Peer Review File

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Reviewer comments:

This study investigated the predictive role of plasma and tissue T790M assays for the efficacy of osimertinib treatment in a group of real-world NSCLC patients with *EGFR* mutation who failed the front-line treatment of first/second generation EGFR-TKI. The finding showcased that the tissue T790M positivity, compared to the plasma T790M positivity, is more relevant to the treatment outcome of osimertinib in terms of DCR and PFS. Higher allele frequency of plasma T790M, however, correlated with a better DCR of osimertinib treatment but not with the PFS.

The finding of this study was generally in line with what we have learnt from the earlier AURA3 trial in which patients with a tissue T790M positivity inclined to have a higher magnitude of osimertinib benefit than patients with plasma T790M positivity. Overall, I have a couple of major concerns about the *EGFR* and T790M assay in this study.

Comment 1: Given that T790M is a subclone of the cancer cells of activating EGFR mutation, the RAF of T790M is reasonably expected to be less than 1.0 which was the case for samples tested by ddPCR as shown in Figure 2. However, a large portion of samples tested by the BEAMing method gave rise to a RAF larger than 1.0, suggesting the specificity issue i.e. a certain amount of false positive call of T790M alleles that should otherwise be called as wild type allele.

Reply 1: As stated in the manuscript, plasma testing for all the patient samples (both BEAMing and ddPCR methods) were carried out by NATA accredited labs. We agree and acknowledge the concern of the reviewer as it is expected for the RAF of T790M to be less than 1.0 as it is a subclone of cancer cells with activating EGFR mutation. We accept that one possible explanation as provided by the reviewer is that the BEAMing assay for T790M may lack specificity (high false positive). Another explanation may be the BEAMing assay for EGFR sensitizing mutation may lack sensitivity (high false negative). Unfortunately, the pre-analytical aspects for the BEAMing assays and quality control of the method by the lab are outside the scope of investigation and discussion for this paper. We acknowledge the limitation of this retrospective study as we do not have the plasma samples for all the patients

for re-testing, or the information on sensitivity and specificity for the BEAMing assays. These are the limitation of this real-world study and we have expanded the discussion to address this limitation.

Changes in text:
Under discussion, page 17 line 21-24 & page 18 line 12, we have added 'We also noted that there was a significant proportion of patient samples with T790M RAF>1 using BEAMing assay as shown in figure 2. This could be related to the suboptimal sensitivity or specificity of EGFR T790M or sensitising mutation assays. However, this accurately reflects the data used for determining patient eligibility for treatment at this point in time and is included as provided by the NATA accredited laboratory'

Comment 2: Also, there was a difference toward the trend of significance between BEAMing and ddPCR. Please clarify.

Reply 2: We thank the reviewer for noting this and agree that although it was not statistically significant, there may be a trend that T790M RAF for individual patients tested using these methods is different in Figure 2. However, this is not unexpected as they are not paired results (different group of patients who had BEAMing vs ddPCR testing). To avoid confusion, we have added 'statistically significant' into the figure legend and removed the following sentence from the discussion 'Further, in our analysis, there was no significant difference in RAF as determined by the two PCR methods (Figure 2)'. Page 17, line 19-21.

Changes in text:
We added the word 'significant' for the figure legend for figure 2. '...There was no statistically significant difference in RAF determined by the two testing methods (p=0.16).' We removed the following 2 sentences: (1) Under result 'There was no difference in the RAF as determined by the two methods using unpaired t-test (mean RAF 2.81 vs 0.43, p=0.16) (figure 2)' page 11, line 8-9. (2) Under discussion 'Further, in our analysis, there was no significant difference in RAF as determined by the two PCR methods (Figure 2)'. Page 17, line 19-21.

Comment 3: Regarding the calculation of RAF, the author stated in Method section: "The T790M RAF was calculated as the ratio of T790M allelic frequency to EGFR driver allelic frequency. In patients where EGFR driver mutation was not detected, we assumed the RAF ratio to be 1.0."
Shouldn't the plasma sample with undetectable EGFR mutation be seen as invalid samples rather than arbitrarily given them an assumed RAF? Furthermore what's the rationale for the number 1.0? Please clarify.

Reply 3: Ten samples had undetectable EGFR sensitizing mutations (9/10 were tested with BEAMing assay). As all patients have previously been determined to carry an EGFR senstising mutation via tissue analysis, we believe that one possible reason that patients had undetectable EGFR driver mutations was due to lower sensitivity of this assay for the EGFR driver mutation assay. While there is the possibility is that the results were invalid with false positive detection of EGFR-T790M, for the purpose of the study we are relying on the interpretation of the diagnostic lab and have made the assumption that the reason for undetectable EGFR sensitizing mutation is due to low sensitivity of the EGFR activating mutation assay which may also contribute/explain the observation that there is a significant proportion of patient with T790M RAF>1 for BEAMing assay (as noted by the reviewer, refer comment 1). Thus we are assuming the T790M result to be true even in absence of detectability of the activating mutation. We assigned RAF ratio of 1.0 for the purpose of this study as we believe that the ratio will be high as VAF for EGFR driver mutation is likely lower (ie undetectable) than T790M VAF, giving a value of >1. Assuming these samples are valid and therefore should be included in this study, they should be categorized as RAF>0.3 rather than <0.3. We have now changed the wording in the manuscript from to make this clearer.

Changes in text: Under Method section, page 9 line 18-21, we have added/changed the original sentence to 'In patients where EGFR driver mutation was not detected (n=10), we determined this was likely due to the low sensitivity of EGFR activating mutation assays to detect low AF in these samples, therefore the AF for EGFR activating mutations will be lower than AF for T790M mutation for these samples. We categorised the RAF ratio to be >0.3.'

Comment 4. Following the line, how many variants of EGFR exon 19 deletion were pick up in the study?

Reply 4: We have contacted individual labs involved in analysis of the plasma samples regarding the variants of EGFR exon 19 deletions being picked up by the individual assays

Lab #1: BEAMing method
The assay detects the EGFR c.2369C>Tp.T790M and EGFR Exon 19del (c2240_2251del12, pL747_T751>S, c.2239_2247del9, pL747_E749del, c2238_2255del18, pE746_S752>D, c2235_2249del15, pE748_A750del, c2238_2250del15, pE748_A750del, c2239_2253del15, pL747_T751del, c2239_2256del18, pL747_S752del, c2237_2254del18, pE746_S752>A, c2240_2254del15, pL747_T751del, c2240_2257del18, pL747_P753>S, c2238_2252>CCA, pL747_T751>Q, c2238_2248>GC, pL747_A750>P, c2237_2251del15, pE746_T751>A, c2238_2253del18, pE746_T751del, c2235_2252>AAT, pE746_T751>I) mutations.

Lab #2: ddPCR method
c.2235_2249del p.Glu746_Ala750del c.2236_2250del p.Glu746_Ala750del c.2582T>A p.Leu861Gln, c.2240_2257del p.Leu747_Pro753delinsSer c.2156G>Cp.Gly719Ala

Lab # 3: ddPCR method
The exon 19 deletion assay was designed to detect the loss of WT sequence.

Changes in text: We have added under method section, page 8 line 11-14 ‘only one laboratory used the EGFR exon 19 deletion assay that is designed to detect the loss of wildtype sequence, which will be more capable of picking up the sequence deletion irrespective of the deletion variants (refer Seki et al 2016).’

Comment 5: As EGFR exon 19 deletion involves multiple, were those plasma samples with undetectable EGFR mutation stated by the author mostly belonged to EGFR exon 19 deletion? Also, there was a lack of details about the primer pair used for picking up the EGFR activating mutation and T790M mutation.

Reply 5: 10 out of 99 samples had undetectable EGFR driver mutation. Seven of them were x19del, two were L858R and one was S768I. We believe that the overall sensitivity for plasma EGFR mutation detection for this study is within the expected range (n=99,

sensitivity=90%). We have previously described the sensitivity of plasma EGFR driver mutation detection using ddPCR assays for our prospective cohort of patient was 80% (range 69-100%) which was consistent with multiple other studies (refer Ding et al, Lung Cancer 2019).

We do not have the details about the primer pair as mutation testing were not done in-house and were sent to external NATA-accredited (Australian diagnostic testing accreditation) labs inter-state. We could not obtain the raw data for the analysis from those labs due to the nature of a retrospective study.

We have added under discussion to discuss the fact that one of the possible reasons for undetectable EGFR activating mutations is because some of the labs may have missed the EGFR exon 19 deletion variants due to the method for exon 19 deletion.

Changes in text: Under discussion section, page 18 line 2-6 we have added "…In addition, not all labs used the most optimal method for plasma EGFR exon 19 deletion detection that was designed to detect the loss of wild-type sequence, which may lead to false negativity. Unfortunately, the pre-analytical aspects of each testing platforms is beyond the scope of this retrospective analysis and we acknowledge that this is a limitation of this study.'

Comment 6: Following the question, was the 19del assay used in this study belonged to the one that designed to detect the loss of WT sequence? As this form of assay are more capable of picking up the sequence deletion irrespective of the deletion variants. (For example in Seki, Y., et al. Oncologist, 2016. 21(2): p. 156-64.)

Reply 6: See reply 4. Only one out of the three labs used the assay that designed to detect the loss of WT sequence. For this particular centre (centre # 3) the EGFR activating assays performed better as there was no undetectable EGFR driver mutation, however the number of samples tested by this lab is small. Regardless, we agree with the reviewer that this form of assay is more capable of picking up the x19del irrespective of the deletion variants and have added this in the revised manuscript ie. method section (see reply 4) and discussion section (see reply 5).

Changes in text: Nil

Comment 7: Lack of the text about the detail statistics of the univariate and multivariate analysis in Result section. Some clinically relevant factors such as smoking history, age, brain metastasis etc. were not included.

Reply 7: The variables included in the multivariate model were those that were found to be associated with survival on univariate analyses or previously reported to be associated with survival in clinical trials or literature. Smoking history, age, brain metastases were not chosen in the multivariate analysis as they were not associated with survival on univariate analysis in this cohort. We accept that on clinical grounds, those factors may be important factors which could affect survival. Unfortunately, due to the relatively small number of patients included in this retrospective study, it will not be appropriate for statistical modeling for too many variables to be included in the multivariate model and therefore decision was made after consulting with the statistician to include only 4 variables reported in table 2.

Changes in text: We have added the following under 'Result' section on the statistics for the univariate and multivariate analyses. Page 12, line 16-22 'The variables included in the multivariate model were those that were found to be associated with survival on univariate analyses or previously reported to be associated with survival in clinical trials. Gender, burden of disease, ECOG performance status and method of T790M detection (plasma vs tissue) were chosen as the variables for the multivariate model. Other clinical factors which may influence survival such as smoking history, age and brain metastases were not included as they were not associated with survival on univariate analysis.'

Comment 8: In 2.4 Statistical Analysis: "Note, because treatment eligibility was determined by mutation screening from tissue or plasma, direct comparison of matching samples is not possible." seems redundant.

Reply 8: We agree that this sentence may be confusing and redundant therefore we have removed it from the section.
Changes in text: Removal of the above sentence in 2.4.

Comment 9: In Result section: "…, as expected for this study (second test only performed if first test negative), these patients results were always discordant" is confusing. A change or

deletion is advised.

Reply 9: We thank the reviewer for this comment and have rephrased the sentence.

Changes in text: Results section, page 11, line 5-8: “For this real-world study, a tissue test was only performed if liquid biopsy was negative (or vice versa). Therefore, patients who were tested T790M positive on a second test always had a discordant result (Figure 1).’