Author's response to reviews

Title: N-nitroso-N-ethylurea Activates DNA Damage Surveillance Pathways and Induces Transformation in Mammalian Cells

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Title: N-nitroso-N-ethylurea Activates DNA Damage Surveillance Pathways and Induces Epithelial to Mesenchymal Transition in Mammalian Cells

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We are grateful to the Editor, the Editorial team and the reviewers for providing an extremely thorough and detailed criticism of our manuscript. It has been extremely valuable to receive these comments.

We are emboldened by the reviewers indicating that the article is of outstanding merit and interest in its field.

We have addressed all the major concerns and significantly, our conclusions remain unchanged. In fact, the suggestions have considerably strengthened the paper and our conclusions. For this, we are grateful to the reviewers.

We have modified the title briefly in our revised version to N-nitroso-N-ethylurea Activates DNA Damage Surveillance Pathways and Induces Transformation in Mammalian Cells to completely incorporate the essence of the manuscript.

A detailed, point-by-point response to the comments by the reviewers is appended with this cover letter.

Response to Reviewer: Shengzhi Wang

1. Figure 1, F and G: Please specify the sample size N, which test is used and p value explicitly for each experiment.

The sample size N for all the comets is 150 cells (50 cells per independent experiment). The sample size as well as the statistical tests and p values for each experiment has been mentioned in the respective figures and figure legends.

2. Second paragraph, page 13 and Figure 2A: “Phosphorylation of Chk1 at serine 345 and of Chk2 at threonine 68 was observed in the absence of Msh2 or Msh6 in cells following NEU damage.”… This is a RNAi depletion experiment and there is still a substantial amount of Msh2 and Msh6 left in the cells. It is not appropriate to use
“in the absence” here. Importantly, to judge whether MMR is dispensable for DDR activation, the authors should compare the amount of phosphorylation of Chk1 and Chk2 in the Msh2 and Msh6 depleted cells to the LacZ RNAi cells, rather than to examine whether there is “Phosphorylation of Chk1 at serine 345 and of Chk2 at threonine 68”. Change the wording should clarify the issue.

Wording has been changed in the text of the manuscript as suggested by the reviewer. Quantification of the blots has assisted in drawing a better conclusion.

**Figure 3E**: to claim no cross-talk or independence between ATM-Chk2 and ATR-Chk1 pathways, the authors need to further show that Chk1 phosphorylation is abolished but ATM and chk2 phosphorylation is not altered by adding the specific inhibitors for ATR (for example, ETP-46464 and Vertex, Gamper et al. Nucl. Acid. Res. 2013). Or the claim should change to “no cross talk from ATM-Chk2 to ATR-Chk1”.

Experiments were performed with VE 821, a potent ATP-competitive inhibitor of ATR [1] in order to show conclusively the absence of any cross-talk between ATM-Chk2 and ATR-Chk1 pathways. As shown in Figure 3F, a time-course study in the presence of 10mM NEU and 10µM VE 821 completely abrogated phosphorylation of Chk1 while phosphorylation of Chk2 or ATM remained unperturbed and was observed at 10 mins following NEU damage.

4. First paragraph, page 16 and Figure 4B: Chk1 phosphorylation was detected at the earliest time-point, which is 5 mins after NEU damage in S phase synchronized HeLa cells (Figure 4B). “… How is this 5 mins determined? At zero mins, seems there is already significant increase of phosphorylation of Chk1 at serine 345 compared to control. (top panel in figure 4B)

With respect to detection of Chk1 phosphorylation in S phase synchronized cells (fig 4B), it is visible at even 0 mins as pointed out by the reviewer and is 1.3 times over the control untreated sample. But following quantifying the blots, a 2-fold increase over control was observed at 20 mins post NEU damage. The necessary changes have been made in the text of the paper.

**Discretionary Revisions**

5. Figure 2A: it would be nice to show the result of ATM phosphorylation in the cells transfected with RNAi against Msh2 or Msh6.
Unfortunately we were not able to perform the experiment as suggested by the reviewer. However, the take home message without this piece of data remains unchanged.

6. Overall, the manuscript is well written and the conclusions are strongly supported by the data. However, I have some concerns about the relevance of adding the story of neoplastic transformation of breast cancer cells to this study, which is not a real surprise per se and seems deviating from the major focus of characterizing the DDR response by NEU treatment.

We have characterized the DDR response in cells following NEU treatment and found the presence of both SSBs and DSBs as well as the activation of both Chk1 and Chk2 checkpoints in a temporal manner. We wanted to investigate whether NEU is also able to bring about transformation in breast epithelial cells and therefore we performed experiments in three-dimensional cultures of breast acini of MCF10A immortalised cells. This is a good and emerging model system to study transformation in cells. On addition of 2 doses of NEU, MCF10A acini showed disrupted polarity and upregulation of vimentin, an EMT marker. Such phenomena are observed in cancerous cells. Therefore, we can speculate that NEU too can cause transformation in cells. Further studies need to be performed to understand which genes are affected upon NEU damage. Also, it will be interesting to check whether NEU is causing chromosome instability, one of the hallmarks of cancer.

Response to Reviewer: Huabing Li

1. It is suggested to avoid to use two acronyms in the manuscript: MMR and DDR, which appear many times and are important for readers to understand/follow the paper.

As suggested by the reviewer, we have removed the acronyms and have written the full forms for MMR and DDR in the manuscript.

2. The authors should quantify the western blot against the controls, to quantitatively and clearly show the increase of the phosphorylation of ATM, Chk2 and Chk1 in all the figures. The numbers may be denoted under each gel, or separately shown, whichever is more straightforward.

All western data have been quantified as suggested by the reviewer and the numbers (average fold differences, N=3) have been shown below the respective blots.
3. As for figure 1, while all the other assays were done after 2hrs treatment, the figure 1D & 1E was done at 1hr time points, which just confuse the readers. Thus to be consistent, 2hr time point is required for figure 1D of pRPA immunostaining, as well as at the concentration of 0.3mM NEU treatment for both 1D and 1E.

NEU treatment for 2 hours was performed along with 0.3mM NEU dose for pRPA immunostaining (fig 1D) and western blot (fig 1E) to make the data consistent with rest of the paper as suggested by the reviewer.

4. As for figure 2, the Msh2 and Msh6 knockdown efficiency should be also quantified by qRT-PCR of their mRNA level. And the pChk2 looks like decreased after MSH6 kd as well as in figure 2C. So all the WB should be quantified.

Unfortunately we were not able to quantify the Msh2 and Msh6 knock-downs by qRT-PCR but have quantified the western data for the knock-downs and it shows that the protein levels of both Msh2 and Msh6 decrease compared to LacZ untreated and NEU –treated controls, thereby signifying that the knock-downs were efficient.

pChk2 levels in figure 2A in the MSH6 knock-down lane following NEU treatment is slightly less than that of the NEU-treated LacZ control. The MSH6 knock down lane following NEU treatment has 0.7 fold difference in protein level when compared to NEU treated LacZ or Msh2 siRNA knocked down lanes. Therefore, the decrease is not significant.

We have earlier mentioned in the text that DLD1 cells are not only Msh6 deficient but also have one CHK2 allele with an inactivating point mutation, while the other allele is transcriptionally silenced [2] and therefore the levels of phosphorylation detected (Figure 2C) was much lower as compared to other Chk2-proficient cell lines, such as MCF7, HeLa or HCT116 (those used in the study).

5. For the important claim that ‘we could detect phosphorylation of ATM and Chk2 10 minutes after initial exposure to NEU while Chk1 phosphorylation was detected 30 minutes after drug damage (Figures 3A and 4A)’, the WB should also be quantified and calculated based on statistics to show agreement with Comet assay.

The western blot data for both figures 3A and 4A were quantified (3 independent experiments) and it was observed statistically that there was significant phosphorylation of Chk1 at 20 mins (3.5 and 2.1 fold difference over control for figures 3A and 4A respectively). This is in contrast to what we mentioned earlier in the manuscript (Chk1 phosphorylation was detected 30 minutes after drug damage)
and therefore we performed alkaline Comet assay to detect single-strand breaks at 20 mins post NEU damage and we were able to observe significant increase in tail length and % tail DNA as shown in figure 3C and Supplementary figure S2D. Necessary changes have also been made in the text of the manuscript.

However, detection of ATM and Chk2 phosphorylation remained unchanged, that is, significant phosphorylation was observed for both ATM and Chk2 at 10 mins as had been cited earlier. This is in agreement with the neutral Comet assay that was performed earlier.

6. For figure 5, the concentration of NEU is very different from all previous assay, and the author need to explain why they chose those number, instead of 0.3 or 10mM, which are used in all previous figures. Another important point is that the authors need to quantify up to 100 cells to claim how many cells have the ‘EMT-like phenotypes’ after NEU treatment.

To study the effect of NEU-induced DNA damage on 3D cultures, doses ranging from 0.5mM to 5mM were used. 2mM NEU was found to be the lowest dose that showed loss of polarity and a visible up-regulation of vimentin. 10mM was found to be too high a dose in 3D cultures as no cells were able to survive to grow to form acini. Hence NEU doses of 2mM, 3mM, and 5mM were used in 3D cultures of breast acini.

NEU causes DNA damage, which may be repaired with or without fidelity by the cell or the cell undergoes apoptosis. Since the first dose of NEU is added at the single cell stage it may be speculated that only those cells that overcome the damage survive and further differentiate during the 20 days of culture.

Also, it is important to note that heterogeneity is introduced into systems while studying effects of chemical agents. Published reports using chemical agents on 3D cultures have analysed 10 spheroids per experiment to prove their hypothesis. SR Mullins *et al* [3], in their paper where they studied the role of cysteine cathepsins in breast cancer progression, have analysed only 10 acini per experiment. Marchese *et al* [4], studied transformation induced by estrogen and parabens, analysed only 10 acini per experiment to make their point. Tsunoda *et al* [5] analysed around 60 acini form three experiments to study the various phenotypes like apical ZO-1 signals, apoptosis and quantification of proliferation.

In our study we have analysed 40-50 acini randomly selected from three independent experiments to make the point that NEU leads to morphological and phenotypical changes in breast acini in 3D.
7. The discussion is really extensive and long, which is difficult for readers to finish. I would suggest the authors to make it more concise.

The discussion section has been shortened to make it more concise as suggested by the referee.

Best regards

Mayurika Lahiri

Reference:


