Author's response to reviews

Title: Kisspeptin and GPR54 immunoreactivity in a cohort of 518 patients defines favourable prognosis and clear cell subtype in ovarian carcinoma

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Author's response to reviews: see over
Dear Dr. Phillips,

We thank the reviewers for their comments that will improve our manuscript. We have tried to address these within the suggested 3 weeks, by revising our manuscript and including additional figures as detailed below. However, we would like to make the following general point, that we feel may have been overlooked – In addition to the mechanistic interests surrounding GPR54/kisspeptin, a key point of our manuscript is a clinical one - hence our motivation for submitting to BMC Medicine. To recap, there have been no cell type specific, clinically applicable prognostic assays that have been validated in large sample sets for clear cell ovarian cancer (and rather few for ovarian cancer overall). We believe an important impact of our findings will be to provide a clinically applicable method for sub-classifying this form of ovarian cancer, in practice and this will have impact in the structure of future ovarian cancer clinical trials. In this respect, immunohistochemistry is the gold standard methodology of choice and the key issue is normally to demonstrate segregation of immunoreactivity, with clinical parameters, in a sufficiently large sample set. This approach has defined many IHC protocols that are in clinical use, where the specificity of the antisera is either not known, is incompletely mapped, or operates with antibodies that are unsuitable for Western blotting - however this does not diminish the clinical validity of the IHC, because the pattern of immunoreactivity identifies clinical subgroups of relevance.

Addressing specific reviewer comments:

(1) Specificity of GPR54 antibody: we have added a figure showing a Western blot with this antibody. The blot (Supplemental Figure 1) shows the major band of correct size in cell lysates derived from human cell lines discovered by us to express/overexpress endogenous GPR54. Reviewer 1 mentions the high dilution factor required to detect a signal. In fact GPR54 is a 7-transmembrane receptor and is normally very difficult to detect by Western blotting and IHC because it’s hydrophobicity (low levels are not detectable by standard Western blot methods). The levels of dilution used with this antibody have been compared with non-expressing cell lines to estimate background. Although 7-TM proteins tend to concentrate on the membrane, they are also recycled through cytosolic pools and it is not uncommon (especially in malignant cells) to see some cytoplasmic expression of these proteins. The TMA scoring protocol evaluated only membrane positive staining (this point has been re-emphasised in the new text).
Specificity of the kisspeptin antibody. Unfortunately none of the commercially available kisspeptide antibodies we have tested (Santa Cruz, Phoenix Peptides) perform well enough on Western blots to address endogenous kisspeptins from human cells. To address the issue of specificity on paraffin embedded tissues, we have therefore used blocking peptides and cell type specificity of staining. Bilban and colleagues (J Cell Sci 2004) were able to clearly show cell type specific isolation and detection of kisspeptin-54 using an antiserum generated by their laboratory (not currently available to us). In this report, it is clear that kisspeptin expression is restricted to synctiotrophoblast, whereas GPR54 is present in extravillous cytotrophoblast and villous cytotrophoblast. Thus, to further clarify the specificity of our IHC, we have modified our Figure 1, showing the effect of blocking with three different kisspeptin sub-peptides. Kisspeptin-54 (68-121aa) and kisspeptin 100-120aa (blocking peptide) fully block the IHC signal when used on control tissues (10 week placenta). Kisspeptin-10 (112-121aa), which only just overlaps the kisspeptin 100-120aa peptide, shows no significant blocking. Furthermore, the signal generated with this IHC protocol is highly cell type specific, with GPR54 and kisspeptin reactivity showing the predicted reciprocal patterns, specific in the same manner as Bilban 2004 (Figure 1). This narrows the region of canonical kisspeptin that could be detected by the antiserum used in this study. Within the 3 weeks allowed by the editorial office this is the closest we can get to addressing immunospecificity. We believe that cell type specificity and blocking by specific peptides reasonably allow a conclusion that the antisera have reactivity against kisspeptides. However we recognize that other peptide fragments of kisspeptin or indeed other proteins may be detected and we have altered our text to reflect that the precise immunoreactivity of the kisspeptin antiserum used in this study, remains to be determined, as a reflection of this uncertainty.

Minor comments from reviewer 1

p4: Metastin was discovered by screening for binding to GPR54 (alternatively named in that paper). So the sentence beginning "Kisspeptins, encoded..." should be written to accurately reflect the situation.

This has been addressed in the text

The authors' PNAS paper reference should be updated

Done

Detailed antigen retrieval methods need to be included in the manuscript since not all antigen retrieval approaches work for all antibodies.

Done

Use of 1:25 titre for the anti-GPR-54 antibody is troubling. Such a high titre is often fraught with ancillary bands in immunoblots.

Automated immunodetection such as the Ventana systems we used often require more
antiserum, but see also the comments and data above re: specificity.

The data should be discussed in the context of the paper earlier this year by Nash et al. whose data suggests that kisspeptin-GPR54 interaction may not be autocrine.

Actually we thought we had done this, indicating that the mechanisms by which kisspeptins might exert anti-metastatic effect remain unclear, but we have further clarified the text.

The figure legends are sparse and do not provide sufficient information for a reader to understand the images without re-reading the text.

Additional text has been added.

There are abbreviations mentioned in the text referring to figures (e.g., p10 and Figure 3). While relatively self-apparent, the manuscript should be made internally consistent with regard to utilization of the abbreviations or eliminate them.

Done

Higher resolution insets that allow the reader to carefully look at subcellular distribution would be helpful. In particular, there is 'concern' that GPR54 is cytoplasmic rather than membrane-associated (Figure 2). (Comment also made by reviewer 2)

Done – note that GPR54 can be both cytoplasmic and membrane linked as receptors are normally recycled through the cytoplasm, so this is expected – however only membrane reactivity was scored in this study.

Comments from reviewer 2.

Reviewer 2 expresses that she feels our manuscript is better suited for BMC Cancer rather than BMC Medicine and feels that additional techniques should have been used.

The main motivation for submitting to BMC Medicine is that both the findings and the technique used, lead to clinical application. Expression microarrays were mentioned, but microarray expression analysis is not a clinically applicable form of analysis. Pathologists and clinicians seeing a clinically applicable (ie. Immunohistochemistry based) assay method that provides prognostic information, can make use of this assay to structure future clinical studies and trials from material that represents clinical practice (formalin fixed, paraffin embedded tissues). To our knowledge this is the first such marker defined for clear cell ovarian carcinoma. We noted and referenced that a recently published small series of ovarian carcinomas analysed by Q-PCR reached conclusions that overlap with, but are not as extensive as, those presented in this study.
1. The authors should make clear why they are investigating the relevance and expression of Kisspeptin and GPR54 in ovarian carcinoma. What lead them to look at these specifically? Was there undisclosed evidence prompting this work?

Our groups discovered the fundamental connection between GPR54 signalling and regulation of the steroid axis through mouse genetics and human genetics in 2003 and we have been systematically investigating the possible relevance of this axis to tumour metastasis and progression in tumours arising from hormonally sensitive epithelia.

2. The discussion is somewhat over-long and should be shortened. Much is made of the short comings of others work, they should better discuss their own results.

The discussion has been shortened, as suggested.

3. Results must be shown for KP-10, not just mentioned in the results section

See notes above on extra figure.

6. The text does not adequately describe Figures 1 and 2: each section is labelled but this is not reflected in the text

Better figure legends have been written as noted above.