Clinical implications of small cell lung cancer molecular and neuroendocrine subtypes

PhD thesis

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1. Introduction

Small-cell lung cancer (SCLC) is an exceptionally lethal and widely metastatic malignancy that kills an estimated 200,000 people worldwide each year. Although tantalizingly responsive to initial chemotherapy (CHT) and seemingly at the cusp of cure at the beginning, it is characterized by rapid recurrence, extensive metastatic spread and a consistently dismal prognosis. The clinical armamentarium for patients with this aggressive type of tumor has changed minimally over the past 40 years, resulting in SCLC being categorized as an extremely frustrating cancer for the oncologists to treat. Moreover, in contrast to the increasingly personalized approaches in other types of lung cancer, SCLC is still treated both in the clinics and in the laboratories as a single disease with no predefined targeted therapeutic options. In the past decade, however, we have witnessed an accelerating pace of biological insights into the genetic landscape and tumoral heterogeneity of SCLC. These genomic profiling studies of human samples together with complementary cell line and *in vivo* data converged on a new model of SCLC subtypes. Accordingly, SCLC tumors can be subdivided by the expression of key neuroendocrine (NE) markers and transcription factors into different NE- and molecular subtypes, respectively. These emerging subtypes might represent a step forward in the implementation of subtype-specific management protocols, yet the diagnostic impact of tumoral heterogeneity on NE subtypes, and the clinicopathological and therapeutic relevance of molecular subtypes are largely unexplored.

2. Objective

Genomic and pathological assessments of both human tumors and murine SCLC models revealed that most tumors harbor substantial heterogeneity in their expression level of NE markers and subtype-defining transcription regulators. Additionally, in small transbronchial-, transthoracic- or mediastinal biopsy specimens, crush artifacts may also be present. Therefore, profiling studies should primarily focus on large tumor samples (i.e., surgical specimens), where the protein-level features are more evident than in small biopsies. Surgery is however rarely performed in SCLC patients and the resulting scarcity of adequate clinical samples still represents an obstacle in SCLC research. Indeed, only a few studies have investigated so far the tissue expression pattern of NE markers and subtype-specific transcription factors in surgically resected SCLC. However, due to the heterogeneity of the study populations and the low number of surgically resected cases included, these studies could not address properly the clinicopathological and prognostic relevance of subtype-defining proteins. In addition, the diagnostic importance of tumoral heterogeneity and the therapeutic relevance of molecular subtypes concerning the efficacy of both targeted- and standard-of-care therapy also warrants further investigation.

In order to gain insights into the diagnostic aspects of inter-tumoral heterogeneity, we aimed to evaluate the gene expression profile of surgically resected primary SCLC tumors and corresponding lymph node (LN) metastases with a special focus on NE subtypes. In addition, within

the framework of an international multicenter study, we also investigated the expression pattern, clinical significance, and prognostic relevance of molecular subtype-defining transcription factors (ASCL1, NEUROD1, POU2F3, and YAP1), as well as P53 and RB1 proteins in a large cohort of surgically treated SCLC patients. Lastly, with the aim of unfolding the specific correlation patterns between subtype-defining proteins and *in vitro* efficacy of targeted and chemotherapeutic agents, we also performed comprehensive in-depth proteomic analyses in a panel of 26 human SCLC cell lines.

3. Methods

Investigating the impact of inter-tumoral heterogeneity on NE subtypes

The effects of inter-tumoral heterogeneity were assessed by using a cohort of 32 LN-metastatic SCLC patients who underwent surgical resection between 1978 and 2013 at the National Koranyi Institute of Pulmonology (Budapest, Hungary). Formalin-fixed, paraffin-embedded (FFPE) tissue samples of both primary tumors and corresponding LN metastases were obtained at the time of lung resection surgery.

FFPE tissue samples were first macrodissected and sent for molecular analysis. RNA expression analysis of 2,560 cancer-related genes was performed by HTG Molecular Diagnostics, Inc. using the HTG EdgeSeq Oncology Biomarker Panel. HTG EdgeSeq is a targeted RNA expression assay that is generated via nuclease protection and consists of hybridization of target RNA to a DNA probe, followed by treatment with a single-strand nuclease. The assay was validated using both negative and positive process controls. All samples were run as singletons.

Both data pre-processing and cluster analysis were performed with ClustVis web tool, which uses several built-in R packages. For data pre-processing, the unit variance scaling method was used dividing the values by standard deviation (SD). SCLC tumor samples were divided into NE high vs. NE low subgroups by agglomerative hierarchical clustering based on the top RNA genes associated with NE differentiation. Gene expression data was analyzed according to the tissue of origin and NE differentiation by Student's t-test and Mann-Whitney U test.

Expression patterns and prognostic relevance of subtype-specific transcription factors in surgically resected SCLC

The expression pattern and prognostic significance of subtype-defining proteins were investigated within the framework of an international multicenter study that included 386 patients with histologically confirmed SCLC who underwent surgical resection in five different Central European medical centers. Based on the type of available samples, we grouped the patients either into a *whole tissue section (WTS) cohort* where complete surgical FFPE blocks were available or a *TMA cohort* (tissue microarray cohort and we analyzed these two cohorts by immunohistochemistry (IHC) separately. All SCLC tumor tissue samples were obtained by surgical resection and all specimens were analyzed for expression of the four markers of SCLC subtypes as well as for P53 and RB1 by using specific antibodies. Expression of the given marker was examined blinded to clinical data by two experienced independent pathologists. All slides were examined with 20× and 40× objective, and the percentage of all tumor cells showing positive staining was determined. For certain analyses, tumor samples were dichotomized into low vs. high expressing subgroups according to their IHC expression levels. To bring the obtained results closer to everyday practice, the cut-off values were defined based on the median protein expression and on the generally used diagnostic thresholds. All statistical analyses were performed using R version 3.6.3 (R Foundation for Statistical Computing, Vienna, Austria).

Proteomic profiling and cell viability assays of human SCLC cell lines

In total, 26 cell lines derived from primary or metastatic human SCLCs were characterized in order to reveal their proteomic profiles. All included cell lines were either purchased from the American Type Culture Collection (ATCC) or kindly provided by our collaborators from the University of Colorado Denver (Aurora CO, USA). During the proteomic analyses, peptides were analyzed by nanoscale liquid chromatography separation combined with tandem mass spectrometry (nLC-MS/MS) using label-free quantification performed on an Ultimate 3000 RSLC nano pump (Thermo Fisher Scientific, Waltham, MA, USA) coupled to a Q-Exactive HF-X (Thermo Scientific) MS equipped with an EASY-Spray ion source. The raw protein intensities were $\log_2$-transformed and the samples were median-normalized by centering all the samples to the global median. Triplicate measurements were averaged, followed by a filter for minimum 80% valid values and left-censored missing data imputation. As for cell viability assays, all experiments were performed at least 3 times in triplicate. IC_{50} values were calculated from dose response curves ranging from 0-100 µM normalized to the vehicle-treated control. Differential expression analyses for the proteomic data were performed via ANOVA, followed by Benjamini-Hochberg multiple testing correction of ANOVA p-values and Tukey's honestly significant difference post-hoc tests. Correlation patterns between proteomic abundance and measured IC_{50} values were assessed by using Pearson correlation.

4. Results

Investigating the impact of inter-tumoral heterogeneity on NE subtypes

The median age of the included patients was 58 years (range 34-78). All patients had Caucasian ethnicity and 22 of them were male (68.7%). With regards to inter-tumoral heterogeneity concerning the gene expression profile, a statistically significant difference was found in the gene expression of 154 genes, including four already reported relevant genes in the SCLC SuperPath. Strikingly, only 13.1% (n=336) of all genes in the entire panel had strong correlation (r value >0.7) between the primary tumor and corresponding LNs. In our gene panel, a total of 6 and 20 NE-high and NE-low genes were identified, respectively. Based on these genes, hierarchical cluster analysis clearly identified SCLC NE subtypes in both primary tumors and LNs. Notably, in five patients we observed a change in the NE pattern of their primary vs. LN metastatic samples: four patients had NE-low-specific gene expression signature in their primary tumor but NE-high-specific expression in their LN metastases, whereas the NE pattern changed from high to low in case of one patient. In fact, because of this heterogeneity between primary tumors and corresponding LN metastases, the correlation between primary and LN samples regarding NE pattern was categorized as moderate (r=0.664), having a match rate of 84.38%. Accordingly, there was a higher number of NE-high and a lower number of NE-low patients theoretically diagnosed when using the LN specimens compared to primary tumors.

Expression patterns and prognostic relevance of subtype-specific transcription factors in surgically resected SCLC

A total of 141 (median age: 63.9 years; range: 41-83) and 245 (median age: 57 years; range, 37-79) surgically treated SCLC patients were included in the *WTS cohort* and *TMA cohort*, respectively. Differential expression of the key transcription regulators clearly distinguished SCLC subtypes in both cohorts. besides SCLC-A (ASCL1-dominant), SCLC-AN (combined ASCL1/ NEUROD1), SCLC-N (NEUROD1-dominant), and SCLC-P (POU2F3-dominant), cluster analyses identified a fifth, quadruple-negative SCLC subtype (SCLC-QN) characterized by the low expression of all four investigated transcription factors. Importantly, this SCLC-QN subtype was not defined by YAP1 expression, and no unique YAP1-defined subtype could be distinguished by IHC in either cohort. With regards to intratumoral heterogeneity, in some tissue specimens, subtype-specific marker expressing and non-expressing cells appeared in a mixed form within a tumorous area, whereas in other cases clusters of these cells were found in spatially distinct regions. This latter phenotype supports the idea that small biopsies might not ubiquitously mirror the expression profile of the entire tumor.

As for the prognostic relevance of subtype-defining proteins in the *WTS cohort*, we found that high ASCL1 expression was associated with impaired survival outcomes in surgically resected patients (vs. low ASCL1 expression; median overall survivals (OSs) were 29.63 versus 42.93 months, respectively; p=0.012). Patients with high NEUROD1-

expressing tumors also had significantly shorter OS (versus those with low NEUROD1 expression; median OSs were 22.88 versus 41.93 months, respectively; p=0.013). In contrast, in our univariate model, high POU2F3 expression was significantly associated with improved OS (versus low POU2F3 expression, median OSs were 69.47 versus 30.07 months, respectively; p=0.046). Next, we grouped the patients according to their dominant molecular subtype, and the highest survival rates were found in SCLC-P and quadruple negative subtypes, whereas the lowest in SCLC-A, -N, and -AN subtypes. Accordingly, the NE phenotype proved to be a sign of poor prognosis in surgically resected SCLC (p=0.003). In order to assess if the prognostic value of ASCL1, NEUROD1, and POU2F3 expression was independent of other variables in the WTS cohort, we performed a multivariate Cox regression analysis. Notably, in our multivariate model adjusted for clinical factors and treatment, high ASCL1 expression remained a significant negative prognosticator for OS (p=0.03), whereas the independent prognostic relevance of POU2F3 proved to be borderline significant (p=0.08). Interestingly, NEUROD1 expression had no significant impact on survival in our multivariate model (p=0.75). Similar tendencies were seen in the *TMA cohort*. Specifically, the OS was also significantly longer in patients with low ASCL1- and high POU2F3-expressing tumors (p=0.027 and p=0.017, respectively). Yet there was no statistically significant difference in OS with regard to NEUROD1 expression.

Proteomic profiling and cell viability assays of human SCLC cell lines

In-depth proteomic analysis identified and quantitated more than 8,000 proteins in each SCLC cell line. Interestingly, unsupervised clustering of samples based on protein abundance levels of ASCL1, NEUROD1, POU2F3, and YAP1 differentiated a distinct YAP1-driven, a mixed SCLC-AN, and a heterogenous SCLC-P cluster. When correlating the proteomic abundance of subtype-defining regulators with the IC_{50} values of examined therapeutic agents, a statistically significant positive linear correlation was found between ASCL1 abundance and alisertib (AURK-inhibitor) IC_{50} values (r=0.493), and also between YAP1 abundance and IC_{50} values of CDK-inhibitors abemaciclib and CGP60474. Furthermore, we also observed that high NEUROD1 proteomic abundance confers *in vitro* sensitivity to alisertib (r=-0.401), the AURK-inhibitor barasertib (r=-0.674), abemaciclib (r=-0.502), CGP60474 (r=-0.536), and the IGF-1R-inhibitor BMS-754807 (r=-0.581). No significant correlations were found with regards to the IC_{50} values of IGF-1R-inhibitor PPP. As for standard-of-care chemotherapeutics, statistically significant negative linear correlations were found between POU2F3 abundance and IC_{50} values for cisplatin (r=-0.585), irinotecan (r=-0.554), topotecan (r=-0.569) and etoposide (r=-0.507). YAP1 abundance positively correlated with IC_{50} values for cisplatin (r=0.628), irinotecan (r=0.611) and topotecan (r=0.589), therefore high YAP1 expression conferred resistance to these agents. The IC_{50} values of epirubicin did not correlate with the proteomic abundance of subtype-defining markers.

5. Conclusions

In our clinical studies, we investigated the impact of inter-tumoral heterogeneity on NE pattern, and assessed the tissue distribution and prognostic relevance of subtype-specific proteins in surgically treated SCLC patients. Moreover, to provide insights into the therapeutic aspects of subtype-defining regulators, we also performed a comprehensive MS-based proteomic analysis in a panel of human SCLC cell lines.

First, our results highlight the gene discordance between primary tumors and corresponding LN metastases in SCLC. These differences are suggestive for a relatively high mutational rate in tumor cells and thus for a potentially higher chance of developing drug resistance-inducing mutations. Furthermore, as a result of this high degree of intratumoral heterogeneity, the NE-phenotype of the LN metastases might not mirror the NE-subtype of the primary tumor. Accordingly, profiling of tumoral metastases might not be sufficient for diagnostic purposes concerning the NE pattern of the primary tumor.

Second, we validated the new molecular subtype classification using the so-far largest cohort of surgically treated patients and, moreover, found that differential expression of ASCL1, NEUROD1, and POU2F3 defines unique SCLC subtypes. However, our IHC analyses did not distinguish a specific YAP1-driven subtype. Instead, we provided evidence for a novel SCLC-QN subtype characterized by low expression of all four transcription regulators. In addition, we also revealed that high ASCL1

expression is an independent negative prognosticator in surgically treated SCLC, whereas high POU2F3 expression is associated with improved survival in a univariate analysis. Consequently, SCLC tumors with NE differentiation have worse prognosis than non-NE tumors.

Lastly, our proteomic analyses of SCLC cell lines provided insight into specific correlation patterns between transcription regulators and the therapeutic efficacy of targeted and CHT agents. Specifically, high we showed that NEUROD1 expression confers sensitivity to AURK- and CDK-inhibitors, while POU2F3-high expressing cells are susceptible to the vast majority of chemotherapeutic agents.

Altogether, our results might help in the development of subtype-specific management protocols and follow-up strategies in this devastating disease.

6. Bibliography of the candidate's publications (∑impact factor: **114.4**)

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

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