

Cadmium induces A549 cell migration and invasion by activating ERK

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Abstract. Cadmium (Cd) is an established carcinogen that is involved in the progression of lung cancer. However, the mechanisms underlying this Cd-induced process have yet to be fully elucidated. The present study explored the potential roles of phosphorylated (p)-ERK in the Cd-induced migration and invasion of lung cancer cells. An MTT assay was performed to evaluate cell viability whilst western blot analysis and reverse transcription-quantitative PCR were used to detect the expression of protein and mRNA, respectively. Migration and invasion assays were performed to assess cell migratory and invasive abilities. The results demonstrated that exposure to Cd increased the expression of p-ERK in A549 cells. Cd also enhanced the migration and invasion of A549 cells, which could be blocked via U0126 treatment (an inhibitor of mitogen activated protein kinase). In addition, it was identified that Cd-induced expression of matrix metalloproteinases 2 mRNA was mediated by p-ERK. In conclusion, the present findings indicated that Cd induced A549 cell migration and invasion by activating ERK, and it was hypothesized that p-ERK could serve as a target in the clinical treatment of Cd-induced lung cancer.

Introduction

Lung cancer is one of the most common malignant tumors that is a major cause of various cancer-related deaths for men and women, worldwide. In 2012, there were ~1.8 million newly

registered cases of lung cancer, with 1.6 million deaths occurring globally (1). Non-small cell lung cancer (NSCLC) accounts for ~85% of all lung-cancer-related deaths and can be classified as adenocarcinoma (40%), squamous cell carcinoma (<40%) and large cell carcinoma (5%). It is well established that tobacco smoke is the main cause of lung cancer; however, a series of studies have identified that occupational and environmental exposure to cadmium (Cd) is another important risk factor (2-5).

Cd is a widely used toxic industrial heavy metal that causes serious environmental health hazards to humans. The International Agency for Research on Cancer has classified Cd as a known human carcinogen (6). The primary means of Cd intake by humans are via food (particularly plant products), habitual tobacco smoking, drinking contaminated sources and Cd-related industry. Increasing evidence has revealed a correlation between Cd exposure and the formation of lung cancer (2,7,8). It has also been reported that oxidative stress, apoptotic resistance, induction of autophagy, decreased DNA repair capacity and genomic instability are involved in the development of lung cancer induced by Cd (9). Recently, exposure to Cd has been correlated with the migration and invasion of lung cancer cells (10,11). However, the underlying mechanisms of Cd-induced cancer are yet to be fully elucidated.

ERK, a member of the mitogen-activated protein kinase (MAPK) family, is a key molecule that transmits signals from surface receptors to the nucleus. It has the capacity to regulate cell growth, survival, mitosis and differentiation. It has also been demonstrated that ERK is involved in cell migration and invasion in several types of cancers (12-16), including lung cancer (17-24). Quintero Barceinas *et al* (17) reported that all-trans retinoic acid promoted growth, survival and migration in A549 lung cancer cells by activating the ERK signaling pathway. Furthermore, Li *et al* (18) reported that angelicin inhibited human lung carcinoma A549 cell growth and migration by regulating the ERK pathway. Zhang *et al* (20) demonstrated that transmembrane protein 17 decreased the invasion and metastasis of lung cancer cells via the ERK signaling pathway.

Based on previous literature, the present study hypothesized that p-ERK may serve an important role in the migration and invasion of A549 cells induced by Cd. Therefore, the aim of the current study was to explore the potential roles of p-ERK in Cd-induced A549 cell migration and invasion to increase

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understanding of the underlying molecular mechanisms and provide a potential therapeutic target for lung cancer treatment.

Materials and methods

Cell culture and treatment. The human lung cancer A549 cell line was obtained from the American Type Culture Collection (25). A549 cells were cultured in RPMI-1640 medium (Beijing Solarbio Science & Technology Co., Ltd.) containing 10% fetal bovine serum (FBS) (GE Healthcare Bio-Sciences), 100 U/ml penicillin and 100 μ g/ml streptomycin in a humidified incubator at 5% CO₂ and 37°C. A549 cells were cultured in serum-free RPMI-1640 medium for 24 h, followed by treatment with different concentrations of Cd [cadmium chloride hemi (pentahydrate); Shanghai Aladdin Bio-Chem Technology Co., Ltd.; 0, 0.1, 0.25, 0.5, 1, 2, 4 or 8 μ M] for various durations (0, 8, 24 or 48 h), as indicated. For certain experiments, 10 μ M U0126 (Sigma-Aldrich; Merck KGaA), an inhibitor of mitogen activated protein kinase (MEK)1/2 that is widely used to inhibit ERK1/2 activation (26-29), was added to the cell culture 1 h before Cd treatment.

MTT assay. A549 cells were harvested and seeded into 96-well plates at a density of 1×10^4 cells per well. Following incubation at 37°C for 24 h, the cells were treated with 0, 0.1, 0.25, 0.5, 1, 2, 4 or 8 μ M Cd for 48 h at 37°C. MTT (10 μ l; 5 mg/ml) was then added to each well and cells were incubated at 37°C for 4 h. Purple formazan was dissolved in 150 μ l of dimethyl sulfoxide, and absorbance was measured on a microplate reader at 492 nm. The group treated with 0 μ M Cd was regarded as the control. Four replicate wells were used for each analysis.

Western blot analysis. Cells were collected and washed three times with cold PBS. RIPA assay (Pierce; Thermo Fisher Scientific, Inc.) solution containing protease and phosphatase inhibitors was subsequently used to lyse cells, after which the protein content was determined using a bicinchoninic acid assay. Proteins (30 μ g) were separated via SDS-PAGE on a 12% gel then transferred onto a nitrocellulose membrane. The membrane was then blocked with 5% bovine serum albumin in Tris-buffered saline and 0.1% Tween-20 (TBST) at room temperature for 1 h. Subsequently, the blots were incubated at 4°C overnight with primary antibodies against phosphorylated (p)-ERK1/2 (1:1,000; Thr202/Tyr204; cat. no. 4730S; Cell Signaling Technology, Inc.), ERK1/2 (1:1,000; cat. no. 4695S; Cell Signaling Technology, Inc.) and tubulin (1:1,000; cat. no. sc-365791; Santa Cruz Biotechnology, Inc.). The blots were then washed with TBST three times, followed by incubation with horseradish peroxidase-conjugated secondary antibodies (Goat anti-rabbit IgG; cat. no. ZB-2301; Goat anti-Mouse IgG; cat. no. ZB-2305; each, OriGene Technologies, Inc.; each, 1:5,000) for 1 h at room temperature. Finally, the protein bands were visualized using an enhanced chemiluminescence reagent (cat. no. #1705060; Bio-Rad Laboratories, Inc.) on a ChemiDoc™ XRS+ image analysis system (Bio-Rad Laboratories, Inc.). Densitometry was performed using Image J software v1.31 (National Institutes of Health).

RNA extraction and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from A549 cells using

RNAiso Plus (Takara Bio, Inc.) according to the manufacturer's protocol. Following the quantification of total RNA concentrations, the PrimeScript™ II 1st Strand cDNA Synthesis kit (Takara, Bio, Inc.) was used to generate cDNA. A SYBR Green PCR master mix kit (SYBR® Premix Ex Taq™-Tli RNaseH Plus; Takara, Bio, Inc.) was used for qPCR. The thermocycling conditions were as follows: 95°C for 30 sec; 40 cycles at 95°C for 5 sec, 60°C for 30 sec and 72°C for 30 sec. mRNA relative expression levels were quantified using the 2^{- $\Delta\Delta C_q$} method (30) and normalized to GAPDH. The following primers were used: MMP2 forward, 5'-TGACATACATCTTTGCTGGAGAC-3' and reverse, 5'-GGCTTGCAGGGGAAGAAGTT-3'; GAPDH forward, 5'-TTCAGGTAATAGGCACCCTT-3' and reverse, 5'-CTTCTCCATGGTGGTGAAGA-3'.

Cell migration assay. A migration assay was performed using Transwell inserts. Cells were maintained at a concentration of 4×10^5 cells/ml in serum-free RPMI-1640. A total of 300 μ l cell suspension was added into the upper chamber, whilst the lower chamber was treated with 600 μ l of RPMI-1640 with 10% FBS. Following incubation at 37°C for 24 h, the medium was discarded and non-migrating cells on the top surface of the upper chamber were removed gently using cotton swabs. Migrated cells were fixed with pre-chilled methanol for 30 min then stained with 0.5% crystal violet at room temperature for 20 min. Representative images were taken under an inverted microscope (magnification, x10 and x20) equipped with a camera (Leica Microsystems GmbH). Subsequently, 33% glacial acetic acid (200 μ l) was added for 10 min to de-stain. Absorbance was measured at 570 nm using a microplate reader.

Cell invasion assay. For the invasion assay, the upper chamber was pre-coated with Matrigel. Ice-cold Matrigel was mixed with ice-cold RPMI-1640 medium at a ratio of 1:1 and spread onto the upper chamber (50 μ l/chamber), which was subsequently incubated at 37°C for 2 h. The following steps including cell plating, incubation, fixing and de-staining were conducted as aforementioned for the migration assay. Representative images were taken under an inverted microscope (magnification, x10 and x20) equipped with a camera (Leica Microsystems GmbH).

Statistical analysis. Statistical analysis was performed using SPSS v13.0 (SPSS, Inc.). Data were presented as the mean \pm standard deviation based on at least three replicates. The differences among groups were analyzed using one-way analysis of variance followed by Least Significant Difference post-hoc test. $P < 0.05$ was considered to indicate statistical significance.

Results

Cd treatment at the highest concentration decreases A549 cell viability. To assess the effect of Cd treatment on cell viability, an MTT assay was performed. The results revealed that the relative cell viability of the 8 μ M Cd treatment group was significantly lower than the solvent control (0 μ M), indicating that 8 μ M Cd had a marked cytotoxic effect (Fig. 1). Cd treatment demonstrated no marked effect on cell viability when the concentration was < 4 μ M (Fig. 1).

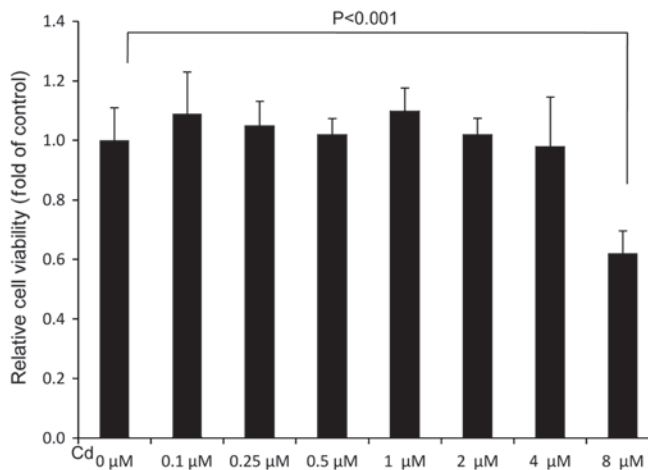


Figure 1. Effects of Cd treatment on A549 cell viability. A549 cells were treated with the indicated concentrations of Cd for 48 h. An MTT assay was subsequently performed. Relative cell viability was determined by normalizing to the control (0 μ M). Cd, cadmium.

Cd treatment increases the expression of p-ERK in A549 cells. Western blot analysis was conducted to investigate whether Cd affected the expression of p-ERK in A549 cells. The results revealed that the expression of p-ERK was markedly increased in a dose-dependent manner when A549 cells were treated with Cd for 48 h (Fig. 2A). When compared with the 0 h group, a significant increase in p-ERK expression was observed when A549 cells were treated with 2 μ M Cd for increasing time durations (Fig. 2B). Cd treatment exhibited no effect on the expression of total ERK protein (Fig. 2). The results indicated that ERK activation was induced by Cd treatment.

Cd accelerates the migration and invasion of A549 cells. The data demonstrated that Cd treatment increased A549 cell migration (Fig. 3A) and invasion (Fig. 3B) compared with the solvent control (0 μ M). The results verified that Cd treatment facilitated the migration and invasion of A549 cells.

p-ERK has an important role in the migration and invasion of A549 cells following Cd treatment. The migration and invasion of A549 cells were assessed following pretreatment with 10 μ M U0126 (an inhibitor of MEK1/2), followed by 2 μ M Cd treatment. The results revealed that the expression of p-ERK was markedly decreased in the U0126-treated group compared with the control group (Fig. 4A). In addition, U0126 blocked the expression of p-ERK following Cd treatment. The data therefore demonstrated that U0126 effectively inhibited the expression of p-ERK (Fig. 4A). It was also determined that U0126 inhibited the migration and invasion of A549 cells following Cd treatment (Fig. 4B and C), indicating that elevated p-ERK activity was required for Cd-induced migration and invasion.

MMP2 mRNA expression following Cd treatment is mediated by p-ERK. An investigation into whether MMP2 was a downstream signaling molecule in the ERK pathway was performed using RT-qPCR. The results revealed that Cd treatment significantly increased the expression of MMP2 mRNA compared with the control group. Furthermore, the Cd-induced expression of MMP2 mRNA was significantly

suppressed in the presence of U0126 (Fig. 5). These results indicated that MMP2 mRNA expression was modulated by p-ERK following Cd treatment and also that MMP2 might be a downstream molecule in the ERK signaling pathway.

Discussion

Cd is a widely used heavy metal environmental pollutant. Previous studies have identified that exposure to Cd is closely associated with the progression of various types of cancer. He *et al* (31) reported that blood Cd levels are positively associated with distant metastasis and clinical stage of human breast cancer. Demir *et al* (32) demonstrated that Cd levels in tumor tissue are significantly correlated with lung cancer TNM staging. Furthermore, Son *et al* (33) reported that chronic Cd treatment enhanced cell migration and invasion in BEAS-2B cells and increased tumor growth in a mouse xenograft model. Person *et al* (7) also determined that chronic Cd-treated lung cells exhibit a marked increase in invasion compared with control cells. Wei and Shaikh (34) reported that increased cell migration and invasion is demonstrated in prolonged Cd-treated triple-negative breast cancer cells. Finally, Zhu *et al* (12) demonstrated that migration and invasion is induced in human follicular WRO and anaplastic FRO thyroid cancer cells when treated with Cd at 250-1,000 nM.

Currently, three published articles have demonstrated that exposure to Cd induces migration and invasion of lung cancer cells. Luo *et al* (10) demonstrated that treatment with Cd increased the expression of certain invasion-associated proteins in the lung tissue of mice and in A549 cells, including MMP9, MMP2 and p-protein tyrosine kinase 2. The study also revealed that high-mobility group AT-hook (HMGA2) serves a significant role in Cd-induced A549 cell migration and invasion (10). Lv *et al* (11) reported that Cd markedly enhances cell proliferation, migration and invasion in lung cancer A549 cells. It was also determined that reactive oxygen species-dependent autophagy and related 4A cysteine peptidase upregulation-mediated autophagy may affect Cd-induced cell growth, migration and invasion. Fujiki *et al* (35) demonstrated that prolonged Cd exposure induced epithelial-mesenchymal transition (EMT), stress-fiber formation and high cell motility in A549 cells, which was partially suppressed by small interfering RNA-mediated Notch1 silencing. These studies indicated that Cd promoted lung cancer cell migration and invasion via different mechanisms.

ERK is activated on both the Thr202 and Tyr204 residues via sequential phosphorylation cascades. It also regulates several downstream events, such as cell proliferation, differentiation, motility and cell death (36-38). It has been reported that the ERK signaling pathway is involved in cell migration and invasion in several types of cancer cell. Peng *et al* (39) reported that anti-migration and anti-invasion activities of oxyfadihalcone C were associated with the downregulation of the MAPK/ERK signaling pathways in melanoma A375 cells. Wang *et al* (40) reported that the chrysin-induced inhibition of proliferation, migration and invasion in glioblastoma cells was mediated by the ERK/Nrf2 signaling pathway. Additionally, Hong *et al* (41) reported that microRNA-508 suppresses the EMT, migration and invasion of ovarian cancer cells via the MAPK1/ERK signaling pathway. Li *et al* (42) also reported that microRNA-23a promoted cell growth and metastasis in

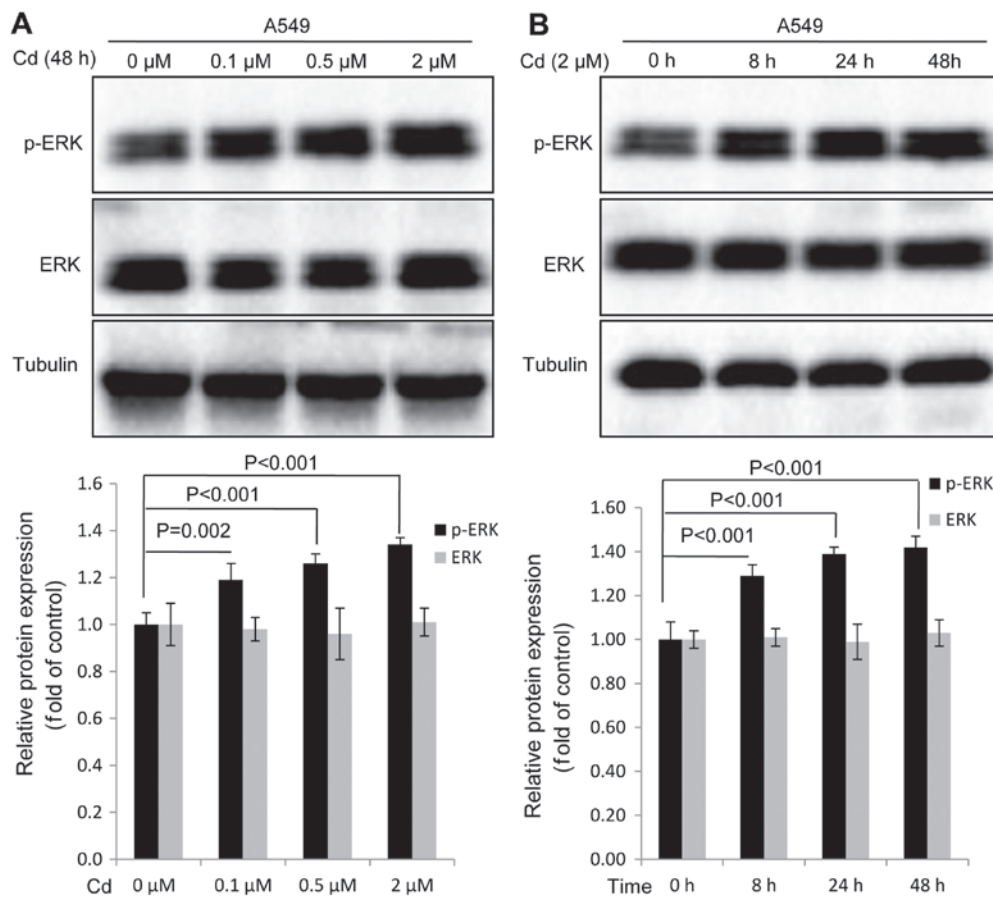


Figure 2. Effects of Cd treatment on p-ERK protein expression. (A) Western blot analysis to measure the expression of p-ERK protein following treatment with indicated concentrations of Cd for 48 h or (B) 2 μM Cd for 8, 24 or 48 h. P<0.05 was considered to indicate a statistically significant difference. Cd, cadmium; p, phosphorylated.

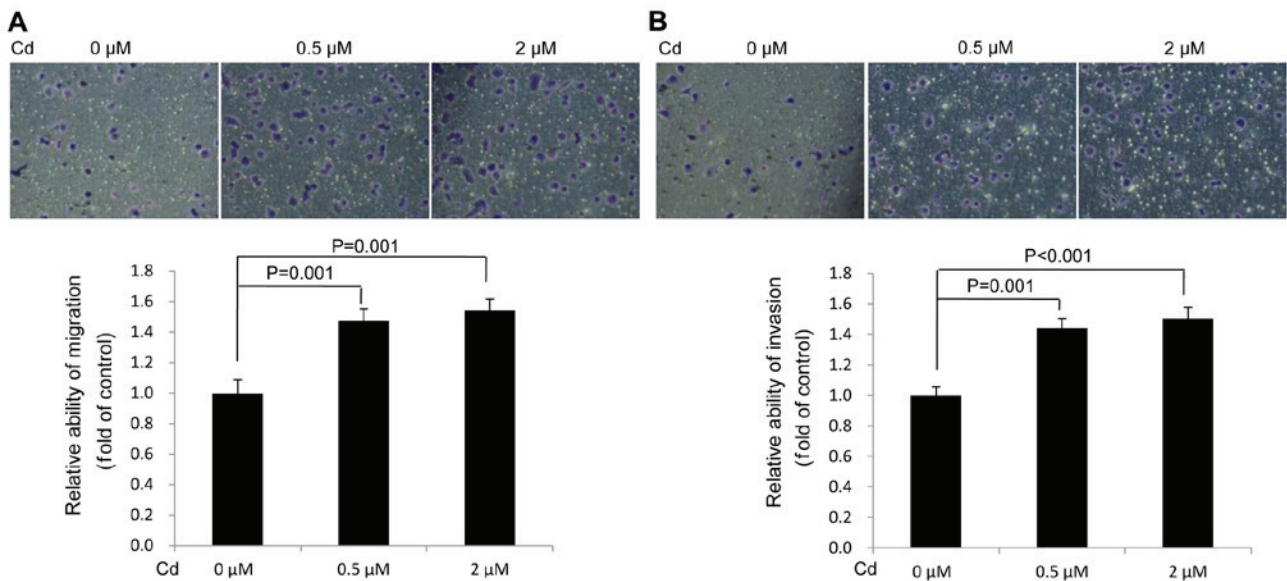


Figure 3. Effects of Cd treatment on A549 cell migration and invasion. (A) Relative cell migration and (B) invasion were quantified as the fold change with respect to the control (magnification, x200). Cd, cadmium.

gastric cancer by targeting sprouty RTK signaling antagonist 2-mediated ERK signaling. A previous study demonstrated that p-ERK serves an important role in benzo(a)pyrene-induced Hep-G2 cell migration and invasion (14).

MMP2, a member of the extracellular matrix degrading proteinase family, is vital for the promotion of tumor metastasis. Previous studies have determined that MMP2 is implicated in the migration and invasion of lung cancer cells. Dong *et al* (43)

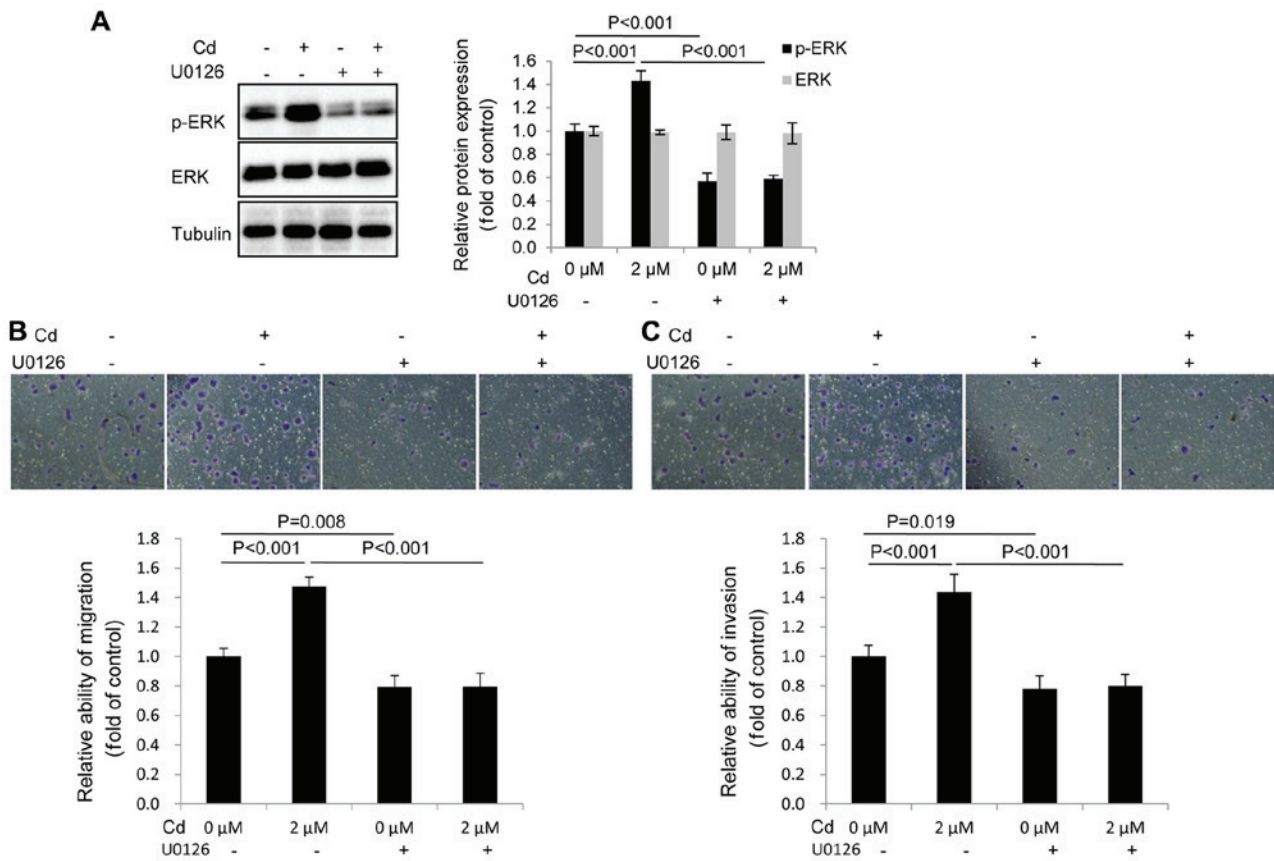


Figure 4. p-ERK has an important role in the Cd-induced migration and invasion of A549 cells. (A) Western blot analysis was conducted to measure the expression of p-ERK in the presence of 2 μ M Cd and 10 μ M U0126. (B) Migration and (C) invasion assays were performed in the presence of 2 μ M Cd and 10 μ M U0126, and quantified as the fold change with respect to the control (magnification, x200). Cd, cadmium; p, phosphorylated.

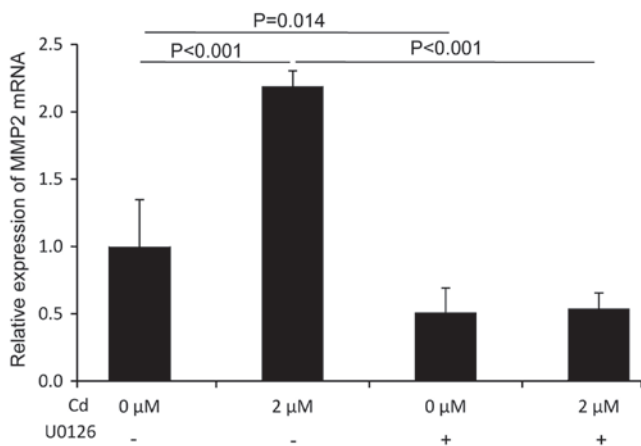


Figure 5. MMP2 mRNA expression induced by Cd is modulated by p-ERK in A549 cells. Reverse transcription-quantitative PCR was performed to detect the mRNA expression of MMP2 in A549 cells in the presence of 2 μ M Cd and 10 μ M U0126. MMP, matrix metalloproteinase; Cd, cadmium; p, phosphorylated.

reported that the inhibition of cell invasion occurs as a result of decreased MMP2 levels in NSCLC cell lines. In addition, it has been reported that MMP2 is a downstream molecule of the ERK pathway. For example, Wu *et al* (44) determined that MMP2 was mediated by the ERK signaling pathway potentially via micro-fibrillar-associated protein 5, which was associated with tissue development and cancer progression. Furthermore, Li *et al* (45)

reported that the activation of the ERK/NF- κ B signaling pathway promoted the expression of MMP2 and induced cell migration and invasion. Yu *et al* (46) also reported that andrographolide enhanced the anti-metastatic effect of radiation in Ras-transformed cells by suppressing ERK-mediated MMP2 activity. Yang *et al* (47) demonstrated that andrographolide suppressed the migratory ability of human glioblastoma multiforme cells by targeting ERK1/2-mediated MMP2 expression.

U0126 is a highly selective inhibitor of MEK1 (IC₅₀, 72 nM) and MEK2 (IC₅₀, 58 nM; a type of MAPK/ERK kinase) (48), and is also a weak inhibitor of proline rich transmembrane protein 2, Raf, JNK, mitogen-activated protein kinase kinase 1, mitogen-activated protein kinase kinase (MKK) 3, MKK-4/EPH receptor A4, MKK-6, cyclin-dependent kinase (Cdk)-2 and Cdk4. Micromolar concentrations of U0126 were used to inhibit ERK1/2 activation in previous studies, including 5 (29), 20 (49) and 40 μ M (26). As the most commonly used U0126 concentration (27,29,50,51), 10 μ M U0126 was selected in the present study to inhibit the activation of ERK1/2.

The present study investigated the effects of Cd on the migration and invasion of A549 cells as well as the potential role of p-ERK in Cd-induced cell migration and invasion. The results demonstrated that Cd-treated lung cancer A549 cell migration and invasion increased compared with the control. Migration and invasion were also decreased when cells were pre-treated with U0126, an inhibitor of MEK1/2. Data also indicated that p-ERK served an important role in Cd-induced A549 cell migration and invasion. In addition, RT-qPCR

determined that the Cd-induced expression of MMP2 mRNA was significantly reduced in the presence of U0126, which indicated that MMP2 might be a downstream molecule in the ERK signaling pathway. Further investigation should be performed to confirm the pattern of A549 cell MMP2 protein expression and activity in the presence of Cd and U0126.

HMGA2 is a driver of tumor cell migration, invasion and metastasis (52-54) and there is a proven link between HMGA2 and p-ERK. Hawsawi *et al* (55) demonstrated that HMGA2 promoted EMT via the MAPK/ERK signaling pathway in prostate cancer. Ping *et al* (56) also reported that angiotensin II type 2 receptor-interacting protein 3a presented potential in suppressing the proliferation and aggressiveness of ovarian carcinoma cells via the HMGA2-mediated ERK/EMT signaling pathway. Additionally, Kao *et al* (57) indicated that Hsp90 indirectly regulated HMGA2 by activating the ERK signaling pathway. HMGA2 upregulation is mediated by Cd-induced A549 cell migration and invasion (10). The present study determined that ERK activation was involved in Cd-induced A549 cell migration and invasion. However, the link between HMGA2 and p-ERK in Cd-induced A549 cell migration and invasion was not investigated and will be the focus of future studies.

To the best of our knowledge, the present study demonstrated for the first time that Cd induced A549 cell migration and invasion by activating the ERK-MMP2 pathway. The results may contribute to the further understanding of the molecular mechanisms of Cd-induced lung-cancer cell migration and invasion, and provide insight to potentially improve lung cancer treatment caused by environmental Cd.

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Availability of data and materials

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YW and HY designed and implemented the current study, performed the experiments and analyzed the data. TP and HZ performed western blotting and RT-qPCR analysis. YW, HZ and HW performed the MTT, migration and invasion assays. HZ and YW wrote the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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