Peer Review File

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Reviewer A

This represents a clear advance with regard to the value of getting both the RNAseq and DNA next gen sequencing done in the lung cancer setting. Very reasonable contribution per above comments to authors. And, the authors have conducted reliable practices with supplementary material and clear indication of institutional ethics approval. While not a huge intellectual advance, the practical advance for lung adenocarcinoma could be helpful to many patients.

Reply 1: We are grateful to the reviewer for this positive opinion about our manuscript.

Reviewer B

Overall, the manuscript entitled "Reflex Testing in NSCLC using DNA- and RNA-based Next-Generation Sequencing – A Single-Center Experience" by Zacharias and colleagues is a fine retrospective analysis of two years worth of NSCLC NGS testing with the addition of RNA-based testing in the second year. The authors demonstrate a high testing success rate, and highlight an increased number of fusions detected with RNA-based NGS employed. This paper provides some valuable real-world testing experience to support the addition of RNA based fusion assays to traditional DNA based NGS panels. The authors should address the following points:

Comment 1-It would be helpful for the authors to provide additional information on what was tested, namely what were the specimens used for NGS testing? Core biopsy, cytology cell block, large biopsy specimens, resection specimens? Does any particular specimen type correlate with testing failure (especially for RNA panels)? It would help the reader place these impressive testing results into the context of their practice if they knew the sample type distribution.

Reply 1: We are thankful for this observation, and completely agree with it. We have now analyzed the type of specimen, and correlated it with testing failure. We also analyzed the association of the sample type and testing failure., and in 6 cases fusion panel “failure” 2 cases were resection specimens, and 4 biopsy. Most commonly the cause was insufficient RNA amount, and in 2 samples was RNA of poor quality. All cytology samples were adequate. All samples for DNA testing were adequate. We have also included the origin of the sample (primary tumor, lymph node, metastasis)

Changes in the text 1: Stratification according to the sample type is now included in Tables 2 and 3. They are also included in Results section (lines 210-215 and 250-255).

Comment 2-Can the authors comment on the turnaround time for the first year vs the second year testing paradigm? Does RNA based NGS testing add any additional time to the DNA NGS

panel in order for the oncologist to have all results available to decide first line therapy?
Reply 2-This is another important point, and we are grateful for bringing this up. Adding RNA based testing prolong the turnaround time from 8 to 10 working days due to some technical/organizational issues in our lab.
Changes in text: We have included a sentence about it in a Results section (lines 320-321), and also in Discussion (lines 418-420).

Comment 3-Admittedly, DNA NGS testing identifies the majority of the currently targetable genomic alterations in this patient cohort, with a relatively low number of patients with additional fusions identified. Although important to find the best drug to treat each patient, could the authors comment on the expanding role of immune checkpoint inhibitor therapy in targetable mutation negative tumors? Are the additional testing and time resources enough to identify so few tumors that could potentially be treated effectively with IO therapy? It would also help to discuss a little more the role of PD-L1 testing in this real-world testing structure (only briefly mentioned in the discussion).
Reply 3-We appreciate this comment very much. We think that identifying targetable mutations is very important, and should be done prior to eventual introduction of IO therapy. Since IO therapy was not in the scope of this paper we did not want to distract readers with too much discussion about it. We really wanted to stress the importance of DNA/RNA based NGS testing in parallel way, and in a reflex manner.
Changes in text 3: We have included a short comment/opinion about it at the very end of discussion (lines 421-425). We sincerely hope that even this short comment will be found suitable by reviewer.

Reviewer C
The paper is generally well written and informative, only a couple minor typographical errors were noted:
Comment 1-Line 148 Qbit should be Qubit
Reply 1-We are thankful for this
Changes in text 1: We have corrected it accordingly (line 163)

Comment 2- Lines 185 /186, the meaning of the first sentence of this paragraph is unclear.
Reply 2- I am not quite certain if I was able to detect the unclear part. However, the first part of the sentence is now deleted
Changes in text 2- The first part of the sentence deleted (lines 195)

The paper is also well referenced with most issues arising already addressed in the discussion. There is also some sound advice on tissue preservation strategy. There are however still a couple of points that I feel it would be beneficial/important to address:
Comment 3-It appears that the findings presented were the ones utilised for actual treatment stratification. Neither assays referenced are CE-IVD, so clarifying if this is an ISO15189 accredited clinical laboratory with both assays being operated under their ISO scope would be important. If the laboratory is not ISO accredited and these were the results used for treatment stratification (i.e. without additional confirmation), some comment regarding how the assays

were validated and deemed appropriate for clinical use by the institution would also be important.

Reply 3- We are grateful for this comment. Indeed, all analyses reported were performed in the diagnostic context and used the Ion Torrent platform in conjunction with Ampliseq and Archer chemistry, neither of which is CE-IVD. However, we are currently preparing ISO 15189 accreditation of the Institute of Pathology and have thoroughly validated the performance of all in-house NGS assays. In detail, assays were tested for specificity, sensitivity, limit of detection as well as repeatability and reproducibility to ascertain concordance to the general safety and performance requirements described in Annex I of the IVDR (EU 2017/746). We use a mix of commercial known-truth samples and patient samples previously analyzed with alternative technologies (qPCR, Pyrosequencing or FISH) at our Institute. We regularly participate in EQA schemes for all our diagnostic analyses, with regards to lung cancer, we have most recently successfully participated in the "2020 ESP External Quality Assessment Scheme for molecular DNA testing" in NSCLC (100% concordance).

Changes in text 3- A new paragraph called Validation is now inserted in Methods, lines 171-180.

Comment 4-The fate of 'all' samples is unclear:

a. 94.2% of samples generated meaningful results with DNA-based NGS, did the rest fail, or was analysis not even attempted? In not attempted, why not? Tumour cellularity too low (what was the cut off?), DNA yield too low etc.

b. Only 62.3% of samples generated meaningful results with RNA-based NGS, yet the authors state very low failure rates, suggesting that there was no attempt made to analyse a significant minority. Some centres undertake DNA-based NGS analysis first and only undertake RNA-based in cases where no recognised early driver event is identified (reduces cost but increase turnaround). However, this would still not explain the 62.3%, plus the authors appear to suggest that both DNA and RNA-based analyses were performed in parallel upon appropriate diagnosis.

Reply 4- We have look at our tables again, and we do agree that the column "all" introduced more confusion than explanation. That is the reason why we have decided to delete it from Suppl Tables 1 and 2. We hope that the tables are now easier to understand. The total number of patients per year is not the same as tested for DNA because the total number includes all patients tested with DNA and RNA based panels. However, some tested samples were tested only with RNA based panel based on clinicians whish, or in search of eventual mutation in samples already tested for DNA (in year before). Important is that all samples tested for DNA were also evaluable.

62.3% seems low, but it resulted out of cumulative presentation of data from both 2019 and 2020 (which is now deleted). In a year 2019 we did not have reflex RNA testing, and therefore we have only 20.8% of the all patients tested, and out of those only 1 failed (1/38). In 2020, after introduction of reflex testing for adenocarcinomas, majority of patients was tested (95.1%), and the rest are non-adenocarcinomas, or samples tested for resistance mutations just with DNA panel, and for few patients at the beginning of 2020 due to the transition to reflex testing with both panels, RNA tests were simply not ordered (pathologist have forgotten to order it). In 2020 we have 5 "failed" cases in RNA based testing, due to the too low content of RNA, or poor quality of RNA.

Changes in text 4- Supplemental Tables 1 and 2 changed accordingly. Additional explanation have been added in the Results (lines 240-244, and 275-278).