Peer Review File

Article information: https://dx.doi.org/10.21037/tlcr-23-505

Reviewer A

Helmut Popper at el. presented the multiple analysis of protein expression to the small cell carcinoma and large cell neuroendocrine carcinoma(LCNEC). This manuscript is interesting in knowing the status of various marker, especially in LCNEC. The writing could be more politely and reorganized before publication.

Major

1. In regards with Table 2, it is so hard to understand the relationship between the expression status. Please add the different figure to clarify this point. Moreover, what did you separate the cases into test-set and validation-set for? If you need to verify the IHC staining in test-set, please write the purpose and method in Materials and Method section.

Response: The expression as summarized in table 2 gives the positive reactions in the cases; for example there were 41 cases of SCLC, where 17 were positive for ASCL1. The reason for using a validation set was two-folded: We were aware that the numbers of ASCL1 positive cases were below those with NeuroD-positivity – in contrast to published results. Primarily we suspected this might be due to a selection bias: the test set was predominantly from resections. Therefore, we added biopsies (validation set). However, a similar distribution was found. In an recent article by Wollenziel et al a similar observation was made and interpreted as a clonal evolution of the carcinoma during time and in addition conferring resistance towards chemotherapy. But we still do not have a full explanation for this phenomenon.

We have added sentences in the material/Method section to clarify this and also to the discussion

2. You mentioned that some biomarker; RB1, PTEN had a promising potency to construct the therapy strategy. You are supposed to be able to present these important data, because subjects of study are collected retrospectively. I'd like to show the clinical outcome, like antitumor response of chemotherapy or progression-free survival, depended on the biomarker which you proposed, if possible.

Response: As pathologists we are not conducting therapy studies, therefore we cannot provide results. We however, cited one Italian study, which also pointed to this aspect. For several decades there was a discussion within the lung cancer community about the best treatment option for LCNEC. In the 1990-ties four studies claimed opposite results by either using a SCLC-like protocol (studies from US), and by using cisplatinum-based therapy like NSCLC (Italian studies). The molecular difference in LCNEC either presenting with a SCLC-like make-up, i.e. RB1-loss, or PTEN-loss/PI3KCA activation should provoke clinical studies proving this possible assumption. Our discussion on this aspect should provoke therapeutic studies.

Minor

1. Some abbreviations that you used in this paper are different from general technical words.

Especially, Neurogenic differentiation factor 1 is suitable for the abbreviation of NEUTOD1.
Response: NeuroD1 is the used abbreviation for neuronal differentiation 1 (gene cards in NCBI)

2. The word of "few" is used many times in your manuscript. Some sentences show discordance in the context. Please ascertain carefully.
Response: we replaced "few" in several sentences using numbers instead.

3. A few language edits and typos that need to be cleaned up during the
editorial phase.
4. In Table 2, the population number tested in SCLC for MYC-L was incorrect; 49.
Response: Thank you, has been corrected

Reviewer B

In this study, Popper et al. evaluated the expression of molecular subtype markers in SCLC and LCNEC and their associations with several other markers. The authors collected 77 (41 resected tumors and 36 biopsies) and 49 cases of SCLC and LCNEC, respectively. They examined several groups of proteins of interest: molecular subtype markers (ASCL1, NeuroD1, YAP/TAZ, POU2F3), MYC family genes (c-MYC, l-MYC, N-MYC), immune marker (PD-L1), tumor suppressor genes (RB1, PTEN, and p53), potentially drug targets (FGFR1, FGFR2, and AURKA), and others (HES1 and vimentin). The authors concluded that both LCNEC can be grouped into three molecular subtypes (ASCL1, NeuroD1, and POU2F3) and there is no clear association between molecular subtype markers and other examined targets.

The expression of molecular subtype and therapeutic markers have been examined in SCLC in previous studies. However, the expression of these markers in LCNEC has not been well evaluated in LCNEC. This study could potentially fill this gap by comparing the expression of these markers between LCNEC and SCLC. However, its relatively small case numbers and a lack of quantitative measures of expression damper my enthusiasm for this study.

1) The previous studies on molecular subtyping of SCLC included 174 (Baine et al., JTO 2020) and 146 cases (Qu et al., JTO 2022). One main issue of this study is low case number. The authors should expand the number of LCNEC cases. This can be done by either collaborating with other institution(s) or using commercial TMAs (e.g., LCNEC tissuearray.com).
Response: It would be worth expanding the number of cases, however, this would be beyond the time limit for a revision of the manuscript (communicate with an outside institution, collect cases, perform immunostains, etc; this would result in a new study). A purchase of commercially TMAs could be an alternative, however, again this would result in a new study, as all the markers have to be tested on these new samples. The importance of our study is that all these cases come from one institution. Another option would be to select more cases from the Archive going back to 1986, when I started to built this Archive. This might be attractive, as SCLC was much more common in these decades (25% of lung cancer). But this creates another problem: Not all antibodies would give a perfect reaction, due to the age of the paraffin

blocks. This could result in different staining intensities or even false negative reactions and therefore all blocks had to be tested for degradation before they could be included into the study.We hope the reviewer can accept this arguments.

2) The expression of each marker should be measured quantitatively, such as using an H-score method. The molecular subtype classification should then be determined based on the predefined H-score cutoffs.

Response: Initially we scored ASCL1, NeuroD1, POU2F3, and ATOH1, for percentage of positive nuclear reaction and staining intensity. As the intensity of nuclear expression for these markers was always +3, we than used only percentages for each. We have reevaluated the therapeutically relevant markers AURAKA, FGFR2, and the MYC proteins semiquantitatively, and included a H-score to each marker; for YAP, TAZ and HES1 we scored only the percentage of positive tumor cells, as these are not therapeutic targets.

3) I noticed that the ASCL1-positive rate is unusually low in SCLC while NEUROD1 expression was much higher than previously reported. In addition, l-MYC, the most commonly amplified MYC family gene in both SCLC and LCNEC was negative in all cases, while N-MYC, which is infrequently expressed in SCLC and NSCLC based on the published RNA-Seq data (George et al. Nature 2015; George et al. Nat Commun. 2018), was positive in all cases. This discrepancy between RNA and protein expression is concerning. I noticed that the antibodies used in this study for the above markers were different from the ones used in Baine et al. 2020 and Qu et al. 2022. Please include the results demonstrating the specificity of these staining.

Response: The in comparison higher number of NeuroD1 cases caused us to include a validation set, as we initially suspected a bias due to the use of resected tissue specimen. However, a similar distribution was seen in the biopsies (see also my response to reviewer 1). Primarily we tested different antibodies for ASCL1, NeuroD1, POU2F3; especially with the Bioscience antibody used by Baine we could not achieve good staining results. As our tests were based on Ventana and DAKO platforms, there might be a difference to a Bond platform used by these investigators. MYC was not established in the report by Baine JTO 2020; the report of Qu was based on commercially purchased samples; different antibodies were used compared to ours. A validation is not shown in this report. Antibody evaluation in our cases was done using tissue microarrays with known positivity of MYC proteins (a TMA with different cancers, predominantly colon carcinoma cases). In addition, the antibodies were selected when the companies could provide a Western blot with a specific band for each of the proteins; the study by George 2015 did RNA sequencing for myc family genes but no confirmation was done at the protein level. Here in one of the reports by George out of 152 cases 1 case showed an amplification for MYC-N, 3 cases for MYC-C, and 6 cases for MYC-L. In the second manuscript there were 5 MYC-L and 2 MYC-C cases out of 75 cases. This means only a minority had amplifications. It is well known, that for oncogenes not only amplifications might be important, but also posttranslational modifications, which can result in protein expression, which might be the cases here.

We have added two sentences in the method section, and a sentence in the discussion referring to this aspect

Reviewer C

Authors retrospectively evaluated the expression of ASCL1, NeuroD1, POU2F3, YAP1, and the other key genes in SCLC and LCNEC by immunohistochemistry staining. This analysis could provide a valuable data for molecular subtypes of not only SCLC but also LCNEC in clinical practice. However, there are several questions and concerns which should be addressed.

1. Authors are encouraged to state the strength of this study more clearly. On first reading, this study appears to be merely a validatory analysis of known findings.
Response: We tried to reformulate the manuscript and pointing to those aspects, which are new. We hope to better present our findings.

2. Clinical information is missing in this study. At least fundamental clinical characteristics of the patients including sex, age, stage, treatment etc. is needed.
Response: we added a table with sex, age range, stages, treatment and the range for overall survival

3. Authors should indicate the criteria for determination of pathologically positive or negative in Material and Methods.
Response: We added a sentence about our evaluation and for the therapy relevant genes we in addition now provide a H-score (staining intensity by percentage of positively stained tumor cells)

4. Table2. Heatmap of the genes expression by sample would be helpful for readers to understand the whole picture.
Response: we have added a suppl.Table with H-score for AURKA and the expression of ASCL1, NeuroD, and POU2F3). High expression of the master genes is highlighted by red, medium expression by yellow, and low by green. We hope this helps

5. Table 2 and 3. If authors discuss the frequency of gene expression comparing SCLC with LCNEC, authors are encouraged to perform statistical analyses.
Response: We think the number of cases is too small to get a meaningful statistical representation. We included the H-score values into table 3 and also calculated mean and standard deviation. We are not sure this is meaningful?

6. Related to question 2, the data for correlation of molecular subtypes with clinical prognosis or the other clinical outcome of each patient would be valuable if available.
Response: Sorry as this was a retrospective study we only can add the overall survival time (see table 4)

7. Did the authors analyze correlation of the molecular subtypes with neuroendocrine markers such as chromogranin A or synaptophysin?
Response: Expression of NCAM (CD56), CGA, and synaptophysin are regularly done during

diagnosis; the POU2F3 cases were all negative, whereas the ASCL1 and NeuroD1 cases were all positive, albeit with some differences in staining intensity

8. Did authors investigate the correlation of the molecular subtypes with tumor markers such as ProGRP or NSE?
Response: No, we do not use anymore NSE and Bombesin (analogon of GRP), as NCAM is now the preferred marker

9. Authors concluded that AURKA be promising target of SCLC and LCNEC. Authors should elaborate its reason. Is this just because AURKA is expressed in two-thirds of samples in this study?
Response: We performed a H-score for those targets, which are targetable; a clinical study is ongoing for a treatment of AURKA inhibitor combined with chemotherapy. In this study no marker evaluation is included; however, we think this might be important for therapeutic decision. An immunohistochemical evaluation could be used for the evaluation.

Reviewer D

Well written except for some grammatical review needed. Well-done figures and tables. While the authors do comment on some limitations at different points, a paragraph in the discussion dedicated to limitations of the study would be good, as well as a paragraph on proposed future studies.
Response: We have added a paragraph on the limitation of this study