Image Analysis–based Assessment of PD-L1 and Tumor-Associated Immune Cells Density Supports Distinct Intratumoral Microenvironment Groups in Non–small Cell Lung Carcinoma Patients

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Abstract

Purpose: We investigated the correlation between immunohistochemical PD-L1 expression and tumor-associated immune cells (TAICs) density in non–small cell lung carcinoma (NSCLC) and correlated them with clinicopathologic variables.

Experimental Design: Tumor tissue specimens from 254 stage I–III NSCLCs [116 adenocarcinomas; 108 squamous cell carcinomas (SCCs)] were examined. PD-L1 expression in malignant cells and macrophages and the density of TAICs expressing CD3, CD4, CD8, CD57, granzyme B, CD45RO, PD-1, FOXP3, and CD68 were evaluated using immunohistochemistry and image analysis.

Results: Malignant cells PD-L1 H-score > 5 was detected in 23% of adenocarcinomas and 31% of SCCs, and no significant differences were detected comparing both histologies; the median H-score in macrophages was significantly higher in SCC than in adenocarcinoma (P < 0.001). In adenocarcinoma, high malignant cells PD-L1 expression and high TAIC density correlated with solid histology, smoking history, and airflow limitation. Multivariate analysis demonstrated that high CD57-positive cell density correlated with better recurrence-free survival (RFS; P = 0.0236; HR, 0.457) and overall survival (OS; P = 0.0261; HR, 0.481) rates for SCC. High CD68-positive cell density in intratumoral compartment correlated with better RFS (P = 0.0436; HR, 0.553) for adenocarcinoma. The combination of low CD4/CD8/CD68-positive cell density and PD-L1 H-score > 5 in malignant cells identified small subset of adenocarcinomas with worse outcomes (RFS: P = 0.036; HR, 4.299; OS: P = 0.00034; HR, 5.632).

Conclusions: We detected different PD-L1 expression and TAIC density patterns in NSCLC. Distinct groups of tumor microenvironment correlated with NSCLC clinicopathologic features, including outcome. Clin Cancer Res; 22(24); 6278–89. ©2016 AACR.

Introduction

Multiple genetic and epigenetic changes in patients with several cancer types cause resistance to immune attack of tumors by inducing specific T-cell tolerance against tumor and expression of ligands that engage inhibitory receptors and block T-cell activation, resulting in T-cell anergy or exhaustion in the tumor microenvironment (1). In this process, programmed cell death protein 1 (PD-1), a T-cell co-inhibitory receptor, and one of the protein’s ligands, programmed cell death ligand 1 (PD-L1; B7-H1 or CD274), play pivotal roles in the ability of tumor cells to evade the host’s immune system. Although virtually absent from normal cells except macrophages, PD-L1 expression can be induced in a variety of cell types, including antigen-presenting, B, T, epithelial, muscle, and vascular endothelial cells and trophoblasts (2). The physiologic role of PD-L1 is to bind to PD-1 receptors expressed on the surface of activated cytotoxic T cells (2). This binding causes inhibition of IL2 production and T-cell activation via reduced phosphorylation of ζ-chain–associated protein kinase 70 and protein kinase C θ (3). PD-1/PD-L1 interaction serves as an important regulatory checkpoint for excessive adaptive immune responses to antigens and autoimmunity. Researchers have observed and evaluated PD-L1 expression in malignant cells of a number of tumor types, including melanoma and breast, colorectal, and lung cancers (4–11). Therapies blocking the PD1/PD-L1 axis have resulted in different rates of tumor response for a variety of cancer types (12). For example, antibody-mediated blockade of PD-L1 induced durable
tumor regression and prolonged disease stabilization in patients with a variety of solid tumors, including non–small cell lung carcinoma (NSCLC; refs. 12, 13). Although these studies demonstrated correlation between immunohistochemical (IHC) PD-L1 expression in NSCLC cells and clinical responses to anti-PD-1 and anti-PD-L1 antibodies (12, 13), some patients with tumors negative for PD-L1 expression had responses to similar to those in patients with tumors positive for PD-L1 expression (14). Recently, investigators showed that across several cancer types, including NSCLC, patients with tumors expressing PD-L1 at high levels in both malignant cells and tumor-associated immune cells (TAIC) had responses to anti-PD-L1 therapy (11). Taken together, these findings suggested that factors other than PD-L1 in the tumor microenvironment, including tumor-infiltrating lymphocytes (TIL; refs. 15, 16) and tumor-associated macrophages (TAM; ref. 17), may drive responses to anti-PD-1 and anti–PD-L1 therapies and be involved in lung cancer pathogenesis and progression (10, 11, 18).

Researchers in a number of studies have characterized PD-L1 protein expression in surgically resected stage I–IV NSCLC tumors using IHC or immunofluorescence (7, 8, 11, 15, 16) with formalin-fixed, paraffin-embedded tumor specimens and correlated the findings with outcome. Although most of the studies demonstrated that high PD-L1 protein expression correlated with better outcome than did low expression (8, 11, 18, 19), some authors reported no association of PD-L1 expression with outcome (9, 15) or that the expression correlated with poor prognosis (10). Most of these studies differed in the type of specimens used [whole histologic sections vs. tissue microarrays (TMA)], type of protein expression analysis (IHC vs. immunofluorescence), and quantification assessment (image analysis vs. microscopic observation; refs. 7–11, 15–19). Researchers in only a few studies have attempted to correlate the expression of PD-L1 with TAIC density and characteristics, particularly TILs, using a limited number of IHC markers (e.g., CD8, CD45RO; refs. 15, 19) or simple histologic evaluation (7, 11). To date, no published studies have included assessment of a comprehensive panel of immune markers in NSCLC cases using image analysis to characterize the association among PD-L1 expression, immune cell response, and clinicopathologic features of tumors to determine distinct subgroups of NSCLC and patients’ outcome. Therefore, in the present study, we characterized the IHC expression of a large panel of immune markers PD-L1, PD-1, and 8 others and assessed the TAIC density in both the peritumoral and intratumoral compartments in surgically resected NSCLC specimens. To assess this objectively, we performed quantitative image analysis and correlated the findings with the patients’ clinical features and their tumors’ pathologic and molecular features.

**Materials and Methods**

**Cases and specimens**

Formalin-fixed, paraffin-embedded histological sections of surgically resected primary NSCLC tumors obtained from 254 patients who underwent surgery with curative intent from 1997 to 2012 at The University of Texas MD Anderson Cancer Center (Houston, TX) were used. The study patients had either adenocarcinoma (n = 146) or squamous cells carcinoma (SCC; n = 108) and did not receive neoadjuvant therapy. Tumor staging was performed using the staging system from the 7th American Joint Committee on Cancer (AJCC; ref. 20). For adenocarcinoma, predominant histologic pattern (solid, lepidic, acinar, papillary, and micropapillary) was determined according to the 2015 World Health Organization classification (21). For SCC grading, we used well, moderate, and poorly differentiated categories. In a subset of cases (n = 130; 97 adenocarcinomas and 33 SCCs), PD-L1 IHC expression was assessed in paired whole tissue sections and TMA samples. Of these, all 3 TMA cores were available in 73 adenocarcinoma and 24 SCCs, whereas the rest of the cases had 2 cores available for analysis. The TMA sections were prepared using three 1.0-mm tissue cores obtained from the center, middle, and periphery of the tumor, as described previously (22). Data on nonreversible airflow limitation, defined as a ratio of the forced expiratory volume in 1 second to the forced vital capacity of less than 0.7 (23), were available for a large subset of our patients (n = 179). EGF and KRAS mutation data obtained using Sanger sequencing were available in 91% (n = 137) of adenocarcinomas. This study was approved by the MD Anderson Institutional Review Board.

**IHC staining**

Four-micrometer-thick sequential histologic tumor sections were obtained from a representative formalin-fixed, paraffin-embedded tumor block and used for IHC analysis. IHC was performed using an automated staining system (BOND-MAx; Leica Microsystems) with antibodies against PD-L1 (clone E1L3N, dilution 1:100; Cell Signaling Technology), CD3 (T-cell lymphocytes; dilution 1:100; Dako), CD4 (helper T cell; Novoceastra; clone 4B12, dilution 1:80; Leica Biosystems), CD8 (cytotoxic T cell; clone D8/144B; dilution 1:20; Thermofisher Scientific), CD57 (natural killer T cell; clone HNK-1, dilution 1:40; BD Biosciences), granzyme B (cytotoxic lymphocytes; clone F1, ready to use; Leica Biosystems), CD45RO (memory T cell; clone UCHL1, ready to use; Leica Biosystems), PD-1 (clone EPR4877t-2, dilution 1:250; Abcam), FOXP3 (regulatory T cell; clone 206D, dilution 1:50; BioLegend), and CD68 (macrophages; clone PG-M1, dilution 1:450; Dako). Expression of all of the markers in cells was detected using a Novoceastra Bond Polymer Refine Detection kit (Leica Microsystems) with a diaminobenzidine reaction to detect antibody labeling and hematoxylin counterstaining. Positive and negative controls were used for PD-L1.
IHC expression (human embryonic kidney 293 cell line transfected and nontransfected with PD-L1 gene and human placenta and tonsil FFPE tissues) during each run IHC staining using autostainers. For the TMC IHC expression, human tonsil FFPE tissues with and without primary antibody were used as positive and negative controls, respectively, with each run IHC staining.

Image analysis
To measure the IHC expression of the different markers and quantify the inflammatory cells expressing the slides, containing whole-tumor sections or immunohistochemically stained TMA sections, were digitally scanned at \( \times 200 \) magnification using a ScanScope Aperio AT Turbo slide scanner (Leica Microsystems). The images were visualized using the ImageScope software program (Leica Microsystems) and analyzed using the Aperio Image Toolbox and GENIE image analysis tool (Leica Microsystems). The pathologist who performed the image analysis was blinded to patients’ outcome. After training, the software by a pathologist (Supplementary Fig. S1), membranous PD-L1 expression in malignant epithelial cells and macrophages was analyzed using a cell membrane staining algorithm, and the staining intensity scored as 0 (no staining), 1+ (weak staining), 2+ (moderate staining), or 3+ (strong staining) and extension (percentage) of expression were determined (Fig. 1). The PD-L1 HI-scores for tumor tissues were determined by multiplying the staining intensity and reactivity extension values (range, 0–300). The densities of cells expressing CD3, CD4, CD8, CD57, granzyme B, CD45RO, PD-1, and FOXP3 were evaluated using the Aperio nuclear algorithm and CD68 using Aperio cytoplasmic algorithm (Fig. 2 and Supplementary Fig. S2) and counting the cells positive for them in 5 random square areas (1 mm\(^2\) each) in both intratumoral and peritumoral compartments. While 5 intratumoral regions were available in all cases and 24 tumors did not have peritumoral regions for analysis. Histologic assessment of each 1 mm\(^2\) was performed to ensure that tumor tissue (at least 80% malignant

Figure 1.
Microphotographs of representative examples of IHC PD-L1 expression in lung adenocarcinoma and SCC specimens. Four levels of staining (brown) are shown: strong (A; staining score, 3+), moderate (B; staining score, 2+), weak (C; staining score, 1+), and negative (D; staining score, 0).
cells and tumor stroma) was included in the selected intratumoral region, and only non-malignant cells were included in the peritumoral compartment. For this analysis, each area examined was overlapped with the sequential IHC slides to quantify each marker at the same location of the tumor specimens. The average total number of cells positive for each marker in the 5 square areas was expressed in density per mm². Similar to PD-L1 \( H \)-score from 5 intratumoral areas, the TMA was scored as the median PD-L1 \( H \)-score average of all cores from each case. In addition, as has been proposed by Teng and colleagues (24), 4 different types tumor microenvironment were able to identify based on the density of TILs and the expression of PD-L1, as follows: type I (adaptive immune resistance), type II (immunologic ignorance), type III (intrinsic induction), and type IV (tolerance). Combining PD-L1 expression in malignant cells (>5% was considered positive) with the density of cells expressing CD3 using 3 levels (tertile) divided on the basis of regular values of distribution by the statistical software (moderate and severe density were considered positive), we were able to identify the frequency of those four subtypes of tumor microenvironment in our NSCLC cases.

PD-L1 mRNA analysis

Total RNA extracted from the NSCLC specimens with fresh-frozen tissue available was used to measure PD-L1 gene expression using an available probe with a human WG-6 v.3 Expression BeadChip (Illumina). Resulting data set for 104 primary adenocarcinomas and 39 primary SCCs (SPORE dataset; GSE41271) were deposited in the Gene Expression Omnibus repository (25).

Statistical methods

The \( \chi^2 \) test or Fisher exact test was used to examine differences in categorical variables, and the Wilcoxon rank-sum test and Kruskal–Wallis test were used to detect differences in continuous variables between groups of patients. The recurrence-free survival (RFS) and overall survival (OS) distributions for the patients were estimated using the Kaplan–Meier method. RFS was defined as the interval from surgery to recurrence or last contact, and OS was defined as the interval from surgery to death or last contact. Both RFS and OS data were censored at 5 years if a patient was alive or died beyond 5 years. A log-rank test was performed to determine the difference in survival between the groups. Regression analysis of the RFS and OS data was performed using Cox proportional hazards model. The statistical software programs SAS (version 9.2; SAS Institute, Inc.) and S-Plus (version 8.04; TIBCO) were used to perform the computations for all analyses.

Results

PD-L1 protein expression

Clinicopathologic and molecular data on the patients are shown in Table 1. Our image analysis–based examination of whole tumor sections obtained from the 146 patients with adenocarcinoma and 108 patients with SCC demonstrated that the median PD-L1 \( H \)-score in malignant cells was low for both tumor histologies (median, 1.29 for adenocarcinoma vs. 0.95 for SCC). We found no significant differences in PD-L1 \( H \)-score between the 2 histologies (median, 0.28 for adenocarcinoma vs. 0.27 for SCC). We found no significant differences in PD-L1 \( H \)-score between the 2 histologies (median, 0.29 for adenocarcinoma vs. 0.27 for SCC). We found no significant differences in PD-L1 \( H \)-score between the 2 histologies (median, 0.29 for adenocarcinoma vs. 0.27 for SCC).
Table 1. Characteristics of the 254 patients with NSCLC

<table>
<thead>
<tr>
<th>Category</th>
<th>Adenocarcinoma (n = 146)</th>
<th>SCC (n = 108)</th>
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</thead>
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<tr>
<td>Age</td>
<td>65.89 y</td>
<td>67.98 y</td>
</tr>
<tr>
<td>Sex</td>
<td>Female: 70 (48)</td>
<td>44 (41)</td>
</tr>
<tr>
<td></td>
<td>Male: 76 (52)</td>
<td>64 (59)</td>
</tr>
<tr>
<td>Tobacco history</td>
<td>No: 18 (12)</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Yes: 128 (88)</td>
<td>108 (100)</td>
</tr>
<tr>
<td>Smoking status</td>
<td>Never: 18 (12)</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>Former: 70 (48)</td>
<td>52 (48)</td>
</tr>
<tr>
<td></td>
<td>Current: 58 (40)</td>
<td>56 (52)</td>
</tr>
<tr>
<td>Tumor size</td>
<td>Median: 3.10 cm</td>
<td>3.85 cm</td>
</tr>
<tr>
<td>Tumor status</td>
<td>T1: 42 (29)</td>
<td>31 (29)</td>
</tr>
<tr>
<td></td>
<td>T2: 76 (52)</td>
<td>49 (45)</td>
</tr>
<tr>
<td></td>
<td>T3: 21 (14)</td>
<td>22 (22)</td>
</tr>
<tr>
<td></td>
<td>T4: 7 (5)</td>
<td>4 (4)</td>
</tr>
<tr>
<td>Nodal status</td>
<td>N0: 104 (71)</td>
<td>61 (56)</td>
</tr>
<tr>
<td></td>
<td>N1: 24 (16)</td>
<td>32 (30)</td>
</tr>
<tr>
<td></td>
<td>N2: 18 (12)</td>
<td>15 (12)</td>
</tr>
<tr>
<td>AJCC stage</td>
<td>I: 84 (58)</td>
<td>43 (40)</td>
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<tr>
<td></td>
<td>II: 36 (25)</td>
<td>41 (38)</td>
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<tr>
<td></td>
<td>III: 26 (18)</td>
<td>24 (22)</td>
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<td>Histologic features</td>
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<tr>
<td></td>
<td>Lepidic</td>
<td>–</td>
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<tr>
<td></td>
<td>Acinar</td>
<td>–</td>
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<td></td>
<td>Papillary</td>
<td>–</td>
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<tr>
<td></td>
<td>Micropapillary</td>
<td>9 (6)</td>
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<tr>
<td>SCC</td>
<td>Well</td>
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<tr>
<td></td>
<td>Moderate</td>
<td>2 (2)</td>
</tr>
<tr>
<td></td>
<td>Poorly</td>
<td>57 (53)</td>
</tr>
<tr>
<td>K-RAS status</td>
<td>Mutant: 47 (32)</td>
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<tr>
<td></td>
<td>Wild-type</td>
<td>97 (66)</td>
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<tr>
<td>EGFR status</td>
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<tr>
<td></td>
<td>Wild-type</td>
<td>117 (80)</td>
</tr>
<tr>
<td>Adjuvant therapy</td>
<td>No: 81 (55)</td>
<td>65 (60)</td>
</tr>
<tr>
<td></td>
<td>Yes: 65 (45)</td>
<td>43 (40)</td>
</tr>
<tr>
<td>Vital status at 5 y</td>
<td>Alive: 91 (62)</td>
<td>45 (42)</td>
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<tr>
<td></td>
<td>Dead: 55 (38)</td>
<td>63 (58)</td>
</tr>
<tr>
<td>Recurrence status at 5 y</td>
<td>No: 89 (61)</td>
<td>62 (57)</td>
</tr>
<tr>
<td></td>
<td>Yes: 57 (39)</td>
<td>46 (43)</td>
</tr>
</tbody>
</table>

*World Health Organization of Lung Cancer.

The difference was statistically significant at only greater than 5% (P = 0.003), greater than 10% (P < 0.0001), and greater than 20% (P = 0.020).

PD-L1 expression in cells with characteristics of TAMs was higher than that in malignant cells for both tumor histologies, whereas SCC specimens exhibited significantly higher PD-L1 H-scores in macrophages than did adenocarcinoma specimens (median, 39.89 and 18.08, respectively; P < 0.001).

Comparison of IHC PD-L1 expression in whole histologic tumor sections and TMA sections

To compare IHC PD-L1 expression levels in whole histologic tumor sections and TMA sections, we looked at the H-scores for malignant cells only and for malignant cells and TAMs together in a subset of adenocarcinoma (n = 97) and SCC (n = 33) specimens. In our analysis of malignant cells only, we found that PD-L1 expression in whole histologic sections correlated positively and significantly with that in TMA sections for both histologies (adenocarcinoma: r = 0.417, P < 0.0001; SCC: r = 0.438, P = 0.0018).

In addition, we found that PD-L1 expression in whole histologic sections correlated positively and significantly with that in TMA sections (adenocarcinoma: r = 0.617, P < 0.0001; SCC: r = 0.502, P = 0.0033). Taken together, these data suggested that the 3 TMA cores were good surrogates for whole tumor sections in PD-L1 expression analysis of NSCLC. In addition, we observed a positive and significant correlation between the expression of PD-L1 in malignant cells and TAMs in whole histologic tumor sections and that of the five 1-mm² areas of the specimens (adenocarcinoma: r = 0.575, P < 0.0001; SCC: r = 0.733, P < 0.0001).

Correlation between PD-L1 protein and PD-L1 gene expression

To assess the correlation between PD-L1 protein and PD-L1 gene expression in NSCLC cases, we studied 104 adenocarcinoma and 39 SCC specimens with available data on PD-L1 mRNA expression. We found that PD-L1 mRNA expression in whole tumor specimens correlated positively and significantly with PD-L1 protein expression for both NSCLC histologies when we assessed the PD-L1 protein expression in the entire population of tumor cells, including both malignant cells and TAMs, in 5 random areas of the whole tumor specimens (adenocarcinoma: r = 0.448, P < 0.0001; SCC: r = 0.634, P < 0.0001) and TMA sections (adenocarcinoma: r = 0.443, P < 0.0001; SCC: r = 0.428, P = 0.02). Similarly, we found positive and significant correlations of PD-L1 gene and protein expression when we examined protein expression in malignant cells only in whole histologic tumor sections (adenocarcinoma: r = 0.323, P = 0.0008; SCC: r = 0.398, P = 0.012). These data indicated that PD-L1 mRNA expression is a good potential surrogate for PD-L1 protein expression in whole NSCLC tumor tissue.

Correlation between PD-L1 expression and clinicopathologic features of NSCLC

In adenocarcinoma specimens, the PD-L1 H-score greater than the median in malignant cells correlated with smoking history, airflow limitation, and tumor differentiation. The PD-L1 H-score was significantly higher in ever-smokers (mean, 11.41; median, 1.45; P < 0.0001) than in never-smokers (mean, 1.31; median, 0.56; P < 0.0001). In comparison, the PD-L1 H-score in adults who have smoked at least 100 cigarettes in their lifetime and quit smoking at least a year before surgery, former smokers (mean, 12.82; median, 1.64; P = 0.918). Also, patients with adenocarcinoma with irreversible airflow limitation (forced expiratory volume in 1 s, forced vital capacity ratio < 0.7) had a significantly higher PD-L1 H-score in malignant cells in whole tumor specimens (median, 1.44 versus 1.11; P = 0.048) and 5 random areas of the tumor specimens (median, 30.78 vs. 15.98; P = 0.014) than did patients without airflow limitation.
(Supplementary Table S1). Adenocarcinoma specimens with a solid tumor histologic pattern had significantly higher PD-L1 H-scores in malignant cells ($P = 0.021$; average, 15.46; median, 1.69) than did specimens with a non-solid tumor histologic pattern (mean, 3.57; median, 1.13). In addition, as we detected at the protein level, we found that PD-L1 gene expression was significantly higher in adenocarcinoma specimens with a solid tumor histology than in those with a non-solid tumor histology ($P < 0.0001$). PD-L1 expression in malignant cells was lower in $EGRF$-mutant adenocarcinoma specimens (mean $H$-score, 5.97; median $H$-score, 1.39) than in adenocarcinoma specimens with $EGRF$ wild-type (mean $H$-score, 9.65; median $H$-score, 1.07), but the difference was not significant ($P = 0.071$). We did not detect an association between PD-L1 expression and KRAS mutation in adenocarcinoma specimens. We observed no other correlations between PD-L1 expression and other clinical and pathologic features for either histological type of NSCLC.

**TAIC density**

Our image analysis–based IHC examination of TAICs included that of immune markers identifying TILs (CD3$^+$), helper T cells (CD4$^+$), cytotoxic T cells (CD8$^+$), natural killer T cells (CD57$^+$), memory T cells (CD45RO$^+$), regulatory T cells (PD-1$^+$ or FOXP3$^+$), and TAMs (CD68$^+$) in 5 randomly selected 1-mm$^2$ areas of tumor specimens. Representative images of immunohistochemical stains of TAICs for these markers are shown in Fig. 2. We found that both adenocarcinoma and SCC specimens had various TIL densities, particularly when we examined the intratumoral and peritumoral compartments separately (Table 2; Supplementary Table S2). Unexpectedly, we found in both tumor types and compartments examined that the number of CD4$^+$CD8$^+$ cells was slightly larger than the number of CD3$^+$ cells and that the number of cells expressing granzyme B was significantly lower than cells expressing CD8. In the peritumoral compartment, SCCs exhibited higher density of cells expressing all markers except PD-1 in TILs, with statistically significantly higher levels of cells expressing CD3 ($P = 0.0010$), CD8 ($P = 0.0020$), and FOXP3 ($P = 0.0010$) than that in adenocarcinomas. In the intratumoral compartment, the differences in TAICs density between the 2 tumor histologies were less noticeable. We did observe that adenocarcinoma specimens had significantly higher densities of CD3$^+$ ($P = 0.0030$), CD4$^+$ ($P < 0.0001$), and CD57$^+$ ($P < 0.0001$) cells than SCC specimens did. Similar to TILs, the density of cells expressing CD68 was significantly higher in the peritumoral compartment than in intratumoral areas for both tumor histologies ($P < 0.0001$) and significantly higher in the intratumoral region in adenocarcinoma than in SCC specimens ($P < 0.0001$). These findings indicated that the densities of TILs and TAMs vary according to the tumor compartment and NSCLC histology.

**PD-L1 expression and TAIC density**

When we examined the correlation of PD-L1 $H$-score in malignant cells with TIL and TAM densities in whole-tumor histologic sections, we found different patterns of correlation according to NSCLC histology and tumor compartment. In adenocarcinoma specimens, density of cells expressing the majority of TAIC markers examined exhibited a positive significant correlation of expression of PD-L1 in malignant cells in the intratumoral (7 of 9 comparisons) and peritumoral (8 of 9 comparisons) compartments (Supplementary Table S3). The cells expressing markers whose level was not significantly correlated with PD-L1 expression in adenocarcinomas were positive for CD68 in both compartments and CD45RO in the intratumoral region. In SCC specimens, we found lower correlation of PD-L1 expression with TAIC markers’ expression, with 4 of 9 markers (CD3, CD8, granzyme B, and CD45RO) significantly and positively correlated with PD-L1 expression in the intratumoral area and one marker (CD3) significantly and positively correlated with PD-L1 expression in the peritumoral region (Supplementary Table S3). The PD-L1 $H$-score in TAMs exhibited a lower rate of correlation with marker expression in TAICs for both NSCLC histologies and in both tumor compartments than did the PD-L1 expression in malignant cells (Supplementary Table S4).

Combining PD-L1 expression in malignant cells with the density of cells expressing CD3 as proposed by Teng and colleges (24), we were able to identify the 4 subtypes of tumor microenvironment in our NSCLC cases as shown in Table 3. This analysis showed that 29% in adenocarcinoma and 26% in SCC had an immune ignorance phenotype and that pattern most frequently observed (adenocarcinoma 48% and SCC 43%) was tumors with immune tolerance pattern defined as positive TILs without PD-L1 expression in malignant cells. Cases with positive PD-L1 expression and positive TILs defined as tumors with adaptive immune resistance pattern were found in 19% of adenocarcinoma and 31% of SCC. Tumors with the intrinsic induction pattern were less frequently detected (adenocarcinoma 3% and SCC 7%). Similar percentages of cases in each group were found when the analysis included CD4$^+$ and CD8$^+$ TILs, as well as CD3$^+$ and CD68$^+$ TAICs (data not shown).

**Correlation between TAIC density and clinicopathologic features of NSCLC**

TAIC density correlated with clinical and pathologic tumor features only in adenocarcinoma specimens. Specifically, these

<table>
<thead>
<tr>
<th>Marker</th>
<th>Intratumoral</th>
<th>Peritumoral</th>
<th>$P^a$</th>
<th>Intratumoral</th>
<th>Peritumoral</th>
<th>$P^a$</th>
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<tbody>
<tr>
<td>CD3</td>
<td>1505.80</td>
<td>1090.00</td>
<td>0.0030</td>
<td>1636.00</td>
<td>2218.70</td>
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<td>1217.50</td>
<td>832.00</td>
<td>&lt;0.0001</td>
<td>1425.40</td>
<td>1648.90</td>
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<td>CD8</td>
<td>866.40</td>
<td>693.20</td>
<td>0.1430</td>
<td>1040.80</td>
<td>1365.60</td>
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<td>475.40</td>
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<td>Granzyme B</td>
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<td>254.10</td>
<td>0.8070</td>
<td>410.60</td>
<td>486.60</td>
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<td>CD45RO</td>
<td>846.37</td>
<td>913.35</td>
<td>0.1370</td>
<td>1199.80</td>
<td>1725.60</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PD-1</td>
<td>525.40</td>
<td>489.63</td>
<td>0.1860</td>
<td>953.60</td>
<td>870.90</td>
<td>0.5800</td>
</tr>
<tr>
<td>FOXP3</td>
<td>357.10</td>
<td>335.70</td>
<td>0.6470</td>
<td>435.00</td>
<td>591.80</td>
<td>0.0010</td>
</tr>
<tr>
<td>CD68</td>
<td>330.81</td>
<td>201.56</td>
<td>&lt;0.0001</td>
<td>445.54</td>
<td>428.69</td>
<td>0.7740</td>
</tr>
</tbody>
</table>

$^a$Kruskal–Wallis test analysis.
specimens with any solid tumor histology had significantly higher levels of TILs in the intratumoral (cells expressing CD3, CD8, granzyme B, and PD-1) and peritumoral (cells expressing CD3, CD4, CD8, granzyme B, CD45RO, PD-1, and FOXP3) compartments than did adenocarcinoma specimens with non-solid tumor histologies (Supplementary Table S5). Tumor specimens obtained from ever-smokers had significantly higher granzyme B expression in both compartments and higher peritumoral CD8 expression than did those obtained from never-smokers, whereas tumor specimens obtained from never-smokers had higher intratumoral CD4 expression. Overall, tumor specimens obtained from current smokers had a significantly higher TAIC density (cells positive for CD3, CD8, CD57, granzyme B, PD-1, and FOXP3), mostly in the peritumoral compartment than did those obtained from former smokers and never-smokers (Supplementary Table S3). Patients with airflow limitation had overall higher numbers of TAICs in both compartments than did patients without airflow limitation (Supplementary Table S1). Furthermore, we observed significantly higher TILs expressing granzyme B and FOXP3 in the intratumoral compartment and cells expressing CD3, CD4, CD8, granzyme B, and CD45RO in the peritumoral compartment in tumors with patients with airflow limitation (Supplementary Table S2). Tumors larger in diameter than the median (3.1 cm) exhibited greater overall TAIC densities in the peritumoral region (cells expressing CD3, CD8, granzyme B, PD-1, or FOXP3) than in the intratumoral region (CD4), whereas smaller tumors had higher densities of CD4-positive cells in the intratumoral compartment than in the peritumoral one. Of note, adenocarcinoma specimens with *EGFR* mutations had more intratumoral cells expressing CD57 and CD45RO and peritumoral cells expressing CD3 and CD4 than did specimens wild-type for *EGFR*. We did not detect a correlation between TAIC density and *KRAS* mutation in adenocarcinoma specimens.

### Correlation between immune markers expression and NSCLC prognosis

PD-L1 expression *H*-score and percentage of expression at various cutoff levels in malignant cells was not significantly correlated with the outcome of NSCLC in our multivariate analysis. However, in adenocarcinoma specimens, our univariate analysis demonstrated that PD-L1 *H*-scores higher than the median (*P* = 0.070; HR, 1.746; 95% confidence interval (CI), 0.955–3.190]) and greater than 5 (*P* = 0.051; HR, 1.128; 95% CI, 1.000–1.274) were nonsignificantly correlated with a poor OS duration (Fig. 3A). In SCC specimens, PD-L1 *H*-scores higher than the median in macrophages correlated significantly with poor OS durations (*P* = 0.036; HR, 0.548; 95% CI, 0.312–0.961) and indicated a nonsignificant trend of poor RFS durations (*P* = 0.063; HR, 0.565; 95% CI, 0.309–1.033) in our univariate analysis (Fig. 3B).

In addition, the density of TAICs expressing several immune markers in tumor specimens correlated with NSCLC outcome in univariate and multivariate analyses. For the patients with adenocarcinoma, intratumoral densities of cells expressing CD4 and FOXP3 greater than the median density were significantly correlated with good RFS and OS durations, respectively, in univariate analysis (data not shown). For the patients with SCC, peritumoral densities of cells expressing CD57 greater than the median density were significantly associated with good RFS and OS durations, respectively, in both univariate (data not shown) and multivariate analyses (RFS: *P* = 0.0236; HR, 0.457; 95% CI, 0.232–0.900; OS: *P* = 0.0261; HR, 0.481; 95% CI, 0.258–0.911) analyses after adjustment for tumor stage and adjuvant therapy (Fig. 3C and D). In addition, our multivariate analysis demonstrated that intratumoral CD68-positive cell densities greater than the median correlated with good RFS for patients with adenocarcinoma (*P* = 0.0436; HR, 0.553; 95% CI, 0.311–0.983; Fig. 3E) and a nonsignificant trend of correlation with poor RFS durations for patients with SCC (*P* = 0.0811; HR, 1.751; 95% CI, 0.33–3.287). Furthermore, the combination of CD4/CD8/CD68-positive cell densities lower than the median and PD-L1 *H*-scores greater than 5 in malignant cells identified a small subset of patients with adenocarcinoma with poor outcomes (RFS: *P* = 0.036; HR, 4.299; 95% CI, 1.415–13.059; OS: *P* = 0.00034; HR, 5.632; 95% CI, 2.015–15.737; Fig. 3F).

### Discussion

In this study, we examined archived tumor specimens obtained from a large cohort of patients with stage I–III NSCLC, both adenocarcinoma (*n* = 146) and SCC (*n* = 108), for IHC expression of PD-L1 in malignant cells and macrophages and density of TAICs (TILs and TAMs) expressing CD3, CD4, CD8, CD57, granzyme B, CD45RO, PD-1, FOXP3, and CD68 in intratumoral and peritumoral compartments using image analysis. We detected PD-L1 *H*-scores > 5 in malignant cells in 23% of the adenocarcinoma specimens and 31% of the SCC specimens and found that the median *H*-score in macrophages was markedly higher in SCC specimens than in adenocarcinoma specimens. In adenocarcinoma specimens, high PD-L1 expression in malignant cells and high TAIC densities correlated with solid tumor histology, airflow limitation, and smoking history. For TAICs expressing most of the immune markers tested, the cell density was significantly higher in peritumoral than in intratumoral compartments for both tumor histologies, and SCCs exhibited higher peritumoral densities than did adenocarcinomas. Multivariate analysis of outcome demonstrated that the combination of low CD4/CD8/CD68-positive cell density and high PD-L1 expression in malignant cells identified a small subset of patients with adenocarcinoma with poor RFS and OS durations. We concluded that the PD-L1 protein expression pattern and TAIC density differ in surgically resected adenocarcinoma and SCC specimens and that TAIC density varies according to tumor compartment.

### Table 3. Distribution of types of microenvironment in NSCLC based on intratumoral malignant cells PD-L1 expression and density of CD3 TILs

<table>
<thead>
<tr>
<th>PD-L1 expression</th>
<th>Adenocarcinoma</th>
<th>Intratumoral TILs</th>
<th>SCC</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Negative (%)</strong></td>
<td><strong>Positive (%)</strong></td>
<td><strong>Total</strong></td>
<td><strong>Negative (%)</strong></td>
<td><strong>Positive (%)</strong></td>
</tr>
<tr>
<td>Negative</td>
<td>45 (29)</td>
<td>70 (48)</td>
<td>113 (77)</td>
<td>28 (26)</td>
</tr>
<tr>
<td>Positive</td>
<td>3 (3)</td>
<td>28 (19)</td>
<td>33 (23)</td>
<td>8 (7)</td>
</tr>
<tr>
<td>Total</td>
<td>48 (33)</td>
<td>98 (67)</td>
<td>146 (100)</td>
<td>36 (33)</td>
</tr>
</tbody>
</table>

**NOTE:** PD-L1 expression (negative, defined as <5% positive malignant cells membranous expression; positive, ≥5%) and intratumoral TILs determined by CD3 cells density (negative, defined as mild (lowest tertile) TIL density; positive, defined as moderate and severe density (middle and highest tertiles)).

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*Clin Cancer Res; 22(24) December 15, 2016*
Importantly, in the present study, we identified distinct tumor environment patterns in NSCLC, and several immune markers whose expression correlated with outcome of surgical resection tumors. As described previously (7, 8, 15, 16, 19), we observed relatively low levels of expression of PD-L1 in malignant cells in NSCLC specimens. In particular, SCCs had slightly higher levels of PD-L1 expression in malignant cells than did adenocarcinomas,

![Graphs illustrating the prognostic effect on OS and RFS of expression of immune markers in lung adenocarcinoma and SCC specimens.](image)

Figure 3.

Kaplan–Meier curves illustrating the prognostic effect on OS and RFS of expression of immune markers in lung adenocarcinoma and SCC specimens.
particularly for cutoff percentages greater than 5%, 10%, and 20%. The clinically relevant threshold for IHC PD-L1 expression in NSCLC cells has yet to be defined, and researchers have examined various cutoffs of PD-L1 positivity, including at least 5% (7, 9, 26), 10% (15), and 50% (8, 16) cells and greater than the median H-score (10, 27). The cutoff most frequently used to assess IHC PD-L1 expression in malignant cells in patients with NSCLC given PD-1/PD-L1 inhibitors has been at least 5% cells, and this cutoff value has been associated with durable tumor regression and prolonged disease stabilization in response to anti-PD-L1 treatment in advanced metastatic NSCLC cases (14). In the present study, 23% of the adenocarcinoma specimens and 31% of the SCC specimens had more than 5% of malignant cells with PD-L1 expression, which was not markedly different from the percentages reported by others (7, 27). In addition, we found high levels of PD-L1 expression in cells with characteristics of macrophages for both NSCLC histologies, with markedly higher expression in SCC than in adenocarcinoma specimens. To the best of our knowledge, we are the first to specifically analyze and quantify TAMs expressing PD-L1 in NSCLC specimens using IHC. PD-L1 expression in macrophages is biologically relevant and may be associated with tumor progression. As shown in a study using an experimental inflammatory macrophages mouse model (28), PD-L1 expression was induced in macrophages using the same cytokines, such as IFNy and VEGF, involved in the PD-L1 upregulation of human cancer cell lines (29), leading to effective suppression of T-cell immunity.

Comparison of different reports of PD-L1 expression in NSCLC cases is in literature is hindered by the use of several analytical methodologies. In most studies, investigators undertook PD-L1 analysis using whole-tissue sections (7, 9, 10, 16, 19, 26, 27) and some studied TAMs (8, 11, 15). Examination of whole-tissue sections may lead to more frequent identification of PD-L1–positive cases than that using more limited sampling with a TMA. However, the present study surprisingly demonstrated that PD-L1 expression in malignant cells in whole-tumor sections correlated markedly and positively with the expression in TMA sections for both NSCLC types. In addition, the data on whole tumor sections correlated with that obtained via examination of 5 randomly selected 1-mm² areas of tumor specimens. Taken together, these data demonstrated for the first time that TMA sections and randomly selected areas of tumor specimens are reliable surrogates for whole-tumor sections in sampling approaches to assessing PD-L1 expression in patients with NSCLC.

We examined PD-L1 in malignant cells and macrophages and TAICs density using a quantitative computer-based image analysis system. In most of the previously reported studies of NSCLC, researchers used semiquantitative PD-L1 and TAIC density scoring systems on the basis of microscopic observation of slides by a trained pathologist (7–10, 15, 16, 19, 26, 27). Visual evaluation of immunohistochemically stained tissue sections remains a rather subjective process characterized by significant intraobserver and interobserver variability and reduced reproducibility, which digital image analysis can overcome (30, 31).

The PD-L1 H-score in malignant cells correlated with smoking history and airflow limitation in patients with adenocarcinoma. The H-score was markedly higher in ever-smokers than in never-smokers, but we detected no difference between former and current smokers. In addition, we found markedly higher PD-L1 expression in malignant cells in patients with airflow limitation, a condition closely associated with chronic obstructive pulmonary disease (23), than in patients without it, suggesting a link between inflammatory processes affecting the lungs of smokers and immune response statuses of tumors.

In our study, specimens of both NSCLC histologies had higher numbers of TAICs in the peritumoral compartment than in the intratumoral region. Also, we found distinct TIL and TAM infiltration patterns in specimens of both histologies when we examined both compartments, with SCC specimens exhibiting higher numbers of TILs expressing immune markers (except PD-1) than those in adenocarcinoma specimens in the peritumoral compartment. We hypothesized that nonhomogeneous or clustered secretion of cytokines and chemokines at the invasive tumor margin may enhance the recruitment of different TAIC subtypes to the tumor microenvironment (32). Interestingly, PD-L1 expression in malignant cells was markedly correlated with the TAIC density in both tumor compartments for both NSCLC histologies. These observations are supported by previous findings that different immune cells, such as CD8+ and CD4+ cells and natural killer cells, are capable of inducing PD-L1 expression in malignant cells via IFNy production (33). Curiously, although we found a significant correlation between PD-L1 IHC expression in malignant cells and infiltration of PD-L1+ cells in adenocarcinoma, this was not observed in SCC suggesting different microenvironment mechanism(s) of immune resistance between these 2 NSCLC histology types. In our study, we characterized the 4 types of tumor microenvironment described by Teng and colleagues (24) using as criteria for TIL density levels of CD3+ cells combined with PD-L1 expression in malignant cells. We found that most tumors demonstrated an immune tolerance phenotype defined as positive TILs without PD-L1 expression and tumors with intrinsic induction pattern defined as positive PD-L1 expression and lack of TILs are rarely observed in surgically resected NSCLCs.

Our TAICs quantitative image analysis of NSCLC encountered a couple of unexpected findings. The number of CD4+ CD8– cells was slightly larger than the number of CD3+ cells in both tumor types and compartments examined. Potential explanations to this finding include that CD4 is expressed in various immune cells such as monocytes/macrophages, eosinophils, CD34+ progenitor cells, and natural killer cells (34) and that double CD4/CD8+ T cells have been described in several pathological conditions as well as in normal individuals (35). Also, we found that the number of CD4+ cells expressing granzyme B was significantly lower than the cells expressing CD8. Interestingly, it has been reported that a decreased proportion of granzyme B+ cells in lung tumors was a result of soluble mediators, not identified yet, secreted probably by cancer cells (36).

In addition, we observed that TAIC density correlated with clinical and pathologic tumor features only in adenocarcinoma specimens. In these specimens, the presence of any solid tumor histology, smoking history, and airflow limitation was correlated with higher overall numbers of TILs in intratumoral and peritumoral compartments than was a non-solid tumor histology. Smoking status and pulmonary disorders with airflow limitation such as chronic obstructive pulmonary disease are associated with greater risk of lung cancer than patients without those characteristics and are characterized by abundant and deregulated inflammation (37). The selective recruitment of TILs (CD3+), helper T cells (CD4+), cytotoxic T cells (CD8+ and granzyme B+), and memory T cells (CD45RO+), to the peritumoral compartment of these lung tumors suggests that these TAICs, which are located around the tumors, are less affected by tumor-derived inhibitory
Factors than are cells located in direct contact with malignant cells in the intratumoral compartment.

Although the present study did not demonstrate that PD-L1 expression in malignant cells is an independent prognostic factor for NSCLC, our univariate analysis demonstrated a nonsignificant trend of correlation of a PD-L1 H-score higher than 5 in adenocarcinoma specimens with poor OS duration as reported previously (15, 26). Notably, we report herein for the first time that a PD-L1 H-score higher than the median in macrophages correlated significantly with poor OS duration and demonstrated a nonsignificant trend of poor RFS rate in patients with SCC in univariate analysis. Accumulating studies have demonstrated that increased TAM density is associated with poor prognosis for NSCLC (38, 39), suggesting that macrophages are targets for therapy for this cancer. Recently, a study showed that a fraction of macrophages in peritumoral stroma from patients with hepatocellular carcinoma expressed PD-L1 and was attenuated by blocking PD-L1 activity in these cells; the accumulation of these macrophages in the peritumoral stroma also predicted poor prognostic survival in these patients (40).

Increased TIL numbers have been associated with increased survival durations for both early-stage and advanced NSCLC (11, 41, 42). In the present study, the TAIC density correlated with NSCLC outcome in univariate and multivariate analyses. The presence of increased numbers of cells expressing intratumoral CD4 or FOXP3 and macrophages in adenocarcinoma specimens and of increased numbers of cells expressing intratumoral and peritumoral CD57 in SCC specimens were associated with OS or RFS. Only the numbers of cells expressing CD4, CD57, or FOXP3 independently indicated better prognosis in patients with adenocarcinoma in multivariate analysis, although. However, studies using IHC analysis of TILs in surgically resected NSCLC specimens have had conflicting results. One study demonstrated an association between increased numbers of cytotoxic (CD8\textsuperscript{+}) but not helper T (CD4\textsuperscript{+}) cells with increased survival duration (43), whereas others had opposite results or did not demonstrate a survival benefit (44, 45). Similar to our findings, increased natural killer cell (CD57\textsuperscript{+}) infiltration in several types of tumors has been associated with good clinical outcome (46, 47), including for lung SCC (48).

A key understudied issue in lung cancer is the potential for TAIC density to predict response to immunostimulatory therapies and patient outcomes. Studying 3 important immune cell populations helps T cells (CD4\textsuperscript{+}), cytotoxic T cells (CD8\textsuperscript{+}), and macrophages (CD68\textsuperscript{+}; ref. 49), we found that the combination of low CD4/CD8/CD68-positive intratumoral density and high PD-L1 expression in malignant cells identified a small subset of adenocarcinomas (6%) with worse outcome than other tumors. This subset of tumors exhibited characteristics of intrinsic induction of PD-L1. Importantly, the majority of adenocarcinomas (58%) exhibited prominent CD4/CD8/CD68-positive cells with high or low PD-L1 expression in malignant cells. Investigators recently showed that in melanoma and NSCLC cases, tumors with high PD-L1 expression in malignant cells and high densities of TAICs exhibited durable tumor regression and prolonged disease stabilization when treated with anti-PD-1/PD-L1 drugs (50), representing a group of patients who would benefit from this type of therapy.

In summary, in this study, we showed that multiple factors may be correlated with PD-L1 expression in NSCLC malignant cells and macrophages. We identified different patterns of PD-L1 protein expression and detected the TAIC density in surgically resected adenocarcinoma and SCC specimens. We found that TAIC density varied according to the compartment of the tumor examined and that adenocarcinoma specimens with a solid tumor histology, obtained from smokers, or obtained from patients with airflow limitation had higher expression of immune markers than patients without these characteristics. Also, we identified several immune markers whose expression correlated with outcome of NSCLC; however, future validation of our finding using similar cohort of patients is needed.

**Disclosure of Potential Conflicts of Interest**

J.V. Heymach reports receiving commercial research grants from AstraZeneca, Bayer, and GlaxoSmithKline and is a consultant/advisory board member for AstraZeneca, Boehringer Ingelheim, Eli Lilly, Exelis, Genentech, GlaxoSmithKline, Novartis, and Symta. N. Kalhor is a consultant/advisory board member for the Bristol-Myers Squibb Immuno-Oncology Pathology Council. No potential conflicts of interest were disclosed by the other authors.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): E.R. Parra, C. Behrens, J.V. Heymach, B. Sepesi, S.G. Swisher, A. Weissenfeld, N. Kalhor, C. Moran


Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): E.R. Parra, J. Rodriguez-Canales, I.I. Wistuba

Study supervision: E.R. Parra

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Immuno-score in NSCLC


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