Author's response to reviews

Title: Prognostic implications of ezrin and phosphorylated ezrin expression in non-small cell lung cancer

Authors:

Tiefeng Jin (jintf@ybu.edu.cn)
Jingchun Jin (jingchun680928@163.com)
Yongjun Shang (syj515@163.com)
Songnan Zhang (zhangsn21@163.com)
Kaiwen Yu (29667091@qq.com)
yingshi Piao (yspiao@ybu.edu.cn)
Xionghu Shen (xim918@sina.com)
Zhenhua Lin (zhlin720@ybu.edu.cn)

Version: 2
Date: 15 January 2014

Author's response to reviews: see over
Responses to the comments

Dear Reviewers,

We appreciate your taking the time to review our manuscript. We have carefully revised the manuscript in accordance with your comments, and hope that our study is now suitable for publication in *BMC Cancer*.

Yours sincerely,
Zhenhua Lin

**Responses to the Reviewer-1’s Comments:**

**Minor Comments:**

1. **Page 2:** The manuscript does not shown that ezrin or p-ezrin play an important role in the progression of NSCLC. It shows only a correlation between these markers and progression. The claim is overblown.

   **ANSWER:** We already modified the conclusion in the manuscript on Page 2, and marked blue.

2. **Page 3.** Third sentence needs clarification.

   **ANSWER:** We already removed the sentence, and revised the introduction (marked blue).

3. **Page 4.** Sentence “Recently, many cancers… is meaningless. Remove.

   **ANSWER:** We already remove the sentence on Page 4.

4. **Page 6.** How are discrepancies between the two pathologists resolved?

   **ANSWER:** For the H&E slides, we only selected cases with no discrepancies for this study. However, in cases of discrepancies for the IHC evaluation, a final score was established by reassessment on a double-headed microscope.

5. **Page 7.** The authors need to demonstrate the specificity of the antibodies
used in this study by Western blot. How does they know what they are staining? Are the antibodies monoclonal or polyclonal? What species?

**ANSWER:** All antibodies are anti-rabbit polyclonal antibodies (*datasheets shown in Fig-S1*), and we have provided this information in the Methods section. To confirm antibody specificity, we performed western blot analysis in A549, H1299, and H460 lung cancer cell lines (*Fig-S2*).

**Fig-S1:**

**Fig-S2:**

6. How can percent total ezrin staining be lower than p-ezrin staining as the latter is a subset of the former? It makes no sense.

**ANSWER:** We reviewed some related references and found that other reports are also consistent with our results. For example, Yasunori et al. reported that in intraductal papillary mucinous neoplasms of pancreas (IPMNs), the expression rate of ezrin was 42.5%, but pTyr353-ezrin was 55.0% and
pTyr354-ezrin was 42.5%. Moreover, in intestinal-type pancreatic neoplasms [Hum Pathol. 2013;44:1487], ezrin expression rate was 32.4%, but pTyr353ezr was higher at 41.2%. Claudio et al. reported a rate of ezrin of 86% in osteosarcoma, but 88% for pThr567-ezrin [Mod Pathol. 2010;23, 1012]. Cui et al. reported that the expression rate of ezrin was also lower (78.6%) than that of pTyr353-ezrin (92.9%) in poorly differentiated pancreatic cancers [Cancer Invest. 2010,28:242]. Thus, our results consistent with these previously published trends. We speculate that the cause might be related to some cases mainly expressing p-ezrin, but showing lower or no ezrin levels (Fig-S3). Verification of the detailed mechanism underlying this phenomenon requires further study.

**Fig-S3:**

7. Were all the cases of NSCLC primary and untreated?

**ANSWER:** Yes, all cases of NSCLC used in this study were primary tumor,
and were not treated before surgery.

8. The discussion is rather dry, being a list of the literature relevant to ezrin in cancer. It would be helpful to synthesize the findings.

9. Please also discuss the known molecular alterations/mutations in NSCLC and how these might alter signaling including ezrin phosphorylation status.

**ANSWER 8 and 9 queries**: We are grateful for the helpful comments. We have rewritten the discussion, with all modified sections marked in blue.

10. The text and graphs in figure 3 are at insufficient resolution.

**ANSWER**: We already change the figure 3 and 4 at sufficient resolution.

11. Micrographs shown have a bar to indicate size.

**ANSWER**: We already added the size bar in Fig-1.
Responses to the Reviewer-2’s Comments:

Major compulsory revisions:

1. Page 12-16:

The discussion needs to be rewritten. It is just a summary of the existing literature and therefore very exhausting to read.

**ANSWER:** We are grateful for the helpful comments. We have rewritten the discussion, with all modified sections marked in blue.

How do you explain that total ezrin staining can be lower than p-ezrin staining?

**ANSWER:** We reviewed some related references and found that other reports are also consistent with our results. For example, Yasunori et al. reported that in intraductal papillary mucinous neoplasms of pancreas (IPMN), the expression rate of ezrin was 42.5%, but pTyr353-ezrin was 55.0% and pTyr354-ezrin was 42.5%. Moreover, in intestinal-type pancreatic neoplasms [Hum Pathol. 2013;44:1487], ezrin expression rate was 32.4%, but pTyr353ezr was higher at 41.2%. Claudio et al. reported a rate of ezrin of 86% in osteosarcoma, but 88% for pThr567-ezrin [Mod Pathol. 2010;23, 1012]. Cui et al. reported that the expression rate of ezrin was also lower (78.6%) than that of pTyr353-ezrin (92.9%) in poorly differentiated pancreatic cancers [Cancer Invest. 2010,28:242]. Thus, our results consistent with these previously published trends. We speculate that the cause might be related to some cases mainly expressing p-ezrin, but showing lower or no ezrin levels (Fig-S3). Verification of the detailed mechanism underlying this phenomenon requires further study.

**Fig-S3:**
Please also comment on already known mutations in NSCLC (especially in adenocarcinomas) and their potential interaction with ezrin.

**ANSWER:** We have included this information and modified the Introduction and Discussion sections, as shown on pages 3 and 15.

**Minor essential revisions:**

1. **Page 2 (Conclusions Abstract):** Omit first sentence since this statement is completely overdrawn.

   **ANSWER:** We already modified the conclusion in the manuscript on Page 2, and marked blue.

2. **Page 5:** Specify accurately how samples were examined. Was it a tissue microarray? If yes, how many punches per tumor? How many punches per healthy tissue?

   **ANSWER:** The tissue microarrays contain 116 cases of lung cancers (*Fig-S4*),
and each case had at least two different punches (usually three). For the healthy tissue (from autopsy samples) and other 34 lung cancers, we used full section slide in this study.

**Fig-S4:**

3. Page 6: Please provide a table for this paragraph to sum up the patients’ characteristics. Age and gender (although I believe the term sex should be used since it refers to the biological state) are biological or clinical parameters, the remaining ones pathological.

**ANSWER:** We changed “Parameter” to “Characteristics” in Table 2, and Table 2 now lists information for all patients’ characteristics.

**Were all tumors untreated prior to surgery?**

**ANSWER:** All cases used in this study were primary tumor, and were not treated before surgery.

In adenocarcinomas, is there a link with EML4/ALK or EGFR-mutation status?

**ANSWER:** According to The International Association for the Study of Lung Cancer (IASLC)/American Thoracic Society (ATS)/European Respiratory Society (ERS) International Multidisciplinary Classification of Lung Adenocarcinoma [Travis WD et al, J Thorac Oncol, 2011;6(2):244-285], molecular target therapy based on EML4/ALK or EGFR-mutation status has been emphasized. Currently in China, EGFR/KRAS mutation and EML4/ALK
fusion status is detected for the target therapy (tyrosine kinase inhibitors, TKI) of advanced-stage lung adenocarcinoma after patient agreement by signed consent form. However, lung adenocarcinomas with positive EML4/ALK or EGFR-mutation status were rare in the specimens used in this study. Thus, we did not analyze the correlation between ezrin expression and EML4/ALK or EGFR-mutation status. If possible, we will perform a study to explore the correlation between ezrin and EML4/ALK or EGFR-mutation status in the future.

Is there any correlation with histological subtype (acinar, micropapillary, mucinous,..)?

**ANSWER:** According to the new classification, we re-reviewed all HE slides of these cases, and divided the slides into SCC, adenocarcinoma, and NSCLC-NOS categories. Only SCC and adenocarcinoma were included in this study (82 adenocarcinoma and 68 SCC). For the adenocarcinoma cases, we confirmed the previous diagnosis based on the HE slides and IHC staining for TTF-1, MUCs, and so on in some cases. Here we did not analyze the correlation between ezrin expression and the detailed histological subtypes of adenocarcinoma because of the limited case numbers of several subtypes.

**Omit the word „carefully“.

**ANSWER:** We already omitted the word.

**Specify „follow-up deadline“ and provide a time range for the follow-up period.**

How many deaths were not attributable to the malignant condition?

Please explain – what was the cause in the remaining cases?

How were discrepancies between these two pathologists resolved?

**ANSWER:** We have included all this information in the “Materials and Methods” section on pages 6 and 7.
Please provide information regarding the specificity of the antibodies used.

Regarding the immunohistochemical staining: I understand that the scoring applied refers to the number of stained cells. Did you take the intensity of the staining into account?

**ANSWER:** All these antibodies are anti-rabbit polyclonal. Here we performed the western blot for confirming the antibodies’ specificity in A549, H1299, and H460 lung cancer cell lines (*Fig-S2*). For the IHC scoring, we combined the intensity of staining, and graded according to the percent of positive staining cells. And we modified the description on page 8.


**Fig-S2:**

<table>
<thead>
<tr>
<th>H1299</th>
<th>A549</th>
<th>EBC-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-EZR^567</td>
<td>81KD</td>
<td></td>
</tr>
<tr>
<td>p-EZR^353</td>
<td>81KD</td>
<td></td>
</tr>
<tr>
<td>EZR</td>
<td>81KD</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>42KD</td>
<td></td>
</tr>
</tbody>
</table>

**5. Page 9:**

Spelling mistake: cytoplasmic

Which structures (pneumocytes, endothelial cells, respiratory epithelium,……..) were stained in non-tumor tissue? Were macrophages included in the scoring?

**ANSWER:** We changed “cytoplasmic” to “cytosolic” in the manuscript.

The slide section of adjacent non-tumor and normal lung tissues include
the structures of pneumocytes, endothelial cells, respiratory epithelium, lymphatic cells, inflammatory cells, and so on, as shown in Fig. 1B in the manuscript. For the macrophage cells, some are positive for these antibodies, but were not counted in the scoring system.

How do you explain the difference between the staining patterns of adjacent and normal lung tissues?

**ANSWER:** There is no significant difference between adjacent and normal lung tissues. The different staining pattern might due to the case selection and individual differences. Despite two groups of normal lung tissues from different population, the staining pattern might be different.

6. Tables 1 and 2:

The P-value is capitalized and has to be in Italics. Please keep the P-value consistent in the main text and the tables (either with or without a zero before the comma, check with the journal’s policies).

**ANSWER:** We already changed in the manuscript (Page-23,24).

7. Table 2:

Differentiation (parameters in the table): it should read „well-moderate-poor“ Last line: it should read „poorly vs well and moderately differentiated tumors“

**ANSWER:** We already changed in the manuscript (Page-24).

8. Figure 1:

Provide a picture of positively stained normal lung tissue (either adjacent or of controls).

**ANSWER:** We already replace one positive staining figure of adjacent control in Fig-1.
9. Figure 2: Provide P-values.

**Answer:** We already added the P-value in fig-2.

10. Figure 3 and 4:
The resolution is not acceptable. Redo both figures.

**Answer:** We already redo the both figures at sufficient resolution.