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Smoking History Predicts High Presence of TILs and Efficacy of PD-1 Blockade in PD-L1 Expression-negative Non-small Cell Lung Cancer Patients

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Abstract. *Background/Aim: Programmed death-ligand 1 (PD-L1) expression on tumor cells is a predictive biomarker of programmed cell death 1 (PD-1) blockade therapy. This study sought to clarify predictors of the efficacy of nivolumab in non-small cell lung cancer (NSCLC) patients with PD-L1 expression-negative tumors. Patients and Methods: We retrospectively reviewed the records of advanced NSCLC patients between January 2016 and April 2019, and investigated the predictive marker of nivolumab including the status of CD8$^+$ tumor infiltrating lymphocytes (TILs). Results: A total of 70 NSCLC patients were included. Overall response rate (ORR) and progression-free survival (PFS) were better in patients with a heavy smoking history (smoking index: SI≥600) than in those without (SI<600) [ORR: 20.6% vs. 2.8%, (p=0.02), and PFS: 2.4 months vs. 1.8 months, (p=0.04)]. A high density of CD8$^+$ TILs was significantly associated with a heavy smoking history (p=0.04). Conlusion: Heavy smoking history (SI≥600), which was correlated with a large number of CD8$^+$ TILs, could be a predictor of the efficacy of nivolumab in NSCLC patients with PD-L1 expression-negative tumors.*

Programmed cell death 1 (PD-1)/programmed death-ligand 1 (PD-L1) checkpoint inhibitors nivolumab, pembrolizumab and atezolizumab have led to improved survival of patients with advanced non-small cell lung cancer (NSCLC) (1-3). PD-L1 expression on tumor cells is a predictive biomarker of a response to PD-1 blockade. Pembrolizumab has been shown to have a significantly better efficacy and to enable a longer survival, compared with platinum doublet chemotherapy, in NSCLC patients with tumours showing strong PD-L1 expression [tumor proportion score (TPS): ≥50%]. The response rate to pembrolizumab among advanced NSCLC patients with PD-L1 expression strongly positive was 44.8% in the KEYNOTE-024 study (1).

The response rates to nivolumab among advanced NSCLC patients who were not selected according to PD-L1 expression were 26% and 19% in the Checkmate-017 and -057 trials, respectively (2, 4). Some patients with PD-L1 expression-negative tumors may benefit from anti-PD-1 inhibitors, since PD-L1 expression is not an entirely reliable predictor of response. Moreover, the clinical characteristics that may predict a response to PD-1 blockade therapy among patients with PD-L1 expression-negative tumors remain unclear.

In addition to PD-L1 expression on tumor cells, the status of tumor infiltrating lymphocytes (TILs) in the immune-related tumor microenvironment (TME) is also a predictor of the efficacy of PD-1 blockade (5-9). Previous studies have reported that PD-L1 expression on tumor cells is correlated with CD8$^+$ TILs and other infiltrating immune cells (10, 11). However, whether CD8$^+$ TILs are associated with the efficacy of PD-1 blockade in PD-L1 expression-negative tumors remains unclear.

The aim of this study was to clarify clinical predictors of the efficacy of nivolumab in advanced NSCLC patients with PD-L1 expression-negative tumors and to evaluate whether such factors are associated with the CD8$^+$ TIL status.

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Key Words: Non-small cell lung cancer (NSCLC), programmed death-ligand 1 (PD-L1), smoking index, nivolumab, and CD8$^+$ tumor infiltrating lymphocytes (TILs).

Patients and Methods

Patient characteristics. We retrospectively reviewed advanced NSCLC patients with PD-L1 expression-negative tumors who had received nivolumab at the National Cancer Center Hospital (Tokyo, Japan) between January 1, 2016, and April 30, 2019. Clinical characteristics including age, sex, Eastern Cooperative Oncology Group (ECOG) performance status (PS), presence of brain metastasis, smoking index (SI), histological type, existence of major mutation, number of metastatic sites, treatment line, history of radiation therapy, and blood tests [neutrophil-to-lymphocyte ratio (NLR), lactate dehydorogenase (LDH), C-reactive protein (CRP), etc.] were collected from a medical database. Computed tomography examinations were performed every 6 to 8 weeks or according to the judgement of the attending physician. The tumor response was assessed using REIST version 1.1 (12). The cut-off date was March 31, 2020. Patients who responded [complete response (CR) or partial response PR)] to nivolumab were defined as responders.

Immunohistochemistry for evaluating PD-L1 expression and TILs. Immunohistochemical staining (IHC) was performed automatically (Autostainer Link 48 platform; Dako, Agilent, Santa Clara, CA, USA) using the following monoclonal antibodies: PD-L1 (clone 22C3; Agilent/DAKO), and CD8 (clone 4B11; Novocastra, Newcastle upon Tyne, UK). PD-L1 expression was evaluated using formalin-fixed, paraffin-embedded (FFPE) specimens containing more than 100 viable tumor cells. Membranous staining was defined as positive staining. The tumor proportion score (TPS) was calculated as the percentage of tumor cells with positive PD-L1 staining. Digital images of $CD8^+$ TILs in the tumor tissues were assessed using the Nanozoomer Digital Pathology (NDP) system (Nanozoomer 2.0-HT Whole Slide Imager; Hamamatsu Photonics, Hamamatsu, Japan). Two pulmonologists and one experienced pathologist with no knowledge of the clinical data evaluated two hot spots of immune cell infiltration on each digital image. The number of $CD8^+$ TILs per mm^2 was counted in the tumor nest. The average number of TILs in the two hot spot areas was then calculated.

Statistical analysis. All the collected data were analyzed using EZR version 1.53 (13) (Kanda Y, Saitama, Japan). Progression-free survival (PFS) was determined as the day from the start of PD-1 blockade to the day of disease progression or death. Overall survival (OS) was determined as the day from the start of PD-1 blockade to the day of death or the end of the follow-up period. We used the Fisher exact test to evaluate differences in categorical data and either the t-test or the Mann–Whitney *U*-test for continuous data. When a significant difference in continuous data was obereved between groups, we used a receiver operating characteristic (ROC) curve analysis to determine an appropriate cut-off value producing the highest sensitivity and specificity for predicting a response to anti-PD-1 therapy. To investigate differences in the PFS and OS between groups, we used the Kaplan–Meier method and compared the two groups using the log-rank test. A Cox proportional-hazard model was used for the univariate and multivariate analyses. The multivariate analysis included prognostic factors with a p-value <0.1 in a univariate analysis. We regarded a p-value <0.05 as being statistically significant. The present study was approved by the Ethics Committee of the National Cancer Center Hospital (2019-123).

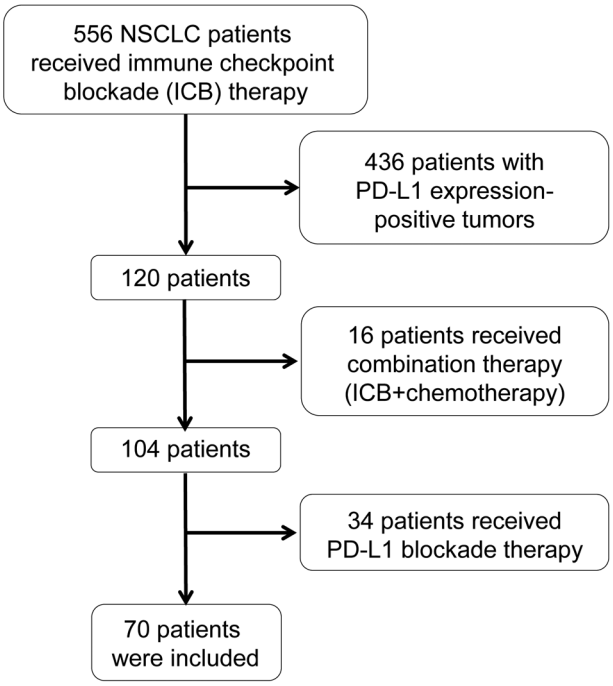

Figure 1. *CONSORT diagram of the study. The CONSORT diagram shows the procedure used to select the patients included in this study.*

Results

Patient characteristics. We identified 556 patients who were treated with anti-PD-1 inhibitors at our hospital between January 1, 2016, and April 30, 2019. Seventy patients were included in this study (Figure 1). The baseline characteristics of the patients are shown in Table I. The median age was 62 years (range=30-83 years). The majority of the patients were male (63%) and had an ECOG PS score of 0-1 (87%). The major histological type was non-squamous cell carcinoma (81%).

Clinical outcomes of nivolumab in all patients with PD-L1 negative tumors. The overall response rate (ORR) and disease control rate (DCR) of nivolumab was 11.4% (95%CI=5.0-21.0) and 24.0% (95%CI=15.08-36.0), respectively. The median PFS and OS were 2.0 months (95%CI=1.8-2.5) and 9.4 months (95%CI=5.5-12.6), respectively (Figure 2A and B). The response to nivolumab was stratified according to the baseline characteristics and is shown in Table I. An SI≥600 was the only clinical predictor of a response to nivolumab. The ORR, DCR and PFS of patients with an SI ≥600 were significantly better than those with an SI<600 [ORR: 20.6% *vs*. 2.8% (p=0.02), DCR: 35.3% *vs*. 13.9% (p=0.05) and PFS: 2.4 months *vs*. 1.8 months (p=0.04)] (Figure 2C).

Next, we performed a univariate and multivariate analysis that included various variables. ECOG PS and SI were

Table I. *Patient baseline characteristics according to response to nivolumab (n=70).*

	Total n=70	Responder n=8	Non-responder n=62	*p*-Value
Median age in years (range)	62 (30-83)	64 (45-68)	62 (30-83)	0.68
Sex (male/female), n (%)	44 (63)/26 (37)	5 (63)/3 (38)	39 (63)/23 (37)	1.00
PS (0-1/2), n (%)	61 (87)/9 (13)	7 (87)/1 (13)	54 (87)/8 (13)	1.00
Brain metastasis (yes/no), n (%)	18 (26)/52 (74)	3 (38)/5 (62)	15 (24)/47 (76)	0.41
Smoking Index (≥600) (yes/no), n (%)	34 (49)/36 (51)	7 (87)/1 (13)	27 (44)/35 (56)	0.02
Histological type (Sq/non-Sq), n (%)	13 (19)/57 (81)	3 (38)/5 (62)	10 (16)/52 (84)	0.16
Major mutation* (yes/no), n (%)	12 (17)/58 (83)	1 (13)/7 (87)	11 (18)/51 (82)	1.0
Number of metastatic sites (<3/≥3), n (%)	22 (31)/48 (69)	3 (38)/5 (62)	19 (31)/43 (69)	0.7
Treatment line (<3/≥3), n (%)	31 (44)/39 (56)	3 (38)/5 (62)	28 (45)/34 (55)	1.00
History of radiation therapy (yes/no), n (%)	54 (77)/16 (23)	7 (87)/1 (13)	47 (76)/15 (24)	0.6
ANC** (/μl)	4835 (1548-21300)	5120 (2450-7510)	4600 (1548-21300)	0.66
ALC** (/μl)	1080 (400-2990)	970 (400-1860)	1100 (460-2990)	0.86
Serum Alb** (g/dl)	3.8 (2.0-4.6)	4.0 (3.2-4.3)	3.8 (2.0-4.6)	0.59
Serum CRP** (mg/dl)	1.1 (0.0-15.6)	2.4 (0.2-9.7)	1.0 (0.0-15.6)	0.91
Serum LDH** (U/l)	211.0 (129-1247)	214.0 (152-1247)	209.5 (129-1071)	0.95

PS: Performance status; Sq: squamous cell carcinoma; ANC: absolute neutrophil count; ALC: absolute lymphocyte count; Alb: albumin; CRP: C-reactive protein; LDH: lactate dehydrogenase. *Del19, L858R and ALK arrangement. **Data presented as median (range).

significantly associated with PFS in patients with PD-L1 expression-negative tumors [PS: HR=0.43, 95%CI=0.21-0.88 (p=0.02); SI: HR=0.58; 95%CI=35-0.96 (p=0.03)] (Table II).

Association between the presence of CD8$^+$ TILs and the efficacy of nivolumab. In addition to the clinical data, we analyzed whether the CD8$^+$ TIL status in the TME affected the response of PD-L1 expression-negative tumors in an exploratory manner (Figure 3). Among the 70 patients, 43 patients had sufficient tissue samples available to allow an analysis of CD8$^+$ TILs in the TME. Responders had significantly more CD8$^+$ TILs in their tumor nests than non-responders [median: 118/mm^2 (range=0-510/mm^2) *vs*. 10/mm^2 (range=0-1250/mm^2), p=0.03, Figure 4A]. A ROC curve analysis resulted in 110/mm^2 as a cut-off value for CD8$^+$ TILs that produced the highest sensitivity and specificity for predicting a response to anti-PD-1 therapy (sensitivity: 0.84, specificity: 0.83, area under the ROC curve: 0.77) (Figure 5). The PFS and OS are shown according to the CD8$^+$ TIL status (≥110/mm^2 *vs*. <110/mm^2) in Figure 4B. A statistically significant difference in PFS was observed between patients with high and low CD8$^+$ TIL statuses [median: 13.2 months (95%CI=2.0-32.6) for ≥110/mm^2 group *vs*. 1.9 months (95%CI=1.1-2.3) for <110/mm^2 group, p<0.01]. The OS of patients with a high number of CD8$^+$ TILs was also significantly longer than that of patients with a low number of CD8$^+$ TILs [median: 29.2 months (95%CI=5.2-NA) for ≥110/mm^2 group *vs*. 10.0 months (95%CI=4.0-12.9) for <110/mm^2 group, p=0.01]. Next, we evaluated the correlation between the CD8$^+$ TIL status (immune-inflamed or non-immune-inflamed phenotype) and baseline clinical characteristics. Heavy smoking history was significantly associated with a high density of CD8$^+$ TILs (Figure 4C).

Discussion

In the present study, 11.4% of the patients with PD-L1 expression-negative tumors responded to nivolumab, similar to the results of previous reports (2, 4). A heavy smoking history had a significant influence on the efficacy of nivolumab in patients with PD-L1 expression-negative tumors. In addition, a heavy smoking history was significantly associated with the relative abundance of CD8$^+$ TILs, which is an immune-inflamed phenotype, even in PD-L1 expression-negative tumors.

In the Checkmate-057 study, nivolumab was associated with a longer PFS, a longer OS, and a higher ORR than docetaxel in patients with PD-L1 expression-positive tumors, but a benefit was not observed in patients with PD-L1 expression-negative tumors (2). However, in a portion of patients with PD-L1 expression-negative tumors, nivolumab showed some clinical activity. The ORR and DOR were 10% and 18.3 months, respectively, in a subgroup analysis of the Checkmate-057 trial. On the other hand, few studies have focused on clinical predictors of the efficacy of PD-1 blockade therapy in patients with PD-L1 expression-negative tumors. We clarified the association between the efficacy of nivolumab and the smoking status in NSCLC patients with PD-L1 expression-negative tumors.

Smoking is associated with harmful adverse effects on human health and is the major cause of lung cancer.

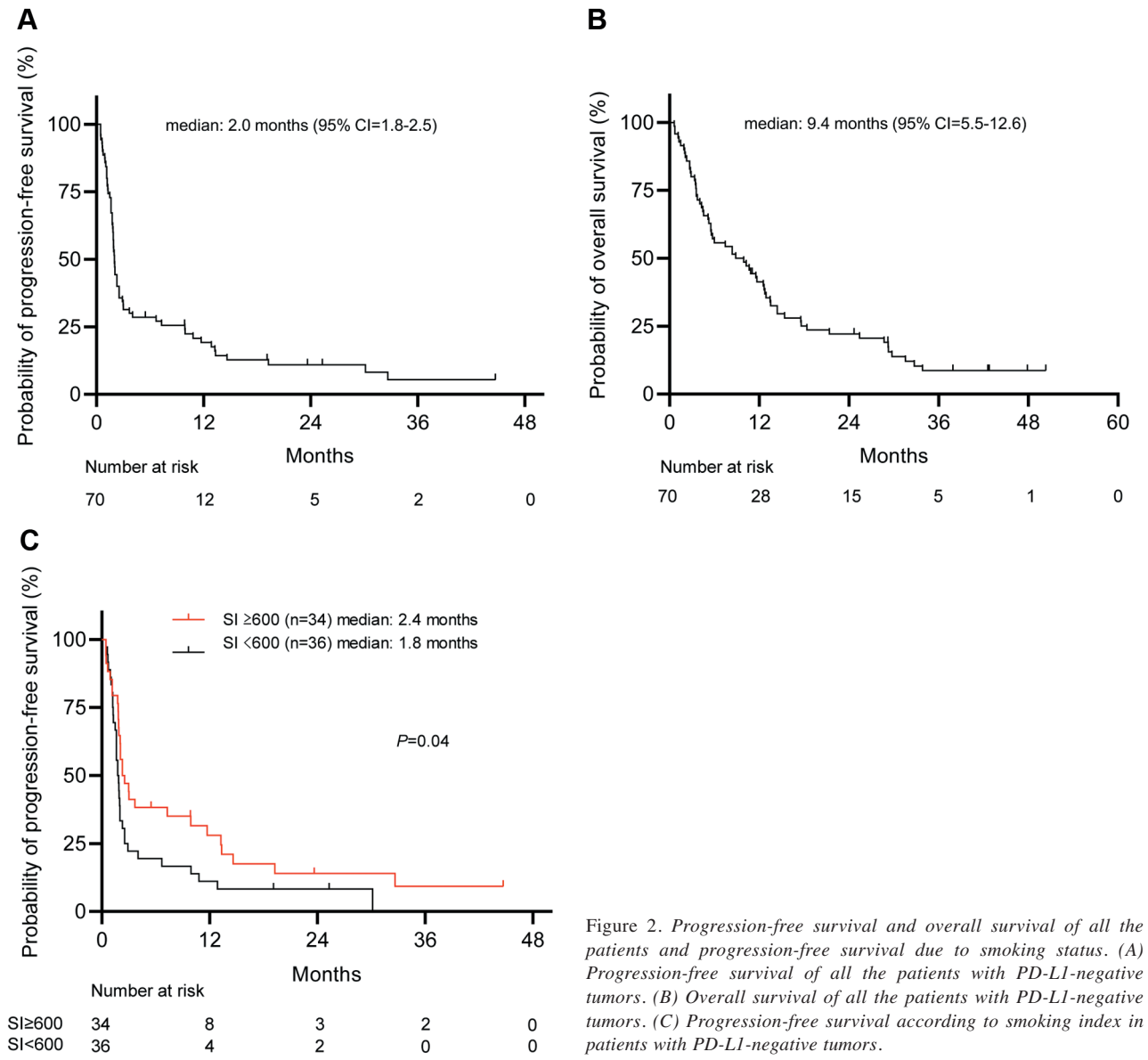

Figure 2. *Progression-free survival and overall survival of all the patients and progression-free survival due to smoking status. (A) Progression-free survival of all the patients with PD-L1-negative tumors. (B) Overall survival of all the patients with PD-L1-negative tumors. (C) Progression-free survival according to smoking index in patients with PD-L1-negative tumors.*

Table II. *Univariate and multivariate analyses for PFS of nivolumab.*

	Univariate analysis			Multivariate analysis		
	HR	95%CI	*p*-Value	HR	95%CI	*p*-Value
Age (<75 *vs.* ≥75)	0.83	0.20-3.43	0.80	-	-	-
Sex (male *vs.* female)	0.82	0.49-1.36	0.43	-	-	-
PS (0-1 *vs.* ≥2)	0.45	0.22-0.93	0.03	0.43	0.21-0.88	0.02
Smoking index (<600 *vs.* ≥600)	0.59	0.36-0.99	0.04	0.58	0.35-0.96	0.03
Tissue (Sq *vs.* non-Sq)	0.87	0.47-1.60	0.65	-	-	-
Brain metastasis (yes *vs.* no)	0.85	0.48-1.51	0.58	-	-	-
LDH (<222 *vs.* ≥222)	0.75	0.45-1.23	0.25	-	-	-

HR: Hazard ratio; CI: confidence interval; PS: performance status; Sq: squamous cell carcinoma; LDH: lactate dehydrogenase.

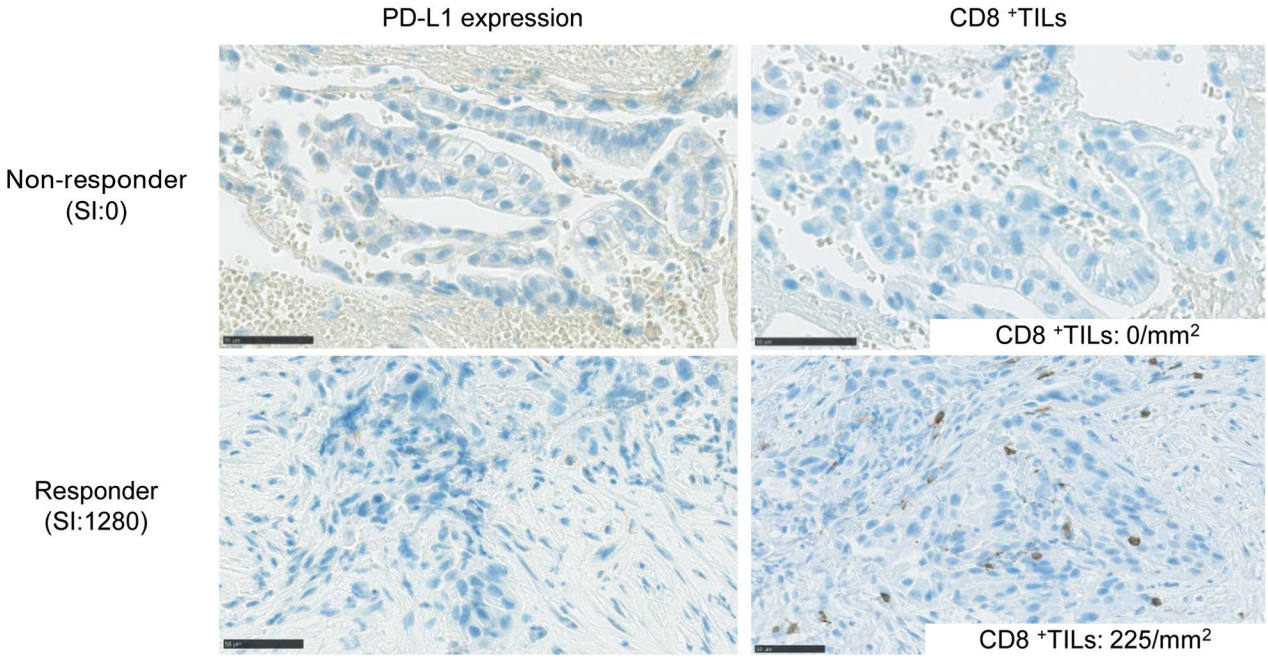

Figure 3. *Representative pathology images. Representative pathology images showing the CD8+ tumor infiltrating lymphocyte status of a non-responder (Smoking index: 0) and a responder (Smoking index: 1280).*

Smoking results in a high DNA mutational load in tumor cells (14-17). In addition, the etiology of smoking-associated cancers can be attributed to both mutagenic and immunomodulatory mechanisms. Lung squamous cell carcinoma, which is a representative of smoking-associated cancers, has been shown to have a strong pro-inflammatory phenotype characterized by an immune-inflamed TME (17).

Regarding the association between smoking history and response to anti-PD-1 therapy, the KEYNOTE-001 trial reported that high PD-L1 expression (TPS ≥50%) was correlated with a current/ever smoking history and was associated with an increased efficacy of pembrolizumab (18). Li *et al*. reported that even among those with high tumor PD-L1 expression levels, smoking status appeared to be an important additional factor associated with response (19). In addition, Rizvi *et al*. reported that a molecular smoking signature was associated with the efficacy of pembrolizumab in patients with advanced NSCLC (20). Therefore, smoking history has been a clinical predictor of the efficacy of PD-1 blockade therapy, probably because of the tumor mutation burden (TMB) in such patients. Our study showed that a heavy smoking habit could predict the response to PD-1 blockade. Therefore, patients with neither PD-L1 expression nor a smoking history are unlikely to benefit from PD-1 blockade therapy.

In the present study, we exploratorily evaluated how the presence of a smoking habit affects the density of $CD8^+$ TILs, which was a predictive biomarker of a response to PD-1 blockade therapy in PD-L1 expression-negative tumors. Indeed, an immune-inflamed phenotype was significantly associated with the SI in PD-L1 negative tumors. Schalper *et al*. reported that the $CD8^+$ TIL status, but not $CD3^+$ or $CD20^+$ TIL status, was significantly associated with smoking history and the prognosis of NSCLC patients, regardless of PD-L1 expression (7). Additionally, in our study, the multivariate analysis showed that the $CD8^+$ TIL status was the best predictive factors of PFS in patients with PD-L1 negative tumors (Table III). On the other hand, the size of biopsy samples in patients with advanced lung cancer is small, and it was difficult to evaluate the status of TILs in all NSCLC patients. Thus, we analyzed the association between the clinical factors and the status of $CD8^+$ TILs, and found that high density of $CD8^+$ TILs was significantly associated with a heavy smoking history.

The present study has several limitations. First, it is retrospective, and the number of patients was limited. Since the tissue size differed depending on the method used for tissue collection, we could not take into account the heterogeneity of PD-L1 expression. Second, a standardized method of evaluating $CD8^+$ TILs to identify the immune-inflamed phenotype has not yet been established. Also, it

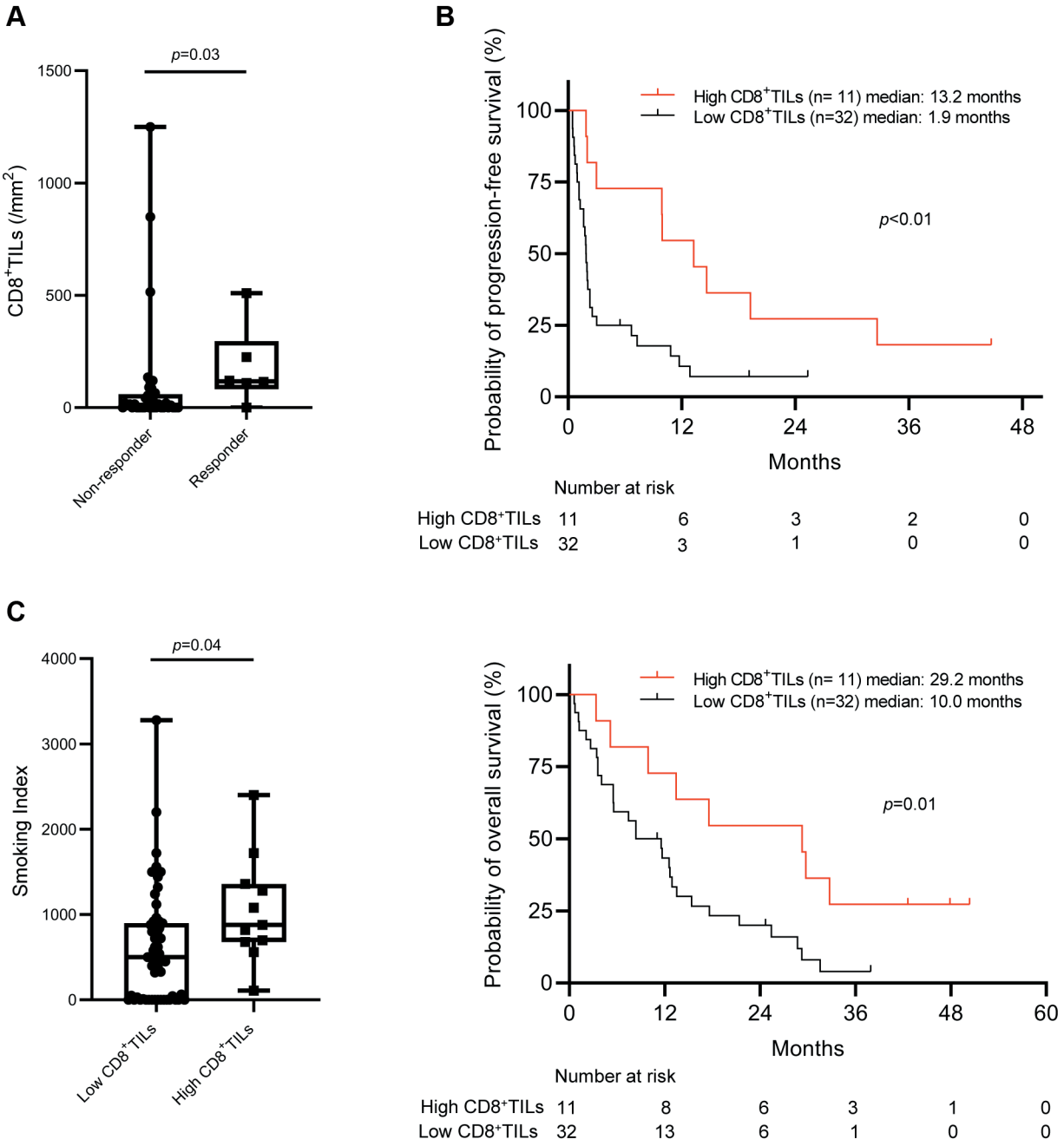

Figure 4. *Evaluation of CD8+ tumor infiltrating lymphocytes (TILs). (A) Difference in CD8+ TILs according to the response to nivolumab. (B) Progression-free survival after nivolumab treatment and overall survival according to the CD8+ TIL status. (C) Difference in smoking history according to the CD8+ TIL status.*

remains unclear whether the cutoff value of SI 600 is relevant. SI 600 is defined as heavy smoking history, and assumed as a high risk of lung cancer (21). Additionally, seven of 8 responders had heavy smoking history (SI ≥600), while none of the responders were non-smokers. Third, other biomarkers known to be associated with the efficacy of PD-1 inhibitors, such as TMB and the mutation profiles for STK11 and KEAP1, were not analyzed. Therefore, further investigations of the associations between TMB, mutation profiles, and the clinical outcomes of PD-1 blockade therapy are needed.

In conclusion, our data suggest a correlation between heavy smoking history and the efficacy of PD-1 blockade in

Table III. *Univariate and multivariate analyses including CD8+ TILs status for PFS of nivolumab.*

	Univariate analysis			Multivariate analysis		
	HR	95%CI	*p*-Value	HR	95%CI	*p*-Value
Age (<75 *vs.* ≥75)	0.83	0.20-3.43	0.80	-	-	-
Sex (male *vs.* female)	0.82	0.49-1.36	0.43	-	-	-
PS (0-1 *vs.* ≥2)	0.45	0.22-0.93	0.03	0.45	0.17-1.19	0.11
Smoking index (<600 *vs.* ≥600)	0.59	0.36-0.99	0.04	1.09	0.51-2.36	0.82
Tissue (Sq *vs.* non-Sq)	0.87	0.47-1.60	0.65	-	-	-
Brain metastasis (yes *vs.* no)	0.85	0.48-1.51	0.58	-	-	-
LDH (<222 *vs.* ≥222)	0.75	0.45-1.23	0.25	-	-	-
CD8+TILs (<110 *vs.* ≥110)	0.33	0.14-0.74	<0.01	0.32	0.13-0.80	0.01

HR: Hazard ratio; CI: confidence interval; PS: performance status; Sq: squamous cell carcinoma; LDH: lactate dehydrogenase; TILs: tumor infiltrated lymphocytes.

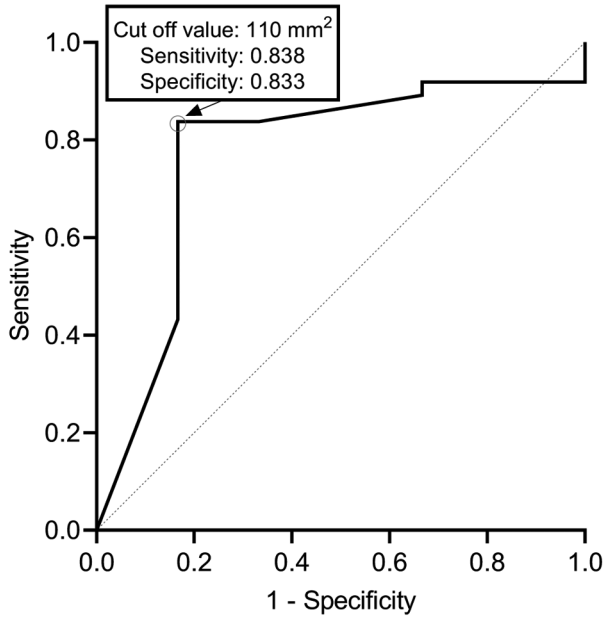

Figure 5. *Receiver operating characteristics curve. Receiver operating characteristics curve for CD8+ tumor infiltrating lymphocytes to predict the response of nivolumab.*

patients with PD-L1 expression-negative tumors. This is the first study to focus on predictors of the efficacy of PD-1 blockade in patients with PD-L1 expression-negative tumors. Even if PD-L1 expression is negative, PD-1 blockade can produce a response in patients with advanced NSCLC who have a heavy smoking history.

Conflicts of Interest

Dr. Yoshida has received grants and personal fees from AstraZeneca, Bristol-Myers Squibb, grants from Abbvie, MSD, Ono Pharmaceutical, Takeda Pharmaceutical, and personal fees from Chugai, Novartis. Dr. Matsumoto has received grants from Grant-in-Aid for Scientific Research on Innovative Areas, Hitachi High-Technologies, Hitachi, Ltd., National Cancer Center Research and Development Fund, and personal fees from AMCO INC., AstraZeneca, COOK, Olympus. Dr. Okuma has received grants from Abbvie. Dr. Goto has received grants and personal fees from Bristol-Myers Squibb, Daiichi- Sankyo, Eli Lilly, Guardant Health, MSD, Novartis, Ono Pharmaceutical, Pfizer, Taiho Pharmaceutical, grants from Kyorin, and personal fees from AstraZeneca, Boehringer Ingelheim, Chugai, Illumina. Dr. Horinouchi has received grants and personal fees from AstraZeneca, BMS, Chugai, Eli Lilly, MSD, Taiho Pharmaceutical, Ono Pharmaceutical, and grants from Astellas, Genomic Health, Merck Serono. Dr. Yamamoto has received grants and personal fees from BMS, Boehringer Ingelheim, Chugai, Eisai, Eli Lilly, Ono Pharmaceutical, Pfizer, Takeda Pharmaceutical, grants from Astellas, Bayer, Chiome Bioscience Inc., Daiichi-Sankyo, GSK, Janssen Pharma, Kyowa-Hakko kirin, MSD, Merck, Novartis, Otsuka, Taiho Pharmaceutical, Quintiles, Sumitomo Dainippon, and personal fees from AstraZeneca, Otsuka, Cimic, Sysmex. Dr. Yatabe has received personal fees from Archer, AstraZeneca, Chugai, Dako-Agilent, MSD, Novartis, Pfizer, Thermo-Fisher Science, Ventana-Roche. Dr. Ohe has received grants and personal fees from AstraZeneca, Bristol-Myers Squibb, Chugai, Eli Lilly, Janssen Pharma, Kyorin, MSD, Nippon Kayaku, Novartis, Ono Pharmaceutical, Pfizer, Taiho Pharmaceutical, Takeda Pharmaceutical, grants from Kissei, personal fees from Boehringer Ingelheim, Celtrion. Dr. Motoi has received grants and personal fees from Ono Pharmaceutical, Roche Diagnostics, grants from NEC, personal fees from AstraZeneca, Beckton Dickinson Japan, Covidien Japan Inc, Miraca Life Sciences, MSD, Novartis, Taiho Pharmaceutical. The remaining Authors declare no competing interests.

Authors' Contributions

YS and TY designed the study. YS, TM and HJ collected the clinical data. YS performed statistical data analyses, interpretation of the results and writing of the manuscript. YS, MS and NM evaluated the pathological findings. YS and TY drafted the manuscript. All Authors discussed the manuscript and approved the final version.

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