Original Article

PD-L1 Expression in Non Small Cell Lung Cancer and Prognosis

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Background: Prognostic significance of programmed death-ligand-1 (PD-L1) status in non-small cell lung carcinoma (NSCLC) is controversial.
Aims: In this study, we aimed to show the PD-L1 expression status in our patient population with NSCLC and its effect on prognosis and relationship with clinicopathologic data.
Study Design: The study was retrospective cross-sectional study and included 208 cases (107 had squamous cell carcinoma (SCC), 72 had adenocarcinoma (AC), and 29 had other types of NSCLC) who underwent surgery between 2001 and 2012.
Methods: PD-L1 (SP142 clone) was applied on the sections acquired from the microarray paraffin block with immunohistochemistry (IHC).
Results: Four different threshold values were used in our study and all clinical and pathologic parameters were compared with the PD-L1 results obtained from these threshold values. PD-L1 expression was observed in patients with NSCLS independent of the histological type or subtype.
Conclusion: In conclusion, PD-L1 expression is observed in NSCLC in parallel to the literature and it can be used as a negative prognostic indicator independent from the treatment method selected.
Keywords: PD-L1, lung, NSCLC, prognosis

Lung cancer is the most commonly observed cancer with the highest death rate in men and women amongst all cancers (1).
Treatment selection for patients with lung cancer is based on the cell type, molecular characteristics of the tumor, tumor stage, and the patient's performance status. Survival rates remain low although important developments have been made in recent years in multimodal treatments with the emergence of targeted therapies (2). New studies are being conducted on lung cancer related to tumor immunotherapy, which has been the subject of studies on many tumors in recent years (3). Impressive and long-term responses have started to be achieved with monoclonal antibodies, which target the checkpoints of the immune system (check-point inhibitors) (4). Effective protective immunity against cancer is dependent on the compatibility of the activity of cytotoxic T lymphocytes. T cell activity is related to the balance of negative and positive signals. CD28 and ICOS (Inducible T cell co-stimulator) are positive co-stimulators and they provide T cell activation and proliferation by binding to the ligand from the B7 family. On the other hand, there are negative regulatory molecules on the cell surface that inhibit T cell activation or prompt apoptosis.PD-L1 and PD-L2 are members of the B7 super family. These decrease the T cell activation by binding to the PD-1 receptors. Normally, it is an important step for the immune response to prevent tissue damage caused by the immune system induced by inflammation. However, PD-L1 and PD-L2 in the cancer cells suppress the T cell attack and provide its escape from the immune system. Thus the tumor cells effectively form an appropriate tumor microenvironment and continue proliferation. (5).

PD-L1 and PD-L2 expression has been shown in activated T cells, B cells, macrophages, and dendritic cells in addition to thymus endothelium, heart, and placenta. Besides these, PD-L1 expression was shown in lung, ovary, breast, head and neck carcinomas, and glioblastoma (6).

In many studies it was detected that prognosis is worse in tumors with PD-L1 expression compared to those without PD-L1 expression (7,8). Monoclonal antibodies that inhibit the PD-1/PD-L1 pathway abolish the inhibitory effect of the tumor cell on the immune system. Immunohistochemically, it was shown that the rate of response to the treatment with this monoclonal antibody in tumors with PD-L1 expression is higher. Besides its significance as a negative prognostic factor, demonstration of PD-L1 expression in the tumor is important for use as a predictive biomarker for therapies targeting this molecule (9).

The aim of the present study was to evaluate PD-L1 expression and its effect on prognosis and relationship with clinicopathologic data in patients with NSCLC.

MATERIAL AND METHOD

The study included 208 cases who were diagnosed with NSCLC and who underwent surgical resection January 1, 2000 and December 31, 2012. Surgical procedure and stage information were retrieved from the archive of the Department of Thoracic Surgery. Survival data were obtained by contacting 113 patients via telephone. Informed consent form was taken from patients.

Microarrays of 4 mm punches were formed from the blocks which best represent the tumor for the immunohistochemical study. The areas surrounded by inflammatory cell infiltration that best represent the tumor were selected.

Immunohistochemical staining was performe dusing an automatic device (BenchMark XT IHK/ ISH Staining Module, Ventana Medical Systems Ins.,Medical Systems,Tucson,AZ,USA). Sections were obtained from the 10% paraffin blocks. Deparaffinization was performed using solutions and they were rehydrated using a series of decreasing alcohol concentrations. They were kept in the 10 mmol/l buffered citrate solution for 30 minutes at 36°C. Afterwards, primary antibody PD-L1 (1/25 dilution, 32 minutes incubation, monoclonal, SP142 clone, Spring Bioscience (Spring) Roche/Genentech) antibody was applied to the slides.

The study was approved by the local ethics committee and was carried out according to the ethical principles of the best clinical applications of the Helsinki Declaration.

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Immunohistochemical Evaluation

Placenta was used as the control tissue. Tumor cells that show membranous staining were counted out of at least 100 tumor cells. Percentage rates were given. Staining intensity was scored as weak (+, 1), moderate (++, 2), and strong (+++, 3). Percentage (0-100%) was multiplied by staining intensity (1-2-3) to calculate the H score. H scores were between 0 and 300.

Four different cut-off values were used; 1: ≥ 1% (independent of intensity), 2: ≥ 5% (independent of intensity), 3: ≥5% moderate/strong staining (except for weak staining), 4: H score≥30 values were considered positive. Additionally, the staining in tumor infiltrating (TIL) or peritumoral inflammatory cells was noted.

Statistical Evaluation

SPSS 21.0 statistical software package was used in the statistical analysis. Fisher'schi-Square test and Pearson's chi-Square test were used in the comparison of categorical data and Mann-Whitney U test was used in the comparison of parameters between the groups. Kaplan Meier Analysis was used to examine the effect of PD-L1 positivity on mortality and survival. The results were evaluated at 95% confidence interval and $p<0.05$ significance level.

RESULTS

Of the cases, 88.5% were male (n=184) and 11.5% were female (n=24). The average age was 60 (range 39-80). Data regarding the clinical and pathological characteristics of the cases are presented in Table 1.

Survival data were available for 184 out of 208 cases. 31 (16, 8%) of these were not included in the survival analysis since they died within 2 months after the surgery. Of the remaining 153 (83.2%) patients, 37 (20%) died, 116 (63%) had survived. The median overall survival time was 30 months (3-142). The median survival time of the died cases was 24 months (3-76).The median survival time of the survivor cases was 33 months (4-142).

Immunohistochemical Findings

Membranous staining was observed in 34 cases (16.3%) in various rates and intensities. Staining was observed in 2 cases at 90%, 1 case at 80%, 1 case at 75%, 1 case at 70%, 2 cases at 60%, 1 case at 50%, 1 case at 40%, 2 cases at 30%, 3 cases at 20%, 8 cases at 10%, 2 cases at 5%, 1 case at 4%, 7 cases at 1%, and 2 cases at less than 1% (Figure 1).

Staining intensity varied between the examined areas of the tumor. In some cases, tumor cell in one area showed strong membranous staining, whereas the neighboring tumor cell showed weak positivity (Figures 2).

PD-L1 staining rates observed in the tumors are presented in Table 2.

Of cases with AC showing PD-L1 expression, solid pattern was predominant in 5 and lepidic pattern was predominant in 2 cases.

Four cases (1.9%) showed staining with PD-L1 antibody in Type 2 pneumocytes.
Various rates of staining were observed in the tumor-infiltrating lymphocytes (TILs) in 38 (18.27%) cases. While no staining was observed in the tumor cells of 11 (28.9%) of these cases, various rates of staining were observed in the tumor tissue in 27 cases (71.1%).
Positivity rates of cases showing PD-L1 expression varied when different cut-off values were used. Thirty-two cases (15.4%) were categorized as positive staining with a cut-off value of ≥ 1%, 24 (11.5%) cases were categorized as positive staining with a cut-off value of ≥5%, 19 cases (9.1%) were categorized as positive staining with a cut-off value of ≥5% with moderate or strong staining (, and 12 cases (5.8%) were categorized as positive staining when the H score was ≥ 30.
Using a cut-off level of ≥5%, the rate of positivity was 8.4% (9/107) in SCC, 8.3% (6/72) in AC, 25% (2/8) in LCNEC, 25% (1/4) in LCC, and 20% (1/5) in PC. The overall rate of positivity was 9.1%.
When the clinical parameters were evaluated according to different cut-off values, mild stromal response was higher than moderate and intense stromal response in cases with moderate and strong ≥5% staining (p=0.019,<0.05). Similarly, mild inflammation accompanying tumor was significantly higher compared to moderate and severe inflammation (p=0.041,<0.05).(Table 3)
PD-L1 positivity was significantly higher in cases with a tumor diameter more than 5 cm when compared to cases with a tumor diameter less than 5 cm (p=0.025,< 0.05).
When the comparison was made by setting the cut-off level to ≥5% and ≥ 1%, PD-L1 positivity was significantly higher in patients over the age of 60 years when compared to patients under the age 60 years (p=0.023 and p=0.015, respectively;<0.05).
Independent from the cut-off level used, there was a positive correlation between PD-L1 positivity in the TILs and PD-L1 positivity in the tumor cell (p=0.00,<0.05).
There was no relationship between PD-L1 expression and other clinicopathologic data (gender, diagnosis, tumor subtype, tumor grade, smoking, pathologic stage, clinical stage, pleura invasion, lymphatic, vascular, perineural invasion, lymph node metastasis status, necrosis).
When the cut-off level was set to ≥5% with moderate and strong staining, the median survival was 45 months (Standard Error (SE):13,752.Confidence interval (Cl):18,047-71,953) in PD-L1- positive cases.According to the Kaplan Meier analysis, the difference between in survival times between PD-L1-positive and PD-L1-negative cases was significant.Survival advantage conferred by PD-L1 negativity was demonstrated in statistical terms (log rank p=0,024;<0.05) (Graphic 1).

DISCUSSION

Immunotherapy has become the new treatment option in many malignancies. Observation of effective treatment responses particularly in malign melanoma and renal cell carcinoma has accelerated the studies regarding the applicability of immunotherapy in lung cancer (3,10).
With the discovery of PD-1/PD-L1 receptors which provide the interaction between the tumor cell and the immune system, studies have shown in full detail as how the tumor blocks immune system and progresses using this receptor signaling (11). The number of patients in whom treatment response was achieved using *check-point inhibitors* was found to be higher in patients with PD-L1 expression compared to those that show no PD-L1 expression (9).
PD-L1 is a transmembrane protein with a cytoplasmic tail . Membranous or cytoplasmic staining can be observed according to the binding point of the PD-L1 antibody. Cytoplasmic staining was shown with quantitative immunofluorescence staining and membranous or cytoplasmic staining can be observed in IMC according to the tumor type and the antibody used. The studies on localization of PD-L1 staining in tumors have shown predominant membranous/perinuclear staining in melanoma and membranous staining in the NSCLC (11,12,13,14). It is suggested that membranous staining pattern should be considered for the PD-L1 SP142 clone used in the present study (15). Cytoplasmic staining was not observed in our cases.
Different cut-off values were used in the literature to evaluate immunohistochemical PD-L1 expression. Only some studies have used the extensiveness of staining. There are also studies that used modified methods besides the H score where the extensiveness of staining and staining intensity are evaluated together (6,7,8,12,13,16,17,18).
We used four different cut-off values in our study (independent of intensity ≥1%, independent of intensity ≥5%, ≥5% moderate/strong staining, H score ≥30). We compared all clinical and pathologic parameters with the PD-L1 results we acquired with these cut-off values. Among the clinical data, we observed a difference in terms of age and survival, whereas no difference was observed in pathological data. In a meta-analysis conducted on this subject, it was stated that even 1% staining with PD-L1 antibody could be sufficient to have a predictive value. Some studies suggest that indicating the absence or presence of staining would suffice, as proposing different cut-off levels while using complex systems may cause intraobserver and interobserver variability (19).
Marti et al. (36) compared their results using different cut-off values. The cut-off values were ≥5%, ≥1% and>1 H score in respective order and the results show high compatibility with each other.

The rate of PD-L1 expression reported in the literature varies depending on the cut-off level used or localization of staining (cytoplasmic and/or membranous). This rate ranges from 7.4% to 72.7% (7,16,17,18).
In our study, staining in a single cell at any intensity was considered to be positive and 34 (16.3%) out of 208 cases were found to be PD-L1 positive. When the cut-off level was set to ≥5% moderate and strong staining, the rate of PD-L1 positivity was found to be 9.1%. When the survival analysis was carried out over this value, an inverse relationship was found between PD-L1 expression and survival.
Independent of its predictive value, some studies have suggested that PD-L1 expression has a prognostic significance. It is reported that prognosis is worse in patients with PD-L1 expression compared to those without PD-L1 expression and hence PD-L1 can be used as a negative prognostic factor (7,8,11,21). In our study, in parallel to the literature, when PD-L1 positivity was based on a cut-off value of ≥5%moderate and strong staining, survival time of PD-L1-positive patients was shorter than PDL-1-negative cases. There are also studies showing that there is no relationship between PD-L1 expression and prognosis, or reporting that patients with PD-L1 expression survive longer (13,16,22). A poor prognosis is expected when suppression of anti-tumoral response around the tumor by PD-L1 and its role in carcinogenesis are considered (23).
Comparing small biopsy and resection materials, Kitazono et al. (24) showed that PD-L1 results in both materials show 92% concordance. In our study, microarray paraffin block was prepared by choosing 4 mm tumor region from resection materials. While evaluating PD-L1 expression in the tumor cells, it was observed that the extent and intensity of staining varied across the areas. When the heterogeneous structure of NSCLC is considered, immunohistochemical evaluation in small biopsy samples may not reflect the entire tumor tissue.
Many studies evaluating the relationship of PD-L1 expression with age and gender have found no significant difference (7,17, 20). However, PD-L1 positivity is significantly higher in women in the studies by D'Incecco et al. (6) and Azuma et al. (11) and in young patients in the study by Cooper et al. (16). In our study, when the cut-off level was set to ≥ 5% or ≥ 1%, PD-L1 expression was higher in patients aged over 60 years. There was no relationship between gender and PD-L1 expression. We believe that the relationship between age and PD-L1 expression may be due to the increase in both tumor burden and the mutation burden with age.
PD-L1 expression status can vary according to the tumor type. Schmidt et al. (18) have found higher PD-L1 expression in 321 cases with NSCLC and SCC compared to other types. In their studies, Mu et al. (8) and Konishi et al. (13) detected higher PD-L1 expression in AC compared to that in SCC. In our study, PD-L1 expression at any intensity was higher in SCC (19.6%, 21/107) compared to that in AC (12.5%, 9/72), but there was no statistically significant difference. Similar results exist in literature (16).
There are studies that have addressed histological pattern and invasion status together with PD-L1 expressions in AC. In their study, Zhang et al. (7) observed higher PD-L1 expression rates in solid AC compared to those in minimally invasive adenocarcinoma (MIA) and adenocarcinoma in situ (AIS), and it was interpreted that PD-L1 expression can increase depending on the invasion status and aggressiveness of the tumor. Microinvasive adenocarcinoma or adenocarcinoma in situ were not included in our study and staining was detected both in cases with solid AC and lepidic pattern adenocarcinoma. There was no relationship between PD-L1 expression and AC patterns.
Sarcomatoid carcinomas are poorly differentiated tumors compared to other NSCLCs and have a poor clinical course. In the study by Velcheti et al. (25), the rate of PD-L1 expression was 69.2% in 13 cases with sarcomatoid carcinoma among 458 cases with NSCLC, and this rate was found to be 27.4% in other histological subtypes. Similarly, in the study by Kim et al. (26), the rate of PD-L1 expression in 41 cases with PC was 90% (37/41) and more positivity was reported in the sarcomatoid regions compared to regions of differentiated carcinoma. In our study, there were a few patients with PC and the rate of PC-L1 expression was 20% (1/5). This rate is higher than the overall rate of positivity. Moreover, it was observed that this case had a diffuse and intense staining. In parallel to this, this patient also showed staining on the TILs. In the literature, high rate of PD-L1 positivity in PC or carcinosarcoma has been explained low differentiation level in the tumor and accompanying intense inflammation. Inflammation inside and around the tumor is related to negative prognosis in sarcomatoid carcinoma. It is thought that this can also be related to the mechanisms suppressing the immune system (PD-1, PD-L1, cytokine, Treg cell, T cell co-inhibitors) (26,26).
Schultheis et al. (27) studied PD-L1 expression using two different clones in 94 cases with small cell carcinoma and observed no staining in the tumor cells. Cases with SCLC were not included in the present study and staining was observed in 25% (2/8) of cases with LCNEC. Although the number of cases is low, PD-L1 expression was detected in 25% (1/4) of LCC cases, a finding consistent with the literature (16).
Other cells accompanying the tumor were also evaluated in terms of PD-L1 expression. While staining is observed with PD-1 on TILs in many studies, different results exist with regard to PD-L1 staining (18,27,28). In two different studies, where the PD-L1 expression in the tumor tissue was found to be 52% and 72%, staining rates in the parenchyma were 4.8% and 9.3%, respectively (8,21). Chen et al. (21), evaluated PD-L1 expression in 120 cases with NSCLC and 10 benign control tissues, and they observed PD-L1 expression in 57.5% of the tumors and no staining in the benign control tissues. In the study by Gettinger et al. (29), PD-L1 staining was observed on the lymphocytes and scoring was performed according to the percentage of staining. As a result of

this study, where a relationship was detected with treatment response, it is suggested that TILs should also be evaluated along with the tumor cells.

In our study, PD-L1 staining on TILs was observed in 38 cases (18.3%) at variable rates. PD-L1 positivity in the tumor cells was higher in cases with PD-L1 positivity on the TILs. In our study without using TIL scoring performed in the literature, PD-L1 positivity on the TILs was observed to be parallel to the positivity in the tumor.

In the evaluation of the relationship between PD-L1 expression and clinical and pathological data, Schmidt et al. (18) observed higher rate of PD-L1 expression in cases receiving adjuvant treatment and in those with a higher tumor size and lymph node metastasis. In our study, PD-L1 positivity was significantly higher in the cases with a tumor diameter larger than 5 cm.

In the studies by Yang et al. (12) and Grosso et al. (30) a positive relationship was detected between PD-L1 expression in tumor and surrounding inflammatory response. In our study, a negative correlation was observed between the peritumoral stromal and inflammatory response and PD-L1 expression. This inconsistent result with the literature can be explained by the fact that PD-L1 expression changes in response to different stimuli and there is also a limited number of studies on this subject.

In the studies conducted with PD-L1 antibodies, the results can vary depending on many non-standardized factors such as different antibody and clone use, different cut-off values, localization of the staining in the cell, disease stage, previous treatments, use of archived or fresh tissue, and working on primary or metastatic tissue. What are accepted in the literature with the current data are that PD-L1 expression is a negative prognostic factor and that PD-L1 expression in the tumor of the patient can be used as a biomarker in the selection of anti-PD-1/PD-L1 antibody (check-point inhibitor) treatment. Common opinion is that treatment response is higher and disease-free survival is longer in patients with PD-L1-positive tumors at any intensity (3). Clinical response was achieved also in PD-L1-negative cases, although the rate of response was lower (9). There is a need for a biomarker that would allow predicting the patients who would achieve better response from the treatment. Immunohistochemical evaluation of PD-L1 receptors is gaining a ground as a biomarker that can be used for this purpose.

In conclusion, immunotherapy appears as a new treatment option in NSCLC to increase survival and support with available treatment methods.

In our study, results on PD-L1 expression and its relationship with survival in NSCLC were in parallel to the literature. Moreover, it can be used as a negative prognostic factor independent from the selected treatment option.

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Table 1: Clinical and Pathological Characteristics

		CASE (n=208)
Age	≤60	106 (51%)
	>60	102 (49%)
Gender	Male	184 (88.5%)
	Female	24 (11.5%)
Smoking Status	Nonsmoker	11 (5.3%)
	Smoker	197 (94.7%)
Pathological Diagnosis	SCC	107 (51.4%)
	AC	72 (34.6%)
	LCC	4 (2%)
	ASC	8 (3.8%)
	LCNEC	8 (3.8%)
	PC	5 (2.4%)
	MEC	4 (2%)
Comorbid Inflammation	Mild	45 (21.6%)
	Medium	122 (58.7%)
	Intense	41 (19.7%)
Stromal response	Mild	54 (26%)
	Medium	99 (47.6%)
	Intense	55 (26.4%)
Tumor stage	1A	29 (13.9%)
	1B	39 (18.8%)
	2A	50 (24%)
	2B	52 (25%)
	3A	38 (18.3%)
Tumor grade	1	3 (1.4%)
	2	63 (30.3%)
	3	142 (68.3%)

Lymph node metastasis	No	118 (56.7%)
	Yes	90 (43.3%)
Tumor diameter	≤5 cm	136 (65.4%)
	>5 cm	72 (34.6%)
Perineural invasion	No	140 (67.3%)
	Yes	66 (32.7%)
Lymphatic invasion	No	38 (18.3%)
	Yes	170 (81.7%)
Vascular invasion	No	128 (61.5%)
	Yes	80 (38.5%)
Necrosis	No	36 (17.3%)
	1-20	66 (31.7%)
	21-40	79 (38%)
	41-60	22 (10.6%)
	61-100	5 (2.4%)
Mortality (n=184)	Survived	116 (63%)
	Not survived	68 (37%)
SCC,Squamous cell carcinoma; AC,Adenocarcinoma; LCC, Large-cell carcinoma; ASC, Adenosquamous carcinoma; LCNEC, Large-cell neuroendocrine carcinoma; PC, Pleomorphic carcinoma; MEC, Mucoepidermoid carcinoma		

Table 2: Distribution of PD-L1 staining ratios according to diagnoses

	SCC	**AC**	**LCC**	**ASC**	**LCNEC**	**PC**	**MEC**	**Total**
NEGATIVE	86	63	3	8	6	4	4	174
	80.4%	87.5%	75.0%	100.0%	75.0%	80.0%	100.0%	83.7%
POSITIVE	21	9	1	0	2	1	0	34
	19.60%	12.5%	25.0%	0.0%	25.0%	20.0%	0.0%	16.3%
SCC,Squamous cell carcinoma; AC,Adenocarcinoma; LCC, Large-cell carcinoma; ASC, Adenosquamous carcinoma; LCNEC, Large-cell neuroendocrine carcinoma; PC, Pleomorphic carcinoma; MEC, Mucoepidermoid carcinoma								

Table 3:Some of the clinicopathologic parameters are associated with PD-L1 relationship

		Negative	Positive	P value*
AGE	≤60	99	7	
		93.40%	6.60%	p=0.015
	>60	85	17	
		83.30%	16.70%	
DIAMETER	≤5 cm	128	8	
		94.10%	5.90%	p=0.025
	>5 cm	61	11	
		84.70%	15.30%	
STROMAL RESPONSE	MILD	44	10	
		81.50%	18.50%	p=0.019
	MODERATE	94	5	
		94.90%	5.10%	
	INTENSE	51	4	
		92.70%	7.30%	
INFLAMMATION	MILD	37	8	
		82.20%	17.80%	p=0.041
	MODERATE	112	10	
		91.80%	8.20%	
	INTENSE	40	1	
		97.60%	2.40%	

Fisher's exact test $p<0.05$

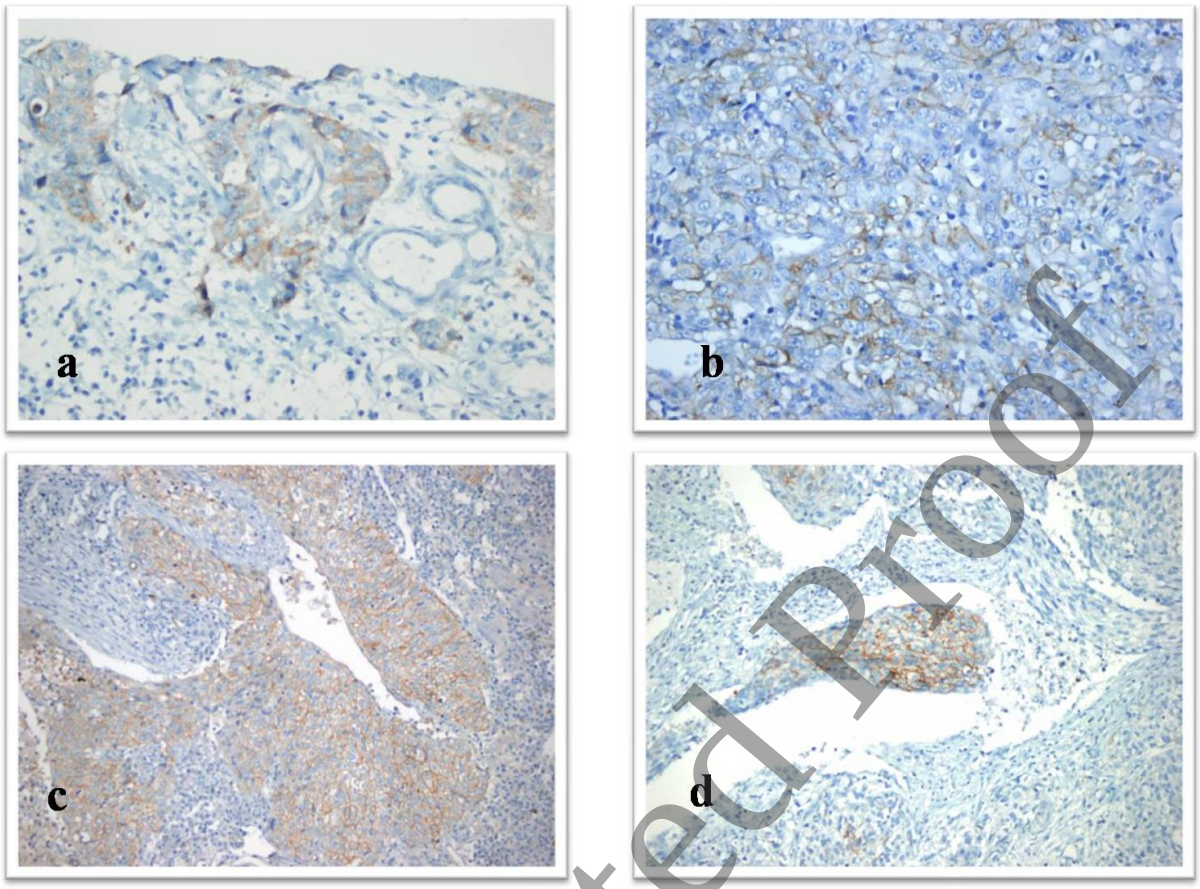


Figure 1. IHK PD-L1 antibody staining at different rates and intensity in tumor cells. a) 20% wear staining(IHC,X400) b)75% moderate staining(IHC,X400) c) 90% strong stainig (IHC,X100) d)30% strong staining(IHC,X200)

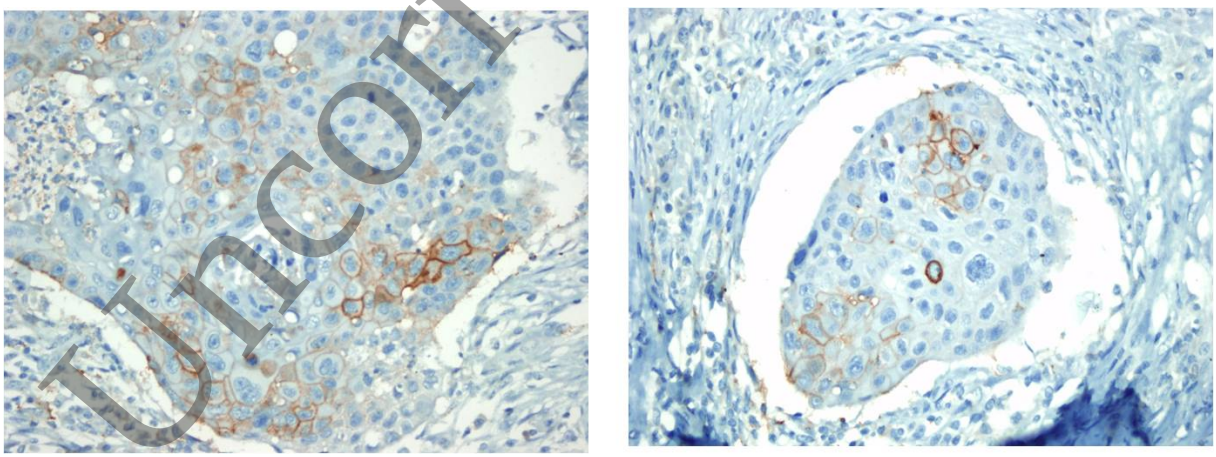
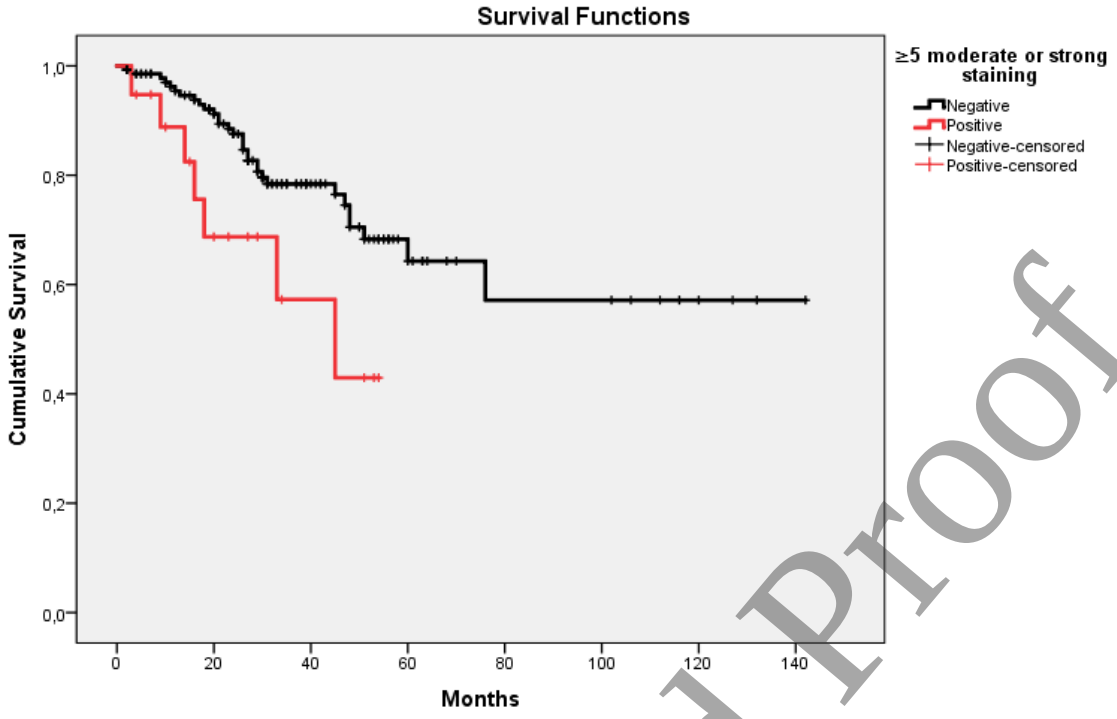


Figure 2. Heterogeneous PD-L1 antibody staining in the same tumor (IHC,X400)



Graphic 1.