

Atmospheric particulate matter_{2.5} promotes the migration and invasion of hepatocellular carcinoma cells

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Abstract. Epidemiological data has demonstrated that particulate matter (PM) with an aerodynamic diameter $\leq 2.5 \mu\text{m}$ (PM_{2.5}) is associated with cancer incidence. However, the precise mechanisms underlying PM_{2.5}-mediated hepatocellular carcinoma cancer (HCC) migration and invasion remain unclear. The aim of the present study was to explore the response of the HCC cell lines HepG2 and HuH-7 to PM_{2.5} exposure. The results revealed that PM_{2.5} treatment promoted the migration and invasion of HCC cells, in addition to increasing protein levels of matrix metalloproteinase (MMP)-13. Additionally, PM_{2.5} induced intracellular reactive oxygen species formation in HCC cells. Further investigation revealed that phosphorylation of RAC- α serine/threonine-protein kinase (AKT) increased in response to PM_{2.5} exposure in HCC cells, and the AKT antagonist LY294002 reduced PM_{2.5}-induced migration, invasion and MMP-13 expression. In addition, the data from the present study demonstrated that high concentrations of PM_{2.5} decreased the proliferation of normal HL7702 hepatocyte cells and promoted apoptosis. These results indicate that the activation of AKT by PM_{2.5} results in MMP-13 overexpression, and stimulates HCC cell migration and invasion. In conclusion, the results from the present study demonstrate that PM_{2.5} promotes HCC development and elucidate a potential underlying molecular mechanism for this effect.

Introduction

Particulate matter (PM), a key type of air pollutant, is regarded as a group 1 human carcinogen by the International Agency for Research on Cancer (1,2). A number of epidemiological studies have demonstrated an association between high concentrations of PM, particularly that with an aerodynamic diameter of $<2.5 \mu\text{m}$ (PM_{2.5}), and an increased risk of cancer development (3). In Northern China, coal combustion is used widely and extensively in rural areas for cooking and heating (4). The high concentration of PM_{2.5} caused by this type of energy production, and chemical and metallurgical industries, in the cities of China may cause serious health problems in the population (5).

Previous studies have demonstrated an association between PM_{2.5} and lung cancer cell metastasis (6,7). The results revealed that PM_{2.5} enhanced lung cancer cell migration and invasion, and promoted reactive oxygen species (ROS) levels-mediated extracellular matrix (ECM) degradation. However, the molecular mechanisms underlying PM_{2.5}-induced carcinogenesis are not yet well understood.

Hepatocellular carcinoma (HCC) is one of the predominant causes of cancer-associated mortality worldwide (8). HCC cell metastasis is the primary cause of HCC development. HCC metastasis occurs through complex processes, including the migration and invasion of tumor cells (9,10). PM_{2.5} induced the metastatic capabilities of lung cancer, including migration and invasion (11). The patient observational reports indicated that PM_{2.5} exposure was associated with HCC via chronic liver inflammation (12). The incidence of HCC may also be associated with PM_{2.5}, therefore the effects of PM_{2.5} on HCC cells require further study.

Aberrant ROS expression may lead to a number of physiological and pathological changes, such as cell cycle progression (13) and apoptosis (14,15). Notably, ROS can stimulate the expression of numerous metastatic factors, which leads to HCC cell migration and invasion (13,16). In addition, ROS production is an important etiological mechanism in PM_{2.5}-induced tissue injury (17-19). However, whether PM_{2.5} affects HCC through the production of ROS is not yet known, to the best of our knowledge.

HCC metastasis occurs through a complex mechanism, during which matrix metalloproteinases (MMPs) are responsible for ECM degradation (20). MMP13 is overexpressed in numerous types of invasive tumors (21-23), suggesting that MMP13 may be associated with the cell migration and invasion induced by PM_{2.5}.

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The phosphoinositide 3-kinase (PI3K)-RAC-alpha serine/threonine-protein kinase (AKT) signaling pathway is important for the development of HCC (24,25). Activated AKT is necessary for the metastasis, proliferation and evasion of apoptosis of tumor cells, therefore the PI3K/AKT signaling pathway may be activated during PM_{2.5}-mediated cancer cell migration and invasion.

The results from the present study demonstrate that PM_{2.5} induces HCC invasion and migration, and revealed that the underlying molecular mechanism involves the PI3K/AKT signaling pathway, which in turn promotes ROS and MMP13 expression.

Materials and methods

Preparation of ambient PM_{2.5} water-soluble extracts. A total of 50 mg of particulate matter 2.5 (PM_{2.5}; SRM[®] 1650b; NIST, Boulder, CO, USA) was suspended in 5 ml PBS for 24 h at 37°C and sonicated at 40 W for 20 min. The PM_{2.5} suspension was centrifuged at 13,000 x g for 10 min at 4°C, and filtered using a 0.22- μ m syringe filter.

Cell culture and exposure. The human HCC cell lines HepG2 and HuH-7, and human normal human HL7702 hepatocytes were purchased from the China Center for Type Culture Collection and were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μ g/ml streptomycin (all Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in a humidified incubator with 5% CO₂ at 37°C. LY294002 was purchased from Sigma Aldrich (#L9908; Merck KGaA, Darmstadt, Germany).

Cell invasion assay. HepG2 and HuH-7 cells (cultured at 37°C) invasion were measured using a 24-well Matrigel[®]-coated Transwell[®] assay, as previously described (26). Briefly, the upper surface of the filter was coated with Matrigel (1 mg/ml) at room temperature. Prior to the assay, HCC cells (5x10⁵ cells/ml) were seeded into plates, 10 μ g/ml PM_{2.5} was added and the plates were cultured for 24 h. The PM_{2.5}-treated HCC cells were harvested, and 8x10⁴ cells in DMEM were added to the upper chamber of the Transwell plate. DMEM medium with 10% FBS was added to the lower chamber. Cells were allowed to migrate through the Matrigel for 24 h. Migrated cells were fixed with 4% paraformaldehyde and stained with crystal violet.

Transwell migration assay. Cell migration was assessed using a Transwell assay. HCC cells (5x10⁴) were incubated with PM_{2.5} at various doses (0-10 μ g/ml) for 24 h prior to seeding into the upper chambers. DMEM containing 10% FBS was placed into the bottom chambers. Following 8 h of incubation, cells in the upper chamber that had not migrated were removed. The migrated cells were fixed with 4% paraformaldehyde and stained with crystal violet. Images were captured using an Olympus light microscope. A total of three independent experiments were performed. The migration index was defined as follows: (the migrated cells number in the experimental group/the migrated cells number in the control group) x100.

ROS assay. A total of 5x10⁵ cells/well of HepG2 or HuH-7 cells were seeded into 35 mm Petri dishes with DMEM containing 10% FBS. The cells were treated with PM_{2.5} at various doses

(0-10 μ g/ml) for 6 h. The cells were harvested, resuspended in DMEM and incubated with 2',7'-dichlorofluoresceindiacetate (DCFH-DA; 10 μ M) at 37°C for 30 min. The intracellular ROS levels were monitored at 488 nm (excitation) and 519 nm (emission) using a confocal fluorescence microscope and analyzed using flow cytometry. The data were processed using the FlowJo Vx 10.0 software (Tree Star Inc., Ashland, OR, USA).

ELISA. MMP13 levels of HepG2 and HuH-7 cells were determined using the Human MMP13 Quantitation ELISA kit (DM1300; R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's protocol. The optical density of the plates was read at 450 nm (excitation) and 540 nm (emission) using a microplate reader. The amount of MMP13 (μ g/ml) was evaluated from a standard curve and expressed as μ g/ml.

Western blotting. Total protein from HepG2 and HuH-7 cells was extracted using radioimmunoprecipitation assay lysis buffer containing 1% protease inhibitor cocktail (#5500; R&D Systems, Inc.). The proteins (40 μ g) were separated by SDS-PAGE and transferred onto a polyvinylidene fluoride membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were incubated for 1 h at room temperature with 5% nonfat milk to block nonspecific binding and then incubated with the primary antibodies, including GAPDH (dilution, 1:100; #2118; Cell Signaling Technology, Inc., Danvers, MA, USA) and p-AKT (dilution, 1:100; #4058; Cell Signaling Technology, Inc.) overnight at 4°C. Following washing with Tris-buffered saline with 0.1% Tween-20, the membranes were incubated with the anti-rabbit (#W4011) or anti-mouse (#W4021) immunoglobulin conjugated to horseradish peroxidase secondary antibody (dilution, 1:500; Promega Corporation, Madison, WI, USA) for 1 h at room temperature. The blots were visualized using enhanced chemiluminescence kit (#32106, Thermo Fisher Scientific, Inc.).

Cytotoxicity assay. HL7702 cell viability was measured using the Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer's protocol. HL7702 cells (5x10³ cells/well) were seeded into 96-well plates overnight and then treated with serial concentrations of PM_{2.5} (0-400 μ g/ml) for 24 h. A total of 10 μ l of CCK-8 solution was added to each well for 1 h, and the absorbance at 450 nm was measured using a microplate reader and analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Shanghai, China).

Apoptosis assay. Cell apoptosis was detected using the Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) assay. HL7702 cells (2x10⁵ cells/well) were seeded into 6-well plates and treated with PM_{2.5} (0-400 μ g/ml) for 24 h. Apoptotic cells were then identified using an Annexin V-FITC apoptosis detection kit (BD Biosciences), according to the manufacturer's protocol. Flow cytometry data were performed using the Cell-Quest software (BD Biosciences, San Jose, CA, USA).

Statistical analysis. The data are presented as the mean \pm standard deviation of three independent experiments. All analyses were performed using analysis of variance tests followed by a Fisher's least significant difference test. P<0.05 was considered to indicate a statistically significant difference.

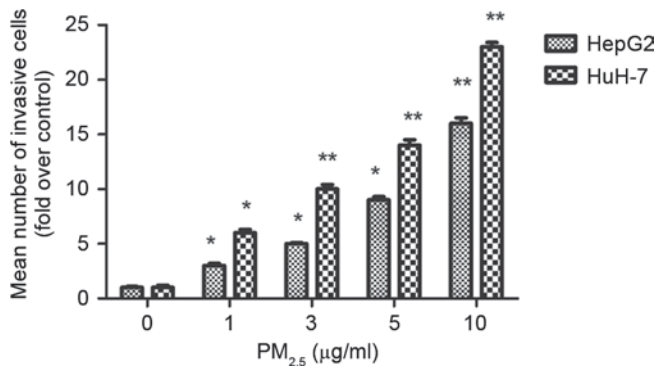


Figure 1. PM_{2.5}-induced hepatocellular carcinoma cell invasion was detected by a Matrigel invasion assay. *P<0.05, **P<0.01 vs. the control group (n=3 independent experiments). PM_{2.5}, particulate matter with an aerodynamic diameter ≤2.5 µm.

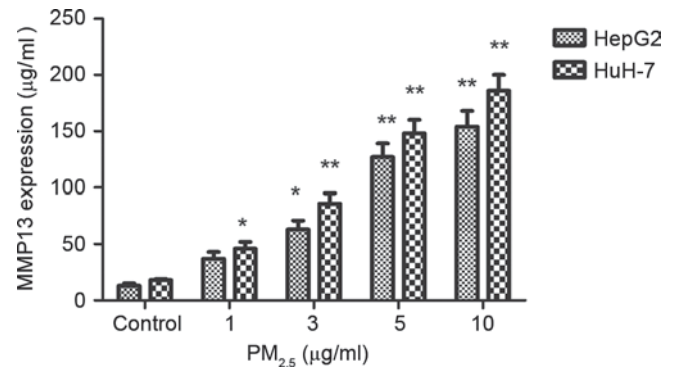


Figure 4. Increases in MMP13 expression were measured using an ELISA in PM_{2.5}-exposed hepatocellular carcinoma cells. *P<0.05, **P<0.01 vs. the control group (n=3 independent experiments). MMP, matrix metalloproteinase; PM_{2.5}, particulate matter with an aerodynamic diameter ≤2.5 µm

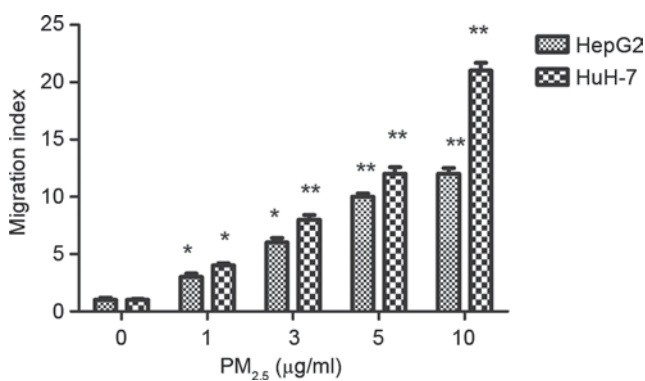


Figure 2. PM_{2.5}-induced hepatocellular carcinoma cell migration was detected using a Transwell assay. *P<0.05, **P<0.01 vs. the control group (n=3 independent experiments). PM_{2.5}, particulate matter with an aerodynamic diameter ≤2.5 µm.

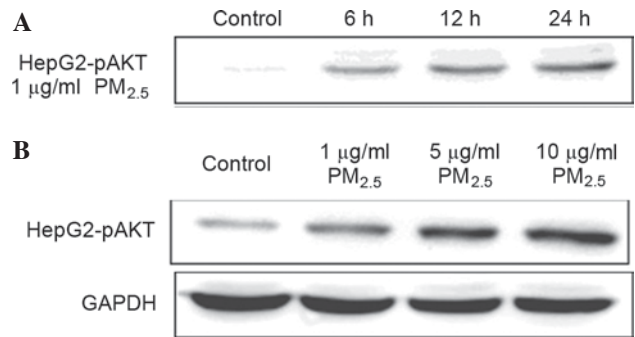


Figure 5. Increases in phosphorylated AKT expression were measured by western blotting in PM_{2.5}-exposed hepatocellular carcinoma cells in a (A) time-dependent manner and in a (B) dose-dependent manner for 24 h. GAPDH was used as a control. AKT; PM_{2.5}, particulate matter with an aerodynamic diameter ≤2.5 µm.

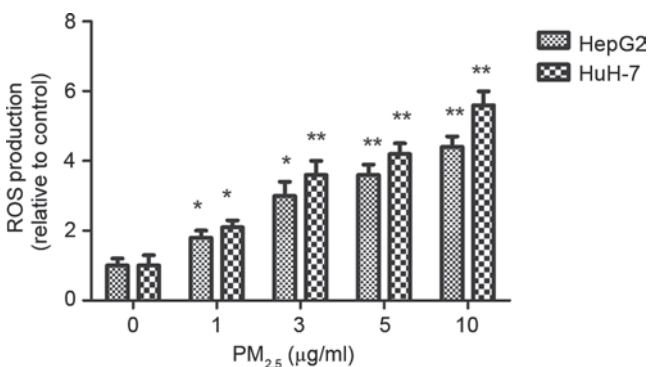


Figure 3. PM_{2.5}-induced ROS expression in hepatocellular carcinoma cells was detected using a 2',7'-dichlorofluoresceindiacetate probe assay. *P<0.05, **P<0.01 vs. the control group (n=3 independent experiments). PM_{2.5}, particulate matter with an aerodynamic diameter ≤2.5 µm; ROS, reactive oxygen species.

Results

PM_{2.5} induces HCC cell invasion. Matrigel chamber assays were used to investigate the role of PM_{2.5} in cell invasion. Following PM_{2.5} exposure, the number of HCC cells that migrated from the upper chamber to the lower chamber compared with the control (untreated cells) was significantly

increased (Fig. 1), which suggested that PM_{2.5} promotes HCC invasion.

PM_{2.5} induces HCC cell migration. To evaluate the effect of PM_{2.5} on cell motility, cell migration assays were carried out using a Transwell assay. Following exposure to PM_{2.5} (1-10 µg/ml) for 24 h, the number of cells that migrated significantly increased compared with the control (Fig. 2). PM_{2.5} stimulated HCC cell migration in a dose-dependent manner.

PM_{2.5} induces ROS production in HCC cells. To investigate the involvement of ROS in PM_{2.5}-induced HCC metastasis, ROS levels were measured following exposure to PM_{2.5} for 6 h. The DCFH-DA staining data demonstrated that PM_{2.5} significantly induces ROS overproduction compared with the control (Fig. 3).

Underlying molecular mechanisms of PM_{2.5}-induced HCC cell migration and invasion. The expression of MMP13 was measured following PM_{2.5} stimulation. The expression of MMP13 was positively associated with the PM_{2.5} dose (1-10 µg/ml) in HepG2 cells (Fig. 4). In addition, MMP13 expression in HCC cells increased significantly following PM_{2.5} treatment compared with the control.

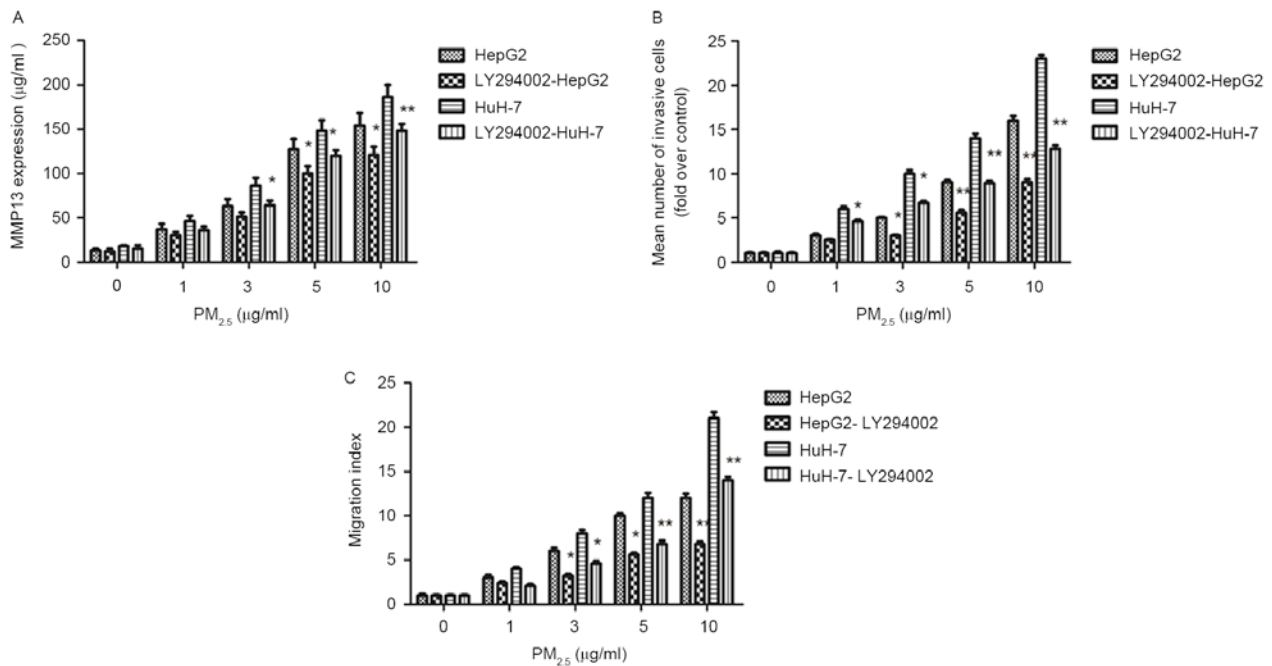


Figure 6. Involvement of the PI3K/AKT signaling pathway in PM_{2.5}-induced (A) MMP13 expression, (B) HCC cell invasion and (C) HCC cell migration. *P<0.05, **P<0.01 vs. the control group. PM_{2.5}, particulate matter with an aerodynamic diameter $\leq 2.5 \mu\text{m}$.

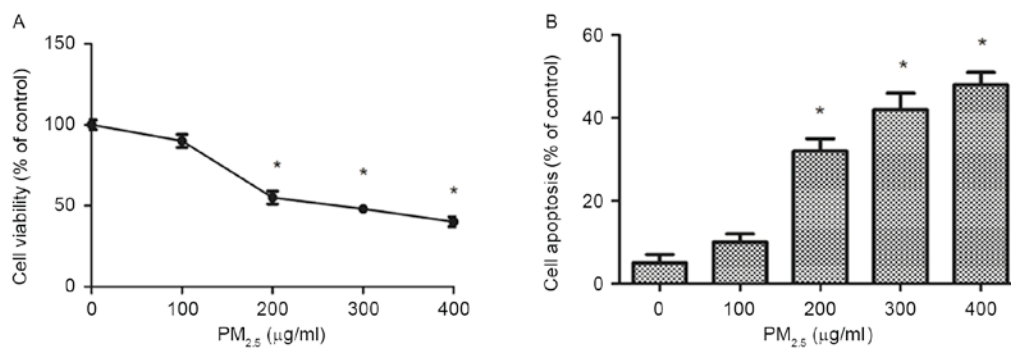


Figure 7. PM_{2.5} inhibits proliferation and induces apoptosis in HL7702 cells. (A) PM_{2.5} inhibited proliferation and (B) induced apoptosis in a dose-dependent manner. *P<0.05 vs. the control group. PM_{2.5}, particulate matter with an aerodynamic diameter $\leq 2.5 \mu\text{m}$.

To test whether the PI3K/AKT signaling pathway was involved in the response to PM_{2.5} exposure in HCC cells, HepG2 cells were treated with PM_{2.5} at different doses and time points. AKT phosphorylation increased 6 h following PM_{2.5} exposure (Fig. 5). The data revealed that PM_{2.5} increased levels of phosphorylated AKT in a dose-dependent manner.

LY294002 significantly suppressed the MMP13 protein expression induced by PM_{2.5} (Fig. 6A), in addition to the increased invasion (Fig. 6B) and migration (Fig. 6C) induced by PM_{2.5}. These data indicate that the inhibition of the AKT signaling pathway reduces MMP13 expression, and may suppress PM_{2.5}-induced HCC migration and invasion.

High concentrations of PM_{2.5} decreases HL7702 proliferation in a dose-dependent manner. The CCK-8 assay results revealed that 200, 300 and 400 $\mu\text{g/ml}$ PM_{2.5} significantly reduced HL7702 viability following exposure for 24 h compared with the control group. The half-maximal inhibitory concentration (IC₅₀) value of PM_{2.5} was 200 $\mu\text{g/ml}$ (Fig. 7A).

High concentrations of PM_{2.5} induces HL7702 apoptosis in a dose-dependent manner. The Annexin V-FITC/PI double staining assays demonstrated that PM_{2.5} induced apoptosis in HL7702 cells in a dose-dependent manner (Fig. 7B). In addition, 200-400 $\mu\text{g/ml}$ PM_{2.5} significantly increased the rate of apoptosis in HL7702 cells following exposure for 24 h compared with the control group.

Discussion

The aim of the present study was to explore the effect of PM_{2.5} on the invasion and migration of HCC cells, and to identify the underlying mechanisms of this effect. The results from the present study demonstrated that PM_{2.5} could induce the migration and invasion of HCC cells. Additionally, PM_{2.5} increased ROS and MMP13 production in a dose-dependent manner. Western blotting results indicated that the activation of the AKT signaling pathway may be involved in these effects of PM_{2.5}. The results from the present study suggest that PM_{2.5}

promotes the development of HCC via inducing cell invasion and migration.

Invasion and metastasis are typical characteristics of HCC and a contributing factor to the poor prognosis of patients with HCC. PM_{2.5} exposure was associated with the risk of developing HCC and PM_{2.5} exposure induced inflammation cytokine levels that may contributed to HCC risk. Considering the frequent occurrence of metastasis in patients with HCC, the association of PM_{2.5} exposure with HCC cell invasion and migration requires further study. HCC cell invasion is the first step for distant metastasis, therefore increased HCC cell invasion may have a significant effect on tumor development. The data from the present study demonstrated that PM_{2.5} exposure significantly promoted HCC migration and invasion in a dose-dependent manner.

ROS production has been revealed to serve an important role in mediating the cytotoxic effects of PM_{2.5}. Exposure to PM_{2.5} is regarded as a cardiovascular risk factor via ROS overproduction (27), but whether it promotes HCC via inducing ROS production remains unclear. In the present study, PM_{2.5} significantly increased HCC cell production of ROS in a dose-dependent manner.

MMP13 serves a crucial role in HCC invasion and metastasis, and has demonstrated to serve a role in the chronic inflammatory response (28). MMP13 mediates the release of inflammatory cytokines (21). Tumor necrosis factor (TNF)- α is a proinflammatory cytokine that serves a role in the pathogenesis of numerous diseases, including HCC (29). Therefore, PM_{2.5} is likely to promote HCC development by affecting MMP13 expression, which could promote cancer invasion and migration, in addition to promoting the expression of inflammatory cytokines. This hypothesis is supported by a previous study, which revealed that PM_{2.5} is associated with inflammatory cytokines as it induces TNF- α expression (30).

Previous studies have demonstrated that the PI3K/AKT signaling pathway is associated with MMP13 expression (31). Additionally, the data from the present study revealed that the PI3K/AKT signaling pathway was activated in PM_{2.5}-treated HCC cells. The AKT inhibitor LY294002 significantly decreased PM_{2.5}-induced MMP13 overexpression in HCC cells. These findings suggest that PM_{2.5}-induced MMP13 upregulation is dependent on the PI3K/AKT signaling pathway. The data also revealed that PM_{2.5} effectively inhibited proliferation of HL7702 cells *in vitro* with an IC₅₀ value of 200 μ g/ml.

In conclusion, the present study demonstrated that exposure to PM_{2.5} promotes the migration and invasion of HCC cells. The present study also highlighted the role of the PI3K/AKT signaling pathway and MMP13 expression in regulating PM_{2.5}-induced HCC cell migration and invasion. The results demonstrating that PM_{2.5} exposure promotes the invasion and migration ability of HCC cells provides an insight into the association between a higher incidence of HCC and PM_{2.5} exposure. Further studies are required to address the chronic exposure to higher PM_{2.5} levels on the effect of public health.

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