, with buds being extremely hard to identify on H&E alone. (D) Pancytokeratin of the same region (C) discloses the morphologic difference of the left upper and right lower part of the tumor and facilitates hotspot identification from an overview perspective, as well as counting of TB (inset). (A-C, H&E; D, CK AE1/AE3). H&E, hematoxylin and eosin.

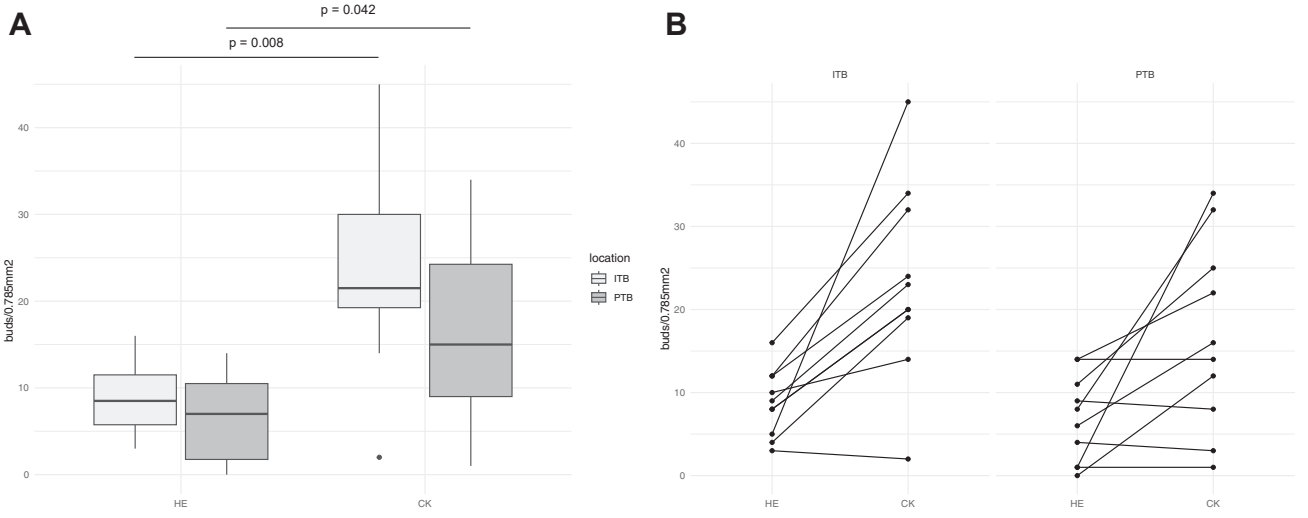


**Figure 6.**
Comparison between budding scores of the same slides using H&E alone and pancytokeratin in difficult cases. (A, B) In difficult cases (n = 18 slides; 9 patients), CK staining led to significantly higher counting of buds. The tumoral budding counts were significantly higher overall ($P < .001$) and when considering PTB ($P = .042$) and ITB ($P = .008$) separately. CK, cytokeratin; H&E, hematoxylin and eosin; ITB, intratumoral budding; PTB, peritumoral budding.

*Declaration of Competing Interest*

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*Ethics Approval and Consent to Participate*

This multicentric retrospective study was conducted according to the REMARK guidelines and approved by the local Ethics Commissions (projects-ID CER-VD: 2020-02354; KEK 200/14), which waived the requirement for written study specific informed consent.

## Supplementary Material

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