Peer Review File

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Reviewer A

I read this paper, and I am very interested in results of this retrospective, single-institutional study. Although I agree with what you concluded in this study, I have a few questions and requests due to improve more this manuscript.

1) In results, you wrote the median follow-up time and average disease-free survival (DFS), presenting days. I guess readers would be more familiar to "months" instead to "days". What do you think it?

→ We added months (see Page 7, Line 157).

2) I believe you had better to make figures about PD-L1 staining, and to site it in method. Since non-pathologists must read the manuscript in this journal, the figures could help them to understand how to score PD-L1 positivity in tumor cells and immune cells, visually. Especially, there may be controversy to judge it in immune cells. As you know, it is well known that there is inter-observational heterogeneity in general when pathological diagnosis is performed. That is why I request you to make figures of PD-L1 staining in SP142 and SP263, and to address the figures.

→ We added Figure 1 to Figure 3 (see Page 7, Line 133).

3) I think that not only I but also readers would like to know the concordance rate of PD-L1 expression between antigens. According to results in Blueprint study 2 (BP2)*, there was no evidence of the interchangeability between SP263 and SP 142 antigens. In their conclusions, BP2 consolidates the analytical evidence for interchangeability of the 22C3, 28-8, and SP263 antigens and lower sensitivity of the SP142 antigen for scoring PD-L1 expression on tumor cells. I think your results also showed the discordance between two antigens, which must correspond to that in BP2. Can you mention the concordance rate?

→ We added in material and methods section, "The concordance rate of PD-L1 expression between SP263 and SP142 antigens were evaluated using Cohen's Kappa Coefficient." (see Page 7, Lines 144-146)

→ We added in results, "3.6. Concordance rate of PD-L1 expression between SP263 and SP142; Using Cohen's Kappa Coefficient, the concordance rate between SP263 and SP142 TC was 0.234. Also taking immune cells into account, concordance rate between SP263 and combined results of SP142 TC or IC was 0.247." (see Page 11, Lines 274-276)

4) In addition, readers would like to know which antigens could be useful in a daily practice, I think. Can you analyze the relationship between clinicopathological factors and the difference of PD-L1 expression between two antigens? I wonder the discordant rate was high between two antigens, but both PD-L1 expression do

not predict any prognosis. Can you explain it in discussion?
→ We added in discussion, **"Meanwhile, different results between SP263 and SP142 were seen, which were compatible with the results of Blueprint Phase 2(BP2) study (50). In our study, concordance rate between SP263 and SP142 TC was 0.234, and concordance rate between SP263 and combined results of SP142 TC or IC was 0.247, both of them revealing relatively poor degree of agreement as analytic methods. Our results support the conclusion in BP2 study that SP142 seems to lack interchangeability with SP263, and it might be related to complex and nonintuitive immune cell scoring methods in SP142. In addition, comparing the relationship between clinicopathological factors and PD-L1 expression between SP263 and SP142, it is evident that SP263 is more sensitive and reveals more correlated factors than SP142. Hence, it is presumed that SP263 would be more eligible for daily practice than SP142, until more simplified and reproducible scoring methods are established for SP142. However, they both failed to reveal correlation with DFS. Although PD-L1 positivity seems to be related to adverse prognostic factors, it also enables more treatment options, namely anti-PD-L1 agents. Indeed, some of the patients with PD-L1 expression received anti-PD-L1 therapies, and they might have influenced on the results." (see Page 15, Lines 367-378).**

*) Tsao MS, Kerr KM, Kockx M, Beasley MB, Borczuk AC, Botling J, Bubendorf L, Chirieac L, Chen G, Chou TY, Chung JH, Dacic S, Lantuejoul S, Mino-Kenudson M, Moreira AL, Nicholson AG, Noguchi M, Pelosi G, Poleri C, Russell PA, Sauter J, Thunnissen E, Wistuba I, Yu H, Wynes MW, Pintilie M, Yatabe Y, Hirsch FR. PD-L1 Immunohistochemistry Comparability Study in Real-Life Clinical Samples: Results of Blueprint Phase 2 Project. J Thorac Oncol. 2018 Sep;13(9):1302-1311. doi: 10.1016/j.jtho.2018.05.013. Epub 2018 May 22. PMID: 29800747; PMCID: PMC8386299.

Finally, I recommend you to revise a bit. Thanks for submitting this manuscript for this journal.

Reviewer B

To justify the present research, the authors stated the controversies of PD-L1 expression regarding immunohistochemistry assays, prognosis and anti-PD-L1 response. Several researchers assumed that PD-L1 expression might serve as a biomarker of an anti-tumor host immune response rather than signaling tumor immune evasion, and that the total stability between the anti-tumor response by the host and immune suppression by the tumor might be related. To test this evidence, the authors hypothesize that these heterogeneous results suffer influence of different companion tests, clinicopathologic characteristics of the patients, and cutoff values used. The cohort included 344 NSCLC cases with PD-L1

assays retrospectively analyzed. PD-L1 expression was detected by immunohistochemistry using SP263 and SP142 in tumor cells and immune cells. PD-L1 expression was associated with several poor clinicopathological factors, including the solid component of adenocarcinoma, lymphatic invasion, and recurrence. Interestingly, low risk of metastasis was associated with high PD-L1 expression of SP142 in tumor-infiltrating immune cells.

1. The research is original as the focus is directed to the patients' clinicopathological factors that may interfere with the PD-L1 profile

2. Reading is clear and understandable. The English language good.

Major Comments

1. I suggest to the authors a more attractive title for the readers: "Revisiting the impact of clinicopathologic characteristics in PD-L1 profile in a large cohort of NSCLC".

→ We changed the title as **"Revisiting the impact of clinicopathologic characteristics in PD-L1 profile in a large cohort of NSCLC" (see Page 1, Line 1)**

2. In MM section, please, include more detail about the immunohistochemistry quantification in TC and IC. Also, an illustrative panel with the cut-offs would be useful for the readers.

→ We added "SP263 (Figure 1), SP 142 TC (Figure 2) and IC (Figure 3) expressions were categorized one of three categories (<1%, 1%-49%, 50%-100%)." (see Page 7, Lines 133-134)

→ We also added Figure 1 to Figure 3 (see Page 7, Line 133).

3. The authors stated "TC expression was calculated as the percentage of tumor cells with membranous staining regardless of intensity. IC expression was calculated as the percentage of immune cells within the tumor area with membranous staining regardless of intensity. The tumor area was set as the area of tumor cells and adjacent stroma". Please, define the area of tumor in a semiquantitative approach and how the percentages were estimated.

→ We revised it as "**TC expression was scored as the percentage of tumor cells with membranous staining regardless of intensity. IC expression was scored as the proportion of tumor area that is occupied by PD-L1 staining immune cells regardless of intensity. The immune cell areas were visually encircled as closely as possible, and combined to estimate the proportion of tumor area occupied by IC aggregates. The tumor area was defined as the area occupied by viable tumor cells, and their associated intra- and contiguous peritumoral stroma. The boundary of peritumor stroma was visually defined without specific distance criteria, since it was well distinguished from surrounding normal tissue." (see Pages 6-7, Lines 125-131)**

→ We added in **material and methods section**, **"Per Ventana's interpretation guide for NSCLC, PD-L1 expression was evaluated. (51 and 52)" (see Page 7, Line 123)**
→ We added reference, **"Interpretation Guide for NSCLC, VENTANA PD-L1 (SP142) Assay, English. Roche Diagnostics 2020:14-15" (see Pages 21-22, Lines 534-537)**

4. Comment about the difficulties to recognize mononucleated immune cells without immunophenotyping.
→ We added in discussion, **"Finally, mononucleated immune cells are composed of CD3+ T and CD20+ B cells, but they cannot be morphologically discriminated via H&E stain. As they are heterogeneous population of variable inflammatory cells, their discrimination could have facilitated further investigation. However, in the setting of observational retrospective study, immunotyping was not implemented." (see Page 16, Lines 390-394)**

Reviewer C
The study cohort consisted of a moderate size of non-small cell lung cancer patients, but it was combined early and advanced stage tumors with both resections and biopsies as specimens. Thus, the study suffers a heterogenous nature of the study cohort.
Nevertheless, the study confirmed previously reported features associated with PD-L1 expression; thus, it lacks novelty.
The only novel finding is the association of high PD-L1 expression in immune cells by SP142 with less frequent metastasis, but the results of IC suffer a statistical issue as the authors have discussed.

Other major issues
Conclusion: The authors note that these findings could help in further establishing criteria for identifying responders and non-responders to anti-PD-L1 therapy and guide treatment approaches. - It is contradictory to what the author indicated in Introduction: PD-L1 expression by immunohistochemistry is not an ideal predictive biomarker for PD-L1 inhibitors due to suboptimal sensitivity and specificity. Thus, many studies have focused on clinicopathological and/or prognostic significance of PD-L1 expression.

- ➔ We intended to point out that current settings and cutoffs of PD-L1 antibodies are not ideal to predict responders from non-responders, but more appropriate cutoff values or criteria might be established in further studies, by taking account of associations of PD-L1 expression with several clinicopathologic factors.

Smoking has been reported to be associated with PD-L1 expression by multiple studies, It should be described and included as a variable of univariable (and

multivariable) analysis.

➔ **We added in discussion, "Fourth, we tried to include smoking history in clinicopathologic factors as correlation of smoking history and PD-L1 expression was reported in some previous studies. However, collecting the clinical data, we found out that smoking history was inappropriately investigated on the medical records of the patients, revealing conflicting comments within each of them. Thus, we decided not to include smoking history for evaluation in this current study, but in the future studies." (see Page 16, Lines 385-389)**

Minor issues

Line 58: I am not certain whether ROS1 alterations were originally listed as an exclusion criterion for pembrolizumab monotherapy in the 2017 NCCN guidelines.

➔ For the 2017 updates (Versions 1 and 2), the NCCN panel recommends pembrolizumab (category 1) as first-line therapy for patients with PD-L1 expression levels of ≥50% and with negative or unknown tests results for EGFR mutations, ALK rearrangements, and **ROS1 rearrangements** based on a phase III randomized trial (Keynote-024) comparing pembrolizumab versus platinum-based chemotherapy. (Ref: ETTINGER, David S., et al. Non–small cell lung cancer, version 5.2017, NCCN clinical practice guidelines in oncology. Journal of the National Comprehensive Cancer Network, 2017, 15.4: 504-535. DOI: https://doi.org/10.6004/jnccn.2017.0050, Page 523)

➔ **We added this reference (see Page 4, Line 58 and Page 21, Lines 530-531)**

Line 82: Thus, many researchers have focused on clinicopathological factors associated with prognosis in NSCLC. – What is described in the prior paragraph does not seem to support this notion.

➔ We revised it as **"As cutoff values for PD-L1 expression have revealed poor prediction for responsiveness to anti-PD-L1 agents, we assumed that immunohistochemical expression of PD-L1 might not be a single independence factor, but confounded by certain conventional clinicopathologic factors. If so, overall prognosis would better be predicted by investigating associated clinicopathologic factors which are already known for its significance on prognosis, rather than investigating PD-L1 expression alone, regardless of responsiveness to anti-PD-L1 agents. Similarly, many researchers have focused on clinicopathological factors associated with prognosis in NSCLC." (see Page 5, Lines 81-86)**

Line 89: Most widely used clones are SP263 and 22C3.

➔ In our institution, SP263 and SP142 are most widely requested PD-L1 analysis items in lung cancer patients. 22C3 also could be widely used as

well, but up to now it has not been widely used.

Line 119: Per company's instructions, immunohistochemistry for SP263 is considered positive when membranous and/or cytoplasmic expression is seen in tumor cells.

➔ In our institution, PD-L1 immunohistochemistry was performed using the Ventana Benchmark Ultra automated staining system. Per VENTANA PD-L1 (SP263) Assay Interpretation Guide for Non-Small Cell Lung Cancer, tumor cell cytoplasmic staining is disregarded for determining PD-L1 expression. (Ref: https://www.rochebiomarkers.be/content/media/Files/PD-L1_SP263_interpretation_guide_NSCLC.pdf, Page 7)

Lines 122-123: What is the definition of tumor stroma? In another word, how many mm from the outer layer of tumor cells is the stroma considered "tumor stroma"?

➔ We added in **material and methods section**, **"The tumor area was defined as the area occupied by viable tumor cells, and their associated intra- and contiguous peritumoral stroma. The boundary of peritumor stroma was visually defined without specific distance criteria, since it was well distinguished from surrounding normal tissue." (see Pages 6-7, Lines 128-131)**

Lines 125-126: The IC expression is typically classified into <1%, >=1%-<10% and >=10%.

➔ We set cutoffs for IC expression as only 1%, for only two cases revealed IC expression >10%. We thought that 10% cutoff would not have statistical significance.

Lines 213-214: no IC-high SP142 IC high-expression case was due to the cut off of 50% set for the high expression?

➔ Previous studies including Shah et al. (Shah M, Hubbard RA, Mamtani R, et al. Very high PD-L1 expression as a prognostic indicator of overall survival among patients with advanced non-small cell lung cancer receiving anti-PD-(L)1 monotherapies in routine practice. Pharmacoepidemiol Drug Saf 2022;31(10):1121-6.) reported that very high PD-L1 expression as a prognostic indicator in lung cancer, using 22C3 clone.
Previous studies tend to set cutoff of 50% for TC and 10% for IC (Herbst RS, Giaccone G, de Marinis F, et al. Atezolizumab for first-line treatment of PD-L1–selected patients with NSCLC. New Engl J Med 2020;383(14):1328-1339.) Since very high PD-L1 expression in SP142 IC has not been studied, we wondered whether 50% cutoff is applicable in SP142 IC, which is same cutoff for TC. However, there was no IC high-expression case and we could not validate it.

Limes 146-149: The distribution of predominant pattern appears skewed toward acinar pattern. How many pathologists were involved in the initial diagnosis and pattern classification? What is their interobserver concordance for pattern classification?

- ➔ In our institution, more than two specialized pathologists have participated in the initial diagnosis of lung pathology. In retrospective review of the slides, three pathologists confirmed the diagnosis. Though the number of papillary subtype is relatively lower than the others, compared to one recent study in South Korea (Lee CH, Jeon YH. The solid predominant subtype as an independent risk factor for recurrence in patients with pathologic stage I lung adenocarcinoma. Kosin Med J 2023;38(2):117-125.), acinar pattern tends to be the most prevalent subtype of lung adenocarcinoma in South Korea.

Lines 159 and 261: Please check the accuracy of OR. It should be >1.0.

- ➔ Lines 159: As we set biopsy samples as a reference parameter, and calculated OR was 0.57, we described that PD-L1 expression rate was significantly higher in biopsy than in resection samples, which could be verified in Table 3.
- ➔ Lines 261: As we set younger age (age<70) as a reference parameter, and calculated OR was 0.48, we described that PD-L1 expression rate was significantly higher in younger age (age<70) than in older age (age>=70), which could be verified in Table 2.

Lines 178-181: The correlation of PD-L1 expression and papillary/lepidic/MIA/AIS was not statistically significant, but OR was 0.27-0.6. It may just be lack of statistical power due to the small number in each category.

- ➔ We agree with your opinion. It is one of the limitations in our study, and we added it in the discussion, **"Fifth, we could not observe statistically significant difference among subtypes of resected adenocarcinoma cases, probably due to small number in each category." (see Page 16, Lines 389-390)**

Line 195: OR should be <1.0.

- ➔ Lines 261: As we set younger age (age<70) as a reference parameter, and calculated OR was 0.47, we described that PD-L1 expression rate was significantly higher in younger age (age<70) than in older age (age>=70), which could be verified in Table 3.

Lines 266-268: The sentence needs to be revised. It does not read well.

- ➔ **We revised the sentence to "Currently, immunohistochemical staining for PD-L1 has become a standard test for evaluating PD-L1 expression, and it is the only validated assay for application of anti-PD-L1 drugs." (See Page 12, Lines 281-283)**

Line 298: in the SP263 and SP142 cells – what do they mean?

- ➔ **We revised "SP263 and SP142 cells" to "SP263 and SP142 positive cells" (See Page 13, Line 313).**
- ➔ **We also revised "combined SP142TC or IC cells" to "combined SP142 positive TC or IC cells" (See Page 13, Lines 304-305), and "SP263 TCs" to "SP263 positive TCs" (See Page 15, Line 356).**

Lines 303-305: In our study, not only the solid-predominant subtype of adenocarcinoma, but also cases with small amounts of solid components were associated with high PD-L1 expression, which is consistent with the previous studies. – The information should be included in the results.

- ➔ We found that solid component (See Table 4), and solid 'predominant' subtype (See Table 6) were associated with PD-L1 expression, and it was included in the results (See Page 8, Line 181; See Page 9, Line 190; See Page 10, Line 217).