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Reviewer A

Considering that histological transformation from original adenocarcinoma to small-cell carcinoma (SCLC) has been detected in many cases after epidermal growth factor receptor tyrosine kinase inhibitor treatment, in the present work, the authors evaluate mutational status in the two different histological types found in three patients and further elucidate the molecular pathogenesis. They found MTOR, JAK1, NOTCH2, CSF1R variants additive to SCLC, and loss of MAP2K2. Both TP53 and Rb1 alterations were detected in adenocarcinoma. Notch2 expression was negative in SCLC in both r-PCR and IHC. ASCL1 expression increased after histological transformation detected using both methods in one case, only these samples were evaluable. They concluded Notch and ASCL1 signaling as the master regulators of neuroendocrine differentiation in SCLC. Our results suggest that the Notch-ASCL1 axis may also play an essential role in the transformation of SCLC under TP53 and RB1 inactivation.

Comments
Although this is a relevant topic now, there are some points that deserve to be discussed further.

1. As this is a translational study, even involving a limited number of cases, from the authors' point of view what would be the practical importance of the results?

Reply 1: Thank you for your crucial review. We contemplated that NOTCH and ACSL1 might be potential therapeutic targets for transformed-small cell carcinoma. We have modified the text as advised (see Page 26, line 453) as follows:

“As described above, the NOTCH-ASCL1 axis might be a potential therapeutic target in transformed small cell carcinoma from adenocarcinoma with oncogenic driver mutation, for which there are a few treatment options except for the regimen applied for classical SCLC.”

2. Since the authors observed enhanced expression of genes involved in NOTCH-ASCL1 pathways, it would be useful to confirm whether pharmacological inhibition of these pathways delayed tumor growth and NE transformation in an EGFR-mutant patient-derived xenograft model.

Reply: We indeed agree with you. We realize the necessity of it, but we are afraid we do not have an adequate technique to do this. We have modified the text as advised (see Page 26, line 456) as follows:

“Further investigation is warranted to confirm whether a pharmacological inhibitor of these pathways would be effective for neuroendocrine transformed tumors of patient-derived xenograft models.”

3. The authors should discuss more about the combined SCLC and adenocarcinoma-histology regarding the treatment used in the patients. Is it possible that core biopsy samples or fine-needle aspirates used to make the initial diagnosis did not provide sufficient pathological material to determine the presence of combined histology to be identified at diagnosis?

Reply: As per your suggestion, the first diagnosis via small biopsy did not clearly show whether the tumor already included a small cell carcinoma component. Although it is difficult to deny this interminglement in case 2, we think that initial diagnosis after surgical resection was definitive for cases 1 and 3. We have modified the text as advised (see Page 11, line 189) as follows:

"In case 1, a woman who was 50 years old at initial diagnosis by surgical resection underwent re-operation upon tumor recurrence. Both resected samples were identified as adenocarcinoma without small cell carcinoma. She had acquired the resistance mutation T790M in exon 20 of *EGFR* after the EGFR-TKI re-challenge with the combination of erlotinib and paclitaxel, and then received osimertinib as the fourth course of treatment."

4. Whether the results from the autopsy studies represent transformation or combined-histology tumors, these prominent observations carriage several practical questions that could affect clinical practice and should be discussed by the authors. First, in localized, early-stage, combined-histology tumors, how does the presence of adenocarcinoma affect the decision on whether to offer surgery? Otherwise, should patients with tumors that have an SCLC component always be given concurrent chemotherapy and radiotherapy? Moreover, in an advanced-stage setting, if tumors do not respond as initially expected or if the responses among two or more different lesions are conflicting, is repeat biopsy indicated to rule out a dominant histology that was not identified at initial diagnosis?

Reply: Despite a few pieces of evidence, surgical resection was one of the most recommended strategy for early-stage pure SCLC. Thus, even if preoperative diagnosis is combined-histology, surgery seems to be reasonable. References are as follows;
Eur J Cardiothorac Surg.2004;26(1):183-8
Eur J Cardiothorac Surg.2004;26(4):782-6.
In a clinical study to estimate the efficacy of targeted therapy, such as osimertinib, in the treatment of naïve metastatic lung cancer with EGFR mutation, the progression rate was reported as about 1% (N Engl J Med 2018;378:113-25.). Moreover, heterogeneity of treatment response among multi-metastatic regions is not rare. Thus, we think that invasive re-biopsy for patients with metastatic cancer is very tough in the clinical setting. Further investigation is needed to grasp the whole status of malignancy maybe by using methods such as cell-free DNA technique. The following changes were added (Page, 27; line 476):

“According to the autopsy in case 1, adenocarcinoma, combined- small cell and adenocarcinoma, and transformed small cell carcinoma were observed during the clinical course of one patient. Thus, tumor progression has heterogeneity among the multi-metastatic lesions. In the clinical setting, comprehensive biopsy of every metastatic site is hard, due to the invasiveness. Further investigation is required to grasp the whole status of malignancy, for example by using cell-free DNA detection technique. We recently observed that cfDNA harbors essential genetic alterations that are representative of the whole cancer; the most detectable tumor-derived genetic alterations in cfDNA are truncation mutations with a high-variant allele frequency (28). Detecting the sign of histological transformation using cfDNA will be possible.

5. The authors showed that genomic sequencing of EGFR from both the original and repeat biopsy samples at the time of resistance showed that every transformed SCLC tumor sample retained its original EGFR-activating mutation, which suggests that these were not independent de-novo cancers, but a transformed phenotype as a mechanism of resistance to treatment instead. However, another hypothesis for this observation is that patients presented tumors with combined histology at the time of initial diagnosis, which was not apparent on the diagnostic biopsy sample, and then the SCLC component became dominant as the adenocarcinoma component was successfully treated with the EGFR inhibitor.

Reply: It is very difficult to completely deny the combined small cell carcinoma at initial diagnosis via clinical small biopsy. As you mentioned, this is fundamental and a limitation to the investigation or interpretation of this phenomenon. We think that surgical resection at initial diagnosis is a strong point of this study. We have modified our text as advised (see Page 19 line 329) as follows:

“To begin with, it is a fundamental problem whether the histological transformation is real or the actual initial diagnosis is just combined small cell carcinoma and adenocarcinoma. Because two out of three cases presented in this study were initially diagnosed by surgical resection, it was suitable that there were no small cell carcinoma component.”

Reviewer B

Neuroendocrine transformation is emerging as a mechanism of resistance to targeted therapies in lung cancers but is also seen is other solid tumours, such as prostate cancer. Indeed, the histologic transformation from adenocarcinoma to small cell lung cancer is an observed mechanism of therapeutic resistance to EGFR-TKIs in up to 14% of patients. The molecular mechanisms underlying this histological plasticity has been for many years illusive, due to the paucity of serial tumour tissue biopsies collected pre- and post-histological transformation. In this manuscript, Koba and colleagues present the molecular and histological characterisation of lung tumour samples from three patients harbouring EGFR-mutant lung adenocarcinoma patients. Importantly, the tumours from these patients had undergone small cell lung cancer transformation, following EGFR-TKI therapy, allowing the molecular mechanisms underpinning tumour cell plasticity to be interrogated.

The authors undertook next generation sequencing analysis of 160 cancer-related genes of and concurrent genetic alteration of key candidate genes were evaluated by qRT-PCR analysis and immunohistochemical staining of tissue sections.

Genetic alteration in five genes (MTOR, JAK1, NOTCH2, CSF1R and MAP2K2) were consistently observed across the three individual patients. In line with previously published studies, RB1 mutations were also observed in the adenocarcinoma patients, highlighting the importance of RB1 loss in driving neuroendocrine cell fate. NOTCH alterations were common across all patients, suggesting a critical role for NOTCH signalling in this process.

This is an interesting study, of a unique source of matched tumour material from lung cancer patients. The authors should also discuss their findings in the context of the recently published manuscript by Quintanal-Villalonga et al. (Cancer Discovery 2021).

Comments:

1. Can the authors please clarify what tissue samples were used for next generation sequencing? Was DNA extracted from fresh tissue biopsies from all patients, or was DNA extracted from FFPE sections? Further details are required in the methods.

Reply: We have modified the text as advised (see Page 7, line 119).

“The tumor biopsy approach used in this study is shown in Fig. 1 and DNA/RNA extraction samples were underlined. All samples were formalin-fixed paraffin-embedded (FFPE).”

2. Related to the above, if FFPE tissue was not micro-dissected for genomic analysis, how was purity of the tumour samples assessed? Specifically, were adenocarcinoma tumour components observed in resistant tumour samples.

Reply: As you mentioned, FFPE tissues were micro-dissected for DNA extraction. Thus, purity of tumor samples were 100%. Although there were many metastatic tissues with various histological properties in case 1 autopsy as shown in Fig.3C, the DNA sample in case 1 was extracted from a pure small cell carcinoma lesion in lung. We have modified our text as advised (see Page =, line =) as follows:

"First, FFPE tissues were micro-dissected. (Page 7, line121).

"Furthermore, FFPE samples were micro-dissected. Thus, purity of tumor samples must have been 100%. (Page 19, line 334)"

3. Figure 1 clearly displays the treatment history of each patient, overlayed with disease state/tumour histopathology. I strongly encourage the following amendments:
- While a broad timeline is shown for each patient, it would be nice to outline in more granular detail the dates corresponding to treatment windows
- Larger scale bars should be included on all histology and IHC-stained images. The current bars generated in the image software are too small to clearly visualise.

Reply: As you pointed out, we have corrected the date and scale bars in Figure 1.

4. Small cell lung cancers have recently been stratified into distinct subtypes, based on the expression of lineage-associated transcription factors, of which ASCL1 makes up the most prevalent subtype (see Rudin et al. Nature Reviews Cancer 2019). Have the authors evaluated whether the SCLC tumours that transdifferentiate from lung adenocarcinoma post-EGFR-TKI exhibit expression of NEUROD1, POU2F3 or YAP1? A recent study by Quintanal-Villalonga et al. (Cancer Discovery 2021) has also shown that SCLC tumours that arise following resistance to TKIs are not restricted to only the ASCL1 SCLC subtype.

Reply: Thank you for your valuable suggestion. We have not evaluated the expressions of these proteins. Based on the comparison of adenocarcinoma and SCLC using next-generation sequencing, we focused on the NOTCH-ASCL1 axis as the key factor for histological transformation. Thus, we validated the expression levels of several proteins related to NOTCH. We have added new text to the discussion on the recent papers. We have modified the text as advised (see Page 21, line 365) as follows:

“In a recent review article, SCLC subtypes were defined by differential expression of four key transcription regulators; ASCL1, neurogenic differentiation factor 1 (NeuroD1), yes-associated protein 1 (YAP1), and POU class 2 homeobox 3 (POU2F3) (14). As YAP1 or POU2F3 expression was present in non-neuroendocrine SCLC (14), transformed small cell tumors in our study were expected to lack both genes.”

5. Somewhat related to the point above, can the authors discuss how their findings (genomic alterations) relate to the concurrent genetic alterations described by Quintanal-Villalonga et al. (Cancer Discovery 2021).

Reply: We additionally searched about the genetic alterations described this paper; PI3K, AKT, and PRC2 complex. We were afraid that our cancer-related panel did not include the PRC region. Thus, the remaining two genes were analyzed, as shown in the new Supplementary Table 3. We have modified the text as advised (see Page =, line =) as follows:

“Furthermore, accumulation or loss of genetic alterations of PI3K and AKT were investigated for each case (Supplementary Table 3). There was no common aberration in three cases among perfectly matching nucleotides. (Page 15, line 255)

“Additionally, Quintanal-Villalonga et al. pointed out the involvement of PRC2 complex, PI3K/AKT, and NOTCH pathways (26). As PI3K and AKT were included in the cancer-panel in this study, both genetic alterations were detected in all three cases (Supplementary Table 3). These results also showed that AKT inhibitor is expected to delay transformed neuroendocrine lung carcinoma (26).” (see Page 26, line 458)

5. As an alternative to listing a summary of the gene alternations in a Table format, the authors could consider displaying the results as a oncoprint.

Reply: We have focused on the genetic alteration in the form of nucleotide change strictly. Thus, we are afraid that an oncoprint is not a proper presentation.

6. Given that the number of genetic alterations identified in transformed SCLC was quite low, the authors may want to consider discussing other mechanisms that may be driving histologic transformation from NSCLC to SCLC (e.g. epigenetic mechanisms, methylation).

Reply: As you pointed out, we have modified the text, adding a limitation paragraph (see Page 28, line 487) as follows:

"The number of genetic alterations were quite low and did not increase after histological transformation in two among three cases. Transcriptional reprogramming, epigenetic mechanisms, or DNA methylation may have another important role in the transformation. A recent report presented that neuroendocrine transformation is primarily driven by transcriptional reprogramming, including loss of the 3p chromosome arm (26)."

Minor comments:

1. Scale bars should be included on the H&E-stained sections and IHC images displayed in Figure 3.

Reply: We have modified the Figure as advised (see Figure 3).

2. Isotype control stains should be shown for the IHC staining of NOTCH2 and ASCL1, as there appears to be some background staining of surrounding normal tissue (Figure 3).

Reply: We have modified and added the isotype control staining figure as advised (see Figure 3).

3. Can the authors indicate from which tumour lesions the histological sections were obtained from in Figure 3, by integrating panels B and C.

Reply: We have modified the Figure and Figure legend as advised (see Figure 3).

Figure 3. Autopsy findings in case 1. (A) Immunohistochemical staining at the border of coexistent metastasis in the left upper lung lobe, indicated with the arrow in Fig. 3C. Adenocarcinoma in the upper region is in contact with the small-cell carcinoma in the lower region. (B) Immunohistochemical staining of isolated histological lesions indicated with arrowheads in Fig. 3C. (C) Schematic representation of tumor distribution in the autopsy of case 1.