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Reviewer A

This manuscript reports the establishment and characterization of a new SDC4-ROS1 lung cancer cell line, from the pleural effusion of non-smoker male patient. The patient was initially treated with cisplatin and pemetrexed but developed systemic multi-organ metastases. The patient was then treated with crizotinib for 2 months, then experienced clinical progression. The ADK-VR2 cell line was from treatment naïve pleural fluid but subsequently, a ADK-VR2 AG143 clone was isolated from cells that was grown in 3D (soft agar) culture in the presence of crizotinib, thus considered a resistant clone. Characterization of the cell lines full exome sequencing, as well as PD-L1, ALK, TTF-1, Ep-CAM IHC and RoS1 FISH on the ADK-VR2 cells, as well as growth in 2D, 3D spheroid, 3D soft agar, in vivo growth in BRG mice, and assessment of lung metastasis by real-time PCR. The authors reported that the ADK-VR2 cells demonstrated discrepant drug sensitivity in 2D vs 3D cultures but able to form tumor in mice and developed spontaneous metastasis. The AG 143 clone was more resistant to crizotinib but remained sensitive to lorlatinib, and showed slower growth rate in mice, although the sensitivity to crizotinib in mice is not reported. The authors concluded that ADK-VR2 cell line is a promising NSCLC preclinical model to investigate novel targeted therapies against ROS1 fusion and mechanism of TKI resistance.

Comments: Very few ROS1 fusion cell lines have been established and available for functional and preclinical studies involving this driver oncogene. With this in mind, a report on the establishment of a novel well-characterized SDC4-ROS1 lung cancer cell line is welcome, especially when this cell line is accessible to wide community of cancer researchers. However, this manuscript can be much improved in term of readability, especially when the characterization has been limited. A summary table documenting the sensitivity (IC50) of the ADK-VR2 and clone AG143 cell lines to various drugs in various conditions (2D, 3D sphere, 3D soft agar) will be useful.

Reply: We included a table reporting IC_{50} of ADK-VR2 and clone AG143 to various drugs in 2D and 3D sphere conditions. 3D soft agar was not included since we tested only one dose for each drug.
Changes in the text: We added to Supplements Appendix the Supplementary Table 2.

Specific comments are:
1). Figure 1: Only panels C, G, H, D are necessary as main figures, the others can be reported as supplementary figures. Higher magnification is preferred for panels A and B.

Reply 1: We moved panels A, B, E and F in Supplements Appendix.
Changes in the text: We added a new Figure 1 and Supplementary Figure 1.

2). Figure 3 and 4 can be combined with only one tumor (HE and perhaps TTF1 at higher magnification) is necessary for 3A. The other sets from additional tumors and PD-L1 can be reported as supplementary figures, as they do not add additional useful info the figure. Figure 3C should include a negative control (mouse lung without tumor cell injection).

Reply 2a: We combined Figure 3 and 4. We changed previous HE and TTF1 pictures with others at a higher magnification.
Changes in the text: We included only one tumor of the previous Figure 3A (excluding PD-L1 staining). Additional tumors and PD-L1 staining were reported in Supplementary Figure 2.

Reply 2b: As reported in Materials the percentage of human cells was calculated by interpolation with a standard curve consisted of a different number of human cells mixed with mouse cells. A negative control was included in each PCR.
Changes in the text: We specified details about PCR negative control in Materials (lines 310-312).

3). Figure 5E: similar to figure 3A, only images for one tumor is necessary and at higher magnification.

Reply 3: We moved extra tumor pictures and pictures of PD-L1 staining to Supplements Appendix. We added HE and TTF1 at a higher magnification
Changes in the text: We added Supplementary Figure 5. Figure 5 was renamed Figure 4.

4). Although ADK-VR2 is not sensitive to larlotinib, DS-6051b (taletrectinib) and entrectinib in 2D culture, its sensitive in soft agar (lorlatinib) and 3D sphere (to all 3 drugs) should prompt the testing of this cell line with all drugs in 3D soft agar and also

in vivo in mice. This would provide a more complete data to compare drug sensitivity in various conditions.

Reply 4: We performed the in vitro tests suggested by the reviewer. In vivo tests could be possibly planned in the future.
Changes in the text: A new Figure 2D was included in the manuscript.

5). Similarly, sensitivity data on the AG143 cell line to all ROS1 TKIs should also be reported.

Reply 5: We performed the in vitro tests suggested by the reviewer. In vivo tests could be possibly planned in the future.
Changes in the text: We included in Supplements Annex the Supplementary Figure 4 reporting the 2D-growth sensitivity of ADK-VR2 AG143 to lorlatinib, entrectinib and DS-6051b**.**
We added to Figure 5B (now Figure 4B) the 3D-growth sensitivity of ADK-VR2 AG143 to entrectinib and DS-6051b.

6). The authors repeated refer to 3D growth (in sphere) as "stemness" phenotype. The concept of 3D sphere representing stem cell phenotype in lung cancer has not been established, thus this reference should be avoided. It is unclear what is meant by the term "stemness".

Reply 6: Stemness has been implicated in tumor initiation, metastasis, recurrence, and treatment resistance in various types of cancer including NSCLC. Stemness refers not only to cancer stem cells (CSC) of tumors but also to cancer cells with a stem cell phenotype, and it plays an essential role in cancer progression and survival (10.1158/0008-5472.CAN-19-3578, 0.3390/cancers13246228). The sphere formation assay is considered a test to measure the stemness ability of cancer cells, including NSCLC cells (10.21037/tlcr-20-633, 10.7150/thno.63627)
Changes in the text: We rewrite text in Results, Paragraph 3.3 (lines 385-397).

7). A whole exome sequencing analysis of the AG143 and comparison to the parent ADK-VR2 cells may add new information on mechanism of resistance to crizotinib.

Reply 7: We are sorry, but we were not able to perform whole exome sequencing analysis of the AG143.
Changes in the text: We included a statement in the Discussion (lines 653-656).

8). The text can be significantly shortened to improve readability.
Reply 8: We revised the manuscript to improve the readability.
Changes in the text: We modified mainly Introduction and Discussion.

9). There should be a statement regarding the accessibility of this cell line to other researchers.
Reply 9: Corresponding authors are available to discuss collaboration proposals for studies with ADK-VR2 and ADK-VR2 AG143 cell lines. generated in this study.
Changes in the text: We added a statement (lines 685-687).

Reviewer B

Francesca et al reported regarding the establishment of a novel cell line of ROS1 lung cancer and its drug sensitivity.

One of the weak points of this paper is why loratinib, which is successful in the 3D system, is not effective in the 2D system? What is the mechanism of resistance of the crizotinib-resistant clone A143 established in the 3D system? is yet to be elucidated. Unfortunately, only observational phenomena was reported.

One of the novelties of this study might be the establishment of an in vitro, in vivo model harboring SDC4-ROS1 fusion, but CUTO-2 cells harboring SDC4-ROS1 fusion have already been established and reported in Cancer Res.
Neel DS Cancer Res 2020
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6359944/

Reply: We are sorry to miss the mention of CUTO-2 cell line. We included the suggested reference and others related to studies with CUTO-2 cell line. Davies and colleagues (10.1371/journal.pone.0082236) derived CUTO-2 cell line from the biopsy of a patient in progression to crizotinib. ADK-VR2 cell lines was derived from the pleural effusion of a patient at the diagnosis. Of note, IC_{50} value of crizotinib on CUTO-2 cultured in 2D was 0.38 μM, quite similar to ADK-VR2 cell line.
Changes in the text: We added a comment in the Discussion (lines 550-558)

Specific comment

Comment1
Introduction Page3
I think it is unnecessary to mention ARRIVE check list in the Introduction section.
Reply 1: We included this statement in accordance with TLCR guidelines.

Comment2
3.1 Patient clinical history and molecular data Page7
Please specify the maximum effect of cisplatin and PEM using RECIST.
Why did you use crizotinib despite disease progression to CNS territory?

I think that next-generation ROS-TKIs may be a better choice for patients with CNS disease.

Reply 2a: done
Changes in the text: A comment has been included in Results, Paragraph 3.1, lines 339-341.
Reply 2b: We explained why new-generation ROS1-TKIs were not considered to treat the patient, taking into account the higher penetrance to CNS of the new TKIs than crizotinib
Changes in the text: A comment has been included in Discussion section, lines 526-531.

Comment3
3.4 Page8
Authors described "tumor showed traits similar to tumor patient sample"
There is no information whether PD-L1 was expressed in patient samples.

Reply 3: PD-L1 expression information was reported in Results, Paragraph 3.1, lines 333-335 “the sample was positive for PD-L1 TPS staining (25%)”.
Changes in the text: We added a statement to recall paragraph 3.1 (lines 413-414 and 517-518).

Comment4
Fig2B
Because the sensitivity of crizotinib to HCC78 differs from that of the previous report, the culture conditions may be different in this paper. Therefore, it is difficult to determine whether the observed phenomenon in this paper is cell line specific or whether culture conditions are involved.

Reply 4: Davies et al. (10.1371/journal.pone.0082236) reported an IC_{50} value for HCC-78 cells treated with crizotinib of 0.79 μM. In our manuscript we reported a IC_{50} value of 0.4686±0.2494 μM. These two IC_{50} values are quite superimposable. Small differences between the two curves can be attributed to the test that was used to measure drug sensitivity. Davies et al. reported in Materials and Methods the use of MTS assays. In our paper cell growth was assessed by vital counting with erythrosine.

Comment5
Fig2C
I think authors should set a positive control.
Otherwise, we cannot determine whether these next-generation ROS1-TKIs were ineffective or whether it is a problem with the experimental system.
Since HCC78 is very sensitive to loratinib, authors should experiment with positive control and should the results at the same time using HCC78

Reply 5a: We performed new experiments. ADK-VR2 AND HCC-78 cell lines were tested in parallel. Cells were treated with lorlatinib, entrectinib and DS-6051b.
Changes in the text: We added a new Figure 2C. New IC_{50} values were calculated and reported in the text and in the Supplementary Table 2. We commented the new figure in Results, lines 399-401.

Fig2D
Data on the sensitivity of loratinib to HCC78 in 3D cultures should also be presented.

Reply 5b: We performed new experiments. ADK-VR2 and HCC-78 cell lines were tested in parallel. Cells were treated with lorlatinib, entrectinib and DS-6051b.
Changes in the text: We added a new Figure 2D. New IC_{50} values were calculated and reported in the text and in the Supplementary Table 2. We commented the new figure in Results, lines 397-399.

Fig3.
IHC staining is too strong and difficult to judge.
Why are some tumors expressing PDL1 and others not?
Is PDL1 expression uniform in IHC in cell blocks of the ADK-VR2 cells?
Is it a heterogeneous cell population with a mix of PDL1+ and PDL1- cells?
FACS examination may also provide the solution.

Reply 5c: We selected the tumor areas with most strong positivity, the PD-L1 immunoreaction is not uniform in the tumors but patchy, we have mixed area of positive and negative tumor cells, as happen in human tissue.
On the other hand, ADK-VR2, and also ADK-VR2 AG143 and HCC-78, grown in 2D-culture showed a homogenous and comparable expression of PD-L1 detected by flow cytometry analysis.
Changes in the text: According to Reviewer 1 suggestions we moved IHC PD-L1 pictures in Supplement Annex. We also added a Supplementary Figure 3 reporting flow cytometry of ADK-VR2, ADK-VR2 AG143 and HCC-78 cell lines.

Fig5A
What was the sensitivity of the A143 clones to next generation ROS-TKI such as loratinib in 2D culture?
Reply 5: We tested ADK-VR2 AG143 cell line in 2D culture in presence of lorlatinib, entrectinib and DS-6051b.
Changes in the text: We added Supplementary Figure 4. We commented the new figure in Results, lines 450-451.

Comment6
mouse experiment

Author described two types of mice were used in material and methods. NSG mice or BRG mice.
In Figs. 3-5, please specify which type of mouse was used for which experiment.

Reply 6: NSG mice were used for the in vivo therapy with crizotinib. Tumor growth and metastatic ability was assessed in BRG mice.
Changes in the text: We added to the Figure Legend of Figure 3 and 4 (ex Figure 5) the specific mice.

Comment7
Suppl Table 1
Based on the WES results, what criteria were used to select the genes?
Please specify the criteria such as the cutoff of the Allele frequency.

Reply 7: Criteria were reported in Supplement Annex, in Supplementary Materials.