EGFR-TKI resistance [22], but the influence of chronic exposure has remained unclear. In the present study, the association between chronic nicotine exposure and EGFR-TKI resistance was investigated experimentally. Furthermore, we investigated whether the smoking status was a predictive and prognostic factor in patients with *EGFR*-mutated NSCLC who were treated with the EGFR-TKI gefitinib.

2. Materials and methods

2.1. Cell culture and reagents

The PC-9 (*EGFR* exon 19 deletion) and 11_18 (*EGFR* exon 21 L858R) cell lines (human NSCLC cell lines) were maintained in RPMI1640 medium with 10% FBS (Sigma-Aldrich, St. Louis, MO). All the cell lines were maintained in a 5% CO_2-humidified atmosphere at 37 °C. The cell lines that were maintained in the presence of 1 μM nicotine (Sigma-Aldrich) for 3 months were designated as PC-9/N and 11_18/N, respectively.

Gefitinib and hexamethonium (nicotinic acetylcholine receptor [nAChR] inhibitor) was purchased from Selleck Chemicals (Houston, TX) and Sigma-Aldrich, respectively.

2.2. In vitro growth inhibition assay

The growth-inhibitory effect of gefitinib was examined using an MTT (Sigma-Aldrich) assay, as described previously [23].

2.3. Western blot analysis

A western blot analysis was performed as described previously [23]. Rabbit antibodies specific for EGFR, phospho-EGFR, phospho-AKT, phospho-ERK1/2, and β-actin were obtained from Cell Signaling (Beverly, MA). A rat antibody for the nicotinic acetylcholine receptor α1 subunit (α1 nAChR) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A rabbit antibody for α7 nAChR was obtained from Abcam (Cambridge, United Kingdom). To evaluate the influence of gefitinib on the phosphorylation, the cells were stimulated with gefitinib for 3 h.

2.4. Short interfering RNA (siRNA) transfection

Cells were transfected with siRNA for *CHRNA1* and 7 or each nonspecific target (scramble) as follows: GAUUUACCUUUAUGUAAGU (siRNA CHRNA1) for *CHRNA1*, GUUCUAUGAGUGCUGCAAA (siRNA CHRNA7) for *CHRNA7*, UAUAUGCGAGUAUAUUUCU for scramble of *CHRNA1* (siRNA Scr1), and GUUUAGCCCUAAUAUGAGG for scramble of *CHRNA7* (siRNA Scr7). siRNA transfection was performed using RNAiMAX (Invitrogen, Carlsbad, CA) as previously described [24].

2.5. Patients

Eighty-two patients who had been diagnosed as having stage IV NSCLC with *EGFR* mutations and who received gefitinib treatment at Kishiwada Municipal Hospital between January 2008 and December 2011 were enrolled. This study was retrospectively performed and was approved by the institutional review board of Kishiwada Municipal Hospital.

2.6. Statistical analysis

Continuous variables were analyzed using the *t*-test, and the results were expressed as the average and standard deviations (SD). The univariate relationship between each independent variable was examined using the χ^2 test. The response to gefitinib was evaluated according to the RECIST ver 1.1. Progression-free survival (PFS) was defined as the time from the initiation of gefitinib treatment until the first observation of disease progression or death from any cause, and the overall survival (OS) was defined as the time from the initiation of first-line treatment until death from any cause. The PFS and OS were analyzed using the Kaplan–Meier method and were compared among groups using the log-rank test. To identify predictive and prognostic factors, a Cox proportional hazards model was used for the univariate and multivariate analyses. Only those variables with *P*-values of <0.15 in a univariate analysis were included in the multivariate analysis. All the tests were two-tailed, and *P* values less than 0.05 were considered statistically significant. All the above-mentioned statistical analyses were performed using JMP 8 software (SAS Institute, Cary, NC).

3. Results

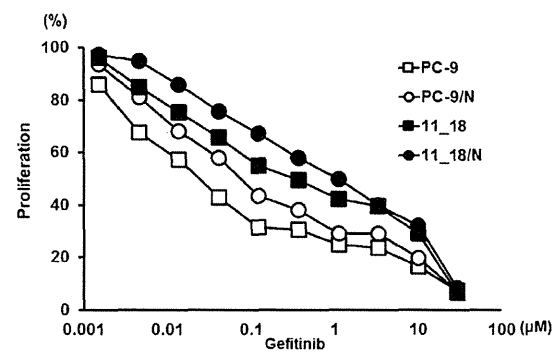
3.1. Resistance of PC-9/N and 11_18/N cell lines to EGFR-TKI

In order to evaluate the influence of chronic nicotine exposure on EGFR-TKI resistance, the PC-9 and 11_18 cell lines were cultured with 1 μM nicotine for 3 months (PC-9/N and 11_18/N, respectively). Both PC-9/N and 11_18/N cell lines were resistant to gefitinib, compared with the controls and the 50% inhibitory concentrations (IC_{50}) of PC-9/N and 11_18/N cell lines became about 3 times higher than the controls (Fig. 1A and B). In contrast, no significant difference in the sensitivity to gefitinib was observed between the cell lines that were temporarily (72 h) cultured with 1 μM nicotine and the controls (Fig. 1B). These results indicate that chronic nicotine exposure mediates the resistance to EGFR-TKI, but short term exposure does not.

3.2. Phosphorylation of EGFR in PC-9/N and 11_18/N cell lines is decreased by gefitinib to a lesser degree than that in the control cell lines

Next, we examined the EGFR signal in the cell lines in the presence of gefitinib using western blot analyses. The cells were

A



B

	PC-9	PC-9 +nicotine (72 h)	PC-9/N	11_18	11_18 +nicotine (72 h)	11_18/N
IC_{50} (μM)	0.024	0.022	0.076*	0.35	0.33	1.09*

Fig. 1. Growth inhibition assay for gefitinib (EGFR-TKI) and the IC_{50} of each cell line. (A) Growth inhibition assay for gefitinib. Growth inhibition in response to gefitinib was evaluated using an MTT assay. Both the PC-9/N and 11_18/N cell lines were resistant to gefitinib, compared with the controls. (B) IC_{50} of each cell line for gefitinib. The IC_{50} of the PC-9/N and 11_18/N cell lines was about 3 times higher than the controls ($P = 0.014^*$ and 0.013^*, respectively). In contrast, no significant difference in the sensitivity to gefitinib was seen between the cell lines cultured with 1 μM of nicotine for 72 h and the controls. Lines, mean of independent triplicate experiments; $^*P < 0.05$.

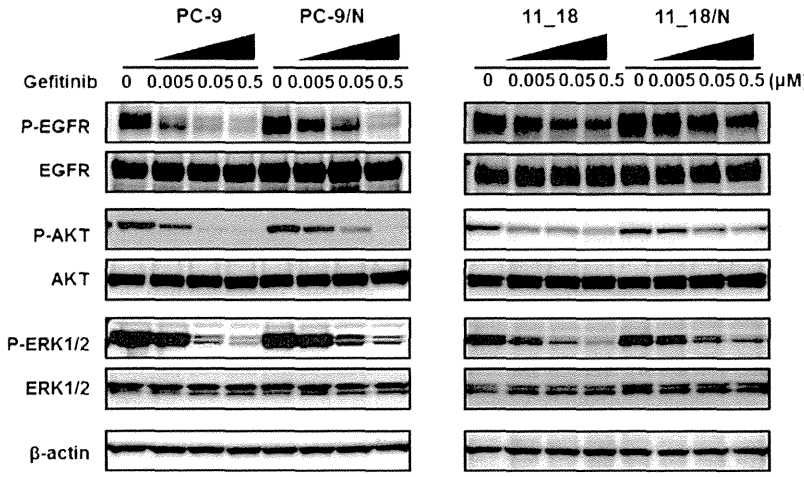


Fig. 2. Western blot analyses for each cell line cultured in the presence of gefitinib (EGFR-TKI). The cells were cultured with gefitinib (0, 0.005, 0.05, or 0.5 μM) for 3 h, and the western blot analyses were then performed. The phosphorylation of EGFR in the PC-9/N and 11_18/N cell lines was decreased by gefitinib to a lesser extent than that observed in the controls. The phosphorylation of AKT and ERK1/2 was also decreased by gefitinib to a lesser extent than that observed in the controls. β-Actin was used as an internal control.

cultured with gefitinib (0, 0.005, 0.05, or 0.5 μM) for 3 h, and a western blot analysis was then performed. As shown in Fig. 2, the phosphorylation of EGFR in the PC-9/N and 11_18/N cell lines was decreased by gefitinib to a lesser degree than that in the control cell lines. The downstream signals (AKT and ERK1/2) were also suppressed by gefitinib to a lesser degree than in the controls. A higher concentration of gefitinib decreased the phosphorylation. In addition, the resistance was cancelled by an nAChR inhibitor, hexamethonium (20 μM) (Fig. 3A). Gefitinib (0.05 μM) greatly decreased the phosphorylation of EGFR when administered in combination with hexamethonium (20 μM), compared with gefitinib alone (Fig. 3B). These results suggest that the resistance induced by chronic nicotine exposure arose from EGFR signal activation and was cancelled by the nAChR inhibitor.

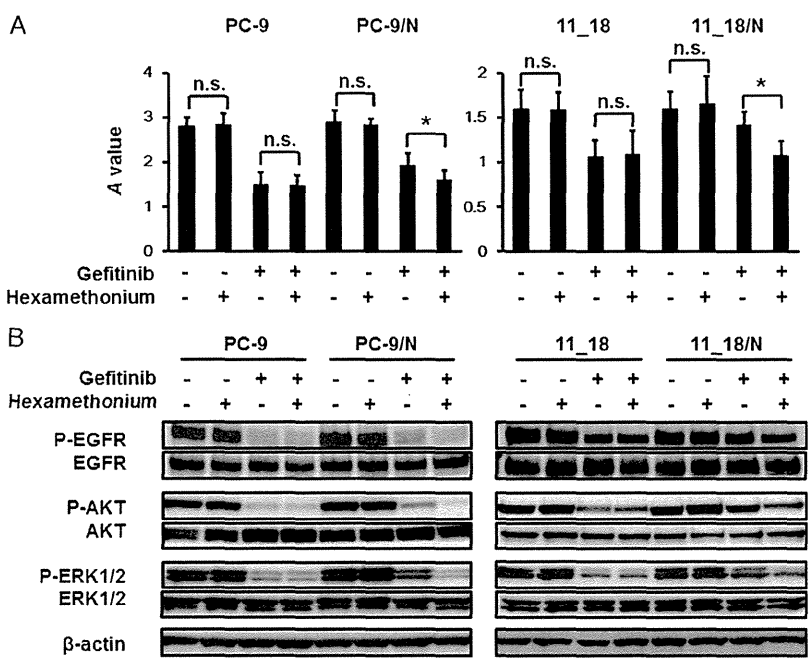


Fig. 3. Effect of an nAChR inhibitor, hexamethonium, on EGFR-TKI resistance induced by chronic nicotine exposure. (A) Growth inhibition of the PC-9 and 11_18 cell lines cultured in combination with hexamethonium. The growth inhibition was evaluated using an MTT assay. Hexamethonium (20 μM) alone did not influence cellular growth in the PC-9, PC-9/N, 11_18, or 11_18/N cell lines ($P = 0.56$, 0.37, 0.96, and 0.45, respectively). The inhibitory effect of gefitinib (0.05 μM) on the PC-9 or 11_18 cell line was not enhanced by hexamethonium ($P = 0.59$ and 0.80, respectively), whereas that for the PC-9/N or 11_18/N cell line was enhanced by hexamethonium ($P = 0.016^*$ and 0.034*, respectively). Columns, mean of independent triplicate experiments; bars, SD; n.s., not significant; * $P < 0.05$. (B) Western blot analyses of the PC-9 and 11_18 cell lines cultured in combination with hexamethonium. Gefitinib (0.05 μM) greatly decreased the phosphorylation of EGFR in cells cultured in combination with hexamethonium (20 μM), compared with those cells cultured in the presence of gefitinib alone. The phosphorylation of AKT and ERK1/2 was also greatly decreased in cells cultured in combination with hexamethonium. β-Actin was used as an internal control.

3.3. PC9/N and 11_18/N cell lines have higher expressions of nAChR α-subunits than cell lines with short exposure periods and controls, and resistance is cancelled by CHRNA-knockdown

To address why chronic nicotine exposure, but not short-term exposure, mediates EGFR-TKI resistance, we examined the influence of nicotine exposure on the expression of α1, 5, and 7 nAChR, which are likely to be involved in the smoking-related pathogenesis of NSCLC [22,25–27]. The mRNA expression of the CHRNA1 gene in the PC-9/N cell line and the mRNA expression of the CHRNA7 gene in the 11_18/N cell line were significantly higher than the levels in cell lines with short-term exposure or the control cell lines, respectively (Supplementary Fig. S1A). Western blot analyses demonstrated similar results (Supplementary Fig. S1B). Next, to examine the influence of α1 and 7 AChR on the EGFR-TKI resistance, the CHRNA1 and 7 genes in the cell lines were subjected to knockdown using siRNA (Fig. 4A). *CHRNA1*-knockdown cancelled the resistance in the PC9/N cell line but not in the 11_18/N cell line, while *CHRNA7*-knockdown cancelled the resistance in the 11_18/N cell line but not in the PC-9/N cell line (Fig. 4B). The inhibitory effect in the PC-9 and 11_18 cell lines were not enhanced by the knockdown (data not shown). These results suggest that the different expressions of the nAChR α-subunits are promoted by chronic nicotine exposure in each cell line and that the promoted expressions are associated with EGFR-TKI resistance.

3.4. Smoking history is an independent predictor of a poor PFS in patients with EGFR-mutated NSCLC treated with gefitinib

To address whether the smoking status was a predictive and prognostic factor for patients with *EGFR*-mutated NSCLC who received gefitinib treatment, the clinical data were analyzed. The clinical characteristics of the 82 patients and a comparison according to smoking history (never-, former-, and current-smoker) are summarized in Table 1. No significant differences were observed among the three groups in terms of age, PS, or *EGFR* mutations. As expected, most of the 55 never-smokers (47/55, 85.5%) were female, while only 8 (14.5%) were male ($P<0.0001$*).

Sixty-two of the 82 patients (75.6%) achieved a partial response or a complete response. The median PFS and OS of all the patients were 310 days and 778 days, respectively (Table 1). Many patients (54/82, 65.9%) received gefitinib treatment as a first-line therapy. The response rate to gefitinib treatment tended to be associated with the smoking status, but this difference was not statistically significant ($P=0.099$; never, 45/55 *vs.* former, 10/14 *vs.* current, 7/13) (Table 1). Significant differences in the PFS were observed among the three group ($P=0.011$*; never, 427 days *vs.* former, 224 days *vs.* current, 149 days) (Fig. 5A and Table 1), and never- or former-smokers had a significantly longer PFS than current-smokers ($P=0.011$*; never or former, 370 days *vs.* current, 149 days). Similarly, significant differences in OS were observed among the three groups ($P=0.045$*; never, 807 days *vs.* former, 787 days *vs.* current, 334 days) (Fig. 5B and Table 1), and never- or former-smokers had a significantly longer OS than current-smokers ($P=0.014$*; never or former, 807 days *vs.* current, 334 days). A multivariate analysis including smoking status (never- or former-smoker/current-smoker), PS (0 or 1/2 or 3), *EGFR* mutation (exon 19 deletion/others), and gefitinib treatment line (first-line/second-line or more) revealed that a never- or former-smoker status (HR, 0.35; 95% CI, 0.18–0.70; $P=0.0045$*), a PS of 0 or 1 (HR, 0.43; 95% confidence interval [CI], 0.22–0.93; $P=0.032$*), and the presence of an *EGFR* exon 19 deletion (HR, 0.56; 95% CI, 0.34–0.91; $P=0.019$*) were significantly associated with a longer PFS (Fig. 5A). Meanwhile, a multivariate analysis including smoking status (never- or former-smoker/current-smoker), PS (0 or 1/2 or 3), and gefitinib treatment line (first-line/second-line or more) revealed that a never- or former-smoker status (HR, 0.40; 95% CI, 0.20–0.85; $P=0.020$*) and a PS of 0 or 1 (HR, 0.44; 95% CI, 0.21–0.99; $P=0.048$*) were significantly associated with a longer OS (Fig. 5B).

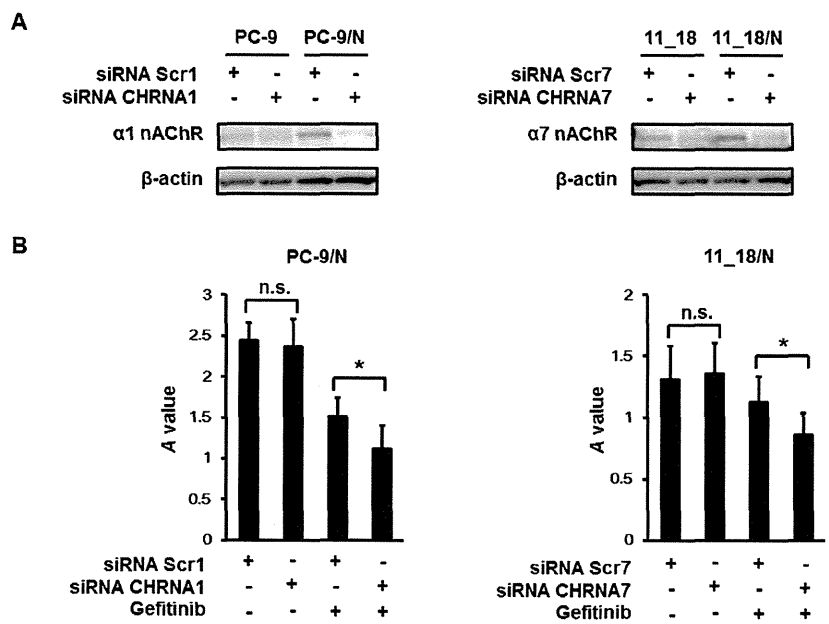


Fig. 4. Effect of *CHRNA*-knockdown on EGFR-TKI resistance induced by chronic nicotine exposure. The CHRNA1 and 7 genes in the cell lines were knocked down using siRNA. (A) Western blot analyses. *CHRNA1* and 7-knockdown were confirmed using western blot analyses. β-Actin was used as an internal control. (B) Growth inhibition of the PC-9 and 11_18 cell lines. *CHRNA1*-knockdown alone did not influence cellular growth in the PC-9/N cell line ($P=0.46$), whereas the inhibitory effect of gefitinib (0.05 μM) on the cell line was enhanced by *CHRNA1*-knockdown ($P=0.026$*). Similarly, *CHRNA7*-knockdown alone did not influence cellular growth in the 11_18/N cell line ($P=0.12$), whereas the inhibitory effect of gefitinib (0.05 μM) on the cell line was enhanced by *CHRNA7*-knockdown ($P=0.0061$*). The inhibitory effect in the PC-9 and 11_18 cell lines were not enhanced by the knockdown (data not shown). Columns, mean of independent triplicate experiments; bars, SD; n.s., not significant; * $P<0.05$.

Table 1
Patient characteristics and associations with smoking history ($n = 82$).

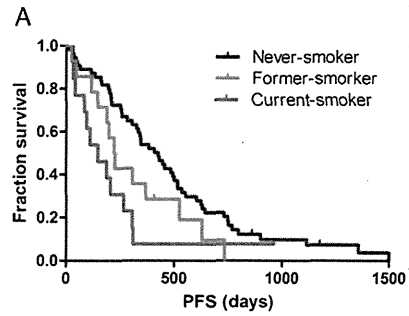
Characteristics	All $n = 82$	Never-smoker $n = 55$	Former-smoker $n = 14$	Current-smoker $n = 13$	P
Age (years)					0.68
Median [range]	71 [32–88]	71 [43–86]	68 [50–82]	72 [32–88]	
Gender					<0.0001*
Male	29	8	11	10	
Female	53	47	3	3	
ECOG PS					0.53
0	13	12	1	0	
1	58	34	12	12	
2	7	6	1	0	
3	4	3	0	1	
EGFR mutation					0.98
Exon 19 deletion	40	27	7	6	
Exon 21 L858R	39	27	6	6	
Others	3	1	1	1	
Gefitinib treatment line					0.10
First-line	54	40	6	8	
Second-line	22	14	5	3	
≥Third-line	6	1	3	2	
Response					0.099
CR	8	6	1	1	
PR	54	39	9	6	
SD	7	3	2	2	
PD	13	7	2	4	
PFS (days)					0.011*
Median	310	427	224	149	
OS (days)					0.045*
Median	778	807	787	334	

ECOG PS, Eastern Cooperative Oncology Group Performance Status; EGFR, epidermal growth factor receptor; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; PFS, progression-free survival; OS, overall survival.
PS was analyzed for 0 or 1 *vs.* 2 or 3, *EGFR* mutation status was analyzed for exon 19 deletion *vs.* others, gefitinib treatment line was analyzed for first-line *vs.* second-line or more, and response was analyzed for CR or PR *vs.* SD or PD.
* $P < 0.05$.

4. Discussion

EGFR mutations occur in 30% of patients with NSCLC who are of East Asian ethnicity, compared with 8% of patients of other ethnicities [28]. In the IRESSA Pan-Asia Study, *EGFR* mutation was a stronger predictor of a response to gefitinib, compared with smoking status [5,29]. However, whether smoking status can serve as a predictor of a response to EGFR-TKI treatment among patients with *EGFR* mutations remained unclear. Our clinical data showed that smoking status tends to be associated with the response rate to gefitinib and is an independent predictor of the PFS among patients treated with gefitinib. These findings were similar to those of a previous meta-analysis [30] and suggest that cigarette smoking mediates EGFR-TKI resistance. Indeed, such experimental data have been previously reported [20,21]. In these previous studies, oxidative stress or an EGFR conformation change induced by cigarette smoking was thought to be associated with the resistance. In addition, our experiments have suggested that chronic nicotine exposure can cause resistance *via* an EGFR signal. Our clinical data showing that current-smokers, who are likely to have persistent serum nicotine levels, have a significantly shorter PFS than never- or former-smokers also supports the concept of nicotine-induced resistance. In general, *EGFR* mutations tend to be found in never-smokers, and cigarette smoking is thought to have a minimal influence on *EGFR*-mutated carcinogenesis [31,32]. As seen in our clinical data, however, a considerable number of patients with *EGFR*-mutated NSCLC had a smoking history and they exhibited a poor outcome in terms of both PFS on gefitinib treatment and OS. Although several studies have identified smoking status as a predictive and prognostic factor in all patients with NSCLC [33], our present study is the first report to indicate an association between smoking status and OS in patients with *EGFR*-mutated NSCLC. Thus, even in patients with *EGFR*-mutated NSCLC, smoking history is an important factor that may be related to the response to EGFR-TKIs and OS.

Our experiments showed that the EGFR signal is decreased by EGFR-TKI to a lesser extent in the PC-9/N and the 11_18/N cell lines than in the control cell lines; furthermore, an nAChR inhibitor cancelled the smaller decrease in the signal. Because a nicotine-activated signal is involved in EGFR, the possibility of cross-talk between EGFR and nAChR has been proposed [18,22], supporting our experimental data showing that chronic nicotine exposure activates an EGFR signal through nAChR. In a clinical setting, gefitinib and erlotinib are the main drugs used as EGFR-TKIs. The most prominent difference between these two drugs is the dose setting. The approved daily dose of erlotinib (150 mg) is equal to the maximum tolerated dose (MTD) of erlotinib. In contrast, the daily dose of gefitinib (250 mg) is approximately one-third of the MTD of gefitinib [34,35]. Therefore, a higher serum concentration of EGFR-TKI can be achieved using erlotinib than using gefitinib. Our experiments have shown that higher concentrations of an EGFR-TKI can inhibit the EGFR signal in the PC-9/N and the 11_18/N cell lines. Of course, smoking cessation is of great importance, but considering the difference between gefitinib and erlotinib, erlotinib might be more effective.



Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	P	HR	95% CI	P
Age (<75 / ≥75 years)	0.89	0.56 - 1.45	0.64			
Gender (female / male)	0.90	0.56 - 1.48	0.68			
Smoking status (never or former / current)	0.45	0.25 - 0.88	0.022*	0.35	0.18 - 0.70	0.0045*
ECOG PS (0 or 1 / 2 or 3)	0.43	0.23 - 0.90	0.028*	0.43	0.22 - 0.93	0.032*
***EGFR* mutation** (ex 19 del / others)	0.56	0.35 - 0.88	0.013*	0.56	0.34 - 0.91	0.019*
Gefitinib treatment line (first / second or more)	1.49	0.91 - 2.49	0.11	1.27	0.74 - 2.21	0.39

Variables	Univariate analysis			Multivariate analysis		
	HR	95% CI	P	HR	95% CI	P
Age (<75 / ≥75 years)	0.91	0.53 - 1.61	0.74			
Gender (female / male)	1.28	0.73 - 2.33	0.40			
Smoking status (never or former / current)	0.42	0.22 - 0.90	0.028*	0.40	0.20 - 0.85	0.020*
ECOG PS (0 or 1 / 2 or 3)	0.38	0.19 - 0.83	0.018*	0.44	0.21 - 0.99	0.048*
***EGFR* mutation** (ex 19 del / others)	0.76	0.44 - 1.33	0.31			
Gefitinib treatment line (first / second or more)	1.92	1.07 - 3.63	0.029*	1.67	0.91 - 3.22	0.10

Fig. 5. Kaplan–Meier curves and univariate and multivariate analyses for PFS and OS. (A) PFS. Significant differences in PFS were observed among the three group ($P=0.011$*; never, 427 days *vs.* former, 224 days *vs.* current, 149 days). A multivariate analysis including the smoking status (never- or former-smoker [never or former]/current-smoker [current]), ECOG PS (0 or 1/2 or 3), *EGFR* mutation (exon 19 deletion [ex 19 del]/others), and gefitinib treatment line (first-line [first]/second-line or more [second or more]) revealed that a never- or former-smoker status (HR, 0.35; 95% CI, 0.18–0.70; $P=0.0045$*), a PS of 0 or 1 (HR, 0.43; 95% confidence interval [CI], 0.22–0.93; $P=0.032$*), and the presence of an *EGFR* exon 19 deletion (HR, 0.56; 95% CI, 0.34–0.91; $P=0.019$*) were significantly associated with a longer PFS. (B) OS. Significant differences in OS were observed among the three groups ($P=0.045$*; never, 807 days *vs.* former, 787 days *vs.* current, 334 days). A multivariate analysis including the smoking status (never- or former-smoker [never or former]/current-smoker [current]), ECOG PS (0 or 1/2 or 3), and gefitinib treatment line (first-line [first]/second-line or more [second or more]) revealed that a never- or former-smoker status (HR, 0.40; 95% CI, 0.20–0.85; $P=0.020$*) and a PS of 0 or 1 (HR, 0.44; 95% CI, 0.21–0.99; $P=0.048$*) were significantly associated with a longer OS.

In the present study, short-term nicotine exposure was not associated with EGFR-TKI resistance, whereas chronic exposure induced EGFR-TKI resistance. As is seen in the mechanism of nicotine addiction, our experiments showed that the expressions of nAChR α subunits are increased by chronic nicotine exposure [36,37]. This resistance was cancelled by their knockdown. These findings suggest that higher expressions of nAChR α subunits might result in EGFR-TKI resistance. nAChRs stimulate intracellular signaling pathways including an EGFR signal in a cell type-specific manner. Indeed, significant smoking-dependent expressions of α1, α5, and α7 nAChR have been reported [25,38], and another previous study has shown that nicotine-induced resistance is caused by α1 nAChR [22]. In the present study, however, α1 or α7 nAChR were elevated by chronic nicotine exposure in each cell line, and both caused EGFR-TKI resistance. Although the detailed mechanism explaining why different subunit expressions are elevated by chronic nicotine exposure in each cell line remains unclear, our study suggests that not only α1 nAChR, but also other subunits can be associated with the resistance.

Several studies have demonstrated that first-line gefitinib treatment is more effective than second-line or more treatments [39]. Our data, however, showed the opposite tendency. This tendency might be explained by the fact that the first-line gefitinib treatment group contained more elderly or poor PS patients, since other therapies were not indicated for such patients. With this in mind, several study limitations were unavoidable because of the retrospective nature of this study and the relatively small study population.

5. Conclusion

Our experiments suggest that chronic nicotine exposure induced by cigarette smoking mediates resistance to EGFR-TKIs *via* an EGFR signal through the α subunits of nAChRs. Smoking cessation is of great importance, and resistance may be overcome by high-dose EGFR-TKI treatment. To confirm this hypothesis, further research is needed.

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Conflict of interest statement

None declared.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.lungcan.2015.01.027.

References

[1] Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. CA Cancer J Clin 2013;63(1):11–30, http://dx.doi.org/10.3322/caac.21166 (PubMed PMID: 23335087).

[2] Siegel R, DeSantis C, Virgo K, Stein K, Mariotto A, Smith T, et al. Cancer treatment and survivorship statistics, 2012. CA Cancer J Clin 2012;62(4):220–41, http://dx.doi.org/10.3322/caac.21149 (Epub 2012/06/16, PubMed PMID: 22700443).

[3] Pao W, Chmielecki J. Rational, biologically based treatment of EGFR-mutant non-small-cell lung cancer. Nat Rev Cancer 2010;10(11):760–74, http://dx.doi.org/10.1038/nrc2947 (PubMed PMID: 20966921; PubMed Central PMCID: PMCPMC3072803).

[4] Pao W, Girard N. New driver mutations in non-small-cell lung cancer. Lancet Oncol 2011;12(2):175–80, http://dx.doi.org/10.1016/S1470-2045(10)70087-5 (PubMed PMID: 21277552).

[5] Mok TS, Wu YL, Thongprasert S, Yang CH, Chu DT, Saijo N, et al. Gefitinib or carboplatin–paclitaxel in pulmonary adenocarcinoma. N Engl J Med 2009;361(10):947–57, http://dx.doi.org/10.1056/NEJMoa0810699 (Epub 2009/08/21, PubMed PMID: 19692680).

[6] Mitsudomi T, Morita S, Yatabe Y, Negoro S, Okamoto I, Tsurutani J, 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(2):121–8, http://dx.doi.org/10.1016/s1470-2045(09)70364-x (Epub 2009/12/22, PubMed PMID: 20022809).

[7] Maemondo M, Inoue A, Kobayashi K, Sugawara S, Oizumi S, Isobe H, et al. Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR. N Engl J Med 2010;362(25):2380–8, http://dx.doi.org/10.1056/NEJMoa0909530 (Epub 2010/06/25, PubMed PMID: 20573926).

[8] Zhou C, Wu YL, Chen G, Feng J, Liu XQ, Wang C, et al. Erlotinib versus chemotherapy as first-line treatment for patients with advanced EGFR mutation-positive non-small-cell lung cancer (OPTIMAL, CTONG-0802): a multicentre, open-label, randomised, phase 3 study. Lancet Oncol 2011;12(8):735–42, http://dx.doi.org/10.1016/S1470-2045(11)70184-X (PubMed PMID: 21783417).

[9] Rosell R, Carcereny E, Gervais R, Vergnenegre A, Massuti B, Felip E, 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(3):239–46, http://dx.doi.org/10.1016/S1470-2045(11)70393-X (PubMed PMID: 22285168).

[10] Sequist LV, Yang JC, Yamamoto N, O'Byrne K, Hirsh V, Mok T, 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(27):3327–34, http://dx.doi.org/10.1200/JCO.2012.44.2806 (PubMed PMID: 23816960).

[11] Jackman D, Pao W, Riely GJ, Engelman JA, Kris MG, Jänne PA, et al. Clinical definition of acquired resistance to epidermal growth factor receptor tyrosine kinase inhibitors in non-small-cell lung cancer. J Clin Oncol 2010;28(2):357–60, http://dx.doi.org/10.1200/JCO.2009.24.7049 (PubMed PMID: 19949011).

[12] Kobayashi S, Boggon TJ, Dayaram T, Jänne PA, Kocher O, Meyerson M, et al. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. N Engl J Med 2005;352(8):786–92, http://dx.doi.org/10.1056/NEJMoa044238 (PubMed PMID: 15728811).

[13] Suda K, Mizuuchi H, Maehara Y, Mitsudomi T. Acquired resistance mechanisms to tyrosine kinase inhibitors in lung cancer with activating epidermal growth factor receptor mutation–diversity, ductility, and destiny. Cancer Metastasis Rev 2012;31(3–4):807–14, http://dx.doi.org/10.1007/s10555-012-9391-7 (PubMed PMID: 22736441).

[14] Remon J, Morán T, Majem M, Reguart N, Dalmau E, Márquez-Medina D, 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(1):93–101, http://dx.doi.org/10.1016/j.ctrv.2013.06.002 (PubMed PMID: 23829935).

[15] Hecht SS, Abbaspour A, Hoffman D. A study of tobacco carcinogenesis. XLII. Bioassay in A/J mice of some structural analogues of tobacco-specific nitrosamines. Cancer Lett 1988;42(1–2):141–5 (PubMed PMID: 3180033).

[16] Hecht SS. Cigarette smoking and lung cancer: chemical mechanisms and approaches to prevention. Lancet Oncol 2002;3(8):461–9 (PubMed PMID: 12147432).

[17] Dasgupta P, Rastogi S, Pillai S, Ordonez-Ercan D, Morris M, Haura E, et al. Nicotine induces cell proliferation by beta-arrestin-mediated activation of Src and Rb-Raf-1 pathways. J Clin Invest 2006;116(8):2208–17, http://dx.doi.org/10.1172/JCI28164 (PubMed PMID: 16862215; PubMed Central PMCID: PMCPMC1513051).

[18] Dasgupta P, Rizwani W, Pillai S, Kinkade R, Kovacs M, Rastogi S, et al. Nicotine induces cell proliferation, invasion and epithelial-mesenchymal transition in a variety of human cancer cell lines. Int J Cancer 2009;124(1):36–45, http://dx.doi.org/10.1002/ijc.23894 (PubMed PMID: 18844224; PubMed Central PMCID: PMCPMC2826200).

[19] Heeschen C, Jang JJ, Weis M, Pathak A, Kaji S, Hu RS, et al. Nicotine stimulates angiogenesis and promotes tumor growth and atherosclerosis. Nat Med 2001;7(7):833–9, http://dx.doi.org/10.1038/89961 (PubMed PMID: 11433349).

[20] Filosto S, Baston DS, Chung S, Becker CR, Goldkorn T. Src mediates cigarette smoke-induced resistance to tyrosine kinase inhibitors in NSCLC cells. Mol Cancer Ther 2013;12(8):1579–90, http://dx.doi.org/10.1158/1535-7163.MCT-12-1029 (PubMed PMID: 23686837).

[21] Filosto S, Becker CR, Goldkorn T. Cigarette smoke induces aberrant EGF receptor activation that mediates lung cancer development and resistance to tyrosine kinase inhibitors. Mol Cancer Ther 2012;11(4):795–804, http://dx.doi.org/10.1158/1535-7163.MCT-11-0698 (PubMed PMID: 22302097).

[22] Wang S, Takayama K, Tanaka K, Takeshita M, Nakagaki N, Ijichi K, et al. Nicotine induces resistance to epidermal growth factor receptor tyrosine kinase inhibitor by (1 nicotinic acetylcholine receptor-mediated activation in PC9 cells. J Thorac Oncol 2013;8(6):719–25, http://dx.doi.org/10.1097/JTO.0b013e31828b51d4 (PubMed PMID: 23625155).

[23] Arao T, Fukumoto H, Takeda M, Tamura T, Saijo N, Nishio K. Small in-frame deletion in the epidermal growth factor receptor as a target for ZD6474. Cancer Res 2004;64(24):9101–4, http://dx.doi.org/10.1158/0008-5472.CAN-04-2360 (PubMed PMID: 15604279).

[24] Kaneda H, Arao T, Tanaka K, Tamura D, Aomatsu K, Kudo K, et al. FOXQ1 is overexpressed in colorectal cancer and enhances tumorigenicity and tumor growth. Cancer Res 2010;70(5):2053–63, http://dx.doi.org/10.1158/0008-5472.CAN-09-2161 (PubMed PMID: 20145154).

[25] Lam DC, Girard L, Ramirez R, Chau WS, Suen WS, Sheridan S, et al. Expression of nicotinic acetylcholine receptor subunit genes in non-small-cell lung cancer reveals differences between smokers and nonsmokers. Cancer Res 2007;67(10):4638–47, http://dx.doi.org/10.1158/0008-5472.CAN-06-4628 (PubMed PMID: 17510389).

[26] Carlisle DL, Liu X, Hopkins TM, Swick MC, Dhir R, Siegfried JM. Nicotine activates cell-signaling pathways through muscle-type and neuronal nicotinic acetylcholine receptors in non-small cell lung cancer cells. Pulm Pharmacol Ther 2007;20(6):629–41, http://dx.doi.org/10.1016/j.pupt.2006.07.001 (PubMed PMID: 17015027).

[27] Wu CH, Lee CH, Ho YS. Nicotinic acetylcholine receptor-based blockade: applications of molecular targets for cancer therapy. Clin Cancer Res 2011;17(11):3533–41, http://dx.doi.org/10.1158/1078-0432.CCR-10-2434 (PubMed PMID: 21444681).

[28] Shigematsu H, Lin L, Takahashi T, Nomura M, Suzuki M, Wistuba II, et al. Clinical and biological features associated with epidermal growth factor receptor gene mutations in lung cancers. J Natl Cancer Inst 2005;97(5):339–46, http://dx.doi.org/10.1093/jnci/dji055 (PubMed PMID: 15741570).

[29] Fukuoka M, Wu YL, Thongprasert S, Sunpaweravong P, Leong SS, Sriurampong V, et al. Biomarker analyses and final overall survival results from a phase III, randomized, open-label, first-line study of gefitinib versus carboplatin/paclitaxel in clinically selected patients with advanced non-small-cell lung cancer in Asia (IPASS). J Clin Oncol 2011;29(21):2866–74, http://dx.doi.org/10.1200/jco.2010.33.4235 (Epub 2011/06/15, PubMed PMID: 21670455).

[30] Zhang Y, Kang S, Fang W, Hong S, Liang W, Yan Y, 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 2014, http://dx.doi.org/10.1016/j.cllc.2014.09.008 (PubMed PMID: 25454007).

[31] Mitsudomi T, Yatabe Y. Mutations of the epidermal growth factor receptor gene and related genes as determinants of epidermal growth factor receptor tyrosine kinase inhibitors sensitivity in lung cancer. Cancer Sci 2007;98(12):1817–24, 10.1111/j.1349-7006.2007.00607.x. (Epub 2007/09/25, PubMed PMID: 17888036).

[32] Ou SH. Lung cancer in never-smokers. Does smoking history matter in the era of molecular diagnostics and targeted therapy? J Clin Pathol 2013;66(10):839–46, http://dx.doi.org/10.1136/jclinpath-2012-201296 (PubMed PMID: 23661716).

[33] Ferketich AK, Niland JC, Mamet R, Zornosa C, D'Amico TA, Ettinger DS, et al. Smoking status and survival in the national comprehensive cancer network non-small cell lung cancer cohort. Cancer 2013;119(4):847–53, 10.1002/cncr.27824. (PubMed PMID: 23023590).

[34] Nakagawa K, Tamura T, Negoro S, Kudoh S, Yamamoto N, Takeda K, et al. Phase I pharmacokinetic trial of the selective oral epidermal growth factor receptor tyrosine kinase inhibitor gefitinib ('Iressa', ZD1839) in Japanese patients with solid malignant tumors. Ann Oncol 2003;14(6):922–30 (Epub 2003/06/11, PubMed PMID: 12796031).

[35] Yamamoto N, Horiike A, Fujisaka Y, Murakami H, Shimoyama T, Yamada Y, et al. Phase I dose-finding and pharmacokinetic study of the oral

epidermal growth factor receptor tyrosine kinase inhibitor Ro50-8231 (erlotinib) in Japanese patients with solid tumors. Cancer Chemother Pharmacol 2008;61(3):489–96, http://dx.doi.org/10.1007/s00280-007-0494-8 (Epub 2007/05/08, PubMed PMID: 17483950).

[36] Benowitz N.L. Neurobiology of nicotine addiction: implications for smoking cessation treatment. Am J Med 2008;121(4 (Suppl. 1)):S3–10, http://dx.doi.org/10.1016/j.amjmed.2008.01.015. (PubMed PMID: 18342164).

[37] De Biasi M, Dani JA, Reward. addiction, withdrawal to nicotine. Annu Rev Neurosci 2011;34:105–30, http://dx.doi.org/10.1146/annurev-neuro-061010-113734 (PubMed PMID: 21438686; PubMed Central PMCID: PMCPMC3137256).

[38] Egleton RD, Brown KC, Dasgupta P. Nicotinic acetylcholine receptors in cancer: multiple roles in proliferation and inhibition of apoptosis. Trends Pharmacol Sci 2008;29(3):151–8, http://dx.doi.org/10.1016/j.tips.2007.12.006 (PubMed PMID: 18262664).

[39] Morita S, Okamoto I, Kobayashi K, Yamazaki K, Asahina H, Inoue A, et al. Combined survival analysis of prospective clinical trials of gefitinib for non-small cell lung cancer with EGFR mutations. Clin Cancer Res 2009;15(13):4493–8, http://dx.doi.org/10.1158/1078-0432.CCR-09-0391 (PubMed PMID: 19531624).