Author’s response to reviews

Title: Silencing microRNA-330-5p increases MMP1 expression and promotes an invasive phenotype in oesophageal adenocarcinoma

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Author’s response to reviews:

Dear Editors,

We thank the reviews for further comments and feedback on our manuscript. We have provided a point by point response letter and have edited the manuscript accordingly. We believe that the manuscript is now ready for publication in BMC Cancer.

Yours sincerely,

Dr Becky Bibby, PhD

Reviewer 1
In their revised manuscript Bibby B et al. have significantly clarified their manuscript according to reviewers comments. Their experimental and theoretical arguments are much more straightforward to follow than in the previous manuscript. Overall, I believe the authors have addressed all of my concerns, and if they can address my relatively simple question about "limitations of in vivo model" in discussion part of the manuscript, I can gladly recommend their manuscript for publication in BMC Cancer. Kind regards.

Author response:
We are happy we were able to address the reviewers concerns. As requested we have included
comments on the limitation of in vivo models in our discussion (pages 14-15, lines 356-367). Further comments on the limitations of the model (for review purposes only) are included below in the response to reviewer 3.

Reviewer 3
The authors have adequately addressed my comments. However, reading the comments of other reviewers, especially reviewer 1 has raised some questions for me, since the authors have NOT adequately addressed all his comments. I will point out the comments that I think need further response and revision of the paper –

Review 1 comment 3. The author’s in vivo experiments have not assessed invasion in their in vivo experiments, focusing on tumor growth. While OAC cells are not that invasive, the authors should have tackled this point - perhaps by immunohistochemical staining for MMP1, measuring protein level in vivo, etc.

Author response:
We are happy we were able to adequately address all of reviewer 3’s comments.

With regard to reviewer 3’s reference to reviewer 1’s comment 3 and our response: Generally, tumour growth is an acceptable proxy for measuring local tumour invasion. The relatively non-invasive cell line used and limitations associated with subcutaneous xenografts in immune compromised mice make the model unsuitable for studies relating to metastatic invasion. Prospectively the authors would be inclined to use the findings from this manuscript to support the development of an alternative in vivo model to study the effect of miR-330-5p on invasive capacity. This would be outside the scope of this manuscript. Here, the authors recognise that ultimately while we know that MMP1 expression is enhanced, it is not possible to state that MMP1 is solely responsible for driving the observed increased tumour growth. This is because our in vitro work demonstrated that miR-330-5p is regulating multiple soluble factors, both identified in our screen and as yet unidentified factors, which could coordinately contribute to the enhanced tumour growth/invasive phenotype. However, what we can state with a high degree of certainty is that miR-330-5p modulation does indeed alter tumour growth. Supporting this line of thinking:
• The mixed population of heterogenous clones (HC) was used for the in vivo experiments because it was considered to be more representative of intratumour heterogeneity than the single clone (SC). In the miRZIP-330-5p HC cell line MMP1 expression increased by approximately 1.75 fold compared to the miRZIP-VC HC control cell line. In comparison, MMP1 expression increased by approximately 7.5 fold in the miRZIP-330-5p SC model compared to the miRZIP-VC SC. The authors do not expect the increase in tumour growth with the HC model in vivo to have occurred as a result of the modest increase in the expression of the MMP1 protein observed in vitro. The change in tumour growth is likely a multi-factorial event driven by multiple miR-330-5p-mediated alterations in tumour cell biology.
• Although we identified MMP1 protein upregulation in vitro we also observed overall higher expression of proteases inhibitors compared to proteases in the antibody profiling array. In particular the profiling array showed a strong signal for TIMP1, which is a recognised inhibitor of MMP1 (1). This is particularly relevant to the in vivo model because the extracellular proteases and protease inhibitors will be acting on the extracellular matrix within the tumour microenvironment. Examining MMP1 expression by IHC, as suggested, without examining its activity, and the relative expression levels of known MMP1 inhibitors, such as TIMP1, and their relative tissue localisation will reveal nothing with any degree of certainty. Indeed, considering we are forcibly overexpressing miR-330-5p in the model, all the IHC will likely show is that MMP1 is overexpressed, which is of little experimental value in this scenario.
• Enhanced tumour growth in this model is unlikely to be attributed to a single protein, the
The authors consider that the increased tumour growth is very likely the result of silencing miR-330-5p, which directly or indirectly modulates the expression of hundreds, if not thousands of gene and protein targets.

- The authors consider that measuring MMP1 expression would not add to the findings of the study because an increase (or decrease) in MMP1 protein expression would not be sufficient evidence to confirm (or dismiss) invasion as the mechanism responsible for enhanced tumour growth. A more appropriate experiment to determine if an increase in invasion is responsible for enhanced tumour growth would involve an assay such as that reported by Hernandez et al, which would be best undertaken in an orthotopic rather than a subcutaneous model (2).

From the in vivo experiments we can conclude that the increase in tumour growth is a consequence of miR-330-5p silencing, which is of significance considering that we have previously identified miR-330-5p as the most downregulated miRNA in a cohort of OAC patients who did not respond to neoadjuvant chemoradiation. It is the myriad changes associated with silencing of the miRNA that is responsible for enhanced tumour growth – the authors do not believe that it is not due to the modest upregulation of a single protein (i.e. MMP1). The take home message from the in vivo experiments is that silencing a single miRNA, miR-330-5p, enhanced tumour growth. Additional studies are needed to determine the mechanism by which miR-330-5p silencing enhances tumour growth, however, it is arguably more worthwhile to determine if rescuing miR-330-5p expression through therapeutic intervention could reverse the increase in tumour growth observed in vivo.

Review 1 comment 5. The article needs to be revised to distinguish between previous result of this group, previous results of other groups, and the results in this current article. I believe the in vivo experiments need to be explained and SUBSTANTIALLY REVISED before acceptance.

Author response:
The manuscript has been edited to clarify those results that are from previous studies and the results in this current article (page 5, lines 101-112).

References: