TRANSLATIONAL LUNG CANCER RESEARCH

Peer Review File

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Reviewer A

Comment 1:

why would it be most interesting to detect activating mutations? We can just use ctDNA for it, with only very few patients being additionally detected by CTC (Sundaresan)

Reply 1: The detection of activating mutation in CTCs could allow identifying patients that could benefit a targeted drug.

Although ctDNA can be exploited to this purpose, as the sum of different neoplastic lesions present at any time in individual patient, we previously reported that sometime EGFR mutation could be detected in EpCAM-positive tumor cells, but not in ctDNA. Since few mutated CTCs did not affect the total amount of ctDNA to result in EGFR detection, we demonstrated that ctcDNA investigation was more useful at that time.

Changes in the text: see, please, Introduction, lines 111-118.

Comment 2:

prognostic value of CTC is clear, predictive value also seems quite clear, but possibly insufficient (Tamminga et al x2).

Reply 2: CTCs are a heterogeneous population, with different metastatic capabilities. We previously reported that quantifying "druggable" CTCs allowed predicting treatment effectiveness.

Changes in the text: see, please, Introduction, lines 118-119.

Comment 3:

why were these CK;s added? why not other mesenchymal markers? Why not lose EpCAM?

Reply 3: The Introduction has been modified to explain that we were focused on implementing CTC assays in clinical. To this purpose, we introduced mAbs to detect much more CKs in EA than SA, since some histotypes of NSCLC lack CK8, 18, 19.

We preferred CS platform, an EpCAM-based technology, since we previously reported that EpCAM-high CTCs predict poor prognosis, whereas the EpCAM-low do not.

Changes in the text: see, please, Introduction, lines 121, 125-131; Discussion, lines 363-366.

Comment 4:

- it is very unclear how the cut off value was determined. Also unclear why it is necessary. why not leave it as a standard value?

Reply 4: As first reported by Cristofanilli et al. in NEJM 2004, the Kaplan-Meir curve results were used to select a cut-off level of CTCs that is capable for stratifying patients into two groups, with a favorable and unfavorable prognosis, respectively.

It was already reported that the cut-off value of poor prognosis differs between malignancies and disease stage, e.g. it is >_5 cells in metastatic breast and prostate cancer, >_3 cells in metastatic colon-rectal cancer; whilst >_1 CTC in early breast cancer predicts higher probability of disease recurrence.

The definition of the proper cut-off level represents a mandatory step of clinical validation of a new assay for quantifying CTCs (see Parkinson et al., 2012).

Changes in the text: No changes have been done in the main text about the testing of different cut-off levels. Requirements for the clinical validation of a new assay have been briefly mentioned in the Discussion (lines 373-374, 377-380).

Comment 5:

- a cox regression never gives a Relative risk.

Reply 5: In the text the RR acronym stands for Hazard Rate Ratio, consistent and accurate terminology with Cox regression.

Changes in the text: In the statistical analysis section Hazard Rate Ratio (RR) was spelled out and its acronym reported in parenthesis.

Comment 6

why were these covariables chosen in the cox regression model?

Reply 6: The co-variables valuated in the Cox Regression model have been previously associated with worst prognosis in NSCLC.

Changes in the text: No changes have been done

Comment 7

The population has a relative high percentage of patients with at least 1 CTC detected. Moist populations only have 20-40% with CTC. Can you explain the higher number of patietns with CTC in the study?

Reply 7: A more severe disease condition may explain this higher prevalence of CTC-positive patients in GR cohort by SA.

Changes in the text: See, please, Discussion (lines 355-356, 360)

Comment 8

a lot of discussion has been placed in the result section, making it hard to read and confusing.

Reply 8: According to reviewer suggestion, the rationale of activities and analyses described in the Results section has been moved to the Discussion.

Changes in the text: See, please, Discussion (lines 363-366, 386-391)

Comment 9

How sure are we that these additionall cells detected by the extended assay really are CTC?

Reply 9: All criteria except the adjunctive anti-CKs mAbs, which we used for defining an event as CTC, were consistent with what was defined by CS manufacturer's instructions and international guidelines, as described in Math&Met Section.

The demonstration that EA recognizes "true" CTCs, i.e. tumor cells with metastatic capacity, was obtained by establishing an adequate cut-off level, capable of stratifying patients in two groups, respectively with favorable and unfavorable prognosis. Using EA, a poorer prognosis was associated with CTC level of 4 or more cells.

This is mandatory to demonstrate the clinical validity of a new assay for CTC enumeration, as EA (Parkinson et al., 2012).

Changes in the text: Requirements for the clinical validation of a new assay have been briefly mentioned in the Discussion (lines 377-380).

Comment 10:

typo line 217

Reply: The typo has been emended.

Changes in the text: See, please, line 26.

Comment 11:

how much did the SA en EA overlap? What was the concordance

For the discordant patients, how did survival look like?

Reply 11: We had only 31 discordant patients, a too small sample size for doing speculations.

Moreover, see please, Reply to Comment 9 of reviewer B.

Changes in the text: No changes have been done.

Comment 12

Why are there more patients in the overall survival cohort than the progression free survival cohort?

Reply: To calculate PFS we needed the date of confirmed (radiographic) clinical progression. This date lack in 30 out of 192 patients (15.6 %), which hence have been excluded from Kaplan-Meier analysis of PFS. However, since 5 out of these 30 patients were still alive the last follow-up, only 25 out of 192 patients (13%) have been excluded from OS analysis.
Changes in the text: No changes have been done.

Comment 13
I have some serious concerns regarding the statistical analysis based on the tables. Additionally the tables are very convuluted, difficult to read and understand.
Reply 13: Explicative Legends have been added to the tables.
Changes in the text: See, please, table legends at the bottom of the tables, files Table 1 and Table 2.

Comment 14:
how can the basic clinical information be missing in a prospective study? gender, age and performance score are basic information? EVen if smoking is missing, there should be an easy workaround?
Reply 14: To determine the prevalence of CTC-positive patients in NSCLC, the full GR cohort has been used (n=192).
The demographic characteristics available for GR cohort have been reported in Table 1. As you can see in Supplementary Table 1, the frequency of CTC-positive and CTC-negative patients according to different clinical pathological parameters, have been analyzed in the group of patients, in which data was available; sample size is indicated for each clinical pathological characteristic. Supplementary Table 2 has been created to present data of CTC status according to smoking habits.
Changes in the text: Supplementary Tables have been updated; a legend has been included to facilitate comprehension. Results of Supplemetary Table 2 have been included in the Results section (lines 263-274), and in the Discussion (370-372).

Comment 15:
In the discussion the lower number of patients is seen as the cause that results were not significant? how can so many patients have missing data? How can the number of included patients in the PFS and OS for the same cohort be so different? These are questions that really need to be answered. In the flow chart it is written that patients are referred to a different hospital??? lost to follow up is common, and no reason to exlude a patient from analysis! And how can data be missing from 25/167 patients eligible for OS analysis? Why are patients eligible for OS analysis but not PFS?

Reply 15: For consideration about patients eligible for PFS and OS analyses, see, please, Reply to comment 12.
All the patients of GR cohort have been considered in the prevalence analysis, as reported in the Results section. Conversely, Kaplan-Meir results have been obtained for patients, for whom PFS and OS data were available.
More generally, there are some considerations to be done, about type and logistic of the study.
Since we were validating a couple new biomarkers, all data except CTC analyses, in our study resembles one based on real-world data.
The clinical information was based on clinicians' notes on the study reporting form. If the information was incomplete (e.g. lack of imaging results), incorrect (e.g. typos in date), or inconsistent, changes could be done, based on verification of the papery medical records, if available. Alternatively, the information could be retrieved by phone call with patient, if patients or familial are willing and capable to do it. There were no alternatives on this, since in Italy we still lack an electronic medical record of individual patients.
The scenario is further complicated by "migration" of patients from one clinical site to another one. In fact, some patients underwent at periodical visit of revaluation at the regional Oncological Hospital, whereas the treatments were performed at the clinical sites closest to their home; alternatively, some patients preferred to change Oncological Clinic. In any case, patients keep imaging discs and reports at home, to show the Oncologist during the next visit.
If the patients migrated toward Hospitals not involved in the study, we had loss of follow-up data, in all cases where we have not been able to contact patients, or they were unable or refused to help us to edit incomplete data recording.
Changes in the text: The sample sizes have been included in the Figure legends (lines 472-479, 506-508) to facilitate comprehension. The Consort diagram has been updated.

Comment 16
I am also missing some more recent articles in the citations. There have been several more groups that have shown that mutations can be identified in CTC.
Reply 16: As suggested, some references have been included.
Changes in the text: see, please, Introduction, lines 104-108, 119).

Reviewer B

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This study evaluated the prognostic utility of CTCs to correlate to PFS and OS using the Cell Search (SA) with the addition of a larger panel of CK, defined as an expanded assay (EA). Also, they quantified the expression of EML4-ALK fusion protein in CTCs. This work presents an interesting concept highlighting limitations in the current FDA approved method of enumerating CTCs in lung cancer.

Comment 1

Several limitations should be addressed before considering acceptance. The use of English must greatly improve if the paper is to be published. Several grammatical and phrasing errors can be found throughout the text (lines 42, 45-46, 61-62, 72, 78-79, 81, 82, 85, 90, 91, 97, 99, 116-117, 156, 194, 207, 212, 224, 243, 294, 308, 309, 338, 342-343, 351, 362). The authors would benefit advice from a native English speaking with an academic background for doing so. In addition, the text appears to be disjointed and the text's flow and syntax should be revised.

Reply 1: The test underwent a second English revision by a professional native English.

Changes in the text: Typos have been edited throughout the text.

Suggested improvements for possible revision:

Comment 2

- The authors should consider using a schematic to accurately define the two methods (this was quite confusing throughout the publication). The two methods have varied titles and description throughout the text making it hard to follow. This also applies for the patient cohort.

Reply 2: As requested by the reviewer, a schematic overview of the assays has been compiled to be posted in supplementary materials. Furthermore, we checked the manuscript to uniform the definition of the assays used in the study. To facilitate the reader, an Alphabetical List of Abbreviations has been added.

Changes in the text: See, please, List of abbreviations at lines 32-46, and the file TLCR_Supplemtary_Mat.

Comment 3

- The quality of the figures needs to be improved. Additionally, the legend needs to be more informative. The same applies to the tables.

Reply 3: According to TLCR authors' instruction, Figures have been prepared at 600 dpi, the maximum quality required. We are afraid that images quality might be reduced during processing of the PDF by the journal platform.

The Figure legends have been updated, and table legends have been now included.

Changes in the text: See, please, updated Figure legends (at lines 472-479, 482-490, 493-501, 506-511), and Tables files.

Comments 4

- It would benefit presenting representative images of CTCs identified using the two methods.

Reply 4: As described in Mat&Meth section, and further detailed in the schematic overview of the CS assays described in the manuscript, SA and EA differ only for the mAbs cocktail. Conversely, since to be scored as CTCs the images must satisfy users' guidelines, for definition SA and EA images are similar. Anyway, representative images obtained with EA are showed in supplementary Figure S1-B and S2, which present the development phases of combining EML4-ALK mAb with the EA.

Changes in the text: No changes have been done.

Comments 5

- It is mentioned in the abstract that this paper presents two novel automatic tests, however, this may need to be rephrased. From reading through the manuscript, the one novelty is the expanded assay.

Reply 5: The schematic overview shows that in the manuscript we used 4 assay, namely the SA and the EA, to detect CTCs with different CK profile, and 2 integrated test for evaluating expression of M30 and EML4-ALK in CTCs. The SA is the commercial test, whilst the development of M30 has been pubblished elsewhere.

Hence, in the manuscript there are two new tests, i.e. EA and EML4-ALK. In fact, despite EML4-ALK mAb is used in combination with EA, the development of the EML4-ALK test is independent from EA, and required specific procedure, that is now described both in Material and Methods section, and in Result section.

Changes in the text: See, please, CS tests description, in Abstarct (lines 61-62), in Mat&Meth Section (lines 188, 195, 199-200) and in Result Section (lines 320-332). See also the schematic overview of the 4 assays, in file TLCR_Supplemtary_Mat.

Comment 6

- In the introduction section, lines 84-87 should be revised. The authors also should clarify reference 14 as it seems they are claiming as their own.

Reply 6: the sentences have been rephrased.

Changes in the text: see, please, lines 98 and 100.

Comment 7

- In methods section - clarification for which test was used to identify EML4-ALK fusion protein in CTCs (SA or EA) as this section was quite vague.

Reply 7: The EML4-ALK mAb has been used in combination with EA. The Mat&Meth Section has been edited. Furthermore a schematic overview of the assays used in the manuscript has been provided, to be posted as supplementary material.

Changes in the text: See, please, Mat&Meth section (lines 188, 195, 199-200) and TLCR_supplementary_mat file.

Comment 8

- In "CTC characterization: EML4-ALK", did CTC and tumour correlate? This needs clarification as this is an important component of this work.

Reply 8: We checked for EML4-ALK translocation all the available biopsy specimens of patients, which expressed EML4-ALK in CTCs (11 out 35 cases). The FISH analysis confirmed the presence of EML4-ALK translocation in all 11 tumour biopsies.

Changes in the text: The FISH analysis has been added in the Mat&Meth Section (lines 201-216). The FISH results in tumor biopsies were already reported in Results Section (lines 336-338) and commented in Discussion (lines 398-401).

Comment 9

- It would be interesting to provide an explanation for the impact of different CK expression profiles. For example, do different CK expression profiles correlate to different outcomes?

Reply 9: Since both SA and EA mAbs cocktails contained CK 8, 18, and 19 (see Supplementary Material), and the two mAbs cocktails have been used in separate tubes, we cannot exclude that the discordant couples (n=31) derived from rarity of CTCs rather than from a different CKs profile.

In other words, to address the reviewer' question we should design a different experimental plan, and employ two different fluorescences for different mAbs cocktails, to directly compare them in the same sample, and avoid false negative results derived from CTC rarity.

The impact of different CK profile on patients' outcome was not included among study objectives. We expanded the CK profile of the SA, because some lung cancer histotype do not express CK 8, 18, 19.

Changes in the text: No changes have been done.

Comment 10)

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- There are couple of papers relevant to this work, which have been missed. Could you please cite these (Kulasinghe et al, Cells, 9, 2020; Kulasinghe et al., Cancers, 2019; Zhou et al., 2019; Kulasinghe et al., cancer medicine, 2018)
Reply 10: As suggested, the references have been included.
Changes in the text: see, please, Introduction, lines 104-108.