Predictive value of morphological features and proliferative markers (mitotic index, Ki-67, PHH3) in bronchopulmonary neuroendocrine tumors

L. M. Zakhartseva [ID][1,2,A,C,E]**, H. Ye. Chytaieva** [ID]*[2,A,B,C,D,F]

[1]Kyiv City Clinical Oncological Center, Ukraine, [2]Bogomolets National Medical University, Kyiv, Ukraine

A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article;
E – critical revision of the article; F – final approval of the article

The aim of this study was to evaluate relations of mitotic index (MI), necrosis, IHC proliferative markers Ki-67 and PHH3, and their predictive value for lung neuroendocrine tumors (NETs) aggressiveness.

Materials and methods. The study used surgical and biopsy material from 64 patients with lung NETs before chemotherapy prescribing. Morphological study and IHC was performed. MI, necrosis, Ki-67 and PHH3 expression and metastatic disease and survival were estimated using nonparametric statistics.

Results. Statistically significant association of necrosis severity and survival rates was found (P = 0.021). This was true for comparing patients with no necrosis in tumor tissue and extensive foci of necrosis (P = 0.023). MI appeared to be associated with metastases in lymph nodes (P = 0.003) and with distant metastatic lesions (P = 0.029). Significant, direct association of Ki-67 and MI (P < 0.001), MI and PHH3 expression (P < 0.001) was found. However, there was no significant link between Ki-67 and PHH3 rates (P = 0.240). Ki-67 didn't show any significant association with necrosis and metastases. Also, Ki-67 rates didn't affect the patient survival. Data on PHH3 expression and their estimation appeared to be rather contradictory. PHH3 expression rates were lower than expected and did not exceed neither Ki-67 rates, nor MI.

Conclusions. MI and necrosis are reliable markers for the assessment of lung NETs aggressiveness. MI is statistically associated with metastatic lesion, while extensive necrosis – with survival rates. Ki-67 expression was significantly associated with MI. No significant association of Ki-67 and PHH3 expression, tumor's morphological features, disease progression and prognosis was found. Contrary to our expectations, PHH3 showed no diagnostic and prognostic value in lung NETs.

Прогностична цінність морфологічних особливостей та проліферативних маркерів (мітотичного індексу, Ki-67, PHH3) у нейроендокринних пухлинах бронхолегеневої локалізації

Л. М. Захарцева, Г. Є. Читаєва

Мета роботи – оцінювання зв'язку мітотичного індексу (МІ), некрозу, імуногістохімічних (ІГХ) маркерів проліферації Ki-67, PHH3 та їхньої цінності як предикторів агресивності нейроендокринних пухлин (НЕП) легень.

Матеріали та методи. Дослідження здійснили, використавши операційний і біопсійний матеріал, що отримали від 64 пацієнтів із НЕП легень до призначення їм хіміотерапії. Виконали морфологічне та ІГХ дослідження. МІ, вираженість некрозу, експресію Ki-67 і PHH3, метастатичні ураження та виживаність хворих оцінювали за допомогою методів непараметричної статистики.

Результати. Виявили статистично вірогідний зв'язок між вираженістю некрозу та виживаністю пацієнтів (р = 0,021). Це стосувалося порівняння груп хворих без вогнищ некрозу у тканині пухлини та з екстенсивними вогнищами некрозу (р = 0,023). МІ достовірно пов'язаний із наявністю метастазів у лімфатичних вузлах (р = 0,003) і віддалених метастазів (р = 0,029). Визначили значущий прямий зв'язок між рівнем Ki-67 і МІ (р < 0,001), МІ та експресією PHH3 (р < 0,001). Але не виявили вірогідний зв'язок між рівнями Ki-67 і PHH3 (р = 0,240), як і між Ki-67, наявністю вогнищ некрозу та метастазів. Рівень Ki-67 також не впливав на виживаність пацієнтів. Експресія PHH3 та результати її оцінювання виявилися доволі неоднозначними. Рівень експресії PHH3 нижчий, ніж очікуваний, не перевищував такий для Ki-67 і МІ.

Висновки. МІ та некроз – надійні маркери оцінювання агресивності НЕП легень. МІ статистично вірогідно пов'язаний із наявністю метастатичного ураження, а екстенсивний некроз – із показниками виживаності. Ki-67 вірогідно пов'язаний із МІ. Не виявили вірогідний зв'язок між експресією Ki-67 і PHH3, а також рівнем Ki-67 і морфологічними особливостями пухлини, прогресією захворювання та прогнозом. Усупереч нашим припущенням, PHH3 при НЕП легень не є діагностичним або прогностичним чинником.

Прогностическая ценность морфологических особенностей и пролиферативных маркеров (митотического индекса, Ki-67, PHH3) в нейроэндокринных опухолях бронхолёгочной локализации

Л. М. Захарцева, Г. Е. Читаева

Цель работы – оценка связи митотического индекса (МИ), некроза, иммуногистохимических (ИГХ) маркеров пролиферации Ki-67, PHH3 и их ценности как предикторов агрессивности нейроэндокринных опухолей (НЭО) лёгких.

Материалы и методы. Исследование провели с использованием операционного и биопсийного материала, полученного от 64 пациентов с НЭО лёгких до назначения им химиотерапии. Выполнили морфологическое и ИГХ исследование. МИ, выраженность некроза, экспрессию Ki-67 и РНН3, метастатическое поражение, выживаемость пациентов оценивали с помощью методов непараметрической статистики.

Результаты. Установлена статистически достоверная связь между выраженностью некроза и выживаемостью больных (p = 0,021). Это касалось групп пациентов без очагов некроза в ткани опухоли и с наличием экстенсивных очагов некроза (p = 0,023). МИ достоверно связан с наличием метастазов в лимфатических узлах (p = 0,003) и отдалённых метастазов (p = 0,029). Выявлена значимая прямая связь между уровнем Ki-67 и МИ (p < 0,001), МИ и экспрессией РНН3 (p < 0,001). Однако не было достоверной связи между уровнями Ki-67 и РНН3 (p = 0,240), как и между Ki-67, наличием очагов некроза и метастазов. Уровень Ki-67 также не влиял на выживаемость пациентов. Экспрессия РНН3 и результаты ее оценки оказались довольно неоднозначными. Уровень экспрессии РНН3 был ниже, чем ожидаемый, не превышал такового для Ki-67 и МИ.

Выводы. МИ и некроз – надёжные маркеры оценки агрессивности ПЭО лёгких. МИ статистически достоверно связан с наличием метастатического поражения, а экстенсивный некроз – с показателями выживаемости. Ki-67 достоверно связан с МИ. Не установлена достоверная связь между экспрессией Ki-67 и РНН3, а также уровнем Ki-67 и морфологическими особенностями опухоли, прогрессией заболевания и прогнозом. Вопреки нашим предположениям, РНН3 при НЭО лёгких не является диагностическим и прогностическим критерием.

Neuroendocrine tumors (NETs) are regarded as rare cancers with a reported incidence of 5–7 per 100.000 population per year and estimated prevalence of 35 per 100.000 [1]. Bronchopulmonary NETs account for 12 % of all NETs and, according to recent studies, make about 20 % of all primary lung neoplasms [2–6]. The annual incidence of lung NETs has substantially increased (in average by 4.5 times) over the past 30 years, mainly due to improvements in detection methods and diagnostic protocols [7].

Lung NETs share neuroendocrine differentiation, but they have heterogeneous morphological, immuno-histochemical (ICH) and molecular characteristics and considerably different clinical and biological behavior [8,9].

Bronchopulmonary NETs encompass four histologic subtypes: TC, AC, LCNEC, and SCLC, with large prevalence of high-grade tumors over carcinoids [4,5,10,11]. TC is considered a low-grade malignant tumor with longer life expectation and time to recurrence, although it also can metastasize. AC is deemed to be an intermediate malignant tumor with more aggressive clinical course, and somewhat unpredictable clinical behavior. LCNEC and SCLC are high-grade malignancies with dismal prognosis [8].

Current classification of bronchopulmonary NETs has its own lights and shadows [12]. The differential diagnosis for subtypes of lung NETs is based on two parameters only: the presence/absence of necrosis and the mitotic index per 2 mm^2. A diagnosis of TC is made when the tumor does not show necrosis and the mitotic count is <2 mitosis per 2 mm^2; the AC shows up with necrosis and/or a number of mitosis between 2 and 10 per 2 mm^2, while high-grade poorly differentiated carcinomas (LCNEC and SCLC) have >10 mitosis per 2 mm^2 and usually present extensive necrotic areas. Focal necrosis in total doesn't make up 20 % of the sample, while extensive necrosis exceeds 50 % of the area [11].

Tumor necrosis is recognized to be a consequence of chronic cellular hypoxia. And both these factors (hypoxia and necrosis) correlate with poor prognosis in some tumors. The extensive necrosis reflects an aggressive tumor phenotype, however, it's prognostic value in most cases remains unknown [13]. LCNEC and SCLC, commonly have histopathological features of tumor necrosis. And according to recent studies, high proportion of tumor necrosis (≥10 %) has a negative prognostic value in high grade lung NETs [14].

Also, a wide constellation of cellular and architectural features is assessed, and ICH markers are applied. But mentioned morphological features and markers' expression are not monitored constantly, especially in small and crushed biopsy specimens [11,15].

WHO's classification for NETs of the digestive system was updated in 2019, while for the bronchopulmonary NETs, due to many inconsistencies and disagreements among researchers, the classification 2015 is still in use. Imperfect diagnostics may lead to a late diagnosis and a disappointing prognosis. Moreover, the majority of lung NETs (over 95 %), are non-functional and consequently the presentation may be incidental or related to metastatic disease [1,2]. More than 20 % of lung NETs present as metastasis, typically to liver and/or bone. The median survival of stage IV bronchopulmonary tumors is about 17 months and less [3,16].

In this situation, it is very important to find reliable predictive factors for lung NETs behavior assessment. And many of them are seemed to be associated with tumor cell proliferation [17,18].

This indicator is very important tool for evaluating the biological characteristics of a malignancy. If the proliferative index is high, it means that the tumor is faster growing or rather aggressive. But despite exhausting amount of literature, the translation of proliferation assessment into daily routine has largely failed [19]. Cell proliferation kinetics depends on the type/subtype of the malignancy, the cell cycle duration, the proportion of proliferating cells, etc. [20]. Using different methods for proliferation assessment also contributes to this confusion. However, researchers do not abandon attempts to estimate the proliferative potential of malignant cells, and in some cases they manage to reveal the significant predictive value of the mitotic index (MI), and ICH proliferation markers (e.g. Ki-67, PHH3, etc.) [21–24].

MI is considered as one of the essential factors for determining the histological grade in different cancers [25,26]. But it also has certain limitations that are due to the heterogeneity of the tumor, used calculation method. Also, the apoptotic bodies or crushed cells may mimic

K-mitoses that falsify counting results [27,28]. Moreover, mitosis counting is rather subjective and time-consuming [29].

Ki-67 may be a potent tool for easy and quick evaluation of the proportion of proliferating cells in a tumor [16]. Ki-67 (proliferation index) is widely used in routine pathological investigation. It is considered "golden standard" in IHC and employed in assessing tumor aggressiveness [30–32]. Although, Ki-67 has been extensively investigated in different cancers, in fact it has not gained widespread clinical acceptance. There is an opinion that no diagnostic role is currently supported in lung NETs for Ki-67 labeling index because of the significant overlap between tumor categories [32,33].

The rate of cellular proliferation counted using MI on H&E slides or proliferative index Ki-67 in ICH is a reflection of tumor cell division. But these parameters measure different phases of the cell cycle, and there is lack of standardized methodology and interobserver variation. Despite all these drawbacks, multiple studies have shown a good correlation between Ki-67 and MI in many neoplasms [34,35]. Moreover, due to some studies, MI can be helpful in prognosis predicting, for instance, in bronchopulmonary NETs is associated with metastatic lesion [21,22,25].

Phosphohistone H3 (PHH3) is relatively new ICH marker which is applied for the objective detection of mitotic activity in different tumors. It allows to identify mitoses, easily-missed by ordinary mitotic count, and is considered more reliable and reproducible than Ki-67 [36–38]. It can help in accurate assessment of tumor cells' proliferation with a perfect agreement among observers, and also it is less time consuming than that on H&E [23,24,39].

IHC findings for the PHH3 have been shown to be reliable mitosis-specific marker in different cancers [40,41]. Some studies emphasize that PHH3 can be used for prediction of metastatic lesions [23] and survival [41].

It is believed that the tumor's histological grade could be assessed more accurately using PHH3, but it would result in increasing proportion of high-grade cancers [41].

But in different tumors, the diagnostic and prognostic value of the MI and proliferative markers Ki-67 and PHH3 varies significantly [38]. And data of PHH3 diagnostic and prognostic value in lung NETs are vastly limited [37].

The lack of strong diagnostic and prognostic criteria significantly complicate the assessment of lung NETs clinical behavior and prognosis. Thus, simple and accurate diagnostics and assessment of proliferation markers predictive value seems to be of essential importance. Also, some morphological features might assist the prediction of prognosis [6].

Aim

Taking into account the inconsistency of the literature data, in the current study we aimed to check if there is a significant correlation between tumor cells division, measured by MI and other proliferation markers (Ki-67 and PHH3), and their impact on the lung NETs aggressiveness. And additionally – whether necrosis used in staging lung NETs has a prognostic value.

Materials and methods

Retrospective and prospective study was conducted. 64 FFPE blocks with lung NETs (resections and biopsies) were chosen randomly from 113 unique patients, treated in Kyiv City Clinical Oncological Center in 2010–2020. Morphological diagnosis was established (including neuroendocrine morphology, grade, TNM, and stage); all cases were classified based on criteria used by 2015 WHO modified classification for lung NETs. Necrosis was detected on H&E slides. TC has no necrosis; in AC small foci of necrosis could be revealed; high-grade NETs usually performed lots of small necroses or extensive necrotic fields, often with hemorrhages. Mitotic count was performed on H&E stains, and MI was considered the average mark per 10 HPFs. Also, ICH was performed (ChrA, Syn, TTF-1, CK7, CK20, CD56, Ki-67 and PHH3) before chemotherapy was prescribed. In current study we mainly focused on necrosis and MI, Ki-67 and PHH3 expression.

All morphological and IHC data were assessed by two different independent pathologists without the knowledge of patient data. Medical records data were used to assess clinical findings and patient survival.

The study was agreed with the commission on bioethical examination of Bogomolets National Medical University (protocol No. 118, 18 Jan 2019).

To determine ICH markers' expression we used anti-Ki-67 monoclonal antibody, clone MIB-1 (Dako, USA), and rabbit polyclonal PHH3 (Diagnostic Biosystems, USA).

Only nuclear ICH staining for Ki-67 and PHH3 was considered. For each sample, 5 microscopic fields at ×200 magnification were selected, and 100 tumor cells in each field were counted to assess the percentage of positive cells.

Samples with Ki-67 ≤3 % was considered grade 1 (TC); Ki-67 4–19 % corresponded grade 2 (AC); Ki-67 ≥20 % was estimated as grade 3 (LCNEC and SCLC).

PHH3 expression was estimated on the same FFPE blocks and compared with MI, Ki-67 level, necrosis extent and severity and metastases presence. Also, there was an attempt to evaluate patient survival depending on MI, Ki-67 and PHH3 rates.

Microsoft Excel was used for all calculations. Statistical analysis was performed using IBM SPSS software Statistics 28 (license No. Z125-3301-14).

MI, Ki-67 and PHH3 distribution was investigated using nonparametric tests (Kruskal–Wallis test, Mann–Whitney test, Spearman's rank correlation). 95 % CI was calculated using adjusted Wald method. Kaplan–Meier estimator and log-rank test were used for survival analysis, survival median, 25th and 75th percentiles and one-year survival rate were calculated.

Results

The patients age ranged from 29 to 76 years, the male (M) / female (F) ratio was 3.92 vs 1.00. There were 7 (10.94 %) patients (5 M/2 F) aged 18–44 years; 19 (29.69 %) (13 M/6 F) aged 45–59 years; and 37 (57.81 %) (32 M/5 F) patients aged 60–74 years; there was 1 man in a group ≥75 years old.

The sample was censored. The follow-up period varied significantly – from 11 days to 7.11 years, the average observation period was 8.9 months. The outcome could not be traced in 15 (23.00 %) cases. 17 (26.56 %) patients died, their life expectancy after diagnosis averaged 12.2 months. Due to medical records, at the end of observation period 32 (50 %) were alive; in this case the follow-up period also varied significantly and averaged 7.1 months.

At the time of diagnosis metastases in lymph nodes were found in 48 (75.00 %) patients, in 11 (22.92 %) of them lymphoma was the preliminary clinical diagnosis. 26 (40.63 %) patients had distant metastases. In 11 (17.19 %) cases local or distant metastatic lesions were not identified clearly. *Fig. 1* shows the distant metastatic lesions in details.

Surgery specimen were available in 23 (35.94 %) cases, other 41 (64.06 %) were biopsies and 21 (51.22 %) of them were rather small, with extensive crush artifact.

All the cases were showing features of neuroendocrine architecture: "nests", "rosettes" and trabeculae and were positive for one or more neuroendocrine markers, other than NSE. Based upon diagnostic criteria, of the 64 selected cases 1 (1.56 %) was estimated as TC, 8 (12.5 %) were AC, 19 (29.69 %) were LCNEC, and 36 (56.25 %) SCLC.

Data on morphological features, MI, Ki-67 and PHH3 expression are given in *Table 1* and *Fig. 2A–D*. G2 and G3 tumors are mentioned since in the current study there was just one case of G1 tumor (T2N0M0, stage IB, lung NET of the lower lobe on the right). This tumor did not perform necrosis, MI was 0.4 (4 mitoses in 10 HPFs), Ki-67 was 2 % and PHH3 expression was estimated 2 %.

G2 NETs were diagnosed in 22 (34.38 %) cases, G3 – in 41 (64.06 %). In G2 tumors there were 7.86 mitoses per 10 HPFs (in average), in G3 – 22.12. The average Ki-67 was 12.09 % in AC NETs and 50.24 % – in high-grade tumors; PHH3 averaged 10.36 % and 12.34 %, respectively. Lymph node metastases at the moment of diagnosis were found in 68.18 % patients with G2 lung NETs and in 87.80 % – with G3 neoplasms; distant metastatic lesions were present in 31.82 % and 46.34 % cases. 31.82 % G2 NETs had no necrosis, 36.36 % performed small necrotic foci, 31.32 % – extensive necroses. Necrosis was not found in 19.51 % of G3 NETs; small foci were present in 29.27 % tumors, and 51.22 % of them extensive necrotic areas were revealed.

MI varied significantly and ranged from 1 to 81 in G2 tumors and from 1 to 114 in G3 NETs. At the same time, the averaged MI in AC appeared to be much lower than in high-grade neuroendocrine carcinomas (7.86 vs 22.12, respectively). However, in some cases it was rather difficult to distinguish K-mitoses and apoptotic bodies from each other, which caused confusion in MI assessment.

Ki-67 level ranged within 2–100 %. Ki-67 expression ≤3 % was observed just in 1 (2 %) tumor sample (T2N0M0); Ki-67 4–19 % – in 22 (34.38 %); Ki-67 ≥20 % – in 41 (64.06 %) cases. Necrosis was found in 75.00 % of tumor samples, and in 43.75 % it was extensive. According to Mann–Whitney test, the difference between that groups was significant, P = 0.02. So, Ki-67 may reflect the tumor's grade, but it is not enough for distinguishing the subclasses of lung NETs in a significant manner.

Modified classification for lung NETs diagnostics includes necrosis characteristics and Ki-67 rates. As a rule, in G2 tumors several small foci of necrosis are found, but in G3 malignancies necrotic areas can be extended and accompanied by massive hemorrhages. However, in current study Ki-67 expression did not differ for groups with different necrosis severity (Kruskal–Wallis test, P = 0.200).

Data on PHH3 expression and its estimation appeared to be rather contradictory. PHH3 expression was lower than expected and did not exceed neither Ki-67 rates, no MI.

Statistically significant, direct association of Ki-67 and MI (Spearman rank correlation ρ = 0.441, P < 0.001) also MI and PHH3 expression (ρ = 0.620, P < 0.001) was found (*Fig. 3*). However, there was no statistically significant link between Ki-67 and PHH3 rates (P = 0.217).

There was no significant association of MI and necrosis (Mann–Whitney test, P = 0.504) and its severity (Kruskal–Wallis test, P = 0.226). But MI appeared to be associated with metastases in lymph nodes (Mann–Whitney test, P = 0.003) and with distant metastatic lesions (Mann–Whitney test, P = 0.029) (*Fig. 4*). At 0–6 mitoses per 10 HPFs the probability of metastases in lymph nodes amounted 62 % (95 % CI: 42–78 %); at ≥7 mitoses per 10 HPFs – 97 % (95 % CI: 83–99 %); at ≥15 mitoses per 10 HPFs it reached 100 % (95 % CI: 87–100 %). The probability of distant metastatic lesion was 32 % at ≤10 mitoses per 10 HPFs (95 % CI: 54–83 %); at ≥10 mitoses per 10 HPFs – 59 % respectively (95 % CI: 41–75 %); and at ≥40 mitoses per 10 HPFs it reached 86 % (95 % CI: 47–99 %) (*Fig. 5*). However, MI did not affect the survival rates (P = 0.062).

Ki-67 didn't show any significant association with necrosis (Kruskal–Wallis test, P = 0.200) and metastatic lesion (Mann–Whitney test, P = 0.065 for local and P = 0.135 – for distant metastases). Also, Ki-67 level didn't affect the patient survival.

And despite literature data and our expectations PHH3 also didn't show any significant association with studied morphological indicators. Neither with necrosis (P = 0.750), and its severity (P = 0.896), nor with regional and distant metastases (P = 0.283 and P = 0.700, respectively). In the current study PHH3 expression did not affect the survival rates also.

Statistically significant relationship of necrosis severity and survival rates was found (log-rank test, P = 0.021) (*Table 2*). We divided all cases into three groups depending on the necrosis severity: mark 0 – tumors without necrosis; 1 – with small foci of necrosis and 2 – with extensive necrotic foci. There were no significant differences in patient survival in groups 0 and 1 (P = 0.365) and in groups 1 and 2 (P = 0.054). But association of necrosis and survival was significant for groups 0 and 2 (for those with no necrosis in tumor tissue and extensive foci of necrosis) (P = 0.023).

Median survival for group 0 was not determined (since more than half of the patients were censored). In group 1 (mild necrosis) the median survival was significantly longer (85 months) than in group 2 (extensive necrosis) (9 months). As group 0 was censored, the 75th percentile was used to compare the survival time (i.e. the time that 75 % of patients will survive). For groups 0, 1, and 2, the 75th percentile was 14, 8, and 3 months, respectively
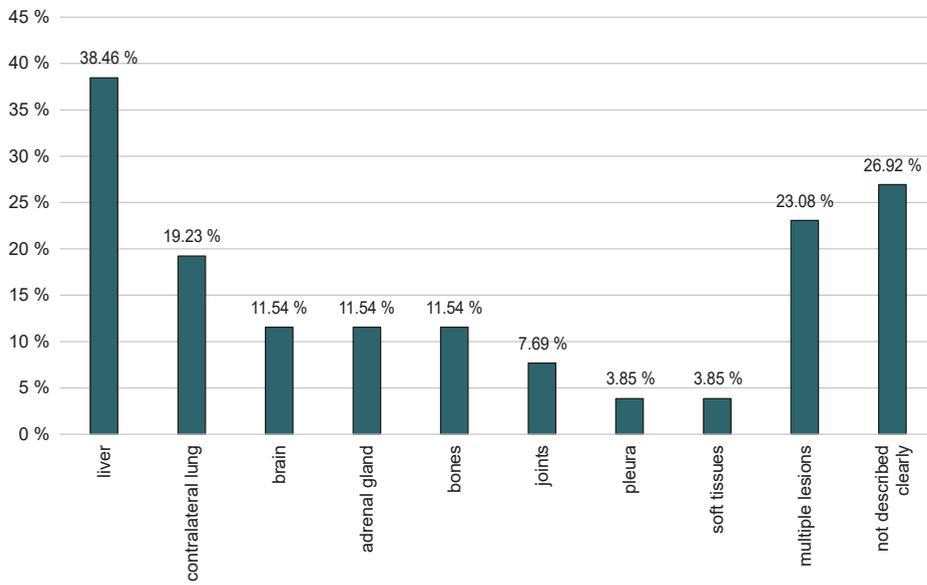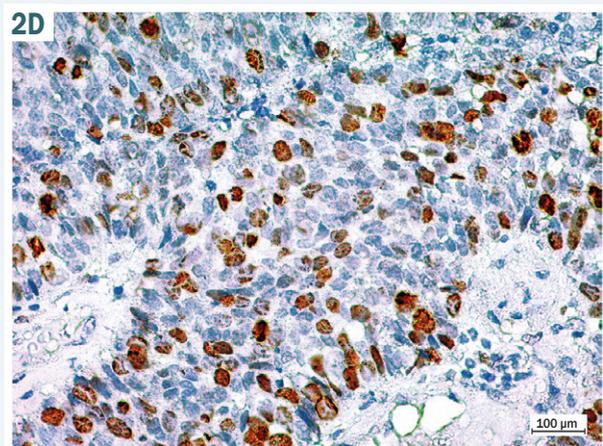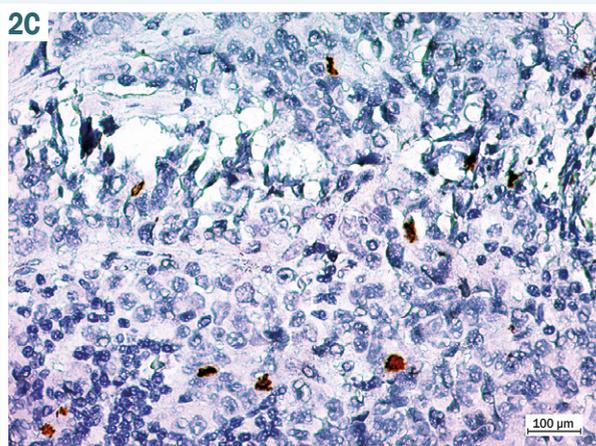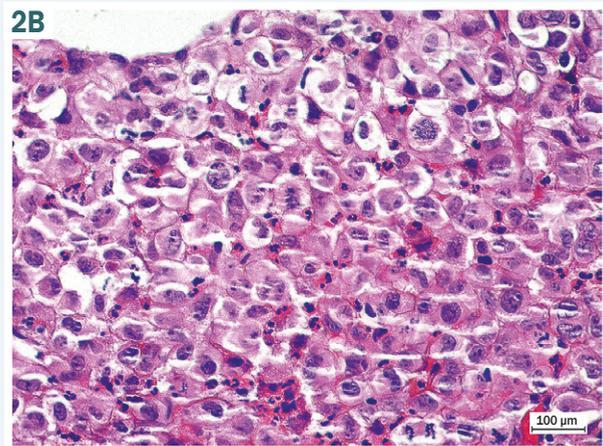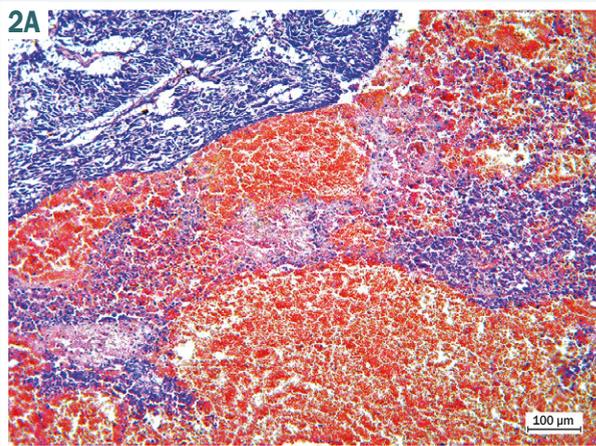
Fig. 1. Distant metastases at the moment of diagnosis of lung NETs (26 cases).

Fig. 2A. SCLC. Extended necrosis and hemorrhages in the tumor tissue. Stained H&E, ×200.

Fig. 2B. LCNEC. Karyorrhexis, K-mitoses and several scattering mitoses in the field of view. Stained H&E, ×400.
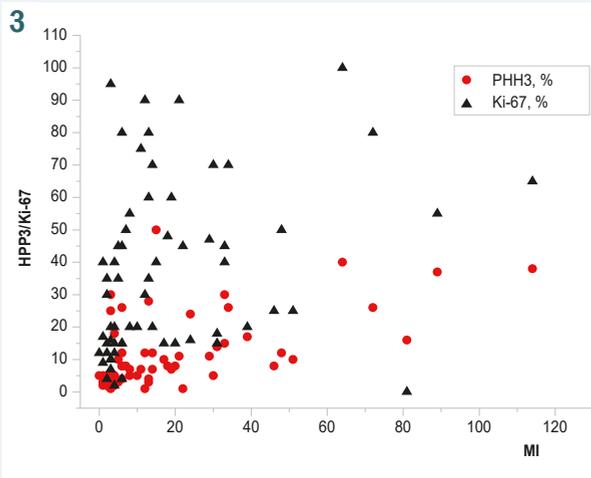
Fig. 2C. PHH3 staining in LCNEC, ×400.

Fig. 2D. Ki-67 staining in lung NET (G3), ×400.

Table 1. Descriptive statistics for MI, Ki-67 and PHH3

| Indicator	Median	25th percentile	75th percentile	Standard deviation	Range	Min	Max
Mitoses per 10 HPFs	10.5	3.25	27.75	23.4	114	0	114
Ki-67	30	15	53.75	26.4	98	2	100
PHH3	8.0	3.25	14.75	11.1	49	1	50

3

4

5

6

Fig. 3. The graph shows the links of Ki-67 and PHH3 expression (**in red**), and MI and Ki-67 (**in black color**).

Fig. 4. Box-plot association of MI and metastatic lesion (without (0) metastases and with metastases (1)) regional – on the left and distant – on the right. Bold line is the median; the bottom and the top of the box are 25th and 75th percentiles respectively; the bottom and the top of the vertical line are the minimal and the maximal values, excluding outliers and extreme values; circles – are outliers, asterisks – extreme values).

Fig. 5. The probability of regional and distant metastatic lesion at different MI rates.

Fig. 6. Survival curves for groups: **0** – no necrotic areas; **1** – small foci of necrosis; **2** – extensive necrosis.
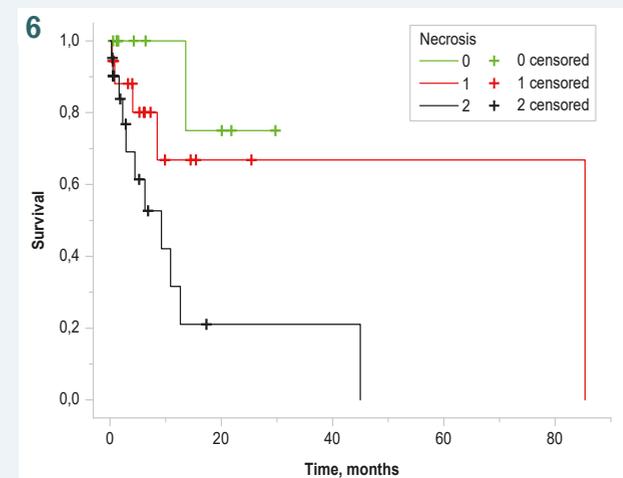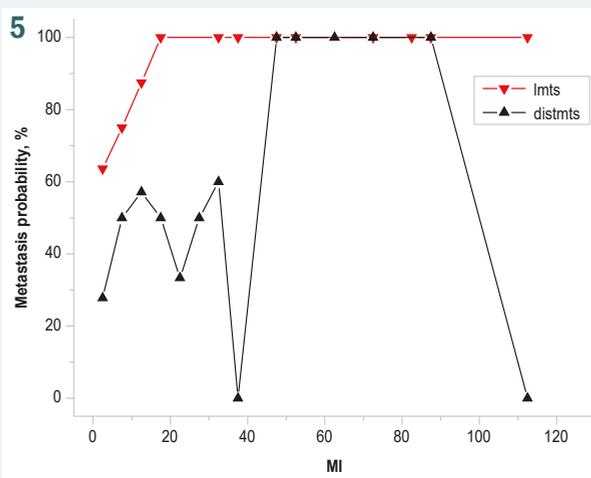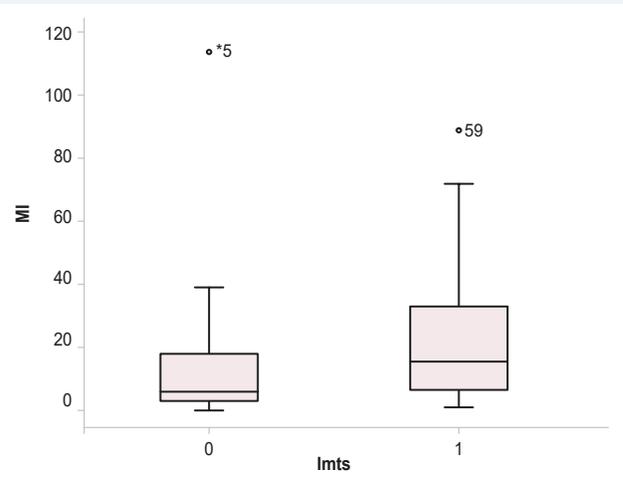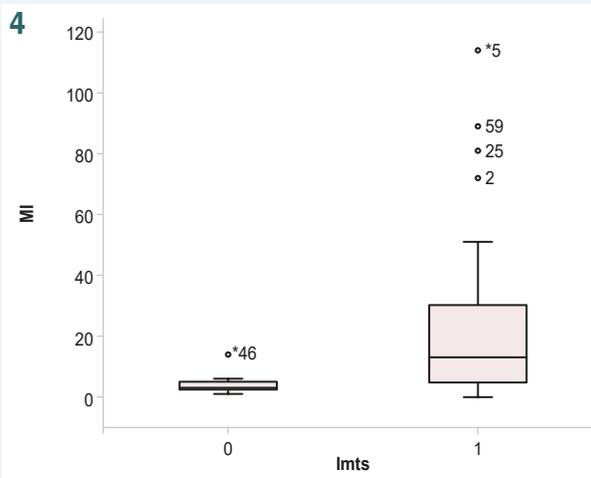
(*Fig. 6*). One-year survival rate was 100 % in group 0, 67 % – in group 1, and 32 % – in group 2, respectively.

Discussion

Data on lung NETs frequency and their subtypes proportion are contradictory: according to different authors, bronchopulmonary NETs comprise about 2–25 % of all

Table. 2. Necrosis severity and percentile for cumulative survival (months)

Necrosis	Percentile		
	25.0 %	50.0 %	75.0 %
0 (no necrotic areas)			
1 (small foci of necrosis)	85.370		82.370
2 (extensive necrosis)	12.630	13.775	9.230
Total	45.000	26.600	13.600

lung neoplasms; their subtypes ratio also varies [6,7,11]. It is believed, that about 9 out of 10 lung carcinoids are typical carcinoids, but we received the opposite data. In the current study we had 1 TC and 22 AC that amounted 4.35 % and 95.65 % of all carcinoids, respectively. In the whole sample (113 cases) there was 1 (2.86 %) TC and 34 (97.14 %) AC.

Assessment of NETs morphological features (i.e. mitotic activity, necrosis that are necessary for modern classification) is not sufficient and is usually difficult in small biopsies, which accounts about 70 % of specimens. IHC markers, widely used in oncomorphology, also give ambiguous results [12,29,32]. There are several publications in this field, and often their results contradict each other. Also, it is inappropriate to extrapolate results of studies on other tumor types to NETs with their specific morphology and behavior. Thus, an adequate, full-fledged diagnostics in bronchopulmonary NETs, using only morphological or just modern IHC markers is impossible. An integrated approach is needed.

In the current study we focused on the most significant morphological parameters and indicators of mitotic activity that could be helpful in assessment of the tumor aggressiveness and the disease prognosis.

Necrosis appeared to be rather confusing indicator. In fact, it's difficult to assess necrosis correctly in small and crashed biopsies. On the other hand, almost 32 % of AC and 20 % of high-grade lung NETs did not show necrosis. But extensive necrosis much more often was detected in G3 malignancies (51.22 % cases). Moreover, severe necrosis was associated with survival rates. For groups 0 (no necrosis), 1 (small foci of necrosis), and 2 (severe necrosis), the 75th percentile was 14, 8, and 3 months. While calculating the median, percentiles and one-year survival, we meant the cumulative survival. In fact, in the group 2 (extended necrosis), only one patient lived up to 7.08 years (85 months). In total there were 21 patients in group 2, and 11 of them died, but due to censored patients at the later stages of the follow-up, the weight of this one case became significant). One-year survival rate was 100 % in group 0, 67 % – in group 1, and almost half as much in group 2 – 32 %. So, necrosis assessment confirmed this indicator predictive value for lung NETs. But obtained results could be affected by wide use of small biopsies and chemotherapy prescribing to some patients (different protocols and number of courses).

Mitoses usually are not assessed accurately in routine practice. MI count is time consumable and requires certain experience. And the counting results are not always reliable, since visually mitoses can be easily confused with apoptotic bodies, macrophages, etc. And it is rather problematic to assess this indicator is small biopsies. In lung NETs MI ranged significantly – from 0.1 to 8.1 in AC and from 0.1 to 11.4 in G3 NETs. The largest number of mitoses (often pathological, including scattering) was detected in LCNEC; MI was rather high – up to 11.4 that is consistent with the data of other studies [9]. In the current study MI was significantly associated with Ki-67 and PHH3 rates (P < 0.001) and also with metastatic lesion (P < 0.003) but didn't affect the survival rates.

Thus, mitoses and necrosis in lung NETs should be estimated at the same time: one of these indicators is related significantly to metastases, another one – to survival rates.

As mitotic count is proportional to Ki-67, it is important to understand if Ki-67 can complement the existing diagnostic guidelines, as well as discover the benefit of these two markers to unravel the biological heterogeneity of primary thoracic NETs. Ki-67 is considered a "golden standard" in ICH and widely used in pathomorphology. The recent WHO classification mentions that Ki-67 rates might have a role in stratifying lung NETS, but it is not officially required. The role of Ki-67 in lung NETs has been widely studied with potential diagnostic, prognostic and grading implications. Since lung NETs may have similar histology but different clinical behavior. But the obtained results are very mixed. In some studies Ki-67 diagnostic role has been denied, except using it for separation of low and high-grade bronchopulmonary NETs, especially on biopsy samples with crush artifacts. Ki-67 predicting role for short-time and overall survival also is a controversial issue [8,42]. We used Ki-67 rates as an adjunct tool for tumors subtyping. Statistically significant direct association between Ki-67 expression and MI was found. Overall, Ki-67 showed better results than PHH3. But current study didn't reveal any significant association of Ki-67 and PHH3 expression, tumor's morphological features, disease progression and prognosis.

PHH3 is a mitotic-specific marker whose value has been validated in several tumor types [24]. In the current study PHH3 index was significantly associated with MI. PHH3 staining highlights mitotic cells and makes easier of rapid grading by driving pathologist's attention on the most mitotically active areas [28]. This ICH marker is easy to use, offers reduced time and improves interobserver reproducibility in mitotic rate assessment. But contrary to data of literature and our expectations PHH3 didn't show any statistically significant links with necrosis, metastasis and survival. Perhaps this is due to use of different methods for PHH3 assessing in different studies. Also, the obtained results could be affected by some limitation of the current study: rather small sample, ratio of different subtypes of lung NETs. So, we can't recommend PHH3 as an additional predictive marker for lung NETs. Some other reliable and easy diagnostic and prognostic features should be considered in lung NETs.

In our study PHH3 staining was not a strong and robust prognostic factor in lung NETs (like Ki-67).

The current study suggests that morphological features remain the main reliable diagnostic and prognostic tool in lung NETs. They are significantly associated with some ICH markers which are very easy to use, however, the results of these studies cannot be interpreted univocal. This may be due to small number of observations, significant heterogeneity of tumors and their irregular clinical behavior, as well as the lack of unified methods for assessing of IHC markers expression. Apparently, the solution to the problem lies in the use of the correct combination of morphological parameters and ICH markers, which will allow to make an accurate diagnosis and reliable prognosis.

260   ISSN 2306-8027   http://pat.zsmu.edu.ua

Патологія. Том 18, № 3(53), вересень – грудень 2021 р.

Conclusions

1. The results of our study confirm the diagnostic and prognostic value of MI and necrosis in lung NETs. Mitotic index is statistically associated with regional and distant metastatic lesion, extensive necrosis – with survival rates. Ki-67 expression was significantly associated with mitotic index.

2. There was no significant association of Ki-67 and PHH3 expression, tumor's morphological features, disease progression and prognosis. And contrary to our expectations, promising marker PHH3 had no diagnostic and prognostic value. However, difficulties arise when interpreting the results of expression of IHC markers of proliferation; theory is not always confirmed in practice. Perhaps this is due to the rarity of pathology, insufficient number of studies, and lack of a unified assessment system.

3. Results, obtained for PHH3 is difficult to compare with other studies due to use of different methods for this marker evaluating.

4. Morphological criteria alone are not enough; full diagnostics and prognosis should also consider tumors' IHC profile.

Further large-scaled prospective studies with standardized methods of PHH3 measuring are required to overcome the limitations of current study and check the utility of PHH3as a predictive marker for metastatic lesion and as a prognostic tool in bronchopulmonary NETs.

Conflicts of interest: authors have no conflict of interest to declare.
Конфлікт інтересів: відсутній.

Information about authors:
Zakhartseva L. M., MD, PhD, DSc, Professor of the Department of Pathological Anatomy, Bogomolets National Medical University; Kyiv City Clinical Oncological Center, Ukraine.
ORCID ID: 0000-0001-6838-9970
Chytaieva H. Ye., Assistant of the Department of Pathological Anatomy, Bogomolets National Medical University, Kyiv, Ukraine.
ORCID ID: 0000-0001-9171-9237

Відомості про авторів:
Захарцева Л. М., д-р мед. наук, професор каф. патологічної анатомії, Національний медичний університет імені О. О. Богомольця; Київський міський клінічний онкологічний центр, Україна.

Читаєва Г. Є., асистент каф. патологічної анатомії, Національний медичний університет імені О. О. Богомольця, м. Київ, Україна.

Сведения об авторах:
Захарцева Л. М., д-р мед. наук, профессор, каф. патологической анатомии №, Национальный медицинский университет имени А. А. Богомольца; Киевский городской клинический онкологический центр, Украина.
Читаева Г. Е., ассистент каф. патологической анатомии, Национальный медицинский университет имени А. А. Богомольца, г. Киев, Украина.

References
[1] Basuroy, R., Bouvier, C., Ramage, J. K., Sissons, M., & Srirajaskanthan, R. (2018). Delays and routes to diagnosis of neuroendocrine tumours. *BMC cancer*, 18(1), 1122. https://doi.org/10.1186/s12885-018-5057-3

[2] Oronsky, B., Ma, P. C., Morgensztern, D., & Carter, C. A. (2017). Nothing But NET: A Review of Neuroendocrine Tumors and Carcinomas. *Neoplasia*, 19(12), 991-1002. https://doi.org/10.1016/j.neo.2017.09.002

[3] Riihimäki, M., Hemminki, A., Sundquist, K., Sundquist, J., & Hemminki, K. (2016). The epidemiology of metastases in neuroendocrine tumors. *International journal of cancer*, 139(12), 2679-2686. https://doi.org/10.1002/ijc.30400

[4] Hung Y. P. (2019). Neuroendocrine Tumors of the Lung: Updates and Diagnostic Pitfalls. *Surgical pathology clinics*, 12(4), 1055-1071. https://doi.org/10.1016/j.path.2019.08.012

[5] Melosky B. (2017). Low Grade Neuroendocrine Tumors of the Lung. *Frontiers in oncology*, 7, 119. https://doi.org/10.3389/fonc.2017.00119

[6] Ichiki, Y., Matsumiya, H., Mori, M., Kanayama, M., Nabe, Y., Taira, A., Shinohara, S., Kuwata, T., Takenaka, M., Hirai, A., Imanishi, N., Yoneda, K., Noguchi, H., Shimajiri, S., Fujino, Y., Nakayama, T., & Tanaka, F. (2018). Predictive factors of postoperative survival among patients with pulmonary neuroendocrine tumor. *Journal of thoracic disease*, 10(12), 6912-6920. https://doi.org/10.21037/jtd.2018.11.115

[7] Hendifar, A. E., Marchevsky, A. M., & Tuli, R. (2017). Neuroendocrine Tumors of the Lung: Current Challenges and Advances in the Diagnosis and Management of Well-Differentiated Disease. *Journal of thoracic oncology*, 12(3), 425-436. https://doi.org/10.1016/j.jtho.2016.11.2222

[8] Righi, L., Gatti, G., Volante, M., & Papotti, M. (2017). Lung neuroendocrine tumors: pathological characteristics. *Journal of thoracic disease*, 9(Suppl 15), S1442-S1447. https://doi.org/10.21037/jtd.2017.01.59

[9] Derks, J. L., Speel, E. J., Thunnissen, E., van Suylen, R. J., Buikhuisen, W. A., van Velthuysen, M. L., & Dingemans, A. M. (2016). Neuroendocrine Cancer of the Lung: A Diagnostic Puzzle. *Journal of thoracic oncology*, 11(3), e35-e38. https://doi.org/10.1016/j.jtho.2015.10.013

[10] Hilal T. (2017). Current understanding and approach to well differentiated lung neuroendocrine tumors: an update on classification and management. *Therapeutic advances in medical oncology*, 9(3), 189-199. https://doi.org/10.1177/1758834016678149

[11] Filosso, P. L., Falcoz, P. E., Solidoro, P., Pellicano, D., Passani, S., Guerrera, F., Ruffini, E., & ESTS Lung Neuroendocrine Working-Group Participating Centers* (2018). The European Society of Thoracic Surgeons (ESTS) lung neuroendocrine tumors (NETs) database. *Journal of thoracic disease*, 10(Suppl 29), S3528-S3532. https://doi.org/10.21037/jtd.2018.04.104

[12] La Rosa, S., & Uccella, S. (2021). Classification of neuroendocrine neoplasms: lights and shadows. *Reviews in endocrine & metabolic disorders*, 22(3), 527-538. https://doi.org/10.1007/s11154-020-09612-2

[13] Swinson, D. E., Jones, J. L., Richardson, D., Cox, G., Edwards, J. G., & O'Byrne, K. J. (2002). Tumour necrosis is an independent prognostic marker in non-small cell lung cancer: correlation with biological variables. *Lung cancer*, 37(3), 235-240. https://doi.org/10.1016/s0169-5002(02)00172-1

[14] Sugimoto, A., Umemura, S., Miyoshi, T., Nakai, T., Kuroe, T., Nosaki, K., Ikeda, T., Udagawa, H., Kirita, K., Zenke, Y., Matsumoto, S., Yoh, K., Niho, S., Tsuboi, M., Goto, K., & Ishii, G. (2021). High proportion of tumor necrosis predicts poor survival in surgically resected high-grade neuroendocrine carcinoma of the lung. *Lung cancer*, 157, 1-8. https://doi.org/10.1016/j.lungcan.2021.05.018

[15] Rindi, G., & Inzani, F. (2020). Neuroendocrine neoplasm update: toward universal nomenclature. *Endocrine-related cancer*, 27(6), R211-R218. https://doi.org/10.1530/ERC-20-0036

[16] Bellizzi A. M. (2020). Immunohistochemistry in the diagnosis and classification of neuroendocrine neoplasms: what can brown do for you?. *Human pathology*, 96, 8-33. https://doi.org/10.1016/j.humpath.2019.12.002

[17] Pelosi, G., Sonzogni, A., Harari, S., Albini, A., Bresaola, E., Marchiò, C., Massa, F., Righi, L., Gatti, G., Papanikolaou, N., Vijayvergia, N., Calabrese, F., & Papotti, M. (2017). Classification of pulmonary neuroendocrine tumors: new insights. *Translational lung cancer research*, *6*(5), 513-529. https://doi.org/10.21037/tlcr.2017.09.04

[18] Raphael, M. J., Chan, D. L., Law, C., & Singh, S. (2017). Principles of diagnosis and management of neuroendocrine tumours. *CMAJ*, *189*(10), E398-E404. https://doi.org/10.1503/cmaj.160771

[19] Chirieac L. R. (2016). Tumor cell proliferation, proliferative index and mitotic count in lung cancer. *Translational lung cancer research*, *5*(5), 554-556. https://doi.org/10.21037/tlcr.2016.10.10

[20] Rekhtman, N., Desmeules, P., Litvak, A. M., Pietanza, M. C., Santos-Zabala, M. L., Ni, A., Montecalvo, J., Chang, J. C., Beras, A., Preeshagul, I. R., Sabari, J. K., Rudin, C. M., Ladanyi, M., Klimstra, D. S., Travis, W. D., & Lai, W. C. (2019). Stage IV lung carcinoids: spectrum and evolution of proliferation rate, focusing on variants with elevated proliferation indices. *Modern pathology*, *32*(8), 1106-1122. https://doi.org/10.1038/s41379-019-0248-2

[21] Joseph, M. G., Shibani, A., Panjwani, N., Arab, A., Shepherd, J., Stitt, L. W., & Inculet, R. (2015). Usefulness of Ki-67, Mitoses, and Tumor Size for Predicting Metastasis in Carcinoid Tumors of the Lung: A Study of 48 Cases at a Tertiary Care Centre in Canada. *Lung cancer international*, *2015*, 545601. https://doi.org/10.1155/2015/545601

[22] Ha, S. Y., Choi, M., Lee, T., & Park, C. K. (2016). The Prognostic Role of Mitotic Index in Hepatocellular Carcinoma Patients after Curative Hepatectomy. *Cancer research and treatment*, *48*(1), 180-189. https://doi.org/10.4143/crt.2014.321

[23] Ozturk Sari, S., Taskin, O. C., Gundogdu, G., Yegen, G., Onder, S., Keskin, M., Saglam, S., Ozluk, Y., Gulluoglu, M., & Mete, O. (2016). The Impact of Phosphohistone-H3-Assisted Mitotic Count and Ki67 Score in the Determination of Tumor Grade and Prediction of Distant Metastasis in Well-Differentiated Pancreatic Neuroendocrine Tumors. *Endocrine pathology*, *27*(2), 162-170. https://doi.org/10.1007/s12022-016-9424-9

[24] Voss, S. M., Riley, M. P., Lokhandwala, P. M., Wang, M., & Yang, Z. (2015). Mitotic count by phosphohistone H3 immunohistochemical staining predicts survival and improves interobserver reproducibility in well-differentiated neuroendocrine tumors of the pancreas. *The American journal of surgical pathology*, *39*(1), 13-24. https://doi.org/10.1097/PAS.0000000000000341

[25] Mirzaiian, E., Tabatabaei Ghods, Z. S., Tavangar, S. M., Emami, B., Oraie, M., Safyari, R., & Saffar, H. (2020). Utility of PHH3 in Evaluation of Mitotic Index in Breast Carcinoma and Impact on Tumor Grade. *Asian Pacific journal of cancer prevention : APJCP*, *21*(1), 63-66. https://doi.org/10.31557/APJCP.2020.21.1.63

[26] Tsuta, K., Liu, D. C., Kalhor, N., Wistuba, I. I., & Moran, C. A. (2011). Using the mitosis-specific marker anti-phosphohistone H3 to assess mitosis in pulmonary neuroendocrine carcinomas. *American journal of clinical pathology*, *136*(2), 252-259. https://doi.org/10.1309/AJCP-DXFOPXGEF0RP

[27] Chan, D. L., Clarke, S. J., Diakos, C. I., Roach, P. J., Bailey, D. L., Singh, S., & Pavlakis, N. (2017). Prognostic and predictive biomarkers in neuroendocrine tumours. *Critical reviews in oncology/hematology*, *113*, 268-282. https://doi.org/10.1016/j.critrevonc.2017.03.017

[28] Elmaci, İ., Altinoz, M. A., Sari, R., & Bolukbasi, F. H. (2018). Phosphorylated Histone H3 (PHH3) as a Novel Cell Proliferation Marker and Prognosticator for Meningeal Tumors: A Short Review. *Applied immunohistochemistry & molecular morphology : AIMM*, *26*(9), 627-631. https://doi.org/10.1097/PAI.0000000000000499

[29] Laflamme, P., Mansoori, B. K., Sazanova, O., Orain, M., Couture, C., Simard, S., Trahan, S., Manem, V., & Joubert, P. (2020). Phospho-histone-H3 immunostaining for pulmonary carcinoids: impact on clinical appraisal, interobserver correlation, and diagnostic processing efficiency. *Human pathology*, *106*, 74-81. https://doi.org/10.1016/j.humpath.2020.09.009

[30] Garg, R., Bal, A., DAS, A., Singh, N., & Singh, H. (2019). Proliferation Marker (Ki67) in Sub-Categorization of Neuroendocrine Tumours of the Lung. Proliferation Marker (Ki67) in Sub-Categorization of Neuroendocrine Tumours of the Lung. *Turk patoloji dergisi*, *35*(1), 15-21. https://doi.org/10.5146/tjpath.2018.01436

[31] Elmaci, İ., Altinoz, M. A., Bolukbasi, F. H., Yapicier, O., & Sav, A. (2017). Paradoxical results obtained with Ki67-labeling and PHH3-mitosis index in glial tumors: a literature analysis. *Clinical neuropathology*, *36*(6), 272-282. https://doi.org/10.5414/NP301028

[32] Metovic, J., Barella, M., Bianchi, F., Hofman, P., Hofman, V., Remmelink, M., Kern, I., Carvalho, L., Pattini, L., Sonzogni, A., Veronesi, G., Harari, S., Forest, F., Papotti, M., & Pelosi, G. (2021). Morphologic and molecular classification of lung neuroendocrine neoplasms. *Virchows Archiv*, *478*(1), 5-19. https://doi.org/10.1007/s00428-020-03015-z

[33] Pelosi, G., Massa, F., Gatti, G., Righi, L., Volante, M., Birocco, N., Maisonneuve, P., Sonzogni, A., Harari, S., Albini, A., & Papotti, M. (2019). Ki-67 Evaluation for Clinical Decision in Metastatic Lung Carcinoids: A Proof of Concept. *Clinical pathology*, *12*, 2632010X19829259. https://doi.org/10.1177/2632010X19829259

[34] Warth, A., Fink, L., Fisseler-Eckhoff, A., Jonigk, D., Keller, M., Ott, G., Rieker, R. J., Sinn, P., Söder, S., Soltermann, A., Willenbrock, K., Weichert, W., & Pulmonary Pathology Working Group of the German Society of Pathology (2013). Interobserver agreement of proliferation index (Ki-67) outperforms mitotic count in pulmonary carcinoids. *Virchows Archiv*, *462*(5), 507-513. https://doi.org/10.1007/s00428-013-1408-2

[35] Kim, J. Y., Jeong, H. S., Chung, T., Kim, M., Lee, J. H., Jung, W. H., & Koo, J. S. (2017). The value of phosphohistone H3 as a proliferation marker for evaluating invasive breast cancers: A comparative study with Ki67. *Oncotarget*, *8*(39), 65064-65076. https://doi.org/10.18632/oncotarget.17775

[36] Huang, W., Nebiolo, C., Esbona, K., Hu, R., & Lloyd, R. (2020). Ki67 index and mitotic count: Correlation and variables affecting the accuracy of the quantification in endocrine/neuroendocrine tumors. *Annals of diagnostic pathology*, *48*, 151586. https://doi.org/10.1016/j.anndiagpath.2020.151586

[37] Duregon, E., Bertero, L., Pittaro, A., Soffietti, R., Rudà, R., Trevisan, M., Papotti, M., Ventura, L., Senetta, R., & Cassoni, P. (2016). Ki-67 proliferation index but not mitotic thresholds integrates the molecular prognostic stratification of lower grade gliomas. *Oncotarget*, *7*(16), 21190-21198. https://doi.org/10.18632/oncotarget.8498

[38] Tracht, J., Zhang, K., & Peker, D. (2017). Grading and Prognostication of Neuroendocrine Tumors of the Pancreas: A Comparison Study of Ki67 and PHH3. *The journal of histochemistry and cytochemistry*, *65*(7), 399-405. https://doi.org/10.1369/0022155417708186

[39] Inoue, T., Nakazato, Y., Karube, Y., Maeda, S., Kobayashi, S., & Chida, M. (2018). Mitosis count and number of cancer cells in cases of primary pulmonary adenocarcinoma: Correlations among phosphorylated histone 3, number of cancer cells, nuclear grade, pathologic features and prognosis. *Pathology international*, *68*(3), 159-166. https://doi.org/10.1111/pin.12635

[40] Villani, V., Mahadevan, K. K., Ligorio, M., Fernández-Del Castillo, C., Ting, D. T., Sabbatino, F., Zhang, I., Vangel, M., Ferrone, S., Warshaw, A. L., Lillemoe, K. D., Wargo, J., Deshpande, V., & Ferrone, C. R. (2016). Phosphorylated Histone H3 (PHH3) Is a Superior Proliferation Marker for Prognosis of Pancreatic Neuroendocrine Tumors. *Annals of surgical oncology*, *23*(Suppl 5), 609-617. https://doi.org/10.1245/s10434-016-5171-x

[41] van Steenhoven, J., Kuijer, A., Kornegoor, R., van Leeuwen, G., van Gorp, J., van Dalen, T., & van Diest, P. J. (2020). Assessment of tumour proliferation by use of the mitotic activity index, and Ki67 and phosphohistone H3 expression, in early-stage luminal breast cancer. *Histopathology*, *77*(4), 579-587. https://doi.org/10.1111/his.14185

[42] Manem, V., Sazonova, O., Gagné, A., Orain, M., Khoshkrood-Mansoori, B., Gaudreault, N., Bossé, Y., & Joubert, P. (2021). Unravelling actionable biology using transcriptomic data to integrate mitotic index and Ki-67 in the management of lung neuroendocrine tumors. *Oncotarget*, *12*(3), 209-220. https://doi.org/10.18632/oncotarget.27874