PD-L1 expression in basaloid squamous cell lung carcinoma: Relationship to PD-1$^+$ and CD8$^+$ tumor-infiltrating T cells and outcome

Marius Ilie[1,2,3,4], Alexander T Falk[2,5], Catherine Butori[1], Emmanuel Chamorey[6], Christelle Bonnetaud[2], Elodie Long[1], Sandra Lassalle[1], Katia Zahaf[1], Nicolas Vénissac[7], Jérôme Mouroux[7], Charlotte Cohen[7], Elisabeth Brambilla[8], Charles Hugo Marquette[2,4,9], Véronique Hofman[1,2,3,4] and Paul Hofman[1,2,3,4]

[1]Laboratory of Clinical and Experimental Pathology, Pasteur Hospital, Nice, France; [2]Institute for Research on Cancer and Ageing, Nice (IRCAN), INSERM U1081/UMR CNRS 7284, Team 3, University of Nice Sophia Antipolis, Antoine Lacassagne Center, Nice, France; [3]Hospital-Integrated Biobank BB-0033-00025, Pasteur Hospital, Nice, France; [4]University Hospital Federation (FHU) OncoAge, CHU de Nice, University of Nice Sophia Antipolis, Nice, France; [5]Department of Radiation Oncology, Antoine Lacassagne Cancer Center, Nice, France; [6]Biostatistics Unit, Antoine Lacassagne Cancer Center, Nice, France; [7]Department of Thoracic Surgery, Pasteur Hospital, Nice, France; [8]Department of Pathology, CHU Albert Michallon, INSERM U823, Université Grenoble Alpes, Grenoble, France and [9]Department of Pneumology, Pasteur Hospital, Nice, France

PD-1/PD-L1 inhibitors demonstrated durable clinical responses in patients with lung squamous cell carcinoma. However, the expression pattern of PD-L1 and the presence of CD8$^+$ and PD-1$^+$ tumor-infiltrating T cells in the basaloid variant of squamous cell carcinoma remain unknown. immunohistochemistry analysis of PD-L1 expression, with three recently validated monoclonal antibodies used in clinical trials (clones SP142, SP263, and 28-8), and detection of CD8$^+$ and PD-1$^+$ tumor-infiltrating T cells was performed on whole-tissue sections from 56 patients following surgery for basaloid squamous cell carcinoma. Data were correlated to clinicopathological parameters and outcome. Fair to poor concordance was observed between the SP142 *vs* SP263 clones, and SP142 *vs* 28-8 (κ range, 0.018–0.412), while the 28-8 and SP263 demonstrated a strong correlation in both the tumor cell and immune cell compartments ($\kappa = 0.883$, and $\kappa = 0.721$). Expression of PD-L1 correlated with a high content of CD8$^+$ and PD-1$^+$ tumor-infiltrating T cells when using SP142 ($P = 0.012$; $P = 0.022$), but not with SP263 or 28-8 ($P = 0.314$; $P = 0.611$). In the multivariate analysis, we found significantly better disease-free and overall survival rates for high PD-L1 expression with SP142, CD8$^+$ and PD-1$^+$ tumor-infiltrating T cells ($P = 0.003$; $P = 0.007$). No significant prognosis value was observed for SP263 and 28-8 clones, except a correlation between improved overall survival and SP263 in the univariate analysis ($P = 0.039$), not confirmed in the multivariate model. In conclusion, we report that the expression of PD-L1 and the content of CD8$^+$ and PD-1$^+$ tumor-infiltrating T cells is an independent indicator of better outcome in basaloid squamous cell carcinoma patients, although the observed effect is dependent on the PD-L1 immunohistochemistry assay.
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Lung cancer is the leading cause of cancer-related deaths in the world.[1] Non-small-cell lung cancer represents 80% of lung cancers. Despite the different therapeutic strategies developed to date, non-small-cell lung cancer has a poor prognosis, as overall survival after 5 years is lower than 20% for all stages.[2] Thus, current prognostic models including the histological subtype, tumor size, and pathological tumor node metastasis stage, urgently need to be improved by the integration of new prognostic biomarkers.[3]

Squamous cell carcinoma represents ~30% of all cases of non-small-cell lung cancer.[4] Among non-small-cell lung cancer and lung squamous cell carcinomas, the basaloid carcinoma variant of lung squamous cell carcinoma shows the poorest prognosis.[5] During the last decade, therapeutic options for advanced non-small-cell lung cancer have increased significantly. However, these achievements have been mostly limited to

Correspondence: Professor Paul Hofman, Laboratory of Clinical and Experimental Pathology, Pasteur Hospital, 30 Voie Romaine, BP69, Nice, Cedex 06001, France.
E-mail: hofman.p@chu-nice.fr
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non-squamous carcinomas, leaving squamous cell carcinoma with an unmet need for new treatments.[6] Interestingly, squamous cell carcinoma is highly immunogenic, thus susceptible to immunotherapeutic approaches.[7] Recently, immune checkpoint PD-1/PD-L1 blocking agents have shown a remarkable clinical efficacy with long response duration in immunogenic tumors, such as melanoma, renal cell carcinoma, and bladder cancer, including both squamous cell carcinoma and non-squamous non-small-cell lung cancer.[8–10] The United States Food and Drug Administration recently approved two PD-1 inhibitors (nivolumab, Opdivo, Bristol-Myers Squibb; and pembrolizumab, Keytruda, MK-3475, Merck) for treatment of patients with advanced squamous and non-squamous non-small-cell lung cancer for whom the disease progressed during or after platinum-based chemotherapy or on approved targeted agents (for those with *EGFR* mutations and *ALK* rearrangements).[11]

Although the expression of PD-L1 on the surface of tumor cells, as measured by immunohistochemistry, may potentially serve as a predictive factor to identify patients who would benefit from anti-PD-1/PD-L1 therapy, not all PD-L1-positive patients respond well.[12,13] Subsequent work has established that across many cancer types, patients with PD-L1–negative tumors show an aggregate 15% response rate, compared with a 48% response rate for patients with PD-L1–positive tumors.[14] Therefore, PD-L1 expression on tumor cells probably may not be the best predictive factor.[11] Interestingly, the presence or absence of $CD8^+$ and $PD\text{-}1^+$ tumor-infiltrating lymphocytes and PD-L1 expression in the tumor microenvironment also correlated with the clinical outcome of anti-PD-1/PD-L1 therapies.[12,15]

Recent studies demonstrated that basaloid squamous cell carcinoma constitute a specific histomolecular entity with dismal prognosis justifying its histological recognition and distinction from non-basaloid SCC.[16] Despite recently approved checkpoint blocking agents in non-small-cell lung cancer, no data are available to support the hypothesis that PD-1/PD-L1 inhibitors may be effective in basaloid squamous cell carcinoma. This prompted us to analyze PD-L1 expression along with content of $CD8^+$ and $PD\text{-}1^+$ tumor-infiltrating T cells infiltrates in association with clinicopathological parameters and clinical outcome of basaloid squamous cell carcinoma patients.

Several PD-L1 diagnostic assays have been recently produced.[11] The specificity, sensitivity, and reproducibility of most commercially available anti-PD-L1 immunohistochemistry antibodies have not been thoroughly evaluated. Moreover, the PD-L1 threshold for positivity is not well defined and is subject to assay and interpretative subjectivity. To overcome such limitations, we report herein the comparison of three specific anti-PD-L1 antibodies, which are regularly used as diagnostic tests in clinical trials, on serial sections of formalin-fixed paraffin-embedded specimens from 56 patients with lung basaloid squamous cell carcinoma.

Patients and methods

Study Population

The retrospective cohort consisted of 56 patients with surgically treated lung pure basaloid squamous cell carcinoma at the Department of Thoracic Surgery, University of Nice, Pasteur Hospital (Nice, France), from March 2007 to October 2014. All tumor specimens were collected, stored, and used with the informed consent from the patients (Hospital-Integrated Biobank BB-0033-00025, Pasteur Hospital, Nice, France). The study was approved by the Ethics Committee of the University of Nice Sophia Antipolis and performed according to the guidelines of the Declaration of Helsinki. The study complied with the REMARK recommendations for tumor marker prognostic studies using biological material.[17]

All samples were independently reviewed according to the current World Health Organization 2015 classification by three pulmonary pathologists (MI, CB, and VH).[4] Tumors had no histological squamous differentiation and expressed p40, p63, and CK34βE12, but did not express TTF-1 or neuroendocrine markers, Chromogranin A, Synaptophysin, and CD56.[16] Moreover, SOX4 expression with low/absent IVL (involucrin) expression confirmed the basaloid phenotype, as previously described (Supplementary Figure S1).[16] A tumor cell content of more than 50% was used as a criterion to select samples. All patients but one were current ($n = 43$) or former smokers ($n = 12$). The main clinical and histopathological characteristics of the patients are summarized in Table 1.

Immunohistochemistry

Immunohistochemistry was performed to estimate the expression of PD-L1 and the density of tumor-infiltrating T cells using the BenchMark ULTRA automated staining instrument (Ventana Medical Systems, Tucson, AZ, USA).

Formalin-fixed paraffin-embedded freshly cut tissue sections of 4-μm thickness, mounted on positively charged slides were stained for PD-L1 with three anti-human PD-L1 rabbit monoclonal antibodies (clones SP142 and SP263, Ventana; and clone 28-8, antibodycam), according to the manufacturer's recommendations, as recently described.[11,18] The OptiView DAB immunohistochemistry Detection Kit (Ventana) was used for the visualization of the bound anti-PD-L1 primary antibodies; sections were counter-stained with haematoxylin. Serial tissue sections were incubated (16 min at 36 °C) with a mouse monoclonal antibody

Table 1 Correlation of PD-L1 protein expression levels and CD8$^+$ and PD-1$^+$ tumor-infiltrating T cells infiltrates with the clinicopathological features of lung basaloid squamous cell carcinoma patients

Feature	*Overall* n *(%)*	*PD-L1 IHC SP142*		P-*value*	*PD-L1 IHC SP263*		P-*value*	*PD-L1 IHC 28-8*		P-*value*	*PD-1$^+$ TILs*		P-*value*	*CD8$^+$ TILs*		P-*value*
		Low	*High*		*Low*	*High*		*Low*	*High*		*Low*	*High*		*Low*	*High*	
Patient number	56 (100%)	31 (55%)	25 (45%)		4 (7%)	52 (93%)		10 (18%)	46 (82%)		29 (52%)	27 (48%)		25 (45%)	31 (55%)	
Age (y), median 70[a]				0.954			1			0.687			0.015			0.119
< 70 years	30 (54%)	16 (53%)	14 (47%)		2 (7%)	28 (93%)		3 (21%)	11 (79%)		11 (37%)	19 (63%)		10 (33%)	20 (67%)	
≥ 70 years	26 (46%)	15 (58%)	11 (42%)		2 (8%)	24 (92%)		7 (17%)	35 (83%)		18 (69%)	8 (31%)		15 (58%)	11 (42%)	
Gender ±				0.369			0.551			0.226			0.044			0.208
Male	42 (75%)	25 (60%)	17 (40%)		4 (10%)	38 (90%)		9 (21%)	33 (79%)		25 (60%)	17 (40%)		21 (50%)	21 (50%)	
Female	14 (25%)	6 (43%)	8 (57%)		0 (0%)	14 (100%)		1 (7%)	13 (93%)		4 (29%)	10 (71%)		4 (29%)	10 (71%)	
Smoking history ±				1			1			0.501			0.330			0.446
Never smoked	1 (2%)	1 (100%)	0 (0%)		0 (0%)	1 (100%)		0 (0%)	2 (100%)		1 (100%)	0 (0%)		1 (100%)	0 (0%)	
Former or current smokers	55 (98%)	30 (55%)	25 (45%)		4 (7%)	51 (93%)		10 (19%)	44 (81%)		28 (51%)	27 (49%)		24 (44%)	31 (56%)	
Tumor size (median, 2 cm)[a]				0.907			0.640			0.118			0.070			0.294
≥ median	33 (58%)	17 (52%)	16 (48%)		2 (6%)	31 (94%)		7 (29%)	17 (71%)		14 (43%)	19 (57%)		13 (39%)	20 (61%)	
< median	24 (42%)	14 (58%)	10 (42%)		3 (13%)	21 (87%)		3 (9%)	29 (91%)		16 (67%)	8 (33%)		13 (54%)	11 (46%)	
pTNM stage ±				0.573			0.071			0.163			0.943			0.375
I	18 (32%)	10 (56%)	8 (44%)		1 (6%)	17 (94%)		4 (22%)	14 (78%)		10 (56%)	8 (44%)		6 (33%)	12 (67%)	
II	20 (36%)	9 (45%)	11 (55%)		0 (0%)	20 (100%)		1 (5%)	19 (95%)		10 (50%)	10 (50%)		10 (50%)	10 (50%)	
III	11 (20%)	7 (64%)	4 (36%)		3 (27%)	8 (73%)		4 (36%)	7 (64%)		5 (46%)	6 (54%)		5 (46%)	6 (54%)	
IV	7 (12%)	5 (71%)	2 (29%)		1 (14%)	6 (86%)		1 (14%)	6 (86%)		4 (57%)	3 (43%)		5 (71%)	2 (29%)	
Neoadjuvant chemotherapy ±				0.358			0.577			0.175			0.700			0.114
No	51 (93%)	26 (51%)	25 (49%)		3 (6%)	48 (94%)		8 (16%)	43 (84%)		26 (51%)	25 (49%)		22 (43%)	29 (57%)	
Yes	5 (7%)	4 (80%)	1 (20%)		0 (0%)	5 (100%)		2 (40%)	3 (60%)		3 (60%)	2 (40%)		4 (80%)	1 (20%)	
Neoadjuvant radiotherapy ±				0.620			0.262			0.080			0.940			0.265
No	52 (93%)	28 (54%)	24 (46%)		3 (6%)	49 (94%)		2 (4%)	50 (96%)		27 (52%)	25 (48%)		24 (46%)	28 (54%)	
Yes	4 (7%)	3 (75%)	1 (25%)		1 (25%)	3 (75%)		1 (25%)	3 (75%)		2 (50%)	2 (50%)		3 (75%)	1 (25%)	

Abbreviation: TNM, tumor node metastasis.
PD-L1 expression was considered to be low *vs* high using the score 0 on tumor and immune cells *vs* 1/2/3 categories. The number of PD-1$^+$ and CD8$^+$ tumor-infiltrating T cells in each case was considered to be low *vs* high using the median value obtained from all cases as a threshold.
[a]χ^2-test; ± Fisher's exact test.

anti-PD-1 (clone MRQ-22, Cell Marque, Rocklin, CA, USA), and a rabbit monoclonal antibody anti-CD8 (clone SP57, Ventana). Each immunohistochemistry run contained a positive control (on-slide tonsil tissue) and a negative antibody control (buffer, no primary antibody).

Staining Evaluation

First, the percentage of PD-L1 expressing tumor cells and tumor-infiltrating immune cells was recorded for correlation purposes, regardless of the staining intensity. Second, PD-L1 expression was stratified based on scoring algorithms described in clinical trials using the corresponding anti-PD-1/PD-L1 inhibitors (Supplementary Material).[12,18–20]

The immunohistochemistry staining was independently assessed by three senior pulmonary pathologists (MI, CB, and VH) blinded to the clinical data. When a discrepancy between the three pathologists was noted, the slides were commonly reviewed in order to obtain a consensus.

Tissue slides stained for PD-1 and CD8 were scanned via digital microscopy using the Nanozoomer HT 2.0 Scanner (Hamamatsu Photonics, Japan). The numbers of PD-1$^+$ and CD8$^+$ tumor-infiltrating T cells per unit area (mm^2) were semi-automatically calculated from the intact tumor areas lacking necrosis using modified nuclear/cytoplasmic immunohistochemistry algorithms in Calopix software (Tribvn, Châtillon, France).[21] This semi-automated calculation was validated by comparative analysis between these data and the data obtained from manual counting in selected cases.

Statistical Analyses

The concordance between the PD-L1 immunohistochemistry assays was determined with the Spearman correlation coefficient (ρ) test, Cohen's kappa index (κ) and Bland-Altman agreement plots. Correlation between PD-1$^+$ and CD8$^+$ tumor-infiltrating T cells was assessed using the Spearman correlation test.

The nonparametric Spearman, χ^2, or Fisher's exact tests were performed for characterization of PD-1$^+$ and CD8$^+$ tumor-infiltrating T cells and the evaluation of PD-L1 expression levels, as continuous or categorical variables respectively, with consideration of the clinicopathological features of the patients. For these statistical analyses, PD-L1 expression assessed with both immunohistochemistry assays was considered to be low *vs* high using the score 0 on both tumor and immune cells (0/0) *vs* 1/2/3 categories. For all statistical analyses, the number of PD-1$^+$ and CD8$^+$ tumor-infiltrating T cells in each case was considered to be low *vs* high using the median value obtained from all cases as a threshold.

Kaplan–Meier survival curves, log-rank test and Cox proportional regression analysis were determined in order to assess the prognostic significance of PD-1$^+$ and CD8$^+$ tumor-infiltrating T cells, and of PD-L1 expression for disease-free survival and overall survival (Supplementary Material).

All statistical analyses and data presentations were performed in R language (version 3.2.2, R Core Team, Vienna, Austria). All statistical tests were two-sided, and *P*-values < 0.05 indicated statistical significance.

Results

The Pattern of PD-L1 Expression and Content of CD8$^+$ and PD-1$^+$ Tumor-Infiltrating T Cells in Lung Basaloid Squamous Cell Carcinoma

On the basis of the SP142 score, tumors were categorized as follows: (i) on tumor cells—score 3 (4%), score 2 (11%), score 1 (9%), and score 0 (77%), as well as on immune cells—score 3 (9%), score 2 (7%), score 1 (21%), and score 0 (63%), when analyzed with the SP142 antibody; (ii) based on the staining with the SP263 antibody on tumor cells—score 3 (18%), score 2 (14%), and score 0 (68%), with no tumors scored 1, while in immune cells there were score 3 (55%), score 2 (21%), score 1 (14%), and score 0 (9%); and finally (iii) when analyzed with the 28-8 antibody on tumor cells—score 3 (18%), score 2 (14%), and score 0 (68%), with no score 1 tumors, as well as score 3 (50%), score 2 (18%), score 1 (13%), and score 0 (20%), on immune cells.

On the basis of the SP263 score, high PD-L1 expression was observed in: (i) 4 (7%) of cases when assessed with the SP142 antibody; (ii) 13 (23%) of cases when evaluated with the SP263 antibody; and iii) 12 (21%) of cases when analyzed with the 28-8 antibody.

On the basis of the 28-8 score, PD-L1 expression was (i) $\geq 1\%$ of tumor cells in 13 (23%) cases, $\geq 5\%$ in 8 (14%) cases, and $\geq 10\%$ in 5 (9%) cases, when analyzed with the SP142 antibody; (ii) $\geq 1\%$ in 18 (32%), $\geq 5\%$ in 18 (32%), and $\geq 10\%$ in 16 (29%), when determined with the SP263 antibody; and (iii) $\geq 1\%$ in 18 (32%), $\geq 5\%$ in 18 (32%), and $\geq 10\%$ in 14 (25%), when tested with the 28-8 antibody.

The concordance analysis of PD-L1 expression on tumor cells showed a poor correlation between the SP142 and SP263 antibodies ($\rho = 0.852$, $\kappa = 0.362$), and the SP142 and 28-8 antibodies ($\rho = 0.860$, $\kappa = 0.412$), while a good correlation was observed between the SP263 and 28-8 antibodies ($\rho = 0.996$, $\kappa = 0.883$; Supplementary Table S1, Supplementary Figure S2). On the basis of the immune cells score, we observed poor agreement between the SP142 and SP263 antibodies ($\rho = 0.568$, $\kappa = 0.018$) and the SP142 and 28-8 antibodies ($\rho = 0.590$, $\kappa = 0.134$), while a

good correlation was noted between the SP263 and 28-8 antibodies ($\rho = 0.880$, $\kappa = 0.721$) (Supplementary Table S1, Supplementary Figure S2, Supplementary Figure S3).

Inter-reader precision in determining the PD-L1 expression in tumor cells resulted in an overall percentage agreement of 92% ($\kappa = 0.910$), 98% ($\kappa = 0.976$) and 96% ($\kappa = 0.935$) for SP142, SP263 and 28-8 assays, while the inter-reader agreement for PD-L1 expression of immune cells was 81% ($\kappa = 0.786$), 87% ($\kappa = 0.832$), and 86% ($\kappa = 0.817$) for SP142, SP263 and 28-8 assays, respectively.

The numbers of $CD8^+$ and $PD\text{-}1^+$ tumor-infiltrating T cells ranged from 0 to 1376 $CD8^+$ cells per mm^2 (median, 194 $CD8^+$ cells per mm^2) and from 0 to 383 $PD\text{-}1^+$ cells per mm^2 (median, 27 $PD\text{-}1^+$ cells per mm^2), respectively, which were significantly positively correlated (Spearman's ρ coefficient, 0.44; $P = 0.004$).

Based on the SP142 assay, high expression of PD-L1 in tumor and immune cells was significantly associated with high levels of $CD8^+$ cells (ρ coefficient, 0.48; $P = 0.012$), and $PD\text{-}1^+$ tumor-infiltrating T cells (ρ coefficient, 0.38, $P = 0.022$).

Based on the SP263 and 28-8 assays, high PD-L1 expression in both tumor and immune cells did not correlate with $CD8^+$ or $PD\text{-}1^+$ tumor-infiltrating T cells (ρ coefficient, 0.25, $P = 0.314$; ρ coefficient, 0.11, $P = 0.611$). Representative immunohistochemistry images of PD-L1 expression, CD8 and PD-1 infiltrates are presented in Figure 1 and Figure 2.

Clinicopathological Association of PD-L1 Expression and the Presence of $CD8^+$ and $PD\text{-}1^+$ Tumor-Infiltrating T cells in Lung Basaloid Squamous Cell Carcinoma

High PD-L1 expression, quantified with all antibodies according to each scoring system, as well as the infiltrates of $CD8^+$ tumor-infiltrating T cells, were not significantly associated with the clinicopathological parameters of lung basaloid squamous cell carcinoma patients (Table 1; data not shown for the SP263 and 28-8 scores). Conversely, the infiltrates of $PD\text{-}1^+$ tumor-infiltrating T cells significantly correlated with younger age at diagnosis ($P = 0.015$), and with female gender ($P = 0.044$), but not with smoking history, tumor size, TNM stage, and neoadjuvant treatment (Table 1).

Association of PD-L1 Expression and Tumor-Infiltrating T cells Infiltrates with Clinical Outcome in Lung Basaloid Squamous Cell Carcinoma Patients

At the last follow-up, 14/56 (25%) patients were dead; including 11/56 (20%) patients who died specifically from basaloid squamous cell carcinoma progression. The median follow-up was 23 months (range, 0–109 months).

Based on the SP142 score, high PD-L1 expression, assessed with the SP142 antibody in the tumor and immune cell compartments, was significantly associated with longer disease-free survival (HR, 0.35; 95% CI, 0.14–0.89; $P = 0.022$) and prolonged overall survival (HR, 0.094; 95% CI, 0.012–0.73; $P = 0.0015$; Figures 3a and b). It is interesting to note

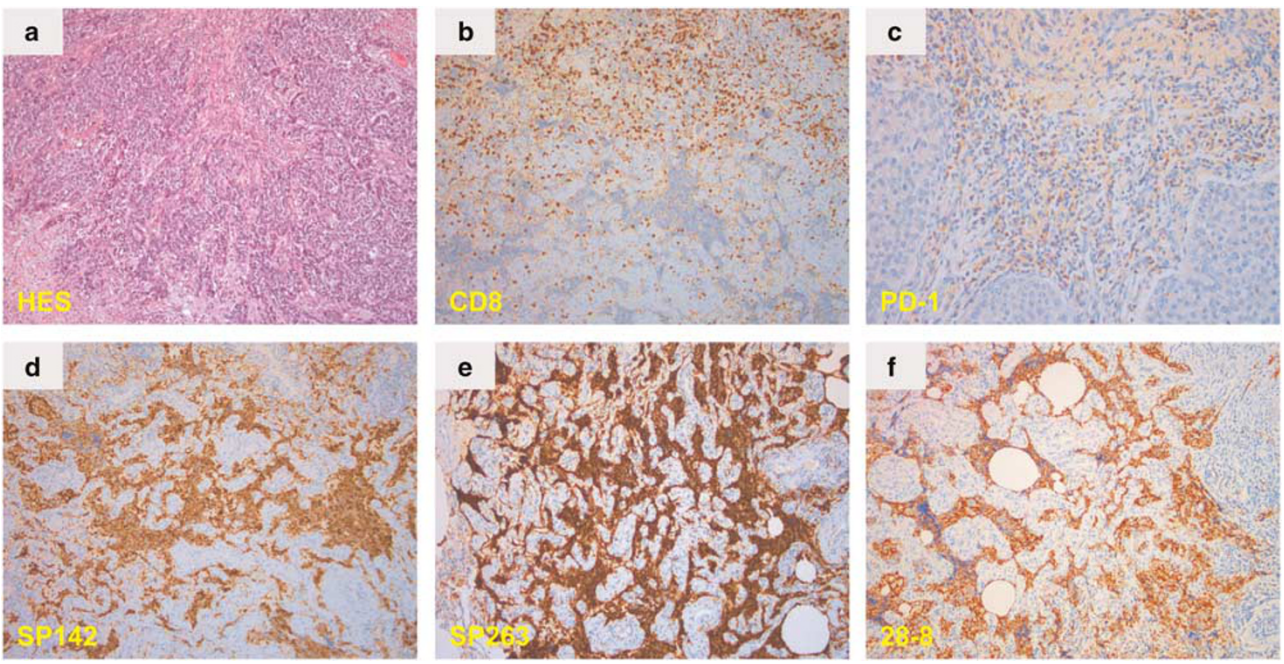

Figure 1 Representative images from matched areas in a selected pulmonary basaloid squamous cell carcinoma showing positive PD-L1 expression and high content of tumor-infiltrating T cells labeled with the indicated assays. Consecutive slides stained with haematoxylin-eosin-safran preparation (**a**, ×10), showing high $CD8^+$ tumor-infiltrating T cells (**b**, ×10), high $PD\text{-}1^+$ tumor-infiltrating T cells (**c**, ×20), and high PD-L1 expression with the SP142 antibody (**d**, ×10), the SP263 antibody (**e**, ×10), and the 28-8 antibody (**f**, ×10).

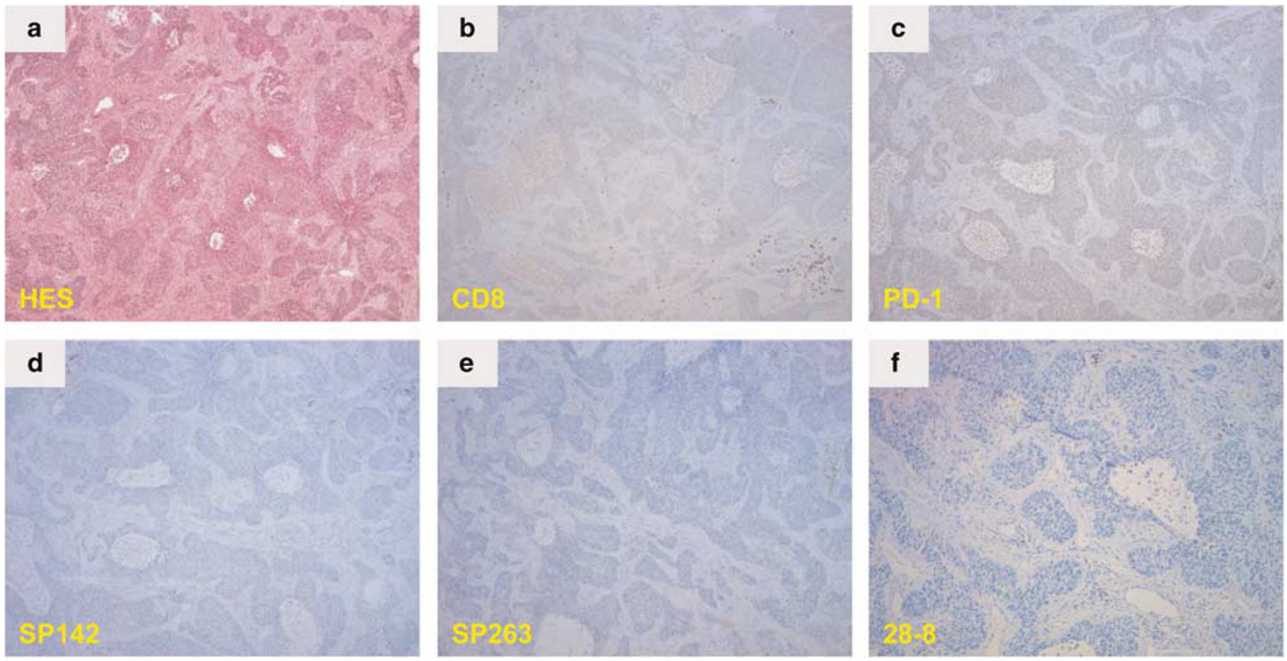

Figure 2 Serial sections from a selected pulmonary basaloid squamous cell carcinoma on haematoxylin-eosin-safran preparation (**a**, ×10), showing the absence of $CD8^+$ tumor-infiltrating T cells (**b**, ×10), with no $PD\text{-}1^+$ tumor-infiltrating T cells (**c**, ×10), as well as no PD-L1 expression with the SP142 antibody (**d**, ×10), the SP263 antibody (**e**, ×10), and the 28-8 antibody (**f**, ×10).

that significant differences were obtained based on the PD-L1 expressing immune cells (disease-free survival analysis, immune cells score 0 *vs* 1/2/3, $P=0.013$, and tumor cells score 0 *vs* 1/2/3, $P=0.118$; overall survival analysis, immune cells score 0 *vs* 1/2/3, $P=0.007$, and tumor cells score 0 *vs* 1/2/3, $P=0.070$). In addition, high PD-L1 expression, based on the SP142 score, assessed with SP263 antibody, was only significantly associated with improved overall survival (HR, 0.27; 95%CI, 0.075–1; $P=0.039$; Figure 3d), but not with disease-free survival (HR, 0.63; 95%CI, 0.19–2.1; $P=0.254$; Figure 3c). For this assay as well, the significant association with overall survival was obtained based on the PD-L1 expressing immune cells (immune cells score 0 *vs* 1/2/3, $P=0.017$, and tumor cells score 0 *vs* 1/2/3, $P=0.191$).

On the basis of the SP142 score no significant association was found between high PD-L1 expression, tested with the 28-8 antibody, and disease-free survival ($P=0.268$; Figure 3e) or overall survival ($P=0.086$; Figure 3f). As the optimal threshold for PD-L1 positivity is still under debated, we further calculated different cutoff values of our cohort for all antibodies, including 5% of positive tumor and immune cells, and 10% of positive immune cells or 50% of positive tumor cells, and found no significant prognostic impact (data not shown).

Based on the SP263 score, all antibodies were not significantly associated either with disease-free survival (SP142 antibody, $P=0.124$; SP263 antibody, $P=0.088$; 28-8, $P=0.281$) or with overall survival (SP142, $P=0.158$; SP263, $P=0.064$; 28-8, $P=0.123$).

The presence of an increased number of $PD\text{-}1^+$ tumor-infiltrating T cells was significantly associated with longer disease-free survival (HR, 0.24, 95% CI, 0.088–0.65, $P=0.0025$, Figure 4a) and overall survival (HR, 0.18; 95% CI, 0.039–0.79; $P=0.011$, Figure 4b). In addition, an elevated number of $CD8^+$ tumor-infiltrating T cells was significantly associated with better disease-free survival (HR, 0.36, 95% CI, 0.15–0.85, $P=0.015$, Figure 4c) and showed mild association with a longer overall survival (HR, 0.35, 95% CI, 0.11–1.2, $P=0.074$, Figure 4d).

The combined survival analysis according to PD-L1 expression analyzed with SP142 and immune type cells showed that the disease-free and overall survival of patients with tumors expressing high PD-L1 expression and high infiltration of $PD\text{-}1^+$ and $CD8^+$ tumor-infiltrating T cells, was significantly more favorable compared with that of patients with low PD-L1 expression and low tumor-infiltrating T cells infiltrates (disease-free survival analysis, HR, 0.24; 95% CI, 0.1–0.55; $P<0.001$; Figure 5a, Supplementary Figure S4A; overall survival analysis, HR, 0.17; 95% CI, 0.05–0.54, $P<0.001$; Figure 5b, Supplementary Figure S4B). In contrast, there was no significant association with disease-free and overall survival based on the SP263 assay (disease-free survival analysis, HR, 0.63; 95% CI, 0.19–2.1; $P=0.448$; Figure 5c, Supplementary Figure S4C; overall survival analysis, HR, 0.27; 95% CI, 0.075–1, $P=0.067$; Figure 5d, Supplementary Figure S4D).

In the multivariate model, after adjusting for tumor size and stage, only patients with a high PD-L1 expression with SP142 together with increased

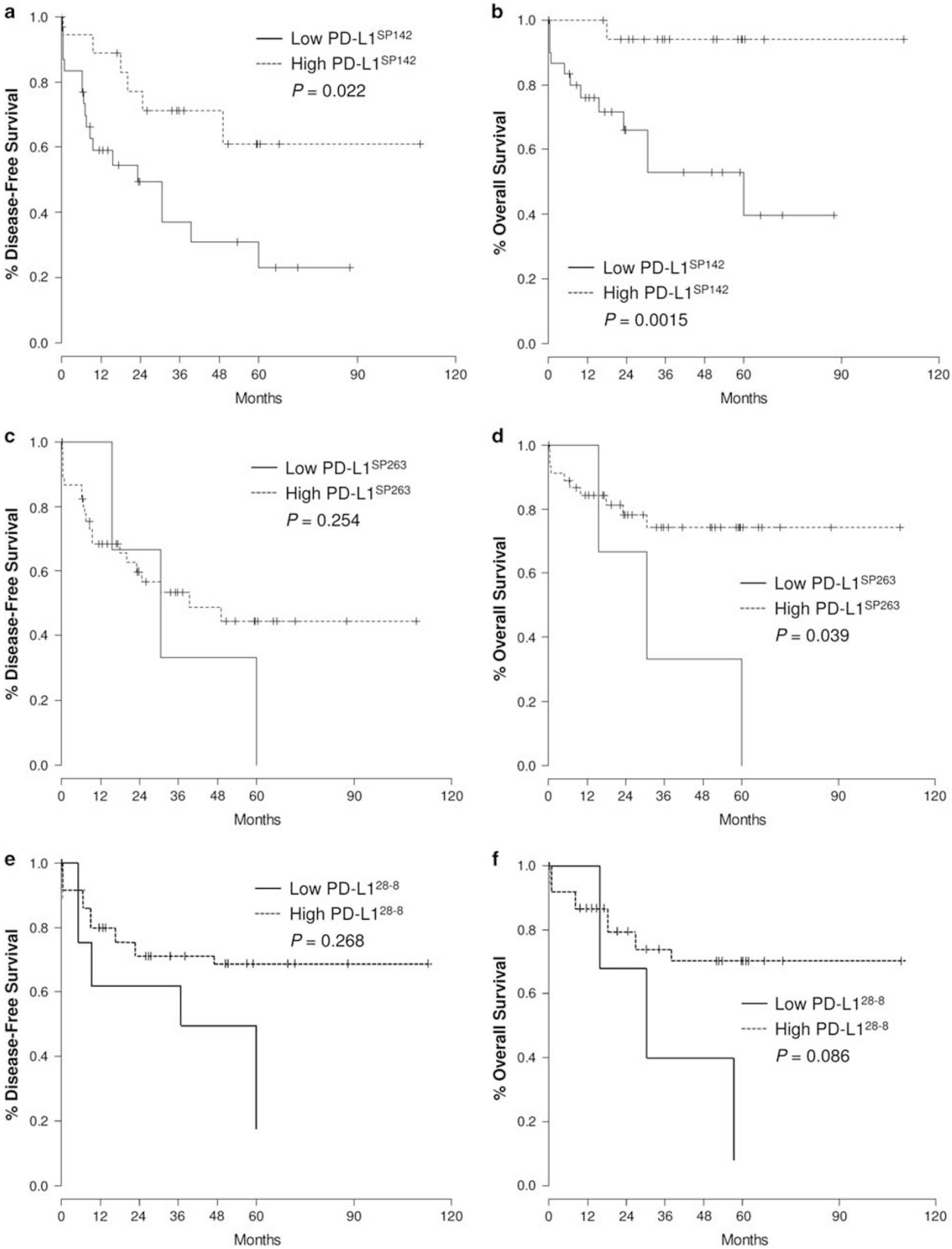

Figure 3 Survival analysis in lung basaloid squamous cell carcinoma patients stratified by the level of PD-L1 expression assessed with SP142, SP263 and 28-8 antibodies. Kaplan–Meier curves for the analysis of the prognostic significance of PD-L1 expression with SP142 for (**a**) disease-free survival and (**b**) overall survival, of PD-L1 expression with SP263 for (**c**) disease-free survival, and (**d**) overall survival, and of PD-L1 expression with 28-8 for (**e**) disease-free survival, and (**f**) overall survival. The *P*-values were calculated using the log-rank test.

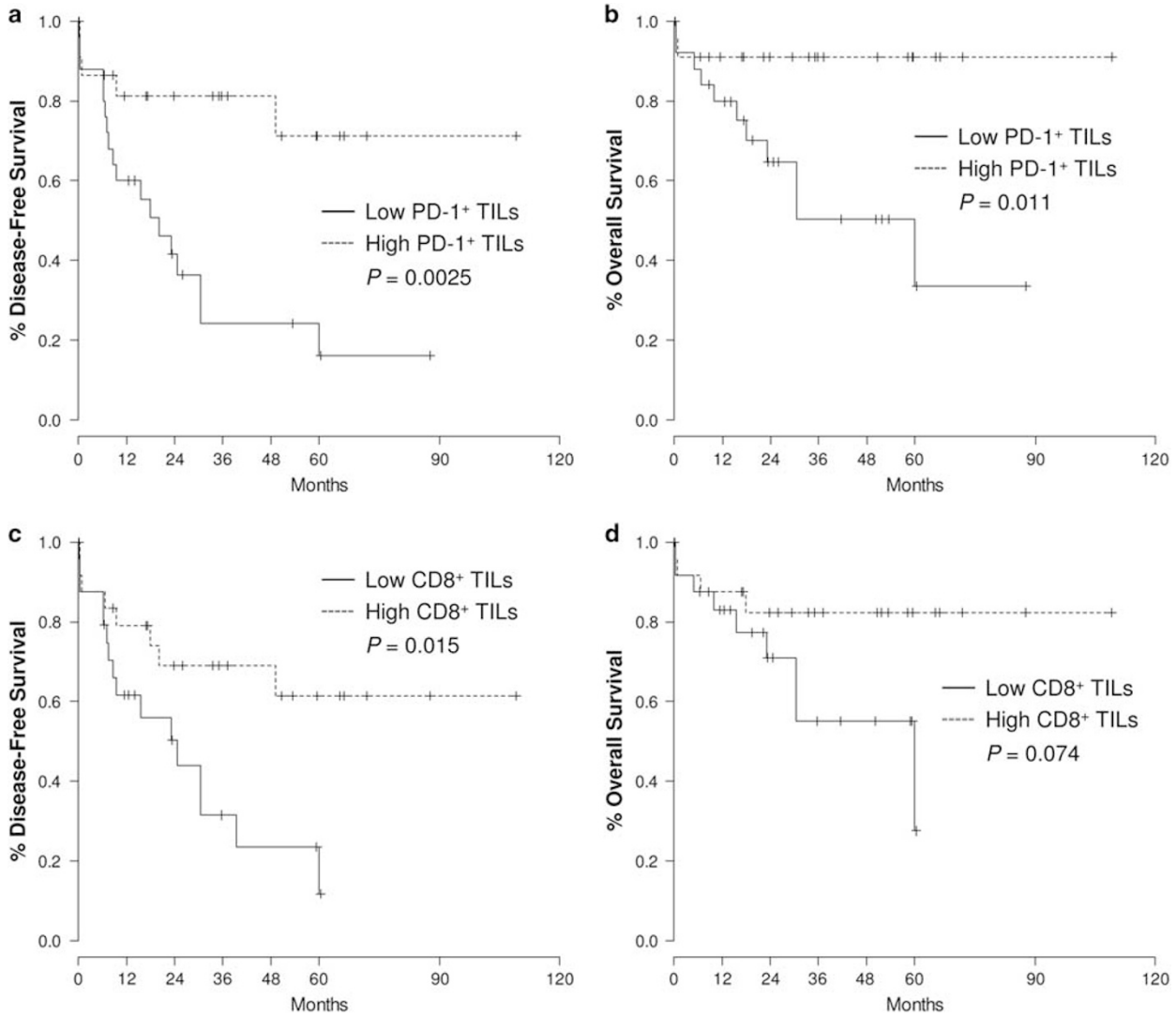

Figure 4 Survival analysis in lung basaloid squamous cell carcinoma patients according to tumor-infiltrating T cells density. Kaplan–Meier curves for the analysis of lung basaloid squamous cell carcinoma patients: (**a**) disease-free survival and (**b**) overall survival stratified based on PD-1$^+$ tumor-infiltrating T cells infiltrates. Kaplan–Meier curves for the analysis of lung basaloid squamous cell carcinoma patients: (**c**) disease-free survival and (**d**) overall survival stratified based on CD8$^+$ tumor-infiltrating T cells infiltrates. The *P*-values were calculated using the log-rank test.

PD-1$^+$ and CD8$^+$ tumor-infiltrating T cells had a significantly prolonged disease-free and overall survival (disease-free survival: HR, 5.277; 95% CI, 0.062–0.577; $P=0.003$; overall survival: HR, 0.136; 95% CI, 0.031–0.584; $P=0.007$; Table 2).

Discussion

This is the first study to evaluate the prognostic impact of PD-L1 expression and tumor-infiltrating T cells infiltrates in lung basaloid squamous cell carcinoma patients. Recent clinical trials suggested that the PD-L1 status assessed by immunohistochemistry on tumor samples could help to select patients for anti-PD-1/PD-L1 immune checkpoint therapies.[22] The United States Food and Drug Administration has recently approved two PD-1 inhibitors (nivolumab, and pembrolizumab) for patients with advanced lung squamous cell carcinoma whose disease progressed during or after platinum-based chemotherapy.[10,23,24] However, recent studies demonstrated that pure basaloid squamous cell carcinoma correspond to a specific non-small-cell lung cancer histomolecular entity at high risk for early death, distinct from non-basaloid squamous cell carcinoma.[16] No data on PD-L1 characterization in these patients are available.

The United States Food and Drug Administration also approved a PD-L1 immunohistochemistry companion diagnostic assay (22C3 pharmDx, Dako) and a complementary immunohistochemistry assay (28-8 pharmDx, Dako North America, CA, USA) to detect PD-L1 protein expression levels.[9,11] Several other PD-1/PD-L1 checkpoint inhibitors are in late-stage clinical testing, such as Atezolizumab

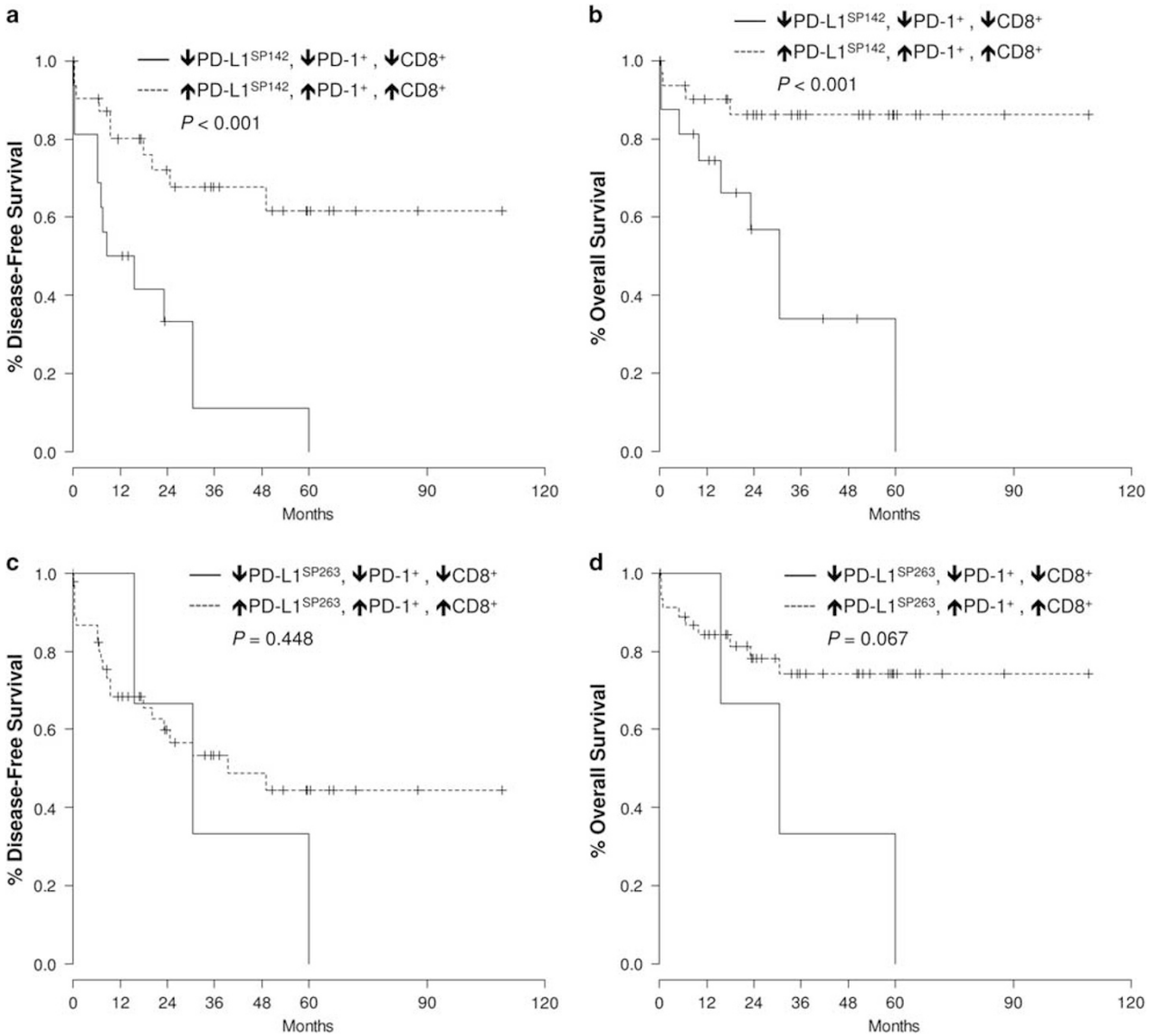

Figure 5 Survival analysis in lung basaloid squamous cell carcinoma patients according to pooled high and low PD-L1 expression and tumor-infiltrating T cells density. Kaplan–Meier curves of disease-free survival and overall survival according to SP142 antibody (**a** and **b**) and to SP263 antibody (**c** and **d**).

(MPDL3280A, Genentech/Roche), for which PD-L1 expression is centrally assayed using the SP142 immunohistochemistry antibody, and Durvalumab (MEDI4736, AstraZeneca/MedImmune), for which the PD-L1 expression level is measured with the SP263 immunohistochemistry assay.[11,20]

Accurate assessment of PD-L1 expression in formalin-fixed paraffin-embedded tumor samples is limited by the absence of studies comparing the different companion diagnostic tests, and interpretative uncertainties (e.g., cutoff for positivity, PD-L1 expression heterogeneity).[25]

To the best of our knowledge, this is the first study to compare three monoclonal anti-PD-L1 antibodies in lung basaloid squamous cell carcinoma (clones SP142, SP263, and 28-8), rigorously validated for immunohistochemistry detection, that are currently used in clinical trials.[12,26] Several collaborative efforts were initiated to evaluate the concordance of the clinical trial PD-L1 assays and commercially available antibodies in non-small-cell lung cancer (eg, Blueprint proposal, National Comprehensive Cancer Network/ Bristol-Myers Squibb study).[27]

Moreover, with regard to the spatial heterogeneity of PD-L1,[28] a major strength of this study is the assessment of PD-L1 expression on whole-tissue formalin-fixed paraffin-embedded sections. Most of the studies that evaluated the prognostic impact of PD-L1 expression in non-small-cell lung cancer measured protein expression in tissue microarray samples, which may hamper translation of these findings into the clinical setting.[29–31] Analysis of whole-tissue slide section samples reflects better the tissue distribution and the type of cells expressing

Table 2 Multivariate Cox analysis of prognostic factors for disease-free survival and overall survival as the end points in patients with lung basaloid squamous cell carcinoma.

Prognostic factor	*HR*	*95% CI*	P-*value*
Disease-free survival			
Tumor size (median, high/low)	1.827	0.160–1.862	0.334
pTNM stage (I+II/III+IV)	0.488	0.731–5.728	0.172
PD-L1^{SP263}, PD-1^{+}, CD8^{+} (low/high)	1.375	0.206–2.561	0.620
PD-L1$^{28\text{-}8}$, PD-1^{+}, CD8^{+} (low/high)	1.490	0.140–1.810	0.277
PD-L1^{SP142}, PD-1^{+}, CD8^{+} (low/high)	5.277	0.062–0.577	0.003
Overall survival			
Tumor size (median, high/low)	1.531	0.141–3.014	0.585
pTNM stage (I+II/III+IV)	0.324	0.850–11.153	0.086
PD-L1^{SP263}, PD-1^{+}, CD8^{+} (low/high)	2.528	0.102–1.521	0.177
PD-L1$^{28\text{-}8}$, PD-1^{+}, CD8^{+} (low/high)	1.450	0.102–2.100	0.294
PD-L1^{SP142}, PD-1^{+}, CD8^{+} (low/high)	0.136	0.031–0.584	0.007

Abbreviations: CI, confidence interval; HR, hazard ratio.

PD-L1, which allows evaluation of the relationship between expression by tumor cells and tumor-infiltrating T cells.[28]

Our results suggest that PD-L1 protein expression is heterogeneous and that different antibody assays may yield variable results. The anti-PD-L1 antibodies SP142 *vs* SP263, and SP142 *vs* 28-8 showed fair to poor concordance, irrespective of the cutoff used, while the 28-8 and SP263 antibodies demonstrated a strong correlation for both the tumor cell and immune cell compartments. In our study, the PD-L1 expression level ranged from 23% to 32% of cases, in at least 1% of tumor cells, as assayed with the SP142, SP263, or 28-8 antibodies. These results were similar to previously reported levels in non-small-cell lung cancer and squamous cell carcinoma populations (from 20 to 56%), whereas PD-L1 expression on immune cells had not been yet explored in lung basaloid squamous cell carcinoma.[29–34] The PD-L1 expression level in $\geq$1% of immune cells ranged from 38% (SP142) to 80% (28-8) and 91% (SP263). Although 28-8 is reported to bind to the extracellular domain of PD-L1, both SP263 and SP142 are directed against the intracellular domain. The difference between these antibodies raises concerns and suggests that antibody validation data should be presented in reports of future clinical trials.[35]

As a consequence, the low level of correlation between the SP142 and SP263 tests as well as SP142 and 28-8 assays yielded discordant results with regard to the immune microenvironment, clinicopathological and outcome. While PD-L1 expression assayed with the SP142 antibody was significantly associated with an increase in CD8^{+} and PD-1^{+} tumor-infiltrating T cells, as recently reported with another validated anti-PD-L1 immunohistochemistry assay,[30] the SP263 and 28-8 antibodies were unable to demonstrate the same relationship. However, we did not find any significant association between PD-L1 expression with all antibodies and clinicopathological features of the patients, in keeping with other recent studies on lung SCC.[30,31,34] We found that a high number of PD-1^{+} tumor-infiltrating T cells was more frequent in younger patients and women, raising the possibility of gender and age differences in anti-tumor host immunity in these patients.[31]

Interestingly, our results demonstrated that the survival effect of PD-L1 expression was dependent on the immunohistochemistry assay. When assayed with the SP142 antibody, PD-L1 expression was significantly associated with longer disease-free and overall survival, whereas the SP263 assay failed to demonstrate significant association with disease-free survival. However, it was associated with favorable overall survival in the univariate survival analysis. No significant association was found between the 28-8 assay and outcome. Moreover, an increase in PD-1^{+} tumor-infiltrating T cells were associated with a favorable disease-free and overall survival, whereas elevated CD8^{+} tumor-infiltrating T cells were associated with a longer disease-free survival, and had a marginal effect on prolonged overall survival.

Moreover, the positive effect of high PD-L1 expression, with the SP142 antibody, along with CD8^{+} and PD-1^{+} tumor-infiltrating T cells infiltration was magnified when the patients were divided into two groups with either all positive or all negative markers. In addition, the positive group was the best independent predictor for better disease-free and overall survival in basaloid squamous cell carcinoma, when compared with other well-known prognostic factors such as tumor size and stage. However, there is a gray zone for patients that do not present all positive or all negative PD-L1 expression, CD8^{+} and PD-1^{+} tumor-infiltrating T cells. These results are in agreement with previous studies showing individually a favorable prognostic effect for PD-L1 expression, CD8^{+} and PD-1^{+} tumor-infiltrating T cells in non-small-cell lung cancer and lung squamous cell carcinoma.[29,31,32,34,36] Moreover, our study suggests that PD-L1 positivity should be comprehensively interpreted with respect to the tumor microenvironment, and that

classification into several groups based on PD-L1, CD8 and PD-1 levels, may be an appropriate model to tailor PD-1/PD-L1-targeted immunotherapy in lung basaloid squamous cell carcinoma patients.[37,38] The results from the current study show that even in patients with a high PD-L1 expression with the SP142 antibody, the overall prognosis differed according to $CD8^+$ and $PD\text{-}1^+$ tumor-infiltrating T cells density (Supplementary Figure S3). It was noteworthy that the significant relationship between PD-L1 expression as assayed with the SP142 antibody with better outcome of basaloid squamous cell carcinoma patients was mostly related to PD-L1 expressing immune cells, with a marginal effect on survival of the PD-L1 expression quantified on the tumor cells.

It is likely that the prognostic and predictive significance relates not to single markers of tumor or immune signals but to the overall balance of the host anti-tumor immune response and tumor-mediated immunosuppression, whether adaptive or related to constitutively upregulated immunosuppressive signals mediated by immune editing. Prognostic significance has been shown to be significantly better in non-small-cell lung cancer with intense lymphocytic infiltration (tumor-infiltrating T cells) regardless of the composition in immune cells.[31,36,39] Therefore, PD-L1 and tumor-infiltrating T cells may point to a sensitive therapeutic window, when there is immune editing and the tumor escapes from immune surveillance in the appropriate CTL/Th1 microenvironment, where immunotherapy may provide increased benefit.[36] Moreover, the determination of PD-L1 expression on immune cells may be pivotal since it has been shown that the response to certain anti-PD-1/PD-L1 drugs correlated with the level of expression of PD-L1 on these components.[12] In addition, the predictive significance of PD-L1 expression on immune cells such as macrophages and lymphocytes may differ from tumor-associated PD-L1 expression in terms of the adaptive immune response.[12] As recently suggested for the neutrophil-to-T cell ratio in non-small-cell lung cancer, PD-L1 expression on both tumor cells and in the immune microenvironment, ie, on $CD8^+$ and $PD\text{-}1^+$ tumor-infiltrating T cells, and the intensity of global tumor-infiltrating T cells infiltration could be effective candidate markers for establishing a TNM immunoscore to refine the predictive value of targeted anti-PD-1/PD-L1 treatment.[21,40–42]

Our study holds several limitations, such as its retrospective design, the relatively low number of cases and importantly the lack of data available for response to anti-PD-1 and anti-PD-L1 monoclonal antibodies in this patient population. However, access to tissue samples from treated patients is still challenging because the PD-1 axis therapies have only recently been approved for lung cancer.

In conclusion, we showed that PD-L1 expressed on both tumor and immune cells has prognostic value in lung pure basaloid squamous cell carcinoma patients, but the effect was strongly dependent on the immunohistochemistry assay used. With respect to PD-1/PD-L1 pathway-targeted immunotherapy, in the absence of standardization, PD-L1 assessment with companion diagnostic tests using different antibodies may be discordant and, thus, the assay for one drug may probably not predict response to others. Moreover, our study demonstrated a significant association between high PD-L1 expression, as assayed with the SP142 antibody, along with increased $CD8^+$ and $PD\text{-}1^+$ tumor-infiltrating T cells and better outcome in lung basaloid squamous cell carcinoma patients. Therefore, assessment of PD-L1 expression and tumor-infiltrating T cells may help substantially to identify a subset of high-risk patients with resectable lung basaloid squamous cell carcinoma. The benefit of active surveillance in this subpopulation of patients with a high risk of recurrence and death should be further considered. Finally, these results raise the question about whether no single but multiple tumor and immune markers should be considered as a stratification factor in trials that test immunotherapy or immunomodulation.

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Disclosure/conflict of interest

Paul Hofman received honorarium from different industrial companies (AstraZeneca, Roche, and Bristol-Myers Squibb). The remaining authors declare no conflict of interest.

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