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Title: Morphological, immune and genetic features in biopsy sample associated with the efficacy of pembrolizumab in patients with non-squamous non-small cell lung cancer

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Abstract

Introduction Usefulness of the histopathology of biopsy samples for predicting the efficacy of immunotherapy in non-squamous, non-small cell lung cancer (NSq NSCLC) patients remains unclear.

Methods We retrospectively investigated the associations between the histopathological features in biopsy samples and survival outcomes in advanced NSq NSCLC patients receiving pembrolizumab. NSq NSCLC was classified histopathologically as morphological adenocarcinoma or non-small cell carcinoma (NSCC: absence of definitive features of either adenocarcinoma or a squamous morphology). We investigated the association between the tumor morphological features and immune/genetic features by examining the tumor PD-L1 expression and tumor mutation burden (TMB).

Results Among 33 advanced NSq NSCLC patients with tumor PD-L1 scores ≥50% receiving pembrolizumab as first-line therapy, a biopsy diagnosis of NSCC was associated with a significantly longer progression-free survival (median, 16.8 vs. 2.3 months; hazard ratio [HR], 0.26; 95% CI 0.10-0.62, *P*=0.01) and overall survival (median, NR vs. 10.1 months; HR, 0.35; 0.12-0.97, *P*=0.04) as compared to that of morphological adenocarcinoma. In an analysis of 367 biopsy samples, the NSCC group showed a higher percentage of samples with PD-L1 scores ≥50%than the morphological adenocarcinoma group (35% vs. 10%). The NSCC group (n=8) also showed a significantly higher TMB than the morphological adenocarcinoma group (n=7) (median 236 vs. 25 mutations/whole-exome, *P*=0.01).

Conclusion Absence of definitive morphological features in a biopsy sample could be a useful predictor of

the efficacy of pembrolizumab in NSq NSCLC patients with tumor PD-L1 scores ≥50%, as these tumors are likely to show high tumor PD-L1 expression and high TMB.

Keywords:

Lung cancer, Pembrolizumab, Morphological features, PD-L1, TMB

Declarations:

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Ethics approval:

This study was conducted with the approval of the Institutional Review Board of the National Cancer Center. The approval number for this study was 2019-098 and 2018-134. All the methods were performed in accordance with the approved guidelines.

Consent to participate:

All the specimens and data were collected after obtaining written comprehensive informed consent from the patients.

Consent to publication:

Consent was obtained

Authors contributions

TS prepared the manuscript, HU designed the concepts and revised the manuscript. All authors read and approved the final manuscript.

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Introduction

Non-small cell lung cancer (NSCLC) is the most common cause of cancer-related death worldwide.(Siegel et al. 2018) Inhibitors of programmed death-1 (PD-1) and programmed death-ligand 1 (PD-L1) have recently become therapeutic options for metastatic NSCLC. Among advanced NSCLC patients with a PD-L1 score ≥50%, pembrolizumab significantly improves the progression-free survival (PFS), compared with platinum-based chemotherapy, and has become a standard of care regimen for first-line treatment.(Reck et al. 2016) Furthermore, the addition of pembrolizumab to standard platinum-based chemotherapy has also shown a survival benefit in untreated, metastatic NSCLC patients regardless of the PD-L1 score (Gandhi et al. 2018); (Paz-Ares et al. 2018).

Several predictive markers of the efficacy of pembrolizumab, such as PD-L1 expression, tumor-mutation burden (TMB) and DNA mismatch-repair (MMR) deficiency, have already been reported (Ott et al. 2019). From the perspective of histopathology, only a few studies have shown that pleomorphic carcinoma diagnosed using “surgical specimens” responds remarkably to pembrolizumab (Kim et al. 2015);(Ikematsu et al. 2017). No study has shown a relationship between histopathological features in “biopsy samples” and the efficacy of pembrolizumab. Depending on the presence of morphological features, biopsy samples of non-squamous (NSq) cell NSCLC are classified as morphological adenocarcinoma or non-small cell carcinoma (NSCC), the latter of which is characterized by the absence of both a definite adenocarcinoma and a squamous morphology. NSCC is further classified as NSCC-favor adenocarcinoma or NSCC not-otherwise-specified (NOS) based on immunohistochemical features (Travis et al. 2015).

However, the relationship between these histopathological features and the efficacy of pembrolizumab is not well understood. The evaluations of PD-L1 expression, TMB and DNA MMR deficiency cost time and money because of the need to perform additional immunohistochemistry (IHC) or next-generation sequencing studies (Ott et al. 2019); (Rizvi et al. 2018);(Rizvi et al. 2015);(Hellmann et al. 2018); (Le et al. 2015). On the other hand, the evaluation of histopathological features is universally available and easily applicable. If histopathological features are associated with favorable factor to PD-1 blockade, and could be a biomarker for the efficacy of pembrolizumab, this finding would have a meaningful impact on clinical practice. Thus, it is very important to evaluate the relationship between histopathological features and the efficacy of pembrolizumab among patients with NSq NSCLC.

Furthermore, several previous studies have reported that the immune and genetic features of the tumors differ depending on the histopathological features in the “resected surgical specimens”; according to these reports, the acinar or solid predominant subtype in resected surgical specimens is associated with a higher PD-L1 score and the solid predominant subtype is associated with a higher TMB than other subtypes (Takada et al. 2016);(Dong et al. 2018; Miyazawa et al. 2019). Nevertheless, in the majority of patients with advanced NSCLC, histopathological diagnoses must generally be performed using “biopsy samples.” Few studies have investigated the relationship between the histopathological features and the immune and genetic features in biopsy samples. Therefore, we evaluated the relationship between the histopathological features in biopsy samples and the tumor PD-L1 expression and TMB in patients with NSq NSCLC.

Materials and methods

This study was conducted with the approval of the Institutional Review Board of the National Cancer Center (approval number 2019-098 and 2018-134).

Patient selection

Patients with unresectable or recurrent NSq NSCLC whose PD-L1 expression levels in biopsy samples were evaluated and who had started pembrolizumab treatment as a first- to third-line therapy at the National Cancer Center Hospital East, Japan, between February 2017 and October 2018 were selected to investigate the predictive impact of histopathological features on survival outcomes after treatment with pembrolizumab. The biopsy methods included transbronchial biopsy, endobronchial ultrasound-guided transbronchial needle aspiration, core needle biopsy, and surgical biopsy of metastatic sites. Because the efficacy of pembrolizumab is related to the intensity of PD-L1 expression and the treatment line,(Lang et al. 2019) patients with a PD-L1 score ≥50% who had been treated with pembrolizumab as a first-line therapy and patients with a PD-L1 score of 1%-49% who had been treated with pembrolizumab as a second- or third-line therapy were analyzed separately. We defined chemotherapy for metastatic disease or recurrence in patients who had completed definitive chemoradiotherapy more than 6 months earlier as first-line therapy, while chemotherapy for metastatic disease or recurrence in patients who had completed definitive chemoradiotherapy within 6 months was defined as second-line therapy. The clinical

characteristics and clinical courses were retrospectively retrieved from the patients' medical records.

In addition, to investigate the association between the histopathological features and the PD-L1 expression, patients with unresectable or recurrent NSq NSCLC whose PD-L1 expression levels in biopsy samples were evaluated using IHC between February 2016 and October 2018 at the National Cancer Hospital East, Japan, were also selected. Furthermore, to examine the association between the histopathological features in biopsy samples and the TMB, TMB data were obtained from the Lung Cancer Genomic Screening Project for Individualized Medicine Immuno-Oncology Biomarker Study (LC-SCRUM-IBIS) (UMIN000026425) database and patients in whom the histopathological features in biopsy samples could be evaluated were selected at the National Cancer Hospital East, Japan, between February 2016 and May 2018.

Histopathological definition

Each patient's hematoxylin-and-eosin (H-E)-stained slides were reviewed independently by two pathologists (T.S and G.I) to determine the histopathological diagnosis (morphological adenocarcinoma or NSCC) based on morphological features according to the 4th edition of the World Health Organization Classification of Lung Tumors (Travis et al. 2015). When there were discrepancies between the two pathologists in the assignment of the histopathological diagnosis based on morphological features were later resolved by consensus on a multiple-headed microscope. Morphological adenocarcinoma was defined

as a tumor with a definite adenocarcinoma morphology, such as the appearance of lepidic, papillary, micropapillary, and/or acinar architectures using H-E staining. NSCC was defined as a tumor with the absence of both a definite adenocarcinoma and squamous morphology using H-E staining. NSCC was further classified based on immunohistochemical features as NSCC-favor adenocarcinoma or NSCC-NOS also according to the 4th edition of the World Health Organization Classification of Lung Tumors (Travis et al. 2015). NSCC-favor adenocarcinoma was defined as an NSCC tumor with positive results for an adenocarcinoma marker, such as TTF-1 and/or mucin. NSCC-NOS was defined as an NSCC tumor with negative results for adenocarcinoma and squamous markers, such as p40 (Supplemental Figure1,2). Possible large cell neuroendocrine carcinoma, which is an NSCC tumor with a neuroendocrine morphology and neuroendocrine marker positivity, was excluded. In morphological adenocarcinoma, the predominant subtype was decided based on the subtype that occupies the largest area of tumors. Subtype was decided based on the International Association for the Study of Lung Cancer/American Thoracic Society/European Respiratory Society classification of lung adenocarcinoma (Travis et al. 2011). The tumor which showed just only solid pattern of growth without a definitive adenocarcinoma morphology was not classified in morphological adenocarcinoma but in NSCC.

Immunohistochemistry

All the samples were stained using alcian blue PAS stain to evaluate the presence of mucin. Samples without definite morphological features were stained using TTF-1 (anti-thyroid transcription factor-1 SP141; Roche

Diagnostics, Indianapolis, IN, USA) and p40 (Anti-p40-DeltaNp63 antibody BC28; Abcam, Cambridge, UK) to distinguish between NSCC-favor adenocarcinoma and NSCC-NOS. PD-L1 IHC was performed for all the samples using the PD-L1 kit (PD-L1 IHC 22C3 pharm DX; Dako, Carpinteria, CA, USA) according to the manufacturer's instructions. The PD-L1 score was evaluated based on the proportion of tumor cells (TCs) exhibiting positive membrane staining of any intensity. The stained slides in each patient were reviewed independently by two pathologists (T.S. and G.I.) and the tumor PD-L1 score was categorized as <1%, 1%-49%, ≥50%, or ≥75%, according to the percentage of cells showing positive staining of the cell membrane.

Whole-exome sequencing

We used whole-exome sequencing (WES) data from the LC-SCRUM-IBIS database and patients in whom the histopathological features could be evaluated in biopsy samples were selected. The WES data were used to generate the TMB, which was defined as the total number of non-synonymous somatic mutations present in the baseline tumor sample.

Statistical analysis

The chi-squared test and *t* test were used to compare the clinicopathological features and the PD-L1 score or TMB among the patients with morphological adenocarcinoma or NSCC. Overall survival (OS) was

defined as the interval between the date of the initiation of pembrolizumab treatment and the date of death from any cause or the last follow-up. PFS was defined as the time between the date of the initiation of pembrolizumab treatment and the date of the detection of disease progression, death from any cause, or the last follow-up. The survival curves were estimated using the Kaplan-Meier method, and differences in OS and PFS were compared using the log-rank test. Univariate and multivariate analyses were performed using the Cox proportional hazards model. The survival data was last updated on August 30, 2019. The objective clinical response was assessed according to the Response Evaluation Criteria in Solid Tumors, version 1.1. The chi-squared test was used to compare the overall response rate (ORR) and the disease control rate (DCR). All differences in *P* values were two sided, and a *P* value of <0.05 was considered as denoting statistical significance. All the statistical analyses were performed using the JMP statistical software program, Ver. 14 (SAS Institute, Cary, NC).

Results

Association between the histopathological features of NSCLC and survival outcomes of pembrolizumab treatment

A total of 52 patients with unresectable or recurrent NSq NSCLC whose PD-L1 expression levels were evaluated using biopsy samples were treated with pembrolizumab monotherapy. Among these 52 patients, 22 patients (42%) were diagnosed as having morphological adenocarcinoma and 30 patients (58%) were

diagnosed as having NSCC, characterized by the absence of definitive morphological features; 33 patients with a PD-L1 score ≥50% were treated with pembrolizumab as a first-line therapy, and 19 patients with a PD-L1 score of 1%-49% were treated with pembrolizumab as a second- or third-line therapy.

Among the patients with a PD-L1 score ≥50% who had been treated with pembrolizumab as a first-line therapy, the proportion of patients with a PD-L1 score quartile of 75%-100% was significantly higher among the patients with NSCC than among those with morphological adenocarcinoma (74% vs. 21%, $P<0.01$) (Table 1). One morphological adenocarcinoma patient harboring an *EGFR* exon20 insertion mutation was treated with pembrolizumab as a first-line treatment. At the time of the data analysis, 15 patients had died, 18 patients were alive (9 patients had disease progression), and only 1 patient was lost to follow-up after disease progression. The median follow-up period of the patients who were still alive was 16.7 months. Among them, NSCC was associated with a significantly longer PFS (median, 16.8 vs. 2.3 months; HR, 0.26; 95% CI, 0.10-0.62; $P=0.01$; Figure 1A) and a significantly longer OS (median, NR vs. 10.1 months; HR, 0.35; 95% CI, 0.12-0.97; $P=0.04$; Figure 1B) compared with morphological adenocarcinoma. On the other hand, when the patients were stratified according to PD-L1 score quartiles, no significant difference in the PFS or OS were observed between patients with PD-L1 scores of 50%-74% and 75%-100% (PFS: median, 7.9 vs. 12.0 months; HR, 1.70; 95% CI, 0.74-3.98; $P=0.20$; Figure 1C; OS: median, NR vs. 17.1 months; HR, 0.66; 95% CI, 0.22-1.84; $P=0.43$; Figure 1D). In a univariate analysis, only NSCC was associated with a longer PFS and OS (Table 2). NSCC was the only independent factor for a longer PFS that remained in the multivariate analysis. Similarly, NSCC was also an independent factor

for a longer OS in a multivariate analysis including other clinical factors, with the exception of brain metastasis (Table 3). The overall response rate and the disease control rate were 68% (95% CI, 43-87) and 84% (95% CI, 60-97), respectively, among the patients with NSCC and 29% (95% CI, 8-58) and 43% (95% CI, 18-71), respectively, among the patients with morphological adenocarcinoma. The response rate and the disease control rates were significantly higher among the patients with NSCC, compared with the patients with morphological adenocarcinoma (P=0.04 and P=0.02, respectively).

A total of 19 patients with a PD-L1 score of 1%-49% who were treated with pembrolizumab as a second- or third-line therapy (Supplemental Table 1). Fourteen patients had died and 5 patients were alive (3 patients had disease progression after pembrolizumab treatment); the median follow-up period of the patients who were still alive was 24.4 months. No significant difference in PFS (median, 4.4 vs. 3.2 months; HR, 0.76; 95% CI, 0.28-2.13; P=0.64; Supplemental Figure 3A) or OS (median, 5.8 vs. 10.4 months; HR, 1.58; 95% CI, 0.41-5.26; P=0.40; Supplemental Figure 3B) was seen between the patients with NSCC and those with morphological adenocarcinoma.

Association between the histopathological features of NSCLC and the PD-L1 expression in biopsy samples

First, we evaluated the association between the histopathological features of NSCLC and the PD-L1 expression in biopsy samples to investigate the reason for the better survival outcomes of pembrolizumab

treatment in patients with a biopsy diagnosis of NSCC. PD-L1 IHC was performed in biopsy samples from a total of 379 patients with unresectable or recurrent NSq NSCLC. Three patients whose samples were diagnosed as possible large cell neuroendocrine carcinoma were excluded. Nine patients were excluded from the analysis because their samples were inadequate for evaluating the PD-L1 score. Finally, 367 patients were included in this study.

Two hundred and twenty patients (60%) were diagnosed as having morphological adenocarcinoma, and 147 patients (40%) were diagnosed as having NSCC, characterized by the absence of definitive morphological features (Supplemental Table 2). Among the patients with NSCC, the proportions of men (70% vs. 50%, $P<0.01$) and ever smokers (80% vs. 60%, $P<0.01$) were significantly higher than those among the patients with morphological adenocarcinoma. The proportion of patients who tested positive for epidermal growth factor receptor (*EGFR*) mutations (21% vs. 43%, $P<0.01$) was significantly lower in the NSCC group than in the morphological adenocarcinoma group. Among the 147 patients with NSCC, 114 patients (78%) were classified as NSCC-favor adenocarcinoma and 33 patients (22%) were classified as NSCC-NOS (Supplemental Table 3).

The NSCC samples had a lower rate of a PD-L1 score <1% (41% vs. 71%) and a higher rate of a PD-L1 score ≥50% (35% vs. 10%) than the morphological adenocarcinoma samples ($P<0.01$, respectively) (Table 4). In the *EGFR*-mutation positive and the negative/unknown patients, the NSCC samples also had lower rates of a PD-L1 score <1% (42% vs. 75% and 41% vs. 67%, respectively) and higher rates of a PD-L1 score ≥50% (29% vs. 7% and 36% vs. 13%, respectively) ($P<0.01$ and $P<0.01$, respectively), compared

with the morphological adenocarcinoma samples. Furthermore, among samples with PD-L1 scores of ≥50%, the group diagnosed as NSCC s showed a higher percentage of samples with PD-L1 scores of 75%-100% as compared to the group diagnosed as morphological adenocarcinoma (49% vs. 26%, $P<0.01$) (Table 4).

There were no significant differences in the rates of a PD-L1 score <1% (40% vs. 46%) and a PD-L1 score ≥50% (37% vs. 24%) between the NSCC-favor adenocarcinoma and the NSCC-NOS samples ($P=0.34$). Among the *EGFR*-mutation positive and negative/unknown patients, there was also no difference in the rates of a PD-L1 score <1% and ≥50% between the NSCC-favor adenocarcinoma and NSCC-NOS samples (Supplemental Table 4). When morphological adenocarcinoma was classified based on the predominant subtype, the rate of a PD-L1 score <1% was highest in the samples with a lepidic predominant subtype, while the rate of a PD-L1 score ≥50% was highest in the samples with a solid predominant subtype (Supplemental Table 5).

Association between the histopathological features of NSCLC and the TMB in biopsy samples

Next, we evaluated the association between the histopathological features of NSCLC and the TMB in biopsy samples to investigate the reason for the better survival outcomes of pembrolizumab treatment in patients with a biopsy diagnosis of NSCC. A total of 15 patients in whom both the TMB and histopathological features in biopsy samples were evaluated were included in this study. Of the 15 samples,

the biopsy diagnosis was morphological adenocarcinoma in 7 patients and NSCC in 8 patients. The TMB was significantly higher in the NSCC group than in the morphological adenocarcinoma group (median 236 vs. 25 mutations/whole-exome; P=0.01; Figure 2).

Discussion

Our results showed that the absence of definitive morphological features in biopsy samples could be a predictive biomarker for the efficacy of pembrolizumab as a first-line therapy in NSq NSCLC patients with a PD-L1 score ≥50%. To our knowledge, this is the first study to investigate the relationship between histopathological features in a biopsy sample and the efficacy of immunotherapy including pembrolizumab in patients with NSCLC. Several previous studies have shown an association between the absence of definitive morphological features in NSCLC and a poor outcome of chemotherapy other than immunotherapy (Pelosi et al. 2014; Shiran et al. 2017; Ujiie et al. 2015; Warth et al. 2012). Our result, that the absence of definitive morphological features in NSq NSCLC was associated with a better outcome, reflects a specific characteristic of immunotherapy. As plausible reasons for the better survival outcomes of pembrolizumab treatment in cases with the absence of definitive morphological features, we found that the group with biopsy samples not showing definitive morphological features showed a lower percentage of samples with a PD-L1 score of <1%, higher percentage of samples with PD-L1 scores of ≥50%, and also a higher TMB than the group with biopsy samples diagnosed as morphological adenocarcinoma. This is the first report to show an association between the histopathological features and the PD-L1 expression and

TMB in biopsy samples. These results were similar to those of previous studies, which showed relationships between the PD-L1 expression and the histopathological features, and between the TMB and the histopathological features in surgical specimens (Takada et al. 2016);(Miyazawa et al. 2019);(Dong et al. 2018).

In this study, we showed that biopsy samples without definitive morphological features were significantly associated with a lower rate of a PD-L1 score <1% and a higher rate of a PD-L1 score ≥50% than biopsy samples with definitive morphological features, regardless of the *EGFR*-mutation status. Furthermore, among morphological adenocarcinoma, we also showed relationships between the PD-L1 score and the predominant subtype. In surgical specimens, the association between the histopathological features and the PD-L1 expression has been analyzed in several studies (Miyazawa et al. 2019; Takada et al. 2016). In those studies, the rate of PD-L1 positivity was higher for the solid predominant subtype than for the other subtypes. Therefore, our result that the absence of definitive morphological features was associated with a higher PD-L1 scoring category was concordant with the findings of previous studies. Thus, these results were observed not only in surgical specimens, but also in biopsy specimens.

A high TMB was identified as biomarkers of the efficacy of immunotherapy in NSCLC. In the CheckMate-568 study, which evaluated the association between the TMB and the efficacy of nivolumab plus ipilimumab as a first-line treatment in 288 NSCLC patients, the patients with a high TMB had a better ORR (44% vs. 12%) and PFS (mPFS, 7.1 vs. 2.6 months) than those with a low TMB.(Ready et al. 2019) Similarly, a high TMB was associated with a longer PFS of pembrolizumab treatment in several studies of

patients with NSCLC (Rizvi et al. 2015; Roszik et al. 2016). Furthermore, a systematic review also reported that a high TMB was associated with greater clinical benefits, particularly in terms of ORR and PFS, among patients receiving immunotherapy (Willis et al. 2019). The relationships between the histopathological features and the TMB were reported in some studies. In surgical specimens, some studied reported that solid predominant adenocarcinoma was associated with a high TMB (Dong et al. 2018; Rekhtman et al. 2013). Moreover, we previously reported an association between morphological features in biopsy samples and the predominant subtype in resected surgical specimens (Matsuzawa et al. 2016). In that study, approximately 70% of the NSCC corresponded to solid predominant adenocarcinoma in the surgical specimens. Furthermore, our WES results demonstrated that NSCC correlated with higher TMB than that with morphological adenocarcinoma. Thus, it is considered that the absence of definitive morphological features in biopsy specimens was associated with a high TMB.

It has been reported previously that patients with tumor PD-L1 scores of 75%-100% showed a significantly longer PFS of pembrolizumab therapy as compared to patients with tumor PD-L1 scores of 50%-74% (Aguilar et al. 2019). The percentage of samples with PD-L1 scores of ≥75% was higher among samples diagnosed as NSCC as compared to those diagnosed as morphological adenocarcinoma in our study. However, no significant difference in the PFS or OS was observed between patients with PD-L1 scores of 75%-100% and 50%-74%. Furthermore, a bivariate analysis of patients with PD-L1 scores of 50%-74% and 75%-100% identified histopathological diagnosis of NSCC as an independent predictor of a longer PFS and OS. Thus, the differences in the PFS and OS of pembrolizumab treatment between patients

with a biopsy diagnosis of NSCC and those with a biopsy diagnosis of morphological adenocarcinoma cannot be considered to be attributable to the distribution of the PD-L1 score between the two groups alone. It is possible that NSCC, which is characterized by the absence of definitive morphological features in biopsy samples, is associated with a high tumor expression of PD-L1 and a high TMB, just like solid predominant adenocarcinoma, which is the histopathological equivalent of NSCC in resected surgical specimens. Thus, we suggest that the absence of definitive morphological features in biopsy samples could be a universally available and easily applicable factor that predicts a high tumor expression of PD-L1 and a high TMB. For that reason, the absence of definitive morphological features in biopsy samples may have been a biomarker of the efficacy of immune checkpoint inhibitors, including pembrolizumab.

On the other hand, among the patients with a PD-L1 score of 1%-49%, the absence of definitive morphological features was not a predictive biomarker of the efficacy of pembrolizumab in our study. It is unclear what caused the difference in the impact of the absence of definitive morphological features as a predictive biomarker of the efficacy of pembrolizumab between patients with a PD-L1 score ≥50% and those with a PD-L1 score of 1%-49%. To our knowledge, a subgroup analysis of CheckMate 026 is the only study to focus on the difference in the predictive impact of biomarkers of immunotherapy, such as TMB or INF-gamma, between patients with a PD-L1 score ≥50% and those with a PD-L1 score of 1%-49%, even though a statistical analysis was not performed. In a subgroup analysis of CheckMate 026, patients with a high TMB and a PD-L1 score ≥50% had a higher response rate to nivolumab (75%) than those with a low TMB and a PD-L score ≥50% (34%), those with a high TMB and a PD-L score of 1%-

49% (32%), and those with a low TMB and a PD-L score of 1%-49% (16%).(Carbone et al. 2017) We considered that the absence of definitive morphological features in biopsy samples might be a meaningful predictive biomarker of the efficacy of pembrolizumab when used in combination with a PD-L1 score ≥50%.

This study had some limitations. This was a single center study with a limited sample size. Owing to the small number of patients with NSCC-NOS, we could not evaluate the difference in the efficacy of pembrolizumab between NSCC-favor adenocarcinoma and NSCC-NOS, and this matter warrants further evaluation using a larger cohort. Furthermore, this study had a retrospective design. Therefore, the possibility of an unintentional selection bias cannot be fully excluded. Consequently, the present findings need to be confirmed in a prospective study. In our study, we only analyzed biopsy samples. It is possible that small biopsy samples will not always result in an accurate morphological classification, because of tumor heterogeneity and potential sampling bias. However, we had decided morphological classification by independent two pathologists according to WHO Classification and our previous study demonstrated that small biopsy sample could represent the morphological feature of entire tumor (Matsuzawa et al. 2016), our classification of morphological adenocarcinoma and NSCC was thought to be reproducible.

Conclusion

The absence of definitive morphological features in biopsy samples was associated with a high tumor

expression of PD-L1 and a high TMB, and could be a predictive biomarker of the efficacy of pembrolizumab monotherapy as first-line therapy in NSq NSCLC patients with PD-L1 scores of ≥50%. This universally available and easily applicable biomarker could enable rational decisions to be made regarding the use of pembrolizumab monotherapy in individual patients.

Figure Legends

Figure 1. Progression-free survival (PFS) and overall survival (OS) in patients with a tumor PD-L1 score of ≥50% treated with pembrolizumab as first-line treatment.

(A) PFS according to the biopsy diagnosis of non-small cell carcinoma (NSCC) or morphological adenocarcinoma (Morpho AD).

(B) OS according to the biopsy diagnosis of non-small cell carcinoma (NSCC) or morphological adenocarcinoma (Morpho AD).

(C) PFS according to tumor PD-L1 scores of 50%-74% and 75%-100%.

(D) OS according to tumor PD-L1 scores of 50%-74% and 75%-100%.

Figure 2. Correlation between the tumor-mutation burden (TMB) and a biopsy diagnosis of morphological adenocarcinoma (Morpho AD) versus non-small cell carcinoma (NSCC).

Supplemental Figure1. Algorithm for histopathological diagnosis

Supplemental Figure 2. Representative slides showing morphological adenocarcinoma, NSCC-favor AD and NSCC-NOS.

(A) Morphological adenocarcinoma with acinar pattern.

(B)(C) NSCC-favor AD. IHC shows TTF-1 positive.

(D)(E) NSCC-NOS. IHC shows TTF-1 negative.

Supplemental Figure 3. Progression-free survival (PFS) and overall survival (OS) in patients with a biopsy diagnosis of non-small cell carcinoma (NSCC) versus morphological adenocarcinoma (Morpho AD).

(A) PFS in patients with a PD-L1 score of 1%-49% who were treated with pembrolizumab as second- or third-line treatment.

(B) OS in patients with a PD-L1 score of 1%-49% who were treated with pembrolizumab as second- or third-line treatment.

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