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Heterogeneity analysis of PD-L1 expression and copy number status in EBUS-TBNA biopsy specimens of non-small cell lung cancer: Comparative assessment of primary and metastatic sites☆

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ABSTRACT

Objectives: Most patients with non-small cell lung cancer (NSCLC) are diagnosed at advanced stages where small biopsy specimens obtained through endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) are sometimes the only available samples for diagnosis. We aimed to determine whether EBUS-TBNA specimens are suitable for the evaluation of PD-L1 protein expression and copy number alterations (CNAs).

Materials and methods: PD-L1 protein expression and CNAs in 71 EBUS-TBNA specimens of NSCLC were assessed. Sixty-eight corresponding transbronchial biopsy (TBB) specimens from primary sites, thirteen resected primary tumors, and six resected metastases were comparatively analyzed. PD-L1 expression in tumor cells was assessed by immunohistochemistry (E1L3N). Positivity of ≥1% was used as the cutoff. *PD-L1* CNAs were assessed with fluorescent *in situ* hybridization and were classified into three categories: amplification, polysomy, and disomy. Concordance between EBUS-TBNA and other specimens was calculated.

Results: The cohort comprised 48 men (67.6%), 15 never-smokers (21.1%), and 39 adenocarcinomas (54.9%). The concordance of PD-L1 positivity between EBUS-TBNA and other specimens was moderate; $\kappa = 0.63$ for EBUS-TBNA vs. TBB, $\kappa = 0.68$ for EBUS-TBNA vs. resected primary tumors, and $\kappa = 1.0$ for EBUS-TBNA vs. resected metastases. The concordance of *PD-L1* CNA status was comparable with that of PD-L1 expression: $\kappa = 0.60$ for EBUS-TBNA vs. TBB and $\kappa = 0.74$ for EBUS-TBNA vs. resected primary tumors. When *PD-L1* copy number was assessed as a continuous variable, the correlation of *PD-L1* CNAs was superior to that of PD-L1 expression. Intratumorally, *PD-L1* copy number was less heterogeneous than protein expression in whole sections of resected tumors.

Conclusion: EBUS-TBNA specimens can be used to assess *PD-L1* CNAs and protein expression. Although spatial heterogeneity should be considered for accurate interpretation, the evaluation of *PD-L1* CNAs provides more reproducible results than that of protein expression levels especially with regard to intratumoral heterogeneity.

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1. Introduction

Immune checkpoint inhibitors (ICIs) including programmed death-1 (PD-1) and programed death-ligand 1 (PD-L1) inhibitors have been one of the mainstream therapies in patients with non-small cell lung cancer (NSCLC) [1–4]. Immunohistochemical staining of PD-L1 is currently used as a biomarker for selecting patients who are likely to benefit from treatment with ICIs; for example, pembrolizumab was approved as a first-line treatment for metastatic NSCLC in cases demonstrating equal to or more than 50% positivity of PD-L1 staining [3]. Therefore, the evaluation of PD-L1 status is critical to determine the most appropriate treatment strategy for patients with advanced NSCLC. Regrettably, most patients with NSCLC are diagnosed at advanced stages, and only tiny biopsy specimens are sometimes often available for histopathological diagnosis and further genetic/molecular analyses.

Immunohistochemistry (IHC) is widely used as a companion/complemental PD-L1 assay for the use of PD1/PDL1 inhibitors [3,5–7]. However, there are several problems with the assessment of PD-L1 expression via IHC. First, there are multiple PD-L1 antibodies for IHC such as clones 28-8, 22C3, SP142, and SP263; of these, the consistency of three PD-L1 antibodies (all except for SP142) has been demonstrated for the assessment of tumor PD-L1 expression in several studies [8–10]. Second, the evaluation methods for PD-L1 expression involving thresholds and target cells differ depending on clinical trials [11,12]. Third, PD-L1 IHC does not satisfactorily identify responders and non-responders to PD-1/PD-L1 inhibitors [3,13]. Finally, one of the most critical issues is the heterogeneity of PD-L1 expression within and between tumors [14–17]. To address these problems, alternative predictive factors of response to ICIs such as tumor mutation burden [18,19] and immune profiling in the tumor microenvironment [11,12] have been proposed. We previously reported the association of *PD-L1* copy number alterations (CNAs) with PD-L1 protein expression levels and immune cell infiltration in NSCLC [20,21], and proposed that *PD-L1* CNAs could potentially complement the predictive performance of PD-L1 expression [22]. Additionally, several reports have demonstrated the link between responses to ICIs and increases in *PD-L1* gene copy number [23–26].

Concordance of PD-L1 positivity between biopsy specimens and resected tumor specimens varied according to reports, primarily due to spatial heterogeneity as well as differences in biopsy methods [14–17,27]. Endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) is a relatively newly developed diagnostic modality that is becoming more relevant and essential in the clinical setting [28,29]. Previous limited studies have demonstrated the utility of the EBUS-TBNA procedure for the evaluation of PD-L1 protein expression [27,30]. However, it remains still elusive whether EBUS-TBNA is suitable for the evaluation of PD-L1 expression, particularly when heterogeneity is considered. Moreover, no data are currently available that demonstrate whether *PD-L1* CNAs can be assessed using EBUS-TBNA-derived specimens and whether PD-L1 protein expression or copy number status is less spatially heterogeneous. Therefore, the first aim of this study was to evaluate the suitability of EBUS-TBNA-derived NSCLC biopsy specimens for the assessment of PD-L1 expression and gene copy number alterations, focusing on intertumoral heterogeneity using corresponding specimens obtained from other sites such as primary and metastasized tumors through different procedures such as surgery and transbronchial biopsy (TBB). The second aim was to compare the intratumoral heterogeneity of PD-L1 expression and CNAs. Our findings provide evidence that both PD-L1 protein expression and CNAs can be effectively assessed using EBUS-TBNA cores and that *PD-L1* copy number status could represent a useful complementary biomarker of PD-L1 protein expression.

2. Material and methods

2.1. Patients and tumor specimen preparation

This study was approved by the ethics committees of Hamamatsu University School of Medicine (#17-067, #G14-260) and Seirei Mikatahara General Hospital (#17-40). The need for patient approval and informed consent was waived because this study was based on reviews of the patients' records. All analyses were conducted in compliance with the ethical standards established by the Declaration of Helsinki. We collected EBUS-TBNA biopsy specimens containing tumor cells from a total of 71 patients with NSCLC (Supplementary Table S1), 17 of whom were from Hamamatsu University Hospital (between January 2013 and June 2017) and 54 were from Seirei Mikatahara General Hospital (between January 2011 and June 2017). Sixty-eight corresponding TBB specimens, thirteen resected primary tumors, and fifteen resected metastatic tumors were also collected. Pathological stages were defined based on the WHO classification [31] and the histology of tumors were classified by senior pathologists (HO, HS, and KS) according to the 2011 IASLC/ATS/ERS adenocarcinoma sub-classification and the 2015 WHO classification [32]. Patient characteristic data such as age, smoking status, sex, neo-adjuvant therapy, and performed lymph node locations were retrospectively collected from medical records. The number of total tumor cells in an EBUS-TBNA derived specimen was calculated on hematoxylin-eosin (HE) stained sections (Fig. 1A). PD-L1 expression and copy number were assessed in both EBUS-TBNA-derived specimens and the corresponding primary/metastatic specimens and the concordance and correlation were calculated. TBB was performed simultaneously with EBUS-TBNA in all cases. Resection and/or sampling of corresponding primary tumors and metastatic tumors were conducted on different days than the EBUS-TBNA procedure; the median interval between EBUS-TBNA and resection of primary/metastatic tumors was 25 days (range, 3–360 days).

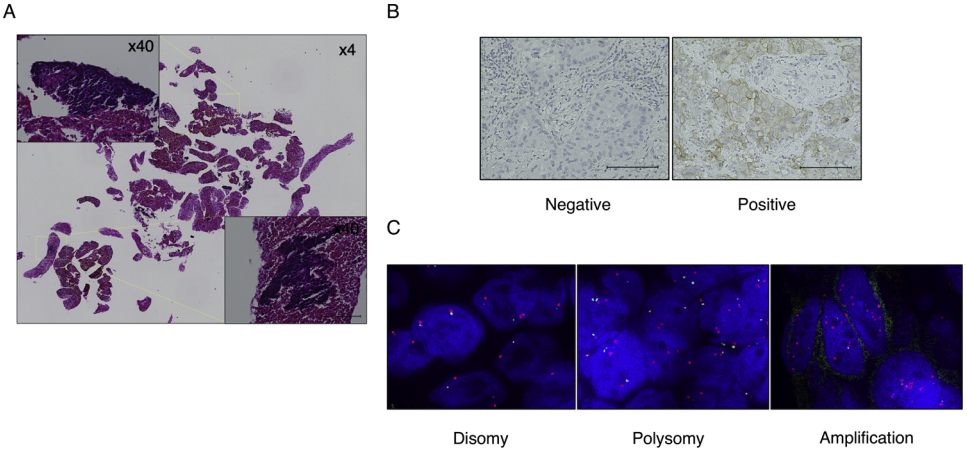

Fig. 1. Representative images of tumor cells derived from EBUS-TBNA with PD-L1 expression and CNAs.
(A) Representative HE-staining image of the EBUS-TBNA-derived specimens. Bars = 100μm.
(B) Immunohistochemical staining of PD-L1 (40× magnification). Positive tumor PD-L1 expression was defined as tumor cell membranous staining regardless of intensity. Bars = 100μm.
(C) FISH with the *PD-L1* probe (red) and chromosome 9 centromere probe (green). Nuclei were stained with 4′,6-diamidino-2-phenylindole (blue) (100× magnification). *PD-L1* copy number was classified as disomy, polysomy, or amplification.

2.2. Bronchoscopic procedures

Bronchoscopy was performed by at least two trained pulmonologists under local anesthesia and mild sedation [33]. A convex probe EBUS (BF-UC260F-OL8; Olympus, Tokyo, Japan) with an endoscopic ultrasonography (EU-C60; Olympus) was used for EBUS-TBNA. The outer diameter of the linear probe was 6.9 mm and the diameter of the bronchoscope was 6.2 mm. Aspiration was conducted using 21- or 22-gauge needles and more than three needle passes were performed per case. Each aspirate was discharged on a glass slide by blowing air/saline, followed by needle stylet replacement. All aspirates were gathered as one sample per lesion and pathologically evaluated. All EBUS-TBNA-derived specimens were treated as tissue specimens of fragmented biopsies. TBB specimens were obtained by disposable biopsy forceps (Radial Jaw 4; Boston Scientific, Marlborough, MA, USA). TBB was carried out to approach primary tumors in all cases.

2.3. PD-L1 IHC analysis

IHC assays were performed as described in our previous study using an autostainer (HISTOSTAINER 48A, Nichirei, Tokyo, Japan) [20,34]. Briefly, formalin-fixed paraffin-embedded (FFPE) tissues were sectioned at a thickness of 4 μm and these FFPE sections were then deparaffinized and rehydrated. After antigen retrieval, endogenous peroxidase was quenched with hydrogen peroxidase before reacting with primary antibodies for 30 min at room temperature. We applied a monoclonal anti-PD-L1 antibody (1:100 dilution, clone E1L3N; Cell Signaling Technology, Danvers, MA, USA). The entirety of the area in each section was screened for PD-L1 evaluation and assessed comparing the corresponding HE section to discriminate tumor cells from the other immune and stromal cells. Positive staining was defined as the presence of membranous staining regardless of the degree of staining intensity (Fig. 1B). Two observers (KY and KT) who were unaware of the clinical data independently assessed protein expression and calculated the percentage of PD-L1-positive tumor cells [tumor proportion score (TPS)] [35,36]. The cutoff value was set at equal to or more than 1% positivity of tumor cells according to previous publications [3,5,9,36]. In addition, two other cutoff values (5% and 50%) were also applied.

2.4. Fluorescent in situ hybridization (FISH) analysis

PD-L1 copy number was assessed via the FISH technique according to previously described protocols [20,37]. Briefly, fluorescent probes for the *PD-L1* gene locus on chr9p24.1 [bacterial artificial chromosome (BAC; Advanced GenoTechs Co., Tsukuba, Japan) RP11-599H20] and a reference locus of *PD-L1* on the centromere (alpha satellite) region of chromosome 9 (CEP9; chr9p24.1; BAC RP11-11O24) were selected. *PD-L1* and the reference probes were conjugated to red and green signals, respectively. For staining of nuclei, 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA) was used. Chromosomal probe localization and specificity were confirmed in normal metaphase lymphocytes. Upon counting probe signals, at least 50 cells per tumor in more than three areas were scored at 100× magnification. The gene signals were quantified and the ratio of the mean targeted signal to the mean centromere enumeration probe (CEP) 9 signal (PD-L1/CEP9) was determined. FISH analysis was evaluated using Z-stack images generated by a fluorescence microscope (BZ-9000; KEYENCE, Osaka, Japan). CNAs were classified into one of three categories: disomy, polysomy, or amplification [38–40]. Amplification was defined as mean *PD-L1*/CEP9 signal ratio ≥ 2 (Fig. 1C). Polysomy was defined as mean *PD-L1* copy number ≥ 3, and mean *PD-L1*/CEP9 signal ratio < 2 [41,42]. Other tumors were classified into the disomy category.

2.5. Intratumoral heterogeneity analysis

We evaluated intratumoral heterogeneity in 17 assessable primary resected tumors. Three cores (A–C) including the centers of tumors and two other marginal areas in a section were selected [38]. The size of each core ranged from 2 to 3 mm in diameter. PD-L1 TPS was assessed under 40× magnification in two or three non-overlapping fields in the three cores individually. Simultaneously, *PD-L1* copy number-per-cell was also assessed under 100× magnification in at least these three spots individually. The diversity index in each assessment was calculated [43,44].

2.6. Statistical analysis

Categorical variables were analyzed using Fisher's exact test. Continuous variables were analyzed using the Mann–Whitney *U* test and multi-group comparisons were performed using the Kruskal–Wallis test followed by a pairwise Mann-Whitney *U* test with Holm's correction. Concordances between two specimens were analyzed using the proportion of agreement and kappa or weighted kappa coefficient. Correlation coefficients were analyzed with Pearson's test. Intratumoral heterogeneity was analyzed using a diversity index assessing differences in PD-L1 status in three spots. The diversity index was defined with the Shannon index, which was calculated by the following formula for each site [43,44]:

$$H = -\Sigma pi \ln (pi)$$

where *p*i was the abundance of clones (i.e., the positive PD-L1 expression clones or the *PD-L1* amplified clones). Statistical analysis was performed using R software version 3.2.0 (The R Foundation for Statistical Computing, Vienna, Austria). P values < 0.05 were considered to be statistically significant.

3. Results

3.1. Clinicopathological characteristics

The clinical characteristics of patients at the time of EBUS-TBNA are shown in Table 1. Most patients were at advanced stages: 67 (94.4%) cases were Stage III or IV. Mediastinal lymph node stations at #4 and

Table 1
Characteristics of patients with non-small cell lung cancer with EBUS-TBNA.

Characteristics	Total N = 71
Age, years	
Median	68.0
Range	[38.0, 90.0]
Sex	
Male	48 (67.6)
Female	23 (32.4)
Smoking status	
Ever	56 (78.9)
Never	15 (21.1)
Histology	
Adenocarcinoma	39 (54.9)
Squamous cell carcinoma	24 (33.8)
Others	8 (11.3)
Stage	
II	4 (5.6)
III	22 (41.0)
IV	45 (63.4)
Neoadjuvant therapy	6 (8.4)
Number of tumor cells in EBUS-TBNA specimens	
Median	477
Range	[60, 24912]
Performed lymphnode stations	
#4	43 (60.6)
#7	32 (45.1)
#10, #11	8 (11.3)
Other stations	1 (1.4)

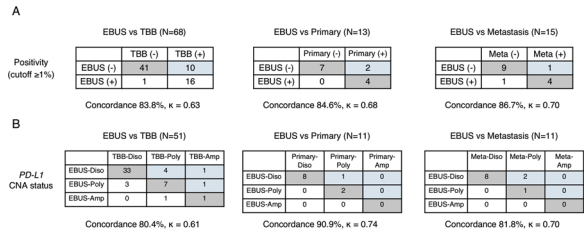

Fig. 2. Concordances of PD-L1 alterations between the EBUS-TBNA and the other derived specimens.
(A) Concordance of PD-L1 expression levels between EBUS-TBNA specimens and the other specimens such as the TBB, the resected primary tumors, and the resected metastases. Positive PD-L1 expression was defined as > 1% TPS.
(B) Concordance of *PD-L1* CNAs between EBUS-TBNA specimens and the other specimens. *PD-L1* CNAs were classified into disomy (Diso), polysomy (Poly), and amplification (Amp).

#7 were most frequently performed by EBUS-TBNA. Neo-adjuvant therapy was prescribed to six (8.4%) patients all of whom received platinum-based chemotherapy prior to the EBUS-TBNA procedure. The median number of tumor cells in an EBUS-TBNA-derived specimen was 477 cells (range, 60—24 912 cells) (Supplementary Fig. S1). More than 1000 tumor cells per EBUS-TBNA procedure were counted in 30 (42.3%) cases.

3.2. Concordance of PD-L1 protein expression

Successful IHC was performed on all EBUS-TBNA-derived and corresponding specimens (Supplementary Table S1). The concordance rates of PD-L1 positivity between EBUS-TBNA-derived specimens and other specimens were 83.8% (vs. TBB), 84.6% (vs. resected primary tumors), and 86.7% (vs. resected metastases) (Fig. 2A and Supplementary Fig. S2A). Kappa coefficients at a cutoff of 1% positivity were moderate: 0.63 (vs. TBB), 0.68 (vs. resected primary tumors), and 0.70 (vs. resected metastases). When PD-L1 TPS was used as a continuous variable, intermediate correlations were observed between two specimens: r = 0.550 (vs. TBB), r = 0.493 (vs. resected primary tumors), and r = 0.693 (vs. resected metastases) (Fig. 3A). PD-L1 TPS was higher in the resected primary and metastatic tumors than in EBUS-TBNA-derived specimens, leading to a proportionally decreased concordance performance when PD-L1 cutoffs were set at higher values such as 5% or 50%; kappa between EBUS-TBNA and TBB specimens was 0.53 and 0.31 at cutoffs ≧ 5% and ≧ 50%, respectively (Supplementary Fig. S3). The number of tumor cells in an EBUS-TBNA specimen was not significantly different between discordant and concordant cases, indicating that the yield of EBUS-TBNA was not likely to affect the concordance of PD-L1 expression with other sites (Supplementary Fig. S4A).

3.3. Concordance of PD-L1 CNAs

PD-L1 CNAs were assessed by FISH, which was successful in 61 (85.9%) of the EBUS-TBNA-derived samples and the following number of corresponding specimens: 55 (75.0%) of TBB, 11 (84.6%) of resected primary tumors, and 11 (73.3%) of metastatic specimens (Supplementary Table S1). FISH analyses were not successful in several cases because of insufficient material or poor signaling intensity. Finally, the following number of paired tissues proceeded for analysis of *PD-L1* CNAs; 51 paired EBUS and TBB specimens, 11 paired EBUS and primary tumor specimens, and 11 paired EBUS and metastatic specimens. The concordance rates of *PD-L1* copy number status between EBUS-TBNA-derived specimens and the others ranged from 80% to 90%: 80.4% (vs. TBB), 90.9% (vs. resected primary tumors), and 81.8% (vs. resected metastases) (Fig. 2B and Supplementary Fig. S2B). Weighted kappa coefficients also showed moderate concordance: 0.61 (vs. TBB), 0.74 (vs. resected primary tumors), and 0.70 (vs. resected metastases). There was no significant difference in the number of tumor cells in EBUS-TBNA-derived specimens between *PD-L1* copy number concordant and discordant cases (Supplementary Fig. S4B). When *PD-L1* copy number per nucleus was analyzed as a continuous variable, strong correlations were observed between EBUS-TBNA-derived and other specimens: r = 0.728 (vs. TBB), r = 0.931 (vs. resected primary tumors), and r = 0.818 (vs. resected metastases) (Fig. 3B). These *PD-L1* CNA correlations were higher than those of PD-L1 expression, and there was no deviation in the *PD-L1* copy number per nucleus from one to another between specimens from different sites.

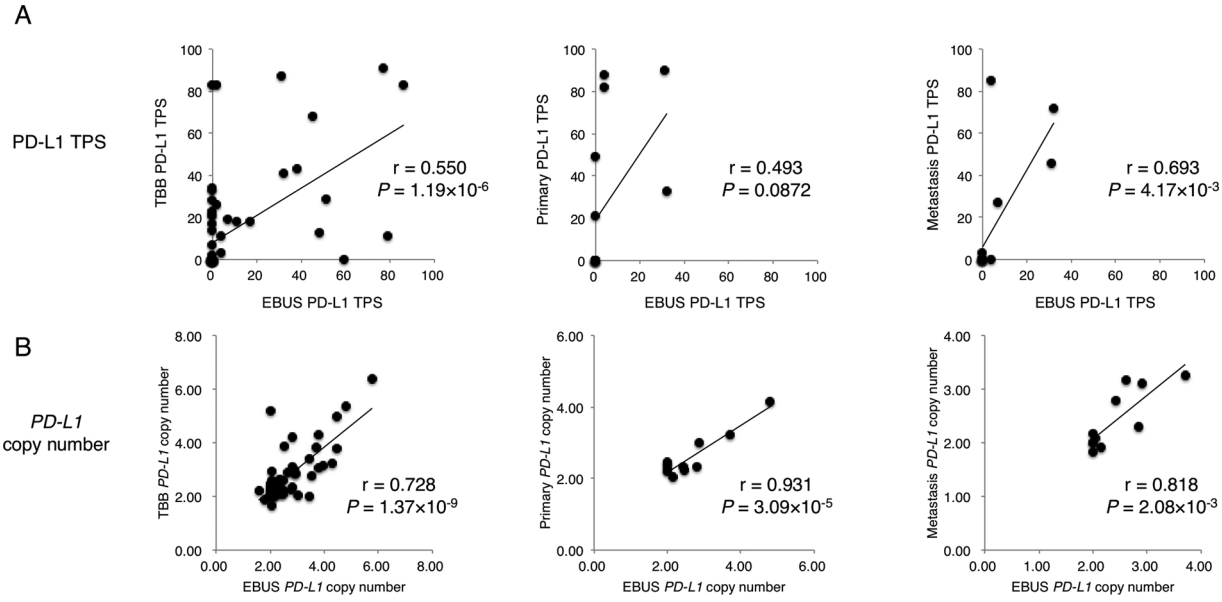

Fig. 3. Correlations of PD-L1 alteration variables between the EBUS-TBNA and the other derived specimens.
(A) Correlations of PD-L1 TPS between EBUS-TBNA specimens and the other specimens (TBB, resected primary tumors, and resected metastatic tumors). r represents Pearson's correlation coefficient.
(B) Correlations of average *PD-L1* copy number per cell between EBUS-TBNA specimens and the other specimens.

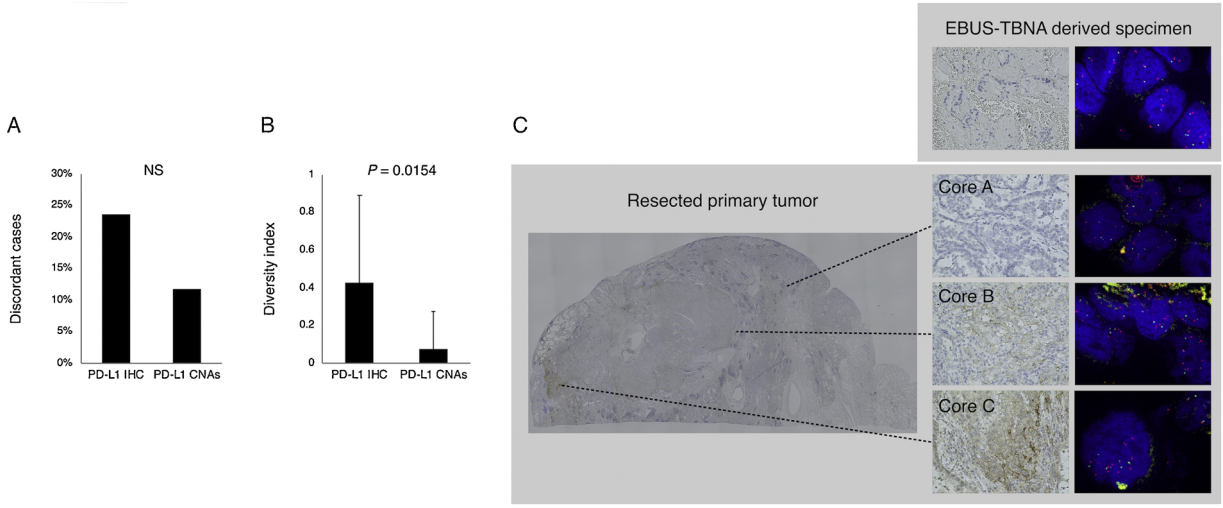

Fig. 4. Intratumoral heterogeneity of PD-L1 alterations in the whole section of resected tumors.
(A) The proportion of discordant cases among the three cores (A, B, and C) of the 17 resected tumors. The number of discordant cases of *PD-L1* CNAs was lower than that of PD-L1 expression.
(B) Diversity index of PD-L1 alterations among the three cores of the 17 resected tumors. The diversity index of *PD-L1* CNAs was significantly lower than that of PD-L1 expression.
(C) Representative images of PD-L1 alteration statuses in cases that intratumorally showed heterogeneous PD-L1 expression. The samples were simultaneously analyzed using PD-L1 IHC and FISH. PD-L1 expressions were discordant between the EBUS-TBNA derived specimen and the resected tumor, and PD-L1 are also heterogeneously expressed in the same primary tumor tissue; PD-L1 TPS were 0% for Core A, 6% for Core B, and 21% for Core C. However, *PD-L1* CNAs were relatively homogenous between two specimens and in the same resected samples; average *PD-L1* copy numbers were 4.00 for Core A, 4.29 for core B, and 3.73 for Core C.

3.4. Association of PD-L1 copy number with protein expression in EBUS-TBNA-derived samples

A total of 61 (85.9%) samples obtained via the EBUS-TBNA procedure were assessed with both PD-L1 IHC and FISH analyses. As shown in Supplementary Fig. S5, there was a weak positive correlation (r = 0.279) between the *PD-L1* gene dosage and protein expression, albeit statistically significant ($P = 0.029$).

3.5. PD-L1 intratumoral heterogeneity within whole sections of resected tumors

The concordance among three cores in the 17 resected tumors including 8 primary tumors and 9 metastatic tumors was analyzed to assess intratumoral heterogeneity. Among 17 resected tumors, 7 (41.1%) tumors were consistently PD-L1-positive in all three cores (Fig. 4A). The average diversity index of PD-L1 expression calculated based on PD-L1 TPS in three cores was 0.427 (Fig. 4B). For *PD-L1* CNAs, there were four polysomic and no amplified cases. Only two (11.8%) cases showed discordance (Fig. 4A). The average diversity index of *PD-L1* CNA was 0.074 (Fig. 4B)—obviously lower than that of PD-L1 expression—indicating that PD-L1 protein expression is more heterogeneous than *PD-L1* CNAs. A representative case is shown in Fig. 4C: PD-L1 expression was completely negative in the EBUS-TBNA derived specimen, while two of three cores in the resected primary tumor were PD-L1-positive (Cores B and C). In contrast, *PD-L1* copy number status was consistent in all three cores.

4. Discussion

We here evaluated the concordance of PD-L1 protein expression and gene copy number between specimens obtained by EBUS-TBNA and other modalities including TBB, biopsy, and surgery. The applicability of EBUS-TBNA for the evaluation for PD-L1 protein expression in patients with NSCLC has been validated previously [27,30]. In addition, we found that *PD-L1* copy number status is also evaluable in EBUS-TBNA-derived biopsy specimens using FISH. The concordance rates of PD-L1 expression and CNAs were comparable intertumorally. However, the concordance of PD-L1 expression decreased based on increasing cutoff values and intratumoral heterogeneity was more prominent in PD-L1 protein expression than in *PD-L1* copy number status. These findings clearly indicated the limitation of the assessment of PD-L1 expression by IHC due to its heterogeneous nature.

Cancer cells are under intense evolutionary pressure and therefore comprise a heterogeneous population. Furthermore, given that PD-L1 expression is induced by IFNγ in adaptive immune resistance [45], heterogeneity of PD-L1 expression is a critical and inevitable problem, particularly when assessed using small biopsy specimens. Because the current decision to use ICIs for the treatment of NSCLC patients is partly dependent on the tumor PD-L1 expression status, this is becoming a more relevant problem that requires urgent attention. Though previous studies have compared the PD-L1 expression status between biopsy samples and primary tumors, EBUS-TBNA-derived specimens were not included [15,17,46]. Only a few studies have reported the utility of biopsy specimens obtained by EBUS-TBNA for the assessment of PD-L1 expression. Sakakibara et al. first reported the concordance among EBUS-TBNA, TBB, and resected specimens [27]. They demonstrated strong correlations between EBUS-TBNA-derived and corresponding specimens. However, the sample size was small, especially for EBUS-TBNA (n = 15). Recently, Sakata et al. compared EBUS-TBNA samples with resected primary tumors in a relatively larger cohort (n = 61) [30]. The concordance rates were 87% with a cutoff of ≧ 1% and 82% with a cutoff of ≧ 50% PD-L1 positivity, respectively. Given that the cutoff of ≧ 50% for PD-L1 positivity resulted in a marked decrease in both sensitivity and positive predictive value of EBUS-TBNA-derived specimens, they concluded that the ≧ 1% PD-L1 positivity was the most appropriate cutoff. The present study corroborated that the concordance of PD-L1 positivity between EBUS-TBNA-derived specimens and corresponding tumor specimens obviously decreased based on increasing cutoff values. This should be largely attributed to the fact that small EBUS-TBNA cores had significantly lower PD-L1 TPSs than resected primary tumors, indicating that PD-L1 expression in EBUS-TBNA

samples is apt to be underestimated when the cutoffs are set at higher values such as 5% and 50%. Indeed, Irie et al. reported a 47% discordance rate between 170 pairs of TBB and resected tumor specimens [15] and claimed that PD-L1 expression in TBB specimens was often underestimated. Nonetheless, PD-L1 expression in EBUS-TBNA specimens was concordant with the corresponding tumors in most cases in our study when the cutoff was defined as ≥ 1%. This result validated the utility of EBUS-TBNA cores for the assessment of tumor PD-L1 positivity when the ≥ 1% of cutoff is applied.

Amplification of the chromosome band 9p.24.1, including the loci encoding the *PD-L1* and *PD-L2* genes, induces PD-L1 expression both as an innate [47,48] and adaptive mechanism [49]. In Hodgkin's lymphoma, tumors with 9p24.1 CNAs are frequently observed and associated with PD-L1 expression and JAK-STAT signaling [26]. Clinical responses to nivolumab on malignant tumors with 9p24.1 amplification were also reported in several case reports [23–26]. A recent study analyzed 118,187 tumor samples by next-generation sequencing and showed that *PD-L1*-amplified tumors tended to show better response to ICIs [23]. Moreover, loss of *PD-L1* amplification was reported as an acquired resistance mechanism to nivolumab in a patient with colon cancer [50]. We previously reported the prevalence of *PD-L1* copy number gains in NSCLC patients and showed that *PD-L1* CNAs were relatively homogenous between resected primary tumors and resected metastatic lymph nodes than PD-L1 protein expression [20]. Evaluation of CNAs using the FISH technique in EBUS-TBNA specimens has been validated for the evaluation of relevant oncogene status such as *EGFR* and *ALK* [29,51]. Accordingly, we here showed that EBUS-TBNA samples were well assessed by FISH analysis for the evaluation of *PD-L1* copy number and that the concordance of *PD-L1* CNAs between EBUS-TBNA-derived and corresponding specimens was comparable with that of PD-L1 expression. In addition, we also showed that *PD-L1* CNAs were intratumorally more homogeneous than PD-L1 protein expression using whole tumor sections. It should be noted that there is currently no evidence established by prospective trials showing that *PD-L1* CNAs have any potential to predict efficacy of ICIs in patients with NSCLC. However, our results indicate that *PD-L1* CNAs could provide useful and reproducible complementary information to predict the efficacy of ICIs, especially in cases where only small biopsy specimens obtained by EBUS-TBNA are available.

There are several limitations in this study. First, the number of cases in which we compared EBUS-TBNA and resected specimens was inadequate due to the fact that most patients who underwent EBUS-TBNA had advanced diseases and thus only a few of them received surgery. Second, the processes of the EBUS-TBNA procedure including the number of samples collected and punctures were not uniformly determined because of the retrospective nature of this study. Also, several cases were not successfully evaluable for *PD-L1* by FISH due to sampling and technical problems. Additional large studies are necessary to confirm our findings. Third, we applied clone E1L3N for the PD-L1 staining in this study. This is a laboratory developed test that is not a companion/complementary diagnostic assay such as 22C3, 28-8, and SP142 for the use of specific PD-1/PD-L1 inhibitors. E1L3N has not been validated in clinical trials dealing with ICIs and it does not have the industrial staining platform. However, the correlation among the aforementioned PD-L1 antibodies has been intensively studied [8–10,52] and E1L3N showed strong correlations with other antibodies [10,52]. Lastly, prospective clinical trials are warranted to confirm the applicability of EBUS-TBNA-derived specimens for the evaluation of PD-L1 IHC and CNAs and to evaluate the predictability of efficacy of *PD-L1* CNAs in the treatment with ICIs in patients with NSCLC.

5. Conclusions

We demonstrated that EBUS-TBNA samples can be assessed by both PD-L1 IHC and FISH assays and that the concordance rate of PD-L1 alterations between EBUS-TBNA and other derived specimens was comparable in both assays. However, spatial heterogeneity should be taken into account, especially to interpret PD-L1 protein expression when using high cutoffs. Further studies and clinical trials are necessary to investigate the utility of EBUS-TBNA for the assessment of PD-L1 alterations when using PD-1/PD-L1 inhibitors to treat patients with NSCLC.

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Conflicts of interest

The authors declare no competing interests.

Author contributions

All authors contributed toward the conception and design, data analysis, drafting, and critically revising the paper, and agree to be accountable for all aspects of the work.

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Appendix A. Supplementary data

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