의학박사 학위논문

# Change in PD-L1 expression after acquiring resistance to gefitinib in EGFR-mutant non-small cell lung cancer

**Gefitinib** 내성 획득 후 **EGFR** 돌연변이 비소세포폐암의 **PD-L1** 발현 변화

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임상의과학과

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**A thesis of the Degree of Doctor of Philosophy**

# Gefitinib 내성 획득 후 EGFR 돌연변이 비소세포폐암의 PD-L1 발현 변화

# Change in PD-L1 expression after acquiring resistance to gefitinib in EGFR-mutant non-small cell lung cancer

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# ABSTRACT

**Introduction:** Therapies targeting the programmed death-1 (PD-1) and its ligand (PD-L1) has been successful in a subset of patients with non-small cell lung cancer (NSCLC). PD-L1 expression in tumor tissues has been suggested as a predictive and prognostic marker. We examined the change of PD-L1 expression after gefitinib treatment in patients with EGFR- mutant NSCLC.

**Materials and Methods:** We established gefitinib-resistant NSCLC cell lines and compared with parental PC-9 cells for regulation of PD-L1 expression. We also collected paired tumor tissues before and after gefitinib from eighteen NSCLC patients. PD-L1 expression on tumor and immune cells was defined by H-score of immunohistochemical stain (range: 0 to 300). The correlations between change of PD-L1 expression and clinicopathologic characteristics were analyzed.

**Results:** The PD-L1 expression level was higher in gefitinib-resistant PC-9 cells (PC9GR1 and PC9GR2) than in parental PC-9 cells. Expression of PD-L1 in gefitinib-resistant PC-9 cells was up-regulated by interferon-γ and down-regulated by MEK inhibitor, selumetinib. In addition, MET and mesenchymal markers were increased in association with PD-L1 protein expression in gefitinib-resistant PC-9 cells. PD-L1 expression on tumor cells showed an increase in the median H-score from 25 to 40 (P = 0.067). Among them, seven patients (38.9%) showed a marked increase in median H-score (80 to 180, group A) and the other eleven patients (61.1%) showed no change in median H-score (0 to 0, group B). In group A and B, the median progression free survival for gefitinib was 13 versus 12 months (P=0.594), and the median overall survival after gefitinib resistance was not

reached versus 16 months (P=0.068), respectively. MET positivity by immunohistochemistry in post-gefitinib biopsies was significantly associated with group A (P = 0.028). PD-L1 expressing immune cells was observed in 3 (16.7%) of pre-gefitinib tumor tissues and 7 (38.9%) of post-gefitinib tumor tissues. PD-L1 expression by immune cells showed statistically significant correlation with pre-gefitinib FOXP3+ TILs (ρ = 0.584, P = 0.014) and with post-gefitinib CD3+ TILs (ρ = 0.547, P = 0.028), CD8+ TILs (ρ = 0.650, P = 0.005), and PD-1+ TILs (ρ = 0.590, P = 0.016), respectively. Patients who had acquired mutation T790M after gefitinib was likely to have higher TILs infiltration score in tumor microenvironments.

**Conclusions:** The level of PD-L1 expression was changed not only in tumor cells but also in tumor infiltrating immune cells after acquiring resistance to gefitinib. Patients who had increased tumoral PD-L1 expression after gefitinib showed longer overall survival than those who did not, which may be associated with increased MET expression or activation of intracellular MEK signaling pathway. Tumor tissues that did not showed increase in tumoral PD-L1 expression after gefitinib had secondary T790M mutation or increased TILs. Re-evaluation of the level of PD-L1 expression is needed after acquiring resistance to gefitinib in tumor and also in tumor infiltrating immune cells. Thus, we can understand the mechanism of drug resistance and improve the outcomes of treatments in NSCLC.

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**Keywords:** EGFR, Gefitinib, Interferon-gamma, Non-small cell lung cancer, Programmed death receptor ligand-1, Tumor infiltrating lymphocytes

**Student number 2013-30828**

# CONTENTS

# LIST OF TABLES

# LIST OF FIGURES

# INTRODUCTION

Lung cancer is the most common cause of cancer death worldwide, including Korea.[1,2] Activating mutations in the *epidermal growth factor receptor* (*EGFR*) gene are found among 50% of lung cancer patients in Asian countries and 10% of lung cancer patients in Western countries.[3] In these patients, EGFR tyrosine kinase inhibitors such as gefitinib or erlotinib produce a higher response and better progression-free survival compared with platinum-doublets.[4-6] However, resistance develops inevitably after a median of 8 to 13 months.[7, 8]

Programmed death-1 (PD-1) and its ligand (PD-L1, also known as B7-H1 or CD274) are known as key molecules for peripheral T cell tolerance. PD-L1 is a member of the B7 superfamily often expressed by activated cells including T cells, B cells, dendritic cells, monocytes/macrophages, natural killer cells, vascular endothelial cells, mesenchymal stem cells, and cultured bone-marrow derived mast cells.[9] Functionally, PD-L1 suppresses the immune system by various mechanisms, such as T cell exhaustion, anergy, and apoptosis.[9] PD-L1 expression is increased in many types of human cancers, including lung cancer.[10] Up-regulation of PD-L1 expression in tumor cells can be explained partly by intrinsic oncogenic signaling.

For example, EGFR pathway activation is associated with PD-L1 overexpression in EGFR-driven lung cancer in animal models.[11] There are very few data on change of PD-L1 expression after acquiring resistance to target agents. In metastatic melanoma with *BRAF* mutation, expression of PD-L1 on tumor cells increased after acquiring resistance to BRAF inhibitors.[12, 13]

In addition to tumor cell-intrinsic regulation of PD-L1, tumor-infiltrating T cells also have been found to upregulate PD-L1 in a paracrine fashion by inflammatory cytokines, including interferon-γ (IFN-γ). Four distinct groups of tumors were suggested in terms of PD-L1 expression and TILs. First group (type I) is described as having the presence of both PD-L1 and TILs, called adaptive resistance. This group includes tumors with ongoing immune response within the tumor microenvironment and tumor adapts to its immune attacks by using PD-L1 expression. Another group (type III) includes the tumor with presence of PD-L1 expression without TILs. This type of PD-L1 expression is involved with intrinsic induction of PD-L1 by oncogenic pathways. The remaining two groups includes the tumors that show absence of PD-L1 expression with TILs (type IV), or absence of both PD-L1 expression and TILs (type II). These two groups represent

immunological ignorance type or tolerance with other immune suppressors, including TIM-3 (T-cell immunoglobulin mucin-3) or LAG-3 (lymphocyte activation gene-3), for example.[14, 15] The blockade of the PD-1 on T cells or PD-L1 on cancer cells with antibodies restored the antitumor activity of T cells in patients with lung cancer.[16, 17] Preliminary reports have demonstrated that the overall response rate of anti-PD-1/PD-L1 antibodies alone was 10-20%.[18-22] Recently, antibodies against PD-1 (nivolumab and pembrolizumab) and PD-L1 (MPDL3280A) has been approved by the U.S. Food and Drug Administration to treat NSCLC.In this study, we found that the PD-L1 expression is increased by gefitinib-resistant PC-9 NSCLC cell lines compared to the parental cell line. From these data, we further investigated whether PD-L1 expression changes by tumor or immune cells (ICs) after acquired resistance to gefitinib in paired tumor tissues obtained from EGFR-mutant lung cancer patients. Finally, we explored the associations between the PD-L1 expression and tumor-infiltrating lymphocytes (TIL) in tumor tissues.

# Materials and Methods

**Generation of gefitinib-resistant PC-9 cells and reagents**

The NSCLC cell line PC-9 was kindly provided by Dr. Mayumi Ono (Kyushu University, Fukuoka, Japan). Gefitinib-resistant sublines (PC9GR1, PC9GR2) were established by exposing parental PC-9 cells to gefitinib from 100 nM to 1 μM. The subcloned GR sublines exhibited 800- to 1000-fold higher $IC_{50}$ values for gefitinib compared with parental PC-9 cells as determined by the cell viability assay (**Figure 1**), and this phenotype was stable for at least 6 months without gefitinib treatment. For viability assay, NSCLC cells were seeded at a density of 3,000 cells per well in 96-well plates, cultured in the presence of drugs or vehicle for 72 hours, and subjected to the CCK-8 colorimetric assay (Dojindo, Japan). The results were measured using an Eon™ Microplate Spectrophotometer (BioTek, Winoosk, VT) in at least duplicate samples according to the manufacturer's instructions. PC-9 and GR sublines were cultured in RPMI-1640 medium supplemented with 10% FBS (GIBCO, Grand Island, NY). Gefitinib was purchased from Selleck chemicals and dissolved in DMSO (Sigma Aldrich, St. Louis, MO) for experiments. Actinomycin D and cycloheximide were

purchased from Calbiochem and IFN-γ was purchased from R&D systems. Selumetinib was purchased from Selleck Chemicals and reconstituted in dimethylsulfoxide (DMSO), and stored at −20°C before adding to culture media.

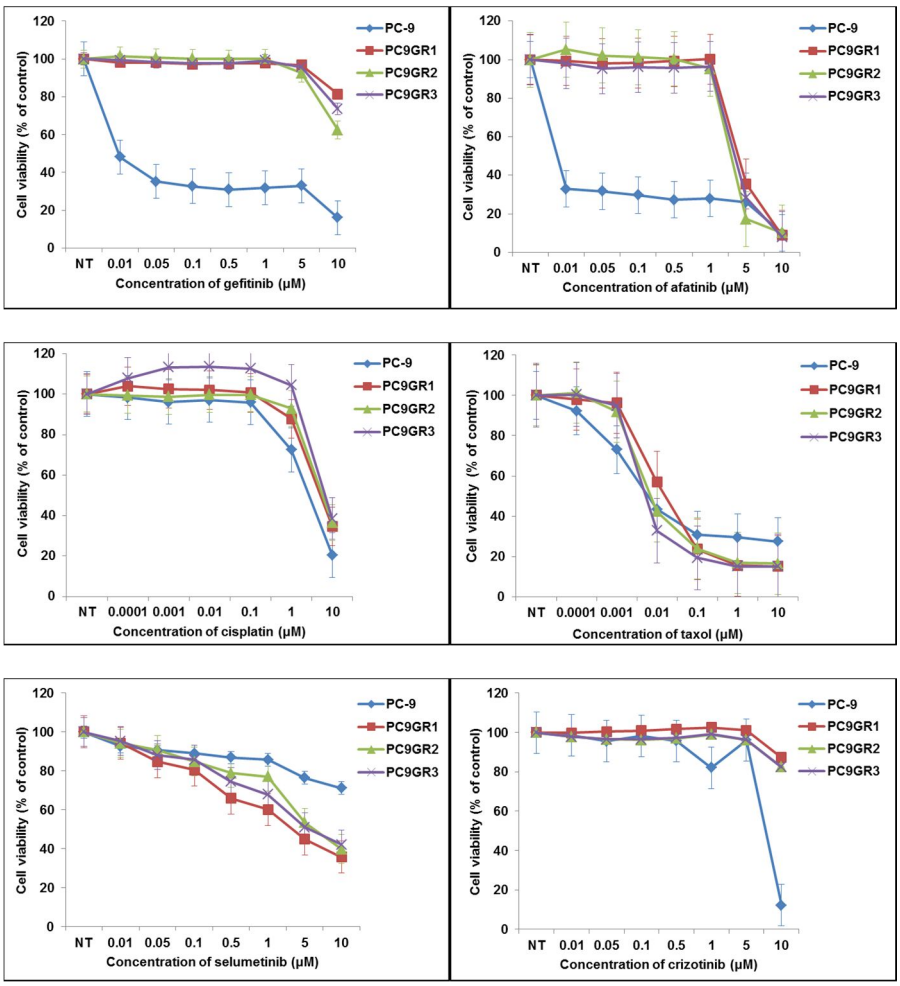



**Figure 1.** The effects of drugs against parental and gefitinib-resistant PC-9 sublines. The parental or gefitinib-resistant PC-9 cell lines were treated with the indicated doses of each drug for 72 hours. Cell survival was measured using CCK-8. Each concentration was measured several times, and the average and SD are shown.

**Flow cytometry analysis**

A total of $2\times10^5$ cells was aliquoted into each assay tube. Next, 2 mL of FACS buffer was added to each tube and rinsed by centrifugation twice. Cells were resuspended in 100 μl of FACS buffer with fixable viability dye (eBioscience, San Diego, CA). Cells were stained with PD-L1 PE (eBioscience) or isotype control for 30 minutes on ice in staining buffer (2% BSA and 0.01% sodium azide). Analysis was conducted on a FACSCalibur instrument (BD Biosciences, San Jose, CA) with CELLQuest software (BD Biosciences). Flow cytometric analysis of PD-L1 expression in PC-9 and GR sublines was conducted. After 24 hours, the cells were harvested and stained with either mouse anti-human PD-L1 (clone 5H1) or a mouse IgG1 isotype control followed by phycoerythrin (PE)-conjugated goat anti-mouse Ig (**Figure 2**).

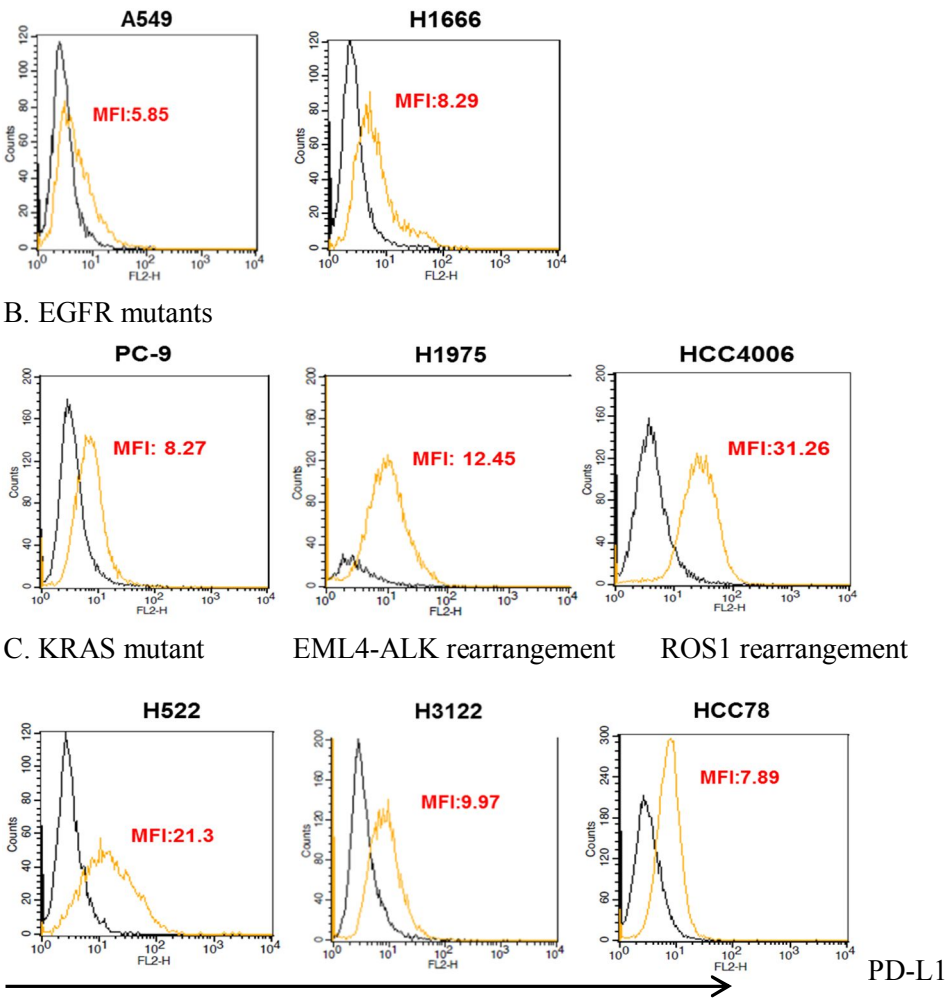



**Figure 2.** PD-L1 expression at the membrane of NSCLC cell lines. Several NSCLC cell lines were harvested and stained against human PD-L1 indirectly. Black color histograms are unstained control.

*Abbreviations:* MFI, mean fluorescence intensity

**Immunoblot analyses**

PC-9 and GR sublines were harvested and lysed for Western blotting. Primary antibodies include p-ERK Thr202/204, ERK, p-MEK Ser217/221, MEK, p-EGFR Tyr1068, EGFR, PD-L1, Vimentin, E-cadherin, Fibronectin and GAPDH (all from Cell Signaling Technology, Danvers, MA). The blots were washed, transferred to freshly prepared enhanced Lumi-Light Western Blotting Substrate (Roche, Indianapolis, IN), and subjected to imaging analysis using an LAS-3000 imaging system (Fuji Photo Film Co., Stamford, CT). PathScan EGFR signaling antibody array kit (Cell Signaling Technology) was used for screening with PC-9 and PC9GR sublines.

**PCR and Direct Sequencing**

Genomic DNA was extracted using the *GenEx*$^{TM}$ Blood/Cell/Tissue kit (Geneall Biotechnology, Seoul, KOREA) according to the manufacturer's protocol. Exons of *EGFR* were amplified from genomic DNA using the High Fidelity plus PCR system (Roche, Indianapolis, IN) and sequenced bi-directionally by Sanger dideoxynucleotide sequencing with the primers for *EGFR* exons 19-21.[23] Direct

sequencing was performed using an ABI3730XL DNA analyzer (Applied Biosystems, Foster City, CA). The reference sequences for *EGFR* were NG_007726.3. (**Figure 3**)

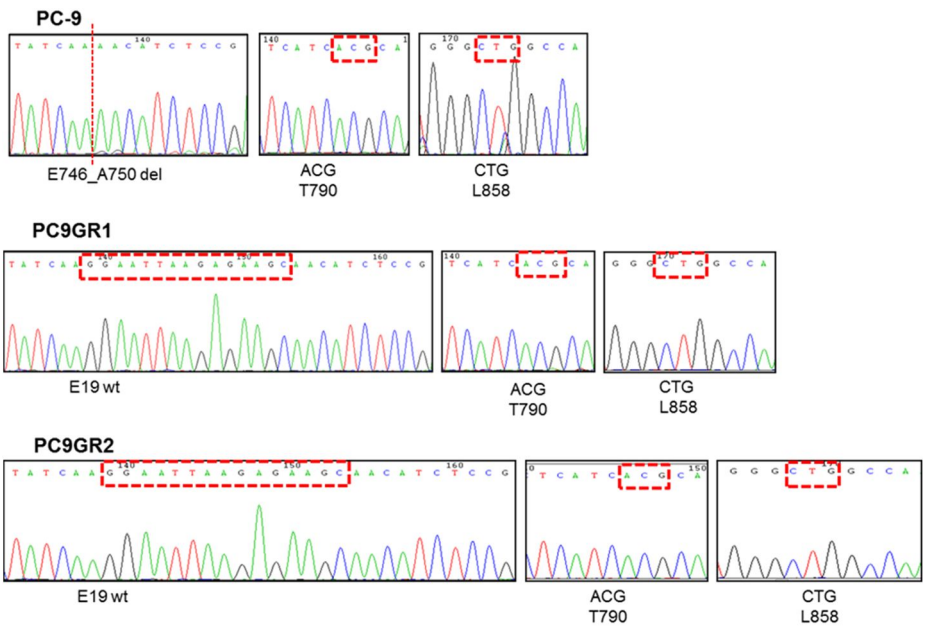



**Figure 3.** Direct sequencing of *EGFR* gene in PC9, PC9GR1, and PC9GR2

The mutation status of *EGFR* in both PC-9 and PC9GR cells was assessed by direct sequencing. Although PC-9 harboring exon 19 deletion is known, PC9GR cells had wild-type *EGFR* without any secondary mutation.

**Real-time quantitative RT-PCR for relative RNA levels of PD-L1**

Total RNA was isolated from PC-9 and gefitinib-resistant PC-9 cells with a PureLink$^{TM}$ RNA Mini kit (Invitrogen, Carlsbad, CA). cDNA was synthesized with SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen). Real-time PCR reactions were performed with an ABI prism 7000 sequence detector (PE Applied Biosystems). Data were analyzed according to the comparative CT method, using the internal control (β-actin) levels to normalize differences in sample loading preparation (StepOne$^{TM}$ Software V2.1). PD-L1 primer sets were 5'- TGG CAT TTG CTG AAC GCA TTT -3' (forward) and 5'- TGC AGC CAG GTC TAA TTG TTT T -3' (reverse).

**Patient selection and tumor tissue samples**

To be eligible for the study, patients had pathologically confirmed adenocarcinoma of lung and required to have an activating *EGFR* mutation at diagnosis, to have been treated with gefitinib, and to have undergone follow-up biopsies after acquiring resistance to gefitinib as a routine practice. The median time from the initial pathologic diagnosis to the first-line chemotherapy was 14 days (range: 0-28

days), and the median time from disease progression to follow-up biopsies was 11 days (range: 2-27 days). The *EGFR* mutation status was determined at diagnosis by direct sequencing of exon 18, 19, 20 and 21.[23] Clinical and pathological data were reviewed retrospectively. This study was approved by the Institutional Review Board at Seoul National University Hospital (H-1404-073-572).

**Immunohistochemical staining**

Individual biopsy blocks were cut into 4-$\mu$m slices using a microtome and were mounted onto silanated slides. The sections were dewaxed in xylene and rehydrated using graded alcohol. Next, antigen retrieval was performed. Commercially available primary antibodies were applied according to the manufacturer's instructions (anti-PD-L1 (E1L3N) XP®, rabbit monoclonal; Cell Signaling Technology, Danvers, MA, USA, anti-CD8, rabbit monoclonal, clone SP16; Neomarkers, Fremont, CA, 1:100 dilution; anti-FoxP3, mouse monoclonal, clone 236A/E7; Abcam, Cambridge, UK, 1:100 dilution; anti-CD3, rabbit polyclonal; DAKO, Glostrup, Denmark, 1:100 dilution; anti-MET, rabbit

monoclonal, clone SP44, Ventana Medical Systems). Antibody binding was detected by an avidin-biotin-peroxidase complex (Universal Elite ABC Kit; Vectastain, Burlingame, CA) for 10 minutes followed by development with diaminobenzidine tetrahydrochloride solution (Kit HK 153-5K; Biogenex, San Ramon, CA) for 5 minutes and counterstaining with hematoxylin. The initial diagnosis and gefitinib-resistant tumors were stained for PD-L1, PD-1, CD3, CD4, CD8, CD68, and FOXP3. The biopsy slides were scanned using a scanner system (ScanScope XT; Aperio Technology, Vista, CA). The densities of positively stained TILs were evaluated in intratumoral areas using an image analysis system (ScanScope XT; Aperio) (cells/$mm^2$). The results of the calculated densities were extracted and put into an Excel file. At the same time, positively stained cells in the stromal and intraepithelial compartments were counted using the Aperio analysis system. For PD-L1 expression analysis, the intensity of staining was evaluated according to the following scale: 0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining (**Figure 4**). The proportion of tumor cells expressing PD-L1 was determined. The H-score was defined and calculated as the product of the intensity score and proportion as

previously described.[21] Immunohistochemical stain was analyzed by two pathologists (JK and YKJ) who were blinded to the identity and clinical data of the specimens.

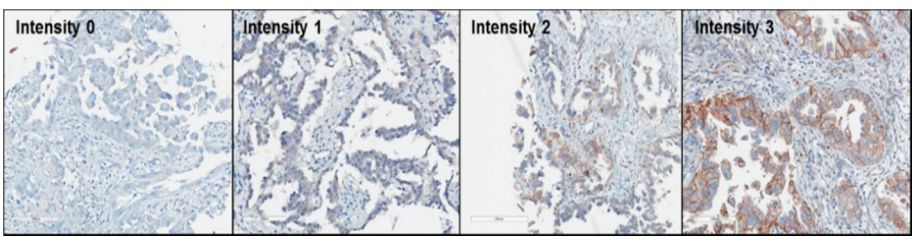



**Figure 4.** Immunohistochemical analyses of tumoral PD-L1 expression in non-small cell lung cancer tissues (200×). Intensity score 0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining.

**Statistical analysis**

Wilcoxon-matched paired test was performed to compare differences in the PD-L1 expression and densities of ICs before and after gefitinib resistance. Correlations between the subtypes of IC were analyzed by Spearman's test. The differences in clinical characteristics according to the PD-L1 expression were analyzed using Fisher's exact test. Progression-free survival (PFS) was measured from the date of initiation of gefitinib to disease progression, death, or the last follow-up. Overall survival (OS) was measured from the date of progression after gefitinib treatment to death or the last follow-up. Survival was analyzed using Kaplan-Meier plots and was compared with the log-rank test. A *P* value less than 0.05 was considered to indicate statistical significance. All statistical analyses were performed using SPSS statistics software (version 20) (IBM Corp., Chicago, IL, USA).

# Results

**Expression of PD-L1 is increased in gefitinib-resistant PC-9 cell lines**

*In vitro* clones with resistance to gefitinib were selected by growing PC-9 cells in increasing concentrations of gefitinib to a final concentration of 1 μM and maintaining them for at least 6 months. PD-L1 expression in PC-9 and GR sublines was analyzed by immunoblotting and flow cytometry to confirm the total and cell surface expression, respectively. GR sublines showed significantly higher levels of PD-L1 than their parental PC-9 cells, a 10-fold to 12-fold increase in the average median fluorescence intensity (MFI) score (6.65 ± 1.53 vs. 76.89 ± 2.45 for PC-9 and PC9GR1, respectively, $P < 0.001$, **Figure 5**). PD-L1 up-regulation in GR sublines persisted in the absence of gefitinib for over 6 months following drug withdrawal. Next, we determined the level of mRNA transcript with real-time RT-PCR. PD-L1 mRNA transcript level was 43-fold higher in PC9GR1 in comparison to PC-9 (**Figure 6**).

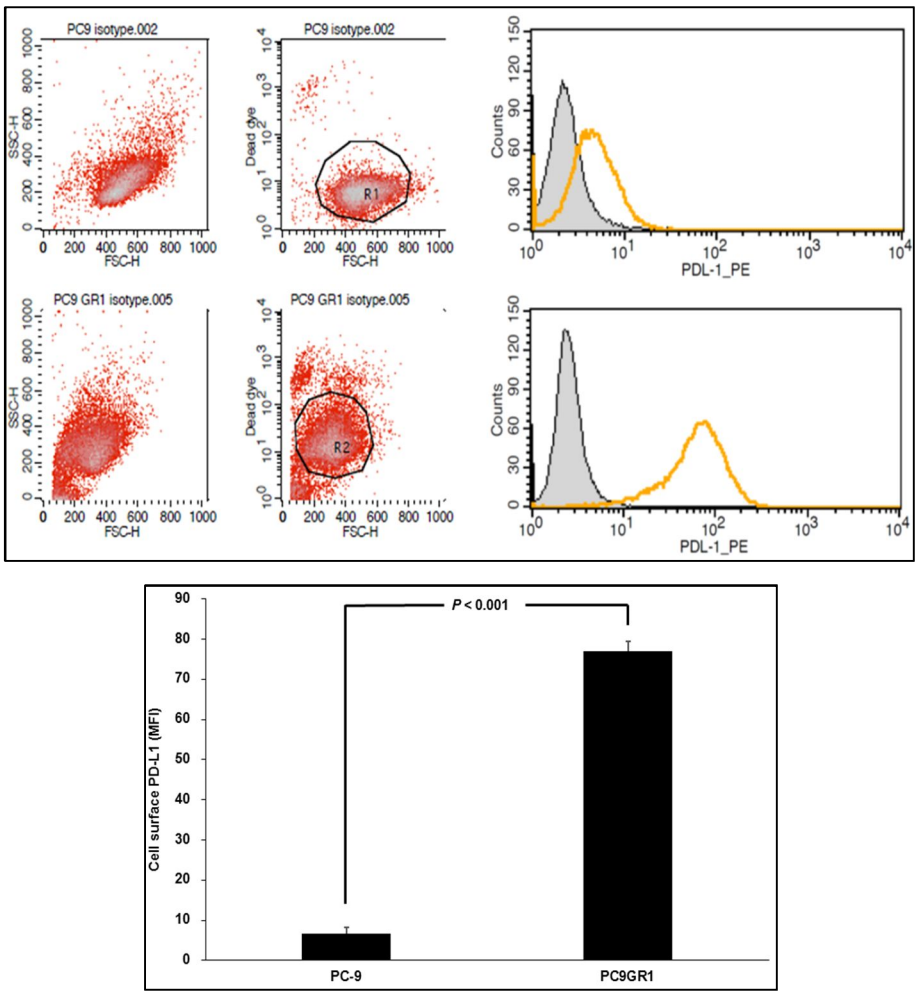



**Figure 5.** Flow cytometry of parental and gefitinib-resistant cell lines confirm changes in PD-L1 expression on the cell membrane. Cells were gated for PD-L1 positivity (x-axis) and ssc-staining (y-axis). A representative sample is shown and histograms depict the mean fluorescent intensity of PD-L1 expression on PC9GR1.

Gray color filled, isotype control; yellow line, PD-L1.

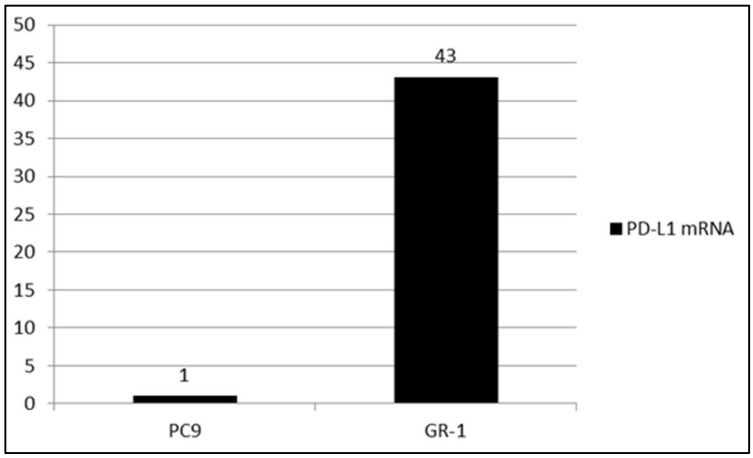



**Figure 6**. PD-L1 mRNA is increased in PC9GR1 cells. Y-axis represents the n-fold relative difference of transcript levels between PC-9 and PC9GR1.

β-actin was used as internal control to normalize differences in sample loading preparations.

**Expression of PD-L1 is increased in gefitinib-resistant PC-9 cell lines independent to EGFR pathway**

It is known from previous reports that *EGFR* mutation in NSCLC cells drive PD-L1 over-expression, we examined the expression of PD-L1 in parental PC-9 (*EGFR* del 19 mutation) and established gefitinib-resistant PC-9 cell lines. Flow cytometric analysis revealed that the level of PD-L1 expression was decreased with various EGFR inhibitors in PC-9 cell line, whereas gefitinib-resistant PC9GR1 and PC9GR2 manifested high expression of PD-L1 at the cell surface without change after challenging various EGFR inhibitors (**Figure 7**). In addition, immunoblot analysis confirmed that gefitinib-resistant cell lines have no change in PD-L1 protein expression in resistant cell lines with or without gefitinib challenge (**Figure 8**). These data confirmed that *EGFR* mutation in PC-9 drive PD-L1 expression and gefitinib-resistant PC-9 cells over-express PD-L1 higher than parental PC-9 cells independent to EGFR pathway.

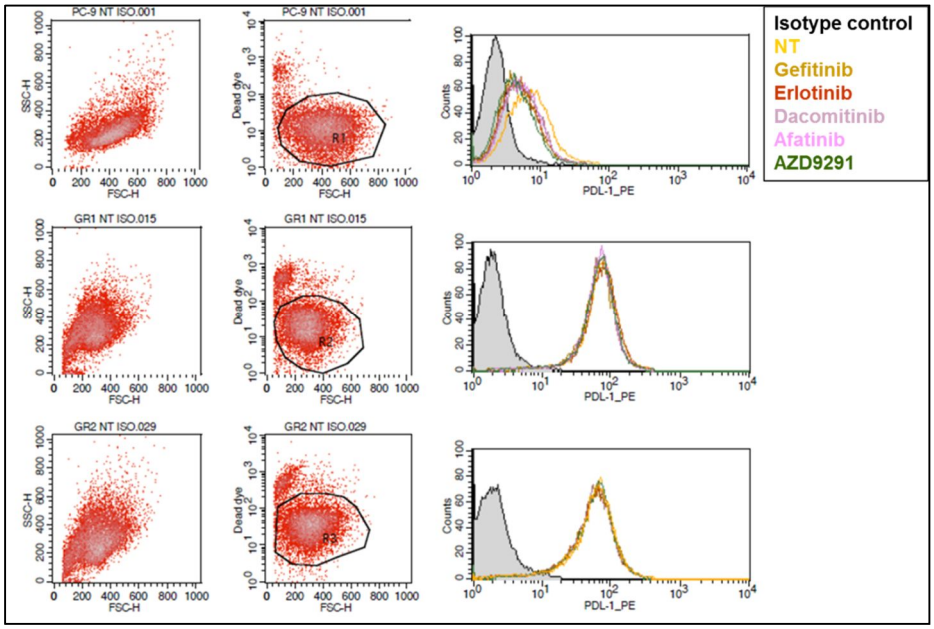



**Figure 7**. Flow cytometry of parental and gefitinib-resistant cell lines with various EGFR inhibitors. PC-9, PC9GR1 and PC9GR2 were seeded into a 6-well plate at $5 \times 10^5$ cells per well and incubated for 24 hours. Then, challenged with 1μM of each EGFR-TKIs or vehicle for 24 hours. Finally, each well was harvested for flow cytometric analysis. Only live cells with PD-L1 surface expression were gated as shown in this figure on the left.

Gray color filled, isotype control; yellow line, no treatment control; colored line, PD-L1 expression after each EGFR inhibitors.

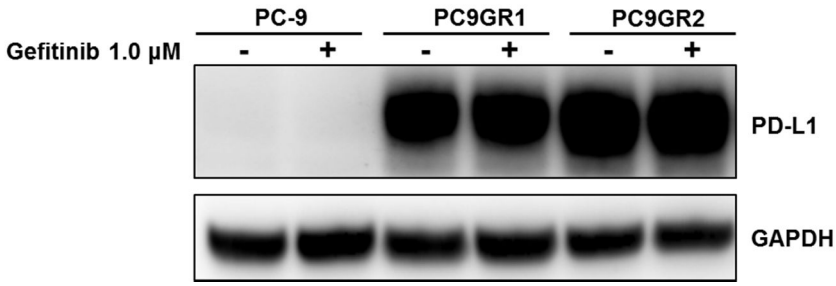



**Figure 8**. Immunoblot analysis of PC-9 parental and gefitinib-resistant cell lines show increased PD-L1 expression in resistant cell lines. Established gefitinib-resistant cell lines PC9GR1 and PC9GR2 were challenged with 10% FBS RPMI-1640 containing gefitinib (1.0 μM) for 72 hours. The parental PC-9 cell lines grew with or without treatment of gefitinib in same conditions as controls.

**IFN-gamma significantly induced PD-L1 expression in gefitinib-resistant PC-9 cell line at translational level**

Because PD-L1 expression is induced by cytokines, such as IFN-γ, we investigated whether PD-L1 expression could be induced with by IFN-γ which is known to have a role in adaptive regulation of PD-L1 expression in lung cancer. After IFN-γ stimulation, PD-L1 protein expression was significantly increased in PC9GR2 cell line, but not in PC-9 cell line (**Figure 9**).

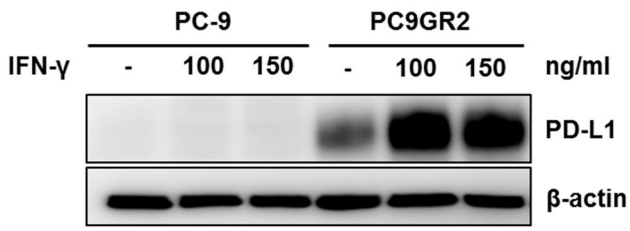



**Figure 9**. IFN-γ significantly induced PD-L1 expression in PC9GR2 cell line, but not in PC-9 cell line. PC-9 and PC9GR2 were seeded into a 6-well plate at 5 × $10^5$ cells per well and incubated for 24 hours. Next, we incubated with IFN-γ for 24 hours, and then harvested for immunoblot analysis.

To understand which process is involved in IFN-γ-induced PD-L1 up-regulation, we blocked RNA synthesis process by actinomycin D (ActD) or blocked protein synthesis process by cycloheximide (CHX). Blocking RNA synthesis with ActD had no effect on the level of IFN-γ-induced PD-L1 expression. But, blocking protein synthesis with CHX could effectively decreased the level of IFN-γ-induced PD-L1 expression in PC9GR2 cell line. Therefore, IFN-γ-induced PD-L1 up-regulation is regulated at the translational level (**Figure 10**).

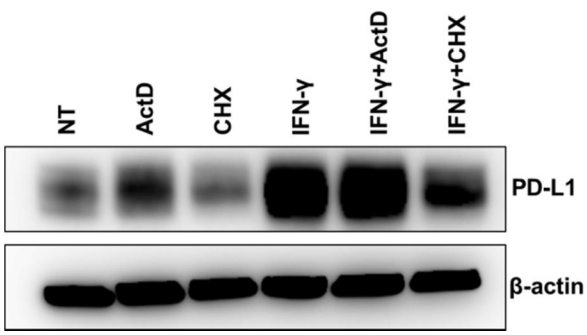
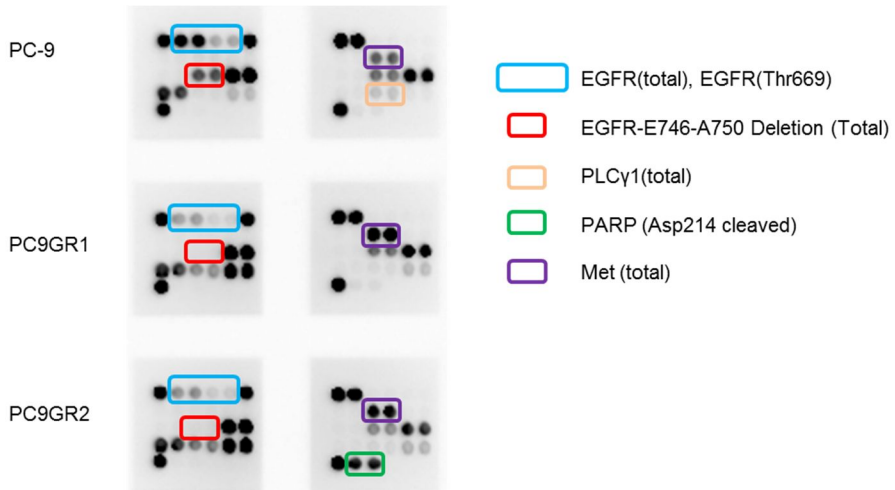



**Figure 10**. IFN-γ-induced PD-L1 expression in PC9GR2 is the result of *de novo* protein synthesis. $5 \times 10^5$ PC9GR2 cells were seeded per well in 6-well plate and incubated for 24 hours. PC9GR2 cells were exposed to IFN-γ 100ng/ml for 6 hours with or without 10μg/ml actinomycin D (ActD) or 10μg/ml cycloheximide for 90 minutes.

**Increased activation of MEK-ERK pathway promotes PD-L1 expression in gefitinib-resistant PC-9 cells**

First, we assessed status of signaling pathways in PC-9 and gefitinib-resistant PC-9 cell lines. A combined analysis of EGFR PAthScan and immunoblot revealed that PC9GR cell lines developed a decrease in EGFR phosphorylation, an increase in total MET, and activation in MEK-ERK pathway compared with parental PC-9 cell line. However, PI3K-AKT signaling pathway was inactivated with high expression of PTEN (phosphatase and tensin homolog) in PC9GR cell lines (**Figure 11**).

A. EGFR PAthScan




B. Immunoblot

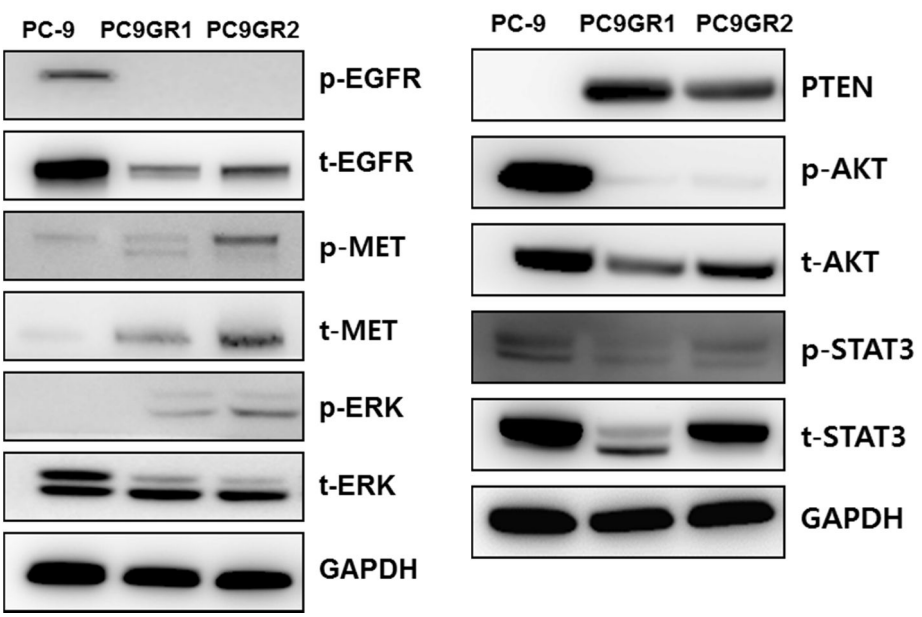



**Figure 11.** EGFR PAthScan and Immunoblot showed activation of downstream signaling pathways mediated by MEK-ERK, but not by PI3K-AKT in gefitinib-resistant cell lines.

Next, we sought pharmacologic effect of MEK inhibition on PD-L1 expression. Treatment of PC9GR cell lines with MEK inhibitor selumetinib decreased phospho-MEK in all lines as expected in a concentration dependent manner. Chemical inhibition of phosphor-MEK also significantly decreased PD-L1 protein expression in PC9GR cell lines (**Figure 12**).

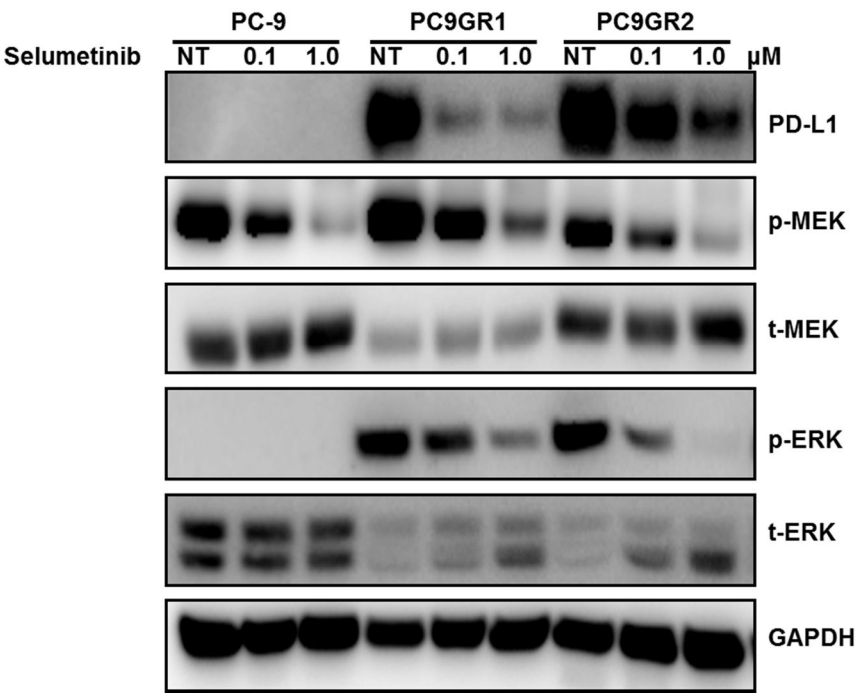



**Figure 12.** Inhibition of MEK-ERK signaling with MEK inhibitor selumetinib down-regulates the PD-L1 protein expression in PC9GR cell lines.

**Epithelial-Mesenchymal Transition (EMT) was observed in association with PD-L1 expression in gefitinib-resistant PC-9 cell lines**

We observed mesenchymal transition with mass-forming characteristics in PC9GR cell lines compared to parental PC-9 cell line. In addition, we evaluated the expression of E-cadherin, β-catenin, vimentin, claudin in association with PD-L1 protein expression using Western blotting. PC-9 express epithelial markers dominantly, in contrast to PC9GR1 and PC9GR2 which express mesenchymal markers (**Figure 13**).

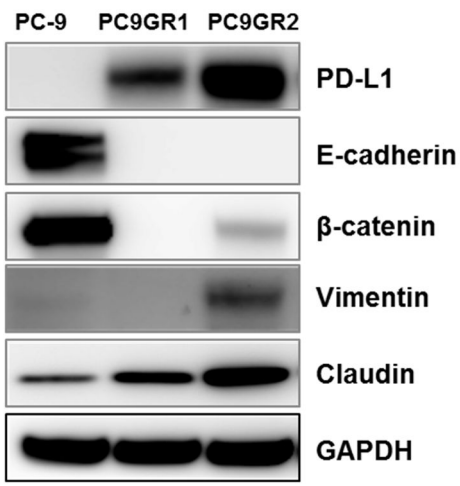



**Figure 13.** Immunoblots analysis of EMT markers confirm that PC9GR cells undergo EMT. PC-9 cells after acquiring resistance to gefitinib was characterized by decreased E-cadherin expression but increased expression of vimenetin and claudin.

**Patient and tumor characteristics**

Patient demographic information is provided in **Table 1**. There were 11 females and 7 males with a median age of 62 years (range: 46-80 years). Eleven patients had an exon 19 deletion and 6 patients had an L858R mutation. One patient had both mutations at diagnosis. A complete response was achieved in one patient (6%), 13 patients (72%) had a partial response, and 4 patients (22%) had stable disease for the best response to gefitinib (evaluated by serial computed tomography (CT) scans and determined by RECIST criteria). The biopsy locations were the lung (13), lymph node (4), or pleura (1) in pre-gefitinib biopsies and lung (15) or lymph node (3) in post-gefitinib biopsies. Overall response rate for gefitinib was 78%. Median PFS and OS were 10 months (95% confidence interval [CI], 5.842-14.158 months) and 48 months (95% CI, 33.955-62.045 months), respectively.

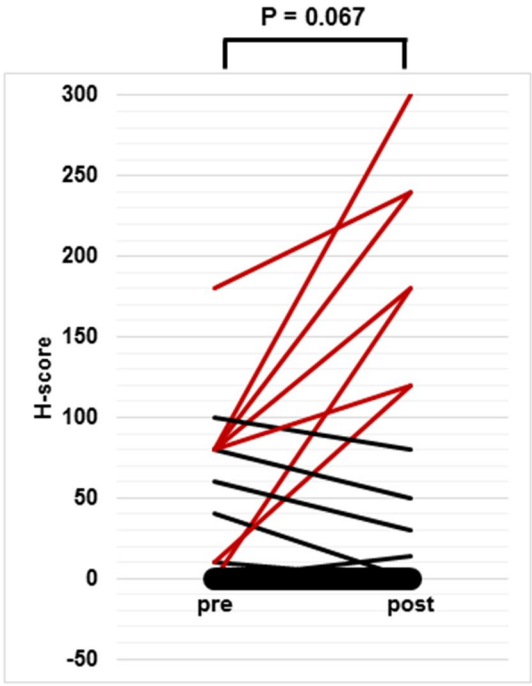
**Table 1.** Clinicopathologic Characteristics of Patients with EGFR-mutant NSCLC

| Characteristic | | N=18 | % |
|---|---|---|---|
| **Age (median 62 years)** | < 62 | 9 | 50 |
| | ≥ 62 | 9 | 50 |
| **Gender** | Male | 7 | 39 |
| | Female | 11 | 61 |
| **Smoking** | Never-smoker | 12 | 67 |
| | Smoker | 6 | 33 |
| **EGFR mutation** | Exon 19 deletion | 11 | 61 |
| | L858R | 6 | 33 |
| | L858R/Exon19 deletion | 1 | 6 |
| **Gefitinib** | First-line | 7 | 39 |
| | ≥ Second-line | 11 | 61 |
| **Best response to gefitinib** | Complete response | 1 | 6 |
| | Partial response | 13 | 72 |
| | Stable disease | 4 | 22 |
| **Duration of gefitinib treatment** | < 12 | 9 | 50 |
| **(months)** | ≥ 12 | 9 | 50 |
| **Number of chemotherapy agents** | 1 (Gefitinib only) | 5 | 28 |
| **between paired biopsy** | 2 | 6 | 33 |
| | ≥ 3 | 7 | 39 |

**Change in PD-L1 expression by tumor cells before and after acquiring resistance to gefitinib in tumor tissues**

PD-L1 expression by tumor cells was observed in the cell membrane in 19 of 36 biopsies (52.8%) and cytoplasm in 3 of 36 biopsies (8.3%). The remaining 14 of 36 biopsies (38.9%) had no PD-L1 expression with an H-score of zero. Pre- and post-biopsies showed a significant correlation in PD-L1 expression by tumor cells (Spearman's test; $Z = 0.717$, $\rho = 0.001$). PD-L1 expression by tumor cells showed an increasing trend in the median H-score from 25 to 40 (paired Wilcoxon signed rank test, $P = 0.067$, **Figure 14A**). The change in PD-L1 expression demonstrated two patterns (**Figure 14A and 14B)**. We considered H-score over 100 (that is 1+ intensity score × 100%) at follow-up biopsy as cut-off value for further analysis. According to this criteria, seven of eighteen patients (38.9%) showed a marked increase in median PD-L1 H-score from 80 to 180 (designated group A). The remaining eleven patients (61.1%) showed no change in median PD-L1 H-score from 0 to 0 (designated group B).

A.


P = 0.067
300
250
200
150
100
50
0
-50
H-score
pre
post

B.

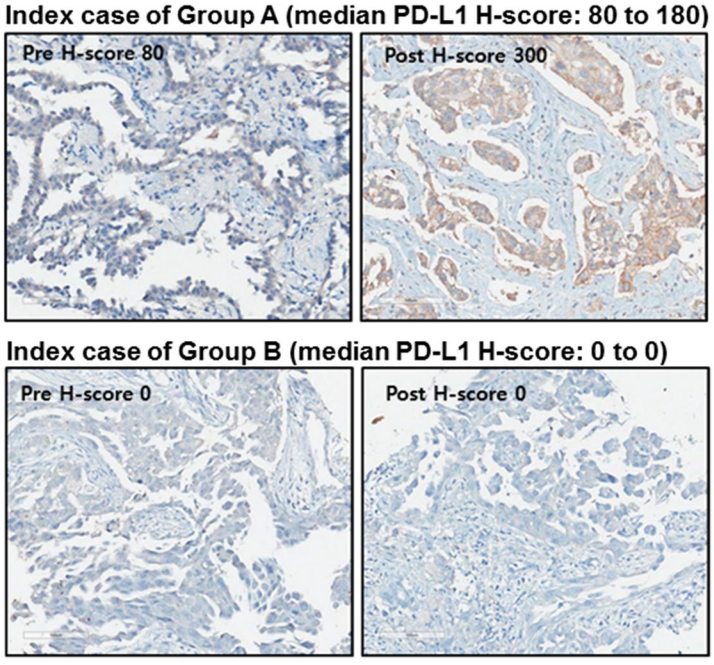

Index case of Group A (median PD-L1 H-score: 80 to 180)
Pre H-score 80
Post H-score 300
Index case of Group B (median PD-L1 H-score: 0 to 0)
Pre H-score 0
Post H-score 0

**Figure 14.** Change of PD-L1 expression on tumor cells.

**A**. H-score showed a trend of increased PD-L1 expression by tumor cells after acquiring gefitinib resistance. The H-score is determined by multiplying the intensity score and proportion of stained tumor cells. Paired biopsies were analyzed using the Wilcoxon signed-rank test. A P value less than 0.05 was considered statistically significant. The thickness of line represents the number of patients. **B**. PD-L1 expression was categorized into two groups. Group A included patients with increased PD-L1 expression in tumor cells (median H-score from 80 to 180). Group B included patients with no change in PD-L1 expression in tumor cells (median H-score from 0 to 0). Immunohistochemical staining of PD-L1 expression in tumor cells for each index case is shown. (40×)

**Change in PD-L1 expression by tumor-infiltrating ICs was also observed**

The number of biopsies with PD-L1-expressing tumor-infiltrating ICs was increased from three (16.7%) in pre-biopsies to seven (38.9%) in post-biopsies (**Figure 15**).

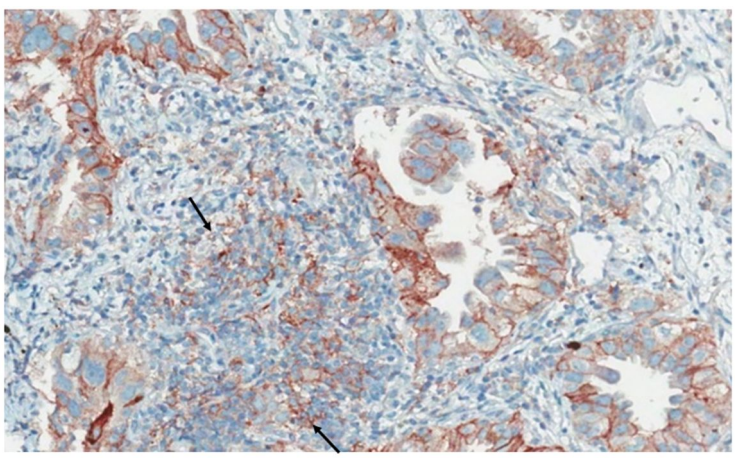


**Figure 15.** Immunohistochemical stain of PD-L1 expression in tumor biopsy showed immune cells with PD-L1 expression (between the arrows). (200×)

**EGFR inhibition is associated with a change in tumor-infiltrating lymphocytes**

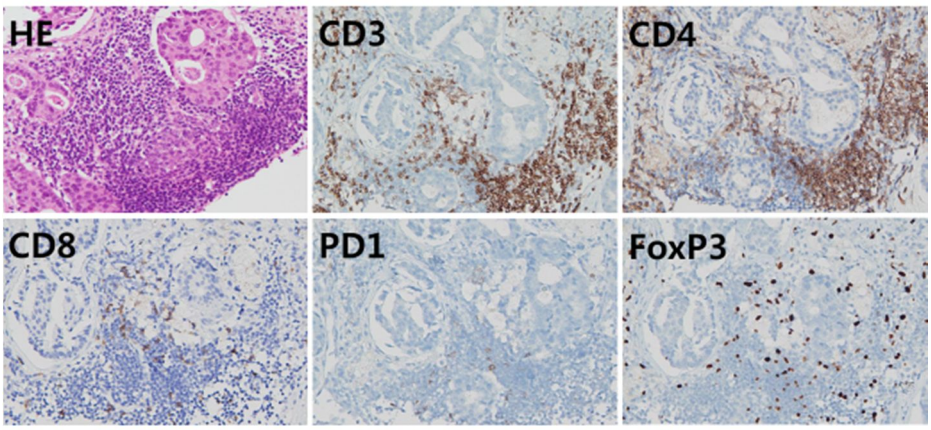
Seventeen of the eighteen study patients were available for tumor-infiltrating lymphocytes analysis. Differences in tumor-infiltrating lymphocytes between pre-and post-gefitinib biopsies were analyzed according to the subtype. After disease progression to gefitinib, FOXP3+ lymphocytes showed an increasing trend after gefitinib (P = 0.055). Correlations between tumor-infiltrating lymphocytes were analyzed in pre- and post-gefitinib biopsies. In pre-gefitinib biopsies, PD-L1 expression by ICs and FOXP3+ lymphocytes was significantly positively correlated (Z = 0.584, $\rho$ = 0.014). In post-gefitinib biopsies, PD-L1 expression by ICs was significantly positively correlated with PD-1+, CD3+, and CD8+ lymphocytes (Z = 0.590, $\rho$ = 0.016 and Z = 0.547, $\rho$ = 0.028, respectively, **Table 2, Figure 16**).

Previously, four distinct groups of tumors were suggested and described as having the presence of both PD-L1 and TILs, presence of TILs without PD-L1, presence of PD-L1 without TILS, or absence of both PD-L1 and TILs. We identified distribution of TIL score in association with tumor PD-L1 expression

(**Figure 17**). Although not statistically analyzable, MET positivity showed a trend in PD-L1 increase and low TIL score after gefitinib treatment. In contrast, T790M mutation showed a trend in low PD-L1 expression and high TIL score after gefitinib. It suggests that PD-L1 expression and TIL score might be different in terms of resistant mechanism to gefitinib in EGFR-mutant NSCLC.

We analyzed the post-gefitinib PD-L1 expression by tumor-infiltrating ICs in association with the two groups according to the change in tumoral PD-L1 expression (Table 3). Of the seven patients with positive PD-L1 expression by immune cells in tumor tissues, three were in group A and four were in group B.

**Before gefitinib treatment at baseline**




**After acquiring resistance to gefitinib**

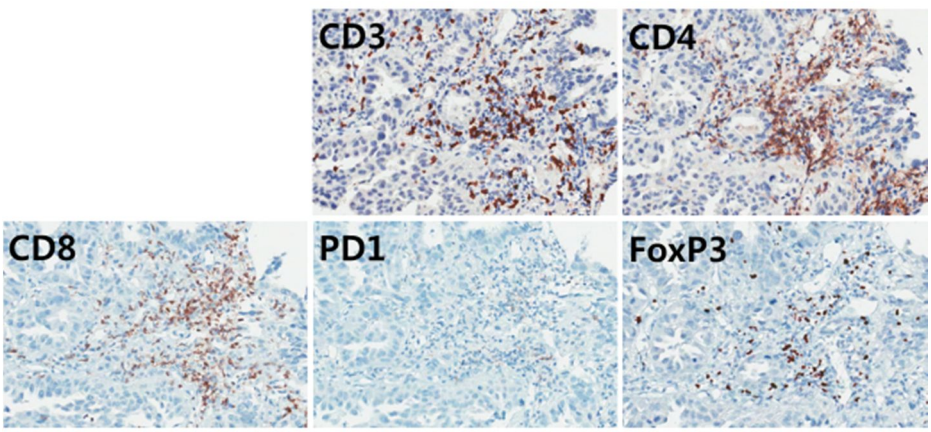
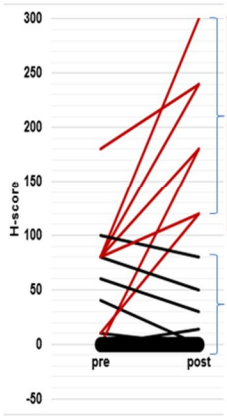



**Figure 16.** Representative immunohistochemical stain showing the relationships between the each tumor-infiltrating lymphocyte before and after treatment with gefitnib. (40×)

**Table 2.** The correlation between PD-L1 expression and tumor-infiltrating lymphocytes.

| | **Pre-gefitinib biopsies** | | **Post-gefitinib biopsies** | |
|---|---|---|---|---|
| | Tumor cell PD-L1 H-score | Immune cell PD-L1 H-score | Tumor cell PD-L1 H-score | Immune cell PD-L1 H-score |
| **Subtypes** | ρ (P-value) | ρ (P-value) | ρ (P-value) | ρ (P-value) |
| **PD-1+ TILs** | 0.380 (0.133) | 0.162 (0.534) | 0.260 (0.330) | 0.590 (0.016*) |
| **CD3+ TILs** | 0.421 (0.092) | 0.214 (0.410) | 0.326 (0.218) | 0.547 (0.028*) |
| **CD4+ TILs** | 0.340 (0.182) | 0.241 (0.351) | 0.344 (0.193) | 0.442 (0.087) |
| **CD8+ TILs** | 0.294 (0.253) | 0.193 (0.457) | 0.439 (0.078) | 0.650 (0.005*) |
| **FOXP3+ TILs** | 0.043 (0.871) | 0.584 (0.014*) | 0.202 (0.436) | 0.349 (0.170) |
| **CD68+ TILs** | -0.132 (0.613) | 0.468 (0.058) | -0.003 (0.992) | 0.429 (0.085) |

*Abbreviations:* CD, cluster of differentiation; FOXP3, forkhead box P3; PD-1, programmed cell death-1; PD-L1, programmed cell death ligand-1; TILs, tumor-infiltrating lymphocytes. The PD-L1 H-score was defined as the product of the intensity score and proportion by immunohistochemical stain. *P < 0.05 was considered statistically significant.




| Post-gefitinib TIL score | 0 | 1 | 2 | 3 |
|---|---|---|---|---|
| GROUP A (secondary mutation, OS in months) | MET, 10 | MET, 17<br>MET, 14<br>MET, 13<br>MET, 3<br>N.A., 13 | MET, 23 | MET, 7<br>N.A., 19 |
| GROUP B (secondary mutation, OS in months) | T790M, 14<br>MET, 13 | T790M, 10<br>MET, 3<br>N.A., 16 | T790M, 16<br>MET, 3 | T790M, 18<br>T790M, 13<br>T790M, 2<br>MET, 7 |

**Figure 17.** Distribution of post-gefitinib TIL score in association with tumor PD-L1 expression groups. TIL score: 0, none; 1, focal; 2, moderate; 3, severe

*Abbreviations:* TIL, tumor-infiltrating lymphocytes; N.A., not available

**Table 3**. PD-L1 expression by immune cells according to the change in PD-L1 expression by tumor cells

| | | **Post-gefitinib PD-L1 expression by immune cells** | |
|---|---|---|---|
| | | Positive | Negative |
| **Two groups according to change in PD-L1 expression by tumor cells (secondary mutation, OS)** | Group A | ● MET, 23<br>● N.A., 19<br>● N.A., 13 | ● MET, 17<br>● MET, 10<br>● MET, 10<br>● MET, 3 |
| | Group B | ● MET, 3<br>● T790M, 18<br>● T790M, 14<br>● T790M, 10 | ● MET, 14<br>● MET, 13<br>● MET, 7<br>● MET, 3<br>● T790M, 16<br>● T790M, 2<br>● N.A., 16 |

Each dot represents a single patient in this study.

*Abbreviations:* N.A., not available.

**Association of PD-L1 expression in tumor tissues with clinicopathologic characteristics and survival**

Clinicopathologic factors were analyzed according to the pattern of PD-L1 expression by tumor cells (**Table 4**). Among them, MET positivity was significantly associated with group A (P = 0.028, **Table 4**). Other parameters, such as gender, age, smoking status, best response to gefitinib, type of *EGFR* mutation at diagnosis, and location of biopsy showed no significant difference between the two groups.

**Table 4.** Associations between the change of PD-L1 expression in tumor cells and clinical characteristics

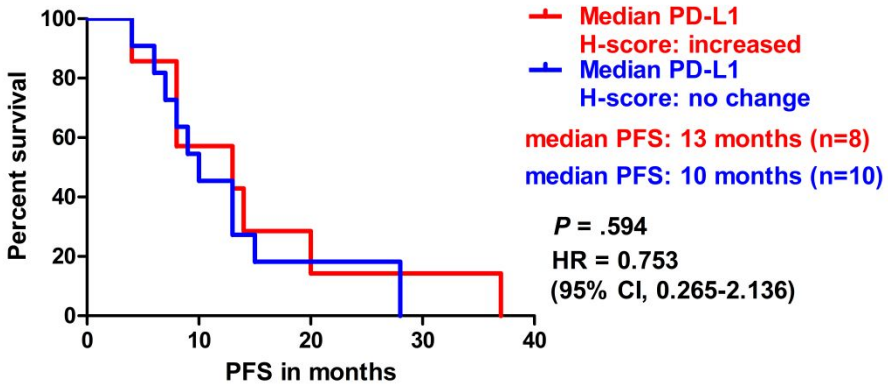
| | | N=18 n (%) | **Group A** n (%) | **Group B** n (%) | ***P* value** |
|---|---|---|---|---|---|
| **Gender** | Male | 7 (39) | 3 (43) | 4 (57) | 0.583 |
| | Female | 11 (61) | 4 (36) | 7 (64) | |
| **Age (median 62 years)** | < 62 | 9 (50) | 2 (22) | 7 (78) | 0.167 |
| | ≥ 62 | 9 (50) | 5 (56) | 4 (44) | |
| **Smoking status** | Never smoker | 13 (72) | 5 (38) | 8 (62) | 0.676 |
| | Current or ex-smoker | 5 (28) | 2 (40) | 3 (60) | |
| ***EGFR* mutation** | 19 del | 11 (61) | 3 (27) | 8 (73) | 0.220 |
| | L858R | 7 (39) | 4 (57) | 3 (43) | |
| **Best response to gefitinib** | Complete response | 1 (6) | 1 (100) | 0 (0) | 0.110 |
| | Partial response | 13 (72) | 6 (46) | 7 (54) | |
| | Stable disease | 4 (22) | 0 (0) | 4 (100) | |
| **Number of chemotherapy between paired biopsies** | 0 (gefitinib only) | 5 (28) | 3 (60) | 2 (40) | 0.209 |
| | 1 | 5 (28) | 3 (60) | 2 (40) | |
| | 2 | 3 (17) | 0 (0) | 3 (100) | |
| | ≥ 3 | 5 (28) | 1 (20) | 4 (80) | |
| **Acquired resistance mechanism (N=15)** | T790M | 6 (40) | 0 (0) | 6 (100) | 0.028 |
| | MET positive | 9 (60) | 6 (67) | 3 (33) | |

*P < 0.05 was considered statistically significant

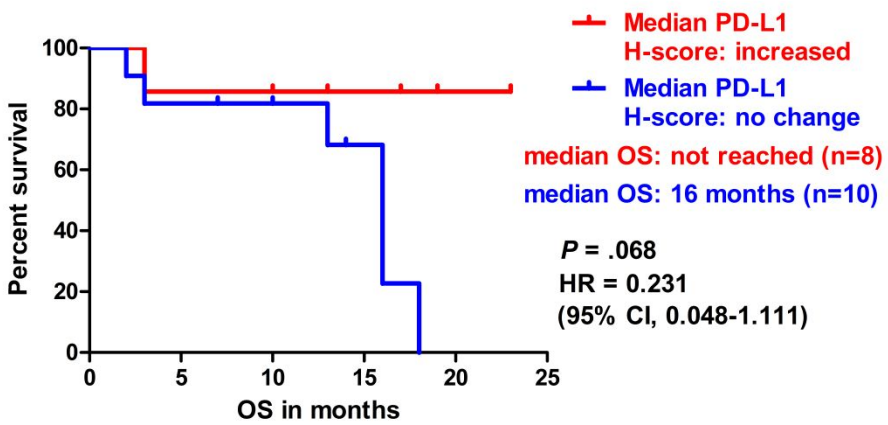
Group A included patients who showed an increase in median PD-L1 H-score on tumor cells from 80 to 180. Group B included patients who showed no change in median PD-L1 H-score on tumor cells from 0 to 0. MET positive was defined as 2+ or 3+ by immunohistochemistry.

Among the 18 patients, group A patients had a better median OS than those in group B, although the difference was not statistically significant (median OS not reached versus 38 months, P = 0.073, **Figure 18A and B**). The median PFS was significantly longer for patients with exon 19 deletions than L858R mutations (39 months versus 2 months, P = 0.005, **Figure 18**) and those with MET positivity than T790M mutations (28 months versus 3 months, P = 0.030). We also analyzed survival according to the post-gefitinib PD-L1 expression by tumor-infiltrating immune cells (**Figure 18 C and D**). PD-L1 expression by tumor-infiltrating immune cells was considered positive if any intensity of immune cells showed PD-L1 expression.

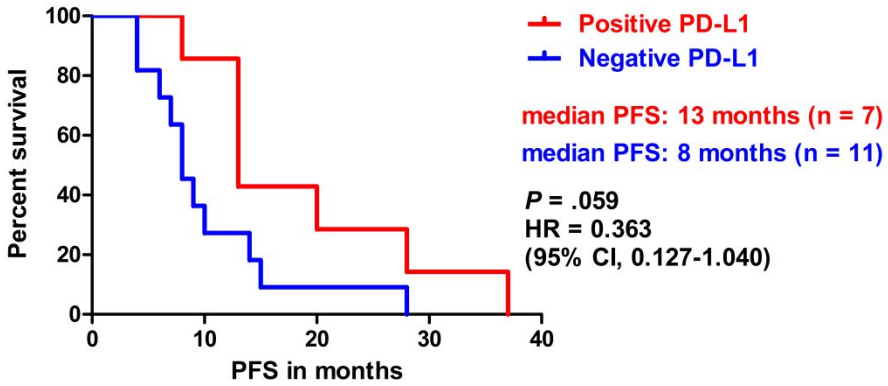
A. Progression free survival by change in tumoral PD-L1 expression




B. Overall survival by change in tumoral PD-L1 expression



C. Progression free survival according to post-gefitinib PD-L1 expression by immune cells.




D. Overall survival according to post-gefitinib PD-L1 expression by immune cells.

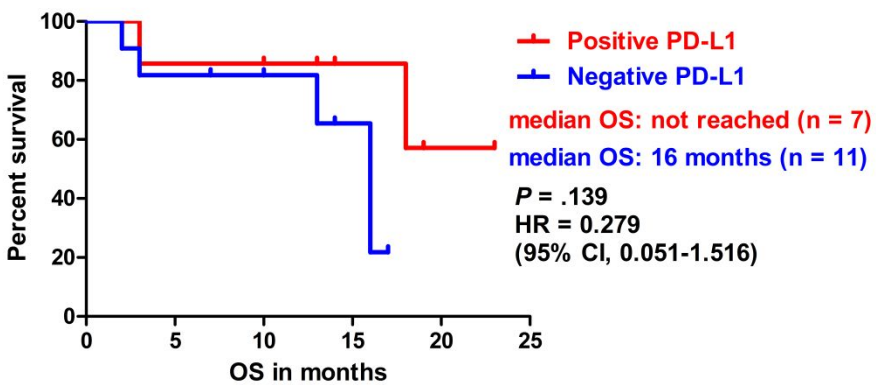


**Figure 18**. Change of PD-L1 expression on tumor cells is associated with survival outcomes. **A**: The median progression-free survivals were not different between the groups according to the change PD-L1 expression in tumor cells. **B**: The median overall survival in patients with increased PD-L1 expression in tumor cells was better, but not statistically significant (P=0.068). **C**. The median progression-free survival of PD-L1 positive group was better than negative group, statistically showed a trend, but not significant. **D**. The median overall survivals were not different between the groups according to the post-gefitinib PD-L1 expression in tumor-infiltraing immune cells. Group A included patients with increased PD-L1 expression in tumor cells (median H-score from 80 to 180). Group B included patients with no change in PD-L1 expression in tumor cells (median H-score from 0 to 0). Positive PD-L1 expression by tumor-infiltrating immune cells was considered positive if any intensity of PD-L1 expression was observed. Log-rank test, $P < 0.005$ considered statistically significant. *Abbreviations:* PD-L1, programmed cell death ligand-1; CI, confidence interval; HR, hazard ratio; OS, overall survival; PFS, progression-free survival

# Discussion

Preliminary clinical results from PD-1 or PD-L1 blockade showed a potential role of immunotherapy in patients with lung cancer.[18-22] Although PD-L1expression in tumor cells has been suggested as a marker for anti-PD-1 therapy,[19] little is known about whether PD-L1 expression changes in association with gefitinib resistance in EGFR-mutant NSCLC. We showed that acquiring resistance to gefitinib could lead to a significant increase in PD-L1 expression on tumor cells in a subset of patients with EGFR-mutant NSCLC. This finding is consistent with findings in melanoma where PD-L1 expression has been shown to be increased after acquiring resistance to BRAF inhibitors.[13] Recently, Gainer *et al.* also reported that tumor cell PD-L1 expression varied between pre- and post-tyrosine kinase inhibitors in 22% of patients with EGFR-mutant NSCLC.[25]

In this study, we observed PD-L1 expression in tumor infiltrating ICs. It is interesting that the number of tumor tissues with PD-L1 expressing ICs increased at post-gefitinib biopsies. In addition, we observed correlations between some types of tumor-infiltrating lymphocytes and the PD-L1 expression of ICs, but not with those of tumor cells. It suggests that immune environment in

tumor tissues could be represented by PD-L1 expression in tumor infiltrating immune cells rather than tumor cells. Maybe the PD-L1 expression in tumor cells had no correlation with TILs because their oncogenic pathways contribute to PD-L1 overexpression independent to the TILs in tumor microenvironment. PD-L1 expression by ICs in tumor tissues was recently reported as a more sensitive predictive marker for specific anti-PD-L1 antibody (MPDL3280A) treatment than PD-L1 expression in tumor cells.[26] These data showed that PD-L1 expression in ICs rather than tumor cells is important in breaking the PD-1/PD-L1 axis in tumor microenvironment. In addition, which types of tumor-infiltrating IC expresses PD-L1 in tumor tissues is not clear. Tumor-infiltrating regulatory T cells functionally inhibit cytotoxic T cells in mouse models,[27] and the regulatory T cell count was associated with worse survival outcomes in 87 surgically resected NSCLC specimens.[28] In our study, regulatory T cells tended to increase in tumor tissue after gefitinib. The role of PD-L1 for the induction, differentiation, or maintenance of regulatory T cells has been reported from *in vivo* studies.[29,30] In patients with stomach or colorectal cancer, correlations between PD-L1 and regulatory T cells were reported.[31,32] However, PD-L1

expressing immune cells showed correlations with tumor-infiltrating lymphocytes either immunosuppressive regulatory T cells or cytotoxic T cells according to pre- or post-gefitinib biopsies. Further study is needed to identify the role of PD-L1 expressing immune cells in tumor tissues.

The activation of the EGFR pathway was associated with increased PD-L1 expression in EGFR-mutant NSCLC.[11] Several studies have reported that *EGFR* mutations contribute to the up-regulation of PD-L1 expression, thus promoting the tumor microenvironment to be immune evasive.[11, 23, 33] Recently, Keiichi Ota *et al*. reported PD-L1 expression is mediated by PI3K-AKT and by MEK-ERK signaling pathway in EGFR-mutant NSCLC cell lines.[34] However, little is known concerning PD-L1 expression after acquiring resistance to target agents. Amanda *et al*. reported in an abstract that B7-H1 confers chemoresistance by enhancing the activation of intracellular MAPK/ERK signaling pathway in a variety of cancer cells.[35] In this study, inhibition of MEK-ERK signaling pathway was involved in PD-L1 down-regulation in PC9GR cells which had wild type *EGFR* (Figure 19). This is similar to the melanoma cells after acquiring resistance to BRAF inhibitor. Jiang *et al*. reported that activation of MAPK

pathway in BRAF resistant melanoma cells promotes PD-L1 expression.[13] MEK inhibitors may have role in down-regulation of immunosuppressive PD-L1 expression by blocking the oncogenic pathway, therefore, should be considered for combination with anti-PD-1/PD-L1 antibodies. PC9GR cells lines showed PTEN overexpression which cannot be understood at this time. PTEN is a well known tumor suppressor which inhibits PI3K/AKT pathway. Loss of PTEN function resulted in PD-L1 upregulation that was inhibited by the inhibition of Akt in triple negative breast cancer and colorectal cancer.[36,37] However, PI3K/Akt pathway regulates PD-L1 expression in a complex way by either transcriptional and posttrancriptional mechanisms in a cell- and tissue-type dependent way[37]. This warrants further studies to elucidate the role of PTEN in PD-L1 expression in NSCLC.

In addition, acquiring MET positivity after gefitinib treatment was significantly associated with increased PD-L1 expression in clinical samples. Cell line data supported that PC9GR cells had no acquired T790M mutation but increased total MET. MET is known as a tyrosine kinase that is activated as an alternative pathway after acquiring resistance to EGFR tyrosine kinase inhibitors.

In skin epithelial cells, up-regulation of PD-L1 promoted EMT and accelerated carcinogenesis.[38] Chen *et al*. reported that EMT and PD-L1 expression are controlled by miR-200/ZEB1 in lung cancer cells.[39] We could not further studied PD-L1 expression in association with mesenchymal markers in tumor tissues due to the lack of available tumor specimen. These findings suggest that some resistant mechanisms to tyrosine kinase inhibitors could be involved in PD-L1 over-expression.

Patients with type III tumor immune environment based on tumor PD-L1 expression and TILs infiltration may be a significant group in NSCLC.[15] In this study, MET positive patients had type III features. Therefore, recruiting the activated lymphocytes into the tumor microenvironment is another issue in addition to blocking the PD-L1 for these patients. For patients who had acquired T790M mutation in their tumor, they showed low PD-L1 expression with high score TILs in tumor (type IV). This type IV is described as immune tolerance type and targeting of non-PD-1/PD-L1 immune suppressive pathways may have a role in the near future.[15]

Currently there are limited data concerning cytotoxic chemotherapy and

PD-L1 regulation. After doxorubicin treatment in a breast cancer cell line and a mouse model, cell surface expression of PD-L1 was decreased due to translocation.[40] In our study, many patients received cytotoxic chemotherapy in addition to gefitinib. However, neither the number of cytotoxic chemotherapy cycles nor interval from last cytotoxic chemotherapy to follow-up biopsy is associated with PD-L1 expression. Further study is needed for PD-L1 expression after cytotoxic chemotherapy in NSCLC.

Survival outcomes according to PD-L1 expression are not consistent among the papers. In 164 patients who had surgically resected stage I-III NSCLC, high PD-L1 expression was significantly associated with worse survival outcomes compared with those with low expression.[23] By contrast, Yang *et al.* found that OS was not different between the PD-L1-positive and -negative groups after 71 months of median follow-up after resection of stage I NSCLC in 163 patients.[41] Another cohort of 304 and 203 cases of stage I-IV NSCLC from Greece and Yale University, respectively, reported that patients who had positive PD-L1 expression had a better survival outcome.[42] In this study, patients who had increased PD-L1 expression on tumor cells after gefitnib treatment showed a

tendency of better OS. Adaptive immune resistance—up-regulation of PD-L1 expression by the surrounding cytokines, such as IFN-γ—may explain the better OS only in part of these patients. Because there were patients who had tumor PD-L1 increased without increasing TILs, other mechanisms must be explored further in these kinds of patients.

Our study has several limitations. First, a small number of patients included in the study. However, it is very difficult to obtain paired biopsies in a patient before and after treatment failure as a routine practice. Second, the semiquantitative nature of the immunohistochemical staining method must be considered. Most of the studies assessed the level of PD-L1 expression in tumor tissues by immunohistochemistry. The cut-off value in lung cancer has not yet been determined, some papers have used 5% of membrane expression, and other papers have used the median value as a cut-off value. Also, there are different antibodies to detect PD-L1 expression in tumor tissues and time interval between biopsy and testing could influence the results. Third, in the metastatic melanoma, change of PD-L1 expression or immune cell populations observed early after exposure to BRAF inhibitor.[43] It is unclear whether PD-L1 expression or TILs changed early

after gefitinib exposure or not from this study, further study is suggested.

In conclusion, we observed that the level of PD-L1 expression changes not only in tumor cells but also in tumor-infiltrating immune cells after acquiring resistance to gefitinib in patients with EGFR-mutant NSCLC. Overall survival tended to be longer in patients with increased PD-L1 expression in part from the increase in MET expression or activation of MEK-ERK pathway. In patients who had no increased level of PD-L1 expression by tumor cells had T790M secondary mutation or increased infiltration of TILs in tumor microenvironment. We suggest that re-biopsy should be considered when using the level of PD-L1 expression as a biomarker for anti-PD-1 or anti-PD-L1 immunotherapy. PD-L1 expression in tumor-infiltrating immune cells in addition to tumor cells should be evaluated to understand the drug resistance and improve the treatment in patients with NSCLC.

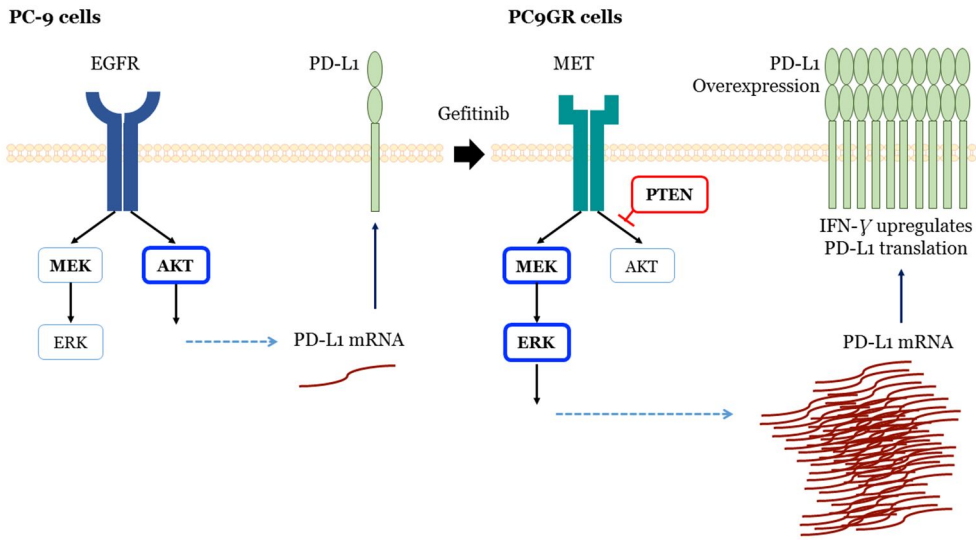



**Figure 19.** Mechanism of PD-L1 overexpression and upregulation in PC9GR cells. Change in signaling pathway from AKT to MEK-ERK is associated with PD-L1 overexpression in PC9GR cells. In addition, PD-L1 upregulation after IFN-γ is regulated in translational step.

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# 국문 초록

서론: Programmed death-1 (PD-1) 또는 PD-1 ligand (PD-L1)을 억제하는 약제는 비소세포폐암 환자들 중 일부에서 효과를 보임이 보고되고 있다. PD-L1 의 종양 내 발현은 이러한 치료의 효과 또는 예후를 예측하는 인자로 제시되고 있다. 그러나, 표적항암치료 후 PD-L1 발현이 변화하는지에 대해서는 아직까지 알려진 바가 거의 없다. 본 연구는 *EGFR* 돌연변이가 있는 비소세포폐암 환자에서 gefitinib 치료에 내성을 획득 후 PD-L1 발현의 변화를 알아보고자 하였다.

방법 및 대상: *EGFR* 돌연변이가 있는 비소세포폐암 세포주 PC-9에 gefitinib을 처리하여 내성을 가지는 세포주 PC9GR을 확립하였다. 이들 세포주들 간에 PD-L1 발현의 차이를 FACS로 관찰하고 그 기전을 PathScan®, Western blot 등으로 연구하였다. Gefitinib 치료 전후로 짝지어진 종양 조직이 있는 18명의 *EGFR* 돌연변이 비소세포암 환자의 종양 조직에서 PD-L1 의 발현을 알아보기 위해 면역염색하였다. 조직내에 침윤하는 면역세포의 PD-L1 발현과 함께 림프구를 면역염색하여 분석하였다.

결과: PC-9 세포주에서 유도한 PC9GR 은 PC-9에 비해 PD-L1 의 단백 발현양이 의미있게 증가하였다. 또한, PC-9 과 달리 IFN-$\gamma$에 의한 자극에 의해 발현양의 증가를 보였으며, 단백질 전사를 억제하는 경우 발현양의 감소를 보였다. 내성 세포주에서는 MEK-ERK 세포내 신호전달 경로가 활성화 되어 있었으며 MEK 을 억제하는 약제인 selumetinib 처리시 PD-L1의 발현이 감소되었다. 또한, 내성세포주에서 MET 발현의 증가를 확인하였다. 전체환자의 짝지어진 조직에서 종양세포가 발현하는 PD-L1 은 gefitinib 치료 후 H-score 의 중앙값이 25에서 40으로 증가하는 경향을 보였다 (P = 0.067). 이들 중 H-score 의 중앙값이 80 에서 180으로 크게 증가하는 7명의 환자들 (A군, 38.9%) 과 중앙값이 0 에서 0으로 변화가 없는 11명의 환자들 (B군, 61.1%) 로 두 군을 나눠 임상 요소와 관련성을 분석하였다. A군의 환자들은 B군의 환자들과 비교하여 전체생존기간이 연장되는 경향을 보였다 (P = 0.068). 또한, A군의 환자들은 gefitinib 치료 후 MET 면역염색에 양성인 환자들이 더 많았다 (P = 0.028). 종양미세환경에 염색되는 PD-L1 양성인 면역세포들이 치료 전 3명 (16.7%), 치료 후 7명 (38.9%)의 환자에서 각각 관찰되었다. 이들 PD-L1 양

성 면역세포들은 치료 전 FOXP3+ TILs ($\rho$ = 0.584, P = 0.014)과 치료 후 CD3+ TILs ($\rho$ = 0.547, P = 0.028), CD8+TILs ($\rho$ = 0.650, P = 0.005), PD-1+ TILs ($\rho$ = 0.590, P = 0.016)과 각각 통계적으로 의미있는 연관성을 나타내었다. 치료 후 T790M 을 획득한 경우 종양미세환경에 더 많은 TILs 이 침윤하는 경향을 보였다.

결론: Gefitinib 에 대한 내성을 획득한 폐암환자의 조직에서 종양세포 뿐만 아니라 침윤된 면역세포 PD-L1 발현이 변화함을 관찰하였다. Gefitinib 내성 획득 후 종양세포가 발현하는 PD-L1 이 증가하는 환자들에서 그렇지 못한 환자들에 비해 생존기간의 연장을 보였는데, MET 발현의 증가나 MEK 세포내 신호 전달의 활성화가 이와 관련될 것으로 추정된다. 종양세포의 PD-L1 발현이 증가하지 않았던 환자들에서 주로 T790M 이차 돌연변이의 획득과 종양침윤림프구의 증가가 관찰되었다. 따라서, 폐암 환자에서 PD-L1 발현 정도는 항암제에 대한 내성 획득 후 재평가가 필요하겠다. 또한, 종양 세포 뿐만 아니라 종양에 침윤된 면역 세포에서도 함께 조사가 이루어져야 약제 내성 기전의 규명과 항암 치료 성적의 향상이 가능하다고 생각된다.

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주요어: EGFR, gefitinib, programmed death receptor ligand-1,인터페론 감마, 비소세포폐암, 종양침윤세포

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