**Research Article**

**Tumor Infiltrating Lymphocyte Expression of PD-1 Predicts Response to Anti-PD-1/PD-L1 Immunotherapy**

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**ABSTRACT**

**Introduction:** Many studies have focused on the role of programmed death receptor ligand 1 (PD-L1) expression in predicting immunotherapy outcomes. Limited clinical data are available regarding the role of programmed death receptor 1 (PD-1; the PD-L1 receptor) expressing tumor-infiltrating lymphocytes (TILs) in PD-1/PD-L1 antibody responsiveness. However, preclinical studies demonstrate that TILs expressing PD-1 contribute to tumor immune evasion. **Methods:** This study analyzed the association between TIL-PD-1 status and outcome after immune checkpoint blockade (ICB) therapy. We evaluated 123 patients with various solid tumors treated with monoclonal antibodies targeting the PD-1/PD-L1 signaling axis. Additionally, 8706 solid tumor specimens were assessed for TIL-PD-1 and tumor mutational burden (TMB) status. **Results:** The presence of PD-1-expressing TILs in tumors was associated with increased median progression-free survival (7.0 vs 1.9 months; \( p = 0.006 \)) and overall survival (18.1 vs 8.0 months; \( p = 0.04 \)) after treatment with ICB. TIL-PD-1-positive patients had an objective response rate (ORR) of 41% (95% CI, 24–61; \( N = 12/29 \)) compared with 17% (95% CI, 4–43; \( N = 3/17 \)) for TIL-PD-1-negative patients (\( p = 0.18 \)). Analyzed as continuous variables, TIL-PD-1 and TMB showed a weak correlation in 8706 solid tumor samples (Pearson \( r = 0.074 \)); when analyzed as categorical variables (cutoffs: TIL-PD-1 \( \geq 1\% \) and TMB \( \geq 10 \) mutations/Mb), the two variables are correlated (\( p < 0.0001 \)). TIL-PD-1-positive status is also associated with enrichment of pathologic variants within several genes, most notably TP53 (adjusted \( p < 0.05 \)). **Conclusion:** TIL-PD-1 positivity in tumors (\( \geq 1\% \)) is associated with significantly longer progression-free and overall survival after ICB. [ClinicalTrials.gov ID: NCT02478931]

**Keywords:** immune checkpoint inhibition, tumor infiltrating lymphocyte, immunotherapy
INTRODUCTION

Immune checkpoint blockade (ICB) therapy, with antibodies targeting the PD-1/PD-L1 signaling axis, is a useful treatment strategy for diverse tumor types, as evidenced by an abundance of US Food and Drug Administration (FDA) approvals. However, despite improved tolerability compared with traditional cytotoxic chemotherapy, these treatments pose significant risks and are not universally efficacious. Thus, efforts have continued to refine the biomarker-based prediction of treatment efficacy.

The hunt for biomarkers has been driven by a paradigm presuming that, as tumors evolve from self-tissue into a neoplasm, they acquire features recognized by the immune system as ‘other’ (e.g., neoantigens) and must also evolve a mechanism to cloak themselves from the immune system’s ongoing surveillance. Tumor expression of PD-L1 is a key cloaking (or checkpoint) signal hiding a tumor from immune surveillance. Disrupting this signal is thought to drive the efficacy of PD-1/PD-L1 binding therapeutic antibodies. Direct observation of tumor PD-L1 expression by immunohistochemical methods has been shown to be a useful biomarker for response to treatment, albeit with some technical challenges in clinical use. Similarly, genomic markers, interpreted as surrogates for neoantigen formation, such as tumor mutational burden (TMB), microsatellite instability, and mismatch repair deficiency, have also shown utility as biomarkers.

Ultimately, tumors that effectively evade the immune system must do so by altering the local quality or quantity of immune effector cells. Such alterations are another potential source of biomarkers. Tumor-infiltrating lymphocytes (TILs) are a diverse set of immune effector cells sharing the common characteristics of being located in or near a tumor and having a stereotypic histologic appearance. In vitro and animal studies have shown that TILs expressing PD-1 are likely a useful biomarker for ICB efficacy because they are known to (1) actively shield tumors from immune surveillance and (2) have their tumor lysis capabilities directly inhibited by tumor cells preventing them from actively disrupting a tumor. To date, a handful of clinical studies have demonstrated that the presence of PD-1-expressing TILs correlates with patient response to PD-1/PD-L1 inhibitor therapy in non–small cell lung cancer (NSCLC), melanoma, and mixed tumor types.

To assess the predictive value of TIL-PD-1 expression as well as its correlation with tumor mutation burden, we examined a cohort of patients with solid tumors treated with anti–PD-1/PD-L1 checkpoint inhibitor therapy at the University of California Moores Center for Personalized Cancer Therapy, as well as a large group of patients from the Foundation Medicine database.

METHODS

Study Approval
All research described was approved by the institutional review board of the University of California at San Diego (UCSD) and followed the guidelines of the Declaration of Helsinki and the UCSD_PREDICT (ClinicalTrials.gov Identifier: NCT02478931) protocol and any investigational therapies for which the patients gave consent.

Patient Selection
For the response to therapy and survival analysis after anti–PD-1/anti–PD-L1 therapy, 123 patients with various solid tumor types (Table 1; Supplemental Fig. S1A, available online) were evaluated (biomarker and clinical data obtained between 2015 and 2020).

Regarding the correlation between TMB and TIL-PD-1 and TMB and TIL-PD-L1 biomarkers, 8706 and 13,740 patients with the described pairs of markers, respectively, from the Foundation Medicine database were included for analysis (Supplemental Fig. S1B) (The 123-patient, clinically annotated dataset from UCSD are a subset of the de-identified Foundation Medicine database).

Immunohistochemistry
Immunohistochemistry (IHC) was performed per manufacturer’s instructions in a Clinical Laboratory Improvement Amendments–certified and College of American Pathologists–accredited laboratory (Foundation Medicine) by trained pathologists. The staining was performed on archival formalin-fixed, paraffin-embedded tissue sections using commercially available antibodies (Cell Marque for PD-1 staining and Ventana SP142 for PD-L1 staining). The median age of archival samples was 67 days (range 8–7091). All cases had two accompanying controls in addition to the PD-1/PD-L1–stained patient slide, including a hematoxylin and eosin (H&E)-stained patient slide and a negative reagent control–stained patient slide. The PD-1/PD-L1–stained patient slides were evaluated for percentage of TILs, defined as the proportion of tumor area that is occupied by PD-1/PD-L1–staining intratumoral and peritumoral lymphocytes of any intensity. The pathologist’s assessment of PD-1/PD-L1 expression was then classified for a sample as either negative (<1% score) or positive (≥1% score) for all subsequent analyses.

Next-Generation Sequencing and Tumor Mutational Burden (TMB)

Hybrid capture-based next-generation sequencing (NGS) was done on all samples using a FoundationOne assay (315, 327, or 405 genes, contingent on the sequencing platform) (foundationmedicine.com). Validation and additional details of the FoundationOne NGS assay are previously described; the mean sequencing depth of coverage was greater than 250×, with >100× at >99% of exons. Concordance between sample replicates in the validation assessment was 97%, and no significant differences between interbatch and intra-
batch replicates were observed. Each assay run includes a control sample that is run in duplicate and is used as a positive mutation detection control. The sample will fail quality control if various germline single-nucleotide polymorphisms are not detected as expected.

The pathologic diagnosis of each tissue sample was confirmed by a review of hematoxylin and eosin–stained slides, and all specimens that advanced to DNA extraction had ≥20% tumor cells. TMB was calculated as the number of non-coding somatic mutations per megabase of genome sequenced.[25]

**Outcome and Statistics**

Responses were assessed by physician notation using RECIST criteria. Progression-free survival (PFS) and overall survival (OS) were calculated by the method of Kaplan-Meier (p values by log-rank test). PFS was defined as the time from initiation of the first line of immunotherapy until patient death. Patients who had not progressed or were alive, respectively, at last follow-up were censored on that date for PFS and OS. Data analysis was performed with R studio (rstudio.com) as described. Survival analyses were performed with the survival (cran.r-project.org/web/packages/survival) and survminer packages (cran.r-project.org/web/packages/survminer). Graphs were created using the ggplot function within the tidyverse package (cran.r-project.org/web/packages/tidyverse). A p ≤ 0.05 was considered significant.

**RESULTS**

### Demographics of UCSD Patient Cohort Treated With Immune Checkpoint Blockade (ICB)

Data analysis was performed on 123 patients with solid tumors treated with anti–PD-1/PD-L1 checkpoint

| Groups | Overall Study, N = 123 | TMB Measured, n = 113 | TIL-PD-1 Measured, n = 46 |
|--------|------------------------|-----------------------|--------------------------|
| Sex, n (%) |                         |                       |                          |
| Men    | 73 (60)                | 68 (60)               | 26 (57)                  |
| Women  | 50 (40)                | 45 (40)               | 20 (43)                  |
| Race or ethnicity, n (%) |                         |                       |                          |
| Black  | 4 (3)                  | 4 (4)                 | 1 (2)                    |
| Asian  | 8 (7)                  | 8 (7)                 | 3 (7)                    |
| Hispanic | 10 (8)               | 8 (7)                 | 3 (7)                    |
| Other or unknown | 2 (2)               | 2 (2)                 | 0 (0)                    |
| White  | 99 (80)                | 91 (81)               | 39 (85)                  |
| Age at diagnosis: mean (min–max)* | 61.2 (16.2–88.5) | 61.7 (22.0–88.8) | 58.5 (22.0–84.8)         |
| Age at ICB treatment start: mean (min–max)* | 64.8 (19.2–92.9) | 65.1 (24.3–90.7) | 61.7 (24.3–89.3)         |
| Diagnosis, n (%) |                       |                       |                          |
| Non–small cell lung cancer | 32 (26)               | 28 (25)               | 9 (20)                   |
| Head and neck squamous cell carcinoma | 19 (15)               | 18 (16)               | 3 (7)                    |
| Skin squamous cell carcinoma | 12 (10)               | 10 (9)                | 4 (9)                    |
| Melanoma | 10 (8)                | 10 (9)                | 2 (4)                    |
| Breast cancer | 6 (5)                 | 5 (4)                 | 3 (7)                    |
| Urothelial carcinoma | 6 (5)                 | 6 (5)                 | 4 (9)                    |
| GI squamous cell carcinoma | 5 (4)                 | 5 (4)                 | 3 (7)                    |
| Appendix adenocarcinoma | 4 (3)                 | 3 (3)                 | 0 (0)                    |
| Glioblastoma | 4 (3)                 | 3 (3)                 | 4 (9)                    |
| GI adenocarcinoma | 4 (3)                 | 4 (4)                 | 3 (7)                    |
| Basal cell carcinoma | 3 (2)                 | 3 (3)                 | 1 (2)                    |
| Hepatocellular carcinoma | 2 (2)                 | 2 (2)                 | 1 (2)                    |
| Renal cell carcinoma | 2 (2)                 | 2 (2)                 | 0 (0)                    |
| Other | 141 (11)               | 141 (12)              | 9 (20)                   |
| Treatment PD-1/PD-L1 targeted antibodies, n (%) |                       |                       |                          |
| Anti–PD-1 monotherapy | 96 (78)               | 87 (77)               | 35 (76)                  |
| Anti–PD-L1 monotherapy | 4 (3)                 | 4 (4)                 | 3 (7)                    |
| Anti–PD-1/PD-L1 combined with other immunotherapy | 4 (3)                 | 4 (4)                 | 0 (0)                    |
| Anti–PD-1/PD-L1 therapy combined with targeted agent(s) | 15 (13)               | 15 (13)               | 7 (15)                   |
| Anti–PD-1/PD-L1 therapy combined with chemotherapy | 4 (3)                 | 3 (3)                 | 1 (2)                    |
*Median value for age at diagnosis was 60 and for age at ICB treatment was 63 years; median number of prior therapies was 2.
†One case each of adrenal cortical carcinoma,† cervical squamous carcinoma,† cholangiocarcinoma, endometrial stromal sarcoma,‡ Kaposi sarcoma,‖ liposarcoma of retroperitoneum, maxillary sinus sarcoma,‖ ovarian adenocarcinoma,‖ papillary thyroid carcinoma, pleomorphic cell carcinoma of leg, prostate cancer,‖ salivary gland adenocarcinoma, spleen angiosarcoma,‖ urethral squamous cell carcinoma.† GI, gastrointestinal; ICB, immune checkpoint blockade; PD-1: programmed death receptor 1; TIL, tumor-infiltrating lymphocyte; TMB, tumor mutation burden (mutation/megabase).
Expression of PD-1 on TILs Predicted a Favorable Outcome After ICB

A subset (N = 46) of the overall patient population had IHC staining of their tumors performed to highlight PD-1 and PD-L1 staining on TILs. Overall, 29 patients had positive staining of PD-1 for TILs (defined as ≥ 1% of TILs staining positive for PD-1); subsequently, 17 patients had negative staining of PD-1 for TILs. The presence of PD-1-expressing TILs was associated with increased PFS (median 7.0 vs 1.9 months; p = 0.006; Fig. 1A) and increased OS (median 18.1 vs 8.0 months; p = 0.04, Fig. 1B) for TIL-PD-1–positive versus –negative. Among TIL-PD-1–positive patients, 2 had a complete response (CR) and 10 had a partial response (PR), leading to an objective response rate (ORR) of 41% (95% CI, 24–61; N = 12/29) compared with 0 patients with a CR, 3 with a PR leading to an ORR of 17% (95% CI, 4–43; N = 3/17) for TIL-PD-1–negative patients (p = 0.18). In contrast, the presence of PD-L1 expression by TILs was not associated with a significant difference in PFS (median 4.9 for TIL-PD-L1–positive [n=15] vs 6.5 months for TIL-PD-L1–negative patients [n = 33], p = 0.91) or OS (median 14.0 vs 14.1 months for TIL-PD-L1–negative patients, p = 0.83) (Supplemental Figs. S2A, B).

TMB ≥ 10 Mutations/mb Associated With Favorable Outcome After ICB

An additional subset (n = 113) of the overall patient population from UCSD treated with ICB had NGS-based TMB determined. Higher TMB (n = 49) (≥ 10 versus < 10 mutations/Mb) was associated with increased PFS (median 6.4 months vs 3.9 months, p = 0.008; Fig. 2A) and increased OS (median 21.0 vs 11.3 months, p = 0.003; Fig. 2B) compared with low TMB (n = 64). Among patients with TMB ≥ 10 mutations/Mb, 4 had CR and 14 had PR leading to an ORR of 36% (95% CI, 23–62; n = 18/49) versus 1 with a CR and 11 with a PR leading to an ORR of 19% in patients with TMB < 10 mutations/Mb (95% CI, 10–30; n = 12/64; p = 0.05).

TIL-PD-1 Correlation With TMB, Somatic Variants, and Tumor Type

To determine the relationship between TIL-PD-1 staining and TMB, we analyzed a larger cohort of 8706 unique clinical samples for which paired comprehensive genomic profiling and TIL-PD-1 expression were obtained during routine clinical care (Supplemental Fig. S1A). The Pearson r between the TIL-PD-1–staining percentage and TMB is 0.074, indicating that the two variables are not strongly correlated (when evaluated continuously; Fig. 3). When analyzed as categorical variables using the cutoffs described (TIL-PD-1 ≥ 1% and TMB ≥ 10 mutations/Mb) the two variables are correlated (odds ratio 2.3; 95% CI, 2.1–2.6; p < 0.0001).

TIL-PD-1 status was significantly associated with the enrichment of pathologic variants within several genes (Supplemental Fig. S3), most notably TP53; BRAF and VHL variants are also enriched in tumors with high TIL-PD-1 status. Low TIL-PD-1–associated variants included GATA3, IDH1, ESR1, TMPRSS2, MEN1, and ATRX (all in TMB low tissues; Supplemental Fig. S3). In addition, tumor types had a variable extent of TIL-PD-1 positivity and TMB ≥ 10 mutations/Mb (Supplemental Fig. S4).
The tumor types with the highest proportion of TIL-PD-1–positive specimens were diffuse large B-cell lymphoma (~97% TIL-PD-1–positive), bladder and peritoneum (~84%), and esophagus, head and neck, melanoma, and mesothelioma (~80%). As expected, the solid tumor type with the most frequent TMB/C21 mutations/Mb was melanoma.

DISCUSSION

The PD-1 receptor is an immune cell-specific surface inhibitor, mainly expressed in the late effector phase on activated CD4+/CD8+ T cells, B cells, natural killer T cells, monocytes, and antigen-presenting cells. Together with its ligands PD-L1 and PD-L2, the PD-1 receptor forms a group of immune checkpoint proteins that can dampen the development of the T-cell response, hence minimizing the possibility of autoimmune inflammation. Tumor cells exploit this checkpoint to evade detection and eradication by the immune system. Antibodies that block PD-1 or PD-L1 can therefore reactivate the immune system and have successfully treated some cancers, although only approximately 15% to 20% of all cancers. There has been a continuous search for biomarkers that predict benefits after ICB. The detection of PD-L1 (B7-H1) IHC on tumors or the combination of tumors, lymphocytes, and macrophages is a predictive biomarker, albeit one that is imperfect because it is confounded by technical and biological issues, including the fact that the reactivated immune system needs to be able to differentiate the malignant cell from its standard counterpart. The latter may be enabled by a high TMB.
and a host major histocompatibility complex (MHC) capable of tumor-specific neoantigen presentation, as well as the fact that the tumor may exploit other checkpoints or ligands to disable the immune response.[4,5,7,26]

Despite the importance of the PD-1 receptor on the lymphocytic populations that invade the tumor (TILS), few clinical studies have evaluated the predictive role of TIL-PD-1 expression for clinical benefit after ICB. Our data show that, in the pan-cancer setting, TIL-PD-1–positive staining by IHC is correlated with a significant increase in median PFS and OS when patients are treated with anti–PD-1/PD-L1 immune checkpoint inhibitors ($p = 0.006$ for PFS and $p = 0.04$ for OS). Other small published patient cohorts of NSCLC patients ($N = 21$)[27] and mixed tumor types ($N = 39$)[28] also demonstrated a correlation between TIL-PD-1 status and favorable response to anti-PD-1/PD-L1 inhibitor therapy.

Though our cohort is relatively small, the data suggest that it is conceivable that the effect size of TIL-PD-1 status on treatment outcome may be comparable to that of TMB. The observed increase in median PFS and OS with TIL-PD-1–positive versus –negative tumors of 5.1 months and 10.1 months, respectively, is similar to that seen with TMB $\geq 10$ versus $< 10$ mutations/Mb tumors (increased median PFS and OS of 2.5 and 9.7 months, respectively) in our patient cohort. In addition, the response rate of 41% for TIL-PD-1–positive tumors seen in our TIL-PD-1–positive cohort compared favorably with the reported response rate of 29% for TMB high ($\geq 10$ mutations/Mb) patients enrolled in the KEYNOTE-158 trial, albeit in slightly different patient populations.[29]

Theoretically, effective therapy with immune checkpoint inhibitors requires both an immunogenic tumor and a potent immune system capable of mounting an effective anti-tumor immune response. TMB is interpreted as a measure of tumor immunogenicity.[6,30] In vitro evidence indicates that TILs expressing PD-1 reflect the presence of a potentially effective immune response when checkpoint signaling is disrupted.[13,14] Analysis of our data in 8706 patient samples for TIL-PD-1 and TMB indicate that the two variables are very weakly correlated as linear variables ($r = 0.074$) (Fig. 3). However, when analyzed as categorical variables using the cutoffs described (TIL-PD-1 $\geq 1\%$ versus $< 1\%$ and TMB $\geq 10$ mutations/Mb versus $< 10$) the two variables are correlated (odds ratio 2.3; 95% CI, 2.1–2.6; $p < 0.0001$). Taken together, these data suggest that the two variables are linearly independent; cut points applied may produce a statistical artifact,[31] though a threshold effect for correlation cannot be ruled out.

Given the mechanistic independence of these two biomarkers, a combination of both should be more predictive of response to therapy than either marker alone. Consistent with this theory, patients with TIL-PD-1–positive/TMB high ($\geq 10$ mutations/Mb) tumors have the longest PFS and OS in our cohort when treated with checkpoint inhibitors (Fig. 4). In addition to their independent clinical utility, testing for both TIL-PD-1 status and TMB for predicting response would decrease the potential patient impact created by analytical challenges for either test. TMB determination methods have not been harmonized, and differing calculation methods can result in significant changes to TMB near clinical decision points.[4,32,33] A multifactorial approach to biomarker-guided immunotherapy would reduce the impact of analytical or biological uncertainty and
provide additional clinical utility compared with the use of any single biomarker.

One final observation of interest was the association of TP53 mutants with TIL-PD-1 positivity in TMB low patients (Supplemental Fig. S3). These patients might be susceptible to ICB despite their low TMB. Prior data has also suggested that differential expression of immunoregulatory molecules coincides with specific genomic alterations and that these data may be exploitable for therapeutic trial design. Our data also indicate that tumor types with the highest proportion of TIL-PD-1–positive specimens were diffuse large B-cell lymphoma (~97% TIL-PD-1–positive), bladder and peritoneum (~84%), esophagus, head and neck, melanoma, and mesothelioma (~80%) (Supplemental Fig. S4), making them potentially amenable to ICB treatment.

Our current study has several limitations. Despite the large cohort in the de-identified database examining correlations between TIL-PD-1 and TMB, the cohort of clinically curated patients was small, and the data accrued retrospectively. Further, TIL are comprised of lymphocytes specifically, although, in some studies, the TIL definition is expanded to include innate cell types, such as natural killer cells. Larger prospective studies are needed. The observations were made in the pan-cancer setting, suggesting their possible generalizability but limiting our ability to determine histologic impact. In addition, further histologic characterization of the spatial relationships between tumor and TILs and pan-TIL quantification were not feasible due to the limited data available for this study. Finally, because of the limited number of patients, we could not evaluate the association between PD-1 TIL and ICB as monotherapy or different cut-offs for PD-1 TIL positivity. Future studies are needed to assess the latter correlation.

Biomarkers of both tumor immunogenicity and immune system potency are continuing to evolve. For tumor immunogenicity, scoring systems incorporating the ability of tumor neoantigens to be presented by MHC proteins have shown promise in providing more precise measurements of tumor immunogenicity compared with TMB. For TIL potency, other cell surface or genetic markers may provide additional utility beyond PD-1 staining. Further studies with multiplexed biomarker identification and larger cohorts will be needed to refine biomarker-guided checkpoint inhibitor therapeutics.

**CONCLUSION**

In our cohort of 46 patients with various solid tumors, the presence of PD-1-expressing TILs in tumors (versus non-PD-1–expressing tumor TILs) was associated with increased median PFS (7.0 vs 1.9 months, \( p = 0.006 \)) and OS (18.1 vs 8.0 months, \( p = 0.04 \)) after treatment with monoclonal antibodies targeting the PD-1/PD-L1 signaling axis. Analyzed as continuous variables, TIL-PD-1 and TMB showed weak correlation in 8706 solid tumor samples (Pearson \( r = 0.074 \)); when analyzed as categorical variables (cutoffs: TIL-PD-1 > 1% and TMB > 10 mutations/Mb), the two variables are correlated (\( p < 0.0001 \)). Our data support several earlier preliminary studies indicating that PD-1 protein expression status is important. Note, although PD-L1 is rarely expressed in some “cold” tumors, such as mesothelioma, PD-1 is expressed in greater than 50% of patients per a prior report, consistent with our finding of expression in approximately 80% of patients. Moreover, nivolumab plus ipilimumab immunotherapy is active and was approved by the FDA for mesothelioma in 2020. In summary, TIL-PD-1 status may be a useful pan-cancer marker predicting response to PD-1/PD-L1–targeted ICB therapy.

**Data Availability**

Complete study data are available upon request by contacting the corresponding author.

**Supplemental Material**

Supplemental materials are available online with the article.

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