



Chronic nicotine exposure affects programmed death-ligand 1 expression and sensitivity to epidermal growth factor receptor-tyrosine kinase inhibitor in lung cancer

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Background: Smoking histories are independently associated with poor response to epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKIs) in non-small cell lung cancer (NSCLC) patients with activating EGFR mutations. The aim of the present study was to determine the effect of nicotine exposure on programmed death-ligand 1 (PD-L1) expression in EGFR mutant lung cancer cells.

Methods: Human lung adenocarcinoma PC9 cells were exposed to 1 μ M nicotine for 3 months designated as PC9/N, and cells were stimulated with gefitinib (0, 0.1, or 1 μ M) for 48 hrs. Cell viability by the MTT assay and morphological changes by immunofluorescence staining were assessed. The protein expression of EGFR, mTOR, AKT, α 1-nicotine acetylcholine receptor (nAChR) and PD-L1 were measured by Western blot. Gene expression of α 1-nAChR and PD-L1 were examined by RT-PCR. Intratumoral levels of PD-L1 expression were compared according to the burden of smoking dosage in 54 EGFR mutant lung cancer patients.

Results: Cellular growth was inhibited by treatment with gefitinib, and PC9 cells were significantly more sensitive to gefitinib than PC9/N cells. Pleomorphic appearance with atypical nuclei and to be detached and shrunken with condensed nuclei in PC9 than PC9/N cells. The gene expression level of α 1-nAChR and PD-L1 gene were higher in PC9/N cells compared to those in PC9 cells after treatment with gefitinib. Phosphorylation levels of EGFR, mTOR, AKT and PD-L1 level were decreased by gefitinib in PC9/N cells, which was to a lesser extent than that in PC9 cells. In tumors, heavy smokers (\geq 30 PY) showed 28.5% of \geq 50% PD-L1 tumor proportion score (TPS) while light smoker and never smokers had 12.5% and 9.7% of \geq 50% PD-L1 TPS, respectively. However, there was no statistical significance (P value =0.628).

Conclusions: Chronic nicotine exposure could increase PD-L1 expression related to intrinsic resistance to EGFR-TKI in NSCLC patients harboring activating EGFR mutation. Considering the clinical importance of inevitable EGFR resistance, further studies regarding the role of anti-PD-1/PD-L1 treatment are needed, especially in EGFR mutant smokers.

Keywords: Nicotine; programmed death-ligand 1 (PD-L1); epidermal growth factor receptor mutation (EGFR mutation)

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Introduction

Somatic mutation of the epidermal growth factor receptor (*EGFR*) gene is a major oncogenic driver in non-small cell lung cancer (NSCLC) (1). Patients with advanced NSCLC harboring *EGFR* mutation can receive *EGFR*-tyrosine kinase inhibitors (TKIs) such as gefitinib, erlotinib, and afatinib with high response rates for first-line treatment (2). However, acquired resistance to *EGFR*-TKIs in metastatic setting is inevitable, although initial responses are dramatic. In addition, ~20–30% of patients have no good initial clinical response to *EGFR*-TKIs, although they harbor sensitizing *EGFR* mutations (3). These primary resistant mechanisms can be explained by *de novo* resistant mutation, suboptimal drug exposure, failure of apoptosis induction, and other somatic alterations on genes encoding components of major signaling pathways (4). After tumor progression, novel third-generation *EGFR*-TKIs such as osimertinib and olmutinib have been designed to overcome resistance due to T790M mutation (5,6). Other strategies to overcome different mechanisms are also under development (7,8).

Although *EGFR* mutations are known to be commonly detected in never or light smokers (9), more than 30% of patients with *EGFR* mutations are currently smoking at the time of diagnosis according to Japanese data (10). Additionally, Kim *et al.* (11) have shown that about 40% of ever smokers with *EGFR* mutant adenocarcinoma have smoking histories of more than 30 pack-years. These smoking histories are independently associated with poor response to *EGFR*-TKIs in terms of progression-free survival in patients with activating *EGFR* mutations (10–13). Poor clinical outcomes are mainly due to cross-talks between nicotine-induced nicotinic acetylcholine receptor (*nAChR*) and *EGFR* pathway, Src activation, and epithelial mesenchymal transition (EMT) both *in vitro* and *in vivo* (14–16).

Recently, immunotherapies based on blocking of immunosuppressive checkpoints have been established as major treatment paradigms for the management of NSCLC. Programmed cell death-1 protein receptor (PD-1) and ligand (PD-L1) pathway is involved in suppressing autoimmunity during T-cell activation, allowing for immune tolerance of PD-L1 expressed cells (17). Multiple solid tumors can adapt these mechanisms and overexpress PD-L1, thereby avoiding immunologic surveillance and promoting cancer growth (17). Given the mechanism of action of anti-PD-1/PD-L1 antibodies, favorable responses have been observed in patients whose tumors express PD-

L1 (18,19). Smoking history also has clinical significance for patients with PD-1/PD-L1 blockade (20,21) which is influenced by specific molecular determinants, including somatic mutational burden and genomic instability (22). However, in real practice, activating *EGFR* mutation is associated with poor clinical response to anti-PD-1/PD-L1 therapy (23). This is explained by low tumoral PD-L1 expression and CD8⁺ tumor-infiltrating lymphocytes within the tumor microenvironment (24).

Immunotherapies are not routinely considered for the management of patients with activating *EGFR* mutations (25). Nevertheless, when we consider a plurality of smokers in these population and favorable effect of smoking history on response to checkpoint inhibitors, the biologic influence of nicotine needs to be investigated clinically. Thus, the aim of the present study was to determine the effect of nicotine exposure on PD-L1 expression in *EGFR* mutant lung cancer cells. Moreover, sensitivity of these cells to *EGFR*-TKIs and biologic changes regarding *EGFR*-related pathways upon nicotine exposure were also evaluated.

Methods

Cell culture

Human NSCLC cell line PC9 (*EGFR* exon 19 deletion) was purchased from Korean Cell Line Bank (Seoul, Korea). Cells were cultured in RPMI-1640 medium (WelGENE, Daegu, Korea) supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin solution (10,000 units/ml penicillin and 10 mg/ml streptomycin) at 37 °C in 5% CO₂. They were split regularly before they attained approximately 80% confluence. PC9 cells maintained in the presence of 1 μM nicotine (Sigma-Aldrich, St. Louis, MO, USA) for three months were designated as PC9/N.

Reagents

Gefitinib was purchased from Cell Signaling Technology (Beverly, MA, USA). It was dissolved in DMSO to obtain a stock solution at 100 mM. The final concentration of DMSO in all conditioned media did not exceed 0.1%. Antibodies specific for *EGFR*, mTOR, AKT, PD-L1, α1-*nAChR*, β-actin, and horseradish peroxidase-conjugated secondary antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Nicotinic acetylcholine receptor α1 subunit (α1-*nAChR*) was obtained from Abcam (Cambridge, United Kingdom). To evaluate the influence

of gefitinib on EGFR related signaling pathway, cells were stimulated with gefitinib for 48 hrs.

Cell morphological observation

Cells were seeded into 6-well plate at density of 3×10^5 cells/well. Following 24 hrs of incubation in room air, cells were treated with gefitinib (0, 0.1, or 1 μM) for 48 hrs. They were then stained with Wright and Giemsa. Morphological changes were observed under a phase contrast inverted microscope (original magnification: 200 \times).

Cell viability assay

Cell viability assay was performed based on the conversion of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] to formazan via mitochondrial oxidation. Briefly, 1×10^4 cells were cultured in 96-well plates (200 μL medium/well). Cells were then treated with gefitinib (0, 0.01, 0.1, 1, 10, and 50 μM) for 48 hrs. MTT solution was added to each well of the plate and incubated in room air for 4 hrs at 37 $^\circ\text{C}$. Formazan crystals were dissolved in acidified and OD value was measured at wavelength of 562 nm.

Reverse transcription (RT)-PCR analysis

To quantify PD-L1 and $\alpha 1$ -nAChR mRNA expression levels, total RNAs were extracted from cells using TRIzol reagentTM (Invitrogen, Carlsbad, CA, USA) and cDNA was synthesized using commercially available RT reagent kit (Invitrogen) according to the manufacturer's instructions. PCR was carried out in reaction mixture containing Taq DNA polymerase, primers, and cDNA. Equal volumes of PCR products were separated by electrophoresis on 2% agarose gels. β -actin was used as an internal control. Quantitative real time PCR (qRT-PCR) was performed using a Rotor-Gene SYBR[®] Green qPCR Kit Master Mix (Qiagen, Valencia, CA, USA) and carried out in a Real Time PCR Rotor-Gene Q Machine (Qiagen). All experiments were performed in triplicates. The relative expression of PD-L1 was normalized to β -actin expression. Fold change of test gene mRNA expression was determined using the $2^{-\Delta\Delta\text{Ct}}$ method (ΔCt = difference in threshold cycles for the test gene, $\Delta\Delta\text{Ct}$ = difference in ΔCt between the control and treatment group with nicotine or gefitinib).

Immunofluorescence

Cells were treated with 0.1 μM gefitinib for 48 hrs. Immunofluorescence staining was then performed according to the manufacturer's protocol. Briefly, cells were washed with cold PBS, fixed with 4% paraformaldehyde, blocked with blocking buffer (PBS plus 0.1% Tween-20 and 3% goat serum), and incubated with primary antibodies (phospho-EGFR or PD-L1) at 4 $^\circ\text{C}$ overnight. After washing with PBS, cells were incubated with fluorochrome-conjugated secondary antibodies (Alexa Fluor 488 anti-rabbit IgG) for 1 hour at room temperature. Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) and then microscopy was performed using by LSM800 w/ Airyscan confocal laser scanning microscope (Carl Zeiss, Germany).

Western blot analysis

Cells were harvested and suspended in RIPA lysis buffer (Thermo, Hercules, CA, USA) containing a mixture of protease inhibitor and phosphatase inhibitor (GenDEPOT, CA, USA) followed by centrifugation at 13,000 rpm for 30 min at 4 $^\circ\text{C}$. Protein content of the supernatant was determined using a BCA kit (Thermo). Proteins (30 μg per well) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a 10% PAGE gel with a Tris-glycine-SDS buffer and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad, MA, USA). These membranes were blocked with 5% skim milk (Sigma, St. Louis, MO, USA) at room temperature for 2 hrs followed by incubation with primary antibodies diluted with 3% BSA in Tris buffered saline containing 0.1% Tween 20 (TBS-T) overnight at 4 $^\circ\text{C}$. Membranes were then washed with TBS-T and incubated with HRP-conjugated secondary antibody at room temperature for 2 hrs. Signals were detected using Western Blotting Luminol Reagent (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

Study population

We consecutively collected 54 patients with pathologically confirmed adenocarcinoma of lung and activating EGFR mutation at diagnosis between January 2016 and December 2017 from Seoul St. Mary's Hospital, Incheon St. Mary's

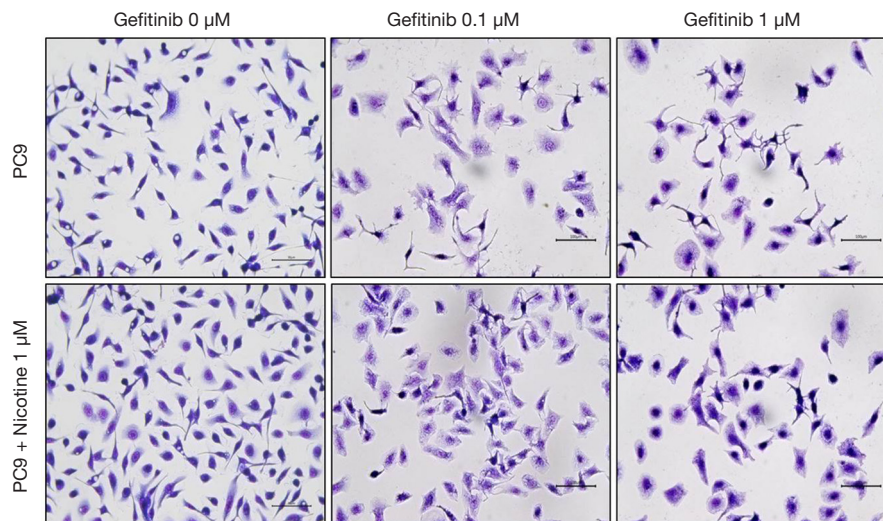


Figure 1 Morphology of cells after treatment with gefitinib in PC9 and PC9/N cells. Cells were seeded in six-well culture plates, and after 24 hrs, the cells were treated with gefitinib (0, 0.1, or 1 μ M) for 48 hrs. Cells were photographed under a phase contrast inverted microscope. Cells were stained with Wright and Giemsa (original magnification 200 \times). These results are representative of technical triplicate.

Hospital, Bucheon St. Mary's Hospital, St. Paul's Hospital, and Uijeongbu St. Mary's Hospital, Korea. Based on pack-year (PY) defined as average number of cigarettes per day/20 \times years of smoking, patients were categorized as never smokers (<100 cigarettes in their life-time), light smokers (0 < PY < 30), or heavy smokers (PY \geq 30). Genotyping of EGFR was done using peptide nucleic acid (PNA)-mediated PCR clamping method such as PNAClamp™ EGFR Mutation Detection Kit (PANAGENE, Inc., Daejeon, Korea) using real-time PCR. Activating EGFR mutation was defined as exon 19 deletion or exon 21 point mutation. PD-L1 immunohistochemistry testing was performing using PD-L1 clone 22C3 pharmDx kit and Dako automated Link 48 platform (Dako, Carpinteria, CA, USA). PD-L1 tumor proportion score (TPS) was calculated as the percentage of at least 100 viable tumor cells with complete or partial membrane staining.

Statistical analysis

The differences in the levels of cell viability between the two study groups in the presence of individual doses of gefitinib were compared using the Mann-Whitney test. The Fisher exact test was applied to compare the high PD-L1 TPS status (\geq 50%) among different burdens of smoking dosage. The cell viability levels were summarized using means and standard errors (SEM), and a P value

<0.05 was used to define statistical significance. All analyses were performed using Graph-Pad Prism version 5.00 for Windows (GraphPad Software, CA, USA).

Results

Morphological changes and growth inhibition by chronic nicotine exposure in EGFR sensitive cells

PC9 cells were exposed to 1 μ M nicotine for 3 months and then treated with gefitinib (0, 0.01, 0.1, 1, 10, and 50 μ M) for 48 hrs. *Figure 1* shows nicotine-induced cellular growth promotion and nonadherent clustered appearance on culture plate under a phase contrast inverted microscopy. Gefitinib treatment decreased cellular proliferation of PC9 and PC9/N cells in a concentration-dependent manner. After treatment with gefitinib, PC9 cells showed more pleomorphic appearance with atypical nuclei and more detached cells shrunken with condensed nuclei than PC9/N cells.

Figure 2 shows effects of gefitinib on the growth of PC9 and PC9/N cells determined by MTT assay. Gefitinib at concentration of 0.01 μ M significantly suppressed the growth of PC9 cells (23.3% \pm 3.0%, **P<0.01 compared to PC9 + gefitinib 0 μ M), but not PC9/N cells. There was a statistical difference in cell viability between the two cells at concentration of 0.01 μ M of gefitinib (P<0.05). DMSO treated cells showed no significant effect on cell morphology

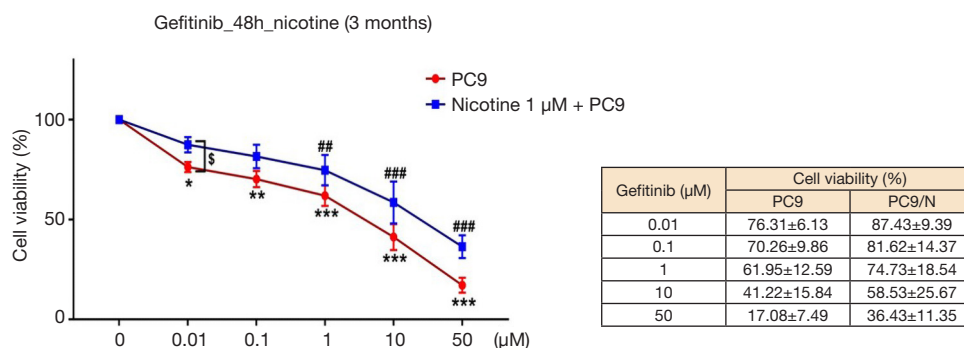


Figure 2 Cell viability assay for PC9 and PC9/N cells cultured in the presence of gefitinib. Cell growth inhibition in response to gefitinib was evaluated by using the MTT assay. Cells were treated with the indicated concentrations of gefitinib and cell viability was determined 48 hrs later. There are mean of independent triplicate experiments. The data are presented as means \pm SEM from technical triplicate. PC9/N: nicotine (1 μ M) expose for 3 months in PC9 cells. *, $P < 0.05$; **, $P < 0.01$ and ***, $P < 0.001$ compared with PC9 + gefitinib 0 μ M. ##, $P < 0.01$ and ###, $P < 0.001$ compared with PC9/N + gefitinib 0 μ M. § , $P < 0.05$ between two cells with gefitinib 0.01 μ M. Data are presented as mean \pm standard error of the mean (SEM). SEM, standard error.

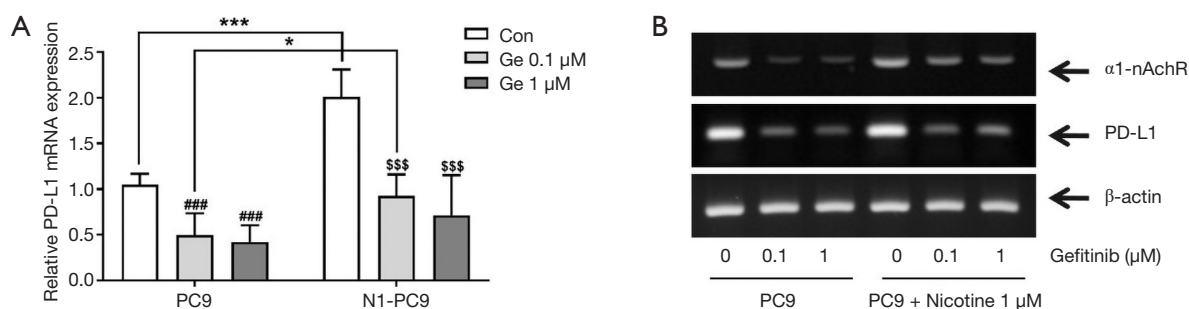


Figure 3 The mRNA expression of cells after treatment with gefitinib in PC9 and PC9/N cells. Cells were treated with gefitinib (0, 0.1, or 1 μ M) for 48 hrs, and the mRNA expressions of $\alpha 1$ -nAChR, and PD-L1 were examined by quantitative reverse transcription (qRT)-PCR (A) and RT-PCR (B). These results are representative of technical triplicate. *, $P < 0.05$ and ***, $P < 0.001$ compared with PC9 cells. ###, $P < 0.001$ compared with PC9 cells + gefitinib 0 μ M and SSS , $P < 0.001$ compared with PC9/N + gefitinib 0 μ M. nAChR, nicotinic acetylcholine receptors; PD-L1, programmed death ligand 1.

or viability (data not shown). Cellular growth was inhibited by treatment with gefitinib in a concentration-dependent manner. Gefitinib significantly suppressed the growth of both cell lines consistent with morphological changes, however, PC9 cells were significantly more sensitive to gefitinib than PC9/N cells.

Effects on PD-L1 and nAChR gene expression by chronic nicotine exposure after treatment with gefitinib in EGFR sensitive cells

mRNA expression levels of PD-L1 and $\alpha 1$ -nAChR in cells after treatment with gefitinib were measured by PCR (Figure 3). Quantitative reverse transcription (qRT)-PCR results

showed that the relative gene mRNA expression of PD-L1 in PC9 and PC9/N cells was significantly decreased after treatment with gefitinib at 0.1 or 1 μ M. The expression level of PD-L1 mRNA tended to be slightly higher in PC9/N cells compared to that in PC9 cells after treatment with gefitinib (0.1 or 1 μ M) (Figure 3A). Reverse transcription (RT)-PCR results also revealed that $\alpha 1$ -nAChR and PD-L1 mRNA expression levels were increased by nicotine exposure but decreased after treatment with gefitinib (Figure 3B). Similar results in their protein expression levels were also observed by Western blot analysis (Figure 4).

The localization of PD-L1 and p-EGFR in PC9 and PC9/N cells was determined by immunofluorescence staining counterstained with DAPI. Higher expression

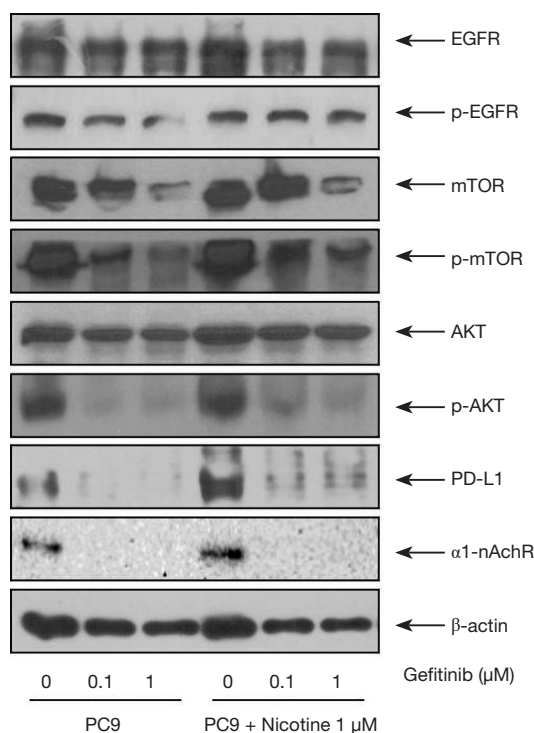


Figure 4 The protein expression levels of cells after treatment with gefitinib in PC9 and PC9/N cells. The cells were cultured with gefitinib (0, 0.1, or 1 μM) for 48 hrs, and EGFR, mTOR, AKT, PD-L1 and $\alpha 1$ -nAChR was detected by Western blot. β -actin was used as an internal control. Western blot was quantified by densitometry and ImageJ. These results are representative of technical triplicate. EGFR, epidermal growth factor receptor; PD-L1, programmed death ligand 1; nAChR, nicotinic acetylcholine receptors.

levels of PD-L1 and p-EGFR on membranes of EGFR sensitive cells after nicotine exposure were observed (red fluorescence, *Figure 5*).

Changes of EGFR-related protein expression by chronic nicotine exposure after treatment with gefitinib

Figure 4 shows protein expression levels of EGFR, nAChR, and related pathway molecules after gefitinib treatment in PC9 and PC9/N cells. Nicotine exposure upregulated protein expressions of p-EGFR, p-mTOR, p-AKT, PD-L1, and $\alpha 1$ -nAChR in EGFR sensitive cells. The phosphorylation of EGFR, mTOR and AKT in the PC9/N cell lines was decreased by gefitinib to a lesser extent than that observed in PC9 cells. PD-L1 expression was also

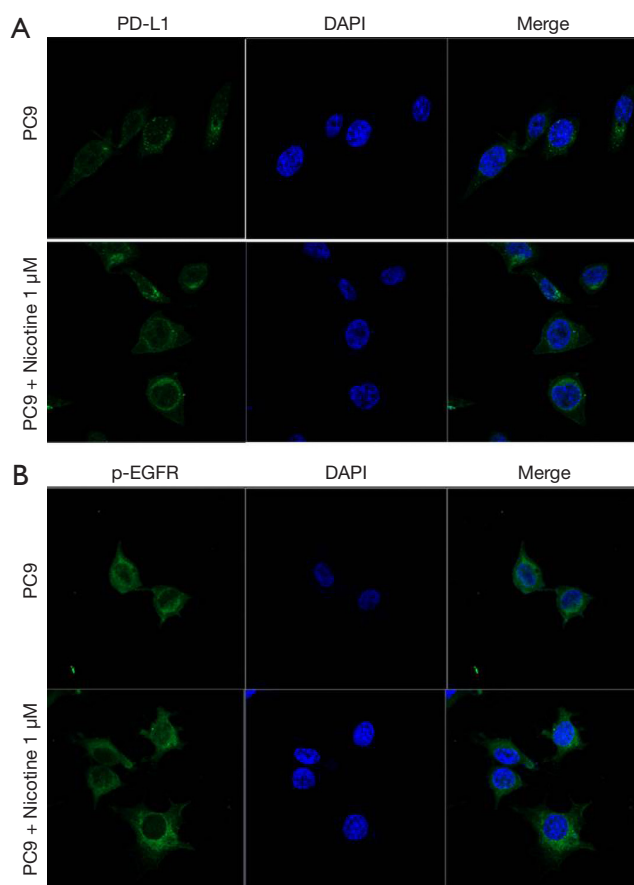


Figure 5 Immunofluorescence staining of PD-L1 and phosphorylation of EGFR in PC9 and PC9/N cells treated with 0.1 μM gefitinib. The localizations of PD-L1 and p-EGFR (green signal; Alexa488) in PC9 and PC9/N cells were shown by immunofluorescence counterstained with DAPI (blue signal) and analyzed by confocal microscopy (original magnification 400 \times). These results are representative of technical triplicate. DAPI, 4',6-diamidino-2-phenylindole; EGFR, epidermal growth factor receptor.

decreased by gefitinib to a lesser extent than that observed in PC9 cells. These results indicate that nicotine induced mTOR-Akt activation is involved in the effect of nicotine on cell survival and cell proliferation following gefitinib treatment.

The overall PD-L1 TPS according to burden of smoking dosage

A total of 54 eligible patients with lung adenocarcinoma harboring activating EGFR mutation were included in

Table 1 Clinicopathological characteristics of EGFR mutant patients

Factors (n=54)	Frequency (%)
Median age, range	62 [39–88]*
Sex	
Male	32 (59.3%)
Female	22 (40.7%)
Smoking history	
Never smoker	31 (57.4%)
Light smoker (<30 PY)	16 (29.6%)
Heavy smoker (≥30 PY)	7 (13.0%)
Clinical stage	
I–II	4 (7.4%)
III–IV	50 (92.6%)
EGFR mutation	
Exon19 deletion	33 (61.1%)
Exon 21 point mutation	21 (38.9%)
PD-L1 TPS in tumor tissues	
0%	20 (37.0%)
1–49%	27 (50.0%)
≥50%	7 (13.0%)

Data are presented as *median [range] for age and number (percentage). PY, pack-year; EGFR, epidermal growth factor receptor; PD-L1, programmed death ligand 1; TPS, tumor proportion score.

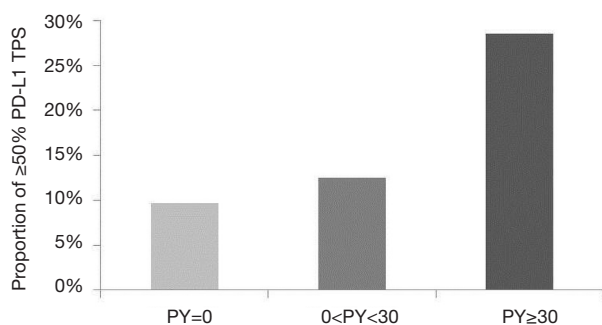


Figure 6 Overall proportion of PD-L1 TPS expression according to pack-year in NSCLC patients harboring activating EGFR mutation. Heavy smokers (≥30 PY) tended to have higher expression (≥50% PD-L1 TPS) than never and light smokers, however, Fisher exact test showed no statistical significance (P value =0.628). PD-L1, programmed death ligand 1; TPS, tumor proportion score; NSCLC, non-small cell lung cancer; EGFR, epidermal growth factor receptor; PY, pack-year.

the present study (Table 1). Their median age at diagnosis was 62 years (range, 39–88 years). Thirty-two (59.3%) of 54 patients were males. Thirty-one (57.4%) were never smokers while seven (13.0%) had heavy smoking history (≥30 PY). Most (92.6%) of these patients had advanced diseases (clinical stage III or IV). With regard to EGFR mutation status, thirty-three (61.1%) had exon 19 deletion mutation and twenty-one (38.9%) showed exon 21 point mutation. According to levels of PD-L1 TPS, seven (13.0%) patients had ≥50% TPS and twenty (37.0%) patients revealed 0% PD-L1 TPS expression. On analysis according to burden of smoking dosage, heavy smokers (≥30 PY) showed 28.5% of ≥50% PD-L1 TPS while light smoker and never smokers had 12.5% and 9.7% of ≥50% PD-L1 TPS, respectively (Figure 6). However, these results were not statistically significant (P=0.628).

Discussion

Our study showed that chronic nicotine exposure could increase the expression of PD-L1 in EGFR mutant lung cancer cells. In addition, nicotine exposure induced nAChR expression and upregulated EGFR downstream pathways such as Akt and mTOR. These biologic changes were associated with reduced sensitivity to EGFR-TKIs, thus conferring nicotine-related resistant mechanisms in EGFR mutant cancer cells.

Nicotine, the major addictive component of tobacco smoke, can induce cell-cycle progression, migration, invasion, and evasion of apoptosis, thus contributing to the progression and metastasis of tumor initiated by tobacco carcinogens (26). These effects occur mainly through the binding of nicotine to cell-surface receptors followed by their activation (especially nAChR, and β-adrenergic receptors to a certain extent) (27). Pallai *et al.* (28) have shown that nicotine can bind to nAChR and induce EGF in NSCLC, resulting in transactivation of EGFR and activation of mitogenic and antiapoptotic pathways. In EGFR mutant cells, chronic nicotine exposure can induce EGFR-TKI resistance via cross-talk between α1-nAChR and EGFR in PC9 cells (16), compatible with our data. In addition, nicotine can activate PI3K/Akt-related pathway and mediate chemotherapy-induced apoptosis in a dose-dependent and time-dependent manner *in vitro* (29). Akt inhibitor can cause reduction in chemotherapy-induced apoptosis and block anti-apoptotic effect caused by nicotine in A549 cells (30). These findings indicate that nicotine-induced activation of EGFR downstream pathways can

mediate EGFR-TKI resistance (31).

Potential therapeutic effect of nAChR antagonists has been experimentally observed in smoking-related malignancies, especially lung cancer (32). Nicotine via nAChR activation can induce invasion, migration, and EMT known to involve MEK/ERK signaling pathway in NSCLC (33,34). Although several novel compounds have recently been developed to overcome acquired EGFR-TKI resistance, little is known about the intrinsic resistance. T790M is a frequent reported mutation of *EGFR* gene. It has been detected in more than 50% of patients with acquired resistance (3). The major signaling pathways targeted by PD-1 are PI3K-Akt and Ras-MET-ERK pathways (35). PD-L1 expression in tumor cells is markedly increased after acquiring resistance to gefitinib in a subset of patients (36). Gefitinib-resistant sublines (PC9GR1, PC9GR2) that had no acquired T790M mutation showed increased PD-L1 expression with activation in MEK and ERK pathways and EMT phenotype. PD-L1 expression was mediated by PI3K-Akt and MET-ERK signaling pathways in EGFR mutant cell lines (37). PD-L1 upregulation promoted EMT and accelerated carcinogenesis in skin epithelial cells (38). Hata *et al.* (39) have demonstrated that T790M status is correlated with lower PD-L1 expression in patients with re-biopsied EGFR mutant tumors. Therefore, our results demonstrating increased PD-L1 expression via nAChR-related EGFR resistance is compatible with high PD-L1 expression with EGFR resistance mechanism by alternative bypass signaling activation, not T790M mutation.

PD-L1 expression and its biologic effect in EGFR mutant lung cancer cells are inconclusive. Previous studies have shown that EGFR activation is involved in the regulation of PD-L1 expression in cell lines (40,41) and that high PD-L1 overexpression is associated with activating EGFR mutation in EGFR mutant cells through surgically resected tumors (42). However, conflicting data have also shown that PD-L1 tumor proportion score $\geq 50\%$ seldom overlaps with the presence of activating EGFR mutation in lung adenocarcinoma (43,44). According to a meta-analysis, EGFR mutation is significantly correlated with high PD-L1 expression. Such discrepancies in results might be due to heterogeneous study populations and variable definitions of PD-L1 expression (45).

Higher PD-L1 expression level was correlated with primary resistance to EGFR-TKIs in advanced EGFR mutant lung cancer (46). In addition, tumor mutation

burden (TMB) is known as one of the predictive biomarkers in immunotherapy, and there exists an association between smoking history and high TMB in NSCLC patients (47). Interestingly, there was no difference in TMB based on smoking status in patients with driver mutations (48,49). These reported findings are similar with our results that PD-L1 TPS does not exhibit significant difference according to smoking history (never, light, heavy smokers).

Uninflamed tumor microenvironment with immunological tolerance and weak immunogenicity has been suggested to explain the inferior response of EGFR mutant NSCLC to anti-PD-1/PD-L1 therapy (50). Clinical responses to PD-1 blockade are associated with PD-L1 expression on tumor-resident cells induced by pre-existing CD8⁺ T cells in “adaptive immune resistance” infiltrating neoplastic tissue (51,52). Even if a tumor expresses PD-L1, anticancer immunosurveillance is unlikely to be reinstated if a tumor is devoid of cytotoxic T cell. Another possible reason is that tumor mutational loads in EGFR mutant-type tumors are lower than those of wild-type tumors (53). Putative biomarkers that can predict the response to anti-PD-1/PD-L1 immunotherapy including tumoral PD-L1 expression, CD8⁺ T-cell infiltration, and tumor mutational burden have been developed (54).

Although the role of anti-PD-1/PD-L1 therapy in TKI naïve EGFR mutant patients is currently unclear (23), clinical trials in settings of smoking-related intrinsic resistance and TKI failure without T790M are clinically challenging. A retrospective study has shown that smokers with EGFR mutant tumor can predict favorable clinical benefit provided by anti-PD-1/PD-L1 therapy (55), supporting our findings. Recent strategies of immunotherapy combined with cytotoxic chemotherapy, other immunotherapeutic agents, and radiotherapy are promising. They have potential to be applied in EGFR mutant patients. The present data did not show other biomarkers rather than PD-L1 expression. In addition, this study had limitation in that *in vitro* experiment was performed. Therefore, the efficacy of anti-PD-1/PD-L1 therapy needs to be tested through *in vivo* study.

In conclusion, chronic nicotine exposure can increase PD-L1 expression related to intrinsic resistance to EGFR-TKI in NSCLC patients harboring activating EGFR mutation. Considering the clinical importance of inevitable EGFR resistance, further studies regarding the role of anti-PD-1/PD-L1 treatment are needed, especially in EGFR mutant smokers.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). The study was approved by the ethics committee at each center (XC14OIMI0070). Informed consent was obtained from all patients.

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