Peer Review File

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Reviewer A

The authors have submitted an analysis of the effects of a missense mutation in NOTCH1 on chemotherapy sensitivity in in two esophageal squamous cancer cell lines. They provide a strong background for these studies by providing references showing that p.E450K missense mutations have been detected in esophageal squamous cell cancer patients who fail to respond to neoadjuvant chemotherapy. This group has previously published a study showing that this missense mutation results in stronger binding between NOTCH1 and the DLL4 receptor resulting in activation of the downstream signaling pathway.

In the current project, the authors sequence several esophageal squamous cancer cell lines and identify two with no mutations in exon 8 where the p.E450K point mutation is located. Point mutations are then created in these cell lines using CRISR/Cas 9 methods and confirmed by sequencing. The mutated cells are compared against their respective wild type cells. The authors how that the mutant cells demonstrate decreased sensitivity to both cisplatin and paclitaxel, as well as decreased proliferation, migration, and invasion. The mutant cells also demonstrate cell cycle changes, with an increase in the proportion of cells in S phase.

Following the elegant creation of the mutated cell lines, the remaining assays are well done and straight forward. My major concern is the apoptosis data shown in Figure 5. The authors show increased rates of spontaneous apoptosis in the mutant cells, although statistical significance is only reached in the KYSE140 cells. One concern is that the apoptotic percentages used in the bar graph seem slightly different than the values shown in the flow cytometry data. This should be checked. More importantly, the finding that the mutant cells demonstrate increased rates of spontaneous apoptosis does not fit with the theme of the paper that the p.E450K missense mutation results in chemoresistance. In the Results section, the authors state that the increased proportion of apoptotic cells did not cause drug resistance in tumor cells although there is no data to support this claim. The authors need to obtain apoptosis data after treating the cells with cisplatin or paclitaxel. If apoptosis rates following exposure to chemotherapy are lower in the mutant cells, this would fit with the theme of the paper. If not, this would call into question the conclusions of the study, and I would favor eliminating the apoptosis data from the manuscript.

Reply: Thank you for your constructive comments in this round of review. Your comments provided valuable insights into how to refine the contents of our manuscript and our analysis. The following is a point-by-point response to the key questions.

After our discussion, it was decided to remove the apoptosis experiment from this paper and to add the experimental analysis of apoptosis after chemotherapy

treatment to the follow-up study protocol.

Other comments:
1.In Figure 3, it would be helpful to 4 create separate curves; one for each cell line with and each chemotherapy agent. Also, in Figure 1b, “Paclitaxel Concentration” should be added as the legend for the x axis.
Reply1: We have modified our Figure as advised (see Figure 3)

2.In Figure 4:
a. In 4a, please provide the actual measurements for the distances between the cells.
b. Also, the images for the KYSE140 are not great. Better quality images would improve the manuscript.ll,
c. In 4e, any statistical significance in the proliferation curves should be shown. There does not seem to be much difference between the curves in Figure 4e.
Reply2: We have modified our text and Figure as advised (see Figure 4；page7-8 line248-252)

3.In Figure 5a, the y axis should be %S phase and not cell umber (%).
Reply3: We have modified our Figure as advised (see Figure 5)

4.Consider changing the title of the manuscript to, “Biofunctional study on chemoresistance in esophageal squamous carcinoma cells induced by missense mutation of NOTCH1 p.E450K.
Reply4: We have modified our title as advised (see page1)

Reviewer B

In the manuscript entitled “Biofunctional study on chemoresistance of esophageal squamous cell carcinoma cell strain induced by missense mutation of NOTCH1 p. E450K”, the authors address the impact of NOTCH1 p.E450K mutations in esophageal cancers through cell line-based research. The research involved introducing the NOTCH1 p.E450K mutations into two HNSCC cell lines, leading to observations of increased chemoresistance and enhanced cellular proliferation and migration in vitro. The study's methodology is very clear, and their findings support that NOTCH1 p.E450K mutations could act as driving factors in esophageal cancer cell lines. Despite NOTCH1 mutations being the second most frequent mutations in ESCC, prior research has primarily centered on transcriptional regulations with a limited focus on mutation-specific studies. My comments are as the following:
Since NOTCH1 mutations are well-recognized mutations in HNSC, the following aspects should be further addressed.

Major comments:
1. I understand that it is challenging to identify mutational hotspot of NOTCH1. The authors rely on their previous findings (Reference #9) to justify focusing on p.E450K

mutations. However, a more detailed exploration of NOTCH1 hotspots and their tumor-type specificity is recommended. One possible explanation is that NOTCH mutations might have bifunctional roles in cancer development, acting as both oncogenes and tumor suppressors.

Reply1: Thank you very much for your comment, the aspect that NOTCH mutations may have a dual role in cancer development cannot be ignored, which is a good guide for our next research, and we plan to explore it in subsequent studies.

2. It would be beneficial to examine the two cell lines used in this study, KYSE450 and KYSE140, for other mutations that might interact synergistically or antagonistically with NOTCH1 mutations. These cell lines are documented in the CCLE database.

Reply2: Thanks for the comment, this is an important question. We searched the CCLE database and found no other mutations in either cell line that could synergistically or antagonistically interact with the NOTCH1 mutation.

3. The authors previously established that the NOTCH1 p.E450K mutation enhances binding with the DLL4 ligand. This interaction requires further experimental investigation, particularly concerning the phenotypic traits of the mutant cells such as chemoresistance.

Reply3: Thank you for your valuable advice, and we will explore the interaction of the NOTCH1 p.E450K mutation with the binding of the DLL4 ligand in a follow-up study.

4. The authors have briefly discussed the clinical implication of NOTCH1 up-regulation. If the authors can access a significant number of cases with NOTCH1 p.E450K mutations from large public databases such as TCGA, they could use this information to support their findings, such as correlations with poor survival rates or other clinical variable.

Reply4: Thank you very much, this is a very meaningful suggestion, and we will further explore the association of the NOTCH1 p.E450K mutation with low survival and other clinical variables in a follow-up study in conjunction with large public databases.

Minor comments.

- Gene names should be italicized throughout the document.
- There are several typographical errors (line 16, page 23).
- The Highlight box requires full clarification regarding 'nCT'.

Reply: we have modified our text as advised.