PRECISION MEDICINE

original reports

Monitoring PD-L1 Expression on Circulating Tumor–Associated Cells in Recurrent Metastatic Non–Small-Cell Lung Carcinoma Predicts Response to Immunotherapy With Radiation Therapy

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abstract

PURPOSE Current diagnostic methods to determine programmed death 1 (PD-1) receptor and its ligand (PD-L1)/PD-1 immunotherapy (immune checkpoint inhibitor [ICI]) efficacy in recurrent or metastatic non–small-cell lung carcinoma (rmNSCLC) are imprecise. Although previously shown that patients with high tumor PD-L1 (≥ 50%) demonstrate clinical benefit in the form of disease reduction and improved survival, patients with low PD-L1 (< 50%) sometimes benefit from treatment. Since the PD-L1/PD-1 pathway is dynamic, monitoring PD-L1 levels during treatment may be more accurate than a static baseline tumor biopsy; however, rebiopsying the primary or metastatic disease is rarely feasible. Liquid biopsies that measure the upregulation of PD-L1 on tumor-associated cells (TACs), ie, cancer-associated macrophage-like cells and circulating tumor cells, have been performed, but their predictive value for ICI therapy efficacy is unknown.

MATERIALS AND METHODS We initiated a single-blind prospective study to evaluate TAC PD-L1 expression changes in rmNSCLC from blood samples before (T0) and after (T1) treatment with ICI (ICI, n = 41) or without ICI (no ICI, n = 41). Anonymized blood was filtered to isolate TACs, which were then quantified for high/low PD-L1 expression. Progression-free survival (PFS) or overall survival (OS) hazard ratios (HRs) were evaluated at 18 and 24 months by censored univariate analysis.

RESULTS Increased TAC PD-L1 expression between T0 and T1 in patients who were not treated with ICI had no relationship with PFS or OS. However, increased TAC PD-L1 expression between T0 and T1 in patients treated with ICI had significantly better PFS (HR, 3.49; 95% CI, 1.5 to 8.3; $P = .0091$) and OS (HR, 3.058; 95% CI, 1.2 to 7.9; $P = .0410$).

CONCLUSION Blood-based monitoring of dynamic changes in PD-L1 in TACs appears to identify patients with rmNSCLC who may benefit from ICI.

JCO Precis Oncol 6:e2200457. © 2022 by American Society of Clinical Oncology

INTRODUCTION

Immune checkpoint inhibitors (ICIs) of the programmed death 1 (PD-1) receptor and its ligand (PD-L1) (eg, pembrolizumab, atezolizumab, and nivolumab) are effective first- or second-line treatments for patients with non–small-cell lung cancer (NSCLC) ineligible for molecularly targeted therapies (eg, epidermal growth factor receptor, anaplastic lymphoma kinase tyrosine kinase inhibitors).[1-4] PD-L1 is a transmembrane protein found on tumor and stromal cells under inflammatory conditions that act as a protumorigenic factor in cancer cells that bind to the coreceptor, PD-1, found on cytotoxic T cells.[5] This PD-1/PD-L1 complex inhibits immune antitumor response and inactivates T cells, which allows cancer cells to evade the immunological response, activating the proliferative and survival signaling pathways.[5] High PD-L1 expression in lung cancer biopsies cells has been shown to predict which patients are likely to respond to treatment with anti–PD-L1 immunotherapies. However, patients with low PD-L1 expression may also benefit from these drugs.[2-4,6,7] In recurrent or metastatic NSCLC (rmNSCLC), patients with high PD-L1 expression treated with pembrolizumab plus chemotherapy observed a 24.9% increase in 12-month overall survival (OS) compared with chemotherapy alone.[6] However, it has been shown that patients with low (< 1%) and medium scores

ASSOCIATED CONTENT

Data Supplement

Author affiliations and support information (if applicable) appear at the end of this article.

Accepted on November 4, 2022 and published at ascopubs.org/journal/po on December 14, 2022: DOI https://doi.org/10.1200/PO.22.00457

CONTEXT

Key Objective

Can liquid biopsy, a trackable predictive companion diagnostic, be used to monitor programmed death 1 receptor and its ligand (PD-L1) changes in both circulating tumor and circulating stromal cells throughout treatment?

Knowledge Generated

We designed a prospective study to evaluate changes in PD-L1 in tumor-derived blood cells (ie, circulating tumor cells and circulating stromal cells) before (T0) and after (T1) treatment induction in N = 82 patients with recurrent or metastatic non–small-cell lung cancer. Our data suggest that upregulation of PD-L1 in circulating cells significantly predict immune checkpoint inhibitor response, including superior progression-free survival ($P = .0091$) and superior overall survival ($P = .0410$).

Relevance

Patients with recurrent or metastatic non–small-cell lung cancer experience the most clinical benefit from PD-L1/programmed death 1 immune checkpoint inhibitors when tumor/stromal cells within immunohistochemistry biopsies express high levels of PD-L1. However, many patients with low PD-L1 may also experience clinical benefit, which can occur as a result of PD-L1 upregulation in response to chemotherapy or radiotherapy.

(1%-49%) also may receive some benefit from pembrolizumab, including a 9.5% and 20.6% increase in 12-month OS, respectively.[6] Similarly, rmNSCLC patients with high PD-L1 expression treated with nivolumab plus chemotherapy had a 12% increase in 24-month OS compared with chemotherapy alone.[7] Similarly, patients with low scores (< 1%) or any score (≥ 1%) also saw a 17% and 7% increase benefit in 24-month OS.[7,8] These results suggest that baseline, pretreatment levels of PD-L1 from tumor biopsies were not fully predictive of ICI benefit.

PD-L1/PD-1 is an active immune-modulating biomarker with dynamic processes that can change over time and with induction of different therapies.[9,10] Specifically, it has been established that PD-L1 expression upregulates in response to immune inflammation caused by chemotherapy and radiation therapy, suggesting that PD-L1 may become active after therapy.[9,10] Monitoring PD-L1 changes sequentially necessitates taking multiple biopsies throughout treatment.[11] However, this is not practical as it is an invasive process with high expense and increased risk of morbidity, even when possible.[12]

Blood-based biopsies are minimally invasive blood draws that can procure both circulating tumor cells (CTCs) or circulating stromal cells (CStCs) for monitoring the changes in the tumor immune microenvironment and track tumor cell dynamics in real time.[13-16] CTCs originate from either the primary or metastatic tumor(s) found in circulation of patients with NSCLC, which have been shown to identify changes in the tumor spread.[17-19] Intrinsic characteristics of CTCs, such as number and biomarker expression, can be indicative of tumor response to therapy.[20] In addition to analyzing CTCs, a specific subtype of CStCs (ie, cancer-associated macrophage-like cells [CAMLs]) have also been identified in patients with NSCLC, appearing as a specialized phagocytic myeloid stromal cells originating from tumor sites, which appear to correlate with the tumor microenvironment.[17-19,21] Studies have shown that PD-L1 expression in both CTCs and CAMLs could be sequentially monitored from patients with local NSCLC and might be prognostic.[18,19,22,23] However, these preliminary studies tracked patients treated with chemoradiation therapy (CRT) alone, CRT alone includes patients treated with chemotherapy and site-directed radiation therapy but not immunotherapy or any targeted therapy, and did not evaluate the predictive value of PD-L1 expression in CTCs or CStCs treated with immunotherapies. Here, we describe a prospective single-blind study that evaluated the predictive value of PD-L1 expression of CAMLs and CTCs (ie, tumor-associated cells [TACs] in patients with advanced rmNSCLC treated either with ICI or without ICI).

MATERIALS AND METHODS

Study Design and Patient Population

Patients (N = 82) with pathologically confirmed rmNSCLC were recruited for this single-blind prospective pilot study. Patients, age older than 18 years, were recruited from July 2013 to October 2021 in accordance with local institutional review board approval with the patients' written informed consent. All patients were treated with standard-of-care therapies on the basis of the NCCN guidelines at the time of recruitment, and treatment regimens were not affected by the sample procurement. Peripheral blood samples (7.5 mL) were drawn sequentially at two separate blood draws: before (T0) and after administration of the first course of therapy (T1) for their respective treatments on the basis of standard-of-care treatment (approximately 30 days). Patients may have had disease that recurred after prior definitive chemoradiotherapy (Data Supplement, online only) or who presented with de novo metastatic disease. The primary end point was progression-free survival (PFS) with a secondary end point of OS. Written informed consent was obtained from all participants on this

study, with local institutional review board approval from MD Anderson Cancer Center and University of Maryland Greenebaum Cancer Center.

Blood Sample Collection

Anonymized blood samples were filtered at MD Anderson Cancer Center or University of Maryland Greenebaum Cancer Center using a commercially available LifeTracDx PD-L1 test, as previously described.[18,19,22] In brief, 7.5 mL were drawn into a CellSave vacutainer (Menarini Silicon Biosystems, Bryn Athyn, PA) and centrifuged at 0.8 rcf for 20 minutes. Plasma (3 mL) was removed and replaced with equal amount of phosphate buffer solution. The sample was set with prefix (7.5 mL) to stabilize and fix the cells. The sample was processed as previously described, with a CellSieve Microfiltration Assay using a low-pressure vacuum system (Data Supplement). The filter was set with post fixation solution and placed in equal amount of permeabilization buffer. The sample was stained with PD-L1 antibody (LifeTracDx), CK18, CK19, and CD45 for 1 hour. The filter was washed and mounted on a slide with Fluoromount-G with DAPI (Southern Biotech, Birmingham, AL). The QUAS-R quenching assay, as previously described,[15,18,24,25] was used to rehydrate and stain the samples with PD-L1 in a subset of 12 slides.

Analysis of Filters

Samples underwent TAC enumeration, as previously described.[15,16,18,19] TACs were quantified and imaged using an Olympus BX51WI fluorescent microscope (Tokyo, Japan) with a Carl Zeiss AxioCam monochrome (Oberkochen, Germany). PD-L1 expression in isolated cells was quantified for by pixel intensity was measured by the Zen2011 Blue software system and scored as previously described.[18] For this study, PD-L1 expression was converted from a quartile score into a binary scoring system (1 = low and 2/3 = high).

Statistical Analysis

MATLAB R2021 software was used to complete analyses from PD-L1 scores from the ICI and no ICI patient populations. Cox proportional hazard regression was used to compute the univariate and multivariate analysis with a statistical significance threshold of $P \leq .05$ for PFS or OS. PFS and OS Kaplan-Meier defined the time to progression/death, as the interval between T0 to the date of progression, by standard RECIST criteria using positron emission tomography/computed tomography scan or death, within 24-month end point. To achieve 90% power two-sided with an alpha of 0.05, on the basis of previous publications,[19,23,26] we calculated that a sample size of 32 was required. Before initiation of the study, we assumed a dropout rate of 20% and set a recruitment goal of 40 patients from each patient population. Patients who dropped off the study or were lost to follow-up were censored at last known clinical follow-up. Significance of Kaplan-Meier plots were determined by log-rank analysis. Median PFS (mPFS) and median OS (mOS) were compared by using the nonparametric k-sample test for equality of medians, with all P values being two-sided.

The data generated in this study are not publicly available as to not compromise patient privacy (ie, dates of birth, dates of death, etc) but are available on reasonable request from the corresponding author.

RESULTS

Patient Demographics

In total, N = 82 patients with rmNSCLC were recruited for this study. Forty-one were treated with ICI and 41 without ICI. The length of time the ICI population received ICI treatment ranged from 0.1 to 15.4 months (Data Supplement). A total of 142 blood samples were collected and processed (Data Supplement). Analysis for cells was possible on 67 samples from the ICI group, 72 from the no ICI group, leaving three samples that failed because of assay clotting. With N = 82 patients, 164 samples were expected; however, 22 samples were incomplete. Specifically, six patients withdrew from study before the T1 time point, and 14 patients were unavailable for phlebotomy at one time point. The median age for patients treated with ICI was 66 years (range = 45-81 years) and for patients treated without ICI was 63 years (range = 45-78 years). Both ICI and no ICI populations had nearly identical distributions for patient sex with males making up 59% (n = 24 of 41) and 56% (n = 23 of 41) of the ICI and no ICI groups, respectively (Table 1).

Both patient populations included patients with local/regional recurrence after prior definitive therapy (ICI 46% and no ICI 49%) and de novo distant disease (ICI 54% and no ICI 51%). Sites of disease included brain bone, liver renal adrenal vertebral and pleural effusion. Both ICI and no ICI populations had similar rates of brain metastases: 46% and 42%, respectively. The average time between detected disease and metastatic relapse for the ICI patients is 15.7 months, the no ICI patients is 15.4 months, and the combined patient population is 15.6 months.

Primary Tissue Biopsy

Primary tumor PD-L1 staining was available for 46% (n = 19 of 41) of the ICI population and 37% (n = 15 of 41) of the no ICI population. In patients with available biopsy and treated with ICI (n = 19), n = 9 of 19 patients were found to have primary tumor PD-L1 $\geq$ 50% and n = 10 of 19 were found to have primary tumor PD-L1 $<$ 50%. In a 24-month analysis of $\geq$ 50% versus $<$ 50% tumor PD-L1 expression, there was neither statistical significance in clinical outcomes in patients treated with ICI for PFS (hazard ratio [HR], 2.24; 95% CI, 0.6 to 7.8; P = .3450) nor OS (HR, 1.88; 95% CI, 0.4 to 8.6; P = .6693) (Table 2 and Data Supplement).

In patients with available biopsy and treated with CRT alone (n = 16), n = 0 of 16 patients were found to have primary tumor PD-L1 $\geq$ 50% and n = 16 of 16 were found to have primary tumor PD-L1 $<$ 50%.

TAC PD-L1 at T0

TACs were identified in 94% (n = 134 of 142) of all samples: 89% (n = 62 of 70) of available T0 samples and

TABLE 1. Patient Demographic Table for N = 82 rmNSCLC Patients Split by Treatment: ICI Patients (n = 41) and No ICI Patients (n = 41)

Variable	ICI Patients (n = 41)	No ICI Patients (n = 41)
Median ages, years	66 (45-81)	63 (45-78)
Sex (male/female)	24 (58.5%)/17 (41.5%)	23(56.1%)/18(43.9%)
Smoking history		
Never smoker	5 (12.2%)	9 (21.9%)
Light smoker (< 50 pks/y)	19 (46.3%)	23 (56.2%)
Heavy smoker (> 50 pks/y)	14 (34.1%)	9 (21.9%)
Unknown	3 (7.4%)	
Race		
Caucasian	33 (80.5%)	32 (78.0%)
Black	5 (12.2%)	5 (12.2%)
Others	3 (7.3%)	4 (9.8%)
Histology		
Adenocarcinoma	21 (51.2%)	27 (65.9%)
NSCLC	12 (29.3%)	5 (12.2%)
Squamous cell	8 (19.5%)	9 (21.9%)
RT modality		
IMRT	13 (31.7%)	21 (51.2%)
VMAT	14 (34.1%)	7 (17.1%)
Proton	6 (14.6%)	11 (26.8%)
SBRT	3 (7.3%)	2 (4.9%)
3D	2 (5.0%)	
Unknown	3 (7.3%)	
Radiotherapy dose		
< 65 Gy	19 (46.3%)	16 (39.0%)
≥ 65 Gy	19 (46.3%)	25 (61.0%)
Unknown	3 (7.3%)	
ECOG		
0	17 (41.5%)	18 (43.9%)
≥ 1	21 (51.2%)	21 (51.2%)
Unknown	3 (7.3%)	2 (4.9%)
Recurrence		
Local	22 (53.7%)	21 (51.2%)
Distant	13 (31.7%)	20 (48.8%)
Unknown	6 (14.6%)	
Distant recurrence	13 (31.7%)	20 (48.8%)
Brain	6 (46.2%)	8 (40.0%)
Bone	2 (15.4%)	7[a] (35.0%)
Other (liver, renal, etc)	5 (38.5%)	9 (45.0%)
Available tumor PD-L1	19 (45.2%)	15 (36.6%)
< 50%	10 (52.6%)	15 (100%)
≥ 50%	9 (47.4%)	
Immunotherapy		None
Pembrolizumab	28 (68.3%)	NA
Nivolumab	10 (24.4%)	NA
Atezolizumab	3 (7.3%)	NA

Abbreviations: ICI, immune checkpoint inhibitor; ECOG, Eastern Cooperative Oncology Group; IMRT, intensity-modulated radiation therapy; NSCLC, non–small-cell lung cancer; PD-L1, programmed death 1 receptor and its ligand; pks, packets; SBRT, stereotactic body radiation therapy; VMAT, volumetric modulated arc therapy; y, year.

[a]Four patients had both brain and bone metastases.

TABLE 2. Multivariate Analysis for Patients Treated With ICI

ICI Patients (n = 41)		PFS				OS		
		Univariate			Multivariate	Univariate		
Variable	**N**	**HR**	**CI**	***P***	***P***	**HR**	**CI**	***P***
T0 PD-L1 expression low *v* high	28 *v* 4	0.35	0.1 to 1.9	.4191		0.36	0.1 to 1.9	.4332
T1 PD-L1 expression low *v* high	21 *v* 14	3.19	1.4 to 7.3	**.0112**	.5425	2.17	0.9 to 5.1	.1221
Tumor PD-L1 < 50% *v* ≥ 50%	10 *v* 9	2.24	0.6 to 7.8	.3450		1.88	0.4 to 8.6	.6693
PD-L1 upregulation T0 to T1	21 *v* 11	3.49	1.5 to 8.3	**.0091**	.2594	2.61	1.0 to 6.6	.0716
Race White *v* not White	33 *v* 8	0.87	0.3 to 2.2	.9616		1.29	0.5 to 3.5	.8161
Male *v* female	24 *v* 17	0.85	0.4 to 1.8	.8159		0.83	0.4 to 1.9	.8354
Age younger than 65 *v* 65 years or older	17 *v* 24	1.48	0.7 to 3.2	.4127		2.42	1.0 to 5.6	.0666
ECOG 0 *v* 1	17 *v* 21	1.09	0.5 to 2.4	.9903		0.98	0.4 to 2.3	.8643
Smoking								
Nonsmoker *v* light (< 50 pks/y)	5 *v* 19	0.93	0.3 to 2.8	.8832		0.67	0.2 to 2.1	.7067
Nonsmoker *v* heavy (≥ 50 pks/y)	5 *v* 14	1.34	0.4 to 4.8	.9039		0.93	0.2 to 3.5	.8166
Nonsmoker *v* light and heavy	5 *v* 33	1.06	0.4 to 3.2	.8611		0.76	0.3 to 2.3	.8479
Light *v* heavy	19 *v* 14	1.46	0.6 to 3.4	.5110		1.39	0.6 to 3.5	.6409
Light *v* heavy and nonsmokers	19 *v* 19	1.36	0.6 to 2.9	.5694		1.43	0.6 to 3.3	.5367
Histology								
Adeno *v* squamous cell (squamous)	21 *v* 8	1.72	0.7 to 4.3	.3598		1.50	0.6 to 3.9	.5607
Adeno *v* NSCLC	21 *v* 12	1.52	0.6 to 3.6	.4677		2.31	0.8 to 6.3	.1708
Adeno *v* squamous and NSCLC	21 *v* 20	1.78	0.8 to 3.9	.2259		2.21	0.9 to 5.4	.1320
Squamous *v* NSCLC	8 *v* 12	1.15	0.4 to 3.4	.9764		2.47	0.7 to 8.8	.2840
Squamous *v* adeno and NSCLC	8 *v* 33	0.79	0.3 to 1.9	.7544		1.17	0.4 to 3.1	.9515
Radiation therapy								
IMRT[a] *v* VMAT[b]	13 *v* 14	1.34	0.5 to 3.4	.7097		2.48	0.9 to 7.0	.1454
IMRT *v* others[c]	13 *v* 11	0.64	0.2 to 1.7	.5373		0.80	0.3 to 2.2	.8692
IMRT *v* VMAT and others	13 *v* 25	1.02	0.5 to 2.3	.8748		1.60	0.7 to 3.9	.4168
VMAT *v* others	14 *v* 11	0.54	0.2 to 1.5	.3631		0.31	0.1 to 1.1	.1239
VMAT *v* IMRT and others	14 *v* 24	0.66	0.3 to 1.5	.4101		0.40	0.2 to 0.9	.0579
T scores 0-2 *v* 3-4	16 *v* 13	0.19	0.1 to 0.6	**.0054**	**.0369**	0.62	0.2 to 1.7	.4930
N scores 0-2 *v* 3-4	26 *v* 7	1.60	0.6 to 4.1	.4644		1.18	0.4 to 3.4	.9725
Recurrence local *v* distant	22 *v* 13	0.69	0.3 to 1.6	.5018		0.84	0.4 to 2.0	.8690
Immunotherapy								
Pembro[d] *v* nivo[e]	28 *v* 10	0.68	0.3 to 1.7	.5497		0.56	0.2 to 1.5	.3898
Pembro *v* atezo[f]	28 *v* 3	0.83	0.2 to 4.0	.8656		0.44	0.1 to 3.0	.7253
Pembro *v* nivo and atezo	28 *v* 13	0.72	0.3 to 1.7	.5769		0.54	0.2 to 1.4	.2904
Nivo *v* atezo	10 *v* 3	1.00	0.2 to 5.0	.6842		0.70	0.1 to 4.2	.9495
Nivo *v* pembro and atezo	10 *v* 31	1.40	0.6 to 3.4	.6152		1.65	0.6 to 4.5	.4645

NOTE. Bold numbers indicate that the univariate analysis showed significant PFS for ICI patients with high PD-L1 expression at T1 ($P = 0.0112$), PD-L1 upregulation ($P = 0.0091$), and with T scores 3-4 ($P = 0.0054$). The multivariate analysis showed significant PFS for ICI patients with T scores 3-4 ($P = 20.0369$).

Abbreviations: Adeno, adenocarcinoma; HR, hazard ratio; ICI, immune checkpoint inhibitor; NSCLC, non–small-cell lung cancer; OS, overall survival; PD-L1, programmed death 1 receptor and its ligand; PFS, progression-free survival; pks, packets; y, year.

[a]IMRT: intensity-modulated radiation therapy.

[b]VMAT: volumetric modulated arc therapy.

[c]Others: 3D, proton, stereotactic body radiation therapy.

[d]Pembro: pembrolizumab.

[e]Nivo: nivolumab.

[f]Atezo: atezolizumab.

TABLE 3. Multivariate Analysis for Patients Treated Without ICI

No ICI Patients (n = 41)		PFS			OS		
		Univariate			Univariate		
Variable	N	HR	CI	*P*	HR	CI	*P*
T0 PD-L1 expression low *v* high	23 *v* 12	1.18	0.5 to 2.5	.8947	0.79	0.3 to 1.9	.7493
T1 PD-L1 expression low *v* high	16 *v* 21	1.10	0.5 to 2.3	.9371	1.44	0.6 to 3.3	.5185
PD-L1 upregulation T0 to T1	16 *v* 10	0.84	0.4 to 2.0	.8569	1.51	0.6 to 4.0	.5529
Race White *v* not White	32 *v* 9	1.40	0.7 to 3.0	.5071	1.01	0.4 to 2.4	.8396
Male *v* female	23 *v* 18	3.24	1.6 to 6.6	**.0021**	1.81	0.9 to 3.8	.1729
Age younger than 65 *v* 65 years or older	21 *v* 20	1.08	0.5 to 2.1	.9606	0.86	0.4 to 1.8	.8405
ECOG 0 *v* 1	18 *v* 21	0.75	0.4 to 1.5	.5291	0.77	0.4 to 1.7	.6534
Smoking							
Nonsmoker *v* light (< 50 pks/y)	9 *v* 23	0.89	0.4 to 2.0	.9374	0.81	0.3 to 1.9	.7982
Nonsmoker *v* heavy (≥ 50 pks/y)	9 *v* 9	0.70	0.3 to 1.9	.6665	1.94	0.6 to 6.4	.4279
Nonsmoker *v* light and heavy	9 *v* 32	0.84	0.4 to 1.8	.8039	1.02	0.4 to 2.4	.8587
Light *v* heavy	23 *v* 9	0.84	0.4 to 2.0	.8668	1.99	0.8 to 4.9	.2096
Light *v* heavy and nonsmokers	23 *v* 18	0.98	0.5 to 1.9	.9078	1.63	0.8 to 3.4	.2758
Histology							
Adeno *v* squamous	27 *v* 9	0.33	0.1 to 0.9	.0551	0.74	0.3 to 1.9	.6940
Adeno *v* NSCLC	27 *v* 5	1.31	0.4 to 3.9	.8370	1.71	0.5 to 5.4	.5254
Adeno *v* squamous and NSCLC	27 *v* 14	0.65	0.3 to 1.4	.3640	1.03	0.5 to 2.3	.8925
Squamous *v* NSCLC	9 *v* 5	2.17	0.7 to 7.1	.3232	2.05	0.5 to 8.0	.4937
Squamous *v* adeno and NSCLC	9 *v* 32	3.05	1.1 to 8.2	.0512	1.49	0.6 to 3.8	.5638
Radiation therapy							
IMRT *v* VMAT	21 *v* 7	0.79	0.3 to 2.3	.8737	0.60	0.2 to 1.9	.5721
IMRT *v* other	21 *v* 13	0.51	0.2 to 1.2	.1618	0.73	0.3 to 1.8	.6338
IMRT *v* VMAT and others	21 *v* 20	0.63	0.3 to 1.3	.2608	0.71	0.3 to 1.5	.4785
VMAT *v* others	7 *v* 13	0.77	0.3 to 2.1	.7957	1.19	0.4 to 3.7	.9870
VMAT *v* IMRT and others	7 *v* 34	1.01	0.4 to 2.6	.8202	1.48	0.5 to 4.4	.6704
T scores 0-2 *v* 3-4	22 *v* 13	1.07	0.5 to 2.3	.9756	0.93	0.4 to 2.1	.9715
N scores 0-2 *v* 3-4	33 *v* 3	1.91	0.7 to 5.4	.3386	1.53	0.5 to 5.1	.7010
Recurrence local *v* distant	21 *v* 20	0.71	0.4 to 1.4	.4067	0.71	0.3 to 1.5	.4740

NOTE. Bold number indicates that univariate analysis showed significant PFS for female No ICI patients ($P = 0.0021$).

Abbreviations: Adeno, adenocarcinoma; HR, hazard ratio; ICI, immune checkpoint inhibitor; IMRT, intensity-modulated radiation therapy; NSCLC, non–small-cell lung cancer; OS, overall survival; PD-L1, programmed death 1 receptor and its ligand; PFS, progression-free survival; pks, packs; VMAT, volumetric modulated arc therapy; y, year.

100% (n = 72 of 72) of available T1 samples, with n = 12 T0 samples and n = 10 T1 samples failing phlebotomy or patient was not available. Specifically, 571 TACs were imaged: 360 TACs in the ICI population and 211 TACs in the no ICI population. Patients treated with ICI had TACs in 88% (n = 30 of 34) of their T0 samples: 87% (n = 26 of 30) with low PD-L1 and 13% (n = 4 of 30) with high PD-L1 and two failed samples. Patients treated without ICI had TACs in 89% (n = 32 of 36) of their T0 samples: 63% (n = 20 of 32) with low PD-L1 and 38% (n = 12 of 32) with high PD-L1 and two failed samples.

At T0, high TAC PD-L1 expression was not significantly correlated with improved survival for either ICI (PFS: HR, 0.35; 95% CI, 0.1 to 1.9; $P = .4191$; OS: HR, 0.36; 95% CI, 0.1 to 1.9; $P = .4332$) or no ICI (PFS: HR, 1.18; 95% CI, 0.5 to 2.5; $P = .8947$; OS: HR, 0.79; 95% CI, 0.3 to 1.9; $P = .7493$) patient populations (Tables 2 and 3 and Data Supplement).

TAC PD-L1 at T1

Patients treated with ICI had TACs in 100% (n = 35 of 35) of their T1 samples: 60% (n = 21 of 35) with low PD-L1 and 40% (n = 14 of 35) with high PD-L1, n = 6 samples were unavailable or failed processing. Patients treated with no ICI had TACs in 100% (n = 37 of 37) of their T1 samples: 43% (n = 16 of 37) with low PD-L1 and 57% (n = 21 of 37) with

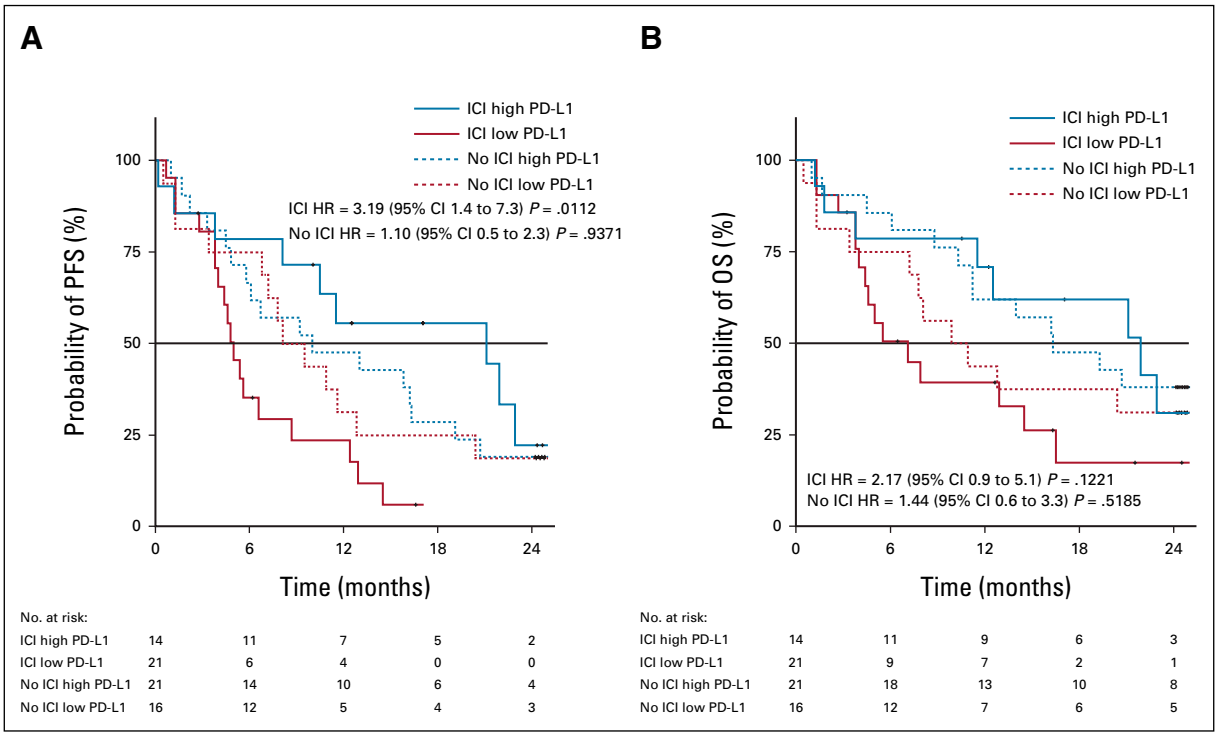

FIG 1. Comparing the survival of patients on the basis of high/low PD-L1 expression after treatment (T1). (A) PFS of ICI patients with high PD-L1 expression (solid blue) *v* ICI patients with low PD-L1 expression (solid red). Median PFS 4.8 *v* 17 months. PFS of no ICI patients with high PD-L1 expression (dotted blue) *v* no ICI patients with low PD-L1 expression (dotted red). Median PFS 8.1 *v* 9.6 months. (B) OS of ICI patients with high PD-L1 expression (solid blue) *v* ICI patients with low PD-L1 expression (solid red). Median OS 5.9 *v* 21 months. OS of no ICI patients with high PD-L1 expression (dotted blue) *v* no ICI patients with low PD-L1 expression (dotted red). Median OS 9.9 *v* 16 months. ICI, immune checkpoint inhibitor; OS, overall survival; PD-L1, programmed death 1 receptor and its ligand; PFS, progression-free survival.

high PD-L1, with n = 4 patients dropping off study before T1 blood draws.

We compared PD-L1 expression in TACs at the T1 time point for patients treated with ICI for PFS (Fig 1 and Table 2). ICI patients with high PD-L1 expression at T1 were significantly less likely to have progressive disease within 18 months (HR, 3.19; 95% CI, 1.4 to 7.3; $P = .0112$) and an identical 24-month PFS (HR, 3.19; 95% CI, 1.4 to 7.3; $P = .0112$), as no additional event occurred after 18 months (Fig 1 and Table 2). Over 24 months, mPFS for ICI-treated patients with low and high TAC PD-L1 scores was 4.8 and 17 months, respectively (Fig 2).

We then compared PD-L1 expression in cells at the T1 time point for patients treated with ICI for OS (Fig 2). ICI patients with high PD-L1 expression at T1 were borderline for significant OS at 18 months (HR, 2.58; 95% CI, 1.1 to 6.3; $P = .06495$) and 24 months (HR, 2.17; 95% CI, 0.9 to 5.1; $P = .1221$) (Fig 1 and Table 2). Over 24 months, mOS for ICI-treated patients with low and high TAC PD-L1 scores was 5.9 and 21 months, respectively (Fig 1).

In contrast, patients treated with no ICI showed no significant differences between high and low TAC PD-L1 expression after CRT (ie, T1) for PFS or OS at 18 (PFS: HR, 1.16; 95% CI, 0.5 to 2.5; $P = .8653$; OS: HR, 1.56; 95% CI, 0.6 to 3.8; $P = .4526$) and 24 months (PFS: HR, 1.10; 95% CI, 0.5 to 2.3; $P = .9371$; OS: HR, 1.44; 95% CI, 0.6 to 3.3; $P = .5185$) (Fig 1 and Table 2). Over 24 months, mPFS for no ICI patients with low and high TAC PD-L1 was 8.1 and 9.6 months, respectively, and mOS was 9.9 and 16 months, respectively (Fig 1).

Upregulation of PD-L1 in TACs

The dynamic nature of PD-L1 and its upregulation in response to cytotoxic therapies therapy is well established. In this study, changes in PD-L1 expression were quantified and analyzed using the two time points in this study, before and after treatment induction. In this study, upregulation was defined as patients who increased from low to high PD-L1 expression between T0 and T1 but excluded patients who (1) had high PD-L1 expression at both T0 and T1 or (2) patients with only a T1 sample without a T0. All other patients who did not increase in PD-L1 expression either decreased PD-L1 or remained with low PD-L1. Changes in TAC PD-L1 expression between T0 and T1, ie, after induction of treatment, were seen in both the ICI and the no ICI groups (Fig 2).

In the ICI group, n = 11 patients increased PD-L1 expression and n = 21 patients did not increase in PD-L1 expression (Fig 2, Tables 2 and 3). The patients who

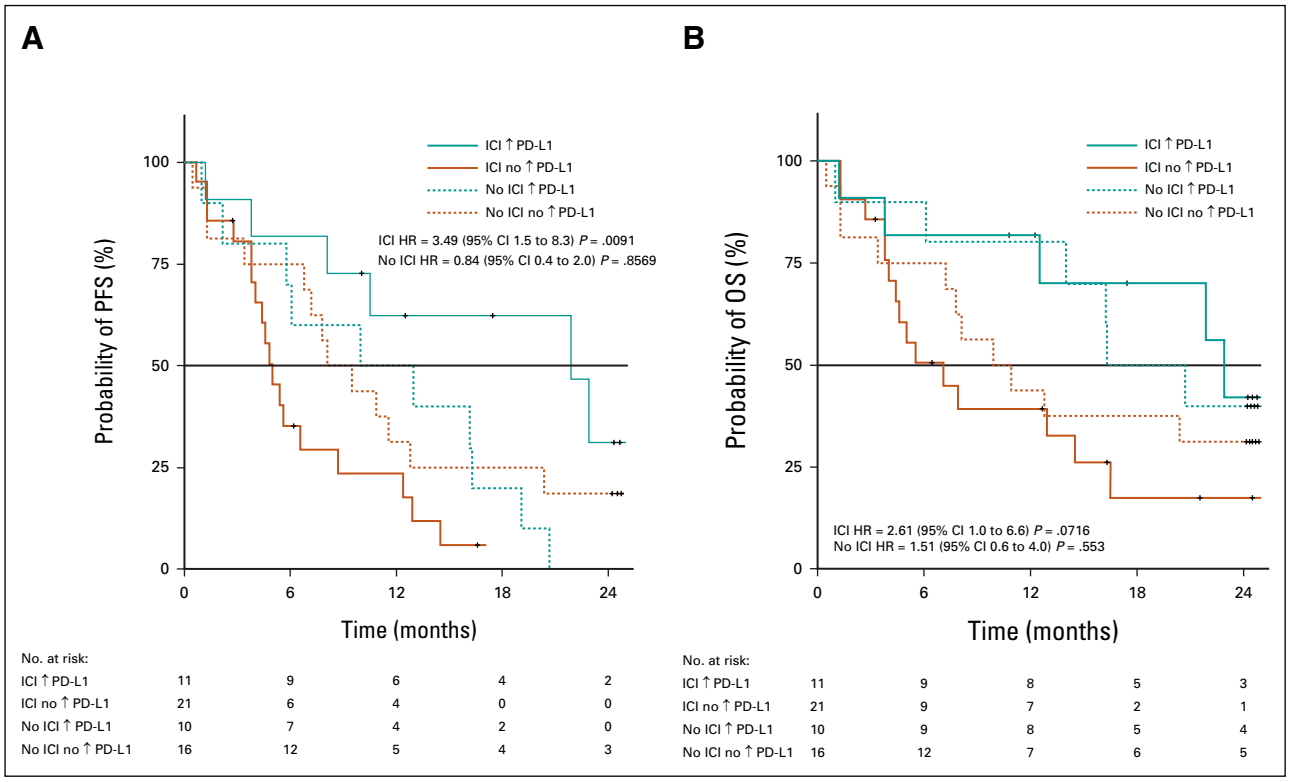

FIG 2. Comparing the survival of patients based on change in PD-L1 expression between T0 and T1. (A) PFS of ICI patients with increased PD-L1 expression (solid green) *v* ICI patients with no increase in PD-L1 expression (solid orange). Median PFS 4.8 *v* 20 months. PFS of no ICI patients with increased PD-L1 expression (dotted green) *v* no ICI patients with no increase in PD-L1 expression (dotted orange). Median PFS 8.1 *v* 10 months. (B) OS of ICI patients with increased PD-L1 expression (solid green) *v* ICI patients with no increase in PD-L1 expression (solid orange). Median OS 5.9 *v* 22 months. OS of no ICI patients with increased PD-L1 expression (dotted green) *v* no ICI patients with no increase in PD-L1 expression (dotted orange). Median OS 9.9 *v* 16 months. ICI, immune checkpoint inhibitor; OS, overall survival; PD-L1, programmed death 1 receptor and its ligand; PFS, progression-free survival.

increased TAC PD-L1 expression showed significantly improved survival with ICI treatments over 24 months for PFS (HR, 3.49; 95% CI, 1.5 to 8.3; $P = .0091$) and borderline significant OS (HR, 2.61; 95% CI, 1.0 to 6.6; $P = .0716$). Patients who increased PD-L1 expression did see significant OS at 18 months (HR, 3.05; 95% CI, 1.2 to 7.9; $P = .0410$) (Fig 2). To clarify, because of missing or failed samples, only 32 patients in the ICI populations had complete pairs of T0 and T1 samples for analysis. Although the previously established sample size, calculated from the statistical power analysis in the methods, required for this study is 32 patients. Over 24 months, mPFS was 4.8 months for ICI patients who did not increase in PD-L1 expression versus 20 months for ICI patients who increased in PD-L1 expression. Similarly, mOS was 5.9 months for ICI patients who did not increase in PD-L1 expression versus 22 months for ICI patients who increased in PD-L1 expression (Fig 2).

In the no ICI group, n = 10 patients increased PD-L1 expression and n = 16 patients did not increase in PD-L1 expression. However, the no ICI patients who had an upregulation in TAC PD-L1 expression did not have significantly different PFS (HR, 0.84; 95% CI, 0.4 to 2.0; $P = .8569$) nor OS (HR, 1.51; 95% CI, 0.6 to 4.0; $P = .5529$) at 24 months (Fig 2). Over 24 months, mPFS was 8.1 months for no ICI patients who did not increase in PD-L1 expression versus 10 months for no ICI patients who increased in PD-L1 expression. Similarly, mOS was 9.9 months for no ICI patients who did not increase in PD-L1 expression versus 16 months for no ICI patients who increased in PD-L1 expression (Fig 2). The patients in the no ICI group with increased PD-L1 expression did do better than the no ICI patients who did not increase PD-L1 expression for OS. It is possible that some patients did receive ICI after cessation of data collection regarding treatment.

Multivariate Analysis

In the ICI group (n = 41), 24-month univariate significance for PFS was identified in the variables: PD-L1 expression at T1 ($P = .0112$), PD-L1 upregulation ($P = .0091$), and T scores ($P = .0054$). Multivariate analysis of these three variables resulted in T scores being the only independent significant variable ($P = .0369$) (Table 2). However, no clinical variables were found to be significant for OS in this grouping.

The no ICI group (n = 41) identified significance in only one variable; thus, multivariate analyses were not needed. In the no ICI group, only sex was highly significant for better PFS in female patients (P = .0021), and none of the variables were significant for OS (Table 3).

In the combined group of all patients (N = 82), PD-L1 expression at T1 was a significant variable for PFS, (P = .0469) and PD-L1 upregulation was significant for OS (P = .0237) (Data Supplement).

Additional Analysis

The entire patient population was further separated by early (T score of 1 and 2) and late (T score of 3 and 4) and tested for survivorship by low and high PD-L1 expression (Data Supplement). The results show that at T0, late T-stage ICI patients with high PD-L1 expression (n = 2) lived significantly longer without progression compared with late T-stage ICI patients with low PD-L1 expression (n = 9) (HR, 0.00075; 95% CI, 1.4 E^{-5} to 0.04; P = .0127) (Data Supplement).

PD-L1 scores were separated into three groups to test for statistical significance in survival outcomes. PD-L1 scores of 1, 2, and 3 were cross compared in log-rank analysis (Data Supplement). At T1, ICI patients with PD-L1 score 2 (n = 5) lived significantly longer without progression or death than ICI patients with PD-L1 score 1 (n = 21) (PFS: HR, 5.21; 95% CI, 1.9 to 14; P = .00343; OS: HR, 6.25; 95% CI, 1.9 to 21; P = .00738) (Data Supplement). At T1, no ICI patients with PD-L1 score 2 had significantly improved OS compared with the no ICI patients with PD-L1 score 1 (HR, 2.88; 95% CI, 1.2 to 6.6; P = .0232) (Data Supplement).

In conclusion, previous studies have established that in rmNSCLC patients with high expression of PD-L1 in their tumor cells (ie, combined positive score) predicts for better ICI responses.[2,5-7,9,11] It has also been hypothesized that some patients with low PD-L1 combined positive score may respond because of upregulation of PD-L1 after chemotherapy or radiotherapy. However, the ability to monitor PD-L1 changes over time and identify PD-L1 upregulation through sequential biopsies, or with a liquid biopsy approach, has not been established. In this study, we evaluated a blood-based assay to sequentially monitor upregulation of PD-L1 in TACs in two parallel groups of patients with rmNSCLC treated with or without ICIs. In this analysis, it was found that at 18 months, patients who had samples that demonstrated an increase in PD-L1 expression had better clinical outcomes after ICI, with longer PFS (P = .0091) and OS (P = .0410) versus patients whose samples did not demonstrate an increase in PD-L1 expression or in the no ICI-treated population (Fig 2).

Primary tumor PD-L1 staining was only available for less than half of both patient populations, which is consistent with previously published values, as up to 80% of patients with NSCLC typically have unresectable and/or smaller cytology samples at diagnosis.[27-29] We tested the available PD-L1 scores from primary tumor biopsies and found no significant association between levels of primary tumor PD-L1 expression and PFS or OS for the ICI patient population. This discrepancy is likely a result of the smaller cohort of patients as only n = 19 ICI patients had available tissue for PD-L1 staining. However, it may also be possible that the dynamic nature of PD-L1 expression in tumors and the microenvironment was different within the pretreatment T0 tissue sample. As PD-L1 is an inflammatory marker, it might have been influenced or changed overtime by the differing therapies, which with the finding of the clinically significant upregulation at the T1 time point, may suggest an importance of sequentially monitoring changes in PD-L1 expression throughout treatment. The data also show a higher presence of distant metastases in the no ICI patient population, which could have led to better survival in the ICI population. The results of the multivariate analyses showed T scores in patients treated with ICI was a significant predictor of PFS (P = .0054) which is interesting and should be further evaluated in larger studies (Table 2).

Overall, sequential monitoring appears possible with the use of liquid biopsies which might provide real-time evaluation of tumor and/or tumor microenvironment changes. Furthermore, monitoring proteomic profiles on TACs have recently been shown to be clinically useful for a variety of tumor-related inflammatory drug targets, such as CXCR4,[15,25] exosome formation,[30] and CCR5 expression,[31] suggesting that CAMLs may provide a previously unknown reservoir of drug targets. Previous studies have described the value of exosomal PD-L1 as a predictor for anti–PD-1 therapy in patients with melanoma via blood analysis.[32] The findings of this study further support our findings of TACs, setting a foundation to analyze exosomes or other extracellular vesicles as they relate to TACs and how both blood-based analytes might be used to better determine predictive values of PD-L1 therapies through liquid biopsies. Irregardless, these data suggest that biological changes of PD-L1 in TACs can be assessed in real time, and a liquid biopsy assay may provide a noninvasive method to sequentially monitor cancer-associated cells in circulation and possibly predict patient outcomes. Although preliminary, this liquid biopsy–based result appears to identify patients with rmNSCLC who are likely receiving clinical benefit from ICI and potentially steer clinicians toward changes in therapy for the patients who are not benefiting from ICI, though larger studies are necessary to verify and validate these preliminary results.

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PRIOR PRESENTATION

Presented in abstract form during General Poster Session of ASCO Annual Meeting, Chicago, IL, June 6, 2022.

SUPPORT

Supported by in part by MD Anderson Cancer Center, University of Maryland Greenebaum Cancer Center, Genentech, a R43 NCI-NIH (grant number R43CA206840), and the Defense Advanced Research Projects Agency (DARPA; grant number W911NF-14-C-0098). The costs of publication of this article were paid for by Creatv MicroTech, Inc.

DATA SHARING STATEMENT

The data generated in this study are not publicly available as to not compromise patient privacy (ie, dates of birth, dates of death, etc) but are available on reasonable request from the corresponding author.

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Final approval of manuscript: All authors
Accountable for all aspects of the work: All authors

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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Patents, Royalties, Other Intellectual Property: Patent application for radiopharmaceutical to treat small cell lung cancer (Inst)
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Travel, Accommodations, Expenses: STCube Pharmaceuticals Inc, Elekta

No other potential conflicts of interest were reported.

ACKNOWLEDGMENT

We would like to thank all the patients who contributed to this study.

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