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## 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 demonstrated 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 regarding 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 (E-cadh) levels and loss of E-cadh is a poor prognostic factor in smokers. Moreover, the downregulation of E-cadh correlates with the number of pack-years. In an attempt to determine the role of long-term cigarette smoking on EMT, we have observed that treatment of lung cell lines with cigarette smoke condensate (CSC) induces EMT through downregulation of epithelial markers including E-cadh and upregulation of mesenchymal markers. CSC decreases E-cadh expression at the transcriptional level through upregulation of LEF1 and Slug, and knockdown of these two proteins increases E-cadh expression. Importantly, chromatin immunoprecipitation assays suggest that LEF-1 and Slug binding to E-cadh promoter is important for CSC-mediated downregulation of E-cadh. The histone deacetylase inhibitor (HDACi) MS-275 reverses CSC-induced EMT, migration and invasion through the restoration of E-cadh expression. These results suggest that recruitment of HDACs by transcriptional repressors, LEF-1 and Slug is responsible for E-cadh suppression and EMT in cigarette smokers and provide a potential drug target towards the treatment of lung cancer.

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## Keywords

E-cadherin; cigarette smoking; EMT; HDAC inhibitor; lung cancer

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## Introduction

The majority of lung cancer related deaths (>85%) are from non-small cell lung cancer (NSCLC), composed of 3 predominant subtypes: adeno, 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 (E-cadh). Reduced expression or loss of membrane localization of E-cadh 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-cadh in tumors. The zinc finger transcriptional repressors of the Snail/Slug family, SIP-1 and ZEB-1, bind to two E-boxes on the E-cadh promoter and interact with the transcriptional corepressor, CtBP, which recruits histone deacetylases to facilitate silencing of E-cadh expression (4, 10, 11). In addition, two types of basic-helix-loop-helix (bHLH) proteins, E12/E47 (12) and Twist (13) have also been shown to repress E-cadh promoter activity and to induce the EMT phenotype. A normal role of E-cadh is to bind and sequester cytoplasmic  $\beta$ -catenin, thus rendering  $\beta$ -catenin unavailable for signaling in the canonical Wnt/LEF/ $\beta$ -catenin signaling pathway (14).

Histone deacetylase inhibitors (HDACi) are potent antiproliferative 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-cadh.

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 demonstrate, for the first time, that cigarette smoking induces the repression of E-cadh by regulating transcription factors LEF-1 and Slug, which leads to EMT. This is supported by the observation that the E-cadh 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-cadh 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% fetal bovine serum (FBS). Human lung adenocarcinoma (ACC-LC-172) and peripheral lung epithelial (HPL1A) (a kind gift from Dr. T. Takahashi) 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, St Louis, MO). 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% CO<sub>2</sub>. 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 (TMA)

Tissues were obtained from surgical specimens through the thoracic center at the Vanderbilt-Ingram Cancer Center. TMA was constructed and the immunohistochemistry (IHC) staining procedure followed as previously described (22). There are 310 tumors represented on the arrays. TMA slides stained for hematoxylin and eosin (H&E) and E-cadh were concurrently evaluated by our pathologist (A.L.G). 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 nM) in each assay. Membranes were probed with primary antibodies and densitometry analyses were performed 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-cadh (1:500) or anti  $\beta$ -catenin (1:500) antibody, and afterward conjugated with Cy3 (Sigma Biochemicals) secondary antibody. The cells were imaged with a Zeiss Axiophot microscope. Images were merged using NIH Image J software.

### RNA isolation and reverse transcription (RT)-PCR analyses

Cells were grown with or without CSC (25 or 125  $\mu$ g/ml) for 4 days, and total RNA was isolated with TriZol™ reagent (Invitrogen). The 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 co-transfected with a firefly E-cadh luciferase promoter-reporter construct (pGL2 E-cadh3/Luc containing E-cadh 5' flanking sequences of 1485 bp) and E-box mutant E-cadh (Addgene plasmid 19291) luciferase construct (kindly provided by Dr. E. R. Fearon) (26, 27) with an equal amount of

total DNA by using Lipofectamine Plus reagents (Invitrogen). Luciferase activity was normalized to  $\beta$ -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 [ $^3\text{H}$ ] thymidine incorporation assays were performed 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 (ChIP) analysis**

A549 cells were grown to confluence before being crosslinked with 1% formaldehyde, lysed and sonicated as described previously (30). Chromatin fragments were co-immunoprecipitated with the LEF-1 or Slug antibodies. Further steps were performed following the protocol from the EZ ChIP kit (Upstate Biotechnology). The PCRs were performed with the E-cadh 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 three independent experiments; and are expressed as the means  $\pm$  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-cadh expression in patients with lung cancer**

Cigarette smoking is strongly correlated with the onset of non-small cell lung cancer, and loss of E-cadh is a poor prognostic factor. To determine the role of smoking on E-cadh 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-cadh in patients with lung cancer by immunohistochemical analyses of TMAs that contain tumor tissues from never, current and former smokers with adeno, squamous cell and large cell carcinoma (Figs. 1B and 1C). The TMA was stained with anti-E-cadh antibody and the index score was calculated. Our data showed significantly reduced ( $p < 0.001$ ) levels of E-cadh expression in current as well as former smokers when compared to never-smokers in

both squamous cell and adenocarcinoma patients (Fig. 1C). The impact of cigarette smoking on E-cadh expression was apparent when we subdivided the former and current smokers based on the pack-years in both adeno and squamous cell carcinoma patients (Fig. 1D). Only never-smokers had a strong E-cadh expression compared with former and current smokers. In adeno and squamous cell carcinoma, the patients with weaker E-cadh expression tended to have higher pack years in both current and former smokers. Low E-cadh level is associated with significantly lower overall survival ( $p=0.0004$ ) (Fig. 1E) and E-cadh score correlated with pack-years (Supplementary Fig. S1). These results suggest that cigarette smoking is directly related to E-cadh downregulation that plays an important role in poor survival.

### **Cigarette Smoke Condensate (CSC) decreases E-cadh expression and promoter activity**

To verify whether cigarette smoke exposure decreases E-cadh in smokers, we performed Western blot analyses for E-cadh 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-cadh in cell lines from smokers as compared to never-smokers (Fig. 2A). To determine the mechanism of regulation of E-cadh 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 h to mimic the conditions of long-term cigarette smoking. The lysates were tested for E-cadh expression by Western analyses. CSC decreased E-cadh expression in both time- and dose-dependent manner (Fig. 2B). We selected HPL1A (non-tumorigenic) 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-cadh expression, cells were treated with CSC for up to 120 days. We observed a gradual decrease in E-cadh expression following CSC treatment starting at 4 days in HPL1A and A549 cells (Fig. 2C). To further test whether E-cadh downregulation by CSC is due to transcriptional regulation, we employed promoter analyses and RT-PCR using total cellular RNA from A549 and HPL1A cells after treating with CSC. We observed that E-cadh was repressed by CSC in a dose dependent manner (Fig. 2D). We further confirmed the reduction in E-cadh expression in the promoter level by transient transfection assays, using both A549 and HPL1A cells pre-treated with CSC for up to 90 days (Fig. 2E). Indeed, E-cadh promoter activity was decreased significantly by CSC in a time dependent manner (Fig. 2E), whereas CSC treatment did not affect the mutant E-cadh promoter level (Fig. 2F). These results indicate that long-term CSC treatment decreases E-cadh expression at the transcriptional level.

### **CSC induces EMT by downregulating E-cadh**

EMT involves the repression of epithelial-specific adhesion molecules like E-cadh and  $\beta$ -catenin with a concomitant expression of proteins like fibronectin and vimentin (32). To test the effect of CSC-mediated downregulation of E-cadh 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-cadh and  $\beta$ -catenin. Interestingly, in the immunofluorescence experiments we observed that CSC decreased membranous E-cadh and  $\beta$ -catenin (Supplementary Fig. S2B). This was supported by the findings that E-cadh and  $\beta$ -catenin expressions were reduced by CSC in a dose- and time-dependent manner in both A549 and Beas2B cells (Fig. 3A), suggesting that CSC induces EMT through the downregulation of E-cadh. Moreover, Fig. 3A shows that CSC-induced EMT is associated with an increase in the levels of mesenchymal markers including fibronectin, N-cadherin and vimentin in both A549 and Beas2B cells. These results suggest that the exposure of lung cancer cells to CSC induces EMT through the downregulation of E-cadh from membrane.



### The epigenetic change, histone deacetylation is important in CSC-induced downregulation of E-cadh expression

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

### 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 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 [ $^3\text{H}$ ] 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-cadh expression

To identify genes that are regulated by CSC treatment and are involved in E-cadh regulation, we performed RT-PCR analysis using specific primers for E-cadh, LEF-1, Slug, ZEB-1, SIP-1, WT-1, and Snail. We observed that CSC treatment suppresses mRNA expression of E-cadh with concomitant upregulation of LEF-1 and Slug (Fig. 5A, top panel). 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-cadh, LEF-1 and Slug (Fig. 5A, bottom panel). Therefore, downregulation of E-cadh 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-cadh, we generated stable knockdown clones using shRNA (Supplementary Fig. S4A). We observed an increase in the E-cadh expression in LEF-1 and Slug knockdown shRNA clones in both A549 (Supplementary Fig. S4B) and HPL1A cell lines (Supplementary Fig. S4C). These LEF-1 and Slug knockdown clones showed an increase in the E-cadh promoter activity (Supplementary Fig. S5). To avoid any effect due to clonal variation, we stably knocked down the expression of LEF-1 and Slug in both A549 and HPL1A polyclonal cells (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-cadh in both A549 and HPL1A cells (for polyclones, Figs. 5D; for single clones, Supplementary Figs. S4B and S4C). In addition, there was no significant decrease in the membranous localization of E-cadh 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-cadh expression by CSC treatment. Similarly, the E-cadh 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 panel). However, LEF1 and Slug knockdown cells did not show any significant change in the mutant E-cadh promoter level (Fig. 5E, right panel). Therefore, these results suggest that CSC decreases E-cadh expression through the induction of LEF-1 and Slug. Next, we performed ChIP assay to determine whether LEF-1 or Slug binds to the E-cadh promoter in these cells (Fig. 5F). PCR was performed with primers specific for the LEF-1 (left panel) and Slug (right panel) binding sites. CSC treatment increases the ability of LEF-1 and Slug to bind to the E-cadh promoter in vector control cells. There was no binding of LEF-1 and Slug on the E-cadh promoter in knockdown cells. Moreover, CSC treatment could not increase their binding to the E-cadh promoter. Collectively, our results suggest an important role of LEF-1 and Slug as transcriptional repressors of the E-cadh gene in CSC-mediated EMT.

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

To determine whether LEF-1 and Slug were responsible for MS-275 mediated E-cadh 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-cadh 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-cadh, and MS-275 treatment of knockdown clones mostly restored E-cadh expression after treatment of CSC in both cell lines. Furthermore, the knockdown polyclonal cells showed significant increase in the E-cadh promoter activity when compare to vector control cells, and MS-275 treatment or CSC treatment showed no significant change (Fig. 5E). Interestingly, the expression of E-cadh was not changed significantly in CSC and MS-275 treated cells when compared to MS-275 treated alone. These data demonstrate that LEF-1 and Slug are involved in CSC-mediated downregulation of E-cadh through histone deacetylation, and MS-275 restores E-cadh 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 compare 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-cadh in relation to the smoking status of lung cancer patients. Interestingly, we observed a decrease in the expression of E-cadh in smokers when compared to never-smokers. Our results suggest that survival outcomes are significantly worse in current or former smokers compared to never-smokers. E-cadh expression was decreased in both former and current smokers when compared to never-smokers and our finding that survival outcomes are significantly improved when E-cadh expression is higher (Fig. 1). This observation is also supported by the finding that the E-cadh level is lower in cell lines from smokers in comparison to those from never smokers (Fig. 2A). In addition to E-cadh (34), alterations in other factors like 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-cadh expression plays a key role in the induction in EMT in lung cancer.

Our finding of a decrease in E-cadh expression in smokers is consistent with the role it plays in lung cancer progression and metastasis (39). Therefore, E-cadh 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 towards a worse prognosis and poor survival compared to current smokers (Fig. 1A), although the E-cadh expression profile in both current and former smokers were similar (Fig. 1D). This study shows that there is a strong correlation of downregulation of E-cadh with smoking status and survival.

We have investigated the pro-oncogenic 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, non-tumorigenic, never smoker cell line) to study the initiation and lung tumor cell line (A549, tumorigenic) to study the pro-oncogenic 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-cadh and  $\beta$ -catenin with a concomitant upregulation of mesenchymal markers like vimentin, fibronectin and N-cadh (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 one component in tobacco smoke like 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-cadh expression is repressed at both mRNA and protein levels by means of changes in transcription events and histone acetylation (Fig. 2). As E-cadh is considered to be a tumor suppressor, the identification of transcription factors that cause E-cadh 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 two factors attenuates E-cadh downregulation by CSC leading to its membranous localization (Fig. 5C and 5D). This is supported by the fact that LEF-1 and Slug knockdown decrease their binding to the E-cadh promoter in control and CSC treated cells in ChIP assays. As a result, E-cadh 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-cadh 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-cadh expression and its localization on the membrane (Figs. 3B and Supplementary Fig. S2C). The restoration of E-cadh 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-cadh promoter in smokers and repressing E-cadh transcription. These studies are in agreement with the observation that E-cadh expression in cell lines from smokers is less than that from non-smokers (Fig. 2A) and that E-cadh 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-cadh expression (Fig. 3). The specificity of the effect of HDACi through E-cadh 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-cadh deregulation that underlie the antitumor effects of these inhibitors in lung cancer. However, this study demonstrates that restoration of E-cadh 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-cadh that leads to poor patient survival. The results presented here also demonstrate the role played by LEF-1 and Slug proteins in E-cadh transcriptional repression. Another significant observation is that downregulation of E-cadh is mediated by epigenetic change, histone deacetylation, which opens up an avenue to target non-small cell lung cancers by HDAC inhibitors in combination with other chemotherapeutic agents.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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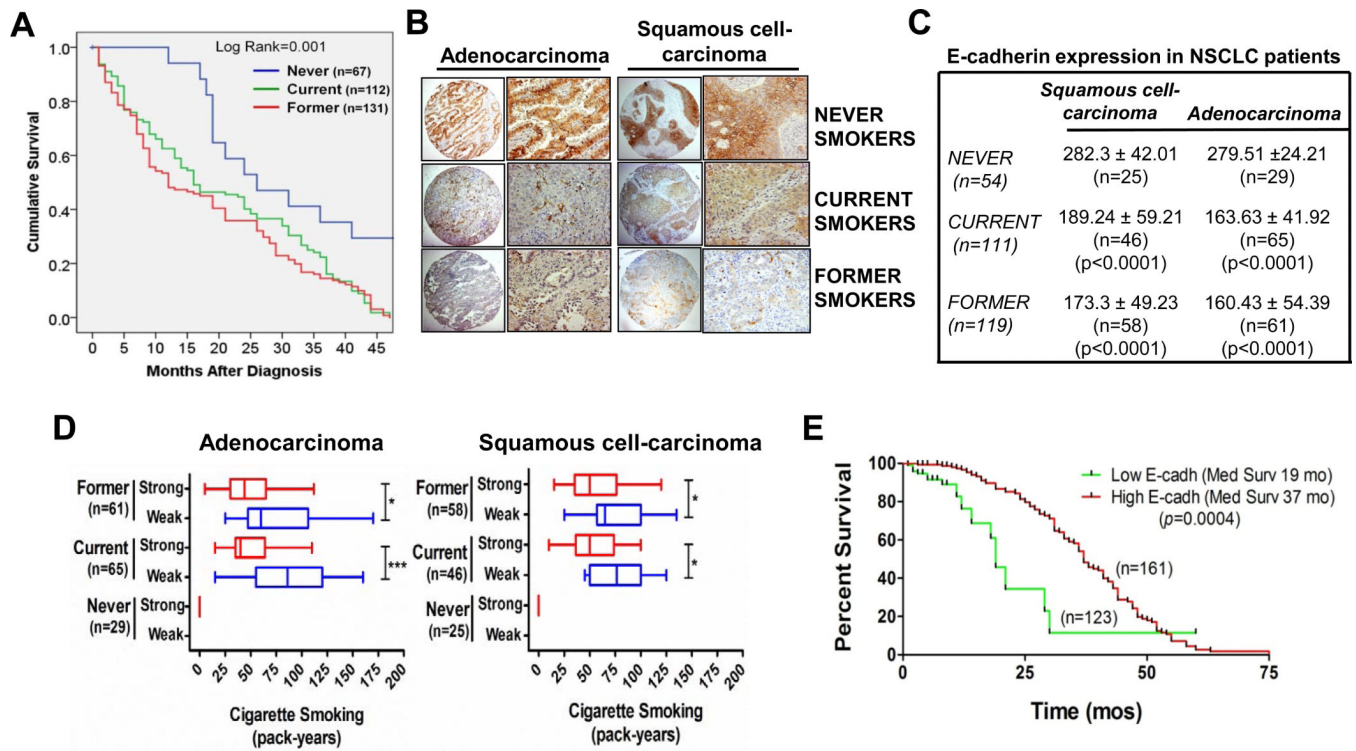
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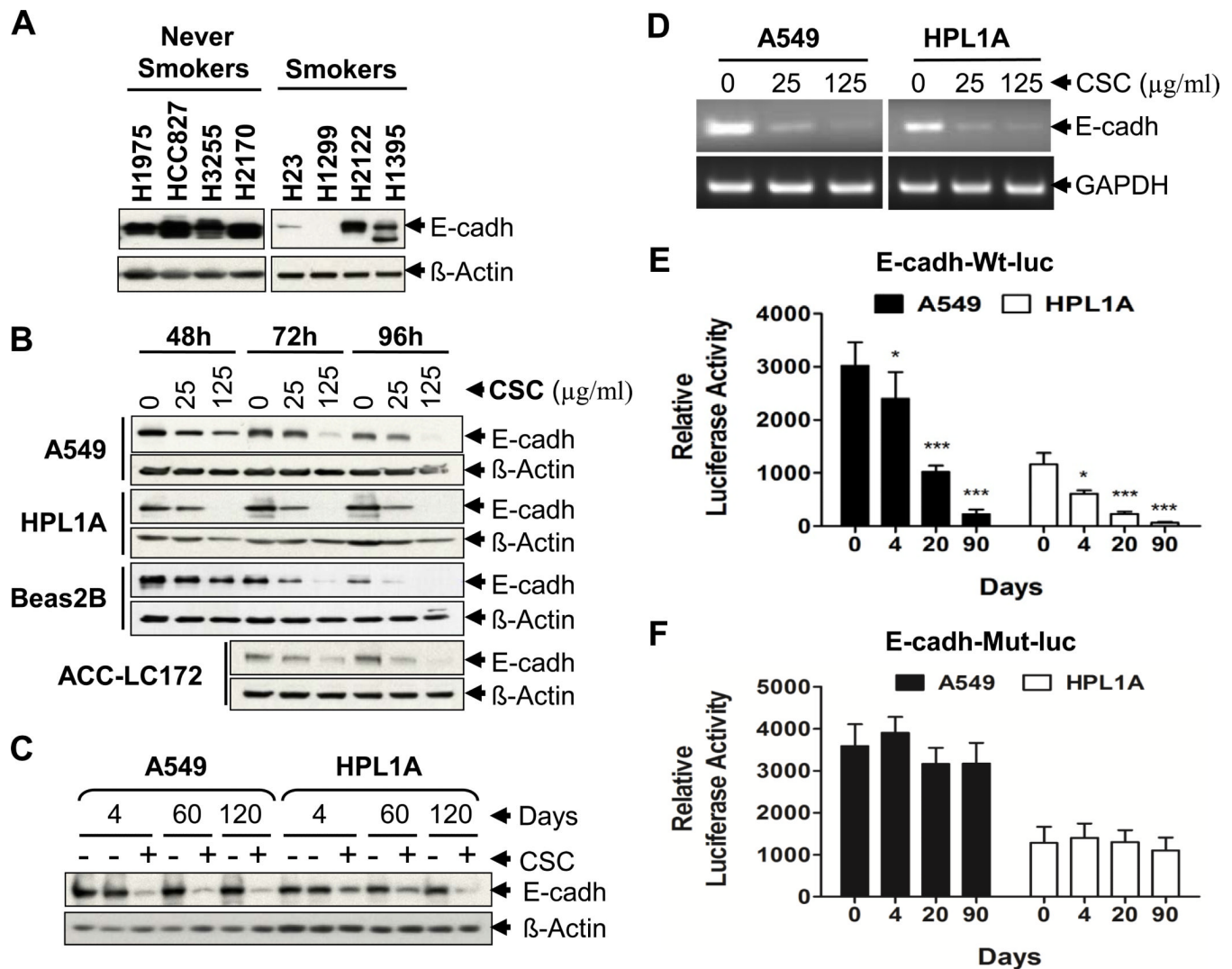
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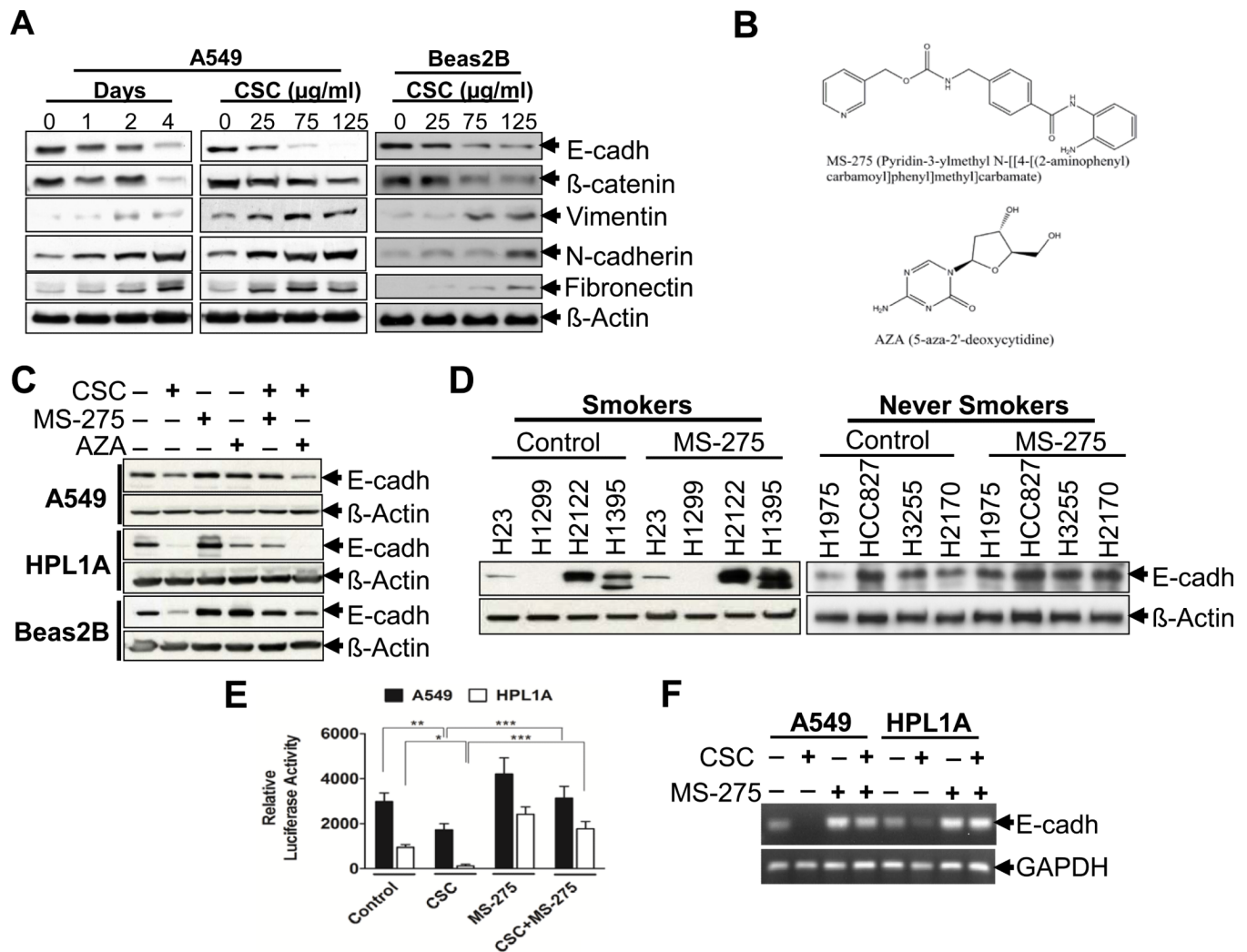


**Figure 1.** Overall survival and E-cadh expression in human lung cancer patients with cigarette smoking status. (A) Kaplan-Meier survival curve for overall survival of lung cancer patients with never-smokers versus current smokers or former smokers. (B) Expression of E-cadh in human lung cancers by IHC analyses of TMAs that contain normal and tumor tissues from patients, either current or former smokers, with adeno and squamous cell carcinoma. Representative of E-cadh staining in one specimen from each group was shown. (C) TMA was stained for E-cadh and the index score was calculated as described in Materials and Methods. (D) Effect of pack-years of cigarette smoking on the expression of E-cadh in adeno and squamous cell carcinoma patients with smoking status. Red color indicates strong and blue color indicates weak E-cadh expression. \*p<0.05; \*\*\*p<0.001. (E) Kaplan-Meier survival curve for overall survival of lung cancer patients according to staining for E-cadh.

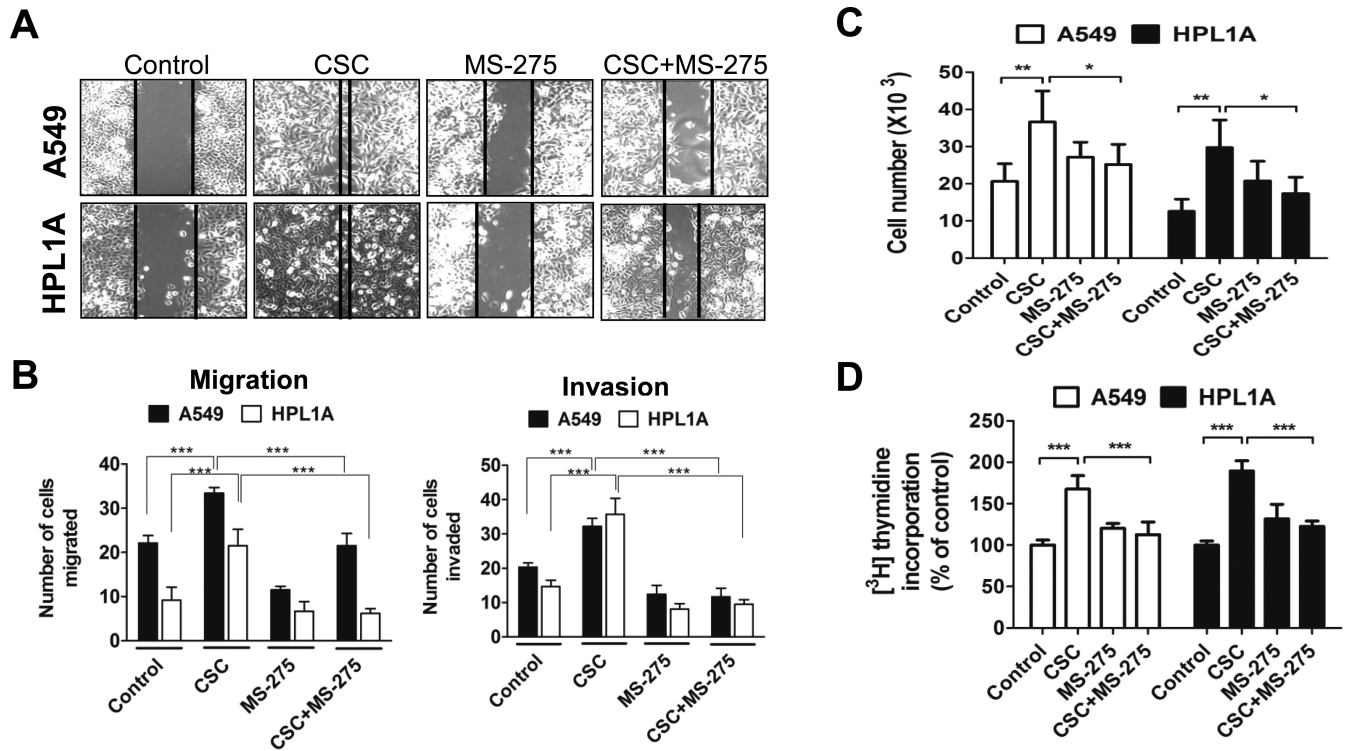


**Figure 2.** CSC decreases E-cadh expression through transcriptional regulation. (A) To verify whether cigarette smoke can decrease E-cadh expression in smokers, performed E-cadh expression by Western blot using cell lysates from lung cancer cell lines from never-smokers (H1975, HCC827, H3255, H2170) and smokers (H23, H1299, H2122, H1395). (B) Cells were treated with CSC in a dose- and time-dependent manner and analyzed for E-cadh protein expression. (C) To test the long-term effect of CSC on E-cadh protein expression, cells were treated with CSC (25  $\mu$ g/ml) for up to 120 days. (D) RT-PCR analyses were performed to verify whether E-cadh downregulation by CSC is at the transcription level. (E) Transient transfection assays using A549 and HPL1A cells pre-treated with CSC (25  $\mu$ g/ml) for indicated time points and E-cadh promoter luciferase activity was analyzed. (F) E-cadh mutant promoter construct was used to assay the promoter activity in CSC treated cells as detailed above in (E). (E) and (F) Individual data point represents the mean  $\pm$  SD of three independent experiments. \* $p$ <0.05; \*\*\* $p$ <0.001.

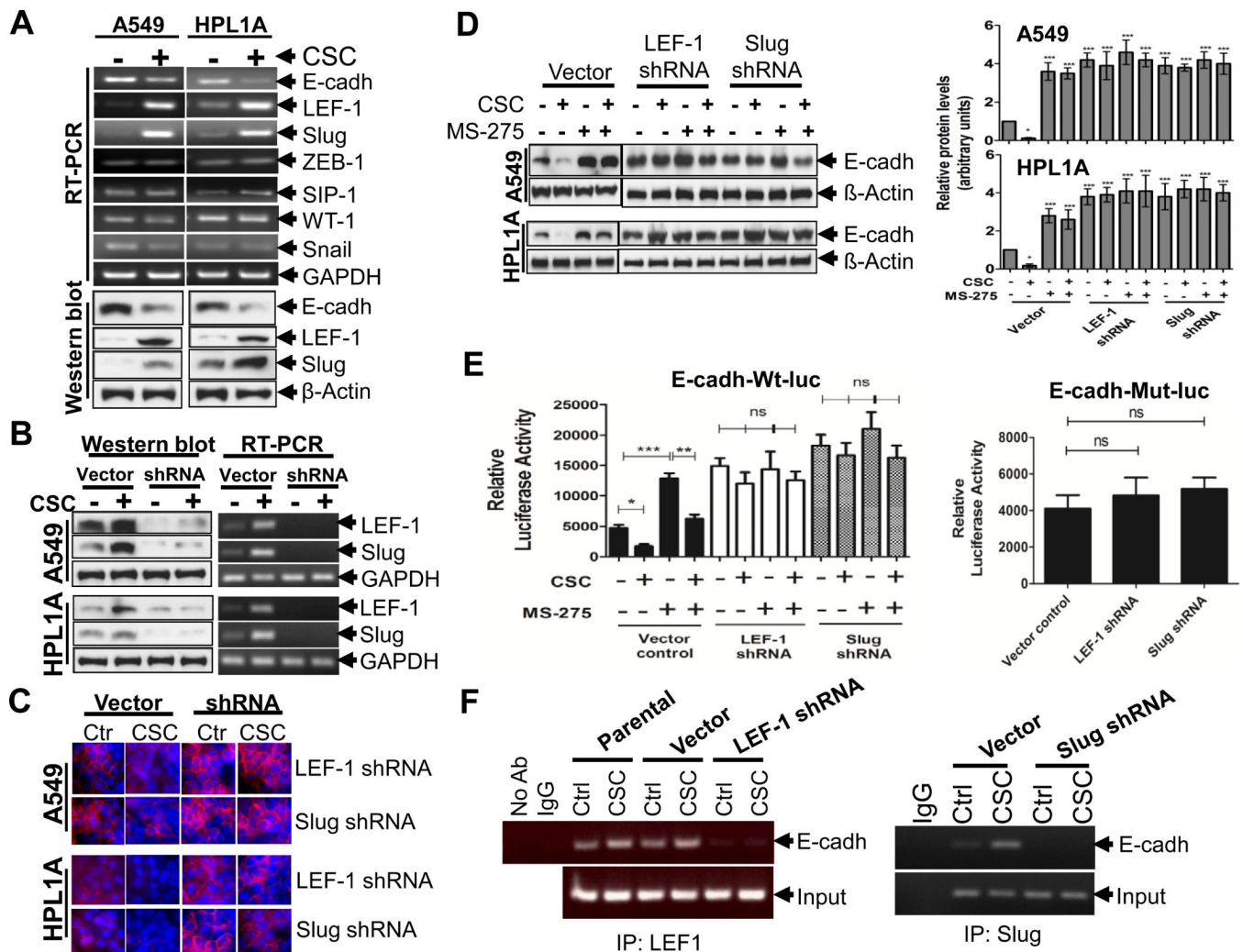


**Figure 3.**

CSC induces EMT and MS-275 reverses the effect of cigarette smoke induced repression of E-cadh expression. (A) A549 and Beas2B cells were treated with CSC (0-125  $\mu\text{g/ml}$ ) for 4 days and for the indicated time points (CSC: 25  $\mu\text{g/ml}$ ) and analyzed for E-cadh,  $\beta$ -catenin, vimentin, N-cadherin and fibronectin protein expression. (B) The chemical structures of MS-275 (top panel) and AZA (bottom panel). (C) Cells were treated with CSC (25  $\mu\text{g/ml}$ ) in presence or absence of either MS-275 (100 nM) or AZA (1 $\mu\text{M}$ ) for 4 days and analyzed for E-cadh protein expression. (D) Cell lines from smokers and never smokers were treated with HDACi, MS-275 (100 nM) for 4 days and analyzed for E-cadh protein expression. (E) The E-cadh promoter level analyzed by transient transcription assays. Individual data point represents the mean  $\pm$  SD of three independent experiments. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$ . (F) E-cadh stabilization was analyzed at the mRNA level.

**Figure 4.**

CSC induced motility, migration and invasion is blocked by HDACi MS-275. (A) Cells were treated with CSC (25  $\mu\text{g}/\text{ml}$ ) with or without MS-275 (100 nM) for 30 h and performed wound healing experiment. (B) CSC stimulates chemotactic migration (left panel) and invasion (right panel); and MS-275 blocked this event. (C) Cell counting and (D)  $[^3\text{H}]$  thymidine incorporation assay was done by treating cells with or without CSC (25  $\mu\text{g}/\text{ml}$ ) or MS-275 (100 nM) for 4 days as detailed in Materials and Methods. (B), (C) and (D) Individual data point represents the mean  $\pm$  SD of three independent experiments. \* $p < 0.05$ ; \*\* $p < 0.01$ ; \*\*\* $p < 0.001$

**Figure 5.**

CSC represses E-cadh expression at transcription level through LEF-1 and Slug. Cells were treated with CSC (25  $\mu$ g/ml) in presence or absence of MS-275 (100 nM) for 4 days. (A) Performed RT-PCR for E-cadh, LEF-1, Slug, ZEB-1, SIP-1, WT-1, and Snail (upper panel); and Western blot for E-cadh, LEF-1 and Slug (lower panel). (B) and (C) Polyclonal cells of vector control, shRNAs of LEF-1 and Slug treated with or without CSC, performed both protein and mRNA expression (B) and immunofluorescence assay for E-cadh 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. E-cadh expression in both A549 and HPL1A knockdown cells by Western blot (left) and quantitative analysis by densitometry of three independent experiments (right) were analyzed (D); E-cadh promoter luciferase activity was analyzed (left panel) and E-cadh mutant promoter construct was used to assay the promoter activity (right panel) in A549 cells. (F) The binding of LEF-1 or Slug with the E-cadh 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  $\pm$  SD of three independent experiments. \* $p$ <0.05; \*\* $p$ <0.01; \*\*\* $p$ <0.001; ns=not significant.