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Comparative expression analysis of immune-related markers in surgically resected lung neuroendocrine neoplasms

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ABSTRACT

Background: Although immunotherapy has led to a paradigm shift in the treatment of lung cancer, the therapeutic approaches for lung neuroendocrine neoplasms (LNENs) are still limited. Our aim was to explore the immunological landscape and the expression of immune checkpoint markers in LNENs.

Methods: Surgically removed tumor samples of 26 atypical carcinoid (AC), 30 large cell neuroendocrine carcinoma (LCNEC) and 29 small cell lung cancer (SCLC) patients were included. The immune phenotype of each tumor type was assessed by using a panel of 15 immune-related markers. As these markers are potentially expressed by immune cells and/or tumor cells, they might serve as putative targets for immunotherapy. Expression patterns were measured by immunohistochemistry and correlated with clinicopathological parameters and prognosis.

Results: Unsupervised hierarchical clustering revealed distinct immunologic profiles across tumor types. Specifically, AC tumors were characterized by high tumor cell CD40 expression and low levels of immune infiltrates whereas SCLC samples had a high CD47 and Inducible T Cell Costimulator (ICOS) expression in tumor cells and immune cells, respectively. High CD70 and CD137 expression by tumor cells as well as elevated expression of CD27, Lymphocyte Activation Gene 3 (LAG3), and CD40 by immune cells were characteristic for LCNEC samples. Overall, SCLC and LCNEC tumors had a more immunogenic phenotype than AC samples. High tumor cell CD47 and CD40 expressions were associated with impaired and improved survival outcomes, respectively.

Abbreviations: AC, Atypical carcinoid; APC, Antigen-presenting cells; ASCL1, Achaete-Scute Homologue 1; CHT, Chemotherapy; DLL3, Delta-like Canonical Notch Ligand 3; FFPE, Formalin-fixed paraffin-embedded; GEPIA2, Gene Expression Profiling Interactive Analysis 2; ICI, Immune checkpoint inhibitor; IHC, Immunohistochemistry; KEAP11, Kelch-like ECH Associated Protein 1; KRAS, Kirsten Rat Sarcoma Virus; LCNEC, Large cell neuroendocrine carcinoma; LNEN, Lung neuroendocrine neoplasm; MEN1, multiple endocrine neoplasia type 1; NEUROD1, Neurogenic Differentiation Factor 1; NCCN, National Comprehensive Cancer Network; NSCLC, Non-small cell lung cancer; OS, Overall survival; PD-1, Programmed Cell Death Protein 1; PD-L1, Programmed Cell Death Ligand 1; PFS, Progression-free survival; POU2F3, POU Class 2 Homeobox 3; RB1, Retinoblastoma 1; SCLC, Small cell lung cancer; STAS, Spread through air spaces; STK11, Serine/threonine Kinase 11; TC, Typical carcinoid; TIM, Tumor immune microenvironment; TIMER 2.0, Tumor Immune Estimation Resource 2.0; TP53, Tumor Protein p53; TMB, Tumor mutation burden; YAP1, Yes-Associated Protein 1.

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Conclusions: By providing insights into the widely divergent immunologic profiles of LNENs, our results might serve as a basis for the development of novel immunotherapy-related approaches in these devastating malignancies.

1. Introduction

Lung neuroendocrine neoplasms (LNENs) mostly originate from neuroendocrine cells of the lung and represent a heterogeneous group of disease [1]. They account for approximately 20% of all lung cancer cases and are characterized by various molecular, morphological, and immunohistochemical features [2].

Histologically, LNENs comprise of four distinct types that are associated with different clinical and biological behaviors [2]. Typical carcinoid tumors (TCs, 1.8% of all lung cancers) are well-differentiated low-grade neoplasms that are usually cured by surgery alone whereas atypical carcinoids (ACs, 0.2%) are intermediate-grade tumors with a dismal clinical course and a 5-year survival rate of 50%. Given its aggressive behavior and high metastatic potential (distant metastases appear in 40–50% of cases), patients with AC tumors often require adjuvant chemo-radiotherapy after surgery [1,3–5]. Of note, all bronchial carcinoids are considered malignant lesions and have the potential to metastasize, even TCs [6]. Large cell neuroendocrine carcinomas (LCNECs, 3%) are poorly differentiated high-grade tumors characterized by high rates of recurrence after surgery. The overall survival (OS) of LCNEC patients is generally dismal (with 5-year survival rates of approximately 15–25%), even for individuals with early stage disease [7,8]. As for its clinical management, therapeutic protocols for LCNEC frequently overlap with the treatment approaches in small cell lung cancer (SCLCs, 15%), the fourth histological type of LNENs [1,9–11]. As SCLC is an exceptionally lethal and metastatic malignancy with a 5-year survival rate way below 7%, it remains one of the deadliest types of cancer. Due to its high metastatic potential and rapid doubling time, surgery is rarely performed in this hard-to-treat cancer. Instead, similar to those with LCNEC tumors, SCLC patients are usually treated with platinum-based chemotherapy (CHT) and etoposide [12–16].

The implementation of immunotherapy into clinical practice over the last few years has fundamentally changed the survival outcomes of non-small cell lung cancer (NSCLC) patients [17]. Nevertheless, progress in the clinical management of LNENs lags far behind the significant developments seen in NSCLC due to immune checkpoint inhibitors [15,18]. Recently, there has been a growing interest in the use of immunotherapeutic agents in advanced or metastatic lung carcinoids [19–22]. Several ongoing clinical trials are investigating the effectiveness of immune checkpoint blockade in these subtypes [23]. As for other LNENs, although the addition of immune checkpoint inhibitors to the standard platinum-based systemic therapy improved survival outcomes in subsets of SCLC and LCNEC patients, the response rates were lower than anticipated [24–26]. The reasons behind these somewhat disappointing results are controversial. However, the tumor immune microenvironment (TIM) might play a pivotal role in immune checkpoint inhibitor efficacy [27]. Past efforts to characterize the TIM revealed its role in cancer development and progression [25]. Additionally, high tumor infiltrating lymphocyte density associates directly with immunotherapy benefit in NSCLC patients and predicts clinical outcomes [28,29].

Evaluating the TIM and specific immune signatures of LNENs is a critical next step in improving the efficacy of currently used immune checkpoint inhibitors and developing next-generation immunotherapies. To date, only a few studies have investigated the expression pattern and clinical relevance of certain immune-related markers in these malignancies (e.g. Programmed Cell Death Protein 1 [PD-1], Programmed Cell Death Ligand 1 [PD-L1], CD8, CD47, Lymphocyte Activation Gene 3 [LAG3]) [21,30–35]. Nevertheless, given the low number of examined immune markers and the scarcity of surgically resected cases, these studies could not comprehensively evaluate the TIM.

In this study, we aimed to investigate the immunological phenotypes and TIM of surgically resected LNENs. Importantly, besides assessing the expression pattern of several immune-related markers, we also evaluated their relationship to clinicopathological parameters and long-term outcomes.

2. Materials and methods

2.1. Study population and treatment

In our two-center retrospective study, we included 85 Caucasian patients with histologically confirmed LNENs who underwent surgical resection either at the National Korányi Institute of Pulmonology (Budapest, Hungary) or at the National Institute of Oncology (Budapest, Hungary) between 2000 and 2020. Of these, 26, 30, and 29 patients were diagnosed with AC, LCNEC, and SCLC, respectively. Clinicopathological data concerning the age at the time of diagnosis, gender, comorbidities, and smoking history were retrospectively collected from medical records. Survival outcomes were provided by the National Health Insurance Office and Central Statistical Office of Hungary. Only individuals with appropriate clinicopathological data and sufficient amount of formalin-fixed paraffin-embedded (FFPE) tumor tissue were included. To compare with the AC samples, we also included 10 additional TC samples in the study.

The present study was conducted in accordance with the guidelines of the Helsinki Declaration of the World Medical Association. The study was approved by the national-level ethics committee of Hungary (Hungarian Scientific and Research Ethics Committee of the Medical Research Council, ETT TUKEB 39249–2/2019/EKU and 52614–4/2013/EKU). The need for individual informed consent for this retrospective study was waived. After clinical information was collected, patient identifiers were removed, thereby disabling direct or indirect patient identification. All patients underwent lung resection surgery (lobectomy or wedge resection surgery), and platinum-based adjuvant CHT was applied when necessary. Systemic therapy was administered in accordance with the current National Comprehensive Cancer Network (NCCN) guidelines in both institutes.

2.2. Immunohistochemistry (IHC)

All tumor tissue samples were obtained by surgical resection. First, each sample was examined as part of the routine pathological check-up to define the histopathological diagnosis for further therapy. This was performed by a board-certified pathologist of the host institute according to the contemporary diagnostic guidelines, using specific IHC stains such as Chromogranin A, Synaptophysin, CD56, Syntaxin, and Ki-67. In addition, in order to ensure correctness of the initial diagnosis and to exclude cases with mixed histology (i.e. combined SCLC-LCNEC/NSCLC), all hematoxylin and eosin (H&E)-stained slides were also reviewed by an independent pathologist prior to inclusion. All included tissue sections were analyzed for the expression of the following 15 immunological markers: PD-L1, PD-1, CD3, CD4, CD8, CD27, CD47, Indolamine 2,3-dioxygenase (IDO), inducible T-cell costimulator (ICOS), CD70, CD137, CD40, CD94/NK Group 2 Member A (NKG2A), LAG3, and OX40. As these markers are expressed by several immune cells and transmit activatory or inhibitory signals, they might represent potential targets for immunotherapy [36–44]. The specific antibodies against these markers are summarized in Supplementary Table S1.

After deparaffinization and rehydration, sections were incubated in a 3% H_2O_2 solution for 20 min in order to reduce nonspecific background staining. Next, tissue samples were heated to 98.0 °C in a 10 mM Citrate buffer (pH = 6.0) or 10 mM Tris-EDTA buffer (pH = 9.0) for 40 min based on the manufacturers' recommendation. Slides were incubated at room temperature with Ultra V Block (Ultravision LP detection system, Lab Vision Corporation, Thermo Fisher Scientific Inc., Pittsburgh, MA, USA) for 5 min, followed by primary antibody incubation overnight at 4 °C. Immunoreaction was detected by the UltraVision LP detection system (Lab Vision Corporation). Primary antibodies were visualized by 3-3′-diaminobenzidine (DAB) and counterstained with hematoxylin. All slides were digitally scanned using PANNORAMIC 250 Flash III (3DHISTECH Ltd., Budapest, Hungary); sections were examined and evaluated by using CaseViewer 2.4 (3DHISTECH Ltd., Budapest, Hungary). During pathological evaluation, the percentage of positive cells was determined and averaged on ten randomly selected areas by two independent pathologists at 20x and 40x magnification. When a discrepancy of more than 20% between the two pathologists' scores arose, cases were re-evaluated by including a third senior lung pathologist. The tumor cells and immune cells were separately scored. Furthermore, in the case of tumor cells, the ratio of positive cells to all tumor cells was quantified. Similarly, the ratio of immune cells showing positive staining as well as the ratio of total immune infiltrates in a given sample were determined. Of note, we choose to analyse each slide "manually" instead of computed-based tools since the evaluated antibodies are not yet part of the routine clinical diagnosis and software-based approaches are mostly optimized for widely-used diagnostic antibodies. Additionally, the training of AI-based algorithms requires a large number of "teaching" sets which were not available in our study.

2.3. Statistical analyses

Statistical analyses were performed using R version 4.0.5 (R Foundation for Statistical Computing, Vienna, Austria). P-values of < 0.05 were considered statistically significant. To compensate for multiple testing, the Bonferroni-correction was applied in certain cases. Adjusted p-values are marked with an asterisk (*) throughout the text. Further details concerning the statistical analyses are presented in the Supplementary Materials and Methods section.

3. Results

3.1. Clinicopathological characteristics and survival outcomes of included patients

Patient characteristics according to their tumor's histological type are presented in Table 1 and Supplementary Fig. S1A. Of note, because bronchoscopy reports were available for all of our patients, we strictly defined the primary tumor location by bronchoscopical visualization. As for the association between clinicopathological parameters and survival outcomes, we found that the lung cancer subtype (log-rank p = 0.059), vascular involvement (log-rank p = 0.0049), and the presence of diabetes as a comorbidity (log-rank p = 0.021) influenced the overall survival (OS) in our univariate model (Supplementary Fig. S1B).

3.2. The expression pattern of immune-related markers by tumor cells in LNENs

Representative IHC images of immune-related markers according to each LNEN subtype are shown in Fig. 1. In order to investigate the key differences in their IHC expression, we evaluated markers with available expression levels in at least one of the NEN subtypes for at least one

Table 1
Clinicopathological characteristics (COPD, Chronic obstructive pulmonary disease; CHT, chemotherapy; NA, not available).

		Atypical carcinoids (AC)	Large cell neuroendocrine lung cancer (LCNEC)	Small cell lung cancer (SCLC)
Total number of patients		26	30	29
Gender	male	11 (42,3%)	18 (60%)	9 (31,03%)
	female	15 (57,7%)	11 (36,67%)	20 (68,9%)
Median age (range)	62 (33–79)	63 (48–76)	65 (50–76)	
Smoking history	current smoker	4 (15,38%)	9 (30%)	9 (31,03%)
	former smoker	6 (23,08%)	11 (36,67%)	13 (44,83%)
	non-smoker	14 (53,85%)	3 (10%)	4 (13,79%)
	NA	2 (7,69%)	7 (23,33%)	3 (10,34%)
COPD	COPD	4 (15,38%)	12 (40%)	13 (44,83%)
	no COPD	22 (84,62%)	17 (56,67%)	14 (48,28%)
	NA	0 (0%)	1 (3,33%)	2 (6,9%)
Hypertension	hypertension	16 (61,54%)	19 (63,33%)	12 (41,38%)
	no hypertension	10 (38,46%)	10 (33,33%)	15 (51,72%)
	NA	0 (0%)	1 (3,33%)	2 (6,9%)
Diabetes	diabetes	4 (15,38%)	5 (16,67%)	7 (24,14%)
	no diabetes	22 (84,62%)	24 (80%)	20 (68,9%)
	NA	0 (0%)	1 (3,33%)	2 (6,9%)
Other malignancy	other malignancy	6 (23,08%)	4 (13,33%)	8 (27,59%)
	no other malignancy	20 (76,92%)	25 (83,33%)	19 (65,52%)
	NA	0 (0%)	1 (3,33%)	2 (6,9%)
Tumor localization	central	13 (50%)	3 (10%)	6 (20,69%)
	peripheral	13 (50%)	24 (80%)	18 (62,07%)
	NA	0 (0%)	3 (10%)	5 (17,24%)
Necrosis	necrosis	10 (38,46%)	24 (80%)	18 (62,07%)
	no necrosis	6 (23,08%)	5 (16,67%)	9 (31,03%)
	NA	10 (38,46%)	1 (3,33%)	2 (6,9%)
Vascular involvement	vascular involvement	10 (38,46%)	10 (33,33%)	13 (44,83%)
	no vascular involvement	15 (57,69%)	19 (63,33%)	11 (37,93%)
	NA	1 (3,85%)	1 (3,33%)	5 (17,24%)
Recurrence	recurrence	2 (7,69%)	3 (10%)	3 (10,34%)
	no recurrence	19 (73,08%)	13 (43,33%)	9 (31,03%)
	NA	5 (19,23%)	14 (46,67%)	17 (58,62%)
Neoadjuvant chemotherapy	neoadjuvant CHT	3 (11,54%)	1 (3,33%)	1 (3,45%)
	no neoadjuvant CHT	23 (88,46%)	28 (93,33%)	26 (89,66%)
	NA	0 (0%)	1 (3,33%)	2 (6,9%)

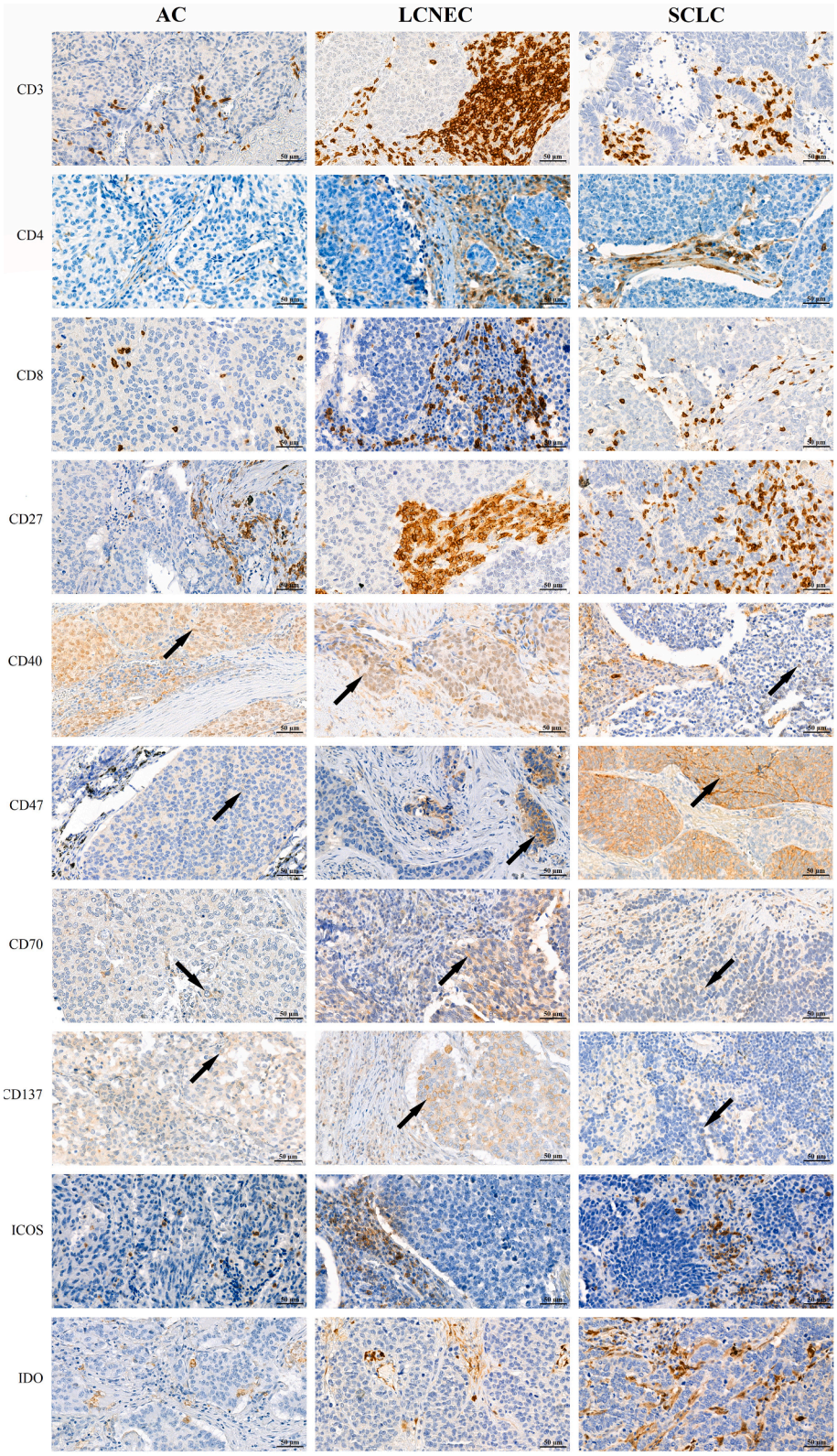

Fig. 1. IHC stainings of formalin-fixed, paraffin-embedded AC, LCNEC and SCLC samples with immune-related markers. The representative images were captured with a 40x objective lens. The positive cells were visualized with 3-3′-diaminobenzidine (DAB), and the nuclei were labeled with hematoxylin. Black arrows point at examples of positive tumor cells. AC, atypical carcinoid; LCNEC, large cell neuroendocrine carcinoma; SCLC, small cell lung cancer; TCs, tumor cells.

patient. Accordingly, the following eight markers were included in the comparative analysis: PD-1, PD-L1, CD47, IDO, CD70, CD137, CD40, and NKG2A. Except for PD-L1, where expression levels were ubiquitously low and resulted in a similar expression pattern across all histological subgroups (only 5 positive cases were found in the LCNEC cohort, median: 0.0), the IHC expression of the other markers showed a

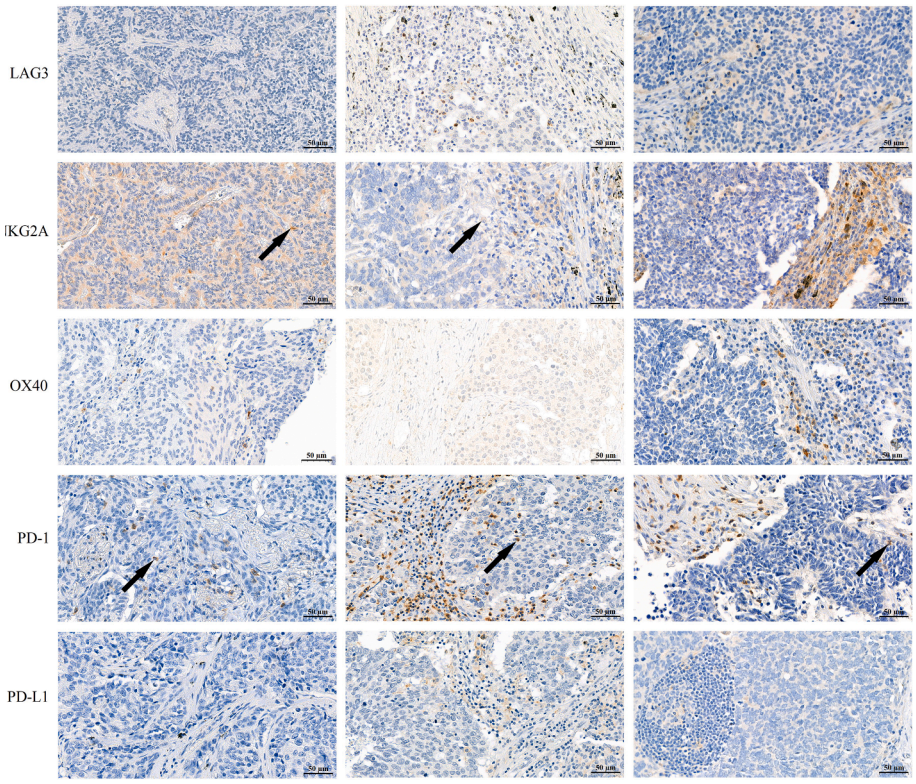

Fig. 1. (*continued*).

different distribution in at least one of the three groups (Fig. 2A). Specifically, tumor cell NKG2A and CD40 expressions were significantly higher ($p < 0.05$) in AC samples compared to the LCNEC and SCLC specimens (the median of NKG2A expressions were 0.015, 0.01, and 0.01 in AC, LCNEC, and SCLC samples, respectively. The median CD40 expressions were 0.275, 0.1, and 0.1 in AC, LCNEC, and SCLC samples, respectively). CD47 expression was the highest in SCLC samples (vs. LCNEC and AC, medians were 0.25 vs. 0.035 vs. 0, respectively). LCNEC tumors expressed both PD-1, CD70, and CD137 at a significantly higher degree than tumors with other histological types ($p < 0.05$). As for their clinical relevance, we found that tumor cell NKG2A expression negatively correlated with the patient's age (Pearson R = -0.38; p*=0.004). Of note, CD40 expression also showed a negative correlation with age, but this outcome did not reach statistical significance after correction for multiple testing (Pearson R = -0.27, p*=0.104; Supplementary Fig. S2A). Other relevant associations between the expression of immune-related markers by tumor cells and categorical clinicopathological variables are shown in Supplementary Fig. S2B.

3.3. Unsupervised hierarchical clustering of LNENs according to the expression pattern of immune-related markers by tumor cells

As shown in Fig. 3A, unsupervised hierarchical clustering based on the IHC expression of different markers of the TIM separated the samples of different histological subgroups fairly well. We found that tumor cell CD40 expression was generally higher in AC tumors (vs. LCNEC and SCLC specimens) whereas high CD47-expressing tumor cells were characteristic for SCLC. CD137 expression by tumor cells was the highest in LCNEC specimens. These results are in line with the above-discussed findings of pairwise comparisons.

3.4. The expression pattern of immune-related markers defined by immune cells varies across LNEN subtypes

First, in order to obtain a comprehensive overview of the TIM concerning each histological subtype, we compared the levels of immune infiltration (i.e. tumor-infiltrating lymphocytes) across the different subgroups (Fig. 2B). The abundance of immune infiltrates was similar in SCLC and LCNEC samples, but notably lower in AC specimens. Likewise, individual expressions of other immune-related markers such as PD-1, ICOS, CD27, CD4, and CD8 were also significantly lower in AC tumors (vs. SCLC and LCNEC specimens). Of note, immune cell expressions of CD27, LAG3, OX40, CD40, and CD8 were highest in LCNEC samples and only these tumors expressed PD-L1. None of the measured parameters correlated with the patients' age. However, as shown in Supplementary Fig. S3, several significant associations between immune cell expression levels of immune-related markers and tumor localization, peritumoral inflammation, and other clinicopathological variables were found.

To examine the immunologic landscape within different carcinoid tumor types, we performed immunohistochemical stainings on ten additional typical carcinoid samples. Supplementary Fig. S4 shows the representative images of immune-related markers in the case of typical carcinoid tumors compared to atypical carcinoids. Supplementary Fig. S5A shows the markers' expression levels by tumor cells in typical- vs. atypical carcinoids. Out of the 15 initial markers, we studied eight markers which had available expression levels in at least the typical or atypical carcinoid subtype of at least one patient. Based on the p-values of the Wilcoxon rank sum test after Bonferroni-correction, median expressions were significantly different between typical and atypical carcinoid cases in case of PD-1 ($p = 0.013$), PD-L1 ($p = 0$), IDO ($p = 0.00003$), CD70 ($p = 0.00014$), and NKG2A ($p = 0.00093$). Supplementary Fig. S5B shows the expression levels of the 15 markers by immune cells. We found a significant difference between typical- and atypical carcinoid cases in case of CD4 ($p = 0.00016$) and PD-L1 ($p = 0.00012$) expression levels.

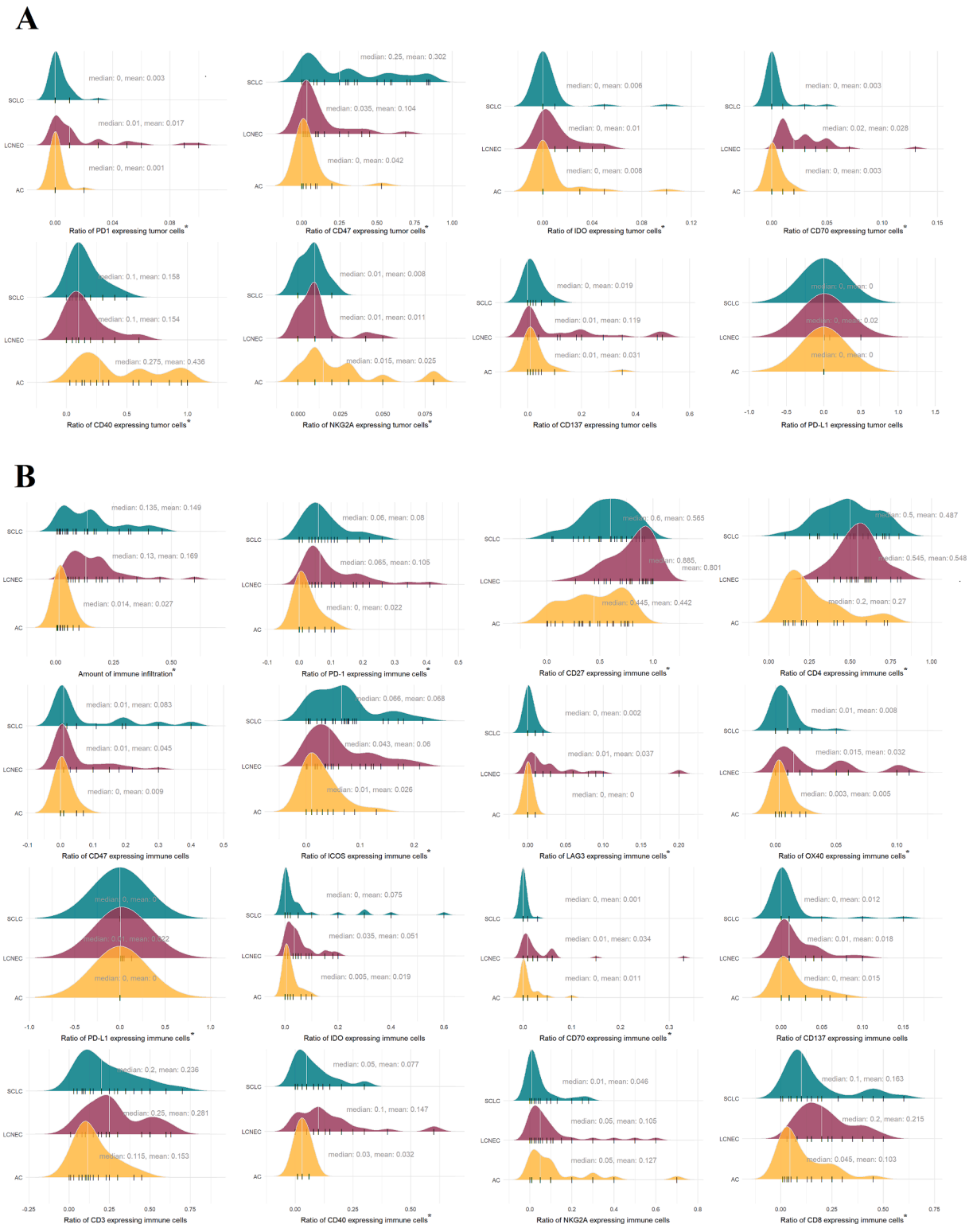

Fig. 2. (A) Expression levels of preselected immune-related markers by tumor cells in different LNEN subtypes. The color-filled curves show the estimated normalized probability density function of the data. Colors indicate the three LNEN subtypes, whereas the short vertical black lines mark the individual samples. We studied 8 markers among the 15 markers which had available expression levels in at least one of the LNEN subtypes for at least one patient. Except for PD-L1, the other 7 markers showed different expressions across the LNEN histological subgroups. Green: SCLC, small cell lung cancer; red: LCNEC, large cell neuroendocrine cancer; yellow: AC, atypical carcinoid. Bonferroni-adjusted significant differences are marked with an asterisk (*). **(B) Expression levels of different immune-related markers by immune cells cells according to the three LNEN subtypes.** The filled curves show the estimated normalized probability density function of the data. Colors indicate the three LNEN subtypes; the short vertical black lines mark the individual samples. The first graph represents the level of immune infiltration in general. Colors indicate different LNEN subtypes, short vertical black lines mark individual samples. Green: SCLC, small cell lung cancer; red: LCNEC, large cell neuroendocrine carcinoma; yellow: AC, atypical carcinoid. Bonferroni-adjusted significant differences are marked with an asterisk (*).

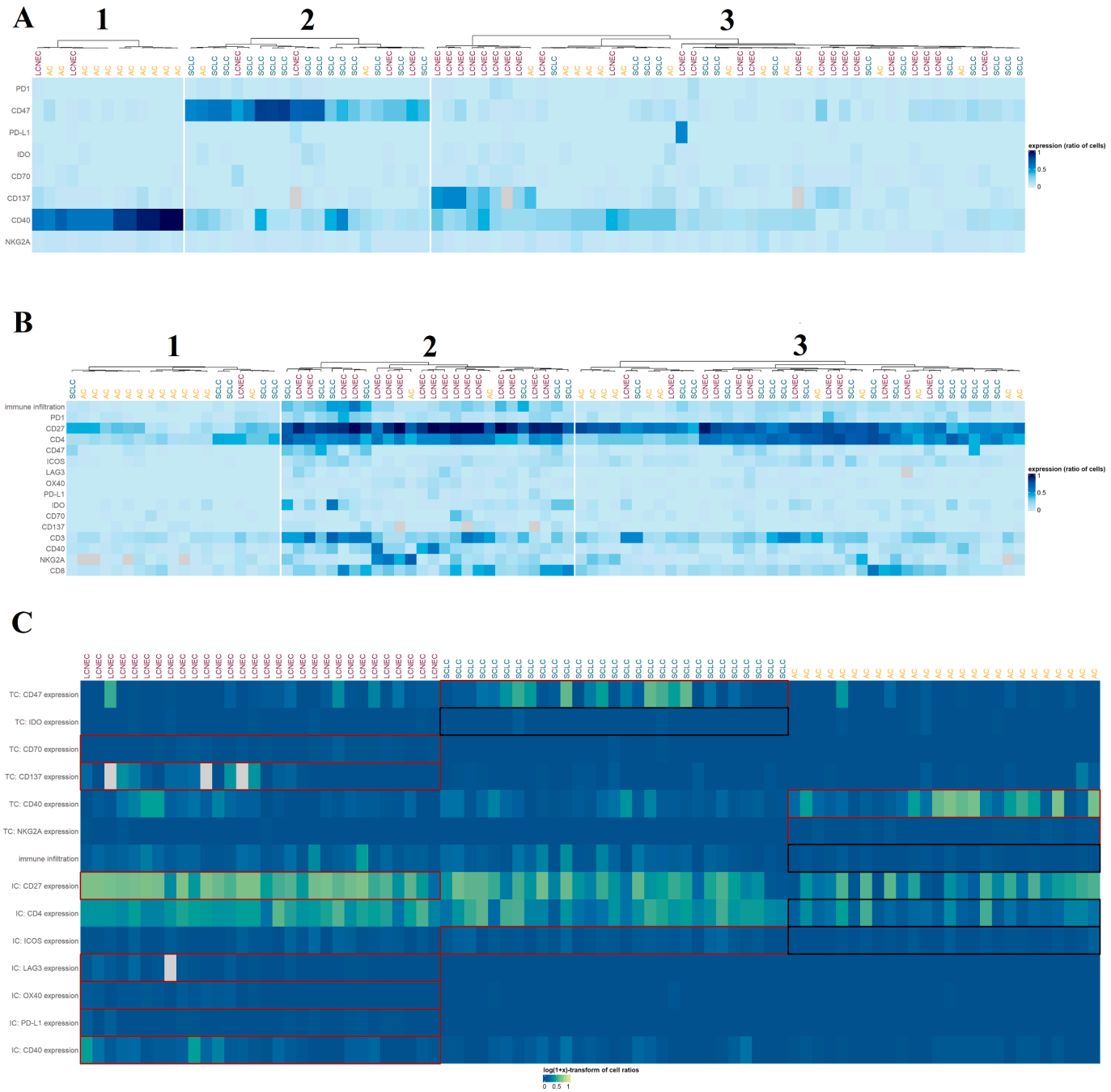

Fig. 3. (A) Hierarchical clustering of LNENs based on the tumor cell expression of immune-related markers. The color bar scale indicates the expression levels of the selected markers (PD-1, CD47, PD-L1, IDO, CD70, CD137, CD40, NKG2A). LCNEC, large cell neuroendocrine lung cancer; SCLC, small cell lung cancer; AC, atypical carcinoid. **(B) Hierarchical clustering based on the expression pattern of immune-related markers defined by the immune cells.** Unsupervised hierarchical clustering of immune cell expression levels separates the samples with different histological subtypes fairly well. The color bar scale indicates the expression levels of the selected markers (PD-1, CD27, CD4, CD47, ICOS, LAG3, OX40, IDO, CD70, CD137, CD3, CD40, NKG2A, CD8). LCNEC, large cell neuroendocrine lung cancer; SCLC, small cell lung cancer; AC, atypical carcinoid. **(C) Heatmap of the expression levels of different markers in tumor and immune cells.** The heatmap contains the covariates that had a non-zero coefficient value in at least one of the three logistic regression submodels of the fitted multinomial penalized linear regression model. Expression levels (x) were transformed with the log (1 + x) transformation to better differentiate between various color hues. Rectangles indicate the variables included in the model (red: positive coefficient, black: negative coefficient). TC: tumor cell, IC: immune cell, LCNEC, large cell neuroendocrine carcinoma; SCLC, small cell lung cancer; AC, atypical carcinoid.

3.5. Unsupervised hierarchical clustering of samples according to the immune cell-based TIM

As shown in Fig. 3B, LNEN samples can be separated fairly well based on the immune cell expression of the examined immune-related markers. AC tumors tended to be less immunogenic than SCLC and LCNEC tumors and the expression levels of CD3, CD8, CD27, and CD4 were also significantly lower in this histological subtype. Differences concerning the immune cell-based expression levels compared to the immune-related marker expression pattern of the tumor cells were less evident between the other two histological subtypes (SCLC and LCNEC). Nevertheless, immune cell expressions of CD27 and CD40 were higher in

LCNEC samples (vs. SCLC).

3.6. The impact of TIM on survival outcomes in LNEN patients

Division of patients into low- and high-expressing categories concerning each immune-related marker was performed by using the median value of the given marker. Univariate Kaplan-Meier survival analysis revealed a tendency towards impaired OS in patients with high (vs. low) CD47-expressing tumors (log-rank $p = 0.096$). In contrast, high tumor cell CD40 expression was associated with favorable survival outcomes (log-rank $p = 0.052$; Fig. 4A). As for the prognostic relevance of immune-related markers concerning immune cells, we found that high (vs. low) CD137 expression cells was associated with significantly improved OS (log-rank $p = 0.0096$, Fig. 4B). Meanwhile, patients with high immune cell ICOS expression tended to have worse survival outcomes compared to those with low ICOS expression (log-rank $p = 0.045$, Fig. 4B). In the case of CD8 and LAG3, a borderline significant tendency was observed between their immune cell-based expression levels and OS (Fig. 4B). Specifically, we found that high CD8 ($p = 0.083$) and LAG3 ($p = 0.15$) expression tended to be associated with impaired and improved OS, respectively. When analysing the SCLC and LCNEC samples only, none of the investigated markers' tumor cell expression demonstrated a significant effect on overall survival in a univariate setting (Supplementary Fig. S6A) As for the immune cells, a significant survival difference was found in the case of PD-1 ($p = 0.048$), CD27 ($p = 0.0043$), LAG3 ($p = 0.023$), CD4 ($p = 0.059$), CD137 ($p = 0.064$) and the amount of immune infiltration ($p = 0.021$) (Supplementary Fig. S6B).

3.7. Multivariate Cox-regression model for OS

A multivariate Cox-regression model fitted to the data revealed that only age ($p = 0.008$) and vascular involvement ($p = 0.012$) influenced the OS independently including all of the three histological subtypes). Accordingly, elderly patients or those with vascular involvement had worse survival outcomes (Supplementary Fig. S7A). Notably, as shown in (Supplementary Fig. S7B), when the model was limited to LCNEC and SCLC patients, only age remained and independent prognostic factor ($p = 0.05$), whereas the prognostic relevance of vascular involvement proved to be borderline significant ($p = 0.06$). None of the immune-related markers which influenced the OS in our univariate models remained significant in our multivariate models (Supplementary Fig. S7A and B).

3.8. Correlation between the expression patterns of immune-related markers defined by tumor cells and immune cells

Fig. 5 shows the statistically significant correlations between the evaluated markers and the general abundance of immune infiltrates. Supplementary Table S2 summarizes the R and p-values in the whole and in filtered dataset (R_{filt} and p_{filt}*). Among others, we found that PD-1 expression of the tumor cells correlates with CD70 expression of both immune and tumor cells ($R = 0.446$, $p^* = 0.0058$ and $R_{filt} = 0.6849$, $p_{filt}^* = <0.0001$, respectively). Likewise, we also observed that the general abundance of immune infiltrates significantly correlates with CD3 expression of immune cells ($R_{filt} = 0.6044$, $p_{filt}^* = 0.0206$). As for PD-L1, a significant positive linear correlation between tumor cell PD-L1 expression and LAG3 expression of the immune cells ($R_{filt} = 0.8294$, $p_{filt}^* = 0.0008$) was seen.

3.9. Multinomial penalized linear regression model predicts the LNENs' histological subtype

In order to evaluate whether the histological subtypes could be defined based on the TIM, a multinomial penalized linear regression model was used. The fitted model was able to predict the histological type of the LNEN with an overall accuracy of 90% in the training set and 77% overall accuracy in the test set. See Supplementary Fig. S8 for confusion matrices and additional performance metrics.

As an additional insight, we aimed to interpret model coefficients. Given that the multinomial model effectively consists of three separate penalized logistic regression submodels, their covariates can be used to differentiate between samples belonging to the given histological type and samples not belonging to that type. To this end, we plotted the expression and immune infiltration patterns of all samples in the dataset ordered by histological type (Fig. 3C) and added a black or red border to covariates that had a negative or positive coefficient in the given submodel, respectively. These results imply that greater tumor cell CD70 and CD137 expression and higher immune cell CD27, LAG3, OX40, PD-L1, and CD40 expression were measured in our LCNEC samples compared to the AC and SCLC cohort. The SCLC cohort was characterized by high expression levels of CD47 and low levels of IDO in tumor cell as well as by a generally high expression level of ICOS in immune cells compared to the AC and LCNEC groups. The AC group showed small amounts of immune infiltrates, high expression levels of CD40 and NKG2A by tumor cells and low expression levels of CD4 and ICOS by immune cells compared to LCNEC and SCLC samples.

4. Discussion

Gaining insights into different aspect of TIM is of clinical importance, as immune cells can impact tumor fate in different stages of disease [45]. Moreover, understanding the interaction between malignant cells and components of the immune system could lead to the development of more effective immunotherapeutic agents. Immunotherapy represents an intriguing weapon in the treatment of NSCLC patients; however, its exact role and mechanisms of action in LNENs have not yet been fully elucidated. Accordingly, in order to gain insight into potential biomarkers and pave the way for future immune checkpoint inhibitor-based strategies, there is an urgent need to study the TIM of these tumors. Here, we investigated the immunological landscape of NENs by assessing the expression pattern of 15 immune-related markers in surgically removed AC, LCNEC, and SCLC tumors.

So far, only a few potential predictive and prognostic biomarkers in LNENs have been proposed. The expression of CD44, the presence and degree of spread through air spaces (STAS) as well as the deletion of chromosome 11q (11q22.3-q25) and the mutations of multiple endocrine neoplasia type 1 (MEN1) have been reported to be negative prognosticators in AC tumors [5,46–49]. SCLC has been formerly considered a homogeneous disease with a single morphological type. Recent profiling studies, however, provide a framework to differentiate biologically distinct SCLC subtypes based on the expression of the following transcription factors: Achaete-Scute Homologue 1 (ASCL1), Neurogenic Differentiation Factor 1 (NEUROD1), POU Class 2 Homeobox 3 (POU2F3), and Yes-Associated Protein 1 (YAP1) [15,50]. Importantly, these biologically specific subgroups show major differences in their morphological features, growth properties, proteomic alterations, and prognosis [15,50–54]. LCNEC was also recently dismembered at the genomic and transcriptomic level, leading to the identification of two distinct subgroups. From a clinicopathological point of view, type I LCNEC tumors are similar to the classic variant of SCLC and are characterized by Retinoblastoma 1 (RB1) and Tumor Protein p53 (TP53) alterations. Meanwhile, type II LCNEC is a NSCLC-like variant frequently associated with Serine/threonine Kinase 11 (STK11), Kelch-like ECH Associated Protein 1 (KEAP1), and Kirsten Rat Sarcoma Virus (KRAS) alterations [7,55,56]. It is also important to mention that a high Ki-67 proliferation index is associated with worse progression-free survival (PFS) and OS in the vast majority of LNENs [57].

In the present study, unsupervised hierarchical clustering separated the samples of different LNEN subtypes fairly well based on the expression pattern of immune-related markers. Importantly, to the best of our knowledge, our study marks the largest immune panel evaluated in these malignancies to date. Although NKG2A is a late immune

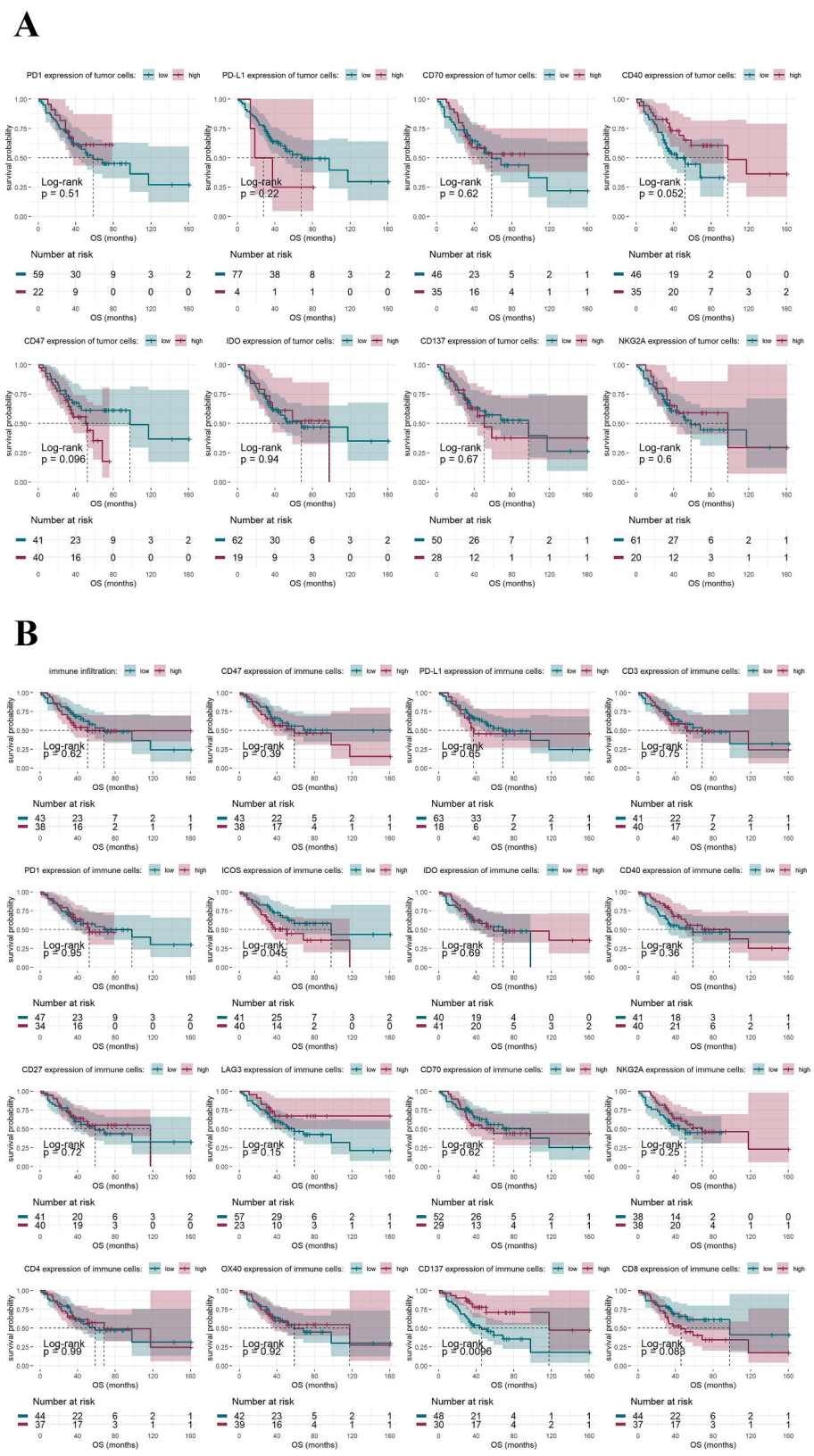

Fig. 4. Kaplan-Meier estimates for OS concerning the expression pattern of immune-related markers by tumor cells (A) and immune cells (B). ICs, immune cells; OS, overall survival; TCs, tumor cells.

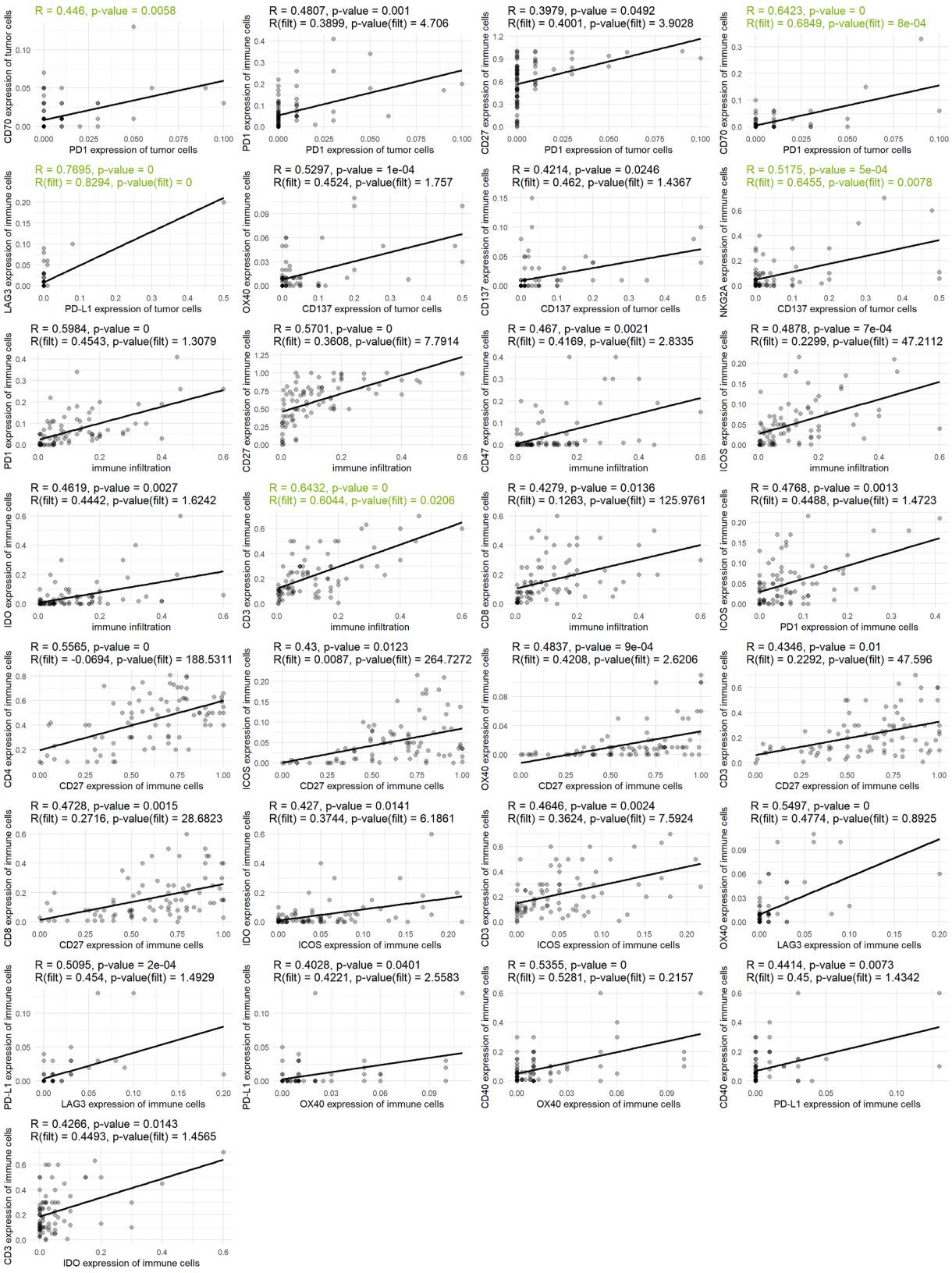

Fig. 5. Correlation between expressions of immune-related markers in tumor cells and immune cells. Only associations significant after Bonferroni-correction are shown. All p-values are adjusted for multiple comparisons. Results obtained for a filtered dataset including only samples with an immune infiltration of 0.1 or larger are indicated with "(filt)". If the observed correlation remained significant on the filtered dataset, the results are highlighted in green. ICs, immune cells; TCs, tumor cells.

checkpoint and marks repeated stimulation and cell division, its expression by tumor cells is still controversial. Importantly, several tumor cells, especially in case of AC samples, showed positivity for NKG2A in this study. This might be of clinical relevance since several antibodies blocking NKG2A are currently being tested in clinical trials [38]. CD47, on the other hand, has been reported to be overexpressed in several malignancies including breast cancer, pancreatic cancer and NSCLC [58–61]. Notably, CD47 expressing tumor cells transmit anti-phagocytic signals which aid in avoiding the antitumor immune response. Accordingly, high CD47 expression is associated with shortened PFS and OS in advanced-stage NSCLC patients [62]. In line with this, our univariate models demonstrated that high tumor cell CD47 expression is a negative prognosticator in LNENs. CD40 is also expressed in several malignancies (i.e. melanoma, colon, prostate, breast, and lung cancer) and its expression is linked with improved survival in lung cancer [63]. Moreover, high CD40 expression correlates with longer OS and enhances the anti-tumor immune response in melanoma patients. Similarly, high CD40 expression by tumor cells conferred a significant survival benefit for our LNEN patients. This may be due to the unique activatory interaction between CD40 expression and a type I anti-tumor response [44,63].

CD137 is another potent immune-modulating molecule that can promote and regulate anti-tumor immunity by interacting with antigen-presenting cells (APC) [64,65]. We found that high expression levels of CD137 by immune cells are suggestive for improved prognosis in LNENs. Importantly, publicly available datasets such as the GEPIA2 (Gene Expression Profiling Interactive Analysis 2) and TIMER 2.0 (Tumor Immune Estimation Resource 2.0) also suggest that high CD137 expression is a favorable prognostic factor in melanoma and HER2 + breast cancer [66,67]. Although CD8 + T lymphocytes were previously reported to have cytotoxic activity against malignant cells, heterogeneity among the various study populations deems these findings rather controversial. Kawai et al. demonstrated that the presence of CD8 + T-cells confers a significant survival benefit for stage IV NSCLC patients [68]. Conversely, others showed that the level of CD8 + T lymphocytes is associated with unfavorable 5-year survival rates in NSCLC [69,70]. In the current study, high CD8 + expression by immune cells tended to be a negative prognostic factor in LNEN patients. Our results are in contrast to the findings of Wang et al. who found that CD8 + TILs are associated with improved PFS and OS in LNEN patients [35]. However, this later study only included Asian individuals who might differ considerably from Caucasian lung cancer patients [71,72]. High CD8 expression was recently reported in inflamed SCLC subtype [52]. In our study, however, no meaningful conclusions could be drawn in this respect due to the small number of each histological subtype.

LAG3 is a novel immune checkpoint molecule that suppresses T cell activation and cytokine secretion and thereby ensures a state of immune homeostasis [73]. Accordingly, targeting LAG3 along with other checkpoints is considerably promising in cancer immunotherapy. Although LAG3 is expressed by a wide range of malignancies such as SCLC, and hepatocellular, gastric, ovarian, and renal cell carcinoma, its prognostic value is rather controversial [33,74]. According to a recent study, SCLC patients with high LAG3-expressing tumors have improved OS (vs. those with LAG-3^{low} tumors) [33]. Similarly, LNEN patients with high LAG3-expressing tumors had favorable survival outcomes in our cohort.

Since ICOS potentiates the CD4 + T cell-mediated immunosuppression, targeting the ICOS/ICOSL pathway holds considerable promise in cancer immunotherapy [41]. Indeed, early-phase clinical trials revealed that ICOS agonist monoclonal antibodies show promising antitumor activity, particularly when given in combination with other immune checkpoint inhibitors such as anti-PD-1 agents [41,75]. ICOS expression has been associated with improved survival outcomes in skin melanoma, head and neck squamous cell carcinoma as well as lung adenocarcinoma. Meanwhile, patients with high ICOS-expressing low-grade glioma and uveal melanoma tend to have a worse prognosis [76]. As for its expression and prognostic relevance in LNENs, we found that ICOS expression is the lowest in AC specimens and, moreover, that high ICOS expression is a negative prognosticator. These findings further support the hypothesis that AC tumors have a bleak immunological landscape. These patients might therefore not be eligible for immunotherapy.

In recent years, targeting the PD-1/PD-L1 axis has revolutionized the therapeutic armamentarium of many solid tumors including melanoma, urothelial carcinoma, and NSCLC [77]. Unfortunately, these promising results with immune checkpoint inhibitors have not been replicated in LNENs. Tumors of neuroendocrine origin, especially those with AC histology, generally have a low PD-L1 expression [21,31,34]. In rare cases when PD-L1 expression was reported, its expression correlated with improved survival in both LCNEC and SCLC patients [31,78]. In line with this, our study demonstrated low or absent PD-L1 expression. Specifically, PD-L1 was only expressed in a subset of LCNEC tumors and was absent in both SCLC and AC samples. Likewise, tumor cell PD-1 expression was also low in all three LNEN subtypes while the immune cells showed slightly higher expression levels. Importantly, neither PD-1 nor PD-L1 expression had a significant impact on survival in our cohort. Given that PD-L1 expression is much lower in these tumors than in NSCLC and that its expression level does not necessarily correlate with immune checkpoint inhibitor efficacy, other predictive biomarkers are needed for LNENs [79]. Among others, the tissue-based tumor mutation burden (TMB) or the tumor's inflammatory phenotype might represent promising alternative biomarkers in these cases [52,79].

It has been recently reported that a molecular link between low- and high-grade neuroendocrine neoplasms can exist [3,56,80]. In support of this, Alcala et al. identified a subgroup of atypical carcinoids (i.e. supracarcinoids) with carcinoid morphological pattern but with molecular characteristics similar to LCNEC [3]. Moreover, various studies suggest that LNENs are not monolithic entities and that combined NE carcinomas containing both SCLC and LCNEC (or even AC) components can exist [45,56,81,82]. This supports the concept of lineage plasticity concerning these tumors. In this context, besides the specific genes influencing NE differentiation and morphological aspects, the immune system might also have an impact on the tumor fate [45,81,82]. Notably, given that based on the used immune panel unsupervised clustering was able to separate tumor samples of different histology relatively well (despite the small study population), our results further support the presumable role of the immune system in influencing tumor fate. Nevertheless, no definitive conclusions could be drawn with regard to LNEN tumor transition based on our results. Doing so would require further investigation of the biological characteristics, molecular profile, and clinical behaviour of the tumors.

Our study has certain limitations that need to be addressed in future settings. Although we managed to collect a relatively large number of surgically treated LNEN samples ideal for profiling studies, the overall size of the study cohort remained small. In addition, our study is also partly limited by its retrospective design concerning the collection of clinicopathological variables and appropriate follow-up data. Thus, cancer-specific survivals were not available in the vast majority of cases. Another limitation may constitute that our study is not appropriate to study the direct effects of immunotherapy since we solely included surgically treated patients where ICIs are not part of the standard-of-care. Nevertheless, our results might be hypothesis-generating and provide a framework for future validation studies [83,84]. Lastly, although we analyzed surgically removed whole tissue sections and ten randomly selected areas in each sample, confounding effects due to tumoral heterogeneity must be considered and our results need to be interpreted accordingly.

5. Conclusions

Our study is among the first to investigate the specific aspects of TIM in surgically resected LNENs. By using a large panel of immune-related markers, we report that NENs have widely divergent immunologic

profiles and the expression pattern of investigated markers varies significantly within the different histological subtypes. These LNEN-specific immune signatures might be a valuable resource for the development of future immune checkpoint inhibitor-based therapeutic strategies.

Author contributions

(I) Study conception and design: Bence Ferencz, Zsolt Megyesfalvi, Kristóf Csende, János Fillinger, Viktória László, Balázs Döme, Judit Berta; (II) Administrative support: All authors; (III) Provision of study materials: Ferenc Rényi-Vámos, Karin Schelch, Viktória László, Balázs Döme, Judit Berta; (IV) Collection and assembly of data: Bence Ferencz, Zsolt Megyesfalvi, Kristóf Csende, János Fillinger, Valentin Poór, András Lantos, Orsolya Pipek, Christian Lang, Anna Schwendenwein, Kristiina Boettiger, Judit Berta; (V) Data analysis and interpretation: Bence Ferencz, Zsolt Megyesfalvi, Kristóf Csende, János Fillinger, András Lantos, Orsolya Pipek, Judit Berta; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Data availability statement

Data were generated by the authors and are available upon reasonable request.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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References

[1] A.E. Hendifar, A.M. Marchevsky, R. Tuli, Neuroendocrine tumors of the lung: current challenges and advances in the diagnosis and management of well-differentiated disease, J. Thorac. Oncol. 12 (3) (2017) 425–436.

[2] Righi, L., et al., *Lung neuroendocrine tumors: pathological characteristics.* J. Thorac. Dis., 2017. **9**(Suppl 15): p. S1442-S1447.

[3] N. Alcala, et al., Integrative and comparative genomic analyses identify clinically relevant pulmonary carcinoid groups and unveil the supra-carcinoids, Nat. Commun. 10 (1) (2019) 3407.

[4] M. Simbolo, S. Barbi, M. Fassan, A. Mafficini, G. Ali, C. Vicentini, N. Sperandio, V. Corbo, B. Rusev, L. Mastracci, F. Grillo, S. Pilotto, G. Pelosi, S. Pelliccioni, R. T. Lawlor, G. Tortora, G. Fontanini, M. Volante, A. Scarpa, E. Bria, Gene Expression Profiling of Lung Atypical Carcinoids and Large Cell Neuroendocrine Carcinomas Identifies Three Transcriptomic Subtypes with Specific Genomic Alterations, J. Thorac. Oncol. 14 (9) (2019) 1651–1661.

[5] Swarts, D.R., et al., *CD44 and OTP are strong prognostic markers for pulmonary carcinoids.* Clin. Cancer Res., 2013. **19**(8): p. 2197-207.

[6] R.F. Herde, K.E. Kokeny, C.B. Reddy, W.L. Akerley, N. Hu, J.P. Boltax, Y. J. Hitchcock, Primary Pulmonary Carcinoid Tumor: A Long-term Single Institution Experience, Am. J. Clin. Oncol. 41 (1) (2018) 24–29.

[7] J. George, et al., Integrative genomic profiling of large-cell neuroendocrine carcinomas reveals distinct subtypes of high-grade neuroendocrine lung tumors, Nat. Commun. 9 (1) (2018) 1048.

[8] S. Lantuejoul, L. Fernandez-Cuesta, F. Damiola, N. Girard, A. McLeer, New molecular classification of large cell neuroendocrine carcinoma and small cell lung carcinoma with potential therapeutic impacts, Transl Lung Cancer Res 9 (5) (2020) 2233–2244.

[9] M.E. Caplin, E. Baudin, P. Ferolla, P. Filosso, M. Garcia-Yuste, E. Lim, K. Oberg, G. Pelosi, A. Perren, R.E. Rossi, W.D. Travis, D. Bartsch, J. Capdevila, F. Costa, J. Cwikla, W. de Herder, G. Delle Fave, B. Eriksson, M. Falconi, D. Ferone, D. Gross, A. Grossman, T. Ito, R. Jensen, G. Kaltsas, F. Kelestimur, R. Kianmanesh, U. Knigge, B. Kos-Kudla, E. Krenning, E. Mitry, M. Nicolson, J. O'Connor, D. O'Toole, U.-F. Pape, M. Pavel, J. Ramage, E. Raymond, G. Rindi, A. Rockall, P. Ruszniewski, R. Salazar, A. Scarpa, E. Sedlackova, A. Sundin, C. Toumpanakis, M.-P. Vullierme, W. Weber, B. Wiedenmann, Z. Zheng-Pei, Pulmonary neuroendocrine (carcinoid) tumors: European Neuroendocrine Tumor Society expert consensus and recommendations for best practice for typical and atypical pulmonary carcinoids, Ann. Oncol. 26 (8) (2015) 1604–1620.

[10] J.L. Derks, L.E. Hendriks, W.A. Buikhuisen, H.J.M. Groen, E. Thunnissen, R.-J. van Suylen, R. Houben, R.A. Damhuis, E.J.M. Speel, A.-M. Dingemans, Clinical features of large cell neuroendocrine carcinoma: a population-based overview, Eur. Respir. J. 47 (2) (2016) 615–624.

[11] J.C. Yao, M. Hassan, A. Phan, C. Dagohoy, C. Leary, J.E. Mares, E.K. Abdalla, J. B. Fleming, J.-N. Vauthey, A. Rashid, D.B. Evans, One hundred years after "carcinoid": epidemiology of and prognostic factors for neuroendocrine tumors in 35,825 cases in the United States, J. Clin. Oncol. 26 (18) (2008) 3063–3072.

[12] A.F. Gazdar, P.A. Bunn, J.D. Minna, Small-cell lung cancer: what we know, what we need to know and the path forward, Nat. Rev. Cancer 17 (12) (2017) 765.

[13] K. Kahnert, D. Kauffmann-Guerrero, R.M. Huber, SCLC-State of the Art and What Does the Future Have in Store? Clin. Lung Cancer 17 (5) (2016) 325–333.

[14] C.M. Rudin, E. Brambilla, C. Faivre-Finn, J. Sage, Small-cell lung cancer, Nat. Rev. Dis. Primers 7 (1) (2021).

[15] A. Schwendenwein, Z. Megyesfalvi, N. Barany, Z. Valko, E. Bugyik, C. Lang, B. Ferencz, S. Paku, A. Lantos, J. Fillinger, M. Rezeli, G. Marko-Varga, K. Bogos, G. Galffy, F. Renyi-Vamos, M.A. Hoda, W. Klepetko, K. Hoetzenecker, V. Laszlo, B. Dome, Molecular profiles of small cell lung cancer subtypes: therapeutic implications, Mol. Ther. Oncolytics 20 (2021) 470–483.

[16] H. Taniguchi, T. Sen, C.M. Rudin, Targeted Therapies and Biomarkers in Small Cell Lung Cancer, Front. Oncol. 10 (2020) 741.

[17] S.M. Lim, M.H. Hong, H.R. Kim, Immunotherapy for Non-small Cell Lung Cancer: Current Landscape and Future Perspectives, Immune Netw 20 (1) (2020) e10.

[18] M. Albertelli, A. Dotto, F. Nista, A. Veresani, L. Patti, S. Gay, S. Sciallero, M. Boschetti, D. Ferone, Present and future of immunotherapy in Neuroendocrine Tumors, Rev. Endocr. Metab. Disord. 22 (3) (2021) 615–636.

[19] Klein, O., et al., Immunotherapy of Ipilimumab and Nivolumab in Patients with Advanced Neuroendocrine Tumors: A Subgroup Analysis of the CA209-538 Clinical Trial for Rare Cancers. Clin Cancer Res, 2020. **26**(17): p. 4454-4459.

[20] Y. Yamamoto, K. Ijichi, A.i. Koike, S. Nakamura, Y. Takahata, Y. Okamatsu, A. Fujita, S. Kawakami, T. Harada, Advanced atypical lung carcinoid tumour successfully treated with carboplatin, etoposide and atezolizumab: A case report, Respirol Case Rep 10 (5) (2022) e0951.

[21] Vesterinen, T., et al., *PD-1 and PD-L1 expression in pulmonary carcinoid tumors and their association to tumor spread.* Endocr Connect, 2019. **8**(8): p. 1168-1175.

[22] J. Nestor, K. Barnaby, M. Esposito, N. Seetharamu, Treatment of atypical pulmonary carcinoid with combination ipilimumab and nivolumab, BMJ Case Rep. 12 (11) (2019) e231029.

[23] S. Di Molfetta, T. Feola, G. Fanciulli, T. Florio, A. Colao, A. Faggiano, Immune Checkpoint Blockade in Lung Carcinoids with Aggressive Behaviour: One More Arrow in Our Quiver? J. Clin. Med. 11 (4) (2022) 1019.

[24] S. Zimmermann, S. Peters, T. Owinokoko, S.M. Gadgeel, Immune Checkpoint Inhibitors in the Management of Lung Cancer, Am. Soc. Clin. Oncol. Educ. Book (38) (2018) 682–695.

[25] A. Pavan, I. Attili, G. Pasello, V. Guarneri, P.F. Conte, L. Bonanno, Immunotherapy in small-cell lung cancer: from molecular promises to clinical challenges, J. Immunother. Cancer 7 (1) (2019).

[26] T. Komiya, N. Ravindra, E. Powell, Role of Immunotherapy in Stage IV Large Cell Neuroendocrine Carcinoma of the Lung, Asian Pac. J. Cancer Prev. 22 (2) (2021) 365–370.

[27] T. Berghmans, A.-M. Dingemans, L.E.L. Hendriks, J. Cadranel, Immunotherapy for nonsmall cell lung cancer: a new therapeutic algorithm, Eur. Respir. J. 55 (2) (2020) 1901907.

[28] R.M. Bremnes, L.-T. Busund, T.L. Kilvær, S. Andersen, E. Richardsen, E.E. Paulsen, S. Hald, M.R. Khanehkenari, W.A. Cooper, S.C. Kao, T. Dønnem, The Role of Tumor-Infiltrating Lymphocytes in Development, Progression, and Prognosis of Non-Small Cell Lung Cancer, J. Thorac. Oncol. 11 (6) (2016) 789–800.

[29] I. Gataa, L. Mezquita, C. Rossoni, E. Auclin, M. Kossai, F. Aboubakar, S. Le Moulec, J. Massé, M. Masson, N. Radosevic-Robin, P. Alemany, M. Rouanne, V. Bluthgen, L. Hendriks, C. Caramella, A. Gazzah, D. Planchard, J.-P. Pignon, B. Besse, J. Adam, Tumour-infiltrating lymphocyte density is associated with favourable outcome in patients with advanced non-small cell lung cancer treated with immunotherapy, Eur. J. Cancer 145 (2021) 221–229.

[30] Y. Fan, et al., Prognostic value of PD-L1 and PD-1 expression in pulmonary neuroendocrine tumors, Onco Targets Ther 9 (2016) 6075–6082.

[31] Kasajima, A., et al., *Inflammation and PD-L1 expression in pulmonary neuroendocrine tumors.* Endocr Relat Cancer, 2018. **25**(3): p. 339-350.

[32] M. Orozco-Morales, et al., Clinicopathological and Prognostic Significance of CD47 Expression in Lung Neuroendocrine Tumors, J. Immunol. Res. 2021 (2021) 6632249.

[33] Sun, H., et al., Lymphocyte activation gene-3 is associated with programmed death-ligand 1 and programmed cell death protein 1 in small cell lung cancer. Ann Transl Med, 2021. **9**(18): p. 1468.

[34] K. Tsuruoka, H. Horinouchi, Y. Goto, S. Kanda, Y. Fujiwara, H. Nokihara, N. Yamamoto, K. Asakura, K. Nakagawa, H. Sakurai, S.-I. Watanabe, K. Tsuta, Y. Ohe, PD-L1 expression in neuroendocrine tumors of the lung, Lung Cancer 108 (2017) 115–120.

[35] H. Wang, Z. Li, B. Dong, W. Sun, X. Yang, R. Liu, L. Zhou, X. Huang, L. Jia, D. Lin, Prognostic significance of PD-L1 expression and CD8+ T cell infiltration in pulmonary neuroendocrine tumors, Diagn. Pathol. 13 (1) (2018).

[36] S. Aspeslagh, S. Postel-Vinay, S. Rusakiewicz, J.-C. Soria, L. Zitvogel, A. Marabelle, Rationale for anti-OX40 cancer immunotherapy, Eur. J. Cancer 52 (2016) 50–66.

[37] J. Borst, T. Ahrends, N. Bąbała, C.J.M. Melief, W. Kastenmüller, CD4(+) T cell help in cancer immunology and immunotherapy, Nat. Rev. Immunol. 18 (10) (2018) 635–647.

[38] L. Borst, et al., NKG2A is a late immune checkpoint on CD8 T cells and marks repeated stimulation and cell division, Int. J. Cancer 150 (4) (2022) 688–704.

[39] D. Dora, C. Rivard, H. Yu, P. Bunn, K. Suda, S. Ren, S. Lueke Pickard, V. Laszlo, T. Harko, Z. Megyesfalvi, J. Moldvay, F.R. Hirsch, B. Dome, Z. Lohinai, Neuroendocrine subtypes of small cell lung cancer differ in terms of immune microenvironment and checkpoint molecule distribution, Mol. Oncol. 14 (9) (2020) 1947–1965.

[40] I. Etxeberria, J. Glez-Vaz, Á. Teijeira, I. Melero, New emerging targets in cancer immunotherapy: CD137/4-1BB costimulatory axis, ESMO Open 4 (2019) e000733.

[41] C. Solinas, C. Gu-Trantien, K. Willard-Gallo, The rationale behind targeting the ICOS-ICOS ligand costimulatory pathway in cancer immunotherapy, ESMO Open 5 (1) (2020) e000544.

[42] A.M. Starzer, A.S. Berghoff, New emerging targets in cancer immunotherapy: CD27 (TNFRSF7), ESMO Open 4 (Suppl 3) (2020) e000629.

[43] Weiskopf, K., et al., *CD47-blocking immunotherapies stimulate macrophage-mediated destruction of small-cell lung cancer.* J Clin Invest, 2016. **126**(7): p. 2610-20.

[44] C. Yan, A. Richmond, Hiding in the dark: pan-cancer characterization of expression and clinical relevance of CD40 to immune checkpoint blockade therapy, Mol. Cancer 20 (1) (2021) 146.

[45] H. Gonzalez, C. Hagerling, Z. Werb, Roles of the immune system in cancer: from tumor initiation to metastatic progression, Genes Dev. 32 (19–20) (2018) 1267–1284.

[46] S. Altinay, J. Metovic, F. Massa, G. Gatti, P. Cassoni, G.V. Scagliotti, M. Volante, M. Papotti, Spread through air spaces (STAS) is a predictor of poor outcome in atypical carcinoids of the lung, Virchows Arch. 475 (3) (2019) 325–334.

[47] D.R.A. Swarts, S.M.H. Claessen, Y.M.H. Jonkers, R.-J. van Suylen, A.-M. Dingemans, W.W. de Herder, R.R. de Krijger, E.F. Smit, F.B.J.M. Thunnissen, C. A. Seldenrijk, A. Vink, A. Perren, F.C.S. Ramaekers, E.-J. Speel, Deletions of 11q22.3-q25 are associated with atypical lung carcinoids and poor clinical outcome, Am. J. Pathol. 179 (3) (2011) 1129–1137.

[48] Swarts, D.R., et al., *MEN1 gene mutation and reduced expression are associated with poor prognosis in pulmonary carcinoids.* J Clin Endocrinol Metab, 2014. **99**(2): p. E374-8.

[49] L.T. Senbanjo, M.A. Chellaiah, CD44: A Multifunctional Cell Surface Adhesion Receptor Is a Regulator of Progression and Metastasis of Cancer Cells, Front. Cell Dev. Biol. 5 (2017) 18.

[50] C.M. Rudin, J.T. Poirier, L.A. Byers, C. Dive, A. Dowlati, J. George, J.V. Heymach, J.E. Johnson, J.M. Lehman, D. MacPherson, P.P. Massion, J.D. Minna, T.G. Oliver, V. Quaranta, J. Sage, R.K. Thomas, C.R. Vakoc, A.F. Gazdar, Molecular subtypes of small cell lung cancer: a synthesis of human and mouse model data, Nat. Rev. Cancer 19 (5) (2019) 289–297.

[51] M.K. Baine, M.-S. Hsieh, W.V. Lai, J.V. Egger, A.A. Jungbluth, Y. Daneshbod, A. Beras, R. Spencer, J. Lopardo, F. Bodd, J. Montecalvo, J.L. Sauter, J.C. Chang, D. J. Buonocore, W.D. Travis, T. Sen, J.T. Poirier, C.M. Rudin, N. Rekhtman, SCLC Subtypes Defined by ASCL1, NEUROD1, POU2F3, and YAP1: A Comprehensive Immunohistochemical and Histopathologic Characterization, J. Thorac. Oncol. 15 (12) (2020) 1823–1835.

[52] C.M. Gay, C.A. Stewart, E.M. Park, L. Diao, S.M. Groves, S. Heeke, B.Y. Nabet, J. Fujimoto, L.M. Solis, W. Lu, Y. Xi, R.J. Cardnell, Q.i. Wang, G. Fabbri, K. R. Cargill, N.I. Vokes, K. Ramkumar, B. Zhang, C.M. Della Corte, P. Robson, S. G. Swisher, J.A. Roth, B.S. Glisson, D.S. Shames, I.I. Wistuba, J. Wang, V. Quaranta, J. Minna, J.V. Heymach, L.A. Byers, Patterns of transcription factor programs and immune pathway activation define four major subtypes of SCLC with distinct therapeutic vulnerabilities, Cancer Cell 39 (3) (2021) 346–360.e7.

[53] Z. Megyesfalvi, N. Barany, A. Lantos, Z. Valko, O. Pipek, C. Lang, A. Schwendenwein, F. Oberndorfer, S. Paku, B. Ferencz, K. Dezso, J. Fillinger, Z. Lohinai, J. Moldvay, G. Galffy, B. Szeitz, M. Rezeli, C. Rivard, F.R. Hirsch, L. Brcic, H. Popper, I. Kern, M. Kovacevic, J. Skarda, M. Mittak, G. Marko-Varga, K. Bogos, F. Renyi-Vamos, M.A. Hoda, T. Klikovits, K. Hoetzenecker, K. Schelch, V. Laszlo, B. Dome, Expression patterns and prognostic relevance of subtype-specific transcription factors in surgically resected small-cell lung cancer: an international multicenter study, J. Pathol. 257 (5) (2022) 674–686.

[54] B. Szeitz, Z. Megyesfalvi, N. Woldmar, Z. Valkó, A. Schwendenwein, N. Bárány, S. Paku, V. László, H. Kiss, E. Bugyik, C. Lang, A.M. Szász, L. Pizzatti, K. Bogos, M. A. Hoda, K. Hoetzenecker, G. Marko-Varga, P. Horvatovich, B. Döme, K. Schelch, M. Rezeli, In-depth proteomic analysis reveals unique subtype-specific signatures in human small-cell lung cancer, Clin. Transl. Med. 12 (9) (2022) e1060.

[55] J.L. Derks, N. Leblay, S. Lantuejoul, A.-M. Dingemans, E.-J. Speel, L. Fernandez-Cuesta, New Insights into the Molecular Characteristics of Pulmonary Carcinoids and Large Cell Neuroendocrine Carcinomas, and the Impact on Their Clinical Management, J. Thorac. Oncol. 13 (6) (2018) 752–766.

[56] Rekhtman, N., et al., Next-Generation Sequencing of Pulmonary Large Cell Neuroendocrine Carcinoma Reveals Small Cell Carcinoma-like and Non-Small Cell Carcinoma-like Subsets. Clin Cancer Res, 2016. **22**(14): p. 3618-29.

[57] Wang, H.Y., et al., Automated quantification of Ki-67 index associates with pathologic grade of pulmonary neuroendocrine tumors. Chin Med J (Engl), 2019. **132**(5): p. 551-561.

[58] Michaels, A.D., et al., *CD47 Blockade as an Adjuvant Immunotherapy for Resectable Pancreatic Cancer.* Clin Cancer Res, 2018. **24**(6): p. 1415-1425.

[59] A. Nigro, et al., Enhanced Expression of CD47 Is Associated With Off-Target Resistance to Tyrosine Kinase Inhibitor Gefitinib in NSCLC, Front. Immunol. 10 (2019) 3135.

[60] J. Yuan, X. Shi, C. Chen, H. He, L. Liu, J. Wu, H. Yan, High expression of CD47 in triple negative breast cancer is associated with epithelial-mesenchymal transition and poor prognosis, Oncol. Lett. (2019).

[61] H. Zhao, et al., CD47 Promotes Tumor Invasion and Metastasis in Non-small Cell Lung Cancer, Sci. Rep. 6 (2016) 29719.

[62] O. Arrieta, A. Aviles-Salas, M. Orozco-Morales, N. Hernández-Pedro, A.F. Cardona, L. Cabrera-Miranda, P. Barrios-Bernal, G. Soca-Chafre, G. Cruz-Rico, M.d.L. Peña-Torres, G. Moncada-Claudio, L.-A. Ramirez-Tirado, Association between CD47 expression, clinical characteristics and prognosis in patients with advanced non-small cell lung cancer, Cancer Med. 9 (7) (2020) 2390–2402.

[63] M.S. Sabel, M. Yamada, Y. Kawaguchi, F.-A. Chen, H. Takita, R.B. Bankert, CD40 expression on human lung cancer correlates with metastatic spread, Cancer Immunol. Immunother. 49 (2) (2000) 101–108.

[64] C. Glorieux, P. Huang, CD137 expression in cancer cells: regulation and significance, Cancer Commun (Lond) 39 (1) (2019) 70.

[65] C. Wang, G.H.Y. Lin, A.J. McPherson, T.H. Watts, Immune regulation by 4–1BB and 4–1BBL: complexities and challenges, Immunol. Rev. 229 (1) (2009) 192–215.

[66] Tang, Z., et al., *GEPIA2: an enhanced web server for large-scale expression profiling and interactive analysis.* Nucleic Acids Res, 2019. **47**(W1): p. W556-W560.

[67] Li, T., et al., *TIMER2.0 for analysis of tumor-infiltrating immune cells.* Nucleic Acids Res, 2020. **48**(W1): p. W509-W514.

[68] O. Kawai, G. Ishii, K. Kubota, Y. Murata, Y. Naito, T. Mizuno, K. Aokage, N. Saijo, Y. Nishiwaki, A. Gemma, S. Kudoh, A. Ochiai, Predominant infiltration of macrophages and CD8(+) T Cells in cancer nests is a significant predictor of survival in stage IV nonsmall cell lung cancer, Cancer 113 (6) (2008) 1387–1395.

[69] C. Tian, S. Lu, Q. Fan, W. Zhang, S. Jiao, X. Zhao, Z. Wu, L. Sun, L. Wang, Prognostic significance of tumor-infiltrating CD8(+) or CD3(+) T lymphocytes and interleukin-2 expression in radically resected non-small cell lung cancer, Chin Med J (Engl) 128 (1) (2015) 105–110.

[70] O. Wakabayashi, K. Yamazaki, S. Oizumi, F. Hommura, I. Kinoshita, S. Ogura, H. Dosaka-Akita, M. Nishimura, CD4+ T cells in cancer stroma, not CD8+ T cells in cancer cell nests, are associated with favorable prognosis in human non-small cell lung cancers, Cancer Sci. 94 (11) (2003) 1003–1009.

[71] M. Izumi, T. Suzumura, K. Ogawa, Y. Matsumoto, K. Sawa, N. Yoshimoto, Y. Tani, T. Watanabe, H. Kaneda, S. Mitsuoka, K. Asai, T. Kawaguchi, Differences in molecular epidemiology of lung cancer among ethnicities (Asian vs, *Caucasian).* J Thorac Dis 12 (7) (2020) 3776–3784.

[72] W. Zhou, D.C. Christiani, East meets West: ethnic differences in epidemiology and clinical behaviors of lung cancer between East Asians and Caucasians, Chin. J. Cancer 30 (5) (2011) 287–292.

[73] Y. He, C.J. Rivard, L. Rozeboom, H. Yu, K. Ellison, A. Kowalewski, C. Zhou, F. R. Hirsch, Lymphocyte-activation gene-3, an important immune checkpoint in cancer, Cancer Sci. 107 (9) (2016) 1193–1197.

[74] L. Long, X. Zhang, F. Chen, Q.i. Pan, P. Phiphatwatchara, Y. Zeng, H. Chen, The promising immune checkpoint LAG-3: from tumor microenvironment to cancer immunotherapy, Genes Cancer 9 (5-6) (2018) 176–189.

[75] F. Amatore, L. Gorvel, D. Olive, Role of Inducible Co-Stimulator (ICOS) in cancer immunotherapy, Expert Opin. Biol. Ther. 20 (2) (2020) 141–150.

[76] G. Wu, M. He, K. Ren, H. Ma, Q. Xue, Inducible Co-Stimulator ICOS Expression Correlates with Immune Cell Infiltration and Can Predict Prognosis in Lung Adenocarcinoma, Int J Gen Med Volume 15 (2022) 3739–3751.

[77] Y. He, H. Yu, L. Rozeboom, C.J. Rivard, K. Ellison, R. Dziadziuszko, K. Suda, S. Ren, C. Wu, L. Hou, C. Zhou, F.R. Hirsch, LAG-3 Protein Expression in Non-Small Cell Lung Cancer and Its Relationship with PD-1/PD-L1 and Tumor-Infiltrating Lymphocytes, J. Thorac. Oncol. 12 (5) (2017) 814–823.

[78] H. Yu, C. Batenchuk, A. Badzio, T.A. Boyle, P. Czapiewski, D.C. Chan, X. Lu, D. Gao, K. Ellison, A.A. Kowalewski, C.J. Rivard, R. Dziadziuszko, C. Zhou, M. Hussein, D. Richards, S. Wilks, M. Monte, W. Edenfield, J. Goldschmidt, R. Page, B. Ulrich, D. Waterhouse, S. Close, J. Jassem, K. Kulig, F.R. Hirsch, PD-L1 Expression by Two Complementary Diagnostic Assays and mRNA In Situ Hybridization in Small Cell Lung Cancer, J. Thorac. Oncol. 12 (1) (2017) 110–120.

[79] B.J. Drapkin, C.M. Rudin, Advances in Small-Cell Lung Cancer (SCLC) Translational Research, Cold Spring Harb. Perspect. Med. 11 (4) (2021) a038240.

[80] G. Pelosi, F. Bianchi, E. Dama, M. Simbolo, A. Mafficini, A. Sonzogni, S. Pilotto, S. Harari, M. Papotti, M. Volante, G. Fontanini, L. Mastracci, A. Albini, E. Bria, F. Calabrese, A. Scarpa, Most high-grade neuroendocrine tumours of the lung are likely to secondarily develop from pre-existing carcinoids: innovative findings skipping the current pathogenesis paradigm, Virchows Arch. 472 (4) (2018) 567–577.

[81] A.S. Ireland, A.M. Micinski, D.W. Kastner, B. Guo, S.J. Wait, K.B. Spainhower, C. C. Conley, O.S. Chen, M.R. Guthrie, D. Soltero, Y.i. Qiao, X. Huang, S. Tarapcsák, S. Devarakonda, M.D. Chalishazar, J. Gertz, J.C. Moser, G. Marth, S. Puri, B.L. Witt, B.T. Spike, T.G. Oliver, MYC Drives Temporal Evolution of Small Cell Lung Cancer Subtypes by Reprogramming Neuroendocrine Fate, Cancer Cell 38 (1) (2020) 60–78.e12.

[82] N. Rekhtman, Lung neuroendocrine neoplasms: recent progress and persistent challenges, Mod. Pathol. 35 (Suppl 1) (2022) 36–50.

[83] https://www.nccn.org/guidelines/guidelines-detail?category=1&id=1448. *NCCN Clinical Practice Guidelines in Oncology: Neuroendocrine and Adrenal Tumors*. Version 2.2022 — December 21, 2022.

[84] https://www.nccn.org/guidelines/guidelines-detail?category=1&id=1462. *NCCN Clinical Practice Guidelines in Oncology: Small Cell Lung Cancer*. Version 3.2023 — December 21, 2022.