Author's response to reviews

Title: The Wnt signalling pathway is upregulated in acquired Tamoxifen resistant breast cancer.

Authors:

Loh Yan Ni (z3231936@student.unsw.edu.au)
Ellen H Hedditch (20120614@my.nd.edu.au)
Laura A Baker (Lbaker@student.unsw.edu.au)
Eve Jary (eve.jary@unsw.edu.au)
Robyn L Ward (robyn@unsw.edu.au)
Caroline E Ford (caroline.ford@unsw.edu.au)

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

Please find attached our revised manuscript #3260892108380641 now entitled “The Wnt signalling pathway is upregulated in an in vitro model of acquired Tamoxifen resistant breast cancer” for consideration for publication as a research article in BMC Cancer.

We thank the Editor and two peer reviewers for their positive and helpful comments and have addressed each point in the revised manuscript text, and below.

Editor’s comment:

First it is difficult to generalize your results as you investigated only a single cell line. Thus, it remains open, if the results are found also in other cell lines and the more also in patients. This point should carefully be handled and discussed. Moreover, this should also be mentioned in the title of your manuscript. This might be THE WNT SIGNALLING PATHWAY IS UPREGULATED IN ACQUIRED TAMOXIFEN RESISTANT BREAST CANCER MCF7 Cell Model.

The title has been altered to reflect that this is one model cell line. The new title is THE WNT SIGNALLING PATHWAY IS UPREGULATED IN AN IN VITRO MODEL OF ACQUIRED TAMOXIFEN RESISTANT BREAST CANCER. In addition, further clarification has been added in the introduction and methods to make it clear this is one cell line.

Second in figure 1b the DMSO value should not be linked to the rest of the curve as it is not part of the variable tamoxifen but an independent test.

Figure 1B has been changed to a bar graph format to more readily distinguish DMSO from the other values.

Third figure 2d is incorrect. The graph implies that you are working with a cardinal sized factor but instead you are using discrete values. Thus, a bar chart is appropriate.

Figure 1D has been changed to a bar graph format as suggested.

Four you are indicating statistical significance by asterisks. In graph 3 c it seems to me that vimentin as well as TWIST 1 show a significant difference but there are no asterisks to be found.

This experiment has been carried out once only (in triplicate wells), therefore statistics are not appropriate. This has been clarified in the text.

Five then and in a while you are writing B- or b-Catenin instead of using the greek letter \( \beta \). Please, (spell-)check your manuscript for that.

Changed throughout.

Six the TOP- and FOP-flash luciferase reporter gene constructs which are usually available (Hans Clevers\( \beta \) or Randy Moon\( \beta \) laboratories) carry four or seven TBEs (TCF4 binding elements) respectively. You are writing that there are three TBEs in your reporter. Please, check for this.
The TOPflash and FOPflash plasmids were purchased for Millipore and the correct details regarding number of binding elements has been added to the methods section.

Seven you are writing ¿¿TamR cells showed significantly increased expression ¿Axin2 and ¿ (Figure 2A). ¿¿ But in figure 2A the relative expression of Axin2 does not display a significant difference.
The word significantly has been removed.

Or ¿¿ at tamoxifen concentrations of ¿ IWP ¿¿ further inhibited cell proliferation ¿ (Figure 3B) ¿¿. But in figure 3B the differences between tamoxifen- and combined tamoxifen IWP-2 treatment are not significant. Thus, there is no additional effect. Please, describe exactly, what was found (also confer to Dr.Vican¿s review).
The addition of IWP-2 further inhibits cell proliferation, but does not reach statistical significance. However, we still believe there is an additive effect which requires further investigation. This has been clarified in the discussion.

Reviewer 1’s report:
Using a laboratory established in vitro culture system; Loh and colleagues provide compelling evidence for a causative role for Wnt signalling in the acquired resistance of breast cancer cells to tamoxifen. Clinically, acquired resistance to tamoxifen is accompanied by detectable changes in the cancer cells; they become more aggressive and metastatic, and these phenotype changes are mirrored in this in vitro model. The study demonstrates increased canonical (b-catenin dependent) and non-canonical (b-catenin independent) Wnt signalling in tamoxifen resistant cells. This increased Wnt signalling was accompanied by concomitant increases in the expression of genes associated with a more aggressive and invasive phenotype (EMT genes) in the Tamoxifen resistant cells. This paper makes an important contribution to our understanding of the mechanisms underlying drug resistance in breast cancer cell but would benefit from some necessary revision.

Major compulsory revision:
1. The data clearly indicate that the non-canonical Wnt pathways are up-regulated in the tamoxifen resistant cells, even more so than the canonical pathway. That is, the largest increases in gene expression are Ror2 and JUN, which are non-canonical Wnt pathway components and strongly indicate an increase in Wnt/PCP signalling. Secondly, DKK, which primarily inhibits canonical Wnt signalling as it binds to LRP, is also dramatically increased in the resistant cells. The increase in Wnt/PCP components and an inhibitor of canonical signalling should be highlighted – this is a very interesting finding. An increase in canonical Wnt signalling is none the less evident (significant increases in reporter activity and c-myc expression etc) but I would conclude that canonical pathway is possibly constrained (by increases in DKK) in the resistant cells. Also, the increase in AXIN2 is relatively small (not sure if it is significant as Fig 2A does not have a * for AXIN2). Near the end of the discussion there is reference to both flavours of Wnt signalling being up regulated, which is clearly supported by the data, however in other sections of the paper interpretation of the results is more single minded, focusing on the canonical pathway. For example, PORCN facilitates secretion of all Wnt glycoproteins yet it is referred to as “…support the activation of the canonical Wnt pathway in response to Tamoxifen.” in the discussion. The increase in CSNK1A1 does indicate increased canonical signalling but increased PORCN does not. Careful revising to ensure only bona fide Wnt target genes are referred to as Wnt target genes, and as already mentioned, canonical vs. non-canonical roles are clarified, as supported by the data, is required. There is emerging evidence that the non-canonical Wnt pathways are involved in tumour invasion, so this is a very timely article as it indicates that the Wnt/PCP pathway might be involved in tamoxifen resistance and the acquisition of migratory and invasive phenotype in breast cancer.
These comments are very helpful, and we have now rewritten sections of our results and discussion to further highlight the involvement of the non-canonical Wnt pathway in acquired Tamoxifen resistance. We
have also speculated about the increased expression of DKK1 in the discussion (“However, while DKK1 is identified as a downstream target of canonical Wnt signalling, this is suspected to be as part of a negative feedback loop as DKK1 is an inhibitor of the canonical co-receptor LRP (40). Therefore it is possible that the upregulation of the canonical Wnt pathway is tempered somewhat by the increased expression of DKK1.”)

We have also separated the discussion of CSNK1A1 and PORCN and included further discussion of PORCN’s importance in both pathways (“A strong increase in expression of Porcupine (PORCN) was also noted, which is essential for secretion of all Wnt ligands from the endoplasmic reticulum. This suggested that the increase in Wnt signalling may be network-wide and not specific to a particular arm of the pathway.”)

**Minor essential revision:**

1. Ensure that \( \mu \) (not u) throughout.
   This has been adjusted throughout.

2. If increase in AXIN2 expression in Fig 2A is not significant (no *) then don’t overstate the results – trend towards increase is fine. AXIN2 is considered a global Wnt target gene, but there are instances where increases are small, so that’s fine.
   Have removed incorrect use of the word “significant” and have adjusted discussion (see answer to major revision).

3. Add * for significance in Fig 3C.
   Inappropriate – please see answer to Editor’s comment.

4. As Mentioned above, PORCN is necessary for Wnt secretion, thus inhibition of PORCN is a global inhibition of Wnt signalling – not specific for the canonical pathway.
   This has been changed in the discussion.

**Level of interest:** An article of importance in its field

**Quality of written English:** Acceptable

**Statistical review:** No, the manuscript does not need to be seen by a statistician.

**Declaration of competing interests:**

I declare that I have no competing interests

**Reviewer 2’s report:**

Breast cancer is one of the few cancers which is sensitive to the hormonal therapy and tamoxifen has been still considered as one of the first options for hormonal therapeutic drugs. However, the drug-resistance is always bothering in this treatment. The authors are trying to characterize one of the proposed characterized Wnt pathway by RT-PCR array and report assay. The question in this study is well focused and the methods are well organized. The data showed that a number of Wnt genes including CCDN1, DKK1, JUN, PORCN, CSNK1A1 and MYC have been increased significantly in the TamR cell line, which was confirmed by further report gene assay. The results also showed epithelial to mesenchymal transition (EMT) was closely related with the Wnt upregulation. The best point of the study is the inhibitor of Wnt pathway suppress both of cell proliferation and EMT, which confirm the previous results. Therefore, in my view, this study is a well focused confirmation study using TamR cell line. The results
confirm that Wnt pathway upregulation is the major part to afford the cell survival for resistance and EMT morphology. It is worthy of publication.

Considering the title, it maybe more appropriate to be “THE WNT SIGNALLING PATHWAY IS UPREGULATED IN ACQUIRED TAMOXIFEN RESISTANT BREAST CANCER Cell Model”

The title has been changed. No other changes requested.

Level of interest: An article of importance in its field
Quality of written English: Acceptable
Statistical review: No, the manuscript does not need to be seen by a statistician.

We believe our manuscript has been considerably improved, and look forward to the opportunity to publish our findings in BMC Cancer.

Yours sincerely,

Dr Caroline Ford
Group Leader,
Wnt Signalling & Metastasis Group