Peer Review File

Article information: http://dx.doi.org/10.21037/atm-20-7900

Reviewer Comments

Fusions involving the ALK, ROS1 and RET genes are the second most common targetable alteration in NSCLC and testing is mandatory in the advanced setting. When tumor tissue is unavailable, liquid biopsies are already a well-established source of material. However, although the sensitivity of the assays used for mutation detection in liquid biopsies are high (usually >80%), this is not the case for gene fusions, and new techniques are needed. In this article, Heeke et al present a novel RT-PCR based assay for detection of ALK and RET fusions in plasma, which can be run in the COBAS platform. Although the results might be of interest, the methodology is poorly described and the manuscript has ample room for improvement.

Reply: We would like to thank the reviewer for their comments and appreciate the opportunity to provide a revision addressing all concerns in the revised manuscript “Detection of ALK fusion transcripts in plasma of non-small cell lung cancer patients using a novel RT-PCR based assay”. Please find our comments below.

Major comments

1. Articles presenting new assays should describe them in detail, even if the assays are “proprietary”. However, the information contained in this manuscript is very scarce and should be significantly expanded. In particular,

Reply: We totally agree with the reviewer that sufficient details for the methods is critical for a reproducible study. We apologize if it was unclear that the aim of the study was to provide performance of a rapid fusion detection assay, which was developed by a manufacturer and might become commercially available. We have added a lot of the requested additional information, but importantly for the interested reviewer we have added a link to the patent

(Ref 7) that the reader can look up all the relevant information. The main aim of the study is to provide assessment of diagnostic performance.

-The sequences of the primers and probes should be provided

Reply: It is very uncommon to provide primer and probe sequences for a proprietary assay, however, all the sequences and additional information like buffer composition and other details can be found in the linked patent (Ref 7).

-The RT-PCR reaction should be described. Volumes of reagents and samples? Cycles? Temperatures?

Reply: We have now added the requested information regarding volumes and thermal cycling profiles in the text, and additional information can be found in the linked patent (ref 7).

-The authors should clarify if they used normalization purposes and, if they did, provide a list

Reply: The assay uses an internal control as a quality check of the cfRNA sample input. However, the internal control is not used for normalization. This is intended to be a qualitative diagnostic assay to detect the presence or absence of the mutation and not a quantitative assay that measures gene expression. We have now added details on the calling of the presence of a mutation in the method section.

-The algorithm used to categorize the samples should be explained. Were Cts or deltaCts used? What were the thresholds for positivity? Which criteria were used to classify a sample as "valid"? Ct of housekeeping genes? (etc)

Reply: We apologize for not describing the algorithm in sufficient detail in the original manuscript. The exact algorithm and details remain intellectual property by the manufacturer. However, we now have added more details on the criteria for a positive call in the Method Section.

-Although ROS1 testing is mandatory in advanced NSCLC, ROS1 fusions were not included in the assay. The authors should clarify why.

Reply: We fully agree with the reviewer that clarification regarding the omission of ROS1 fusion testing is needed. The aim of this evaluation was to rely on a very distinct and clinically defined cohort of *ALK* patients as part of our STALKLUNG clinical cohort. Therefore, we were only able to evaluate the assay performance for the detection of *ALK* positive samples and the conclusions of the manuscript are consequently limited for the detection of *ALK* fusions. We have provided an explanation of this in the manuscript in lines 255-257.

-The specific fusions found in the positive samples should be presented in the results section

Reply: We totally agree with the reviewer that it would be informative to specify the exact *ALK* fusions. Unfortunately, the multiplex assay is not designed to distinguish the precise fusion, and likewise, our tissue analysis based on FISH and IHC is not able to distinguish the different fusions. We have clarified this in the manuscript. Interestingly, the administration of anti-ALK TKI is recommended independent of the underlying fusion, and thus the clinical impact of knowing the specific fusion is limited.

-The authors claim that ALK in tissue was analyzed by FISH and/or IHC. However, in Figure 2, there are 2 ALK+ patients identified by "other" techniques.

Reply: We apologize for the missing information. We have reverified the information and those patients were indeed tested by IHC. We have corrected the information in the revised version of the manuscript.

2. Preanalitical steps are critical in the case of liquid biopsies. The authors should describe them in more detail. What centrifugation steps were performed? Which columns they used for RNA purification? Were samples immediately processed? Also, in the results section, they should provide data about the purified cfRNA, such as concentration or Ct of housekeeping genes (if used)

Reply: We totally agree with the reviewer on the importance of pre-analytics and that inclusion of more details are necessary. Aside from the double centrifugation during the plasma separation step, no additional pre-clearing of the plasma sample was done. We have consequently added those details in the method/results section. Additionally, we have added

information on the cfRNA concentration after extraction in the new Figure 2. We have also added additional information of the Ct value of the internal control and the relationship with cfRNA input in the new Supplementary Figure 1.

3. The sensitivity of the RT-PCR presented by the authors (33%) is too low for the technique to be used in the clinical setting. The assay needs further refinement and authors should discuss how they plan to do it.

Reply: We fully agree with the reviewer that further discussion on how to improve assay performance needs to be provided. We have added additional proposals on how the assay could be improved in lines 265-276, with a special emphasis on the importance of evaluating multiple pre-analytical conditions.

Minor comments

-Line 88. References about multiplex testing of gene fusions should be provided

Reply: We have provided the missing reference.

-Line 111. The authors should specify which FISH probe they used

Reply: We have added the respective IHC and FISH assays used for the detection of ALK (line 112-113). Additionally, we have referenced the main publication of the STALKLUNG study which gives additional information on the methodology and study inclusion (Ref 4)

-Line 143 and others. The word “to highlight” is incorrectly used instead of “to present” or “to show”

Reply: we have replaced “highlighted” with “presented”

-Line 148. Cohen’s kappa values should be calculated

Reply: we have added Cohen’s Kappa in the analysis (Line 188)

-Line 163. It should be “no significant difference”

Reply: we have added “significant” to the sentence (Line 202).

-Line 203-205. Not all NGS panels have the “potential to discover unreported variants”. They need to include imbalance probes

Reply: We have added the information and references on the imbalance probes to detect novel variants.

-Line 220. Centrifugation at the xg usually employed to separate plasma will not precipitate exosomes

Reply: We agree with the reviewer that for the precipitation of exosomes, typically ultracentrifugation at >100.000xg is used. However, larger tumor cell derived extracellular vesicles might be affected even at lower speed and we have consequently clarified this in the manuscript (Line 270 -272).

-Table 2. The number of patients positive by FISH and/or IHC should be included in the table

Reply: We have included the information in Table 2.

-Figure 2 is hard to understand. Patients positive in tissue should be grouped to the right of the figure and sub-grouped in baseline, treated and progression. The blue color should be defined (not analyzed?). Age is irrelevant and it's never presented in a bar plot, it should be deleted.

Reply: We have adapted Figure 2 accordingly.