Original Study

Assessing PDL-1 and PD-1 in Non–Small Cell Lung Cancer: A Novel Immunoscore Approach

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Abstract

Novel immune biomarkers could complement the TNM classification for non–small cell cancer (NSCLC), improving the prognostic accuracy. The present study evaluated the prognostic significance of the immune checkpoint molecules programmed cell death protein 1 (PD-1) and PD-1 ligand (PD-L1) in 536 patients with stage I to IIIA NSCLC using an Immunoscore approach. Independently, and in combination, the infiltration of immune cells expressing PD-L1 and PD-1 predicted patient survival, supplementing the TNM classification in each stage.

Introduction: Immune checkpoint inhibitors targeting programmed cell death protein 1 (PD-1) or its ligand, PD-L1, have gained momentum in the treatment of non–small cell lung cancer (NSCLC). However, their prognostic significance remains controversial. The present study evaluated the expression of PD-L1 and PD-1 and their potential role in an Immunoscore, supplementing the TNM classification of NSCLC. **Materials and Methods:** Tissue microarrays constructed from tumor tissue samples from 2 cohorts of a total of 536 patients (University Hospital of North Norway, n = 285; Nordland Hospital, n = 251) with primary resected stage I to IIIA NSCLC. PD-L1 and PD-1 were evaluated by immunohistochemistry in the primary tumor and metastatic lymph node tissue. **Results:** In univariate analysis, a high density of PD-L1$^+$ immune cells in the stromal compartment (S-PD-L1) and PD-1$^+$ intraepithelial tumor infiltrating lymphocytes (T-PD-1) was associated with favorable disease-specific survival (DSS; S-PD-L1, $P = .004$; T-PD-1, $P = .012$), both limited to the squamous cell carcinoma histologic subgroup (S-PD-L1, $P = .002$; T-PD-1, $P = .034$). A combined low S-PD-L1 and T-PD-1 was associated with poor survival in all patients (DSS: hazard ratio [HR], 1.81; 95% confidence interval [CI], 1.37-2.40; $P < .001$) at both centers and for all pathologic stages. In multivariate analysis, S-PD-L1 and T-PD-1 were independent positive prognostic factors, and combined low scores remained an independent prognosticator for poor survival (DSS: HR, 1.72; 95% CI, 1.29-2.28; $P < .001$; disease-free survival, $P = .001$; overall survival, $P = .005$). **Conclusion:** Our study identified S-PD-L1 and T-PD-1 as independent positive prognostic factors for NSCLC patients. Their combination added significant prognostic impact within each pathologic stage and hence are feasible to include in a TNM Immunoscore.

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Introduction

The TNM classification system is currently the most important predictor of survival and is an essential guide to treatment decision-making for non–small cell lung cancer (NSCLC), supplemented by histologic and genetic characteristics. However, significant differences in survival within each TNM stage hamper its prognostic accuracy.[1] Accumulating evidence that the immune contexture, defined as the type, density, location, and organization of immune cell subtypes, constitutes a major influence on cancer patient outcomes[2,3] has inspired efforts to identify immunologic factors that can aid in the prognostic assessment of cancer patients. Analysis of the presence and

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subtypes of tumor infiltrating lymphocytes (TILs) in the tumor microenvironment adds significantly to the prognostic yield of the TNM classification in colorectal[4] and breast cancer.[5] In colorectal cancer, the Immunoscore has even surpassed the prognostic impact of the TNM classification. To explore the Immunoscore concept in NSCLC patients, our group identified candidate immune markers (CD8, CD45RO) for an NSCLC TNM−Immunoscore.[6-8] In search of other prognostic markers of immunologic importance that could potentially contribute to an NSCLC Immunoscore, immune checkpoint molecules are of great interest.

Immune checkpoint pathways induce costimulatory and inhibitory signals crucial for regulating the physiologic T cell immune response and maintaining self-tolerance.[9] Activation of the programmed cell death protein 1 (PD-1) pathway mediates inhibitory signals in T cells in the peripheral effector phase of T-cell activation.[9] PD-1 is expressed by activated T cells, B cells, natural killer T cells, and myeloid cells and is often highly expressed by TILs. PD-1 ligand 1 (PD-L1) is expressed by a wide range of hematopoietic and non-hematopoietic cell types.[10] Also, PD-L1 is frequently highly expressed by human cancer cells, induced intrinsically by oncogenic pathways or extrinsically by inflammatory mediators, of which interferon-γ is the most potent.[11] Ligation of PD-L1 with PD-1 mediates suppression of T cell function, differentiation, and survival.[11] Termed "adaptive immune resistance," increased tumor PD-L1 expression as a response to the secretion of cytokines from activated TILs is considered a major mechanism of immune evasion in cancer.[9]

Immune checkpoint inhibitors targeting the PD-1 pathway have demonstrated impressive clinical benefit in several cancers, including NSCLC, although for a limited fraction of patients.[12] This has fueled great interest in the potential prognostic and predictive value of PD-L1 and PD-1 expression. Although some studies have reported high PD-L1 expression in tumor cells or TILs to be predictive of the response to PD-1 pathway inhibition,[13-16] it has not proved adequately reliable as a single biomarker.[17,18] The results regarding its prognostic effects in NSCLC are conflicting.[19] Also, the variability in assays and definitions of biomarker positivity have definitely challenged its validity as a biomarker.[20] Few studies have reported the prognostic effect of stromal expression of PD-L1 and PD-1$^+$ TILs. To the best of our knowledge, no study has compared its expression in primary tumors and lymph node metastases. To assess their prognostic impact and potential as Immunoscore candidate markers, we analyzed the expression of PD-1 and PD-L1 in the tumor epithelium and stromal compartments of 536 primary resected tumors from patients with stage I to IIIA NSCLC and in 142 matched metastatic lymph nodes.

Materials and Methods

Patients and Clinical Samples

Primary tumor tissue specimens from patients who had undergone radical resection for NSCLC pathologic stage I to IIIA at the University Hospital of North Norway (UNN) and the Nordland Hospital (NH) from 1990 through 2010 were retrospectively collected for the present study. The tumors were staged according to the current International Union Against Cancer TNM classification, seventh edition,[21] and histologically classified according to the 2011 World Health Organization guidelines on the classification of lung cancer.[22] According to the hospital pathologic databases, 633 cases of NSCLC were diagnosed during the study period. Of the 633 patients, 97 were excluded because of (1) radiotherapy or chemotherapy before surgery (n = 15); (2) other malignancy within 5 years before the NSCLC diagnosis (n = 39); (3) inadequate paraffin-embedded formalin-fixed tissue blocks available (n = 25); or (4) the presence of adenocarcinoma in situ, which, before 2011 was classified as bronchioloalveolar carcinoma ≤ 3 cm (n = 18).[23] Thus, 536 patients with complete medical records and adequate paraffin-embedded tissue blocks available were eligible, including 142 patients with available lymph node specimens of the 172 patients with node-positive disease. The present report includes follow-up data to October 1, 2013. The median follow-up time of the survivors was 86 months (range, 34-267 months). The Norwegian Data Protection Authority and the Regional Committee for Medical and Health Research Ethics approved the present study (protocol ID, 2011/2503) and waived the need for patient consent. The reporting of the clinicopathologic variables, survival data, and biomarker expression was conducted in accordance with the REMARK (reporting recommendations for tumor marker prognostic studies) guidelines.[24]

Microarray Construction

Two pathologists histologically reviewed all the tissue specimens. The most representative areas of viable neoplastic epithelial cells and tumor stroma in the primary tumors and matched positive lymph nodes (LNs) were carefully selected for the tissue microarrays (TMAs). The TMAs were assembled using a tissue-arraying instrument (Beecher Instruments, Silver Springs, MD). The detailed method has been previously reported.[25] In brief, we used a 0.6-mm diameter stylet, and the study specimens were routinely sampled with 2 replicate core samples from different areas of the tumor epithelium and 2 from the tumor stroma in primary tumors and metastatic LNs. Multiple 4-μm sections were cut using a Microm microtome (HM355S; Thermo Fisher Scientific Life Sciences, Waltham, MA) and stained using specific antibodies for immunohistochemistry (IHC) analysis.

IHC Analysis

For analysis of PD-L1 expression with IHC, we used the following antibodies: mouse monoclonal (catalog no. MAB1561; R&D Systems, Minneapolis, MN), rabbit polyclonal (catalog no. ab58810; Abcam, Cambridge, UK), and rabbit monoclonal (catalog no. 13684; clone, E1L3N; Cell Signaling Technology, Danvers, MA). For analysis of PD-1 expression with IHC, mouse monoclonal antibody (catalog no. ab52587; clone, NAT105; Abcam) was applied.

The specificity of the antibodies was verified by staining multiorgan TMAs as positive and negative tissue controls and by corresponding transfectant plasmid cell lysates (see Antibody Validation). The positive tissue controls were tonsil for PD-1 and placenta for PD-L1. The negative tissue controls were samples of normal brain and ventricle for both PD-1 and PD-L1. These antibodies fulfilled the standards for evaluation in our lung cancer population: PD-L1 (clone E1L3N) and PD1 (clone NAT105).

IHC analysis was performed using the Discovery-Ultra immunostainer (Ventana Medical Systems, Tucson, AZ). The slides were deparaffinized in three 8-minute cycles. For on-board antigen

retrieval, PD-L1 and PD-1 were incubated with CC1 for 64 and 32 minutes, respectively. Endogenous peroxidase was blocked by Discovery inhibitor (catalog no. 760-4840; Ventana Medical Systems) for 8 minutes for both antibodies. The PD-L1 primary antibody in 1/25 dilution was loaded, and the slides were incubated for 32 minutes at 37°C. The slides were developed using UltraMap anti-rabbit horseradish peroxidase (HRP; catalog no. 760-4315) for 20 minutes, followed by 8 minutes of HRP amplification, and detection using ChromoMap DAB (catalog no. 760-159; Ventana Medical Systems). The PD-1 primary antibody in 1/50 dilution was incubated for 60 minutes at 37°C. Next, OmniMap anti-mouse HRP (catalog no. 760-4310) was applied for 16 minutes, followed by 16 minutes of HRP amplification. The primary antibodies were visualized using a purple detection kit (catalog no. 760-229) with a 32-minute incubation time. Finally, to detect the nuclei, the slides were counterstained with hematoxylin II reagent (Ventana Medical Systems) for 32 minutes, followed by a bluing reagent for 8 minutes. The slides were then dehydrated, cleared, and mounted using routine processing. Control staining with an isotype-matched antibody by omission of the primary antibody was also performed for each antibody. For each antibody, staining was performed as a single experiment.

Antibody Validation

Transient overexpressed human HEK293T cell lysates were used for PD-L1 (catalog no. LY415473), PD-1 (catalog no. LY401555), and HEK293 (catalog no. LY500001/negative control; all from OriGene Technologies, Rockville, MD). They were incubated with 2 × sodium dodecyl sulfate sample buffer (OriGene Technologies) for 10 minutes at 100°C. Equal amounts of protein lysates were resolved onto a 4% to 12% Bis-Tris gel (catalog no. NP0322; Life Technologies, Carlsbad, CA). The resolved proteins were transferred onto an Odyssey nitrocellulose membrane (catalog no. 926-31092; LI-COR Biosciences, Lincoln, NE), and the membrane was subsequently blocked for 1 hour at room temperature using the Odyssey blocking buffer (catalog no. 927-40000; LI-COR Biosciences). PD-L1 antibody in a 1/1000 dilution and PD-1 antibody in a 1/50 dilution were applied, and the membrane was incubated overnight at 4°C. IRDye 800CW secondary antibodies for PD-L1 (catalog no. 926-32213; LI-COR Biosciences) and PD-1 (catalog no. 926-32212; LI-COR Biosciences) in 1/10,000 dilution were incubated for 1 hour at room temperature. Rabbit anti-actin (catalog no. A2066; Sigma-Aldrich, St. Louis, MO), 1:1000, was used as the internal control, and all lanes showed 42-kDa molecular weight protein load (Supplemental Figure 1; online version). Between antibody incubations, the membrane was washed 3 times for 5 minutes each in Tris-buffered saline containing 0.05% Tween 20 (Sigma-Aldrich). MagicMark XP Western Protein Standard (catalog no. LC5603; Invitrogen, Carlsbad, CA) and SeeBlue Plus2 prestained standard (catalog no. LC5925; Invitrogen) were used as molecular weight ladders. The most prominent bands represent the observed molecular weight of the detected protein, which corresponded intimately with the predicted weight provided by the manufacturer.

IHC Scoring

The samples were anonymized and independently scored by 2 of us (PD-L1: T.K.K. and E.-E.P.; PD-1: M.R.K. and E.-E.P.), under the supervision of an experienced pathologist (E.R.), who established a semiquantitative score for each marker. When assessing a given core, the observers were blinded to the other's findings, the clinical variables, and the outcomes.

The NSCLC tumor stroma consists mainly of immune cells of myeloid and lymphoid origin, in addition to basement membranes, fibroblasts, extracellular matrix, and vasculature. The tumor epithelial and stromal compartments were scored separately.

PD-L1 staining was typically cytoplasmatic, with a variably strong component of membranous staining. In general, the staining intensity of PD-L1 in tumor epithelial cells (T-PD-L1) was relatively homogenous, although in some cases, and more commonly in solid tumor aggregates, the intensity was stronger in the tumor–stroma interface. The intensity of T-PD-L1 was scored as 0, no staining; 1, weak; 2, moderate; and 3, strong.

In the stromal compartment, PD-L1 staining (S-PD-L1) was predominantly found in cells morphologically consistent with immune cells, with homogenous, moderate to strong intensity. The percentage of stromal PD-L1$^+$ cells compared with the total number of nucleated cells (density) was scored as 0, absent; 1, 1% to 49%; 2, 50% to 75%; or 3, > 75%.

The PD-1 antibody stained cells morphologically consistent with TILs, with homogenous intensity. The percentage of intraepithelial PD-1$^+$ TILs completely enclosed by tumor epithelial cells compared with the total number of nucleated TILs (density) in the tumor epithelial compartment (T-PD-1) was scored as 0, absent; 1, 1% to 9%; 2, 10% to 50%; or 3, > 50%. In the stromal compartment (S-PD-1), it was scored as 0, absent; 1, 1% to 24%; 2, 25% to 50%; or 3, > 50%. Scoring of the density of CD8$^+$ and CD45RO$^+$ TILs has been previously reported.[8] Identical scoring approaches were used in the primary tumors and metastatic LNs for T-PD-L1 and T-PD-1. However, the stromal component of metastatic tissue in LNs is difficult to discern from LN tissue and, therefore, was not scored.

Two cores were sampled from each compartment (tumor epithelial and stromal) and scored by 2 of us. Both cores from primary tumors were missing in 5% to 6% of cases, and 1 core was missing in 3% to 7% of cases. For metastatic LN tissue, both cores were missing in 12% to 15%, and 1 core was missing in 12% to 18% of cases. The mean value of the 4 or 2 (if 1 TMA core was missing) scores available from each patient was used as the basis for dichotomization of the patients' scores into categories of high and low. All possible cutoff thresholds were tested. For each marker, the cutoff chosen for dichotomization was the one yielding the minimum P value when analyzing the association between each marker with disease-specific survival (DSS) in primary tumors, keeping the categories balanced in size (close to the mean value). Accordingly, a high score was defined as follows (mean value of single scores provided in parentheses): T-PD-L1 > 1.25 (1.12), S-PD-L1 > 1.5 (1.45), T-PD-1 > 0.25 (0.68), S-PD-1 > 1 (1.21), S-CD8 > 1.5 (1.55), and T-CD45RO > 0.5 (0.66). We applied the same cutoffs for the LN metastases. IHC scoring of PD-L1 and PD-1 in the primary tumor is illustrated in Figure 1. IHC scoring of the metastatic LNs is presented in Supplemental Figure 2 (online version).

Statistical Analysis

All statistical analyses were performed using the SPSS, version 22 (SPSS, Chicago, IL). The IHC scores from each observer were

Figure 1 Programmed Cell Death Protein 1 (PD-1) and PD-1 Ligand (PD-L1) Immunohistochemical Analysis in Primary Tumors. Immunohistochemical Analysis of Non–Small Cell Lung Cancer Representing Different Scores for Tumor Cell and Stromal Expression in Primary Tumors. (A) Low Intraepithelial PD-1$^+$ Tumor Infiltrating Lymphocytes (TILs) (T-PD-1) Score (Squamous Cell Carcinoma [SCC]). (B) High T-PD-1 Score (SCC). (C) Low Stromal PD1$^+$ TILs (S-PD-1) Score (SCC). (D) High S-PD-1 Score (Adenocarcinoma [ADC]). (E) PD-1$^+$ Tissue Control (Tonsil). (F) Low Intraepithelial PD-L1 (T-PD-L1) Score (SCC). (G) High T-PD-L1 Score (SCC). (H) Low Stromal PD-L1$^+$ Immune Cell (S-PD-L1) Score (SCC). (I) High S-PD-L1 Score (ADC). (J) PD-L1$^+$ Tissue Control (Placenta). Original Magnification ×20. In Most Tumor Cores and in Some Stromal Cores, a Mixture of Stromal Cells and Tumor Cells Was Found. Using Morphologic Criteria, We Scored Intraepithelial Cells Only if Tumor Tissue Was Present and Stromal Cells Only in Stromal Tissue

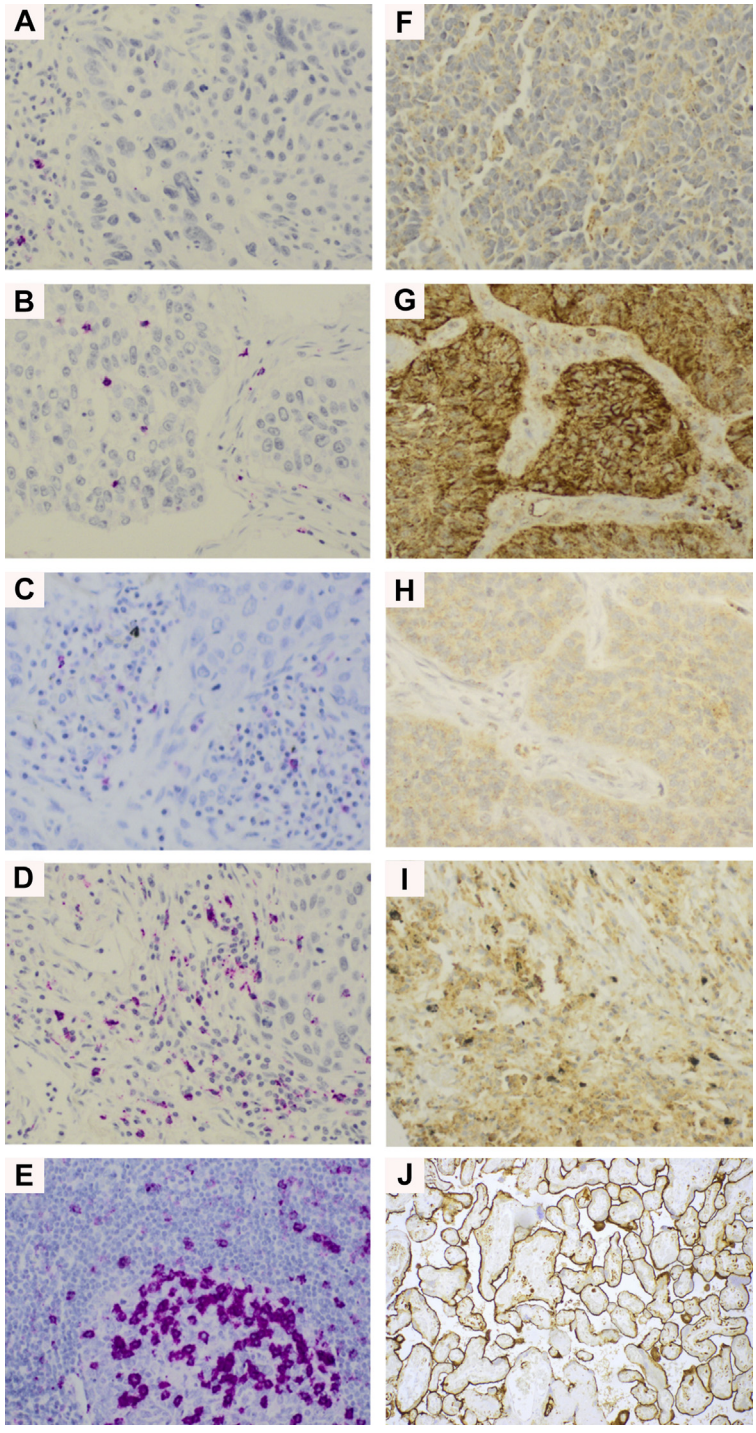


Table 1 Clinicopathologic Variables as Predictors of Disease-Specific Survival in 536 NSCLC Patients (Univariate Analyses; Log-Rank Test, Unadjusted Cox Proportional HRs)

	All Patients					SCC Patients					ADC Patients				
Variable	n (%)	5-Year DSS (%)	Median DSS (mo)	HR (95% CI)	*P* Value	n (%)	5-Year DSS (%)	Median DSS (mo)	HR (95% CI)	*P* Value	n (%)	5-Year DSS (%)	Median DSS (mo)	HR (95% CI)	*P* Value
Age (years)					.711					.654					.505
≤65	227 (42)	57	127	1		106 (37)	64	235	1		102 (51)	48	54	1	
>65	309 (58)	58	NA	0.95 (0.73-1.24)		183 (63)	66	NA	0.91 (0.61-1.36)		99 (49)	49	57	0.87 (0.59-1.3)	
Sex					.026[a]					.108					.050[a]
Female	170 (32)	63	190	1		73 (25)	73	NA	1		83 (41)	56	190	1	
Male	366 (68)	55	88	1.4 (1.06-1.84)		216 (75)	63	235	1.49 (0.96-2.31)		118 (59)	43	51	1.5 (1.01-2.23)	
ECOG PS					.015[a]					.158					.003[a]
0	310 (58)	62	235	1		158 (55)	69	235	1		122 (61)	56	NA	1	
1	190 (35)	52	71	1.45 (1.09-1.93)		110 (38)	61	114	1.47 (0.97-2.23)		67 (33)	40	50	1.57 (1.02-2.4)	
2	36 (7)	48	36	1.61 (0.83-3.09)		21 (7)	67	NA	1.08 (0.45-2.6)		12 (6)	17	25	3.25 (0.96-11.03)	
Smoking					.039[a]					.19					.68
Never	17 (3)	44	20	1		7 (2)	50	19	1		9 (5)	44	21	1	
Previous	342 (64)	62	235	0.56 (0.25-1.24)		182 (63)	69	235	0.58 (0.14-2.37)		125 (62)	50	68	0.69 (0.26-1.84)	
Present	177 (33)	51	71	0.75 (0.33-1.7)		100 (35)	60	114	0.82 (0.2-3.41)		67 (33)	45	57	0.73 (0.27-1.99)	
Weight loss					.961					.689					.536
<10%	480 (90)	58	127	1		257 (89)	66	235	1		184 (92)	49	57	1	
≥10%	55 (10)	59	NA	0.99 (0.63-1.56)		32 (11)	62	NA	1.14 (0.57-2.28)		17 (8)	40	47	1.24 (0.59-2.63)	
Surgical procedure					<.001[a]					<.001[a]					<.001[a]
Wedge or lobectomy	394 (74)	63	190	1		197 (68)	72	235	1		161 (80)	54	104	1	
Pneumonectomy	142 (26)	42	30	1.98 (1.43-2.74)		92 (32)	50	35	1.99 (1.28-3.09)		40 (20)	25	24	2.66 (1.46-4.84)	
Surgical margins					.129					.252					.018[a]
Free	489 (91)	59	190	1		257 (89)	67	235	1		189 (94)	50	68	1	
Not free	47 (9)	47	57	1.39 (0.85-2.29)		32 (11)	57	114	1.39 (0.73-2.63)		12 (6)	0	35	2.33 (0.81-6.69)	
T stage					<.001[a]					<.001[a]					<.001[a]
1	168 (31)	72	235	1		83 (29)	78	235	1		74 (37)	67	190	1	
2	265 (49)	57	91	1.74 (1.3-2.32)		147 (51)	66	NA	1.88 (1.22-2.89)		94 (47)	43	47	1.94 (1.27-2.95)	
3	97 (18)	36	30	2.84 (1.87-4.31)		56 (19)	46	33	2.93 (1.62-5.31)		31 (15)	16	25	3.48 (1.76-6.9)	
4	6 (0)	20	15	4.89 (0.89-26.9)		3 (1)	0	10	17.41 (0.22-1371.77)		2 (1)	50	13	1.76 (0.23-13.27)	
N stage					<.001[a]					<.001[a]					<.001[a]
0	364 (68)	69	235	1		198 (69)	77	235	1		133 (66)	60	190	1	
1	118 (22)	36	35	2.76 (1.93-3.94)		73 (25)	45	35	3.26 (1.99-5.35)		39 (19)	25	30	2.41 (1.38-4.2)	
2	54 (10)	21	19	4.23 (2.43-7.37)		18 (6)	18	13	7.12 (2.44-20.77)		29 (15)	23	24	2.88 (1.42-5.82)	

Table 1 Continued

Variable	All Patients					SCC Patients					ADC Patients				
	n (%)	5-Year DSS (%)	Median DSS (mo)	HR (95% CI)	*P* Value	n (%)	5-Year DSS (%)	Median DSS (mo)	HR (95% CI)	*P* Value	n (%)	5-Year DSS (%)	Median DSS (mo)	HR (95% CI)	*P* Value
Pathologic stage					<.001[a]					<.001[a]					<.001[a]
I	256 (48)	72	235	1		127 (44)	82	235	1		105 (52)	65	190	1	
II	194 (36)	53	84	1.89 (1.42-2.51)		126 (44)	60	114	2.5 (1.66-3.77)		56 (28)	34	43	2.07 (1.3-3.28)	
IIIA	86 (16)	20	17	4.58 (2.87-7.32)		36 (12)	23	15	7.15 (3.23-15.84)		40 (20)	16	24	3.37 (1.8-6.33)	
Histologic type					.040[a]										
SCC	289 (54)	65	235	1											
ADC	201 (37)	48	57	1.43 (1.08-1.89)											
LCC	46 (9)	50	83	1.29 (0.8-2.08)											
Differentiation					<.001[a]					.033[a]					.006[a]
Poor	231 (43)	49	51	1		104 (36)	57	84	1		81 (40)	38	43	1	
Moderate	240 (45)	63	190	0.67 (0.5-0.89)		155 (54)	70	235	0.63 (0.41-0.97)		85 (42)	50	68	0.69 (0.44-1.07)	
Well	65 (12)	70	NA	0.44 (0.29-0.66)		30 (10)	72	NA	0.47 (0.24-0.94)		35 (18)	69	NA	0.36 (0.21-0.63)	
Vascular infiltration					<.001[a]					.029[a]					.012[a]
No	437 (82)	62	235	1		231 (80)	69	235	1		172 (86)	52	71	1	
Yes	97 (18)	38	35	1.89 (1.29-2.78)		58 (20)	53	71	1.65 (0.97-2.82)		27 (13)	26	27	1.9 (1-3.62)	
Missing	2 (0)										2 (1)				

Abbreviations: ADC = adenocarcinoma; CI = confidence interval; DSS = disease-specific survival; ECOG PS = Eastern Cooperative Oncology Group performance status; HR = hazard ratio; LCC = large cell carcinoma; NA = not applicable; NSCLC = non–small-cell lung cancer; SCC = squamous cell carcinoma.
[a]Statistically significant.

Table 2 Prognostic Effect of Tumor Epithelial and Stromal PD-1 and PD-L1 Expression in Primary Tumors and Metastatic Lymph Nodes on Disease-Specific Survival (Univariate Analyses; Log-Rank Test, Unadjusted Cox Proportional HRs)

	All Patients					SCC					ADC				
Variable	n (%)	5-Year DSS (%)	Median DSS (mo)	HR (95% CI)	*P* Value	n (%)	5-Year DSS (%)	Median DSS (mo)	HR (95% CI)	*P* Value	n (%)	5-Year DSS (%)	Median DSS (mo)	HR (95% CI)	*P* Value
S-PD-L1					.004[a]					.002[a]					.501
High	182 (34)	67	235	1.00		105 (36)	80	NR	1.00		67 (33)	50	57	1.00	
Low	323 (60)	53	73	1.55 (1.15-2.1)		173 (60)	57	235	2.09 (1.31-3.32)		120 (60)	47	57	0.75 (0.10-1.79)	
Missing	31 (6)					11 (4)					14 (7)				
T-PD-L1					.313					.037[a]					.632
High	130 (24)	63	190	1.00		64 (22)	79	235	1.00		55 (27)	47	47	1.00	
Low	373 (70)	56	104	1.19 (0.85-1.63)		211 (73)	63	NR	1.79 (1.03-3.11)		131 (65)	48	57	0.90 (0.58-1.40)	
Missing	33 (6)					14 (5)					15 (8)				
N^+ LN T-PD-L1					.773					.674					.544
High	36 (25)	32	21	1.00		15 (20)	13	40	1.00		17 (29)	25	37	1.00	
Low	84 (59)	31	24	0.93 (0.57-1.52)		47 (64)	44	25	0.85 (0.41-1.79)		30 (52)	13	24	1.25 (0.61-2.59)	
Missing	23 (16)					12 (16)					11 (19)				
S-PD-1					.080					.182					.251
High	253 (47)	62	253	1.00		134 (46)	72	253	1.00		98 (49)	52	73	1.00	
Low	253 (47)	53	91	1.28 (0.97-1.68)		142 (49)	60	NR	1.32 (0.88-1.99)		90 (45)	44	50	1.27 (0.84-1.91)	
Missing	30 (6)					13 (5)					13 (6)				
T-PD-1					.012[a]					.034[a]					.281
High	282 (53)	63	235	1.00		155 (54)	73	235	1.00		103 (51)	52	88	1.00	
Low	225 (42)	51	64	1.42 (1.08-1.86)		122 (42)	57	NR	1.55 (1.03-2.32)		85 (42)	43	52	1.25 (0.83-1.88)	
Missing	29 (5)					12 (4)					13 (7)				
N^+ LN T-PD-1					.570					.168					.781
High	38 (27)	34	27	1.00		14 (19)	64	105	1.00		20 (34)	16	13	1.00	
Low	87 (61)	31	21	1.15 (0.70-1.90)		48 (65)	42	33	1.93 (0.74-5.00)		34 (59)	15	23	0.91 (0.47-1.76)	
Missing	17 (12)					12 (16)					4 (7)				
S-PD-L1 + T-PD-1					<.001[a]					<.001[a]					.045[a]
Other	339 (63)	63	235	1.00		195 (68)	74	235	1.00		117 (58)	54	189	1.00	
Low + low	157 (29)	43	43	1.81 (1.37-2.40)		78 (27)	47	37	2.06 (1.36-3.13)		68 (34)	38	50	1.52 (1.01-2.31)	
Missing	40 (8)					16 (5)					16 (8)				

Abbreviations: ADC = adenocarcinoma; CI = confidence interval; DSS = disease-specific survival; HR = hazard ratio; NA = not applicable; N^+ LN = metastatic lymph node; NR = not reached; NSCLC = non–small-cell lung cancer; PD-1 = programmed cell death protein 1; PD-L1 = PD-1 ligand; S = stromal; SCC = squamous cell carcinoma; T = tumor.

[a]Statistically significant.

compared for interobserver reliability using a 2-way random effects model with absolute agreement definition, yielding an intraclass correlation coefficient (reliability coefficient) and Cohen's kappa. DSS, disease-free survival (DFS), and overall survival (OS) were defined as the time from surgery to lung cancer death, first lung cancer relapse, and death from any cause, respectively.

The χ^2 test or Fischer's exact test was used to examine the association between molecular marker expression and various clinicopathologic parameters. Spearman's rank correlation coefficient was used to examine the associations among the markers' expression. Univariate analysis of survival according to each immune marker was visualized using the Kaplan-Meier method, and statistically significant differences between survival curves were assessed using the log-rank test. For univariate analyses, unadjusted Cox proportional hazard ratios (HRs) were calculated. Multivariate analysis was performed using the Cox proportional hazards model, testing the simultaneous influence on survival of all relevant, by expert opinion, covariates found to be significant on the univariate analyses. All clinicopathologic variables significant in the initial multivariate analysis were included in the multivariate analyses, including the immunologic markers assessed in the present study. The backward conditional method was used for model fitting. The probability for stepwise entry and removal was set at .05 and .10, respectively. P values $< .05$ were considered statistically significant.

Results

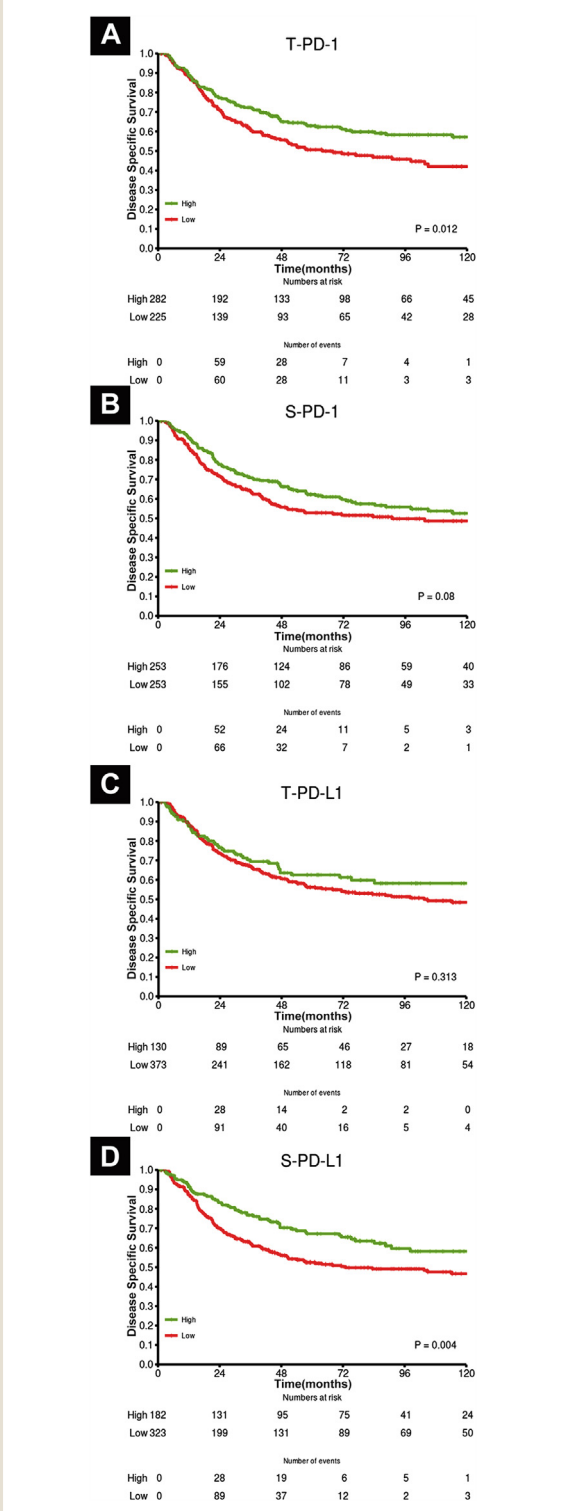
Patient Characteristics

The demographic, clinical, and histopathologic variables are listed in Table 1. The median age was 67 years (range, 28-85 years), and 68% of the patients were men. Because of LN metastasis or nonradical surgical margins, 76 patients (14%) received postoperative radiotherapy. After its introduction into the Norwegian national guidelines in 2005 (stage II-IIIA disease), 43 patients (8%) received adjuvant therapy. Of the 172 patients with N^+ disease, 91 were diagnosed with squamous cell carcinoma (SCC), 68 with adenocarcinoma (ADC), and 13 with large cell carcinoma.

Correlations Between PD-1/PD-L1 and Clinicopathologic Variables, Interobserver Reliability, and Between-Core Heterogeneity

No significant associations were found between the expression of PD-L1 or PD-1 and age, sex, Eastern Cooperative Oncology Group status, smoking, N status, pathologic stage, histologic subgroup, or vascular infiltration. The level of heterogeneity was low (assessed using intraclass correlations), and the agreement between the 2 cores sampled from 1 tumor was greater for markers analyzed in primary tumor nests (T-PD-L1, 0.844; T-PD-1, 0.805) than in primary tumor stroma (S-PD-L1, 0.792; S-PD-1, 0.726). A similar tendency was found in the metastatic LNs (LN T-PD-L1, 0.917; LN T-PD-1, 0.797). Extensive correlations were found between the mean scores of PD-L1 and PD-1 in the intraepithelial and stromal compartments. Between the primary tumor and LN tissue scores, we found a significant correlation only for T-PD-1 (Supplemental Table 1; online version). The between-scorer agreement was excellent, with an intraclass correlation > 0.83 for all markers (Supplemental Table 2; online version).

Figure 2 Survival Curves. Disease-Specific Survival Curves Are Shown According to Intraepithelial Programmed Cell Death Protein 1-Positive ($PD\text{-}1^+$) Tumor Infiltrating Lymphocytes (TILs) (T-PD-1) (A), Stromal $PD\text{-}1^+$ TILs (S-PD-1) (B), Intraepithelial PD-L1 (T-PD-L1) (C), and Stromal $PD\text{-}L1^+$ Immune Cells (S-PD-L1) (D) in Primary Tissue for All Patients



Univariate Analysis

The results of the univariate analyses of the prognostic impact regarding stromal and tumor PD-1 and PD-L1 expression on DSS are presented in Table 2 and Figure 2. For S-PD-L1 and T-PD-1, several different thresholds for high and low expression resulted in a significant association with DSS. The results were largely similar for the DFS and OS endpoints (Supplemental Table 3; online version).

In the entire patient material, a low density of PD-L1$^+$ cells in the stromal compartment (S-PD-L1) was associated with unfavorable DSS (HR, 1.55; 95% confidence interval [CI], 1.15-2.10; $P = .004$). A low density of intraepithelial PD-1$^+$ immune cells (T-PD-1) was also significantly and negatively associated with DSS (HR, 1.42; 95% CI, 1.08-1.86; $P = .012$).

When assessing the outcome by histologic subgroup, low S-PD-L1 was significantly associated with DSS (HR, 2.09; 95% CI, 1.31-3.32; $P = .002$) in the SCC patient subgroup in both cohorts (UNN, $P = .017$; and NH, $P = .033$). Also, low tumor epithelial expression of PD-L1 (T-PD-L1; HR, 1.79; 95% CI, 1.03-3.11; $P = .037$) and PD-1 (T-PD-1; HR, 1.55; 95% CI, 1.03-2.32; $P = .034$) had a significant negative prognostic impact on DSS in SCC patients. PD-L1 or PD-1 status was not associated with survival for patients with large cell carcinoma or ADC.

Metastatic LNs

No significant associations were found between the expression of PD-L1 and PD-1 in the metastatic LNs and survival (DSS, DFS, or OS; Table 2; Supplemental Table 1; online version). The frequency of high T-PD-L1 was 29% in both primary tumors and metastatic LNs. Nearly 69% displayed identical scores in the primary tumor and LN metastasis, 16% had a high score in the primary tissue and a low score in the LNs, and 15% had the opposite. The frequency of T-PD-1$^+$ TILs was lower in metastatic LNs (30%) than in primary tumors (56%; $P < .001$); 53% maintained the score in the metastatic LNs, 38% had a high score in the primary tissue only, and 9% in the metastatic LNs only.

PD Immunoscore

Combining the scores of the 2 variables with the strongest prognostic impact for all patients in the univariate analyses, S-PD-L1 and T-PD-1, allowed for stratification of patients with low scores for both markers compared with all other score combinations (PD Immunoscore; Figure 3). This combination yielded a highly significant negative association of "low + low" scores with DSS in the entire patient material (HR, 1.81; 95% CI, 1.37-2.40;

Figure 3 From TNM to TNM-I. Disease-Specific Survival (DSS) Curves of All Patients According to (A) Pathologic Stage (TNM) and (B) Programmed Cell Death Protein (PD) Immunoscore According to the Combination of the PD-1$^+$ Intraepithelial Tumor Infiltrating Lymphocyte (T-PD-1) and Stromal PD-L1 (S-PD-L1) Score. The Combination of Pathologic Stage and PD Immunoscore Resulted in a TNM Immunoscore Table of 5-Year DSS (C), Adding Significant Prognostic Impact Across Each Pathologic Stage (Pstage). Furthermore, Patients Were Grouped by 5-Year Survival According to the TNM Immunoscore (< 10%, 10%-40%, 40%-70%, > 70%), and the Resulting Survival Curves (D) Illustrate the Increased Stratification

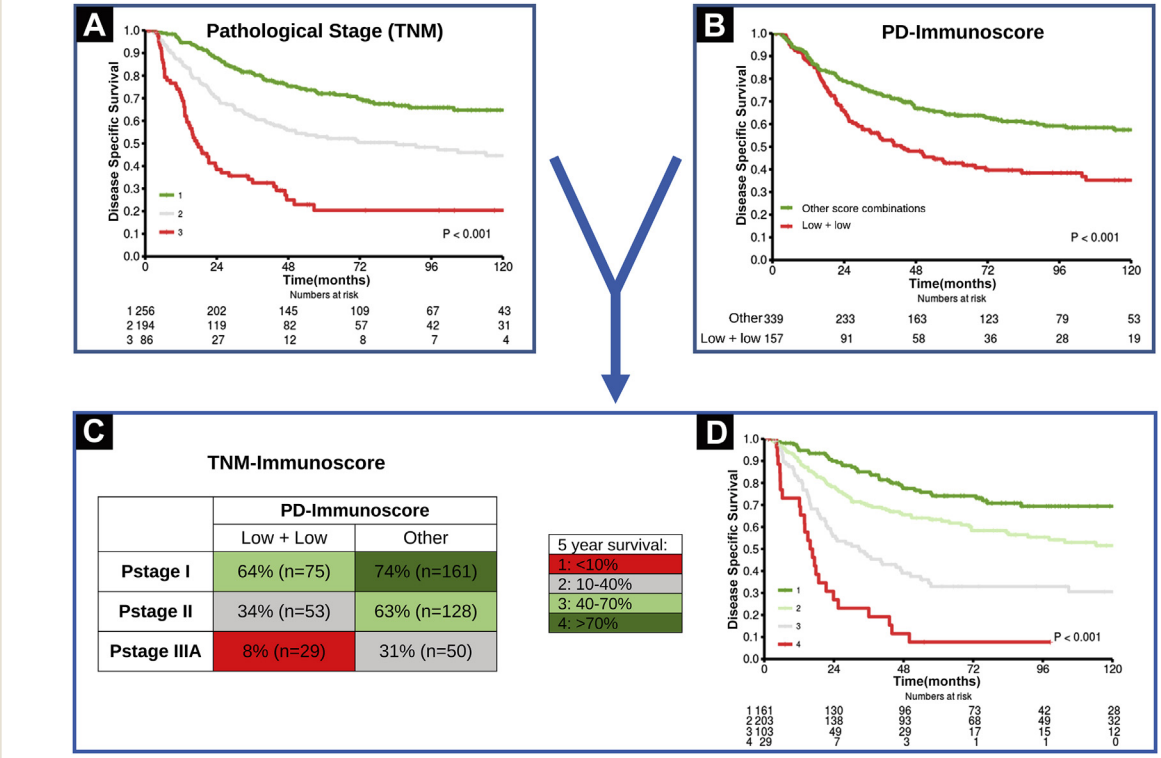


Table 3 Results of Cox Regression Analysis Summarizing Significant Independent Prognostic Factors for Disease-Specific Survival

Variable	All Patients		SCC		ADC	
	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value
Clinicopathologic variables[a]						
Pathologic stage		<.001[b,c]		<.001[b,c]		<.001[b,c]
I	1.00		1.00		1.00	
II	1.79 (1.31-2.46)	<.001[b]	2.49 (1.53-4.06)	<.001[b]	2.20 (1.37-3.53)	.001[b]
IIIA	4.08 (2.83-5.89)	<.001[b]	7.38 (4.17-13.07)	<.001[b]	3.33 (1.94-5.72)	<.001[b]
Histologic type		.003[b,c]				
SCC	1.00					
ADC	1.62 (1.21-2.19)	.001[b]				
LCC	1.00 (0.60-1.67)	.996				
Vascular infiltration						
No vs. yes	1.79 (1.29-2.50)	.001[b]	1.51 (0.95-2.41)	.081	1.72 (1.01-2.94)	.048[b]
Differentiation		.006[b,c]		.303[c]		.098[c]
Well	1.00		1.00		1.00	
Moderate	1.75 (1.02-3.01)	.042[b]	1.31 (0.59-2.89)	.201	1.99 (0.98-4.03)	
Poor	2.32 (1.35-3.99)	.001[b]	1.69 (0.76-3.78)	.507	2.13 (1.06-4.26)	
Sex						
Female vs. male	1.68 (1.24-2.29)	.001[b]		NE	1.64 (1.07-2.52)	.023[b]
ECOG PS		.005[b,c]		NE		.003[b,c]
0	1.00				1.00	
1	1.54 (1.16-2.03)	.003[b]			1.64 (1.08-2.50)	.021
2	1.78 (0.99-3.18)	.052			3.59 (1.58-8.16)	.002
Smoking		.013[b,c]		NE		NE
Never	1.00					
Present	0.36 (0.18-0.71)	.003[b]				
Former	0.41 (0.20-0.82)	.012[b]				
Surgical margins						
Free vs. not free		NE		NE	1.42 (0.67-2.93)	.362
PD variables assessed in separate models[d]						
T-PD-L1						
High vs. low	1.72 (0.99-3.00)	.055	1.68 (0.97-2.92)	.066	1.05 (0.66-1.67)	.842
S-PD-L1						
High vs. low	1.47 (1.08-1.97)	.014	2.16 (1.36-3.44)	.001	1.16 (0.75-1.79)	.517
T-PD-1						
High vs. low	1.48 (1.12-1.96)	.005[b]	1.71 (1.13-2.58)	.011[b]	1.33 (0.88-1.02)	.173
S-PD-L1 + T-PD-1						
Other scores vs. low + low	1.72 (1.29-2.28)	<.001[b]	2.14 (1.41-3.26)	<.001[b]	1.52 (1.00-2.32)	.049[b]
PD variables assessed in same model[d]						
S-PD-L1[a]						
High vs. low	1.41 (1.03-1.93)	.031[b]	2.05 (1.28-3.3)	.003[b]	1.04 (0.66-1.65)	.853
T-PD-1[a]						
High vs. low	1.39 (1.04-1.85)	.025[b]	1.55 (1.03-2.35)	.038[b]	1.39 (0.89-2.15)	.145
PD Immunoscore assessed with S-CD8 and T-CD45RO[d]						
S-PD-L1 + T-PD-1[a]						
Other scores vs. low + low	1.48 (1.10-2.00)	.010[b]	1.57 (1.00-2.46)	.049[b]	1.52 (1.00-2.32)	.049[b]

$P < .001$) both centers (UNN, $P = .005$; NH, $P = .001$), and all pathologic stages (I, $P = .022$; II, $P = .009$; IIIA, $P = .026$) and for DFS ($P < .001$) and OS ($P = .002$).

Although the PD Immunoscore was marginally significant for DSS in ADC ($P = .045$), the prognostic impact for SCC patients was strong (DSS: HR, 2.06; 95% CI, 1.36-3.13; $P < .001$) and

Table 3 Continued

Variable	All Patients		SCC		ADC	
	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value
S-CD8[a]						
High vs. low	1.54 (1.15-2.08)	.004[b]	1.93 (1.24-2.99)	.004[b]	1.20 (0.76-1.89)	.435
T-CD45RO[a]						
High vs. low	1.36 (0.99-1.87)	.060	1.78 (1.13-2.79)	.012[b]	0.593 (0.51-1.47)	.593

Abbreviations: ADC = adenocarcinoma; CI = confidence interval; HR = hazard ratio; PD-1 = programmed cell death protein 1; PD-L1 = PD-1 ligand; S = stromal; SCC = squamous cell carcinoma; T = tumor.
[a]In the same model.
[b]Statistically significant.
[c]Overall significance as a prognostic factor.
[d]All clinicopathologic covariates significant on multivariate analysis were included in each model.

was present for the other survival endpoints (DFS, $P < .001$; OS, $P = .019$) and confirmed at both centers (UNN, $P = .004$; NH, $P = .042$).

Multivariate Analysis

The results from the multivariate Cox regression analysis are presented in Table 3. Assessed as separate markers included in the same model, both T-PD-1 and S-PD-L1 had an independent prognostic effect on DSS (S-PD-L1: HR, 1.41; 95% CI, 1.03-1.93; $P = .031$; T-PD-1: HR, 1.39; 95% CI, 1.04-1.85; $P = .025$) in the total material. In the SCC patient subgroup, the prognostic effect of S-PD-L1 (HR, 2.05; 95% CI, 1.28-3.30; $P = .003$) surpassed the effect of T-PD-1 (HR, 1.55; 95% CI, 1.03-2.35; $P = .038$).

The PD Immunoscore yielded a significant independent prognostic effect for all patients for DSS, DFS, and OS (HR, 1.72 [Table 3]; HR, 1.57 and 1.36 [Supplemental Table 4; online version]; $P < .001$ for all). This finding was enhanced in the SCC patient subgroup for all endpoints. In contrast, for the ADC patients, a trend was found for DSS and OS. When adjusted for S-CD8 and T-CD45RO, the "low + low" PD Immunoscore remained an independent prognostic factor for all the patients (HR, 1.48; 95% CI, 1.10-2.00; $P = .010$) in both histologic subgroups: SCC (HR, 1.57; $P = .049$) and ADC (HR, 1.52; $P = .049$; univariate analyses of S-CD8 and T-CD45RO; Supplemental Table 5; online version).

Discussion

In our large, unselected patient material of surgically resected stage I to IIIA NSCLC, we demonstrated an independent negative prognostic effect of low stromal PD-L1$^+$ immune cells (S-PD-L1) and of PD-1$^+$ TILs infiltrating the tumor epithelial compartment (T-PD-1) in primary tumors. Combined low scores for S-PD-L1 and T-PD-1 (PD Immunoscore) predicted a negative outcome for all endpoints (DSS, DFS, and OS) in the total cohort and especially in the SCC histologic subgroup. The finding was consistent in the UNN and NH cohorts both and for all pathologic stages. The PD Immunoscore was an independent prognostic factor in multivariate analysis for DSS, DFS, and OS and remained so when adjusting for S-CD8 and T-CD45RO.

The mechanisms regulating the expression of PD-L1 and PD-1 are incompletely understood. Particularly for PD-L1, the lack of standardized assays is a major challenge in the assessment of its predictive and prognostic role.[26] Although its validity has recently been challenged,[20] the PD-L1 antibody applied in our study (E1L3N) is commercially available, has been carefully validated by us and others, and has been used in several NSCLC studies.[27-32] Most clinical studies have used the proportion of membrane-positive tumor cells as a cutoff for tumor positivity after it was associated with the clinical response to anti-PD-1 therapy.[33] However, the functional significance of cytoplasmatic PD-L1 expression remains unclear, and it can be difficult to distinguish membranous from cytoplasmatic staining using IHC.[32] Hence, we, and others, have described and analyzed the combination of cytoplasmatic and membranous staining of PD-L1.[29-31,34] Nevertheless, it is important to be aware that IHC analysis can only capture a snapshot in the dynamic nature of the antitumor response.

Although acceptable in primary tumor tissue, the frequency of missing cores might have limited the statistical power of our analyses of the metastatic LNs, and our results must be considered in light of this. A heterogeneity of PD-L1 expression has been demonstrated in NSCLC tumor tissue,[20] and TMAs might underrepresent the heterogeneity. This was to some extent compensated for in the TMA by the inclusion of 2 representative areas of tumor and 2 of stroma from each tumor specimen. Also, we found a relatively high degree of agreement between the cores from the same tumor. However, in future validating studies, staining should also be performed of whole tissue sections, because that is what is currently being used for diagnostic purposes and also allows for further assessment of heterogeneity.

We observed high tumor epithelial PD-L1 (T-PD-L1) in 26% of patients, similar to recent studies using the same antibody[29,31] in patients with stage I-IIIA NSCLC. Numerous studies have reported a wide range of NSCLC tumor cell PD-L1 positivity rates, and an association with high T-PD-L1 has been reported for both poor[30,34-37] and favorable[29,38-41] outcomes. In contrast, other studies,[27,31,42-44] including a recent meta-analysis,[45] found no associations. Recently, a Korean study reported no association between T-PD-L1 expression and OS in patients with SCC (n = 331).[31] In contrast, 1 German[29] and 1 Australian[39] study (SCC cohort sizes of 149 and 271, respectively) observed a positive prognostic effect, in line with our findings. The heterogeneity with respect to assays, definitions of positivity, and baseline characteristics such as ethnicity might explain some of the differences.[45] Smoking status, mutational load, and oncogene driver mutation status seem to influence the activity of PD-1 inhibitors in NSCLC patients.[46,47] We found no

association between PD-1 and PD-L1 expression or the PD Immunoscore and smoking status. However, our data set did not include an analysis of mutational status.

Although some reports have described the prevalence of PD-L1 expression in immune cells infiltrating stage I to IIIA NSCLC tumors,[20,28,48,49] we are the first to describe the prognostic value of stromal expression of PD-L1 (S-PD-L1). Although our method does not allow for the distinction of stromal cell subtypes, morphologically, PD-L1$^+$ stromal cells were consistent with immune cells. Performing multiplexed IHC should clarify the significance of different subsets of stromal cells and would be of great interest in future similar studies. We found high S-PD-L1 expression in 36% of patients, which was significantly associated with a positive outcome in all patients and in the SCC subgroup, in particular. Our findings contrast with the "adaptive immune resistance" as a major immune evasion mechanism and supports the hypothesis that high levels of S-PD-L1 induced by inflammatory mediators such as interferon-γ produced by immune cells reflect the presence of an ongoing and, at least to some degree, functional tumor microenvironmental immune response. In line with this, Herbst et al[50] found immune cell expression of PD-L1 was predictive of the response to anti−PD-L1 treatment in patients with advanced NSCLC, emphasizing the importance of pre-existing immunity that is further amplified during treatment.

As the first to assess PD-1 positivity in TILs in the intraepithelial (T-PD-1) and stromal (S-PD-1) compartments separately, we observed that high T-PD-1 expression was associated with improved prognosis for all patients and particularly for the SCC histologic subgroup. Three previous studies (2 Korean and 1 German) have investigated PD-1, combined stromal and intraepithelial, in patients with stage I to III NSCLC, using different assays and scoring methods.[29-31] The Korean studies found that PD-1$^+$ TILs were positively associated with OS in patients with SCC[31] and with DFS in patients with ADC.[30] In contrast, the German investigators did not observe any association with OS.[29] Our results might reflect the importance of previously activated immune cells infiltrating tumor tissue and not residing solely around the outer edge, thus avoiding "immunologic ignorance" and "excluded infiltrate," 2 of the patterns described in patients who don't respond to anti-PD-L1 treatment.[51]

A novel finding in the present investigation was the lack of a significant association between survival and the expression of tumor epithelial PD-L1 and intraepithelial PD-1$^+$ TILs in LN metastasis (n = 143), regardless of the histologic subgroups, even when multiple cutoffs were tested. We found the frequency of high T-PD-L1 identical in N$^+$ LN and primary tumors, and the score was maintained from primary tumor to LN metastasis in 69% of cases, in line with the findings from a previous study.[31] The frequency of high T-PD-1 TILs was lower in N$^+$ LNs than in primary tumors; 38% of patients displayed a high score in the primary tumor and a low score in the N$^+$ LNs. Others have found functional differences in the immune infiltrates of primary tumors and metastatic sites and indications of more tolerogenic and tumor-promoting microenvironments in metastatic LNs versus tumor-free LNs.[52-54] Hence, our study findings shed light on the differences in the characteristics of the immune response in primary tumor and metastatic tissue, although the limited N$^+$ study size reduced our ability to draw conclusions. If confirmed in larger studies, this finding supports that primary tissue samples, rather than samples from metastatic LNs, should be used for analysis of these biomarkers in the prognostic setting.

By combining the PD Immunoscore with the pathologic stage, the stratification of patient survival might be considerably improved (Figure 3). Although the TNM classification alone stratifies patient 5-year DSS as 20% to 72% according to pathologic stage IIIA and I, respectively, the addition of a PD Immunoscore increases the stratification of patient survival, ranging from 8% (pathologic stage IIIA and PD Immunoscore "low + low") to 74% (pathologic stage I and PD Immunoscore of "other score combinations"). For patients with pathologic stage I, the 5-year DSS varied from 64% to 74% according to the PD Immunoscore "low + low" versus "other score combinations". For pathologic stage II, it varied from 34% to 63% and for pathologic stage IIIA, from 8% to 31%. Thus, the PD Immunoscore has the potential to stratify patients with regard to survival even within each pathologic stage.

The retrospective nature of the present study introduced a potential bias from variable diagnostic accuracy and treatment. However, the PD Immunoscore was a significant prognostic factor independent of adjuvant chemotherapy or radiotherapy and of whether patients were treated before or after the introduction of adjuvant chemotherapy in accordance with the national recommendations (2005; data not shown).

Measuring the expression of PD-1 and PD-L1 in the tumor microenvironment might be of considerable clinical relevance, representing facets of the immune response seen in "inflamed cancers" versus "noninflamed cancers." If confirmed in prospective studies, this type of prognostic stratification might assist in the selection of patients for adjuvant treatment and the choice of order and type of treatment (eg, chemotherapy vs. immunotherapy). Also, the PD Immunoscore could be tested as a predictor of anti-PD-pathway treatment response in future studies.

Conclusion

We attempted to predict the outcomes of patients with NSCLC stage I to IIIA according to the PD-1 and PD-L1 expression levels in primary tumors and metastatic LNs. We identified the density of stromal PD-L1$^+$ immune cells and intraepithelial PD-1$^+$ TILs in primary tumors as independent positive prognostic factors, mainly attributed to the SCC subgroup. The combined low scores added significantly to the prognostic effect, revealing substantial differences in survival within each pathologic stage. The combination remained an independent prognostic factor for DSS, DFS, and OS for all patients, even after adjustment for immunologic markers with strong independent prognostic effect. The evaluation of PD-1 and PD-L1 expression in the tumor microenvironment, combined in a novel Immunoscore approach to supplement the TNM classification in predicting patient prognosis, seems feasible. If confirmed in prospective studies, this could translate into changes in future treatment decision-making.

Clinical Practice Points

- For NSCLC patients, significant differences in survival within each TNM stage impede its prognostic accuracy, and novel prognostic biomarkers are warranted.

- Analysis of the presence and subtypes of TILs in the tumor microenvironment supplement the prognostic yield of the TNM classification in other cancers and exploring an Immunoscore concept in NSCLC is of great interest.
- Treatment targeting the immune checkpoint molecules PD-1 and PD-L1 has proved effective in some NSCLC patients; however, their potential prognostic value remains uncertain. Thus, exploring the expression in both tumor epithelial cells and stromal immune cells and the association with the outcomes is relevant.
- Our findings showed that a low density of PD-L1$^+$ stromal immune cells and PD-1$^+$ intraepithelial TILs independently predicted for unfavorable survival outcomes, especially for patients with SCC. Low scores for both of these 2 markers combined (PD Immunoscore) independently predicted poor survival (DSS, DFS, and OS) in patients with TNM stage I, II, and IIIA resected NSCLC, allowing the stratification of patient 5-year DSS ranging from 8% to 74%.
- These results suggest that high expression of PD-L1 and PD-1 indicates the presence of an ongoing tumor microenvironment immune response influencing patient survival. Evaluating their expression in the tumor microenvironment might in the future assist treatment decision-making by allowing individualized risk stratification and aiding in the selection of patients for adjuvant treatment and choice of order and type of treatment.

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Disclosure

The authors declare that they have no competing interests.

Supplemental Data

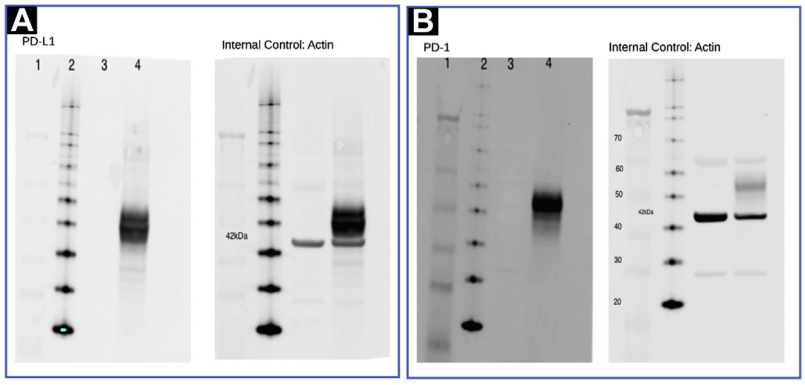
Supplemental figures and tables accompanying this article can be found in the online version at http://dx.doi.org/10.1016/j.cllc.2016.09.009.

References

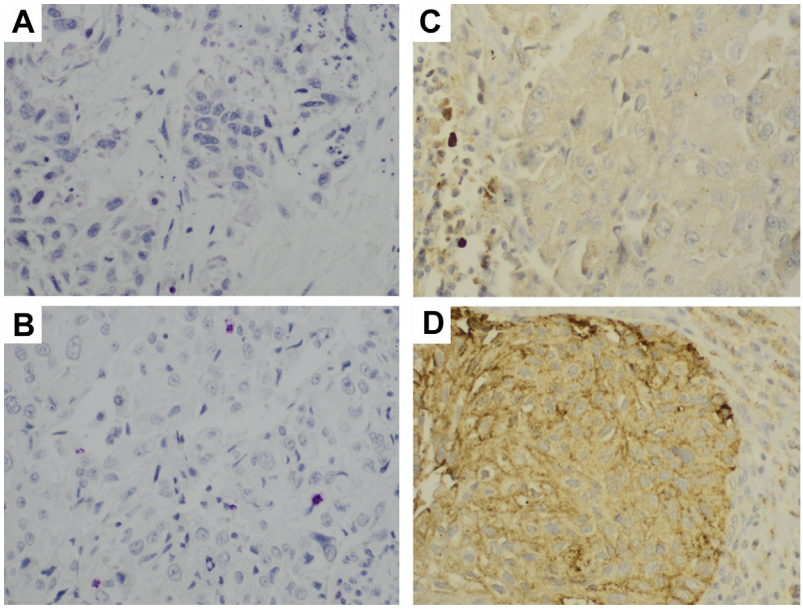
1. Goldstraw P, Crowley J, Chansky K, et al. The IASLC Lung Cancer Staging Project: proposals for the revision of the TNM stage groupings in the forthcoming (seventh) edition of the TNM Classification of malignant tumours. *J Thorac Oncol* 2007; 2:706-14.
2. Fridman WH, Pages F, Sautes-Fridman C, Galon J. The immune contexture in human tumours: impact on clinical outcome. *Nat Rev Cancer* 2012; 12:298-306.
3. Bremnes RM, Busund LT, Kilvaer TL, et al. The role of tumor-infiltrating lymphocytes in development, progression, and prognosis of non-small cell lung cancer. *J Thorac Oncol* 2016; 11:789-800.
4. Angell H, Galon J. From the immune contexture to the Immunoscore: the role of prognostic and predictive immune markers in cancer. *Curr Opin Immunol* 2013; 25:261-7.
5. Salgado R, Denkert C, Demaria S, et al. The evaluation of tumor-infiltrating lymphocytes (TILs) in breast cancer: recommendations by an International TILs Working Group 2014. *Ann Oncol* 2015; 26:259-71.
6. Donnem T, Hald SM, Paulsen EE, et al. Stromal CD8+ T-cell density—a promising supplement to TNM staging in non-small cell lung cancer. *Clin Cancer Res* 2015; 21:2635-43.
7. Donnem T, Kilvaer TK, Andersen S, et al. Strategies for clinical implementation of TNM-Immunoscore in resected nonsmall-cell lung cancer. *Ann Oncol* 2016; 27: 225-32.
8. Paulsen EE, Kilvaer T, Khanehkenari MR, et al. CD45RO(+) memory T lymphocytes—a candidate marker for TNM-Immunoscore in squamous non-small cell lung cancer. *Neoplasia* 2015; 17:839-48.
9. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer* 2012; 12:252-64.
10. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. PD-1 and its ligands in tolerance and immunity. *Annu Rev Immunol* 2008; 26:677-704.
11. Sanmamed MF, Chen L. Inducible expression of B7-H1 (PD-L1) and its selective role in tumor site immune modulation. *Cancer J* 2014; 20:256-61.
12. Anagnostou VK, Brahmer JR. Cancer immunotherapy: a future paradigm shift in the treatment of non-small cell lung cancer. *Clin Cancer Res* 2015; 21:976-84.
13. Garon EB, Rizvi NA, Hui R, et al. Pembrolizumab for the treatment of non-small-cell lung cancer. *N Engl J Med* 2015; 372:2018-28.
14. Borghaei H, Paz-Ares L, Horn L, et al. Nivolumab versus docetaxel in advanced nonsquamous non-small-cell lung cancer. *N Engl J Med* 2015; 373:1627-39.
15. Herbst RS, Baas P, Kim DW, et al. Pembrolizumab versus docetaxel for previously treated, PD-L1-positive, advanced non-small-cell lung cancer (KEYNOTE-010): a randomised controlled trial. *Lancet* 2016; 387:1540-50.
16. Fehrenbacher L, Spira A, Ballinger M, et al. Atezolizumab versus docetaxel for patients with previously treated non-small-cell lung cancer (POPLAR): a multicentre, open-label, phase 2 randomised controlled trial. *Lancet* 2016; 387:1837-46.
17. Patel SP, Kurzrock R. PD-L1 expression as a predictive biomarker in cancer immunotherapy. *Mol Cancer Ther* 2015; 14:847-56.
18. Brahmer J, Reckamp KL, Baas P, et al. Nivolumab versus docetaxel in advanced squamous-cell non-small-cell lung cancer. *N Engl J Med* 2015; 373:123-35.
19. Zhou ZJ, Zhan P, Song Y. PD-L1 over-expression and survival in patients with non-small cell lung cancer: a meta-analysis. *Transl Lung Cancer Res* 2015; 4:203-8.
20. McLaughlin J, Han G, Schalper KA, et al. Quantitative assessment of the heterogeneity of PD-L1 expression in non-small-cell lung cancer. *JAMA Oncol* 2016; 2:46-54.
21. Rami-Porta R, Chansky K, Goldstraw P. Updated lung cancer staging system. *Future Oncol* 2009; 5:1545-53.
22. Travis WD, Brambilla E, Muller-Hermelink HK, Harris CC, World Health Organization Classification of Tumours. *Pathology and Genetics of Tumours of the Lung, Pleura and Heart*. Lyon: IARC Press; 2004.
23. Travis WD, Brambilla E, Van Schil P, et al. Paradigm shifts in lung cancer as defined in the new IASLC/ATS/ERS lung adenocarcinoma classification. *Eur Respir J* 2011; 38:239-43.
24. McShane LM, Altman DG, Sauerbrei W, et al. Reporting recommendations for tumor marker prognostic studies (REMARK). *J Natl Cancer Inst* 2005; 97:1180-4.
25. Donnem T, Al-Saad S, Al-Shibli K, et al. Inverse prognostic impact of angiogenic marker expression in tumor cells versus stromal cells in non small cell lung cancer. *Clin Cancer Res* 2007; 13:6649-57.
26. Kerr KM, Tsao MS, Nicholson AG, et al. Programmed death-ligand 1 immunohistochemistry in lung cancer: in what state is this art? *J Thorac Oncol* 2015; 10: 985-9.
27. Tang Y, Fang W, Zhang Y, et al. The association between PD-L1 and EGFR status and the prognostic value of PD-L1 in advanced non-small cell lung cancer patients treated with EGFR-TKIs. *Oncotarget* 2015; 6:14209-19.
28. Sheng J, Fang W, Yu J, et al. Expression of programmed death ligand-1 on tumor cells varies pre and post chemotherapy in non-small cell lung cancer. *Sci Rep* 2016; 6:20090.
29. Schmidt LH, Kummel A, Gorlich D, et al. PD-1 and PD-L1 expression in NSCLC indicate a favorable prognosis in defined subgroups. *PLoS One* 2015; 10: e0136023.
30. Koh J, Go H, Keam B, et al. Clinicopathologic analysis of programmed cell death-1 and programmed cell death-ligand 1 and 2 expressions in pulmonary adenocarcinoma: comparison with histology and driver oncogenic alteration status. *Mod Pathol* 2015; 28:1154-66.
31. Kim MY, Koh J, Kim S, Go H, Jeon YK, Chung DH. Clinicopathological analysis of PD-L1 and PD-L2 expression in pulmonary squamous cell carcinoma: comparison with tumor-infiltrating T cells and the status of oncogenic drivers. *Lung Cancer* 2015; 88:24-33.
32. Mahoney KM, Sun H, Liao X, et al. PD-L1 antibodies to its cytoplasmic domain most clearly delineate cell membranes in immunohistochemical staining of tumor cells. *Cancer Immunol Res* 2015; 3:1308-15.
33. Topalian SL, Hodi FS, Brahmer JR, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med* 2012; 366:2443-54.
34. Mu CY, Huang JA, Chen Y, Chen C, Zhang XG. High expression of PD-L1 in lung cancer may contribute to poor prognosis and tumor cells immune escape through suppressing tumor infiltrating dendritic cells maturation. *Med Oncol* 2011; 28:682-8.
35. Chen YB, Mu CY, Huang JA. Clinical significance of programmed death-1 ligand-1 expression in patients with non-small cell lung cancer: a 5-year-follow-up study. *Tumori* 2012; 98:751-5.
36. Azuma K, Ota K, Kawahara A, et al. Association of PD-L1 overexpression with activating EGFR mutations in surgically resected nonsmall-cell lung cancer. *Ann Oncol* 2014; 25:1935-40.
37. Mao Y, Li W, Chen K, et al. B7-H1 and B7-H3 are independent predictors of poor prognosis in patients with non-small cell lung cancer. *Oncotarget* 2015; 6: 3452-61.
38. Velcheti V, Schalper KA, Carvajal DE, et al. Programmed death ligand-1 expression in non-small cell lung cancer. *Lab Invest* 2014; 94:107-16.
39. Cooper WA, Tran T, Vilain RE, et al. PD-L1 expression is a favorable prognostic factor in early stage non-small cell carcinoma. *Lung Cancer* 2015; 89:181-8.
40. D'Incecco A, Andreozzi M, Ludovini V, et al. PD-1 and PD-L1 expression in molecularly selected non-small-cell lung cancer patients. *Br J Cancer* 2015; 112: 95-102.

41. Lin C, Chen X, Li M, et al. Programmed death-ligand 1 expression predicts tyrosine kinase inhibitor response and better prognosis in a cohort of patients with epidermal growth factor receptor mutation-positive lung adenocarcinoma. *Clin Lung Cancer* 2015; 16:e25-35.
42. Konishi J, Yamazaki K, Azuma M, Kinoshita I, Dosaka-Akita H, Nishimura M. B7-H1 expression on non-small cell lung cancer cells and its relationship with tumor-infiltrating lymphocytes and their PD-1 expression. *Clin Cancer Res* 2004; 10:5094-100.
43. Boland JM, Kwon ED, Harrington SM, et al. Tumor B7-H1 and B7-H3 expression in squamous cell carcinoma of the lung. *Clin Lung Cancer* 2013; 14:157-63.
44. Yang CY, Lin MW, Chang YL, Wu CT, Yang PC. Programmed cell death-ligand 1 expression in surgically resected stage I pulmonary adenocarcinoma and its correlation with driver mutations and clinical outcomes. *Eur J Cancer* 2014; 50: 1361-9.
45. Zhong A, Xing Y, Pan X, Shi M, Xu H. Prognostic value of programmed cell death-ligand 1 expression in patients with non-small-cell lung cancer: evidence from an updated meta-analysis. *Onco Targets Ther* 2015; 8:3595-601.
46. Rizvi NA, Hellmann MD, Snyder A, et al. Cancer immunology: mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. *Science* 2015; 348:124-8.
47. Akbay EA, Koyama S, Carretero J, et al. Activation of the PD-1 pathway contributes to immune escape in EGFR-driven lung tumors. *Cancer Discov* 2013; 3: 1355-63.
48. Ilie M, Long-Mira E, Bence C, et al. Comparative study of the PD-L1 status between surgically resected specimens and matched biopsies of NSCLC patients reveal major discordances: a potential issue for anti-PD-L1 therapeutic strategies. *Ann Oncol* 2016; 27:147-53.
49. Calles A, Liao X, Sholl LM, et al. Expression of PD-1 and its ligands, PD-L1 and PD-L2, in smokers and never smokers with KRAS-mutant lung cancer. *J Thorac Oncol* 2015; 10:1726-35.
50. Herbst RS, Soria JC, Kowanetz M, et al. Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. *Nature* 2014; 515:563-7.
51. Taube JM, Klein A, Brahmer JR, et al. Association of PD-1, PD-1 ligands, and other features of the tumor immune microenvironment with response to anti-PD-1 therapy. *Clin Cancer Res* 2014; 20:5064-74.
52. Muller P, Rothschild SI, Arnold W, et al. Metastatic spread in patients with non-small cell lung cancer is associated with a reduced density of tumor-infiltrating T cells. *Cancer Immunol Immunother* 2016; 65:1-11.
53. Viguier M, Lemaitre F, Verola O, et al. Foxp3 expressing CD4+CD25(high) regulatory T cells are overrepresented in human metastatic melanoma lymph nodes and inhibit the function of infiltrating T cells. *J Immunol* 2004; 173:1444-53.
54. Heeren AM, Koster BD, Samuels S, et al. High and interrelated rates of PD-L1+CD14+ antigen-presenting cells and regulatory T cells mark the microenvironment of metastatic lymph nodes from patients with cervical cancer. *Cancer Immunol Res* 2015; 3:48-58.

Supplemental Figure 1 Antibody Validation. (A) Programmed Cell Death Protein 1 Ligand (PD-L1; Catalog No. 13684; Clone, E1L3N; Cell Signaling Technology, Danvers, MA) and (B) Programmed Cell Death Protein 1 (PD-1; Catalog No. ab52587; Clone, NAT105; Abcam, Cambridge, UK). Lanes 1 and 2, Molecular Weight Markers (1, SeeBlue; 2, Magic Marker XP); Lane 3, Empty Vector (Catalog No. LY5000001/Negative Control); Lane 4, Transient Overexpressed Human HEK293T Cell Lysates for PD-L1 (Catalog No. LY415473) and PD-1 (Catalog No. LY401555). The Most Prominent Bands Represent the Observed Molecular Weight of the Detected Protein, Which Corresponded Intimately With the Predicted Weight (PD-L1, 40-50 kDa; PD-1, 47 kDa) Provided by the Manufacturer



Supplemental Figure 2 Programmed Cell Death Protein 1 (PD-1) and PD-1 Ligand (PD-L1) Immunohistochemistry in Metastatic Lymph Nodes. Immunohistochemical Analysis of Non—Small-Cell Lung Cancer Representing Different Scores for Tumor Cell (T) and Stromal (S) Expression in Metastatic Lymph Nodes. (A) Low T-PD-1 Score (Squamous Cell Carcinoma [SCC]). (B) High T-PD-1 Score (Adenocarcinoma [ADC]). (C) Low T-PD-L1 Score (SCC). (D) High T-PD-L1 Score (ADC). Original Magnification ×20



Supplemental Table 1 Spearman Rank Correlations Between Mean Scores of PD-L1 and PD-1 in Tumor and Stromal Primary Tissue and Lymph Node Tissue

Variable	S-PD-L1	T-PD-L1	N^+ LN T-PD-L1	S-PD-1	T-PD-1	N^+ LN T-PD-1
S-PD-L1	—					
T-PD-L1	.384[a]	—				
N^+ LN T-PD-L1	NS	.232[b]	—			
S-PD-1	.354[a]	.195[a]	NS	—		
T-PD-1	.279[a]	.113[b]	NS	.537[a]	—	
N^+ LN T-PD-1	NS	NS	NS	NS	.225[b]	—

Abbreviations: N^+ LN = metastatic lymph node; NS = not significant ($P \geq .05$); PD-1 = programmed cell death protein 1; PD-1L = PD-1 ligand; S = stromal; T = tumor.
[a]Statistically significant ($P < .001$).
[b]Statistically significant ($P < .05$).

Supplemental Table 2 Intraclass Correlations and Cohen's Kappa Between Scorers for Investigated Prognostic Markers

Variable	S-PD-L1	T-PD-L1	N^+ LN T-PD-L1	S-PD-1	T-PD-1	N^+ LN T-PD-1
ICC (A,2)	0.879	0.939	0.951	0.900	0.891	0.826
P value	<.001	<.001	<.001	<.001	<.001	<.001
Kappa	0.563	0.754	0.742	0.602	0.582	0.593
P value	<.001	<.001	<.001	<.001	<.001	<.001

Abbreviations: A,2 = 2-way random effects model with absolute agreement; ICC = intraclass correlation; N^+ LN = metastatic lymph node; PD-1 = programmed cell death protein 1; PD-1L = PD-1 ligand; S = stromal; T = tumor.

Supplemental Table 3 Prognostic Effect of Tumor Epithelial and Stromal PD-1 and PD-L1 Expression in Primary Tumors and Metastatic Lymph Nodes on Disease-Free Survival and Overall Survival (Univariate Analyses; Log-Rank Test, Unadjusted Cox Proportional Hazard Ratios)

	All Patients					SCC					ADC				
Variable	n (%)	5-Year Survival (%)	Median Survival (mo)	HR (95% CI)	*P* Value	n (%)	5-Year Survival (%)	Median Survival (mo)	HR (95% CI)	*P* Value	n (%)	5-Year Survival (%)	Median Survival (mo)	HR (95% CI)	*P* Value
DFS															
S-PD-L1					.039[a]					.007[a]					.710
High	182 (34)	59	87	1.00		105 (36)	77	229	1.00		67 (33)	36	36	1.00	
Low	323 (60)	47	44	1.33 (1.01-1.74)		173 (60)	53	119	1.76 (1.16-2.68)		120 (60)	43	38		
Missing	31 (6)					11 (4)					14 (7)				
T-PD-L1					.470					.457					.389
High	130 (24)	54	65	1.00		64 (22)	72	178	1.00		55 (27)	40	41	1.00	
Low	373 (70)	51	68	1.11 (0.84-1.47)		211 (73)	60	NR	0.84 (0.53-1.33)		131 (65)	39	33	1.19 (0.80-1.87)	
Missing	33 (6)					14 (5)					15 (8)				
N^+ LN T-PD-L1					.461					.278					.374
High	35 (25)	33	15	1.00		15 (20)	46	15	1.00		17 (29)	29	18	1.00	
Low	84 (59)	31	15	1.15 (0.72-1.84)		47 (64)	42	22	1.36 (0.65-2.83)		30 (52)	17	13	0.73 (0.36-1.47)	
Missing	23 (16)					12 (16)					11 (19)				
S-PD-1					.043[a]					.138					.202
High	253 (47)	56	83	1.00		134 (46)	69	229	1.00		98 (49)	43	43	1.00	
Low	253 (47)	47	42	1.29 (1.01-1.66)		142 (49)	55	178	1.33 (0.91-1.95)		90 (45)	37	31	1.28 (0.88-1.86)	
Missing	30 (6)					13 (5)					13 (6)				
T-PD-1					.021[a]					.007[a]					.466
High	282 (53)	57	119	1.00		155 (54)	71	229	1.00		103 (51)	44	40	1.00	
Low	225 (42)	45	41	1.34 (1.04-1.72)		122 (42)	51	65	1.68 (1.15-2.45)		85 (42)	34	33	1.15 (0.79-1.67)	
Missing	29 (5)					12 (4)					13 (7)				
N^+ LN T-PD-1					.763					.267					.686
High	38 (27)	34	13	1.00		14 (19)	58	105	1.00		20 (35)	21	11	1.00	
Low	87 (61)	30	15	0.90 (0.56-1.44)		48 (65)	41	15	0.51 (0.20-1.32)		34 (59)	19	18	1.14 (0.60-2.16)	
Missing	17 (12)					12 (16)					4 (7)				
S-PD-L1 + T-PD-1					<.001[a]					<.001[a]					.174
Other	339 (63)	58	119	1.00		195 (68)	71	229	1.00		117 (58)	44	44	1.00	
Low + low	157 (29)	37	28	1.65 (1.28-2.14)		78 (27)	40	26	2.04 (1.38-3.01)		68 (34)	32	31	1.31 (0.89-1.92)	
Missing	40 (8)					16 (5)					16 (8)				

Supplemental Table 3 Continued

Variable	All Patients					SCC					ADC				
	n (%)	5-Year Survival (%)	Median Survival (mo)	HR (95% CI)	*P* Value	n (%)	5-Year Survival (%)	Median Survival (mo)	HR (95% CI)	*P* Value	n (%)	5-Year Survival (%)	Median Survival (mo)	HR (95% CI)	*P* Value
Overall survival															
S-PD-L1					.053					.085					.344
High	182 (34)	52	70	1.00		105 (36)	58	78	1.00		67 (33)	44	52	1.00	
Low	323 (60)	40	40	1.24 (1.00-1.54)		173 (60)	43	33	1.29 (0.96-1.72)		120 (60)	36	45	1.44 (1.19-1.70)	
Missing	31 (6)					11 (4)					14 (7)				
T-PD-L1					.136					.064					.530
High	130 (24)	51	62	1.00		64 (22)	61	84	1.00		55 (27)	44	47	1.00	
Low	373 (70)	43	45	1.20 (0.94-1.53)		211 (73)	45	40	1.37 (0.98-1.92)		131 (65)	38	50	1.13 (0.77-1.67)	
Missing	33 (6)					14 (5)					15 (8)				
N^+ LN T-PD-L1					.804					.853					.180
High	35 (25)	27	19	1.00		15 (20)	33	19	1.00		17 (29)	25	37	1.00	
Low	84 (59)	20	17	1.06 (0.69-1.60)		47 (64)	25	18	0.95 (0.52-1.72)		30 (52)	9	15	1.58 (0.81-3.09)	
Missing	23 (16)					12 (16)					11 (19)				
S-PD-1					.279					.089					.747
High	253 (47)	47	57	1.00		134 (46)	54	71	1.00		98 (49)	41	52	1.00	
Low	253 (47)	41	41	1.12 (0.91-1.37)		142 (49)	43	33	1.27 (0.96-1.68)		90 (45)	37	45	1.06 (0.75-1.48)	
Missing	30 (6)					13 (5)					13 (6)				
T-PD-1					.027[a]					.052					.359
High	282 (53)	49	57	1.00		155 (54)	55	71	1.00		103 (51)	43	47	1.00	
Low	225 (42)	38	36	1.22 (0.80-1.86)		122 (42)	41	31	1.13 (1.00-1.73)		85 (42)	34	47	1.17 (0.85-1.64)	
Missing	29 (5)					12 (4)					13 (7)				
N^+ LN T-PD-1					.358					.554					.659
High	38 (27)	26	19	1.00		14 (19)	36	35	1.00		20 (34)	15	12	1.00	
Low	87 (61)	21	17	1.18 (0.82-1.70)		48 (65)	29	15	1.21 (0.64-2.32)		34 (59)	11	15	1.15 (0.62-2.11)	
Missing	17 (12)					12 (16)					4 (7)				
S-PD-L1 + T-PD-1					.002[a]					.019[a]					.071
Other	339 (63)	50	59	1.00		195 (68)	54	71	1.00		117 (58)	46	54	1.00	
Low + low	157 (29)	32	31	1.40 (1.13-1.73)		78 (27)	35	26	1.42 (1.06-1.90)		68 (34)	27	41	1.37 (0.97-1.94)	
Missing	40 (8)					16 (5)					16 (8)				

Abbreviations: CI = confidence interval; HR = hazard ratio; N^+ LN = metastatic lymph node; NR = not reached; PD-1 = programmed cell death protein 1; PD-1L = PD-1 ligand; S = stromal; T = tumor.
[a]Statistically significant.

Supplemental Table 4 Results of Cox Regression Analysis Summarizing Significant Independent Prognostic Factors for Disease-Free Survival and Overall Survival

Variable	All Patients		SCC		ADC	
	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value
Disease-free survival						
Clinicopathologic variables[a]						
Pathologic stage		<.001[b,c]		<.001[b,c]		<.001[b]
I	1.00		1.00		1.00	
II	1.67 (1.26-2.14)	<.001[b]	1.85 (1.21-2.83)	.004[b]	2.05 (1.34-3.12)	.001[b]
IIIA	3.35 (2.39-4.70)	<.001[b]	5.36 (3.17-9.06)	<.001[b]	2.98 (1.82-4.90)	<.001[b]
Histologic type		<.001[b,c]				
SCC	1.00					
ADC	1.81 (1.38-2.38)	<.001[b]				
LCC	1.51 (0.97-2.36)	.069				
Vascular infiltration						
No vs. yes	1.44 (1.06-1.95)	.019[b]	1.46 (0.94-2.27)	.092	1.33 (0.81-2.19)	.264
Differentiation		.016[b,c]		NE		.012[b,c]
Well	1.00				1.00	
Moderate	1.81 (1.11-2.94)	.017[b]			2.45 (1.29-4.64)	.006[b]
Poor	2.04 (1.26-3.32)	.004[b]			2.58 (1.36-4.89)	.004[b]
Sex						
Female vs. male		NE		NE		NE
ECOG PS		.004[b,c]		NE		<.001[b,c]
0	1.00				1.00	
1	1.50 (1.16-1.93)	.002[b]			1.73 (1.18-2.55)	.005[b]
2	1.65 (0.96-2.84)	.068			4.83 (2.25-10.39)	<.001[b]
Smoking		NE		NE		NE
Never						
Present						
Former						
Surgical margins						
Free vs. not free		NE		NE	1.26 (0.62-2.59)	.522
PD variables assessed in separate models[d]						
T-PD-L1						
High vs. low	0.91 (0.68-1.22)	.522	1.12 (0.71-1.76)	.640	0.99 (0.65-1.51)	.967
S-PD-L1						
High vs. low	1.24 (0.95-1.63)	.118	1.80 (1.19-2.74)	.006[b]	0.88 (0.59-1.32)	.542
T-PD-1						
High vs. low	1.41 (1.09-1.81)	.009[b]	1.94 (1.32-2.87)	.001[b]	1.40 (0.94-2.07)	.096
S-PD-L1 + T-PD-1						
Other scores vs. low + low	1.57 (1.21-2.05)	.001[b]	2.20 (1.49-3.26)	<.001[b]	1.39 (0.93-2.07)	.104
PD variables assessed in the same model[d]						
S-PD-L1[a]						
High vs. low	1.20 (0.91-1.58)	.204	1.71 (1.12-2.61)	.013[b]	0.77 (0.51-1.18)	.232
T-PD-1[a]						
High vs. low	1.42 (1.10-1.84)	.007[b]	1.83 (1.24-2.71)	.002[b]	1.44 (0.97-2.14)	.073
PD Immunoscore assessed with S-CD8 and T-CD45RO[d]						
S-PD-L1 + T-PD-1[a]						
Other scores vs. low + low	1.34 (1.01-1.77)	.040[b]	1.73 (1.14-2.63)	.010[b]	1.24 (0.81-1.90)	.327

Supplemental Table 4 Continued

Variable	All Patients		SCC		ADC	
	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value
S-CD8aS-CD8[a]						
High vs. low	1.50 (1.14-1.96)	.003[b]	1.68 (1.12-2.54)	.013[b]	1.48 (1.00-2.57)	.051
T-CD45RO[a]						
High vs. low	1.40 (1.04-1.88)	.025[b]	1.55 (1.01-2.39)	.045[b]	0.86 (0.53-1.39)	.528
Overall survival						
Clinicopathologic variables[a]						
Pathologic stage		<.001[b,c]		<.001[b,c]		<.001[b,c]
I	1.00		1.00		1.00	
II	1.12 (0.90-1.40)	.318	1.26 (0.94-1.70)	.118	1.48 (1.00-2.20)	.051
IIIA	2.73 (2.07-3.59)	<.001[b]	2.91 (1.96-4.32)	<.001[b]	3.04 (1.97-4.69)	<.001[b]
Histologic type		NE				
SCC						
ADC						
LCC						
Vascular infiltration						
No vs. yes	1.71 (1.32-2.23)	<.001[b]	1.46 (1.04-2.05)	.029[b]	1.76 (1.11-2.80)	.017[b]
Differentiation		NE		NE		.257[c]
Well					1.00	
Moderate					1.44 (0.87-1.29)	.156
Poor					1.62 (1.14-2.30)	.107
Sex						
Female vs. male	1.53 (1.22-1.93)	<.001[b]		NE	1.69 (1.20-2.40)	.003[b]
ECOG PS		<.001[b,c]		NE		<.001[b,c]
0	1.00				1.00	
1	1.33 (1.08-1.64)	.008[b]			1.31 (0.92-1.87)	.140
2	2.16 (1.46-3.18)	<.001[b]			3.87 (2.01-7.48)	<.001[b]
Age						
≤65 year vs. >65 year	1.52 (1.24-1.88)	<.001[b]	1.73 (1.29-2.32)	<.001[b]		NE
Surgical margins						
Free vs. not free		NE		NE		NE
PD variables assessed in separate models[d]						
T-PD-L1						
High vs. low	1.14 (0.98-1.46)	.277	1.35 (0.96-1.89)	.085	1.30 (0.87-1.96)	.200
S-PD-L1						
High vs. low	1.19 (0.96-1.48)	.123	1.26 (0.94-1.69)	.117	1.19 (0.83-1.71)	.346
T-PD-1						
High vs. low	1.25 (1.02-1.54)	.032[b]	1.37 (1.04-1.81)	.027[b]	1.24 (0.88-2.13)	.213
S-PD-L1 + T-PD-1						
Other scores vs. low + low	1.36 (1.10-1.69)	.005[b]	1.48 (1.10-1.99)	.010[b]	1.41 (0.99-1.99)	.055
PD variables assessed in same model[d]						
S-PD-L1[a]						
High vs. low	1.18 (0.95-1.47)	.144	1.23 (0.92-1.65)	.167	1.15 (0.79-1.68)	.462
T-PD-1[a]						
High vs. low	1.27 (1.03-1.56)	.026[b]	1.37 (1.03-1.81)	.029[b]	1.28 (0.90-1.80)	.165
PD Immunoscore assessed with S-CD8 and T-CD45RO[d]						
S-PD-L1 + T-PD-1[a]						
Other scores vs. low + low	1.26 (1.01-1.58)	.044[b]	1.27 (0.93-1.73)	.140	1.42 (1.00-2.02)	.047[b]

Supplemental Table 4 Continued

Variable	All Patients		SCC		ADC	
	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value	HR (95% CI)	*P* Value
S-CD8[a]						
High vs. low	1.32 (1.06-1.65)	.013[b]	1.67 (1.25-2.39)	<.001[b]	1.05 (0.71-1.56)	.795
T-CD45RO[a]						
High vs. low	1.39 (1.09-1.78)	.009[b]	1.58 (1.14-2.19)	.006[b]	1.09 (0.72-1.66)	.687

Abbreviations: ADC = adenocarcinoma; CI = confidence interval; ECOG = Eastern Cooperative Oncology Group; HR = hazard ratio; LCC = large cell carcinoma; NE = not entered; PD-1 = programmed cell death protein 1; PD-1L = PD-1 ligand; PS = performance status; SCC = squamous cell carcinoma; S = stromal; T = tumor.
[a]In the same model.
[b]Statistcally significant.
[c]Overall significance as a prognostic factor.
[d]All clinicopathologic covariates significant in multivariate analysis were included in each model.

Supplemental Table 5 Prognostic Impact of S-CD8 and T-CD45RO on Disease-Specific Survival, Disease-Free Survival, and Overall Survival (Univariate Analyses; Log-Rank Test, Unadjusted Cox Proportional Hazard Ratios)

	All Patients					SCC					ADC				
Variable	n (%)	5-Year Survival (%)	Median Survival (mo)	HR (95% CI)	*P* Value	n (%)	5-Year Survival (%)	Median Survival (mo)	HR (95% CI)	*P* Value	n (%)	5-Year Survival (%)	Median Survival (mo)	HR (95% CI)	*P* Value
DSS															
S-CD8					<.001[a]					<.001[a]					.160
High	306 (57)	66	235	1.00		163 (56)	77	235	1.00		117 (58)	52	76	1.00	
Low	212 (40)	46	51	1.73 (1.32-2.27)		118 (41)	49	44	2.23 (1.49-3.35)		77 (38)	41	50	1.34 (0.89-2.01)	
MD	18 (3)					8 (3)					7 (4)				
T-CD45RO					.002[a]					<.001[a]					.636
High	371 (69)	60	189	1.00		201 (70)	72	235	1.00		141 (70)	47	57	1.00	
Low	133 (25)	47	50	1.61 (1.19-2.16)		71 (25)	44	35	2.32 (1.52-3.56)		50 (25)	51	71	1.12 (0.70-1.80)	
MD	32 (6)					17 (6)					10 (5)				
DFS															
S-CD8					<.001[a]					<.001[a]					.099
High	306 (57)	60	124	1.00		163 (56)	74	229	1.00		117 (58)	45	41	1.00	
Low	212 (40)	39	29	1.62 (1.26-2.08)		118 (41)	45	29	2.00 (1.37-2.92)		77 (38)	30	31	1.37 (0.94-1.99)	
MD	18 (3)					8 (3)					7 (4)				
T-CD45RO					.001[a]					<.001[a]					.786
High	371 (69)	54	85	1.00		201 (70)	79	229	1.00		141 (70)	40	33	1.00	
Low	133 (25)	38	28	1.58 (1.20-2.08)		71 (25)	41	26	2.10 (1.40-3.14)		50 (25)	37	42	1.06 (0.69-1.64)	
MD	32 (6)					17 (6)					10 (5)				
OS															
S-CD8					<.001[a]					<.001[a]					.550
High	306 (57)	51	64	1.00		163 (56)	58	83	1.00		117 (58)	43	54	1.00	
Low	212 (40)	36	32	1.44 (1.18-1.77)		118 (41)	36	25	1.74 (1.32-2.29)		77 (38)	34	44	1.11 (0.79-1.56)	
MD	18 (3)					8 (3)					7 (4)				
T-CD45RO					<.001[a]					<.001[a]					.163
High	371 (69)	47	54	1.00		201 (70)	54	71	1.00		141 (70)	40	49	1.00	
Low	133 (25)	34	28	1.54 (1.23-1.95)		71 (25)	29	24	1.79 (1.31-2.45)		50 (25)	38	33	1.31 (0.90-1.92)	
MD	32 (6)					17 (6)					10 (5)				

Abbreviations: ADC = adenocarcinoma; CI = confidence interval; DFS = disease-free survival; DSS = disease-specific survival; HR = hazard ratio; MD = missing data; N^+ LN = metastatic lymph node; NR = not reached; OS = overall survival; S = stromal; SCC = squamous cell carcinoma; T = tumor.

[a]Statistically significant.