ARAŞTIRMA MAKALESİ / *RESEARCH ARTICLE*

Analysis of PD-L1 Immunohistochemistry Results for Different Sampling Procedures of Non-small Cell Lung Carcinoma. Can We Use Cell Blocks for Evaluation of PD-L1?

Küçük hücre Dışı Akciğer Karsinomlarında Tümörün Farklı Örnekleme Prosedürlerine Göre PD-L1 Immünohistokimyasal Sonuçlarının Değerlendirilmesi. Hücre Blokları PD-L1 Değerlendirilmesinde Kullanılabilir mi?

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ABSTRACT

Aim: *Current guidelines recommend evaluating the expression of PD-L1 immunohistochemically in most tumors, especially lung cancer. However, it is not easy to evaluate the immunohistochemical staining of PD-L1 because of the tumor heterogeneity. Therefore, we aim to evaluate the different patterns of PD-L1 in the lung's non-small cell carcinoma and the relationship with clinicopathological features. We also wanted to see if cell blocks obtained from cytology materials can be an alternative for PD-L1 immunohistochemistry.*

Material and Method: *We retrospectively analyzed the immunohistochemical patterns of PD-L1 performed between 2018 and 2019. Biopsy/aspiration procedure of tumors, tumor type, patient's age, and gender were reviewed. Positive tumor cells (percentage) were categorized according to density and distribution as negative (<1%), low expression (1–49%), and high expression (>50%).*

Results: *Material adequacy was found to be satisfactory in evaluating PD-L1 in the cases of cell blocks. Positive staining with PD-L1 was detected even with a small number of tumor cells in the cell block. For optimal evaluation of PD-L1 expression in the cell block, the tumor cell ratio of 10% is significant (p=0.002). The cases with negative PD-L1 expression mostly belonged to small biopsy samples (48.3%). However, the difference in PD-L1 expression in histological and cytological samples was insignificant (p=0.79). Besides this, expression of PD-L1 was negative in almost half of the cases (48.3%) diagnosed with squamous cell carcinoma. In adenocarcinoma cases, the PD-L1 expression rate was between 1–49 % in more than half (51.8%) of them. The difference in histological subtype was not significant in PD-L1 staining (p=0.009).*

Conclusion: *In conclusion, we can use cell blocks for immunohistochemical evaluation of PD-L1 expression. Analysis of PD-L1 staining in cytological and histological tissue samples may be a guide for other studies.*

Key words: *PD-L1; tumor; lung carcinoma; cell block*

ÖZET

Amaç: *Güncel kılavuzlar, akciğer kanserleri başta olmak üzere çoğu tümörde PD-L1 ekspresyonunun immünohistokimyasal olarak değerlendirilmesini önermektedir. Ancak, tümör heterojenitesi nedeniyle PD-L1'in değerlendirilmesi kolay değildir. Çalışmamız, akciğerin küçük hücre dışı karsinomlarda PD-L1'in farklı boyanma paternlerini ve bu ekspresyonun klinikopatolojik özelliklerle ilişkisini değerlendirmeyi amaçlamaktadır.*

Materyal ve Metot: *Bu amaçla 2018–2019 yıllarında immünohistokimyasal olarak çalışılan PD-L1'in ekspresyon paternleri retrospektif olarak analiz edildi. Yapılan işlemin prosedürü (biyopsi/aspirasyon), tümör tipi, hastanın yaşı, cinsiyeti gözden geçirildi. PD-L1 pozitif tümör hücreleri, yoğunluk ve dağılım baz alınarak yüzdelerine göre negatif (<%1), düşük ekspresyon (%1–49) ve yüksek ekspresyon (>%50) olarak kategorize edildi.*

Bulgular: *Çalışmamızda, hücre bloğunda PD-L1'in değerlendirmesi için gereken hücresellik tatmin edici bulundu. Hücre bloğunda az sayıda hücre bile olsa PD-L1 ile pozitif boyama elde edilebildi. Hücre bloğunda PD-L1 ekspresyonunun optimal değerlendirilmesi için tümör hücre oranı %10 sınırı anlamlı bulundu (p=0,002). PD-L1 ekspresyonu negatif olan olgular çoğunlukla küçük biyopsi örneklerine (%48,3) aitti. Ancak histolojik ve sitolojik örneklerdeki PD-L1 ekspresyonu farkı anlamlı değildi (p=0,79). Bunun yanında skuamöz hücreli karsinom tanısı alan olguların neredeyse yarısında (%48,3) PD-L1 ekspresyonu negatif olarak saptandı. Adenokarsinom olgularının yarısından fazlasında (%51,8) PD-L1 ekspresyon oranı %1–49 arasındaydı. Histolojik alt tip PD-L1 boyanmasında anlamlı farklılık göstermedi (p=0,009).*

Sonuç: *Analizimize göre, PD-L1 ekspresyonunun immünohistokimyasal olarak değerlendirilmesinde hücre blokları kullanılabilir. Hem sitolojik ve hem de histolojik doku örneklerinde PD-L1 ekspresyonlarının analizi diğer çalışmalar için yol gösterici olabilir.*

Anahtar kelimeler: *PD-L1; tümör; akciğer kanseri; hücre bloğu*

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Introduction

The pathway of PD-1/PD-L1 plays an important role in immune regulation by balancing T cell activation, tolerance and immune-mediated tissue damage. Binding PD-L1 to PD-1 causes the formation of PD-1/ TCR. This formation suppresses T cell activation by increasing apoptosis, inhibing proliferation and decreasing cytokine secretion[1]. The formation of this complex causes depletion of effector T cells and thus tumor cells escape from the immune system. PD-L1 also transmit the anti-apoptotic signal to tumor cells. So tumor cells can be protected from apoptosis. Also according to Shi et al., PD-L1 may act as an oncogenic molecule in colorectal carcinogenesis[2]. Mechanism of PD-1/ PD-L1 binding on the tumor development has been illustrated in Fig. 1.

PD-L1 is not expressed on the surfaces of normal tissues. Therefore, antibodies targeting PD-1 and PDL-1 is novel therapeutic option in cancer treatment. Monoclonal antibodies targeting the PD-L1 or PD-1 receptor prevent the suppressive effects of this pathway on T cells, thereby increasing T cell function. Clinical studies have demonstrated that these antibodies leads to impressive outcomes in many cancer types such as renal cell carcinoma, bladder, melanoma and non-small cell lung cancers[3,4].

Besides oncogenic potential, PD-L1 has different prognostic effects in different malignancies. It is associated with poor prognosis in cancers of stomach, esophagus, pancreas, ovarian, bladder, kidney and liver. Rarely, the high rate of PD-L1 expression in tumor cells is associated with a better clinical course. Patients with breast cancer and Merkel cell carcinoma expressing PD-L1 have longer disease-free survival times. The prognostic value of PD-L1 for lung cancer, colorectal carcinoma and melanoma is controversial[1].

Evaluation of tumor PD-L1 expression (PD-L1 exp.) has become a predictive biomarker for selecting patients for immunotherapy. However, it is difficult to evaluation of PD-L1 by immunohistochemistry. PD-L1 shows heterogeneity within the tumor. This makes immunohistochemical evaluation difficult. Therefore, evaluation of PD-L1 exp. can be much more difficult in small tissue samples. This situation causes problems especially in lung cancers which have typically limited samples. Current guidelines recommend determining

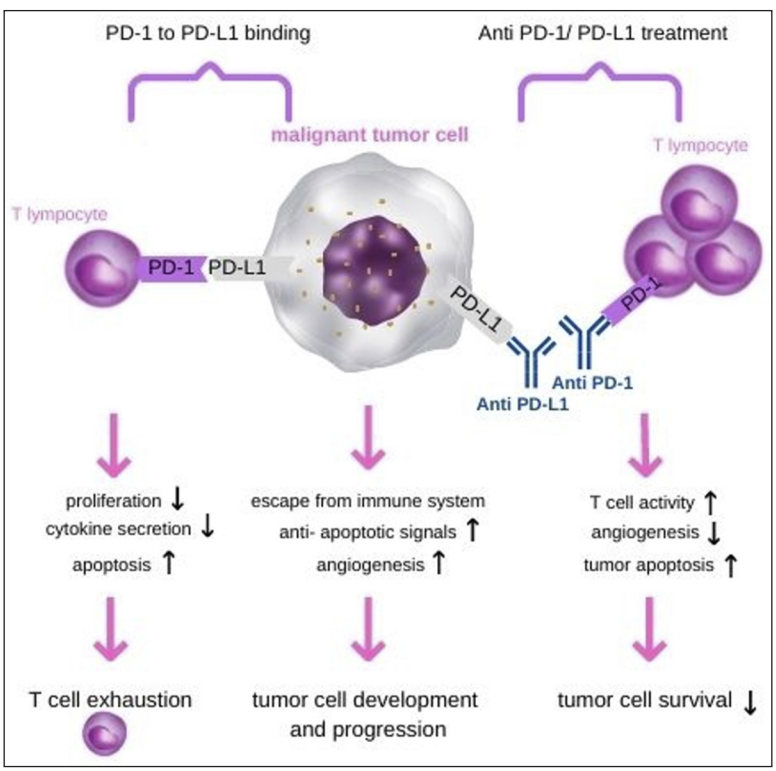

Figure 1. *Mechanism of PD-1/ PD-L1 pathway and anti PD-1/PD-L1 treatment. (Image was prepared with Canva for Education Program by Esin Kaymaz)*

PD-L1 exp. levels with immunohistochemistry in advanced lung cancers[5]. The diagnosis of lung tumors is mostly based on endobronchial / transbronchial aspiration biopsies or pleural effusion materials. Cell blocks obtained from cytological materials has recently been proposed as an alternative way of evaluating PD-L1 exp.

Current study aims to identify clinicopathologic features and expression patterns of PD-L1 in primer and metastatic non-small cell lung tumors. Also we investigated the usability of the cell block for PD-L1 immunohistochemistry.

Materials and Methods

We retrospectively reviewed the data of PD-L1 immunohistochemical analysis of patients between 2018 and 2019 in the Pathology Laboratory. PD-L1 immunohistochemical stain (Dako Clone 22 c3) were applied to tissue samples or cell blocks. The procedure of sampling (biopsy / aspiration), tumor type, gender and also the percentages tumor cells with PD-L1 positivity which were categorized according to density and distribution were recorded. A total of 89 cases between these years have been documented, regardless tumor localization. Here, we wanted to see the distribution of PD-L1 studied cases. But the evaluation and statistical analysis were performed just for tumors localized to lung.

Formalin fixed paraffin embedded tissue blocks have been prepared for all cases. 4 micrometer thick sections were cut and stained with Hematoxylin and Eosin for evaluation. Also cell block was prepared from effusion or aspiration samples. Effusion samples were centrifuged, and the supernatant was tossed away. The sediment was placed on a slide and mixed with 4–5 drops of plasma and 4–5 drops of thromboplastin. The sample was centrifuged again and topped with 10% formalin. The clumped sample is then placed into a cassette, processed through the routine processing protocol. For consultation cases, each block were analyzed for if they have sufficient tumor cell.

PD-L1 was performed if the sample had more than 100 tumor cells. In cases with sufficient tumor immunohistochemical staining were performed with PD-L1 antibody, Dako Clone 22 c3 in dense 1/50.

The percentage of positive staining was determined by an experienced pathologist. Membrane staining (local/global) at any intensity greater than background staining was evaluated, and only viable tumor cells were scored. For non-small cell lung carcinoma, PD-L1 expression was divided into three categories according to the percentage of staining: <1% (negative), 1–49% (low expression) and ≥50% (high expression)[6]. An example for each category has demonstrated in Fig. 2.

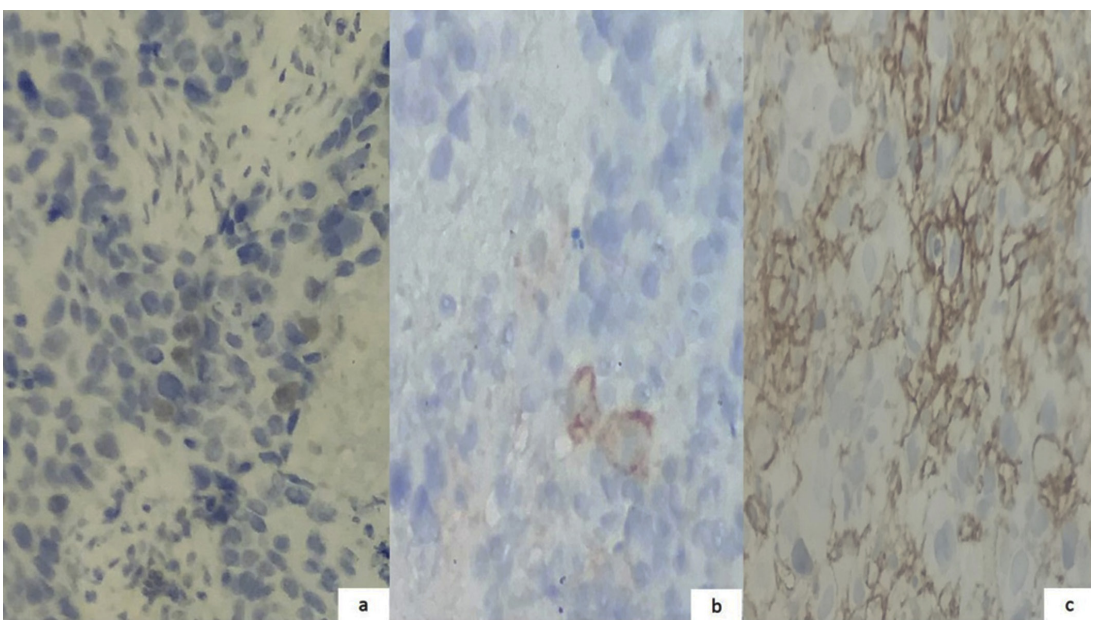

Figure 2. *PD-L1 expression according to percentage of staining: a) <1% (negative) (×400), b) 1–49% (low expression) (×400) and c) ≥50% (high expression) (×400) (Dako Clone 22 c3) (1/50 dilution).*

For the statistical evaluation we considered PD-L1 expression in two groups as negative (<1%) and positive (≥1%). In other word we grouped score 2 and 3 as just positive.

Evaluating the difference of the present/absent or the weak/intense staining of PD-L1 among specimen types was one of the main points we focused on in our study. For statistical analysis, we classified the specimen types into two groups as cytology materials (cell block) and histology materials (biopsy, resection).

We used JASP Team (2022) (Version 0.16.1) program for statistical evaluation. Categorical variables were compared using Chi-Square test. Duo to the small number of cases, comparison between groups were made using Fisher's exact analysis. P value of less than 0.05 was considered statistically significant for all tests.

Results

There were 89 cases in which PD-L1 immunohistochemical staining was studied between 2018 and 2019 in our department.

While 83 of these cases were lung tumors, 2 of them were colon, 2 of them among stomach, 1 of them was belonging to breast and 1 of them to the nasopharynx. Colon and stomach samples were in the form of small biopsies and the pathological diagnosis of them were adenocarcinoma. The diagnosis of the mastectomy material and the biopsy of nasopharynx were as follows; invasive breast carcinoma and malignant melanoma. As can be seen, most of the cases studied with PD-L1 belong to the lung.

We evaluated the tumors of lung. Among them 18 were female and 70 were male. Of the 83 cases belonging to lung, 60 were tissue samples and 23 were cell blocks prepared from aspiration / pleural effusion. Of the 60 tissue samples, 27 were in the form of wedge/segmental resection or lobectomy, and 33 were transbronchial biopsies, transbronchial or small biopsies of metastatic lesion. Regardless of the sampling method, it was determined that 29 cases were reported as squamous cell carcinoma and 54 cases as adenocarcinoma. The majority of squamous cell carcinomas showed moderate differentiation. Acinar pattern was dominant in samples diagnosed with adenocarcinoma. Also 76 of the cases were diagnosed with lung samples, while 7 were samples from the brain, lymph node or bone tissues metastases of lung cancer. However, the location of the metastatic tumor was not of significance for the study. The main thing here was that the tumor was of lung origin.

We evaluated PD-L1 exp. in 3 categories. In 29 of the cases diagnosed with non-small cell lung cancer, either no or <1% staining was observed with PD-L1. These cases were evaluated as negative by PD-L1. Staining was low (1–49%) with PD-L1 in 41 cases while it was high in 13 cases. In Fig. 3a, a squamous cell carcinoma with score 3 of PD-L1 (80% percentage) is demonstrated. In Fig. 3b, we can see a strong expression pattern in an adenocarcinoma.

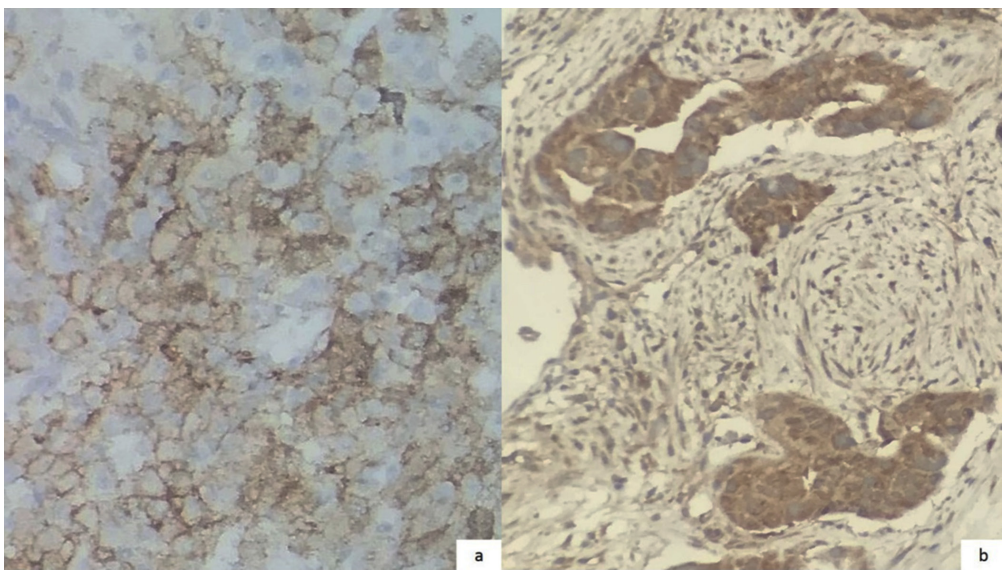

Figure 3. *a) Squamous cell carcinoma of lung with 80% PD-L1 staining (×200) b) PD-L1 staining strong in an adenocarcinoma with score 3 (×200) (Dako Clone 22 c3) (1/50 dilution).*

Evaluation results of PD-L1 exp. according to patient gender, primary or metastatic tumor and the subtype of tumor diagnosis are summarized in Table 1. PD-L1 exp. was negative in almost half of the cases (48.3%) diagnosed with squamous cell carcinoma. In the cases of adenocarcinoma PD-L1 exp. rate was between 1–49 % in more than half (51.8%) of them.

In this study, we performed a statistical analysis of PD-L1 expression in primary and metastatic tumors of the lung, excluding 6 tumors localized in other organs. We evaluated PD-L1 expression statistically between primary and metastatic tumors and also between two histological subtypes (squamous cell carcinoma and adenocarcinoma).

PD-L1 positivity or negativity was not statistically significant between primary and metastatic tumors (p=1). Same way it was not significant between squamous cell carcinoma and adenocarcinoma (p=0, 09). For histological subtypes we also evaluated the difference of low or high expression. The p value was determined as 0.76 for this analysis. So we can say that different histological subtypes did not affect the expression density.

Sampling type and PD-L1 exp. results in cases diagnosed with non-small cell lung carcinoma are summarized in Table 2. The case distributions were similar, if not equal. Also low and high PD-L1 rates were similar in different sampling procedures. When we compared PD-L1 expression as present/absent or low/strong for cytology and histology samples, no significant difference was found between the groups (p=0.79, p=0.74 respectively). We subdivided the histology materials into two main groups as small biopsy materials and large resection materials. We have analyzed that the cases with negative PD-L1 exp. mostly belonged to small biopsy samples (48.3%). But it was not significant with statistically when we compared these groups with PD-L1 expression status. We determined that small or large material does not affect the positivity or density of PD-L1. (p=0.42, p=1).

Material adequacy was found to be satisfactory in the cases of cell blocks obtained from the fluid. Sufficient numbers of tumor cells were observed in the samples to assess PD-L1 expression. The tumor cell rate varied between 5 and 80% in these cases. Positive staining with PD-L1 was detected even with a small number of tumor cells in the cell block (Fig. 4a, b). Figure 4c belongs to an adenocarcinoma of lung in cell block with score 3 of PD-L1. We have found similar proportions of tumor cells in small biopsy samples.

Table 1. *PDL-1 expression and clinicopathological parameters*

	Tumor proportion score of PD-L1					
Specimen type	<1%, n (%) (negative)	1–49%, n (%)	≥50%, n (%)	≥1%, n (%) (positive)	Total (n)	P*
Cell block of pleural or bronchial fluid	7 (30.4)	12 (52.2)	4 (17.4)	16 (69.6)	23	0.79
Histology sample	22 (36.7)	29 (48.3)	9 (15)	38 (63.3)	60	
– Large biopsy (resection)	8 (29.6)	15 (55.6)	4 (14.8)	19 (70.4)	27	0.42
– Small biopsy	14 (42.4)	14 (42.4)	5 (15.2)	19 (57.6)	33	

* P value of less than 0.05 was considered significant

Table 2. *PDL-1 expression in cell blocks and histology samples that belong to non-small cell lung carcinoma*

	Tumor proportion score of PD-L1					
Parameters	<1%, n (%) (negative)	1–49%, n (%)	≥50%, n (%)	≥1%, n (%) (positive)	Total (n)	P*
Diagnosis						
SCC	14 (48.3)	10 (34.5)	5 (17.2)	15 (51.7)	29	0.09
Adenocarcinoma	15 (27.8)	31 (57.4)	8 (14.8)	39 (72.3)	54	
Sex						
Male	23 (35.4)	32 (49.2)	10 (15.4)	42 (64,6)	65	1
Female	6 (33.3)	9 (50)	3 (16.7)	12 (66,7)	18	
Origin						
Primary	27 (35.5)	38 (50)	11 (14.5)	49 (54.5)	76	1
Metastatic	2 (28.6)	3 (42.8)	2 (28.6)	5 (71.4)	7	
Total	29 (34.9)	41 (49.4)	13 (15.7)	54 (65.1)	83	

* P value of less than 0.05 was considered significant

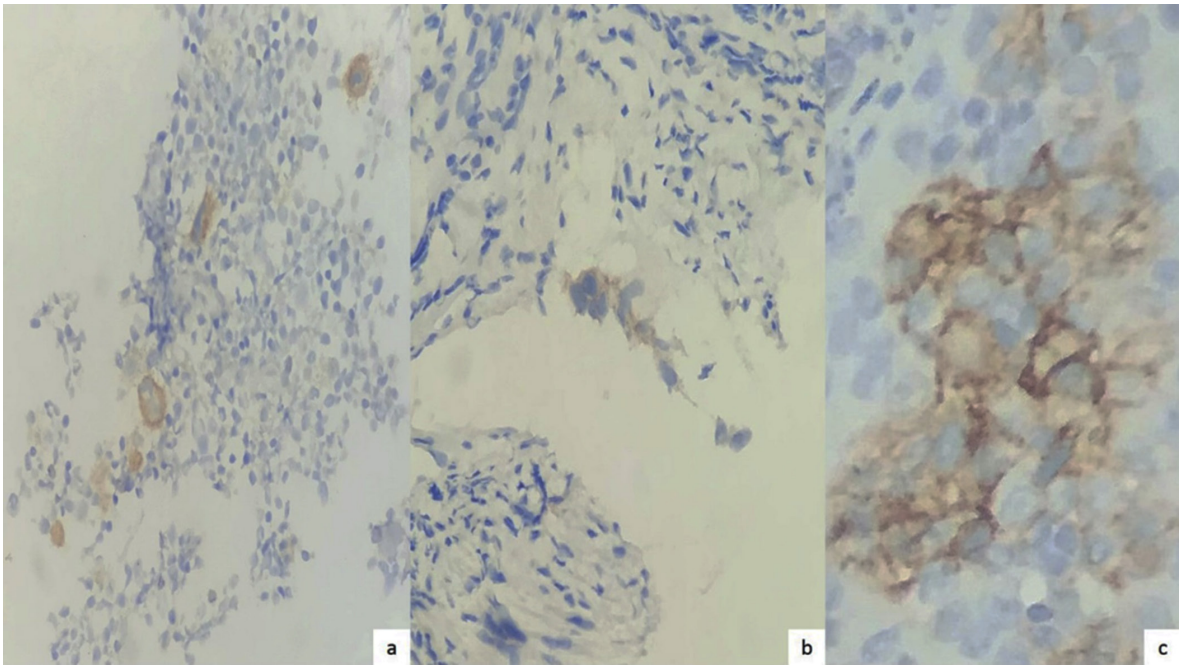

Figure 4. *Tumor cells with positive PD– L1 staining in the cell block. a, b) Low PD-L1 staining with a ratio 1–2% (×100) c) PD-L1 staining with a ratio 20% in adenocarcinoma (×200) (Dako Clone 22 c3) (1/50 dilution).*

We evaluated statistically the effect of cellularity in the cell block on PD-L1 expression status. We separately compared the presence or absence of PD-L1 according to whether the cell block contains more than 10% tumor cells or more than 50% tumor cells. If tumor cells constituted more than 10% of the material, PD-L1 expression was significantly different as negative or positive (p=0.02). The 50% rate of tumor cell amount was not statistically significant for PD-L1 (p=0.5). These datas are summarized in Table 3.

In addition the cellularity in the PD-L1 negative group belonging all specimen types was not lower from the other groups, it was similar.

Discussion

Bubendorf et al. compared PD-L1 exp. in small biopsy specimens and resection specimens and they have found much lower PD-L1 in small biopsy specimens. In our study although it is not significant statistically, half of the PD-L1 negative cases belonged to small biopsy samples, similarly. Bubendorf et al. suggested that this difference may be due to the heterogeneity and the sampling procedure as well as the scoring algorithm and even inter-observer variability[7]. Also different antibody clones may explain the reason for the low PD-L1 expression in some studies[1].

Table 3. *PDL-1 expression in cell blocks according to cellularity*

	Tumor proportion score of PD-L1			
Proportion of cells in a cell block	<1%, n (%) (negative)	≥1%, n (%) (positive)	Total (n)	P*
<10% cells	***6 (28.5)***	***4 (19.1)***	***21***	***0.02***
≥10% cells	***1 (4.8)***	***10 (47.6)***		
<50% cells	6 (28.5)	13 (61.9)	21	0.5
≥50% cells	0 (0)	2 (9.6)		

* P value of less than 0.05 was considered significant

Scoring systems and cut-off values used for PD-L1 evaluation also vary for different types of tumors. The triple scoring system currently accepted for lung cancer is such as <1%, 1–49% and >50%. A cut-off value of 10% is used for pancreatic, esophageal and stomach cancer[1].

Aspiration, a noninvasive procedure, is preferred for diagnosis in many tumors, especially for lung tumors. Cytological preparations allow for definitive diagnosis as well as many molecular tests. It is known that cytological samples are used for PD-L1 immunohistochemical evaluation. Studies demonstrating the usability of the cell block for this purpose are limited. However, according to current data, PD-L1 exp. in cell block and biopsy samples overlaps.

Zou et al. have suggested to prefer cell blocks for the assessment of PD-L1 exp. instead of small biopsy samples[6]. Considering the intra-tumor heterogeneity, it is not surprising to observe lower PD-L1 exp. in small biopsy samples[8,9]. They have considered that cytology samples have stronger PD-L1 exp. However, the reason for this is not very clear. One of the reason according to Zou et al. is decrease of tumor heterogeneity due to the distribution of tumor cells in cytology samples[6]. Another hypothesis for strong expression is the close contact between tumor cells and the immune microenvironment in pleural effusions. So, it is believed that T lymphocytes and macrophages in this environment increase PD-L1 exp. on the tumor cell surface[10].

In our study, cell block samples with high level of positive PD-L1 staining were higher than small biopsy samples. The statistical inconsistency in our study may be to the small number of cases. However, in general, we can say that cell blocks obtained from aspiration material may be a good idea to evaluate PD-L1. Here, the main point to be considered in order for optimal is that the number of cells, and therefore the tumor cell, is sufficient. For this, evaluation of PD-L1 expression in a cell block containing 10% tumor cells may be satisfactory. In this case, the need for a small biopsy and also for an interventional procedure to the patient may be eliminated.

Zou et al. also evaluated many factors that may be effect the strong PD-L1 exp. in the cell block[6]. They suggested that the fixation period (15–20 minutes for cell block, 2–24 hours for histological samples) may be a factor that may affect PD-L1 staining. Pinar et al., on the other hand, considered that the preservative solution used for cell block can affect the PD-L1 exp. in these samples. In their study, they compared the distribution and intensity of PD-L1 expression in the cell blocks formed with different preservative solutions. They concluded that –direct application of formalin with dripping 96% alcohol have the best results[11].

There are also some difficulties in the evaluation of immunohistochemical staining in the cell block. It can be difficult to determine the rate of positive tumor cell, especially if the tumor cells are dispersed as single. In addition, false positivity may occur in histiocytes and it is not easy to distinguish between histiocytes and single tumor cells in the cell block[12]. This evaluation should be done by an experienced pathologist to avoid from misleading[12]. Dual immunohistochemical staining (CD68 for histiocytes or TTF-1 for tumor cells) with PD-L1 may also help for this discrimination[1].

There are also studies investigating clinicopathologic data that may affect PD-L1 expression. However, no definite results could be obtained in this regard. A meta-analysis study evaluating PD-L1 in patients with lung cancer showed that PD-L1 expression was stronger in adenocarcinoma than in squamous cell carcinoma[13]. However, East Asian data differ from these results[14]. We could not have any significant difference between PD-L1 expression and clinicopathological datas such as gender, histological subtypes of the tumor and the status of primer or metastatic.

Our study has some limitations. One of them is the retrospectivity of the study. Another limitation was that the cytology and biopsy materials in this study belonged to different cases. So we could not perform cytology– histology comparison on the same tissue. However, we believe that our study will provide an idea about PD-L1 expression in cell blocks.

As we know cell blocks contain a limited number of cells. Finding the maximum number of cells to be obtained in the cell block should be the point to be noted. According to our study, it is more meaningful to evaluate PD-L1 expression in cell blocks containing tumor cells in at least 10% of the material. In other words, with the maximum number of cells that can be obtained, we can reach more optimal results in the PD-L1 evaluation.

As conclusion; in this retrospective analysis samples consisted of small biopsy samples and cell blocks prepared from cytological samples as well as resection materials with similar ratio. According to our analysis,

cell blocks prepared from cytological samples may be a good alternative for immunohistochemical evaluation of PD-L1 exp. When we evaluated the results of PD-L1 exp. of cell blocks, it was not at all different from the resection materials. As we know cell blocks contain a limited number of cells. Finding the maximum number of cells to be obtained in the cell block should be the point to be noted. According to our study, it is more meaningful to evaluate PD-L1 expression in cell blocks containing tumor cells in at least 10% of the material. In other words, with the maximum number of cells that can be obtained, we can reach more optimal results in the PD-L1 evaluation. We think that revealing the data on PD-L1 exp. in cytological and histological tissue samples is valuable and also may be a guide for other studies.

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