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Please cite this article TUMOR BUDDING IN TUMOR TISSUE AMONG OPERATIVELY TREATED PATIENTS WITH LUNG ADENOCARCINOMA

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UDC:

DOI: https://doi.org/10.2298/VSP190522091V

When the final article is assigned to volumes/issues of the Journal, the Article in Press version will be removed and the final version appear in the associated published volumes/issues of the Journal. The date the article was made available online first will be carried over.
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Abstract

Background/Aim. The histological phenomenon of tumor budding is being recognized as an important determinant of disease progression and poor prognosis in various types of carcinoma. We aimed to evaluate the clinicopathological significance of tumor budding in adenocarcinoma of the lung. Methods. The study included 114 patients, operatively treated for lung adenocarcinoma in one-year period. Microscopic analysis of routine histological slides was performed to establish the presence and density of tumor buds. These results were compared to gender, age, tumor size, nodal status and pathological stage. Results. The budding-positive group included 34 men (53.1%) and 27 women (54%). There were 30 men (46.9%) and 23 women (46%) in the budding-negative group. There was no statistical significance found between males (64.3 ± 6.59) and females (63.1 ± 6.53) in the budding-positive group, nor in the budding-negative group (males 63.3 ± 6.02; females 63.2 ± 6.72), age considering. Statistically significant result in tumor size was found in females with the presence of tumor budding (p<0.05). Budding-positive group of patients in nodal status N1 and in stage III of the disease pointed to the statistical significance (p<0.05). Conclusion. With statistical significance confirmed between higher nodal status, higher pathological stage and tumor budding found in this study, this histological phenomenon is still relatively new for diagnostics domain of pathology, but receiving increasing attention as an adverse prognostic factor. These results may help tumor budding to incorporate into existing staging systems as it is associated with other factors known to portend worse outcome.

Key words: budding; size; nodal status; stage.

Apstrakt

na pol, u grupi ispitanika sa potvrdenim prisustvom tumorskog pupljenja bilo je 34 (55,7%) muškaraca i 27 (44,3%) žena. U grupi ispitanika bez prisustva tumorskog pupljenja bilo je 30 (56,6%) ispitanika muškog i 23 (43,4%) ženskog pola. Nije uočena statistički značajna razlika u godinama između muškog (64,3 ± 6,59) i ženskog pola (63,1 ± 6,53) kod pacijenata sa prisustvom tumorskih pupoljaka u tumorskom tkivu, kao ni kod muškog (63,3 ± 6,02) i ženskog pola (63,2 ± 6,72) kod pacijenata bez prisustva tumorskog pupljenja. Statistički značajna razlika nije pronađena ni u veličini tumoru među pacijentima sa (4,4 ± 2,34) i bez prisustva tumorskog pupljenja (4,2 ± 2,18). Dominantna je grupa ispitanika sa nodalnim statusom N1 u čijem je tumorskom tkivu dokazan fenomen pupljenja (p<0,05). Nađeno je statistički značajno postojanje tumorskog pupljenja u stadijumu III (p<0,05).

Zaključak. Sa potvrđenom statističkom značajnošću između tumorskog pupljenja, višeg nodalnog statusa i stadijuma bolesti, dokazuje se da ovaj fenomen, iako relativno nov u dijagnostičkom domenu patologije, privlači dodatnu pažnju kao značajn progresijski faktor. Dobijeni rezultati bi mogli pomoći u integriranju ovog fenomena u postojeće skoring sisteme, zajedno sa ostalim faktorima koji naznačavaju lošiju prognozu bolesti.

Ključne reči: pupljenje; veličina; nodalni status; stadijum.

Introduction

Lung cancer is confirmed to be the leading cause of cancer-related deaths worldwide, with a generally unfavorable outcome, even with a successful surgery. In the recent years, the number of lung adenocarcinoma cases has been increasing. Lung adenocarcinoma often comes with an aggressive biological behavior, according to many studies noting an experience of recurrence or distant metastasis soon after curative resection [1-5]. A better understanding of changes in malignant neoplasms biology that result in a more aggressive neoplastic behavior may help identifying patients with high risk for recurrent disease and influencing treatment algorithms.

In an arsenal of parameters that are essential in the outcome of patients, the target area in the present study was the phenomenon of tumor budding, investigated in terms of having an important role in risk stratification in lung adenocarcinoma.
The term tumor budding has been applied to the detachment and migration of single tumor cells or small clusters of cells from the neoplastic epithelium on the invasive front of tumor [6]. With unknown molecular background, tumor budding is associated with high incidence of local invasion and distant metastasis. Previously known as tumor dedifferentiation, this phenomenon has been likened to an epithelial-mesenchymal transition, thereby increasing cell migration and invasion [7-10]. This phenomenon is speculated to be a morphological expression of invasive growth process that include detachment between tumor cells, migration and active invasion of surrounding stroma. From a morphological point of view, these groups of tumor cells tend to appear more atypical than cells in the main tumor body, and may be visualized with difficulties on H&E routine slides. In addition, these cells may be obscured by peritumoral inflammatory reaction and hardly distinguished from reactive stromal cells [11, 12]. There are no well-established criteria used to determine how many cells should be in a cluster so it can be called a tumor bud. To date, the majority of studies used the 5-cell cutoff value. This criterion is often regarded to the presence and intensity of tumor budding in colorectal adenocarcinoma, while the number of studies referred to the budding in lung adenocarcinoma is rather small [13].

Tumor budding cannot be identified as tumor dedifferentiation [14]. The pattern composed of solid areas with numerous detached cells is often found in poorly differentiated tumors. Furthermore, high grade tumors do not have or may have an insignificant number of tumor buds [15].

The differences in morphological features between cells in tumor buds and cells in the main tumor body are major. Budding cells have a tendency to progressively lose epithelial-cell features and resemble mesenchymal cells, therefore, they get long and spindle. In addition, these cells may degrade extracellular matrix. Markers of motility, chemotaxis and angiogenesis may be present on the cell surface. By performing immunohistochemistry, positive reaction on mesenchymal cell markers may be confirmed. All the features mentioned are not found among cells in the central tumor area [9, 10, 15].

Limited number of studies, mostly in colorectal oncology domain, recognizes the presence of tumor budding as an important determinant of disease progression and poor prognosis [16-18]. Nevertheless, many disagreements are present among authors. The majority of authors consider budding as an indicator that drives aggressiveness and affects the disease-
free and overall survival, opposed to other authors who reported the presence of tumor budding in cases with already anticipated unfavorable prognosis due to the lymphatic and vascular invasion, as well as the infiltration of serosa [17].

As budding is often thought to have an independent prognostic value in patients with primary operable lung adenocarcinoma, the purpose of this study was to evaluate the clinicopathological significance of budding in adenocarcinoma of the lung.

Methods

During the period from January 1 to December 31 2018, a total of 114 patients with primary lung adenocarcinoma were treated by surgical resection in the regional hospital for pulmonary diseases. The cases of 64 male patients and 50 female patients who had undergone complete resection of lung adenocarcinoma were reviewed in this retrospective study. The study protocol was approved by the Research Ethics Committee of our hospital (January 26, 2018, No. 73-I/23). These cases were selected sequentially. The patients who were diagnosed with lung adenocarcinoma, with histological slides available for histological evaluation, and whose follow-up data was complete were included in this study. The exclusion criteria that was used in this study referred to patients who received neoadjuvant chemotherapy before the surgery and patients whose follow-up data was incomplete. The patients’ characteristics that were assessed included sex, age, tumor size, stage of the disease and histological subtypes of lung adenocarcinoma. The clinicopathological data were obtained from routine medical reports.

The histological diagnosis of primary lung adenocarcinoma was based the 2015 World Health Organization Classification of Lung Tumors [19]. Tumor size was measured as the maximal diameter on the cut sections of the lung. The tumor subtypes, as well as the pathological stage were determined according to the newest, 2015 World Health Organization Classification of Lung Tumors and 2014 IASLC/ATS/ERS Lung Adenocarcinoma Classification [20].

The lung tissue surgical specimens for the histological analysis were fixed by 10% neutral formalin, then routinely paraffin-embedded. The tumors were cut at approximately 5mm intervals, sliced to 4µm thick sections and stained with hematoxylin and eosin (H&E). Full-section H&E slides were used to evaluate the presence and intensity of tumor budding,
characterized by isolated tumor cells or small clusters that migrate a short distance into the neoplastic stroma, at the advancing edge of neoplasms. Tumor budding was evaluated semiquantitatively, using a 20x objective lens by two pulmonary pathologists (AL and MP). In the first step, all of the slides were evaluated to determine the most representative tumor area. A histological section the maximal intensity of tumor budding was selected on the slide, and the number of tumor buds in that field was counted using a 20x objective lens. According to the presence of tumor buds per field, 2 major groups of patients were formed: 1. a budding-positive group of patients (Figure 1), and 2. a budding-negative group of patients.

In order to investigate the relationship between tumor budding and clinicopathological characteristics of the patients, we compared these results with demographic parameters (sex, age), as well as with histological subtypes, tumor size, nodal status and pathological stage.

The data were processed in the IBM SPSS (Statistical Package for Social Sciences) program, version 23. Data analysis methods used descriptive and inferential statistic. Numerical variables were presented by the arithmetic mean and standard deviation, and the categorized variables through the frequencies and percentages. To determine the existence of a difference in variables between study groups, Student's t-test and Chi-Squared test were used. Cumulative survival rates were calculated by the Kaplan-Meier method. The log-rank test was used to evaluate differences between the survival curves. All the differences were considered significant when the $p$ value was less than 0.05. The results were shown as tables and figures.

**Results**

*Clinicopathological characteristics and histologic examination*

Summarized characteristics of all 114 cases that were presented through percentages. Within both groups, male patients were dominant regarding to tumor budding (Table 1). 61 case was classified as the acinar subtype, 42 as the solid subtype, 5 as the papillary subtype, 4 as the mucinous subtype, and 2 as the lepidic subtype. Tumor budding was found in 61 case, as well as most frequently detected in acinar subtype of lung adenocarcinoma (Table 1).
Table 2 shows the distribution of 114 cases by age and by tumor size within budding-positive and budding-negative groups. The arithmetic mean and standard deviation were calculated for these numerical variables, but, as the results show, average age and tumor size are not significantly associated with the presence of tumor budding.

Table 3 shows the results of Chi-Squared test used to determine the existence of a difference in nodular stage and pathological stage between study groups. These two parameters and the presence of tumor budding were analyzed for associations, and significant associations were found between N1 status and stage III and the presence of tumor budding.

Survival Analysis

From the Kaplan-Meier plots, it can be concluded that the cumulative survival proportions vary between the examined parameters. The cumulative survival proportion appears to be much higher in the population without tumor budding compared to the population with tumor budding. It would appear that patients without tumor budding have better survival (Figure 2A). Secondly, the cumulative survival proportion appears to be equal in all nodular stages (Figure 2B). Also, the cumulative survival proportion appears to be much higher in the stage II compared to the stage I and stage III, which do not appear to differ considerably. It would appear that patients with the second stage of the disease have better survival (Figure 2C). A log rank test was run to determine if there were differences in the survival distribution for these three parameters. The survival distributions were not significantly different (for tumor budding: χ²(2)=1.556, p=0.212; for nodular stage: χ²(2)=1.236, p=0.539; for pathological stage: χ²(2)=5.939, p=0.051).

Discussion

The histological phenomenon of tumor budding was first described in the Japanese medical literature in 1949 [21], but revised after more than 2 decades among patients with colorectal adenocarcinoma. It is still not the part of the routine medical access and does not have a definite role in evaluating the prognosis of patients with different types of carcinoma, because no consensus for the finest and most precise definition of tumor budding
and the unique methodology for scoring has been formed [22]. The desire to conduct this retrospective study was based on findings of multivariate analysis studies that show the stronger relationship between tumor budding and poor overall prognosis, unlike the singly used TNM classification [18]. In spite of these results, budding has still not been fully accepted as a factor that correlates directly with the biological behavior of the tumor.

Various ways can be used to define a histological structure as a tumor bud and to exclude bud-looking structures that are not true buds. Ueno and colleagues defined buds as isolated malignant cells or ≤4 clustered malignant cells in the stroma at the invasive front of the tumor [16]. Some authors slightly changed this definition and increased the cutoff value to foci of ≤5 clustered malignant cells [23, 24], thus they set the value many other authors tend to favor [25-27]. Along with the 2002 original publication of Ueno and colleagues that was widely used in literature, there are 4 most cited methods for tumor budding assessment: Hase et al (1993) [28], Nakamura et al (2005) [29], conventional and rapid Wang (2009) [30].

Total of 114 patients in this study were divided in two groups based on budding-positive or budding-negative findings. Result of a dominant male distribution between study groups may be related to the conventional fact of men being more frequently diagnosed with lung carcinoma than women. However, the study from 2015 indicated to a relationship between females and low-grade budding in lung adenocarcinoma [31]. In the present study, no association between gender and the presence of tumor budding has been confirmed. The median of age in our study was 63, with range from 46 to 78, which is consistent with the observations of 2016 study, where the median was 66 (66 ± 9.9) [32], but the consistency between age and tumor budding has not been found.

Our attention was also dedicated to histological subtype analysis, and it was proved that the acinar subtype was dominant in male patients in both study groups, while the acinar and solid subtype were equally found in female patients, which makes these results corresponding to reports of Kadota K. et al study [31]. Tumor budding was not found in lepidic subtype of lung adenocarcinoma [33]. These results suggest that the biological mechanism by which tumor budding is induced may vary with histological subtype.

Mean tumor size in the budding-positive group of patients was 4cm (4.4 ± 2.34), as well as in the budding-negative group (4.2 ± 2.18), so the statistical significance was not
confirmed. Yamaguchi Y. et al reported the findings of tumor budding in cases with adenocarcinoma bigger than 3cm [33].

One of the most significant parameters that tumor budding is connected with is nodal status. The current result revealed that N1 status was significantly associated with the presence of tumor budding. In contrast, the absence of lymphatic invasion resulted in other studies conducted on N0 status, opposed to our study [31]. Also, we analyzed the presence of tumor budding and pathological stage for associations, and significant associations were found between stage III and tumor budding. Comparing our results to other studies' results is difficult due to different stage analysis in other studies (mostly stage I) [33]. The reason why tumor budding is significantly associated with parameters that lead to a poor prognosis is not clarified. One of the satisfactory explanations is that budding cells phenotype represents a component of distant tumor invasion [22]. Taken together may explain the more aggressive behavior of the tumors that show this feature.

The overall number of studies demonstrating the presence and intensity of tumor budding in primary lung adenocarcinoma is rather small, especially because the use of corresponding immunohistochemistry methods is often required. The biggest obstacle for considering tumor budding as an integrated category in pathology reports is not having enough well-defined criteria for its evaluation. In addition, it has been pointed out in various types of carcinoma. In this manner, budding aspect as a prognostic factor has been attracting interest [34-39]. Furthermore, it is believed that budding represents a histological basis for tumor cells to detach and invade locally and sistematically [22]. In keeping with the data reported previously, budding has been strongly linked to adverse clinicopathological features, poor overall prognosis and disease-free survival.

**Conclusion**

With statistical significance confirmed between higher nodal status, higher pathological stage and tumor budding found in our study, this histological phenomenon is still relatively new for diagnostics domain of pathology, but receiving increasing attention as an adverse prognostic factor. It is imperative to add more clinicopathological features used to assess the risk of overall prognosis and to facilitate optimal clinical menagement through planning the treatment prior to surgery. These results may help tumor budding to incorporate into existing staging systems as it is associated with other factors that are known to portend
worse outcome, such as infiltrating tumor border, schirrhous stromal type, lymphatic, vascular, perineural and pleural invasion, nodal and distant metastases.

It is widely noted that additional studies will be needed to further define the methodology and uniform reporting of tumor budding through the most reproducible scoring method. The significance of tumor budding will need to be further evaluated in a multidisciplinary setting, until further data become available.

Acknowledgments

This study was financed by the Serbian Ministry of Science and Technological Development (Project Grant No. 175006).

Konačno sredena literatura EUR 21845


Tables and figures

Table 1. Clinicopathological characteristics of patients with tumor budding

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>All cases (%)</th>
<th>Budding (+) (%)</th>
<th>Budding (-) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of patients</td>
<td>114</td>
<td>61 (53.5)</td>
<td>53 (46.5)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>64</td>
<td>34 (53.1)</td>
<td>30 (46.9)</td>
</tr>
<tr>
<td>Female</td>
<td>50</td>
<td>27 (54)</td>
<td>23 (46)</td>
</tr>
<tr>
<td>Histological subtypes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acinar</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Solid</td>
<td>61</td>
<td>35 (57.3)</td>
<td>26 (42.7)</td>
</tr>
<tr>
<td>Papillary</td>
<td>42</td>
<td>22 (52.3)</td>
<td>20 (47.7)</td>
</tr>
<tr>
<td>Mucinous</td>
<td>5</td>
<td>3 (60)</td>
<td>2 (40)</td>
</tr>
<tr>
<td>Lepidic</td>
<td>4</td>
<td>1 (25)</td>
<td>3 (75)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0 (0)</td>
<td>2 (100)</td>
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</table>
Table 2. Distribution of patients by age and by tumor size in study groups

<table>
<thead>
<tr>
<th></th>
<th>Budding (+)</th>
<th>Budding (-)</th>
<th>Student’s t- test</th>
<th>p value</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>64</td>
<td>64.3 ± 6.59</td>
<td>63.3 ± 6.02</td>
<td>0.627</td>
<td>0.533</td>
</tr>
<tr>
<td>Female</td>
<td>50</td>
<td>63.1 ± 6.53</td>
<td>63.2 ± 6.72</td>
<td>-0.053</td>
<td>0.958</td>
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<tr>
<td><strong>Tumor size</strong></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>64</td>
<td>4.12 ± 1.93</td>
<td>4.78 ± 2.50</td>
<td>-1.227</td>
<td>0.224</td>
</tr>
<tr>
<td>Female</td>
<td>50</td>
<td>4.79 ± 2.77</td>
<td>3.33 ± 1.32</td>
<td>2.304</td>
<td>0.026</td>
</tr>
</tbody>
</table>

Ns – not significant; S- significant.

Table 3. Association between nodal status and pathological stage and the presence of tumor budding

<table>
<thead>
<tr>
<th></th>
<th>Budding (+)</th>
<th>Budding (-)</th>
<th>χ² test</th>
<th>p value</th>
<th>Statistical significance</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nodular stage</strong></td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td>N0</td>
<td>78</td>
<td>35 (44.9)</td>
<td>43 (55.1)</td>
<td>7.407</td>
<td>0.08</td>
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<tr>
<td>N1</td>
<td>20</td>
<td>17 (85)</td>
<td>3 (15)</td>
<td>9.669</td>
<td>0.02</td>
</tr>
<tr>
<td>N2</td>
<td>16</td>
<td>9 (56.2)</td>
<td>7 (43.8)</td>
<td>0.056</td>
<td>1</td>
</tr>
<tr>
<td><strong>Pathological stage</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>50</td>
<td>23</td>
<td>27</td>
<td>2.019</td>
<td>0.109</td>
</tr>
<tr>
<td>II</td>
<td>31</td>
<td>15</td>
<td>16</td>
<td>0.449</td>
<td>0.323</td>
</tr>
<tr>
<td>III</td>
<td>33</td>
<td>23</td>
<td>10</td>
<td>4.893</td>
<td>0.022</td>
</tr>
</tbody>
</table>

Ns – not significant; S- significant.
Figure 1. Tumor budding in lung adenocarcinoma, A, B. H&E, x20; C, D. H&E, x40.
**Figure 2A** - Cumulative overall survival curves stratified by the presence or absence of tumor budding.

![Survival Functions](image)

**Figure 2B** - Cumulative overall survival curves stratified by the nodular stage N0, N1 and N2.

![Survival Functions](image)

**Figure 2C** - Cumulative overall survival curves stratified by the pathological stage I, II and III.
Received on May 22, 2019.
Accepted July 18, 2019.
Online First September, 2019.