The immunologic landscape of intermediate- and high-grade neuroendocrine lung tumors

Ph.D. thesis

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1. Introduction

Lung cancer is one of the most common malignancies and the leading cause of cancer-related deaths worldwide. Lung neuroendocrine neoplasms (LNENs) are a heterogeneous group of tumors that mostly originate from the neuroendocrine cells of the lung. They account for approximately 20% of primary lung cancer. According to the current World Health Organization (WHO) classification 4 histological types are distinguished. Typical carcinoids (TC) are well differentiated tumors, that account for 1.8% of pulmonary malignancies. In contrast, atypical carcinoids (AC) are moderately differentiated lung tumors comprising 0.2% of all lung cancers. Carcinoids usually appear between the 4th and 6th decade of life, with a median age of 45 years. Other histological types of LNENs (as per WHO definition) include large cell neuroendocrine lung cancer (LCNEC) and small cell lung cancer (SCLC). LCNEC account for 3% of lung cancers and represent poorly differentiated, high-grade tumors with complex biological features that share many similarities to SCLC. SCLC is a heterogeneous malignancy characterized mainly by genetic instability, early metastasis and high proliferative activity. These malignancies are frequently detected at an advanced stage, after several metastases have developed, making surgical treatment rarely feasible

2. Objectives

Intermediate- and high-grade neuroendocrine lung neoplasms, especially LCNEC and SCLC are aggressive tumors, with high metastatic potential and poor prognosis. Due to the fact that they are relatively rare entitites with complex biology, our knowledge is still very limited. Accordingly, the therapeutic arsenal has not changed significantly over the last 30 years. Although targeted therapies, and immunotherapy in particular, have gained ground in recent years for several malignancies, this remarkable progress has been somewhat lagging behind in the case of pulmonary neuroendocrine neoplasms. Since AC is less sensitive to currently used chemotherapeutic agents, while LCNEC and SCLC are sensitive but become resistant relatively quickly, implementing targeted- and immunotherapeutic approaches for these tumors would be crucial. Therefore, investigating the immunological phenotypes and specific immune signatures, as well as the tumor immune microenvironment of surgically resected LNENs might be the first step to develop effective therapeutic approaches for these devastating diseases as soon as possible. As a result, it is expected that the efficacy of currently available immune checkpoint inhibitors (ICIs) will be improved and the use of these agents can be optimized based on TIM. In addition, our

results would also contribute to the development of second-generation ICIs in the future.

We also investigated whether the immune marker expression signature of different tumors can be used to classify a tumor into its appropriate histological category. This has a particularly important relevance in case of small biopsy specimens, as sometimes there are serious diagnostic pitfalls in establishing the accurate diagnosis by using these small tissue samples. Finally, in order to also provide insights into the applicability of immunotherapy, we also aimed to examine the expression levels and distribution patterns of 4 novel immunotherapeutic markers (OX40L, VISTA, TIM3, GITR) of potential therapeutic relevance in SCLC, AC, and LCNEC patients.

3. Methods

In our multicenter retrospective study, we have included 156 Caucasian patients with histologically confirmed LNENs who underwent surgical resection at one of the four Central European centers between 1997 and 2021. The four centers were the National Korányi Institute of Pulmonology (Budapest, Hungary), the National Institute of Oncology (Budapest, Hungary), Medical University of Graz (Graz, Austria) and Palacky University Olomouc (Olomouc, Czech Republic). Of these, 26, 64, and 66 patients were diagnosed with AC, LCNEC and SCLC, respectively. Clinicopathological data were retrospectively collected from the medical records of each center. An important inclusion criterion was that only whole-tissue specimens were included to avoid bias due to intratumoral heterogeneity.

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 contemporary NCCN guidelines.

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 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. In the next step, tissue sections were analyzed for the expression of the following 19 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), lymphocyte-activation gene 3 (LAG3), OX40, OX40L, V-domain Ig suppressor of T cell activation (VISTA), glucocorticoid-induced TNF receptor (GITR) and T cell immunoglobulin and mucin domain 3 (TIM3). The expression patterns of the first 15 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 were examined in 26 AC, 30 LCNEC, and 29 SCLC. In our second cohort, the expression patterns of OX40L, GITR, TIM3, and VISTA were examined in 26 AC 49 LCNEC and 66 SCLC.

Unfortunately, due to low tissue sample size, in case of 21 SCLCs, only VISTA staining was performed. Totally, 75 cases were overlapping between the two cohorts. Briefly, after deparaffinization and rehydration, sections were incubated in a 3% H2O2 solution, in order to reduce nonspecific background staining. Next, tissue samples were heated in a 10 mM Citrate buffer (pH = 6.0) or 10 mM Tris-EDTA buffer (pH = 9.0). Slides were incubated at room temperature, followed by primary antibody incubation overnight at 4^{o}C. Primary antibodies were visualized by 3-3′-diaminobenzidine (DAB) and counterstained with hematoxylin. Of note, the staining protocol was validated by appropriate positive tissue controls. Expression of the given marker was examined blinded to clinical data by two experienced independent lung pathologists. Therefore, during pathological evaluation, we determined the percentage of positive tumor cells in at least 20 randomly selected areas at 20x and 40x magnifications. Two experienced pulmonary pathologists performed the evaluation process, and if a discrepancy of more than 20% occurred in their results, a third pulmonary pathologist was also involved.

Tumor cells were evaluated separately from immune cells. In the case of tumor cells, the ratio of positive cells to all tumor cells was also quantified. Similarly, the ratio of immune cells

showing positive staining and the ratio of total immune infiltrates in a given sample was determined.

All statistical analyses were performed in R version 4.2.1 (R Foundation for Statistical Computing, Vienna, Austria).

Expression levels of immune markers of the tumor cells or and the amount of immune infiltration, dividing the patients into low and high categories for each marker was performed by using the median of the measured values (for the given marker) as a cutoff value. Patients with a measured level not larger than the median were categorized into the “low” group, while patients with a measured level higher than the median were included in the “high” category. Expression levels of immune-related markers were compared between tumors of different histological types in a pairwise manner with Wilcoxon signed-rank tests and Bonferroni-correction was used to adjust for multiple testing. Whenever at least one of the three pairwise comparisons resulted in a corrected p-value of 0.05 or lower, the association was considered significant.

Hierarchical clustering of samples based on expression levels was performed with the Complex Heatmap R package (version 2.10.0) with the “ward.D2” clustering method using Eucledian-distance measure.(96) The distance matrix was calculated using Manhattan distance measure and the dendrograms were created

using the ward:D clusterin method. 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.

As an exploratory approach, we additionally built a multinomial penalized linear regression model using the glmnet R package (version 4.1-4) to predict histological type by observing all measured expression levels and the amount of immune infiltration.(97) Penalization was necessary to avoid over-fitting due to the relatively low number of samples and large number of covariates. The dataset was assigned to training and test sets by a random 60-40% split. Missing expression levels and data on immune infiltration were imputed with zeros. The model was trained on the training set and hyperparameter tuning was performed by the default 10-fold cross-validation technique of the cv.glmnet() function with the family = "multinomial" setting. Model performance was measured on both the training and test sets, the latter of which serves as a more reliable indicator of goodness-of-fit.

To investigate which expression levels of OX40L, TIM3, VISTA and GITR are most indicative of LNEN subtype, a

principal component analysis (PCA) was performed (with the factoextra R package (version 1.0.7)) to find linear combinations („principal components”) of the measured variables (expression levels) that most effectively explain the variance in the data.

4. Results

The vast majority of SCLC and LCNEC patients in our cohort were smokers, whereas the majority of individuals diagnosed with AC were never-smokers. SCLC tumors tended to be centrally located contrasting the peripheral localization of LCNEC. Due to the availability of bronchoscopic findings in the majority of cases, the localization of the primary tumor was determined on the basis of bronchoscopical visualization.

First of all, in order to investigate the key differences of immune-related markers in their IHC expression, we evaluated markers with available expression levels in at least one of the LNEN subtypes for at least one patient. 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, the expression patterns and of the other markers showed a different distribution in at least one of the three groups. Specifically, NKG2A and CD40 expressions were significantly higher ($p<0.05$) in tumor cells of 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 ($p<0.05$). We also evaluated the differences and similarities in case of the four immunotherapeutic markers, and we found, that OX40L expression of AC tumor cells were significantly lower than in SCLC tumors ($p<0.001$). Meanwhile, ACs tended to demonstrate significantly higher tumor cell GITR expression levels than SCLC or LCNEC tumors ($p<0.001$). Of note, tumor cell GITR expression was also considerably higher in SCLC than in LCNEC ($p=0.011$). As for TIM3, its TC expression was significantly higher in ACs (vs. LCNEC and SCLC tumors; $p=0.047$ and $p<0.001$, respectively). No significant differences were observed for VISTA expression.

The unsupervised hierarchical clustering based on the IHC expression of the immune-related 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.

We also examined also, whether LNEN subtypes can be distinguished solely by their tumor cell VISTA, GITR, OX40L, or TIM3 expression. Although cluster analysis differentiated three distinct subgroups with divergent immunologic phenotypes, these clusters did not conclude with the histological subtypes.

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. The abundance of immune infiltrates was similar in SCLC and LCNEC samples, but notably lower in AC specimens ($p<0.001$). 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.

In case of therapeutic target markers ACs expressed significantly lower levels of immune cell VISTA ($p<0.001$) and GITR ($p=0.002$) than LCNEC or SCLC tumors. Meanwhile,

TIM3 expression by immune cells was significantly lower in SCLCs compared to ACs ($p<0.001$) or LCNECs ($p<0.001$).

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).

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. 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.

It has become clear, that based on a cluster analysis was not able to distinguish LNEN subtypes based solely on VISTA, GITR, OX40L, or TIM3 expression by immune cells.

A principal component analysis was also performed, which revealed, that ACs can be distinguished from both LCNEC and SCLC tumors based on their immune cell and tumor cell marker expression. In this context, ACs express high levels of tumor cell TIM3 and GITR, and low levels of immune cell GITR; both tumor cells and immune cells of SCLCs express high levels of GITR, and their immune cells express low levels of TIM3; and immune cells of LCNECs express high levels of GITR and TIM3.

Over the course of our two projects, a total of 19 immune markers were investigated. Expression pattern of 15 immune-related markers (PD-1, CD27, CD4, CD47, ICOS, LAG3, OX40, PD-L1, IDO, CD70, CD137, CD3, CD40, NKG2A,

CD8) and 4 novel immunotherapeutic targets (OX40L, GITR, TIM3, VISTA) was analyzed in a representative number of AC, LCNEC, and SCLC samples. Since 69 cases of the first patient cohort overlapped with the second cohort, the datasets were merged in order to examine whether the overall marker expression distinguishes LNEN subtypes. Unsupervised clustering revealed unique marker expression patterns in the different histological samples. Importantly, the results demonstrate that the applied immune-related markers are highly effective in classifying tumors into their respective subgroups.

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.

5. Conclusions

Our study is among the first to investigate the specific aspects of TIM in surgically resected LNENs, using a large panel of immune-related markers. We report that LNENs 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.

By investigating the expression pattern of potential immunotherapy targets in intermediate- and high-grade LNENs, the current multicenter study aimed to aid the future implementation of novel immunotherapeutic approaches. We report that high tumor cell TIM3 expression is characteristic of AC tumors, whereas elevated GITR levels in tumor cells could be found in both ACs and SCLCs. OX40L expression by tumor cells is the highest in SCLCs and the lowest in ACs. Immune cell infiltration is the least pronounced in AC lesions, and immune cell VISTA and GITR expressions are also considerably lower in these intermediate-grade malignancies. Altogether, these results might open alternative diagnostic approaches and new immunotherapeutic horizons in these hard-to-treat malignancies.

6. Bibliography of the candidate's publications

Cumulative impact factor: 49.385

List of publications that served as a basis for the current thesis

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