Author's response to reviews

Title: Decreased expression of Yes-associated protein is associated with outcome in the luminal A breast cancer subgroup and with an impaired tamoxifen response

Authors:

Sophie Lehn (sophie.lehn@med.lu.se)
Nicholas P Tobin (nick.tobin@ki.se)
Andrew H Sims (andrew.sims@ed.ac.uk)
Olle Stål (olle.stal@liu.se)
Karin Jirström (karin.jirstrom@med.lu.se)
Håkan Axelson (hakan.axelson@med.lu.se)
Göran Landberg (goran.landberg@gu.se)

Version: 2 Date: 20 December 2013

Author's response to reviews: see over
Dear Editor,

We are very grateful for the comments associated with the manuscript "Decreased expression of Yes-associated protein is associated with outcome in the luminal A breast cancer subgroup and with an impaired tamoxifen response" which we feel have contributed to improvement of the manuscript, and we thank the reviewers for the time spent revising our manuscript. Below is a copy of the reviewers’ comments, followed by a point-by-point response.

Referee 1. Alexander Hergovich

Major compulsory revisions (major points)

1) Comment:
In the first paragraph of the introduction, the authors cite three very selective and kind of outdated reviews on YAP. Instead of these references the reviewers should use a more recent and outstanding reviews on YAP (e.g. Hong et al. 2012). In the context of references, the authors should also include all relevant references on YAP/TEAD functions in Hippo signalling (e.g. Wu et al. 2008; Zhang et al. 2008; Ota et al. 2008; and maybe also Li et al. 2010; Zhao et al. 2009; Tian et al. 2010; Chen et al. 2010), since currently they cite only one relevant paper (reference 6). Furthermore, the authors also should include the following references in the introduction and/or discussion section: Matallanas et al. (Mol. Cell 2007) and Chen et al. (Nature Med 2012), since these studies showed that YAP levels are decreased in breast cancer samples and that YAP plays a significant role in breast cancer metastasis, respectively.

1) Response:
A The suggested review, which we agree is much more up to date than previously cited references, has been cited.
B The suggested articles regarding YAP/TEAD functions (Wu et al. 2008; Zhang et al. 2008; Ota et al. 2008) have been added to the already included reference Zhao et al. 2008 in the introduction of the manuscript on page 5. In addition, the suggested references Zhao et al. 2009 and Chen et al. 2010 have been cited in the paragraph following.
C The Matallanas paper from 2007 has been cited in all parts of the manuscript which refer to “decreased YAP1 protein expression in breast cancer”, on page 6 and page 20. Chen et al. 2012 is cited in the discussion on page 19, in the paragraph discussing the possibility that YAP1 has oncogenic functions in ER negative breast cancers [ref 56].

2) Comment:
The clinical data look very convincing and are very interesting. However, the data presented in Figure 4 and 5 using the T47D ER+ breast cancer cell line could be expanded with respect to the tamoxifen response phenotype. Personally, it would be interesting to know how MCF7 (another ER+ breast cancer cell line) are affected by YAP siRNA and how YAP
overexpression in a standard model cell lines such as MCF10A (either parental ER- or manipulated to express ER) affects the tamoxifen response (maybe YAP overexpression in the T47D cells should also be considered). However, I would also be happy when the authors could explain why they decided to focus on only one cell line in these settings. In my opinion, this point is rather important, since the authors mention the tamoxifen response phenotype also in the title of this manuscript. Moreover, the authors should also speculate more on the observed discrepancy between the ER+ cell line model and primary breast cancer data (see end of 2nd paragraph in the discussion section).

2) Response:

We have performed growth curve experiments downregulating YAP1 in both T47D and MCF7 cells which shows that proliferation is decreased in the MCF7 cell line when YAP1 is downregulated. This information has been added to Additional file 9. Wang et al. 2012 PMID: 22056638 also report decreased proliferation in MCF7 cells upon YAP1 depletion, using a stable transfected YAP1 shRNA MCF7 cell line. Yuan et al. 2008 PMID: 18617895 report that decreasing YAP1 protein expression in MCF-7 cells leads to increased invasion. Please also see response C below for a further discussion on why we did not choose MCF-7 for further experiments.

We agree with the reviewer that it would be of great interest to study the MCF10A (ER manipulated) cell line in the context of tamoxifen response and YAP1 expression. However, since the overexpression of YAP1 in MCF10A cells has been reported to result in cell transformation including induction of EMT, increased proliferation and inhibition of apoptosis (Overholtzer et al. 2006, PMID: 16894141) delineating the effects of YAP1 with regards to tamoxifen response is likely beyond the scope of this manuscript. In short, the aforementioned oncogenic changes in response to YAP1 overexpression render elucidation of the tamoxifen response highly challenging in this cell line.

The T47D cell line was chosen for several reasons. First, T47D cells have a higher gene and protein expression of YAP1 compared to other commonly used luminal breast cancer cell lines, which makes T47D a suitable cell line for YAP1 downregulating experiments. Second, downregulating YAP1 in T47D cells has no significant effect on their proliferation. An additional file [Additional file 9] with growth curves of YAP1 downregulation in T47D and MCF7 cell lines has been added, as well as western blots comparing YAP1 protein expression in the two cell lines, among others [see Additional file 9c]. Gene and protein expression levels of YAP1 in breast cancer cell lines may also be found in references Moleirinho et al. 2012 PMID:22614006 and Wang et al. 2012 PMID:22056638, which are also cited on page 17. The arguments for choosing T47D have been added to the result section, in the subsection YAP1 downregulation in the luminal cell line T47D results in a weaker tamoxifen response (page 17), which now reads: The T47D cell line was chosen to further investigate the role of YAP1 in tamoxifen response due to its relatively high expression of YAP1 compared to other luminal cell lines such as MCF7 [Additional file 9] [Moleirinho, 2012 PMID:22614006 and Wang, 2012 PMID:22056638], and also since proliferation in this cell line was not significantly affected by YAP1 downregulation. A section regarding this matter has also been added to the discussion (page 22). For the reviewer’s interest, we also note that the only other commonly used cell line with high YAP1 expression (of luminal origin) is BT-474 (Moleirinho et al. 2012 PMID: 22614006), but as BT-474 cells are HER+, endocrine resistance is likely to be driven by other pathways in this cell line.
We have extended the discussion regarding this matter, please see the discussion section, page 20. It now reads as follows:

“The effects of protein downregulation in cell line models are usually assessed after days or weeks, whereas primary tumours evolve over a period of years. As YAP1 loss is proposed to be an early event in breast cancer [Yuan, 2008 #147], YAP1 downregulation in cell lines such as MCF-7 might have to be assessed at much later time points in order to better correlate findings between primary tumour data and cell lines. The microenvironment might also constitute a critical parameter for understanding and modelling YAP1 in breast cancer, and stable downregulation of YAP1 in MCF-7 has been reported to result in increased invasion in a matrigel transwell assay [Yuan, 2008 #147].”

3) Comment: In the context of Figure 4, I would also recommend to control for changes in cell cycle progression as a consequence of YAP manipulations by siRNA. It could be that the general cell cycle is changing in these cells, which might result in a different tamoxifen response, which would indicate that the observed effect is not really directly linked. Therefore, cell cycle profiles and cell cycle markers (such as cyclins D/A/B) should be also examined in these settings.

3) Response:
A western blot showing expression levels of cyclin D1 and cyclin A2 have been added to figure 4a. We note modest changes in the expression levels of these cell cycle proteins in response to YAP1 silencing (please also see response 2C).
In addition, we have attempted to analyse the cell cycle phase distribution in the T47D cell line by flow cytometry, but the hypertetraploid genome of T47D rendered it difficult to analyse the results correctly, as the G2/M peak of the 4n population merges with the G0/G1 peak of the 8n population (this phenomenon has also been addressed in Graham et al. 1989, PMID2660983).
We hope that the reviewer will agree that the growth curves in Additional file 9 and the western blots of cyclin D1 and A2 in figure 4a is sufficient for assessing the cell cycle progression. We do believe that there is a slight effect on cell cycle progression upon YAP1 downregulation in T47D cells, but we are confident that this minor cell cycle effect is not completely responsible for the decrease in tamoxifen sensitivity. In addition, the increase in PgR (Progesterone receptor) and ER (Estrogen receptor) expression levels in figure 5 is an important result to consider in relation to figure 4, as it links YAP1 downregulation to changes in the ER/PgR signalling pathway, important for tamoxifen response.

Minor compulsory revisions (minor points)
A) In the materials and methods section please include the catalogue numbers of the antibodies used in this study, to facilitate the translation of these findings into other ongoing studies.
Response to A: Catalogue numbers of the used antibodies have been added to the methods section.
B) Please improve the labelling of Figure 1, since currently this figure is basically unlabelled.
Response to B: The staining intensities absent, weak, intermediate and strong have been added to Figure 1.
Referee 2: Sigurd Lax

1) One minor point of critique: The definition of luminal A breast carcinoma needs to be more clearly stated, in the method section and the abstract.

Response: We have revised the result section in the abstract to address the Luminal A type of tumours more clearly and it now reads:

Notably, low YAP1 mRNA was independently associated with decreased recurrence-free survival in the gene expression dataset, specifically for the luminal A subgroup \( p<0.001 \) which includes low proliferating tumours of lower grade associated with a good prognosis.

In the methods section, we have added information on what type of gene expression signature (Norway/Stanford) was applied to distinguish the different molecular subtypes, and the reference Sims et al. 2008 PMID:18803878 was added to this section.

2) The photomicrographs should be increased in size although the resolution is excellent. The weak intensity is really very weak.. Maybe it would be worth to stratify the staining into 2 groups: negative and weak versus moderate and strong.

Response: The photomicrographs in figure 1 have been increased in size and clearly labelled. We have considered stratifying the staining into the two proposed groups of negative and weak vs. moderate and strong. The significant correlations are the same as when four groups are analysed. However, since it is clear from figure 3d that patients with tumours lacking YAP1 expression have the worst prognosis, we concluded that it would be more interesting to analyse patients from the four intensity groups separately, rather than using two groups. Comparing the two analyses (two groups and four groups) reveals that p-values in general are lower in the four group analysis, also indicating the importance of analysing the tumours lacking YAP1 separately (for example, for significant p-values in table 2; two groups vs. four groups; \( p=0.002 \) vs. \( p<0.001 \) (ER\( \alpha \)); \( p=0.009 \) vs. \( p=0.002 \) (PgR); \( p=0.006 \) vs. \( p<0.001 \) (CCND1 amplification) and so on). Furthermore, we believe that for future studies in the same field, it is an advantage if our data is presented as informative and detailed as possible by openly showing the data in four separate groups instead of only two.

3) There are some minor typos such as adjustment for.. in the middle of page 14

Response: This has been corrected.

All authors of this paper have read and approved the final version submitted. The contents of this manuscript have not been copyrighted or published previously. The contents of this manuscript are not now under consideration for publication elsewhere. The contents of this manuscript will not be copyrighted, submitted, or published elsewhere while acceptance by BMC Cancer is under consideration. There are no directly related manuscripts or abstracts, published or unpublished, by any author of this paper. There are no conflicts of interests associated with the paper.

Kind regards,

Sophie Lehn PhD
Center for Molecular Pathology
Lund University
205 02 Malmö, Sweden
Phone +46 (0) 70 270 3242

Göran Landberg MD, PhD
Sahlgrenska Cancer Center
University of Gothenburg
405 30 Gothenburg, Sweden
Phone +46 (0) 31 342 16 53