Peer Review File

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Reviewer A

The paper titled "Dissecting the genetic variations associated with response to first-line chemotherapy in patients with small cell lung cancer" is interesting. Increased EGFR gene CNVs may be involved in the pathophysiology of PR in SCLC patients receiving standard first-line chemotherapy. However, there are several minor issues that if addressed would significantly improve the manuscript.

1) The abstract is not adequate and needs further revisions. The research background does not indicate the clinical needs of this research focus. The study results need to show the clinical characteristics of the two groups of patients.

Reply: Thank you for your comment. We have changed relevant content in accordance to your suggestions (see Page 1-2, lines 29-35, lines 46-59).

"**Background**: Chemotherapy has been the standard treatment for small-cell lung cancer (SCLC) for decades. Nonetheless, patients are usually responsive to initial chemotherapy but quickly suffer from relapse, resulting in a poor long-term outcome. Treating advances that greatly ameliorate survival outcomes are historically finite, and credible biomarkers for therapeutic evaluation are deficient. As the genetic biology emerges, investigating biomarkers to optimize individualized treatment for SCLC is necessary.

Results: For the clinical characteristics of enrolled SCLC patients, except for significant differences in sex, age, clinical stage, and limited or extensive stage, PD patients showed distinctly shorter overall survival than those with PR (6.5 vs. 14.0 months, respectively, P=0.007). Genetic variations analysis discovered several common genes with CNV mutations between the PR and PD groups, and increased epidermal growth factor receptor (*EGFR*) gene copy numbers gain was found in PR groups in comparing with PD patients (P=0.006). However, no significant differences in terms of SNVs, indels, genotypes associated with first-line chemotherapy, CNI of tumor tissue-derived DNA, and tumor mutational burden of tumor tissues were observed between two groups. Additionally, the relationship between EGFR gene mutation and clinicopathological features of SCLC indicated that EGFR gene mutation may be an independent indicator for SCLC patients."

2) What is the relationship between EGFR gene mutation and clinicopathological features in SCLC? It is recommended to add relevant content.

Reply: Thank you for your comment. We have descripted the relationship between EGFR gene mutation and clinicopathological features in the text. (see Page 7, lines 226-230).

"Additionally, we also analyzed the relationship between EGFR gene mutation and clinicopathological features of SCLC. Surprisingly, we found that EGFR gene mutation had no

obvious connections with clinicopathological features in SCLC patients (Table 2). This observation indicated that EGFR gene mutation may be an independent indicator for SCLC patients."

Table 2 the relationship between EGFR gene mutation and clinicopathological features in SCLC.

3) How to provide specific maintenance for individual patients under the guidance of PS, EGFR mutation status, histology, and induction response? Suggest adding relevant content.

Reply: Thank you for your comment. In accordance to your advice, we searched the online database and found a similar paper "Bayesian network meta-comparison of maintenance treatments for stage IIIb/IV non-small-cell lung cancer (NSCLC) patients with good performance status not progressing after first-line induction chemotherapy: Results by performance status, EGFR mutation, histology and response to previous induction" (PMID: 26364517). We carefully read the paper and found that due to different study aim, we did not collect relevant information, such as PS, histology, and induction response. Thereby, it was unable to carry out similar research. However, thank you again for your constructive suggestions, we will consider conduct this meaningful work in the future to provide specific maintenance for individual SCLC patients.

4) What drives the predicted value of EGFR gene copy number? Suggest adding relevant content (see Page 9, lines 290-299).

Reply: Thank you for your comment. We have added relevant content into the discussion part.

"The normal physiological function of EGFR is to regulate epithelial tissue development and homeostasis. However, under pathological environment, such as lung, breast cancer as well as glioblastoma, it was demonstrated to be a driver of tumorigenesis (35). As research deepens, abnormally activated EGFR in tumor mainly accounts for amplification and point mutations at the genomic locus, which lead to unfavorable survival (36, 37) and tolerance to various chemotherapy drugs (38). These findings drive the predicted value of EGFR gene copy number. Therefore, given that EGFR mutations are detected in up to 50% of NSCLC, EGFR has also become a critical target in NSCLC treatment (39)."

5)

EGFR gene copy number	Gain	Non Gain	P value
Clinical features	(N=8)	(N=16)	
Gender			
Female	1 (12.5%)	0 (0%)	0.718
Male	7 (87.5%)	16 (100%)	
Age			
Mean (SD)	63.3 (5.63)	63.1 (8.74)	0.95
Median [Min, Max]	62.5 [57.0, 70.0]	62.5 [48.0, 76.0]	
Stage			
IIb-IIIa	2 (25.0%)	3 (18.8%)	1
IIIb-IV	6 (75.0%)	13 (81.3%)	
Limited.or.extensive.stage			
Extensive stage	4 (50.0%)	10 (62.5%)	0.884
Limited stage	4 (50.0%)	6 (37.5%)	

How about EGFR mutations in advanced SCLC? How does the mutation status of this gene affect the efficacy of different chemotherapy methods? It is recommended to add relevant content.

Reply: Thank you for your comment. In this research, we enrolled 8 and 11 advanced SCLC patients in PD and PR groups, respectively (Table 1). Through Figure 2, we could see that most SCLC patients in PR groups displayed EGFR mutations accompanied with complex mutations. A previous study "Gene copy number gain of EGFR is a poor prognostic biomarker in gastric cancer: evaluation of 855 patients with bright-field dual in situ hybridization (DISH) method" (PMID: 25487305) reported that patients with EGFR gene copy number gain but not amplification, including those exhibiting polysomy, also exhibited poorer prognosis than gene copy number non-gain patients". These study indicated that the higher mutation status may result in greater drug tolerance.

6) The introduction part of this paper is not comprehensive enough, and the similar papers have not been cited, such as "Concurrent use of metformin enhances the efficacy of EGFR-TKIs in patients with advanced EGFR-mutant non-small cell lung cancer—an option for overcoming EGFR-TKI resistance, Transl Lung Cancer Res, PMID: 33889509". It is recommended to quote this article.

Reply: Thank you for your comment. We have supplemented more relevant background information into the paper and also cited the reference you recommended. (see Page 3, lines 86-104).

"Because of recent advances in high-resolution detection technology, a new understanding of the genetic biology of SCLC has led to the development of more selective and targeted therapies, the most promising of which is that the genetic variability in individual patients may predict drug response and therapeutic efficacy or susceptibility to adverse drug reactions (16). At common RNA levels, an upregulated miR-27a expression after chemotherapy was seen in partial response (PR) patients than in those who exhibited no response (NR), and further survival analysis indicated that patients with reduced miR-27a levels displayed inferior outcomes than those with raised miR-27a levels (17). Furthermore, in EGFR-mutant NSCLC patients, EGFR-TKIs was adopted for considerable therapeutic effects (18). Genetic variation was also related to response to dutasteride for male undergoing androgenetic alopecia (19) as well as long-term therapeutic response in bipolar depression (20). What's more, the combination of genomic variation with other immunotherapy related indicators has been thought to be meaningful for precise immunotherapy decisions for advanced lung squamous cell carcinoma (21). All this findings highlight the importance of genetic variation in drug treatment. Nonetheless, similar studies on SCLC are rare (22, 23). A few consistent associations have been reported for some individual susceptibility genes, but no general recommendations have been formulated to date (24-26)."

7) It is recommended that further clinical studies be added to validate the results.

Reply: Thank you for your comment. In the discussion part, we have complemented several clinical studies validate present results. (see Page 9-10, lines 289-312).

"*EGFR* is a member of the erbB family of tyrosine kinase receptors, and the *EGFR* gene coding the receptor is localized at chromosome 7 (34). The normal physiological

function of *EGFR* is to regulate epithelial tissue development and homeostasis. However, under pathological environment, such as lung, breast cancer as well as glioblastoma, it was demonstrated to be a driver of tumorigenesis (35). As research deepens, abnormally activated EGFR in tumor mainly accounts for amplification and point mutations at the genomic locus, which lead to unfavorable survival (36, 37) and tolerance to various chemotherapy drugs (38). These findings drive the predicted value of EGFR gene copy number. Therefore, given that *EGFR* mutations are detected in up to 50% of NSCLC, *EGFR* has also become a critical target in NSCLC treatment (39). Higaki and colleagues futher observed that patients with *EGFR* gene copy number gain but not amplification, including those exhibiting polysomy, also exhibited poorer prognosis than gene copy number non-gain patients. These study indicated that the higher mutation status may result in greater drug tolerance (37). In contrast, *EGFR* gene mutations are rare in SCLC, accounting for only 2.6–7.1% of SCLC patients in China (40). In 2006, Okamoto *et al.* were the first to report an *EGFR* mutation (heterozygous in-frame 15-base pair deletion) in a gefitinib-responsive SCLC patient (41). Two years later, Tatematsu *et al.* examined the *EGFR* gene copy number in five SCLC patients with *EGFR* mutations and found gene amplification in four cases (42). Since then, some SCLC cases with *EGFR* mutations have been reported successively, showing that *EGFR* mutations are sensitive to EGFR-tyrosine kinase inhibitors (TKIs) and may suggest a positive prognostic efficacy (43-45). Conversely, one prior report has clarified that *EGFR* is low expressed in SCLC, suggesting that EGFR-TKIs are ineffective against SCLC even when *EGFR* is mutated (42).”

Reviewer B

1) First, the title needs to indicate the clinical research design of this study, i.e., a retrospective cohort study.

   Reply: Thank you for your comment. We have changed the title in accordance to your suggestions (see Page 1, lines 2-3).

   “Dissecting the genetic variations associated with response to first-line chemotherapy in patients with small cell lung cancer, a retrospective cohort study”

2) Second, the abstract is not adequate. The background did not briefly describe the knowledge gaps and the objective of this study. The methods need to describe the inclusion criteria of the subjects, how they were followed up, and how the treatment outcome was assessed. The results need to describe the baseline comparability between the two groups and report the distribution of genes. The conclusion “involved in the pathophysiology of PR” is still overstated, since authors only cross-sectionally compared the two groups.

   Reply: Thank you for your comment. We have added and changed content you mentioned in the Abstract part (see Pages1-2, lines 28-62).

   “**Abstract**

   Background: Chemotherapy has been the standard treatment for small-cell lung

cancer (SCLC) for decades. Nonetheless, patients are usually responsive to initial chemotherapy but quickly suffer from relapse, resulting in a poor long-term outcome. Treating advances that greatly ameliorate survival outcomes are historically finite, and credible biomarkers for therapeutic evaluation are deficient. As the genetic biology emerges, investigating biomarkers to optimize individualized treatment for SCLC is necessary.

Methods: Based on following inclusion criteria: (I) patients diagnosed as SCLC by pathology; (II) patients treated with first-line EP chemotherapy; (III) patients who received long-term follow-up and signed informed consent, atotal of 24 SCLC patients receiving first-line standard chemotherapy were divided into progressive disease (PD) and partial remission (PR) groups. They were regularly followed every 3 months with computed tomography (CT) scan until recurrences determined by CT scan results. Next-generation sequencing (NGS) with a panel of 1,406 cancer-related genes was conducted on the tumor tissue-derived DNA of patients to compare genetic variations, including deletions (indels), single nucleotide variations (SNVs), copy number variations (CNVs), and copy number instability (CNI) between the two groups.

Results: For the clinical characteristics of enrolled SCLC patients, except for significant differences in sex, age, clinical stage, and limited or extensive stage, PD patients showed distinctly shorter overall survival than those with PR (6.5 vs. 14.0 months, respectively, P=0.007). Genetic variations analysis discovered several common genes with CNV mutations between the PR and PD groups, and increased epidermal growth factor receptor (*EGFR*) gene copy numbers gain was found in PR groups in comparing with PD patients (P=0.006). However, no significant differences in terms of SNVs, indels, genotypes associated with first-line chemotherapy, CNI of tumor tissue-derived DNA, and tumor mutational burden of tumor tissues were observed between two groups. Additionally, the relationship between EGFR gene mutation and clinicopathological features of SCLC indicated that EGFR gene mutation may be an independent indicator for SCLC patients.

Conclusions: Increased *EGFR* gene CNVs may be an independent indicator influencing the survival time and PR in SCLC patients receiving standard first-line chemotherapy."

3) Third, in the introduction of the main text, the authors need to review what has been known on the genetic variations in treatment response and analyze the limitations of prior studies.

Please also explain whether the methodology of comparing the genetic variants between response and non-response groups is appropriate to answer the research question, i.e., without clinical and functional validations.

Reply: Thank you for your comment. We have added and changed content you mentioned in the introduction part (see Pages 3-4, lines 86-104).

"Because of recent advances in high-resolution detection technology, a new understanding of the genetic biology of SCLC has led to the development of more selective and targeted therapies, the most promising of which is that the genetic variability in individual patients may predict drug response and therapeutic efficacy or susceptibility to adverse drug reactions (16). At common RNA levels, an upregulated miR-27a expression after chemotherapy was seen in partial response (PR) patients than in those who exhibited no response (NR), and further survival analysis indicated that patients with reduced miR-27a levels displayed inferior outcomes than those with raised miR-27a levels (17). Furthermore, in EGFR-mutant NSCLC patients, EGFR-TKIs was adopted for considerable therapeutic effects (18). Genetic variation was also related to response to dutasteride for male undergoing androgenetic alopecia (19) as well as long-term therapeutic response in bipolar depression (20). What's more, the combination of genomic variation with other immunotherapy related indicators has been thought to be meaningful for precise immunotherapy decisions for advanced lung squamous cell carcinoma (21). All this findings highlight the importance of genetic variation in drug treatment. Nonetheless, similar studies on SCLC are rare (22, 23). A few consistent associations have been reported for some individual susceptibility genes, but no general recommendations have been formulated to date (24-26)."

4) Fourth, in the methodology of the main text, please indicate the clinical research design, sample size estimation, and the assessment of baseline clinical characteristics. In statistics, please first describe the test of the baseline comparability between the two groups and ensure $P<0.05$ is two-sided.

Reply: Thank you for your comment. We have complemented the content you adviced into the method part. (see Pages 4-5, lines 114-143).

"##Patients and data collection

In this study, all procedures involving human genes were strictly performed according to the Declaration of Helsinki (as revised in 2013).

Clinical research design: A total of 24 SCLC patients who underwent first-line standard chemotherapy (EP regimen) from October 2009 to February 2012 were enrolled in this retrospective study. For enrolled patients, they were regularly followed every 3 months with computed tomography (CT) scan until recurrences determined by CT scan results. They were allocated into a PD group (n=10) or a PR group (n=14) based on the curative effect of chemotherapy assessed by CT scan results. All participants gave written informed consent for the provision of clinical information, biospecimen collection, and

analysis. PD was defined as the appearance of new lesions or a >25% increase in the size of lesions, while PR was defined as a >50% reduction in the size of the lesions. The inclusion criteria were as follows: (I) patients diagnosed as SCLC by pathology; (II) patients treated with first-line EP chemotherapy; (III) patients who received long-term follow-up and signed informed consent. Patients were excluded based on the following criteria: (I) history of other malignancies; (II) history of myocardial infarction, unstable angina pectoris, stroke, or uncontrollable arrhythmias; (III) pregnant or lactating patients; (IV) history of mental illness; (V) poor compliance. The clinical characteristics of SCLC patients are summarized in Table 1.

Samlpe size estimation: The sample size of the trial was determined by the analysis of overall survival. We calculated that 26 deaths in the chemotherapy treated SCLC population would be needed to provide 90% power at a two-sided significance level of 0.05 to detect a significance between treatment-resistance and treatment-sensitive group.

Assessment of baseline clinical characteristics: Tumor specimens were acquired by surgery (>2% of total tissue mass and >150 cells). Diagnosis of SCLC was confirmed by pathologists using Formalin-fixed and paraffin-embedded tissues. TNM staging system of International Association for the Study of Lung Cancer (version 7) was used to determine the clinical staging.Chi-square test or Fisher's exact test were analyzed categorical variables for baseline comparability."

5) Finally, please cite several related papers: 1. Xu Y, Li H, Huang Z, Chen K, Yu X, Sheng J, Zhang HH, Fan Y. Predictive values of genomic variation, tumor mutational burden, and PD-L1 expression in advanced lung squamous cell carcinoma treated with immunotherapy. Transl Lung Cancer Res 2020;9(6):2367-2379. doi: 10.21037/tlcr-20-1130. 2. Xie E, Lin M, Sun Z, Jin Y, Zhang S, Huang L, Sun R, Wang F, Pan S. Serum miR-27a is a biomarker for the prognosis of non-small cell lung cancer patients receiving chemotherapy. Transl Cancer Res 2021;10(7):3458-3469. doi: 10.21037/tcr-20-3276.

Reply: Thank you for your comment. We have cited the papers you suggested into the introduction part.