Adverse prognostic value of PD-L1 expression in primary resected pulmonary squamous cell carcinomas and paired mediastinal lymph node metastases

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Immunohistochemical assessment of programmed cell death (PD)-ligand 1 (PD-L1) expression in lung cancer in the context of therapeutically targeting the PD1/PD-L1 axis is still controversially discussed. This includes the comparability of antibody clones, prognostic value, and discrepancies between primary tumors and metastases. We assessed tumoral PD-L1 expression using clones E1L3N and SP142 in 372 primary resected pulmonary squamous cell carcinomas, including 40 paired N2 lymph node metastases, in relation with clinico-pathological parameters. PD-L1 expression was negative ($< 1\%$) in 163/372 (44%, E1L3N) or 231/370 patients (62%, SP142). Positivity of $1-< 50\%$ was observed in 135 (36%, E1L3N) or 92 patients (25%, SP142) and $\geq 50\%$ in 74 (20%, E1L3N) or 47 patients (13%, SP142). PD-L1 staining correlated significantly between both antibodies ($r = 0.781$; $P < 0.001$). Scores correlated significantly between full-slide sections ($N = 40$) and tissue microarrays, and between primaries and N2 metastases ($P < 0.001$ all). CD8$^+$ tumor infiltrating lymphocyte counts positively correlated with PD-L1 expression ($P < 0.001$). PD-L1 $\geq 50\%$ showed the best prognostic discrimination using the split-sample validation method. It was associated with shorter disease-specific survival in the observation group (E1L3N: $P = 0.035$, SP142: $P = 0.002$) and validation group (E1L3N: $P = 0.024$, SP142: $P = 0.101$) and shorter time to recurrence (observation group: E1L3N: $P = 0.056$, SP142: $P < 0.001$; validation group: E1L3N: $P = 0.036$, SP142: $P = 0.247$). Multivariate analysis showed that PD-L1 expression $\geq 50\%$ determined by clone E1L3N was an independent prognostic factor in the observation group regarding disease-specific survival (HR = 2.768; 95% CI = 1.149–6.666; $P = 0.023$) and time to recurrence (HR = 2.164; 95% CI = 1.056–4.436; $P = 0.035$) and in the validation group (disease-specific survival: HR = 1.978; 95% CI = 0.928–4.214; $P = 0.077$ and time to recurrence: HR = 1.571; 95% CI = 0.838–2.944; $P = 0.159$). High PD-L1 expression was associated with adverse prognosis in pulmonary squamous cell carcinoma. Clone E1L3N was more sensitive than SP142 and superior regarding prognostication. PD-L1 expression correlated significantly between primary tumor and N2 metastases, rendering mediastinal lymph node metastases adequate for immunohistochemical assessment.

Lung cancer, comprising non-small cell lung cancer (NSCLC) as the largest subgroup, belongs to the most frequent malignancies worldwide.[1] In advanced stages, radiation and chemotherapy render unsatisfactory clinical results, making it the leading cause of cancer-related deaths in the Western world.[1,2] In contrast to adenocarcinomas, for squamous cell carcinomas no targeted therapy is available to date.[3]

Targeting the programmed death protein 1 (PD1)/programmed death-ligand 1 (PD-L1) interaction proved impressively efficacious in NSCLC, and rapidly translated into clinically approved therapeutics.[2,4–8] PD-L1 on the tumor cell binds to PD1 on the effector T cell, thereby protecting the tumor by shutting down the T-cell response. PD1/PD-L1 inhibitors block this immunoescape, leading to elimination of the malignant cells through

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reinvigorated immune surveillance.[9,10] Predictive markers are needed, as only around 20% of unselected NSCLC patients respond to therapy and immunotherapy has already arrived in the first-line setting, competing with effective standard therapies.[11,12]

Immunohistochemical staining for PD-L1 is technically well applicable in histopathology laboratories, but presents many uncertainties to date. Its prognostic but also its predictive role, or the congruence between the expression in primary tumors and metastases are controversial. Nevertheless, PD-L1 expression on the tumor cells is still regarded the best single predictive biomarker for anti-PD1/PD-L1 therapy in NSCLC.[9,13,14]

We aimed to validate the prognostic value and heterogeneity of PD-L1 expression in primary tumors and lymph node metastases in squamous cell carcinomas of the lung in a large Western cohort.

Materials and methods

Patient Cohort

We retrospectively analyzed 378 consecutive patients with primary resected pulmonary squamous cell carcinomas, diagnosed at the Institute of Pathology, University of Bern, January 2000–December 2013, without previous or concomitant diagnosis of squamous cell carcinomas of other organs to reliably exclude metastatic lung disease. The study was conducted according to the *REMARK guidelines*,[15] and was approved by the Cantonal Ethics Commission of the Canton of Bern (KEK 200/14), which waived the requirement for written informed consent. Initial inclusion criteria were matched by 402 patients according to the pathology files.[16] Re-evaluation of clinical files and histology led to exclusion of 24 additional patients, 13 due to neoadjuvant treatment not mentioned in the pathology files, 3 due to previous squamous cell carcinomas of other organs, 1 due to lack of sufficient available tissue, and 7 due to TTF1 positivity in the tumors, suggestive of adenocarcinoma.[17] All tumors were re-staged according to the UICC 2009 7th edition TNM classification (Table 1).[18]

Survival data were available for 278 patients. Time to recurrence was measured from the time of resection to loco-regional or metastatic recurrence or disease-specific death. Mean time to recurrence in the total cohort was 105 months (95% CI = 93–116 months). Disease-specific survival was measured from the time of resection to disease-specific death. Patients who died due to unrelated causes or secondary malignancies were censored at the time of death. Mean disease-specific survival in the total cohort was 139 months (95% CI = 126–151 months) and mean overall survival was 83 months (95% CI = 74–92 months). Of note, 100/159 (63%) deaths were unrelated to pulmonary squamous cell carcinoma (eg, heart attack, secondary lung adenocarcinoma) and 59/159 (37%) were disease-specific deaths.

Tissue Microarray and Full-Slide Analysis

A next-generation tissue microarray, with digital annotation of scanned slides and automatic transferal of the punches was constructed from formalin-fixed and paraffin-embedded tissue blocks archived in the Institute of Pathology, University Bern, Switzerland, as described before.[16,19] One representative tissue block was selected for each tumor after reviewing all available slides per case (hematoxylin and eosin stained), and eight tumor cores (diameter 0.6 mm) were randomly selected from the block by placing digital annotations on the scanned slide. It has been shown before that one block/tumor is sufficient to represent the spatial heterogeneity of PD-L1 expression of the tumor.[20] The eight cores/tumor were placed on four different tissue microarray blocks to exclude technical staining bias.

Additionally, full tissue sections were used in the pN2 subcohort for comparison between tissue microarray cores and full tissue sections and between primaries and lymph node metastases.

Immunohistochemistry

Immunohistochemical staining was performed using an automated immunostainer (Bond III, Leica Biosystems, Muttenz, Switzerland) using the following antibodies: anti-CD3 (Abcam Cambridge, UK, clone SP7, Tris-buffer, 95 °C 30 min, 1:400), anti-CD8 (Dako, clone C8/144B, Tris-buffer, 95 °C 20 min, 1:100), as described before.[19] The anti-TTF1 clone 8G7G3/1 (Cell Marque, Rocklin, CA, USA) was used at a dilution of 1:400 after antigen retrieval performed with Tris-EDTA, pH 9 for 30 min at 95 °C. PD-L1 immunohistochemistry was performed using laboratory-developed tests using clone SP142 (Spring Bioscience, Pleasanton, CA, USA) and clone E1L3N (Cell Signaling Technology, Danvers, MA, USA) at a dilution of 1:400 each, as described before.[21] Both PD-L1 clones have been validated for specificity.[21–23] Deparaffinized sections were rehydrated in Dewax dilution (Leica Biosystems) for 20 min, and antigen retrieval was performed with Tris-HCl, pH 9 for 40 min at 95 °C. Endogenous peroxidase activity was blocked with H_2O_2 solution (Leica Biosystems). Samples were incubated with primary antibodies at room temperature for 15 min, followed by incubation with the secondary antibody using the Bond Polymer Refine Kit with 3-3′-diaminobenzidine–DAB as chromogen (Leica Biosystems), counterstained with hematoxylin and mounted in Aquatex (Merck, Darmstadt, Germany).

PD-L1 expression was assessed as proportion of tumor cells showing membranous staining of any intensity (tumor proportion score) by a pathologist

Table 1 Clinico-pathological characteristics of the patient cohort in relation with PD-L1 expression (clone E1L3N)

| Factors	Number of patients		PD-L1 expression			P-value
	Total cohort	PD-L1 evaluation	< 1%	1–< 50%	≥ 50%	
Total	378	372	163 (44%)	135 (36%)	74 (20%)	
Gender						
Male	320 (85%)	315	144 (46%)	111(35%)	60 (19%)	0.217
Female	58 (15%)	57	19 (33%)	24 (42%)	14 (25%)	
Age, median (min–max)	69 (36.5–85.1)		69.5 (43.2–85.1)	67.8 (43.8–83.9)	70.9 (36.5–84.9)	0.825
pT stage						
T1a	38	36	12 (33%)	19 (53%)	5 (14%)	0.110
T1b	53	53	29 (55%)	15 (28%)	9 (17%)	
T2a	123	120	53 (44%)	49 (41%)	18 (15%)	
T2b	56	56	23 (41%)	16 (29%)	17 (30%)	
T3	79	78	31 (40%)	29 (37%)	18 (23%)	
T4	29	29	15 (52%)	7 (24%)	7 (24%)	
pN stage						
N0	220	216	94 (44%)	81 (37%)	41 (19%)	0.571
N1	116	115	55 (48%)	38 (33%)	22 (19%)	
N2	42	41	14 (34%)	16 (39%)	11 (27%)	
M						
M0	371	365	160 (44%)	132 (36%)	73 (20.0%)	0.905
M1	7	7	3 (43%)	3 (43%)	1 (14%)	
Stage						
IA	69 (18%)	68	31 (46%)	26 (38%)	11 (16%)	0.703
IB	80 (21%)	77	32 (42%)	31 (40%)	14 (18%)	
IIA	77 (20%)	77	38 (49%)	29 (38%)	10 (13%)	
IIB	51 (14%)	51	21 (41%)	17 (33%)	13 (26%)	
IIIA	87 (23%)	85	34 (40%)	27 (32%)	24 (28%)	
IIIB	7 (2%)	7	4 (57%)	2 (29%)	1 (14%)	
IV	7 (2%)	7	3 (43%)	3 (43%)	1 (14%)	
Grade						
G1		10	8 (80%)	2 (20%)	0	0.073
G2		140	58 (41%)	58 (41%)	24 (17%)	
G3		176	73 (42%)	62 (35%)	41 (23%)	
Resection						
R0	330	326	144 (44%)	117 (36%)	65 (20%)	0.966
R1	45	43	18 (42%)	17 (39%)	8 (19%)	
R2	3	3	1 (33%)	1 (33%)	1 (33%)	
Smoking						
Pack-years, median (min–max)	50 (2–200)		50.0 (5.0–150.0)	45.0 (2.0–200.0)	50.0 (16.0–150.0)	0.425

experienced in lung pathology and trained for PD-L1 evaluation (SB). Examples of the staining are shown in Figure 1. The tumor proportion score was assessed using the currently most relevant cutoffs applied in clinical studies (in %): < 1, 1–< 50, and ≥ 50.[24] Each tissue microarray core was scored separately. A final score was calculated as mean percent across all tissue microarray cores/tumor.

The numbers of CD8$^+$ and CD3$^+$ tumor infiltrating lymphocytes were determined using image analysis (Aperio Image Scope), and adjusted for core completeness. The algorithm (provided in the supplementary Table S1) was previously determined by comparison with manual scoring on a training set of 70 cases.

Statistical Analysis

Statistical analysis was performed using IBM SPSS Statistics 24 (IBM Corporation, Armonk, USA). For group comparisons, crosstabs, χ^2-tests, and Fisher's exact tests were performed. For associations between continuous variables, Spearman's rho correlation analysis was used. Associations between immunohistochemical staining patterns and the number of tumor infiltrating lymphocytes were evaluated using the non-parametric Mann–Whitney *U*-test. For survival analysis, disease-specific survival and time to recurrence were assessed instead of overall survival, due to the high number of non-tumor-related deaths.
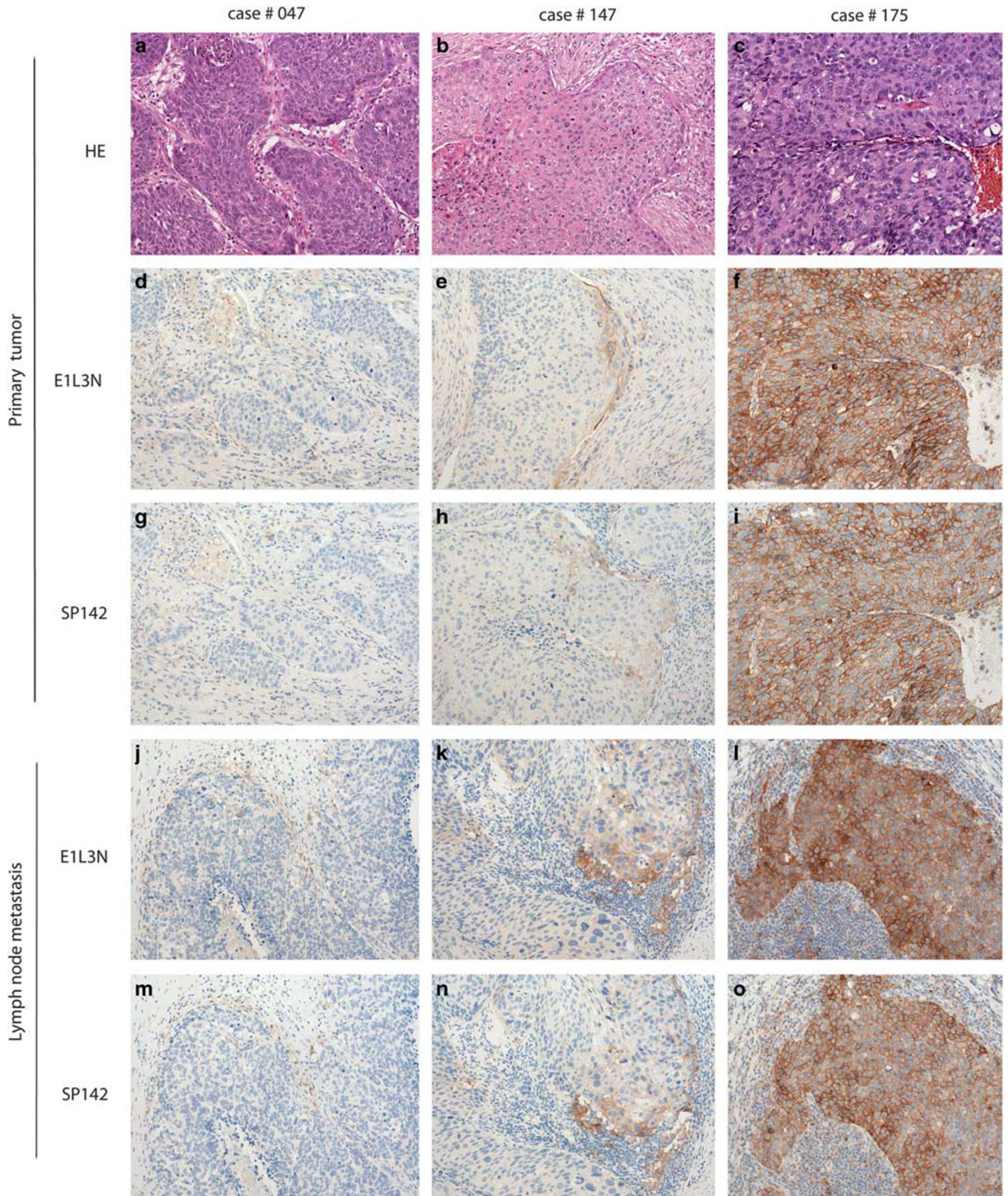
Figure 1 Examples of different increments of membranous expression of PD-L1 using both antibodies in the primary tumors and paired mediastinal lymph node metastases. Primary tumors (**a–c**, hematoxylin and eosin; **d–i**, PD-L1) and lymph node metastases (**j–o**, PD-L1). Case #047 (left column): < 1% PD-L1, case #147 (middle column): 1– < 50% PD-L1; case #175 (right column): ≥ 50% PD-L1. (Total magnification × 200).

We determined the prognostically most relevant cutoff of PD-L1 expression using the split-sample validation method, whereby we identified the relevant cutoff on an observation group and validated it on the validation group. For this, the total cohort was divided into two groups of equal size that were homogenized according to UICC/AJCC TNM stage and date of surgery, but otherwise randomly separated (observation group: $N = 139$; validation group: $N = 139$). Kaplan–Meier plots were first generated for the three-tier classification ($< 1\%$, $1 - < 50\%$, and $\geq 50\%$) and for two groups using a two-tier classification with 50% cutoff. The prognostically most relevant grouping was then applied for further analysis, including Cox regression analysis for multivariate analysis. *P*-values were two sided and regarded as significant, if $P < 0.05$.

Results

PD-L1 Expression Patterns

PD-L1 expression was evaluable in 372 (E1L3N) and 370 tumors (SP142). PD-L1 was negative ($< 1\%$) in 163 (44%, E1L3N) or 231 tumors (62%, SP142). Positivity of $1 - < 50\%$ was observed in 135 (36%, E1L3N) or 92 tumors (25%, SP142) and reached $\geq 50\%$ in 74 (20%, E1L3N) or 47 tumors (13%, SP142) (Figure 1). The tumor proportion score correlated significantly between the two antibodies ($r = 0.781$; $P < 0.001$), although clone E1L3N showed higher sensitivity, especially in cases with low PD-L1 expression. Detailed values for all increments and comparing both antibody clones are provided in Table 2.

The tumor proportion score correlated significantly between full tissue sections and corresponding tissue microarray cores ($P < 0.001$, both antibodies, $N = 41$), validating our tissue microarray-based approach, with congruence rates of 29/41 (E1L3N and SP142) for the three-tier classification ($< 1\%$, $1 - < 50\%$, and $\geq 50\%$) and 39/41 (E1L3N) and 40/41 (SP142) for the two-tier classification with 50% cutoff. The discrepant results were predominantly seen within the group of $1 - < 50\%$ PD-L1 expression initially assessed ($1-4$, $5 - < 9$, $10-24$, and $25-49\%$; data not shown), whereas staining patterns were very homogeneous in cases with scores $< 1\%$

or $\geq 50\%$. This was also seen in the whole tissue microarray cohort across the eight cores/tumor: For E1L3N, 127/163 (78%) cases were negative (ie, $< 1\%$) in all cores, and 65/74 (88%) cases reached $\geq 50\%$ in all cores. Similar results were seen for SP142, with 197/231 (85%) cases homogenous for score $< 1\%$ and 41/47 (87%) cases homogenous for score $\geq 50\%$.

Correlation of PD-L1 Expression with Clinico-Pathological Parameters

There was an antibody-dependent correlation between higher tumor proportion scores and higher pT categories (three-tier classification; E1L3N: $P = 0.11$; SP142: $P = 0.032$) but not for other clinical and pathological parameters (Table 1). However, higher PD-L1 scores correlated significantly with higher CD8$^+$ tumor infiltrating lymphocytes count ($P < 0.001$ for both antibodies and all increments; Supplementary Figure S1).
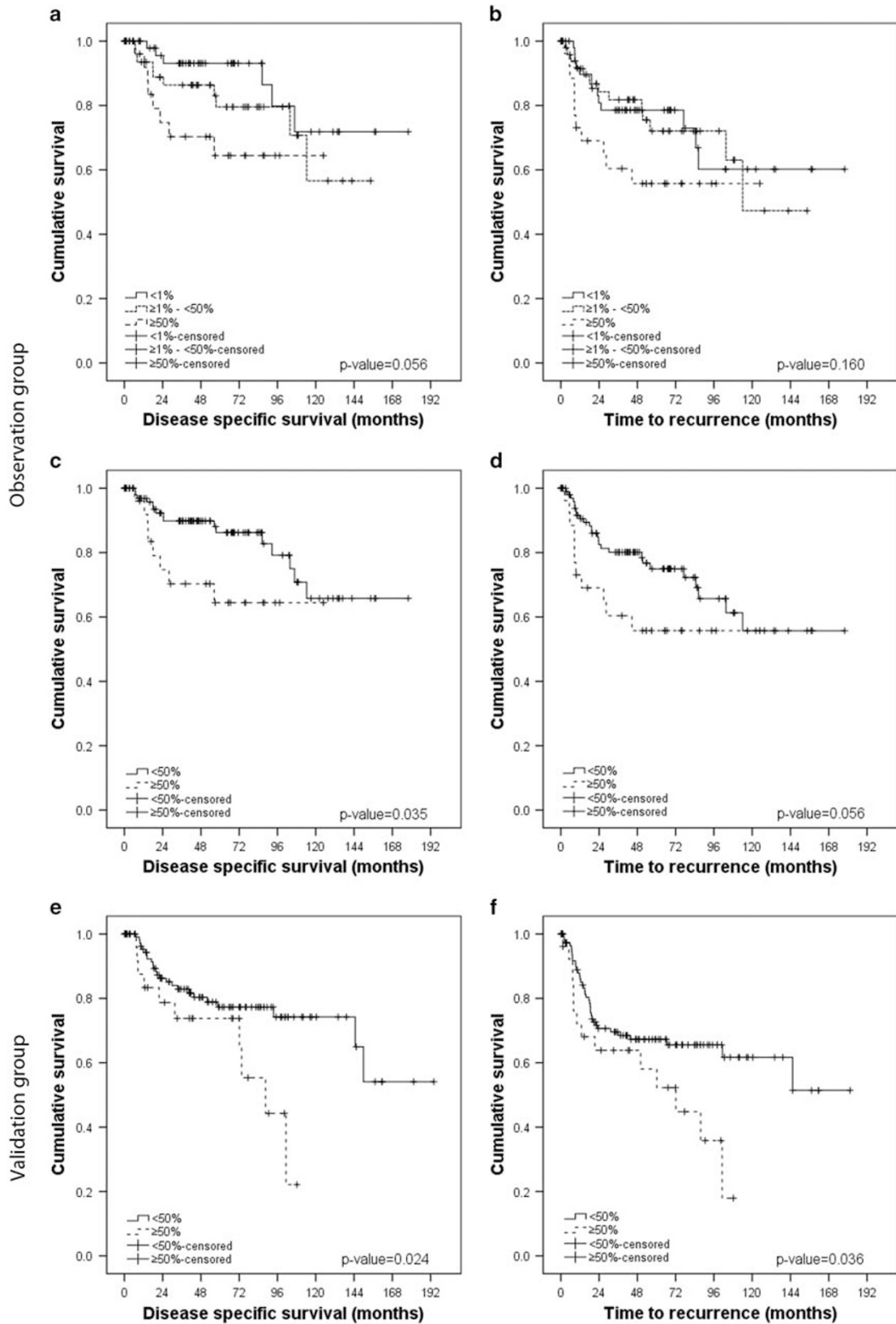
Survival Analysis—Observation Group

In univariate analysis, the following clinico-pathological factors were associated with longer disease-specific survival and time to recurrence in the observation group: lower UICC/AJCC stage ($P < 0.001$ each), complete tumor resection ($P < 0.001$; $P = 0.15$), and in trend female gender ($P = 0.129$; $P = 0.215$). Tumor grading, patient's age (cutoff: median), and the number of tumor infiltrating lymphocytes assessed as mean over all tissue microarray cores had no prognostic value and were not included in further multivariate analysis nor survival analysis on the validation cohort.

The prognostically relevant cutoff for PD-L1 expression was determined using Kaplan–Meier plots that constantly showed that PD-L1 scores $\geq 50\%$ were associated with the worst disease-specific survival and time to recurrence (Figure 2 for E1L3N, Supplementary Figure S2 for SP142). This was seen for both PD-L1 antibodies used for expression analysis, and was also demonstrated by log rank analysis (disease-specific survival: $P = 0.035$ for E1L3N and $P = 0.002$ for SP142; time to recurrence: $P = 0.056$ for E1L3N and $P < 0.001$ for SP142). Multivariate analysis showed that PD-L1 expression was an independent prognostic factor for disease-specific survival (E1L3N: HR = 2.768; 95% CI = 1.149–6.666; $P = 0.023$ and SP142: HR = 4.153; 95% CI = 1.564–11.022; $P = 0.004$) and time to recurrence (E1L3N: HR = 2.164; 95% CI = 1.056–4.436; $P = 0.035$ and SP142: HR = 3.559; 95% CI = 1.652–7.668; $P = 0.001$), besides UICC/AJCC stage (Table 3 for E1L3N and Supplementary Table S2 for SP142).

Survival Analysis—Validation Group

Application of the 50% cutoff on the validation group showed similar prognostic values for PD-L1

Table 2 Correlation of tumoral PD-L1 stainings using clones E1L3N and SP142 for the different increments

	PD-L1 (SP142) tumor proportion score			Total
	< 1%	*1– < 50%*	*≥ 50%*	
PD-L1 (E1L3N) tumor proportion score				
$< 1\%$	160	2	0	162
$1 - < 50\%$	69	66	0	135
$\geq 50\%$	2	24	47	73
Total	231	92	47	370

Figure 2 Kaplan–Meier plots for the development (**a–d**) of the prognostically most relevant cutoff for PD-L1 expression using clone E1L3N in the observation cohort (*N* = 139; **a–b**, three-tier; **c–d**, two-tier) and its application in the validation cohort (**e–f**, *N* = 139) for disease-specific survival (DSS) and time to recurrence (TTR).

Table 3 Multivariate analysis on the observation group (PD-L1 expression using clone E1L3N)

Parameter	HR	95.0% CI		P-value
		Min	Max	
Disease-specific survival				
UICC/AJCC stage	1.498	1.042	2.155	0.029
Gender	0.277	0.036	2.129	0.217
Resection status (complete *versus* incomplete)	1.661	0.539	5.124	0.377
PD-L1 (50% cutoff)	2.768	1.149	6.666	0.023
Time to recurrence				
UICC/AJCC stage	1.350	1.030	1.768	0.030
Gender	0.520	0.156	1.733	0.287
Resection status (complete *versus* incomplete)	1.013	0.369	2.780	0.980
PD-L1 (50% cutoff)	2.164	1.056	4.436	0.035

Table 4 Multivariate analysis on the validation group (PD-L1 expression using clone E1L3N)

Parameter	HR	95.0% CI		P-value
		Min	Max	
Disease-specific survival				
UICC/AJCC stage	1.533	1.162	2.022	0.003
Gender	0.862	0.325	2.285	0.765
Resection status (complete *versus* incomplete)	1.332	0.545	3.254	0.530
PD-L1 (50% cutoff)	1.978	0.928	4.214	0.077
Time to recurrence				
UICC/AJCC stage	1.432	1.164	1.761	0.001
Gender	0.813	0.377	1.753	0.597
Resection status (complete *versus* incomplete)	1.272	0.612	2.647	0.519
PD-L1 (50% cutoff)	1.571	0.838	2.944	0.159

expression for disease-specific survival ($P = 0.024$ for E1L3N and $P = 0.101$ for SP142) and time to recurrence ($P = 0.036$ for E1L3N and $P = 0.247$ for SP142) (Figure 2 for E1L3N and Supplementary Figure S2 for SP142). In multivariate analysis, UICC/AJCC stage was independent prognostic factor for disease-specific survival and time to recurrence. PD-L1 expression ≥ 50% determined by clone E1L3N did not reach statistical significance but retained a higher HR compared to UICC/AJCC stage (disease-specific survival: HR = 1.978; 95% CI = 0.928–4.214; $P = 0.077$ and time to recurrence: HR = 1.571; 95% CI = 0.838–2.944; $P = 0.159$; Table 4). PD-L1 expression determined by clone SP142 was not an independent prognostic factor in the validation cohort (Supplementary Table S3).

The Kaplan–Meier plots for the total cohort and the respective multivariate analyses are provided for completeness as Supplementary Material (Supplementary Figure S3 and Supplementary Tables S4 and S5).
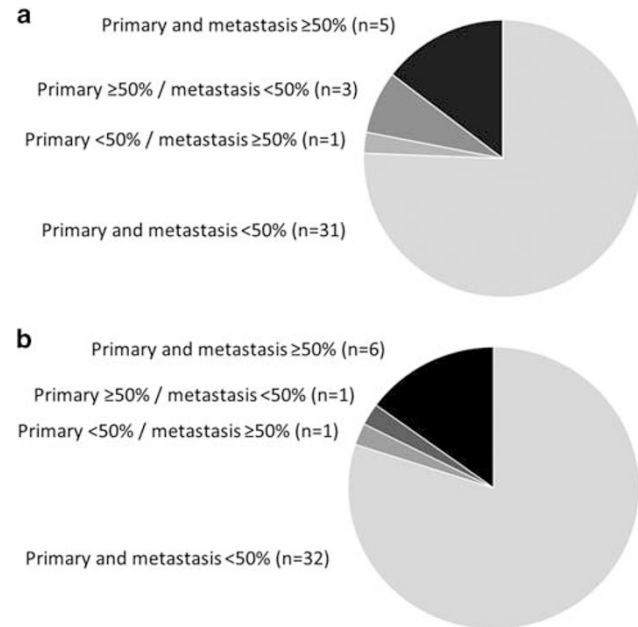
Figure 3 Correlation of PD-L1 expression using the cutoff 50% (**a**, clone E1L3N; **b**, clone SP142) between primary tumors and paired N2 lymph node metastases.

Survival Analysis—Subgroup with Adjuvant Radio- or Chemotherapy

A subgroup analysis of patients who were treated with adjuvant radio- or chemotherapy according to standard clinical guidelines, taking into account postoperative stage and patients' risk factors ($N = 144$; survival data available for $N = 120$), showed also significant associations of PD-L1 expression with disease-specific survival (E1L3N: $P = 0.041$, SP142: $P = 0.028$ and in trend for time to recurrence (E1L3N: $P = 0.121$, SP142: $P = 0.091$) (Supplementary Figure S4).

Congruent PD-L1 Expression in N2 Lymph Node Metastases

Using full-slide sections, PD-L1 scores ≥ 1% were detected in 19/40 (48%, E1L3N) or 16/40 (40%, SP142) N2 lymph node metastases, with score ≥ 50%, in six cases (15%, E1L3N) and seven cases (18%, SP142), respectively. As in primary tumors, staining of both antibodies showed a significant correlation ($P < 0.001$) with a complete congruence of 34/40 (85%) cases in the three-tier classification and 39/40 (98%) cases in the two-tier classification with the 50% cutoff.

PD-L1 tumor proportion scores correlated significantly between primary tumors and N2 metastases ($P < 0.001$ both antibodies). The scores were completely congruent in 24/40 (60%, E1L3N) and 28/40 cases (70%, SP142) for the three-tier increments, and 36/40 (90%, E1L3N) and 38/40 cases (95%, SP142) for the two-tier increments using the prognostically relevant 50% cutoff (Figure 3).

Discussion

Data regarding the prognostic value of PD-L1 expression in NSCLC are remarkably heterogeneous. As recently reviewed in detail, some studies show an association with worse prognosis, while others report the opposite or no prognostic impact.[25,26] Discrepancies may be explained by methodological variances, eg, differences in the collectives investigated, which may be quite heterogeneous regarding histology, stage, and ethnicity, confounding factors like previous therapy, staining heterogeneity, and detection methodology. Here we reliably validate the adverse prognostic value of tumoral PD-L1 expression $\geq 50\%$ in pulmonary squamous cell carcinoma, a cutoff which is relevant for selecting patients for first-line therapy with pembrolizumab.[12] In our retrospective, single center study on a large and clinically well-annotated European cohort of primary resected squamous cell carcinomas, we have included only true pulmonary squamous cell carcinomas, reliably excluding pulmonary metastases by excluding patients with previous or concomitant squamous cell carcinomas of other sites. Histology was re-evaluated according to current guidelines.[2,27] Because there is evidence that chemotherapy and radiotherapy may alter PD-L1 expression, we reliably included only therapy-naive tumors.[28] Patients with pulmonary squamous cell carcinomas often have significant comorbidities, reflected by the high number of non-tumor-associated deaths in our cohort (63%). Therefore, we assessed disease-specific survival and time to recurrence rather than overall survival, showing the independent adverse prognostic value of PD-L1 expression for both parameters. Recent studies suggested that prognostic value of PD-L1 expression may be weakened or increased when adjusting for postoperative therapy, and thus overwhelmed by treatment effect.[29] We show that PD-L1 expression remains a prognostic marker for poor outcome in the adjuvantly treated subcohort.

Positivity for PD-L1 has been very heterogeneously defined in the different studies and clinical trials as expression over 1, 5, 10, 25, or 50%.[4–8,24,26,30] The cutoffs 1 and 50% are currently the most relevant ones and show the best reproducibility with regard to the evaluation of the immunohistochemical staining.[20] Assessing these three increments, we validated the best prognostic discrimination for a PD-L1 tumor proportion score of $\geq 50\%$, using the split-sample size validation method by dividing our total cohort into the observation group and an internal validation group.

We assessed heterogeneity of PD-L1 staining in the primary by using eight tissue microarray cores/tumor and comparing the results gained by tissue microarray core scoring with those from full-slide scoring in the N2-positive subcohort. For the clinically and prognostically most significant two-tier approach (50% cutoff), for both antibodies the correlation of full slides with tissue microarray cores reached $>95\%$ and complete homogeneity over all cores/case was $>87\%$. This validates our PD-L1 scores assessed on the tissue microarray for the whole cohort and thus our results regarding the prognostic value of PD-L1 expression. On the other hand, by showing homogeneity of less than 100% over all tissue microarray cores assessed, our data also corroborate a recently published study reporting a sensitivity of a single tissue microarray core for scoring PD-L1 expression $\geq 50\%$ of around 95% for squamous cell carcinomas.[22] The authors conclude that assessing PD-L1 status on a single small sample, as it is done on small biopsies of advanced tumors in clinical routine, may be unreliable.[22]

Immunohistochemical assessment of PD-L1 can be performed using different PD-L1 clones and different detection systems,[13] and pre-set kits are associated to the different compounds used in clinical studies.[4,5,7,31] Most cross-validation studies compare the commercially available kits.[24,32] Recently, Adam *et al*[33] compared a large number of kits and laboratory-developed tests and presented the feasibility of an equally high sensitivity of PD-L1 staining using clone E1L3N. PD-L1 staining using clone E1L3N has been shown to render equivalent results to DAKO clones 22C3 and 28-8 by others.[34] Our PD-L1 staining and readout using E1L3N has been duly validated by external quality assessment. Quantification of PD-L1 was performed by light microscopy. A recently published excellent study on NSCLC compared the PD-L1 reading of pathologists with quantitative immunofluorescence using automated morphometric evaluation, and showed a very high concordance of 94% for tumor cell staining.[20] Moreover, PD-L1 scores were assessed for each of the tissue microarray cores separately, and the mean value/case was used for further evaluations, denoting an additional level of reliability. Therefore, and given the fact that pathologist-read evaluation of PD-L1 staining was used in the clinical studies and is used in daily routine, we consider our approach as valid and reliable.

In daily clinical settings, only tissue from lymph node metastases may be available for biomarker testing. We focused on mediastinal lymph node metastases (N2)[18] to assure true lymphatic spread, and showed high concordance ($\geq 90\%$) between primaries and paired metastases for the two-tier approach (50% cutoff). The congruence seems to be significantly lower in adenocarcinoma, where discrepant results were noted in up to 38% of paired samples of primaries and lymph node metastases (N1: $N = 33$, N2: $N = 32$, N3: $N = 1$), presumably due to the heterogeneity of growth patterns.[35] Lower concordance rates of 70.3% have also been described in a Korean cohort of pulmonary squamous cell carcinomas and associated pN-unspecified local lymph node metastases, but this may be due to the different cutoff value of 10% set do define PD-L1 positivity, and assessment in only one tissue microarray core/tumor.[30]

CD8^{+} T cell counts are also in the focus when PD-L1 expression is assessed. They are the effector T cells disabled in their anti-tumoral function by activation of the PD1/PD-L1 pathway, but they also can induce PD-L1 expression through production of interferon-gamma.[36] Concordant to previous reports,[30,35] PD-L1 expression and the number of tumor infiltrating lymphocytes correlated positively in our cohort.

In summary, we demonstrated an adverse prognostic value of PD-L1 expression in a clinically well-annotated Western patients' cohort of primary resected pulmonary squamous cell carcinomas. PD-L1 expression was highly concordant in primary tumors and N2 lymph node metastases, which may be the only tumor tissue available in clinical settings, implicating they are suitable for assessment of PD-L1 status in pulmonary squamous cell carcinomas. The results of this study add further knowledge to the ardently discussed field of PD-L1 expression in lung cancer.

Acknowledgments

Disclosure/conflict of interest

SB and RL have conflicts of interest outside the present work: Consultancy honoraria for MSD, Roche, and Bristol-Myers Squibb. All remaining authors have declared no conflicts of interest.

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Supplementary Information accompanies the paper on Modern Pathology website (http://www.nature.com/modpathol)