Author's response to reviews

Title: Identification and validation of dysregulated MAPK7 (ERK5) as a novel oncogenic target in squamous cell lung and esophageal carcinoma

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Dear Sir/Madam,

I am grateful for BMC Cancer’s efforts in soliciting reviewers comments to our manuscript. Please find below our point-by-point responses to these comments.

Referee 1:

• “In Figure 2, authors need to show an image of 2+ and 1+”.  
  We have now added representative MAPK7 FISH and IHC images for 1+ and 2+ cases in a Supplemental Figure 1.

• “Figure 3. In line 111 is claimed that NCIH193 cell s have low Erk5 levels. To support this conclusion all the controls of the 3 lines should be loaded in parallel (in the same gel)”  
  We have included 2 further Western blots which show Erk5 levels across a panel of cell lines which includes all three lines (SNU449, KYSE30 and NCI-H1793). This data now forms Supplemental Figure 2.

• “The rational for the ELISA assays is correct. However, what is the connection with the role of erk5 in lung or esophageal cancer? This is an interesting methodology approach but does not fit with the rest of the paper.”  
  Thank you for this comment. From a pharmaceutical industry perspective, we have shown that MAPK7 represents a bona fide oncogenic target in a small number of esophageal and lung cancer cases. Having established disease linkage for the MAPK7 target, a logical next step was to build a screening assay to allow us to test potential drug candidates for MAPK7 enzyme inhibitory activity. An ELISA assay represents one type of assay which has higher throughput and allows screening of large numbers of test drugs. The authors believe it important to not just explore the basic biology, but also to demonstrate that there is a viable way forward to screen for inhibitory drugs. Furthermore, the use of a robust artificial co-transfection system here is relatively novel approach.

• “In addition, in Figure 4a no endogenous Erk5 is detected in 293T cells….any explanation?”. 
• As these are co-expression studies which introduce over-expressed MAPK7 and MEK5, these Western blots were only exposed for a short time. We expect that if we were to expose these Western blots for a longer duration, we would likely see endogenous low-level expression of MAPK7 in this cell background. Indeed, we see low level endogenous MAPK7 expression across a number of the cell lines shown in Supplemental Figure 2.

• “For Figure 5, a nice control that is missed is to the same experiment in NCIH1793 and demonstrate if the target blocked by the XMD8-92 treatment are affected or not in a cell system with low Erk5 levels. This experiment will support the specificity of the inhibitor as well as the target described in the phosphorylation antibody array.”

• Thank you for this suggestion. We agree that this would be a good additional negative control to confirm specificity of target modulation. Unfortunately however, the cost of doing this type of experiment was a factor in limiting this analysis to only KYSE30 cells. For the KYSE30 cells, we did include treated and untreated groups and so we believe that the modulation by XMD8-92 is genuine and that this experiment is well controlled.

• “Minor issue: Authors need to evaluate if other MAPK (Erk1/2) is also affected at least in the experimental model of cell culture to support the specificity of Erk5”
• Thank you for this comment. Although we did not check modulation of Erk1/2 by XMD8-92 in our cell system, previous publications have evaluated this compound using biochemical and cell assay systems and have found no activity versus Erk1/2 (Yang Q et al. 2010. Cancer Cell. 18:258-267). In addition, we have also checked the biochemical activity of XMD8-92 versus Erk1 and Erk2 enzymes in vitro and find no activity (Enzyme IC_{50} values >10μM). Therefore, we believe that this compound is relatively specific for Erk5.

• “Minor issue: In discussion section, round line 229, authors should consider other possibilities as Erk5 should be consider other possibilities such as Erk5 degradation or stabilization”.
• Thank you for this suggestion. We have added the following statement to our discussion section and also cited the publications you refer to: “Although our data demonstrate coordinate MAPK7 gene amplification and protein overexpression, it should be noted that previous studies have identified additional mechanisms through which MAPK7 activity can be regulated [Arias-Gonzalez L et al, Buschbeck M et al]. Detailed exploration of the precise molecular mechanisms of MAPK7 dysregulation was outwith the scope of our work here, however our data do suggest that dysregulated MAPK7 may provide a further level of disease segmentation within squamous cell lung cancer, which crucially, is a disease with high unmet need and currently has no specific approved targeted therapeutics.”

Referee 2:

• “Is MAPK7 activated (I mean phosphorylated) in the tissue specimens?”. 
This is a great question and one which unfortunately we do not yet know the answer to. In this study we used the pMAPK7 antibody \textit{in vitro} to show activation of MAPK7 in cell lines overexpressing MAPK7 and MEK5 protein (Figure 4A), however we are currently in the process of validating this (and other antibodies) for immunohistochemistry to enable analysis of our tissue sections. We hope to document this data in a future manuscript.

“Are the results shown with XMD8-92 in Figure 5B reproducible with Erk5 knock-downs?”. This is a good question but unfortunately, we have not done this experiment. As Erk5 is a big protein with a significantly extended C-terminal domain (compared to other Erk’s), we were keen to focus only on the consequences of inhibiting the Erk5 kinase activity using XMD8-92 (published data shows that this is a selective Erk5 inhibitor). Knockout of the Erk5 gene may have led to other effects (which of course would include abolishing Erk5 kinase activity) but which could have been unrelated solely to the kinase activity of Erk5. As the purpose of this work was to identify a downstream marker of Erk5 signaling, we focused on kinase inhibition.

“Figure 4A: the GAPDH controls are seem to be derived from a different blot (First three lanes seem to be from one gel and the next three are from another one). Why? It’s better the authors provide the original blots here”. Our sincere apologies and thank you for pointing this out. We have checked this figure and discovered an error during the electronic image capture step. We have now replaced this image with a corrected version of the actual GAPDH image from the Western blotting experiment.

“Include Mol.weight markers in the blots and scale bars in the immunofluorescence images”. Thank you. We have amended all figures now to include these details.

“4B: Are the data obtained from a technical replicate or a biological replicate? What does the error bar mean here?”. These ELISA data are obtained from 1 experiment which is representative of a total of 3 experiments. In brief, different co-transfected cell populations (referred to in Figure 4A as ‘lane’ groups) were seeded into 96-well plates and used to generate cell lysates for ELISA assay. The error bars here represent the standard error of the mean across multiple wells from the same transfection group (ie. Degree of signal variation within a transfection group).

We thank you for your useful and constructive comments and sincerely hope that we have answered these in sufficient detail and clarity.

Yours faithfully,

Paul R.Gavine. PhD.