Original Article
Incense burning smoke sensitizes lung cancer cells to EGFR TKI by inducing AREG expression

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Abstract: Incense burning is common in Asian countries due to the religious beliefs. Environmental exposure to incense burning smoke is a potential risk factor for tumor development and progression of non-small cell lung cancer (NSCLC). Eastern Asia ethnic origin is strongly associated the clinical benefits of epidermal growth factor receptor (EGFR) tyrosine kinase inhibitors (TKIs) in NSCLC patients. However, the impact of the oriental custom of incense burning on the cancer progression and the EGFR TKI-sensitivity of NSCLC remains unclear. Our results showed that long-term exposure to incense burning extract (IBE) increases the cellular proliferation with S phase accumulation and the motility activity of NSCLCs. Interestingly, IBE enhances EGFR signaling activity without affecting its genetic status, and increases the cellular sensitivity of NSCLC cell lines to EGFR TKIs. Auramine, a yellow dye for making incense sticks, was identified as a residual composition in the burning incense smoke, and showed similar EGFR TKI-sensitizing effects. Furthermore, IBE or auramine transcriptionally induce EGFR ligand amphiregulin (AREG) expression for the enhancement of EGFR activity. Neutralization of AREG reduced the viability of IBE-treated cells. These results indicated that exposure to incense smoke may enhance NSCLC progression and their sensitivity to EGFR TKIs through increasing their oncogenic addiction to AREG-induced EGFR signaling.

Keywords: Non-small cell lung cancer, EGFR, AREG, incent burning smoke

Introduction
Lung cancer is the most common cancer type worldwide both in terms of numbers of cases and deaths, and has high case fatality (ratio of mortality to incidence, 0.87) [1, 2]. Most of lung cancers are carcinomas originated from epithelial cells. Lung cancer is broadly divided into small-cell lung cancer (SCLC, comprising 15-20% of lung cancers), and non-small-cell lung cancer (NSCLC, comprising 80-85% of lung cancers) [3]. Non-small-cell lung cancer is the major type of epithelial lung cancer. The most common sub-types of NSCLC are adenocarcinoma, squamous cell carcinoma, and large cell carcinoma. NSCLC can be treated with surgery, radiotherapy, chemotherapy, or a combination, depending on their stage. Some patients with advanced lung cancer may be responsive to tyrosine kinase inhibitors (TKIs) of epidermal growth factor receptor (EGFR).

EGFR is a member of cell-surface ErbB receptor family, which includes EGFR (ErbB-1), HER2/c-
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neu (ErbB-2), HER3 (ErbB-3), and HER4 (ErbB-4). EGFR is activated by binding with its specific ligands, such as epidermal growth factor (EGF), transforming growth factor α (TGF-α), Heparin-binding EGF-like growth factor (HB-EGF), and Amphiregulin (AREG) [4]. Upon activation, EGFR goes through a transition from an inactive form to an active homo-dimer. In addition to forming homo-dimers after ligand binding, EGFR may also create an activated heterodimer by pairing with another member of ErbB receptor family and thisimerization stimulates its intracellular protein tyrosine kinase activity. As a result, autophosphorylations in the C-terminal domain of EGFR occur at several tyrosine (Y) residues, including Y992, Y1045, Y1068, Y1148 and Y1173. These autophosphorylations lead to the activation of downstream signaling by recruiting several proteins which associate with these phosphorylated tyrosines through their SH2 or PTB domains. These downstream signaling proteins initiate several signal transduction cascades, mainly the MAPK (RAS-RAF-MEK-ERK), PI3K (PI3K-AKT-mTOR), and JNK pathways, leading to DNA synthesis and cell proliferation. Such signaling pathways also mediate other malignant phenotypes such as cell migration and adhesion [4]. The identification of EGFR as an oncogene leads to the development of anti-cancer therapeutics directly against EGFR, including gefitinib, erlotinib, afatinib, and icotinib for lung cancer targeted therapies [5]. Clinical trials have indicated the significant variability in response to EGFR TKIs, and found higher response rate in Japanese patients than in European derived population (27.5% vs. 10.4%) [6]. The better clinical responses to EGFR TKIs have been observed most frequently in women, in non-smokers, and in NSCLC patients with adenocarcinomas [7-9]. Consequently, lung cancer patients with these specific characteristics are associated with EGFR mutations, and showed better clinical outcome through therapies with these drugs [10, 11].

EGFR mutations mostly are identified in these NSCLC patients, and lead to EGFR over activation and association with the poor prognosis of lung cancer patients [12]. Most of the somatic activating EGFR mutations were found in the adenosine triphosphate (ATP)-binding pocket of the receptor tyrosine kinase domain. Sequencing of the EGFR gene revealed that a majority of tumors responding to EGFR TKIs harbored mutations in the TK domain [13, 14]. Overall, the frequency of EGFR mutations is 5-40% [15]. For patients whose tumors bearing EGFR mutations, the response rate to erlotinib and gefitinib is approximately 75%, suggesting that these mutations play a critical role in driving malignant transformation [15, 16]. Approximately 45% of NSCLC patients in the East Asia and 10% of NSCLC patients in US bear EGFR mutations [13, 17, 18]. These somatic mutations, particularly exon 18 G719X point mutation, exon 19 deletion, and exon 21 L858R point mutation, occur within the kinase domain of EGFR [13], and increase the kinase activity and subsequent hyper-activation of its downstream survival signaling pathways [19]. These activating EGFR mutations are commonly observed in patients with the characters of adenocarcinomas, female, non-smoker and Asian offspring, and are positively associated with the responsiveness to EGFR TKIs [20, 21]. By contrast, the exon 20 T790M mutation of EGFR is associated with acquired resistance to TKI targeted therapy [22]. Therefore, EGFR mutations are the criteria to decide the use of EGFR TKIs for lung cancer patients, and have been used as the predictive biomarker for the therapeutic efficacy. However, it remains unknown why Asian patients express activating EGFR mutants at high incidence.

There are many religions practiced in Asia, and the most common types are Hinduism (centered around India), Buddhism (centered around China), Islam (mainly in the Middle East), and Christianity (in Russia). Especially, Buddhism, Taoism, and Shinto are widely distributed religions in Asian region, and incense burning is the common way by which people delivered their faith. A typical composition of stick incense consists of herbal and wood powder, fragrance material, staining matter, adhesive powder, and bamboo stick [23]. Upon combustion of incense stick, smoke from incense burning contains particulate matter (PM), gas products and volatile organic compounds (VOCs). Particulate products from incense burning (45 mg/g) on averagely are greater than that from cigarette burning (10 mg/g) [23]. The gas products from incense burning include CO, CO₂, NOx, SO₂ etc.; smoke from incense stick combustion in religious and ritual places also produces a large number of health-damaging and
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carcinogenic air pollutant such as benzene, toluene, xylene, formaldehyde, 1,3 butadiene, styrene, as well as aldehyde and polycyclic aromatic hydrocarbons (PAHs) [23-27].

Because of the region factor, religion in Asian and incense burning are inseparable. In addition, some studies showed that incense smoke induces blood lead level and cardiovascular disease mortality [28, 29]. Long-term use of incense is also associated with the increasing risk of PAHs toxicity, including DNA adducts formation and development of squamous cell carcinoma [30-32]. Some studies even demonstrated that cigarette smoking and frequency of exposure to incense facilitates the development of lung cancer [33]. However, it remains unclear whether exposure to incense burning smoke impacts the lung cancer incidence and tumor progression. Also, Japanese research also reported that the response rate of Asian lung cancer patients bearing EGFR mutant to EGFR inhibitor-gefitinib was approximately 75-90% [34-37]. Although EGFR mutation has been implicated in the responsiveness of Asian NSCLC patients to anti-cancer EGFR targeted therapies, it is still not clear whether incense-burning smoke is related to sensitivity of these patients to EGFR targeted therapies.

Materials and methods

Preparation of incense burning extract (IBE) medium

One incense stick was used to prepare 10 mL of IBE medium. Incense-burning smoke was dissolved into RPMI-HEPES medium by suction with pump. The pH value of IBE medium was adjusted to 7.4. The IBE medium was filtrated by 0.22 μm filter to remove large particles, and was defined as 100% IBE stock medium storage at -30°C before use.

Cell culture

Human H292 lung cancer cell line, derived from a lymph node metastasis of a pulmonary mucoepidermoid carcinoma, was cultured in RPMI (Roswell Park Memorial Institute) 1640 (HyClone) supplemented with 10 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 10 mM sodium pyruvate, 10% heat-inactivated FBS (fetal bovine serum) (GeneDriex), 100 units/ml penicillin, and 100 μg/ml streptomycin (Thermo). HCC827 lung adenocarcinoma cell line expressing EGFR mutant (E746-A750 deletion) was cultured in RPMI 1640 (HyClone) supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin, and 100 μg/ml streptomycin (Thermo). Both of them were gifts from Prof. Mien-Chie Hung (M.D. Anderson Cancer Center, Tx). Lung cancer cell lines were treated with IBE medium (H292 IBE 1%, H292 IBE 5%) or with 1 μM auramine for over one month to establish the stable clones. All cells were incubated at 37°C in a humidified incubator containing 5% CO2.

Cell counting

After wash with PBS, cells were detached by incubation with trypsin-EDTA, and then collected and centrifuged at 1,000 rpm for 5 minutes. The cells were re-suspended in fresh medium and loaded onto hemocytometer. The cell numbers were counted under an Inversion Microscope.

MTT and WST-1 assay

Cells were trypsinized to seed at density of 4 × 10^3-8 × 10^3 cells/well in 96-well plate followed by treatment with EGFR TKIs for indicated time. After TKI treatment, the culture medium was removed and cells were washed and incubated with 100 μL of serum-free medium with 5 mg/mL MTT solution (Sigma) or WST-1 for 3 hours. Then, 100 μL of DMSO was added to lyse the cells and the absorption was detected by using ELISA reader at the wavelength of O.D. 550.

Western blot

The concentration of total protein lysate was measured by Bradford protein assay (Bio-Rad protein assay). Protein lysates (10-40 μg) were heated in 1X sample buffer at 95°C for 5 minutes, and loaded into SDS-PAGE under 300 V, 30 mA/each gel until protein sample separated to the bottom. The used 1X running buffer was composed of 3.03 g Tris-base, 14.4 g Glycine, and 0.1% SDS. Then, the separated protein was transferred to a PVDF membrane (0.45 μM, millipore) or a NC membrane (0.22 μM, GE Healthcare) in transfer buffer under 150 V, 300 mA for 3 hour. The transfer buffer composed of 700 mL of ddH2O, 200 mL of Methanol, 100 mL of 10X running buffer, and 3.75 mL of SDS. After these steps, the transferred membrane
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was blocked in 5% milk in Tris-buffered saline-Tween (10 mL of 2 µM Tris-HCl pH 7.4, 100 mL of 5 M NaCl, 0.5 mL of 100% Tween-20, and 890 mL of ddH₂O) for one hour at room temperature. The membranes were hybridized with primary antibodies at 4°C overnight, and then incubated with HRP-labeled secondary antibodies at room temperature for 1 hour. The chemiluminescence signal was catalyzed by ECL (GE Healthcare or millipore) to detect the level of protein expression.

Clonogenic formation assay

Cells were seeded less than 15000 cells/well and then treated with gefitinib or erlotinib for 7 days. 1% Crystal violet (buffered with 30% ethanol) staining was then performed.

Migration and invasion assay

The migration and invasion assays were performed using trans-wells plate (0.8 µm costar) without or with matrix gel. Approximately 2 × 10⁵ cells in 200 ul of serum free medium were placed in the upper-chamber, 300 ul of different condition medium were placed in the lower-chamber. The plate was incubated for 24 hours at 37°C in 5% CO₂, and then cells were fixed in 4% formaldehyde for 15 minutes and stained 0.05% crystal violet in PBS for 30 minutes. Cells on the upper slide of the filters were removed with cotton-tripped swabs, and the filters were washed with PBS. Cells on the down slide of the filters were examined and counted under a microscope.

Flow cytometry

H292 cells were treated with mitomycin C to arrest cell cycle for 3 hours. After treatment, cells were washed with PBS for two times and trypsinized. Next, cells were collected and centrifuged at 1,000 rpm for 5 minutes, and then were fixed with 75% Ethanol at room temperature followed by staining with PI buffer (1% Triton X-100, 0.1 mg/mL PureLink™ RNase A (Cat no. 12091-021), and 20 µg/mL Propidium iodide in 1X PBS) for 30 minutes. The cell cycle population was analyzed by using Flow Cytometry.

RNA extraction and reverse transcription (RT)

After treatments, cells were washed with PBS for two times and total RNA was isolated with

Figure 1. IBE treatment induced cell proliferation in NCI-H292 cells. A. H292 parental and IBE stable clone were seeded in 6-well for 7 days followed by 1% crystal violet staining. The result was quantitated according to the absorbance at 570 nm followed by dissolving the crystal violet staining with acetic acid. B. H292 parental and IBE stable clone were seeded in 12-well for 3 days, and then cell viabilities were determined by automated cell counter. C. The cell cycle status of H292 parental and IBE stable cells were examined by FACS. Cells were pre-treated with mitomycin C for 3 hours for synchronization followed by PI staining for 1, 3, 6, 12, 24 hours, respectively. Results were expressed as mean ± S.E.M. of two independent experiments. *: P < 0.05; **: P < 0.01 as compared with control group.
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0.5 mL of Trizol™ reagent (Roche) followed by addition of 0.1 mL of chloroform and centrifugation at 13,000 rpm for 15 minutes to separate aqueous phase, interphase, and organic phase. Next, the precipitated RNA from the aqueous phase was mixed with 0.25-0.5 mL of isopropanol and centrifuged at 13,000 rpm for 15 minutes. After removal of supernatant, gel-like pellet was washed with 1 mL of 75% ethanol twice after centrifugation at 7500 rpm for 15 minutes. Finally, after removal of supernatant, RNA pellet was air-dried for about 30 minutes and then dissolved in DEPC-treated water for storage at 4°C for 24 hours. Sample concentration and purity were determined at OD 260 nm and 280 nm. The OD\textsubscript{260/280} ratio should be in the range between 1.8-2.0. Then, the first-strand cDNA synthesis was performed with 500 ng RNA, dNTP, Oligo-dT and distilled DEPC H\textsubscript{2}O. The mixture was heated to 65°C for 5 minutes and quick chilled to 4°C for 5 minutes. Then 5X First-Strand Buffer, DTT, M-MLV RT Enzyme were added to the mixture, incubated at 37°C, 25°C then back to 37°C again for 2, 10, 50 minutes, respectively, and then at 70°C for 15 minutes. The cDNA products were used for amplification in PCR.

**Statistic analysis**

Data were displayed as mean ± S.E.M. The significance of difference between the experimental and control groups was assessed by Student’s t test. The difference is significant if \( p \) value is < 0.05.

**Results**

*IBE induced proliferation, migration, and invasion of NSCLCs*

To investigate the effect of incense burning on NSCLC cancer cell progression and drug sensitivity, wild-type EGFR-expressing H292 lung cancer cell line was treated with different concentrations of IBE medium for 24 hours followed by the examination of cell viability. The results of MTT assay showed that H292 cells were died significantly in a dose-dependent manner after exposure to 20% IBE medium (Supplementary Figure 1). To mimic the scenario of religious belief, H292 cells were chronically treated with the sub-lethal concentration (1% and 5%) of IBE medium for at least one month to establish IBE stable clone. The data from both crystal violet staining and cell count-
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ing assays showed that IBE stable clones showed much higher cell proliferation ability as compared with their parental cells (Figure 1A and 1B). In parallel, the S phase distribution of IBE stable clones after 12 hours of release from synchronization by mitomycin C was also higher than that of parental cells (Figure 1C). Next, we examined the effect of IBE on the motility of lung cancer cells. To exclude the enhancement of cell growth by IBE, IBE stable clones were treated with mitomycin C for 3 hours to arrest cell cycle at G1 phase prior to migration and invasion assays. The migration (Figure 2A) and invasion (Figure 2B) abilities of 1% and 5% IBE clones are dramatically higher than that of parental cells within 24 hours. Taken together, these findings suggest that exposure to IBE renders cancer cells much more malignant.

**IBE and its ingredient auramine induced EGFR kinase activation through upregulating AREG expression**

Tumor progression involves diverse regulatory events especially those signaling pathways driven by receptor tyrosine kinases. To elucidate the underlying mechanisms of IBE-induced tumor progression, we tested whether any receptor tyrosine kinase was activated in response to the IBE exposure. To this end, general tyrosine phosphorylation was examined by using 4G10 anti-phosphotyrosine antibody, and the results showed that some increased signals
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were observed around 100-190 kDa in H292 IBE clones (Figure 3A). EGFR is a 185 kDa receptor tyrosine kinase, and its signaling pathway is frequently amplified and crucial for malignancy in many cancer types, including NSCLC. Therefore, the roles of EGFR and its downstream signaling in IBE-induced tumor progression were then examined, and we found that IBE induced the kinase activations of EGFR and its downstream effectors Akt and Erk by detecting their activating phosphorylations at Y1068, Y845 for EGFR, at T202 and Y204 for Erk1/2, and T308 for Akt in the absence or presence of EGF stimulation (Figure 3B). We also examined the tyrosine kinase activity of c-Met and ErbB2 (HER2), whose molecular weight are 140/170 and 185 kDa, respectively, but their kinase activities were suppressed and not increased respectively by IBE treatment (Figure 3C). These data suggested that IBE induced kinase activation of EGFR signaling for tumor progression, but meanwhile suppressed c-Met in H292 cells. Short-term treatment (3 days) with IBE also slightly induced kinase activation of EGFR signaling in H292 and H322 cells (Figure 3D). In addition to lung cancer cell expressing wild-type EGFR, we also examine the effect of IBE on EGFR activation.
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We treated EGFR exon19 deletion HCC827 cells and EGFR exon21/T790M double mutation H1975 cells with IBE for 7 days. However, the data showed that IBE did not induce EGFR kinase activation significantly in these two cells (Figure 3E), suggesting that IBE treatment may specifically induce EGFR signaling pathway in wild-type NSCLC cells but not mutant EGFR-expressing NSCLC cells.

Next, we addressed whether IBE cells release cytokines to activate EGFR. The IBE conditioned medium was collected from the 1% IBE clones, which were cultured in IBE-free medium for 24 hours before the harvest. The parental H292 lung cancer cells were then cultured with the parental or IBE conditioned medium for 30 minutes as illustrated in Figure 4A. Our data from Western blot analysis showed that, like direct IBE treatment, the conditioned medium from IBE clones induced EGFR, Akt and Erk phosphorylations, suggesting an autocrine regulation to activate EGFR by IBE exposure (Figure 4B). We examined the effect of IBE treatment on the expressions of some common EGFR ligands including EGF, TGF, HB-EGF and AREG, and found that the mRNA level of AREG

Figure 5. Auramine induced the cell proliferation of H292 cells through AREG-dependent EGFR signaling. (A) The cell viabilities of H292 cells and its auramine-stable clone were measured by MTT assays. (B) Total lysates prepared from H292 cells treated with 0.1, 0.5, 1 mM auramine for 5 days were subjected to Western blot with indicated antibodies. (C, D) Auramine stable clone of H292 cells were treated with indicated concentration of anti-AREG antibody for 6 hours. The level of secreted AREG in the culture medium was determined by ELISA assay (C). The total lysates of these cells were prepared and subjected into Western blot analysis with indicated antibody (D). (E) Auramine stable clone of H292 cells were infected with lentivirus expressing AREG shRNA, and their viabilities were then determined by MTT assays.
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was increased in IBE-treated H292 cells (Figure 4C). The secretion of AREG from IBE cells was also detected (Figure 4D). These findings suggest that IBE may activate EGFR signaling pathway through induction of AREG.

In the process of making incense stick, auramine O is a dye diarylmethane used for surface staining to make incense stick better looking. We found that auramine, a residual composition from the smoke after burning incense sticks in mass spectrometry analysis (Supplementary Figure 2 and [38]), increases the metastatic abilities and stemness characters of NSCLCs [38]. Also, treatment with auramine increased the proliferation of H292 cells (Figure 5A). Similar to IBE, auramine also induced AREG secretion of H292 cells into the culture medium, and the induction was suppressed by the anti-AREG neutralizing antibody (Figure 5B). Activations of EGFR signaling and its downstream Akt and ERK activities by auramine treatment were also found in H292 cells (Figure 5C). Treatment with anti-AREG antibody further suppressed the auramine-induced EGFR and ERK activations (Figure 5D), indicating that AREG mediated IBE- and auramine-induced EGFR activation. Silence of AREG also reduced the viability of auramine-treated H292 clone (Figure 5E).

Exposure to IBE enhances EGFR-TKI sensitivity in H292 cells

Previous studies suggested that Asian NSCLC patients bearing EGFR mutants showed better responses to EGFR TKIs therapies [1, 34, 36]. But it is not clear whether incense-use custom in Asia is associated with the higher sensitivity of lung cancer cells to EGFR TKIs. In comparison to parental cells, IBE- or auramine-treated H292 cells were much more sensitive to EGFR TKI gefitinib (Figure 6A) or erlotinib (Figure 6B and 6C) in MTT assays. To examine the possibility of EGFR mutation in IBE-treated cells, gene sequencing of EGFR was performed. However, after alignment of the EGFR sequence expressed in 1% IBE clones and the parental H292 cells, activating mutations, including exon 19 deletion or L858R substitutions, were not found in H292/IBE clones (Supplementary Figure 3). Furthermore, TKI treatment induced PARP and Caspase 3 cleavages in IBE clones more than in their parental cells (Figure 6D). Also, erlotinib-induced sub-G1 population was higher in IBE clones than in parental cells (Figure 6E), indicating that IBE treatment sensitizes H292 cells to TKI-induced apoptosis. The data from clonogenic formation (Figure 6F) and cell counting (Figure 6G) assays also showed that IBE-treated H292 cells were much more sensitive to various EGFR TKIs including gefitinib, erlotinib or afatinib. In addition, we also found treatment with gefitinib can suppress the spheroid formation of IBE-treated H292 clones but not their parental cells (Figure 6H). These results demonstrated that exposure to IBE may render lung cancer cells more sensitive to EGFR TKIs. However, the effect of IBE on TKI sensitization was not found in HCC827 cells (data not shown), suggesting that exposure to IBE enhances EGFR-TKI sensitivity in wild-type NSCLC cells but not in mutant EGFR NSCLC cells.

Discussion

Lung cancer is one of the most common cancer types worldwide, and its incidence and mortality are very high, especially in the developed countries. It might attribute to multiple risk factors, such as lung cancer family gene history, cigarette smoke, air pollution or cooking smoke, and incense-burning smoke, which can induce lung cancer formation. Among these risk factors, incense-burning smoke, a part of daily life of Asian people, might be a very important factor in lung cancer tumorigenesis [30-32] and other diseases [28, 29]. However, it is not clear whether incense-burning smoke can induce tumor progression of lung cancer. In this study, we demonstrated that IBE induced cell progression in proliferation, migration and invasion abilities. These findings are paralleled to some case reports that people long-term exposure to incense smoke environment, such as temple worker or Buddhism, Taoism and Shinto believer, may increase the risk of squamous cell carcinoma of the respiratory tract or lung cancer, and cigarette smoke may further increase their risk [39]. Similar to the inducing effect of cigarette smoke on DNA synthesis [40], our data that population of NSCLC in S phase was increased by IBE also supports the role of incense smoking in promoting lung cancer proliferation.

Cell proliferation as a result of S phase accumulation involving various reasons, and overexpression of growth factor receptors on cell membranes was the main event contributing
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Figure 6. IBE/Auramine treatments render EGFR wt-expressing lung cancer cells more sensitive to EGFR TKI. (A and B) H292 parental and IBE stable clone cells were seeded at the density of 2500 cells/well and treated with 0.1, 0.5, 1, 5, 10 μM of gefitinib (A) or erlotinib (B) for 3 days. Cell viabilities were determined by WST-1 assay. (C) H292 parental cells and auramine stable clone were seeded at the density of 2500 cells/well and treated with indicated concentration of erlotinib for 3 days. Cell viabilities were determined by WST-1 assay. (D) H292 parental cells and
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IBE clones were treated with 1 μM of EGFR TKI erlotinib for 3 days. Whole-lysates extracted from these cells were subjected to Western blot analysis with indicated antibodies. (E) H292 parental cells and their IBE stable clone were treated with 1 μM of EGFR TKI erlotinib for 3 days. The sub-G1 population was determined by using flow cytometry analysis with PI staining. (F) H292 parental cells and IBE stable clone were treated with 0.5 μM EGFR-TKI gefitinib, erlotinib or afatinib for 7 days followed by 1% crystal violet staining. The result was quantitated determined according to the absorbance at 570 nm followed by dissolving crystal violet with acetic acid. (G) H292 parental cells and IBE stable clone were seeded in 12-well and then treated with 1 μM of gefitinib or erlotinib for 3 days. Cell numbers were counted. (H) H292 parental cells and IBE clones were seeded and suspended in sphere formation medium in non-coated dishes with 0.5 μM EGFR-TKI gefitinib for 7 days. Results were expressed as mean ± S.E.M. of two independent experiments. * or #: P < 0.05; ** or ##: P < 0.01 as compared with control group.

Figure 7. Hypothetical working model of incense burning smoke render NSCLC more aggressive and sensitive to EGFR TKIs. Exposure to IBE or its residue composition auramine can induce tumor progression and enhance EGFR TKI sensitivity through modulation of EGFR signaling. AREG is upregulated by IBE and auramine transcriptionally and is secreted as an autocrine ligand for EGFR activation, rendering NSCLC cells more addicted to the increased EGFR signaling for survival.

to cell survival [41]. EGFR is overexpressed approximately in 40-80% of lung cancers and drives important signaling pathways for cancer progression [42]. EGFR mutations lead to its kinase activation and are associated with development of non-small cell lung cancer [43]. Our data indicated that IBE not only induced the cell progression and spheroid formation but also the sensitivity to EGFR TKI gefitinib or erlotinib. Previous studies showed that EGFR activating mutation is associated with EGFR TKIs sensitivity [15, 16]. Our data showed that EGFR and its downstream signaling Akt and Erk activations in IBE- and auramine-treated cells. However, EGFR mutations were not found in the IBE-treated cells in this study. On the other hand, EGFR mutants were found in many NSCLC cell types, and may represent loss-of-functional domain mutations or enhance tyrosine kinase activity intrinsically [44, 45]. Somatic mutations of EGFR, such as ex- on19 deletions or L858R substitution can be detected in NSCLC cells, which are highly responsive to EGFR TKIs [46]. However, EGFR secondary T790M mutation was determined to cause TKIs resistance [46]. In this study, EGFR-activating and TKI-sensitizing effects of IBE was not observed in EGFR mutant-expressing NSCLC cells, suggesting that EGFR activity in these cells is too high to be further enhanced by IBE.

Autocrine regulation in cancer is recognized as a major mechanism providing self-sufficient growth signals, including EGFR activation [47]. Several EGFR ligands, including EGF, transforming growth factor α (TGF-α), amphiregulin (AREG), heparin-binding EGF-like growth factor (HB-EGF), betacellulin, and epiregulin, were identified for EGFR activation [47]. The involvement of EGFR activation in diseases or normal tissue homeostasis depends on the expression levels of both ligand and receptor [47]. Among these well-known EGFR ligands, our data from this study showed that autocrine cytokines AREG mediates the IBE-induced activation of EGFR signaling, leading to NSCLC progression and sensitization to EGFR TKIs. AREG is associated with lower survival rate of patients with NSCLC and poor prognosis [48, 49]. In contrast to our findings, however, high serum level of AREG has also been proposed as a diagnostic marker for predicting a poor response of advance NSCLC patients to
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gefitinib [50]. The anti-apoptotic activity of AREG through inactivation of BAX has further been proposed to mediate the gefitinib resistance in NSCLCs [51]. In addition to the classic ligands for EGFR, angiogenin/Ribonuclease 5 has been recently identified as a novel EGFR ligand and a serum biomarker for erlotinib sensitivity in pancreatic cancer [52]. Interestingly, our data from mass spectrum analysis (see Supplementary Materials and Methods) showed the significant changes in the gene expressions, which are involved in RNA process, in response to IBE and Auramine treatment (Supplementary Figure 4). It is worthy to further address whether Ribonuclease 5 or other potential molecules are also regulated by IBE to affect the sensitivity to EGFR TKIs.

In addition to EGFR, co-regulation of other pathways by IBE and auramine may account for sensitizing effect of AREG on the response to EGFR TKIs. Although AREG has also been reported to activate HER2 activity through dimerization with EGFR for tumor proliferation and migration of breast cancer cells [53], HER2 tyrosine kinase activity was not affected by IBE treatment in NSCLCs in our study. c-Met amplification has been demonstrated as one of the reasons causing the acquired EGFR TKIs resistance, and therefore combination with c-Met inhibitors was employed to improve survival rate of lung cancer patients who failed to EGFR TKIs [54, 55]. Interestingly, our data showed that IBE not only increases EGFR activation but also suppresses the kinase activity of c-MET. It seemingly exhibits the switch of survival dependence from multiple oncogenic drivers, including c-MET, to EGFR mainly, revealing a possible “Achilles’ heel” that can be targeted therapeutically in incent smoke-associated NSCLC cells. Thus, exposure to IBE or auramine may render H292 cells more oncogenic addictive to EGFR signaling, resulting in EGFR TKIs sensitization. However, it remains further studies to further investigate how IBE and auramine induce AREG expression and suppress c-MET activation.

In summary, IBE and its ingredient auramine increased EGFR activation through AREG induction but suppressed c-Met activity to not only promote tumor progression but also enhance EGFR TKIs sensitivity in EGFR wild-type cells (Figure 7). These findings provide another plausible explanation of high response rate to EGFR TKIs in eastern Asian NSCLC patients, but further clinical studies are required to prove this notion.

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Disclosure of conflict of interest

None.

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References

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Supplementary Figure 1. Cell viability of H292 cells in response to short-term IBE treatment. H292 cells were treated with different concentration of IBE as indicated for 24 hours, and then the cell viabilities were determined by MTT assay.

Supplementary Figure 2. Mass spectrometry analysis of incense burning smoke solution. Incense-burning smoke was dissolved into de-ionized water by suction with pump. The relative abundance of IBE compositions was performed in the Orbitrap LC-MS with a mass resolution of 60,000 at $m/z$ 200 (A). The survey scan of IBE compositions at 268.12 was similar with commercial standard Auramine O [38] (B).
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Supplementary Figure 3. Activating mutations of EGFR were not detected in IBE stable clone of H292 cells. EGFR sequence at Exon 19 deletion (A) and L858R point mutation (B) in 1% IBE-treated clone of H292 cells was aligned and compared with that in the parental cells.
Supplementary Materials and Methods

Mass spectrometry analysis

Incense-burning smoke was continuously dissolved into water by suction with pump. The survey scan of IBE compositions was performed in the Orbitrap LC-MS (ThermoFisher scientific) with a mass resolution of 60,000 at \( m/z \) 200. The flow rate of HPLC was 50 µL/min, and solution gradient was from 0% to 100% B (B = 80% acetonitrile, 0.1% formic acid) over 30 min minutes.

Total proteins (40 µg) lysate of parental, IBE-treated, or auramine-treated H292 cells were separated by 10% SDS-PAGE and divided into eight gel fractions. After in-gel trypsin digestion, the tryptic peptides of each fraction were injected into the linear ion trap-Fourier transform ion cyclotron resonance mass spectrometer (LTQ-FTICR MS) (Thermo Electron) for mass identification. The survey scan (\( m/z \) range: 320-2,000) was performed in FTICR MS with a mass resolution of 100,000 at \( m/z \) 400. The top ten most abundant multiply charged ions were sequentially isolated for MS/MS assay by LTQ. Protein identification and label-free quantitative analysis were performed by MaxQuant and MaxLFQ softwares, respectively. The significance threshold for the identification was set as \( P < 0.01 \).
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Supplementary Figure 4. Enrichment of Gene Ontology (GO) terms in up- or down-regulated genes in H292 IBE/Auramine cells vs. parental cells. GO terms belong to cellular components (GOCC), biological processes (GOBP) and molecular functions (GOMF) of H292 IBE- (A) or auramine- (B) treated cells were shown in red, green, and blue, respectively. GO terms were analyzed by David functional annotation tools (https://david.ncifcrf.gov/tools.jsp).