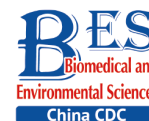


Original Article

Exosomes from PM_{2.5}-treated Human Bronchial Epithelial Cells Increase Lung Cancer Metastatic Potential*YU Heng Yi¹, GUO Hua Qi^{1,2}, FENG Yan¹, CHENG Wei¹, and WANG Yan^{1,2,#}

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Abstract

Objective Fine particulate matter (PM_{2.5}) is an air pollutant that has become of great concern in recent years. Numerous studies have found that PM_{2.5} may contribute to lung cancer, but the pathogenesis has not yet been fully elucidated. In this study, we explored the roles of exosomes from bronchial epithelial cells in PM_{2.5}-promoted lung cancer metastasis.

Methods Exosomes were isolated from cell supernatants. An animal model of lung metastasis (established by tail vein injection of A549-luc) and *in vitro* studies with lung cancer cell lines were used to investigate the effects of exosomes derived from PM_{2.5}-treated human bronchial epithelial cells (PHBE-exo).

Results The animal experiments revealed that PHBE-exo-treated mice showed stronger luciferase activity and a larger relative metastatic region in the lungs, thus indicating that PHBE-exo promoted the metastatic potential of lung cancer. Additionally, PHBE-exo promoted the migration, invasion and epithelial-to-mesenchymal transition of lung cancer cells, in a manner mediated by activation of c-Jun N-terminal kinase.

Conclusion These results implied that PM_{2.5} may promote the development of lung cancer through exosomes derived from bronchial epithelial cells, thus providing a potential interventional target for lung cancer. These findings broadened our understanding of cancer-promoting mechanisms of environmental pollutants.

Key words: Fine particulate matter; Exosome; Lung cancer; C-Jun N-terminal kinase; Bronchial epithelial cell

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INTRODUCTION

During the course of industrialization, particulate matter has become a common pollutant. The International Agency for Research on Cancer has classified the particulate matter in outdoor air pollution as a group 1 carcinogen^[1]. In urban areas, motor vehicle emissions and fuel burning are major sources of particulate matter air pollution^[2,3]. Fine particulate matter (PM_{2.5}), whose aerodynamic diameter is

smaller than 2.5 μm, can adsorb a wide variety of toxic compounds (such as organic compounds and poisonous metals). PM_{2.5} can penetrate deeply into the lungs through breathing, cross the blood-air barrier and cause lesions in multiple organs, particularly the lungs^[4,5].

According to the World Cancer Report published by the International Agency for Research on Cancer, lung cancer was the leading cause of cancer-associated death worldwide in 2020^[6]. In addition, lung cancer is the most common cause of cancer

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disability-adjusted life years^[7]. Metastasis is a major cause of the high mortality in lung cancer; however, the mechanisms of lung cancer metastasis have not been fully elucidated^[8,9]. Many studies have reported adverse effects of PM_{2.5} on lung cancer occurrence and development. The Harvard Six Cities study has shown that PM_{2.5} exposure increases the risk of lung cancer death (*RR*, 1.27; 95% *CI*, 0.96–1.69), and decreasing PM_{2.5} concentrations is critical to diminish lung cancer mortality^[10]. Chao et al. have found that PM_{2.5} exposure contributed to proliferation, migration and invasion in lung cancer cell lines, and promoted tumor growth and metastasis *in vivo*^[11].

Exosomes are extracellular vesicles with a diameter of approximately 40–160 nm, containing many constituents of cells, such as DNA, RNA, lipids and metabolites^[12]. Exosomes are secreted by most cells and have been detected in a variety of body fluids including blood, urine, saliva, lymph and bronchoalveolar fluids^[13-15]. Exosomes secreted by cells can be taken up by adjacent cells or can enter the circulation and affect distant cells^[15,16]. Numerous studies have focused on the biology and function of exosomes in cancers. Exosomal miR-21-5p derived from gastric cancer cells has been found to promote peritoneal metastasis^[17]. The prometastatic secretome can be delivered by exosomes and lead to breast cancer multi-organ metastasis^[18]. Moreover, exosomal miRNAs have been found to boost lung metastasis *via* STAT3-induced epithelial-to-mesenchymal transition (EMT) *in vitro* and *in vivo*^[19]. In a review on the function of exosomes in tumors, Zhang et al. have noted that exosomes played crucial roles in tumor pathogenesis and progression^[20].

Bronchial epithelial cells are located at the lung-environment interface and directly contact PM_{2.5}. We hypothesized that signals of PM_{2.5} exposure might be transduced to other lung cells through exosomes derived from human bronchial epithelial (HBE) cells. In the present study, HBE cells were treated with PM_{2.5} originating from traffic. The exosomes were isolated, and their roles in the malignant transformation of lung cancer were investigated.

METHODS

Cell lines, Cell Culture and Reagents

HBE cells (human bronchial epithelial cells), and A549 and H1975 cells (human lung adenocarcinoma cells) were obtained from the Shanghai Institute for

Biological Sciences, Chinese Academy of Sciences (Shanghai, China). A549-luciferase (A549-luc) cells were purchased from Shanghai Zhong Qiao Xin Zhou Biotechnology (Shanghai, China). HBE cells were cultured in MEM (BasalMedia Technologies, China) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, USA). A549, A549-luc and H1975 cells were cultured in DMEM (BasalMedia Technologies, China) supplemented with 10% FBS (Sigma-Aldrich, USA). Cells were grown at 37 °C under 5% CO₂. SP600125 (Sigma-Aldrich, USA) was dissolved in DMSO (Sigma-Aldrich, USA). Antibodies against E-cadherin (#3195), N-cadherin (#D4R1H), ZEB1 (#3396), JNK (#9252), P-JNK (#4668), ERK (#4370), P-ERK (#4695) and P38 (#8690) were purchased from Cell Signaling Technology, USA. Antibodies against GAPDH (AF1186) and P-P38 (AM063) were purchased from Beyotime Institute of Biotechnology, China. Antibodies against CD9 (EXOAB-CD9A-1) and CD81 (EXOAB-CD81A-1) were purchased from System Biosciences, USA.

PM_{2.5} Collection and Preparation

A large-volume PM_{2.5} sampler (Intelligent 2031, Qingdao Laoying Inc., China) was set on top of a five-story building in the vicinity Chongqing South Road, a two-lane, two-way road with heavy traffic in Shanghai, China. Before collection, the quartz filter membrane was dried in a muffle furnace for 2 h at a temperature of 500 °C to remove any organic matter attached to the filter membrane. Sampling was performed for 3–5 days per week, 24 h per day. After the filter membrane was saturated with adsorption, it was folded in half, wrapped in aluminum foil and stored at –20 °C. During fall/winter 2018 (from 1st Nov. to 31st Dec. 2018), the main components of PM_{2.5} were sampled. The method of sample preparation has been described in our previous study^[21]. Briefly, ultrasonic treatment of the sampling filter membranes was performed to obtain PM_{2.5} suspensions. Then the suspensions were stored at –80 °C. When the suspension had solidified, the samples were freeze-dried under vacuum to obtain PM_{2.5} particles (Labconco, USA). Finally, the PM_{2.5} samples were weighed and stored at –20 °C. The particles were resuspended in sterilized ultrapure water before experiments. The sampling conditions (including location, equipment and season), collection methods and extraction methods were as previously reported; therefore, the composition of PM_{2.5} samples was expected to be the same as that in the previous study, including several toxic heavy metals (such as lead and nickel)

and a variety of polycyclic aromatic hydrocarbons^[21].

Cell Viability Assays

Cells were seeded in 96-well plates (3,000 cells per well). After treatment with PM_{2.5} or exosomes, cell viability was assessed with Cell Counting Kit-8 assays (Beyotime Institute of Biotechnology, Shanghai, China) according to the manufacturer's protocol. The absorbance at 450 nm was measured with a microplate reader (BioTek, Winooski, VT).

Exosome Isolation

Because PM_{2.5} exposure in real life is long-term and chronic, we treated HBE cells with PM_{2.5} for 20 passages in this study. Considering the possible adverse effects of chronic PM_{2.5} exposure on cell viability, we used 25 µg/mL of PM_{2.5} as the experimental dose for treating HBE cells, which showed no overt toxic effects and was more beneficial than other dose of PM_{2.5} for cells (Supplementary Figure S1, available in www.besjournal.com). First, HBE cells were treated for 19 passages with PM_{2.5} at a dose of 25 µg/mL. Then cells were cultured in MEM with PM_{2.5} and 5% exosome-depleted FBS (by ultracentrifugation at 100,000 ×g at 4 °C for 12 h) for 48 h to collect exosomes. The cell supernatant was centrifuged at 3,000 ×g for 15 min and passed through a 0.22-µm filter (Millipore, USA) to remove floating cells and cellular debris; this was followed by concentration with 100 k ultrafiltration tubes (Millipore, USA). Finally, Exoquick-TC reagent (System Biosciences) was added to the concentrated solution of the supernatant according to the protocol. The mixture was incubated at 4 °C overnight and then centrifuged at 1,500 ×g for 30 min. The precipitate at the bottom, containing exosomes, was resuspended in phosphate buffered saline (PBS) and stored at -80 °C. The exosome samples were quantified with bicinchoninic acid (BCA) assays.

Transmission Electron Microscopy and Nanoparticle Tracking Analysis

The exosomes were diluted in PBS and fixed with 4% paraformaldehyde and 4% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4). Then 10 µL of exosome sample was placed on a carbon-coated copper grid and negatively stained with phosphotungstic acid (pH 7.0) for 30 s. Finally, the exosomes were visualized under a transmission electron microscope (JEM-1200EX; JEOL Ltd., Tokyo, Japan). Nanoparticle tracking analysis was performed to measure the size distribution and concentration of exosomes with a ZetaView particle

tracker (Particle Metrix, Munich, Germany) according to the instruction manual.

Western Blotting

Proteins were extracted from cells with cell lysis buffer for western blotting containing protease inhibitors (Beyotime Institute of Biotechnology). A BCA protein assay kit (Beyotime Institute of Biotechnology) was used for quantification. The standard protocol for western blotting was as previously described^[22]. The proteins (35 µg per well) were separated and transferred to polyvinylidene difluoride membranes (Millipore, USA). Primary antibodies (diluted at 1:1,000) were incubated with membranes at 4 °C overnight. After washing and incubation of the membrane with secondary antibodies (diluted at 1:1,000) for 1 h at room temperature, the protein band density was detected with a ChemiDoc MP system (Bio-Rad, USA), and quantified in Image J Software.

Migration and Invasion Assays

Transwell assays were used to assess the migration or invasion of A549 and H1975 cells. For migration assays, 2×10^4 A549 cells or 5×10^4 H1975 cells were suspended in 200 µL serum-free medium and seeded into the upper chambers, and 600 µL medium containing 10% FBS was added to the lower chamber. The cells were incubated at 37 °C. After 24 h, the non-migrating cells were removed with cotton swabs. The cells that migrated to the bottom of the membrane were fixed with 4% paraformaldehyde and stained with crystal violet. Migrated cells were visualized under a microscope (Olympus, Japan) and quantified by counting of the average number of cells in four random fields. The invasion assay protocol was the same as that of the migration assay except that A549 and H1975 cells were seeded into the upper chambers, which had been coated with Matrigel (Millipore, USA).

Animal Experiments

Seven-week-old female BALB/c nude mice were purchased from Shanghai Jihui Laboratory Animal Care Co., Ltd. (Shanghai, China). The mice were raised according to the institutional guidelines in the SPF level animal experimental center of the Ninth People's Hospital of Shanghai. The mice were divided into four groups randomly: a, control group; b, A549 group; c, A549+HBE-exo group; and d, A549+PHBE-exo group. The mice in the control group did not receive any treatment. The mice in the other three groups were injected with 5×10^6 A549-luc cells via

the tail vein to establish a mouse model of lung metastasis. Additionally, the mice in the A549+HBE-exo group and A549+PHBE-exo group were injected with 200 μ g exosomes derived from HBE cells or PM_{2.5}-treated HBE cells via the tail vein twice per week. At four weeks after inoculation of A549-luc cells, the luciferase activity in the mice was evaluated through bioluminescence imaging (IVIS Lumina II; PerkinElmer, USA). The mice were then sacrificed, and the lung tissues were collected for histological analysis with hematoxylin and eosin staining. This experiment was approved by the Animal Care and Use Committee of Shanghai Jiao Tong University School of Medicine, China.

Statistical Analysis

The statistical analyses were performed in SPSS 19.0 software (IBM, USA), and the graphs were generated in GraphPad Prism 8.0 software (Graphpad, USA). The data for cell experiments are shown as mean $\pm$ SD of three independent experiments, whereas the metastatic characteristics of

experimental mice were assessed according to the median in each group. Statistics were analyzed with one-way ANOVA with the LSD post hoc test. The significance level was defined as ≤ 0.05 .

RESULTS

Isolation of Exosomes Derived from HBE Cells

Exosomes were isolated with Exoquick-TC reagent, and qualitative analyses were then performed. As indicated by the electron micrographs, the cup-shaped nano-vesicles had a diameter of approximately 50–150 nm (Figure 1A). Nanoparticle tracking analysis indicated no clear difference in the size distribution between exosomes derived from HBE cells (HBE-exo) and PM_{2.5}-treated HBE cells (PHBE-exo) (Figure 1B). Furthermore, exosomal markers, including protein CD9 and CD81, were positively expressed in exosome samples (Figure 1C). Collectively, these results demonstrated that the exosomes were extracted successfully.

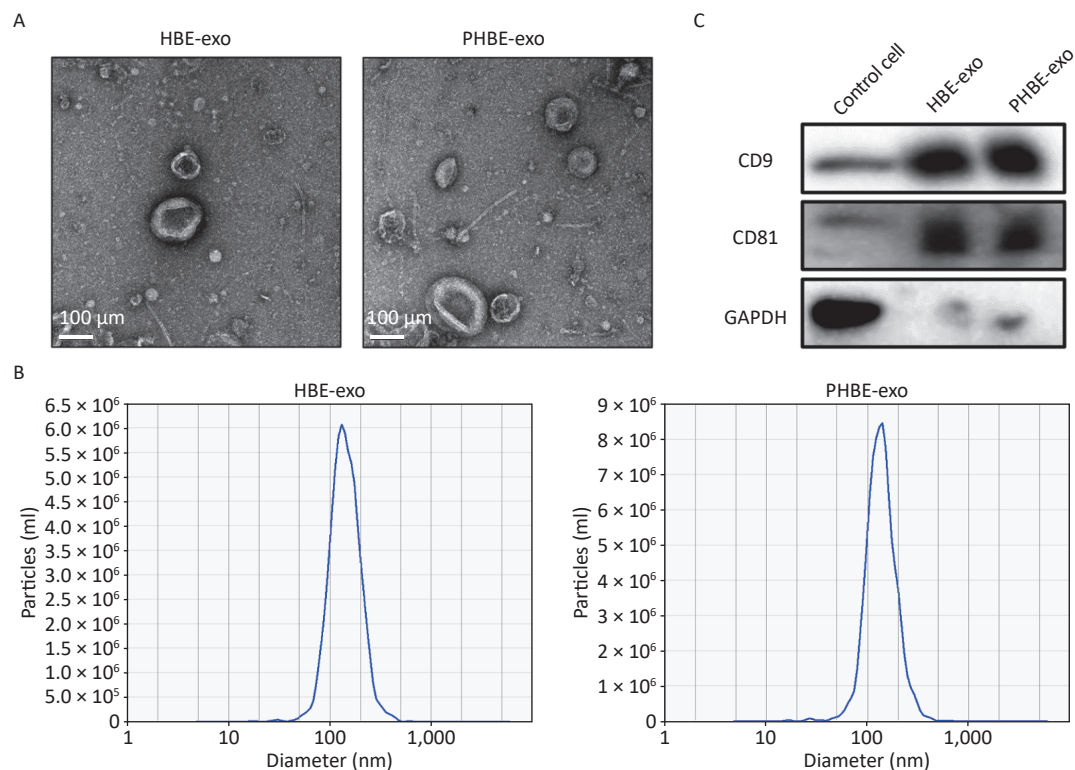


Figure 1. Identification of exosomes derived from HBE cells (HBE-exo) and exosomes derived from PM_{2.5}-treated HBE cells (PHBE-exo). (A) Transmission electron microscopy (TEM) pictures of HBE-exo and PHBE-exo. Scale bar: 100 μ m. (B) Nanoparticle tracking analysis (NTA) tested the size distribution of HBE-exo and PHBE-exo. (C) Western blotting results of exosomal markers CD9 and CD81 of control cells, HBE-exo and PHBE-exo.

Exosomes Derived from PM_{2.5}-treated HBE Cells Promote Lung Cancer Metastasis In Vivo

To explore the role of PHBE-exo in metastasis of lung cancer, we used an animal model of lung metastasis. Figure 2A shows the schedule of the animal experiment. The body weight of each mouse was measured before every treatment (injection of cells or exosomes). As shown in Supplementary Figure S2 (available in www.besjournal.com), no clear difference in mouse body weight was observed among the four groups. Moreover, the lung weights and organ coefficients were also similar among the four groups (Supplementary Figure S3, available in www.besjournal.com).

The characteristics of lung metastasis were assessed with two methods: *in vivo* imaging and histopathological analysis. Considering the individual differences in mice, we assessed the metastatic characteristics of experimental mice according to the median in each group. As shown in Figure 2, higher luciferase activity was observed in mice in the A549+PHBE-exo group (total luciferase: 2.20×10^5) than the A549 group (total luciferase: 0.50×10^5) and A549+HBE-exo group (total luciferase: 1.24×10^5) (Figure 2B and 2C). Histopathological analysis showed no clear difference in metastatic nodule

numbers among the mice with lung cancer (A549 group: 4.5; A549+HBE-exo group: 2; A549+PHBE-exo group: 4.5) (Figure 2D and 2E). Notably, more large metastatic nodules were observed in the lungs of mice treated with PHBE-exo than in the mice in the other groups. The calculated ratio of tumor to lung tissue (relative metastatic region) was higher in the A549 + PHBE-exo group than the other groups (A549 group: 0.004; A549+HBE-exo group: 0.005; A549+PHBE-exo group: 0.016) (Figure 2F). These results implied that PHBE-exo might promote metastasis of lung cancer *in vivo*.

Effects of Exosomes Derived from PM_{2.5}-treated HBE Cells on A549 and H1975 Cells

To assess the mechanisms through which PHBE-exo promoted the lung metastatic potential, we treated A549 and H1975 cells with HBE-exo or PHBE-exo at a dose of 200 $\mu\text{g}/\text{mL}$, at which no clear change in cell viability was observed (Supplementary Figure S4, available in www.besjournal.com).

Exosomes Derived from PM_{2.5}-treated HBE Cells Promote the Migration and Invasion of A549 and H1975 Cells Through Transwell assays, we evaluated cell migration and invasion to determine the effects of PHBE-exo on lung cancer cell lines. As shown in Figure 3, more A549 and H1975 cells

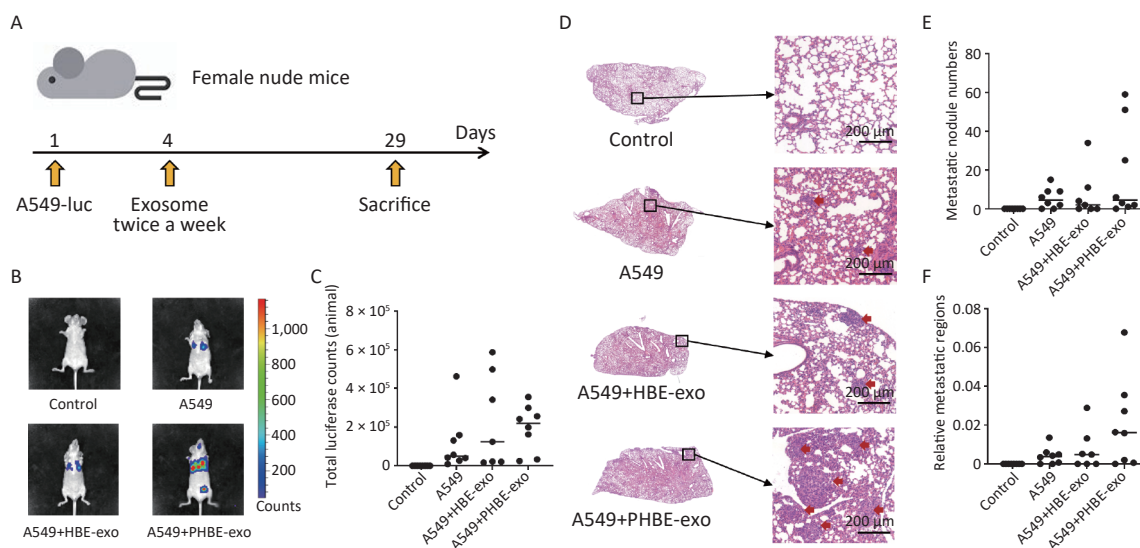


Figure 2. Exosomes derived from PM_{2.5}-treated HBE cells promoted metastasis of lung cancer. (A) The procedure of animal experiment. (B&C) The representative images of the mice in each group (B) and total luciferase counts of each mouse (C) were assessed by biofluorescence image system. (D–F) Hematoxylin and Eosin (H&E) staining analysis of lung tissues. The representative histopathological pictures of each mice group (D): left panel, magnification $\times 10$; right panel, magnification $\times 100$. Red arrows: metastatic nodules. Scale bars: 200 μm . Metastatic nodule numbers (E) and relative metastatic regions (tumor region / tissue region) (F) of each mouse were calculated and shown as scatter graph with median, $n \geq 7$.

migrated to the bottom of the membrane in the PHBE-exo group than the PBS or HBE-exo group ($P < 0.05$). Moreover, the effects of exosomes on the invasive ability of A549 and H1975 cells were examined. More cells treated with PHBE-exo passed through the Matrigel ($P < 0.05$) (Figure 3), thus indicating that PHBE-exo enhanced the invasive potential of A549 and H1975 cells.

Exosomes Derived from $PM_{2.5}$ -treated HBE Cells Regulate the Protein Expression of EMT Markers in A549 and H1975 Cells

EMT is considered an important process in tumor metastasis^[23]. In the present study, western blotting results indicated that (Figure 4) the protein levels of the epithelial marker E-cadherin were downregulated ($P < 0.05$), whereas the protein levels of the mesenchymal markers N-cadherin and ZEB1 were up-regulated ($P < 0.05$) after treatment with PHBE-exo, compared with PBS or HBE-exo, thus indicating that EMT was induced by PHBE-exo.

Exosomes Derived from $PM_{2.5}$ -treated HBE Cells Affect the Protein Levels of Phosphorylated c-Jun N-terminal Kinase (P-JNK)

Mitogen-activated protein kinase

(MAPK) signaling pathways regulate a variety of cellular functions including gene transcription and protein biosynthesis^[24]. To determine whether PHBE-exo might affect MAPK activation in lung cancer cell lines, we assessed the JNK, extracellular regulated protein kinase (ERK) and P38 mitogen-activated protein kinase (P38) activity. As shown in Figure 5, treatment with PHBE-exo significantly increased the protein level of P-JNK ($P < 0.05$) but not those of P-ERK ($P > 0.05$) and P-P38 ($P > 0.05$) in A549 cells. The increase in the protein level of P-JNK was also observed in H1975 cells (Supplementary Figure S5, available in www.besjournal.com). These results indicated that PHBE-exo activated JNK in lung cancer cells.

Exosomes Derived from $PM_{2.5}$ -treated HBE Cells Enhance the Malignant Transformation of Lung Cancer Cells via JNK

We further explored the role of JNK in promoting lung cancer induced by PHBE-exo. A549 and H1975 cells were treated with DMSO or 20 $\mu\text{mol/L}$ SP600125, a selective inhibitor of JNK

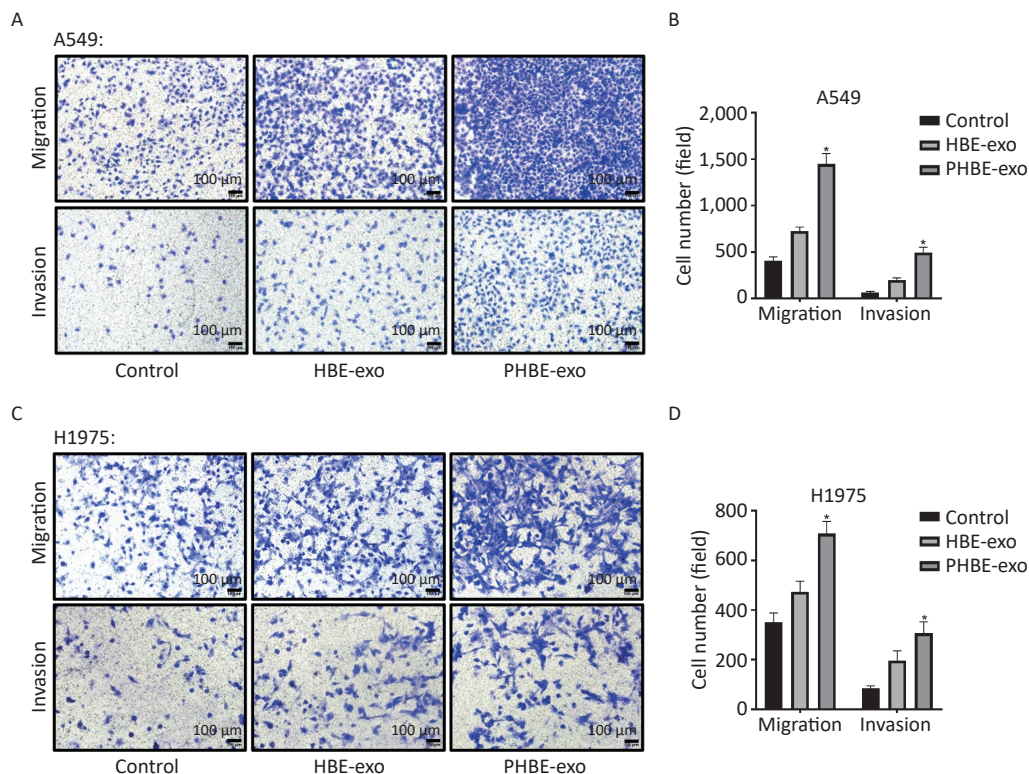


Figure 3. Exosomes derived from $PM_{2.5}$ -treated HBE cells promoted the migration and invasion capacity of lung cancer cells. Lung cancer cells were treated with PBS, exosomes derived from HBE cells (HBE-exo) or exosomes derived from $PM_{2.5}$ -treated HBE cells (PHBE-exo). Representative figures of A549 (A) and H1975 (C) cells in transwell assays. Scale bar: 100 μm . A549 (B) and H1975 (D) cell numbers were quantified by image J (mean $\pm$ SD, $n = 3$). * $P < 0.05$, different from HBE-exo-treated group.

phosphorylation, then treated with PHBE-exo. As shown in Supplementary Figure S6 (available in www.besjournal.com), the increase in protein levels of P-JNK mediated by PHBE-exo was effectively inhibited by SP600125 ($P < 0.05$).

Enhancement of Migration and Invasion by Exosomes Derived from PM_{2.5}-treated HBE Cells is Suppressed by SP600125

To elucidate whether PHBE-exo might promote lung cancer migration and invasion through activating JNK, we exposed A549 and H1975 cells with or without SP600125 treatment to exosomes. As shown in Figure 6, SP600125 diminished the cell migration and invasion promoted by PHBE-exo ($P < 0.05$), thus indicating that JNK plays a key role in these processes.

EMT Induced by Exosomes Derived from PM_{2.5}-treated HBE Cells Is Suppressed by SP600125

Next, we investigated the effect of JNK on PHBE-exo-induced EMT. Western blotting analysis indicated that the levels of EMT-associated proteins in PHBE-exo treated A549 cells were modulated by

SP600125. As shown in Figure 7, the protein levels of the epithelial marker E-cadherin were up-regulated ($P < 0.05$) and those of the mesenchymal marker N-cadherin were down-regulated ($P < 0.05$) in cells initially treated with SP600125, compared with A549 cells cultured with only PHBE-exo. The same results were observed in H1975 cells (Figure 7). These results indicated that inhibition of JNK restrained the PHBE-exo-induced EMT.

DISCUSSION

In the past century, the harm of PM_{2.5} has been widely reported^[25]. Hundreds of millions of people live in environments with excessive PM_{2.5}^[26]. Numerous studies have revealed that PM_{2.5} contributes to lung cancer^[10,27,28]. To date, the lung cancer promotion mechanism of PM_{2.5} has not yet been fully elucidated. In the current study, the animal experiment results indicated that exosomes derived from PM_{2.5}-treated human bronchial

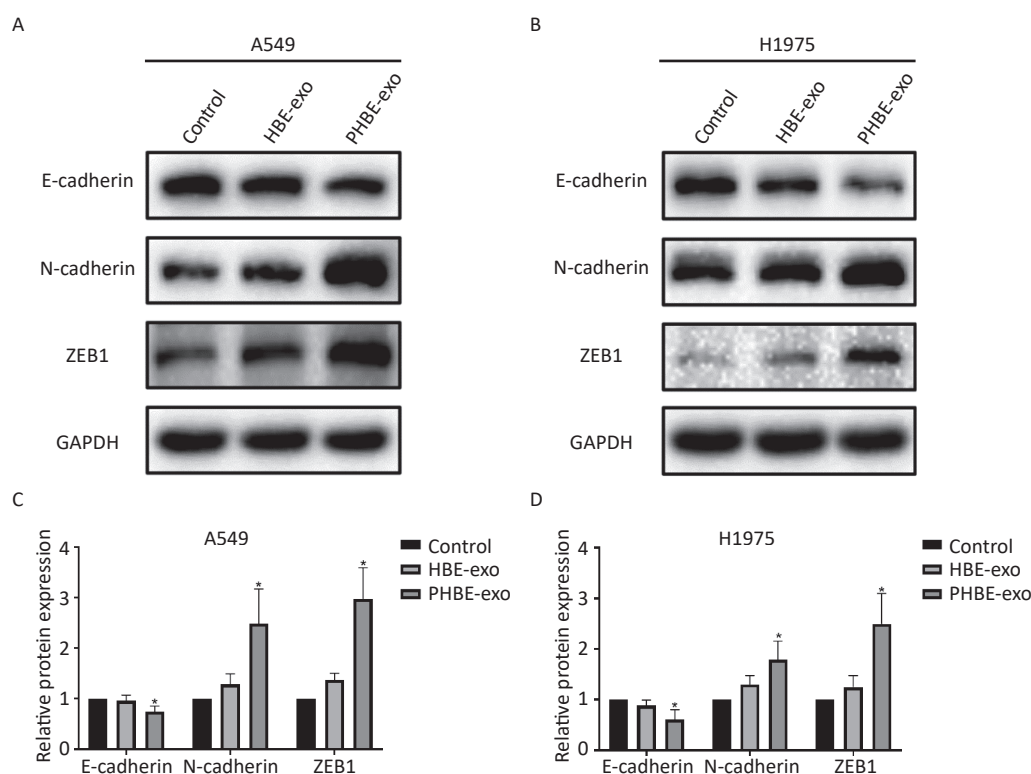


Figure 4. Exosomes derived from PM_{2.5}-treated HBE cells changed the protein expression of epithelial-to-mesenchymal transition (EMT) markers of lung cancer cells. Lung cancer cells were treated with PBS, exosomes derived from HBE cells (HBE-exo) or exosomes derived from PM_{2.5}-treated HBE cells (PHBE-exo). Protein expression of EMT markers in A549 (A&C) and H1975 (B&D) cells were quantified by western blotting (mean $\pm$ SD, $n = 3$). The intensities of protein bands were analyzed and normalized to GAPDH. * $P < 0.05$, different from HBE-exo-treated group.

epithelial cells (PHBE-exo) might promote lung cancer metastasis *in vivo*. Furthermore, PHBE-exo promoted the migration, invasion and EMT of lung cancer cells, but these effects were partially suppressed by inhibition of JNK activation. These findings broadened understanding of the cancer-promoting mechanisms of environmental pollutants.

Although the functions of exosomes have been extensively studied, the roles of exosomes secreted by cells exposed to environmental pollutants remain to be investigated. Bronchial epithelial cells are a major source of exosomes in the lungs^[29]; consequently, bronchial epithelial cell-derived exosomes may be involved in the pathogenesis of a wide range of lung diseases. Some studies have concluded that exosomes derived from HBE cells (HBE-exo) played important

roles in several lung diseases including tumor and inflammatory diseases. Xu et al. have reported that, through intercellular communication, exosomal miRNAs derived from arsenite-transformed HBE cells participated in carcinogenesis induced by environmental chemicals^[30]. Exosomal miRNA-21 derived from cigarette smoke extract treated HBE cells has been reported to be involved in cigarette smoking induced chronic obstructive pulmonary disease, pulmonary fibrosis and carcinogenesis^[31-33]. Environmental pollutants can induce or promote lung diseases *via* exosomes from HBE cells. Therefore, we wondered whether exosomes from HBE cells might be involved in PM_{2.5}-mediated promotion of lung cancer.

Using a lung cancer animal model, we observed

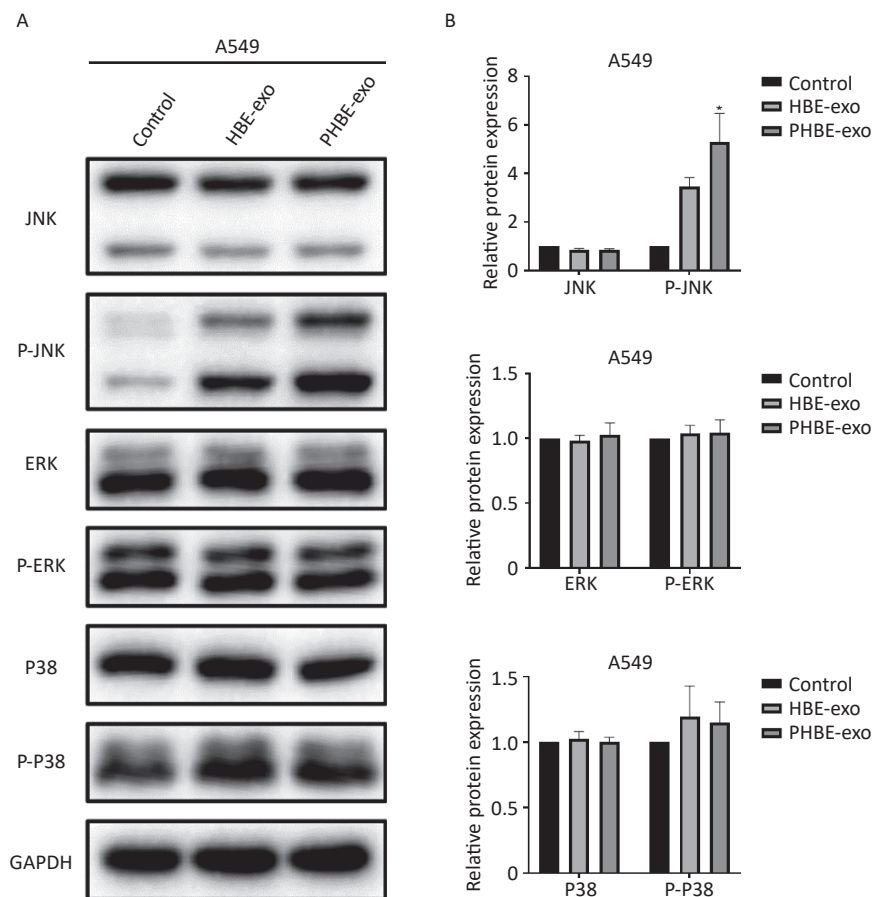


Figure 5. Exosomes derived from PM_{2.5}-treated HBE cells affected the activation of MAPKs. A549 cells were treated with PBS, exosomes derived from HBE cells (HBE-exo) or exosomes derived from PM_{2.5}-treated HBE cells (PHBE-exo). Representative figures of western blotting (A) and the protein expression of JNK, P-JNK, ERK, P-ERK, P-38, and P-P38 were quantified by image J (B) (mean $\pm$ SD, $n = 3$). The intensities of protein bands were analyzed and normalized to GAPDH. * $P < 0.05$, different from HBE-exo treated group.

that the exosomes from PM_{2.5}-treated HBE cells increased the luciferase activity and the relative metastatic region in the lungs of mice. These results indicated that PHBE-exo promote lung metastasis. Xu et al. have reported that exosomes derived from PM_{2.5}-treated A549 cells increase proliferation of A549 cells *in vitro* and increase tumor growth *in vivo*, thus demonstrating that exosomes derived from PM_{2.5} treated cells might be involved in lung cancer development^[34]. Several studies have reported that, after PM_{2.5} exposure, the composition of exosomes derived from lung cells is altered^[35,36]; on the basis of bioinformatics analysis of exosomal miRNAs from A549 and BEAS-2B cells, the authors have speculated that exosomes might be involved in the tumorigenesis and development of PM_{2.5}-associated cancer. In the present study, exosomes from bronchial epithelial cells (HBE-exo) also promoted lung cancer cell migration and invasion, and exosomes

from PM_{2.5}-treated cells (PHBE-exo) enhanced these effects (Figure 3). In addition, EMT was induced by PHBE-exo. Therefore, we propose that PM_{2.5} might promote lung cancer metastasis through exosomes derived from human bronchial epithelial cells.

MAPK signaling pathways are widely known to mediate cellular responses to various stimuli. Activation of MAPK signaling pathways often occurs in PM_{2.5}-treated cells, and MAPK signaling pathways are associated with stress and inflammation^[37-39]. In the present study, we observed the upregulation of P-JNK protein expression induced by PHBE-exo. Xu et al. have also reported that exosomal miRNAs of PM_{2.5}-treated BEAS-2B cells might target MAPK signaling pathways^[35].

JNK is a member of the MAPK family that has been reported to be involved in carcinogenesis in many studies. Exosomes can accelerate cancer

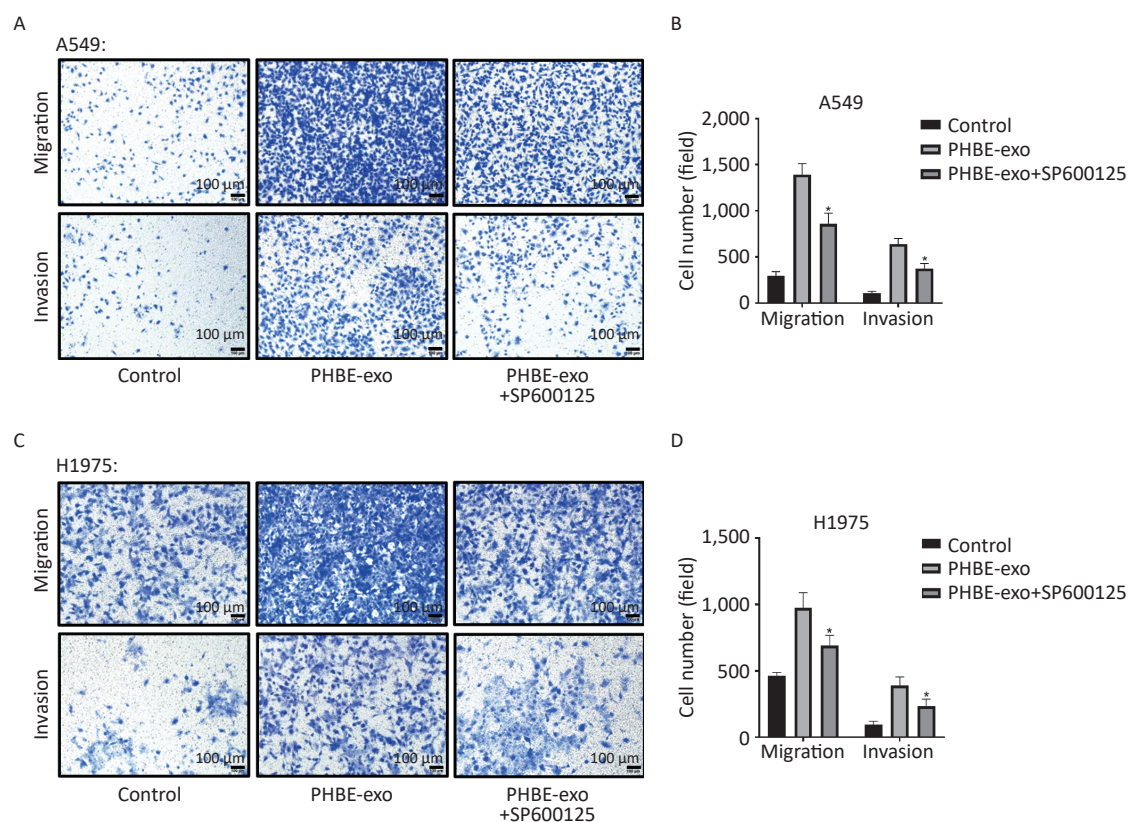


Figure 6. Inhibition of the JNK signaling pathway suppressed the migration and invasion capacity enhanced by exosomes derived from PM_{2.5}-treated HBE cells. Lung cancer cells were treated with DMSO, exosomes derived from PM_{2.5}-treated HBE cells (PHBE-exo), or SP600125+PHBE-exo. Representative figures of A549 (A) and H1975 (C) cells in transwell assays. Scale bar: 100 μ m. Cell numbers of A549 (B) and H1975 (D) were quantified by image J (mean $\pm$ SD, $n = 3$). * $P < 0.05$, different from PHBE-exo-treated group.

development, and the JNK signaling pathway is involved in this process^[40-42]. Wagner et al. have reported hyperactivation of JNK proteins in biological samples from patients with cancer, thus indicating that the JNK signaling pathway is associated with cancers in humans^[43]. JNK plays multiple roles in many types of cancer progression including proliferation, migration and invasion^[44-47]. Moreover, the JNK signaling pathway is involved in cancer cell growth, tumor formation and metastasis in the lungs^[24,48]. Furthermore, the EMT and cell migration ability of mouse transformed keratinocytes caused by TGF- β 1 rely on activation of the JNK signaling pathway^[49]. In the present study, we further explored the effects of JNK in promoting lung cancer induced by PHBE-exo. The results showed that exosomes from PM_{2.5}-treated HBE cells promoted migration, invasion and EMT of lung cancer cells, which were accompanied by activation of JNK in the cells. SP600125, a specific inhibitor of JNK phosphorylation, diminished the migration, invasion and EMT induced by PHBE-exo. Thus, we speculate that PHBE-exo might facilitate the malignant transformation of lung cancer cells,

partially through JNK.

DECLARATION OF COMPETING INTERESTS

All authors declare that they have no competing interests.

AUTHORSHIP CONTRIBUTION STATEMENT

YU Heng Yi: conceptualization, methodology, formal analysis, investigation, writing the original draft. GUO Hua Qi: conceptualization, methodology, investigation. FENG Yan: methodology, resources. CHENG Wei: resources. Wang Yan: supervision, funding acquisition, writing the review & editing.

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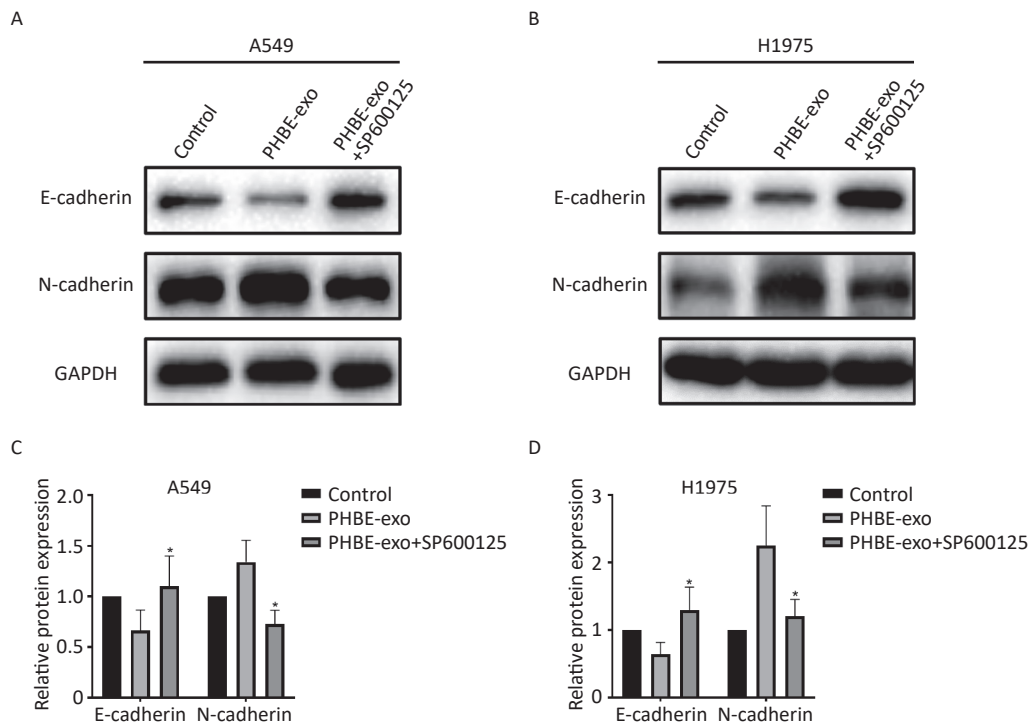


Figure 7. Inhibition of the JNK signaling pathway suppressed EMT induced by exosomes derived from PM_{2.5}-treated HBE. Lung cancer cells were treated with DMSO, exosomes derived from PM_{2.5}-treated HBE cells (PHBE-exo), PHBE-exo+SP600125. A549 (A&C) and H1975 (B&D) cell protein expression of EMT markers were quantified by western blotting (mean $\pm$ SD, $n = 3$). The intensities of protein bands were analyzed and normalized to GAPDH. * $P < 0.05$, different from PHBE-exo-treated group.

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