TLCR TRANSLATIONAL LUNG CANCER RESEARCH
AN OPEN ACCESS JOURNAL FOCUSING ON CLOSING THE GAP BETWEEN "BENCH AND BEDSIDE"
IMPACT FACTOR 6.498

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Reviewer A

I commend authors for this work, which is very important for the future studies of the role of TAMs in lung cancer. The manuscript is well written but there are a lot of data here to get through and I find some of the results confusing. Also, I think that the results and discussion should be adjusted to the context of the rapidly evolving field of the operable lung cancer. Here are some of my comments and suggestions:

1) I think the introduction section is comprehensive but way too long. I think some of the component could be placed in the discussion.

Reply:

Thank you very much for your comment. We have revised as suggested.

Chang in the text:

Page 23-24, line 486-500: "Meanwhile, several limitations in previous reports should also be noted, such as small sample size and homogeneous cohorts (i.e., utilizing a specific TNM stage), which may lead to the contradictory findings. Moreover, the wide variation of methods used in evaluating the infiltrating patterns of TAMs in previous studies made it difficult to draw a definitive conclusion concerning their associations to prognosis. Despite that immunohistochemistry (IHC) is a helpful tool in diagnostic settings and has been widely used for decades, some drawbacks limit its ability to assess the complex components in TME. For instance, the subjectivity in the interpretation of IHC stain by pathologists may significantly affect the reproducibility of CD68 expression levels [1]. Additionally, although multiplex IHC (mIHC) can simultaneously identify colocalized markers, it is limited by quantitative assessment (i.e., appraise expression levels as positive vs. negative simply or a semiquantitative H-score) [2]. The multiplex immunofluorescence (mIF) approach is an emerging and powerful approach, based on the principle that various protein targets can be dyed apiece by a particular antibody labeled with a disparate fluorophore [3]. Given that the fluorophores have a sizeable dynamic scope and be captured in situ by the multispectral microscope, the mIF tool can characterize cells phenotypically and facilitate quantitative and spatial analysis."
"

2) The patient cohort should be described in greater detail. Patients had stage I-III lung cancer. Were these all upfront resections? Any chemotherapy in neo or adjuvant setting?

Reply:

Thank you very much for your valuable comment. All of the samples used for multiplex immunofluorescence were from radical resection cases (i.e., lobectomy/sub-lobectomy and lymph node dissection). The treatment information is missing because it was not recorded systematically from 2009 to 2011. We have added more details of the patient cohort in the main text, which can also be found in table 1.

Change in the text:

Page 9, line 157-164: "Six hundred and eighty-one patients of stage IA to IIIB NSCLC who underwent radical resection (i.e., lobectomy/sub-lobectomy and lymph node dissection) were enrolled over the years 2009-2011 at the First Affiliated Hospital of Guangzhou Medical University, China. Additional inclusive criteria were: (1) all resected tissues and lymph nodes were confirmed by pathology finally and (2) sufficient resected tissues for MIF test. Patients were excluded if any of the following criteria were met: (1) multiple LC; (2) small cell lung cancer (SCLC) or non-invasive LC like lung adenocarcinoma (LUAD) in situ and minimally invasive LUAD; (3) diagnostic biopsy in pre-operation; and (4) preoperative neoadjuvant therapy, as we previously described [4]."

3) I did not see the definition of the DFS, was this by imaging or biopsy prove?

Reply:

Thank you very much for your valuable comment. The definition of disease-free survival in the present study is the time from radical resection to local recurrence as measured by computed tomography (CT) scan. Patients received CT scans routinely every three months after the radical resection of lung cancer. Contrast-enhanced magnetic resonance imaging of the head and radionuclide bone scanning were also done annually for detecting the recurrence.

Chang in the text:

Page 9 line 167-171: “DFS is defined as the time from radical resection to local recurrence as measure by computed tomography (CT) scan. Patients received CT scan routinely every three months after the radical resection of lung cancer. Contrast-enhanced magnetic resonance imaging of head and radionuclide bone scanning were also done annually for detecting the recurrence [5].”

4) Authors should comment on the practicality of their findings? is it possible to use this nomogram analysis from biopsy samples such as FNA or core biopsy pre-therapy. is this possible only on fully resected tumors? And how would neoadjuvant therapy for example influence TAMs within the tumor environment?

Reply:

Thank you very much for your valuable comment. To the best of our knowledge, this is the first macrophage-based immune-related risk score (IRRS) and nomogram system for predicting disease-free survival (DFS) of resectable non-small cell lung cancer, with good accuracy performance. However, due to the fact that the multiplex immunofluorescence was applied only on formalin-fixed paraffin-embedded (FFPE) lung cancer tissues [3], our IRRS and nomogram system are only applicable using lung cancer surgery pathological specimens after radical resection. Hence, our findings' application is compatible with radical resection specimens, as pre-surgery biopsy specimens such as FNA or core biopsy were not used to construct the IRRS and nomogram system.

Patients were enrolled over the years 2009-2011 in the present study, during which the adjuvant therapy was the mainstream treatment rather than the neoadjuvant setting. As a result, it was difficult to interpret how neoadjuvant therapy influences TAMs within the tumor environment, which is a limitation in our current study. As for the effect of how neoadjuvant therapy influences TAMs within the tumor environment, Parra et al. [6] discovered that the density of CD68+ tumor-associated macrophages (TAMs) was higher in neoadjuvant

chemotherapy (NCT) non–small cell lung carcinoma (NSCLC) than in non-NCT-NSCLC cases. Besides, a recent study conducted by Gaudreau et al. [7] also revealed that patients with NSCLC who received NCT followed by surgery, as compared with patients who received upfront surgery (US), had overall higher levels of immune infiltration, including higher densities of CD68+TAMs in their tumors. However, both studies failed to interrogate the polarization of TAMs subtypes (including M1 and M2, which may exert differential effects in the tumor microenvironment in NSCLC patients who received NCT. As suggested by Blankenstein [8], in the tumor epithelial and stromal compartments, the activation of TAM class M1 and helper T cells (CD3+ CD4+) mediate tumor suppression factors in NCT-treated NSCLC patients.

Chang in the text:

Page 23 line 475-483: "As for the effect of how neoadjuvant therapy influences TAMs within the tumor environment, Parra et al. [6] discovered that the density of CD68+ TAMs was higher in neoadjuvant chemotherapy (NCT) NSCLC than in non-NCT-NSCLC cases. Besides, a recent study conducted by Gaudreau et al. [7] also revealed that patients with NSCLC who received NCT followed by surgery, as compared with patients who received upfront surgery (US), had overall higher levels of immune infiltration, including higher densities of CD68+TAMs in their tumors. As suggested by Blankenstein [8], in the tumor epithelial and stromal compartments, the activation of TAM class M1 and helper T cells (CD3+ CD4+) mediate tumor suppression factors in NCT-treated NSCLC patients."

5) I am a bit confused about the findings. On one hand authors mention that M1 are more favorable than M2 but it was the M1 macrophages that were found to be associated with worse outcome. also it was the macrophages with negative PD-L1 expression, which goes against some other findings in the literature which suggest that patients with TAMs with PD-L1 + expression have worse outcomes.

Reply:

Thank you very much for your valuable comment. It is worthy of acknowledging that the increased TAMs infiltration is positively correlated with human lung cancer stage and metastatic lymph node involvement [9, 10]. The reason why the results you have mentioned were inconsistent with previous studies may consist of two aspects. On the one hand, although there was no statistical significance, a positive association was found between M2 (CD68+CD163+) and shorter disease-free survival of patients in our study. Compared to M1 (CD68+CD163-), the odds ratio of M2 was higher in both tumor nest (1.05 vs. 1.04) and tumor stroma (1.05 vs. 1.02) in multivariable Cox regression. On the other hand, since the proportion of TAMs expressing PD-L1 is low among TAMs, the increment of TAMs with PD-L1 + expression may correlate with an increased number of overall TAMs as well as antitumoral immune cells infiltration, leading to a longer DFS in some patients who underwent surgery. Besides, the prognostic effect of PD-L1 remained inconsistent according to the study by Takada et al. [11], which summarized data of previous reports on the association between the PD-L1 expression and clinical outcomes in NSCLC patients. Hence, further studies of the predictive and prognostic roles and underlying mechanisms of PD-L1 expressed on macrophages are required.

I think this is an important work, but I would suggest to simplify this paper for the major findings and put them in the context of currently FDA approved adjuvant therapies based on the ADAURA and IMPower 010 trials. Also, in the discussion, authors should comment on the value of TAMs in EGFR population etc. They mentioned this in the limitations, however, placing their findings in the context of the latest discoveries is important.

Reply:

Thank you very much for your comment. We have revised as suggested.

Chang in the text:

Page 23, line 467-475: "In the field of epidermal growth factor receptor (EGFR)-mutant LC patients, the prognostic value of macrophages has been a research focus in recent years. Saxon et al. reported that epithelial NF-κB signaling sustained carcinogenesis in EGFR-mutant LC by recruiting pro-tumorigenic macrophages [12]. Wang et al. further found that EGFR oncogene–dependent progression was correlated with the expansion of alveolar macrophages and the presence of activated signature of macrophages indicated unfavorable OS in patients receiving resection for EGFR-mutant LUAD [13]. Yin and colleague showed that nanomedicine remodeling the tumor microenvironment (e.g., M2-macrophage repolarization) could reverse the resistance of EGFR-tyrosine kinase inhibitor and enhance treatment outcomes in mouse model [14]"

Page 25-26, line 530-532: In the context of the success of ADAURA [15] and IMPower 010 [16] that adjuvant therapies brought pronounced benefit for resected early-stage NSCLC cases, targeting TAMs is emerging as an attractive and effective strategy (ClinicalTrials.gov identifier: NCT05053750, NCT01765790, NCT00317603, et al.) for therapeutic intervention [17].

Reviewer B

This is a large cross sectional study of stage I-III NSCLC who underwent surgical resection.

Specific comments:

1. Please include ethics approval numbers (hospital/institution)

Reply:

Thank you very much for your valuable comment. The study protocol was approved by the Ethics committee of the First Affiliated Hospital of Guangzhou Medical University, and the approval number is KLS-17-03.

Change in the text:

Page 9, line 171-172: Ethics committee of the First Affiliated Hospital of Guangzhou Medical University approved this study (approval number: KLS-17-03).

2. Was the Helsinki convention used?

Reply:

Thank you very much for your valuable comment. As mentioned on Page 8, line 162, Our study was performed in strict adherence with the Declaration of Helsinki (as revised in 2013) [18]

3. One of my concerns is the lack of orthogonal validation for this study. Have the authors

considered IHC to confirm a number of the tumour/stroma markers?

Reply:

Thank you very much for your valuable comment. In the chromogenic IHC approach, multiplexing is relatively difficult. When multiplex IHC (mIHC) stain is performed on a single slide simultaneously, only a few existing chromogens are very effective in allowing markers' co-expression, and the dynamic range of marker intensity is also limited. Compared to immunohistochemistry (IHC) [3], multiplex Immunofluorescence (mIF) has the notable benefit of being able to characterize a large dynamic range of co-expression on a single slide simultaneously. Hence, mIHC was not considered as the means of orthogonal validation of our study.

In our study, a machine learning algorithm was developed by training 10 to 15 typical multispectral images in batch analysis, and tissue segmentation was implemented by the algorithm subsequently. Due to the limitations of current technology, we could not label tumor cells directly using MIF technique. Therefore, in order to fully solve the problem of tumor and stroma division during the use of MIF, antibodies to label tumor cells are needed with sufficient sensitivity and specificity. Instead, we introduced fresh whole-tissue slides cut from normal human tonsils enrolled in each staining batch as positive control and evaluated the experiment's reproducibility, reducing the bias of labeling and segmentation to a certain extent.

4. Please cite recent papers using spatial profiling by Rimm et al., 2020; Monkman et al., Cancers 2020; medrxiv 2021

Reply:

Thank you for your valuable comment. We have cited the papers within the revised manuscript.

5. The h-score using mIF can be challenging and dependent on antibody concentrations - how have the authors controlled for this?

Reply:

Thank you very much for your valuable comments. Indeed, we agreed that the H-score as assessed by mIF may not be precise enough because it is also correlated with antibody concentrations and the intensity of the fluorescent dye itself. To overcome this issue, as described by our previously published article [4], we have set both positive control (i.e., normal human tonsil tissue with both primary and secondary antibody incubation) and the negative control (i.e., normal human tonsil tissue with secondary antibody but without primary incubation) in each batch and then determined the appropriate positive threshold for each biomarker according to the signal intensity in the inForm software. The inForm software can subsequently automatically determine the expression levels of different biomarkers across the slides using the same positive threshold set by the pathologist. We defined X, 2X, and 3X as the threshold of the signal intensity of low fluorescence intensity (+), median fluorescence intensity (++), and high fluorescence intensity (+++), respectively. The H-score was analyzed with the formula of H-score = (high fluorescence intensity)% × 3+ (median fluorescence intensity)% × 2+ (low fluorescence intensity)% × 1.

6. Was cross validation performed

Reply:

Thank you very much for your valuable comment. In random forests, there is no need for cross-validation or a separate test set to get an unbiased estimate of the test set error. It is estimated internally. [19] During the run, each tree is constructed using a different bootstrap sample from the original data. About one-third of the cases are left out of the bootstrap sample and not used in the construction of each tree. In principle, since it randomizes variable selection during each split of the trees, it is not prone to overfitting. [19] Hence, cross validation was unnecessary to perform in our RF model.

References:

1. Prichard. Overview of automated immunohistochemistry. Arch Pathol Lab Med. 2014;138(12):1578-82.
2. Gibney, Weiner, Atkins. Predictive biomarkers for checkpoint inhibitor-based immunotherapy. Lancet Oncol. 2016;17(12):e542-e51.
3. Taube, Akturk, Angelo, et al. The Society for Immunotherapy of Cancer statement on best practices for multiplex immunohistochemistry (IHC) and immunofluorescence (IF) staining and validation. J Immunother Cancer. 2020;8(1).
4. Peng, Wu, Zhong, et al. Profiling Tumor Immune Microenvironment of Non-Small Cell Lung Cancer Using Multiplex Immunofluorescence. 2021;12(4221).
5. National Comprehensive Cancer Network: NCCN Clinical Practice Guidelines in Oncology: Non-Small Cell Lung Cancer, V.4, (2019) [Available from: https://www.nccn.org/professionals/physician_gls/pdf/nscl.pdf.
6. Parra, Villalobos, Behrens, et al. Effect of neoadjuvant chemotherapy on the immune microenvironment in non-small cell lung carcinomas as determined by multiplex immunofluorescence and image analysis approaches. J Immunother Cancer. 2018;6(1):48.
7. Gaudreau, Negrao, Mitchell, et al. Neoadjuvant Chemotherapy Increases Cytotoxic T Cell, Tissue Resident Memory T Cell, and B Cell Infiltration in Resectable NSCLC. Journal of thoracic oncology : official publication of the International Association for the Study of Lung Cancer. 2021;16(1):127-39.
8. Blankenstein. The role of tumor stroma in the interaction between tumor and immune system. Curr Opin Immunol. 2005;17(2):180-6.
9. Schmall, Al-Tamari, Herold, et al. Macrophage and cancer cell cross-talk via CCR2 and CX3CR1 is a fundamental mechanism driving lung cancer. Am J Respir Crit Care Med. 2015;191(4):437-47.
10. DeNardo, Ruffell. Macrophages as regulators of tumour immunity and immunotherapy. Nature reviews Immunology. 2019;19(6):369-82.
11. Takada, Toyokawa, Shoji, et al. The Significance of the PD-L1 Expression in Non-Small-Cell Lung Cancer: Trenchant Double Swords as Predictive and Prognostic Markers. Clin Lung Cancer. 2018;19(2):120-9.
12. Saxon, Sherrill, Polosukhin, et al. Epithelial NF-κB signaling promotes EGFR-driven lung

carcinogenesis via macrophage recruitment. Oncoimmunology. 2016;5(6):e1168549.
13. Wang, Lee, Yoon, et al. Progression of EGFR-Mutant Lung Adenocarcinoma is Driven By Alveolar Macrophages. Clin Cancer Res. 2017;23(3):778-88.
14. Yin, Yu, Kang, et al. Remodeling Tumor-Associated Macrophages and Neovascularization Overcomes EGFR -Associated Drug Resistance by PD-L1 Nanobody-Mediated Codelivery. Small. 2018;14(47):e1802372.
15. Wu, Tsuboi, He, et al. Osimertinib in Resected -Mutated Non-Small-Cell Lung Cancer. N Engl J Med. 2020;383(18):1711-23.
16. Felip, Altorki, Zhou, et al. Adjuvant atezolizumab after adjuvant chemotherapy in resected stage IB-IIIA non-small-cell lung cancer (IMpower010): a randomised, multicentre, open-label, phase 3 trial. Lancet. 2021;398(10308):1344-57.
17. Yang, Zhang. Tumor-associated macrophages: from basic research to clinical application. J Hematol Oncol. 2017;10(1):58.
18. World Medical. World Medical Association Declaration of Helsinki: Ethical Principles for Medical Research Involving Human Subjects. JAMA. 2013;310(20):2191-4.
19. Tibshirani. Bias, variance and prediction error for classification rules: Citeseer; 1996.