Peer Review File

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Reviewer A

Comment: The authors assessed levels of three serum markers (CYFRA21-1, NSE, and CEA) in lung cancer patients with brain metastases. All tests have been examined for a long time but have never found clinical application in lung cancer due to their low utility. The present study included only a subset of patients with brain metastases but adds little to the current knowledge. Elevations of CYFRA21-1 and NSE were found to carry unfavorable prognoses, but their value in treatment monitoring was minor, if any. The study value is significantly biased by the joint analysis of different types of lung cancer, especially non-small-cell and small-cell lung cancer. Such an approach does not allow for meaningful conclusions, as studied markers are generally specific for particular subtypes. The study contains several methodological and interpretative shortcomings, but this is less important in view of the study's general low scientific and applied value.

Reply: We greatly appreciate your comments in guiding the revision of the article. Your insights have proven to be tremendously helpful in refining our work. We will address your concerns about the study`s scientific and applied value the following three points.

1) **The value of three (CYFRA21-1, NSE, and CEA) serum markers in lung cancer:**

Although the use of serum biomarkers in lung cancer is currently controversial, their potential value should not be dismissed arbitrarily. International guidelines, such as those from NACB, as well as specific guidelines for combined serum biomarker testing in China, provide guidance on the clinical application of serum biomarkers in lung cancer practice, including three aspects: screening and early diagnosis, disease monitoring and prognosis assessment, and personalized therapy. M. Grunnet summarized over twenty studies on the application of CEA in lung cancer, and reported that CEA has considerable potential for monitoring treatment of NSCLC in advanced disease as well as for the detection of recurrent disease after primary therapy, particularly in NSCLC (Reference: “National Academy of Clinical Biochemistry Guidelines for the Use of Tumor Markers in Lung Cancer” and “DOI: 10.1038/s41392-024-01823-2”). Moreover, serum biomarkers (CYFRA21-1, NSE, and CEA) testing is simple, non-invasive, and cost-effective, making their value crucial in the practice of lung cancer management.

In the past, the prognosis for patients with lung cancer brain metastases (BM) was indeed very poor, with a natural average survival time of only 1-2 months. Consequently,

the significance of studying disease course monitoring markers in these patients was limited. However, with the advent of radiotherapy, immunotherapy and targeted therapy, there has been a shift, and long-term survival for patients with BM is now achievable. In light of this progress, follow-up monitoring has become crucial.

The original aim of our study was to investigate whether serum tumor markers (STMs) could serve as baseline predictive or prognostic factors throughout the course of lung cancer with BM, and to assess their relationship with prognosis based on dynamic changes or baseline levels. Regardless of whether the findings turn out to be negative or positive, there is inherent value in presenting them. This research could potentially contribute valuable insights into optimizing treatment strategies and improving outcomes for these patients.

2) Number of lung cancer brain metastases participants:

The total number of studies we included was advantageous compared to previous studies of lung cancer tumor markers, which amounted to 1,169. (Reference: Table 3,"DOI: 10.1038/s41392-024-01823-2")

3) Markers target specific subtypes:

Our understanding of the reviewer` concerns. Joint analysis of non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) may indeed be somewhat biased. However, we chose to jointly analyze these lung cancer subtypes based on the following considerations: First, certain markers may share important biological features in the disease course of multiple lung cancer subtypes. A recent report published in 2024 Nature (DOI: 10.1038/s41586-024-07177-7) indicates that a highly proliferative and dominant subclone exists when SCLC is first diagnosed; after therapeutic intervention new dominant subclones emerges and tumor heterogeneity increases. Thus, there may be changes in multiple tumor markers during disease surveillance, not just CYFRA21-1 or NSE alone. Second, the aim of our study was to explore the potential application of these markers in lung cancer with BM as a whole rather than being limited to a single subtype. Moreover, the larger the sample size, the more reasonable the fit trajectory, and the better the model fit can reflect the true situation. Although this approach may limit precise conclusions about specific subtypes, it can help to reveal the potential diagnostic or therapeutic significance of these markers in a broader group of lung cancers. Of course, we further added separated analyses to articulated the role of baseline levels and dynamic changes in STMs in predicting prognosis of NSCLC or SCLC patients with BM, respectively.

We provided supplement Figure 1-5.

Changes in Figure 3 and the text: Lines 162-165,199-201.

Reviewer B

Overall Comment: In this retrospective longitudinal monocentric cohort study the Authors investigate the possible prognostic role of serum tumor markers (STMs) in lung cancer patients with brain metastases. Thus, the paper deals with a major clinical challenge in thoracic oncology, focusing on a population frequently characterized by poor survival and low quality of life.

STM levels and other clinical data from 1169 lung cancer patients with brain metastasis collected in a single Hospital from 2013 to 2020 were analyzed.

The aim of this study was to understand the possible prognostic role of STMs (CEA, Cyfra, NSE), for patients with brain metastases from lung cancer. Furthermore, the authors tried to demonstrate if the trajectory of these markers could have a prognostic role too.

After analysis of baseline STMs levels of the selected population, the authors proved that levels of Cyfra and NSE have an independent prognostic role.

Moreover, collection and analysis of longitudinal STMs levels lead to the identification of 3 trajectories classes for each marker, which however were not associated with OS at regression analysis.

In the end, ALK and EGFR status were identified as independent prognostic factors and a subgroup analysis for non-oncogene addicted patients showed CYFRA dynamic trajectory as an independent prognostic factor.

Several positive points could be underlined in this study. In particular, the number of enrolled patients is certainly a positive element. In addition, the use of dynamic alterations of marker levels in addition to the evaluation of their baseline level to identify patients with a worst prognosis is original, which is hindered by the lack of significant after correction for confounding factors.

Additionally, the discussion is fairly self-critical and adequately underlines some of the limitations of the study.

However, there are several major concerns that need to be addressed prior to recommending this article for publication.

Reply: Thank you for your pertinent comments and recognition! We are committed to addressing all your concerns comprehensively in the revised version of our manuscript, as follows:

1. Comment: The most critical point is the pooled analysis of all histology types. As addressed in the discussion, this is a main limitation of the study, as well as a major confounder that can be only partially corrected with statistical methods. Indeed, different lung cancer histology types (and among ADCs, oncogene-addicted vs wild-type), have dramatically different clinical characteristics and, most of all,

prognosis. For this reason, analyses should be conducted separately for each tumor type (small cell lung cancer, oncogene addicted ADC, non-oncogene addicted ADC, and SCC is desireable).

Reply: Thank you for your nice suggestions and reminders. Joint analysis of non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC) may indeed be somewhat biased. However, we chose to jointly analyze these lung cancer subtypes based on the following considerations: First, certain markers may share important biological features in the disease course of multiple lung cancer subtypes. A recent report published in 2024 Nature (DOI: 10.1038/s41586-024-07177-7) indicates that a highly proliferative and dominant subclone exists when SCLC is first diagnosed; after therapeutic intervention new dominant subclones emerges and tumor heterogeneity increases. Thus, there may be changes in multiple tumor markers during disease surveillance, not just CYFRA21-1 or NSE alone. Second, the aim of our study was to explore the potential application of these markers in lung cancer with BM as a whole rather than being limited to a single subtype. Although this approach may limit precise conclusions about specific subtypes, it can help to reveal the potential diagnostic or therapeutic significance of these markers in a broader group of lung cancers. Of course, we still further conducted separate analyses for NSCLC, SCLC and other types to articulated the role of baseline levels and dynamic changes in STMs in predicting prognosis of different types patients with BM, respectively. We provided Figure 3 and supplement Figure 2-4, Changes in the text: Lines 162-165,199-201.

2. Comment: Since the focus of the paper is on STMs more specifications should be provided about them in the methods of the study. In particular, the paper should list the determination method of serum markers, the standard cut-off values and if there was a minimal number of determinations for each patient to be entered in the dynamic trajectory.

   Reply: Thank you for your very helpful suggestions to improve our paper. We acknowledge the importance of providing detailed specifications regarding STMs in the methods section of the study. We added the section titled "2.3 STMs measurement", and included our hospital's standard cut-off values "Normal reference values of CEA, CYFRA21-1 and NSE are < 3.4 ng/mL, < 3.0 ng/mL and < 15.0 ng/mL, respectively". Moreover, each patient underwent at least three follow-up measurements before being included in the analysis.

   Changes in the text: Lines 99-104.

3. Comment: The authors properly point out that from 2013 to 2020 immunotherapy and target therapy drastically changed the therapeutic landscape for these

malignancies. However, in the methods of the study there is no mention of provided treatments. Therefore, I recommend to mention in the 2.2 “patients”" paragraph if the patients underwent therapy and which options were provided (chemotherapy, chemo-immunotherapy or target therapy) AFTER BM identification (were all baseline BM or also during disease history?). These data should then be described in the 3.1 “Participants” characteristics, and if possible, entered the Cox regression analysis.

Moreover, since the study focuses only on patients with brain metastasis and considering that radiotherapy (RT) is a fundamental part of treatment of these patients, I suggest taking this treatment into account as well. Methods of the study should mention if patients underwent RT or not and this factor should be considered in the Cox regression models if a consistent part of the study population did not undergo this treatment.

Reply: Thank you for your thoughtful suggestions that you made on our original submission. We corrected the method in 2.2 “patients”" paragraph and mentioned “The treatment regimen (radiotherapy, chemotherapy, immunotherapy, and targeted therapy) for all of our included patients was used after diagnosis of BM.”

Changes in the text: Lines 96-98.

In addition, we have incorporated the treatment distribution into Table 1 as described in Section 3.1 “Participant Characteristics”: “In terms of treatment, 485 cases (41.5%) underwent targeted therapy, 39 cases (3.3%) underwent immunotherapy, 627 cases (53.6%) underwent radiotherapy and 954 cases (81.6%) underwent chemotherapy.”

Changes in the text: Lines 130-132.

We also have included the radiotherapy, chemotherapy, immunological and targeted therapies received by the patients in the Cox regression models in Figures 1D, 1E and 1F.

Changes in the text: Figure 1.

4. Comment: There is a high prevalence of patients aged less than 50 years (~27%): could there be a selection bias? Please clarify or explain this potential abnormality

Reply: Thank you for your suggestions. Patient age was not purposely selected in our article, so there was no selection bias. The median age of our included patients was 58 years, which is roughly in line with the median age of brain metastases from lung cancer in other studies. (Reference: “DOI: 10.1016/j.esmoop.2023.102069; 10.1200/JCO.2011.40.1174; 10.1245/s10434-022-11365-y”.)

5. Comment: In Figure 1D, 1E, and 1F, STMs are analyzed as continuous variables

rather than categorically defined (high vs low). Please clarify, what is the methodological reason to adopt two different classification modalities and provide the Cox models with categorical STM.

Reply: Thank you for your suggestions. Initially, considering the practical clinical application requires specific numerical values of STM, we utilized continuous type of STMs for Cox model analysis. Indeed, by converting STM from a continuous variable to a categorical variable, we can better understand the impact of different STM groups (high and low levels at baseline) on survival time. We re-analysed STM as a categorical definition (high vs. low) and corrected Figures 1D, 1E and 1F. The conclusions remain consistent with our previous conclusions.
Changes in the text: Lines 156-165, Figure 1D-E and Supplement figure 1.

6. Comment: STM trajectory analysis enormously suffer of the joining of all histology type, e.g., NSE class 1 has the worst prognosis because it is likely enriched in SCLC, which is the histology type with the worst prognosis, compared to other classes, which are enriched with the non-SCLC histology types.
Reply: I apologize profusely for the study's limitation. The data set evaluated was a heterogeneous cohort of patients with BM. We collected the survival data of patients with BM from different treatment models and histology, and we did not study the prognosis of patients with different treatment models separately. However, based on the consideration: the larger the sample size, the more reasonable the fit trajectory, and the better the model fit can reflect the true situation. So, we included all patients (NSCLC OR SCLC) into the trajectory analysis. During this revision process, we conducted COX analysis separately on the dynamic trajectories of STM related to NSCLC and SCLC. Pay attention to Figure 3. The conclusions remain consistent with our previous conclusions: “After excluding confounding factors using multivariate Cox regression analysis, dynamic changes of STMs were not significantly correlated with survival”. All in all, we for the first time investigated the relationship between dynamic changes of STMs (CEA, CYFRA21-1 and NSE) in lung cancer patients with BM and prognosis. Regardless of whether the findings turn out to be negative or positive, there is inherent value in presenting them.
Changes in Figure 3 and the text: Lines 196-203.

7. Comment: Lines 99-101: described histology types do not sum up to 100%, which are the other types? Some seem to be included in Table 1, but there isn’t a “Total population” column, nor an explanation for the abbreviations used. Also, rows are not aligned, and the title seems wrong ([…] “with and without lung cancer brain metastases”.). Furthermore, contingency table analyses will be useful to check

whether there are significant imbalances betweek the STM-defined categories.

Reply: Thank you for your suggestions. We corrected errors as follows:

1) **Lines 99-101: described histology types do not sum up to 100%, which are the other types?**

   We have added the remaining histological types: "Association for the Study of Lung Cancer (WHO/IASLC) classification criteria for lung tumors, squamous cell carcinoma (SCC) accounted for 10.5%, adenocarcinoma (AD) for 65.3%, small cell lung cancer (SCLC) for 13.2%, and not otherwise specified (NOS) for the remaining 11%."

   Changes in the text: Lines 128-130.

2) **Correction of errors in Table 1**

   We have re-added the totals, re-aligned the table, added abbreviations, and changed the title name to "Baseline characteristics of lung cancer brain metastases participants."

   Changes in the text: Table 1 marked in red.

3) **Contingency table analyses will be useful to check whether there are significant imbalances betweek the STM-defined categories?**

   We added T-test for baseline characteristics of lung cancer brain metastases participants.

   Changes in the text: Table 1 marked in red.

8. Comment: Lines 105-109: I suggest to replace "increase" and "decrease" with other terms since the used ones seem to reflect a time-related difference of dynamic change instead of an intergroup difference.

   Reply: Thank you for pointing out the need for more accuracy in the words. We have corrected the content as follows: "In the high NSE levels group (≥15.5 ng/mL), there was a higher proportion of male individuals (63.4% vs 56.7%), a lower BMI, a higher prevalence of SCLC pathology type (20.7% vs 8.2%), a higher prevalence of smoking habits (27.1% vs 19.9%), and a lower Graded Prognostic Assessment (GPA) score (40.7% vs 30%)."

   Changes in the text: Lines 136-139.

9. Comment: Line 158: change "diver" with "driver"?

   Reply: We are sorry for our careless mistakes. Thank you for your reminder.

   Changes in the text: Lines 160-162.

10. Comment: Line 170: correct with serum CEA instead of serum STMs

    Reply: Thank you for your nice suggestions and reminders. In order to make it clearer, we'd like to make the following revision: Several studies have shown that

STMs are biomarkers and associated with brain metastasis development in lung cancer.

Changes in the text: Lines 213.

11. Comment: Lines 196-197: the sentence should be rephrased or removed. The interaction might solely be due to the fact that Cyfra21-1 high group is enriched with SCC which have worst prognosis of ADCs and are seldomly oncogene-addicted.

    Reply: Thanks for your valuable suggestions. The text has been revised by deleting the following sentence: This suggests an interaction between smoking and CYFRA21-1 and the status of driver genes.