PD-L1 expression on circulating tumor cells may be predictive of response to Pembrolizumab in advanced melanoma: Results from a pilot study

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PD-L1 Expression on Circulating Tumor Cells May Be Predictive of Response to Pembrolizumab in Advanced Melanoma: Results from a Pilot Study

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ABSTRACT

Background. PD-1 inhibitors are routinely used for the treatment of advanced melanoma. This study sought to determine whether PD-L1 expression on circulating tumor cells (CTCs) can serve as a predictive biomarker of clinical benefit and response to treatment with the PD-1 inhibitor pembrolizumab.

Methods. Blood samples were collected from patients with metastatic melanoma receiving pembrolizumab, prior to treatment and 6–12 weeks after initiation of therapy. Multi-parametric flow cytometry was used to identify CTCs and evaluate the expression of PD-L1.

Results. CTCs were detected in 25 of 40 patients (63%). Patients with detectable PD-L1+ CTCs (14/25, 64%) had significantly longer progression-free survival (PFS) compared with patients with PD-L1− CTCs (26.6 months vs. 5.5 months; p = .018). The 12-month PFS rates were 76% versus 22% in the PD-L1+ versus PD-L1− CTCs groups (p = .012), respectively. A multivariate linear regression analysis confirmed that PD-L1+ CTC is an independent predictive biomarker of PFS (hazard ratio, 0.229; 95% confidence interval, 0.052–1.012; p = .026).

Conclusion. Our results reveal the potential of CTCs as a non-invasive real-time biopsy to evaluate PD-L1 expression in patients with melanoma. PD-L1 expression on CTCs may be predictive of response to pembrolizumab and longer PFS.

Implications for Practice: The present data suggest that PD-L1 expression on circulating tumor cells may predict response to pembrolizumab in advanced melanoma. This needs further validation in a larger trial and, if proven, might be a useful liquid biopsy tool that could be used to stratify patients into groups more likely to respond to immunotherapy, hence leading to health cost savings.

INTRODUCTION

Advanced melanoma is an aggressive cancer with poor prognosis. However, the survival outcomes have improved recently for a proportion of these patients with the introduction of new immune modulating agents [1]. Biomarkers identifying these patients are lacking. Furthermore, these agents are expensive and can lead to substantial immune-related toxicity that can place a huge economic burden on the health system.

A number of tumor and immune biomarkers are currently under development in an effort to better predict...
treatment responders to immunotherapy [2]. Despite its caveats, PD-L1 expression is the most studied and developed biomarker so far in immuno-oncology. Tissue PD-L1 expression assessed via immunohistochemistry (IHC) is currently being used in the management of advanced lung cancer [3]. A number of studies have demonstrated higher response in patients with high PD-L1 expression in their tumors [3–5].

Tissue biopsy is the current gold standard for cancer diagnosis and for determining prognosis in certain cases [6]. However, this can be invasive and uncomfortable to the patients and lead to complications. A single biopsy only provides a limited snapshot of cancer at that particular time. As our understanding of tumor biology has improved over the last decade, we now know that cancer evolves with time and can undergo escape mutations and epigenetic alterations with dynamic molecular changes [7, 8]. This can lead to resistance to therapy and disease progression, factors that cannot be determined from a single biopsy.

Liquid biopsies can provide useful genomic information that could be complementary to tissue biopsy. They are relatively noninvasive and can track tumor evolution longitudinally through serial sampling at various time points [9]. Tumor cells spread through blood vessels and can be captured using various techniques. Molecular analysis of these circulating tumor cells (CTCs) can provide useful molecular information regarding the parent tumors [10–12]. Such information can enhance our understanding of response and resistance mechanisms to immunotherapy.

Previously we reported on the heterogeneity of melanoma CTCs and the prognostic value of CTC subpopulations in patients undergoing mitogen-activated protein kinase inhibiting therapies [13]. Here we use the same multiparametric flow cytometry panel to detect CTCs in patients commencing pembrolizumab therapy and evaluate the expression of PD-L1 on CTCs in relation with response to treatment and survival. The primary objective of the study was to assess the predictive significance of pretreatment CTC PD-L1 expression for response and progression-free survival (PFS). Secondary objectives included predictive significance of pharmacodynamic changes in total CTC count and in the percentage of PD-L1–expressing CTCs (CTC PD-L1+) during treatment and its impact on response to pembrolizumab.

**Materials and Methods**

**Patients, Treatment, and Blood Collection**

Patients were recruited from three clinical sites in Perth, Western Australia. Patients were diagnosed and staged according to the guidelines of the American Joint Committee on Cancer, 8th edition. Participants signed informed consent with the clinician in accordance with protocols safeguarding patient rights. All procedures were accepted by the Human Research Ethics Committees at Edith Cowan University (no. 11543) and Sir Charles Gairdner Hospital (no. 2013-246).

Peripheral blood samples were obtained from each patient prior to commencement of treatment (baseline) with pembrolizumab and then every 6–12 weeks. Blood was drawn into K2-EDTA tubes (BD Biosciences, San Jose, CA, USA) after discarding the first 2–3 mL to avoid epithelial contamination and refrigerated at 4°C until use. Samples were processed within 24 hours from collection.

**Flow Cytometric Staining**

Peripheral blood mononuclear cells (PBMCs) were isolated from 2 × 8 mL of blood using Ficoll-Paque (GE Healthcare, Chicago, IL), washed in FACS buffer (0.1% bovine serum albumin, 100 mM EDTA, 10 mM HEPES, and phosphate-buffered saline), and stained immediately for flow cytometry analysis as described previously [13]. Prior to antibody staining, cells were incubated for 10 minutes with Fc-Blocking Reagent (Miltenyi Biotec, Bergisch Gladbach, Germany). The total number of PBMCs isolated from 8 mL of blood was stained with antibodies to MCAM-PECy7, MCSP-APC, ABCB5-PE.TxR, CD271-PerCP.Cy5.5, RANK-PE, PD-L1.AF488, CD45-APC.A750, and CD34-APC.F700 (supplemental online Table 1). PBMCs from the second 8 mL of blood were stained using isotype controls for PECy7, APC, PE.TxR, PerCP.Cy5.5, PE, and AF488 and stained with CD45-APC.A750 and CD34-APC.F700. Samples were incubated for 30 minutes at 4°C in the dark. After two washes with FACS buffer to remove unbound antibodies, cells were incubated with Live/Dead Fixable Aqua Dead Cell Stain Kit (Thermo Fisher Scientific, Waltham, MA), fixed, and stained with Hoechst 33342 (Thermo Fisher Scientific). The cell suspension was then acquired to exhaustion in a Galios Flow Cytometer (Beckman Coulter, Brea, CA). Data were analyzed using the Kaluza analysis software (version 1.2, Beckman Coulter; gating strategy exemplified in supplemental online Fig. 1). The sample stained with isotype controls was used to define the gates. A cell was identified as a CTC if it was Aqua Vital negative (live) and Hoechst positive (nucleated) and demonstrated positive staining for MCAM, MCSP, ABCB5, CD271, or RANK and negative stains for CD45 and CD34. PD-L1 expression was evaluated on the cells identified as CTCs.

**Treatment Response and Disease Progression Assessment**

Tumor responses were assessed radiologically by computed tomography (CT) and/or positron electron tomography (PET) scans at two to three monthly intervals. Response (decrease in standardized uptake value/size/number of lesions on PET or decrease in size/number of lesions on CT scan as per RECIST 1.1) to treatment was defined on the basis of individual PET or CT scan reports by a radiologist blinded to clinical data) and the treating oncologist’s interpretation of imaging findings correlated with clinical benefit from therapy. PFS was defined as the time interval between the start of therapy and the date of first progression. Overall survival (OS) was defined as the time interval between the start of therapy and death.

**Immunohistochemistry for Assessment of PD-L1 Expression in Tumor Tissue**

Immunohistochemistry for PD-L1 expression was performed as described previously [14], using the PD-L1 IHC 22C3 pharmDx (Dako, Carpinteria, CA) and approved by the U.S. Food and Drug Administration for use in non–small-cell lung
cancer. Verification of successful reaction on each slide was assessed with tonsil and placenta as external tissue controls. PD-L1 expression was assessed based on the Tumor Proportion Score (TPS) by an experienced pathologist (B.A.). Only viable tumor cells were assessed. Positivity is defined as any perceptible linear cell membrane staining (partial or complete), the score reflects percentage of positive tumor cells, and any associated immune cells are excluded from scoring.

Statistical Analysis

Patients were dichotomized into those with at least one PD-L1–positive CTC (CTC PD-L1+) and those with PD-L1–negative CTCs (CTC PD-L1−). Receiver operating characteristic (ROC) curve was used to determine the best cutoff value to discriminate between responder and nonresponders. Univariate logistic regression model was then used to establish the odds ratio and 95% confidence interval (CI) at the optimal cutoff. Fisher’s exact tests were used to assess the association between PD-L1+ CTCs or PD-L1 expression in tumors and response to treatment, as well as the association between changes in total and PD-L1+ CTCs upon treatment initiation and response to treatment. Survival curves were plotted using the Kaplan-Meier method and hazard ratios computed through a Mantel-Cox analysis. Univariate and multivariate Cox proportional hazards regression models were used to evaluate the association of PD-L1+ CTCs and progression-free and overall survival. Analyses were performed using GraphPad Prism 8.2.0 (GraphPad Software, San Diego, CA) and IBM SPSS Statistics version 25 (IBM, Armonk, NY).

RESULTS

Patient Characteristics and PD-L1 Detection on CTCs

A total of 58 patients treated with pembrolizumab either as first- or second-line therapy were recruited between September 2014 and September 2017. Here we present the data of 40 patients for whom blood samples were collected prior to treatment initiation (baseline) and any associated immune cells are excluded from scoring.

Table 1. Patient characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>40</td>
</tr>
<tr>
<td>Age (mean, 71 years)</td>
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</tr>
<tr>
<td>&lt;70</td>
<td>17 (43)</td>
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<tr>
<td>&gt;70</td>
<td>23 (58)</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>M</td>
<td>28 (70)</td>
</tr>
<tr>
<td>F</td>
<td>12 (30)</td>
</tr>
<tr>
<td>M stage</td>
<td></td>
</tr>
<tr>
<td>M1b</td>
<td>3 (8)</td>
</tr>
<tr>
<td>M1c</td>
<td>29 (73)</td>
</tr>
<tr>
<td>M1d</td>
<td>8 (20)</td>
</tr>
<tr>
<td>Line of treatment</td>
<td></td>
</tr>
<tr>
<td>First</td>
<td>25 (63)</td>
</tr>
<tr>
<td>Second</td>
<td>14 (35)</td>
</tr>
<tr>
<td>Third</td>
<td>1 (3)</td>
</tr>
<tr>
<td>BRAF status</td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>26 (65)</td>
</tr>
<tr>
<td>V600E</td>
<td>9 (23)</td>
</tr>
<tr>
<td>V600K</td>
<td>1 (3)</td>
</tr>
<tr>
<td>V600R</td>
<td>2 (5)</td>
</tr>
<tr>
<td>Others</td>
<td>2 (5)</td>
</tr>
<tr>
<td>NLR ≥5</td>
<td>11 (28)</td>
</tr>
<tr>
<td>&lt;5</td>
<td>29 (72)</td>
</tr>
<tr>
<td>Liver metastases</td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>9 (23)</td>
</tr>
<tr>
<td>No</td>
<td>31 (77)</td>
</tr>
</tbody>
</table>

Abbreviations: F, female; M, male; NLR, neutrophil-to-lymphocyte ratio; WT, wild type.

Prognostic Value of PD-L1–Expressing CTCs

Within the 25 patients with detectable CTCs, those with PD-L1+ CTCs had significantly longer PFS compared with patients with PD-L1− CTCs (p = .009; Fig. 2A), with a hazard ratio of 0.162 (95% CI 0.042–0.631). The median PFS for the PD-L1− CTCs group was 5.5 (5.2–5.8) months, whereas median PFS was not reached for the group with PD-L1+ CTCs. The 12-month PFS rates were 81% versus 22% in the PD-L1+ versus PD-L1− CTCs groups, respectively (p = .034). Interim overall survival analysis did not reveal statistically significant differences between the groups, although survival rates were lower in patients with PD-L1− CTCs (Fig. 2B), with median OS not reached in the group with PD-L1+ CTCs.

Multivariate Cox regression analysis controlling for age, sex, line of therapy, disease stage, BRAF status, Eastern Cooperative Oncology Group (ECOG) status, neutrophil-to-lymphocyte ratio, and presence of liver metastases confirmed that CTC PD-L1 positivity is an independent predictive biomarker of PFS (hazard ratio, 0.11; 95% CI, 0.01–0.81; p = .03; Table 2).
There were 21 responders and 19 nonresponders to pembrolizumab monotherapy. The total number of CTCs was similar and did not significantly differ between responders and nonresponders, and there were no differences in PFS or OS between patients with detectable and nondetectable CTCs. However, the number of PD-L1+ CTCs was significantly higher in responders (p = .005; Fig. 3). We applied a univariate logistic regression model and ROC curve to assess whether CTC PD-L1 positivity distinguished responders from nonresponders to pembrolizumab monotherapy (Fig. 3C). Using a cutoff of at least one PD-L1+ CTC, we observed a 61.9% sensitivity with an 84.2% specificity. Among patients with detectable CTCs, patients with PD-L1+ CTCs were eight times more likely to be responders.

**Figure 1.** CTCs in detected in patients with advanced melanoma prior to treatment with pembrolizumab. (A): Number of CTCs in 8 mL of blood corresponding to each of the CTC subpopulations identified. Each bar represents a single patient with melanoma. Absent bars represent patients in whom CTCs were not detected. (B): Proportion of total CTCs (full bars) that express PD-L1 (red bars) at baseline in patients treated with pembrolizumab monotherapy. Patients were grouped based on therapeutic objective response. Tumor Proportion Scores indicating PD-L1 expression in the tumor tissue are indicated for each patient. (-) indicates not available tissues. Abbreviation: CTC, circulating tumor cell.

**Figure 2.** Kaplan-Meier plots of progression-free survival and overall survival according to PD-L1 expression on CTCs prior to treatment initiation. (A): Progression-free survival. (B): Overall survival. Abbreviations: CI, confidence interval; CTC, circulating tumor cell; HR, hazard ratio.

PD-L1+ CTCs and Response to Pembrolizumab
There were 21 responders and 19 nonresponders to pembrolizumab monotherapy. The total number of CTCs was similar and did not significantly differ between responders and nonresponders, and there were no differences in PFS or OS between patients with detectable and nondetectable CTCs. However, the number of PD-L1+ CTCs was significantly higher in responders (p = .005; Fig. 3). We applied a univariate logistic regression model and ROC curve to assess whether CTC PD-L1 positivity distinguished responders from nonresponders to pembrolizumab monotherapy (Fig. 3C). Using a cutoff of at least one PD-L1+ CTC, we observed a 61.9% sensitivity with an 84.2% specificity. Among patients with detectable CTCs, patients with PD-L1+ CTCs were eight times more likely to be responders.
responders compared with patients with undetectable PD-L1+ CTCs (OR, 8.67; 95% CI, 1.19–342.96; p = .017; Table 3; supplemental online Fig. 2A).

Comparison of CTCs detected at baseline and after 6–12 weeks after treatment initiation (follow-up) showed that the total number of CTCs, as well as the proportion of CTCs expressing PD-L1, decreased upon treatment in most responders and increased or remained the same in most nonresponders (Fig. 4).

**PD-L1 Expression in Matching Tumor Samples**

Available archival melanoma specimens were tested for PD-L1 analysis in 25 of the 40 patients. Fourteen cases had PD-L1 status data available for both the tumor and CTCs samples (Fig. 1B). Six patients were PD-L1+ in both tumor and CTCs, and all of them responded to therapy. Three patients were PD-L1+ in tumor (≤10% TPS) but not on CTCs, and two of them responded to treatment. Two patients were PD-L1+ on CTCs but not in tissue, with one responding and the other not responding to treatment. Finally, three patients were PD-L1− in both tissue and CTCs, and all failed to respond. Representative images of PD-L1 staining are shown in supplemental online Figure 3.

Table 2. Progression-free survival univariate and multivariate Cox regression analysis

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group</th>
<th>Univariate</th>
<th></th>
<th></th>
<th>Multivariate</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>PD-L1+ CTCs</td>
<td>Positive vs negative</td>
<td>.020</td>
<td>0.20 (0.05–0.77)</td>
<td>.030</td>
<td>0.11 (0.01–0.81)</td>
<td></td>
</tr>
<tr>
<td>ECOG</td>
<td>≥1 vs 0</td>
<td>.408</td>
<td>1.26 (0.73–2.18)</td>
<td>.050</td>
<td>11.00 (1.00–120.83)</td>
<td></td>
</tr>
<tr>
<td>Age</td>
<td>Continue</td>
<td>.378</td>
<td>0.98 (0.94–1.03)</td>
<td>.328</td>
<td>0.97 (0.90–1.03)</td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td>M vs. F</td>
<td>.652</td>
<td>0.87 (0.48–1.59)</td>
<td>.226</td>
<td>0.18 (0.01–2.92)</td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td>M1c/d vs. M1a/b</td>
<td>.279</td>
<td>1.54 (0.71–3.35)</td>
<td>.111</td>
<td>9.65 (0.59–157.13)</td>
<td></td>
</tr>
<tr>
<td>BRAF</td>
<td>Mutant vs. WT</td>
<td>.219</td>
<td>1.41 (0.81–2.45)</td>
<td>.026</td>
<td>164.05 (1.86 to 1.45E+04)</td>
<td></td>
</tr>
<tr>
<td>Line of therapy</td>
<td>1st vs. 2nd/3rd</td>
<td>.217</td>
<td>2.03 (0.66–6.26)</td>
<td>.945</td>
<td>1.8E-04 (6.5E-112 to 4.7E+103)</td>
<td></td>
</tr>
<tr>
<td>NLR</td>
<td>&gt;5 vs. &lt;5</td>
<td>.003</td>
<td>0.12 (0.03–0.49)</td>
<td>.909</td>
<td>5.6E-07 (2.1E-114 to 1.5E+101)</td>
<td></td>
</tr>
<tr>
<td>Liver metastases</td>
<td>Yes vs. no</td>
<td>.713</td>
<td>0.75 (0.17–3.42)</td>
<td>.256</td>
<td>0.20 (0.01–3.19)</td>
<td></td>
</tr>
</tbody>
</table>

**Table 3. Comparison of response to pembrolizumab relative to PD-L1 detection in CTCs**

<table>
<thead>
<tr>
<th>Response</th>
<th>PD-L1+ CTCs (n = 16), n (%)</th>
<th>PD-L1− CTCs (n = 9), n (%)</th>
<th>CTC− (n = 15), n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Best overall response</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complete response</td>
<td>6 (38)</td>
<td>1 (11)</td>
<td>4 (27)</td>
</tr>
<tr>
<td>Partial response</td>
<td>7 (44)</td>
<td>2 (22)</td>
<td>1 (7)</td>
</tr>
<tr>
<td>Stable disease</td>
<td>0 (0)</td>
<td>2 (22)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Progressive disease</td>
<td>3 (19)</td>
<td>4 (44)</td>
<td>10 (67)</td>
</tr>
<tr>
<td>Objective response rate</td>
<td>13 (81)</td>
<td>3 (33)</td>
<td>5 (33)</td>
</tr>
</tbody>
</table>

Abbreviation: CTC, circulating tumor cell.

PD-L1 positivity in tumor tissue (≥1% TPS) was significantly associated with response to treatment (p = .027; supplemental online Fig. 2B). Furthermore, there were more responders in the PD-L1+ cohort irrespective of PD-L1 assessment through...
patients with melanoma at baseline is prognostic of survival.

Ongoing studies have shown that the number of CTCs in other isolation and detection methodologies [18, 19]. The heterogeneity of melanoma CTCs has also been demonstrated using flow cytometry–based PD-L1 status was superior to other baseline clinical parameters associated with response and prognosis, including lactate dehydrogenase, disease stage, and ECOG performance status.

A number of biomarkers, including tumor PD-L1 expression, tumor mutational burden, tumor-infiltrating lymphocytes, and immune gene signature, have been evaluated in various studies with encouraging results [2]. In particular, PD-L1 expression in tumors has been shown to be associated with response to anti–PD-1 therapies in melanoma and other cancers [3–5, 15]. Thus, efforts have been made to investigate the performance of CTCs as a surrogate to assess PD-L1 expression in the bloodstream of several tumor types [16, 17]. Our study is the first to evaluate the predictive significance of blood-based CTC PD-L1 expression for response to anti–PD-1 therapy in advanced melanoma.

The results further validated our previous observations regarding the heterogeneity of melanoma CTCs using this flow cytometry–based method [13]. Similarly, the heterogeneity of melanoma CTCs has also been demonstrated using other isolation and detection methodologies [18–20]. Previous studies have shown that the number of CTCs in patients with melanoma at baseline is prognostic of survival [21]. However, in our study we did not find that the pretreatment total CTC number was associated with survival or response to treatment. This may be affected by the effectiveness of pembrolizumab to divert the natural disease progression regardless of tumor burden. Similarly, in a recent study by Hong et al. [22], using a combination of a microfluidic device and 19 transcripts for detection of melanoma CTCs, no correlation was found between baseline CTC scores and survival or response to treatment. In addition, the authors noted that using serial monitoring of patients with melanoma treated with immune checkpoint inhibitors, rapid decline in CTC score preceded response by standard clinical assessment and was highly predictive of long-term clinical outcome. This is highly concordant with our observation that a decline in total CTCs upon treatment initiation was strongly associated with response to treatment. Thus, longitudinal blood collection for CTC PD-L1 analysis may assist with differentiation between responders and nonresponders or pseudoprogressors early in therapy.

Our results indicate better response rates in patients with PD-L1+ CTCs at baseline and in those in whom the proportion of PD-L1+ CTCs decreased a few weeks into treatment. This study also demonstrated better median and 12-month PFS for PD-L1+ CTC compared with PD-L1− CTC. Currently, there is no biomarker in routine use for guiding treatment of patients with melanoma with immunotherapy and differentiating between responders and nonresponders. Validation of these results in larger prospective trials in the future might assist clinicians to stratify their patients into potential responders and nonresponders and change treatment earlier to alternative therapies or combination therapies for those less likely to respond.

**Figure 4.** Changes in total CTCs and in the percentage of PD-L1–expressing CTCs during treatment and relative to response to pembrolizumab. (A): Total CTCs. (B): PD-L1–expressing CTCs. Green indicates changes in CTC numbers in responders and red in nonresponders. Fisher’s exact test was used to compare the association between changes in CTCs and response to therapy. Values of p are indicated.

Abbreviations: CTC, circulating tumor cell; R, responder.

**DISCUSSION**

Here we evaluated the expression of PD-L1 on melanoma CTCs. Our results indicated that PD-L1 positivity in CTCs is an independent predictor of response and prolonged PFS in patients with melanoma treated with pembrolizumab. CTC-based PD-L1 status was superior to other baseline clinical parameters associated with response and prognosis, including lactate dehydrogenase, disease stage, and ECOG performance status.

A number of biomarkers, including tumor PD-L1 expression, tumor mutational burden, tumor-infiltrating lymphocytes, and immune gene signature, have been evaluated in various studies with encouraging results [2]. In particular, PD-L1 expression in tumors has been shown to be associated with response to anti–PD-1 therapies in melanoma and other cancers [3–5, 15]. Thus, efforts have been made to investigate the performance of CTCs as a surrogate to assess PD-L1 expression in the bloodstream of several tumor types [16, 17]. Our study is the first to evaluate the predictive significance of blood-based CTC PD-L1 expression for response to anti–PD-1 therapy in advanced melanoma.

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We compared PD-L1 detection on CTCs with that of matching tumor tissues. PD-L1 expression was assessed as a percentage of tumor cells in tissue for better comparison with CTCs, rather than using the melanoma scoring system (MEL) as reported by Daud et al. [23]. Patients with PD-L1 positivity on CTCs or tumor tissue have a high probability of response, suggesting that the methods could be complementary. Of note, concordant results in tissue and CTCs (n = 9) predicted response to treatment with a 100% accuracy.

There are a number of limitations to this study. Sample size is small, and these results need to be validated in a larger independent, prospective cohort. CTCs were not detectable in about a third of patients, which could be related to disease biology or technical limitations. For comparison, circulating tumor DNA is detectable in around 43%–76% of patients with advanced melanoma [24–26], suggesting that circulating markers are below detection in some patients with melanoma despite disseminated disease. There was no standardization of imaging modalities used to assess response to treatment. However, our data are reflective of the real-world setting and routine clinical practice outside the context of a clinical trial where RECIST 1.1 is not formally used and the choice of imaging modality varies among clinicians.

**Conclusion**

Our research suggests that the presence of PD-L1–expressing CTCs is associated with treatment response to pembrolizumab. Patients with one or more PD-L1+ CTCs had a higher response rate to pembrolizumab as well as longer PFS. This study provides a proof of concept that detecting PD-L1 status through a liquid biopsy could provide clinically relevant information.

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