Peer Review File

Article information: https://dx.doi.org/10.21037/jtd-23-818

Reviewer A

Chen et al. propose a role of OPN-induced EGFR TKI-resistance. Overall, the manuscript contains a lot of data which merits publication.

1、There are however a couple of major concerns. The conclusions rely on one NSCLC cell line pair and on resistance to gefitinib, a first-generation EGFR TKI. Given that only one cell line pair is used as well as a TKI that has been largely replaced by osimertinib in several parts of the world, it is questionable what the community may gain from this study unless a similar mechanism is shown in other models of EGFR mutant NSCLC as well as in models of osimertinib resistance.

Reply: We appreciate the positive and encouraging comments from the referee and we are honored to get the support from the referee. Although gefitinib has been has been largely replaced by osimertinib in several parts of the world, there are still a few resistive mechanisms remain elusive. This experiment is only a preliminary study to explore the regulatory roles of OPN in EGFR-TKI resistance in EGFR mutant non-small cell lung cancer. As suggested by the professor, we are currently collecting the samples and clinical informations of osimertinib resistant patients to establish the resistance mechanisms and identify potential therapeutic targets. These data will be shown in our later research.

Experimental concerns

2、The authors propose OPN to mediate gefitinib resistance in the tested cell line. However, the only experiments addressing OPN in gefitinib resistance (Fig. 2E-G) are not too convincing. Knocking down OPN reduces the IC50 of gefitinib to 3,78uM from 7.11uM. However, the fully sensitive cell line has an IC50 of only 0.04uM. Moreover, the authors decide to investigate apoptosis using a concentration of 10uM gefitinib which will likely target other kinases than mutant EGFR. The apoptosis assay should be conducted at maximum 1uM to ensure relatively specific targeting of mutant EGFR.

Reply: Thanks for the pertinent comments. Given that knocking down OPN only reduces the IC50 of gefitinib to 3,78uM from 7.11uM, there may also be some other signaling mechanisms. We then explored the signaling pathways and found that the combination of OPN silencing and LY294002 inhibited PC9GR cell (a gefitinib-resistant cell line) growth more than either drug alone. These results showed that the knockdown of OPN at least partially mediate gefitinib resistance in PC9GR cell. Considering that the IC50 of gefitinib is 7.11uM in PC9GR cell, we decide to investigate apoptosis using a concentration of 10uM gefitinib. There may indeed be some confounding factors, and we can further explore the concentration to ensure relatively specific targeting of mutant EGFR.

3、The authors state that the cell lines were obtained from the Shanghai Institute of Biological Science. The authors need to clarify whether the obtained gefitinib resistant cells have been sequenced for additional mutations in the EGFR kinase domain and provide a reference to such data. If not, targeted sequencing of the EGFR kinase domain should be carried out to rule out a T790M-mutation as the main resistance mechanism in these cells.

Reply: The PC9 and PC9 gefitinib resistance (PC9GR) human lung cancer cell lines were obtained from the Shanghai Institute of Biological Science and had been sequenced for additional mutations in the EGFR kinase domain.

Reviewer B

This is a very well carried out preclinical research work, there are no major caveats.

Minor aspects:

Figure 5 - the proposed model should be included in the Discussion instead of Conclusions.
Keep in consideration the article of Ichihara et al. SFK/FAK signaling attenuates Osimertinib efficacy in both drug-sensitive and drug-resistant models of EGFR-mutant lung cancer. Cancer Res 2017. See in this article the signaling pathway model in Figure S/ with the roles of integrins as well the Western blot on figure 3E showing the activation of several B (beta simbolo griego) integrins. See also figure 4A where inhibition of Src (dasatinib) is stronger that with PI3K inhibitor). Please at least consider in the discussion this study in comparison with your results.
OPN is an abbreviation also employed to define optineurin. I advise not to use the abbreviation for osteopontin and leave the word ostepontin through the manuscript.
Since the secretion or presence of osteopontin is present in the supernatant. I suggest that these findings can be better reflected in the manuscript in results and in the discussion.

Reply: Thanks for the pertinent comments. We have added some relevant contents in the discussion of revised manuscript.
Changes in the text: Discussion/Paragraph 2, page 11.

Reviewer C

This is a nice study with a comprehensive series of experiments. The manuscript is also well written. The following points could help improve the article more:

1. could you please provide some information about the clinical samples used? Which EGFR mutation was detected, how was initial testing performed? Which TKI was used? Was any molecular retesting done at the time of TKI failure? Any T790M or other resistance? Any comutations at initial diagnosis (because TP53 comutations are known to modulate emergence of resistance in EGFRmut NSCLC, e.g., https://pubmed.ncbi.nlm.nih.gov/32871455/)

Reply: 8 pairs of tumor tissue samples that harbor activating EGFR mutations (deletions in exon 19) were used in our study and all of them were treated with gefitinib. These 8 patients have been retested at the time of TKI failure and no common drug resistance mechanisms (e.g., EGFR-T790M secondary mutation, TP53 comutation, human epidermal growth factor receptor 2 amplification, MET amplification) have been found. So, we selected these 8 pairs of tumor tissue samples for further research.

2. Figure 5: the EGFR is displayed with a structure like an antibody, please consider modifying.

Reply: Figure 5 has been modified according to the above requirements in the revised manuscript.
Changes in the text：Figure 5-revised/page 21-22.

Reviewer D

The manuscript entitled "Epithelial-mesenchymal transition is associated with OPN-induced EGFR-TKI r esistance in EGFR mutant non-small cell lung cancer" by Chen et al described that OPN drives EMT occurrence in lung cancer cells by reducing E-cadherin and increasing vimentin levels in response to stimulation, which provides further evidence that OPN has a regulatory function in EGFR-TKI resistance.

This manuscript is well written, the results are clear and the concussions are based on robust experimental results.

This reviewer has some minor comments.

1. From 8 pairs of human NSCLC tissues, the authors showed elevated OPN expression in tissues after TKI treatment than tissues before TKI. The resolution of figures need to be improved. Do authors analyze H-index of OPN expression?
I'd recommend semiquantitation of OPN expression in 8 pairs of tissues.
Reply: Thanks for the pertinent comments. We have analyzed the semiquantitation of OPN expression in 8 pairs of tissues, as illustrated in Figures 1B. Furthermore, the protein levels of OPN were also elevated in NSCLC tissues following the development of EGFR-TKI resistance, as illustrated in Figures 1D.

2. The legend of figure itself in the manuscript should be understandable to the reader. In Figure 3, the authors described as below (for example)
[[.(I) The expression of E-cadherin was measured in the PC9 and PC9GR cells by qRT-PCR.(J) The qRT-PCR results showed that both PC9 and PC9GR cells produced vimentin. (K) The 531 western blot results showed that both E-cadherin and vimentin were produced by the PC9 and PC9GR cells.]]

---> I recommend more specific description of figure legend would be better to understand their results.

Reply: Thanks for the pertinent comments. The legend of figure has been modified according to the above requirements in the revised manuscript.

Changes in the text: legend/Figure 3-4, page 19-20.

Reviewer F

1. Figure 1

In F-H, there's no * in the figure, please remove the explanation.

617 P<0.01, respectively, compared to NSCLC tissues before EGFR-TKI resistance. (F-H)

618 *, P<0.05 and **, P<0.01, respectively, compared to the PC9 cells. GS, Gefitinib

Reply: We have revised in the text.

Changes in the text: Page 18.

2. Figure 4

There's no ++ in the figure, please remove the explanation.

689 P<0.05 and ++, P<0.01, respectively, compared to the OPN treatment group.When

Reply: We have revised in the text.

Changes in the text: Page 20.

3. Table S1

Please explain all the abbreviations in the table footnote.

Reply: We have revised in the text.

Changes in the text: Page 21.