Smoking Induces Epithelial-to-Mesenchymal Transition in Non–Small Cell Lung Cancer through HDAC-Mediated Downregulation of E-Cadherin

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Abstract

Epidemiological studies have shown that most cases of lung cancers (85%–90%) are directly attributable to tobacco smoking. Although association between cigarette smoking and lung cancer is well documented, surprisingly little is known about the molecular mechanisms of how smoking is involved in epithelial-to-mesenchymal transition (EMT) through epigenetic changes. Here, we show that lung cancer patients with a smoking history have low E-cadherin levels and loss of E-cadherin is a poor prognostic factor in smokers. Moreover, the downregulation of E-cadherin correlates with the number of pack years. In an attempt to determine the role of long-term cigarette smoking on EMT, we observed that treatment of lung cell lines with cigarette smoke condensate (CSC) induces EMT through downregulation of epithelial markers, including E-cadherin and upregulation of mesenchymal markers. CSC decreases E-cadherin expression at the transcriptional level through upregulation of LEF1 and Slug, and knockdown of these two proteins increases E-cadherin expression. Importantly, chromatin immunoprecipitation assays suggest that LEF-1 and Slug binding to E-cadherin promoter is important for CSC-mediated downregulation of E-cadherin. The histone deacetylase (HDAC) inhibitor MS-275 reverses CSC-induced EMT, migration, and invasion through the restoration of E-cadherin expression. These results suggest that recruitment of HDACs by transcriptional repressors LEF-1 and Slug is responsible for E-cadherin suppression and EMT in cigarette smokers and provide a potential drug target toward the treatment of lung cancer.

Introduction

The majority of lung cancer related deaths (>85%) are from non–small cell lung cancer (NSCLC), composed of 3 predominant subtypes: adenocarcinoma, squamous cell, and large cell carcinoma (1). Cigarette smoking alone accounts for approximately 30% of all cancer deaths in the United States and 80% of these smoking-attributable cancer deaths involve lung cancer (2). Although association between cigarette smoking and diseases such as lung cancer is well documented, little is known about the mechanism of cigarette smoking-induced lung cancer at the cellular level. This is due, in part, to the fact that cigarette smoke is a complex and dynamic mixture of more than 4,000 individual chemical constituents (3).

Epithelial-to-mesenchymal transition (EMT) is one of the possible mechanisms behind the initiation of cancer progression during staged metastasis, resulting in invasive cancers that possess the migratory characteristics of mesenchymal cells (4, 5). Evidence suggests that EMT is important in tumor cell aggressiveness in terms of cell migration, invasiveness, and metastatic behavior (5). During EMT, cell–cell junctions are disrupted by mechanisms involving loss of expression or delocalization of the adherens junction protein, E-cadherin. Reduced expression or loss of membrane localization of E-cadherin has been reported in several carcinomas including lung cancer (6–8), and is associated with invasion, metastasis, and worse prognosis (4, 9). Transcriptional repression has been shown to be an important mechanism for downregulation of E-cadherin in tumors. The zinc finger transcriptional repressors of the Snail/Slug family, SIP-1 and ZEB-1, bind to 2 E-boxes on the E-cadherin promoter and interact with the transcriptional corepressor, CtBP, which recruits histone deacetylases to facilitate silencing of E-cadherin expression (4, 10, 11). In addition, 2 types of basic-helix-loop-helix proteins, E12/E47 (12) and Twist (13) have also been shown to repress E-cadherin promoter activity and to induce the EMT phenotype. A normal role of E-cadherin is to bind and sequester cytoplasmic...
β-catenin, thus rendering β-catenin unavailable for signaling in the canonical Wnt/lymphoid enhancer factor (LEF)/β-catenin signaling pathway (14).

Histone deacetylase inhibitors (HDACi) are potent anti-proliferative agents, where they exhibit striking effects on cell proliferation, cell cycle arrest, migration, apoptosis, autophagy, angiogenesis, and differentiation in cultured and transformed cells from both hematologic and epithelial tumors (15–18). Clinical trials assessing several HDACi are underway, including butyrates, depsipeptide, pyroxamide, CI-994, SAHA, and MS-275 used either alone or in combination with other agents (18–20). The precise mechanisms whereby HDACi induce tumor cell growth arrest, differentiation, and/or apoptosis are currently the focus of intensive research. However, it is not known mechanistically how these inhibitors function to reverse smoking-induced EMT through the regulation of E-cadherin.

Although association between cigarette smoking and lung cancer is well documented, the molecular mechanisms underlying cigarette smoke-induced EMT processes that are critical for the progression and metastasis of lung cancer are not well understood. Our studies show, for the first time, that cigarette smoking induces the repression of E-cadherin by regulating transcription factors LEF-1 and Slug, which leads to EMT. This is supported by the observation that the E-cadherin level in lung tumors from smokers is lower than that from never smokers. The observation that the E-cadherin level in lung tumors from smokers is lower than that from never smokers. The mechanisms underlying cigarette smoke-induced EMT processes are critical for the progression and metastasis of lung cancer are not well understood. Our studies show, for the first time, that cigarette smoking induces the repression of E-cadherin by regulating transcription factors LEF-1 and Slug, which leads to EMT. This is supported by the observation that the E-cadherin level in lung tumors from smokers is lower than that from never smokers. The HDACi, MS-275 can reverse cigarette smoke-induced migration and invasion through restoration of E-cadherin expression.

Materials and Methods

Cell lines and reagents

Human lung adenocarcinoma (A549) and human bronchial epithelial lung (Beas2B) cell lines were purchased from American Tissue Culture Collection (ATCC) and maintained in RPMI 1640 and DMEM media, respectively, supplemented with 10% FBS. Human lung adenocarcinoma (ACC-LC-172) and peripheral lung epithelial (HPL1A; a kind gift from Dr. T. Takahashi, Nagoya University Graduate School of Medicine, Showa-ku, Japan) cell lines were used in the study, and these cells were Mycoplasma negative tested by a polymerase chain reaction detection method (April 10 and October 8, 2010, testing; Sigma Venor-Gem). Other human lung carcinoma cell lines used—H1975, H23, H1395, HCC827, H2170, H1299, and H2122—were purchased from ATCC. All cell lines were cultured and maintained at 37°C with 5% CO2. ATCC cell lines were characterized and were free of Mycoplasma contamination, tested by Hoechst DNA stain (indirect) and agar culture (direct) methods. Cigarette smoke condensate (CSC) was purchased from Murty Pharmaceuticals Inc. and was prepared as discussed previously (21). The CSC was diluted in DMSO and aliquots were kept at −80°C. Cell lines, inhibitors, and primary and secondary antibodies are listed in Supplementary Materials.

Tissue microarrays

Tissues were obtained from surgical specimens through the thoracic center at the Vanderbilt-Ingram Cancer Center. Tissue microarray (TMA) was constructed and the immunohistochemistry staining procedure followed as previously described (22). There are 310 tumors represented on the arrays. TMA slides stained for hematoxylin and eosin and E-cadherin were concurrently evaluated by our pathologist (A.L. Gonzalez). Staining, scoring, and analysis are detailed in Supplementary Methods.

Immunoblot analysis

Western analyses were done using standard methods (23). Cells were grown in complete media overnight and then treated with DMSO as a control or CSC in different dosage and time intervals as required with or without MS-275 (100 nmol/L) in each assay. Membranes were probed with primary antibodies and densitometry analyses were conducted as described previously (23).

Immunofluorescence staining

Cells were fixed and stained according to the published method (24). Treated cells were fixed and incubated with anti-E-cadherin (1:500) or anti β-catenin (1:500) antibody, and afterward conjugated with Cy3 (Sigma Biochemicals) secondary antibody. The cells were imaged with a Zeiss Axiohtop microscope. Images were merged using NIH Image J software.

RNA isolation and reverse transcription-PCR analyses

Cells were grown with or without CSC (25 or 125 μg/mL) for 4 days, and total RNA was isolated with TRizol reagent (Invitrogen). The reverse transcription (RT) reaction in detail and the primer sequences are presented in Supplementary Materials.

Transient transfection and promoter study

Transfection was as described previously (25). Cells were cotransfected with a firefly E-cadherin luciferase promoter-reporter construct (pGL2 E-cadherin3/Luc containing E-cadherin 5’ flanking sequences of 1,485 bp) and E-box mutant E-cadherin (Addgene plasmid 19291) luciferase construct (kindly provided by Dr. E. R. Fearon, University of Michigan, Ann Arbor, MI; refs. 26, 27) with an equal amount of total DNA by using Lipofectamine Plus reagents (Invitrogen). Luciferase activity was normalized to β-galactosidase activity and the relative luciferase activity was presented.

Migration, invasion, wound healing, cell proliferation, and cell counting assay

Experiments were carried out as described in Supplementary Methods. Migration and invasion assays were done using standard methods (28) and [3H]thymidine incorporation assays were conducted as detailed before (29).
LEF-1 and Slug gene knockdown by shRNA
Open Biosystems pGIPZ-based short hairpin RNA (shRNA) lentiviral vectors were used to deplete LEF-1 and Slug expression. Lentiviral shRNA vector pGIPZ with either targeting sequences for knocking down human LEF-1 (Clone IDs: 115188, 115189, 115191, 115192, and 224400) and Slug (Clone IDs: 153125 and 153128) or non-silencing control sequence was obtained from Vanderbilt University Microarray Core and transfected cells with FuGENE 6 transfection reagent (Roche) following the manufacturer's instruction. Stable clones were maintained in the presence of puromycin and the expression of LEF-1 and Slug were verified by RT-PCR.

Chromatin immunoprecipitation analysis
A549 cells were grown to confluence before being cross-linked with 1% formaldehyde, lysed, and sonicated as described previously (30). Chromatin fragments were coimmunoprecipitated with the LEF-1 or Slug antibodies. Further steps were conducted following the protocol from the EZ Chromatin Immunoprecipitation (ChIP) Kit (Upstate Biotechnology). The PCRs were conducted with the E-cadherin primers as described above. The PCR products were resolved on a 1.5% agarose gel and visualized with ethidium bromide.

Statistical analysis
Descriptive statistics including mean values and SD were calculated using Microsoft Excel and Prism software (Graphpad). All data represent 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 unless otherwise indicated.

Survival analysis was done by using SPSS PC package (SPSS, Inc.). Overall survival time was calculated from the date of diagnosis until death or the last follow-up contact. The survival was assessed using the Kaplan–Meier method and compared using the log rank test.

Results
Cigarette smoking decreased survival and E-cadherin expression in patients with lung cancer
Cigarette smoking is strongly correlated with the onset of NSCLC, and loss of E-cadherin is a poor prognostic factor. To determine the role of smoking on E-cadherin expression, we analyzed 310 patients with lung cancer (never smokers, 67; current smokers, 112; and former smokers, 131) between the years 1996–2008 for survival analysis. The demographic characteristics of the patients are summarized in Supplementary Table S1. Never smokers had a longer median survival than current and former smokers (26 months vs. 16 and 12 months, respectively; Fig. 1A). We then tested the expression of E-cadherin in patients with lung cancer by immunohistochemical analyses of TMAs that contain tumor tissues from never, current and former smokers with adenocarcinoma, squamous cell, and large cell carcinoma (Fig. 1B and C). The TMA was stained with anti–E-cadherin antibody and the index score was calculated. Our data showed significantly reduced (P < 0.001) levels of E-cadherin expression in current as well as former smokers when compared with never smokers in both squamous cell and adenocarcinoma patients (Table 1). The impact of cigarette smoking on E-cadherin expression was apparent when we subdivided the former and current smokers based on the pack years in both adenocarcinoma and squamous cell carcinoma patients (Fig. 1C). Only never smokers had a strong E-cadherin expression compared with former and current smokers. In adenocarcinoma and squamous cell carcinoma, the patients with weaker E-cadherin expression tended to have higher pack years in both current and former smokers. Low E-cadherin level is associated with significantly lower overall survival (P = 0.0004; Fig. 1D) and E-cadherin score correlated with pack years (Supplementary Fig. S1). These results suggest that cigarette smoking is directly related to E-cadherin down-regulation that plays an important role in poor survival.

CSC decreases E-cadherin expression and promoter activity
To verify whether cigarette smoke exposure decreases E-cadherin in smokers, we conducted Western blot analyses for E-cadherin using cell lysates from lung cancer cell lines from never smokers (H1975, HCC827, H3255, and H2170) and smokers (H23, H1299, H2122, and H1395). We observed an overall lower level of E-cadherin in cell lines from smokers as compared with never smokers (Fig. 2A). To determine the mechanism of regulation of E-cadherin by cigarette smoking, we treated immortalized lung epithelial cells (HPL1A and Beas2B) and lung carcinoma cells (A549 and ACC-LC 176) with CSC for up to 96 hours to mimic the conditions of long-term cigarette smoking. The
lysates were tested for E-cadherin expression by Western blot analyses. CSC decreased E-cadherin expression in both time- and dose-dependent manner (Fig. 2B). We selected HPL1A (nontumorigenic) and A549 (tumorigenic) cell lines based on our previous observation that A549 cells become more tumorigenic after chronic CSC treatment, whereas HPL1A cells are not (31). To test the long-term effect of CSC on E-cadherin expression, cells were treated with CSC for up to 120 days. We observed a gradual decrease in E-cadherin expression following CSC treatment starting at 4 days in HPL1A and A549 cells (Fig. 2C). To further test whether E-cadherin down-regulation by CSC is because of transcriptional regulation, we used promoter analyses and RT-PCR using total cellular RNA from A549 and HPL1A cells after treating with CSC. We observed that E-cadherin was repressed by CSC in a dose-dependent manner (Fig. 2D). We further confirmed the reduction in E-cadherin expression in the promoter level by transient transfection assays, using both A549 and HPL1A cells pretreated with CSC for up to 90 days (Fig. 2E). Indeed, E-cadherin promoter activity was decreased significantly by CSC in a time-dependent manner (Fig. 2E), whereas CSC treatment did not affect the mutant E-cadherin promoter level (Fig. 2F). These results indicate that long-term CSC treatment decreases E-cadherin expression at the transcriptional level.

**CSC induces EMT by downregulating E-cadherin**

EMT involves the repression of epithelial-specific adhesion molecules such as E-cadherin and β-catenin with a concomitant expression of proteins such as fibronectin and vimentin (32). To test the effect of CSC-mediated downregulation of E-cadherin on EMT, we treated lung cell lines with CSC. We observed that treatment with CSC induced EMT in A549 cells (Supplementary Fig. S2A). We further confirmed this by verifying whether CSC-mediated EMT is through the loss of expression of membranous E-cadherin and β-catenin. Interestingly, in the immunofluorescence experiments we observed that CSC decreased membranous E-cadherin and β-catenin (Supplementary Fig. S2B).
The epigenetic change, histone deacetylation is important in CSC-induced downregulation of E-cadherin expression

Recent data indicate that cigarette smoke induces cancer-associated epigenetic alterations in cultured respiratory epithelia (33). To test which epigenetic change (histone deacetylation or DNA methylation) is playing a role in CSC-induced downregulation of E-cadherin, lung cell lines were treated with in the presence of either histone deacetylation inhibitors (MS-275 or trichostatin A) or the methylation inhibitor [5-aza-2'-deoxycytidine (AZA)] (Fig. 3B). Treatment of MS-275 suppressed CSC-mediated downregulation of E-cadherin, whereas AZA had no significant effect (Fig. 3C and Supplementary Fig. S3A). Next, we observed that MS-275 treatment blocked CSC-mediated membranous E-cadherin downregulation and increased E-cadherin level to nearly normal (Supplementary Fig. S2C). These results suggest that CSC-mediated downregulation of E-cadherin is through histone deacetylation and E-cadherin expression can be restored by HDACi. To test whether histone deacetylation is playing a role in the downregulation of E-cadherin in lung tumor cell lines from smokers, we treated these cells with the HDACi, MS-275 for 4 days. Interestingly, we observed enhanced E-cadherin expression in 3 of 4 cell lines (Fig. 3D, left). This study was also conducted in lung tumor cell lines from never smokers, which had marginal increase in E-cadherin expression (Fig. 3D, right). We next determined whether the HDACi, MS-275 was playing a role in restoring E-cadherin at the transcriptional level. CSC treatment significantly decreased E-cadherin promoter activity in both A549 and HPL1A cell lines, and MS-275 treatment restored E-cadherin promoter activity (Fig. 3E).
To further test whether E-cadherin upregulation by MS-275 is in the mRNA level, we conducted RT-PCR analyses after treating the cells with MS-275 in presence of CSC. We observed that the E-cadherin expression was induced by MS-275 (100 nmol/L) in the mRNA level, we conducted RT-PCR analyses to further test whether E-cadherin upregulation by MS-275 is in the mRNA level, we conducted RT-PCR analyses.

The HDACi, MS-275 inhibits CSC-induced cell motility, migration, and invasion

As CSC promotes EMT, we tested whether it can affect cell motility of lung A549 and HPL1A cells by wound healing experiment. CSC treatment accelerated wound closure in both cell lines (Fig. 4A). MS-275 suppressed CSC-induced motility of these cells and as a result, the wound remained open. Moreover, CSC stimulated...
Figure 5. CSC represses E-cadherin expression at transcription level through LEF-1 and Slug. Cells were treated with CSC (25 μmol/L) for 4 days. A, conducted RT-PCR for E-cadherin, LEF-1, Slug, ZEB-1, SIP-1, WT-1, and Snail (top), and Western blot analysis for E-cadherin, LEF-1, and Slug (bottom). B and C, polyclonal cells of vector control, shRNAs of LEF-1 and Slug treated with or without CSC, conducted both protein and mRNA expression (B) and immunofluorescence assay for E-cadherin expression (C). D and E, polyclonal cells from knockdown of LEF-1 and Slug were treated with or without CSC in presence or absence of MS-275. D, E-cadherin expression in both A549 and HPL1A knockdown cells by Western blot (left) and quantitative analysis by densitometry of 3 independent experiments (right) were analyzed. E, E-cadherin promoter luciferase activity was analyzed (left) and E-cadherin mutant promoter construct was used to assay the promoter activity (right) in A549 cells. F, the binding of LEF-1 or Slug with the E-cadherin promoter was determined by ChIP assay using parental (A549), vector control, and LEF-1 or Slug knockout clones as described in Materials and Methods. D and E, individual data point represents the mean ± SD of 3 independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001. ns, not significant. E-cadh, E-cadherin.

chemotactic migration and invasion (Fig. 4B) of A549 and HPL1A cells. The number of migrating and invading cells was significantly decreased when treated with MS-275. CSC significantly increased the growth of A549 and HPL1A cells (P < 0.001), whereas MS-275 significantly inhibited CSC-induced cell growth (Fig. 4C). This is supported by [3H] thymidine incorporation assay (Fig. 4D), where CSC induced thymidine incorporation in both cell lines that was inhibited by MS-275 (P < 0.0001). CSC induced the expression of HDAC1 but reduced the expression of HDAC2 and HDAC3 in A549 cells (Supplementary Fig. S3B). MS-275 induced the accumulation of acetylated histones H3 and H4 (Supplementary Fig. S3C). Therefore, these results indicate that smoke condensate induces migration, invasion, and cell proliferation of lung cells, and the HDACi, MS-275 can inhibit these CSC-induced effects.

LEF-1 and Slug is involved in CSC-mediated downregulation of E-cadherin expression

To identify genes that are regulated by CSC treatment and are involved in E-cadherin regulation, we conducted RT-PCR analysis using specific primers for E-cadherin, LEF-1, Slug, ZEB-1, SIP-1, WT-1, and Snail. We observed that CSC treatment suppresses mRNA expression of E-cadherin with concomitant upregulation of LEF-1 and Slug (Fig. 5A, top). However, there were no significant changes in the mRNA expression of ZEB-1, SIP-1, WT-1,
and Snail. To confirm these results at the protein level, we conducted Western blot analyses for E-cadherin, LEF-1, and Slug (Fig. 5A, bottom). Therefore, downregulation of E-cadherin by CSC may be associated with the upregulation of LEF-1 and Slug. To determine the specific involvement of LEF-1 and Slug in the regulation of E-cadherin, we generated stable knockdown clones using shRNA (Supplementary Fig. S4A). We observed an increase in the E-cadherin expression in LEF-1 and Slug knockdown cells with CSC did not show any increase in the expression of LEF-1 and Slug (Fig. 5B). The polyclonal cells were treated with CSC and were analyzed for protein and mRNA expression of LEF-1 and Slug. The knockdown cells did not show any increase in their level of expression with CSC. Interestingly, when the knockdown clones were treated with CSC, there was no significant decrease in the expression of E-cadherin in both A549 and HPL1A cells (polyclones, Figs. 5D; for single clones, Supplementary Fig. S4B and C). In addition, there was no significant decrease in the membranous localization of E-cadherin by CSC in the LEF1 or Slug knockdown polyclonal population from both cell lines (Fig. 5C). In contrast, the vector control cells showed a decrease in their E-cadherin expression by CSC treatment. Similarly, the E-cadherin promoter activity did not change with CSC treatment in LEF1 and Slug knockdown cells but vector control cells showed significant decrease in the promoter activity (Fig. 5E, left). However, LEF1 and Slug knockdown cells did not show any significant change in the mutant E-cadherin promoter level (Fig. 5E, right). Therefore, these results suggest that CSC decreases E-cadherin expression through the induction of LEF-1 and Slug. Next, we conducted ChIP assay to determine whether LEF-1 or Slug binds to the E-cadherin promoter in these cells (Fig. 5F). PCR was conducted with primers specific for the LEF-1 (left) and Slug (right) binding sites. CSC treatment increases the ability of LEF-1 and Slug to bind to the E-cadherin promoter in vector control cells. There was no binding of LEF-1 and Slug on the E-cadherin promoter in knockdown cells. Moreover, CSC treatment could not increase their binding to the E-cadherin promoter. Collectively, our results suggest an important role of LEF-1 and Slug as transcriptional repressors of the E-cadherin gene in CSC-mediated EMT.

The HDACi, MS-275 reverses the downregulation of E-cadherin by LEF-1 and Slug

To determine whether LEF-1 and Slug were responsible for MS-275-mediated E-cadherin expression, we initially did the protein expression of LEF-1 and Slug with CSC and/or MS-275 (Supplementary Fig. S6A). The slug knockdown cells with CSC did not show increase in the expression of mesenchymal markers (Supplementary Fig. S6B). As expected, CSC increased expression of LEF-1 and Slug, but addition of MS-275 did not change these expressions. Then knocked down LEF-1 and Slug clones from A549 and HPL1A cells were treated with CSC and/or MS-275, and analyzed for E-cadherin expression (Supplementary Figs. S6C and S6D). The individual cell clones of knockdown of LEF-1 or Slug led to an increase in the expression of E-cadherin, and MS-275 treatment of knockdown clones mostly restored E-cadherin expression after treatment of CSC in both cell lines. Furthermore, the knockdown polyclonal cells showed significant increase in the E-cadherin promoter activity when compared to vector control cells, and MS-275 treatment or CSC treatment showed no significant change (Fig. 5E). Interestingly, the expression of E-cadherin was not changed significantly in CSC and MS-275 treated cells when compared with MS-275 treated alone. These data show that LEF-1 and Slug are involved in CSC-mediated downregulation of E-cadherin through histone deacetylation, and MS-275 restores E-cadherin expression. Interestingly, the LEF-1 and Slug knockdown polyclonal cells showed significant decrease in cell migration (Supplementary Fig. S7A) and proliferation (Supplementary Fig. S7B) when compared to vector control cells, and MS-275 treatment or CSC treatment showed no significant change in these knockdown cells. These data suggest that MS-275 has no effect on CSC-induced cell migration and proliferation in LEF-1 and Slug knockdown cells.

Discussion

An important adverse effect of smoking on lung cells is DNA alteration and mutation, which if not repaired, may lead to an aberrant irreversible cell proliferation. The reversible changes mostly involve EMT, migration, and invasion through changes in the molecular expression of tumor suppressor genes and/or oncogenes. We began our present study with the goal of evaluating the expression of E-cadherin in relation to the smoking status of lung cancer patients. Interestingly, we observed a decrease in the expression of E-cadherin in smokers when compared with never smokers. Our results suggest that survival outcomes are significantly worse in current or former smokers compared with never smokers. E-cadherin expression was decreased in both former and current smokers when compared with never smokers and our finding that survival outcomes are significantly improved when E-cadherin expression is higher (Fig. 1). This observation is also supported by the finding that the E-cadherin level is lower in cell lines from smokers in comparison to those from never smokers (Fig. 2A). In addition to E-cadherin (34), alterations in other factors such as p38MAPK (35), twist (36), cyclin D1 (37), and DNA repair capacity (38) are also involved in smoking related reduced survival in cancer patients. Our results reveal, for the first time, that smoking-mediated decrease in E-cadherin expression plays a key role in the induction in EMT in lung cancer.
Our finding of a decrease in E-cadherin expression in smokers is consistent with the role it plays in lung cancer progression and metastasis (39). Therefore, E-cadherin is an important molecular marker in smokers. Our data are in agreement with the previous study that never smokers had longer survival than smokers (34). Surprisingly, former smokers had a higher propensity toward a worse prognosis and poor survival compared with current smokers (Fig. 1A), although the E-cadherin expression profiles in both current and former smokers were similar (Fig. 1C). This study shows that there is a strong correlation of downregulation of E-cadherin with smoking status and survival.

We have investigated the prooncogenic effects of smoking on the initiation and progression of lung cancer that mimic the conditions of long-term cigarette smoking. We have chosen immortalized bronchial epithelial cell line (HPL1A, nontumorigenic, never smoker cell line) to study the initiation and lung tumor cell line (A549, tumorigenic) to study the prooncogenic effects during tumor progression. Results presented here show that chronic treatment of HPL1A and A549 cells with CSC induces EMT through the downregulation of E-cadherin and β-catenin with a concomitant upregulation of mesenchymal markers such as vimentin, fibronectin, and N-cadherin (Fig. 3A). This study shows mechanistically the combined and long-term effects of all carcinogenic components of cigarette smoke on the EMT of both immortalized and tumor cells, whereas the previous studies suggested the effects of 1 component in tobacco smoke such as nicotine (40) or benzopyrene (41) in higher concentrations on the EMT of tumor cells. The ability of CSC to induce EMT of HPL1A cells suggests that cigarette smoking may be involved in initiating EMT in NSCLC.

E-cadherin expression is repressed at both mRNA and protein levels by means of changes in transcription events and histone acetylation (Fig. 2). As E-cadherin is considered to be a tumor suppressor, the identification of transcription factors that cause E-cadherin repression and EMT induction has been the topic of intensive investigation. We have shown that LEF-1 and Slug are upregulated by CSC treatment, and knockdown of these 2 factors attenuates E-cadherin downregulation by CSC leading to its membranous localization (Fig. 5C and D). This is supported by the fact that LEF-1 and Slug knockdown decrease their binding to the E-cadherin promoter in control and CSC treated cells in ChIP assays. As a result, E-cadherin promoter activity is increased in LEF-1 and Slug knockdown clones in both A549 and HPL1A cell lines. Therefore, it is possible that induction in LEF-1 and Slug is important in inducing EMT through the downregulation of E-cadherin in smokers.

In an attempt to further understand the mechanism of cigarette smoke-induced EMT in lung cell lines, we observed that HDACi, MS-275 reverses EMT through the restoration of E-cadherin expression and its localization on the membrane (Figs. 3B and Supplementary Fig. S2C). The restoration of E-cadherin expression in CSC treated cells is concomitant with the increase in its promoter activity in response to MS-275 (Fig. 3E). These results suggest a role of LEF-1 and Slug in recruiting HDACi activity to the E-cadherin promoter in smokers and repressing E-cadherin transcription. These studies are in agreement with the observation that E-cadherin expression in cell lines from smokers is less than that from nonsmokers (Fig. 2A) and that E-cadherin expression in cell lines from smokers is increased in response to HDACi (Fig. 3D). Previous studies suggest that smokers tend to have more invasive and metastatic cancer than never smokers, which is in consistent with our findings that CSC treatment makes the cells more motile and invasive, most likely through EMT (Fig. 4). Treatment with HDACi, MS-275 inhibits CSC-induced cell migration and invasion through E-cadherin expression (Fig. 3). The specificity of the effect of HDACi through E-cadherin expression was tested using LEF-1 and Slug knock down cells, where MS-275 treatment has no significant effect on CSC-mediated cell migration and proliferation (Supplementary Fig. S7). Although the HDACi is in several clinical trials (17, 18, 20), nothing is known about smoking related molecular processes including E-cadherin deregulation that underlie the antitumor effects of these inhibitors in lung cancer. However, this study shows that restoration of E-cadherin expression by MS-275 in smokers with lung carcinoma may have a broad impact in the development of successful therapeutic strategies.

In summary, we have shown for the first time, how cigarette smoking plays an important role in promoting EMT, cell migration and invasion in NSCLC through deregulation of E-cadherin that leads to poor patient survival. The results presented here also show the role played by LEF-1 and Slug proteins in E-cadherin transcriptional repression. Another significant observation is that downregulation of E-cadherin is mediated by epigenetic change, histone deacetylation, which opens up an avenue to target NSCLCs by HDAC inhibitors in combination with other chemotherapeutic agents.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: N.S. Nagathihalli, P.P. Massion, P.K. Datta
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