Comprehensive analysis of tumor heterogeneity in neuroendocrine lung cancers

Ph.D. thesis

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1. Introduction

Lung cancer was the leading cause of cancer-related deaths in 2022, accounting for an estimated 1.8 million deaths. This represents 18.7% of all cancer types globally. A commonly overlooked fact is that in Europe, these types of cancers are the second leading cause of maternal orphanhood (11-18%). The loss of a parent has a profound and lasting negative impact on both the child and society. Lung neuroendocrine neoplasms are diverse. The subtypes differ in grade, appearance, mitotic activity, and necrosis. SCLC has been categorized as a NE malignancy; it has the worst prognosis, with a 5-year mortality rate of 90% or more. SCLC in a clinical setting is currently considered a uniform disease. Recent preclinical progress has shown different main types within an underlying molecular heterogeneity, which are inferior subtypes to the High-NE and Low-NE groups. These biologically subtypes are based on the expression of transcription factors: Achaete-Scute Homologue 1 (ASCL1) SCLC-A subtype, Neurogenic differentiation factor 1 (NEUROD1) SCLC-N subtype, and POU class 2 homeobox 3 (POU2F3) SCLC-P subtype, as well as on inflammatory characteristics (SCLC-I subtype).

2. Objectives

In our first study, we investigated the spatial heterogeneity of SCLC between PTs and corresponding LN metastases, whereas our second study aimed to assess the key differences in the immunological profiles of LNENs (SCLC, LCNEC and AC).

Our objective was to explore the inter-tumoral heterogeneity between SCLC PTs and their corresponding LN metastases using surgically resected representative tissue samples. Following the pathological preparations, we analyzed RNA-Seq and IHC data to identify unique molecular and cellular characteristics. Additional statistical tests were used to compare the differences between the PTs and matched LNs metastases. This research should provide insights into the extent of inter-tumoral heterogeneity and tumor plasticity in SCLC. The study was conducted by the Helsinki Declaration of the World Medical Association and received approval from the Hungarian Scientific and Research Ethics Committee of the Medical Research Council (ETT-TUKEB-7214-1/2016/EKU).

Our second study focused on intermediate-grade (AC) and high-grade (SCLC and LCNEC) NE lung cancers and their immunophenotypic differences, using IHC methods. The cohort involved four European centers. Statistical tests were used to assess differences in the expression levels of immune

markers. The study was approved by the national-level ethics committee of Hungary, by the Hungarian Scientific and Research Ethics Committee of the Medical Research Council (ETT TUKEB 39249–2/2019/EKU and 52614–4/). Our findings may have diagnostic significance for these highly aggressive tumor types and could aid in designing and implementing novel immunotherapeutic clinical trials.

3. Methods

In the first phase, FFPE tissue samples from PTs and corresponding LNs metastases were macrodissected for molecular analysis. RNA expression profiling was conducted using the HTG EdgeSeq Targeted Oncology Biomarker Panel (HTG Molecular Diagnostics Inc., Tucson, AZ, USA), enabling simultaneous and quantitative detection of 2,560 genes associated with tumor biology. This targeted RNA expression assay employs a nuclease protection method involving the hybridization of target RNA to a complementary deoxyribonucleic acid probe followed by single-strand nuclease treatment. The panel was specifically designed to identify well-established cancer-specific therapeutic targets and markers of drug response. Assay validation was performed using appropriate positive and negative controls. Before enrolment, all hematoxylin and eosin (H&E)-stained slides were re-evaluated by a board-certified pulmonary pathologist to confirm the diagnosis of SCLC. Quality control of older blocks was achieved within our previous study framework by performing confirmatory IHC staining with routinely used diagnostic antibodies. Tissue microarray (TMA) construction was performed at the University of Colorado, Denver (Aurora, CO, USA), as previously described. Notably, two 1-mm tissue

punches were taken from each donor tissue block and placed into a recipient paraffin block in a positionally encoded array format (MP10 1.0-mm tissue punch on a manual TMA instrument, Beecher Instruments). Tissue cores were retrieved from the most viable tumor areas defined on H&E-stained slides. In addition to subtype markers (ASCL1, NEUROD1, POU2F3, and YAP1), the expression of the following clinically relevant proteins were also evaluated: CD47, c-myc, l-myc, delta-like ligand 3 (DLL3), enhancer of zest homolog 2 (EZH2), lysine-specific demethylase 1 (LSD1), mammalian target of rapamycin proteins (mTOR), PD-L1, and phosphoinositide 3-kinase (PIK3). Concerning IHC staining, after deparaffinization and rehydration of the four μm-thick sections, slides were heated for 20 minutes in either 10 mM citrate buffer (pH 6.0) or 10 mM Tris-EDTA buffer (pH 9.0) according to antibody protocols. Slides were incubated in a 0.3% hydrogen peroxide (H_2O_2) solution to reduce background staining. Signal amplification was performed according to the manufacturer's recommendation of the Novolink TM Polymer Detection System kit from Leica Biosystems (RE7150-K; Wetzlar, Germany), followed by antibody incubation at room temperature for one hour. The used antibodies are listed in Table 1. Antibody binding was detected with the ImmPACT

3-3′-diaminobenzidine (DAB) substrate Kit from Vector Laboratories (NC9567138; Newark, CA, USA). Nuclei were counterstained using hematoxylin. All antibodies were validated with appropriate tissue controls. IHC-labeled and H&E-stained slides were digitally scanned using PANNORAMIC 250 Flash III (3DHISTECH Ltd., Budapest, Hungary); sections were evaluated with CaseViewer 2.4 (3DHISTECH Ltd., Budapest, Hungary). The expression level of the given marker was examined by two experienced, independent pathologists, blinded to clinical data, and the staining index (percentage of TCs showing positive staining relative to all TCs) was determined.

In the second phase, tumor tissue samples were obtained through surgical resection and examined by a board-certified pathologist using routine diagnostic protocols and IHC staining for markers such as Chromogranin A, Synaptophysin, CD56, Syntaxin, and Ki-67. To ensure diagnostic accuracy and exclude mixed histology cases, all H&E-stained slides were independently reviewed before inclusion. Tissue sections were analyzed for 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),

lymphocyte-activation gene 3 (LAG3), and tumor necrosis factor receptor superfamily, member 4 (OX40), which are potential immunotherapy targets. (88-92)

After deparaffinization and rehydration, tissue sections were treated with a 3% H_2O_2 solution for 20 minutes to minimize nonspecific background staining. Following this, samples were heated to 98°C in a 10 mM Citrate buffer (pH 6.0) or a 10 mM Tris-EDTA buffer (pH 9.0) for 40 minutes, according to the manufacturer's guidelines. The slides were then incubated at room temperature with Ultra V for 5 minutes, followed by overnight incubation with primary antibodies at 4°C. Immunoreactions were visualized using the UltraVision LP detection system and stained with DAB, followed by hematoxylin counterstaining. Pathological evaluation was performed by averaging the percentage of positive cells across ten randomly selected areas, assessed independently by two pathologists at 20x and 40x magnification. A third senior lung pathologist reviewed the slides to see if the scores differed by more than 20%. Separate scoring was conducted for tumors and ICs, with the proportion of positive cells calculated relative to the total tumor and IC populations, including the overall immune infiltrates in each sample.

4. Results

The first study included 32 surgically treated patients with histologically confirmed SCLC. The median age of the patients was 58 years (range: 34–78), with 22 male participants. All patients were of Caucasian descent. The median OS was 20.7 months, while the median disease-free survival was 14.9 months. Protein-level expression patterns were subsequently evaluated using IHC. DLL3 expression was significantly higher in PTs than in LN metastases ($p = 0.008$). Similarly, the mean expression levels of CD47, LSD1, mTOR, and POU2F3 were higher in PTs, though these differences were not statistically significant. Conversely, NEUROD1 expression was significantly lower in PTs compared to LN metastases ($p < 0.001$), while c-myc expression showed a non-significant reduction in PTs. Next, we explored whether protein expression patterns could differentiate between PTs and LN metastases. While cluster analysis identified two distinct subgroups with differing protein expression profiles, these clusters did not align with the site of origin. Notably, the transcription factor YAP1 consistently exhibited low expression levels across all sample types. In contrast, TIGIT, an immune checkpoint involved in immune suppression, was overexpressed in most primary and LN metastatic tumors. Interestingly, mTOR and

DLL3, emerging as potential therapeutic targets in SCLC, showed variable expression levels among tumors. Differential expression analysis of subtype-defining proteins identified five major SCLC subgroups across the tumor samples. These subgroups included SCLC-A, SCLC-AN, SCLC-N, SCLC-P, and the previously proposed quadruple-negative SCLC subtype (SCLC-QN), which was characterized by low expression of all four transcriptional regulators (ASCL1, NEUROD1, POU2F3, and YAP1). Most samples (61%) exhibited a NE phenotype, encompassing the SCLC-A, SCLC-AN, and SCLC-N subgroups. Next, we investigated whether the subtype of PTs matched that of their corresponding LN metastases or if there were notable differences in subtype-defining protein expression. The analysis revealed that LN metastases often differed from their PT counterparts, with primary and metastatic lesions frequently clustering into different subgroups. Molecular subtype changes were observed in 21 cases. Most commonly, SCLC-QN PT transitioned to SCLC-AN (n=5) or SCLC-N (n=5) in their LN metastases. Other changes included transitions from SCLC-A to SCLC-AN (n=4), SCLC-N (n=1), or SCLC-QN (n=3). Interestingly, the majority of SCLC-AN PTs retained their subtype during metastatic spread, with only one case switching to SCLC-N.

Notably, none of the PTs exhibited an SCLC-N subtype, yet it was the second most common subtype in LN metastases. Both SCLC-P PTs transitioned to SCLC-N in their LN metastases. In the second study, patients were categorized into low- and high-expression groups for each immune-related marker using the median value as the cutoff. Univariate Kaplan-Meier survival analysis indicated a trend toward worse OS in patients with high CD47-expressing tumors compared to those with low expression. Conversely, high tumor cell CD40 expression was linked to better survival outcomes. For immune-related markers in ICs, high CD137 expression was significantly associated with improved OS. In contrast, high IC ICOS expression was associated with poorer survival outcomes. Borderline significance was observed for IC-based expression of CD8 and LAG3 concerning OS, with high CD8 expression showing a trend toward worse OS and high LAG3 expression suggesting a trend toward improved OS. When analyzing SCLC and LCNEC samples separately, none of the tumor cell marker expressions significantly affected OS in the univariate analysis. However, in ICs, significant survival differences were observed for PD-1 ($p = 0.048$), CD27 ($p = 0.0043$), LAG3 ($p = 0.023$), CD4 ($p = 0.059$), CD137 ($p = 0.064$), and the extent of immune infiltration ($p = 0.021$).

5. Conclusions

Firstly, we gained insights into the molecular changes during metastatic spread by analyzing a cohort of surgically resected primary SCLCs and their LN metastases. Different expressions of DLL3 and NEUROD1 were seen in the PTs and paired LN metastases. DLL3 was significantly higher in PTs, whereas NEUROD1 was more strongly expressed in LN metastases. Our results imply that the molecular subtype of LN metastases does not always align with the same PT, pointing to potential subtype transitions during lymphatic dissemination. Changes from both NE subtypes to non-NE lesions and non-NE subtypes to NE subtypes were seen. Through these lenses, potential diagnostic challenges emerge when determining the molecular profile and classification of SCLCs based solely on LN biopsies. Secondly, we obtained a large panel of immune-related markers and investigated the immunologic profiles and expression patterns of LNENs. TCs elevated CD47 expression showed impaired survival, while high CD40 expression correlated with improved outcomes. As we investigated immune cells, high expression of CD137 improved patient survival, whereas high expression of ICOS was associated with worse patient outcomes.

6. Bibliography of the candidate's publications

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

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