Peer Review File

Article information: https://dx.doi.org/10.21037/tau-23-101

Reviewer Comments

Hello dear Editors, thank you very much for your time and help. All your comments will be addressed below in blue:

- In lieu of your comments and suggestions, the name of the manuscript has been re-phrased (lines 1-2)

The authors analyzed the expression and subcellular localization of NHERF1/EBP50 in a limited number of renal tumor samples by immunohistochemical (IHC) staining. They also examined the presence and localization of microlumen-like structures in clear cell renal cell carcinoma (ccRCC) by electron microscopy. The authors included 14 cases of benign renal tumor oncocytoma in the study. Furthermore, they split the remaining still small number of renal cell cancer (RCC) samples into 64 samples (including 34 ccRCC) obtained from 2010 to 2019, and the last twelve samples, obtained later. Taken together, there were only 46 ccRCC samples analyzed altogether. There is only one previous report on the expression of NHERF1/EBP50 in RCC (DOI: 10.1016/j.acthis.2021.151717). Thus, the present manuscript is potentially interesting. The author's idea to analyze intracellular microlumen-like structures is also very attractive. However, besides the small number of cases and questionable grouping, the manuscript has several serious drawbacks.

The authors mention that the samples “were selected”. If the samples included in the study were not sequential what were the inclusion criteria?

- Thank you for this question.

  All cases managed by the department were sequentially included in the study without any exclusion criteria, with the exception of the last group of clear cell carcinomas (where other types of kidney tumors were excluded). First of all, we performed a retrospective search using our pathology database for all primary renal epithelial tumors managed by the department from 2010 to 2019. A total of 66 cases were identified. Stored FFPE tissue blocks were retrieved, and 4 μm tissue sections were immunostained with NHERF1/EBP50 (lines 165-167, 183-184).

  Since the dot/ring-like structures highlighted by IHC were identified only in ccRCC, the need for ultrastructural analysis arose. Fresh tumor tissue was required as the best source for EM. At the time of this decision, all new kidney tumors admitted to the department were considered as potential cases of ccRCC. If the tumor volume allowed, we collected three representative sections from heterogeneous areas for each newly accessioned case (Figure 1). Of the 19 newly received tumor cases with suspected ccRCC, only 12 were diagnosed as ccRCC (lines 186-187), cases other than ccRCC were rejected.

The rationale for including oncocytoma samples in this study is questionable. In the present manuscript, the authors concentrate on the ccRCC and oncocytoma originates

from the same cells (collecting duct intercalating cells) as chromophobe RCC and both tumors share morphological and ICH features. I was unable to find the intensity and staining pattern of NHERF1/EBP50 in oncocytoma samples (maybe it is there and I missed it).

- Thank you for your question.
The reason for inclusion of oncocytomas was that we did not know (and this has not been studied before) whether any distinctions/abnormalities in NHERF1/EBP50 immunoexpression in renal tumors can be found (differ among themselves), and if so, we did not know what type of tumors they could be related to. This prompted us to study all available kidney tumors (benign or malignant) under the department's care.
- The paragraph was added (lines 127-132)

You can also ask a reasonable question: "Why didn't we use stored FFPE tissue blocks for electron microscopy?" In our experience, the best source for ultrastructural analysis was only fresh tissue fixed in glutaraldehyde solution (difference in tissue processing processing, long-term storage, paraffin melting and reprocessing gave undesirable results). This prompted us to look for fresh tissue from concurrently diagnosed renal tumor/ccRCC cases.

Sample #7 in Table 2 was multifocal. Was it the case of vHL disease?

- Two tumor nodes (9.6 x 8.5 x 6.9 cm (lower half) and 1.8 x 1.2 x 1.2 cm (upper pole)) were identified in the nephrectomy specimen of patient #7. Despite being multifocal, there was no family history of von Hippel-Lindau syndrome, and the patient had no other associated lesions, so genetic testing was not performed.
- Added to the manuscript (lines 285-288)

For clarity, the authors might consider summarizing the clinicopathological features (also including the type of surgery; nephron-sparing surgery, nephrectomy, open, laparoscopic, and robot-assisted) of the patients in a separate table. There is no data on the N and M status of the tumors. Did the authors include only non-metastatic RCC in the study? The authors might also perform a correlation analysis between the staining intensity or staining pattern of NHERF1/EBP50 and the clinicopathological features of the patients.

- Clinicopathological features (type of surgery, status of regional lymph nodes, presence of metastatic disease, and condition of resection margins) are added. In order to avoid an increase in the number of tables/figures, it was decided to modify table 2 and add a few lines to the manuscript (see Table 2 and lines 285-294).

The presentation is not consistent. The objective is vague and confusing.

- Addressed in the manuscript to resolve confusion points (please see in blue, thank you).

If the aim of the study is to characterize the IHC expression of NHERF1/EBP50, the

authors could perform immunoelectron microscopy. If their aim was to analyze the subcellular localization of microlumen-like structures, they might better add the pertinent statement in the introduction part of the manuscript.

- Thank you for your concern and suggestion.

Our institution does not conduct immunoelectron microscopy and, unfortunately, has some budget limitations. We have encountered the fact that, having a voluminous cytoplasm, ccRCC tumor cells become "fragile" (especially in areas close to hemorrhages or necrosis). We ran into an issue where some samples that passed the technical steps required for EM analysis lost their cytoplasmic content and only cell membranes were present. Particular attention was required when cutting and staining the tissue for the new EM cycle.

The authors mention the “heterogeneity of immunoexpression” in their samples (line 294). Still, they use only intensity (0 to 3) to evaluate NHERF1/EBP50 IHC. Any kind of scoring system (like multiplying the intensity of staining by the percent of stained cells etc.) could improve this drawback.

- Thank you for a good point. However, as you noted, NHERF1/EBP50 expression was patchy/heterogeneous. In some sections, rare “microlumens” were found on stained tissue sections in multiple foci, in some – a single focus with “microlumen” condensation was seen.
- To make it clear, “only hot-spot areas of NHERF1/EBP50 expression were subjected to semi-quantitative analysis” (lines 231-232).

Generally speaking, RCC tumor cells lack polarity. Thus, it seems incorrect to repetitively mention “apical”, ”apical/lateral”, or “brush border” membranous staining referring to tumor samples. In lines 305 to 306, the authors mention that “normal expression was found in samples where tumor cells formed tubular structures…” This should be illustrated by a figure of IHC staining.

- Thank you for your question. “Apical”, ”apical/lateral”, or “brush border” has been replaced by “luminal” throughout the manuscript.
- A photomicrograph of NHERF1/EBP50 IHC was added showing tubule formation by tumor cells and the pattern of expression (see Figure 2B)

In lines 307 to 310, the authors mention that they combined nuclear grade 1 and 2 tumor samples into the “low-grade group” and the nuclear grade 3 and 4 tumor samples into the “high-grade group”. However, 12 samples is still a small number for correlation analysis.

- Thanks for this comment. Of course, our work is not without flaws. The number of samples studied is small and bias cannot be completely ruled out, but despite this, we were able to identify unique findings in clear cell renal cell carcinoma, trace the relationship between the grouped samples, and present these findings that have not studied so deeply for these types of tumors.
- A new paragraph was added at the end of the discussion (lines 407-413)

Data on various tissue components presented in lines 299 to 301 and Figure 3 does not add any additional information to the main idea of the manuscript. The authors might want to omit it for consistency.

- Figure 3 was deleted.

In Figure 2 the authors use various magnifications in the 4 panels (scale bar ranging from 20 to 100 micrometers). HE staining of RCC seems to be unnecessary because it doesn't add any valuable information. If the authors prefer to present HE staining in the manuscript, they might want to use serial sections instead (HE and IHC with the same scale).

- Figure 2 has been modified and reorganized.
- H&E photomicrograph was removed.
- All images were taken at x400 resolution.
- IHC photomicrograph of tubules formed by ccRCC cells was added (answering the concern above).
- Updated legend for Figure 2 (lines 547-552)
- WHO Classification of Tumours Editorial Board, 5th edition, was added to the reference list (reference #25).

After the revision, this valuable information on NHERF1/EBP50 in RCC could be of interest to oncologists, pathologists, and urologists.

- Thank you very much for your comments!
- Please let us know if you have any questions or concerns.
- We appreciate all your hard work.
- And we would be honored if Your Journal would help us present our findings to the medical community.