Peer Review File

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Reviewer A

In this manuscript the authors describe their findings of a retrospective study to explore the incidence of NTRK IHC positive lung cancer in a real world data set. While the use of NTRK IHC has been proposed as a means to screen for NTRK fusions, it has some limitations, which also hold implications for this study.

Comment 1. In the first sentence of the discussion, it is stated that 'we found the frequency of NTRK fusion in NSCLC to be only 0.35%. This is not true, as we do not know what the level of sensitivity of NTRK IHC is for screening for these fusions. In our hands, the sensitivity is limited, and we have therefore not implemented screening by IHC. The authors have not tested sensitivity in their series, so this statement cannot be made.

Reply 1: Thank you for your comment, we have modified this opening sentence
Changes in the text: In this study we found *NRTK* fusions are extremely rare in NSCLC, which is in concordance with other studies (8-12). (See page 9, line 185-186)

Comment 2. In line 220-222 the author state that the study reflects real-world challenges for NTRK testing in NSCLC. However, this is a retrospective study and thus we do not know in how many case there might have been enough material for RNA testing if this had been incorporated into the routine workflow, which is now the standard in many labs.

Reply 2: Thank you for your comment. We acknowledge that RNA-based sequencing is the gold standard to detect *NTRK* gene fusions and performed in many laboratories as part of routine workflow. However, screening with IHC particularly in cancers with low prevalence of NTRK gene fusions, is an acceptable tool and has been recommended by international guidelines (11-13) In Australia, testing for *NTRK* fusions in adult patients with solid tumors is not routine practice, except in locally advanced or metastatic solid tumors with risk of being caused by *NTRK* gene fusion including mammary analogue secretory carcinoma of the salivary gland, secretory breast carcinoma and sarcomas without apparent line differentiation. Furthermore, molecular sequencing methods are not available in all pathology laboratories, particularly in smaller regional centres. We have included these points in the discussion.
Changes in text: Screening with IHC is in line with international recommendations (13, 17, 18), and can be particularly useful in cancers with low prevalence of NTRK gene fusions and in laboratories where molecular sequencing methods are not readily available or NTRK testing is not part of routine workflow. (See page 9, line 199-202)

Comment 3. In the methods, it should be stated what type of FFPE material was used, i.e. how many biopsies, resections, cytology blocks etc.

Reply 3: Thank you for your suggestion. We have included this information in the methodology.
Changes to text: A total of 289 samples were analyzed and included 81 cytology cell blocks, 30 resection specimens and 178 biopsies. (See page 7, line 143-144)

Reviewer B

Comment 1. The results were not confirmed by sequencing except for one of the 10 cases. Only additional one case was confirmed by FISH. Therefore, 8 cases (or 80% of cases) were not confirmed. It is widely known that IHC produces false-positive results and that the cases cannot be considered positive if not confirmed by molecular methods.

Reply 1: Thank you for your comment. Of the 10 cases that were positive on IHC, one sample underwent RNA-sequencing, while the other 9 samples were tested by FISH. All the cases tested by FISH were negative (Figure 2). We acknowledge the limitations of FISH including the inability to detect *NTRK* fusion partners and as some of the *NTRK*1 and *NTRK*3 fusion partners are intrachromosomal this can lead to false negative FISH break-apart results. We have included these limitations in our discussion.
Changes to text: In addition, a positive FISH result does not provide information on the functional significance nor fusion partner and a false negative FISH result may occur as some of the NTRK1 and NTRK3 fusion partners are intrachromosomal. (See page 10, line 211-214)

Comment 2. One of the 10 positive cases was large-cell neuroendocrine cancer. Tumors with neuroendocrine differentiation are often positive for NTRK by IHC, but often there is no underlying translocation. The expression is based on neural differentiation. This case should not be included especially if this is one of the 8 cases that was not confirmed to have a translocation.

Reply 2: Thank you for your comment. We agree that false-positive results are seen especially in cases with mesenchymal tumors that show neural and smooth muscle differentiation. This is due to the physiological cytoplasmic expression of pan-TRK in neural and smooth muscle tissue and therefore, the general recommendation is not to screen tumors with neural and smooth muscle differentiation using pan-TRK IHC. We are not aware of such recommendations for large cell neuroendocrine tumors of the lung. In study by Strohmeier et al (6) 31 cases of large cell neuroendocrine tumors of which two cases (2/31, 6.5%) demonstrated a positive reaction.

Changes to text: No changes

Comment 3. This study contributes no new evidence.

Reply 3: Thank you for your comment, however we respectfully disagree. *NTRK* fusions are an extremely rare occurrence in NSCLC. This study adds to the limited real world data about the frequency of this fusion in NSCLC as well as some of the challenges and limitations in testing methodologies.
Changes to text: No changes

Comment 4. It is problematic that IHC was used for screening. Although this approach is reasonable for clinical practice (but only if IHC is appropriately validated), it is not acceptable for the study because it is known that IHC could easily produce false-negative (as well as false-positive) results. However, if we accept to use IHC for screening, the IHC assay needs to be highly diagnostically sensitive and specific. Unfortunately, there is no evidence that the authors used such IHC assay. See point 5.

Reply 4: Thank you for your very helpful comment. We made an error in reporting the platform used for IHC. IHC was performed on the VENTANA BenchMark ULTRA platform using the VENTANA® pan-TRK (EPR17341) assay as per manufacturer's instructions. As has been reported the EPR17341 is a widely investigated pan-TRK clone and has demonstrated to be an efficient and reliable screening method for NTRK fusions. This has been corrected in the methodology.
Changes to text: Immunohistochemistry was performed on the Benchmark Ultra platform (Ventana Medical Systems, Tucson, AZ) using the VENTANA® pan-TRK (EPR17341) assay as per manufacturer's instructions. (See page 6, line 118-119)

Comment 5. Major issue with methods for IHC staining – it is not possible to use VENTANA pan-TRK assay on Bond instrument from Leica. This could only be done if the pre-diluted primary Ab from the VENTANA pan-TRK assay is taken out of the original container and used for laboratory developed assay on Bond instrument. If this was done, such new IHC protocol needs to be diagnostically validated for which the authors should have used a series of positive and negative cases with NTRK translocations. Clearly this was not done and the diagnostic sensitivity and specificity of this IHC protocol is completely unknown. If for no other reason, this is sufficient basis to reject this paper.

Reply 5: Thank you for your very helpful comment. We made an error in reporting the platform used for IHC. IHC was performed on the VENTANA BenchMark ULTRA platform using the VENTANA® pan-TRK (EPR17341) assay as per manufacturer's instructions. We have

corrected this in the methodology.

Changes to text: Immunohistochemistry was performed on the Benchmark Ultra platform (Ventana Medical Systems, Tucson, AZ) using the VENTANA® pan-TRK (EPR17341) assay as per manufacturer's instructions. (See page 6, line 118-119)

Comment 6. Criteria for the readout of stained slides – authors developed their own criteria for the readout in order to distinguish between positive and negative cases. Since there are no international guidelines for the pan-TRK readout yet, this could be done. However, this is acceptable with the new IHC protocol only if the protocol is properly diagnostically validated, which it is not.

Since we have established that the authors used IHC protocol that was not validated, all reported results are irrelevant.

Reply 6: Thank you for your feedback. As literature suggest the interpretation of pan-TRK IHC is challenging compared to many other IHC-based biomarkers. Currently there is no consensus or interpretation guide for the available Pan-TRK IHC clones and there is no clear cut level of IHC positivity as reliable threshold to proceed to genomic confirmation. A positive cut-off has been defined in other large pan-tumour series as staining above background in at least 1% of tumour cells (10,11). Pan-TRK IHC–positive cases usually show diffuse strong cytoplasmic staining in tumour cells, similar to the only ISH proven case in our study. There were 61 cases with weak (1+) cytoplasmic staining, many of which with similar discernible staining in background cells, therefore to provide a pragmatic approach we chose all cases with 2+ or 3+ staining in at least 1% of tumour cells as immunohistochemistry positive.

Changes to text – No changes.