Associations of immune checkpoint inhibitor therapy efficacy with clinical parameters and tumor-infiltrating CD68-positive cell counts in patients with EGFR-mutant non-small cell lung cancer

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Abstract. Immune checkpoint inhibitor (ICI) therapy has been less effective in patients with non-small cell lung cancer (NSCLC) harboring epidermal growth factor receptor (EGFR) mutations than in patients with EGFR wild-type NSCLC. This retrospective study was conducted to investigate the associations of clinical parameters with the efficacy of ICI therapy in patients with EGFR-mutant NSCLC. Clinical information was retrieved from the medical charts, and immunohistochemical analysis was performed in some cases to determine the tumor-infiltrating CD68-positive cell count. Data from 46 patients were included in the analysis. The median (95% confidence interval) progression-free survival and overall survival from the initiation of ICI therapy was 1.4 months (1.0-1.7 months) and 6.4 months (3.9-19.0 months), respectively. Analysis using a Cox proportional hazards model revealed that tumor programmed death-ligand 1 expression was associated with the overall survival of patients with EGFR-mutant NSCLC after ICI treatment. The tumor-infiltrating CD68-positive cell count was evaluated in 11 patients. Comparison using the log-rank test revealed that the progression-free survival time after ICI treatment was longer in the patients with lower tumor-infiltrating CD68-positive cell counts than those with higher tumor-infiltrating CD68-positive cell counts. The present analysis demonstrated that PD-L1 expression and the tumor-infiltrating CD68-positive cell count may be associated with the efficacy of ICI therapy in patients with NSCLC harboring EGFR mutations.

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Introduction

Numerous studies have reported more favorable survival outcomes of immune checkpoint inhibitor (ICI) therapy, either alone or in combination with cytotoxic agents, as compared with cytotoxic agent therapy alone, in patients with non-small cell lung cancer (NSCLC) (1-5). However, ICI therapy has also been reported to be relatively less effective in patients with NSCLC harboring epidermal growth factor receptor (EGFR) mutations than in those with tumors harboring wild-type EGFR (2,6).

While tumor programmed death-ligand 1 (PD-L1) expression may be associated with the efficacy of ICI treatment in patients with non-squamous NSCLC (2), the reports about the efficacy of ICI therapy in patients with EGFR-mutant NSCLC are not consistent (7-12). Furthermore, although the serum level of lactate dehydrogenase (LDH), the peripheral blood neutrophil-lymphocyte ratio (NLR), and serum C-reactive protein (CRP) level may be associated with survival after the initiation of ICI treatment in patients with NSCLC (13-16), it remains unclear if the same associations can also be observed in patients with EGFR-mutant NSCLC.

Regarding the influence of the tumor microenvironment in patients with cancer, the number of tumor-infiltrating macrophages can affect the clinical course in patients with malignancies (17). Notably, macrophages contribute to the early elimination of cancer. However, tumor progression is associated with skewing of macrophage function (18), and macrophages recruited by the cancer cells promote the survival and proliferation of cancer cells (19,20). Such macrophages can also be a therapeutic target in patients with cancer (18). However, their prognostic impact is dependent on the treatment employed (18), and the association between the tumor-infiltrating macrophage count and the efficacy of ICI

treatment in patients with EGFR-mutant NSCLC has not been clarified.

We conducted this retrospective study to investigate the associations of some clinical parameters and tumor-infiltrating CD68-positive cell counts with the efficacy of ICI therapy in patients with EGFR-mutant NSCLC.

Patients and methods

Patients. We conducted a retrospective analysis of the data of consecutive patients who had been diagnosed as having EGFR-mutant advanced NSCLC and had received ICI monotherapy between 2016 and 2022 at Toyama Prefectural Central Hospital or Toyama University Hospital. No exclusion criteria were established.

This study was conducted in accordance with the Declaration of Helsinki and the Ethical Guidelines for Medical and Health Research Involving Human Subjects (Ministry of Health, Labour and Welfare, Japan). The requirement for informed consent was waived and we disclosed the study information on our website to the patients, under the approval of the Ethics Committee, University of Toyama (approval number R2019040).

Clinical information. Clinical information on the patient background characteristics and the clinical course was retrieved from the medical charts. Results of blood tests performed at the onset of ICI treatment or the most recent tests performed within the previous month were evaluated, and the patients were subdivided by the median value (NLR: 5; serum LDH: 220 U/l; serum CRP: 0.5 mg/dl). The NLR was calculated by dividing the number of neutrophils by the number of lymphocytes. Tumor PD-L1 expression was determined using the 22C3 antibody in tumor specimens obtained at any time during the entire clinical course of the patient, either before or after the EGFR-tyrosine kinase inhibitor (EGFR-TKI) therapy. The proportion of PD-L1-positive tumor cells was calculated as the tumor proportion score (TPS). A positive history of radiation therapy was defined as radiation therapy administered within 6 weeks prior to the initiation of ICI therapy or during the ICI therapy.

Immunohistochemistry. The tumor immunohistochemistry was commissioned to Mediridge Co., Ltd (Tokyo, Japan) and performed on primary or metastatic tumor specimens obtained from 11 NSCLC patients who had received treatment at Toyama University Hospital between 2016 and 2019.

For immunohistochemical staining, formalin-fixed paraffin-embedded tumor tissues were deparaffinized using xylene and an 80-100% downgraded ethanol series. Antigen retrieval treatment was performed with 0.1% trypsin (T4799-25G; Sigma-Aldrich Corporation, St. Louis, MO, USA)/PBS (pH 7.6) at 37˚C for 15 min. Endogenous peroxidase was blocked with 3% hydrogen peroxide, and the non-specific reaction was blocked with Blocking One (#03953-95; Nacalai Tesque, Kyoto, Japan). For the primary antibody, we incubated the sections with anti-human CD68 mouse IgG1 monoclonal antibody (clone: Kp-1, M0814, at 1:200 dilution, Agilent Inc., Santa Clara, CA) overnight at 4˚C. Positive reactions were visualized using horse radish peroxidase-conjugated secondary

Table I. Patient characteristics.

Variable	No. of patients (%)
Sex	
Male	21 (45.7)
Female	25 (54.3)
Age, years	
<70	23 (50.0)
≥70	23 (50.0)
Smoking history	
Yes	18 (39.1)
No	28 (60.9)
PS	
0-1	31 (67.4)
≥2	15 (32.6)
Histology	
Adenocarcinoma	43 (93.5)
Others	3 (6.5)
EGFR	
Exon 19 del	19 (41.3)
L858R	19 (41.3)
Others	8 (17.4)
PD-L1, %	
<1	16 (34.8)
1-49	11 (23.9)
≥50	10 (21.7)
Unknown	9 (19.6)
History of radiation therapy	
Yes	9 (19.6)
No	37 (80.4)
ICI	
Nivolumab	16 (34.8)
Pembrolizumab	13 (28.3)
Atezolizumab	17 (37.0)
ICI treatment line	
2	4 (8.7)
3	10 (21.7)
4	13 (28.3)
5	9 (19.6)
≥6	10 (21.7)
NLR	
<5	25 (54.3)
≥5	21 (45.7)
LDH, U/l	
<220	21 (45.7)
≥220	25 (54.3)
CRP, mg/dl	
<0.5	24 (52.2)
≥0.5	22 (47.8)

CRP, serum C-reactive protein; EGFR, epidermal growth factor; ICI, immune checkpoint inhibitor; LDH, serum lactate dehydrogenase; NLR, neutrophil-lymphocyte ratio; PD-L1, programmed death ligand-1; PS, performance status.

Table II. Associations between clinical parameters and progression-free survival after initiation of immune checkpoint inhibitor therapy according to the Cox proportional hazards model.

Variable	HR	95% CI	P-value
PS			
0-1	1.00		
≥2	0.82	0.37-1.82	0.625
EGFR status			
Exon 19 del	1.15	0.54-2.46	0.713
L858R	1.00		
Others	1.41	0.55-3.61	0.471
PD-L1, %			
≥50	0.63	0.23-1.72	0.368
<50	1.00		
Unknown	0.96	0.38-2.43	0.926
History of radiation therapy			
Yes	0.61	0.23-1.61	0.319
No	1.00		
NLR			
<5	1.00		
≥5	2.32	1.00-5.38	0.051
LDH, U/l			
<220	1.00		
≥220	1.02	0.51-2.05	0.947
CRP, mg/dl			
<0.5	1.00		
≥0.5	0.67	0.31-1.47	0.319

CRP, serum C-reactive protein; EGFR, epidermal growth factor; HR, hazard ratio; LDH, serum lactate dehydrogenase; NLR, neutrophil-lymphocyte ratio; PD-L1, programmed death ligand-1; PS, performance status.

Table III. Associations between clinical parameters and overall survival after initiation of immune checkpoint inhibitor therapy according to the Cox proportional hazards model.

Variable	HR	95% CI	P-value
PS			
0-1	1.00		
≥2	2.30	0.95-5.60	0.066
EGFR status			
Exon 19 del	2.12	0.84-5.37	0.113
L858R	1.00		
Others	1.75	0.60-5.14	0.308
PD-L1, %			
≥50	0.16	0.04-0.62	0.008
<50	1.00		
Unknown	0.36	0.10-1.32	0.124
History of radiation therapy			
Yes	2.06	0.70-6.06	0.188
No	1.00		
NLR			
<5	1.00		
≥5	1.68	0.73-3.88	0.221
LDH, U/l			
<220	1.00		
≥220	1.80	0.74-4.37	0.193
CRP, mg/dl			
<0.5	1.00		
≥0.5	0.82	0.32-2.11	0.676

CRP, serum C-reactive protein; EGFR, epidermal growth factor; HR, hazard ratio; LDH, serum lactate dehydrogenase; NLR, neutrophil-lymphocyte ratio; PD-L1, programmed death ligand-1; PS, performance status.

antibody (HISTOFINE #424134, Nichirei Bioscience Inc., Tokyo, Japan) and 3-3' diaminobenzidine as the substrate.

Determination of the tumor-infiltrating cell count was performed by two investigators who were blinded to the clinical courses of the patients. The number of CD68-positive cells in the tumor tissue or stroma in contact with the tumor tissue per field of view (400x magnification) were counted in up to 10 fields per section, and the mean CD68-positive cell count was used for the analysis.

Statistical analysis. The endpoint of the present study was the progression-free survival (PFS) and overall survival (OS). The PFS was calculated from the initiation date of ICI therapy to the detection date of disease progression. Disease progression was defined according to the clinical judgment or computed tomography evidence of progressive disease (PD) and censored at the last visit without disease progression. PD was defined as a 20% or greater increase in the diameter of the target lesion, emergence of new lesions, deterioration of the general condition of the patient, or death. The OS was calculated from the day that ICI therapy was initiated to the day of death and censored at the last visit during the life of the patient. Survival curves were drawn by the Kaplan-Meier method and survival was compared by the log-rank test between patient groups subdivided according to categorical variables.

A Cox proportional hazards model was used to evaluate the associations between the clinical parameters and the PFS or OS. We included the patient performance status (PS), EGFR status (9), tumor PD-L1 expression status (2), values of NLR, LDH, and CRP (13-16), and history of radiation therapy (21) as independent variables because they may be associated with the efficacy of ICI treatment. Fisher's exact test was used to compare the patient characteristics. $P<0.05$ was considered to indicate a statistically significant difference. Statistical analysis was performed using JMP ver. 14.0.2 (SAS, Cary, NC).

Results

Patient characteristics. Table I shows the patient characteristics. A total of 46 patients with EGFR-mutant NSCLC received ICI monotherapy at Toyama University Hospital

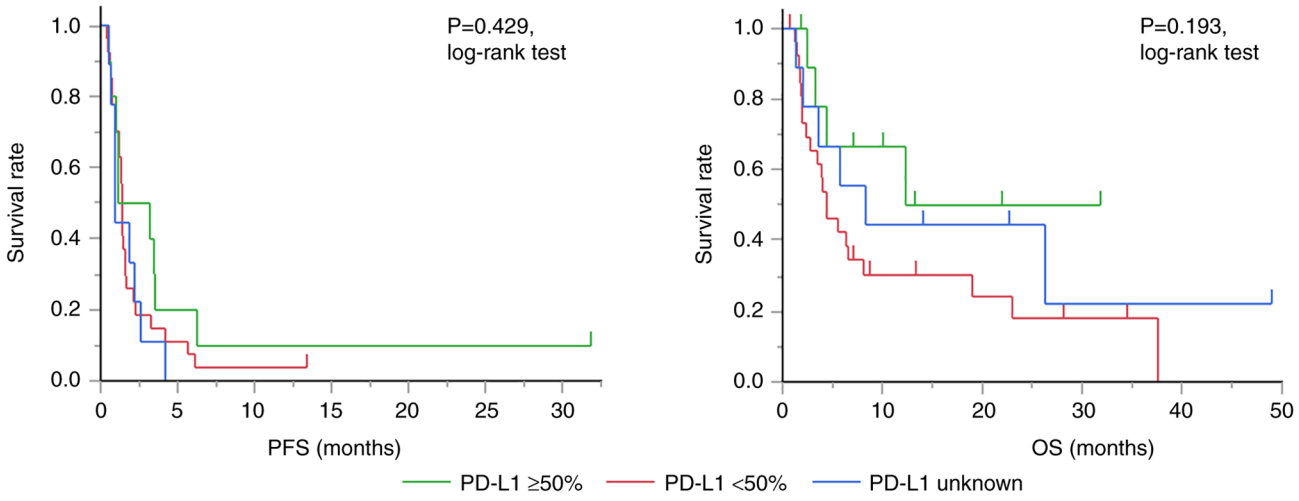

Figure 1. Comparison of PFS and OS after the initiation of immune checkpoint inhibitor therapy in patients with epidermal growth factor receptor-mutant non-small cell lung cancer according to tumor PD-L1 expression level. OS, overall survival; PD-L1, programmed death-ligand 1; PFS, progression-free survival.

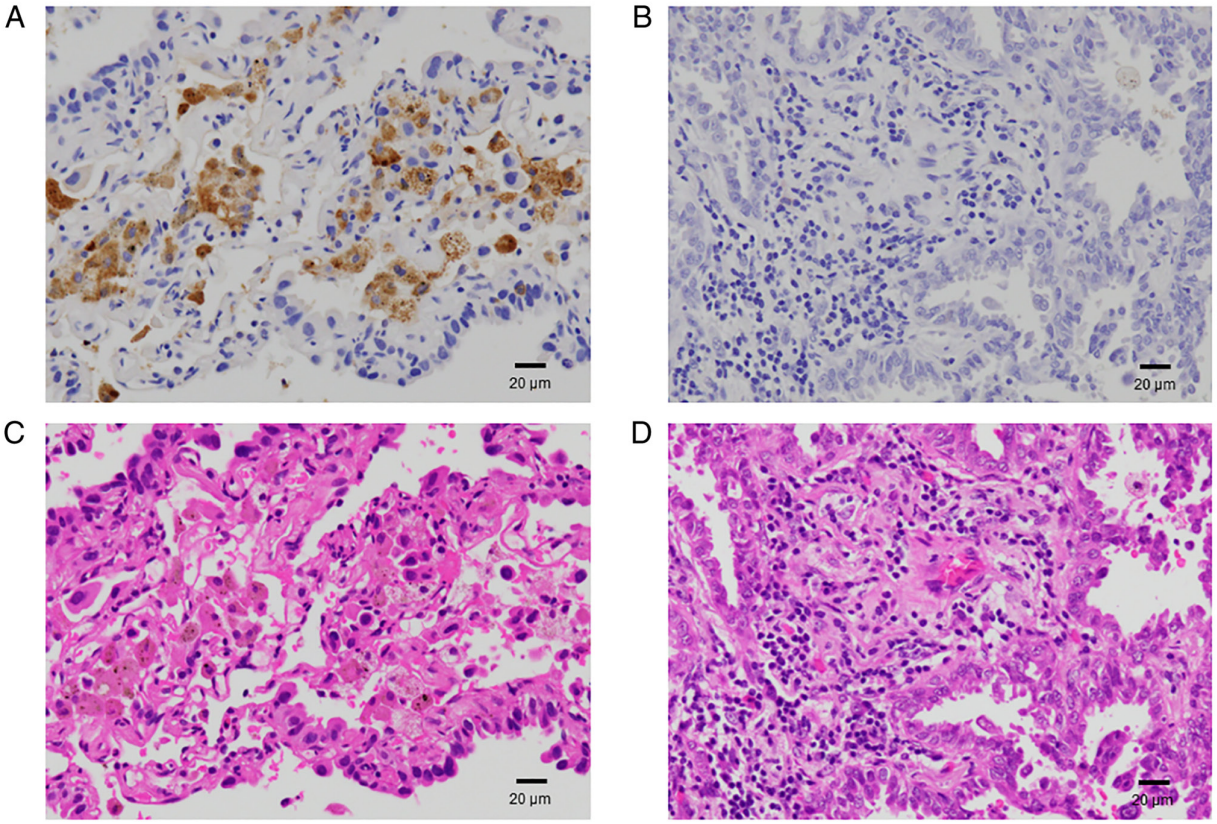

Figure 2. A representative image of high (mean CD68-positive cells ≥5/field) and low (mean CD68-positive cells <5/field) CD68-positive cells in the tumor tissue or tumor stroma in contact with the tumor tissue. (A) CD68 staining of specimen with high CD68-positve cell count (CD68-positive cell count is 23.2). (B) CD68 staining of specimen with low CD68-positive cell count (CD68-positve cell count is 2.0). (C) Hematoxylin-eosin staining of specimen with high CD68-positive cell count. (D) Hematoxylin-eosin staining of specimen with low CD68-positve cell count.

or Toyama Prefectural Central Hospital. Of the 46 patients, 43 (93.5%) were diagnosed with adenocarcinoma, 2 (4.3%) with NSCLC (not otherwise specified), and 1 (2.2%) with squamous cell carcinoma. PD-L1 TPS was ≥50% in 10 (21.7%) patients and was not evaluated in 9 (19.6%) patients. Of the 46 patients, 43 (93.5%) had a previous history of EGFR-TKI therapy prior to ICI therapy, including gefitinib (n=10), erlotinib (n=4), erlotinib plus bevacizumab (n=6), afatinib (n=19), or osimertinib (n=4). Of these, 36 patients had received EGFR-TKI therapy as first-line therapy, and 7 (15.2%) patients as second-line therapy. The EGFR-TKI therapy was discontinued because of acquired resistance to the drug in 41 patients and because of the emergence of adverse events in 2 patients. T790M mutation was detected in 12 (30.8%) patients out of

Table IV. Information on the specimens for evaluation of the CD68-positive cell count.

Age, years	Sex	Histology	EGFR	Organ	Procedure	Duration, months	Before/after the TKI therapy	CD68, /field
68	F	Adeno	L858R	Bone	Biopsy	Not assessed[a]	Before	1.2
81	M	Adeno	del 19	Lung	Surgical resection	56.1	Before	2.0
74	M	Adeno	del 19	Lymph node	Biopsy	22.3	Before	3.1
60	F	Adeno	L858R	Lung	Biopsy	35.1	After	4.1
57	M	NOS	del 19/ins	Lung	Biopsy	5.1	After	4.7
80	F	Adeno	L858R	Bone	Biopsy	3.2	After	5.4
69	F	NOS	del 19	Brain	Surgical resection	26.8	After	5.8
87	F	Adeno	L858R	Lung	Biopsy	0.7	After	6.7
77	F	Adeno	L858R	Lung	Biopsy	67.9	After	6.9
65	M	Adeno	L858R	Lung	Biopsy	9.5	Before	18.5
60	M	Adeno	del 19	Lung	Biopsy	18.0	Before	23.2

[a]The only patient who underwent biopsy after the initiation of immune checkpoint inhibitor therapy. Duration denotes the period from the biopsy to the initiation of immune checkpoint inhibitor therapy. Before/after the TKI therapy denotes that biopsy was performed before or after the initiation of the treatment with EGFR-TKIs. Adeno, adenocarcinoma; EGFR, epidermal growth factor receptor; F, female; M, male; NOS, not otherwise specified, TKI, tyrosine kinase inhibitor.

39 patients treated with first- or second-generation EGFR-TKIs prior to ICI therapy.

Clinical parameters. The median (95% confidence interval) PFS and OS after the initiation of ICI treatment was 1.4 (1.0-1.7) and 6.4 (3.9-19.0) months, respectively. Tables II and III show the results of analyses performed using the Cox proportional hazards model. The PD-L1 expression level was significantly associated with the OS. Fig. 1 shows the Kaplan-Meier curve comparing the PFS and OS after the initiation of ICI therapy according to PD-L1 expression levels.

Immunohistochemistry. We conducted an immunohistochemical analysis to evaluate the degree of infiltration of the tumor tissue and tumor stroma in contact with the tumor tissue by CD68-positive cells. Representative images of positive and negative immunohistochemistry results are shown in Fig. 2. The patient characteristics are shown in Table IV. The average number of CD68-positive cells per field of view varied from 1.2 to 23.2 in the patients, with a median of 5.4. Fig. 3 shows a comparison of the PFS between the groups with low (CD68-positive cells <5/field) and high tumor-infiltrating CD68-positive cell (CD68-positive cells ≥5/field) counts. The PFS was significantly worse in the group with a high tumor-infiltrating CD68-positive cell count than that in the group with a low tumor-infiltrating CD68-positive cell count.

Table V shows a comparison of the patient background characteristics between those with high and low CD68-positive cell counts. There were no apparent differences between the two groups, and none of the patients, except one, had received radiation therapy prior to ICI therapy.

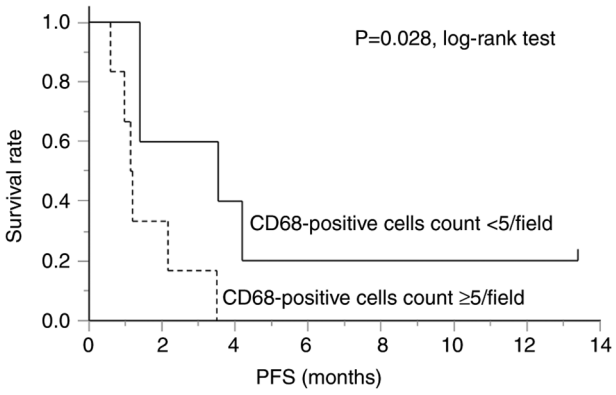

Figure 3. Comparison of the PFS after the initiation of immune checkpoint inhibitor therapy in patients with epidermal growth factor receptor-mutant NSCLC with high and low tumor-infiltrating macrophage counts in the tumor specimens. PFS, progression-free survival.

Discussion

In the present study, the median of PFS after the initiation of ICI therapy was 1.4 months in patients with EGFR-mutant NSCLC, suggesting that ICI therapy is relatively less effective in this subset of NSCLC patients. Conversely, the results suggested that the PD-L1 expression level and CD68-positive cell count in the tumor microenvironment are significantly associated with the efficacy of ICI therapy.

The present study revealed an association between the OS after the start of ICI therapy and the tumor PD-L1 expression status. Although there is an opposing report (9), several authors also reported the association between positive tumor PD-L1 expression and survival benefits in patients with EGFR-mutant NSCLC (7,11,12). However, it has been reported that the tumor PD-L1 expression status can change during EGFR-TKI therapy (6,12). Furthermore, tumor PD-L1 expression may be induced by EGFR signaling (22) and interferon γ (23). Given that the CD8-positive T cell density is significantly higher in PD-L1-positive EGFR-mutant tumors than in PD-L1-negative or low-positive tumors after EGFR-TKI treatment (12), tumor

Table V. Characteristics of the patients evaluated for CD68-positive cell count.

Variable	CD68 <5/field, n (%)	CD68 ≥5 /field, n (%)	P-value
Sex			
Male	3 (60.0)	2 (33.3)	0.567
Female	2 (40.0)	4 (66.7)	
Age, years			
<70	3 (60.0)	3 (50.0)	>0.999
≥70	2 (40.0)	3 (50.0)	
Smoking history			
Yes	3 (60.0)	2 (33.3)	0.567
No	2 (40.0)	4 (66.7)	
PS			
0-1	3 (60.0)	3 (50.0)	>0.999
≥2	2 (40.0)	3 (50.0)	
Histology			
Adenocarcinoma	4 (80.0)	5 (83.3)	>0.999
Others	1 (20.0)	1 (16.7)	
EGFR			
Exon 19 del	3 (60.0)	2 (33.3)	0.567
L858R	2 (40.0)	4 (66.7)	
Others	0 (0.0)	0 (0.0)	
PD-L1 TPS, %			
<1	3 (60.0)	4 (66.7)	>0.999
≥1	2 (40.0)	2 (33.3)	
Unknown	0 (0.0)	0 (0.0)	
ICIs			
Nivolumab	1 (20.0)	2 (33.3)	>0.999
Pembrolizumab	2 (40.0)	2 (33.3)	
Atezolizumab	2 (40.0)	2 (33.3)	
NLR			
<5	2 (40.0)	3 (50.0)	>0.999
≥5	3 (60.0)	3 (50.0)	
LDH, U/l			
<220	1 (20.0)	4 (66.7)	0.242
≥220	4 (80.0)	2 (33.3)	
CRP, mg/dl			
<0.5	1 (20.0)	4 (66.7)	0.242
≥0.5	4 (80.0)	2 (33.3)	
History of radiation therapy			
Yes	1 (20.0)	0 (0.0)	0.455
No	4 (80.0)	6 (100.0)	

CRP, serum C-reactive protein; EGFR, epidermal growth factor; ICI, immune checkpoint inhibitor; LDH, serum lactate dehydrogenase; NLR, neutrophil-lymphocyte ratio; PD-L1, programmed death ligand-1; PS, performance status; TPS, tumor proportion score.

PD-L1 expression may reflect the infiltration of CD8-positive T-lymphocytes. However, EGFR signaling also suppresses tumor immunity by increasing the production of C-C motif chemokine 22 (CCL22), which recruits regulatory T cells, and decreasing the production of C-X-C motif chemokine ligand 10 and CCL5 which are known to induce CD8+ T cell infiltration (24).

In the present study, the NLR, LDH, and CRP did not exhibit a significant association with survival after the initiation of ICI treatment in patients with EGFR-mutant NSCLC. Alternatively, a meta-analysis of patients with various solid tumors has shown an association between the NLR and patient survival across disease stages (25). Furthermore, an elevated

NLR has been reported to be associated with poor survival after ICI treatment in patients with NSCLC (26). Tumor-infiltrating lymphocytes and neutrophils (CD15-positive) have been reported as favorable and poor prognostic factors, respectively, in cancer patients (27,28). Moreover, cytotoxic T-lymphocyte cell-lytic activity was observed to be inhibited by neutrophils in-vitro (29). These findings may explain the association between the NLR and the prognosis in NSCLC patients treated with ICIs. The results of the present study suggest that this association may not be found in patients with EGFR-mutant NSCLC. Therefore, it may be difficult to predict the efficacy of ICI therapy based on the NLR in patients with EGFR-mutant NSCLC.

Additionally, γ-irradiation can cause immunogenic cell death of tumor cells, which reportedly induce the release of tumor antigens and danger-associated molecular patterns, triggering tumor immunity (21). However, it remains unclear if radiotherapy enhances the clinical effectiveness of ICI therapy. A phase II trial conducted to investigate the effect of radiotherapy in enhancing the response to pembrolizumab in patients with NSCLC failed to meet the prespecified endpoint criteria (30). In contrast, the results of a subgroup analysis in patients with PD-L1-negative tumors suggested a beneficial effect of the addition of radiotherapy. Furthermore, a secondary analysis of the KEYNOTE-001 phase 1 trial suggested that radiotherapy in patients with advanced NSCLC may yield a longer survival in patients treated with pembrolizumab (31). We failed to show any association between radiation therapy and the efficacy of ICI treatment. However, the possibility of decreased statistical power due to the small sample size affecting the results of the analysis cannot be excluded.

In the present study, higher tumor-infiltrating CD68-positive cell counts were found to be associated with a shorter PFS after the initiation of ICI therapy. Previously, it was reported that the number of tumor-infiltrating macrophages can affect the clinical course in patients with malignancies (17). Furthermore, infiltration of macrophage was reported to be associated with PFS in patients with EGFR/ALK wild type NSCLC treated with ICI therapy (32). As for patients with EGFR mutant NSCLC, it was reported that infiltration of CD8-positive T cells (7), CD4-positive T cells and Foxp3-positive cells (10) were associated with PFS after the initiation of ICI therapy. However, CD68-positive cells were not investigated in these previous studies. Macrophages are recruited and polarized to the M2 phenotype to promote the survival and proliferation of cancer cells (19,20). However, because the timing at which the specimens were obtained varied among the patients, we could not evaluate the tumor microenvironment immediately before the start of the ICI therapy in all cases. Thus, the findings in this study need to be interpreted with caution, and further studies are warranted to elucidate the association between the tumor microenvironment and the efficacy of ICI therapy.

The major limitations of the present study were the small sample size and retrospective design. Thus, bias or random error may have affected the statistical analysis.

In conclusion, the present study showed that the PD-L1 expression level and tumor-infiltrating CD68-positive cell count might be associated with the efficacy of ICI therapy. Further investigation is required to verify this association.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

MI and KaT contributed to conception and design of the study. TT, KS and MI contributed to the analysis and interpretation of data. MI, TT, KS, KH, IM, KoT, CT, SO, KK, SI, TM, RH, SM, YM and HT contributed to data acquisition. MI and TT wrote the main manuscript, and KH, IM, KoT, CT, SO, KK, SI, TM, RH, SM, YM, HT and KaT were involved in revising the manuscript. MI and TT confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was conducted in accordance with the Declaration of Helsinki and Ethical Guidelines for Medical and Biological Research Involving Human Subjects (Ministry of Health, Labour and Welfare, Japan), and approved by the Ethics Committee, University of Toyama (Toyama, Japan, approval no. R2019040). The need to obtain informed consent from the study subjects was waived under the approval of the Ethics Committee, University of Toyama, and information about the study was disclosed to the subjects on the Toyama University Hospital website (http://www.hosp.u-toyama.ac.jp/guide/index.html).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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