

Impact of EGFR-TKI Treatment on the Tumor Immune Microenvironment in *EGFR* Mutation-Positive Non-Small Cell Lung Cancer



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ABSTRACT

Purpose: The impact of EGFR tyrosine kinase inhibitors (TKI) on the tumor immune microenvironment (TME) in non-small cell lung cancer (NSCLC) is unclear.

Experimental Design: We retrospectively identified 138 patients with *EGFR*-mutated NSCLC who underwent rebiopsy after progression during EGFR-TKI treatment. PD-L1 and CD73 expression in tumor cells and tumor-infiltrating lymphocyte (TIL) density at baseline and after progression were determined by IHC. Tumor mutation burden (TMB) was determined by next-generation sequencing.

Results: The proportion of patients with a PD-L1 expression level of $\geq 50\%$ (high) increased from 14% before to 28% after EGFR-TKI ($P = 0.0010$). Whereas CD8⁺ and FOXP3⁺ TIL densities were significantly lower after EGFR-TKI treatment than before, CD8⁺

TIL density was maintained in tumors with a high PD-L1 expression level. Expression of CD73 in tumor cells after EGFR-TKI treatment was higher than that before in patients with a high PD-L1 expression level. TMB tended to be higher after EGFR-TKI treatment than before (3.3→4.1 mutations/Mbp, $P = 0.0508$). Median progression-free survival for subsequent treatment with antibodies to PD-1 was longer for patients with a high than for those with a low PD-L1 expression after EGFR-TKI (7.1 vs. 1.7 months, $P = 0.0033$), and two of five patients whose PD-L1 expression level changed from low to high after EGFR-TKI treatment achieved a PFS of >6 months.

Conclusions: EGFR-TKI treatment was associated with changes in the TME of *EGFR*-mutated NSCLC, and such changes may provide clues for optimization of subsequent PD-1 inhibitor treatment.

Introduction

Lung cancer is the leading cause of cancer-related death worldwide (1). Recent advances in the development of molecularly targeted drugs such as EGFR tyrosine kinase inhibitors (TKI) and immune-checkpoint inhibitors (ICI), including programmed cell death-1 (PD-1) and programmed cell death-ligand 1 (PD-L1) inhibitors, have greatly improved therapeutic outcome in non-small cell lung cancer (NSCLC).

The tumor immune microenvironment (TME) as characterized by indicators such as the expression of PD-L1 in tumor cells and the tumor nonsynonymous mutation burden (TMB) is an emerging

predictor of ICI response in NSCLC (2, 3). ICIs were recently found to be of limited benefit for patients with NSCLC harboring *EGFR* mutations (4, 5). Furthermore, recent studies have shown that *EGFR* mutation-positive NSCLC has a low TMB (6) and a high frequency of inactive tumor-infiltrating lymphocytes (TIL; ref. 7). Nevertheless, our previous study suggested that patients with *EGFR* mutation-positive tumors might receive benefit from PD-1 inhibitor treatment after acquiring resistance to conventional EGFR-TKIs if they are negative for the T790M TKI resistance-conferring mutation of *EGFR* and have a favorable TME for ICI therapy such as a high PD-L1 expression level and a high TMB (8). Although changes in the TME induced by EGFR-TKI treatment are likely to be an important determinant of the efficacy of subsequent ICI therapy, only a few previous studies have focused on such changes (5, 9).

We have now evaluated changes in the TME for patients with *EGFR* mutation-positive NSCLC between before and after the development of resistance to EGFR-TKI treatment.

Materials and Methods

Patients

We reviewed the medical records of all patients with *EGFR* mutation-positive advanced or recurrent NSCLC treated at Kindai University Hospital, Kanagawa Cardiovascular and Respiratory Center, Kishiwada City Hospital, and Kurashiki Central Hospital between February 2012 and May 2018. *EGFR* mutations were detected with commercial assays such as the cobas *EGFR* Mutation Test (Roche Molecular Diagnostics). The current study was performed according to the Declaration of Helsinki and protocols approved by the Institutional Review Board (IRB) of each hospital. All patients provided written informed consent, where applicable, or such informed consent

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Translational Relevance

Previous studies have found limited clinical benefit of treatment with immune-checkpoint inhibitors (ICI) in patients with non-small cell lung cancer harboring activating mutations of the EGFR gene. We examined the effects of EGFR tyrosine kinase inhibitor (TKI) therapy in such patients on the tumor immune microenvironment, a potentially important determinant of subsequent ICI treatment efficacy. The expression of programmed cell death-ligand 1 (PD-L1) in tumor cells was significantly increased and tumor mutation burden tended to be increased after the development of resistance to EGFR-TKI treatment. ICI efficacy tended to be higher in patients with a high level of PD-L1 expression in tumor cells after EGFR-TKI treatment. However, ICI efficacy was unsatisfactory in some patients, possibly as a result of immune suppressive factors, such as an increase in regulatory T-cell density and CD73 expression in tumor cells.

was waived by IRB-approved protocols for aggregate deidentified data analysis. We identified 138 patients who had baseline tissue specimens available and who underwent rebiopsy after disease progression during EGFR-TKI therapy including that with osimertinib. Patients who were diagnosed on the basis of cytology only or for whom the amount of residual tumor tissue specimens was insufficient for analysis were excluded (Supplementary Figure S1).

Data collection

Data regarding clinicopathologic features and treatment history were extracted from medical records. Data were updated as of 31 May, 2018. Responses were assessed according to RECIST v1.1. Progression-free survival (PFS) was measured from treatment initiation to clinical or radiographic progression or death from any cause. Overall survival (OS) was measured from treatment initiation to death from any cause. Patients without documented clinical or radiographic disease progression or who were still alive were censored on the date of last follow-up.

IHC

Tumor histology was classified according to WHO criteria. IHC was performed with mAbs to PD-L1 (kit with clone 22C3 and clone 28-8, Agilent Technologies), to CD8 (clone C8/144B, Agilent Technologies), to FOXP3 (clone 236A/E7, Abcam), and to CD73 (clone D7F9A, Cell Signaling Technology) and with the use of an automated stainer (Dako). The stained slides were evaluated by board-certified pathologists who were blinded to clinical outcome.

PD-L1 immunostaining was optimized with human placenta and tonsil as positive controls. We defined PD-L1 positivity as membranous staining of tumor cells at any intensity with cutoffs of $\geq 1\%$ and $\geq 50\%$ ($< 1\%$ defined as negative, 1 to 49% as low positive, and $\geq 50\%$ as strong positive) of tumor cells in a section that included at least 100 evaluable tumor cells (10). The percentage of PD-L1-positive tumor-infiltrating immune cells occupying the tumor was also recorded with cutoffs of $\geq 1\%$, $\geq 5\%$, and $\geq 10\%$ (11, 12). Tumor CD73 expression was graded on the basis of a semiquantitative H-score, which was calculated by multiplying the cytomembrane staining intensity (0, absent; 1, weak; 2, moderate; 3, strong) by the percentage of stained cells (0%–100%) to yield a value of 0 to 300 (13).

The number of TILs was evaluated at an absolute magnification of $\times 200$ ($0.0625 \text{ mm}^2/\text{field}$). At least one and a maximum of five scanned fields of tumor regions were randomly chosen for each TIL count. TILs were counted by one pathologist, and the density of TILs in the tumor was calculated by dividing the number of TILs by the sum of the area (mm^2) of the viewed fields (8, 13). TILs were defined as cells positive for CD8, FOXP3, or CD73 regardless of staining intensity. Cytology specimens and fragmented biopsy specimens were excluded from quantitative TIL assessment because of the lack of an adequate stromal component for analysis. For TIL analysis, all fields were evaluated as intratumoral regions.

TMB analysis

DNA was extracted from formalin-fixed paraffin-embedded tumor specimens with the use of a QIAamp DNA Micro Kit (Qiagen). Samples for which the concentration of extracted DNA was $< 20 \text{ ng/mL}$ were excluded. Whole-exome capture libraries were constructed with the use of Agilent Sure-Select Human All Exon v5.0. Samples with a library concentration of $> 20 \text{ ng}/\mu\text{L}$ were used for further analysis. Enriched exome libraries were sequenced with the HiSeq 2500 platform (Illumina), yielding an average of 115 million reads (11.6 Gb). Nonsynonymous mutations were identified with the CLC whole-exome pipeline (ver. CLC 5.0) followed by additional annotation for SIFT, polyphen2, and GERP as described previously (8). Samples with $> 20\%$ C \rightarrow T transitions, potentially resulting from DNA damage, were excluded from further analysis. For comparison with previous findings (14), TMB was calculated as the total number of nonsynonymous single-nucleotide or insertion-deletion mutations divided by the coding region captured in the latest version of the Memorial Sloan Kettering-Integrated Mutation Profiling of Actionable Cancer Targets (MSK-IMPACT) panel of 468 genes (1.22 Mb; ref. 9). Only those bases with sufficient coverage in the sequencing run and with an allele frequency of $\geq 10\%$ were included in the calculation of TMB.

Statistical analysis

Fisher exact test and the Mann-Whitney *U* test were applied to compare categorical or continuous variables, respectively. Bowker test of symmetry was used for categorical comparisons of PD-L1 expression. Paired data were compared with the Wilcoxon signed rank test. Differences in PFS or OS curves constructed by the Kaplan-Meier method were assessed with the log-rank test, and the Cox proportional hazard regression model was adopted to determine HRs. All *P* values were based on a two-sided hypothesis, and a *P* value of < 0.05 was considered statistically significant. Given the exploratory nature of the study, multiplicity adjustment was not performed. Statistical analysis was performed with JMP software version 10.0.2 (SAS Institute, Cary, NC).

Results

Patient characteristics

A total of 138 patients with EGFR mutation-positive NSCLC who had been treated with EGFR-TKIs and who had both baseline and postprogression tumor samples available was identified (Supplementary Figure S1). Of these 138 patients, paired analysis of pre-EGFR-TKI treatment and postprogression samples was possible for 134 individuals in the case of PD-L1 expression and CD8⁺ TILs, 133 individuals in the case of FOXP3⁺ TILs, 70 individuals in the case of

Table 1. Characteristics of the study patients.

N = 138	
Median age at rebiopsy (range), years	68 (42-86)
Sex	
Male	57 (41)
Female	81 (59)
Smoking status ^a	
Current or former	49 (36)
Never	87 (63)
Unknown	2 (1)
ECOG performance status at rebiopsy	
0-1	104 (75)
2-4	21 (15)
Unknown	13 (9)
Stage at initiation of first EGFR-TKI	
Recurrence	33 (24)
IIIB-IV	103 (75)
Other	2 (1)
Metastasis at baseline	
CNS	43 (31)
Intrathoracic only	48 (35)
Extrathoracic	80 (58)
None	9 (7)
Unknown	1 (1)
Histology at first biopsy	
Adeno/adenosquamous	136 (99)
Small cell	0
Squamous	0
Pleomorphic	1 (1)
Not otherwise specified	1 (1)
Not examined	0
EGFR mutation status at first biopsy	
Ex19del	82 (59)
L858R	47 (34)
T790M	4 (3)
Minor ^b	8 (6)
Wild type	0
Site of first biopsy	
Primary focus	119 (86)
Lung metastasis	1 (1)
Lymph node	6 (4)
Pleura	3 (2)
Liver	0
Skin	0
Muscle	0
Bone	1 (1)
Brain	2 (1)
Body fluid ^c	6 (4)
First EGFR-TKI	
Gefitinib	76
Erlotinib	37
Afatinib	22
Dacomitinib	3
Treatment before rebiopsy	
No cytotoxic agent	72 (52)
Cytotoxic drug	66 (48)
Treatment with osimertinib ^d	61 (44)
Treatment with antibody to PD-1 ^e	28 (20)

(Continued on the following column)

Table 1. Characteristics of the study patients. (Cont'd)

N = 138	
T790M positive at rebiopsy	11
T790M negative at rebiopsy	17
Pretreatment lines before antibody to PD-1	
Median (range)	3 (1-10)
1	3
2	8
3	3
4	3

Note: Data are N (%) unless indicated otherwise.

Abbreviations: CNS, central nervous system; ECOG, Eastern Cooperative Oncology Group; Ex19del, exon-19 deletion.

^aCurrent smokers, individuals who had smoked ≥100 cigarettes, including at least one within the previous year; former smokers, those who had smoked ≥100 cigarettes but had quit >1 year before rebiopsy; never-smokers, those who had smoked <100 cigarettes.

^bIncluding G719A, G719C, G719S, G719X, L861Q, L861X, and exon-20 insertions.

^cIncluding pleural, ascitic, pericardial, and cerebrospinal fluid.

^dIncluding four patients who were positive for T790M after progression during conventional EGFR-TKI treatment by liquid biopsy only.

^eIncluding one patient who underwent rebiopsy after progression on osimertinib without collection of a sample after conventional EGFR-TKI treatment.

CD73⁺ TILs, and 15 individuals in the case of TMB. Clinicopathologic characteristics of the patients included in the study are shown in **Table 1**. A total of 28 patients (20%) received the ICIs nivolumab or pembrolizumab intravenously after the development of EGFR-TKI resistance.

Change in PD-L1 expression and its association with T790M status

We first investigated changes in PD-L1 expression in tumor cells between before EGFR-TKI treatment and the development of drug resistance. PD-L1 expression was significantly changed after EGFR-TKI treatment ($n = 134, P = 0.0007$; **Fig. 1A**), and the proportion of patients with PD-L1 strong positive tumors increased from 14% at baseline to 28% after EGFR-TKI treatment ($n = 134, P = 0.0010$; **Fig. 1B**; Supplementary Table S1). A similar significant increase in the proportion of PD-L1 strong positive tumors was apparent for individuals negative for the T790M TKI resistance-conferring mutation at progression ($n = 70, P = 0.0125$) but not for those positive for this mutation ($n = 64, P = 0.0620$; **Fig. 1C**). In addition, the proportion of PD-L1 strong positive tumors postprogression tended to be greater for T790M-negative patients than for T790M-positive patients ($P = 0.0565$; Supplementary Table S1). The proportion of PD-L1 strong positive tumors was also significantly increased or tended to be increased after EGFR-TKI treatment regardless of smoking status or the type of EGFR-activating mutation (Supplementary Fig. S2A and S2B). We also investigated the change in PD-L1 expression by tumor-infiltrating immune cells between baseline and the development of EGFR-TKI resistance, which was evaluable in 101 cases. Although no significant change was detected ($n = 101, P = 0.1393$), the proportion of patients with a PD-L1 expression level in tumor-infiltrating immune cells of ≥10% increased from 11% at baseline to 25% after EGFR-TKI treatment (Supplementary Fig. S2C).

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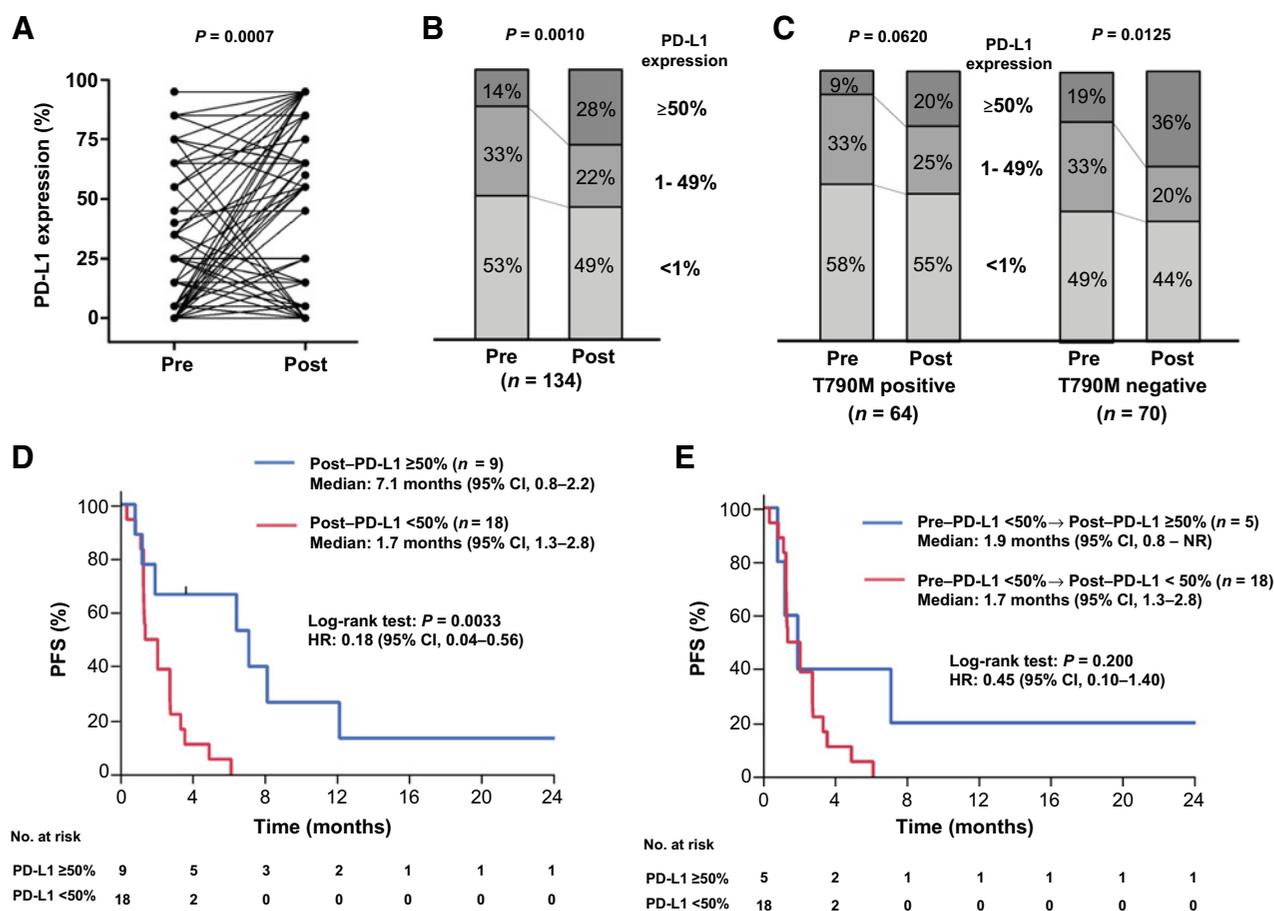


Figure 1. Changes in PD-L1 expression and ICI efficacy with regard to PD-L1 expression status. **A–C**, Changes in PD-L1 expression between before (pre) and after (post) EGFR-TKI treatment for all study patients with available paired tumor samples (**A** and **B**) as well as for those classified according to T790M status at the time of EGFR-TKI resistance development (**C**). PD-L1 expression is classified as $\geq 50\%$, 1% to 49%, or $< 1\%$ in **B** and **C**. The *P* values were determined with the Wilcoxon signed rank test (**A**) and Bowker’s test of symmetry (**B** and **C**). **D** and **E**, Kaplan–Meier curves of PFS for patients treated with antibodies to PD-1 after disease progression during EGFR-TKI treatment. PFS is plotted according to PD-L1 expression level with a cutoff of 50% in tumor samples obtained after EGFR-TKI treatment (**D**) or according to a change in PD-L1 expression status from $< 50\%$ to $\geq 50\%$ versus no change from $< 50\%$ (**E**). Tick marks represent censored data. CI, confidence interval; NR, not reached.

Association of PD-L1 expression postprogression with efficacy of subsequent ICI treatment

Twenty-seven patients with paired samples assayed for PD-L1 expression in tumor cells were subsequently treated with antibodies to PD-1. We evaluated PFS for ICI treatment according to PD-L1 expression in tumor cells in both pre- and post-EGFR-TKI treatment samples to examine which result was more related to ICI efficacy. Consistent with our previous findings (8), the median PFS was longer for patients with PD-L1 strong positive tumors than for those with a PD-L1 expression level of $< 50\%$ (negative or low positive) with regard to the post-EGFR-TKI treatment samples (**Fig. 1D**). A significantly better PFS was also apparent for patients with PD-L1 strong positive tumors with regard to the pre-EGFR-TKI treatment samples (Supplementary Fig. S2D). A similar trend was observed for OS relative to the initiation of ICI treatment (Supplementary Fig. S3A and S3B). Two of five patients whose PD-L1 expression level changed from negative or low positive at baseline to strong positive after the development of EGFR-TKI resistance showed a PFS for ICI treatment of > 6 months (**Fig. 1E**). We also found that patients negative for T790M and strongly positive for PD-L1 expression in post-EGFR-TKI treatment samples

showed the most favorable PFS and OS outcomes (Supplementary Fig. S3C and S3D). In addition, ICI treatment showed a durable response with regard to PFS in patients with a PD-L1 expression level in tumor-infiltrating immune cells in the post-EGFR-TKI treatment sample of $\geq 10\%$ (Supplementary Fig. S4A and S4B), consistent with previous findings (12). Although recent studies have suggested that *EGFR*-mutated tumors differ in their response to ICI treatment according to the type of mutation (15, 16), we detected no significant difference in PFS or OS between patients with exon-19 deletions and those with the L858R point mutation (Supplementary Fig. S5A and S5B).

Change in TIL density and its relation to PD-L1 expression

We next evaluated CD8⁺ and FOXP3⁺ TIL densities with paired tissue samples obtained before and after the development of EGFR-TKI resistance. The densities of both CD8⁺ and FOXP3⁺ TILs were significantly lower after EGFR-TKI treatment compared with before (median of 292.8→224.0/mm², *P* = 0.0274, and of 249.6→150.4/mm², *P* < 0.0001, respectively; **Fig. 2A** and **B**; Supplementary Table S1).

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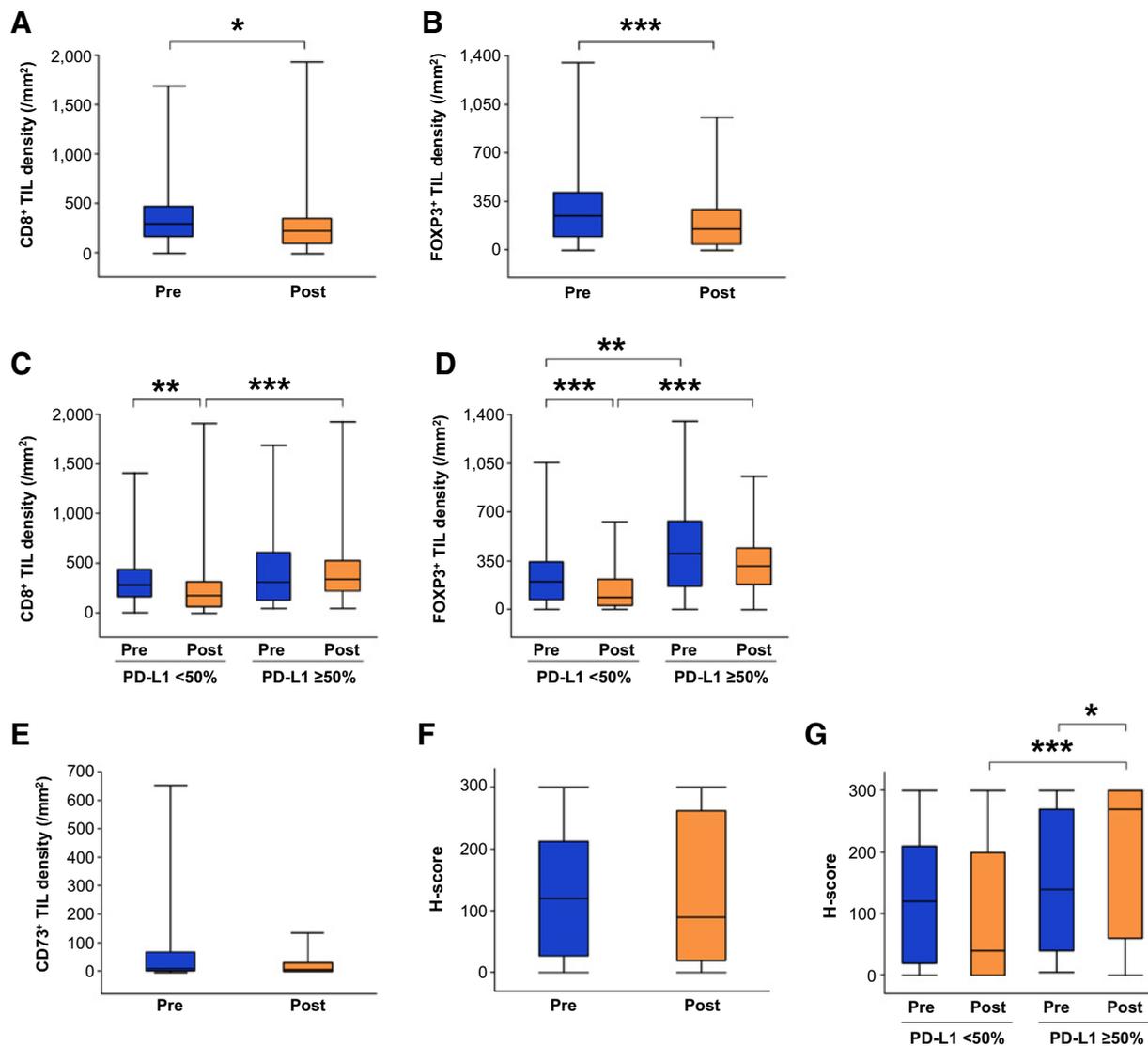


Figure 2.

Comparison of TIL densities and CD73 expression in tumor cells between before (pre) and after (post) EGFR-TKI treatment. **A** and **B**, CD8⁺ (**A**) and FOXP3⁺ (**B**) TIL densities for all study patients with available paired tumor samples. **C** and **D**, CD8⁺ (**C**) and FOXP3⁺ (**D**) TIL densities in pre- and post-EGFR-TKI treatment samples according to PD-L1 expression level with a cutoff of 50%. **E** and **F**, CD73⁺ TIL density (**E**) and CD73 expression in tumor cells (**F**) before (pre) and after (post) EGFR-TKI treatment for all study patients with available paired tumor samples. **G**, CD73 expression in pre- and post-EGFR-TKI treatment samples according to PD-L1 expression level with a cutoff of 50%. Each box plot shows the full range of variation, the median, and the interquartile range (top and bottom borders of the box). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by the Wilcoxon signed rank test for pairwise comparisons and by the Mann-Whitney U test for unpaired comparisons.

In the case of specimens obtained after EGFR-TKI treatment, CD8⁺ TIL density was significantly higher in PD-L1 strong positive tumors than in PD-L1 negative or low positive tumors (median of 337.6 vs. 172.8/mm², $P < 0.0001$), whereas there was no such difference between the corresponding pretreatment samples (**Fig. 2C**; Supplementary Table S2). In the case of both pre- and post-EGFR-TKI treatment samples, FOXP3⁺ TIL density was significantly higher in PD-L1 strong positive tumors than in PD-L1 negative or low positive tumors (median of 403.2 vs. 203.2/mm², $P = 0.0015$, and of 316.8 vs. 89.6/mm², $P = 0.0001$, respectively; **Fig. 2D**; Supplementary Table S2).

With regard to the change in TIL density according to PD-L1 expression status, both CD8⁺ and FOXP3⁺ TIL counts were significantly lower after EGFR-TKI treatment than before in PD-L1 negative or low positive tumors (median of 281.6→172.8/mm², $P = 0.0016$, and of 203.2→89.6/mm², $P < 0.0001$, respectively), whereas CD8⁺ TIL density essentially did not change (median of 312.0→337.6/mm², $P = 0.4014$) and FOXP3⁺ TIL density also showed a nonsignificant change (median of 403.2→316.8/mm², $P = 0.1544$) between before and after EGFR-TKI treatment in PD-L1 strong positive tumors (**Fig. 2C** and **D**; Supplementary Table S2). Together, these findings suggested that any improvement in the

TME in terms of CD8⁺ cell infiltration induced by EGFR-TKI treatment may occur only in PD-L1 strong positive tumors, despite the higher FOXP3⁺ TIL density in such tumors both before and after treatment compared with PD-L1 negative or low positive tumors.

We next evaluated CD73 expression in both tumor cells and TILs to explore why ICI efficacy is unsatisfactory in some patients with a favorable TME as characterized by a high PD-L1 expression level with CD8⁺ cell infiltration. CD73 is a mediator of immune suppression (17), and its expression in tumor cells was found to be high in *EGFR* mutation-positive NSCLC (18). CD73⁺ TIL density was significantly higher in tumors with a high FOXP3⁺ TIL density (with a cutoff of the median value for all study patients) than in those with a low FOXP3⁺ TIL density after EGFR-TKI treatment (median of 9.6 vs. 3.2/mm², $P = 0.0318$; Supplementary Fig. S6), consistent with a previous finding that CD73 is expressed on Treg cells (19). Unexpectedly, CD73⁺ TIL density was not significantly changed after EGFR-TKI treatment compared with baseline (median of 9.6→6.4/mm², $P = 0.0653$; Fig. 2E; Supplementary Table S1). Also, evaluation of CD73 expression in tumor cells by determination of H-score values revealed no significant change between before and after EGFR-TKI treatment (median of 120→90, $P = 0.9012$; Fig. 2F). Of note, however, expression of CD73 in tumor cells at the time of disease progression during EGFR-TKI treatment was significantly higher for PD-L1 strong positive tumors than for PD-L1 negative or low positive tumors (median of 270 vs. 40, $P = 0.0004$). In addition, a significant increase in CD73 expression was observed after EGFR-TKI treatment com-

pared with baseline in PD-L1 strong positive tumor (median of 140→270, $P = 0.0155$; Fig. 2G; Supplementary Table S2).

Representative IHC data and CT images for a patient who showed marked changes in the TME including an increase in PD-L1 expression level in tumor cells (5%–85%) after the development of EGFR-TKI resistance but who showed an unsatisfactory response to subsequent pembrolizumab treatment (PFS of 1.9 months) are shown in Fig. 3. The densities of CD8⁺ and FOXP3⁺ as well as the expression of CD73 in tumor cells changed from negative or low at baseline to high after the development of EGFR-TKI resistance, suggestive of possible immunosuppressive effects of regulatory T (Treg) cells and CD73 expression in tumor cells.

Change in TMB between before and after EGFR-TKI treatment

We determined TMB for paired tissue samples collected before EGFR-TKI treatment and at the time of resistance development. TMB tended to be higher after EGFR-TKI treatment than before for the 15 patients with paired samples available (median of 3.3→4.1 mutations/Mbp, $P = 0.0508$; Fig. 4A). Given that we found that, among patients with PD-L1 strong positive tumors after EGFR-TKI treatment, those who were T790M negative at the time of resistance development showed a more durable PFS response during subsequent ICI treatment than did those who were T790M positive (Supplementary Fig. S3C), we speculated that this difference in PFS might be attributable to a difference in TMB. However, we did not detect a significant difference in TMB according to T790M status at the time of EGFR-TKI resistance development

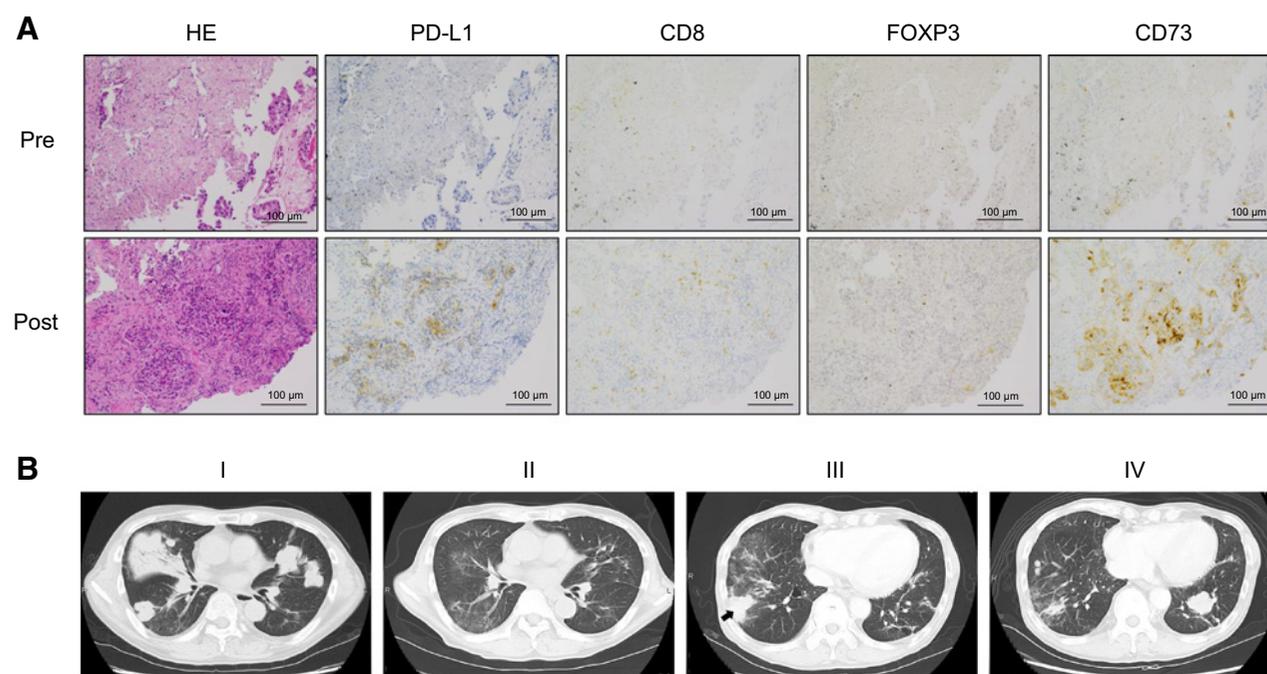


Figure 3.

IHC and computed tomography findings for a patient showing marked changes in the TME after EGFR-TKI treatment and an unsatisfactory response to subsequent pembrolizumab treatment. **A**, Hematoxylin-eosin (HE) staining and IHC staining of PD-L1, CD8, FOXP3, and CD73 in pre- and post-EGFR-TKI treatment samples. PD-L1 expression level in tumor cells (5% to 85%), CD8⁺ TIL density (51.2 to 425.6/mm²), FOXP3⁺ TIL density (73.6–339.2/mm²), and the CD73 H-score (40 to 210) were all increased after EGFR-TKI treatment. Scale bars, 100 μ m. **B**, CT images before (I), at the time of best response to (II), and at progression during (III) EGFR-TKI treatment as well as at progression during subsequent pembrolizumab treatment (IV). The initial biopsy sample was obtained from a right upper lobe tumor (not shown). The rebiopsy specimen was obtained from the right lower lobe tumor (arrow).

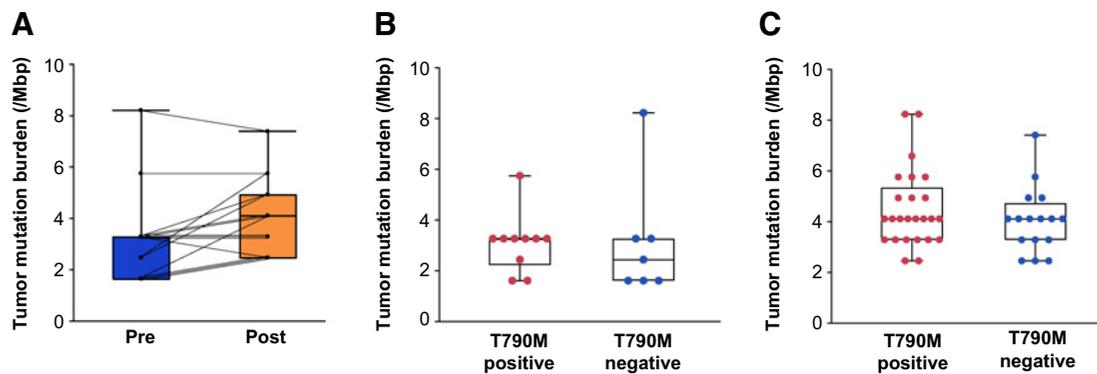


Figure 4.

TMB in the study patients. **A**, Changes in TMB between before (pre) and after (post) EGFR-TKI treatment for 15 patients with available paired samples. Paired samples are linked by black lines. **B** and **C**, TMB according to T790M status at the time of development of EGFR-TKI resistance for the 17 and 41 patients for whom TMB was determined in pre-EGFR-TKI treatment samples (**B**) or posttreatment samples (**C**), respectively. No significant differences were apparent between the two groups in each panel.

for tumor samples obtained either before (median of 3.1 vs. 3.2 mutations/Mbp for T790M positive or negative, respectively, $n = 17$, $P = 0.4430$; **Fig. 4B**) or after (median of 4.1 vs. 4.1 mutations/Mbp, $n = 41$, $P = 0.3866$; **Fig. 4C**) EGFR-TKI treatment.

Impact of osimertinib on the TME

Finally, we investigated nine patients who underwent rebiopsy after progression on osimertinib (**Table 2**). Among the eight individuals for whom paired samples from before and after osimertinib treatment were evaluable, PD-L1 expression was increased in two (25%). No trend for changes in CD8⁺, FOXP3⁺, and CD73⁺ TIL densities between before and after osimertinib treatment were apparent, whereas five of the seven patients for whom TMB was evaluated in paired pre- and posttreatment samples showed a decrease in this parameter.

Discussion

We have here retrospectively assessed whether EGFR-TKI therapy might improve the TME of *EGFR* mutation-positive NSCLC by analysis of paired tissue samples collected before and after such treatment. As far as we aware, our study is the first to evaluate the impact of EGFR-TKI therapy on the TME by comprehensive analysis of PD-L1 expression, TMB, as well as CD8⁺, FOXP3⁺, and CD73⁺ TIL densities in such a relatively large number of patients. We found that targeted therapy was associated with a significant increase in PD-L1 expression in tumor cells in post-progression tumor samples compared with those obtained at baseline, especially in the case of T790M-negative patients, consistent with previous findings that T790M-negative status was associated with higher PD-L1 expression (8, 20). In addition, our results suggested that EGFR-TKI treatment also might lead to an increase in PD-L1 expression in immune cells. Furthermore, patients who showed an increase in PD-L1 expression in tumor cells tended to manifest a durable response to subsequent ICI treatment, suggestive of a favorable impact of EGFR-TKI therapy on the TME with regard to ICI efficacy.

Little information has been available regarding whether PD-L1 expression changes in association with the development of EGFR-TKI resistance in patients with *EGFR*-mutated NSCLC. Studies have suggested that inhibition of the EGFR-MEK-ERK signaling pathway results in increased tumor cell expression of MHC class I molecules

(MHC-I), which are important for the presentation of antigens to CD8⁺ cytotoxic T cells (21, 22). Such upregulation of MHC-I might thus be expected to increase the availability of recognizable antigens and to promote the antitumor activity of CD8⁺ TILs. Although there was a significant decrease in CD8⁺ TIL density between before and after EGFR-TKI treatment in our study patients, CD8⁺ TIL density was significantly higher in PD-L1 strong positive tumors than in PD-L1 negative or low positive tumors after EGFR-TKI treatment, consistent with the previous finding that PD-L1 expression in cancer cells was induced by IFN γ released by CD8⁺ TILs (23).

FOXP3⁺ TIL density was increased in PD-L1 strong positive tumor specimens before and after EGFR-TKI treatment compared with corresponding PD-L1 negative or low positive tumor specimens. Although antibodies to PD-1 have shown efficacy in patients with *EGFR* mutation-positive NSCLC, especially in those with tumors strongly positive for PD-L1 (median PFS of 7.1 months in the current study), the results are unsatisfactory compared with the efficacy of these agents in patients with tumors wild type for *EGFR* (24). Immunosuppression mediated via the adaptive induction of Treg cells might contribute to this lower efficacy of PD-1 blockade therapy in *EGFR*-mutated NSCLC, even that strongly positive for PD-L1. Live and apoptotic Treg cells express the ectoenzymes CD39 and CD73 that convert extracellular ATP to adenosine (19), the latter of which mediates immunosuppression via interaction with the A2A receptor (17). Actually, a significant association between FOXP3 and CD73 expression in TILs was observed, but we did not detect a significant difference in the density of CD73⁺ TILs between before and after progression with EGFR-TKI treatment. Interestingly, however, we observed a higher CD73 expression in PD-L1 strong positive tumor cells after EGFR-TKI treatment compared with baseline. Tumor-derived CD73 was previously shown to suppress T cell and NK cell-mediated antitumor immunity in preclinical models (25). Therefore, the expression of CD73 in tumor cells might contribute in part to this insufficient efficacy of ICIs. The relevance of CD73 expression in both *EGFR*-mutated tumor cells and TILs to the efficacy of PD-1 blockade therapy remains unclear, with further investigation of the conversion of ATP to adenosine by CD39 and CD73 in *EGFR* mutation-positive NSCLC being warranted. Moreover, given that several clinical trials of antibodies that target CD73 or the A2A receptor are ongoing in patients with cancer (26), such agents are also

Table 2. Tumor immune microenvironment for nine patients with paired samples obtained before (pre) and after (post) osimertinib treatment.

Patient	Age (years)	Smoking status	EGFR activating mutation	Treatment lines before osimertinib	PFS on osimertinib (months)	PD-L1 expression level (%)		TMB (/Mbp)		CD8 ⁺ TILs (/mm ²)		FOXP3 ⁺ TILs (/mm ²)		CD73 ⁺ TILs (/mm ²)		CD73 H-score	
						Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
1	70	Never	Ex19del	1	2.8	1-49	<1	3.3	3.3	224.0	198.4	147.2	54.4	NA	NA	NA	NA
2	56	Never	Ex19del	7	20.3	<1	<1	NA	2.5	208.0	307.2	44.8	198.4	NA	NA	NA	NA
3	66	Never	Ex19del	6	5.0	1-49	≥50	4.1	2.5	313.6	22.4	288.0	16.0	NA	NA	NA	NA
4	67	Never	Ex19del	7	2.6	1-49	1-49	5.7	4.1	307.2	131.2	268.8	86.4	NA	NA	NA	NA
5	80	Never	Ex19del	1	10.3	≥50	≥50	4.1	4.9	57.6	128	76.8	83.2	NA	NA	NA	NA
6	64	Former	Ex19del	7	5.8	NA	1-49	NA	4.1	NA	1,232.0	NA	536.0	NA	NA	NA	NA
7	50	Never	Ex19del	3	8.1	<1	1-49	5.7	2.5	163.2	166.4	3.2	217.6	54.4	3.2	285	80
8	69	Former	L858R	2	3.6	≥50	≥50	5.7	4.9	425.6	147.2	339.2	150.4	0	9.6	210	40
9	73	Never	Ex19del	5	6.0	<1	<1	2.5	1.6	309.3	448.0	168.0	400.0	53.3	16.0	0	20

Abbreviations: Ex19del, exon-19 deletion; NA, not available.

candidates for combination therapy with ICIs that target the PD-1–PD-L1 system in selected populations such as patients with *EGFR*-mutated NSCLC strongly positive for PD-L1.

NSCLC harboring *EGFR* mutations has been shown to have a lower TMB compared with tumors without oncogenic drivers (6), but TMB was recently found to be significantly increased after EGFR-TKI treatment (9). Another recent study of a large cohort of patients with *EGFR*-mutated NSCLC with longitudinal cell-free DNA samples and clinical context showed that EGFR-TKI treatment resulted in enrichment of cooccurring genetic alterations (27). We have now also shown with paired tissue samples that TMB tended to be higher at the time of development of EGFR-TKI resistance compared with baseline, consistent with these previous findings. However, we were not able to investigate the relation between TMB and the efficacy of antibodies to PD-1 because of the lack of a sufficient number of tissue samples from treated cases. On the other hand, we did not detect a significant difference in TMB with regard to T790M status that was shown in previous study (9). As the number of patients was small in both studies, however, further investigation with a large number of patients is necessary to resolve this issue.

There are several limitations to our study. First, it was retrospective in nature. Second, a substantial number of patients received cytotoxic chemotherapy between the termination of EGFR-TKI treatment and rebiopsy. Third, despite the recent adoption of osimertinib in the first-line setting (28, 29), we focused mainly on those patients initially treated with conventional EGFR-TKIs. Further studies with more patients are needed to clarify the change in the TME between before and after osimertinib treatment. Another important limitation of our study relates to the heterogeneity of PD-L1 expression. Previous evaluations of PD-L1 expression in primary tumors and their metastases have shown that PD-L1 expression varies among tumor sites in the majority of cases (30–32). In the current study, pre- and post-TKI samples for evaluation of PD-L1 expression in tumor cells were obtained from the same anatomic lesions in only 65 of the 134 cases (49%). In addition, tissue samples for IHC analysis of PD-L1 are intrinsically nonhomogeneous (30), with the result that any change in PD-L1 expression observed might have been due to such heterogeneity.

In conclusion, we have shown that there were significant changes in the TME, including PD-L1 expression in tumor cells and TIL densities, between baseline and the time of development of EGFR-TKI resistance. The observed increase in PD-L1 expression suggests that EGFR-TKI therapy might confer a promising TME for subsequent ICI treatment. Nevertheless, unsatisfactory results with PD-1 inhibitors were obtained even in patients with tumors that express PD-L1 at a high level, possibly as a result of immunosuppressive factors such as an increase in Treg cell density and CD73 expression in tumor cells. Future investigations of the efficacy of combination immune therapy to overcome such immunosuppressive factors are warranted.

Disclosure of Potential Conflicts of Interest

K. Haratani reports receiving commercial research grants from AstraZeneca K.K., and reports receiving speakers bureau honoraria from AstraZeneca K.K., Ono Pharmaceutical Co. Ltd., AS ONE Corp., Bristol-Myers Squibb Co. Ltd., Chugai Pharmaceutical Co. Ltd., MSD K.K., and Pfizer Japan Inc. H. Hayashi reports receiving commercial research grants from AstraZeneca, Boehringer Ingelheim Japan Inc., and Ono Pharmaceutical Co. Ltd., and reports receiving speakers bureau honoraria from AstraZeneca K.K., Ono Pharmaceutical Co. Ltd., Boehringer Ingelheim Japan Inc., Bristol-Myers Squibb Co. Ltd., Chugai Pharmaceutical Co. Ltd., Eli Lilly Japan K.K., MSD K.K., Pfizer Japan Inc., Shanghai Haihe Biopharma, and Taiho Pharmaceutical Co. Ltd.

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