Clinicopathological features and immune microenvironment of non-small cell lung cancer with primary resistance to epidermal growth factor receptor-tyrosine kinase inhibitors

Yuta Takashima[a], Jun Sakakibara-Konishi[a], Yutaka Hatanaka[b], Kanako C. Hatanaka[b], Yoshihito Ohhara[c], Satoshi Oizumi[d], Yasuhiro Hida[e], Kichizo Kaga[e], Ichiro Kinoshita[c], Hirotoshi Dosaka-Akita[c], Yoshihiro Matsuno[b], Masaharu Nishimura[a]

[a]First Department of Medicine, Hokkaido University Hospital, Sapporo, Japan

[b]Department of Surgical Pathology, Hokkaido University Hospital, Sapporo, Japan

[c]Department of Medical Oncology, Hokkaido University Graduate School of Medicine, Sapporo, Japan

[d]Department of Respiratory Medicine, National Hospital Organization Hokkaido Cancer Center, Sapporo, Japan

[e]Department of Cardiovascular and Thoracic Surgery, Hokkaido University Graduate School of Medicine, Sapporo, Japan

Correspondence and request for reprints should be addressed to:

Jun Sakakibara-Konishi, MD, PhD; First Department of Medicine, Hokkaido University Hospital,

North 15, West 7, Kita-ku, Sapporo 060-8638, Japan

Phone: +81-11-716-1161 (Ext. 5911)

Fax: +81-11-706-7899

E-mail: konishj@med.hokudai.ac.jp

Abbreviations: CI, confidence interval; DCR, disease control rate; EGFR, epidermal growth factor receptor; EGFR-TKI, epidermal growth factor receptor-tyrosine kinase inhibitor; ERK, extracellular signal-regulated kinase; IHC, immunohistochemical; KRAS, v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog; MET, mesenchymal-epithelial transition; NSCLC, non-small cell lung cancer; ORR, objective response rate, OS, overall survival; PFS, progression free survival; PTEN, phosphatase and tensin homolog

MicroAbstract

We evaluated the clinical and immunopathological features of non-small cell lung cancer with primary resistance to epidermal growth factor receptor-tyrosine kinase inhibitors. The rate of smoking was significantly higher in primary resistance. The immune microenvironment characterized by low total tumor infiltrating lymphocytes and negative programmed death ligand 1 correlated significantly with primary resistance.

Abstract

Background: Approximately 20-30% of non-small cell lung cancer (NSCLC) patients with epidermal growth factor receptor (*EGFR*) activating mutations are not responsive to EGFR-tyrosine kinase inhibitors (TKIs). Although, primary resistance to EGFR-TKI is attributed to various genetic alterations, little is known about the clinical and immunopathological features of patients with primary resistance. The tumor immune microenvironment including tumor infiltrating lymphocytes (TILs) and programmed death ligand 1 (PD-L1) has been reported to play an important role in tumor progression in NSCLC. However, few studies have directly focused on the relationship between the tumor immune microenvironment and primary resistance to EGFR-TKI.

Materials and Methods: Characteristics of 124 NSCLC patients with *EGFR* mutations who received EGFR-TKI were analyzed. Primary resistance was defined as disease progression within 3 months

after EGFR-TKI treatment. Tumor specimens obtained before EGFR-TKI treatment were assessed for the density of TILs expressing CD4 or CD8, and for the expression rate of PD-L1 on tumor cells and tumor-infiltrating immune cells, immunohistochemically.

Results: Primary resistance was observed in 13.7% (17/124) of patients. Significant difference in smoking history was observed between patients with primary resistance and those with non-primary resistance. Lower density of total TILs and negative PD-L1 expression as per immunohistochemical analysis correlated significantly with primary resistance, in contrast to that with non-primary resistance. Moreover, immune ignorant phenotype of tumor microenvironment, negative PD-L1 expression with low TIL density, was significantly observed in primary resistance.

Conclusion: Smoking and immune ignorance in the tumor microenvironment might result in primary resistance to EGFR-TKIs.

Keywords: NSCLC, EGFR-TKI, primary resistance, tumor infiltrating lymphocyte, PD-L1, smoking

Introduction

Lung cancer is a leading cause of cancer-related death worldwide [1] and non-small cell lung cancer (NSCLC) accounts for approximately 80% of all lung cancers [2]. Epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKIs) are mainstay in the treatment of advanced unresectable NSCLC. The *EGFR* activating mutations are the primary predictive factors of EGFR-TKI therapy outcome [3,4]. Among patients with *EGFR* activating mutations, approximately 70-80% respond to EGFR-TKIs [5-7], however, acquired drug resistance is frequently observed, leading to disease progression. The mechanisms of acquired resistance have been revealed [8]. On the other hand, approximately 20-30% of patients with *EGFR* activating mutations are not responsive to EGFR-TKIs [4]. Little is known about the clinical and immunopathological features of patients with primary resistance, although possible mechanisms of primary resistance such as coexisting de novo T790M mutation [9], v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*) mutation [10], mesenchymal-epithelial transition (*MET*) amplification [11], and phosphatase and tensin homolog (*PTEN*) loss [12] have been investigated in some preclinical and retrospective studies. Therefore, further studies are required to elucidate the mechanisms of primary resistance.

Previous clinical trials in NSCLC indicated that high expression of programmed death ligand 1 (PD-L1) on tumor cells and/or tumor infiltrating lymphocytes (TILs) was a predictive factor of clinical outcome for programmed death 1 (PD-1)/PD-L1 inhibitor treatment [13-15]. Previously, we

have shown that the number of TILs in B7-H1 (PD-L1) positive tumor regions is significantly lower than that in B7-H1 negative tumor regions, indicating that B7-H1 expression on tumor cells might contribute to negative regulation against TILs in NSCLC [16]. Moreover, Akbay *et al*. showed that the activation of EGFR signaling induced the PD-1/PD-L1 pathway, leading to repression of T-cell function, thereby facilitating escape of tumor cells from the host immune system [17]. Although the tumor immune microenvironment including TILs and PD-L1 has been reported to play an important role in tumor progression in NSCLC, the relationship between the tumor immune microenvironment and primary resistance to EGFR-TKI is as yet unclear.

In this study, we evaluated the clinical features of patients with primary resistance to EGFR-TKI. Furthermore, we assessed the density of TILs and the expression of PD-L1 on tumor cells (TC) and tumor-infiltrating immune cells (IC) immunohistochemically to analyzed immune microenvironmental features of the primary resistance.

Materials and methods

Study patients

This study was approved by the Medical Ethics Committee of the Hokkaido University School of Medicine. Informed consent was obtained from all patients. *EGFR* mutations were identified in tumor tissues using the peptide nucleic acid-locked nucleic acid polymerase chain reaction (PNA-LNA PCR) clamp method or the Scorpion amplification refractory mutation system

(Scorpion-ARMS) method. A total of 124 patients with *EGFR* mutations who received EGFR-TKI therapy at the Hokkaido University Hospital, Japan, from January 2004 to January 2015, were screened. Medical records of patients were reviewed to obtain clinical and pathological information. Additional tumor tissue from the same sample previously used for EGFR assessment was available for analysis in all cases. We defined primary resistance as disease progression within 3 months after EGFR-TKI treatment based on a previous report [18].

Immunohistochemical staining

Four micrometer-thick sequential histologic tumor samples were obtained from representative formalin-fixed, paraffin-embedded tumor blocks. Samples were deparaffinized with xylene and rehydrated with graded alcohol. After rinsing with Tris-buffered saline, samples were processed for antigen retrieval with the Dako EnVision FLEX Target Retrieval Solution (pH 9.0) at 97^{o}C for 20 min using the Dako PT Link instrument, according to manufacturer's instructions. Staining for CD4 and CD8 was performed using an automated staining system (Autostainer Link 48) with antibodies against CD4 (clone 4B12, Ready-to-Use; Dako, Glostrup, Denmark), and CD8 (clone C8/144B, Ready-to-Use; Dako, Glostrup, Denmark). We used the anti-PD-L1 antibody clone SP142 (1:100; Spring Bioscience, Pleasanton, CA, USA) to manually stain for PD-L1. Samples were incubated with SP142 for 30 min, and staining was performed using the Dako EnVisio FLEX

system. Hematoxylin was used as a counterstain for all samples.

Evaluation of immunostaining

Since both biopsy samples and surgically resected samples were analyzed in this study, two different algorithms were used to evaluate density of TILs, based sample type. For surgically resected samples, we counted TILs expressing CD4 or CD8 in 3 random square areas (1 mm^2 each) in tumor compartments, and calculated the average. For biopsy samples, we counted TILs expressing CD4 or CD8 in all tumor areas on the microscope slide, and calculated density using the entire tumor area. Tumor area was measured using image analysis software (ImageJ). We scored TC expressing PD-L1 as a percentage of total TC, and IC expressing PD-L1 as a percentage of tumor area, as previously described (TC staining score: TC0: <1%, TC1: ≥1% to <5%, TC2: ≥5% to <50%, and TC3: ≥50%; IC staining score: IC0: <1%, IC1: ≥1% to <5%, IC2: ≥5% to <10% and IC3: ≥10%; Figure 2) [14,19]. We stratified PD-L1 staining based on intensity as follows: score 0: no staining, score 1: weak, score 2: moderate, score 3: strong. We further defined all samples with a score above score 1 as positive for PD-L1. Cell counting and scoring were performed by two observers, including one pathologist (K.C.H), who were blinded to clinical and pathological information.

Statistical analysis

Differences between primary resistance and non-primary resistance were compared using the Fisher's exact test. We used logistic regression models for performing multivariate analysis. The relationship between TIL density and clinicopathological variables was analyzed statistically using Wilcoxon's rank-sum test. Overall survival (OS) was defined as time from the treatment with EGFR-TKI to death, and was estimated using the Kaplan-Meier method. Differences in survival distributions were evaluated using the log-rank test. Two-sided p-values < 0.05 were considered statistically significant. All analyses were performed using the JMP software (JMP Pro12.2.0; SAS Institute Inc, USA).

Results

Patient characteristics

The characteristics of patients at the initiation of EGFR-TKI therapy are summarized in Table 1. The median age of all patients was 65 (37-85) years, and 58.1% (72/124) of patients were women. In addition, 47.1% of patients were never-smokers. Most of the patients had adenocarcinoma (96.0%). The most common mutation (50.8%) was the L858R mutation in exon 21, followed by a deletion in exon 19 (39.5%). 86.3% (107/124) of patients responded to EGFR-TKI treatment (non-primary resistance), while 13.7% (17/124) of patients exhibited primary resistance.

None of the patients had T790M at the diagnosis. Significant differences between primary resistance and non-primary resistance were found in sex and smoking history ($p<0.001$). We carried out multivariate analysis to adjust impact of sex on smoking history. In multivariate analysis, smoking history correlated significantly with primary resistance ($p=0.040$). Incidentally, 52.9% (9/17) of primary resistance and 53.3% (57/107) of non-primary resistance cases received systemic therapy after EGFR-TKI treatment, respectively (data not shown). The OS was significantly shorter in primary resistance cases compared to that in non-primary resistance cases (median: 11.6 months (95% confidence interval (CI): 3.2-19.0) in primary resistance vs. median: 31.6 months (95% CI: 24.9-37.4) in non-primary resistance, $p<0.001$) (Figure 1).

TIL density

To clarify whether TIL density was related to primary resistance, we immunohistochemically assessed the density of CD4 positive (CD4+) TILs and CD8 positive (CD8+) TILs in 79 tumor specimens. Total TIL density was defined as the sum of CD4+ and CD8+ TIL densities. Positive staining for CD4 and CD8 was observed in the cytoplasm of lymphocytes (Figure 2A, B). Median densities of CD4+ TILs and CD8+ TILs were 18.7 cells/mm^2 (0-257.7) and 33.7 cells/mm^2 (0-271.1), respectively (Supplementary Figure S1). We used the median density of CD4+ TILs, CD8+ TILs, and total TILs as cutoff points to divide the 79 patients into ‘high’ or ‘low’

infiltration groups classified by T-cell subsets in tumor areas. The relationship between TIL density and primary resistance is presented in Table 2. No significant correlation was observed between primary resistance and density of CD4+ TILs. Lower density of CD8+ TILs was observed in patients with primary resistance compared to those with non-primary resistance, although there was no statistical significance (p=0.087). In addition, lower density of total TILs correlated significantly with primary resistance in comparison with non-primary resistance (p=0.014). In this study, there was no significant correlation between smoking status and density of total TILs [median 63.7 cells/mm^2 (0-417.3) in never-smoker vs. median 56.7 cells/mm^2 (0-313.2) in smoker, p=0.832].

PD-L1 expression on tumor cells and tumor-infiltrating immune cells

Immunohistochemical assessment of PD-L1 expression was performed in 84 specimens. Positive staining for PD-L1 was observed in the membrane of TC and IC (Figure 2C, D). Distribution of PD-L1 expression patterns on TC and IC are summarized in Figure 3. In all cases, 81% (68/84), 2% (2/84), 11% (9/84), and 6% (5/84), samples were designated to be TC0, TC1, TC2, and TC3, respectively, and 52% (44/84), 27% (23/84), 13% (11/84), and 7% (6/84) were designated to be IC0, IC1, IC2, and IC3, respectively. The relationship between PD-L1 expression and primary resistance is listed in Table 3. We defined TC1/2/3 or IC1/2/3 (PD-L1 expression on ≥1% of TC or IC) as positive PD-L1 expression, as per a previous study [20]. Negative PD-L1 expression correlated

significantly with primary resistance in comparison with non-primary resistance (p=0.046). In this study, there was no significant correlation between smoking status and PD-L1 expression (p=0.827) (Supplementary Table S1). While analyzing the correlation between PD-L1 expression and density of total TILs, the density of total TILs was significantly higher in samples with PD-L1 positive compared to those with PD-L1 negative [median 87.0 cells/mm^2 (0-417.4) in PD-L1 positive vs. median 28.7 cells/mm^2 (0-327.4) in PD-L1 negative, p=0.002]. Based on a previously reported classification of the tumor microenvironment according to the TILs and PD-L1 status [21], we divided patients into two groups: “negative PD-L1 expression with low TIL density (density of total TILs)”, and “others”. We observed that a combination of negative PD-L1 expression and a low TIL density correlated significantly with primary resistance in comparison with non-primary resistance (p=0.006) (Table 4).

Discussion

This study shows that the rate of smoking was higher in primary resistance to EGFR-TKI compared to non-primary resistance. Moreover, based on the immunohistochemical analysis, lower density of TILs and PD-L1 negative status was observed in primary resistance.

This study revealed that PD-L1 expression on TC and IC was lower in primary resistance compared to non-primary resistance. Several studies have examined the correlation

between PD-L1 expression on TC and treatment outcomes in EGFR-TKI treated NSCLC patients with *EGFR* mutations [22-24], though the results were contradictory. It has been reported that in NSCLC patients with *EGFR* mutations, high PD-L1 expression on TC was associated with better response to EGFR-TKI and longer progression free survival (PFS) and OS [22,23]. On the other hand, Tang *et al*. reported that there was no correlation between the expression of PD-L1 and EGFR-TKI efficacy in NSCLC with *EGFR* mutations [24]. Moreover, the expression of PD-L1 on TC and IC, and its impact on response to EGFR-TKI in NSCLC with *EGFR* mutations have not been adequately investigated. Soo *et al*. reported that PD-L1 expression on TC and IC analyzed using SP142, similar to our study, was not associated with response to EGFR-TKI in NSCLC with *EGFR* mutations [25]. Comparison of results between the above mentioned study and the current study showed: TC0 status: 43% cases and 81% cases, respectively, and IC3 status: 0% cases and 7% cases, respectively. Furthermore, in the previous study, all patients were newly diagnosed and treatment-naive. This difference in distribution of PD-L1 expression patterns and patient characteristics might reflect the inconsistency of the association between PD-L1 expression on TC and IC and the efficacy of EGFR-TKI.

The status of tumor infiltrating lymphocytes (TILs) is considered to be an important prognostic parameter in a variety of cancers. Previously, high incidence of CD4+ and CD8+ TILs was reported to be a significant prognostic factor in NSCLC [26]. This study showed that low total TILs

(CD4+ TILs and CD8+ TILs) were significantly associated with primary resistance compared to non-primary resistance. Previous studies have shown conflicting results pertaining to the association between TILs and response to EGFR-TKIs. Soo *et al*. reported that CD3 positive (CD3+) TILs were associated with worse PFS in NSCLC with *EGFR* mutations [25]. On the other hand, it was reported that a subset of CD4+ and CD8+ TILs were not associated with PFS and OS [23]. Inconsistency between our results and other reports might be due a difference in T-cell subsets examined, in addition to differences in patient characteristics.

Further, our immunohistochemistry analysis revealed that a tumor microenvironment with a low total TIL density and an absence of PD-L1 expression was significantly linked with primary resistance. To the best of our knowledge, this is the first study to investigate the association between primary resistance to EGFR-TKIs and tumor microenvironment parameters such as TIL density and/or PD-L1 expression. Classification of tumors into four groups based on their PD-L1 status and presence or absence of TILs has already been proposed, and an assessment has been made to identify the best type of immunotherapy for each tumor class [21,27]. The tumor classification includes type I (PD-L1 positive with TILs driving adaptive immune resistance), type II (PD-L1 negative with no TILs indicating immune ignorance), type III (PD-L1 positive with no TILs indicating intrinsic induction), and type IV (PD-L1 negative with TILs indicating the role of other suppressors in promoting immune tolerance) tumors. Some studies about melanoma have reported that patients

with a type II microenvironment are predicted to have very poor prognosis based on a lack of detectable immune reaction [21]. In this study, patients with primary resistance also showed poor survival compared to those with non-primary resistance, indicating that a type II microenvironment may underlie primary resistance to EGFR-TKIs and poor prognosis. Recently, several immune checkpoint inhibitors including an anti-PD-1/PD-L1 antibody and an anti-CTLA-4 antibody have been reported to show efficacy in NSCLC [14,15,28-30]. Gainor *et al*. reported that patients with *EGFR* mutations showed low colocalization of PD-L1 expression and TILs, and showed low response to PD-1/PD-L1 inhibitors [31]. Our data suggests that primary resistance was significantly associated with an immune ignorant status among patients with *EGFR* mutations. Thus, in such patients, combination therapy that is designed to promote T-cell infiltration into tumors and further help preserve their activity, such as a combination of anti–CTLA-4 and anti–PD-1 antibodies, would be an effective immunotherapeutic modality. However, the detailed relationship between type II microenvironment and primary resistance is still unknown, and further investigations will be required to validate our results and proposed mechanisms.

In this study, we found that smoking history was significantly linked to primary resistance, which is consistent with the result of previous studies that cigarette smoke decreased objective response rate (ORR), disease control rate (DCR), and PFS in NSCLC patients treated with EGFR-TKIs [32-35]. A potential explanation for the poor response to EGFR-TKIs in smokers is the

possibility of activating *EGFR* mutations that can result from smoking. A previous report has suggested that activation of the nicotinic acetylcholine receptor by cigarette smoking may have increased extracellular signal-regulated kinase (ERK) and AKT signaling, leading to EGFR-TKI resistance [36]. In addition, it has been reported that the rate of genetic alteration in smokers with NSCLC was higher than that in never-smokers and activated the pathway that was not inhibited by EGFR-TKI [37]. Although this study is the first comprehensive analysis of smoking status, the expression of PD-L1 and TILs density in primary resistance to EGFR-TKI and further investigation will be required, smoking may have negative impact on tumor immune microenvironment in primary resistance.

There are several limitations and possible biases in our study. First, Because of limited small sample size, the statistical significance was marginal. Secondly, Because of its retrospective nature, patient selection and time trend biases regarding the diagnosis of disease progression and treatment might be inevitable. Additionally, we have not investigated the potential genetic or molecular alterations related to the primary resistance (such as *MET* amplification, *PTEN* loss etc.) and the correlation between these alterations and the tumor immune microenvironment.

In conclusion, we showed that smoking history correlated significantly with primary resistance to EGFR-TKI. We found that a tumor immune microenvironment characterized by low total TIL density and/or negative PD-L1 expression correlated significantly with primary resistance.

These results suggest that immune ignorance in the tumor microenvironment might result in primary resistance to EGFR-TKI therapy.

Clinical Practice Points

- Few studies have directly focused on the relationship between the tumor immune microenvironment and primary resistance to EGFR-TKI.
- We found that a tumor immune microenvironment characterized by low total TIL density and/or negative PD-L1 expression correlated significantly with primary resistance.
- This is the first study to investigate the association between primary resistance to EGFR-TKIs and tumor microenvironment parameters such as TIL density and/or PD-L1 expression.
- Our results suggest that immune ignorance in the tumor microenvironment might result in primary resistance to EGFR-TKI therapy. Thus, in such patients, combination therapy that is designed to promote T-cell infiltration into tumors and further help preserve their activity, such as a combination of anti–CTLA-4 and anti–PD-1 antibodies, would be an effective immunotherapeutic modality.

Conflict of interest statement

There is no conflict of interest to declare.

Acknowledgements

We thank M. Maeda (Department of Respiratory Medicine, Faculty of Medicine and Graduate School of Medicine, Hokkaido University), A. Nange and A. Okumura (Department of Surgical Pathology, Hokkaido University Hospital) for providing special support and experimental assistance during this study.

References

1. Siegel RL, Miller KD, Jemal A. Cancer Statistics, 2017. *CA Cancer J. Clin.* 2017; 67:7-30 doi: 10.3322/caac.21387.

2. Rothschild SI. Targeted Therapies in Non-Small Cell Lung Cancer-Beyond EGFR and ALK. *Cancers (Basel).* 2015; 7:930-949 doi: 10.3390/cancers7020816.

3. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *N. Engl. J. Med.* 2004; 350:2129-2139 doi: 10.1056/NEJMoa040938.

4. Maemondo M, Inoue A, Kobayashi K, et al. Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR. *N. Engl. J. Med.* 2010; 362:2380-2388 doi: 10.1056/NEJMoa0909530.

5. Mitsudomi T, Morita S, Yatabe Y, et al. Gefitinib versus cisplatin plus docetaxel in patients with non-small-cell lung cancer harbouring mutations of the epidermal growth factor receptor (WJTOG3405): an open label, randomised phase 3 trial. *Lancet Oncol.* 2010; 11:121-128 doi: 10.1016/S1470-2045(09)70364-X.

6. Rosell R, Carcereny E, Gervais R, et al. Erlotinib versus standard chemotherapy as first-line treatment for European patients with advanced EGFR mutation-positive non-small-cell lung cancer (EURTAC): a multicentre, open-label, randomised phase 3 trial. *Lancet Oncol.* 2012; 13:239-246 doi: 10.1016/S1470-2045(11)70393-X.

7. Sequist LV, Yang JC, Yamamoto N, et al. Phase III study of afatinib or cisplatin plus pemetrexed in patients with metastatic lung adenocarcinoma with EGFR mutations. *J. Clin. Oncol.* 2013; 31:3327-3334 doi: 10.1200/JCO.2012.44.2806.

8. Remon J, Moran T, Majem M, et al. Acquired resistance to epidermal growth factor receptor tyrosine kinase inhibitors in EGFR-mutant non-small cell lung cancer: a new era begins. *Cancer Treat. Rev.* 2014; 40:93-101 doi: 10.1016/j.ctrv.2013.06.002.

9. Su KY, Chen HY, Li KC, et al. Pretreatment epidermal growth factor receptor (EGFR) T790M mutation predicts shorter EGFR tyrosine kinase inhibitor response duration in patients with non-small-cell lung cancer. *J. Clin. Oncol.* 2012; 30:433-440 doi: 10.1200/JCO.2011.38.3224.

10. Takeda M, Okamoto I, Fujita Y, et al. De novo resistance to epidermal growth factor receptor-tyrosine kinase inhibitors in EGFR mutation-positive patients with non-small cell lung cancer. *J. Thorac. Oncol.* 2010; 5:399-400 doi: 10.1097/JTO.0b013e3181cee47e.

11. Cappuzzo F, Janne PA, Skokan M, et al. MET increased gene copy number and primary resistance to gefitinib therapy in non-small-cell lung cancer patients. *Ann. Oncol.* 2009; 20:298-304 doi: 10.1093/annonc/mdn635.

12. Sos ML, Koker M, Weir BA, et al. PTEN loss contributes to erlotinib resistance in EGFR-mutant lung cancer by activation of Akt and EGFR. *Cancer Res.* 2009; 69:3256-3261 doi: 10.1158/0008-5472.CAN-08-4055.

13. Rizvi NA, Mazieres J, Planchard D, et al. Activity and safety of nivolumab, an anti-PD-1 immune checkpoint inhibitor, for patients with advanced, refractory squamous non-small-cell lung cancer (CheckMate 063): a phase 2, single-arm trial. *Lancet Oncol.* 2015; 16:257-265 doi: 10.1016/S1470-2045(15)70054-9.

14. Rittmeyer A, Barlesi F, Waterkamp D, et al. Atezolizumab versus docetaxel in patients with previously treated non-small-cell lung cancer (OAK): a phase 3, open-label, multicentre randomised controlled trial. *Lancet.* 2017; 389:255-265 doi: 10.1016/S0140-6736(16)32517-X.

15. Herbst RS, Baas P, Kim DW, et al. Pembrolizumab versus docetaxel for previously treated, PD-L1-positive, advanced non-small-cell lung cancer (KEYNOTE-010): a randomised controlled trial. *Lancet.* 2016; 387:1540-1550 doi: 10.1016/S0140-6736(15)01281-7.

16. Konishi J, Yamazaki K, Azuma M, Kinoshita I, Dosaka-Akita H, Nishimura M. B7-H1 expression on non-small cell lung cancer cells and its relationship with tumor-infiltrating lymphocytes and their PD-1 expression. *Clin. Cancer Res.* 2004; 10:5094-5100 doi: 10.1158/1078-0432.CCR-04-0428.

17. Akbay EA, Koyama S, Carretero J, et al. Activation of the PD-1 pathway contributes to immune escape in EGFR-driven lung tumors. *Cancer Discov.* 2013; 3:1355-1363 doi: 10.1158/2159-8290.CD-13-0310.

18. Lee JK, Shin JY, Kim S, et al. Primary resistance to epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) in patients with non-small-cell lung cancer harboring TKI-sensitive

EGFR mutations: an exploratory study. *Ann. Oncol.* 2013; 24:2080-2087 doi: 10.1093/annonc/mdt127.

19. Herbst RS, Soria JC, Kowanetz M, et al. Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. *Nature.* 2014; 515:563-567 doi: 10.1038/nature14011.

20. Hirsch FR, McElhinny A, Stanforth D, et al. PD-L1 Immunohistochemistry Assays for Lung Cancer: Results from Phase 1 of the Blueprint PD-L1 IHC Assay Comparison Project. *J. Thorac. Oncol.* 2017; 12:208-222 doi: 10.1016/j.jtho.2016.11.2228.

21. Teng MW, Ngiow SF, Ribas A, Smyth MJ. Classifying Cancers Based on T-cell Infiltration and PD-L1. *Cancer Res.* 2015; 75:2139-2145 doi: 10.1158/0008-5472.CAN-15-0255.

22. D'Incecco A, Andreozzi M, Ludovini V, et al. PD-1 and PD-L1 expression in molecularly selected non-small-cell lung cancer patients. *Br. J. Cancer.* 2015; 112:95-102 doi: 10.1038/bjc.2014.555.

23. Lin C, Chen X, Li M, et al. Programmed Death-Ligand 1 Expression Predicts Tyrosine Kinase Inhibitor Response and Better Prognosis in a Cohort of Patients With Epidermal Growth Factor Receptor Mutation-Positive Lung Adenocarcinoma. *Clin. Lung Cancer.* 2015; 16:e25-35 doi: 10.1016/j.cllc.2015.02.002.

24. Tang Y, Fang W, Zhang Y, et al. The association between PD-L1 and EGFR status and the prognostic value of PD-L1 in advanced non-small cell lung cancer patients treated with EGFR-TKIs. *Oncotarget.* 2015; 6:14209-14219 doi: 10.18632/oncotarget.3694.

25. Soo RA, Kim HR, Asuncion BR, et al. Significance of immune checkpoint proteins in EGFR-mutant non-small cell lung cancer. *Lung Cancer.* 2017; 105:17-22 doi: 10.1016/j.lungcan.2017.01.008.

26. Hiraoka K, Miyamoto M, Cho Y, et al. Concurrent infiltration by CD8+ T cells and CD4+ T cells is a favourable prognostic factor in non-small-cell lung carcinoma. *Br. J. Cancer.* 2006; 94:275-280 doi: 10.1038/sj.bjc.6602934.

27. Taube JM, Anders RA, Young GD, et al. Colocalization of inflammatory response with B7-h1 expression in human melanocytic lesions supports an adaptive resistance mechanism of immune escape. *Sci. Transl. Med.* 2012; 4:127ra137 doi: 10.1126/scitranslmed.3003689.

28. Brahmer J, Reckamp KL, Baas P, et al. Nivolumab versus Docetaxel in Advanced Squamous-Cell Non-Small-Cell Lung Cancer. *N. Engl. J. Med.* 2015; 373:123-135 doi: 10.1056/NEJMoa1504627.

29. Borghaei H, Paz-Ares L, Horn L, et al. Nivolumab versus Docetaxel in Advanced Nonsquamous Non-Small-Cell Lung Cancer. *N. Engl. J. Med.* 2015; 373:1627-1639 doi: 10.1056/NEJMoa1507643.

30. Hellmann MD, Rizvi NA, Goldman JW, et al. Nivolumab plus ipilimumab as first-line treatment for advanced non-small-cell lung cancer (CheckMate 012): results of an open-label, phase 1, multicohort study. *Lancet Oncol.* 2017; 18:31-41 doi: 10.1016/S1470-2045(16)30624-6.

31. Gainor JF, Shaw AT, Sequist LV, et al. EGFR Mutations and ALK Rearrangements Are Associated with Low Response Rates to PD-1 Pathway Blockade in Non-Small Cell Lung Cancer: A Retrospective Analysis. *Clin. Cancer Res.* 2016; 22:4585-4593 doi: 10.1158/1078-0432.CCR-15-3101.

32. Hasegawa Y, Ando M, Maemondo M, et al. The role of smoking status on the progression-free survival of non-small cell lung cancer patients harboring activating epidermal growth factor receptor (EGFR) mutations receiving first-line EGFR tyrosine kinase inhibitor versus platinum doublet chemotherapy: a meta-analysis of prospective randomized trials. *Oncologist.* 2015; 20:307-315 doi: 10.1634/theoncologist.2014-0285.

33. Igawa S, Sasaki J, Otani S, et al. Impact of Smoking History on the Efficacy of Gefitinib in Patients with Non-Small Cell Lung Cancer Harboring Activating Epidermal Growth Factor Receptor Mutations. *Oncology.* 2015; 89:275-280 doi: 10.1159/000438703.

34. Kim MH, Kim HR, Cho BC, et al. Impact of cigarette smoking on response to epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors in lung adenocarcinoma with activating EGFR mutations. *Lung Cancer.* 2014; 84:196-202 doi: 10.1016/j.lungcan.2014.01.022.

35. Zhang Y, Kang S, Fang W, et al. Impact of smoking status on EGFR-TKI efficacy for advanced non-small-cell lung cancer in EGFR mutants: a meta-analysis. *Clin. Lung Cancer.* 2015; 16:144-151.e141 doi: 10.1016/j.cllc.2014.09.008.

36. Wang S, Takayama K, Tanaka K, et al. Nicotine induces resistance to epidermal growth factor receptor tyrosine kinase inhibitor by alpha1 nicotinic acetylcholine receptor-mediated activation in PC9 cells. *J. Thorac. Oncol.* 2013; 8:719-725 doi: 10.1097/JTO.0b013e31828b51d4.

37. Govindan R, Ding L, Griffith M, et al. Genomic landscape of non-small cell lung cancer in smokers and never-smokers. *Cell.* 2012; 150:1121-1134 doi: 10.1016/j.cell.2012.08.024.

Figure Legends

Figure 1:

Kaplan-Meier survival curves of association between epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI) response and overall survival time.

Figure 2:

Representative immunohistochemical (IHC) staining of CD4, CD8 and programmed death ligand 1 (PD-L1) in tumor area. (A) Positive IHC staining pattern of CD4 (x 100), (x 400). (B) Positive IHC staining pattern of CD8 (x 100), (x 400). (C) Positive IHC staining pattern of PD-L1 on tumor cells (x 200). (D) PD-L1 on immune cells (x 200), (x 400). Arrows indicate cells expressing PD-L1.

Figure 3:

Prevalence of programmed death ligand 1 (PD-L1) expression on tumor cells (TC) and/or immune cells (IC) in all patients. (A) Percentages in Venn diagrams represent the prevalence of PD-L1 expression in non-overlapping subgroups. (B) Prevalence of each TC group. (C) Prevalence of each IC group. (D) Prevalence of each TC/IC combination subgroup.

Supplementary Figure Legend

Supplementary Figure S1:

Histograms of tumor-infiltrating lymphocyte (TIL) density assessed by immunohistochemistry (IHC).

(A) Density of TILs expressing CD4. (B) Density of TILs expressing CD8. (C) Density of total TILs.

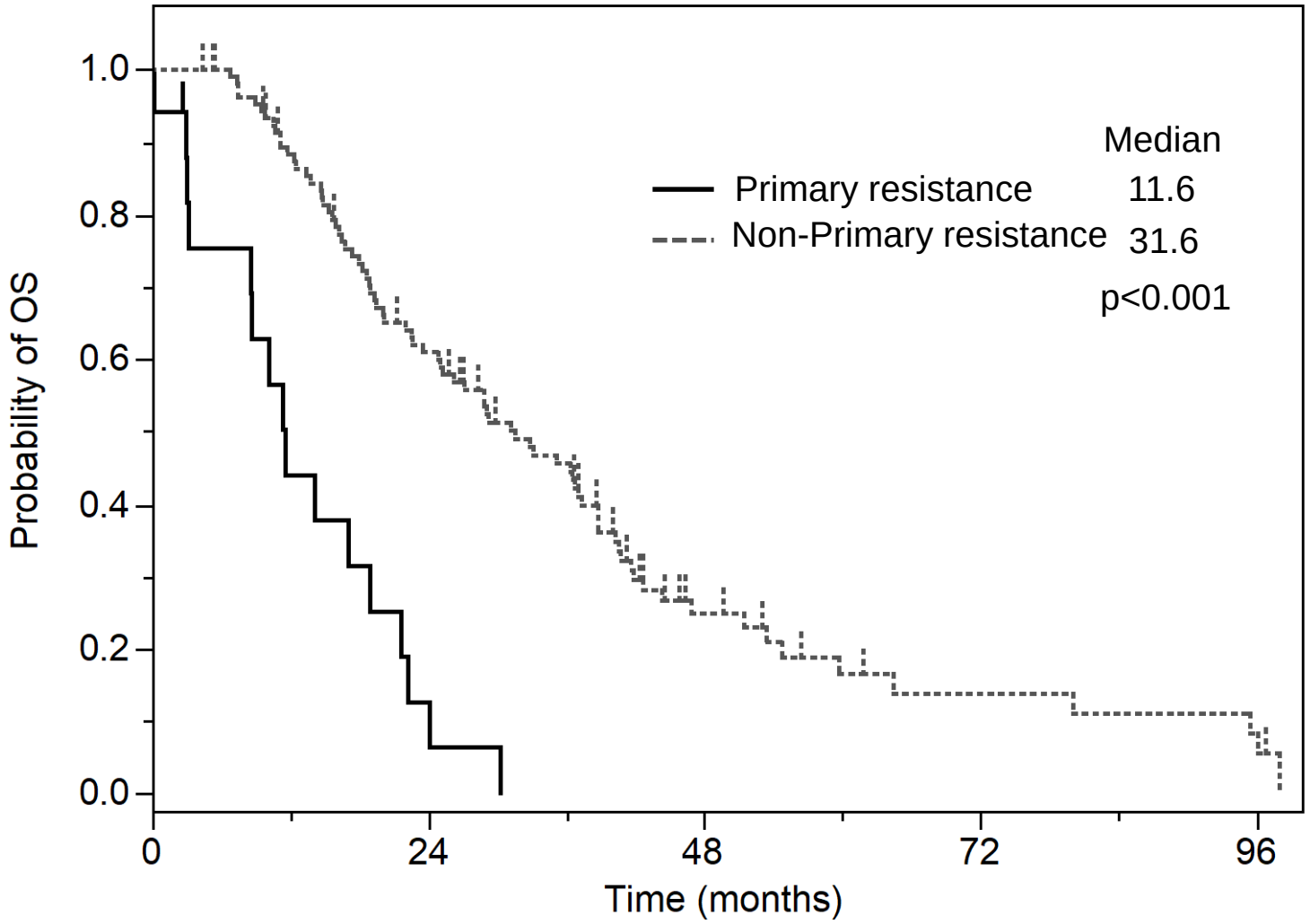

Probability of OS
1.0
0.8
0.6
0.4
0.2
0.0
0
24
48
72
96
Time (months)
Median
Primary resistance 11.6
Non-Primary resistance 31.6
p<0.001

Figure 1.

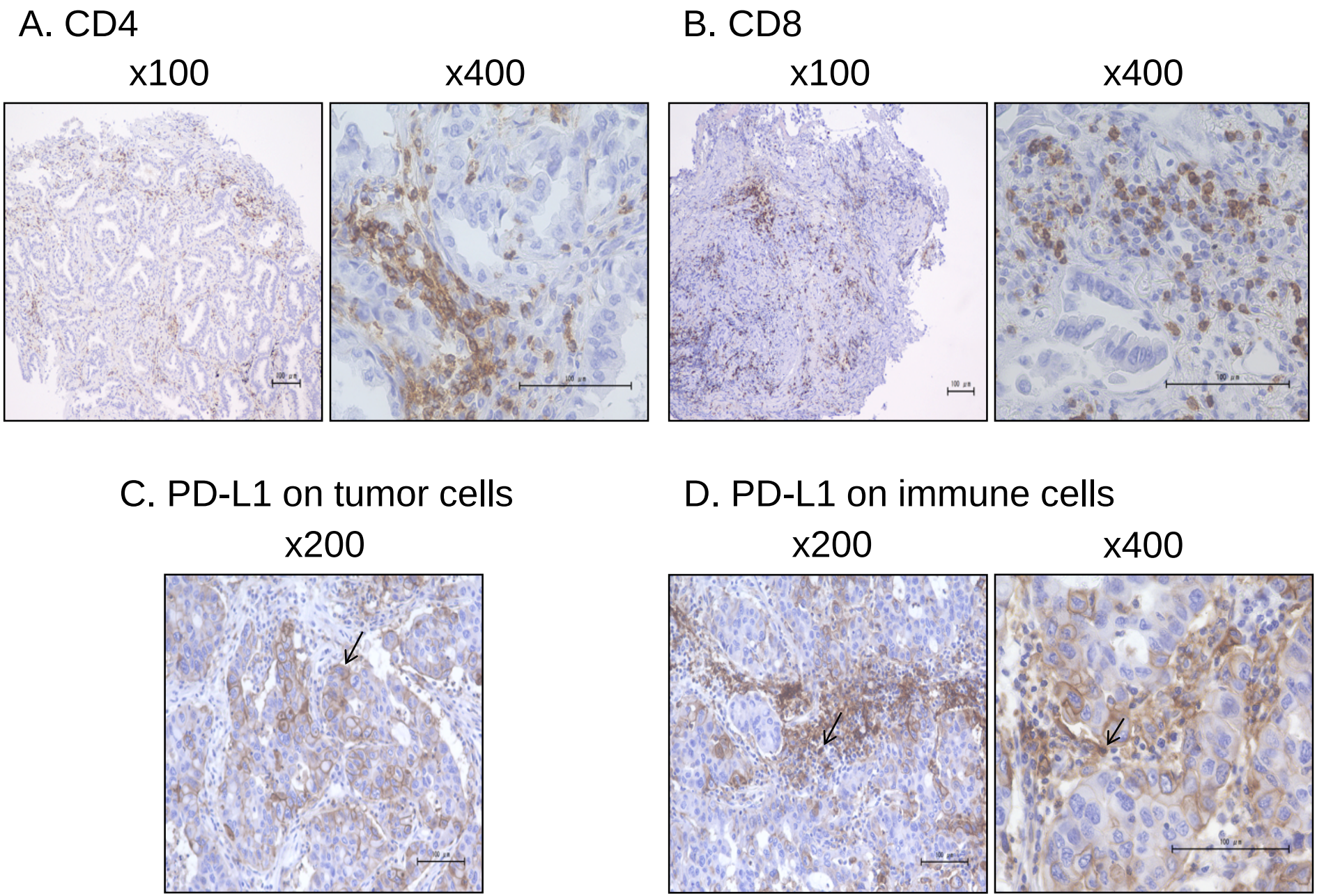
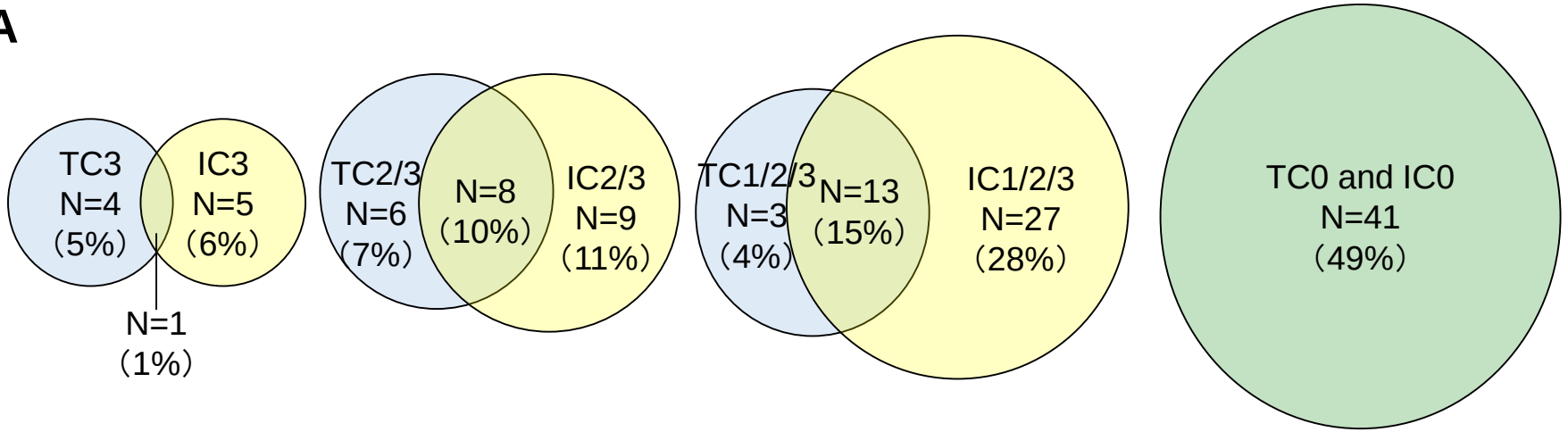

A. CD4
x100
x400
B. CD8
x100
x400
C. PD-L1 on tumor cells
x200
D. PD-L1 on immune cells
x200
x400

Figure 2.

A



B

Score	Percentage of PD-L1 expressing on TC	n（%）
TC3	≥50%	5（6%）
TC2	≥5% and <50%	9（11%）
TC1	≥1% and <5%	2（2%）
TC0	<1%	68（81%）

C

Score	Percentage of PD-L1 expressing on IC	n （%）
IC3	≥10%	6（7%）
IC2	≥5% and <10%	11（13%）
IC1	≥1% and <5%	23（27%）
IC0	<1%	44（52%）

D

Subgroup	%
TC3 or IC3	11%
TC2/3 or IC2/3	29%
TC1/2/3 or IC1/2/3	51%
TC0 and IC0	49%

Figure 3.

Table 1. Clinical characteristics of patients according to response to EGFR-TKI therapy

	Primary resistance (n=17)	Non-primary resistance (n=107)	Univariate analysis P-value	Multivariate analysis P-value
Sex				
Male	14	38	**<0.001**	0.077
Female	3	69		
Age (years)				
Median (range)	58 (45-82)	65 (37-85)		
≤65	11	55	0.434	
>65	6	52		
ECOG PS				
0-1	14	97	0.386	
≥ 2	3	10		
Smoking				
Smoker	16	49	**<0.001**	**0.040**
Never-Smoker	1	58		
Histology				
ADC	16	103	0.528	
Non-ADC	1	4		
Stage [a]				
Ⅳ	13	69	0.416	
others	4	38		
EGFR mutation status				
exon 19 del	3	46	0.054 [b]	
exon 21 L858R	12	51		
exon 18 G719X	0	3 [c]		
Unknown [c]	2	7		
EGFR-TKI				
1st line	10	80	0.240	
≥2nd line	7	27		

[a] Clinical stage at the time of initial diagnosis was determined according to American Joint Committee on Cancer (Six edition) guidelines.

[b] exon 19 del vs. exon 21 L858R. [c] The records of *EGFR* mutation subtype were missing.

ADC, Adenocarcinoma; ECOG PS, Eastern Cooperative Oncology Group Performance Status; EGFR, Epidermal growth factor receptor; TKI, tyrosine kinase inhibitor.

Table 2. The relationship between TIL density and EGFR-TKI primary resistance

		Primary resistance (n=9)	Non-primary resistance (n=70)	P-value
CD4+ TILs	Low	6	33	0.145
	High	3	37	
CD8+ TILs	Low	7	32	0.087
	High	2	38	
Total TILs *	Low	8	31	**0.014**
	High	1	39	

* Total TILs defined as the total amount of CD4+ TILs and CD8+ TILs.

TILs, Tumor infiltrating lymphocytes.

Table 3. The relationship between programmed death ligand 1 (PD-L1) expression and epidermal growth factor receptor-tyrosine kinase inhibitor (EGFR-TKI) primary resistance

		Primary resistance (n=10)	Non-primary resistance (n=74)	P-value
TC	<1%	9	59	0.678
	≥1%	1	15	
IC	<1%	8	36	0.092
	≥1%	2	38	
TC and IC	TC0 and IC0	8	33	**0.046**
	TC1/2/3 or IC1/2/3	2	41	

Tumor cell staining score represents staining positivity: TC0, <1%, TC1, ≥1% to <5%, TC2, ≥5% to <50%, and TC3, ≥50%.

Immune cell staining score represents staining positivity: IC0, <1%; IC1, ≥1% to <5%; IC2, ≥5% to <10%; and IC3, ≥10%.

TC, Tumor cell; IC, Immune cell.

Table 4. The relationship between combination of TIL density/PD-L1 expression and EGFR-TKI primary resistance

	Primary resistance (n=9)	Non-primary resistance (n=70)	P-value
Total TILs * low/ TC0 and IC0	7	20	**0.006**
Others	2	50	

* Total TILs defined as the total amount of CD4+ TILs and CD8+ TILs.

TC0, Tumor cell staining score representing <1% positivity.

IC0, immune cell staining score representing <1% positivity.

TILs, Tumor infiltrating lymphocytes; TC, Tumor cell; IC, Immune cell.