Author’s response to reviews

Title: Activation and Function of Receptor Tyrosine Kinases in Human Clear Cell Renal Cell Carcinomas

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Author’s response to reviews:

Dear Editor:

Thank you for your letter and the reviewers’ comments concerning our manuscript “Activation and Function of Receptor Tyrosine Kinases in Human Clear Cell Renal Cell Carcinomas”. We have read the comments carefully and have made corrections accordingly. We hope we have addressed all the concerns and comments satisfactorily in our revised manuscript. The revised portion are marked in red.

The itemized responses to the reviewer’s comments are listed below:

Shanshan Wang (Reviewer 1):

Major comments:

Introduction
1. Line 65, spell the percentage out when start a sentence, also "the kidney cancer" should be "kidney cancer", the sentence should be "Ninety percentage of kidney cancers……". Line 66, sentence "originate from…., and are subdivided into…” is too long, should be "originate from tubular structures of the kidney. They are subdivided……". Line 70, "metastasized cancers" should be "metastasized cancer", also this sentence needs to be re-written to make it easy to understand, e.g. "surgery for
localized kidney cancer, targeted therapies and immunotherapies for metastasized cancer". Line 71, again, spell the percentage out when start a sentence.
Answer: We have made corrections according to the reviewer’s suggestions (new line65,66,70,71).

2. Redundant use of words such as 'of the RTKs' in line 80. Line 82, "the current studies on ..." is really confused, is it should be "the previous study" or "recently published studies from other groups"?
Answer: We have made corrections according to the reviewer’s suggestions (new line79,82).

3. Why spend a whole paragraph talk about VHL mutation and its treatment? If its related to RTKs mutation, this needs to be described also in the opening Discussion paragraph to set the narrative of the study, since it's a little bit confused when read through the abstract part.
Answer: We have revised the paragraph according to the reviewer’s suggestions (new line84,85).

Methods
1. Line 105, "were" should be "are".
Answer: Correction has been made, new line 102.

2. Line 126, "was" should be "were".
Answer: Correction has been made, new line 124.

3. Line 129, "for 2 h" should be "for 2 hours".
Answer: Correction has been made, new line 127.

4. Line 130, one should not start a sentence with "AND".
Answer: Correction has been made, new line 128.

5. Line 146, primary antibodies are "anti-phospho-EGFR……".
Answer: Corrections have been made, new lines 144-148.

6. Line 172, "tumor measured" should be "tumor was measured".
Answer: Correction has been made, new line 160.

7. Line 177, "Mice were euthanized in a CO2 chamber 2 hours after last treatment" should be "Mice were euthanized in a CO2 chamber for 2 hours after the last treatment".
Answer: Corrections have been made, new line 165.

Results
1. Line 193, "There was a lot of extravasated red blood cells" should be "An abundance of extravasated red blood cells were observed in the tumors".
Answer: Corrections have been made, new lines 192-193.

2. Line 220, "To determine if …. were specific, we evaluate…” Should be "To determine whether...are specific, we evaluated ...".
Answer: Corrections have been made, new lines 222, 223.

3. Line 229, when describe phosphorylation pattern in papillary RRC, oncocytoma, etc, should add sample number here also, it's confused because author will need to go back to table 2 to find the correlated information of these sample numbers.
Answer: Corrections have been made, new lines 232, 234.

4. Line 239, the "only one" should be more clarified by add the sample number here also lable in the figure9 A and the legend.
Answer: Corrections have been made, new lines 243, 245. We reorganized the figures according to the reviewer’s suggestion. Please see figure 7A. The sample number has been added in the figure and the legend.

5. Line242, author described that EGFR and HGFR were maintained high level while others decreased, but according to Figure 9A, ERBb2 seems also maintained high level of phosphorylation as EGFR. Quantification graph is needed here to demonstrate authors conclusion.
Answer: Corrections have been made according to the reviewer’s comments, new line 246. A quantification graph has been added in figure 7A.

6. Line 248, "tumorigenicity seemed to correlate with...." may not accurate without any correlation analysis, also it should not appear in result, should move this part to discussion since it is not a solid result from analysis.
Answer: The sentence has been revised according to the reviewer’s comments, new lines 251-253.

7. Line 256, from the poor-quality pictures provided here, hard to tell the result that "PDGFR was absent..."
Answer: We have added higher-magnification pictures in figure 9 (old figure 8) and also reorganized the results according to the comments from reviewer 2, new line 277-280.

8. Line 264. "Fig. 9C" appears without any describe of Figure 9B. Is it missing in Line 267, here "Fig. 9D,9E" should be "Fig.9B,9D,9E"? Figure 9B readout is missing here. Line 274, "As shown in figure 9" is not consistent with previous paragraph, it should be "Fig. 9F, 9G, 9H". This paragraph seems lack of organization, which make it hard to read, the whole paragraph should be organized more definitively with describe each result in figure 9 and re-arranged by order.
Answer: We have reorganized figure 9 (new figure 8) and revised the text accordingly (new line258,260,264,268).

9. Line 267, when compare these 3 group, lapatinib, crizotinib and combination, should use "In comparison..." instead of "But...".
Answer: Corrections have been made, new line 260,261.

Discussion
1. Line 286, "These data are consistent with several previous studies on the roles of RTKs in ccRCCs. For example, the activation of VEGF/VEGFR… the expression of HGFR mRNA was upregulated in patients with ccRCCs" needs to be re-written, such as "these data are consistent with several previous studies on the roles of RTKs in ccRCCs that showed VEGF/VEGFR activation and HGFR mRNA upregulation in patients with ccRCCs.
Answer: Corrections have been made, new line 290, 291.

2. Line 290, "correlate" should change to "relate to" or "due to".
Answer: Correction has been made, new line 292.

3. Line 292, author cited one report but saying "there were reports". Line 295, should add "but not adjacent normal tissues" after the sentence "insulin R was significantly phosphorylated in the ccRCC
samples”.
Answer: Corrections have been made, new lines 294, 297.

4. Line 298, 6 RTKs? Its 9 RTKS through the whole paper.
Answer: We identified 9 activated RTKs in the primary ccRCC tissue samples and 6 of them, Insulin R, HGFR, PDGFRβ, M-CSFR, VEGFR1, and VEGFR2, were specific to the ccRCCs comparing to their adjacent normal tissues. We revised the discussion to make this point clearer, new line 301-304.

5. General comment - Discussion seems lack to conceptual organization and should be organized more definitively with concluding sentences after each paragraph. E.g. Line 242. Author just stated that not clear which of these RTKs were activated in the ccRCC cells, and some of them may be expressed in endothelial cells while some of them are expressed in cancer cells, and PDGFR is associated with poor prognosis, so the conclusion "targeting all of these RTKs…” is not accurate, this conclusion needs to be more specific, such as "Thus, targeting these activated RTKs that associated with poor prognosis may be an efficient way to inhibit tumor growth."
Answer: We have revised the discussion according to the reviewer’s comments.

Figures& Legends

1. Figure 4. Needs to be re-labeled by each set of pictures. Also WB bands needs to be quantified, graphs are missing here. In the method, quantification by software of Azure system was described.
Answer: Figure 4 have been reorganized. WB bands were quantified and graphs were added. Quantification by software of Azure system is described in the methods section, new line 150,151.

2. Figure 8. Immunostaining figure is poor quality, needs higher resolution.
Answer: We added new higher-magnification pictures. We also rearranged the figures according to the second reviewer’s suggestions. The old figure 8 now is figure 9.

3. Figure 9A. Which ccRCC sample is this? should absolutely lable the number of this sample because it is the only one grew successfully. that so Quantification graph is needed here to demonstrate authors conclusion as mentioned above.
Answer: The figures have been rearranged. The old Figure 9A now is Figure 7A. We have added the number and the quantification graph of this sample in the new Figure 7A.

4. Figure 9 legend, Figure C and D were measure before Figure B, should be rearranged by time order. Figure 9F, what is the number (2,4,5) underneath the group mean? Should be described here. P-akt quantification is missing here, even it's a negative read out. Line 561, Last sentence, "Error bars, means±SEM" is this a full sentence?!!!
Answer: We have rearranged the figures. The old figure 9 now is figure 8. Please see figure 8. The numbers (2, 4, 5) underneath the group represent the serial number of mice in the group. P-Akt quantification is now added. The legend of the figure has been corrected according to the reviewer’s comments.

Peter Schraml (Reviewer 2):

1. A-498 and ACHN are considered papillary RCC cell lines (see Brodaczewska et al. and refs herein, Mol Cancer 2016, 15:83). This should be corrected throughout the manuscript accordingly.
Answer: Corrections have been made according to the reviewer’s comments in Abstract section, line 27; Background section, line 89; Methods section, line 109,110; and Results section, line 220, 223, 225,258, figur5, figure 7.
2. The images shown in Fig. 1A/B are of bad quality. The size of "adjacent" normal tissue is uncommon.
Answer: We are sorry for the quality of the pictures. We now reduced the magnification of the pictures so that the resolution may looked better. The ccRCC sample and its adjacent tissue in figure 1 was a portion from a total nephrectomy sample, which is the reason for the size of the adjacent normal tissue. We mentioned this point in the revised manuscript.

3. Despite the known heterogeneous phenotype and genotype of ccRCC it is surprising that the RTK phosphorylation patterns are similar among ccRCCs. This suggests that some RTKs may be preferentially bound. 2-3 cell lines or tumor tissue samples other than RCC should be included as additional controls to show activity of other RTKs (e.g. EphA, ALK).
Answer: We have several comparison controls for the data in figure 2. First is the array data from the adjacent tissues, which showed a totally different pattern of RTK phosphorylation. We also showed data from other kidney cancer samples and from kidney cancer cell lines, which also showed different patterns of RTK phosphorylation (Fig. 5 and Fig.6). Therefore, we believe that the RTK phosphorylation patterns we observed are true reflection of the RTK phosphorylation patterns in vivo.

It's a bit hard to follow the sequence of figures and paragraphs.
Fig 9A should be combined with Fig 7. The paragraph "PDGFRb was expressed..." should be placed to the end of the results section and the Figure numbers should be corrected accordingly.
Answer: We have rearranged the figures and revised the text accordingly following the reviewer’s suggestions.

6. The authors claim that PDGFRb is present in glomeruli, interstitium, peritubular and stroma cells but not in ccRCC cells. In contrast, in Figure 3 PDGFRb is much higher than in the adjacent tissue suggesting the tumors might be strongly contaminated with non-tumorous material? This should be explained.
Answer: The absence of the PDGFRb phosphorylation signals in the adjacent tissues suggested that the PDGFRb phosphorylation signals were from tumors, not from contaminated non-tumorous material (Fig. 2). Our interpretation of this data is that the stroma cells in the ccRCCs were also abnormal and that it was the abnormally activated PDGFRb in the abnormal tumor stroma cells that contributed to the phosphorylation signals on the array. We discussed this issue in the revised manuscript.

7. A supplementary schematic illustration of the RTK array showing the localization of the RTKs would help the reader to compare and interpret the RTK phosphorylation patterns.
Answer: We have added the schematic illustration of the RTK array as the supplementary Figure 1.

Minor:
What is meant by the VHL gene and its partners?
VHL loss of function prevents HIFa from proteasome degradation, which leads to HIFa stabilization rather than overproduction.
Answer: We have revised the paragraph to simplify the statement, because it is a indirectly mechanism to activate the VGEFR and the mechanism is not relevant to the topic of our manuscript (new line 84,85).
Thank you again for your consideration of our manuscript.

Sincerely yours,

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