# Peer Review File

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**Reviewer A**

The manuscript by Yu et al. is relevant because it deals with a still incompletely understood mechanism of resistance to TKI-treatment in EGFR-mutated pulmonary adenocarcinomas, such as the transformation to SCLC. Despite previous studies have shed some light on the molecular pathogenesis and clinical features of EGFR-mutated NSCLCs undergoing this transformation, additional information is needed to better pinpoint the patients, who are at risk of this transformation during treatment with EGFR-TKIs and how to treat them. In that respect, the results by Yu et al., although generated in only 9 patients from a single institution, are able to complement what has been published on the subject so far. However, some parts of the manuscript need revision and clarification before it is acceptable for publication. Some, mostly minor corrections/adjustments and missing parts should be taken care of.<br />
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SPECIFIC POINTS<br />
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Comment 1: Abstract, line 32, "We performed a retrospective review of cases …": please indicate also in the abstract, how many cases were retrospectively reviewed. <br />
Reply 1: We performed a retrospective review of 964 cases at the University of California, San Diego of patients with EGFR sensitizing mutations.
Changes in the text: we added "964" to this sentence (see Page 2, line 35).
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Comment 2: Line 46, "Six patients resumed/continued TKIs …": the authors should better specify that the patients resuming EGFR-TKIs (TKI rechallenge) did so after terminating chemotherapy, while those continuing the TKI therapy (TKI continuation), did so concomitantly with etoposide/platinum. Moreover, in order to justify rechallenge/continuation of EGFR-TKI treatment, it should be mentioned that these patients retained the founder EGFR mutation, as described in the text.<br />
Reply 2: Six patients, as they retained the initial EGFR mutations, resumed (did so after terminating chemotherapy)/continued (did so concomitantly with chemotherapy) TKIs with a median duration of 13.8 months (IQR: 3.8 - 27.7).
In our study, 2 patients resumed EGFR-TKIs (TKI rechallenge) at the completion of chemotherapy, and 4 patients continued TKIs therapy (TKI continuation) concomitantly with chemotherapy.
Changes in the text: we have modified our text as advised (see Page 3, line 49-52; Page 13, line 237-240).
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Comment 3: Line 50, "… transformation had poorly differentiated tumors at baseline": it would be appropriate to specify that they were poorly differentiated adenocarcinomas, as both "NSCLC" and "tumor" are generic terms and comprise other histological subtypes of pulmonary carcinomas.<br />

Reply 3: In our series, most patients with small cell transformation had poorly differentiated adenocarcinomas at baseline.

Changes in the text: we have modified our text as advised (see Page 3, line 53-54).

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Comment 4: Line 50, “in this series, though p53 mutation was present …”: since it was detected by NGS, the gene name TP53 should be used.<br />

Reply 4: RB1 loss was not universal in transformed patients in this series, though TP53 mutation was present in all tumor samples.

Changes in the text: we have modified our text as advised (see Page 3, line 55).

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Comment 5: Methods, line 99, “consent for this retrospective analysis was waived.”: presumably, the reason for that is that many/all patients were deceased at the time of the study. However, it would be appropriate to specify it.<br />

Reply 5: individual consent for this retrospective analysis was waived because 6 out of 9 patients were deceased at the time of the study.

Changes in the text: we have specified it as advised (see Page 7, line 113-114).

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Comment 6: Line 101-102, “The SCLC transformation has been confirmed by a histologic diagnosis”: did the diagnosis comprise IHC for neuroendocrine markers (synaptophysin, chromogranin, CD56) according to WHO guidelines (WHO classification of lung tumors) or only histological evaluation of H&E-stained slides? Other cancer types may mimic SCLC histologically, therefore IHC is useful to confirm the transformation to SCLC.<br />

Reply 6: The SCLC transformation has been confirmed by the histologic diagnosis comprising immunohistochemistry (IHC) for neuroendocrine markers according to WHO classification guidelines of lung tumors

Changes in the text: we have modified our text as advised (see Page 7-8, line 116-118).

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Comment 7: Line 126-8, “The histological types of all the 9 patients were ADC, in which 7 patients with poor differentiation at diagnosis, 2 were moderately-to-poorly differentiated#. The sentence should be rephrased more properly.<br />

Reply 7: The histological types of all the 9 patients were ADC, in which 7 patients were poorly differentiated at diagnosis, 2 were moderately-to-poorly differentiated.

Changes in the text: we have rephrased the sentence as advised (see Page 9, line 154-156).

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Comment 8: Line 149-152, “Seven cases had NGS results, among which, 5 cases had NGS both at baseline and after transformation, in which, 4 cases had retinoblastoma 1 (RB1) loss both at baseline and after transformation, but one case did not have RB1 loss”: The sentence is quite convoluted, not reader-friendly. Please simplify, for ex. by splitting it up in two parts. <br />

Reply 8: Seven cases had NGS results, among which, 5 cases had NGS both at baseline and after transformation. In these five cases, 4 cases had retinoblastoma 1 (RB1) loss both at baseline and after transformation, but one case did not have RB1 loss.

Changes in the text: we have modified our text as advised (see Page 10-11, line 178-181).

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Comment 9: Line 155-157, “common unique genetic mutation for WNK1 (c.2567C>A p.T856K Missense variant and c.2176_2219delins (46) p. I726fs Frameshift, respectively.”: what analyses and db did the authors use to assess that these two WNK1 variants are pathogenic and not a VUS?<br />

Reply 9: A CLIA approved panel was utilized with the bioinformatic classification of these mutations. While no “normal” sequencing was done, PolyPhen-2 was used, and both mutations were classified as “possibly damaging”.

Changes in the text: we have specified this in the “Methods” as advised (see Page 8, line 130-135).

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Comment 10: Discussion, line , “Upon transformation, despite retention of the EGFR-mutation, EGFR protein expression decreases, and patients have limited benefit from EGFR-TKIs(7, 15)”: This is true, however Marcoux et al. (ref 9) found also in their study 5/67 cases with EGFR-amplification after SCLC transformation, in addition to the founder EGFR-mutation, suggesting that both EGFR-downregulation and -upregulation can contribute to the unresponsiveness of transformed tumors to EGFR-TKIs. <br />

Reply 10: Upon transformation, some researchers found, despite the retention of the EGFR-mutation, EGFR protein expression decreases, and patients have limited benefits from EGFR-TKIs (7, 16). However, Marcoux et al. (9) found 5/67 cases with EGFR-amplification after SCLC transformation, in addition to the founder EGFR-mutation, suggesting that both EGFR-downregulation and -upregulation can contribute to the unresponsiveness of transformed tumors to EGFR-TKIs.

Changes in the text: we have modified our text as advised (see Page 13, line 228-234).

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Comment 11: In this respect, in figure 1 the authors show that 3 out of 7 cases with NGS data exhibited EGFR-amplification (“EGFR amp”) at baseline (together with CCNE mutation), while 1 patient displayed “EGFR Copy number gain” after SCLC transformation. These data could be commented upon in the Discussion, with regards to the previous point. <br />

Reply 11: our data showed that 3 out of 7 cases with NGS data exhibited EGFR-amplification at baseline (together with CCNE mutation), while 1 patient displayed “EGFR copy number gain” after SCLC transformation (Table 3 and 4).

Changes in the text: these data have been commented upon in the Discussion (see Page 13, line 234-237).

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Comment 12: Line 192-194: “Chemotherapy has poor penetration into the central nervous system (CNS), while osimertinib is bioavailable and effective for CNS metastases”: it would be appropriate to mention how many of the 9 cases showed CNS metastasis after SCLC transformation.<br />

Reply 12: In our study, 3 of the 9 cases showed CNS metastasis after SCLC transformation. Chemotherapy has poor penetration into the central nervous system (CNS), while Osimertinib is bioavailable and effective for CNS metastases, which is an issue in this patient population.

Changes in the text: we have modified our text as advised (see Page 13, line 224-228).

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Comment 13: Line 201-205, “In our cases, 2 patients (one patient had high tumor mutational

burden (TMB) 15 Muts/Mb and programmed cell death-ligand 1 (PD-L1) tumor proportion score (TPS) >50%) received immunotherapy, also had no clinical improvement, which indicated that post-transformation, immunotherapy may not be effective". Please reformulate the sentence more properly. <br />

Reply 13: In our cases, 2 patients also had no clinical improvement after receiving immunotherapy, even though one patient had high tumor mutational burden (TMB) 15 Muts/Mb and programmed cell death-ligand 1 (PD-L1) tumor proportion score (TPS) >50%, which indicated that post-transformation, immunotherapy may not be effective.

Changes in the text: we have reformulated the sentence as advised (see Page 14, line 245-249).

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Comment 14: Line 212-214, "There were 2 cases without RB1 loss, suggesting that RB1 loss is not universal in SCLC transformed patients.": similar results were reported in ref. 9 (mutations in TP53 in more than 90% of their SCLC-transformed cases tested by NGS, RB1 in 58%, and PIK3CA in 27%, which are all mutations detectable in the conventional EGFR-wt SCLC). Was PIK3CA not mutated at all in the authors' 9 cases? If that is the case, the authors could a comment on the lack of this mutation as compared to previous studies and classic SCLC.<br />

Reply 14: There were 2 cases without RB1 loss, suggesting that RB1 loss is not universal in SCLC transformed patients, similar to Marcoux's research results(9).

PIK3CA was mutated in 2 cases. Please see Table 3 and 4.

Changes in the text: we added this ref. as advised (see Page 14, line 256-258).

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Comment 15: Line 217-218, "… showed that WNK1 altered in 1.25% to 30% of SCLC cases, and only 2.32% to 6.09% of patients with lung ADC(25, 26).": the references to be cited here seem to be # 24 and 25.<br />

Reply 15: the references to be cited here should be # 24 and 25, however, the reference list has been updated, therefore, the references to be cited here are still # 25 and 26.

Changes in the text: we have modified our text as advised (see Page 15, line 263; Page 21, line 401-406).

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Comment 16: Line 220-221, "WNK pathway has been implicated in numerous human diseases including cancer(27).": ref. 27 is not in the reference list, probably it should have been ref. 26 here.<br />

Reply 16: the reference to be cited here should be # 26, however, the reference list has been updated, therefore, the reference to be cited here is still # 27.

Changes in the text: we have modified our text as advised (see Page 15, line 267; Page 21, line 407-408).

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Comment 17: Figure 1: To make sure it is understandable for the readers: did patients with multiple written gene names harbored co-mutations in all the listed genes in their tumors? How was it verified that mutations were pathogenic and not VUS? There seem to be single VUS in the lists. Maybe, for clarity, they should be removed and stated in the legend that VUS are not shown. <br />

Reply 17: Yes. The patients had multiple co-mutations of the listed genes in their tumors. The

other genes were bioinformatically determined to be potentially pathogenic and, if question, confirmed by PolyPhen-2 (please see Reply 9). The single VUS in the lists has been removed and we stated in the legend that VUS is not shown.
Changes in the text: we have modified our text as advised (see Page 10, line 169-170; Page 8, line 130-135; Page 24, line 447; Page 28, Table 4).
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Comment 18: Table 1, Patients' characteristics: it would be appropriate to indicate also which TKIs each patient received. This is relevant especially if sequential treatment with 1st gen. + 2nd gen. and/or 3rd gen EGFR-TKIs was used and to see when SCLC occurred with respect to Osimertinib, which is now used as 1st line for EGFR-mutated NSCLC.
Reply 18: We have added Table 2 to indicate which TKIs each patient received.
Changes in the text: we added these data as advised (see Page 23, line 433-438; Page 26, Table 2).

**Reviewer B**

Li Yu et al reported 9 cases with transformed small cell lung cancer from EGFR-mutant lung adenocarcinoma. They found that RB1 loss was not universal and TP53 mutation was universal in transformed cases.

Comment 1: NGS methods were unclear in this manuscript. In addition, RB1 gene coverage is unknown. Therefore, one patient without RB1 loss might have false negative result regarding RB1 loss. Please clarify these methods.
Reply 1: NGS was performed using whole exon coverage of at least 73 genes in a CLIA (clinical laboratory improvement amendments)-certified laboratory. Matched "normal" sequencing was not available to further characterize germline or variant of undetermined significance (VUS), and thus bioinformatic tumor only approaches were utilized, and if question on nature of the mutation, PolyPhen-2 was utilized.
Changes in the text: we have specified this in the manuscript to add clarity (see Page 8, line 130-135).

Comment 2: Figure 1 is uninformative. Please delete these and provide a schematic table or an alternative figure.
Reply 2: We have deleted Figure1 and provided Table 3-5.
Changes in the text: we have modified our text as advised (see Page 10, line 169; Page 10, line 174; Page 13, line 237; Page 23-24, line 440-450; Page 27-29, Table 3, 4 ,5).

Comment 3: Authors should adhere to patients with paired NGS data before and after transformation.
Reply 3: We agree that optimally the identical platform should be utilized pre and post-transformation, however, given the rare nature of the event and the limited number of patients, we felt if baseline changes portended transformation to SCLC (similar to Rb) would be important not to miss. If reviewers feel strongly, we can remove, but that will further limit the number of cases amongst this already rare cohort.

Changes in the text: we described this limitation in the Discussion (see Page 15, line 270-275).

Comment 4: They concluded WNK1 mutation may be a new resistance mechanisms to EGFR TKIs. However, its rationale is very weak. In addition, please provide the details of WNK1 mutation. Do you have any functional data regarding WNK1 mutation? In addition, WNK1 mutation affects survival outcomes in lung cancer?
Reply 4: We have added information in the manuscript about WNK1 in other cancer types, though there is limited data on the mechanism despite being seen as recurrent mutation. We do not have data, and the goal of this paper was to stimulate discussion around larger data sets with well-annotated survival data to see if our findings could be replicated.
Changes in the text: we have added additional commentary and citation (see Page 15, line 267-269; Page 22, line 409-411).

Comment 5: In transformed cases, all are transformed fully or partially? Especially, if you have these data in 6 cases who resumed or continued EGFR TKIs, please provide these.
Reply 5: It is unclear as that would require a biopsy of each lesion, and many patients have dozens of lesions. Unfortunately, there is no imaging test or other means to discern which lesion on a scan is NSCLC vs. transformed SCLC.
Changes in the text: we described this limitation in the Discussion (see Page 11-12, line 198-202).

**Reviewer C**

This succinct case series reports a single-institution observation of the NGS-derived somatic mutations in the small cell carcinoma transformation as acquired resistance to the EGFR-TKI for nine patients with poorly differentiated, activating EGFR-mutant adenocarcinoma of the lung. It is interesting to read while the authors bring in the newly acquired understanding of the mechanistic transformation. However, I think there are two areas in the manuscript that require a mild revision:
Comment 1: Table 1 should include a column showing the extent of disease of the small cell carcinoma, either limited or extensive stage at the transformation.
Reply 1: We have added a column to Table 1 showing the stage of disease at SCLC transformation.
Changes in the text: we added these data as advised (see Page 25, Table 1).

Comment 2: Line 182: Please also cite a remarkable case report showing durable remission. (Kok et al. Extensive-Stage Small Cell Carcinoma Transformation From EGFR Del19-Mutant Lung Adenocarcinoma on Gefitinib at the Twelfth-Year Follow-Up Case Report. Front Oncol 2021 Mar 18;11:564799.) This is a relevant citation.
Reply 2: Some patients did respond well to these regimens (9, 13, 14)
Changes in the text: we added the citation of the case report as advised (see Page 12, line 216; Page 20, line 365-368)

Comment 3: Line 196: the same reference above may be considered citing here.

Reply 3: Some cases also showed clinical benefit from re-challenge/continue TKIs treatment(9, 14, 17, 18).

Changes in the text: We have added the citation of the case report as advised (See Page 14, line 242-243).