Author's response to reviews

Title: The heat shock protein-90 co-chaperone, Cyclophilin 40, promotes ALK-positive, anaplastic large cell lymphoma viability and its expression is regulated by the NPM-ALK oncoprotein

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Author's response to reviews: see over
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Re: Manuscript # 4047953569730457. "The heat shock protein-90 co-chaperone, Cyclophilin 40, promotes ALK-positive, anaplastic large cell lymphoma viability and its expression is regulated by the NPM-ALK oncoprotein".

Dear Dr. Rao,

We were extremely encouraged by the positive reviews of our manuscript. While Reviewer 1 offered no suggestions to improve the manuscript, Reviewer 2 raised some good points. We have made several changes to the manuscript to address Reviewer 2’s comments which are outlined below.

Reviewer 2

Comment number 1 “Standard deviations are demonstrated in every figure from every experiment. In the methods, the authors mention experiments performed in triplicate for the Cyp40 luciferase assay and the MTS assay. Please state in the methods the number of times the Western blot and the RT-PCR experiments were performed”

We have made the following additions to the Methods section:

- On page 10, 1st paragraph, 7th line we have included the statement: “The number of independent replicates for each experiment are indicated in the figure legends.”

- On page 10, 2nd paragraph, 12th line we have changed the statement: “Results are displayed relative to control siRNA-transfected cells”, to read: “Results are displayed relative to control siRNA-transfected cells and represent the mean and standard deviation of three independent experiments.”
- On page 11, 1st paragraph, 6th line we added the statement: “Three independent replicates were performed for each experiment.”

- On page 12, 1st paragraph, 2nd line we have included the statement: “Each experiment was performed in quadruplicate.”

Comment number 2, “Please state the statistical methods that were used in the Methods section”

We have included a section titled “Statistical analysis” in the Methods section and stated the statistical methods used (see page 12).

Comment number 3, “In the experiments outline in Figure 3, the authors show quite nicely that NPM-ALK knock-down leads to significant inhibition of both Cyp40 and FKBP52. It is not clear to me whether the Cyp40 inhibition is due to JunB being inhibited as an intermediary. Is the NPM-ALK effect on Cyp40 merely another manifestation of JunB inhibition? Can this pathway be further clarified, Perhaps, NPM-ALK inhibition in the setting of JunB over expression or NPM-ALK over expression in the setting of JunB inhibition. Which promoter is more important? What happens when both are inhibited”

Comment number 4, “Knock-down of NPM-ALK led to more efficient suppression of Cyp40 than JunB knock-down (comparison of figures 2 and 3). Can the authors comment on whether this suggests that JunB is secondary in promoting Cyp40 as compared to NPM-ALK? Or could this be an artificial result due to incomplete JunB knock-down as compared to NPM-ALK knock-down?”

We feel these comments are related, and therefore we have dealt with them together. We think it likely that reduced JunB expression is largely responsible for reduced Cyp40 expression in NPM-ALK siRNA-treated cells, and we stated this in the Discussion of the previous manuscript (See Discussion; page 18, 1st paragraph, 7th line of current manuscript). However, the reviewer’s question as to whether NPM-ALK regulates Cyp40 expression exclusively through JunB, or primarily through JunB with some contribution of JunB-independent pathways is a good one. We favour the latter the scenario based on the fact the Cyp40 promoter contains consensus binding sites for many transcription factors. Moreover, our previously published findings demonstrated that the reduction in JunB expression was comparable in JunB and NPM-ALK siRNA treated cells (Pearson et al. Int J Clin Exp Pathol. 2011 Jan 30;4(2):124-33). This results argues that the greater reduction in Cyp40 protein and mRNA levels in NPM-ALK siRNA-treated cells, compared to cells treated with JunB siRNA, is likely due to other pathways activated by NPM-ALK. We have added a panel to Figure 3A to demonstrate the reduction in JunB levels in NPM-ALK siRNA treated cells, and added the following statement to the Results (page 14; paragraph 2, line 6) “NPM-ALK knock-down also resulted in a substantial reduction in JunB levels,”
that was comparable to reduction in JunB observed after JunB siRNA treatment (compare Figure 3A and Figure 1).” We have also added the following statement to the Discussion (page 18, paragraph 1, line 10) to address potential mechanisms by which NPM-ALK and JunB and Cyp40 expression.

“However, it is unresolved whether NPM-ALK regulates Cyp40 transcription exclusively through JunB or via a combination of JunB-dependent and independent pathways. NPM-ALK knock-down results in a greater reduction in Cyp40 expression that JunB knock-down (compare Figures 1 and 2 to Figure 3), despite a similar reduction in JunB levels in both instances, so we believe it likely that other signalling pathways activated by NPM-ALK also contribute to Cyp40 expression.”

Comment number 5, “The authors demonstrate that Cyp40 knock-down leads to decreased cell viability as shown by MTS assay. It would be helpful to explore this further with the addition of apoptosis assays (caspase, Tdt), and if positive, follow-up with the examination of expression levels of apoptotic related genes, i.e. p53, p21.”

We investigated whether Cyp40 knock-down affected apoptosis by performing Annexin V/Propidium Iodide (PI) staining and observed no difference when compared to cells treated with control siRNA. We also examined whether Cyp40 knock-down influenced cell cycle status/proliferation by performing PI staining and examining BrdU incorporation. While we did see a modest difference in PI staining between control and Cyp40 siRNA-treated cells in one experiment, this was not reproducible and we saw no difference in BrdU incorporation between the different treated cell lines. We feel that these findings are not too surprising given the relatively modest reduction in viability due to Cyp40 knock-down measured in our MTS assay. Thus, while Cyp40 has an important role in promoting viability, we cannot distinguish at this time whether this is due to promoting proliferation, preventing apoptosis, or both these processes. To make this point more clear, we have added a statement to the Discussion “Specific experiments to determine whether this is an increase in apoptosis, a decrease in proliferation, or combination of both of these processes have been inconclusive.” (page 19, paragraph 2, line 3)

With regard to the discretionary revisions:

Comment number 6, “Elegantly shown is the pathway that JunB inhibition leads to decreased expression of Cyp40. Using similar assays, have the authors knocked-up JunB to determine if over-expression of JunB leads to increased Cyp40?”

To address this question we have included data demonstrating that over-expression of JunB promotes transcription from the Cyp40 promoter luciferase construct (Figure 2C). On page 13, 2nd paragraph, 7th line we included these results: “In addition, over-expression of Myc-tagged JunB was found to promote transcription from this luciferase promoter construct, further demonstrating that JunB promotes transcription of Cyp40 (Figure 2C).” Minor changes to the Methods section and Figure Legends were also made to accommodate this additional data.
Comment number 7, "In Figure 4B, over time, the NPM-ALK inhibitor led to decreased pALK and ALK in the Karpas299 cell line. In the SUP-M2 cell line, pALK clearly goes down but ALK goes up. Can the authors offer an explanation of the difference in ALK expression after being treated with an ALK inhibitor?"

This was not something we observed in other experiments, and we do not have good explanation for the apparent modest change in ALK levels in this experiment, and since this observation was not reproducible, and not observed in Karpas 299 cells, we doubt it is significant observation.

Comment number 8, "The in vitro work is clear and sound, however, have the authors examined any of these effects in vivo?"

While the in vivo role of Cyp40 in ALK+ ALCL is an important, unanswered question, we respectively feel these experiments are beyond the scope of the current study.

We hope these revisions have adequately addressed the concerns of Reviewers, and that the manuscript is now acceptable for publication in BMC Cancer.

Regards,

Robert Ingham