Am J Transl Res 2015;7(10):2026-2035
www.ajtr.org /ISSN:1943-8141/AJTR0008342

Original Article

Cigarette smoking impairs the response of EGFR-TKIs therapy in lung adenocarcinoma patients by promoting EGFR signaling and epithelial-mesenchymal transition

Ming Liu[1], Chenzhi Zhou[1], Jian Zheng[2]

[1]State Key Laboratory of Respiratory Disease, Guangzhou Institute of Respiratory Disease, The First Affiliated Hospital of Guangzhou Medical University, Guangzhou 510120, China; [2]Department of Paediatrics & Adolescent Medicine, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong SAR 000000, China

Received March 23, 2015; Accepted August 17, 2015; Epub October 15, 2015; Published October 30, 2015

Abstract: Cigarette smoking represents for the highest risk-factor for non-small cell lung cancer (NSCLC), and a growing body of evidence suggested that smoking was associated with a high recurrence and poor therapeutic response of NSCLC as well. On the other hand, epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKIs), such as gefitinib, has been proved to be an efficient and safe strategy for treating NSCLC. Although accumulating clinical data suggested that smoking history might influence the therapeutic effects of EGFR-TKIs even in NSCLC patients harboring sensitive EGFR mutation, the exact effects of cigarette smoking on the efficacy of EGFR-TKIs treatment in NSCLC patients remain exclusive. In this study, we firstly identified the adverse effect of smoking exposure on the efficacy of EGFR-TKIs treatment against lung adenocarcinoma in mutation-positive patients by retrospective analysis of clinical data. The hypo-responsiveness of smoking patients on the therapy was accompanied with persistent activation of EGFR-downstream signal molecules ERK1/2 and AKT, which could not be inhibited by gefitinib and thus lead to the failure of EGFR-TKIs treatment. Based on our in vitro data, it was also found that long-term cigarette smoking extract (CSE) exposure induced epithelial-mesenchymal transition (EMT), which might also contribute to acquired resistance to EGFR-TKIs. Taken together, our findings suggested that cigarette smoking negatively regulated the clinical outcome of EGFR-TKIs therapy in lung adenocarcinoma patients, which was correlated with the activation of EGFR signaling and the induction of EMT.

Keywords: NSCLC, adnocarcinoma, cigarette smoking, EGFR-TKIs, EMT

Introduction

Lung cancer is the most common and leading cause of cancer deaths, resulting in over 1.4 million worldwide deaths per year. Smoking history represents for a high risk of the occurrence of lung cancer and about 80% of lung cancer cases in males and 50% in females arouse in ever-smokers [1]. Despite progress in loco regional and systemic therapies, improvement in the prognosis of advanced lung cancer remained a great challenge. Besides its role in increasing the risk of cancer occurrence, continuous smoking during the treatment of lung cancer could also lead to a higher incidence of recurrence, development of drug resistance and significantly increased mortality as shown by retrospective studies [2-4].

In recent years, epidermal growth factor receptor (EGFR)-tyrosine kinase inhibitors (TKIs) therapy emerged as a breakthrough for treating non-small cell lung cancer (NSCLC) and has been widely used in clinical practice. Currently EGFR-TKIs drugs (gefitinib and erlotinib) have been recommended to be the first-line treatment on advanced NSCLC patients harboring somatic EGFR mutations [5, 6]. In concert with that, the responsiveness of patients to EGFR-TKIs largely depends on the somatic mutations occurring in EGFR kinase domain of cancer cells [7]. Exon 19 in-frame deletions (delE746-751) and exon 21 substitution (L858R) have been shown to be the mostly observed types of EGFR mutations, and thus were used as biologic predictors during patients selection for EGFR-TKIs treatment [8, 9]. Unfortunately,

Table 1. Patients' characteristics

	Smokers (n=92)	Never-smokers (n=103)	*P* value
Pathological stage			0.173
III	34	48	
IV	58	55	
EGFR mutation			0.000
Positive	30	63	
Negative	62	40	
Sex			0.000
Male	84	23	
Female	8	80	
Age, years [median (Y)]	60.6±13.5	61.3±11.9	0.752
PFS (M)	5.8 (4.6-6.7)	6.6 (5.2-8.3)	0.315

EGFR mutations were mainly found in non-smokers and plenty of studies reported that the frequency of EGFR mutations was inversely associated with the amount of tobacco abducted in smoking population, which might represent for the main cause leading to the failure of EGFR-TKIs therapy in smoking population. More importantly, another study [10] demonstrated that the history of environmental tobacco smoke (ETS) stood as an important predictor of poor response to EGFR-TKIs as well. Therefore, it is critical to verify the impacts of smoking on the efficacy of EGFR-TKIs among lung adenocarcinoma patients carrying EGFR mutations and the corresponding molecular mechanisms.

In this study, we studied the correlations between ETS exposure and the outcome of lung adenocarcinoma patients by retrospect analysis and found that smoking history was conversely correlated with the therapeutic efficacy of EGFR-TKIs on NSCLC independent of somatic mutations in EGFR. We then investigated the mechanisms underlying smoking-related resistance against EGFR-TKIs treatment and found that cigarette smoking could activate ERK1/2 and AKT, downstream molecules of EGFR signal pathway, in lung adenocarcinoma. Importantly, ERK1/2 and AKT activation could not be inhibited by gefitinib and thus led to the failure of EGFR-TKIs treatment. Moreover, CSE (cigarette smoking extract) exposure could induce the epithelial-mesenchymal transition (EMT), which might also be critically associated with acquired resistance to EGFR-TKIs. Collectively, our study demonstrated that the influences of smoking on EGFR-TKIs treatment might be mediated by the activation of ERK1/2 and AKT and induction of EMT, which provided important information and potential targets for improving the outcome of lung adenocarcinoma therapy.

Materials and methods

Cell cultures and reagents

EGFR mutant human lung adenocarcinoma cell line PC9 (del E746_A750) from American Type Culture Collection (Manassas, VA, USA) were maintained in DMEM (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) under 5% CO_2 in 37°C. 10 mM stocks of gefitinib (AstraZeneca, Cheshire, UK) were dissolved in dimethyl sulfoxide (DMSO) and stored at -80°C. Cells at a confluence of 80% were treated with CSE with or without gefitinib, while the cell viability was assessed using the CCK8 kit (Dojindo, Japan).

Preparation of cigarette smoke extract

Cigarette smoke extract was prepared by a modified method of Carp [11]. In brief, two cigarettes (Hongmei, China) without filters were combusted with a modified syringe-driven apparatus. The smoke was bubbled through 50 ml of serum-free DMEM, and the solution was adjusted to pH 7.4 and then filtered through a 0.20-μm filter for use immediately or stored at -80°C.

Patients and data collection

A total of 195 patients who carried histologically-confirmed lung adenocarcinoma from 1120 NSCLC patients at The First Affiliated Hospital of Guangzhou Medical University, from January 2007 to November 2010 were enrolled in the present study (**Table 1**). All the patients received at least one month EGFR-TKIs treatment (gefitinib or erlotinib). Clinical data of patients involved were obtained from the inpatient and outpatient medical records. Smoking status was confirmed based on records at patients' first clinic visit while those who had smoked more than 1000 cigarettes were identified as smokers. Pathology stages of adenocarcinoma were determined using the current tumor-node-metastasis classification system (UICC, 1997).

Computed tomography (CT) scans were applied after 8 weeks of treatment while drug responses were analyzed following the Response Evaluation Criteria in Solid Tumors guidelines. Briefly, objective response is defined as patients with complete response or partial response, and progression-free survival (PFS) is defined as the time from the initial administration of second-line therapy to the earliest occurrence of disease progression or death from any cause. A written informed consent to receive genetic analyses was obtained from all patients before surgery, and the study was approved by the Ethics Committee of Guangzhou Medical University.

Mutant-enriched PCR assays for EGFR exons 19 and 21 mutation screening

EGFR exons 19 and 21 mutations were detected by mutant-enriched PCR assays [12]. Briefly, a restriction enzyme digestion (MseI for exon 19, and MscI for exon 21) was performed between the two round of amplification to enrich the mutant alleles, then the second round exon 19 PCR product and Sau96I digested second exon 21 product were analyzed on polyacrylamide gel electrophoresis (PAGE) with silver staining.

Immunoblotting

PC9 cells were lysed in RIPA buffer [50 mM Tris (pH 7.5), 150 mM NaCl, 1% NP-40, 0.1% SDS, 1 mM EDTA, 1 mM NaN_3, 1 mM PMSF, 2 μg/ml aprotinin, 2 μg/ml leupeptin] and then quantified using a BCA assay kit (Pierce, USA). The same amount of protein from each sample (30 μg) was separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. After blocking for 1 h in blocking buffer [1×Tris buffered saline (TBS), 5% milk and 0.2% Tween-20], Membrane was blotted for primary antibody of total or phosphorylated EGFR, STAT3, AKT, ERK1/2 and GAPDH (Cell Signaling, Cambridge, MA, 1:1000) overnight at 4°C followed by washing three times. Then the membrane was incubated with secondary antibody (Cell Signaling, Cambridge, MA) for one additional hour and visualized with enhanced chemiluminescent detection system ECL (Pierce, Rockford, USA). The digital scanning was performed in an Image Station 2000 (Kodak, US). All Immunoblot experiments were performed at least three times.

Immunohistochemistry

Immunohistochemical analysis was performed following the standard avidin-biotin immunoperoxidase staining procedure. Briefly, slides were de-paraffinized and then submitted to antigen retrieval by steamer treatment for 15 minutes in 10 mM citrate buffer at pH 6.0, followed by primary antibody incubation overnight at 4°C. Then slides were incubated with horseradish peroxidase (HRP)-Polymer anti-mouse/rabbit immunoglobulins (Max-Vision; China) for 30 min and visualized by diaminobenzidine (DAB) and counterstained with hematoxylin. Primary antibodies used here included anti-p-EGFR (Ab-6, mouse clone 6H2.1; Neomarkers, Thermo Scientific; 1:50), anti-p-AKT (Ser473, rabbit polyclonal; Cell Signaling Technology; 1:50), anti-p-STAT3 (Ser 2448, rabbit clone 49F9; Cell Signaling Technology; 1:50), and anti-p44/42 MAPK (rabbit clone 137F5; Cell Signaling Technology; 1:100). Histopathologic score was analyzed by two independent observers who were blind to the treatment. P-AKT and p-ERK staining were considered as positive if >10% of tumor cells showed positive (2+) cytoplasmic and/or nuclear staining.

Immunofluorescence

PC9 cells grown on chamber slides (BD Bioscience, San Diego, CA) were fixed with 10% buffered-formalin for 15 min, and double immunofluorescence was performed as the protocol published previously. Primary antibodies used here included monoclonal anti-E-cadherin (24E10, Cell Signaling, Cambridge, MA, 1:100) and anti-vimentin (Cell Signaling, Cambridge, MA, 1:100). Secondary antibodies were Alexa Fluor-546 goat anti-rabbit and Alexa Fluor-488 anti-mouse Ab (Molecular Probes, Carlsbad, CA) respectively. No cross-reactivity between anti-mouse and anti-rabbit antibodies were identified. DAPI (Vector labs, Burlingame, CA) was used to stain the nuclei. Cells were visualized with a Nikon TE2000 confocal microscope (Nikon Microsystems, Japan).

Statistical analysis

Statistical analysis was carried out using SPSS 19.0 (SPSS, Inc., Chicago, IL). Fisher's exact test, χ^2 and t tests were used to compare the differences in data obtained from distinct groups where appropriate. PFS was constructed by the Kaplan-Meier method and was ana-

Table 2. Effect of smoking history on the response of EGFR-TKIs therapy in lung adenocarcinoma patients with different *EGFR* status

	EGFR mutation(-)			EGFR mutation(+)		
	Smoking (N=72)	No-smoking (N=30)	*P* Value	Smoking (N=20)	No-smoking (N=73)	*P* Value
OR (%)	16.7	26.7	0.246	40.0	65.8	0.037
PFS (M)	3.247 (2.734-3.759)	3.514 (2.981-4.046)	0.358	5.889 (4.168-7.160)	9.039 (7.642-10.542)	0.031

Table 3. Effect of smoking history on the response of EGFR-TKIs therapy in male lung adenocarcinoma patients with different *EGFR* status

	EGFR mutation(-)			EGFR mutation(+)		
	Smoking (N=70)	No-smoking (N=8)	*P* Value	Smoking (N=14)	No-smoking (N=15)	*P* Value
OR (%)	22.9	25	0.892	28.6	73.3	0.027
PFS (M)	3.148 (2.624-3.671)	3.578 (2.834-4.322)	0.316	5.373 (3.539-7.207)	8.759 (6.618-10.901)	0.033

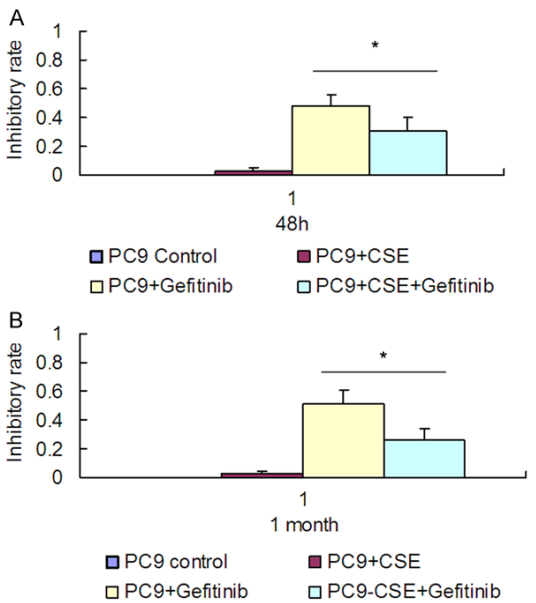

Figure 1. Cigarette smoke exposure abolished the efficiency of EGFR-TKI in vitro. Serum-starved PC9 cells were incubated with CSE or gefitinib (0.01 μM) alone, or in combination of both of them for 48 hours (A) or 1 month (B), CCK8 assay was used to assess the cell viability. *P<0.05.

lyzed by log-rank test. A two-tailed *p* value of <0.05 was considered statistically significant.

Results

Profiles of patients

The profiles of patients were summarized in **Table 1**. A total of 195 eligible lung adenocarcinoma patients were enrolled. 92 patients (47.2%) were smokers while 103 patients (52.8%) were non-smokers. There were no differences in the baseline characteristics between the groups with and without CS exposure except the gender and EGFR genotype. All the patients are at advanced stage of adenocarcinoma, while the frequency of EGFR mutations were higher in non-smokers compared to smokers (61.2% v.s. 32.6%, P=0.000).

Smoking ruined the therapeutic effects of EGFR-TKIs in NSCLC patients with EGFR mutations

Firstly, we examined the association between smoking history and overall response rate to EGFR-TKIs. As presented in **Table 2**, the overall response (OR) in total 195 patients treated with gefitinib was 39.0% (76/195). Logistic multivariate analysis showed that only EGFR mutation was significantly associated with the objective response rate of gefitinib therapy, while EGFR gene mutation and smoking history represented for the major risk factors evidenced by progression-free survival revealed by Cox regression model multivariate analysis (P<0.05). More importantly, although no difference was found in OR between the smokers and non-smokers who have no EGFR mutation, the EGFR mutation-positive non-smokers had a significantly higher OR (65.8%) compared with the smokers (40.0%) (P<0.05). Meanwhile, hierarchical analysis showed that the PFS of smokers was significantly shorter than that of non-smokers in patients with EGFR-mutated lung adenocarcinoma (P<0.05). Finally, we performed OR and PFS analysis in male population to clarify the influence of smoking states on survival devoid of the gender-bias. It was found that EGFR-TKIs treated smoking patients had a significantly low OR (28.6% v.s. 73.3%; p=0.027)

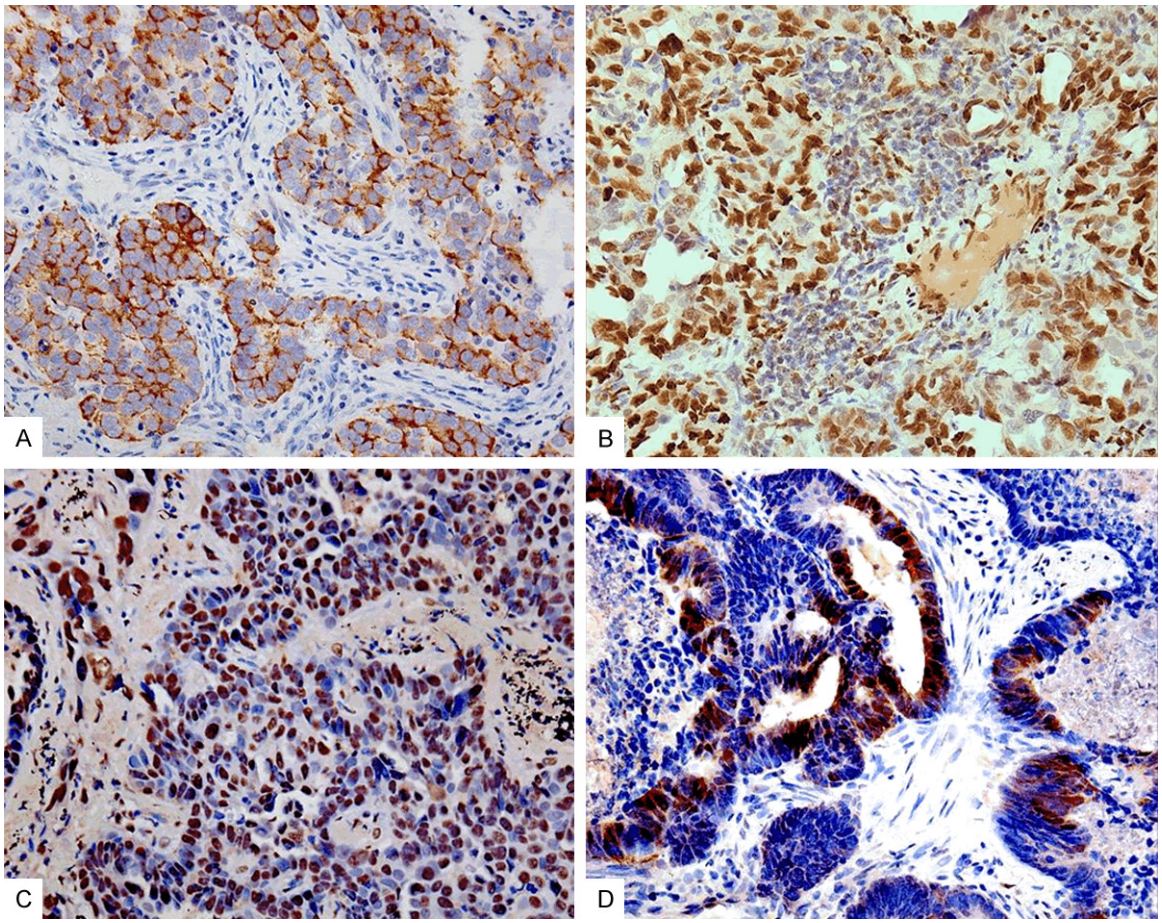

Figure 2. Characteristic expression of p-EGFR, p-AKT, p-SATA3, and p-ERK in lung adenocarcinoma tissue sample. The expressions of p-EGFR (A), p-AKT (B), p-SATA3 (C), and p-ERK (D) in 68 primary lung adenocarcinomas were determined by immunohistochemical assay. All pictures were taken at a magnification of 200.

and a shorter PFS (5.373 v.s. 8.759 months; p=0.033) compared to non-smoking patients (**Table 3**). Taken together, these data indicated that smoking ruined the therapeutic effects of EGFR-TKIs in NSCLC patients carrying EGFR mutation.

Smoking exposure abolished EGFR-TKIs effects in EGFR mutant cell lines

To investigate the influence of cigarette smoking on the sensitivity of cells to gefitinib in vitro, PC9 cells were cultured with CSE for 48 hours or 1 month, and then were treated with gefitinib. As shown in **Figure 1**, with the addition of CSE, the inhibitory rate of gefitinib (0.01 μM) on PC9 growth reduced from 43±8% to 35.1±10% (48 h) or 26%±8% (1 month) respectively as determined by CCK8 assay. These data indicated that both short and long cigarette smoke exposures might abolish the EGFR-TKIs sensitivity in EGFR mutant cell line.

Smoking changed the expression of EGFR-related signal molecules

Of the 195 lung adenocarcinoma patients, 68 patients had evaluable tissue specimens. Using immunohistochemical assay, we firstly analyzed the expression of some EGFR related downstream signaling molecules (p-EGFR, p-AKT, p-ERK, and p-STAT3) (**Figure 2**). It was found that positive immunostaining of p-EGFR were detected in 35 out of 68 specimens of lung adenocarcinoma (51.5%), p-STAT3 in 32 of 68 (47.1%), p-AKT in 30 of 68 (44.1%), and p-ERK in 28 of 68 (41.2%). Though there was no significant correlation of the expression of p-EGFR, p-AKT, p-ERK, and p-STAT3 between smoke status, further analysis revealed that the expressions of p-ERK and p-AKT protein were higher in smokers compared to non-smokers in mutant-positive patients (72.7% v.s. 26.3%, P=0.023; 63.6% v.s. 21.1%, P=0.047,

Table 4. Expression of p-EGFR, p-SATA3, p-AKT and p-ERK in lung adenocarcinoma with different *EGFR* mutation and smoking status

	EGFR mutation(-) n=38			EGFR mutation(+) n=30		
	Smoking	Non-smoking	*P* Value	Smoking	Non-smoking	*P* Value
p-EGFR	(8/21)	(10/17)	0.513	(9/11)	(8/19)	0.057
p-STAT3	(9/21)	(8/17)	1.000	(5/11)	(10/19)	1.000
p-AKT	(8/21)	(9/17)	0.513	(8/11)	(5/19)	0.023
p-ERK	(9/21)	(8/17)	1.000	(7/11)	(4/19)	0.047

Table 4). In vitro, CSE could induce the phosphorylation of ERK1/2 and AKT in a time-dependent manner in PC9 cell lines (**Figure 3A**), which could be inhibited by gefitinib. However, the persist phosphorylation of ERK1/2 and AKT signaling induced by CSE treatment which could not be inhibited by gefitinib (**Figure 3B**, **3C**).

Smoking exposure induced epithelial-mesenchymal transition

Though persist activating the EGFR downstream signaling could partially explain the effect of CSE on the EGFR-TKIs sensitivity, especially in the short-term exposure, no significant difference of p-ERK1/2 and p-AKT expression level in PC9 could be observed between the long-term CSE exposure group and control group (**Figure 4A**). This phenomenon implied that there might be some other mechanisms involved in the formation of resistance. As EMT has recently proved to be one of the mechanisms of acquired resistance to EGFR-TKIs, we then examined whether long term treatment with CSE could induce EMT in EGFR mutant PC9 cells. As shown in **Figure 4B**, the treatment with CSE for 1 month caused an increased expression of mesenchymal proteins vimentin accompanied with downregulated epithelial marker E-cadherin in PC9, which were also confirmed in immunofluorescence experiments (**Figure 4C**). Taken together, these data suggested that the negative effects of cigarette smoking on the outcome of EGFR-TKIs therapy in lung adenocarcinoma might be mediated by both the activation of EGFR signaling and the induction of EMT.

Discussion

As well known, lung cancers harboring EGFR mutations led to a high sensitivity to EGFR-TKIs. During the past ten years, targeting the EGFR pathway has become the mainstream of treatment for advanced NSCLC. Though the first-generation reversible EGFR-TKIs (gefitinib and erlotinib) have been recommended as the first-line drugs in treating EGFR mutant-carried patients, their therapeutic efficiency may be influenced by many factors, especially smoking history. Currently, direct evidence on the relation between smoking history and EGFR-TKI treatment response is still in lack, and the evaluation on smoking history as an independent factor for predicting the response to EGFR-TKIs treatments is in urgent need. Here, by conducting a retrospective study, we found that smoking history could represent for a predictor of the responsiveness to EGFR-TKIs therapy. Meanwhile, our in vitro experiments confirmed the impairment of EGFR-TKIs efficacy on NSCLC caused by smoking exposure due to the persistent activation of ERK1/2 and AKT induced by CSE, which could not be inhibited by gefitinib and thus led to the failure of EGFR-TKI treatment. Moreover, long-term CSE exposure contributed to the acquisition of EMT phenotype, which might be critically associated with acquired resistance to EGFR-TKIs as well.

Previously, a meta-analysis conducted by Mitchell et al. suggested that the impact of cigarette smoking history and dosage on the treatment outcomes of EGFR-TKIs might be influenced by multiple factors [13]. On the other hand, Kim et al's work showed that cigarette smoking dosage of ≥30 pack-years could be an independent negative predictive impact on EGFR-TKIs treatment outcome in lung adenocarcinoma patients with activating EGFR mutation [14]. Here, our retrospective study also found that smoking history might ruin the therapeutic effects of EGFR-TKIs in lung adenocarcinoma patients harboring sensitive EGFR mutations independent of the gender of patients, although there was no different of OR and PFS between the smokers and non-smokers who have no EGFR mutations. Through in vitro assays, we further confirmed that both short-term and long-term CSE treatment could induce EGFR-TKIs-resistance in the TKI-sensitive EGFR mutants. This result was consistent with Filosto and Wang's findings, though

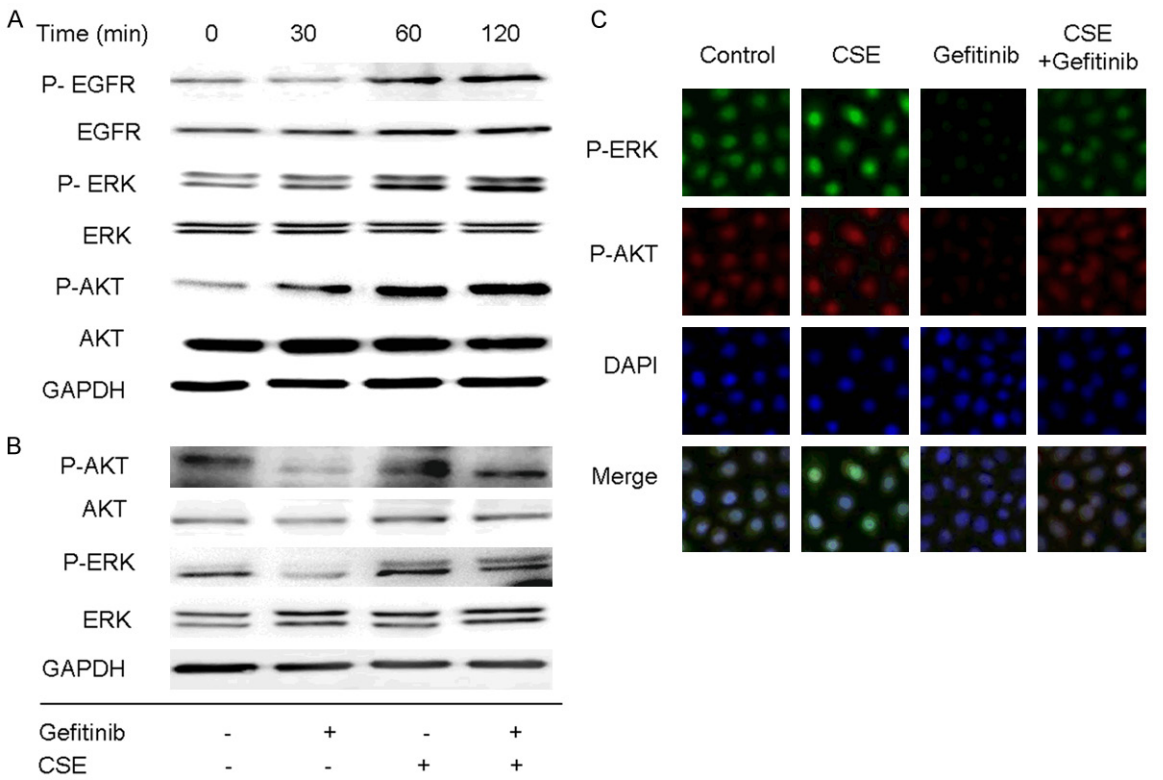

Figure 3. The activation of EGFR downstream signals caused by cigarette smoke could not be inhibited by gefitinib. Serum starved PC9 cells were incubated with CSE for 30 min, 1 hour and 2 hours respectively, while p-EGFR, EGFR, ERK1/2, p-ERK1/2, p-AKT, AKT and GAPDH expressions were examined by western blot (A). (B) ERK1/2, p-ERK1/2, p-AKT, AKT were further detected in PC9 cells treated with CSE or gefitinib (0.02 μM) alone, or in combination of both of them for 48 hours. (C) Representative immunofluorescence photos were shown. n=3.

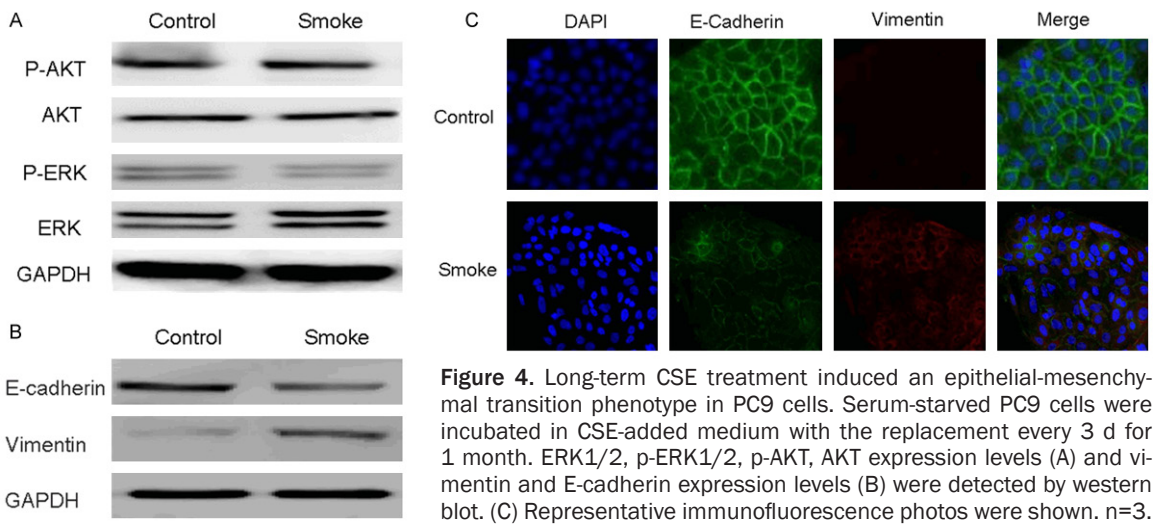

Figure 4. Long-term CSE treatment induced an epithelial-mesenchymal transition phenotype in PC9 cells. Serum-starved PC9 cells were incubated in CSE-added medium with the replacement every 3 d for 1 month. ERK1/2, p-ERK1/2, p-AKT, AKT expression levels (A) and vimentin and E-cadherin expression levels (B) were detected by western blot. (C) Representative immunofluorescence photos were shown. n=3.

they used the different cell lines (NCI-H3255 with L858R mutation) or treated cells directly with nicotine, the major component in cigarette smoke [15, 16]. These results may explain the unexpected poor outcome of some patients with EGFR mutations, and could be a valuable index in predicting the treatment outcomes in individual patients.

Mutant EGFR activates the Ras/ERK, PI3K/Akt and STAT pathways that are crucial to the growth, survival, and migration of NSCLC cells

[17]. Previous studies had demonstrate that nicotine or CS exposure resulted in prolonged EGFR signaling and might contribute to uncontrolled lung cell growth. Among these molecules, ERK1/2 and AKT were regarded as the major components involved in nicotine-induced cell proliferation and tumorigenesis [18, 19]. The ERK1/2 and AKT pathway is a critical pathway in the lifecycle of cancer and contributes to tumorigenesis, tumor growth and therapeutic resistance. The activation of ERK and AKT accompanied with the promotion of lung cancer cell proliferation and survival induced by tobacco components thus underlie the poor clinical outcomes for patients with tobacco-related cancers who continued to smoke. Consistently, here we also demonstrated that the CSE-induced persistent phosphorylation of ERK and AKT could not be inhibited by gefitinib, although EGFR and short-term CSE treatment could be transduced by similar downstream signaling pathway.

Several groups have noted that cell lines undergoing EMT were intrinsically resistant to EGFR inhibitors, and the mesenchymal phenotype was more resistant to EGF-TKIs than the epithelial phenotype [20, 21]. Further studies found the EMT was associated with not only primary sensitivity but also acquired resistance to EGFR-TKIs in vitro [22-24]. Here, our in vitro study directly showed that long-term CSE treatment could promote the EMT phenotypic changes and the development of resistance to gefitinib in PC9 cells. Consistent with these results, both clinical and epidemiological studies have suggested that long-term exposure to nicotine could alter the phenotype of epithelial and endothelial cells, while nicotine might directly induce the changes consistent with EMT [25, 26]. Actually, chronic treatment with nicotine was found to result in the down-regulated expression levels of E-cadherin and β-catenin with the concomitant increase of fibronectin and vimentin. Meanwhile, tobacco-specific carcinogen 4-(n-methyl-n-nitrosamino)-1-(3-pyridyl)-1-butanone (NNK) was found to promote EMT in human bronchial epithelial cells and lung alveolar epithelial type II cells as well [27, 28]. Similarly, some recent investigations suggested that smoking could cause EMT through an Src-mediated oxidative pathway [29]. On the other hand, two other reports suggested that smoking induced EMT in NSCLC was mediated through HDAC-mediated down-regulation of E-cadherin [30], while N-cadherin expression was associated with the acquisition of EMT phenotype and enhanced invasion in Erlotinib-resistant lung cancer cell lines [31]. Nevertheless, mechanisms underlying CS-induced EMT and its effects on EGFR-TKIs sensitivity still need further study.

In conclusion, cigarette smoke could impair the responsiveness on EGFR-TKIs therapy in lung adenocarcinoma, which was mediated by the activation of EGFR signaling and the induction of EMT. These findings provided some novel insights into the development of more effective strategies for treating EGFR-TKIs insensitive patients.

Acknowledgements

This work was supported by National Natural Science Foundation of China (Grant No. 81200050), Natural Science Foundation of Guangdong (Grant No. 2014A030313505), Science and Technology Program of Guangzhou (Grant No. 201510010249), Science Program of Higher Education of Guangzhou (Grant No. 2012C157) and Project for Young Scholars of Stat Key Laboratory of Respiratory Disease (2011A8).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Jian Zheng, Department of Paediatrics & Adolescent Medicine, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong SAR 000000, China. Tel: 852-39179355; Fax: 852-28198142; E-mail: teddy629@hku.hk

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