Peer Review File

Article Information: https://dx.doi.org/10.21037/tlcr-22-418

Reviewer A

In this study, the authors described the development and validation of two immunohistochemistry clones for OTP and reported their findings in a cohort of pulmonary neuroendocrine tumors. Overall, this is a well-conducted and well-written study.

Response to reviewer:
We would like to thank this reviewer for the thorough and critical review of our manuscript and commenting on the conduction of the study.

Remark 1: In the method section "tumor samples", the authors described using 19 cases diagnosed as carcinoid tumor, NOS. It was unclear why these cases were not diagnosed as typical or atypical carcinoids instead if these are all FFPE resection specimens as the authors reported. Were these samples from lung biopsies or resections from metastatic sites? Please clarify.

Answer 1: Thank you for your question. All tumor samples were FFPE resection specimen of patients with a pulmonary neuroendocrine neoplasm. For this study we used the original pathology report diagnosis as final diagnosis. Hence, a carcinoid diagnosis is established, and the aim of this study was not to delineate subtypes of carcinoid. The aim was to test the monoclonal antibody staining validity.

Remark 2: Are these two OTP antibodies specific for pulmonary carcinoids among carcinoids of all sites? Does their performances differ when used in gastrointestinal carcinoids, thymic carcinoids, or carcinoids from other sites? Even a pilot test with several cases if available may be helpful.

Answer 2: Thank you for your suggestion, several papers have investigated OTP expression in different neuroendocrine tumors concluding that OTP is specific for pulmonary carcinoids. We are referring to these papers in the introduction on page 5, line 122-124. However, we agree with you that a small pilot test may be helpful to support this observation, and we therefore stained several head and neck NENs (n=7), GEPNETs (n=12), paragangliomas (n=2), NE breast tumors (n=2), merkel cell carcinomas (n=2), and insulinomas (n=15). All tumors were negative for both monoclonal antibodies. We have added this in the results section page 11, line 306-310: ‘’ To investigate whether the two mAbs were specific for pulmonary carcinoids, we performed immunostaining on n=40 neuroendocrine neoplasms of non-

pulmonary origin (i.e., gastroenteropancreatic neuroendocrine tumors (NETs), insulinomas, head and neck NETs, breast NETs, paragangliomas, and merkel cell carcinomas). All cases stained negative for both mAbs.‘’

Reviewer B

The authors have presented a well-conducted study developing and validating a new monoclonal antibody directed against OTP for use in clinical histopathology laboratories to assist in the diagnosis of pulmonary carcinoids. Two clones were developed and assessed on two different staining platforms, with one clone demonstrating a higher false negative rate and less inter-observer concordance when used on the Dako platform. The performance of both clones was acceptable with regards to clear nuclear staining of pulmonary carcinoids, inter-observer variability, antibody stability and performance on older FFPE tissues. The results showing positive staining in 73.7% (n=28/38) of TC, 64.3% (n=9/14) of AC, and 89.5% (n=17/19) of carcinoid NOS reflects the acceptance of imperfect immunohistochemical biomarkers for routine pathological diagnosis and the need for a panel approach. The apparently platform related difference in the performance of one of the clones is a reminder that assays must always be validated to local laboratory conditions. It is unfortunate that staining on the Ventana BenchMark Ultra platform was not assessed in this study, as this is the most commonly used platform in other regions.

Response to reviewer:
We would like to thank the reviewer for carefully reading our manuscript, commenting on the impact, and addressing relevant points of improvement and discussion. To respond on the last sentence, we are currently working on a protocol for the Ventana staining platform. However, we are currently observing problems with the staining on the Ventana platform resulting in an unfavourable false negative rate. Together with Atlas Antibodies, we are trying to address this problem.

Remark 1: A main concern is in relation to the statement made in the introduction that 57% of diagnoses are discordant between the pre-operative biopsy specimen and the paired resection specimen, and the conclusion that this antibody may improve classification and prognostication. The paper referenced regarding the discordant results (Moonen et al 2021) relates only to typical vs atypical carcinoids. From the results presented, OTP immunohistochemistry does not seem to differentiate between typical and atypical carcinoids, which is the critical issue in this situation. There are, in the majority of cases, sufficient morphological differences between carcinoids and SCNEC or LCNEC for distinction on biopsy specimens, and OTP would appear to be useful in the few challenging cases that remain. The aim of this current study does not appear to be to

examine the usefulness of OTP monoclonal antibodies in distinguishing between typical and atypical carcinoids, and this conclusion does not reflect the results.

Answer 1: The aim of this study was not to delineate subtypes of carcinoid but to provide a reference for the monoclonal antibody of OTP as this is currently not available and this is becoming a relevant marker also addressed by the recent WHO [1]. The aim was to test the monoclonal antibody staining validity. Several studies have confirmed OTP's prognostic relevance [2, 3] also on biopsies, and recent molecular studies have shown that OTP is relevant to define molecular subtypes of lung carcinoid [4]. Because further studies need to be conducted to assess the prognostic relevance of this marker, we have modified the conclusion sentence by adding ''may'' on page 13, line 388.

Remark 2: Further description of the origin of the utilised tumour tissues would be useful to the reader to aid in implementation planning and local laboratory validation – did they originate from one institution or many? Storage conditions? Resection specimens or small biopsies?

Answer 2: Thank you for your question. In the methods ''tumor samples'' section, we mention in line 196 that the study was conducted on FFPE resection specimen of patients with a pulmonary neuroendocrine neoplasm. Because we describe that the samples are from the Netherlands, we agree with you that it is not clear whether this is one institute or more institutes. Therefore, we have added on page 8, line 201, ''in medical centers of'', to explain that samples are derived from multiple medical centers. All centers use their own fixation and processing, though, we did not observe any difference between centers.

Remark 3: How was the cut-off for "positive" determined? How many cases scored between 0 and 50? Did changing the cut-off impact the results?

Answer 3: The cut-off for OTP positivity was determined by plotting all different H-scores. Based on the scatterplot of the reference rabbit pAb, a cut-off value of H≥50 was determined as ''positive'' since this defined a clear separation and a H-score of 50 is easy to determine in daily clinical practice. We have added the scatterplot as a supplementary figure to the manuscript and refer to the figure on page 9, line 252 ''Supplementary figure 1''.

Remark 4: A more in depth discussion of the staining results would be of benefit to the reader who may wish to implement this assay in their laboratory e.g. intensity of expected staining, criteria for acceptance or rejection.

Answer 4: Thank you for your suggestion, we have added a figure to declare the different intensities (see reference on page 9, line 249, ‘’(Figure 1)’’) and the figure title on page 15, line 481-482. In addition, we have generated a scatterplot of all the H-scores using the pAb and mAbs on the different platforms and added this figure to the manuscript as a supplementary figure (page 9, line 252 ‘’(Supplementary Figure 1)’’.

Reviewer C

In this study the authors Moonen et al. developed monoclonal antibodies for orthopedia homeobox (OPT) which has shown to be a promising prognostic biomarker in pulmonary carcinoid tumors. Two clones were selected for production and the authors tested these on normal tissues as well as on pulmonary neuroendocrine tumors, both manually and with automated staining protocols. The main finding was, that clone CL11225 performed similarly to the previously developed polyclonal antibody and it worked well on automated immunohistochemical staining platforms allowing application in routine diagnostics.

The manuscript is well-written, and the conclusions seem appropriate based on the presented data. The approach is of interest and clinically valid. Although I find the manuscript relevant for publication, I have some matters that require revisions of the manuscript, table, and figure in order to further improve the manuscript.

Response to reviewer:

We would like to thank the reviewer for the effort of reviewing this manuscript carefully and for the useful remarks to improve the manuscript.

Remark 1: Lines 50-51: Sentence Histology-based grading of pulmonary carcinoids is subject to a high interobserver variation seems to be unnecessary.

Answer 1: Thank you for the remark, we have removed this sentence from the abstract.

Remark 2: Line 53: Please correct spelling of the World Health Organization

Answer 2: We have corrected the spelling of the World Health Organization

Remark 3: Line 104: Please correct spelling of the biomarker

Answer 3: We have corrected the spelling

Remark 4: Line 192: Were FFPE tissues collected from different hospitals or from one? I am wondering if differences between centers in tissue fixation and processing were considered.

Answer 4: Thank you for your question. While we indicate that the samples are from the Netherlands, we agree upon you that it is not clear whether this is one institute or more institutes. Therefore, we have added on page 8, line 201, ‘’in medical centers of’’, to declare that it consists of multiple medical centers. All centers use their own fixation and processing, though, we did not observe any difference between centers.

Remark 5: Line 205: How were the three cores selected? Randomly or e.g., from the middle of the tumor and from tumor border?

Answer 5: The representative tumor regions were marked by a pathologist and subsequently three different cores from both central and peripheral parts of the tumor (e.g. upper part, middle part, and lower part) were selected to allow intratumor heterogeneity analysis. To clarify the TMA core selection, we have added on page 8, line 216-217, ‘’The cores were taken from both central and peripheral parts of the tumor to allow intratumor heterogeneity analysis.’’.

Remark 6: Line 239: I am missing here a panel of figures to show examples on different staining intensities. Please also add that you evaluated nuclear or cytoplasmic intensity or both.

Answer 6: Thank you for the suggestion, we agree upon your opinion that examples of the different staining intensities can be helpful. Therefore, we have constructed a figure and added this figure to the manuscript. In addition, we have added a reference to the figure in the manuscript at page 9, line 249, ‘’(Figure 1).’’ As OTP is a transcription factor that should be present in the nuclear, we have only scored nuclear intensity. We have added this on page 9, line 248 ‘’nuclear’’.

Remark 7: Line 242: Please justify why you selected H score 50 to be the cut-off line between positive and negative staining.

Remark 7: The cut-off for positive was determined by plotting all different H-scores. Based on the scatterplot of the reference rabbit pAb, a cut-off value of H≥50 was determined as ‘’positive’’ since this defined a clear separation and a H-score of 50 is easy to determine in daily clinical practice. We have added the scatterplot as a supplementary figure to the manuscript and refer to the figure on page 9, line 252 ‘’Supplementary figure 1’’.

Remark 8: Line 274: Please correct Figure reference to be Figure 1B.

Answer 8: We have corrected the figure reference.

Remark 9: Lines 287-288 and Figure 2. You write that highest H scores were observed in pulmonary carcinoids. Do you mean in typical carcinoids? Based on the figure 2, AC and SCLC expressed OTP similarly.

Answer 9: Thank you for the remark, highest H-scores were indeed observed within pulmonary carcinoids as you can see in table 2, this included both TC and AC (ranging between 0-300) of which the percentage positive was highest in TC and carcinoid NOS. However, as we also state in the introduction in line 113-115 OTP is a marker to identify patients with a favorable prognosis regardless of histopathological diagnosis as also mentioned in a recent paper of Alcala *et al*. [4] and stated by the WHO 2021 criteria [1]. A TC diagnosis does not guarantee for long-life recurrence free survival. The aim of this manuscript was not to delineate subtypes of carcinoid. The aim was to test the monoclonal antibody staining validity. Figure 2 illustrates the differences between the different antibodies for different diagnosis. However, as may be seen in Table 2, both TC and AC show OTP positivity. The main message of this table is that OTP is absent in high-grade neuroendocrine carcinomas (e.g., LCNEC and SCLC) while present in the low-grade pulmonary neuroendocrine tumors which again underlines the possibility of OTP to select patients with a favorable prognosis since HGNECs are overall highly malignant with worse survival rates.

Remark 10: Line 289, Table 2. Since this is a comparison study between old and new antibodies, it would be useful to see exact H scores, too. Was any of the ACs strongly positive? Did ACs also express nuclear OTP? In the figure 2, AC seems to be more or less negative.

Answer 10: As shown in table 2, both TC and AC show OTP positivity. We have only scored nuclear positivity since OTP is a transcription factor which should be present in the nucleus. To give an overview of the different H-scores we have generated a scatterplot of all the H-scores using the pAb and mAbs on the different platforms and added this figure to the manuscript as a supplementary figure (page 9, line 252 ‘’(Supplementary Figure 1)’’. However, again we want to emphasize that the aim of this study was not to delineate subtypes of carcinoid. The aim was to test the monoclonal antibody staining validity.

Remark 11: Line 320-321: Sentence No difference in staining intensity and patterns after storage at 4 degrees Celsius was observed is not clear. Based on the title, the primary mAbs were stored but for how long?

Answer 11: Thank you for your question, we have removed this sentence as the mAbs are best stored at -20 degrees Celsius as also recommended by Atlas Antibodies.

Remark 12: Line 345: Why did you prefer using Autostainers and not e.g. Ventana's instruments?

Answer 12: Thank you for your question, we are currently working on a protocol for the Ventana staining platform. However, we are currently observing problems with the staining on the Ventana platform resulting in an unfavourable false negative rate. Together with Atlas Antibodies, we are trying to address this problem.

Remark 13: Line 366: You state that intratumor heterogeneity of OPT expression is very low, thus indicating OTP to be a very homogeneous stain. I would rather say that intratumor heterogeneity is very low, thus indicating that OPT is homogenously expressed through the tumor (which results in a homogenous staining pattern).

Answer 13: Thank you for the suggestion, we have adjusted the text accordingly on page 13, line 378-380, '' thus rather indicating that OTP is homogeneously expressed through the tumor resulting in a homogeneous staining pattern''.

Remark 14: Line 369: Please remove whether or not

Answer 14: We have removed ''whether or not''

Remark 15: Line: 374-375: Based on the data presented, I would not state that these antibodies can now be used in routine diagnostics. More validation with external samples is needed, i.e., introducing a larger validation cohort would be useful.

Answer 15: Thank you for your suggestion, we have modified the sentence by adding ''may'' on page 13, line 388.

Remark 16: Discussion in general: I am missing a future plan: what are the next steps to make these antibodies commercially available for diagnostics? Also, study limitations should be discussed.

Answer 16: Thank you for your remark, to answer the question regarding the commercially availability of the monoclonal antibodies. Clone 2 (CL11225) is now commercially available via Atlas Antibodies

see the following link (https://www.atlasantibodies.com/products/antibodies/primary-antibodies/precisa-monoclonals/otp-antibody-amab91696/).
Regarding the study limitations, the use of TMAs is often considered as a study limitation due to tumor heterogeneity. However, we have minimized this by using serial sections and by proving that the intra tumour heterogeneity for OTP is very low resulting in a homogeneous staining. However, to address this possible limitation we have added a sentence on page 12-13, line 374-376 '' The use of TMAs is often considered as a study limitation due to tumor heterogeneity. However,''.

Remark 17: STARD 2015 form: line numbers provided here don't match the manuscript, but all information given seem to be correct except 12) rationale for test positivity cut-offs, and 26) study limitations, including sources of potential bias.

Answer 17: Thank you for your remark, we have adjusted the line numbers and corrected the rational for test positivity cut-offs and referred to the study limitations line as adjusted following revisions.

References

1. Borczuk AC. WHO Classification of Tumours: thoracic Tumours. International Agency for Research on Cancer, 2021.
2. Swarts DR, Henfling ME, Van Neste L, van Suylen R-J, Dingemans A-MC, Dinjens WN, Haesevoets A, Rudelius M, Thunnissen E, Volante M. CD44 and OTP Are Strong Prognostic Markers for Pulmonary CarcinoidsPrognostic Markers for Lung Carcinoids. *Clinical Cancer Research* 2013: 19(8): 2197-2207.
3. Papaxoinis G, Nonaka D, O'Brien C, Sanderson B, Krysiak P, Mansoor W. Prognostic significance of CD44 and orthopedia homeobox protein (OTP) expression in pulmonary carcinoid tumours. *Endocrine pathology* 2017: 28(1): 60-70.
4. Alcala N, Leblay N, Gabriel A, Mangiante L, Hervás D, Giffon T, Sertier A-S, Ferrari A, Derks J, Ghantous A. Integrative and comparative genomic analyses identify clinically relevant pulmonary carcinoid groups and unveil the supra-carcinoids. *Nature communications* 2019: 10(1): 1-21.