Peer Review File

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Reviewer A

In the manuscript "Molecular, biological characterization and drug sensitivity of chidamide-resistant MCF7 cells," the authors generate and characterize a cell line that shows phenotypic resistance to the approved HDAC inhibitor chidamide. This work is relevant to clinical use of the inhibitor in ER+ breast cancer and could inform treatment choices in patients treated with chidamide. While the system is potentially informative, the characterization of the resistance model is incomplete and the manuscript is not suitable for publication in its current form. Several key limitations need to be addressed before the manuscript can be reconsidered for publication.

General comments
One key question is the clinical relevance of an acquired resistance model from a single cell line. The authors could address this limitation by looking at evidence of similar molecular changes in clinical examples of chidamide resistance; if that is not possible, they could look at biomarkers of sensitivity to the inhibitor in a panel of ER+ breast cancer cell line to see if any of the potential resistance mechanisms in MCF7-CHI-R cells are observed more generally. Related to this concern, the authors should indicate whether the concentrations used in this study are achievable and observed in the clinic; if the concentrations used here are not observed in patients it would be hard to generalize these findings to the context of clinical resistance.
Reply 1: We apologize for not taking into account the clinical relevance of drug resistance and appreciate your reminder and advice. Through literature review and reading, clinical studies have shown that CHI can induce drug resistance in relapsed/refractory diffuse large B-cell lymphoma patients and the cell cycle mechanism plays an important role in drug resistance.
Changes in the text: We have added the words about the clinical relevance as advised (see Page 5, line 83).

Another critical experiment that is absent is transcriptomic characterization of the parental and resistant cell line, as well as changes in gene expression after chidamide treatment. HDAC inhibitors act by modifying chromatin state and gene expression, and examination of mRNA changes should be used to support the observed changes in protein expression. Even in the absence of transcriptomics analysis, the authors could carry out RT-qPCR with key genes to support their conclusions.
Reply 2: We took your great suggestion and the transcriptome data was generated from MCF7cells and MAF7-CHI-R cells after CHI treatment using RNA-seq technique. The representative distributions of genes up-or downregulated are shown in the volcano in supplementary Fig 8A. Our data revealed that 2572 genes were upregulated, 2155 genes were downregulated in MCF7 cells after CHI treatment. And 1448 genes were upregulated, and 1316 genes were downregulated in MCF7-CHI-R cells compared to MCF7 cells. However, 713 genes were upregulated and 231 genes were downregulated in MCF7-CHI-R cells after CHI treatment. In addition, 1992 genes were upregulated, and 2093 genes were downregulated in MCF-CHI-R cells after CHI treatment compared to MCF7 cells after CHI treatment. Transcriptome data showed that resistant cells were significantly different from parental cells. By analyzing the mRNA changes in the transcriptome data (supplementary Fig

8B), the results showed that the changes of BCL2, CDKN1A, H2AX, GPX4, KEAP1, HMOX1, HDAC10 and HDAC3 were basically consistent with the changes of protein levels in MCF7 and MCF7-CHI-R cells after CHI treatment (supplementary Table 1). Based on the transcriptome data, we will further explore the resistance mechanism of CHI and develop anti-resistance strategies.
Changes in the text: We have added the figure and table as advised (see supplementary Figure 8 and supplementary Table 1).

Another limitation of the study is that the authors use changes in protein expression to draw functional conclusions about the resistance mechanism. In order to draw conclusions about the role of apoptosis and ferroptosis in defining response to chidamide, the authors should treat cells with appropriate concentrations of caspase inhibitors or ferroptosis inhibitors to test whether either treatment can rescue the phenotypic effects of chidamide.
Reply 3: We are very grateful for your rigorous advice on the use of antagonists and we chose ferroptosis antagonists Ferrostatin-1 to administer to the cells. Preliminary results showed that Ferrostatin-1 alleviated CHI-induced cell death in MCF7 cells, and a slight effect was also observed inMCF7-CHI-R cells. More experimental schemes and more in-depth research will be carried out in the future, and we plan to complete a new article on the mechanism of ferroptosis in CHI resistance and submit it to **Translational cancer research** again.
Changes in the text: We have added the figure as advised (see supplementary Fig 9).

Similarly, in order to make the case for oxidative stress, the authors should measure reactive oxygen species in parental and resistant cells rather than relying on changes in NRF2 protein expression or its downstream genes.
Reply 4: We apologize for the neglect of the measurement of reactive oxygen species and then we took a Beckman flow cytometer to measure the concentration of reactive oxygen species. And the results showed that CHI treatment induced significantly elevated concentrations of ROS in MCF7 cells but not in MCF7-CHI-R cells.
Changes in the text: We have modified our text as advised (see Page 11, line 229)

Finally, there are a few issues with the examination of cross-resistance to other therapeutic agents. As with the use of chidamide, the authors should justify the concentrations of the drugs tested by comparing to clinically relevant doses. The authors should also test for induced expression of MDR1 as a general resistance mechanism to pharmacologic agents. Finally, the parental cells are overall not very sensitive to these agents, and the difference in the resistant cell line is subtle. It is hard to draw conclusions based on these small differences.
Reply 5: Thank you for your rigorous consideration and we totally understand the reviewer's concern. But through statistical analysis of the data, we draw a conclusion that the uses of GEM, ADM, DXT, nab-PTX and PTX at the same concentration would indeed cause different degrees of death in MCF7 and MCF7-CHI-R cells. The results of the final analysis were statistically significant and so we thought we should remind the choice of chemotherapy drugs for the patients with CHI resistance in the future. We will keep the above problems in mind in future research and experiments to make our research results more rigorous and persuasive. We really appreciate your reminding.
Specific points
- In the initial characterization of the resistant line, the authors should provide evidence (STR or SNP profiling) that the cells retain their identity as MCF7 cells.

Reply 6: We gratefully appreciate for your valuable comment and we have performed STR identification of resistant cell lines. The results showed that the resistant cells retain the identity as MCF7 cells.
Changes in the text: We have modified our text as advised (see Page 6, line 114)

- The authors should compare 2D growth rates of the parental and resistant cells.

Reply 7: Thank you for your rigorous suggestion. We have tested growth rates of MCF7 and MCF7-CHI-R cells and the result showed that the growth of MCF7-CHI-R cells was lower than that of MCF7 cells.
Changes in the text: We have modified our text as advised (see Page 9, line 168)

- The authors should test whether the resistant cells are still dependent on estrogen/ER for growth.

Reply 8: We sincerely appreciate the valuable comments and we performed a CCK8 assay to examine the effect of estrogen receptor antagonist tamoxifen on cell viability.
Changes in the text: We have modified our text as advised (see Page 9, line 169)

- On page 11, the authors say that HDACs are "inhibited" by treatment. This should say "downregulated."

Reply 9: We feel sorry for our carelessness and thanks for your careful checks.
Changes in the text: We have modified our text as advised (see Page 15, line 303)

- In figure 2, the authors should comment on the reduced baseline expression of p21 in the resistant cells.

Reply 10: We appreciate your careful observation of the image. But after our re-analysis of the images, there was no statistical difference in the expression of p21 protein between the MCF7-CHI-R cells and the MCF7 cells. We think that this result is due to the low expression of p21 in the second sample of MCF7.

- In figure 3, the authors should show more of the PARP Western blot, as the important species for apoptosis induction is cleaved PARP, not full length.

Reply 11: We are very sorry for the failure to provide cleaved PARP due to our negligence and we have added it in the article.
Changes in the text: We have modified our text as advised (see Page 10, line 195)

- In figure 3, there is a clear decrease in BCL2 upon chidamide treatment in parental MCF7 cells, and a clear increase in BCL2 in the resistant cells; the authors should comment on this, as it does not support their conclusion that apoptosis does not play a role in resistance to chidamide.

Reply 12: We think this is an excellent suggestion and we discussed it seriously. In our opinion, the express of BCL2 was decreased significantly after CHI treatment and it is upregulated in MCF7-CHI-R cells, but other proteins related to apoptosis did not show the same trend. So, we draw the conclusion that apoptosis was involved in CHI treatment but not played a key role in MCF7 and MCF7-CHI-R cells after CHI treatment.
Changes in the text: We have modified our text as advised (see Page 9, line 186)

- The authors should more clearly indicate the time points and concentrations used for inhibitor treatment throughout the manuscript.

Reply 13: We apologize for the lack of detail of time points and concentrations and we have supplement it in the text.

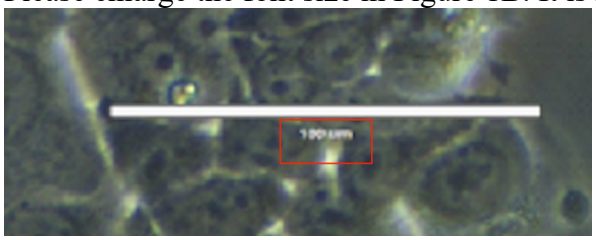
Changes in the text: We have modified our text as advised (see Page 7, line 120)

Reviewer B:

1. Please check if more/any reference should be cited in the following sentences since you mentioned "studies":
   - Previous studies have indicated that the treatment of HR+ advanced/metastatic breast cancers has historically been endocrine therapies, including aromatase inhibitors, selective ER modulators, and selective ER down-regulators (5).
   - Previous studies have shown that CHI induces cell proliferation inhibition and apoptosis in hematologic malignancies (15).
   - … but a few studies have reported CHI resistance in breast cancer.
   - Extensive studies suggest that ferroptosis plays a pivotal role in tumor suppression and is correlated with cancer therapy resistance (19).
   - In addition, previous studies have identified that HDACIs could induce apoptosis through many ways, including selective upregulation of death receptors and ligands in tumor cells, downregulation of pro-survival proteins, and upregulation of pro-apoptotic proteins (25).
   - previous studies identified that HDACIs suppressed tumor by ferroptosis induction (26).

   Reply: We have modified our text as advised (see Page 5, line 78) (see Page 6, line 102) (see Page 9, line 181) (see Page11, line 231) (see Page 14, line 298)

2. Figures
   1) Please enlarge the font size in Figure 1B. It is hard to tell and read.



   Reply: We have modified our text as advised (see Page 21, Figure 1B)
   2) There is no "**" in Figure 1, please remove its explanation.
   Reply: We have modified our text as advised (see Page 21, line 463)
   3) Please indicate the staining method in Figure 1B-C.
   Reply: We have modified our text as advised (see Page 21, line 461), and the cells in Figure 1B were not stained.
   4) Please check if the citation here should be Figure 2C.
      - The p16 was reduced in MCF7 cells after CHI treatment (Fig. 2B, P = 0.040),

   Reply: We have modified our text as advised (see Page 10, line 206)
   5) There is no "cleaved PARP" in Figure 3, hut it is indicated in the legend.

after the treatment of CHI were determined by Western blot analysis, and the quantification was analyzed. (C-F) The expression of PARP, cleaved PARP, p53, bax, bcl2 and γH2AX were determined by Western blot analysis, and the quantification was

   Reply: We have modified the figure as advised (see Page 23, Figure 3)
   6) There is no "**" "#", "&&&" in Figure 4, please remove their explanations.
   Reply: We have modified our text as advised (see Page 24, line 489 and 490)
   7) There is no "&&" in Figure 5, please remove its explanation.

Reply: We have modified our text as advised (see Page 25, line 499)