Smoking Attenuates Transforming Growth Factor-β–Mediated Tumor Suppression Function through Downregulation of Smad3 in Lung Cancer

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Abstract

Epidemiologic studies have shown that most cases of lung cancers (85%–90%) are directly attributable to cigarette smoking. Although much information has been gained about the effects of cigarette smoking on various signaling pathways causing lung cancer, nothing is known about the effect of cigarette smoking on the TGF-β–induced tumor suppressor function in lung cancer. To address this issue, lung adenocarcinoma A549 and immortalized bronchial epithelial HPL1A cells were chronically treated with cigarette smoke condensate (CSC) and dimethyl sulfoxide (as a control) to mimic the conditions of long-term cigarette smoking. Prolonged exposure of these cells to CSC resulted in a decrease in Smad3 and Smad4 complex formation and TGF-β–mediated transcription due to reduced expression of Smad3. Long-term CSC treatment reduced apoptosis, increased cell viability, decreased TGF-β–mediated growth inhibition, and enhanced tumorigenicity. The decrease in apoptosis is due to the upregulation of Bcl-2, which is a downstream target of Smad3. Re-expression of Smad3 in the CSC-treated cells restored TGF-β signaling, increased apoptosis, and decreased cell viability and tumorigenicity. Withdrawal of CSC treatment resulted in the restoration of Smad3 expression, reduction in cell viability, and increased TGF-β–mediated growth inhibition. Expression of Smad3 is lower in lung tumors of current smokers than that observed in never-smokers. Collectively, these data provide evidence that cigarette smoking promotes tumorigenicity partly by abrogating TGF-β–mediated growth inhibition and apoptosis by reducing expression of Smad3. Cancer Prev Res; 1–11. ©2012 AACR.

Introduction

Cigarette smoking is the main risk factor for lung cancer, accounting for 90% of cases in men and 70% to 85% of cases in women. Approximately 1.3 billion people smoke cigarettes worldwide, which contributes to 5 million preventable deaths per year (1). The vast majority of lung cancers are strongly correlated with tobacco consumption, and smoking cessation remains the only known way of reducing cancer risk in smokers (2). Cigarette smoking has been found to induce a number of genetic and molecular changes in the respiratory tract, including cellular atypia, LOH (3), and promoter hypermethylation (4). Microarray studies of bronchial epithelial cells have indicated that cigarette smoke not only induces, primarily, the expression of xenobiotic-metabolizing and redox-regulating genes but also points to the effects on tumor suppressor genes, oncogenes, and genes involved in the regulation of inflammation (5). Systematic analysis of mRNA and protein expression levels among thousands of genes has also contributed to defining the molecular network of lung carcinogenesis. Despite the fact that enormous progress in the understanding the molecular mechanisms leading to lung cancer has been made, how intrinsic Smad-dependent TGF-β signaling is affected by smoking has not yet been investigated.

Alterations in TGF-β signaling are linked to a variety of human diseases including cancer, inflammation, and tissue fibrosis (6, 7). The disruption of TGF-β signaling occurs in several human cancers, and the pathway generally possesses a tumor suppressor function (8). However, in the later stages of carcinogenesis, the tumor cells become resistant to TGF-β–induced growth arrest. Although alterations in TGF-β receptors have been implicated in the resistance to its tumor suppressor function, tumors may use various...
mechanisms anywhere along its primary cytoplasmic signal transducers, the Smad proteins, to circumvent the growth-inhibitory effects of TGF-β (9, 10).

The majority of the studies conducted thus far were analyzed on short-term (11–13) or long-term (14) exposure of epithelial cells to high doses of tobacco constituents. Interestingly, despite the fact that cigarette carcinogens mediate inactivation of numerous tumor suppressor genes, the relevance of cigarette smoking as to the overall prognosis of patients with lung cancer remains controversial (15, 16). The present study was undertaken to ascertain if cigarette smoke induces any alterations in the Smad-dependent TGF-β signaling, which directly enhance the malignant phenotype of lung cancer cells.

Here, we describe the results of a study in which immortalized human bronchial epithelial cells, HPL1A and lung adenocarcinoma cell line A549, were chronically exposed to low doses of cigarette smoke condensate (CSC) for approximately 1 year. Therefore, we intended to mimic, in vitro, the long-term exposure of human lung epithelium to smoke. We found that cells exposed to smoke for a long-term makes them tumorigenic or more tumorigenic by decreasing the levels of tumor suppressor protein Smad3.

Materials and Methods

Cell lines

Human lung adenocarcinoma cells (A549) obtained from American Type Culture Collection and human lung immortalized epithelial cells (HPL1A; a kind gift of Dr. T. Takahashi), Mycoplasma negative by a PCR detection method (April 4, 2010 and October 8, 2010, testing Sigma Venor-Gem), were maintained in RPMI with 10% FBS supplemented with penicillin/streptomycin and L-glutamate. All cell lines were cultured and maintained at 37°C in a humidified incubator in the presence of 5% CO₂. Cells were treated chased from Sigma Bio chemicals. TGF-β1 was purchased from Cell Signaling Technology. Mouse anti-Smad3, anti-Smad4, and anti-Bcl-2 antibodies were purchased from Santa Cruz Biotechnology. Rabbit anti-Smad2 and anti-Smad3 were from Zymed Laboratories, Inc. Mouse anti-Smad3, anti-Smad4, and anti-Bcl-2 were purchased from Santa Cruz Biotechnology. Anti-phospho-Smad2, 3, Bax, Bcl-xl, and Bcl-w antibodies were purchased from Cell Signaling Technology. MTT kit and ChIP assay kits were purchased from Millipore.

Immunoprecipitation and immunoblot analysis, transcriptional response assay, cell viability, Bcl-2 siRNA, apoptosis by ELISA, apoptosis by FACS, quantitative real-time PCR, and stable overexpression of Smad3

All the above experiments were carried out as described in Supplementary Materials and Methods.

DNA laddering

Cells were serum-starved for 72 hours to induce apoptosis. Cells (floating and adherent) were collected and lysed. DNA laddering was conducted as described in the work of Halder and colleagues (17).

Soft agarose assay and xenograft studies

A total of 1 × 10⁶ cells were plated for soft agarose assay as discussed previously (18). For xenograft studies, 1 × 10⁶ cells were injected subcutaneously in athymic nude mice. The animals were monitored for tumor formation every week for a total of 7 weeks. If found, tumors were measured as described previously (18).

Immunohistochemistry

Immunohistochemistry was conducted as described in (19) with mouse monoclonal Smad3 incubated for 2 hours (dilution 1:100). Smad3 expression was evaluated semiquantitatively on the basis of the intensity of staining and was scored as weak (+1), moderate (+2), and intense (+3). Samples with no staining were considered negative, and samples with weak-to-intense staining were considered positive.

Statistical analysis

Descriptive statistics including mean values and SD were calculated using Prism software (GraphPad). All data are representative of at least 3 independent experiments and are expressed as the means ± SD unless otherwise indicated. ANOVA was used to assess the differences between experimental groups and survival curves, unless otherwise indicated.

Results

CSC treatment inhibits Smad-dependent TGF-β signaling through downregulation of Smad3

To test the effect of CSC on TGF-β signaling, we looked at the functional complex formation between Smad2 or Smad3 and Smad4 by immunoprecipitation assays. A549 and HPL1A cells were treated with CSC (25 µg/mL) for 4, 100, and 300 days together with and without TGF-β for 1 hour. Lysates were subjected to immunoprecipitation with either anti-Smad2 or anti-Smad3 antibody followed by immunoblotting with anti-Smad3 antibody. We observed that TGF-β-induced Smad3/Smad4 but not Smad2/Smad4 complex formation was significantly reduced in chronically CSC-treated cells for 300 days, suggesting a biased role of CSC in blocking the Smad3/Smad4 complex formation in both the cell lines. The reduced Smad3/Smad4 complex...
formation in the long-term CSC-treated cells (300 days) was due to reduced levels of Smad3. There was no change observed in the levels of Smad2 or Smad4 (Fig. 1A). We observed same results when we conducted the reverse experiment, namely immunoprecipitation with anti-Smad4 and immunoblotting for Smad3 (Supplementary Fig. S1A). We observed the complex formation between Smad2, Smad3, and Smad4 going down even when the lysates were prepared similarly as above and were subjected to immunoprecipitation with both anti-Smad2 and anti-Smad3 together (Supplementary Fig. S1B). To test whether the inhibition of Smad complex formation affects downstream transcriptional responses mediated by TGF-β, we conducted transient transfection assays using TGF-β-responsive reporters, p3TP-Lux and (CAGA)₉-MLP-Luc. Both the reporters (CAGA)₉-MLP-Luc (Fig. 1B) and p3TP-

![Figure 1](https://www.aacajournals.org)
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whether the decrease in the Smad-dependent TGF-β signaling has any effect on cell viability, we checked for cell viability by assaying a number of important apoptotic regulators including Bcl-2, Bcl-xl, Bcl-w, and Bax in CSC-treated A549 and HPL1A cells. Immunoblot analyses indicated that the level of anti-apoptotic Bcl-2 was upregulated in cell extracts prepared from the 300-day CSC-treated cells. No detectable change of other members of the Bcl-2 family was observed (Fig. 2D) and the proapoptotic members namely Bax remain unchanged. To validate whether Bcl-2 is the cause of increase in apoptosis, we conducted Bcl-2-siRNA knockdown in the 300-day CSC-treated A549 and HPL1A cells. We assayed for apoptosis 72 hours after transfection of 20 nmol/L of each Bcl-2 siRNA and scrambled control. We observed that apoptosis goes up (Fig. 2E) upon Bcl-2 knockdown. Simultaneously, we also checked the protein expression of Bcl-2 to confirm whether Bcl-2 levels are decreasing (Supplementary Fig. S2A). Because NF-κB is known to upregulate Bcl-2 and to rule out the possibility that NF-κB is responsible for upregulating Bcl-2, we checked the expression of NF-κB in the long-term CSC-treated cells. We do not see an increase of NF-κB (p65) levels (Supplementary Fig. S2B) due to long-term CSC treatment, suggesting that upregulation of Bcl-2 is not dependent on NF-κB in our model system. These results suggest that CSC treatment increases cell viability by decreasing apoptosis through upregulation of antiapoptotic Bcl-2.

Withdrawal of CSC treatment restores TGF-β signaling and apoptosis

After smoking cessation, the cumulative death risk from lung cancer decreases. Peto and colleagues showed that the earlier cigarette smoking is stopped, the greater the decline in lung cancer mortality (21). There is a pressing need to clarify the role of smoking cessation in the care of patients with lung cancer. To mimic the conditions of smoking cessation, we withdrew cells from the long-term (300-day) CSC treatment and tested the effects on TGF-β signaling. We observed that Smad3/Smad4 complex formation goes up with the withdrawal of CSC treatment in the 300-day CSC-treated samples. Thus, the Smad-dependent TGF-β signaling is restored together with Smad3

CSC treatment inhibits TGF-β-induced growth suppression and attenuates apoptosis by upregulation of Bcl-2

To determine whether long-term CSC treatment has any effect on cell viability, we checked for cell viability by MTT assay in both A549 and HPL1A cells. We also tested whether the decrease in the Smad-dependent TGF-β signaling has any effect on TGF-β-induced growth suppression. We found that the long-term CSC-treated cells grew faster than the corresponding DMSO-treated cells. We also observed that the TGF-β-induced growth suppression was diminished in the long-term CSC-treated cells (Fig. 2A). These data suggest that long-term CSC treatment increases cell growth and decreases TGF-β-induced growth suppression. The increase in cell viability could be due to an increase in cell proliferation or decrease in apoptosis. We tested the cell proliferation by thymidine incorporation assay. We did not see a significant change in cell proliferation by long-term CSC treatment (data not shown). To determine whether CSC treatment has any effect on apoptosis, we conducted quantitative ELISA assay, which detects the cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) after cell death. We found that long-term CSC treatment (300-days) of both A549 and HPL1A cells led to less apoptosis in CSC-treated cells compared with their corresponding DMSO controls (Fig. 2B), suggesting a role of long-term smoking in inhibiting apoptosis. This was confirmed by a qualitative DNA laddering assay in which the 300-day DMSO-treated cells showed much more DNA laddering than the corresponding CSC-treated cells (Fig. 2C). To understand the mechanism of decreased apoptosis, we assayed a number of important apoptotic regulators including Bcl-2, Bcl-xl, Bcl-w, and Bax in CSC-treated A549 and HPL1A cells. Immunoblot analyses indicated that the level of anti-apoptotic Bcl-2 was upregulated in cell extracts prepared from the 300-day CSC-treated cells. No detectable change of other members of the Bcl-2 family was observed (Fig. 2D) and the proapoptotic members namely Bax remain unchanged. To validate whether Bcl-2 is the cause of increase in apoptosis, we conducted Bcl-2-siRNA knockdown in the 300-day CSC-treated A549 and HPL1A cells. We assayed for apoptosis 72 hours after transfection of 20 nmol/L of each Bcl-2 siRNA and scrambled control. We observed that apoptosis goes up (Fig. 2E) upon Bcl-2 knockdown. Simultaneously, we also checked the protein expression of Bcl-2 to confirm whether Bcl-2 levels are decreasing (Supplementary Fig. S2A). Because NF-κB is known to upregulate Bcl-2 and to rule out the possibility that NF-κB is responsible for upregulating Bcl-2, we checked the expression of NF-κB in the long-term CSC-treated cells. We do not see an increase of NF-κB (p65) levels (Supplementary Fig. S2B) due to long-term CSC treatment, suggesting that upregulation of Bcl-2 is not dependent on NF-κB in our model system. These results suggest that CSC treatment increases cell viability by decreasing apoptosis through upregulation of antiapoptotic Bcl-2.

CSC treatment decreases Smad3 mRNA, and the promoter activity and the expression are correlated to histone deacetylation

To determine whether the Smad3 mRNA levels correlate with the protein levels, we carried out quantitative real-time PCR in the various time points of CSC-treated cells along with the controls. We observed that the Smad3 mRNA goes down in the 300-days CSC-treated cells compared with the control cells (300 days DMSO; Fig. 1D) in both the cell lines. To determine whether the reduction in Smad3 expression by CSC is at the promoter level or due to posttranscriptional changes, we transiently transfected Smad3 promoter reporter (−1,878/+13) in both HPL1A and A549 cells pretreated with CSC for 4, 100, and 300 days. Smad3 promoter activity was decreased by CSC in a time-dependent manner (Fig. 1E). The Smad3 expression can go down through several epigenetic mechanisms namely histone deacetylation and DNA methylation. To test which epigenetic change, histone deacetylation or the DNA methylation, is playing a role in CSC-induced downregulation of Smad3. 300-day CSC-treated A549 and HPL1A cells were treated with either histone deacetylase inhibitor, sodium butyrate TSA or the methylation inhibitor, azacytidine. Treatment of sodium butyrate (Fig. 1F) suppressed CSC-mediated downregulation of Smad3, whereas azacytidine had no significant effect (data not shown). When we preformed ChIP assay using Sp1, a known transcription repressor shown to be involved in Smad3 transcription (20), we observed an increase in occupancy of Sp1 on the Smad3 promoter in the 300-days CSC-treated cells and it vanishes due to sodium butyrate treatment (Supplementary Fig. S1C). These results suggest that long-term CSC treatment decreases Smad3 mRNA and Smad3 promoter activity, and histone deacetylation may play a role in reducing the Smad3 expression.
expression (Fig. 3A). To test whether this restoration in TGF-β signaling translates to biologic outcomes namely a decrease in cell viability, MTT assay was conducted using both A549 and HPL1A cells after withdrawing CSC treatment. We saw that cell viability decreases and TGF-β-mediated growth inhibition increases with withdrawal of CSC treatment (Fig. 3B). To test whether withdrawal of CSC treatment has any effect on apoptosis of cells, we conducted cell death with the ELISA assay. Withdrawal of CSC treatment brings back apoptosis (Fig. 3C). These results suggest that the effect of CSC treatment is reversible and that the withdrawal of CSC treatment restores TGF-β signaling, apoptosis and reduces cell viability.

Stable expression of Smad3 restores TGF-β signaling, apoptosis and reduces cell viability in long-term CSC-treated cells

CSC contains thousands of compounds comprising 5 known human carcinogens and many toxic agents. These may affect many different pathways ultimately leading to increased cell viability and altered apoptosis. To verify that the increase in cell viability is due to the decrease in Smad3 expression, we carried out rescue experiments, where we overexpressed Smad3 in 300-day CSC-treated cells (Fig. 4A). Stable overexpression of Smad3 in both the 300-day CSC-treated cell lines, HPL1A and A549, brought TGF-β signaling back as shown by the luciferase assays with
TGF-β-responsive reporter elements both p-3TP-Lux and (CAGA)₉-MLP (Supplementary Fig. S3A and S3B). Similarly, the biological outcomes of the long-term CSC treatment namely, increased cell viability and decreased apoptosis were also rescued by the ectopic overexpression of Smad3 in the 300-day CSC treated A549 and HPL1A cells. We observed that clones of Smad3 overexpressing in both 300-day CSC-treated HPL1A and A549 cell lines underwent more apoptosis than the 300-day CSC-treated HPL1A and A549 cell lines and vector controls when we conducted cell death by ELISA (Fig. 4B and C, left). We observed that the same clones were less viable compared with the 300-day CSC-treated HPL1A and A549 cell lines and vector controls (Fig. 4B and C, right). These combined results suggest that the increase in cell viability by decreasing apoptosis is imparted via long-term CSC treatment, partly through downregulation of Smad3.

Long-term CSC treatment enhances tumorigenicity of the cell both in vitro and in vivo partly by downregulating Smad3

Because the increase in cell viability and decrease in apoptosis by chronic exposure of cells to CSC are reminiscent of the phenotypes characteristic of oncogenically transformed cells, we next examined whether CSC-treated cells were capable of anchorage-independent growth by culturing them in soft agar. We conducted soft agar assay using CSC-treated A549 cells along with corresponding DMSO controls. The size and the number of colonies in the 300-day CSC-exposed cells were increased significantly when compared with the corresponding DMSO-treated cells (Fig. 5A). We also observed that 300-day CSC-treated A549 cells formed tumors bigger and faster than the 300-day DMSO and parental A549 cells (Fig. 5B). Taken together, these data suggest that chronic CSC treatment not only enhances oncogenic transformation as elucidated by the soft agar assay but also enhances the tumorigenicity in the A549 cells.

To verify that the increase in anchorage-independent growth and tumorigenicity is due to the decrease in Smad3 expression, we carried out rescue experiments, where we overexpressed Smad3 in 300-day CSC-treated cells. We conducted soft agar assay using Smad3 overexpression clones and the corresponding vector controls. The number and the size of colonies were fewer in the Smad3 overexpression clones when compared with the vector controls and 300-day CSC-treated cells (Fig. 5C). We also observed...
that the tumors formed in nude mice by Smad3 overexpression clones were smaller and slower than the vector controls and 300-day CSC-treated cells (Fig. 5D). Taken together, these data suggest that long-term CSC treatment partly enhances the tumorigenicity of the cells, and thus, partly contributes to smoking-induced lung cancer development by reducing the levels of Smad3.

Smad3 status and its correlation with patient survival and smoking

To elucidate the clinical relevance of our in vitro results that Smad3 expression goes down due to long-term CSC treatment, we conducted immunohistochemical staining of tissue microarray (TMA) using the anti-Smad3 antibody. We observed that the staining of Smad3 in cancer cells was mostly nuclear (Fig. 6A). We also observed strong staining of Smad3 in the stroma and macrophages. The frequency of positive staining (>0) was more in the case of adenocarcinoma than in the other subcategories of lung cancer, namely, small cell lung cancer, squamous cell lung cancer, and carcinoma. We plotted the intensity scores of smokers and nonsmokers and we observed that the Smad3 intensity scores was less in smokers than nonsmokers (Fig. 6B). When the staining intensities were correlated with survival status of the corresponding patients, the tumor-related survival was much better for the tumors with a high intensity (>2) of Smad3 staining than for tumors with a low intensity (<2; Fig. 6C). To further strengthen our observations, we looked into a public database (22) to check the Smad3 mRNA expression in smokers, never-smokers, and former-smokers in patients with lung cancer. We found that Smad3 is expressed in lower levels in smokers than in never-smokers (P = 0.0396; Smad3 expression is lower in former-smokers than in never-smokers (P = 0.0254) (Fig. 6D).

Taken together, these data suggests that Smad3 expression is lower in patients with lung cancer who are current smokers and former-smokers compared with never-smokers.

Discussion

Lung carcinogenesis involves the accumulation of genetic and epigenetic changes that accumulate over a long course due to chronic smoking or other genetic susceptibility factors (23). One of the putative problems in all previous studies is that the cells were treated with tobacco constituents for a maximum of 10 days. As a result, short-term exposure to cigarette smoke cannot be a true representation of lung cancer, which mainly occurs in long-term smokers.

In this study, we have shown that long-term exposure to CSC can damage lung epithelial cells, inducing hyperplastic growth and carcinogenesis. Our results reveal that SV40-immortalized bronchial epithelial HPL1A cells and lung adenocarcinoma cell line A549 surviving 300 days of repeated CSC exposure are endowed with some phenotypic changes that are characteristic of oncogenic transformation: alteration in growth kinetics, decrease in the tumor-suppressing effects of TGF-β, and decrease in apoptosis due to an increase in oncogenic antiapoptotic Bcl-2. In addition, the A549 cell line with 300 days of CSC treatment has increased anchorage-independent growth, and more importantly, the ability to produce tumors in nude mice (Fig. 5). Therefore, this approach of treating cells long-term with CSC at low concentration, offers a new tool to study the biology of lung cancer in long-term smokers. One putative issue in culturing the HPL1A cells could be culturing these cells in media supplemented with 10% FBS instead of 1% FBS that was used for establishing the cell line (24). We have used 10% FBS containing medium to culture these cells for...
several reasons, (i) there are no changes in TGF-β signaling when these cells are grown in 10% serum (25), (ii) 10% FBS containing medium was used to grow HPL1A cells before (26), (iii) HPL1A cells cannot survive long-term (300 days) in culture without serum, and (iv) TGF-β–induced transcriptional regulation and growth inhibition (Figs. 1B and C right and 2A) were not affected and these cells did not become tumorigenic when grown in 10% serum-containing medium (data not shown).

In the present study, an in vitro model system was used to gain insight about how Smad-dependent TGF-β signaling alterations potentially contribute to the initiation and early
progression of smoking-induced lung cancers. We show that long-term CSC treatment results in the reduced expression of Smad3 (Fig. 1). We have observed decreased Smad3/Smad4 complex formation and reduced TGF-β–induced transcription from p3TP-Lux and (CAGA)$_9$-MLP-Luc reporters (Fig. 1B and C). This suggests that reduced expression of Smad3 inhibited TGF-β signaling. Functionally, Smad3 reductions lead to an increase in cell viability. The increase in cell viability was because of decreased apoptosis. This decrease in apoptosis was due to upregulation in antiapoptotic Bcl-2, as seen in the chemically induced model of hepatocellular carcinoma (ref. 27; Fig. 2E). We also observed that the long-term CSC treatment increases the tumorigenicity of the cells (Fig. 5A and B). Cigarette smoke contains about 4,800 chemical agents including more than 60 known human carcinogens (28). The increase in tumorigenicity could be due to the activation of oncogenic pathways and/or inactivation of the tumor-suppressive signaling cascade. In an attempt to elucidate the role of reduced expression of Smad3 in response to CSC treatment, we have observed that restoration of Smad3 expression in long-term CSC-treated cells reduces tumorigenicity (Fig. 5C and D). These results suggest that smoking-induced lung cancer progression is partly due to the reduction in Smad3.

Figure 6. Smad3 status and tumor-related survival in patients with lung cancer. A, representative images of immunohistochemical staining for Smad3 in patients with lung cancer (200× magnification). B, Kaplan–Meier curve for tumor-related survival status based on Smad3 status. Patients with high Smad3 tumors (intensity ≥ 2) were compared with patients with moderate or low expressions of Smad3 (intensity = 0, 1, and 2). $P = 0.75$ by log-rank test. C, box plot for correlating Smad3 and smoking: smokers (including current smokers and former-smokers) and never-smokers were categorized and Smad3 intensity scores (intensity × percentage of cells) were plotted and the box plot was generated. $P = 0.15$ for intensity scores of Smad3 by Student t test. D, scatter plot for Smad3 from Shedden and colleagues (2008): Gene expression normalized z-scores of Smad3 from patients with lung cancer are plotted categorized by smoking status into current smokers, never-smokers, and former-smokers. $P$ values were calculated for the pairs shown by Student t test.
expression and attenuated TGF-\(\beta\) tumor suppressor function. This is in agreement with the previous data that ectopic expression of Smad3 reduces the susceptibility to hepatocellular carcinoma in a chemically induced murine model, consolidating the evidence for the tumor suppressor function of Smad3 (27).

The failure of the long-term CSC-treated HPLA cells to grow in soft agar or orthotopically in nude mice (data not shown) indicates that additional alterations are required to facilitate tumor formation. The ability to confer a complete malignant phenotype would likely require additional epigenetic or genetic changes. Only A549 cells bear a \(K\)-ras codon 12 mutation (29). We observed enhanced tumorigenicity in long-term CSC-treated A549 cells both in vitro and in vivo (Fig. 6A and B). Therefore, one plausible explanation is that the \(K\)-ras mutation, which is present only in A549 cells, is contributing to the tumorigenicity in vitro and in vivo.

We next validated the conclusions drawn from the in vitro model in real-life smokers using lung tumors of patients and public database, which provides the largest available set of microarray data with extensive pathologic and clinical annotation for lung adenocarcinomas (Supplementary Material). In our TMA staining, we saw a trend that the expression of Smad3 is less in smokers than in non-smokers. Similarly, in public database, we found that Smad3 expression is less in current smokers than in the never-smokers and former-smokers. Thus, we showed the relevance of Smad3 expression, which is downregulated in our in vitro model, to lung adenocarcinoma gene expression pattern found in smokers. To our knowledge, this is first time; Smad3 is correlated with the smoking status of patients with lung cancer.

Our results reveal, for the first time, that a smoking-mediated decrease in Smad3 expression plays a key role in the induction of lung cancer by increasing cell viability and decreasing apoptosis. This is in accordance with the expression of Smad3 from clinical patient samples which correlates with the results obtained from our in vitro system. Because the decrease in Smad3 expression is reversible, it would suggest that it happens in the early phases of smoking-induced lung cancer. Hence, it can be potentially used as a biomarker for smoking-induced lung cancer.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interests were disclosed.

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