Programmed Death-ligand 1 Expression Analysis for Non-small Cell Lung Cancer in Tissues Sampled Using Different Methods

Farklı Yöntemlerle Örneklenen Akciğer Küçük Hücreli Dışı Karsinom Dokularında Programlı Ölüm Ligandı 1 Ekspresyon Analizi

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──────── **Abstract** ────────

Aim: This study aims at investigating the concordance of programmed death-ligand 1 (PD-L1) expression in non-small cell cancer tissues that have been sampled using different methods and its relationship with the pathologic parameters of tumors.

Methods: PD-L1 expression assays were made on the cell blocks taken using fine needle aspiration, the small tissue samples representing endoscopic biopsy and the large tissue samples representing the resected tumor taken from the tumors of 100 subjects diagnosed with non-small cell lung cancer; their percentage values were evaluated and their groups were determined based on these values.

Results: The average difference in expression rates found through different sampling methods was close to 0, and the values of such differences changed mostly in a narrow range. In paired comparisons, a good level of concordance was observed between all the sampling methods. The values were 0.8865 (SE: 0.0306, CI: 0.8264-0.9466) in the comparison of tumor tissue (TT) and small biopsy (SB): 0.8637, (SE: 0.033, CI: 0.7989-0.9285) TT to cell block (CB): 0.8916, (SE: 0.0272, CI: 0.8383-0.9449) SB tissue to the CB.

Conclusion: Therefore, it can be concluded that the PD-L1 expression does not differ between the tissues sampled through various methods in non-small cell lung cancers.

Keywords: Non-small cell carcinoma, PD-L-1, cell block, different expression

──────── **Öz** ────────

Amaç: Çalışmada amacımız farklı yöntemlerle örneklenmiş küçük hücreli dışı karsinomda programmed death-ligand 1 (PD-L1) ekspresyon uyumunu ve tümör patolojik parametrelerle ilişkisini araştırmaktır.

Yöntemler: Küçük hücreli dışı akciğer karsinom tanısı alan 100 olgunun tümöründen ince iğne aspirasyonu hücre bloğu, endoskopik biyopsiyi temsil eden küçük doku ve rezeksiyon tümör temsil eden büyük doku örneğinde PD-L1 ekspresyon tayini yapılıp yüzde değerleri ve buna göre grupları değerlendirildi.

Bulgular: Örnekleme yöntemleri tarafından saptanmış ekspresyon oranlarının ortalama farkının 0'a yakın olduğu gibi, fark değerlerinin, büyük oranda, dar uyum içinde olduğu bulundu. İkişerli karşılaştırmalarda, tüm örnekleme yöntemleri arasında, PD-L1 ekspresyon oranı açısından iyi düzeyde uyum olduğu izlendi. Tümör dokusu-biyopsi dokusu karşılaştırmasında 0,8865 (SE: 0,0306, CI: 0,8264-0,9466), tümör dokusu-hücre bloğunda 0,8637 (SE: 0,033, CI: 0,7989-0,9285); biyopsi dokusu-hücre bloğunda 0,8916 (SE: 0,0272, CI: 0,8383-0,9449) idi.

Sonuç: Sonuçta akciğerin küçük hücreli dışı karsinomlarında PD-L1 ekspresyonu farklı yöntemlerle örneklenmiş dokular arasında fark göstermemektedir.

Anahtar Sözcükler: Küçük hücreli dışı karsinom, PD-L1, hücre bloğu, farklı yöntemler

Introduction

The treatments of non-small cell lung cancer have shown fast improvements in recent years, the most outstanding being cancer immunotherapy (1,2). In carcinogenesis, suppression of the host immune system by tumor cells facilitates tumor proliferation. Blocking the programmed death-ligand 1 (PD-L1) receptors on the cell surface that create immune tolerance is an activating cancer immunotherapy method (3). Removal of the blockage triggers T lymphocyte immunization against cancer cells.

The decision for immunotherapy is made upon evidencing the presence of a PD-L1 expression in the tissue. The samples obtained using various methods can be used to identify the ligand. Moreover, the distribution of PD-L1 expression in tissues is variable in non-small cell cancers (4). This raises the question whether the expression values of different intervention methods used in the diagnosis of cancer have the same proliferation value.

This study aims at investigating the concordance of PD-L1 expressions in non-small cell cancer tissues sampled using different methods and its relationship with the pathologic parameters of tumors.

Methods

This study was conducted in the Department of Pathology in Yedikule Chest Diseases and Thoracic Surgery Training and Research Hospital. One hundred subjects diagnosed with non-small cell lung cancer based on the preoperative or intraoperative frozen sections were enrolled in the study.

The tissues whose routine pathological macroscopic assessments and samplings in their pulmonary tumor resection material had been completed were included in this study. Tumors larger than 2.9 cm and diagnosed with non-small cell lung cancer were the inclusion criteria. Patients with a tumor ≤2.9 cm, those with undetectable tumor tissue (TT) after neoadjuvant therapy and those diagnosed with a disease other than non-small cell cancer were excluded.

First, cell block (CB) were sampled using fine needle aspiration from the residual tumor that remained on the resection and was not yet involved in buffered formalin fixation. Secondly, 2-5 pieces of tissue 2-3 mm in diameter, representing endoscopic small biopsy (SB) were sampled from the residual tissue. Finally, after the completion of the macroscopic TT sampling, a piece of TT at least 1-2 cm² in diameter and 2-3 mm in thickness was taken.

The demographic characteristics of the subjects, anatomic resection types, tumor diameters, histopathological diagnoses, tumor diameters, lymphovascular involvements, peritumoral lymphocytic inflammation and lymph node metastasis statuses (no metastasis=N0, inter-and intra-lobular lymph node metastasis=N1, mediastinal unilateral lymph node metastasis=N2) were explored.

Study designing has been approved by Local Ethical Committe of University of Health Sciences, İstanbul Training and Research Hospital Ethics Committee. The study protocol number and date are 1058/04.08.2017.

Immunohistochemical Analysis

A commercial product approved for lung cancer was chosen for the immunohistochemical analysis of PD-L1 (5). After deparaffinization, the slides were subjected to immunohistochemical analysis using the PD-L1 antibody (SP263 Roche/Ventana) and an Optiview DAB IHC detection kit (Roche/Ventana) was used as a universal kit.

While assessing the PD-L1 expression, complete or incomplete membranous staining was accepted as a positive value. The intensity of staining was not considered in this assessment. The tumor cells in the entire area of each sample were counted. Further, the tumor cells exhibiting PD-L1 expression were counted; and PD-L1 percentage value was found based on the percentage value, they were grouped as none (0), less than 1, equal to or greater than 1, less than 5, equal to or greater than 5, less than 10, equal to or greater than 10, less than 50 and equal to or greater than 50.

Statistical Analysis

The analyses were conducted using the R software and interactive software. The one-sample t-test was used to compare the mean values. To demonstrate the concordance, Lin's concordance correlation coefficient and Cronbach's alpha coefficient were used for measured variables and weighted kappa and Fleiss' kappa values for ordinal variables. To show correlations, Pearson's correlation coefficient was used for measurable variables and Spearman's correlation coefficient for ordinal variables. Additionally, any probability level less than 0.05 was considered significant.

Results

A total of 100 patients had a preoperative or intraoperative diagnosis of non-small cell cancer. The demographic characteristics and surgical data of the subjects and histopathological findings are shown in Table 1.

The surgical procedures included 70 lobectomies, four bilobectomies, 26 pneumonectomy, four chest wall resections and one carinal sleeve resection.

The PD-L1 expression was found to be less than 1 in 35 subjects (35%) and 1 or more in 65 (65%) (Figure 1). The PD-L1 expression value groups and histopathological tumor distribution are shown in Table 2.

The general distribution of PD-L1 expression (Figure 2), and its distribution in squamous cell carcinoma (Figure 3) and in adenocarcinoma (Figure 4), were shown. Moreover, its highest value among TT, SB and CB were considered.

The least PD-L1 expression was found in the other carcinomas group. No expression was found in mucinous adenocarcinoma, large-cell neuroendocrine carcinoma and

Table 1. The demographic and histopathological findings	
Total cases	**n=100**
Female/Male	15/85
Age	60.2 (39-78)
Histopathological diagnosis	
Squamous cell carcinoma	58
Keratinizing squamous cell carcinoma	40
Nonkeratinizing squamous cell carcinoma	17
Basaloid squamous cell carcinoma	1
Adenocarcinoma	31
Mucinous adenocarcinoma	3
Adenosquamous cell carcinoma	1
Carcinoid tumor	4
Typical carcinoid tumor	3
Atypical carcinoid tumor	1
Large-cell neuroendocrine carcinoma	1
Pleomorphic carcinoma	2
Tumor diameter (cm)	2.9-11
Lymphovascular invasion	
Positive/Negative	71/29
Peritumoral lymphocytic inflammations	
No/minimal/medium/intense	43/30/20/7
Lymph nodal metastasis	
N0/N1/N2	56/33/11

N0=No metastasis, N1=Inter-and intra-lobular lymph node metastasis, N2=Mediastinal unilateral lymph node metastasis, n: Number
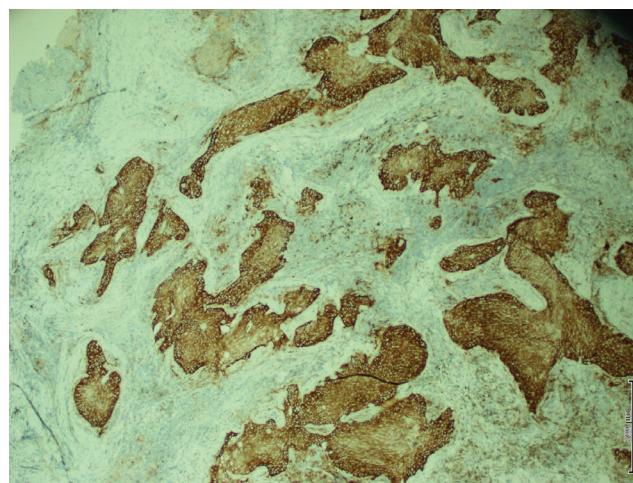
typical and atypical carcinoid tumors. The expression was found to be 5-10% in adenosquamous cell carcinoma and over 50% in one of the pleomorphic carcinomas. It was limited only to the carcinoma component in that tumor. Its value was 0 in the other pleomorphic carcinoma.

The concordance of the PD-L1 expression percentage values was explored in the samples of TT, SB and CB.

Assessment of the expression values showed that 39% of the tumors expressed PD-L1 at a low level and 46% of them at a high level in the TT; a moderate level of expression was hardly encountered (Table 3).

Discussion

The concordances of the PD-L1 expression values obtained with the three sampling methods were compared. A graphical evaluation of the concordance (Figure 5), showed that the expression values found by the sampling methods were very close to each other in each paired comparison as seen in the scatter plots, and for this reason, the best fit line determined for the distribution

Figure 1. Programmed death-ligand 1 expression in small biopsy

Table 2. Distribution of programmed death-ligand 1 expression								
Histologic type	**0 (%)**	**<1 (%)**	**≥1<5 (%)**	**≥5<10 (%)**	**≥10<50 (%)**	**>50 (%)**	**Total (%)**	**Discordant case number**
Squamous cell carcinoma	8	6	1	2	10	15	58	16
Adenocarcinoma	7	5	1	0	6	6	31	6
Mucinous adenocarcinoma	3	-	-	-	-	-	3	-
Adenosquamous cell carsinoma	-	-	-	-	-	-	1	1
Typical carcinoid tumor	3	-	-	-	-	-	3	-
Atypical carcinoid tumor	1	-	-	-	-	-	1	-
Large-cell neuroendocrine carcinoma	1	-	-	-	-	-	1	-
Pleomorphic carcinoma	1	-	-	-	-	1	2	-
Total	24	11	2	2	16	22	77	23

was close to 45 degrees. The Bland-Altman plots revealed that in each paired comparison, the mean difference of the expression values found in the sampling methods was close to 0 in each paired comparison and the difference values were within the narrow concordance limits (limits of agreement-LOA); there was no trend showing that the difference changed as the values changed; the difference remained consistently low even when the values changed.

The levels of concordance between the sampling methods were calculated with reference to the PD-L1 expression values that were found. A good degree of concordance in terms of PD-L1 expression values was observed between all sampling methods in paired comparisons [Lin's correlation coefficient: pc was 0.9371 (CI: 0.9081-0.9572)] for the concordance between TT-SB, 0.9522 (CI: 0.9301-0.9675) for the concordance

between TT-CB and 0.9689 [(CI: 0.9542-0.9789) for the concordance SB-CB)]. The Cronbach's alpha correlation coefficient for the concordance between all the three methods was calculated to be 0.9837 (CI: 0.977-0.989), which indicates a good level of concordance.

The concordance was also analyzed based on the results obtained through classification of the expression values. All methods revealed the same expression values in 77 samples. In the other 23 samples, the expression values were different from the others in at least one sampling method.

The concordance of the sampling methods with each other was analyzed with respect to their expression values in pairs. A very good concordance between the sampling methods was observed in this analysis [(Weighted Kappa level was 0.8865 (SE: 0.0306, CI: 0.8264-0.9466)] for the TT-SB comparison, 0.8637 (SE: 0.033, CI: 0.7989-0.9285) for the TT-CB comparison and 0.8916 (SE: 0.0272, CI: 0.8383-0.9449) for the SB-CB comparison). Even when the three sampling methods were assessed together, there was a good concordance among them (Fleiss' Kappa: 0.7823 (SE: 0.0326, CI: 0.7184-0.8463).

The factors that may affect the concordance of the sampling methods were analyzed. For this, the correlation of the differences in the expression values found in each pair of sampling methods with the variables was explored. Further, the same exploration was repeated by making the expression groups ordinal and finding the difference between the rates obtained from the sampling methods (Table 4).

Through this analysis, it was observed that although significant, a weak difference occurred only between the expression rates of TT and CB and a stronger difference between the expression rates found in the two methods
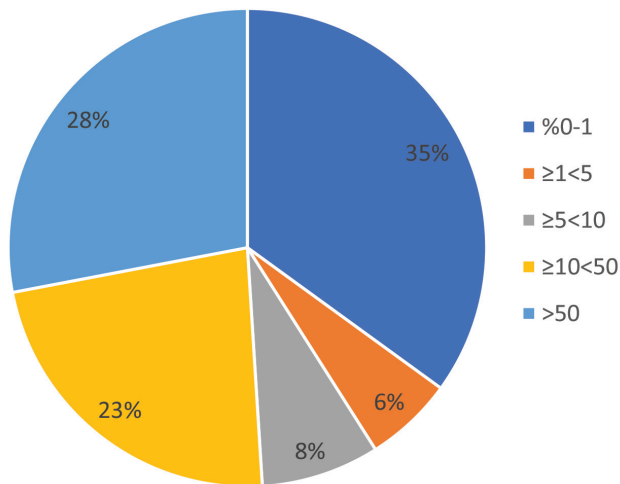

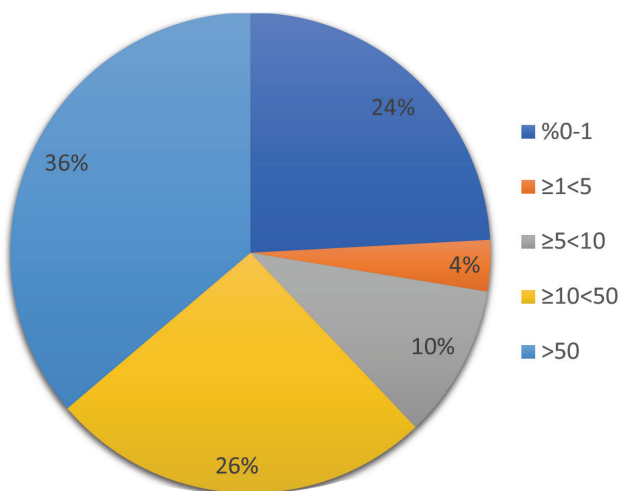**Figure 2.** General programmed death-ligand 1 expression

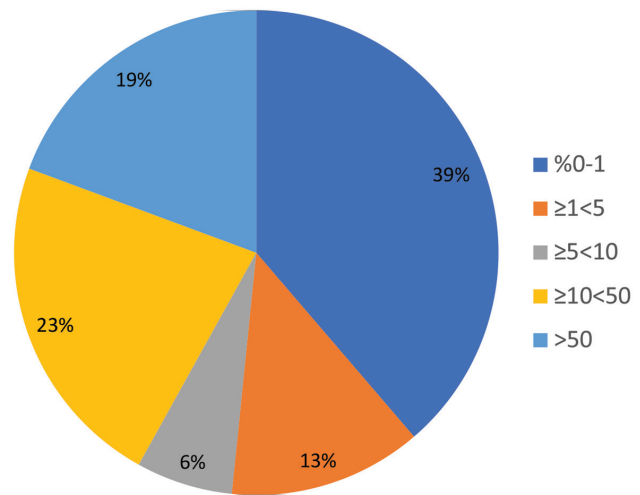**Figure 3.** Programmed death-ligand 1 expression in squamous cell carcinoma

Figure 4. Programmed death-ligand 1 expression in adenocarcinoma

in squamous cell carcinomas than in adenocarcinomas (r=0.2, p=0.04); this difference found between the expression rates in the two methods increased as the tumor diameter increased (r=0.2, p=0.045). Similarly, the difference between the expression rates in the two methods decreased as the N stage advanced (r=0.22, p=0.03).

Conclusion

The PD-L1 expression is critically important in lung cancer checkpoint inhibitory therapy. The identification of PD-L1 in tumor cells is standardized using immunohistochemical methods (6). The coding of the selected tissue at a preanalytical stage, fixation, processing, section thickness, determination of priorities in molecular and immunohistochemical analyses, selection of PD-L1 clone at the analytic stage, and the correct methods of conducting assessments and quality control have been specified in practice (7,8). The use of different PD-L1 clones has also been shown in non-small cell lung cancer (9,10). In fact, the administration of immunotherapy according to the threshold values of the expression has created a new vision in the treatment of non-small cell lung cancer.

The aim in determining the treatment option is the presence and rating of an in vitro PD-L1 expression in the TT. In this study, the presence of expression was explored in tissues that were sampled using different methods from the same tumor. Instead of taking samples from the tumor resection tissue through the microarray method, three different tissues that were taken simultaneously but separately from the same tumor were examined. Although studies comparing the expression in tumor anatomic resection tissue in the samples taken by way of endoscopic biopsy, transthoracic fine needle aspiration and mediastinoscopic lymph node biopsy are available, in this study, the concordance of expression was found to be very high in the tissue samples (11). As a result, it was found that CB representing the tumor and limited tissue samples give correct and reliable results in the assessment of PD-L1 expression. Moreover, there are also studies comparing the expression value in tumor cells in biopsy and resection samples (12,13). In fact, the presence of immune reaction varying across regions in tumors is a known fact (14). A study conducted by Munari et al. (15) has shown that the minimum amount of tissue should be 3-4 pieces of core biopsies for a maximum yield. The concordance was found to be 92.4% at an expression score of 1% and over, but there was a marked difference in stratified score groups (16). Further, SB samples were argued not to be sufficient for assessment due to strikingly non-concordant results. The reason for these different results obtained from the methods may be the combination of the intensity of membranous staining and the proportion of tumor cells showing positivity. On the other hand, the presence of a weak concordance can be associated with the chosen PD-L1 clone (11). Similarly, the presence of expression in the same cell can vary in the pre-and post-chemotherapeutic stages (17). In fact, mucinous adenocarcinoma and signet-ring cell carcinoma also involve difficulties in the assessment of expression.

Table 3. Frequency of programmed death-ligand 1 expression values by sample types and their distribution

Sample type	Expression values						
	<1 (%)	≥1<5 (%)	≥5<10 (%)	≥10<50 (%)	>50 (%)	Median	IQR
Tumor tissue	39	7	8	21	25	3	1-4.75
Small biopsy	39	10	6	19	26	3	1-5
Cell block	36	5	12	22	25	3	1-4.75

IQR: Interquartile range

Table 4. The difference between the expression values and histpathologic parameters

Histologic type			Grade		Diameter		N status	
PD-L1 expression values	CC	p	CC	p	CC	p	CC	p
TT-SB	-0.03	0.74	0.03	0.79	0.16	0.10	-0.09	0.35
TT-CB	-0.2	0.04	-0.06	0.62	0.2	0.045	-0.22	0.03
SB-CB	-0.11	0.29	-011	0.29	0.014	0.89	-0.14	0.17
PD-L1 expression group								
TT-SB	-0.019	0.85	-0.02	0.82	0.11	0.26	-0.009	0.93
TT-CB	-0.09	0.38	-0.02	0.87	0.16	0.11	-0.09	0.37
SB-CB	-0.10	0.31	0.006	0.95	0.07	0.48	-0.10	0.31

PD-L1: Programmed death-ligand 1 TT: Tumor tissue SB: Small biopsy CB: Cell block
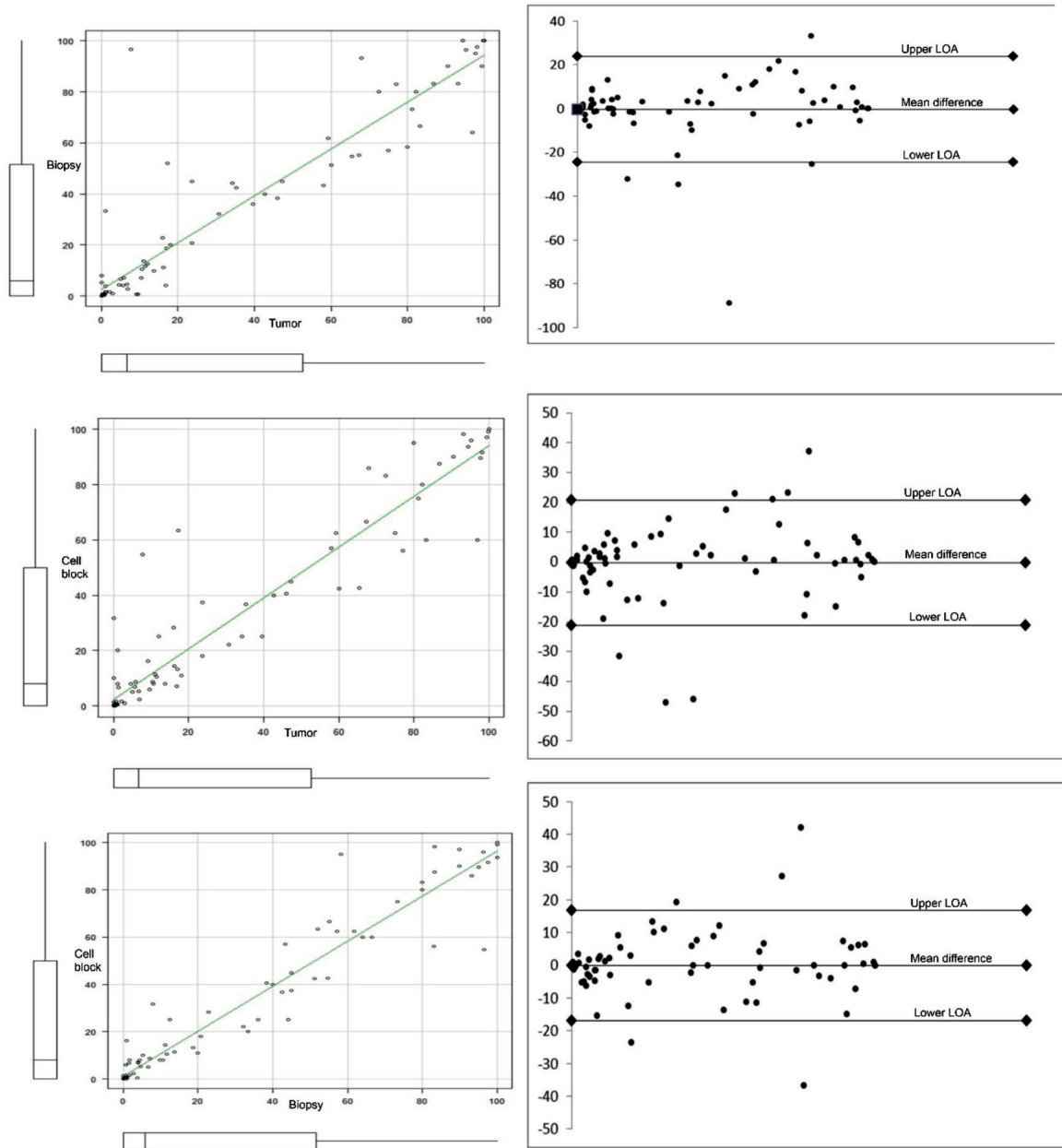*CC: Correlation coefficient, N: Nodal status

Figure 5. Concordance between samples
LOA: Limits of agreement

PD-L1 expression has been explored in poorly-differentiated lung cancers. The expression rate was found to be over 90% in pleomorphic carcinoma (18). The reaction is present more evidently in the sarcomatoid component of the tumor than in its carcinoma component. Along with adenocarcinoma, squamous cell carcinoma and neuroendocrine tumors, this study also considered pleomorphic carcinoma and adenosquamous cell carcinoma. The expression appeared at a rate of 2/3

in these tumors. Although the result of this study would require further support by further studies, it helps in creating an immunotherapy option in poorly-differentiated non-small cell lung cancer with a high probability.

The diameter, as one of the tumor parameters, was included in this study to create a difference, although insignificantly, in the expression between TT and CB. Accordingly, cytological samples may represent the PD-L1 expression lesser than expected because of the diameter

in T3 and T4 tumors. In a study conducted by Sakata et al. (19), the EBUS needle aspiration was compared to tumor resection. This is supported by the fact that cytological samples are less sensitive than large tissues. However, this difference can be eliminated by combining needle aspiration with tru-cut biopsy.

The PD-L1 expression does not show any difference among tissues sampled using different methods in non-small cell lung cancer. It can also be concluded that small biopsies and cytological samples with completed CB are able to represent the tumor itself. However, it should be noted that the tumor parameters may have an effect on expression.

Authorship Contributions

Concept: H.N.Ü. Design: H.N.Ü. Data Collection or Processing: H.N.Ü, L.C., M.A.B. M.Z.G. Analysis or Interpretation: H.N.Ü. Literature Search: H.N.Ü. Writing: H.N.Ü.

Conflict of Interest: The authors declare that they have no conflict of interest.

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