Clinical Cancer Research

Tumor Characteristics Associated with Benefit from Pembrolizumab in Advanced Non–Small Cell Lung Cancer

Siwen Hu-Lieskovan[1,2], Aaron Lisberg[1], Jesse M. Zaretsky[1], Tristan R. Grogan[1], Hira Rizvi[3], Daniel K. Wells[2], James Carroll[1], Amy Cummings[1], John Madrigal[1], Benjamin Jones[1], Jacklin Gukasyan[1], I. Peter Shintaku[1], Dennis Slamon[1], Steven Dubinett[1], Jonathan W. Goldman[1], David Elashoff[1], Matthew D. Hellmann[3,4], Antoni Ribas[1,2], and Edward B. Garon[1]

Abstract

Purpose: Several biomarkers have been individually associated with response to PD-1 blockade, including PD-L1 and tumor mutational burden (TMB) in non–small cell lung cancer (NSCLC), and CD8 cells in melanoma. We sought to examine the relationship between these distinct variables with response to PD-1 blockade and long-term benefit.

Experimental Design: We assessed the association between baseline tumor characteristics (TMB, PD-L1, CD4, and CD8) and clinical features and outcome in 38 patients with advanced NSCLC treated with pembrolizumab (median follow-up of 4.5 years, range 3.8–5.5 years).

Results: PD-L1 expression and CD8 infiltration correlated with each other and each significantly associated with objective response rate (ORR) and progression-free survival (PFS). TMB was independent of PD-L1 and CD8 expression, and trended towards association with ORR and PFS. There was no association between CD4 infiltration and outcomes. Only PD-L1 expression was correlated with overall survival (OS). Among 5 patients with long-term survival >3 years with no additional systemic therapy, PD-L1 expression was the only discriminating feature. The increased predictive value for PFS and OS of composite biomarker inclusive of PD-L1, CD8, CD4, and TMB was limited.

Conclusions: In patients with NSCLC treated with PD-1 blockade with long-term follow up, TMB, PD-L1, and CD8 were each associated with benefit from PD-1 blockade. Pretreatment PD-L1 expression was correlated with T lymphocyte infiltration and OS, whereas models incorporating TMB and infiltrating CD4 and CD8 lymphocytes did not substantially add to the predictive value of PD-L1 alone for OS.

Introduction

The success of PD-1 checkpoint inhibition in treating patients with non–small cell lung cancers (NSCLC) is an important milestone in the history of cancer therapy (1). The hallmark of cancer immunotherapy is the durability of the tumor-specific immune response, but this durability has only been achieved in a minority of patients, highlighting the need for biomarkers to predict long-term response to therapy. Further, biomarkers can identify factors to help guide the study of the combination of immunotherapies (2).

Tumor PD-L1 expression is correlated with clinical benefit in NSCLC, and is now routinely used as a biomarker in clinical practice (3–8). Still, PD-L1 is an imperfect biomarker, as many high expressors are nonresponders, and responders with negative or low tumor PD-L1 expression are often observed. Tumor mutational burden (TMB) has also been associated with objective response rate (ORR) and progression-free survival (PFS) to PD-1 checkpoint inhibitors in NSCLC (9–12). Application of TMB in clinical practice requires ongoing efforts for harmonization of computation approaches for quantification, solutions for expeditious return of results, cost, and intra- and intertumoral heterogeneity. A correlation of TMB with overall survival (OS) in analyses to date is either not seen or limited by relatively short follow-up (11, 13).

Studies in melanoma patient–derived tumor specimens revealed that responses to PD-1/L1 blockade rely on pretherapy tumor infiltration of activated CD8 T-effector cells (14). The role of CD4 T lymphocytes in response to anti-PD1 therapy has not been well studied, with no clear correlation identified to date. In addition, no previous evaluation has examined the relationship between PD-L1, TMB, and infiltrating CD4 and CD8 T cells in a single patient cohort and the composite power of these biomakers

[1]David Geffen School of Medicine at the University of California Los Angeles, Los Angeles, California. [2]Parker Institute for Cancer Immunotherapy, San Francisco, California. [3]Memorial Sloan Kettering Cancer Center, New York, New York. [4]Weill Cornell Medical College, New York, New York.

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S. Hu-Lieskovan, A. Lisberg, and J.M. Zaretsky contributed equally in this article.

Corresponding Author: Edward B. Garon, David Geffen School of Medicine at UCLA, 2825 Santa Monica Blvd., Suite 200, Santa Monica, CA 90404. Phone: 310-586-2098; Fax: 310-586-0841; E-mail: egaron@mednet.ucla.edu

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Translational Relevance

Our analysis of baseline tumor characteristics (tumor mutational burden, PD-L1 expression, CD4 and CD8 infiltration) in 38 patients with non–small cell lung cancer (NSCLC) treated by pembrolizumab with long-term follow-up indicated a significant correlation of tumor PD-L1 expression with tumor infiltrating lymphocytes, response, and progression-free/overall survival, especially among long-term survivors (overall survival longer than 3 years without need of subsequent systemic therapy). Although the cohort is small, it is the largest reported cohort that evaluates all 4 tumor characteristics in the same treated with anti-PD-1 therapy with long-term follow up. These data help to advance our understanding of response to PD-1 checkpoint inhibitors and guide selection of patients most likely to experience long-term benefit from therapy.

to predict long-term outcomes in patients with NSCLC treated with PD-1 checkpoint inhibitors.

Materials and Methods

Study design and treatment

Patients were identified with advanced NSCLC treated at 1 of 2 institutions [University of California, Los Angeles (UCLA) and Memorial Sloan Kettering Cancer Center (MSK)] with pembrolizumab as part of KEYNOTE-001 (3). The study was performed in accordance with the Declaration of Helsinki and informed written consent was obtained from each subject, or each subject's guardian, prior to enrollment on trial. The patient eligibility criteria, study schema, and treatment schedules have been previously described.

All patients were consented to institutional review board approved protocols for tissue banking and sample analysis. Efficacy was determined by investigator assessed immune-related response criteria (irRC), with imaging performed per protocol every 9 weeks. PFS and OS were defined from the date the patient began pembrolizumab. Patients who had not progressed/still alive were censored for PFS at the date of the last scan and for OS at the date of last contact.

Whole-exome sequencing

Tissue from 25 patients was used for whole-exome sequencing (WES). DNA was extracted and quality controlled from tumor and patient-matched blood or other noncancerous tissue. Data for 10 patients were performed as described previously (9). For the additional 15 patients, WES was performed at the UCLA Clinical Microarray Core using the Roche Nimblegen SeqCap EZ Human Exome Library v3.0 targeting 65 Mb of genome. Paired-end (2 × 100 or 2 × 150 base-pair) sequencing of the enriched exome libraries was performed on a HiSeq platform (Illumina) to a goal mean depth of 150× overtargeted regions. Reads were aligned to genome build GRCH37 with bwa 0.7.12, followed by duplicate removal (Picard Tools 1.137), indel realignment, and base recalibration using the Genome Analysis Toolkit (GATK v3.4, Broad Institute) with reference files from the b37 GATK resource bundle.

Tumor content was assessed from sequencing data using Sequenza (v2.1.2, http://www.cbs.dtu.dk/biotools/sequenza/). Those below Sequenza's minimum sensitivity of 30% tumor cellularity were assessed for tumor content by IHC. Cases were only included if they met sufficient overall quality criteria for coverage (>50× tumor and >30× normal) and tumor content (>10%).

Mutation calling from mapped BAM files of all 25 samples was performed by a uniform pipeline incorporating MuTect (v1.1.7), Varscan (v2.3.9), and a Fisher exact test of alternate read-counts between tumor/normal samples from calls made by the GATK Haplotypecaller (v3.4, jointly genotyped at the cohort level from gvcfs), as described previously (15, 16). Variant sites were considered if identified by 2 of 3 programs, covered by a minimum 10 reads in both the tumor and normal sample, and if the variant allele was supported by at least 4 reads. Functional consequence of mutations was determined using Oncotator (Broad Institute, v1.5, Dec112014 data corpus). Only nonsynonymous mutations (Nonsense, Missense, Splice_Site, Frameshift indels, In-frame indels, Start_Codon indels or SNPs, and Stoploss/Nonstop variants) were counted toward tumor mutational burden to minimize differences between exon-capture kits. A final filter was applied to exclude variants at sites of known germline variation with a population allele frequency >0.0005 in the Exome Aggregation Consortium (ExAC) database v0.3.1.

HLA typing was performed on 25 patients in the correlative cohort. Of these patients, 5 had HLA typing previously performed (17). For the remaining 20 patients, HLA zygosity was determined by inference of HLA alleles from whole exome sequencing by ATHLATES as previously described (17).

IHC analyses

Patients with adequate, nonlymph node pretreatment FFPE tissue samples were stained with hematoxylin and eosin, anti-CD4, anti-CD8, anti-PD-L1, anti-CD45, and anti-FOXP3 at the UCLA Anatomic Pathology Immunohistochemistry and Histology Laboratory (CLIA-certified). Readers were blinded to patient outcomes. Antibodies used included rabbit polyclonal CD4 (Clone SP35, 1:100 dilution, low pH retrieval; Cell Marque), CD8 (clone C8/144B, 1/100, low pH retrieval; DAKO), PD-L1 (SP142, 1/200 dilution with High pH retrieval Spring Biosciences), CD45 (Clone 2B11+PD7/26, 1:600 dilution, low pH retrieval; DAKO), and FOXP3 (Catalog no. 14-4776-82, 1:200 dilution, high pH retrieval; eBioscience). IHC was optimized and performed on Leica Bond III autostainer using Bond ancillary reagents and Refine Polymer Detection system. Slides were examined for the presence of CD4, CD8, and PD-L1 within the tumor parenchyma. All slides were scanned at an absolute magnification of ×200 (resolution of 0.5 5 μm mu;m per pixel). The percentage of positively IHC stained cellularity against all nucleated cells including both tumor cells and stromal cells/immune infiltrates (% positive cells/all nucleated cells) was calculated using the Halo platform (Indica Labs; ref. 18). Although the primary analysis was based on all nucleated cells, a secondary analysis evaluated whether the PD-L1 staining was tumor predominant or immune infiltrate predominant by calculating the percentage of positively stained cells in the tumor by H&E using 50% as the cutoff (i.e., if more than 50% of the PD-L1 expression comes from the tumor cells, then it is defined as tumor predominant). A subset of specimens for which slides were available were also evaluated with CD45 to verify the identity of cells described as immune cells by H&E was CD45 positive. Tumors from lymph nodes were not used in this analysis due to the inability to differentiate antitumor

versus resident immune cells and the high background PD-L1 expression. Of note, PD-L1 expression reported in this analysis is distinct from the PD-L1 testing performed as part of KEYNOTE-001; testing was re-done here to permit analysis of multiple marker expression from a single tissue sample, stained at the same time. In addition, it should be recognized that SP142 was used for the PD-L1 IHC analysis performed using an optimized semimanual staining procedure, different from the commercial kit developed for this antibody.

Statistical analysis

Patient characteristics were summarized descriptively using median (min/max or Q1/Q3) or frequencies (percentages). Survival curves were plotted using the Kaplan–Meier method and compared between groups using the log-rank test. Comparisons between response groups and TMB, PD-L1, CD8, CD4 were assessed using the Wilcoxon test for 2 groups or the Kruskal–Wallis test for 3 groups. The ORR was reported as proportion along with Clopper–Pearson exact CIs. The chi-square or Fisher exact test were used to test for differences between groups for categorical variables. Associations between continuous or ordinal measures were assessed using the Spearman rank correlation coefficients. These analyses were exploratory and not powered for statistical comparison across subgroups. Using the planned significance level of 0.05, each group of primary analyses was estimated to have a FDR no more than 12%.

Univariable and multivariable Cox regression models for PFS and OS were constructed using TMB, CD8, PD-L1, and CD4 with all combinations of markers (including interaction terms) to identify the most predictive model. In order to assess the prognostic ability of each of these models, the survival c-statistic for survival models was computed (19). Similar methodology was carried out for the binary outcome (PR vs. SD/PD per irRC) using logistic regression models. These regression analyses were exploratory given the limited sample size.

All tests were 2-sided; P values < 0.05 were considered statistically significant. The FDRs associated with the primary analyses was estimated using the Benjamini–Hochberg step-up procedure. Statistical analyses were performed using GraphPad Prism and R v3.3.3 software (www.r-project.org).

Results

Demographics of the correlative cohort

We identified 38 patients (33 from UCLA; 5 from MSKCC) with available baseline fresh or archival tumor adequate for WES and IHC studies including PD-L1, CD8, and CD4. Best response in this cohort was partial response in 16 patients (42%), stable disease in 10 patients (26%), and progressive disease in 12 patients (32%; Table 1). Thirty-two patients had tissue for PD-L1, CD8, and CD4 evaluation and 25 patients had TMB determined. Twenty-one patients had a complete set of all 4 parameters evaluated.

Demographic features of the correlative cohort are largely representative of the clinical cohort [all patients with NSCLC treated in KEYNOTE-001 trial at UCLA ($N = 97$, Table 1)] except a higher percentage of responders. The population treated at UCLA was generally similar to the overall study population with the exception of a greater percentage of *EGFR*-mutation positive patients treated at UCLA (31%) compared with the total study population (15%; ref. 20). Median follow-up in the UCLA clinical cohort was 4.4 years (range 38–1,995 days), based on an internal database lock on December 31, 2017. A total of 12 patients had an OS duration ≥3 years (up to 65.6 months). Of those, 7 long-term benefiters survived >3 years from the initial dose of pembrolizumab with no additional systemic therapy after pembrolizumab (Table 1), whereas 5 received additional systemic therapy after pembrolizumab.

Table 1. Patient characteristics of all patients, patients included in the correlative cohort, and long-term benefiters (5 of the 7 LTBs are in the correlative cohort)

Characteristics	UCLA clinical cohort ($N = 97$)		Correlative cohort ($N = 38$)		Long-term benefiters ($N = 7$)	
Age-year						
Median	65		67.5		59	
Range	(32–83)		(48–82)		(51–68)	
Gender, no. (%)						
Male	50	52%	22	58%	6	86%
Female	47	48%	16	42%	1	14%
Previous therapy lines, no. (%)						
0	13	13%	8	21%	2	29%
1–3	61	63%	20	53%	5	71%
>3	23	24%	10	26%	0	0%
Smoking status, no. (%)						
Ever	54	56%	21	55%	6	86%
Never	43	44%	17	45%	1	14%
PD-L1 proportion score by Merck, no. (%)						
Unknown	21		4		3	
<1	21	22%	10	29%	0	0%
1–49	38	39%	15	44%	1	25%
≥50	17	17%	9	27%	3	75%
Histology, no. (%)						
Nonsquamous	78	80%	30	79%	5	71%
Squamous	19	20%	8	21%	2	29%
Targetable mutations, no. (%)						
EGFR mutation	30	31%	9	24%	0	0%
ALK translocation	2	2%	1	3%	0	0%
Best response, no. (%)						
PR	20	21%	16	42%	6	86%
SD	28	29%	10	26%	1	14%
PD	47	48%	12	32%	0	0%

The median PD-L1 expression was 26.5% (range of 0.5–98). PD-L1 was ≥ 50% in 9 of 32 patients (28%), similar to other reports (5). PD-L1 level was correlated with prior smoking history ($P = 0.04$) and squamous histology ($P = 0.025$), but not *EGFR* mutational status ($P = 0.271$; Fig. 1; Supplementary Table S1). Median TMB was 104 (range 9–1,616) and TMB was significantly correlated with smoking status ($P = 0.008$) but not with other clinical features. The median CD8 and CD4 T-cell infiltration was 4.5% and 3.0%, respectively (ranges 0%–23% and 0%–31%). CD8 infiltration was numerically higher in patients who were smokers ($P = 0.06$; Fig. 1) and those who were treatment naïve ($P = 0.03$; Supplementary Table S3). Specifically, when CD8 was analyzed as categorical variable, patients with at least one prior line of therapy were more likely to have low (<5%) CD8 than treatment naïve patients (65.4% vs. 16.7%, $P = 0.030$; Supplementary Table S3).

Individual variables and clinical benefit

ORR by irRC. PD-L1 expression was significantly higher in the responders compared with those with SD/PD group (median 66% vs. 15%, $P = 0.002$). Although the primary PD-L1 analysis

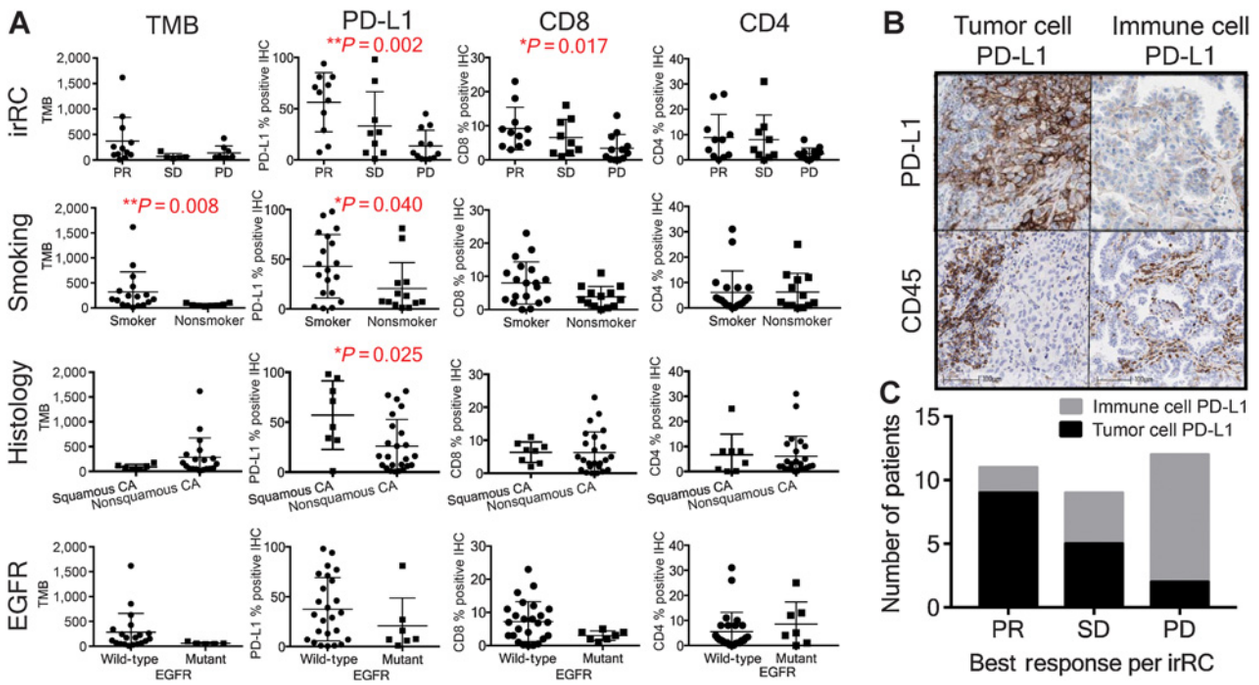

Figure 1.

Correlative factors and clinical characteristics. **A,** Scatter plot of correlative factors against clinical characteristics. **B,** Examples of PD-L1 expression on tumor vs. immune cells. **C,** Baseline PD-L1 expression in tumor vs. immune cells by best response per irRC ($P = 0.007$, Fisher exact test).

assessed all nucleated cells, 2 patterns were seen based on whether the majority of PD-L1 positive cells were in the tumor- or immune-infiltrating cells. To confirm the accuracy of this assessment, a subset were evaluated by CD45 staining to confirm the assessment of immune cells as shown in Fig. 1B. There was an association between responses and the majority of PD-L1 expression being on tumor cells ($P = 0.007$, Fisher exact test; Fig. 1C). A trend was observed of higher TMB in responders compared with those with SD/PD (median 189 vs. 55, $P = 0.08$). Of the 25 patients with WES data available for analysis, only 2 exhibited HLA homozygosity and there was no correlation found between HLA zygosity and response (data not

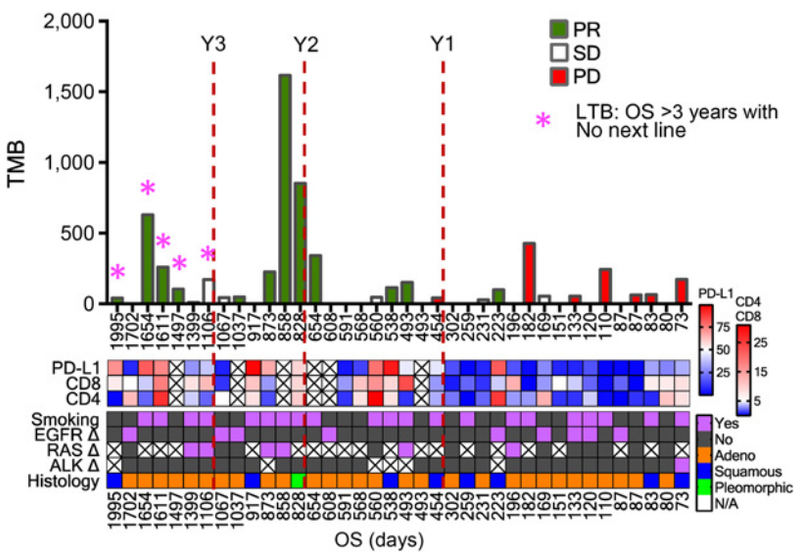

Figure 2.

Schematic presentation of the analyzed parameters in the correlative cohort per OS. TMB, total mutational burden; PR, partial response; SD, stable disease; PD, progressive disease; Δ, mutation; LTB, long-term benefiter; Y1, year 1; Y2, year 2; Y3, year 3.

Table 2. Hazard Ratio of PFS, OS, and ORR in all categories of clinical and tumor characteristics

	PFS		OS		ORR (PR vs. SD/PD)	
Characteristics	**HR (95% CI)**	***P* value**	**HR (95% CI)**	***P* value**	**OR (95% CI)**	***P* value**
TMB	1.00 (0.99–1.00)	0.065	1.00 (1.00–1.00)	0.775	1.00 (0.99–1.00)	0.127
PD-L1	0.98 (0.96–0.99)	0.002	0.99 (0.97–1.00)	0.032	0.96 (0.93–0.99)	0.007
CD8	0.90 (0.83–0.99)	0.026	0.94 (0.87–1.02)	0.131	0.86 (0.74–1.00)	0.052
CD4	0.96 (0.90–1.01)	0.120	0.98 (0.93–1.03)	0.397	0.94 (0.85–1.03)	0.184
Age	1.00 (0.96–1.04)	0.992	0.99 (0.95–1.03)	0.487	0.98 (0.91–1.05)	0.498
Male	0.97 (0.46–2.02)	0.930	0.92 (0.46–1.87)	0.825	1.12 (0.31–4.14)	0.861
EGFR mutations	1.56 (0.68–3.61)	0.298	1.08 (0.48–2.43)	0.846	1.38 (0.28–6.70)	0.694
ALK	–	–	–	–	–	–
EGFR or ALK	1.85 (0.82–4.18)	0.138	1.27 (0.58–2.77)	0.548	1.71 (0.36–8.15)	0.500
Ever smoker	0.83 (0.40–1.71)	0.613	1.02 (0.51–2.06)	0.955	0.93 (0.26–3.41)	0.917
Squamous	1.52 (0.65–3.59)	0.338	1.36 (0.58–3.15)	0.480	1.28 (0.26–6.33)	0.767
≥One line therapy	1.19 (0.51–2.79)	0.689	1.46 (0.60–3.58)	0.403	2.88 (0.57–14.44)	0.199

shown). CD8 cell infiltration was significantly higher in responders compared with patients in the SD/PD group (median 8% vs. 3%, $P = 0.02$). CD4 infiltration was not different between responders and nonresponders (median 8% vs. 2%, $P = 0.17$; Fig. 1A and 2; Supplementary Fig. S1; Supplementary Table S2). Additional FOXP3 staining in 6 available cases with CD4 infiltration suggested Tregs were less than 5% of the $CD4^+$ cells in these cases (data not shown).

PFS and OS. Consistent with prior reports, baseline tumor PD-L1 expression was associated with improved PFS ($P = 0.002$), as well as with improved OS assessed either as a continuous or categorical variable (PD-L1<50% vs. ≥50%; $P = 0.03$; Table 2; Figs. 2 and 3; Supplementary Table S3; Supplementary Fig. S1 and S2). When assessed as a continuous variable, TMB showed a trend towards improved PFS (HR =1; 95% CI, 0.99–1.00; $P = 0.065$). When assessed by quartiles, increasing TMB correlated with improved

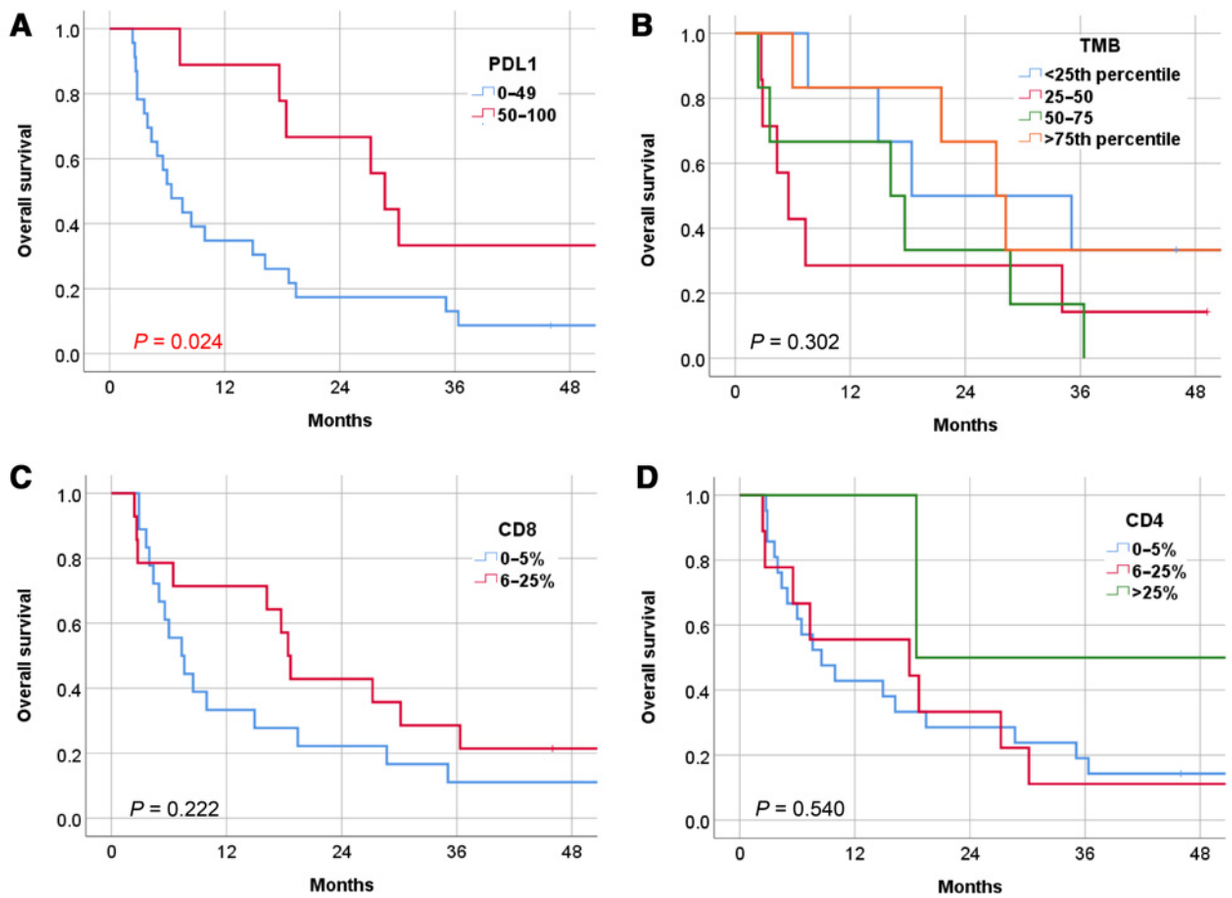

Figure 3.
OS and subgroup survival analysis of the correlative cohort ($N = 38$) by log-rank (Mantel–Cox) test. **A,** OS By PD-L1 categories (0%–49% $n = 23$ vs. >49% $n = 9$, $P = 0.024$). **B,** OS By TMB percentile (<25th percentile $n = 6$ vs. 25–50th percentile $n = 7$ vs. 50–75th percentile $n = 6$ vs. >75th percentile $n = 6$, $P = 0.302$). **C,** OS By CD8 categories (0%–5% $n = 18$ vs. 5%–25% $n = 14$, $P = 0.222$). **D,** OS By CD4 categories (0%–5% $n = 21$ vs. 5%–25% $n = 9$ vs. >25% $n = 2$, $P = 0.540$).

Table 3. Univariate/multivariate models for PFS, OS, and best response (BR) using combinations of TMB, PD-L1, CD8, and CD4

	PFS	OS	ORR (PR vs. SD/PD)
Univariate models			
($n = 25$ or $n = 32$)	**c-stat**	**c-stat**	**c-stat**
TMB ($n = 25$)	0.64	0.51	0.71
CD8 ($n = 32$)	0.64	0.59	0.76
PD-L1 ($n = 32$)	0.69	0.62	0.82
CD4 ($n = 32$)	0.58	0.54	0.65
Univariate models (have both, $n = 21$)	**c-stat**	**c-stat**	
TMB ($n = 21$)	0.63	0.54	0.65
CD8 ($n = 21$)	0.64	0.61	0.77
PD-L1 ($n = 21$)	0.75	0.66	0.91
CD4 ($n = 21$)	0.55	0.52	0.57
Two variable models ($n = 21$ for all)	**c-stat**	**c-stat**	**c-stat**
TMB	0.75	0.66	0.92
PD-L1			
TMB	0.71	0.62	0.79
CD8			
PD-L1	0.71	0.63	0.89
CD8			
TMB	0.65	0.55	0.62
CD4			
CD8	0.64	0.59	0.78
CD4			
PD-L1	0.71	0.62	0.95
CD4			

PFS ($P = 0.02$; Table 2; Supplementary Table S4), similar to previous publications (9, 12), which was mostly driven by the top quartile (Supplementary Fig. S2). However, in this limited dataset, TMB was not associated with OS (Table 2; Fig. 2). Baseline tumor CD8 (but not CD4) infiltration was significantly higher in patients with longer PFS when assessed as a continuous variable ($P = 0.026$; Table 2; Fig. 2) and with longer OS when assessed as a categorical variable ($P = 0.048$; Supplementary Table S4).

Relationship between biomarker variables. Consistent with previous data (10–12), TMB and PD-L1 were not correlated with each other (Spearman $\rho = 0.19$; $P = 0.406$; Supplementary Table S1; Supplementary Fig. S3). No significant correlation was found between TMB and infiltrating CD4 or CD8 lymphocytes. PD-L1 was correlated with CD8 (Spearman $\rho = 0.66$; $P < 0.001$) and CD4 expression (Spearman $\rho = 0.48$; $P = 0.005$).

Multivariable modeling. In univariate/multivariable analysis of PFS, OS, and ORR using combinations of TMB, CD8, PD-L1, and CD4, univariate PD-L1 expression had the highest c-index for benefit prediction either when all 38 correlative tumors were evaluated or when only the 21 tumors with all 4 parameters available were assessed (Table 3). Additional combinations of 2, 3, or 4 variables (data not shown) and 2 variables with interaction modeling did not significantly increase the predictive value as compared with single variable PD-L1(Table 3).

Characterization of long-term survivors. Five patients had long-term benefit, defined as survival >3 years from the initial dose of pembrolizumab with no additional systemic therapy. PD-L1 expression $\geq$50% was seen in 4 of these 5 patients (80%, 3 was evaluated at UCLA, and 1 was evaluated by Merck proportion score) and the fifth had 39% PD-L1 expression. Out of all correlative analyses assessed, only baseline PD-L1 expression level was significantly higher in the long-term benefiter group compared with the remaining patients in the correlative cohort (median of 72% vs. 16%; $P = 0.029$; Fig. 2; Supplementary Table S5).

Discussion

Checkpoint inhibitors unleash a patient's immune system to fight cancer and have transformed the management landscape of NSCLC. It is an exciting proof of principle that cancer immunotherapy can be effective and durable beyond indications traditionally considered immunotherapy sensitive (21). However, only a minority of patients benefit, and data on long-term benefit is particularly lacking, as correlative analyses are typically generated on patients with immature follow-up based on surrogate endpoints, rather than OS.

T-cell–based antitumor response can be influenced by many factors in different immune compartments, including tumor foreignness from the normal counterpart, sensitivity to effectors, the tumor immune suppressive contexture, T-cell priming, and activation as well as exhaustion status (21, 22). Developing reliable biomarkers to predict response to checkpoint inhibitors is critical in selecting the most effective therapy to maximize clinical benefit. Our study in a NSCLC cohort treated with pembrolizumab on KEYNOTE-001 with long-term follow up suggest that baseline PD-L1 expression correlates with lymphocyte infiltration in NSCLC, and in our data, is the most reliable biomarker to predict survival with PD-1 checkpoint blockade.

High PD-L1 expression in tumors can be either constitutive due to a genetic alteration, or induced by IFNγ released by activated immune cells (23). The latter scenario is a strong indication that an active antitumor immune response blocked by the PD-1 checkpoint is occurring. Indeed in our study, high PD-L1 expression is significantly correlated with ORR, PFS, OS, squamous histology, and history of smoking. It is also highly correlated with the tumor-infiltrating lymphocytes (both CD4 and CD8 cells), indicating an active adaptive immune response. Although our primary PD-L1 analysis looked at all nucleated cells, to address questions regarding PD-L1 staining in infiltrating immune cells versus tumor cells, we sought differences in response based on location of PD-L1 staining, showing that tumor PD-L1 staining was the most relevant in our dataset. In addition, PD-L1 is the only parameter that correlated with long-term benefit from pembrolizumab, and multivariate modeling did not indicate much improved predictive value with the addition of other parameters. Of note, all of the long-term benefiters had high pretreatment PD-L1 expression (>50% in 4 cases and 39% in the remaining 1 case).

The efficacy of PD-1 checkpoint inhibitors is hypothesized to rely on a preexisting antitumor response, which is specifically blocked by the PD-1 checkpoint (23, 24). PD-L1, and to some extent infiltrating CD4 and CD8 lymphocytes, serve as a surrogate marker for this scenario. TMB assesses the neoantigens that could potentially be recognized by a patients' immune system. Indeed, the median TMB of different histologies in which anti-PD1/L1 therapies have been approved has a nearly linear relationship with their corresponding response rates (18, 25). Both PD-L1 and TMB have been associated with ORR and PFS in NSCLC, but these 2 biomarkers are assessing different things as evidenced in our study by the observed lack of association of TMB with PD-L1 expression or infiltrating CD4 and CD8 lymphocytes. The lack of correlation of TMB with an active immune response could be responsible for the inability to use TMB to predict OS to date, although other data sets assessing TMB have immature follow-up for OS (11, 13).

Limitations of our study, in addition to relatively small sample size, include a cohort with higher ORR relative to unselected NSCLC population and a lower TMB than has been seen in other studies. Although partly explained by more conservative mutation calling methods, another possible reason could be the presence of more never smokers, particularly patients with *EGFR* mutant tumors. This could decrease the generalizability of our data to a more typical NSCLC patient population. Yet, as inferior outcomes have been noted with *EGFR* mutant disease treated with a PD-1 inhibitor as compared with *EGFR* wild-type disease (26), the lack of OS correlation with TMB despite the inclusion of *EGFR* mutation positive patients could also be considered an expected outcome. Another potential limitation is that our study utilized the SP142 antibody to assess PD-L1, rather than the 22C3 antibody, which is more commonly used in clinical practice. Of note our staining methods used a semimanual procedure optimized in our laboratory, and the (at the time) commercially available SP142 antibody did not perform less well than 22C3 using this procedure. Our protocol is different from the commercial kits using automated strainers, which was utilized in the Blueprint Programmed Death Ligand 1 (PD-L1) Immunohistochemistry (IHC) Assay Comparison Project (27). In addition, we previously evaluated the relationship between the percentage of PD-L1–stained tumor cells with 22C3 and SP142, as well as the association between the PD-L1 levels identified by each antibody and clinical outcomes in patients from this same correlative cohort, finding excellent concordance between both antibodies, which somewhat mitigates concern regarding the use of SP142 (28). Also, although in cutaneous melanoma, pretreatment high CD8 infiltration in the tumor invasive margin correlates with clinical response to anti-PD1 therapy (14), we could not adequately assess invasive margins as the majority of the samples were from core biopsies. Despite our efforts, we were not able to see a strong value to adding the other markers to PD-L1 to predict OS. In the case of infiltrating CD4 and CD8 lymphocytes, this was because the markers were highly correlated with PD-L1. In the case of TMB, this could be due to the small sample size, and of note, increasing predictive value of the composite of PD-L1 plus TMB has been reported in other larger studies for outcomes including ORR and PFS (10, 12, 13). Interestingly, although CD4 lymphocyte infiltration was associated with CD8 and PD-L1 expression, it did not show correlation with response or survival to pembrolizumab. It is possible that the functionality of CD4 does not depend on numbers, or the primary location of functionality is not in the tumors. Further studies with multiplex immunofluorescent staining or nanostring are required to further elucidate the tumor milieu and the mechanism of response to PD-1 blockade.

In conclusion, long-term follow up of patients with NSCLC treated with pembrolizumab demonstrated the robustness of pretherapy PD-L1 expression to predict OS, including long-term benefit. Models incorporating TMB and infiltrating CD4 and CD8 lymphocytes did not substantially add to the predictive value of PD-L1 alone for OS. Whether the addition of other therapies, such as chemotherapy or immunotherapies including CTLA-4 inhibitors, to PD-1 checkpoint inhibitors will change the relative predictive benefit from these biomarkers will be learned from emerging data in ongoing or recently completed clinical trials.

Disclosure of Potential Conflicts of Interest

S. Hu-Lieskovan is a consultant/advisory board member for Amgen, Merck, and Genmab. A. Lisberg reports receiving commercial research grants from Dracen Pharmaceuticals, other commercial research support from Daiichi Sankyo, Calithera Biosciences, and AstraZeneca, and is a consultant/advisory board member for AstraZeneca, Bristol-Myers Squibb, and Leica Biosystems; he also reports that an immediate family member is an employee of and holds ownership interest (including patents) in Boston Scientific. J.M. Zaretsky is an employee of and holds ownership interest (including patents) in PACT Pharma. D.K. Wells is an employee of, holds ownership interest (including patents) in, and is a consultant/advisory board member for Immunai. D.J. Slamon reports receiving commercial research grants, speakers bureau honoraria, and is a consultant/advisory board member for Pfizer and Novartis, and holds ownership interest (including patents) in Pfizer and BioMarin. S.M. Dubinett reports receiving commercial research grants from Johnson & Johnson, and is a consultant/advisory board member for Johnson & Johnson, T-Cure Bioscience, Inc., EarlyDx, Inc., and Cynvenio Biosystems, Inc. J.W. Goldman reports receiving commercial research grants from Merck Pharmaceuticals, Bristol-Myers Squibb, Roche/Genentech, Pfizer, Vaccinex, and Astra Zeneca/Medimmune, speakers bureau honoraria from Merck Pharmaceuticals, and is a consultant/advisory board member for Astra Zeneca. M.D. Hellmann reports receiving commercial research grants from Bristol-Myers Squibb, holds ownership interest (including patents) in Shattuck Labs and a patent filed by MSK related to the use of tumor mutation burden to predict response to immunotherapy (PCT/US2015/062208), which has received licensing fees from PGDx, and is a consultant/advisory board member for Merck, Bristol-Myers Squibb, AstraZeneca, Genentech, Janssen, Nektar, Syndax, Mirati, and Shattuck Labs. A. Ribas holds ownership interest (including patents) in Lutris, Tango, PACT Pharma, Advaxis, Arcus, Bioncotech, Compugen, Cytomix, Five Prime, FLX-Bio, Imaginab, Isolplexis, Kite-Gilead, Merus, and Rgenix, and is a consultant/advisory board member for Amgen, Chugai, Genentech, Merck, Novartis, and Roche. E.B Garon is a consultant/advisory board member for Dracen and EMD Serono. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: S. Hu-Lieskovan, A. Lisberg, D. Slamon, M.D. Hellmann, E.B. Garon

Development of methodology: S. Hu-Lieskovan, A. Lisberg, I.P. Shintaku, E.B. Garon

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Hu-Lieskovan, A. Lisberg, J.M. Zaretsky, H. Rizvi, J. Carroll, J. Madrigal, I.P. Shintaku, J.W. Goldman, M.D. Hellmann, A. Ribas, E.B. Garon

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Hu-Lieskovan, A. Lisberg, J.M. Zaretsky, T.R. Grogan, D.K. Wells, A. Cummings, J.W. Goldman, D. Elashoff, M.D. Hellmann, A. Ribas, E.B. Garon

Writing, review, and/or revision of the manuscript: S. Hu-Lieskovan, A. Lisberg, J.M. Zaretsky, T.R. Grogan, H. Rizvi, A. Cummings, S. Dubinett, J.W. Goldman, D. Elashoff, M.D. Hellmann, A. Ribas, E.B. Garon

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Hu-Lieskovan, A. Lisberg, J. Carroll, J. Madrigal, B. Jones, J. Gukasyan, I.P. Shintaku, D. Slamon, E.B. Garon

Study supervision: S. Hu-Lieskovan, A. Lisberg, M.D. Hellmann, E.B. Garon

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