Nicotine Induces Resistance to Epidermal Growth Factor Receptor Tyrosine Kinase Inhibitor by α1 Nicotinic Acetylcholine Receptor–Mediated Activation in PC9 Cells

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Introduction: Nicotine, the major component among the 4000 identified chemicals in cigarette smoke, binds to nicotinic acetylcholine receptors (nAChRs) on non–small-cell lung cancer (NSCLC) cells and regulates cellular proliferation by activating mitogen-activated protein kinases [AQ: MAPK has been expanded to mitogen-activated protein kinases. Please approve.]and PI3K/Akt pathways. In patients with smoking-related lung cancer who continue smoking, the anticancer effect of epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) is weaker than that in nonsmokers; however, the precise reason for this difference remains unclear. We investigated the role of α1 nAChR subunit in this phenomenon.

Methods: We screened for α1 nAChR mRNA in three NSCLC cell lines and analyzed the protein in resected primary NSCLC tissues. We used Western blot and RNA interference (siRNA) methodology to confirm the results.

Results: We determined that α1 nAChR plays an essential role in nicotine-induced cell signaling and nicotine-induced resistance to EGFR-TKI. In addition, we showed that silencing of α1 nAChR subunit in NSCLC may suppress the nicotine-induced resistance to EGFR-TKI.

Conclusions: These results further implicate nicotine in lung carcinogenesis, and suggest that α1 nAChR may be a biomarker for EGFR-TKI treatment and also a personalizing target molecule for patients with smoking-related lung cancer.

Key Words: Nicotinic acetylcholine receptors, Non–small-cell lung cancer, Nicotine, Epidermal growth factor receptor tyrosine kinase inhibitor, Targeting therapy.

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Cigarette smoking is one of the major risk factors associated with non–small-cell lung cancer (NSCLC), which accounts for 80% of all lung cancers.[1–4] Nicotine, the main psychoactive component of cigarette smoke,[5] can induce cell proliferation, angiogenesis, and resistance to apoptosis,[6–9] and may also facilitate the development of lung cancer by sustained activation of growth-promoting pathways.[10–13] These effects of nicotine are mediated by nicotinic acetylcholine receptors (nAChRs) that are widely expressed in the lung as well as the brain and neuromuscular junctions.[5,10,13–15] Nicotine also reduces the effectiveness of cancer chemotherapy[14,16,17]; however, the mechanism by which this occurs remains unclear.

nAChRs are either homopentamers or heteropentamers consisting of 10 α-subunits (α1–α10), four non–α-subunits β (β1–β4), γ, δ, and ε, and are classified into neuronal and muscle forms.[5,18] Although the combinations of nAChRs have not been completely characterized, it is possible that each subtype has distinct pharmacologic properties.[19] In addition, nAChRs stimulate intracellular signaling pathways in a cell type–specific manner, thus cell type–specific oncogenesis occurs in response to different subunits of nAChRs.[13,19] Furthermore, significant smoking-dependent declines in expression of α1, α5, and α7 nAChR have been reported.[13,19] These nAChR subunit genes could be playing roles in nicotine-induced lung cancer. Among the subunit genes, α7 nAChR is well studied[14,20,21]; however, little is known about the others. Further evaluation of the functional roles played by these nAChR subunit genes in nicotine exposure and lung carcinogenesis is needed.

NSCLC is characterized by its poor prognosis and resistance to anticancer drugs.[22] Now, more than ever, clinicians and NSCLC patients are struggling to optimize treatments. Although clinical trials have revealed epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI) as the most promising therapeutic agent (e.g., erlotinib, gefitinib) in NSCLC,[23,24] there are still clinical questions to be addressed. Recently, smoking (nicotine) exposure has been shown to have a negative effect on EGFR-TKI therapy in lung cancers,[25] although the mechanisms that contribute to the drug resistance remain unknown. It has been reported that exposure to nicotine increases EGFR expression in lung cells by activating survival pathways.[10,13,26,27] In addition, differential expression of nAChR subunits have been observed between

smokers and nonsmokers.[19] Therefore, we hypothesized that interaction between nicotine and nAChRs may contribute to the process that generates resistance against EGFR-TKI treatment.

In this study, we focused on the functionality of the muscle-type α1 nAChR subunit. We examined the expression of α1 nAChR mRNA and protein in human NSCLC cell lines and human NSCLC tissues. We also analyzed the role of nicotine in activations of ERK and Akt (Ser-473) pathways through the α1 nAChR in PC9 cells. Finally, we studied the ability of nicotine-α1 nAChR signaling to protect NSCLC cells from EGFR-TKI treatment.

MATERIALS AND METHODS

Cell Culture

NSCLC cell lines (A549, H2122, and PC9), HEK293, and BEAS 2B cells were purchased from American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco's Modified Eagle Medium or Roswell Park Memorial Institute media supplemented with 10% fetal bovine serum and incubated at 37°C in 5% carbon dioxide.

Real-Time Reverse Transcription Polymerase Chain Reaction of α1 nAChR

Total RNA was isolated with an RNeasy Mini kit from Qiagen (Valencia, CA), and RNA (1μg) was reverse-transcribed to generate cDNA with PrimeScript RT reagent kit (Perfect Real Time, Takara, Japan) according to the manufacturer's protocol. The primers and conditions for real-time reverse-transcriptase polymerase chain reaction (RT-PCR) were described elsewhere.[19] The relative quantitation value for α1 nAChR gene compared with the calibrator was expressed as $2^{-(Ct-Cc)}$ (Ct and Cc are the mean threshold cycle differences after normalizing to 18S). A 10-μl aliquot of each reaction was analyzed on 2% agarose gels. glyceraldehyde-3-phosphate dehydrogenase (GADPH) (5′-ACCTACCAAATATGATGACATCA-3′, 5′-CGCTGTTGAAGTCAGAGGA-3′) was used as a positive control for RNA integrity.

Western Blot Analysis

A549, PC9, and H2122 cells were rinsed with ice-cold phosphate-buffered saline and scraped into lysis buffer with protease inhibitors and phosphatase inhibitor. Protein was quantitated with the BCA assay (Thermo Scientific, Pierce). Whole-cell lysates were used for Western blot analysis. In brief, cell lysates adjusted for protein concentration were separated on 10% sodium dodecyl sulfate polyacrylamide gels and electrotransferred to polyvinylidene fluoride membranes. The transferred membranes were blocked with 3% nonfat milk and incubated with appropriate primary antibody (Sigma-Aldrich, St. Louis, MO) overnight at 4°C. Membranes were then washed briefly and incubated with horseradish peroxidase-conjugated secondary antibody (Cell Signaling Inc., Beverly, MA).

Immunohistochemistry for α1 nAChR

For immunohistochemistry (IHC), 43 paraffin-embedded lung cancer samples were obtained from the Department of Pathology in Kyushu University Hospital. The patients' characteristics are summarized in Table 1. Clinicopathological factors, including sex, smoking history, and tumor type were evaluated. The samples included 32 adenocarcinomas, seven squamous cell carcinomas, two large-cell carcinomas, and two small-cell carcinomas. Formalin-fixed and paraffin-embedded tissue blocks were cut into 4-μm sections. The slides were deparaffinized and hydrated and then pretreated for microwaving. Endogenous peroxidase was quenched with methanol/peroxide. The slides were blocked with rabbit serum, incubated with primary α1 nAChR antibody overnight (Salk Institute by Sigma), and then incubated with secondary antibody (Dako). Immunodetection was performed with an avidin-biotin horseradish peroxidase method and visualized with 3,3′-diaminobenzidine (DAB) as the chromogen. The slides were then counterstained with hematoxylin.

Scoring of α1 nAChR Expression

Immunoreactivity was scored according to the percentage (P) of tumor cells showing characteristic membrane staining (0, undetectable; level 1, few; level 2, <10%; level 3, 10%–50%; and level 4, >50%) and the intensity (I) of staining (1, weak; 2, moderate; and 3, strong). Expression of α1 nAChR in each section was scored by multiplying P by I, the so-called quick score (Q) (Q = P × I; maximum = 12). We classified staining as low-positive (Q ≤ 6; $n = 16$, [including negative Q = 0]) versus high-positive (Q > 6; $n = 27$). Assessments were made by two independent observers blinded to histologic diagnoses. The correlation between clinicopathological factors was analyzed using $\div^2$ tests.

RNA Interference

Chemically synthesized double-stranded siRNA specific for nAChR α1 was purchased from Dharmacon Research (Thermo Fisher Scientific, Lafayette, CO). The siRNA was transfected (100 nmol/L) with DharmaFECT reagents according to the manufacturer's instructions. A nontargeting (NT) siRNA sequence (Dharmacon Research) was used as nonspecific control.

Cell Survival after EGFR-TKI Treatment

Intact PC9 cells and PC9 cells pretreated with nicotine (Sigma) were seeded at a density of 5,000 cells per well in a 96-well plate in complete medium overnight. Cells were treated with various concentrations of EGFR TKI (Calbiochem EMD Biosciences, La Jolla, CA) for 48 hours, and then 20 μl of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) reagent (Promega, Madison, WI) was added per well for 1 hour. Survival was determined by comparison of the absorbance at 490 nm with that of the control.

Statistical Analysis

Data are presented as the mean ± SEM for three independent experiments. The $\div^2$ tests were used to compare two groups, and one-way analysis of variance was used to analyze data among groups for significant difference. A p value less than 0.05 indicated a statistically significant difference. JMP version 9 (SAS Institute Inc., Cary, NC) software was used for all analyses.

TABLE 1. Association between α1 nAChR Expression and Clinicopathological Factors in NSCLC

Factors	Total Patients N = 43	α1 nAChR-High Positive *n* = 27	α1 nAChR-Low Positive *n* = 16	*p*
Sex				
Male	25	18	7	0.2035
Female	18	9	9	
Smoking history				
Smoker	27	13	14	0.0206
Never smoked	16	14	2	
Tissue type				
Adenocarcinoma	32	24	8	0.0138
Nonadenocarcinoma[a]	11	3	8	

[a]Seven squamous cell carcinomas; two large-cell carcinomas; two small-cell carcinomas.
nAChR, nicotinic acetylcholine receptor.

RESULTS

Expression of the α1 nAChR Subunit in NSCLC Cell Lines and in Human NSCLC Tissues

Three NSCLC cell lines (A549, PC9, and H2122) were examined for expression of α1 nAChR subunit mRNA by RT PCR. BEAS 2B cells were used as a positive control,[13,15] and GAPDH was included in each panel as a reference. As shown in Figure 1*A*, all three cell lines expressed α1 nAChR mRNA. Expression of the receptor proteins in the four cell lines was confirmed by Western blot with HEK293 cells as the negative control.[3] All four cell lines expressed detectable levels of the α1 nAChR subunit, as shown in Figure 1B.

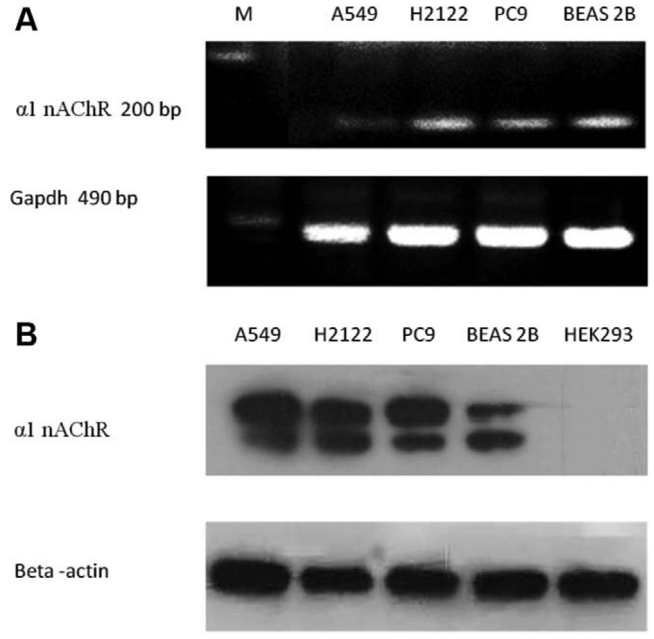

FIGURE 1. *A,* NSCLC cell lines express nAChR mRNA and protein. Detection of reverse-transcriptase polymerase chain reaction products for α1 nAChR subunits in A549, H2122, and PC9 cell lines. The BEAS 2B cell line was a positive control. Products were sequenced and in each case confirmed to be the expected authentic sequence for each subunit. M, 100 bp molecular marker with the brightest band at 600 bp. *B,* Western blot results for α1 nAChR subunits. NSCLC cell lines express nAChR protein. BEAS 2B cell line or HEK293 cell line served as the positive or negative control, respectively. nAChR, nicotine acetylcholine receptors; NSCLC, non–small-cell lung cancer.

We also examined paraffin-embedded specimens of human NSCLC tumors for expression of α1 subunit by IHC. Our series of NSCLC types included 32 adenocarcinomas, seven squamous cell carcinomas, two large-cell carcinomas, and two small-cell carcinomas. Although a certain level of expression of α1 nAChR was shown in almost all types of lung cancer, statistical analysis showed that the adenocarcinoma tissues were stained more strongly than the nonadenocarcinoma tissues (Table 1). Representative photographs of positive immunostaining for each histological type of lung cancer are shown in Figure 2. The results suggested that the α1 nAChR might have a function in lung tumors, especially in adenocarcinoma.

Roles of the α1 nAChR Subunit in Nicotine-Induced Cell-Signaling Pathways of NSCLCs

These studies led to the realization that the α1 nAChR may have functional roles in NSCLC cells. Indeed, given the fact that nicotine could induce cell proliferation, angiogenesis, and growth of tumors,[10–13,28] we next examined the ERK1/2, Akt (Ser-473), and STAT3 pathways induced by nicotine in PC9 cells. In Western blot analysis, we found that phosphorylated ERK1/2 and PI3K/ Akt (Ser-473) increased in a time-dependent manner after treatment with nicotine (Fig. 3*A*). In contrast, phosphorylated STAT3 was not detected at all. To further determine whether α1 nAChR subunit was directly involved in the ERK1/2 and Akt (Ser-473) pathways, PC9 cells were transfected with siRNA targeting α1 nAChR (si-α1) or NT siRNA (si-NT). Markedly down-regulated expression of phosphorylated Akt (Ser-473) and phosphorylated ERK1/2 protein was seen by Western blot (Fig. 3*B*). Taken together, these results suggested that α1 nAChR contributed, at least in part, to nicotine-induced activation of the ERK1/2 and Akt (Ser-473) pathways in PC9 cells.

Effects of the α1 nAChR Subunit in Nicotine-Induced EGFR Signaling Pathways of NSCLCs

The experiments with siRNA targeting α1 nAChR showed that phosphorylation of Akt (Ser-473) and ERK1/2 can be partially blocked in PC9 cells. EGFR has been reported to activate the same downstream pathways as α1 nAChR.[26] Considering the cross-talk between downstream signaling by EGFR and α1 nAChR, it is possible that nicotine activates EGFR through the activation of ERK1/2 and PI3K/Akt (Ser-473). In the studies that followed, we observed that nicotine caused time-dependent phosphorylation of EGFR in PC9 cells (Fig. 3*C*), and that the knockdown of α1 nAChR expression by siRNA could reduce the protein levels of both EGFR and phosphorylated EGFR (Fig. 3*D*). Gene silencing of α1 nAChR by siRNA was validated by real-time RT PCR (Fig. 4*A*, *B*). These results suggest that α1 nAChR might act as an upstream regulator of the EGFR pathway in PC9 cells.

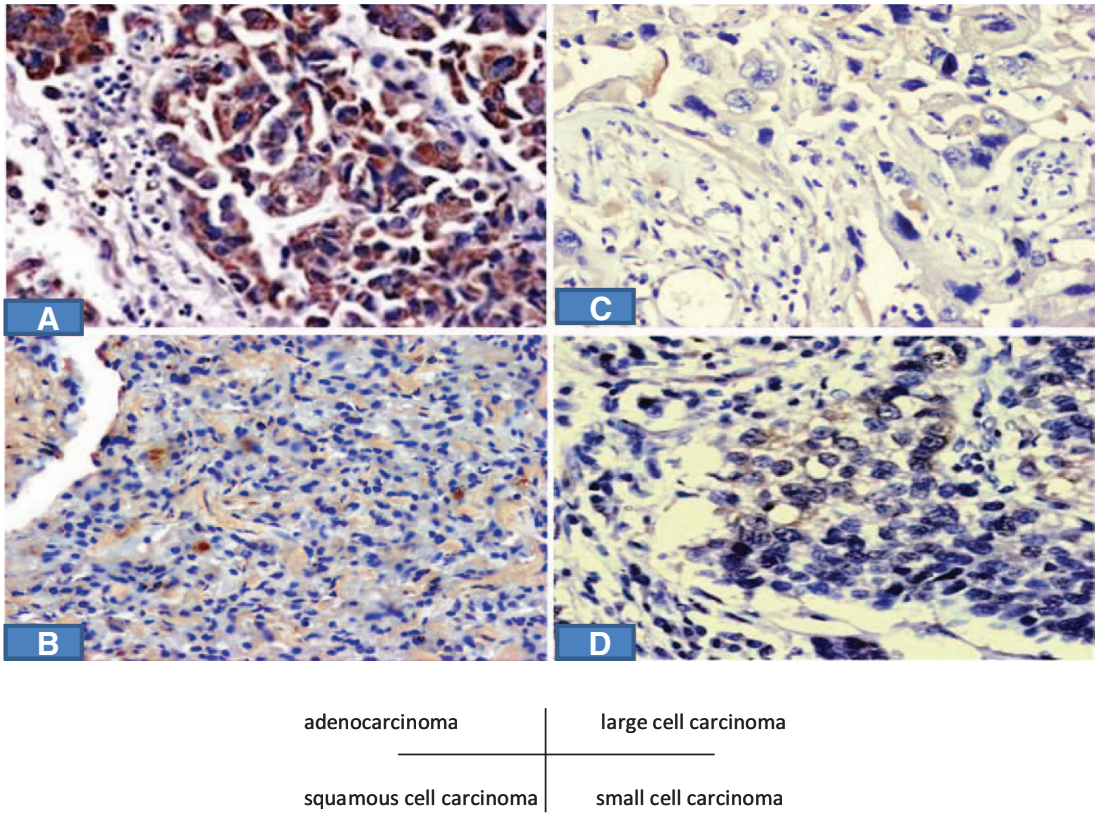

FIGURE 2. Representative photographs of tissue microarrays showing that non–small-cell lung cancer tissue expressed α1 nicotine acetylcholine receptors. Dark-brown staining was indicative of the presence of α1 protein, and tissue was counterstained to indicate nuclei with dark-blue staining. *A*, adenocarcinoma ×400; (*B*), squamous cell carcinoma ×400; (*C*), large-cell carcinoma ×400; and (*D*), small-cell carcinoma ×400.

Nicotine-Induced Resistance to EGFR-TKI

Nicotine-induced activation of pathways related to proliferation (PI3K/Akt, ERK1/2, and EGFR) suggested that exposure to nicotine might protect cancer cells against the cytotoxic effects of EGFR-TKI. Therefore, we investigated whether nicotine exposure caused resistance to EGFR-TKI in PC9 cells (Fig. 5*A*). EGFR-TKI had a dosage-dependent killing effect in PC9 cells as shown in Figure 5*A* (open bar). However, PC9 cells pretreated with nicotine were not killed as effectively by EGFR-TKI. When PC9 cells were pretreated with nicotine for 24 hours, larger numbers survived treatment with 5 or 10 μmol of EGFR-TKI as compared with unexposed PC9 cells. When PC9 cells were pretreated with nicotine for 1 month, the resistance against EGFR-TKI was clear at a concentration of EGFR-TKI as high as 20 μmol. Finally, restoration of sensitivity to EGFR-TKI was examined by α1 nAChR knockdown in nicotine-exposed PC9 cells. PC9 cells were incubated in the presence or absence of 1 μmol of nicotine for 1 month,[19] then transfected with siRNA α1 or NT siRNA for 72 hours. As shown in Figure 5*B*, siRNA against α1 nAChR decreased the resistance to EGFR-TKI induced by nicotine. In contrast, the resistance persisted in cells stimulated by nicotine and transfected with NT siRNA. These results suggested that the enhanced resistance to EGFR-TKI in PC9 cells stimulated by long-term nicotine exposure was, at least in part, dependent on α1 nAChR expression.

DISCUSSION

More than one billion people around the world smoke, and cigarette smoking and second-hand smoking account for nearly 90% of lung cancer deaths.[29] Studies in recent years raise the possibility that exposure to nicotine might lead to increased risk of lung cancer[30]; however, the detailed molecular mechanisms remain largely unknown. The fact that nicotine promotes lung cancer by activating different nAChR subunits, thus leading to the activation of several pathways,[10,12,13] gives reason to believe that the nAChR family may play important roles in lung tumorigenesis.

In the present study, expressions of α1 nAChR mRNA and protein were determined in NSCLC cell lines. The results also proved to be applicable to human NSCLC by analysis of lung cancer human tissues with IHC. Consistent with previous reports,[10,12,19] all these data suggested that α1 nAChR is likely to be involved in the smoking-related pathogenesis of NSCLC.

In accordance with the roles of nicotine in multiple signaling pathways of NSCLC,[10–13] we found that exposure to nicotine stimulated phosphorylation of ERK1/2 and Akt (Ser-473) in a time-dependent manner. The phosphorylation was evident within 5 minutes and reached a peak within 30 minutes, suggesting that the triggering of these pathways could play a role in the early development of nicotine-induced NSCLC (failure of detection of STAT3 phosphorylation may be because of the inappropriate cell line).[28] Because the nicotine-activated

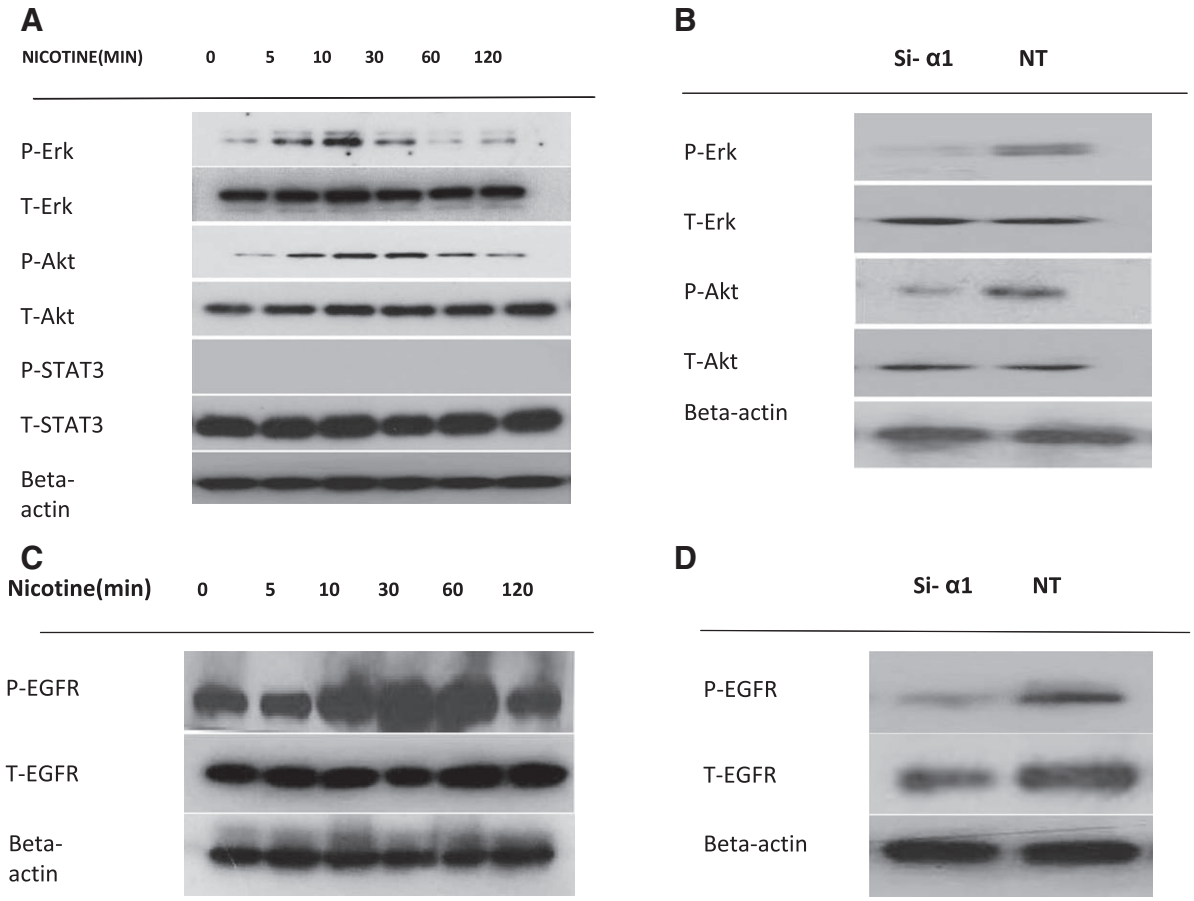

FIGURE 3. *A,* Induction of Erk, Akt (Ser-473) and STAT3 phosphorylation by nicotine. PC9 cells were serum-starved for 24 hours then incubated with 10 μM of nicotine for the number of times indicated (upper panel). Western blot analysis was used to reveal the time-dependent phosphorylation of ERK and Akt (Ser-473); (*B*) Induction of Erk and Akt (Ser-473) phosphorylation was dependent on α1 nAChR. PC9 cells were transfected with siRNA against α1 nAChR subunit (si-α1) or NT siRNA for 72 hours. Western blot analysis revealed the difference in phosphorylated levels of ERK1/2 (p-ERK1/2) and Akt (p-Akt) between cells treated with siRNA α1 and NT; (*C*) Induction of EFGR phosphorylation by nicotine. PC9 cells were serum-starved for 24 hours then incubated with 10 μM of nicotine for the number of times indicated (upper panel). Western blot analysis shows the EGFR and p-EGFR protein levels; (*D*) Role of α1 nAChR subunit in the EFGR signaling pathway. PC9 cells were transfected with siRNA against the α1 nAChR subunits (si-α1) or NT siRNA for 72 hours. Western blot analysis shows EGFR and p-EGFR protein levels. EFGR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; nAChR, nicotinic acetylcholine receptor; NT, nontargeting.

signaling pathways were also thought to be involved with EGFR,[26,31,32] cross-talk between EGFR and nAChR was proposed. In support of this hypothesis, we found in our present study that nicotine caused phosphorylation of EGFR in a time-dependent way. Therefore, we next attempted to identify the specific nAChR subunit responsible for these effects. Although

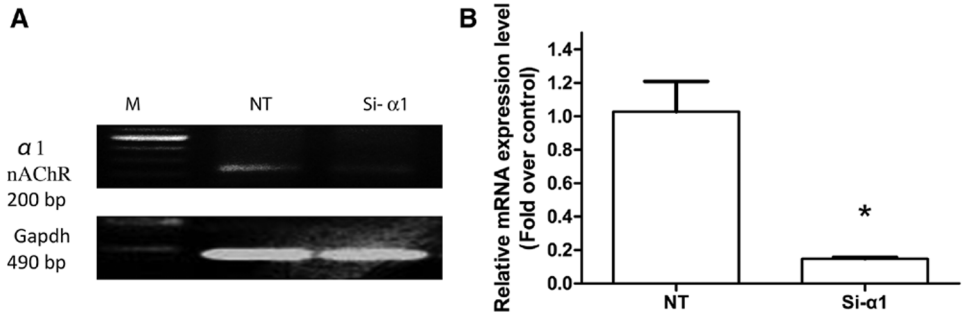

FIGURE 4. *A,* Gene silencing of α1 nAChR was validated by reverse transcription PCR in PC9 cells. α1 nAChR mRNA expression was knocked down by siRNA; (*B*) The relative levels of α1 nAChR mRNA measured by real-time-PCR in control NT and α1 nAChR knockdown (si-α1) PC9 cells. Data are presented as mean ± standard error mean of three independent experiments carried out in triplicate. *$p < 0.05$. nAChR, nicotinic acetylcholine receptor; NT, nontargeting; PCR, polymerase chain reaction.

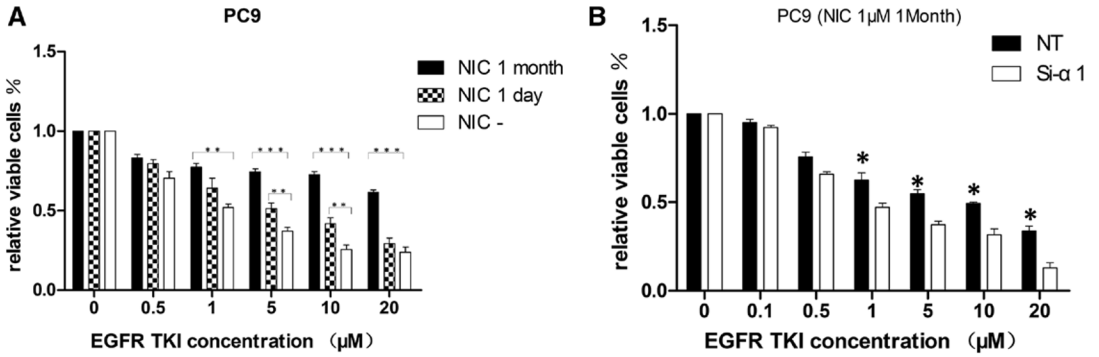

FIGURE 5. *A,* Nicotine protects NSCLC cells from cytotoxicity induced by EGFR-TKI. NSCLC PC9 cells were pretreated with 1 μM of nicotine for 1 month or 10 μM of nicotine for 1 day. Cells were then treated with EGFR-TKI for 48 hours. Cell survival was measured with the MTS assay and was expressed as a percentage of the untreated control. The concentrations of EGFR-TKI were increased in increments as indicated. Data are presented as mean ± standard error mean of three independent experiments carried out in triplicate. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. *B,* α1 nAChR subunit mediated EGFR-TKI resistance induced by long-term nicotine exposure. PC9 cells were pretreated with 1μM of nicotine for 1 month, then transfected with siRNA against α1 nAChR subunit (si-α1) or nontargeting siRNA NT for 72 hours. Cell survival was measured with the MTS assay and is expressed as a percentage of the untreated control. The concentrations of EGFR-TKI were increased in increments as indicated. Data are presented as mean ± standard error mean of three independent experiments carried out in triplicate. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. EFGR-TKI, epidermal growth factor receptor tyrosine kinase inhibitor; NSCLC, non–small-cell lung cancer; NT, nontargeting.

several functional subunits of nAChRs have been reported recently,[20,33] our IHC results suggest the possibility that nicotine could act through a pathway from α1 nAChR to EGFR as well.

Consistent with these findings, our blocking experiments (siRNA) and Western blot analysis proved that α1 subunit was responsible for EGFR release, and that siRNA down-regulated phosphorylation of EGFR by reducing phospho-ERK and phospho-Akt (Ser-473). Thus, we provide the following new information about nicotine-induced signaling pathways in PC9 NSCLC cells. First, nicotine was suggested as inducing EGFR-signaling pathways. Second, nicotine increased expression and phosphorylation of EGFR, which was coupled with increased ERK and AKT phosphorylation. These findings suggest that in PC9 cells, nicotine-stimulated cell proliferation and survival may be partially dependent on EGFR signals transmitted through AKT and ERK pathways. Third, the phosphorylation of EGFR, ERK, and Akt (Ser-473) were down-regulated by treatment with si–α1 nAChR, suggesting that α1 nAChR may act as an upstream regulator of the EGFR pathway in PC9 cells and may be responsible for mediating the proliferative and apoptotic effects caused by nicotine exposure. Here we identified a novel nicotine-stimulated survival signaling pathway mediated by α1 nAChR through EGFR phosphorylation.

It has been shown that never-smokers with lung cancer are more likely to respond to EGFR-TKI treatment than smokers.[34] Although one major mediator of responsiveness to EGFR-TKI is the mutation status of EGFR that is often shown in never-smokers,[35] we conjectured that nicotine–α1 nAChR signaling pathways may contribute to differences in responsiveness to EGFR-TKI therapy between never-smokers and active smokers or exsmokers who use nicotine replacement therapy. Our results from the MTS assay showed that both short-term (10 μm of nicotine for 1 day) and chronic nicotine stimulation (1 μm of nicotine for 1 month) did confer protection from EGFR-TKI-induced cytotoxicity in PC9 cells. Notably, under conditions of chronic stimulation by nicotine, the resistance to EGFR-TKI was significantly abrogated by silencing α1 nAChR (siRNA). These observations suggested that in addition to nicotine exposure or smoking status, continued or chronic exposure to nicotine (usually taken to be >10 days of continuous exposure to nicotine)[19] increased the resistance to EGFR-TKI compared with immediate or short-term exposure to nicotine; furthermore, the α1 nAChR subunit was responsible for mediating the resistance of EGFR-TKI induced by chronic nicotine exposure in PC9 cells. Therefore, elimination of α1 nAChR subunit or treatment with an α1 nAChR–specific inhibitor could provide highly tailored treatments for smoking NSCLC patients.

In summary, the results of our report, together with previously published reports suggest that the EGFR system shares significant cross-talk with the nAChR system in NSCLC cell lines. Our results indicate that the α1 nAChR subunit mediates resistance to EGFR-TKI therapy, induced by chronic nicotine exposure, through activation of the ERK and Akt (Ser-473) pathways. Thus, our findings might provide a mechanistic basis for the resistance to EGFR-TKI therapy, observed in patients who continue to smoke. A major problem of current NSCLC therapy is the fact that this cancer expresses many different signaling pathways. Inhibition of the activities of ERK, AKT, or EGFR pathways and other angiogenic regulators are therefore, currently targeted therapies that require treatment with multiple agents. Given the potential role of α1 muscle-type nAChR in several of the important pathways, targeted inhibition of α1 nAChR might become a novel treatment in NSCLC. In addition, the notion that α1 nAChR functions to promote lung carcinogenesis raises questions regarding the safety and appropriateness of nicotine-replacement therapies. Also, as smoking cessation is the most effective way to prevent nicotine-induced EGKR-TKI resistance, the study gives us a substantial reason to advocate smoking cessation for the cancer patients who smoke.

Finally, future exploration of nicotine–α1 nAChR-induced lung carcinogenesis should address the question of whether lung cancer risk is directly influenced by α1 nAChR or indirectly influenced by smoking behaviors; the effects of cigarette exposure on the pharmacokinetics involved in the EGFR-TKI resistance actions[36] should also be raised. Moreover, other nAChR subunit genes in chronic nicotine exposure warrant further evaluation.

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