Author's response to reviews

Title: Non-Small Cell Lung Cancer with EML4-ALK Translocation in Chinese Male Never-Smokers is Characterized with Early-Onset

Authors:

Yongjun Guo (yongjunguo@hotmail.com)
Jie Ma (majie@medmail.com.cn)
Wenjing Bao (baowi_7@163.com)
Bing Wei (weibing1999@hotmail.com)
Jiuzhou Zhao (Jiuzhou5578@163.com)
Nan Zhang (nzhang012@163.com)
Shuang Fu (fushuangtt@126.com)
Lu Ding (dingludlc@126.com)
Jihong Zhang (zhangjihong2014@126.com)

Version: 7
Date: 17 August 2014

Author's response to reviews: see over
Aug 16, 2014

Dear Dr. Dafne Solera or editor of the *BMC Cancer*

We would like to thank you for considering our manuscript (MS: 4124372561153266) entitled “Non-Small Cell Lung Cancer with EML4-ALK Translocation in Chinese Male Never-Smokers is Characterized with Early-Onset and Less-Differentiation” as potentially suitable for publication in the *BMC Cancer* and inviting us to resubmit it with revision. We would also like to thank the Reviewers for their helpful comments. In the revised manuscript enclosed, we have fully addressed the reviewers’ concerns, as detailed below in the section “Response to the Reviewers’ Comments”.

Having made these changes, we respectfully request that the revised manuscript be accepted for publication in the *BMC Cancer*.

Sincerely,

Zhang Jihong
Hematology laboratory of Hematology malignancy treatment center
Shengjing Hospital of China Medical University
No.39 Huaxiang Road, Tiexi District
Shenyang, Liaoning 100022
China
Email: zhangjihong2014@126.com
Telephone: 024-96615-24811

**Response to the Reviewers’ Comments:**

---

NOTE: In this round of review, two reports were provided to us, from Reviewers #1 and #2.

**Reviewer 1 (Haruhiko Sugimura)**

Major compulsory revisions

1. Pathological or morphological description is too simplified. Several authors from Asia described ALK or ROS1 rearrangement lung cancer often has "mucinous cribriform pattern" (Takeuchi et al. Nat Med, Rodig SJ et al. CCR, 2009, and others)

Response: Following this Reviewer’s comments, we have added the following sentence in the Results section and Fig. 4C accordingly: “As reported previously [9, 24, 25], we found a mucinous cribriform pattern in two of EML4-ALK-positive adenocarcinomas (Fig. 4C)”. In addition, we added a more detailed description of pathological evaluation in the Methods section.

2. Fig4B legend should be "positive case".

Response: We have made the correction per this Reviewer’s request.

3. The method of IHC is not appropriate. Try the other ones more consistently with RT-PCR results (Shinmura K et al. Experimental and Therapeutic Medicine 1: 271-275, 2010)

Response: We thank the Reviewer for this insightful comment. We apologize that we didn’t describe clearly the method of IHC used in this manuscript. In this study, we performed IHC using a primary rabbit monoclonal antibody against ALK (clone number D5F3, purchased from Cell Signaling Technology, Inc., USA), which was approved for the purpose of clinical application by the China Food and Drug Administration (CFDA) in China and by the Food and Drug Administration (FDA) in the United States. We have added a more detailed description of the IHC protocol in the Methods section and related reference (Kwak EL et al., 2010, N Engl J Med 363:1693-1703). We believe this method is one of the widely used and accepted methods for detecting ALK expression in lung cancers. Therefore, it should be fine in this study if we use either this method or the
Response: We have deleted word “large” in “Abstract” per this Reviewer’s request.

Minor essential revisions:

If the 8.8% of female NSCLC being positive for ALK rearrangements, drastic change in therapeutic strategy toward crizotinib and other target therapy must follow. What do the authors think about therapy for these population.

Response: We have identified 8.4% male never smokers with NSCLC being positive for ALK rearrangements. Its significance was briefly discussed in the Discussion section (the second last paragraph). To better accommodate this Reviewer’s comment, we have added an additional sentence in the Discussion section to emphasize the importance of our finding: “It is then possible that nearly one-tenth of male never-smokers with NSCLC (8.42%) would likely respond to crizotinib, but not to EGFR TKIs (gefitinib and erlotinib) or drugs targeting other tyrosine kinases”.

Level of interest: An article of insufficient interest to warrant publication in a scientific/medical journal

Response: We respectively disagree with this Reviewer on this aspect. As indicated by the other Reviewer (An article whose findings are important to those with closely related research interests), this paper will provide invaluable information to the investigators working in the field related to ALK rearrangement and lung cancers in general based on the argument below. Although the influence of both smoking habit and gender in the frequency and characterization of ALK rearrangement were reported in a number of papers, most of these reports did not provide enough information to determine these characteristics in a gender specific manner in never-smokers. To overcome that, we have specifically determined the frequency and characterization of ALK rearrangement in male never-smokers, which is complementary with a recent study reporting the frequency of ALK arrangement in female never-smokers (Reference 18).

Quality of written English: Acceptable

Response: Our manuscript has been proofread by native English speakers.

Statistical review: No, the manuscript does not need to be seen by a statistician.

Response: We made changes to accommodate the comment from the other Reviewer.

Reviewer 2 (Maria Wong)

This manuscript reports EML4-ALK translocations in 95 primary lung cancers from Chinese male never smokers. Multiple analysis methods including RT-PCR-sequencing, FISH and immunohistochemistry were used. The authors found EML4-ALK translocations in 8/95 (8.42%) of the study subjects including 6 adenocarcinomas and 2 squamous cell carcinomas. Amongst the 8 positive cases, only 1 case showed well-differentiation. The mean age of patients was 50.63. Comparing with 5 other series of Chinese patients, the authors found male never-smokers in their series showed higher EML4-ALK translocation frequencies than Chinese males and smokers in other reported series. The relatively large case number and the unusual subgroup of male never-smokers are merits of this study, providing data that supplement a gap in other studies that usually include much fewer male never smoking subjects.

Major compulsory revision:

1. One of the main correlates found in this study – that EML4-ALK translocations are more common in “less differentiated” tumors should not be emphasized and thus it is not advisable to include this term in the title. Tumor differentiation is a very subjective morphological feature. The criteria for defining “well”, “moderate” or “poor” differentiation are not stated in the manuscript and there is no pathologist in the authorship to ensure consistent criteria are used throughout the entire cohort. The number of cases in the respective subgroups is also not balanced. Thus the significance of “tumor differentiation” is unclear. The term “less differentiation” is also not an established pathological term. These limitations should also be discussed.
Response: We thank the Reviewer for this insightful remark and below is our response to this multiple-part comment.

--We have removed “less-differentiation” from the title per this Reviewer’s request.
--We have added more content about our criteria for defining “well”, “moderate” or “poor” differentiation in the Methods section.
--Drs. Jie Ma and Bing Wei, who are co-authors in this paper, are pathologists. They have performed tumor evaluation including grading tumor differentiation levels for patients enrolled in this paper.
--We agree with the Reviewer for her comment on “The number of cases in the respective subgroups is also not balanced” although consecutive patients were selected under the criteria that they are male never-smokers diagnosed with NSCLC. To accommodate this Reviewer’s advice, we added the following sentence at the end of 3rd paragraph in the Discussion section to address the limitation of our results “It should be noted that although we enrolled consecutive patients who met our criteria, our samples are predominantly collected from adenocarcinomas (88.4%), which may bias our results and interpretations”. It is also worth it to mention that non-adenocarcinomas are under-represented in other studies. For example, over 70% patients are diagnosed as adenocarcinomas in a different cohort (see ref 17).

2. The optimal approach for EML4-ALK testing is not universally established. Since 3 different methods of analyses were used in this study, the authors should state clearly the criteria for calling a case “positive”, whether only one test was determinant or an agreement of all 3 methods was required.

Response: We thank the Reviewer for this comment. In this study, all positive cases from RT-PCR were verified by DNA sequencing and by Vysis ALK Break Apart FISH Probe Kit as indicated in the Results section. To make it clearer in the Methods section, we added this sentence: “All positive cases identified by RT-PCR were confirmed by both DNA sequencing and Vysis ALK Break Apart FISH analysis”. Meanwhile, we performed IHC in both negative and positive cases to show the expression of ALK protein in these carcinomas.

Minor essential revisions:

1. The specific clone name of antibody used for IHC is required.

Response: We have included the clone name of the antibody (D5F3), which was purchased from Cell Signaling Technology, Inc., USA.

2. Genomic DNA is not used in the testing and this should be amended in the “Methodology”.

Response: We have removed the sentence “Genomic DNA was extracted by the QIAamp DNA FFPE kit (Qiagen, CA, USA) according to the manufacturer’s instructions” in the Methods section to avoid the confusion. The following sentence “These RNA samples were then subjected to one-step RT-PCR to detect EML4-ALK fusion transcripts…..” in the same section states that total RNA was used to detect ALK fusion variants.

3. The reference numbers (particularly studies quoted for clinicopathological comparison) used in the text and table are inconsistent and needs to be unified.

Response: We thank this Reviewer for pointing out our mistakes and have made the corrections accordingly.

4. The study

Discretionary revision

The finding of around 8% of EML4-ALK translocation is similar to reported frequencies in studies of non-selective patients, inferring gender differences probably plays a limited role in the occurrence of the translocation. It is a pity this study has not included females or male smokers to enable regression analysis which is needed to delineate the effects of these factors. The term “meta-analysis” is wrongly used as strict statistical methodology is required for this type of analysis but is not utilized in this study. It is advisable to emphasize these points in the “Discussion” as limitations of this study.

Response: This is a two-part comment. We will address each separately.
--Including female patients in the study: We admit that it would be better if we also included female patients in the current study. However, due to limited funding, we are unable to do so, as it would increase the budget significantly. In addition, the frequency of EML4-ALK in lung cancer in female patients has already been reported in a recent study published in 2013 (see reference 18 for more details).
--The use of the term “meta-analysis”: We thank this review for her/his comment. We have removed the “meta” in the phrase and revised the Results and Discussion sections accordingly to reflect the nature of our analysis.
Level of interest: An article whose findings are important to those with closely related research interests
Response: We are grateful for the comment.

Quality of written English: Needs some language corrections before being published
Response: Our manuscript has been proofread by native English speakers.

Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.
Response: We had our work evaluated by an experienced statistician, Dr. Chaowei Fu at the School of Public Health, Fudan University, Shanghai, China, as requested (credited in the Acknowledgements section). Some minor changes were made accordingly as described below.

1. It is appropriate to compare between positive patients and negative patients, but not between positive patients and all patients. For that, we removed the results related to the later comparison in Table 5 and modify the text accordingly.

2. For the difference in the expression ALK mRNA between EML4-ALK+ and EML4-ALK- samples in Figure 3, the statistical significance was originally determined by Student’s t test. As per our statistician’s suggestion, it is now determined by a non-parametric Mann-Whitney test.