Author's response to reviews

Title: Clinicopathologic features and prognostic implications of NOK/STYK1 protein expression in non-small cell lung cancer

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Author's response to reviews: see over
Dear Ms. Roselyn Remoto:

Thank you very much for your attention and the referee’s evaluation and comments on our paper “Clinicopathologic features and prognostic implications of NOK/STYK1 protein expression in non-small cell lung cancer”. We have revised the manuscript according to your kind advice and referee’s detailed suggestions. Enclosed please find the response to the referees. We sincerely hope this manuscript will be finally acceptable to be published.

Thank you very much for all your help and looking forward to hearing from you soon.

Best regards

Sincerely yours

Xiao-Fei Li

Please find the following response to the comments of referees:

Referee Naoki Watanabe

Comment:
There might be the patients treated with chemotherapeutic drugs after surgery. The authors should describe the information regarding the relationship between outcome (recurrence etc.) after adjuvant chemotherapy and STYK1/NOK expression level.

Response:
There were some difficulties in accurate determination of patient’s recurrence time in the follow-up process: the start time of recurrence was unable to be determined in many patients who are found to have recurred during a particular review; in the telephone follow-up process, the recurrence conditions were difficult to be clearly described by some patients. However, the time of death could be accurately obtained. Thus, for the accuracy of the data, the time of death was used for studying the outcome.

To investigate the relationship between outcome after adjuvant chemotherapy and STYK1/NOK expression level, 39 cases of patients with stage II squamous cell carcinoma (SCC) and 31 cases of patients with stage II adenocarcinoma (ADC) were respectively extracted (the remaining stages were not given statistical analysis due to the small sample size). These patients were all received postoperative chemotherapy with cisplatin and docetaxel. COX risk model was used for the determination of the effect of NOK expression level on the outcome of chemotherapy. The results showed that HR (95%CI) was 1.914 (0.829-4.423) and P-value was 0.129 in patients with squamous cell carcinoma, and HR (95%CI) was 2.957 (0.385-22.719) and P-value was 0.297 in patients with adenocarcinoma. In conclusion, the effect of NOK on the outcome of lung cancer chemotherapy (adjuvant chemotherapy) was not found by analysis of samples into the group. However, since this was a small sample size of patients with the same stage underwent the same chemotherapy, the effect of NOK on the outcome of chemotherapy needed to be further validated in future studies with larger sample sizes.
Referee Radostina Cherneva

Comment 1:
The distribution of the tumors regarding some of their clinicopathological features (grade, tumor stage and lymph node metastases) is uneven, which makes the statistics unreliable.

Response:
In this study, patients with lung cancer hospitalized from 2007 to 2010 were randomly selected (patients who received preoperative chemotherapy, radiotherapy or epidermal growth factor receptor (EGFR)-targeted therapy were excluded). Affected by local economic and health conditions, the patients were most in stage II or III lung cancer but rare in stage I or IV when admitted, leading to that clinicopathological features (grade, tumor stage and lymph node metastases) were uneven.

Comment 2:
The second drawback is the follow-up period – it is too short and limits the Kaplan -Meier in retrieving the median survival period. Mean survival period is mostly mentioned and this, we know, is not as a reliable measure for survival and thus for prognosis. Moreover, if we look the data in detail, we shall see that the median survival is not achieved in any of the NOK negative tumors, which makes the prognostic value of the marker doubtful. I am not sure that the general reader knows exactly what mean and median survival is, this may cause confusion and may deter the comprehensibility of the results.

Response:
As a single-center study, this experiment was affected by the number of cases into groups and the duration of follow-up, so that the median survival period was often unable to be calculated. To describe the survival of patients, we had to use the mean survival period in the article for many times. As the reviewer described, it might be not quite clear for some readers to know the difference between the mean survival time and median survival time. For this reason, it was described by the author in the revised manuscript.

Comment 3:
The method as I already mentioned is not an appropriate one.

Response:
The immunohistochemistry method was used in this trial, mainly based on the following considerations that there had been a report on the expression level of NOK gene in lung cancer but no report on the expression of NOK protein in lung cancer; that the specimens used in this study were from the specimen library in our department, in which many specimens were kept by embedding in the paraffin blocks, thus more suitable for immunohistochemical studies. The reviewer noted that “It usually starts with proteomics-gene expression and real-PCR studies and finally comes to Western blotting and immunohistochemistry”, which was agreed with by the author. Currently, we are collecting fresh tissue specimens for detection of gene expression of NOK and its possible effectors, but it will takes a long time to complete
the experiment.

Comment 4:

Another drawback, which is very important, is the semi-quantitative way of interpreting the results of the immunochemical study of NOK - it is very subjective. The reason for the choice of these cut-off points is not well described. As this is the first immunostudy of NOK the choice of the cut-off points is of major importance for the interpretation of the results. The ROC analysis that is mentioned deserves a more detailed description.

Response:

For the problem of interpreting the results of the immunochemical study of NOK, we considered other options before, for example, more detailed classification by using ",-, +, ++, +++". However, due to the limitations of sample size and other factors, these options were often undesirable in assessing the outcome. To reduce the subjectivity in judging results, all slides were assessed by 3 independent investigators who were blinded to the clinical features and outcomes.

Concerning the selection of positive standard, what we adopt is ROC test. The results can be seen in the chart as follows: the area under the curve AUC is 0.901 ($z=27.419, \ P<0.0001$), that is to say, this index has a better diagnostic value; after ROC curve analysis, 3 points is determined as the best diagnostic critical value, the sensibility is 70.68, the specificity is 90.58, and Youden index is 61.26%. It was described in the revised manuscript.

![](image)

<table>
<thead>
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<th>Criterion</th>
<th>Sensitivity</th>
<th>95% CI</th>
<th>Specificity</th>
<th>95% CI</th>
<th>+LR</th>
<th>95% CI</th>
<th>-LR</th>
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<td>0.00</td>
<td>0.0 - 1.9</td>
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<td></td>
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<td>35.8 - 50.3</td>
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<td>1.4 - 2.0</td>
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<tr>
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<td>91.2 - 97.8</td>
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<td>50.8 - 65.2</td>
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<tr>
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<td>68.7 - 81.3</td>
<td>85.34</td>
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<td>5.14</td>
<td>4.7 - 5.7</td>
<td>0.29</td>
<td>0.2 - 0.4</td>
</tr>
</tbody>
</table>
A fifth limitation is the cut-off point of Ki 67- 31%. In NSCLC, in contrast to breast cancer, there is not a consensus about the cut-off of Ki-67 for the division of tumors up to their proliferation rate. If a cut-point should be used for this study, it should be reconsidered up to the mitotic index and based on it. It ought not be the median or average Ki-67 LI, that should be applied as a cut-off. This again is very important for the future interpretation from the view point of NOK expression.

Response:
In this study, as an indicator for determination of cell proliferation, Ki-67 was used for analysis of the effect of NOK on the proliferation. To determine the cut-point of ki-67, we reviewed similar reports on lung cancer [1, 2] and found that the median ki-67 LI was usually used as the cut-point. To make the results easier to understand for readers, we used the same method as previously described.


Other comment:
Please note that the adjacent tumor tissue is rarely normal – it always has some dysplasia. It should be adjacent non-cancerous tissue instead.

Response:
The corresponding modification has been made in the article.