Author's response to reviews

Title: Relationship between EGFR expression, copy number and mutation in lung adenocarcinomas

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Version: 2 Date: 3 May 2010

Author's response to reviews: see over
Dear Editors,

The manuscript (MS: 7645180083387644, Relationship between EGFR expression, copy number and mutation in lung adenocarcinomas, written by Zhiyong Liang, Jing Zhang, Xuan Zeng, Jie Gao, Shafei Wu, Tonghua Liu*.Department of Pathology, Peking Union Medical College Hospital, Chinese Academy of Medical Science, Beijing 100730, China) was revised according to reviewers’ opinion.

We have carefully read the reviewers’ advice, and made corresponding answers to each question. This is the revised manuscript, the results/data/figures in this manuscript have not been published elsewhere, nor are they under consideration by another publisher.

We used standard methods and interpretation criteria to investigate EGFR expression, mutation and copy number in lung adenocarcinoma.

There is no conflict of financial and personal interest exists.

All authors and acknowledged contributors have read and approved the manuscript.

This paper is proofread by editors of American Journal Expert.

Tonghua Liu, MD
Professor,
Department of Pathology,
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The following is point-by-point response to reviewers’ suggestion.

**Answer to reviewer Robert Clifford**

**1. Major compulsory revisions**

**Question 1)** In their statistical analysis, Liang and coworker do not make adjustment for multiple tests. In some cases, this changes the interpretation of the data. The data in Table 1 should be Bonferroni corrected for 5 tests (association with age, gender, smoking status, lymph node metastasis and tumor stage). After this correction, only the association between mutation and smoking status is statistically significant. The p-values in Table 3 should Bonferroni correct for 2 tests (association with mutation and FISH). After this correction, the association between third criteria and FISH approaches significance (p = .054).

**Answer:** Bonferroni correction were used in our statistical analysis, the results were corrected according to reviewer’s suggestion, the corrected part were red colored in our manuscript. After Bonferroni correction, the association between mutation and gender was not statistically significant, and the association between FISH positivity and tumor stage was not statistically significant in this study, these may be caused by small sample size, and were corrected in result part and discussion part.

**Question 2)** One goal of the study is to provide insight into which molecular feature “is more suitable for selection of patients for EGFR-targeted therapies.” Previous studies have shown that tyrosine kinase inhibitor therapy is most effective against lung cancers with activating kinase mutations. The authors do demonstrate a correlation between EGFR copy number, mutation and protein expression. But since 50% of protein expression-positive tumors are negative for EGFR mutations (Table 3), as are 29% of FISH-positive tumors (Table 2), it seems that mutation detection is the most useful predictor of whether a cancer will respond to TGI therapy. The authors, however, recommend that all three assays be used to prior to treatment. What evidence supports the idea that copy number and protein expression information - alone or in combination with mutation status – are useful predictors of response to treatment? The paper would benefit greatly from a more comprehensive discussion of the benefits (or lack of benefits) of incorporating EGFR copy number and protein expression in the determining how to treat lung cancer.

**Answer:** No analysis was done to relate EGFR protein and DNA changes to the treatment response and patient survival data in this study, the goal of the study was modified in the revised manuscript. We deleted the sentence ‘and to try to determine which one is more suitable for selection of patients for EGFR-targeted therapies’ .We focused on discussing the relationship of the three markers. We agree with reviewer’s opinion that mutation is the most useful predictor of whether lung adenocarcinoma will respond to anti-EGFR therapy, this was largely demonstrated by previous studies. Whether EGFR copy number is a useful predictor of anti-EGFR treatment is controversial.
in previous studies, EGFR copy number was associated with EGFR mutation has been reported in several studies. It is still not clear whether EGFR protein expression could be a predictor of successful EGFR-targeted therapy. We have no evidence support the idea that copy number and protein expression information - alone or in combination with mutation status – are useful predictors of response to treatment, we deleted the recommendation that all three assays be used to prior to treatment in revised manuscript.

**Question 3)** Liang et al argue for the need to establish standard methods for assaying molecular features of tumor samples. The tissue fixation protocol, which impacts these assays, should be described in greater detail in section 2.2.

**Answer:** The tissue fixation was described in detail in red color in section 2.2.

**Minor essential revisions**

**Question 1)** The authors should provide a more thorough explanation of the Scorpion ARMS data shown in Figures 1C, 1F, 2C, 2F, and 2I. To readers unfamiliar with the assay, it is unclear what the curves in these panels represent and what the colors of the lines mean.

**Answer:** Scorpion ARMS mutation detection kit detect mutation in real-time PCR reactions, reaction curves represent the result, if only one ascending curve represent the wide-type of EGFR, more than two ascending curves indicate mutation(one curve represents wild type, the other one represents mutant), the colors of the lines are randomly generated, there are no sense. Legends to Figures 1C, 1F, 2C, 2F, and 2I were corrected in red color in manuscript.

**Question 2)** In section 3.2, the phrase “... also exists exon …” should be changed to “...also had an exon …”

**Answer:** In section 3.2, the phrase “... also exists exon …” has been changed to “...also had an exon …”

**Discretionary revisions**

**Question 1)** In section 3.3, the phrase “showed EGFR mutations” should be moved to the end of the sentences.

**Answer:** In section 3.3, the phrase “showed EGFR mutations” has been moved to the end of the sentences.

**Question 2)** In section 3.5, “Application of the latter two criteria, statistically similar results were yielded” should be changed to “Similar results were obtained using either of the latter two criteria” to improve clarity.

**Answer:** In section 3.5, “Application of the latter two criteria, statistically similar results were
yielded” has been changed to “Similar results were obtained using either of the latter two criteria”

**Answer to reviewer Maxwell Lee**

**1 Major Compulsory Revisions**

**Question:** The manuscript entitled “Relationship between EGFR expression, copy number and mutation in lung adenocarcinomas” by Liang et al. characterized EGFR protein expression, DNA copy number, and mutations in 133 lung adenocarcinomas. The authors found positive association among increased protein expression and DNA copy and mutations. My major concern is that no analysis was done to relate these protein and DNA changes to the treatment response and patient survival data, which were apparently the stated goal of the study. The other issue is patient selection. The authors need to provide explicitly the selection criteria.

**Answer:** No analysis was done to relate EGFR protein and DNA changes to the treatment response and patient survival data in this study, the goal of the study was modified in the revised manuscript. The goal was changed to ‘This study was designed to investigate EGFR protein expression, EGFR copy number and EGFR mutations in lung adenocarcinomas, to explore the relationship of the three markers’. Only patients with primary lung adenocarcinoma were selected, intrapulmonary metastases and recurrent disease were not included in this study. And the patients who were not previously treated with chemotherapy, radiation or anti-EGFR therapy were selected. Only cases with available EGFR immunohistochemistry, mutational status, and EGFR FISH data were analyzed. Clinical information included gender, age, smoking status, tumor stage and lymph node metastasis status. The selection criteria were described in detail in the revised manuscript.

**2 Minor Essential Revisions**

**Question:** I would also like to see some discussion regarding the molecular basis for the positive association between mutations and DNA copy number changes.

**Answer:** We have added some discussion regarding the molecular basis for the positive association between mutation and DNA copy number changes in revised manuscript. Three references were added in the manuscript and the following references’ numbers were changed.
Answer to reviewer Robert Kratzke

1. Major Compulsory Revisions

Question 1) More information on how the patients for analysis were selected is needed. The paper says that only 133 of 886 were selected. The paper only says that patient with clinical data intact were selected. What does this mean? A median follow up, survival data, and such would be criteria that one would like to see mentioned.

Answer: Only patients with primary lung adenocarcinoma were selected, intrapulmonary metastases and recurrent disease were not included in this study. And the patients who were not previously treated with chemotherapy, radiation or anti-EGFR therapy were selected. Only cases with available EGFR immunohistochemistry, mutational status, and EGFR FISH data were analyzed. Clinical information included gender, age, smoking status, tumor stage and lymph node metastasis status. The selection criteria were described in detail in the revised manuscript. Follow up, survival data were not analyzed in this study.

Question 2) If the authors insist on "intact clinical data", why are there no correlations with the clinical data? No response to therapy, survival, progression free survival?

Answer: Because follow up, survival data were not analyzed in this study, the sentence “patient with clinical data intact were selected.” was deleted. All patients in this study have not received anti-EGFR treatment, so the response to therapy, survival, etc were not analyzed.

Question 3) The positive correlation between EGFR mutation and IHC is contrary to many other published reports. This makes one less confident in the conclusion that IHC may be an adequate marker for determining response to EGFR targeted therapies.

Answer: Whether IHC may be an adequate marker for determining response to EGFR targeted therapies need to be demonstrated in further studies. The positive correlation between EGFR mutation and IHC in our study was contrary to previous reports, there may be caused by several reasons. First, strict selection criteria for samples were used in our study, only the surgical resected primary lung adenocarcinomas were selected, each specimen was large enough for molecular analysis, small biopsy specimens were excluded. All the patients did not receive chemotherapy, radiation and anti-EGFR therapies, these treatments may cause molecular change. Second, all the specimens in this study were fixed in 10% neutral buffered formalin within 6-48 hours. Third, the DAKO EGFR PharmDx kits were used in this study. Forth, different interpretation criteria were used. Fifth, microdissection and Scorpions ARMS methods were used in mutation detection in this study. Sixth, small sample size was analyzed in this study.

Question 4) The statement that “no single test was sufficient to predict response to EGFR TKI” is not supported by the data in the paper. There was no attempt to correlate the results in this
study with response to EGFR targeted therapies. There are amply published data that EGFR mutations are strongly associated with response to EGFR TKIs including the recently published IPASS trial data (N Engl J Med. 2009 Sep 3;361(10):947-57.).

Answer: We agree with the reviewer that there was no attempt to correlate the results in this study with response to EGFR targeted therapies. We deleted the sentence “no single test was sufficient to predict response to EGFR TKI” in revised manuscript.