Author's response to reviews

Title: Expression and Significance of HMGB1, TLR4 and NF-kappaB p65 in Human Epidermal Tumors

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The Biomed Central Editorial Team
Thank you for your kind letter and for the reviewers’ comments concerning our manuscript.

We have studied the comments carefully and revised the manuscript accordingly.

Reviewer # 1 (Erina Vlashi)

Minor revisions:

The authors state that they counted 10 fields per slide. Were 10 fields also counted for endothelial and inflammatory cells as well, in order to get a total score for these tumor areas?

Re:

Yes, 10 fields for endothelial and inflammatory cells were also counted.

It is not very clear from the slides chosen in fig. 1, why these slides have the scoring that the authors describe in the text. For example, nuclear staining of Fig.1g (SCC) appears stronger than in Fig. 1i, but the authors state in the text the opposite, as well as in Fig. 1k. This may just be a result of not choosing a good representative slide for this particular SCC. It would help if there was a panel of slides representing scoring
1-6, in order for the reader to have a better idea of what intensity of the staining corresponds to what score.

Re:

- We apologize for our negligence in choosing Fig.1g (SCC), and we changed the field with a more representative one according to the average scoring.

- In order to get a total idea of inflammatory mediators for the tumors, we tried to choose the slides representing average scoring of nucleus, cytoplasm, cell or intercellular space as well in one slide. Additionally, we truncated the representative sections from 400x original figures in the manuscript otherwise the specific regions depicted by the arrows would be difficult to visualize. As such, instead of providing a panel of slides representing scoring 1-6, we present the 400x image of larger field for suggesting read on line which may exhibited more all roundly in the other hand considering the reviewer’s kind comment, and hope it would be helpful.

Fig. 1:

Bar graphs: please include lines connecting the bars that differ significantly from each other, with an asterisks above showing the level of significance. I.e. one asterisks (*) for p<0.05, two asterisks (**) for p<0.01 etc.

Re:
We have included lines connecting the bars that differ significantly from each other with (**) for p<0.01 in the revised Fig. 1j, Fig. 1k, and Fig. 1l according to the reviewer’s constructive comments.

Arrows: there is no brown-red arrow, I think the authors are referring to the purple arrow for epithelial cell cytoplasmic staining.

Re:

We have changed brown-red arrow to orange arrow for epithelial cell cytoplasmic staining to make it more well-defined in the revised Fig. 1c (PCL).

Fig. 2:

The authors state in the text that TLR4 expression is seen in basal cell and acanthocyte membranes. Please use different color arrows to distinguish between the two.

Re:

We apologize for our redundancy in describing TLR4 expression in basal cell and acanthocyte membranes.

As we mainly focus on the expression of TLR4 on epithelial cell membranes in the study, and both basal cell and acanthocyte belong to epithelial cells, so we use purple arrow to show both the two kind of cells, and changed
“Positive TLR4 expression was predominantly seen on basal cell and acanthocyte membranes in benign seborrheic keratosis (SK) and precancerous lesions (PCL)” into “In benign seborrheic keratosis (SK) and precancerous lesions (PCL), there were diffuse positive expression of TLR4 on epithelial cell membranes” in the revised manuscript which we hope to meet with the reviewer’s satisfaction.

Fig. 2c: seems out of focus. Please replace. Bar graphs: make the same changes regarding significance levels as suggested for Fig. 1.

Re:

- The figure focalized on epithelium membranes has been changed into the revised Fig. 2c according to the reviewer’s comment.

- Lines connecting the bars that differ significantly from each other and (**) for p<0.01 have been added to the revised Fig. 2f.

Fig. 3:

Arrows: there is no brown-red arrow, I think the authors are referring to the purple arrow for epithelial cell cytoplasmic staining.

Re:

- We have changed brown-red arrow to orange arrow for epithelial cell cytoplasmic staining to make it more well-defined in the revised Fig.3a (SK),
Fig. 3c (PCL), Fig. 3d (BCC), in Fig. 3e (SCC), Fig. 3f (SCC), and Fig. 3g (NS).

Bar graphs: make the same changes regarding significance levels as suggested for Fig. 1.

Re:
- Lines connecting the bars that differ significantly from each other and (** *) for $p<0.01$ have been added to the revised Fig. 3h and Fig. 3i.

Fig. 4

Fig. 4e (normal skin): does not seem to be the same magnification as the others (400x)

Re:
- The figure (normal skin) of the same magnification (400x) with truncation as the others has been changed into the revised Figure 4e.

Bar graphs: make the same changes regarding significance levels as suggested for Fig. 1.

Re:
- Lines connecting the bars that differ significantly from each other and (** *) for $p<0.01$ have been added to the revised Fig. 4f.

Reviewer #2: Chann Lagadec
Major concerns:

The figures presented are not convincing. The “representative” slides exhibited cannot really be useful for the reader, who has to trust graphs from the authors. The authors should present at least 2 magnifications for each slide.

Re:

- We apologize for our negligence in choosing some slides such as Fig.1g (SCC) and Fig.1i (NS), and we have changed the field with a more representative one according to the average scoring.

- In order to get a total idea of inflammatory mediators for the tumors, we tried to choose the slides representing average scoring of nucleus, cytoplasm, cell or intercellular space as well in one slide. Additionally, we truncated the representative sections from 400x original figures in the manuscript otherwise the specific regions depicted by the arrows would be difficult to visualize. As for presenting at least 2 magnifications for each slide, we provide the appendant of 400x without truncation for suggesting read on line which may exhibited another magnification practically being with more fields of vision considering the reviewer’s constructive comment.

Since the question is to study if diffuse HMGB1 and activation of NF-kB via TLR4, it
would have been really interesting to perform a dual staining... or at least correlate
diffuse HMGB1 and active p65 in ec.. and not HMGB1 ec n and p65 ec n... since
HMGB1 should be release in the extracellular component to activate TLR4. All cross
correlation should have been done...
Again, correlation graphs are not convincing at all, but the figure 5a.

Re:

- Instead of a dual staining, all cross correlation including active p65 in ec (p65
  ec n) with diffuse HMGB1 (HMGB1 ics) have been added to the Correlation
  analysis, DISCUSSION and FIGURE LEGENDS in the revised manuscript
  according to the reviewer’s constructive comments.

- As for figure 5b and figure 5c seemed relatively less convincing than figure 5a,
  these may be due to the expression of p65 in inflammatory cell nuclei in SK
  was the highest, while in NS and PCL showed relatively low expression with
  its own biology significance. As for TLR4 on epithelial cell membranes,
  TLR4 showed the highest in SCC, while in BCC exhibited relatively low
  expression. As such, the tendency of (p65 inflam n) in figure 5b or (TLR4 ec
  m) in figure 5c was not increased progressively or decreased progressively
  respectively.

  From above all, the correlation analysis showed p65 in epithelial nuclei of
  normal skin and different tumors was negatively correlated with p65 levels in
  the inflammatory cell nuclei (r=-0.2496, *P <0.05) (Fig. 5b), and was
positively correlated with TLR4 levels on the epithelial cell membranes

(r=0.3212, **P <0.01) (Fig. 5c).

HSP70 staining is at the edge of the article, or at least not well incorporate. Level of HSP70 staining is not used for any correlation... these data are coming for free...

Re:

As we mainly investigated the involvement of HMGB1, TLR4 and NF-κB in the different types of skin tumors in the study, and however, the result exhibited HMGB1 may be one of mediators resulting in the development of inflammation in epidermal tumors, but it did not play a central role in highly malignant epidermal tumors. As such, we supposed it is possible for other ligands engaging TLR4 such as HSP70, and then we added the supplementary data of HSP70 staining which indicated it may be another mediator of local inflammation in high malignancy epidermal tumors. This actually help us to explain some HMGB1 questions as we mentioned in the DISCUSSION.

Maybe this is why HSP70 seemed somewhat at the edge of the article.

In order to better incorporate HSP70 in the manuscript, we have added the correlation analysis of HSP70 in epithelial intercellular spaces with p65 in the epithelial nuclei in Correlation analysis, DISCUSSION and FIGURE LEGENDS in the revised manuscript according to the reviewer’s constructive comments.
Authors speak about “significantly higher”, which means there is difference, and “numerically higher” which means not significant... this confuses the reader...

Re:

We have added “P <0.01” or “P <0.05” after “significantly higher”, and changed “was numerically higher than in SCC” into“was higher than in SCC but without statistical significance” in the ABSTRACT and DISCUSSION of the revised manuscript according to the reviewer’s kind comments.

Minor comments:

Magnification of slide are different from figure to figure, and even in figure 4... this adds to the confusion

Re:

Figures truncated from the 400x magnification as the others have been changed into the revised Fig.2a, Fig.2b, Fig.2c, Fig.2d, and Fig.2e.

The figure truncated from the 400x magnification as the others has been changed into the revised Figure 4e.

No scale

Re:
The scale has been added to the revised Fig.1, Fig. 2, Fig.3, and Fig.4.
according to the reviewer’s constructive comments.

p value have to be real values and not <0.01 or <0.05

Re:

P real values have been added to the RESULTS in the revised manuscript.

We have attempted to improve the manuscript and make appropriate changes which
do not influence the content and framework of the paper. We are for
Editors/Reviewers’ input and advice and hope that the corrections will meet with
approval. Should you have any questions, please contact us without hesitation. Once
again, thank you and all the reviewers for the kind advice.

Yours sincerely,

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