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ORIGINAL ARTICLE

Expression patterns of programmed death-ligand 1 in esophageal adenocarcinomas: comparison between primary tumors and metastases

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Abstract Expression analysis of programmed death-ligand 1 (PD-L1) may be helpful in guiding clinical decisions for immune checkpoint inhibition therapy, but testing by immunohistochemistry may be hampered by heterogeneous staining patterns within tumors and expression changes during metastatic course. PD-L1 expression (clone SP142) was investigated in esophageal adenocarcinomas using tissue microarrays (TMA) from 112 primary resected tumors, preoperative biopsies and full slide sections from a subset of these cases ($n = 24$), corresponding lymph node ($n = 55$) and distant metastases ($n = 17$). PD-L1 expression was scored as 0.1–1, >1, >5, >50% positive membranous staining of tumor cells and any positive staining of tumor-associated inflammatory infiltrates and/or stroma cells. There was a significant correlation with overall PD-L1 expression between the full slide sections and the TMA ($p = 0.001$), but not with the corresponding biopsies. PD-L1 expression in tumor cells >1% was detected in 8.0% of cases (9/112) and 51.8% of cases (58/112) in tumor-associated inflammatory infiltrates and/or stroma cells of primary tumors. Epithelial expression in metastases was found in 5.6% of cases (4/72) and immune cell expression in 18.1% of cases (13/72), but did not correlate with the expression pattern in the primary tumor. Overall PD-L1 expression in the primary tumor did not influence survival. However, PD-L1 expression was correlated with the number of CD3$^+$ tumor-infiltrating lymphocytes in the tumor center, and a combinational score of PD-L1 status/CD3$^+$ tumor-infiltrating lymphocytes was correlated with patients' overall survival.

Abbreviations
EAC Esophageal adenocarcinoma
MMR Mismatch repair
PD1 Programmed cell death protein 1
PD-L1 Programmed death-ligand 1
TAI Tumor-associated inflammatory infiltrates and/or stroma cells
TC Tumor cells
TIL Tumor-infiltrating lymphocytes
TMA Tissue microarray

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Introduction

Esophageal adenocarcinoma (EAC) has shown an increase in incidence in Western countries over the last 40 years. Despite improved treatment regimens, e.g., combining surgery and neoadjuvant chemo- or chemoradiotherapy for locally advanced tumors, the overall 5-year survival rate remains below 20% [1]. Immune checkpoint inhibition, targeting the PD1 (programmed cell death protein 1)/PD-L1 (programmed death-ligand 1) axis, has become a promising therapeutic option for many solid tumors, including EAC and other gastrointestinal malignancies [2–4]. The receptor PD1 is expressed on T- and B-lymphocytes and represses the activation and cytokine secretion of T-lymphocytes upon binding to its ligands PD-L1 and PD-L2. The activated PD1/PD-L1 axis thus maintains peripheral T-cell tolerance and prevents autoimmunity [5]. Tumors exploit this mechanism by overexpressing PD-L1, which induces immunological tolerance of potential anti-tumoral lymphocytes, thus escaping immunological surveillance [6]. Inhibition of the PD1/PD-L1 axis has been shown to evoke robust responses in a variety of solid tumors, including gastrointestinal carcinomas, with response rates correlating only partially with PD-L1 expression [3].

Esophageal carcinomas and other gastrointestinal adenocarcinomas express PD-L1, rendering them potential candidates for immune checkpoint inhibition [4, 7, 8]. In contrast to esophageal squamous cell carcinomas, however, data about PD-L1 expression in esophageal adenocarcinomas are limited [7, 8].

Expression of PD-L1 in tissue can be investigated by immunohistochemistry, which allows the analysis of individual cell populations and to perform semiquantitative measurements. For PD-L1, several antibodies are commercially available, as well as companion diagnostic kits related to PD1/PD-L1-directed therapies [9, 10]. Despite the inconsistencies between different antibodies that have been reported, e.g., for the application on lung cancer, the staining and detection methods generally deliver comparable results [9, 11]. In addition to methodological aspects, evaluating PD-L1 staining comprises the definition of cutoffs (percentage of positive cells) in the particular tissue compartment, i.e., tumor cells (TC), and of tumor-associated inflammatory infiltrates and/or stroma cells (TAI).

Here, we comprehensively investigate the immunohistochemical expression of PD-L1 with two antibody clones using a tissue microarray (TMA) of 112 cases of primary resected well-characterized EAC, including full slide sections and preoperative biopsies for a subset of cases. As several studies have reported heterogeneous expression patterns within tumors and dynamic expression along tumor progression and metastases, corresponding lymph node and distant metastases were analyzed.

Materials and methods

Patients

Buffered formalin-fixed paraffin-embedded tumor tissue from 112 EAC patients treated at the Department of Surgery, Inselspital Bern, University of Bern, Switzerland, was used for this study. We selected those patients from a consecutive series between 1990 and 2011 who did not undergo neoadjuvant therapy, and enough material and detailed clinical and histopathological data were available. An overview of the clinicopathological features of the case collection is given in Table 2.

Tissue material

A TMA containing all cases was constructed as described before [12]. The TMA consists of three tissue cores (core size 0.6 mm) each of the tumor center, the tumor periphery, corresponding lymph node ($n=55$) and distant metastases ($n=17$). Duplicates of the TMA containing tumor center and tumor periphery were stained, resulting in a total of 12 TMA cores for the primary tumors. All lymph node metastases and 15 out of 17 distant metastases represented resection specimens. The remaining two distant metastases were obtained from needle core biopsies. In comparison with the primary tumor, the overall tumor volume of the metastases was much lower, resulting in an increased ratio of TMA cores to the overall tumor volume, despite a lower number of total cores. For 24 cases, sufficient preoperative biopsy material was available. From these cases, additional full slide sections were investigated. Moreover, conventional TMAs with one core of the tumor center (core size 1.0 mm) from 377 primary resected colon carcinomas, 125 primary resected gastric carcinomas and 30 non-gastrointestinal tumors were used for comparison. These TMAs were retrieved from the Tissue Bank of the University of Bern, Switzerland, and the Institute of Pathology of the Technical University of Munich, Germany. The local ethics commissions had approved the use of archival tissue for molecular analysis and TMA analysis, respectively (Technical University of Munich, No. 2136/08; University of Bern, No. 200/14).

Immunohistochemistry

Immunohistochemistry for PD-L1 was established for this study on an automated immunostainer (Bond III, Leica Biosystems, Newcastle, UK) for the two clones SP142 (Spring Bioscience, Pleasanton, CA, USA) and E1L3N (Cell Signaling Technology, Danvers, MA, USA). Tissue from human tonsils and Hodgkin's lymphoma as well as a TMA containing solid tumors known for their high-frequency of

PD-L1 expression (seminomas, melanomas, pulmonary carcinomas) and placenta was used as a positive control [4]. Deparaffinized sections were rehydrated in Dewax dilution (Leica Biosystems, Wetzlar, Germany) for 20 min. 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 at a dilution of 1:400 for 30 min. The slides were incubated with the secondary antibody using the Bond Polymer Refine Kit (with 3-3′-diaminobenzidine-DAB as chromogen) (Leica Biosystems). Finally, samples were counterstained with hematoxylin and mounted in Aquatex (Merck, Darmstadt, Germany).

Immunohistochemistry for mismatch repair (MMR) proteins was performed on the Leica Bond III autostainer as described elsewhere [13]. The expression data were obtained from a previous study [14]. In line with this study, tumors were classified as MMR proficiency if expressing MLH1, MSH2, PMS2 and MSH6, whereas MMR deficiency was defined as absence of the expression of one or more of these proteins.

Tumor-infiltrating lymphocytes (TIL) were determined by immunohistochemistry as described before [14]. In short, the stainings were performed on the Bond III immunostainer using antibodies against CD3 (clone SP7, 1:400, TRIS buffer, 95 °C for 30 min; Abcam, Cambridge, UK) and CD8 (clone C8/144B, 1:100, TRIS buffer, 95 °C for 20 min; Dako, Glostrup, Denmark) [12]. Slides were scanned with a high-resolution scanner and processed by an image analysis software (ScanScope CS, Aperio Image Scope; Leica, Wetzlar, Germany) to determine the TIL counts. The median of the total number of lymphocytes in the TMA cores of the tumor center was used as cutoff for classification into low and high levels of intratumoral lymphocytes.

Evaluation of PD-L1 stainings

PD-L1 expression in the primary tumors was scored as positive for tumor cells if a membranous staining pattern was detected. TIL or stromal cells were considered positive if any (membranous or cytosolic) staining was detected. Positivity was categorized according to the percentage of positive cells amongst all cells in the compartment (0.1–1, >1, >5, >50%). The same approach was applied for tumoral PD-L1 expression in lymph node and distant metastases. For the inflammatory infiltrate of metastases, only cells immediately adjacent to tumor tissue were analyzed. Necrotic or ulcerated areas were excluded from the analysis [15]. The TMA of the primary tumor was first scored by two independent reviewers (B. Dislich and J. Galvan), with a third reviewer (R. Langer)

on a multi-headed scope to reach consensus for divergent cases. This approach was chosen as there is a marked training effect when evaluating PD-L1 immunohistochemistry, which was apparent in the discussion about divergent cases in the second round [15].

Statistical analyses

Statistical analysis was carried out using the IBM SPSS 23.0 Statistics software (IBM, Chicago, IL, USA). Associations between immunohistochemical staining patterns and the absolute number of TIL were evaluated using the Mann–Whitney U test. Correlations between categorical variables were conducted using χ^2-square and Fisher's exact tests. Survival analysis was performed using Kaplan–Meier estimates and log-rank tests. Multivariate analysis was carried out using Cox proportional hazard models. p values were two-sided and regarded as significant if $p < 0.05$.

Results

PD-L1 immunohistochemistry

PD-L1 expression was initially scored as positive membranous staining of TC or any positivity in TAI for 0.1–1, >1, >5, >50% of all cells within the given compartment, respectively. We observed a very good concordance between the two antibodies for overall (TC and/or TAI positivity) PD-L1 staining in full slide and TMA sections ($p < 0.001$; overall concordance rate 89.5%). Staining intensity and staining patterns of the two different antibody clones (SP142, E1L3N) were comparable, which has also been observed by others (Fig. 1) [16]. Staining was generally very weak compared to our positive control tissue and tumors that have been reported to show high PD-L1 expression such as non-small cell lung cancers and seminomas (Fig. 1). We selected clone SP142 for further analysis of our cohort, because (a) in our hands it proved to be more robust with regard to staining intensity in between different runs, (b) it has been used in various clinical studies, (c) it is applied in a Food and Drug Administration-approved biomarker assay for urothelial cancer and (d) it has recently been included in an immunohistochemistry harmonization study [15, 17]. We defined TC or TAI PD-L1 positivity as >1% of positive cells in the given compartment, a cutoff value which has been used by various studies and which showed the best correlation with pathological features and survival as described in the following sections [18, 19].
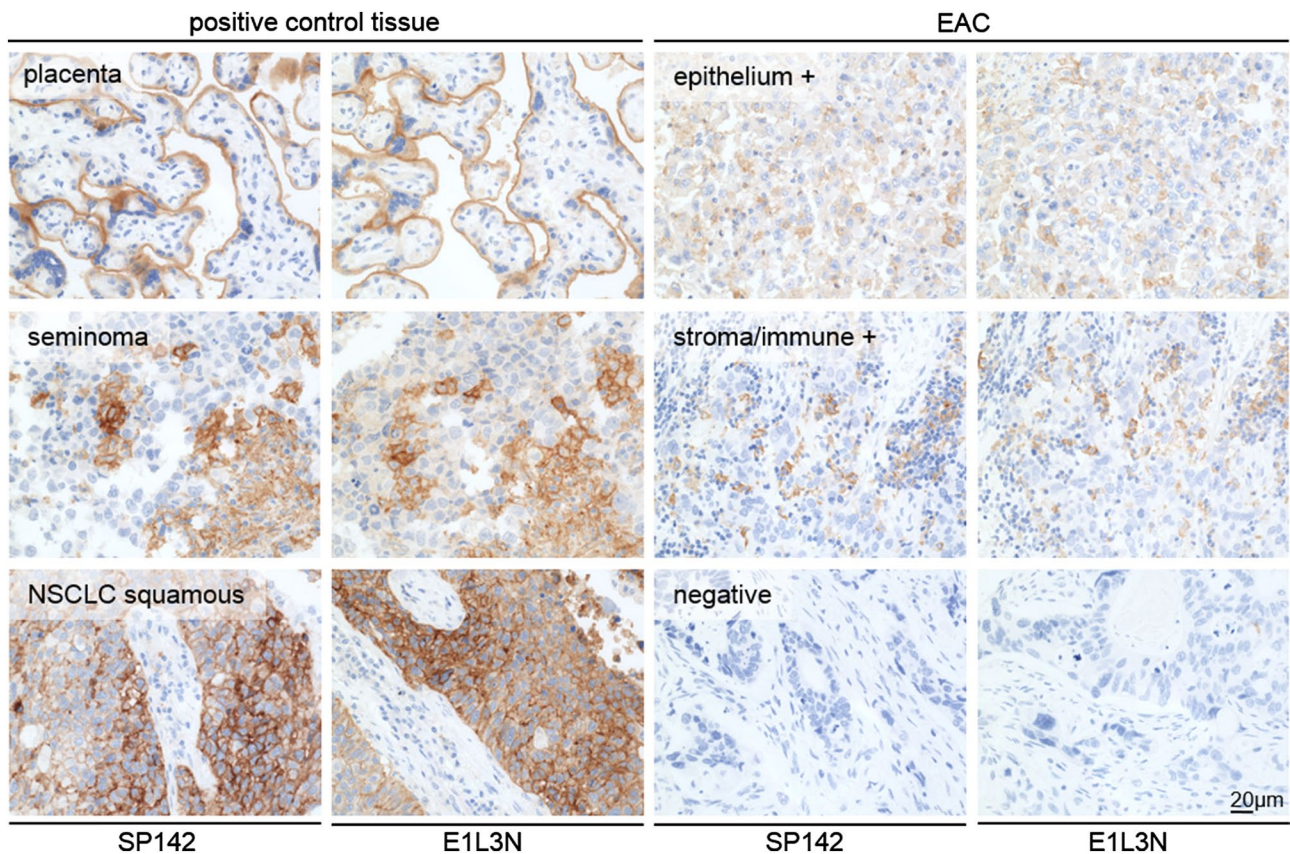
Fig. 1 Representative images of immunohistochemical stainings against PD-L1 with two different antibody clones (E1L3N and SP142). Placental tissue, unselected cases of lung squamous cell carcinoma (epithelial positivity) and seminoma (stromal positivity) were used as positive controls. Both antibody clones yielded similar results. For EAC, three cases each with epithelial, stromal or absent positivity for PD-L1 (stained with SP142) are shown. Note the overall weak PD-L1 positivity in EAC in comparison with the positive controls

PD-L1 expression patterns in primary EAC and corresponding metastases

There was a significant correlation between overall PD-L1 expression observed in full slide sections and the 12 cores/tumor containing TMA ($p = 0.001$), but not with the corresponding biopsies. Thus, we considered our 12 cores/tumor TMA as representative for PD-L1 expression with regard to the analyzed full slides, despite the patchy epithelial PD-L1 staining observed in a subset of cases (Supplementary figure 1). In our 12 cores/tumor TMA, PD-L1 expression in TC was detected by SP142 in 9/112 cases (8.0%) of primary EAC, whereas expression in TAI was observed in 58 cases (51.8%). All cases with PD-L1 TC positivity also showed PD-L1 positivity in TAI. PD-L1 expression was found in 11.8% (2/17) of distant and 3.6% (2/55) of lymph node metastases, whereas intratumoral lymphocytes were found in 11.8% (2/17) and 14.5% (8/55) of cases, respectively (Table 1). Epithelial PD-L1 expression in distant metastases was unrelated to the primary tumor in both cases, whereas epithelial expression in lymph node metastasis was found in one case with positive and one case with

Table 1 Frequencies of PD-L1 expression in gastrointestinal adenocarcinomas

PD-L1 positivity	Epithelial	Stroma/immune	Overall
EAC primary	9/112 (8.0%)	58/112 (51.8%)	58/112 (51.8%)
EAC lymph node metastasis	2/55 (3.6%)	8/55 (14.5%)	9/55 (16.4%)
EAC distant metastasis	2/17 (11.8%)	2/17 (11.8%)	4/17 (23.5%)
Gastric adenocarcinoma	10/125 (8.0%)	31/125 (24.8%)	37/125 (29.5%)
Colorectal adenocarcinoma	24/377 (6.4%)	128/377 (34.0%)	135/377 (35.8%)

negative epithelial PD-L1 expression in the primary tumor. Representative images of two divergent cases of metastatic PD-L1 expression are shown in Fig. 2.

PD-L1 correlates with clinicopathological parameters and the inflammatory environment

Overall PD-L1 expression was observed more often in tumors with higher pT category (>pT1; $p = 0.008$), vascular involvement ($p = 0.032$) and in trend in tumors with lymph node metastases ($p = 0.136$) or lower differentiation ($p = 0.123$). As a positive correlation between the number of tumor-infiltrating lymphocytes (TIL) and the expression of PD-L1 had been described for several cancer types before, we scored the number of TIL for our EAC cohort [20, 21]. Overall PD-L1 positivity was associated with higher counts of intratumoral CD3$^+$ TIL ($p = 0.003$) and in trend with CD8$^+$ TIL ($p = 0.092$). Tumors with TC PD-L1 positivity were more frequent of poor differentiation grade ($p = 0.076$) and non-intestinal type according to the Laurén's Classification ($p = 0.004$), which was not apparent for the PD-L1 overall positivity. Moreover, the percentage of lymph node metastases was significantly higher in tumors with TC PD-L1 positivity ($p = 0.027$). Mismatch repair

(MMR) deficiency was not associated with PD-L1 status. The correlations between overall and TC PD-L1 expression with pathological features are shown in Table 2.

Prognostic value of the overall PD-L1 status in relationship to CD3$^+$ tumor-infiltrating lymphocytes

TC, TAI or overall PD-L1 expression alone in the primary tumor was not associated with overall survival ($p = 0.675$). Tumors were then classified according to their overall PD-L1 status in relationship to CD3$^+$ TIL (highlighting the total number of T-lymphocytes) in analogy to a previously published classification proposal that subgroups tumors according to the relation of tumoral PD-L1 expression and TIL [22]. Thirty-six tumors (32%) scored overall positive for PD-L1 and had a high number of CD3$^+$ TIL (class I, PD-L1$^+$/CD3$^+$high); 36 tumors (32%) were class II (PD-L1$^-$/CD3$^+$low); 22 tumors (20%) were class III (PD-L1$^+$/CD3$^+$low); and 18 tumors (16%) were class IV (PD-L1$^-$/CD3$^+$high). A similar approach was also used for combining PD-L1 expression and CD8$^+$ TIL levels (data not shown).

Using this subclassification, the four different classes showed significant prognostic differences in univariate

Fig. 2 Two selected cases of EAC with conversion to a positive epithelial PD-L1 staining in distant or lymph node metastases (*upper panels*) are shown. The corresponding primary tumors that were scored negative for PD-L1 are included for comparison (*lower panels*). All stainings shown were carried out using the SP142 antibody

EAC case #1 — distant metastasis / primary

EAC case #2 — lymph node metastasis / primary

20μm

SP142
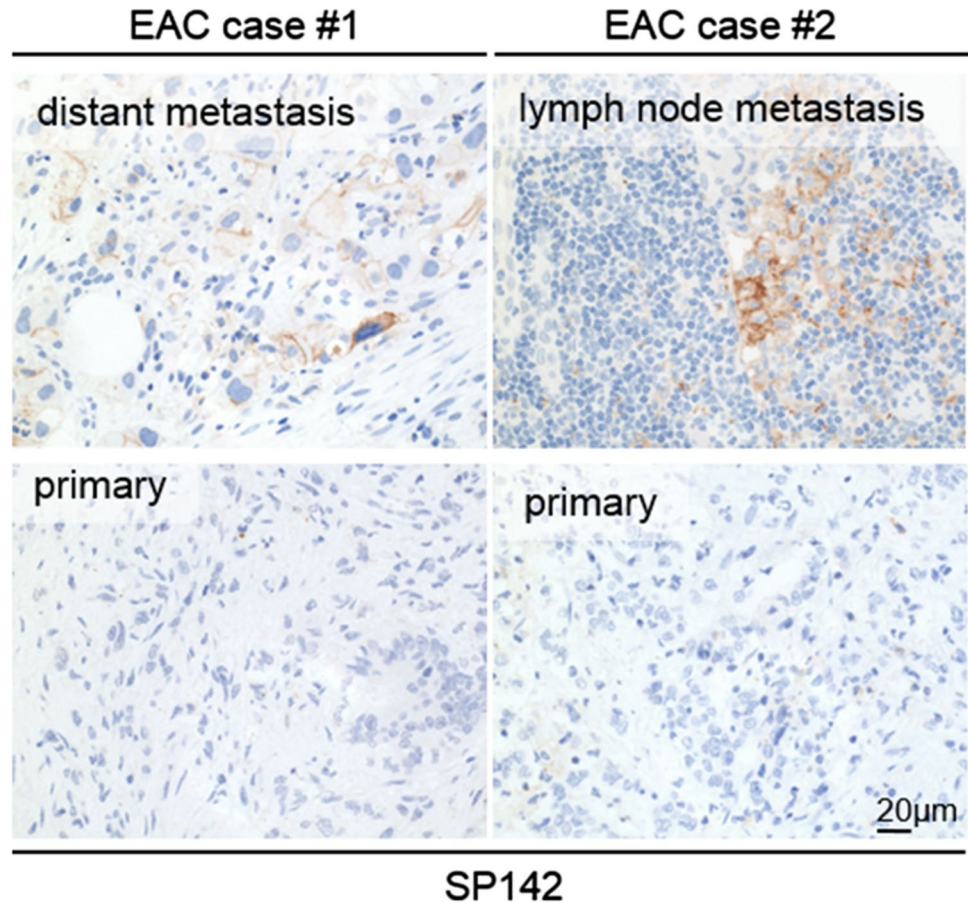
Table 2 Clinicopathological features and overall PD-L1 status

| Feature	Category	Total	PD-L1 TIA/overall Neg.	Pos.	p value	PD-L1 TC Neg.	Pos.	p value
pT category	pT1	33	21	12	0.008	33	0	0.106
	pT2	10	1	9		9	1	
	pT3	66	32	34		59	7	
	pT4	3	0	3		2	1	
Lymph node metastases	Absent	52	29	23	0.136	51	1	0.027
	Present	60	25	35		52	8	
Lymphatic invasion	Absent	33	18	15	0.386	32	1	0.208
	Present	79	36	43		71	8	
Blood vessel invasion	Absent	82	45	37	0.032	77	5	0.212
	Present	30	9	21		26	4	
Perineural invasion	Absent	66	33	33	0.651	63	3	0.104
	Present	46	21	25		40	6	
Distant metastases	Absent	107	52	55	1	98	9	0.5
	Present	5	2	3		5	0	
Tumor grade	G1	16	10	6	0.123	16	0	0.076
	G2	48	26	22		46	2	
	G3	48	18	30		41	7	
Lauren's type	Intestinal	74	37	37	0.598	72	2	0.004
	Non-intestinal	38	17	21		31	7	
Resection status	R0	105	49	56	0.259	96	9	0.419
	R1	7	5	2		7	0	
MMR status	Deficient	11	51	50	0.143	93	8	0.892
	Proficient	101	3	8		10	1	
CD3+ tumor center	Low	58	36	22	0.003	56	2	0.064
	High	54	18	36		47	7	
CD8+ tumor center	Low	59	33	26	0.092	54	5	0.857
	High	53	21	32		49	4	
Total		112	54	58		103	9	

analysis, with class III (PD-L1+/CD3+low) harboring the worst and class IV (PD-L1−/CD3+high) the best outcome (p = 0.047; Fig. 3). This prognostic stratification could be demonstrated for the combination PD-L1/CD3+ TIL, but not for PD-L1/CD8+ TIL (p = 0.161). Since the prognostic impact of CD3+ TIL alone in our case cohort had been shown before we analyzed the subgroups of high and low CD3+ TIL in relation to their PD-L1 status [14]. Although PD-L1 seemed to further stratify the patients with a worse prognostic impact in both subgroups, the difference was statistically not significant (p = 0.569 in low CD3+ TIL tumors and p = 0.252 in high CD3+ TIL tumors, see Supplementary figure 2). This comparable low prognostic effect of PD-L1 status also influenced the results of a multivariate analysis that included the combination PD-L1/CD3+ TIL and the most relevant prognostic factors in EAC as shown elsewhere [14]: Only pT category, pN category and tumor grading, but not PD-L1/CD3+ TIL, were independent prognostic parameters (Table 3). Interestingly,

PD-L1 expression in lymph node metastases was associated with a trend to better outcome (p = 0.06), similar to distant metastases (p = 0.198).

PD-L1 expression in gastric and colorectal adenocarcinomas

In order to set the staining results in correlation with other gastrointestinal cancers, especially with regard to staining intensities and focality, we applied PD-L1 immunohistochemistry on multi-tumor TMAs with various gastrointestinal adenocarcinomas, and a small number of non-small cell lung carcinomas. Similar to our observations on EAC, only 10/125 gastric carcinomas (8.0%) and 24/377 colon carcinomas (6.4%) showed membranous PD-L1 staining of TC. PD-L1 expression was detected to a higher degree in TAI in 159/502 cases (31.7%; Table 1, Supplementary figure 3). As for EAC, stainings for TC and TAI were rather weak and focal.
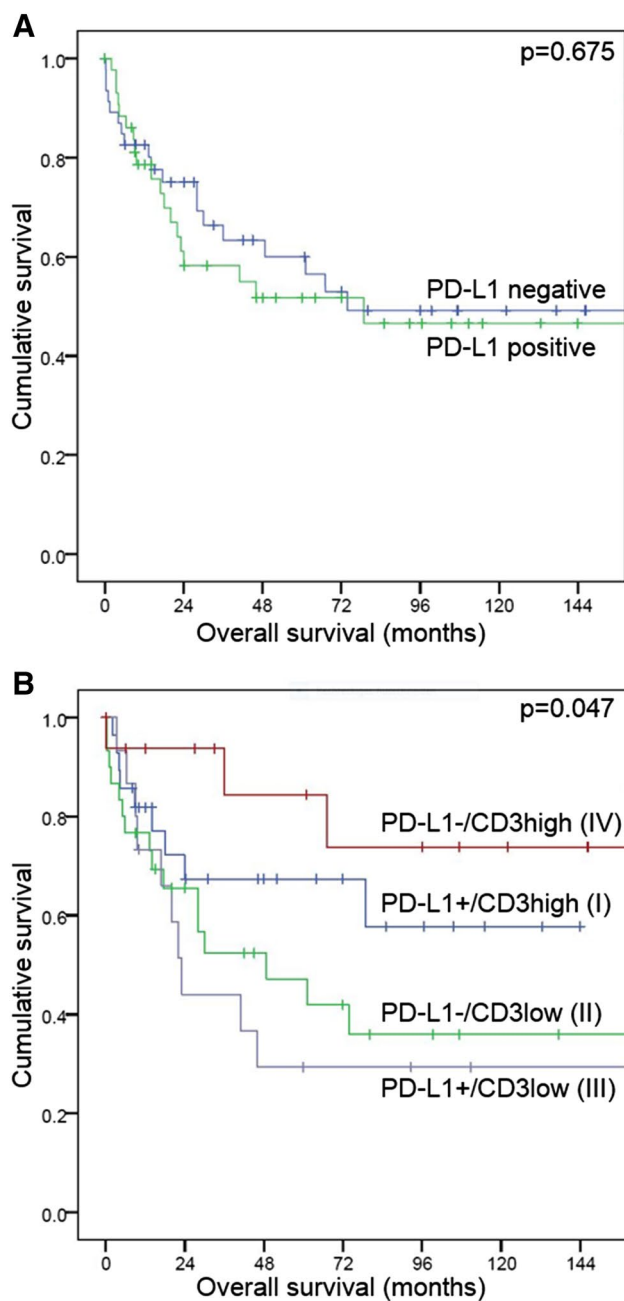
Fig. 3 Kaplan–Meier survival curves and log-rank tests illustrating the association of overall survival with overall PD-L1 status (**a**) and the overall PD-L1 status in relationship to the amount of CD3$^+$ tumor-infiltrating lymphocytes, separated into four classes (*class I* PD-L1$^+$/CD3high, *class II* PD-L1$^-$/CD3low, *class III* PD-L1$^+$/CD3low, *class IV* PD-L1$^-$/CD3high) (**b**)

Discussion

In this study, we investigated the expression patterns of PD-L1 in primary resected EAC and show that TC and/or TAI expression is found in the primary tumors and metastases, but does not necessarily correlate with each other.

We show that expression in the TC is rather low (8.0%), whereas expression in the TAI is higher (51.8%). In addition, our data demonstrate that overall PD-L1 expression in the primary tumor is not associated with overall survival. However, we show that PD-L1 expression is correlated with the number of CD3$^+$ TIL in the tumor center and that a combinational score of PD-L1 status in relationship to CD3$^+$ TIL correlates with patient survival.

In primary EAC, we detected a higher frequency of TC and TAI PD-L1 expression as compared to a recent TMA-based study relying on a different antibody clone (clone 405.9A11, Gordon Freeman's Lab, Dana-Farber Cancer Institute) observing 2% TC and 18% immune cell expression [8]. This could be due to the fact that our combination of antibody clone, dilution and protocol is more sensitive. In addition, our TMA consisted of a large number of cores per tumor, thus minimizing the chance of missing the sometimes patchy PD-L1 expression. This had been the case in the cited study, where a higher number of PD-L1-positive immune cells were found after analyzing a subset of cases with full slide sections. Furthermore, using a multi-tumor TMA we observed that the staining patterns for PD-L1 in EAC, especially with regard to the weak staining intensity, were comparable to other gastrointestinal adenocarcinomas, such as gastric or colon cancers.

Previous studies reported epithelial PD-L1 staining in EAC in up to 73% of tumors and as high as 89% for colorectal cancer or 50% for gastric adenocarcinomas, which is in contrast to our findings [7, 23–26]. This discrepancy may be due to differences in antibody sensitivity or the use of unspecific antibodies, with the lack of antibody binding sites in PD-L1 representing a general challenge in the generation of specific anti-PD-L1 antibodies or the use of different antibody dilutions accepting a higher background staining [4, 6, 15, 25]. The antibodies used in our study have been validated and compared to other commercially available antibodies by others, supporting the specificity of our PD-L1 expression analysis [15, 16, 27]. However, as the TMAs for gastric and colorectal adenocarcinoma that were used in our study contained only one core/tumor, our study might underestimate the amount of PD-L1 expression in comparison with studies relying on full slide sections or multi-core TMAs.

Due to the inconsistencies in PD-L1 expression testing and the low number of clinical trials focusing on gastrointestinal cancer, especially of the upper gastrointestinal tract, it is currently unclear whether there is a reliable relationship for gastrointestinal adenocarcinomas between PD-L1 expression and outcome after PD1/PD-L1-directed therapy [4]. Such a relationship exists for other tumor entities, such as melanoma, where increasing the cutoff value for positive PD-L1 expression results in better response rates [28]. Despite these uncertainties and the low PD-L1 expression

Table 3 Multivariate analysis of clinicopathological features and the PD-L1 to CD3$^+$ TIL ratio

Feature	HR	95.0% CI for HR		p value
		Lower	Upper	
pT category	1.727	1.044	2.857	0.033
pN category	1.459	1.015	2.098	0.041
Lymphatic invasion	0.817	0.293	2.276	0.699
Blood vessel invasion	0.646	0.244	1.711	0.379
Perineural invasion	0.751	0.294	1.915	0.549
Distant metastases	1.310	0.255	6.746	0.746
Tumor grade	2.028	1.013	4.061	0.046
Lauren's type	0.586	0.248	1.387	0.224
Resection status	0.920	0.252	3.355	0.899
PD-L1/CD3$^+$ TIL class	1.030	0.709	1.498	0.876

HR hazard ratio, *CI* confidence interval

in TC in our cohort, others have shown that gastrointestinal adenocarcinomas still benefit from PD1/PD-L1-directed therapy, which might be due to several reasons [29, 30]: First, our and other studies demonstrate that metastases may express PD-L1 in the presence of PD-L1-negative primary tumors, due to several possible mechanisms [31]. On the one hand, increases in mutational load during the metastatic course could drive constitutive PD-L1 expression. On the other hand, increased tumor immunogenicity due to a higher mutational burden as well as differences in the tumor microenvironment in the metastatic setting could lead to an adaptive immune resistance driven PD-L1 expression [32]. Second, positive responses to checkpoint inhibition have been shown in PD-L1-negative tumors [3]. Third, PD-L2, an alternative ligand of PD1, was reported to be preferentially expressed in EAC, thus indicating that PD1 receptor blockade could be beneficial in PD-L1-negative tumors [8].

Our descriptive results may also provide valuable information about the biological mechanisms of PD-L1 expression in EAC and other gastrointestinal carcinomas. PD-L1 expression in the tumor cells was associated with less tumor differentiation and non-intestinal histological type. This goes mostly in line with other studies in gastric and colon cancer [26, 33, 34]. Interestingly, we could not demonstrate an association between mismatch repair deficiency and PD-L1 status. Moreover, the association between EBV positivity and PD-L1 expression as described in gastric cancer does not play a role in EAC, as EBV is not involved in carcinogenesis of EAC [34, 35].

Despite the uncertainties toward the influence of overall PD-L1 status in EAC on PD1/PD-L1-directed therapy, our study suggests that patients can be grouped into four classes depending on the amount of CD3$^+$ TIL and their PD-L1 status and that within the groups with low or high amounts of CD3$^+$ TIL, overall PD-L1 expression is correlated with shorter survival. As most of the overall PD-L1 expression found in our cohort of EAC was scored for TAI, our data suggest that PD-L1 expression in the tumor microenvironment impacts survival. Although the overall impact of the host immune response, highlighted by a higher degree of CD3$^+$ TIL, may dominate over the impact of PD-L1 expression in our case cohort, the observation of the probable negative influence of PD-L1 expression further stratifying the subgroups of high and low CD3$^+$ TIL is in line with data from other solid carcinomas (breast invasive ductal carcinoma and renal cell carcinoma) and has raised the hypothesis that PD-L1 expression on TAI has an immunosuppressive/protumoral effect [36]. As more than half of EAC in our study featured PD-L1 positivity in TAI, these patients could benefit from PD-L1-directed therapy to counteract the putative anti-tumoral immunosuppressive effect of PD-L1 in the tumor microenvironment.

Finally, our study reiterates that the assessment of the PD-L1 status of tumors in the preoperative setting remains cumbersome, as we failed to show a correlation between the TMA and or full slide sections of EAC with the corresponding biopsies. PD-L1 expression in superficial small biopsies may be hampered not only by intratumoral heterogeneity, differential expression between primary tumor and metastases, but also by fixation artifacts or false-positive staining products due to vicinity to ulceration. In addition, the evaluation of the tumor microenvironment in the superficial biopsies is limited, as the inflammatory infiltrate of the tumor center end tumor front cannot be assessed. Furthermore, as PD-L1 expression is also involved in the immune response to non-tumoral conditions, a possible local effect on the regulation of PD-L1 apart from tumor intrinsic factors has to be discussed [37]. The above-mentioned factors have to be considered when determining the PD-L1 status in EAC. Finally, PD-L1 status might be influenced by neoadjuvant therapy, as chemotherapeutic agents have been shown to have a significant impact on PD-L1 expression levels ex vivo and during neoadjuvant chemotherapy for other solid tumors [38, 39].

Compliance with ethical standards

raria from Roche. There is no conflict of interest for the other authors of this study.

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