TLCR TRANSLATIONAL LUNG CANCER RESEARCH
AN OPEN ACCESS JOURNAL FOCUSING ON CLOSING THE GAP BETWEEN "BENCH AND BEDSIDE"
IMPACT FACTOR 5.132

Peer Review File

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Reviewer A

Comment 1: Methods: very short high-level description. A lot of details are missing.

Reply 1:
We added descriptions of the parts that were lacking in the explanation in the method. A list of genes that can be examined by comprehensive genetic analysis has been added as supplementary data.
Changes in the text:
Page 7 Line 5-Page 10 Line 13; added description
Also added supplemental methods 1 and 2

Comment 2: Results: The sample size is very small and comes close to a case study. To underpin their hypothesis at least some in vitro functional assays have to be performed to give evidence that the CDK4 expression is somehow related to the acquired resistance against Dabrafenib.

Reply 2:
Lung adenocarcinoma with BRAF_V600E is a rare disease, accounting for about 1% of all cases. Furthermore, the number of patients receiving molecularly targeted therapy is even smaller, and it is currently difficult to analyze resistance mutations on a large scale in clinical samples.
As you highlighted, experiments in cell culture systems are important, but at the time of the initial submission, the BRAF_V600E cell lines known in the literature were HCC-364 and NCI-H854, but they were difficult to obtain.
However, in our laboratory, we succeeded in creating a cell line from primary culture. Using these cells, we genetically transfected them with CDK4 We could generate a cell line with CDK4 gene transfer and forced expression of the protein, which was resistant to BRAF inhibitors (Figure 3). Further studies were conducted to determine how increased expression of CDK4 is involved in dabrafenib resistance. We screened for major phosphoproteins using RTK antibody arrays and did not find any phosphoproteins that were attenuated or enhanced before or after CDK4 introduction. The weakness of this study is that the ON cells established in this study are partially resistant to BRAF inhibitors, and the inhibition of cell proliferation by dabrafenib above 100 nM is about 70%. This partial resistance suggests that survival signals other than BRAF contribute to the process from primary culture to cell line. Figure 3C shows that phosphorylation of EGFR and MET is observed in this cell line. It is unclear whether the phosphorylation is due to the expression and proliferation of phosphoproteins in vivo, or phosphorylation acquired during cell passaging. However, considering that the inhibition of

cell proliferation, which was initially about 50%, gradually increased with each cell passaging, it could be an artifact of cell culture.

Changes in the text:
Page 13 Line 2-Page 13 Line 11; added results
Also added Figure 3A-C

Comment 3: Discussion: a good but not exhaustive overview of possible resistance mechanism. The biological role or any understanding why CDK4 might be involved is missing.
The dataset is very small. From my perspective it is not possible to draw any conclusion at that point. Some in vitro data or at least a comparison with other clinical datasets is immanent.

Reply 3:
Thank you for your comments.
Although it has not been fully elucidated how CDK4 is involved in resistance, we thought the fact that BRAF_V600E cells become resistant to BRAF inhibitors when CDK4 expression is increased was novel and we reported it here. The mechanism of resistance in this case could be that the altered expression of CDKs and their regulators regulated the activity of CDKs and promoted proliferation, which may have acted as oncogene mutations independent of the growth stimulation by BRAF. Discussion has been added to the main text.

Changes in the text:
Page 14 Line 18-Page 16 Line 3; added discussion

Reviewer B

Comment 1: The manuscript is well written clarifying a common mechanism of resistance to dabrafenib plus trametinib BRAF V600E- mutated lung cancer patients.
It could be of great interest if the authors can further explain the mechanism by which CDK4 is amplified an up-regulated. The authors commented that in KRAS mutant NSCLC rather often there is over-expression of CDK4-. What about AMBRA1? Mutations and loss of expression of AMBRA1 occur frequently in NSCLC. See for example Maiani et al. Nature 2021, Chaikovsky et al. Nature 2021.
I agree that BRAF cell lines derived from patient tumor samples are difficult but some work from BRAF established cell lines could be adequate to see that dabrafenit with or without trametinib increase CDK4 protein expression.
Above all keep in consideration AMBRA1.
If you can revise the NGS analysis for alterations in AMBRA1 could be of great significance.

Reply 1:
As you highlighted, experiments in cell culture systems are important, but at the time of the initial submission, the BRAF_V600E cell lines known in the literature were HCC-364 and NCI-

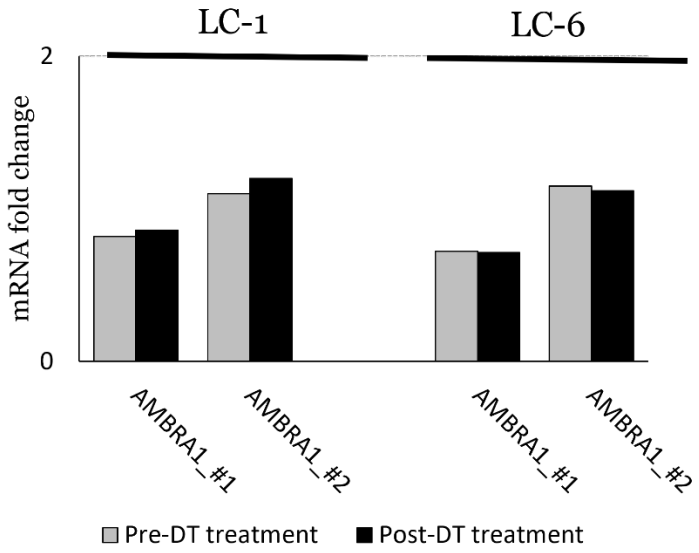
H854, but they were difficult to obtain.

However, in our laboratory, we succeeded in creating a cell line from primary culture. Using these cells, we genetically transfected them with CDK4 We could generate a cell line with CDK4 gene transfer and forced expression of the protein, which was resistant to BRAF inhibitors (Figure 3). Further studies were conducted to determine how increased expression of CDK4 is involved in dabrafenib resistance. We screened for major phosphoproteins using RTK antibody arrays and did not find any phosphoproteins that were attenuated or enhanced before or after CDK4 introduction. The weakness of this study is that the ON cells established in this study are partially resistant to BRAF inhibitors, and the inhibition of cell proliferation by dabrafenib above 100 nM is about 70%. This partial resistance suggests that survival signals other than BRAF contribute to the process from primary culture to cell line. Figure 3C shows that phosphorylation of EGFR and MET is observed in this cell line. It is unclear whether the phosphorylation is due to the expression and proliferation of phosphoproteins in vivo, or phosphorylation acquired during cell passaging. However, considering that the inhibition of cell proliferation, which was initially about 50%, gradually increased with each cell passaging, it could be an artifact of cell culture.

We think that the genes you pointed out related to AMBRA1 and autophagy are important.

Unfortunately, AMBRA1 was not included in our research panel. In an additional experiment, we analyzed the expression of AMBRA1 individually with two TaqMan probes.

The results showed no overexpression, downregulation, or changes before and after treatment, so we did not include the data in the text at this time.



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Added Figure 3A-C

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