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PD-L1 EXPRESSION IN BRONCHOPULMONARY NEUROENDOCRINE TUMORS: CORRELATION WITH MORPHOLOGICAL FEATURES AND PROGNOSIS

Resume. *The purpose of the study was to estimate the programmed death-ligand 1 (PD-L1) expression in lung NETs (Grade 2 and Grade 3) and it's possible relation to clinicopathological features and patients outcome.*
Materials and methods. The study was performed using surgical material and biopsies from 40 patients with lung neuroendocrine tumors (NETs) before chemotherapy prescribing. Morphological study and immunohistochemistry (IHC) were applied. Necrosis, fibrosis, lymphocytic infiltration, neoangiogenesis, Ki-67 and PD-L1 rates, and metastatic lesions and patients' survival were estimated using nonparametric statistics.
Results. PD-L1 didn't show any significant association with studied morphological indicators and tumors grade. PD-L1 and Ki-67 expression rates were not significantly associated. But there was a significant difference in median survival rates at different levels of PD-L1 expression. In group 0 (PD-L1 negative tumors) the median survival was 85.37 months; in group 1 (PD-L1 rates 1–5%) it decreased sharply to 8 months, and in group 2 (PD-L1 expression 6–20%) it critically decreased again and did not exceed 1 month. The probability that a patient in group 1 will die earlier than a patient in group 0 was 71%. The same risks were observed while comparing the events in group 2 and group 1. The probability that a patient in group 2 will die earlier than a patient in group 0 was 86%.
Conclusions. PD-L1 expression is considered a prospective target for immunotherapy, but it also may be closely related to prognosis. In the current study 72.5% lung NETs expressed PD-L1. Moderately differentiated atypical carcinoids showed substantial aggressiveness, 69.23% of them were PD-L1 positive. There was no significant association of PD-L1 rates with tumors Grade, proliferative index (Ki-67) and morphological features (necrosis, fibrosis, lymphocytic infiltration and angiogenesis). But patients' life expectancy was closely connected to the level of PD-L1 expression in lung NETs Grade 3. The higher the expression rate of PD-L1, the shorter the patients' life expectancy. In PD-L1 negative cases median survival was 85 months; at low PD-L1 expression (1–5%) it dropped sharply to 8 months, and at moderate PD-L1 expression (6–20%) it decreased even more — to 0,8 months. The probability of an earlier death increased significantly (71%) even at low PD-L1 expression (1–5%) and exceeded 80% at PD-L1 rate 6–20%. PD-L1 should be considered as additional negative prognostic factor in lung NETs Grade 3.
Key words: *lung neuroendocrine tumors, metastasis, morphological features, immunohistochemistry, Ki-67, PD-L1 expression, life expectancy.*

Neuroendocrine tumors (NETs) are uncommon epithelial malignancies accounting for 2–6% of all neoplasia and less than 1% of all lung cancers [1–3]. According to other sources, bronchopulmonary NETs account up to 20% of all primary lung tumors [4–6].
As a rule, clinical signs of lung NETs are faint, nonspecific, the disease is diagnosed to late, in more than a half of patients it usually presented at stage III / IV. The prognosis of metastatic lung NETs is poor; in such case the patient's life expectancy rarely exceeds 12 months [7–9].

Lung NETs comprise a heterogeneous group of malignancies, histologically divided into 4 subtypes (typical carcinoid (TC), atypical carcinoid (AC), large cell neuroendocrine carcinoma

(LCNEC) and small cell carcinoma (SCLC)) with different morphology and clinical behavior [10, 11]. Bronchial carcinoids are generally considered indolent, but in fact they are malignant and have metastatic potential [12, 13]. Mainly studies deal with poorly differentiated highly aggressive NETs and underestimate lung carcinoids, that also can be rather aggressive [2, 3, 13, 14]. Due to the literature, metastases are found and in about 16–24,8% cases at AC and even in 8,4% patients at TC [13, 15].

The initial accurate diagnostics and an understanding of the tumor biology are key in making management decisions. Modern differential diagnostics of bronchopulmonary NETs is based on morphological and IHC-criteria. IHC plays a decisive role, since very often the diagnosis of lung NETs is made on biopsies (about 70% of cases), many of them are small or even crushed, that makes impossible the accurate and complete assessment of tumors morphological features. And expression rates of certain markers have not only diagnostic, but a significant prognostic value.

Proliferation features play an important role in tumor's aggression. But the ability of tumor cells to avoid immune control seems to be equally significant [9, 16, 17].

Programmed cell death ligand (PD-L1) and it's receptor — programmed cell death protein (PD-1) are the key immune checkpoint molecules that promote tumor progression via negative regulation of immune responses. PD-L1 is highly expresses on the surface of tumor cells and binds to PD-1 on the surface of activated T-cells, leading to their suppression, which consequently enables cancer cells to escape antitumor immunity [2, 18, 19].

Blocking PD-L1 pathways is seemed to be a promising therapeutic option in various aggressive malignancies with limited treatment alternatives and poor prognosis (such as melanoma, renal and lung cancers, etc.) [2, 20–27]. Also, in some studies, a small, but significant overall survival benefit with the addition of a PD-L1 antibody to standard chemotherapy in the treatment of SCLC [28]. Some studies discuss the role of PD-1 and PD-L1 as possible predictive biomarkers [29, 30].

According to the literature, up to 50% of NSCLC show positive PD-L1 staining. But in lung NETs PD-L1 expression is mainly revealed in small portion of LCNEC (about 10%) and SCLC (about 5%), and almost is not observed in carcinoids [31–33].

Increased PD-L1 expression of cancer cells has an important role for immune escape [34, 35]. In experimental models the metastatic cell line had a significant increase in expression of PD-L1 compared to the non-metastatic cell line [19]. According to the literature, PD-L1 can be thought of as an independent positive prognostic factor in patients with lung cancer [35]. Also, some studies suggest that in other malignancies PD-L1 correlates with metastatic lesions and poor prognosis [27, 32, 33–38]. The mean overall and progression free survival in cases with positive PD-L1 are lower than negative cases. Although, information on PD-L1 association with tumors biological characteristics or behavior is contradictory [27, 39, 40].

PD-L1 prognostic value in bronchopulmonary NETs needs clarification; data of literature are rather scant and controversial [32, 35]. But according to recent studies, a comprehensive characterization of the tumor microenvironment is lacking in NETs, while PD-L1 expression correlates with T-cell exhaustion independent of tumor hypoxia and suggests with tumors progression.

The **purpose of the study** was to evaluate the programmed death-ligand 1 (PD-L1) expression in atypical carcinoids (Grade 2) and high-grade lung NETs (Grade 3) and it's possible relation to clinicopathological variabilities and patients outcome.

Materials and methods. The retrospective and prospective study was conducted. We enrolled 113 patients who had been diagnosed with lung NETs and had been treated at the Kyiv City Clinical Oncological Center in 2010–2020 years. Morphological diagnosis was established (including neuroendocrine morphology, grade, TNM, and stage). Also, ICH was performed (ChrA, Syn, TTF-1, CK7, CK20, CD56, Ki-67 and PD-L1) before chemotherapy was prescribed. All the cases were showing features of neuroendocrine architecture: "nests", "rosettes" and trabeculae and were positive for one or more neuroendocrine markers. Due to morphological features and Ki-67 rates (clone MIB-1, Dako, USA), all cases we ranged into 3 Grades. NETs without necrotic areas, mitoses <2×10 hpf and Ki-67 ≤3% were considered TC (Grade 1). Specimens with focal necroses, mitoses 2–10×10 hpf and Ki-67 4–19% were estimated as AC (Grade 2). And samples with numerous or large necrotic foci, mitoses >10×10 hpf and Ki-67 ≥20% were assessed as Grade 3. Results of morphological and IHC studies were assessed by two different independent pathologists without the knowledge of patient's data. In addition, medical records were used to assess clinical findings and patients' survival.

In current study we mainly focused on PD-L1 expression and it's links with clinicopathological features of lung NETs Grade 2 and Grade 3 and the outcome. IHC, using PD-L1 antibody (clone 22C3, Dako, USA) was performed on 40 biopsy specimens of metastatic lung NETs: 13 (32.5%) cases of Grade 2 and 27 (67.5%) — of Grade 3. Tumor PD-L1 expression scores were calculated taking into account staining intensity and stained area (0–100%) [33, 39, 41]. The threshold for PD-L1 positivity or negativity was that PD-L1 stained cell accounted for ≥1% of tumor cells, assayed ICH staining [42].

PD-L1 immunostaining was evaluated as the percentage of tumor cells showing positive membrane staining, ranged into groups: group 0 —PD-L1 <1%; group 1 — PD-L1 was 1–5%; group 2 — PD-L1 ≥6%. The highest PD-L1 rate was 20% PD-L1 overexpression (>30%) was not seen in the current study. PD-L1 staining intensity was local, weak, or moderate in all cases.

Microsoft Excel was used for all calculations. Statistical analysis was performed using IBM SPSS software "Statistics 28" (license # Z125-3301-14).

We evaluated correlation of PD-L1 expression and some clinicopathological features: patients' sex, age, NETs grade, Ki-67 scores, necrotic foci, fibrosis, lymphocytic infiltration, neoangiogenesis, distant metastases and survival rates using nonparametric tests (Mann-Whitney test, Kruskal-Wallis test, Kendall's and Spearman's rank correlation). Cox regression was used for survival analysis.

The study was agreed with the commission on bioethical examination of Bogomolets National Medical University (protocol #118, 18 Jan 2019).

Results. In the sample the patients' age ranged from 33 to 76 years, the male / female ratio was 4.71:1. There were 3 (7.5%) patients (2M/1F) under 44 years; 10 (25%) (8M/2F) aged 45–59 years; and 26 (65%) (22M/4F) patients aged 60–74 years; and one male patient 76 years old.

Tumors samples with Ki-67 expression rates from 4 up to 19% were considered as Grade 2 (AC); Ki-67 ≥20% — as lung NETs Grade 3 (LCNEC and SCLC).

There were 14 (35%) patients with AC (Grade 2) and 27 (65%) with lung NETs Grade 3 (among them 12 LCNEC and 14 SCLC). All patients performed metastases in the lymph nodes at the time of diagnosis; distant metastases were found in 6 (42.86%) cases at AC and in 16 (61.53%) cases at LCNEC and SCLC, and in 6 observations they were multiple. Sample's characteristics are given in details in the tab. 1.

The sample was censored. The follow-up period varied significantly — from 11 days to 7.11 years, the observation period varied significantly and averaged 9.71 months. Due to medical records, at the end of observation period 21 (51.22%) patients were alive. 19 (47.5%) patients died; life expectancy in 15 (78.95%) cases did not exceed 10.9 months.

Slight to moderate and only local PD-L1 membrane staining was revealed in 29 (72.5%) cases: in 9 (64.29%) AC and in 20 (76.92%) samples of high-grade neuroendocrine carcinomas (in 7 (58.33%) LCNEC and 12 (85.71%) SCLC samples). 11 (27.5%) specimens of lung NETs did not show PD-L1 expression: among them 4 (28.57%) AC, and 7 (26.92%) NETs Grade 3 (5 (41.67%) LCNEC and 2 (14.29%) SCLC respectively). In 20 (50%) samples PD-L1 expression was estimated 1–5% (group 1): in 5 (35.71%) AC and in 15 (57.69%) samples of NETs Grade 3 (in 5 (41.67%) LCNEC and in 10 (71.43%) SCLC). In 9 (22.5%) observations PD-L1 expression was ≥6%: in 4 (28.57%) AC and 5 (19.23%) NETs Grade 3 (including 2 (16.67%) LCNEC and 3 (21.43%) SCLC). In the current study the highest PD-L1 rate was estimated 20% and it was seen in a sample of LCNEC and of SCLC.

PD-L1 and Ki-67 expression rates were not significantly associated (fig. 3).

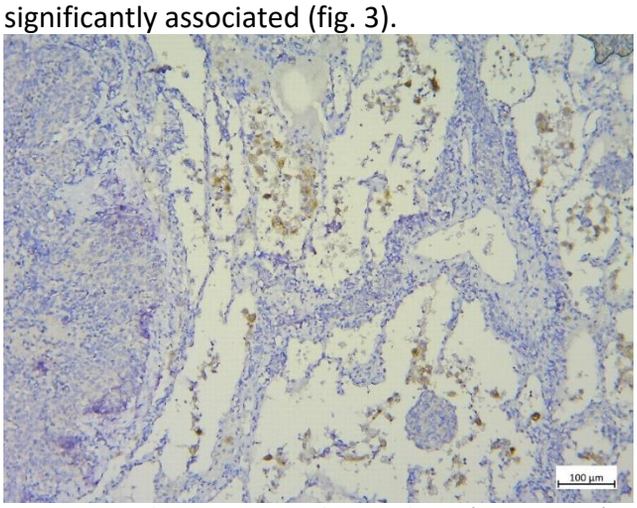

Fig. 1. Male patient aged 62 with AC (Ki-67=15%), stage IIIB. Local moderate PD-L1 expression, 12%.

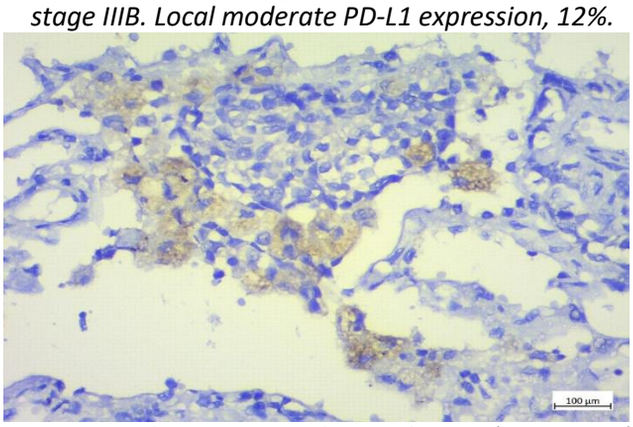

Fig. 2. Female patient aged 63 with SCLC (Ki-67=45%), stage IVA. Local weak PD-L1 expression, 6%.

Table 1.

Sample's characteristics.

Indicator		Lung NETs		Total score (40 cases)
		AC Grade 2 (14 cases)	LCNEC and SCLC Grade 3 (26 cases)	
Male patients		12 (30%)	21 (52.5%)	33 (82.5%)
Female patients		2 (5%)	5 (12.5%)	7 (17.5%)
Patient's age	≤ 44 years	2 (5%)	1 (2.5%)	3 (7.5%)
	45–59 years	5 (12.5%)	5 (12.5%)	10 (25%)
	60–74 years	7 (17.5%)	20 (50%)	27 (67.5%)
	≥ 75 years	0	1 (2.5%)	1 (2.5%)
Distant metastases		6 (15%)	16 (40%)	22 (55%)
PD-L1, %	group 0 (no expression)	4 (10%)	7 (17.5%)	11 (27.5%)
	group 1 (1–5%)	5 (12.5%)	14 (35%)	19 (47.5%)
	group 2 (6–20%)	5 (12.5%)	5 (12.5)	10 (25%)
Ki-67, %		4–19% (on the average 14.7%)	20–100% (on the average 52.5%)	4–100% (on the average 39.3%)
Necrosis	no	2 (5%)	4 (10%)	6 (15%)
	small foci	5 (12.5%)	9 (22.5%)	14 (35%)
	large areas	7 (17.5%)	13 (32.5%)	20 (50%)
Fibrosis	no	0	2 (5%)	2 (5%)
	slight	8 (20%)	14 (35%)	22 (55%)
	moderate	6 (15%)	10 (25%)	16 (40%)
Lymphocytic infiltration	no	5 (12.5%)	3 (7.5%)	8 (20%)
	slight	8 (20%)	18 (45%)	26 (65%)
	moderate	1 (2.5%)	5 (12.5%)	6 (15%)
Neoangiogenesis	no	2 (5%)	1 (2.5%)	3 (7.5%)
	slight	6 (15%)	14 (35%)	20 (50%)
	moderate	6 (15%)	11 (27.5%)	17 (42.5%)

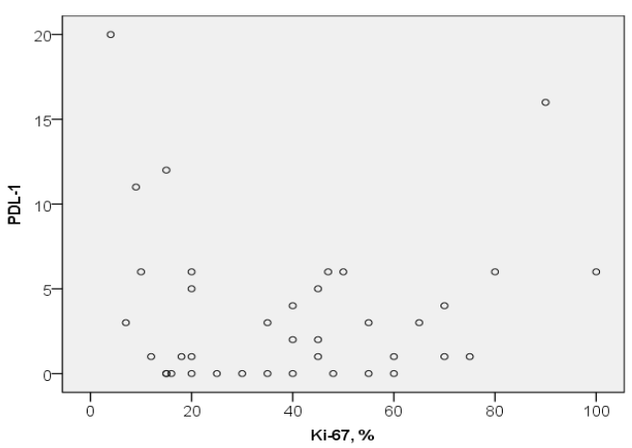

Fig. 3. Correlation between Ki-67 and PD-L1 is not significant. Kendall's rank correlation, τb=0.042, p>0.729.

There was no notable difference in PD-L1 expression in lung NETs Grade 2 and Grade 3 considering different severity of necrosis, fibrosis, neongiogenesis, and lymphocytic infiltration.

In 5 (38.46%) AC single small foci of necrosis were detected, in 7 (50%) — multiple or large foci, 2 (15.38%) samples didn't show necrotic changes. Large necrotic areas were seen in 3 (25%) cases of PD-L1-negative AC, in 2 (16.67%) AC with PD-L1 rate 1–5% and in 2 (16.67%) AC with PD-L1 expression 6–20%. 13 (50%) lung NETs Grade 3 showed excessive necrosis, in 9 (34.61%) tumors small foci of necrosis were detected, in 4 (15.38%) necrosis were absent. Large foci of necrosis were found in 2 (7.69%) NETs Grade 3 with negative PD-L1 expression, in 7 (26.92%) cases with PD-L1 level 1–5%, and in 4 (15.38%) — with PD-L1 expression rate 6–20%.

Light fibrosis was seen in 20 (50%) cases, moderate — in 18 (45%). It was found in all AC and in 24 (92.31%) NETs Grade 3. Fibrosis was detected in 10 (25%) PD-L1 negative samples, in 18 (45%) tumors with low PD-L1 expression (1–5%) and in 10 (25%) — with moderate PD-L1 level (6–20%).

Light lymphocytic infiltration was detected in 26 (65%) cases, moderate — in 6 (15%). Lymphocytic infiltration was found in 9 (64.29%)

AC and in 23 (88.46%) NETs Grade 3. 9 (22.5%) samples were PD-L1 negative, 12 (30%) tumors showed low PD-L1 expression (1–5%), in 9 (22.5%) specimens PD-L1 expression was moderate (6–20%).

Signs of light neoangiogenesis were seen in 20 (50%) cases, of moderate — in 17 (42.5%). Neoangiogenesis was detected in 12 (85.71%) AC and in 25 (96.15%) NETs Grade 3. 11 (27.5%) tumors were DPL-1 negative, in 18 (45%) low rates of PD-L1 expression (1–5%) were found, in 8 (20%) — PD-L1 expression estimated 6–20%.

PD-L1 didn't show any significant association with studied morphological indicators and tumors grade (fig. 4).

But the average survival differed significantly

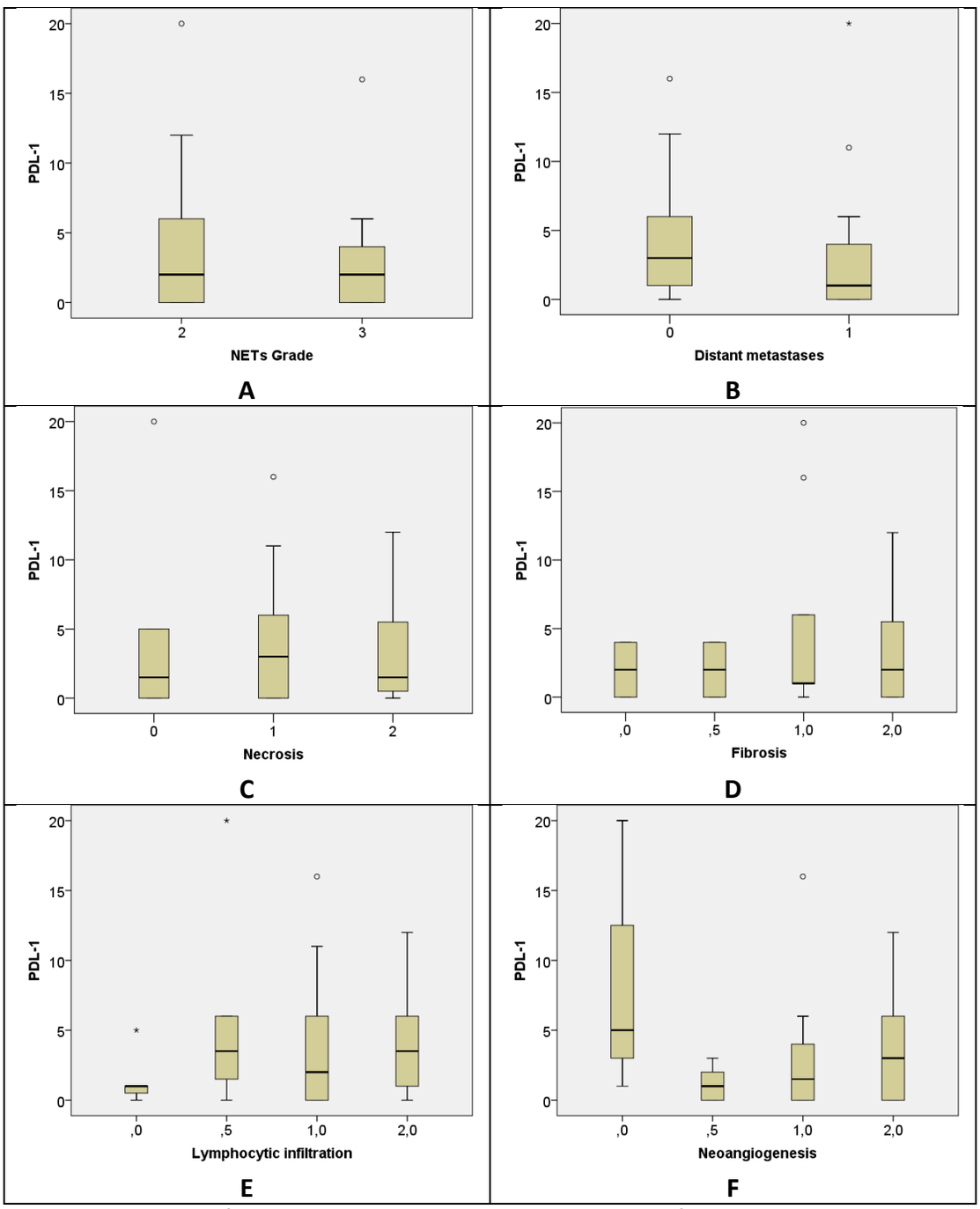

Fig. 4. Box-plot association of PD-L1 expression and NETs morphological features. 4A — PD-L1 rates and NETs Grade. 4B — PD-L1 rates and presence of distant metastases. 4C — PD-L1 rates and necrosis (0 — no necrosis, 1 — small foci of necrosis, 2 — large necrotic areas). 4D — PD-L1 rates and fibrosis in the tumor tissue (0 — no fibrosis, 1 — very slight, 1 — slight, 2 — moderate fibrosis). 4E — PD-L1 rates and lymphocytic infiltration in the tumor tissue (0 — no lymphocytes, 1* — very slight lymphocytic infiltration, 1 — slight, 2 — moderate). 4F — PD-L1 expression and neoangiogenesis in the NETs tissue (0 — no neoangiogenesis was found, 1* — very slight, 1 — slight, 2 — moderate). Bold line is the median; the bottom and the top of the vertical line are the minimal and the maximal values, circles — are outliers, asterisks — extreme values. Mann-Whitney and Kruskal-Wallis tests were applied. In all compared groups, the median values didn't differ significantly.*

depending on the PD-L1 expression rates (fig. 5, tab. 2). Group 0 — samples were PD-L1 negative, group 1 — PD-L1 expression was estimated 1–5%, group 2 — PD-L1 expression rate was 6–20%.

There was a significant difference in median survival rates at different levels of PD-L1 expression. In group 0 (PD-L1 negative tumors) the median survival was 85.37 months; in group 1 (PD-L1 rates 1–5%) it dropped sharply to 8 months, and in group 2 (PD-L1 expression 6–20%) it critically decreased again and did not exceed 1 month.

PD-L1 group variable was statistically significant in survival analysis (Cox regression, p=0,041). Hazard ratio was 2.47 (95% CI 1.04–5.90). Hazard ratio suggests that with an increase

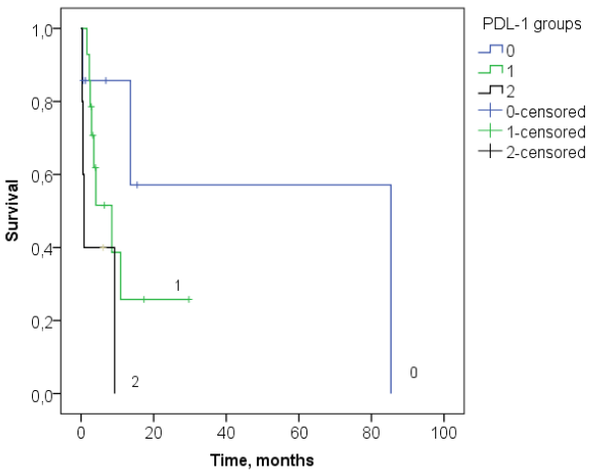

Fig. 5. Survival curves for patients at lung NETs Grade 3.

Table. 2

There was a significant difference in survival (months) for different rates of PD-L1 expression.

PD-L1	Percentile					
	25.0%		50.0%		75.0%	
	value	standard error	value	standard error	value	standard error
group 0 (no expression)	85.370		85.370		13.600	11.548
group 1 (1–5%)			8.470	3.346	2.900	0.638
group 2 (6–20%)	9.230		0.830	0.329	0.530	0.252
total	85.370		9.230	4.212	2.530	0.892

in the PD-L1 group number by 1 point the risk of an earlier death of the patient increased by 2.47 times, and with an increase by 2 points (from group 0 to the group 2) — it increased by 6.10 times. The probability that a patient in group 1 will die earlier than a patient in group 0 was 71%. The same risks were observed while comparing the events in group 2 and group 1. The probability that a patient in group 2 will die earlier than a patient in group 0 was 86%.

Discussion. Lung NETs are rather heterogeneous cancers, and all of them have malignant potential despite different morphological features, proliferative activity, and clinical behavior. In most cases these malignancies are diagnosed too late mainly because of nonspecific clinical manifestations and lack of clearly agreed criteria for diagnosis and prognosis.

In recent decades, the frequency of lung NETs has increased. Modern studies in pathology are devoted to diagnostics improvement and prognosis clarification.

Mainly studies deal with Grade 3 tumors (LCNEC and SCLC) that make up the largest group of bronchopulmonary NETs and up to 18–23% of all primary lung malignancies. AC is much rare tumor (about 2% of all primary lung cancers), but it's aggressiveness is often underestimated. Despite the low proliferative activity, at the moment of diagnosis patients with AC may perform metastases, including distant lesions that is associated with poor prognosis, and critically decreased life expectancy.

High aggressiveness of the tumor and poor prognosis are associated with increased proliferative activity and escape from immune control. According to some studies, PD-L1 expression considered as negative prognostic factor in many aggressive malignancies [31, 33]. However, these data are uncertain, especially in NETs. Many malignant tumors express PD-L1 in a small number of cases.

In the current study the sample was rather small: 13 (32.5%) cases of lung NETs Grade 2 (AC) and 27 (67.5%) Grade 3 (LCNEC and SCLC) with local metastases. Distant metastases were found in 22 (55%) cases: in 6 (15%) patients with AC and in 16 (40%) with lung NETs Grade 3; the percentage of PD-L1 positive cases corresponds to the data of literature [8, 15]. The maximal PD-L1 rate seen in the current study was 20%.

The distribution of PD-L1 rates in AC was almost uniform: 30.77% samples were PD-L1 negative, 38.46% samples showed low PD-L1 expression (1–5%), in 30.77% specimens

moderate PD-L1 expression was detected. In lung NETs Grade 3 the distribution of PD-L1 rates differed: 25.92% tumors were PD-L1 negative, in most samples (55.56%) low PD-L1 expression was seen, moderate PD-L1 rates were seen just in 18.52% specimens.

Statistical analysis didn't show any significant association of PD-L1 expression and proliferative index (Ki-67 rates), tumor's Grade, distant metastases and morphological features (necrosis, lymphocytic infiltration, fibrosis, neoangiogenesis) in lung NETs.

However, the patients' life expectancy at lung NETs Grade 3 significantly depended on the level of PD-L1 expression.

Median survival rates varied significantly at different levels of PD-L1 expression. In PD-L1 negative cases median survival was 85 months; at low PD-L1 expression (1–5%) it was 8 months, and at moderate PD-L1 expression (6–20%) it was critically decreased and estimated at 0,8 months.

The probability to die earlier exceeded 70% while comparing the survival rates of patients in groups 0 and 1 (PD-L1 negative samples and low PD-L1 expression) and also while comparing groups 2 and 1 (moderate and low PD-L1 expression). But the probability that a patient in group 2 (PD-L1 expression 6–20%) will die earlier than a patient in group 0 (no PD-L1 expression) was higher — more than 80%.

Thus, PD-L1 rates may be used as additional independent negative prognostic factor in bronchopulmonary NETs Grade and Grade 3. The higher the PD-L1 rate, the worse the prognosis.

Conclusions.

1. High rates of the proliferation index are associated with increased aggressiveness of the malignancy, but the ability of tumor cells to avoid immune control also plays significant role. PD-L1 expression is considered a prospective target for immunotherapy, but it also may be closely related to prognosis.

2. In the current study 72.5% aggressive bronchopulmonary NETs expressed PD-L1, and in 69.97% cases the expression was low and local. Moderately differentiated atypical carcinoids showed substantial aggressiveness, 69.23% of them were PD-L1 positive.

3. There was no significant association of PD-L1 rates with tumors Grade, proliferative index (Ki-67) and morphological features (necrosis, fibrosis, lymphocytic infiltration and angiogenesis). But patients' life expectancy was closely connected to the level of PD-L1 expression in lung NETs Grade 3. The higher the expression rate of PD-L1, the shorter the patients' life expectancy. In PD-L1 negative cases median survival was 85 months; at low PD-L1 expression (1–5%) it dropped sharply to 8 months, and at moderate PD-L1 expression (6–20%) it decreased even more — to 0,8 months. The probability of an earlier death increased significantly (71%) even at low PD-L1 expression (1–5%) and exceeded 80% at PD-L1 rate 6–20%.

4. PD-L1 should be considered as additional negative prognostic factor in lung NETs Grade 3.

The prospects for further research. It is somewhat difficult to match our results with other studies because of rather small samples, different studies design, use of different ICH markers for PD-L1 staining and lack of a unified assessment system. Further studies on PD-L1 expression are desirable for more correct characterization of it's effects.

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