Peer Review File

Article Information: http://dx.doi.org/10.21037/tlcr-20-1277

Reviewer A

Authors developed a PD-L1+ EV detection assay based on the Simoa technology and identified a significant correlation of PD-L1 expression between T-EVs and tissues. Their findings were well documented. Thus, I have only minor comments.

Comments:
1. line 63: programmed death-1 (PD-1) -> programmed cell death-1 (PD-1)

Reply 1:We thank the reviewer for this correction.

Changes in the text:

We changed "Several antibodies against programmed death-1 (PD-1) and programmed death ligand-1 (PD-L1) have been approved as treatments in monotherapy or in combination with chemotherapy" to "Several antibodies against programmed cell death-1 (PD-1) and programmed cell death ligand-1 (PD-L1) have been approved as treatments in monotherapy or in combination with chemotherapy" (see Page5 Line 4)

2. line 244: Simoa Epcam-PD-L1 assay on plasma samples from 35 patients with lung cancer was performed. Were all plasma taken before surgery? If so, were PD-L1 EV/exosome levels decreased after surgery?

Reply 2: Thanks for the question. Yes, these samples were all taken before surgery, thus the comparison between Simoa results and tumor proportion score (TPS) could make sense. However, because these samples are retrospective blood samples obtained from the hospital biobank, where no blood sample after surgery is available. If Simoa Epcam-PD-L1 assay could monitor tumor treatment is a very good point. We would like to investigate this point in future studies with a systematical design.

3. line 248: How did authors define deep stromal invasion?

Reply 3: We thank the reviewer for this comments. Actually it was a mistake and we are very sorry for that. The "deep stromal invasion" should be "vascular invasion", the same as described in the legend of Table 1 "vascular invasion"

Change in text: We changed "deep stromal invasion" --> "vascular invasion" on Page 15

Line 26 in the manuscript. We also made a clear definition as "vascular invasion" instead of "invasion" in the Table 1.

4. Did patients receive ICIs after relapse? If so, were PD-L1 EV/exosome levels correlated with response to ICI treatment?

Reply 4: We thank the reviewer for this question. In these 35 patients, only 1 of them received ICIs treatment immediately after surgery as an adjuvant therapy. And it is same as the question 2, the sample after ICIs is not collected routinely in the hospital biobank.

5. P21, line 2: glow -> flow?

Reply 5: We thank the reviewer for this correction.

Change in the text:

We changed "Exosomal PD-L1 levels in SK-MES1 and A549 cells with and without IFNγ treatment were evaluated using glow cytometry (C)" to "Exosomal PD-L1 levels in SK-MES1 and A549 cells with and without IFNγ treatment were evaluated using flow cytometry (C) in the legend of Figure 2 on Page 25 Line 21.

Reviewer B

The authors explored the utility of single molecule array, Simoa, to detect PD-L1 expression on tumor-derived extracellular vesicles (T-EVs) using cell lines and matched samples (plasma and tissue) from 35 patients with NSCLC.

Although this research has some fundamental limitations considering future applications in clinical practice, the topic itself is quite interesting and it may worth considering for publication in TLCR. However, in my opinion, the authors need to several corrections in descriptions of the research, answering the inquiries as below.

Major comments

1. The authors set the cutoff value of TPS at 1% or 10%. I understand the positivity of PD-L1 itself has certain meaning, considering the results from KEYNOTE-042 trial, however, TPS cutoff at 50% would have more clinical values based on preceding reports. The authors do not show the result when the cutoff was set at 50%. Even if it was negative data, the authors are encouraged to show it to help the readers appropriately interpret this research.

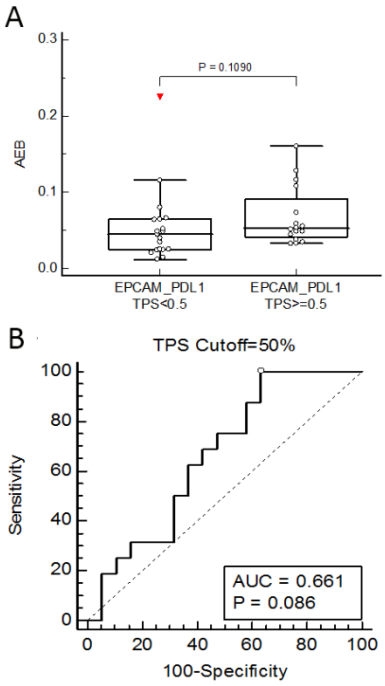
Reply 1: We thank the reviewer for this suggestion. In keynote-042, 1% is the cutoff for PD-L1 IHC positive patient and 50% is for PD-L1 high expression patients who would be response positive to Pembrolizumab. In this proof of concept study, the first objective is to evaluate if our method has a potential to distinguish PD-L1 expressing and non-expressing patients. Therefore, we used 1% and 10% (the optimal cutoff evaluated, by which the two methods have the closest PD-L1 expression results). It would be great if we can also compare ICIs treatment response by using 50% (the cutoff evaluated in keynote-042) or even to evaluate the optimal cutoff of Epcam-PD-L1 for ICIs positive response, because different PD-L1 antibodies used or different methods might lead to a variant on the cutoffs sensitive to ICIs [1]. Unfortunately, the information of ICIs treatment results is not available in this study, which is the major limitation as we discussed in the manuscript. That is the reason we did not include the result when the cutoff was set at 50% in the current manuscript. However, we agreed with the review, the result when the cutoff was set at 50% would give the readers more interpretation on the comparison of the two methods. We therefore provided this result as a supplementary data and a corresponding description was also added in the manuscript.

Changes in the text:

The following sentence was added into the result part (Page 16 Line 5). "It has been demonstrated that TPS ≥ 50% is for PD-L1 high expression patients who would be response positive to ICIs treatment. We therefore also evaluated a discrimination of the Epcam-PD-L1 expression on these PD-L1 high expression patients. At 50% cutoff, Epcam-PD-L1 showed an increase in positive samples but not statistically significant (P value=0.109), AUC was at 0.661 with sensitivity at 100% specificity at 36.84% at the highest Youden index (Supplementary Figure 2)."

In the discussion, a corresponding sentence was also added as "At TPS cutoff set as 50%, the performance of Epcam-PD-L1 assay decreased to AUC=0.661." on Page 18 Line 6.

The results as below were added as Supplementary Figure 2.



2. The descriptions on patient characteristics were not shown enough. Were all the patients' diagnosed at early or resectable stage? Were all tissues obtained by surgery? etc. As authors stated in introduction section, the tumor size might have impact on the T-EVs. If so, the authors are encouraged to show the information on tumor size or T stage in Table 1, not only their median and range.

Reply 2: We thank the reviewer for this suggestion. All the plasma samples were obtained before surgery. The information about T stage was added into Table 1 (also listed below) and other information were also stated clearly and classified further.

Changes in the text: On Page 15 Line 8, additive descriptions were added according to the updated Table1 as below.

"Clinicopathological characteristics of the patients are summarized in Table 1. The median age of the patients was 64 years (range, 45-76 years). Twenty-four of 35 (68.6%) patients were male. More than half (65.7%) of the patients had tumor smaller than 3cm in largest dimension. Approximately 45.7% (16/35) of the patients presented with lymph node metastasis. TNM stage showed that more T1 (42.9%) and non-metastasis (91.4%) patients were enrolled. Vascular invasion was observed in 65.7% (23/35) of patients."

The Table 1 and the corresponding legends (Page 26 Line5) were changed as below.

Table 1. Clinicopathological features of patients with lung adenocarcinoma

Characteristic	No. (%)
Age, median (range)	64 (45-76)
Sex:	
Male	24 (68.6%)
Female	11 (31.4%)
Tumor Size, median (range)	2.50 (1.20; 12.0)
≤3cm	23 (65.7%)
>3cm	10 (28.6%)
T Stage	
T1	15 (42.9%)
T2	5 (14.3%)
T3	5 (14.3%)
T4	8 (22.9%)
Lymph Node	
N0	19 (54.3%)
N1-3	16 (45.7%)
Metastasis:	
M0	32 (91.4%)
M1	2 (5.7%)
Vascular Invasion	
Negative	11 (31.4%)
Positive	23 (65.7%)
TPS, median (range)	40% (0.5-98%)
≥1%	n=28
<1%	n=7

Table 1. Clinicopathological features of patients with lung adenocarcinoma. Clinicopathological features of the patients, including age, sex, tumor size in the largest dimension, TNM stage, vascular invasion, and Tumor Proportion Score of PD-L1 expression."

3. The results from this research would not be able simply to apply to advanced stage patients, for whom the PD-L1 detection has clinical meaning. This point needs to be addressed as a limitation in the article.

Reply 3: We thank the reviewer for this comment. Actually we did not understand very well what is the specific meaning of this comment by the review. As we understand, the liquid biopsy method developed in the current study would give benefits especially to the advanced stage patients. Because for those patients, target therapies (such as ICIs) are

the most efficient treatments to them other than surgery to the early stage patients. Besides, it is usually more difficult to obtain tumor tissues from advanced stage patients and therefore a non-invasive method of liquid biopsy would be ideal to them. Moreover, in patients with multiple lesion sites, the biopsy site could be another issue leads to biased result, the liquid biopsy provides a non-heterogeneous method to evaluate PDL1 expression level. The other advantages including potential of dynamic measurements and its quantitative nature. All might benefit to the companion diagnostics of advanced stage patients.

We guess if the reviewer would like to point out that in the current study, we used limited sample size to show an association between circulating Epcam-PD-L1 positive exosome and PD-L1 IHC results. The advantages of this liquid biopsy, such as dynamic measurements and association with ICIs treatment response, are necessarily proved in series of further clinical trials and in larger populations. We totally agree with this point. As we discussed in last paragraph, the limitations about the current study should be explored in the future studies. We modified this paragraph to make it more clear.

Changes in the text:

The limitations of the current study was rewritten in the last paragraph to make it more clear. (Page 20 Line 2)

"The encouraging results obtained with the Simoa PD-L1^{+}T-EVs assay were based on a population with a limited size. The current results must now be confirmed in a larger patient cohort. Additionally, other assays might be performed to obtain a better understanding of the technical issues raised above, including Epcam specificity and the different effects of anti-PD-L1 antibodies and finally to standardize procedures before clinical usage. At last, additional clinical trials should be conducted to determine whether PD-L1 expression on the circulating T-EVs has a similar value to tissue PD-L1 IHC in predicting the tumor response to ICI therapies and its expression cutoff sensitive to ICIs therapy should be evaluated."

Minor comments

1. The authors introduced Simoa assay as highly sensitive immunoassay. Adding some more explanation on the differences from preceding assays would help the readers to understand its value. Why Simoa assay is more sensitive over conventional ones? etc.

Reply 1: We thank the reviewer for this suggestion. Some detail descriptions were added in the Method part to introduce the mechanism of "ultrasensitivity" of Simoa.

Changes in the text:

On Page 9 Line 12, we added a paragraph in the Materials and Methods as below:

"Simoa signal is expressed in AEB as previous described [31, 33]. In short, AEB is determined by counting the number of wells containing both a bead and fluorescent signal ("on" well) relative to the total number of wells containing beads, using Poisson statistics and the digital or analog methods based on high or low concentrations of captured analyte. At low concentrations, the ratio of analytes to beads is small resulting in statistical distribution of individual molecules on the beads, giving Simoa its single molecule sensitivity."

2. Who evaluated the TPS of tissue samples? Experienced pathologist? Please add the explanation on quality control on this point.

Reply 2: We thank the reviewer for this suggestion. The TPS of tissue samples were evaluated by the experienced pathologist, who has a certificate to give TPS assessment for the patient.

Changes in the text: PD-L1 expression was evaluated by 2 board-certified pathologists in FUSCC, who were blinded to clinical data and patient outcomes, via calculating the Tumor Proportion Score (TPS)), which is defined as the percentage of PD-L1-positive tumor cells (TCs) relative to the total number of TCs. (Page 12, Line 13)

3. The authors stated they used Origene antibody due to its good performance. Please add the explanations on the specific reasons, not just as "good performance".

Reply 3: We thank the reviewer for this suggestion. We totally screened 3 commercial monoclonal antibodies for PD-L1 detection (showed as below) on the Simoa platform. In the preliminary results for antibody pair screening, the Origene TA507087 gave the highest signal/background ratio among all the antibodies tested, when combined with capture antibody for Epcam (MAB9601, R&D Systems, which was selected in our previous publication [2])

Changes in the text:

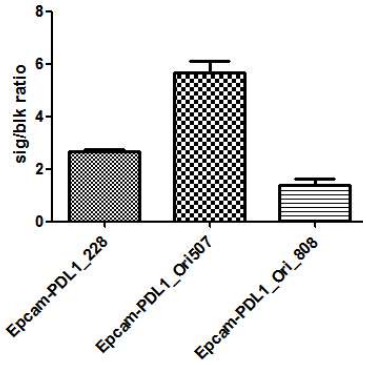
The following description was added to the Method (see Page 8 Line 11)

In order to screen the best antibody pair for Simoa prototype, three monoclonal antibodies of PD-L1 were purchased from Abcam and Origene (Supplementary Table 1). An antibody for Epcam (MAB9601, R&D Systems) confirmed in a previously study [33] was taken as the capture antibody. Exosomes were collected from cell culture supernatant of HCT-116 for antibody pair testing due to its positive expression of Epcam and PD-L1 [35, 36]. Finally, the antibody pair of MAB9601- TA507087 gave the highest signal/background ratio among all the antibodies tested, thus was selected for the further study (Supplementary Figure 1).

Supplementary Table 1: Tested PD-L1 antibody

Name	Company	Catalogue Number	Source
PD-L1-1	Abcam	ab205921	Monoclonal Rabbit IgG Clone # 28-8
PD-L1-2	Origene	TA507087	Monoclonal Mouse IgG1 Clone #OTI2C7
PD-L1-3	Origene	TA808771	Monoclonal Mouse IgG1 Clone #OTI2C7

Supplementary Figure 1



Reviewer C

The manuscript by wu et al. evaluates the application of a single molecule array (Simoa) Epcam-PD-L1 as an alternative, non-invasive method for the identification of lung cancer patients eligible for treatment with immune checkpoint inhibitors (ICIs). The immunoassay is based on the capture of tumor-derived extracellular vesicles (T-EVs) from plasma samples of patients with lung cancer using bead-conjugated anti-EpCAM antibodies and the detection of PD-L1 levels using specific antibodies. The method was initially tested for the detection of PD-L1 in T-EVs isolated from culture supernatant of A549 and SK-MES1 cells by ultracentrifugation. Subsequently, the technique was validated on 35 plasma samples from patients with lung cancer and the results were compared with the levels of PD-L1 obtained by immunohistochemistry, represented by the Tumor Proportion Score (TPS). The manuscript brings very promising results and demonstrates the potential for applying the evaluated methodology. However, some points could be better explored:

- SiMoa signal was expressed in AEB (average enzymes per bead), however, there is no definition of this abbreviation in the manuscript. It would be important to include the definition of this abbreviation and an explanation of it in the current manuscript.

Reply: We thank the reviewer for this suggestion. A description of AEB was added to the Materials and Method.

Changes in the text:

On Page 9 Line 12, we added a paragraph in the Materials and Methods as below:

"Simoa signal is expressed in AEB as previous described [31,33]. In short, AEB is determined by counting the number of wells containing both a bead and fluorescent signal ("on" well) relative to the total number of wells containing beads, using Poisson statistics and the digital or analog methods based on high or low concentrations of captured analyte. At low concentrations, the ratio of analytes to beads is small resulting in statistical distribution of individual molecules on the beads, giving Simoa its single molecule sensitivity."

- It would be important to include a western blot for the detection of an exosomal markers, Epcam and PD-L1 in the vesicles isolated from the culture supernatant of A549 and SK-MES1 cells, in order to demonstrate that the signals detected by SiMoa EpCam-PD-L1 reflect the tumor-derived EVs released from the cell lines.

Reply: We thank the reviewer for this suggestion. We agreed this point is very important. Instead to use western blot, we actually use a previously validated and widely used protocol of on-bead flow cytometry [3,4] to detect the expression of PD-L1 in the EVs isolated from the culture supernatant of A549 and SK-MES1 cells. This protocol used aldehyde/sulfate latex beads (S37225, Thermo Fisher) to capture exosomes and sequentially incubated with PD-L1 antibody (clone 28-8, ab205921, Abcam) and the HRP-Goat Anti-Rabbit IgG H&L (ab97051) secondary antibody, which is actually similar to the process of western blot. In addition, exosomes/EVs flow cytometry reflects only the surface PD-L1 expressions, while the western blot on exosome/EVs may also include PD-L1 expressions inside of the exosomes/EVs. Because Simoa platform is also to detected exosomes/EVs by its surface biomarkers (use Epcam to detect exosomes/EVs has been confirmed in our previous publication, see ref. 33 in the manuscript), we think on-bead flow cytometry is an ideal method to compare in parallel with Simoa. From Figure 2C and 2D, we could see no matter the baseline levels of PD-L1 on A549 and SK-MES1 or the increased PD-L1 expressions after IFNγ treatment, Simoa and exosomes/EVs flow cytometry showed consistent results.

Changes tin the text:

The sentence as bellow and 2 representative references to the method of on-bead flow cytometry for EVs detection were added On Page 11 Line 8.

An on-bead flow cytometry is used to detect exosomes/EVs [38, 39].

- The data demonstrate the potential for applying Simoa as a non-invasive alternative method for the identification of lung cancer patients eligible for treatment with ICIs. However, as recognized by the authors themselves, the number of patient's samples analyzed is limited (35 in total; 28 with TPS> 1% and 7 with TPS <1%), which impacts the power of the analysis. Are there any major difficulties or limitations for the collection and analysis of a larger number of samples that justify the limited number of the population included in the study?

Reply: We thank the reviewer for this question. We understood the small sample size is a limitation for the current study. However, this study is a part of work for an exosome detection study by using Simoa, the aim of this part of work is to perform a "proof of concept" study to demonstrate if Simoa has a potential to detect PD-L1-positive EVs and then use as a companion diagnosis for cancer immunotherapy. Due to the limitation in budget, time and the partnership, we didn't enlarge the sample size for the validation. We think even at this stage, the technology, study design and results of this part of work have a certain degree of value for an inspiration to people working in the field.

Reviewer D

First, I would like to thanks the authors for having the opportunity to review this work and congrats F. Wu and Y. GU et al. for their work. This is a really interesting work that show that exosomes applications are closer to the clinics each day.
The manuscript is well conducted and clear, however I would like to solve some concerns prior to their publication.
1. Please, review the verb tenses. For example, in the abstract, the present is used for presenting the results and the past tense is more accurate for this concern.

Reply 1: We thank the reviewer for this suggestion. The verb tenses in the abstract have been modified to the past tense.

Changes in the text: The verb tenses were modified to the past tenses in the Abstract (Page 3 Line 14-20).

2. During the methodology, the authors used nM for nanometers and nanomolar. Please use only nm for nanometers and nM for concentration.

Reply 2: We thank the reviewer for this correction. The mistake was corrected in the manuscript.

Changes in the text:

Two “nM” refer to “nanometers” were modified to “nm”. (Page 6 Line 4, 5)

3. Please, provide the complete list of antibodies used in a supplementary table, although they were not selected at the end.

Reply 3. We thank the reviewer for this suggestion. We totally screened 3 commercial monoclonal antibodies for PD-L1 detection (showed as below) on the Simoa platform. In the preliminary results for antibody pair screening, the Origene TA507087 gave the highest signal/background ratio among all the antibodies tested, when combined with capture antibody for Epcam (MAB9601, R&D Systems, screened in our previous publication (ref. 33))

Changes in the text:

The following description was added to the Method (Page 8 Line 11)

In order to screen the best antibody pair for Simoa prototype, three monoclonal antibodies of PD-L1 were purchased from Abcam and Origene (Supplementary Table 1). An antibody for Epcam (MAB9601, R&D Systems) confirmed in a previously study [33] was taken as the capture antibody. Exosomes were collected from cell culture supernatant of HCT-116 for antibody pair testing due to its positive expression of Epcam and PD-L1 [35, 36]. Finally, the antibody pair of MAB9601- TA507087 gave the highest signal/background ratio among all the antibodies tested, thus was selected for the further study (Supplementary Figure 1).

Supplementary Table 1: Tested PD-L1 antibody

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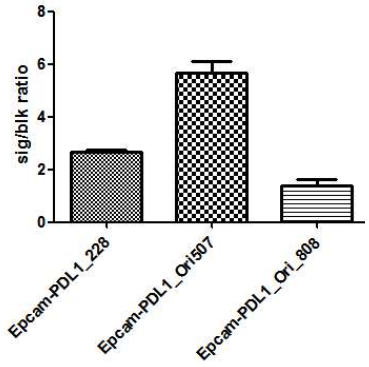
Supplementary Figure 1



4. Please, provide information about the starting plasma material used from patients. This value is crucial for the clinical applications.

Reply 4: We thank the reviewer for this suggestion. More information about the starting plasma material was added in the Materials and Methods.

Changes in the text:

On page 10 line 8, the paragraph was modified to:

"Clinical samples analyzed by Simoa platform were EDTA plasma samples collected from lung cancer patients before surgery. Three milliliter of patient whole blood were collected into the EDTA tube. Plasma were separated within 2 hours under 3000 rpm centrifugation and transferred to the tissue bank of Fudan University Shanghai Cancer Center (FUSCC). The plasma samples used in this study were mainly collected in 2018 and stored at -80°C.

5. During the work, the authors compared the measurements of flow cytometry and the simoa system, I would really appreciate during the discussion a paragraph showing why is better to use this system respect to flow cytometry and their main advantages for the clinic (as device/system).

Reply 5: We thank the reviewer for this suggestion. A paragraph was added in the Discussion to compare and discuss the Simoa platform with conventional EVs detection systems, such as on-bead flow cytometry.

On Page 17 Line 13, we added a paragraph in the Discussion as below:

In addition, the identification and quantification of biomarkers on EVs/exosomes in clinical samples remains challenging due to the complex isolation process. For example, to evaluate exosomes by the flow cytometry technology requires isolation of exosomes before detection, which is not feasible in clinical setting. Due to the small size of EVs/exosomes, an

aldehyde/sulfate latex bead need to be used in the conventional flow cytometry to capture exosomes but in a non-specific way [37, 38]. In contrast, Sioma platform might provide an ultrasensitive, non-invasive, fully automated, and high-throughput EV detection assay with double EVs biomarkers targeting [33].