

Original Paper

LncRNA LCPAT1 Mediates Smoking/Particulate Matter 2.5-Induced Cell Autophagy and Epithelial-Mesenchymal Transition in Lung Cancer Cells via RCC2

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Key Words

Lung cancer mortality • Cigarette smoking • PM2.5 • Additive interaction • Autophagy • Epithelial–mesenchymal transition (EMT) • LCPAT1

Abstract

Background/Aims: Ecological studies have shown that air pollution and prevalence of cigarette smoking are positively correlated. Evidence also suggests a synergistic effect of cigarette smoking and PM2.5 exposure (Environmental Particulate Matter $\leq 2.5 \mu\text{m}$ in diameter) on lung cancer risk. We aimed to evaluate the interaction between smoking prevalence and PM2.5 pollution in relation to lung cancer mortality and determine its underlying mechanisms *in vitro*. **Methods:** “MOVER” method was used to analyze the interaction between smoking prevalence and PM2.5 pollution in relation to lung cancer mortality. Cell autophagy and malignant behaviors induced by cigarette smoke extract (CSE) and PM2.5 exposure were examined *in vitro*. Gene expression was examined by qRT-PCR and western blot. RNA and protein interaction was determined using a RNA binding protein immunoprecipitation assay. **Results:** An increased risk for lung cancer death (RERI (the relative excess risk) = 0.28) was observed with a synergistic interaction between cigarette smoking and PM2.5 pollution. Cell migration, invasion, EMT (epithelial-mesenchymal transition) and autophagy were elevated when lung cancer cells were treated with CSE and PM2.5 in combination. A lncRNA, named lung cancer progression-association transcript 1 (*LCPAT1*), was up-regulated after the treatment of CSE and PM2.5, and knocking down the lncRNA impaired the effect of CSE and PM2.5 on lung cancer cells. In addition, *LCPAT1* was shown to bind to RCC2, and RCC2 mediated the effect of *LCPAT1* on cell autophagy, migration, invasion and EMT in lung cancer. **Conclusions:** Our results suggest that combined exposure to CSE and PM2.5 induces *LCPAT1* expression, which up-regulates autophagy, and promotes lung cancer progression via RCC2.

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Introduction

Lung cancer is the most common cancer worldwide, with an estimated 1.82 million new lung cancer cases and 1.59 million deaths in 2012 globally [1]. According to the International Agency for Research on Cancer (IARC), smoking is a major risk factor of lung cancer [2]. Although many countries have implemented tobacco control programs and smoking prevalence has significantly declined, smoking still accounted for 11.5% of deaths worldwide in 2015 [3] and ranked as the second major risk factor for early death and disability [4]. Air pollution by Particulate Matter ≤ 2.5 μm in diameter (PM2.5) is another strong risk factor for lung cancer. PM2.5 has been classified as Group 1 carcinogens and caused 223,000 lung cancer deaths in 2010 [5]. In addition, according to the Global Burden of Disease Study in 1990–2015, PM2.5 air pollution increased by 11.9%, especially during 2010–2015 [6].

A large epidemiologic study of 387,195 adults showed that air pollution was positively associated with cigarette smoking, suggesting that smokers were more likely to encounter PM2.5 pollution than non-smokers [7]. Moreover, compared to pregnant non-smokers, a stronger relationship between outdoor air pollution exposure and micronuclei (MN) frequencies was observed in pregnant smokers [8]. A recent meta-analysis reported that the association between PM2.5 exposure and lung cancer risk was more evident among former smokers than never-smokers or current smokers [9]. The interaction between cigarette smoking and PM2.5 exposure has been studied for its effect on lung cancer risk. A higher risk of lung cancer was observed among individuals who were exposed to both risk factors [10, 11]. Despite strong epidemiologic evidence in support of a possible joint effect of cigarette smoking and PM2.5 exposure, no studies have evaluated the underlying mechanism involved in the influence of these risk factors in combination on lung cancer etiology.

Long non-coding RNAs (lncRNAs) are long non-protein coding transcripts with 200 nucleotides or more in length [12]. Although lacking protein-coding function, lncRNAs participate in various biological processes, such as stem cell differentiation [13], and cell invasion [14, 15] and metastasis [16], by interacting with DNA, RNAs [15, 17, 18], or proteins. Increasing evidence has shown that lncRNAs play important roles in lung cancer. Knocking down *HOTAIR* [19], *SOX21-AS1* [20] and *LINC00342* [21] could decrease cell proliferation in lung cancer cells, and silencing of *MALAT1* inhibit lung cancer cell invasion and migration [22]. Furthermore, lncRNAs have been identified to play roles in environmental carcinogen-induced cancer. *CCAT1* was shown to be involved in malignant transformation of human bronchial epithelial cells induced by cigarette smoke extract (CSE) [23]. We previously found that lncRNA *LOC146880* was up-regulated in lung cancer cells after treatment with PM2.5. *LOC146880* knockdown could reverse PM2.5 induced autophagy and malignant behaviors of lung cancer cells [24]. In the previous study, we also found that lncRNA *ENST00000439577* located at 1p36.13 was associated with lung cancer survival and *ENST00000439577* knockdown in lung cancer cells led to decreased cell proliferation, migration, and invasion (we termed it *LCPAT1*). Furthermore, expression of *LCPAT* was associated with RCC2 [25].

How *LCPAT1* is involved in lung cancer and whether it plays a role in malignant cells exposed to environmental carcinogens are still unknown. In the present study, we analyzed the correlation between cigarette smoking and PM2.5 exposure in association with lung cancer mortality in China, and evaluated the joint effects of PM2.5 and cigarette smoke extracts (CSE) on lung cancer cell migration, invasion, epithelial–mesenchymal transition (EMT), and autophagy. We also investigated the roles of *LCPAT1* in regulating EMT and autophagy in PM2.5/CSE-treated lung cancer cells.

Materials and Methods

Data acquisition

Lung cancer mortality was obtained from the 2012 Chinese Cancer Registry. The average mortality of urban areas was assigned to the rural areas. Female smoking rate in each province was obtained from

the report 'Smoking and Passive Smoking in Chinese, 2002 [26]. The smoking rates were grouped into four categories based on their quartile distributions. The highest lung cancer mortality was observed in the regions with the highest smoking rate (Table 4). PM2.5 concentration was estimated according to satellite observations of aerosol optical depth by the NASA Moderate Resolution Imaging Spectroradiometer (MODIS; <https://modis.gsfc.nasa.gov/>) between 2001-2009. During that time, PM2.5 concentration ranged from 51-101 $\mu\text{g}/\text{m}^3$ (Table 4). PM2.5 concentrations were grouped into high and low categories using the median as a cutoff. Lung cancer mortality was higher in the high PM2.5 regions than in the low areas.

Chemicals and reagents

Anti-vimentin (#ab92547), anti-LC3 (#ab48394), anti-N-cadherin (#ab18203), anti-Atg5 (#ab108327), anti-RCC2 (#154708) and Alexa Fluor 568-conjugated IgG secondary antibody (#ab175694) were purchased from Abcam (Cambridge, UK). Anti-actin (A5316) was purchased from Sigma (St. Louis, MO). IRDye® 800CW Goat anti-Rabbit IgG (#926-32211) was obtained from LICOR (Lincoln, NE). Dimethylsulfoxide was purchased from Sigma (St. Louis, MO). TRIzol and Dulbecco's modified Eagle's medium (DMEM) were from Thermo fisher scientific (Waltham, MA). Transwells were obtained from BD Biosciences (Heidelberg, Germany). Matrigel was purchased from Corning, Inc. (Corning, NY). Fetal bovine serum (FBS) was purchased from Sigma. Poly-vinylidene difluoride (PVDF) membranes and ultra-pure water system were obtained from Millipore (Billerica, MA). All other chemicals, including RIPA lysis buffer, penicillin, streptomycin, 4',6-diamidino-2-phenylindole (DAPI), and phenylmethanesulfonyl fluoride (PMSF) were obtained from Sigma.

PM2.5 sampling and preparation

PM2.5 samples were collected from nitrocellulose filters (pore size = 0.45 μm , size = 90 mm, Pall Life Sciences, New York, NY) using a high-volume sampler (Beijing Geology Device Company, Beijing, China). The collection protocol was described in our previous study [27]. A PM2.5 suspension was prepared according to the Imrich et al. method [28]. All suspensions were stored at -80°C until cell experiments. Suspensions were prepared in deionized water at 10 mg/ml and vortexed for 1 min prior to use.

Preparation of CSE

CSE was prepared using commercially available filter cigarettes (tar = 8 mg, nicotine = 0.6 mg in each cigarette; Zhongnanhai brand, Beijing Tobacco Company, Shanghai, China) according to the method described by Wirtz and Schmidt [29]. In brief, the mainstream smoke of one cigarette, completely combusted within 5 min, was bubbled into a flask containing 15 ml of DMEM medium using a respiratory pump. The CSE solution was adjusted to pH 7.4 and sterilized by filtering through a 0.22 μm membrane. The solution was measured by a spectrometer at the wavelength of 320 nm to standardize CSE, with DMEM as the blank. The absorbance of CSE was 1.79 ± 0.11 . This CSE preparation was regarded as 100% CSE solution. CSE was freshly prepared within 30 min of each experiment.

Cell culture and exposure

Human lung adenocarcinoma cell line, H1299, and squamous cell carcinoma cell line, H520, were obtained from the American Type Culture Collection (ATCC, Rockefeller, MD). All cells were maintained in DMEM supplemented with 10% fetal bovine serum, 80 IU/ml penicillin, and 50 $\mu\text{g}/\text{ml}$ of streptomycin in a humidified atmosphere of 5% CO₂ and 95% air at 37°C . All cell exposure experiments were performed at 70% of cell confluence with viability $\geq 90\%$ determined by trypan blue staining. Freshly dispersed PM2.5 and CSE suspensions were applied to the cells for the indicated concentrations in culture medium. Cells were exposed to 2% CSE and 25 $\mu\text{g}/\text{ml}$ PM2.5 for 24 h either individually or jointly.

RNA isolation and quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis

Cells were washed twice with ice-cold phosphate buffered solution (PBS) and then mixed with TRIzol, according to the manufacturer's instructions. Total RNA (2 μg) was reverse transcribed into cDNA using the High-Capacity cDNA Reverse Transcriptase kit (Applied Biosystems, Foster City, CA). *LCPAT1*, *CDH2*, *Vimentin*, *CTNNB1*, *LC3II*, *Atg5*, and *Beclin1* expression were determined by qRT-PCR using the 7500 Fast Real-Time PCR System (Applied Biosystems) with the SYBR green super mix (Applied Biosystems). The PCR protocol included: initial denaturation at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for

10 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. Gene expression levels were analyzed using 2^{-ΔΔCT}, and β-actin was used as a reference to normalize the RNA input. The qRT-PCR primers were designed according to the sequence of genes (the sequence of *LCPAT1* was shown in Table 1, while the primers were shown in Table 2).

Cell invasion assay

Invasion assays were performed with the use of 8 μm pore Transwell inserts (Millipore). The upper chambers of the Transwells were pre-coated with diluted Matrigel (BD Biosciences, Sparks, MD). A total of 10⁴ cells were seeded onto the upper chamber in serum-free medium, and medium containing 10% serum was added to the lower chamber as a chemoattractant. After 24 h incubation, the upper surface of the insert was wiped with a cotton swab and cells that migrated to the lower surface were fixed by 4% paraformaldehyde and stained with crystal violet. The number of cells were counted in three random fields per well, and the quantity is expressed as mean ± SD of triple assessments.

Si-LCPAT1, si-Atg5, and RCC2 transfection experiments

The siRNA and pEX1-*RCC2* plasmids were transfected with Lipofectamine 2000 according to the manufacturer's protocol. In short, H1299 and H520 cells at approximately 70% confluence were incubated with DMEM containing pEX1-*RCC2* plasmid (2.5 μg), pEX1-vector plasmid (a negative control, 2.5 μg), si-*Atg5* (20 nM), si-*LCPAT1* (20 nM), or scramble siRNA (20 nM) for 24 h, followed by exposing the cells to CSE and PM2.5 together for another 24 h. To determine the efficiency of transfection, transfected cells were harvested to evaluate the levels of *Atg5*, *LCPAT1*, and *RCC2* expression. The sequences of siRNA were shown in Table 3.

Wound healing assay

H1299 and H520 cells were seeded into six-well plates. When the cell density reached > 90%, scratch wounds were made by scraping the cell layer in each culture well using the tip of a 200 μl pipette. Then, the cells were washed with PBS and cultured in medium containing 25 μg/ml of PM2.5 and 2% CSE for 24 h, individually or together, after which three fields were randomly chosen from each scratch wound and visualized by microscopy to evaluate the ability of cell migration. The experiments were performed in triplicate.

Western blot analysis

Cell lysate preparation and western blot analysis were performed as previously described [25]. Briefly, cells were lysed in lysis buffer (50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 150 mM NaCl, 0.5% NP-40, and 1 mM PMSF) for 30 minutes. The lysates were centrifuged for 10 min at 10,000 g, and the supernatant was collected for SDS-PAGE separation and western blot analysis.

Immunofluorescence staining

Immunofluorescence staining was performed as described in detail previously [30]. Briefly, cells were fixed in 4% formaldehyde, and immunostained for 2 h with primary antibodies followed by a 2 h exposure to Alexa Fluor 568-conjugated secondary antibodies. Immunofluorescence was visualized by confocal microscopy (Zeiss, Sachsen, DE).

Table 1. The sequence of *LCPAT1*

RNA	Sequence
LCPAT1 (also known as ENST00000439577)	GACAGTAGACAATGTTGTTTATTTAAATGTTTACTCCAAGAAA
	TATATATATAAAAAAATAAATAAGACAATTACAGCACTAAACCAAGCA
	CCTTCGAGCAAAATCAGAACCTCCTCTTTGATTCCCTTCACGCTAAGC
	CTCTTTCAAATCTTTTTCCTGAGCTGGAAGACAGTCAGATGCCCGC
	AGGGTCAGCGCAAGCATTCCCAACGGGGCAACTGTGTACTCTTCT
	CTAGGAGTGCCTGGGCTCATTCAGGAAAGAGACCTGTCCAGGGAAA
	CGGATCAGGCTGTGCGATGGAAGC

Table 2. The sequence of qRT-PCR primers

Genes	Primer Sequences (5' to 3')
CDH2-F	TGGGTACAGTGAAGTGGG
CDH2-R	GAAACGGGCTATCTGCTCG
CTNNB1-F	CATCTACACAGTTTGTAGTGTCT
CTNNB1-R	GCAGTTTTGTCAAGTTCAGGGA
Vimentin-F	AGTCCACTGAGTACCGGAGAC
Vimentin-R	CATTTCAAGCATCTCGGGTTC
Beclin1-F	GGTGTCTCTCGCAGATTCATC
Beclin1-R	TCAGTCTTCGGCTGAGGTTCT
LC3II-F	AACATGAGCGAGTTGGTCAAG
LC3II-R	GCTCGTAGATGTCGGGAT
Atg5-F	AAAGATGTGCTTCGAGATGTGT
Atg5-R	CACTTTGTGAGTTACCAACGCTCA
LCPAT1-F	TACAGCACTAAACCAAGGCACT
LCPAT1-R	TCCAGCTCAGGAAAAGAAAT
ACTIN-F	CTGGAACGGTGAAGGTGACA
ACTIN-R	CGGCCACATTGTGAACCTTTG

Table 3. The sequences of siRNA

Genes	siRNA Sequences (5' to 3')
negative control siRNA-F	UUUCCGAAAGGUGUCAGGUTT
negative control siRNA-R	ACGUGACAGUUCGAGAAAT
Atg5 siRNA-1-F	GACGUUGUAACUGACAAAT
Atg5 siRNA-1-R	UUUGUCAGUUAACAAGGUCTT
Atg5 siRNA-2-F	GUCCAUUAAGGAUGCAAUUT
Atg5 siRNA-2-R	AUUGCAUCCUUAUGGAGACTT
Atg5 siRNA-3-F	GACCUUUAUUAAGAAAGGUCTT
Atg5 siRNA-3-R	AGCUUCUGAAUGAAAGGUCTT
LCPAT1 siRNA-F	AGACAUAUACAGCACUAUAAAC
LCPAT1 siRNA-R	UUUAGUGUCUUAUUUGUCUUA

RNA binding protein immunoprecipitation

On the basis of the EZ-magna RNA immunoprecipitation kit (#17-701; Millipore), we used RCC2 antibody to bind RCC2 protein, and then detected whether RCC2 binds to *LCPAT1* by qPCR.

Statistical analyses

Lung cancer mortality rates and risk differences were calculated according to categories of cigarette smoking prevalence ($\geq 4.71\%$ vs $< 4.71\%$) and PM2.5 concentration (high $\geq 92 \mu\text{g}/\text{m}^3$ vs low $< 92 \mu\text{g}/\text{m}^3$). The relative excess risk due to interaction on an additive scale was estimated according to RERI (RR11-RR00-RR01+1), attributable proportion (AP) (RERI/RR11), and synergy index (SI) ((RR11-1)/(RR10+RR01-2)) with 95% confidence intervals (95% CIs), using the "MOVER" method [31]. Cell experiment results were expressed as mean \pm SEM, as indicated in the Fig. legends. Statistical significance in mean difference was determined by two-tailed Student t-tests or one-way ANOVA. P values < 0.05 were considered significant.

Results

Synergistic effect of cigarette smoking and PM2.5 pollution on lung cancer

The contributions of smoking prevalence and PM2.5 pollution to lung cancer mortality are shown in Fig. 1. Lung cancer mortality attributable to smoking was higher in northern China than in other regions. In the northeast and eastern coastal areas, where levels of PM2.5 pollution were high, the PM2.5 attributed lung cancer mortality was also high. The relative risks (RRs) and 95% confidence intervals (CIs) for lung cancer mortality according to smoking prevalence and PM2.5 pollution are presented in Table 4. The RR for high versus low smoking prevalence was 1.32 (1.20, 1.46), and for high versus low PM2.5 was 1.03 (0.97, 1.09). The two risk factors had a synergistic interaction at an additive scale (RERI = 0.28, AP = 0.17 and SI = 1.81).

Increased cell autophagy, migration, invasion, and EMT after joint exposure to CSE and PM2.5

As shown in Fig. 2A, increased LC3 dot formation was observed in H1299 and H520 cells after treatment with CSE and PM2.5 individually for 24 h, compared to the control cells. Simultaneous treatment with CSE and PM2.5 for 24 h resulted in more LC3 dots in H1299 and H520 cells when compared to cells treated individually with CSE or PM2.5. The size and signal of LC3 puncta were larger and stronger in the co-treatment cells, suggesting that CSE and PM2.5 may have a synergistic effect on autophagy.

We further examined whether CSE and PM2.5

Table 4. Association of lung cancer mortality with cigarette smoking and PM2.5 exposure

Exposure	No. of Persons	No. of Deaths	RR (95% CI)	Interaction (95% CI)
Smoking prevalence				RERI=0.28 (0.10,0.46)
Low (<4.71%)	27,863,461	4,424	1.00	AP=0.17 (0.06,0.28)
High ($\geq 4.71\%$)	5,175,539	1,222	1.32 (1.20,1.46)	SI=1.81 (1.56,2.16)
PM2.5 concentration rank				
Low (<92 $\mu\text{g}/\text{m}^3$)	19,703,382	3,206	1.00	
High ($\geq 92 \mu\text{g}/\text{m}^3$)	13,335,618	2,440	1.03 (0.97,1.09)	

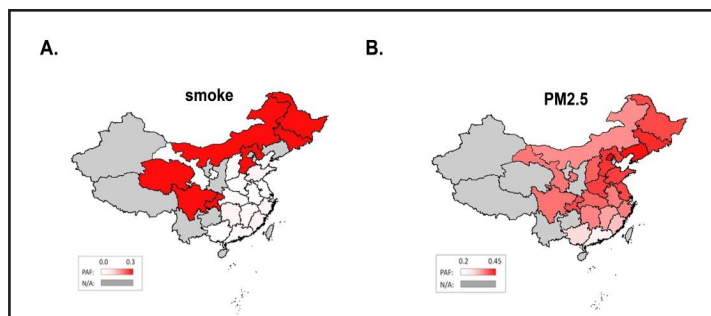


Fig. 1. Lung cancer mortality attributed to smoking prevalence and PM2.5 pollution. The population-attributed fraction (PAF) of (A) Smoking prevalence and (B) PM2.5 concentration.

Fig. 2. Increased cell autophagy, migration, invasion, and EMT after joint exposure to CSE and PM2.5. (A) Joint exposure increased LC3 dot formation in H1299 and H520 cells. Cells were treated with 2% CSE, 25 $\mu\text{g}/\text{cm}^2$ PM2.5, and both CSE and PM2.5 for 24 h. Cell activities after exposure to CSE and PM2.5 were determined by wound-healing assay (B) and invasion assays (C). Expression of EMT-related genes (*CDH2*, *Vimentin*, and *CTNNB1*) and autophagy-related genes (*LC3II*, *Atg5*, and *Beclin1*) were determined by qRT-PCR (D) and western blot (E). Actin was used as a reference. Data presented were mean \pm SEM of three independent experiments. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ compared with control groups; # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ compared with CSE treatment groups; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the PM2.5 treatment group.

affected other activities of lung cancer cells, such as migration, invasion and EMT. After 24 h of treatment with CSE and PM2.5, either individually or in combination, cell migration and invasion were significantly increased when compared to the control cells (Fig. 2B and 2C). The increases in migration and invasion were more evident in the cells treated with both CSE and PM2.5 jointly compared to those treated with these agents individually.

In addition, we also found that the joint treatment of CSE and PM2.5 could influence the expression autophagy-related genes (*LC3II*, *Atg5* and *Beclin1*) as well as EMT-associated genes (*N-cadherin*, *Vimentin* and *CTNNB1*) as shown in Fig. 2D and 2E. These results further demonstrate that CSE and PM2.5 may have a synergistic effect on lung cancer progression.

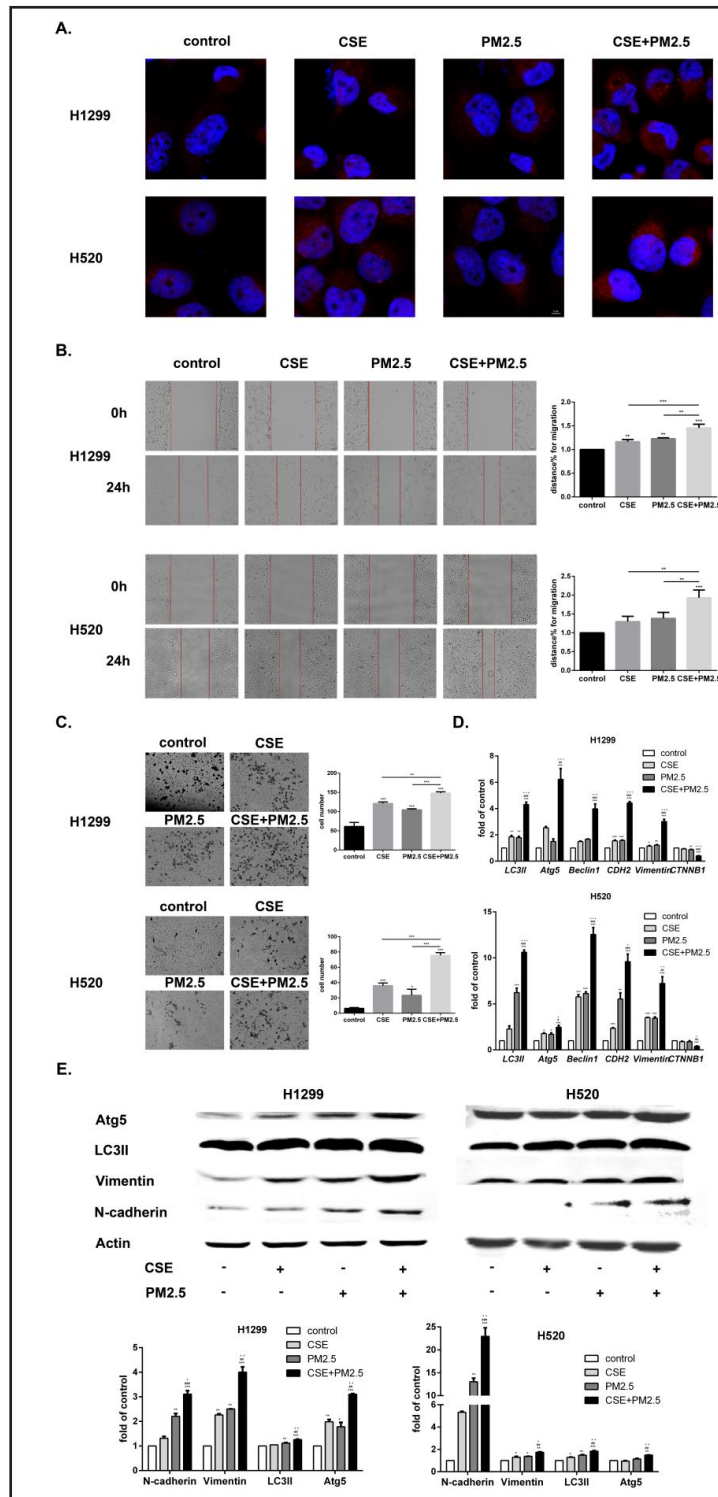


Fig. 3. *Atg5* knockdown (si-*Atg5*) on autophagy inhibition. (A) The efficiency of si-*Atg5* on *Atg5* expression (mRNA/protein) after joint exposure of CSE and PM2.5. (B) Decreased LC3 dots after *Atg5* knockdown in the CSE/PM2.5-treated cells. Changes of autophagy-associated genes (LC3II and Beclin1) were determined by qRT-PCR (C) and western blot (D). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Involvement of autophagy in cell migration, invasion and EMT induced by joint exposure

Previous studies suggest that autophagy may play a role in cell migration, invasion and EMT. To evaluate the involvement of autophagy in cell migration, invasion and EMT induced by CSE and PM2.5 exposure, we knocked down the expression of *Atg5*, a protein necessary for autophagy [32]. The efficiency of *Atg5* knockdown by si-*Atg5* reflected by autophagy inhibition is shown in Fig. 3. Earlier we showed that treatment of H1299 cells with both CSE and PM2.5 for 24 h resulted in increased cell migration, invasion and EMT (Fig. 2). These effects, however, were abolished after we blocked autophagy by knocking

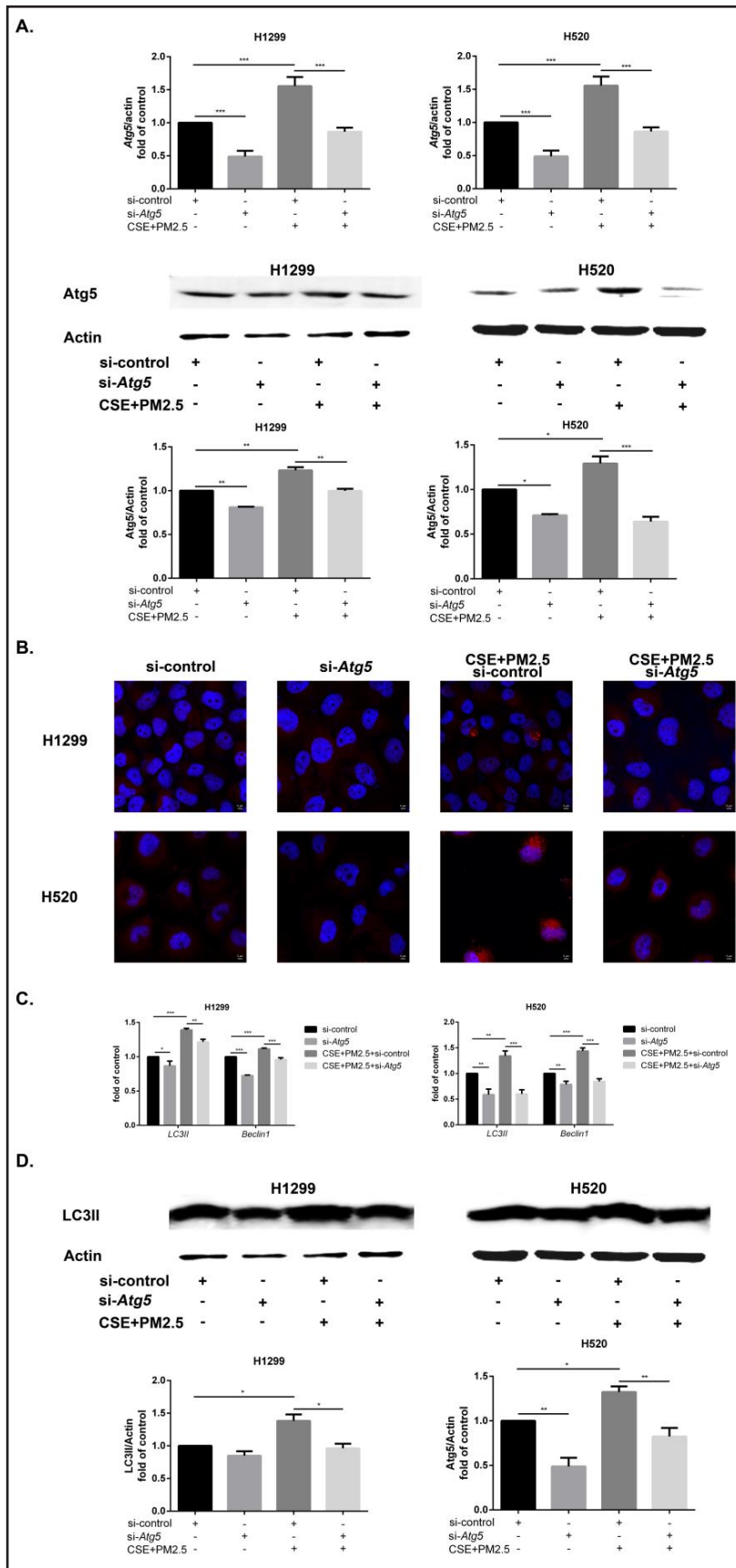


Fig. 4. Involvement of autophagy in cell migration, invasion and EMT induced by joint exposure to CSE and PM2.5. Cells were pretreated with *si-Atg5* for 24 h, after which CSE and PM2.5 were added to the cells for another 24 h incubation. The ability of cell migration (A), invasion (B), and expression of EMT genes (C) and proteins (D) were determined. All experiments were repeated three times and data presented were mean \pm SEM. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

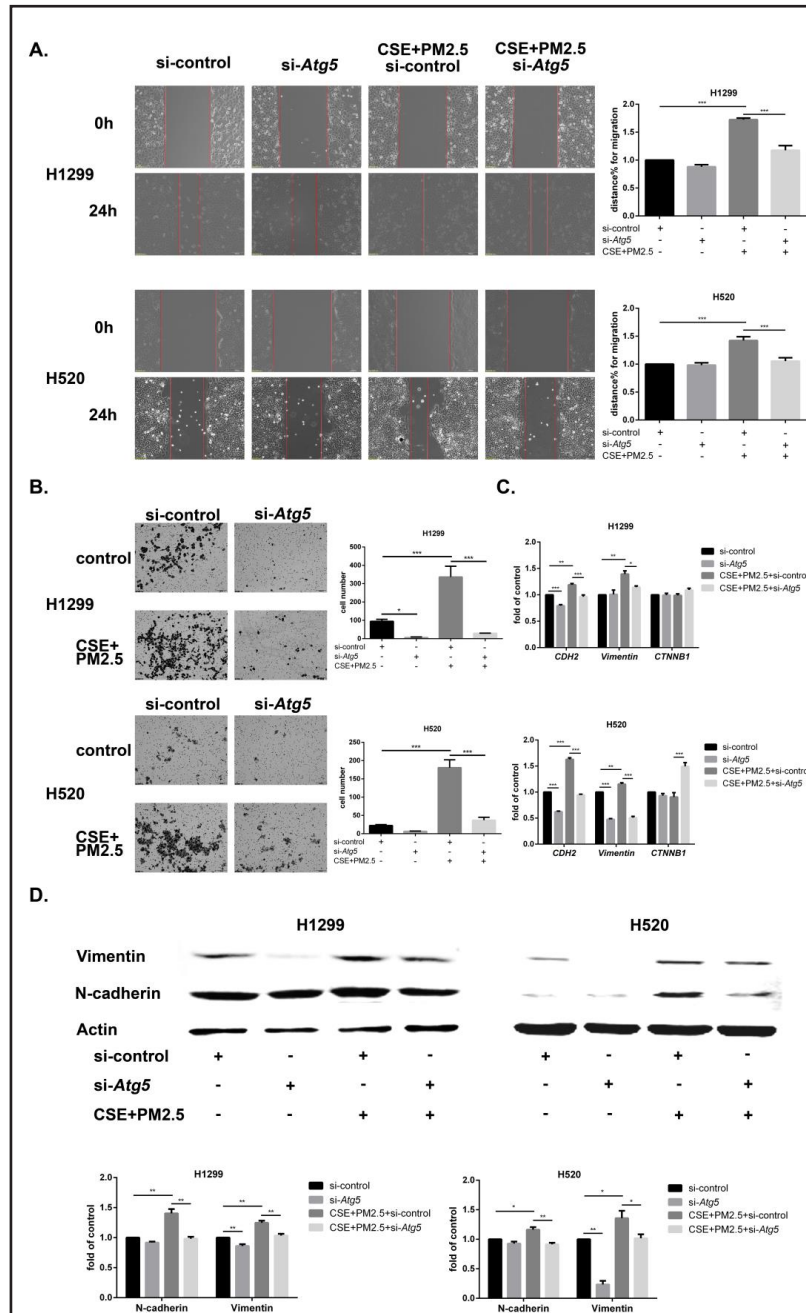
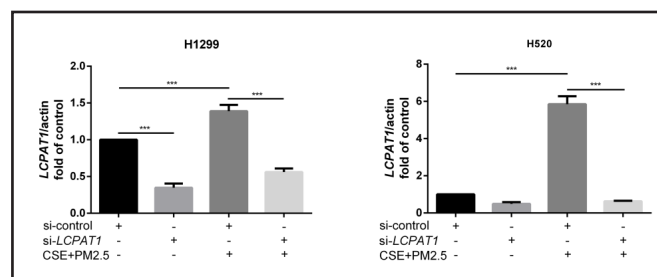


Fig. 5. Efficiency of *si-LCPAT1* on *LCPAT1* expression after CSE/PM2.5 exposure in H1299 and H520 cells.



down *Atg5* (Fig. 4A and 4B). Similar changes were also observed in H520 cells. Furthermore, increased expression of autophagy- and EMT-related genes induced by CSE and PM2.5 exposure were reversed when autophagy was suppressed by knocking down *Atg5* (Fig. 4C

Fig. 6. Role of *LCPAT1* in CSE/PM2.5-induced autophagy, migration, invasion and EMT. (A) Joint exposure increased the expression of *LCPAT1*. H1299 and H520 cells were transfected with scrambled siRNA and *LCPAT1* siRNA for 24 h. Then cells were incubated with or without CSE/PM2.5 for 24 h. Knocking down *LCPAT1* decreased the LC3B dots (B), cell migration (C), invasion (D), autophagy, and EMT-associated gene transcripts (E) and proteins (F) expression after joint exposure. All experiments were repeated three times and data presented as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

and 4D). These results suggest that autophagy is involved in the migration, invasion and EMT of lung tumor cells after they are exposed to CSE and PM2.5.

Role of LCPAT1 in CSE/PM2.5-induced autophagy, migration, invasion and EMT

Fig. 6A shows that CSE or PM2.5 could significantly induce the expression of *LCPAT1* in H1299 and H520 cells, and the induction was significantly enhanced when the cells were exposed to both CSE and PM2.5 together compared to

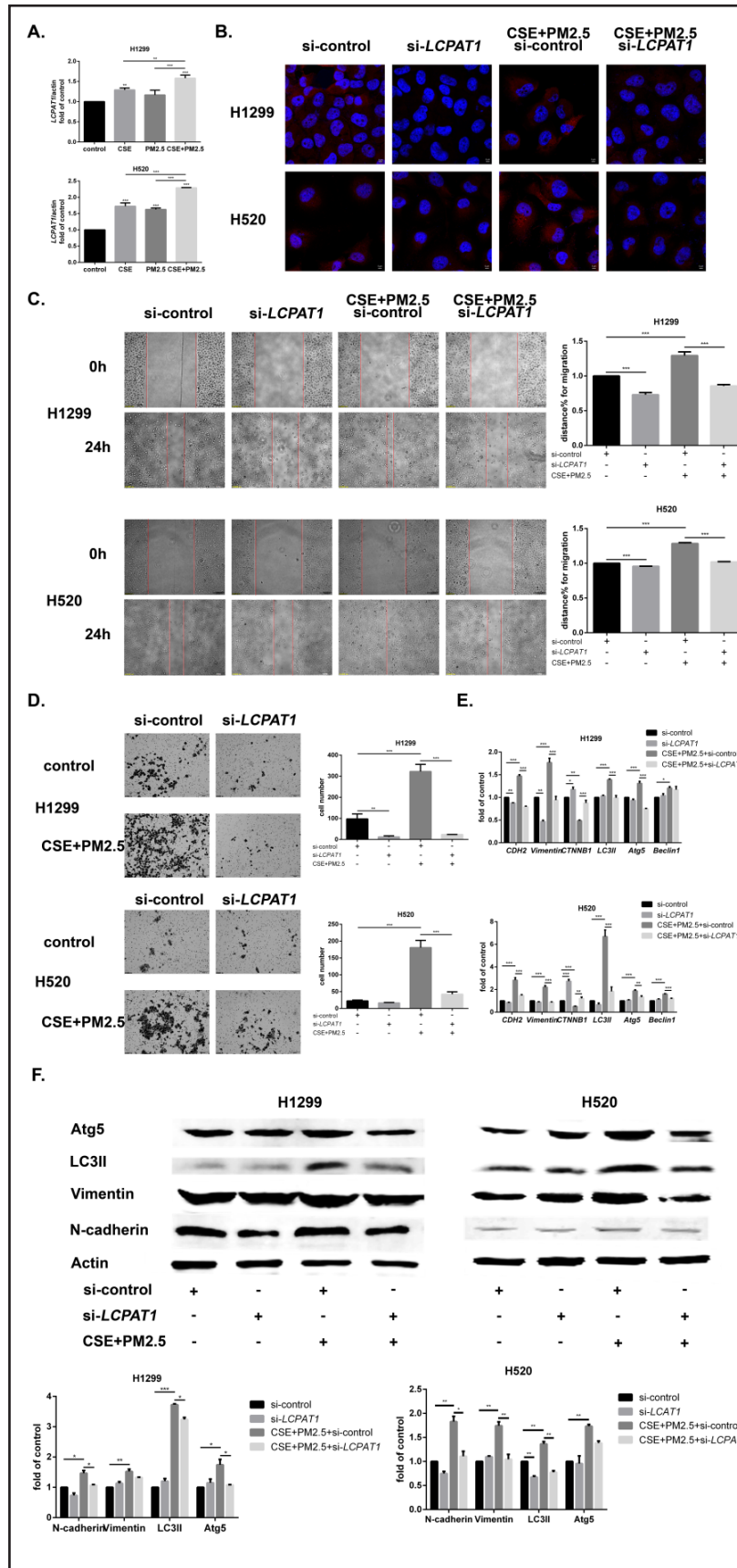
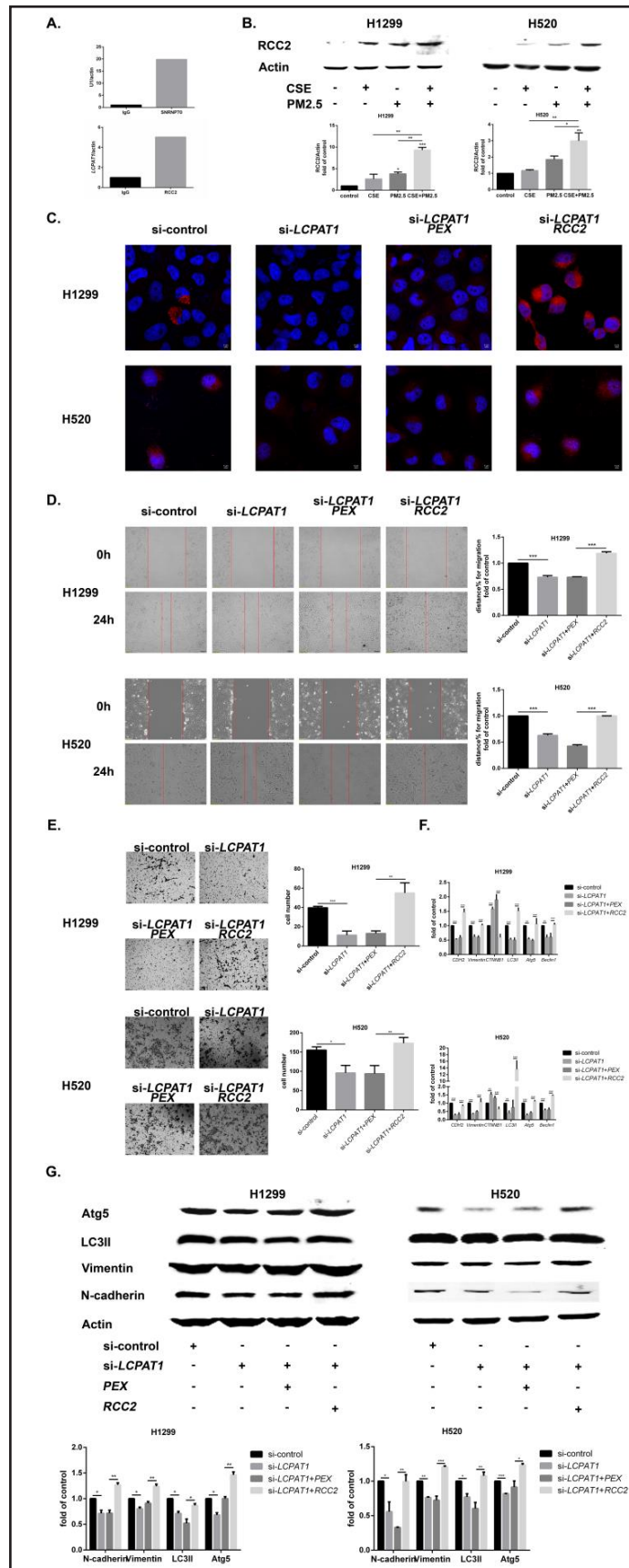


Fig. 7. Interaction between *LCPAT1* and *RCC2* in regulation of cell autophagy, migration, invasion and EMT in CSE/PM2.5-treated cells. (A) RIP assay results to show the interaction between *LCPAT1* and *RCC2*. (B) western blot results to show increased *RCC2* after cells were treated with CSE/PM2.5. Changes of cell activities after H1299 and H520 cells were co-transfected with si-*LCPAT1* and *RCC2* plasmid for 24 h and incubated with CSE and PM2.5 for another 24 h, LC3 dot formation (C), migration (D), and invasion (E). Expression of EMT and autophagy-related genes (F) and proteins (G) were also determined. All experiments were repeated three times and data presented as mean \pm SEM. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

single exposure. To investigate whether *LCPAT1* was involved in tumor progression caused by CSE and PM2.5 exposure, we analyzed CSE/PM2.5-induced cell migration, invasion, EMT and autophagy after knocking down *LCPAT1* in H1299 and H520 cells. The efficiency of *LCPAT1* knockdown by siRNA is shown in Fig. 5. After CSE and PM2.5 treatment for 24 h, cell migration and invasion were decreased in *LCPAT1* knockdown cells (Fig. 6C and 6D). Autophagy was not increased after CSE/PM2.5 exposure when *LCPAT1* was knocked down (Fig. 6B). Assessing the expression of genes involved in EMT and autophagy, we found no changes in their expression after *LCPAT1* knockdown in cells treated with CSE and PM2.5 (Fig. 6E and 6F). These results indicate that *LCPAT1* may play a crucial role in lung cancer progression after CSE and PM2.5 exposure.



Interaction between LCPAT1 and RCC2 in regulation of cell autophagy, migration, invasion and EMT in CSE/PM2.5-treated cells

We previously reported a positive correlation between *RCC2* and *LCPAT1* expression in tumor samples of lung cancer patients. In the present study, we found that *LCPAT1* was able to bind to *RCC2* in H1299 cells (Fig. 7A). In addition, treatment with CSE and PM2.5 increased the expression of *RCC2* in lung cancer cells (Fig. 7B). To investigate the role of *RCC2* in the CSE/PM2.5-treated H1299 and H520 cells, we knocked down *LCPAT1* and overexpressed *RCC2*. The transfection efficiency of a *RCC2*-overexpressing plasmid was shown in Fig. 8A. In the experiment, we found that when *RCC2* was overexpressed, there was no significant increase or decrease in *LCPAT1* expression (Fig. 8B), suggesting the lncRNA being upstream of *RCC2*. Our experiment also showed that autophagy inhibition by *LCPAT1* knockdown was reversed when *RCC2* was overexpressed (Fig. 7C). The size and signal of LC3 puncta were significantly greater and stronger in cells with *RCC2* overexpression than in those without. Cell migration (Fig. 7D) and invasion (Fig. 7E) were also recovered after *RCC2* overexpression. Furthermore, the expression of autophagy- and EMT-related genes were restored after *RCC2* overexpression, whether at the transcriptional (Fig. 7F) or translational level (Fig. 7G).

Discussion

In this study, we observed a synergy between cigarette smoking and PM2.5 exposure in association with lung cancer mortality, and the synergistic interaction was further indicated in our *in vitro* experiments where CSE and PM2.5 exposure were found to promote cell autophagy, invasion, migration and EMT of lung cancer. We also found in our experiments that autophagy played a key role in cell migration, invasion and EMT. Knockdown of *Atg5* could abolish the effects of CSE/PM2.5 on cell migration, invasion and EMT. Our experiments also indicated that lncRNA, *LCPAT1*, was involved in the CSE/PM2.5-induced cell behaviors and the effect was mediated through *RCC2*.

Tobacco smoke and PM2.5 contain thousands of components, many of which are carcinogens, such as PAHs (polycyclic aromatic hydrocarbons) [2, 33]. It was shown that PAH-bound PM could induce the migration and invasion of lung cancer cells through the reactive oxygen species (ROS)-mediated EMT activation [34]. ROS are also involved in CSE-induced EMT [35]. Exposure to CSE has been shown to induce autophagy and increase LC3II or Beclin1 expression via activation of the ERK/p38 MAPK pathway [36] and inhibition of the PI3K/AKT/mTOR pathway [37] in alveolar macrophages [38], pulmonary epithelial cells [36, 37], and adult male mice [39]. Additionally, when human corneal epithelial cells were exposed to PM2.5 for 24 h, autophagy was also activated as levels of autophagy-associated markers, including LC3II, Atg5, and Beclin1, increased [40]. The PI3K/AKT/mTOR pathway has been reported to be involved [41]. Few studies have been designed to examine the joint effects of multiple environmental exposures on lung cancer risk. One cohort study, the Cancer Prevention Study II, suggested an interaction between smoking and PM2.5 in lung cancer

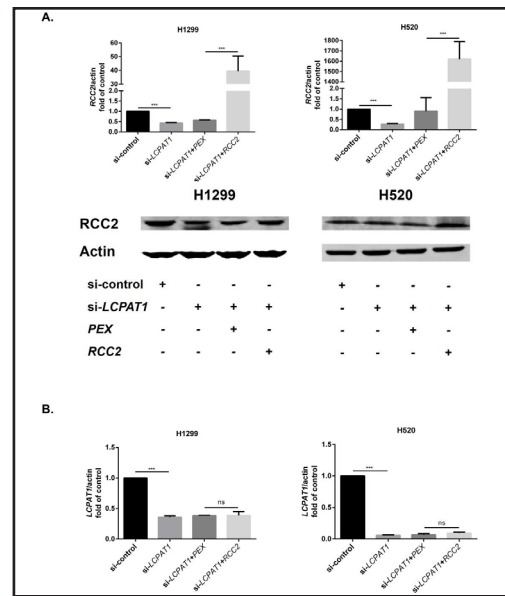


Fig. 8. Expression of *RCC2* and *LCPAT1* after transfection with a *RCC2* plasmid. (A) The expression of *RCC2* measured by qRT-PCR and western blot was increased after transfection with a *RCC2* plasmid. (B) There was no alteration in *LCPAT1* expression after *RCC2* overexpression.

mortality [11]. In the current study, we also observed a significant interaction between these two exposures and found that CSE and PM2.5 could synergistically increase cell autophagy, migration, invasion and EMT.

Autophagy was previously reported to be involved in cell migration, invasion and EMT. One study of 1400 tumor samples found that high LC3II expression in tumor tissues was associated with tumor invasion, metastasis, and worse outcomes [42]. Overexpression of N-myc downstream regulated gene1 (NDRG1) could inhibit the initiation of autophagy, and NDRG1 was known to function as a metastasis suppressor in human pancreatic, colorectal, and prostate cancer cells [43]. Mouse models, created by injecting cells with or without silencing *atg5* and *beclin1*, were used to investigate the role of autophagy in hepatocellular carcinoma metastasis. The experiments showed less pulmonary metastasis in autophagy-inhibited mice [44]. In hepatoma cells, inhibition of autophagy by treatment with 3-methyladenine (3-MA) could increase the expression of *CDH1* (an epithelial marker) and reduce cell migration and invasion [45]. Treatment of 3-MA could also inhibit cell migration and invasion in lung cancer cells, A549 and H460 [46]. We previously reported that in lung cancer cells, ROS-activated autophagy promoted EMT, cell migration and invasion, and PM2.5 could increase ROS-activated autophagy. In addition, a lncRNA, *LOC146880*, was found to play a crucial role in this process [24]. In the current study, by adding CSE and PM2.5 together with si-*Atg5*, we found that si-*Atg5* abolished the effects of CSE and PM2.5 on cell migration, invasion and EMT. These results support the hypothesis that autophagy promotes cell migration, invasion and EMT. Furthermore, we demonstrate that another lncRNA, *LCPAT1*, is involved in CSE/PM2.5-induced cell autophagy, migration, invasion and EMT via RCC2.

LCPAT1 was first reported by our group in lung cancer. In the present study, we found that exposure to CSE and PM2.5 could increase the expression of *LCPAT1* in lung cancer cells. Induction of cell autophagy, migration, invasion and EMT by joint exposure of CSE and PM2.5 was shown to be reversed after *LCPAT1* knockdown. These results suggest that *LCPAT1* may increase autophagy which further promotes EMT, migration, and invasion of lung cancer cells after the cells were exposed to CSE and PM2.5. How *LCPAT1* affects autophagy and EMT is still unknown. We previously reported that in lung tumor tissues the expression of *LCPAT1* was positively correlated with RCC2 [25], and in this study we further demonstrated that *LCPAT1* could bind to RCC2 in our RIP assay. On the basis of these results, we speculate that *LCPAT1* may mediate CSE/PM2.5-induced autophagy and EMT by increasing RCC2 expression. RCC2, also known as TD-60, plays an important role in cell cycle regulation through several signaling pathways. RCC2 binds to the small G protein, Rac1, and recruits Aurora B, which is associated with microtubules [47]. Studies investigating the role of RCC2 in cancer are limited. RCC2 knockdown inhibits gastric carcinoma cell viability [48] and reduces colon cancer cell proliferation. Increased apoptosis due to mutation at RCC2 5' UTR led to better outcomes in patients with microsatellite instable colorectal cancer [49]. In lung cancer cells, overexpression of RCC2 could significantly induce cell migration and invasion through the MAPK-JUN signaling-induced EMT [50]. Furthermore, RCC2 could be an independent marker for poor prognosis of lung adenocarcinoma [50]; however, no evidence has been reported on the induction of RCC2 by environmental carcinogens. Here, we observed that RCC2 was up-regulated in CSE/PM2.5-treated lung cancer cells. We also found that overexpression of RCC2 abolished the effect of *LCPAT1* knockdown on autophagy and EMT. These results suggest that RCC2 is involved in *LCPAT1*-regulated autophagy and EMT. Shen et al. reported that Rac1 mediated the smoking-induced autophagy [51]. Another study found that RCC2 could bind to Rac1 [47] and acted as a dual regulator of Rac1 and Arf6 to further influence cell migration and movement [52]. Our study suggests that RCC2 may promote autophagy, and further increase EMT, migration and invasion in CSE/PM2.5-treated lung cancer cells.

In summary, we found that CSE and PM2.5 had a synergistic effect on lung cancer cells by stimulating cell autophagy which further resulted in increased cell migration, invasion and EMT. Our experiments also showed that lncRNA *LCPAT1* mediated the CSE/PM2.5-induced autophagy that upregulated cell migration, invasion and EMT through interacting with RCC2. RCC2 was involved in CSE/PM2.5-induced cell autophagy, migration, invasion and EMT.

These results suggest a new mechanism that involves *LCPAT1* and *RCC2* in environmental carcinogen-induced lung cancer progression and metastasis. How CSE and PM2.5 affect *LCPAT1* expression is unknown. Further studies are needed to explain the mechanisms involved in *LCPAT1*-promoted autophagy, EMT, migration and invasion in connection to *RCC2*.

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Disclosure Statement

The authors declare to have no competing interests.

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