Benzo(a)pyrene promotes Hep-G2 cell migration and invasion by upregulating phosphorylated extracellular signal-regulated kinase expression

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Abstract. Benzo(a)pyrene (BaP), a carcinogenic component of cigarette smoke, has been reported to activate extracellular signal-regulated kinase (ERK) in cancer cells. Furthermore, activated ERK is associated with liver cancer cell invasion and metastasis. Therefore, the aim of the present study was to investigate the potential role of phosphorylated (p)-ERK in BaP-induced Hep-G2 cell migration and invasion. An MTT assay was used to determine the effects of BaP treatment on Hep-G2 cell proliferation. Wound-healing and Transwell invasion assays were employed to assess the migration and invasion abilities of Hep-G2 cells. Western blot analysis was applied to detect the expression of proteins. The results of the present study demonstrated that BaP treatment was able to increase the level of p-ERK protein expression in Hep-G2 cells. BaP treatment promoted Hep-G2 cell migration and invasion. The ERK inhibitor, U0126, was able to block the migration and invasion abilities of Hep-G2 cells induced by BaP. The results of the present study demonstrated that BaP treatment promoted the migration and invasion of Hep-G2 cells by upregulating p-ERK expression.

Introduction

Benzo(a)pyrene (BaP) is a polycyclic aromatic hydrocarbon derived from incomplete combustion of organic materials (including cigarette smoke). BaP is listed as a group I carcinogen by the International Agency for Research on Cancer based on the data from animal experiments and epidemiological studies (1). Numerous studies have documented the associations between BaP exposure and the formation of different types of cancer (2-7), including liver cancer (8-10). It has been reported that gene mutations, chromosomal aberrations and epigenetic alterations are involved in the process of BaP-induced hepato-carcinogenesis (11-15). Recently, Ba *et al* (16) reported that BaP exposure had effects on the metastasis of human liver cancer cell, but the underlying mechanisms of this are not well understood.

Extracellular regulated protein kinase (ERK), a pivotal regulator of the mitogen-activated protein kinase (MAPK)/ERK pathway, has been implicated in the regulation of cell proliferation, differentiation and survival (17,18). The ERK cascade reaction may be activated by various stimuli, including receptor tyrosine and G-protein-coupled receptors (19), and activated ERK may phosphorylate various downstream molecules (20). An increasing volume of evidence has demonstrated that the activated ERK signaling pathway is associated with the development and progression of liver cancer (21-25). For example, Jiang *et al* (26) reported that calcium binding protein 39 promoted the metastasis of liver cancer by activating the ERK signaling pathway. Dang *et al* (27) reported that loss of protocadherin-17 promoted the metastasis and invasion of liver cancer through hyperactivation of the epidermal growth factor receptor (EGFR)/ERK signaling pathway.

Based on the observations of previous studies that phosphorylated (p)-ERK served an important role in hepatocarcinoma cell migration and invasion, and that BaP promoted hepatocarcinoma cell migration and invasion, we hypothesized that ERK activation may serve a pivotal role in BaP-induced migration and invasion of hepatoma cells. In the present study, the most commonly studied cell line of human hepatoblastoma, Hep-G2 (28) was used to investigate the potential role of p-ERK in the BaP-induced migration and invasion of hepatoma cells.

Materials and methods

Cell culture and BaP treatment. The human hepatoblastoma Hep-G2 cell line was purchased from the Cell

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Resource Center, Peking Union Medical College (National Infrastructure of Cell Line Resource, Beijing, China). Hep-G2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (GE Healthcare Bio-Sciences, Pittsburgh, PA, USA) supplemented with 10% fetal bovine serum (FBS) (GE Healthcare Bio-Sciences) and antibiotics (penicillin 100 U/ml and streptomycin 100 μg/ml) in an incubator with a humidified atmosphere of 5% CO_2 at 37˚C. For BaP exposure, Hep-G2 cells were treated with different concentrations (0, 2, 4, 8, 16, 32 or 64 μM) of BaP (B1760; >96% HPLC; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) at different time points (0, 24, 48 or 72 h), as described previously (29,30). The final concentration of dimethyl sulfoxide (DMSO) used as solvent control was 0.1% (v/v) or less.

Cell proliferation assay. To observe the effects of BaP on Hep-G2 cell proliferation, an MTT assay was performed as described previously (30). In brief, $1x10^4$ cells in 100 μl DMEM (GE Healthcare Bio-Sciences) were plated into each well of 96-well plates (6 wells per group). A total of 24 h after plating, cells were exposed to different concentrations of BaP (0, 2, 4, 8, 16, 32 or 64 μM) for 0, 24, 48 or 72 h. A total of 10 μl of 5 mg/ml MTT was added to each well, and then the plates were incubated at 37˚C for 4 h. Finally, 150 μl DMSO was added to each well to dissolve the purple formazan. The optical density was read on a micro-plate reader (Multiskan Ascent Software, Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 492 nm. Relative cell proliferation rates were determined by normalizing with that of the solvent control at 0 h.

Wound healing assay. A Wound-healing assay was conducted to evaluate the migratory ability of Hep-G2 cells in accordance with our previous study (30). In brief, cells were seeded onto 6-well plates and were wounded by scratching the surface of plates with a sterile 200-μl-pipette tip. Floating cells were removed by washing with phosphate-buffered saline (PBS) three times. A total of 2 ml DMEM (GE Healthcare Bio-Sciences) with 4 μM BaP or 20 μM ERK inhibitor, U0126 (U120-1MG; ≥98% HPLC; Sigma-Aldrich; Merck KGaA), was added to each well. Images of the process of cell migration into the wound were captured using an inverted microscope (magnification, x10 and x20) equipped with a camera (Leica Microsystems GmbH, Wetzlar, Germany). The healing width was calculated as the wound width at 0 h minus wound width at 48 h, and was normalized to the control.

Invasion assay. A Matrigel invasion assay was performed using a 24-well Transwell chamber (Corning Incorporated, Corning, NY, USA). A total of $5x10^4$ cells in DMEM (GE Healthcare Bio-Sciences) without FBS were seeded into the upper chamber with Matrigel. A total of 4 μM BaP or 20 μM U0126 were added to the upper chamber. A total of 600 μl DMEM supplemented with 10% FBS (both GE Healthcare Bio-Sciences) were added to the lower chamber. After 24 h of incubation at 37˚C, non-migrated cells on the upper side of the membrane were removed with cotton swabs. Cells on the lower surface of the membrane were fixed with pure methanol at room temperature for 10 min and stained with crystal violet at room temperature for 30 min. The number of invaded cells was counted in six randomly selected fields. Relative ability of invasion was calculated by normalizing with control.

Western blot analysis. Western blot analysis was performed as previously described (30). In brief, cells were harvested and washed with ice-cold PBS three times. Whole-cell lysates were prepared in radioimmunoprecipitation assay buffer with protease inhibitors and phosphatase inhibitors (Pierce; Thermo Fisher Scientific, Inc.) at 4˚C for 15 min, followed by 15 min of centrifugation (14,000 x g) at 4˚C. Total protein was determined by a bicinchoninic acid kit (Pierce; Thermo Fisher Scientific, Inc.). Equal amounts of protein (50 μg) were electrophoresed on 10% sodium dodecyl sulfate polyacrylamide gel. Following electrophoresis, proteins were transferred onto nitrocellulose membranes. The membranes were washed and blocked with 5% bovine serum albumin in Tris-buffered saline and 0.1% Tween-20 at room temperature for 1 h. The membranes were incubated with primary antibodies against the following: p-ERK1/2 (1:2,000; cat. no. 4370S), ERK1/2 (1:2,000; cat. no. 4695S), p-STAT3 (1:1,000; cat. no. 9145S), vimentin (1:1,000; cat. no. 5741S; all Cell Signaling Technology, Inc., Danvers, MA, USA), tubulin (1:1,000; cat. no. sc-365791), β-catenin (1:1,000; cat. no. sc-7199), p-protein kinase B (Akt; 1:1,000; cat. no. sc-33437), MMP2 (1:1,000; cat. no. sc-10736), N-cadherin (1:1,000; cat. no. sc-7939), TGIF (1:1,000; cat. no. sc-9084), STIM1 (1:1,000; cat. no. sc-68897), Twist (1:500; cat. no. sc-81417) and E-cadherin (1:1,000; cat. no. sc-7870; all Santa Cruz Biotechnology, Inc., Dallas, TX, USA) overnight at 4˚C. The members were washed and incubated with Peroxidase-Conjugated Goat anti-Rabbit IgG (1:5,000; cat. no. ZB-2301) or Peroxidase-Conjugated Goat anti-Mouse IgG (1:5,000; cat. no. ZB-2305; all ZSGB-BIO; OriGene Technologies, Inc., Beijing, China) for 1 h at room temperature. The signals were captured in the ChemiDoc™ XRS^+ imaging system (Bio-Rad Laboratories, Inc., Hercules, MA, USA) by a Bio-Rad Clarity™ western enhanced chemiluminescence substrate (Bio-Rad Laboratories, Inc.).

Statistical analysis. Data were analyzed by one-way analysis, followed by the least significant difference test, using SPSS 13.0 software (SPSS, Inc., Chicago, IL, USA). All values are expressed as the mean ± standard deviation (SD). $P<0.05$ was considered to indicate a statistically significant difference.

Results

The effects of BaP treatment on Hep-G2 cell proliferation. The results of the present study demonstrated that Hep-G2 cell proliferation was significantly suppressed by 16, 32 and 64 μM BaP treatment at 48 and 72 h, compared with the solvent control (Fig. 1). When the concentration of BaP treatment was <8 μM, the cell proliferation was not significantly affected (Fig. 1). Therefore, the highest dose of BaP treatment did not exceed 8 μM for the subsequent experiments, as it had no obvious toxicity to cells at this concentration.

The effects of BaP treatment on the expression of p-ERK. The results of the present study demonstrated that the expression of p-ERK protein was markedly increased in the BaP-treated

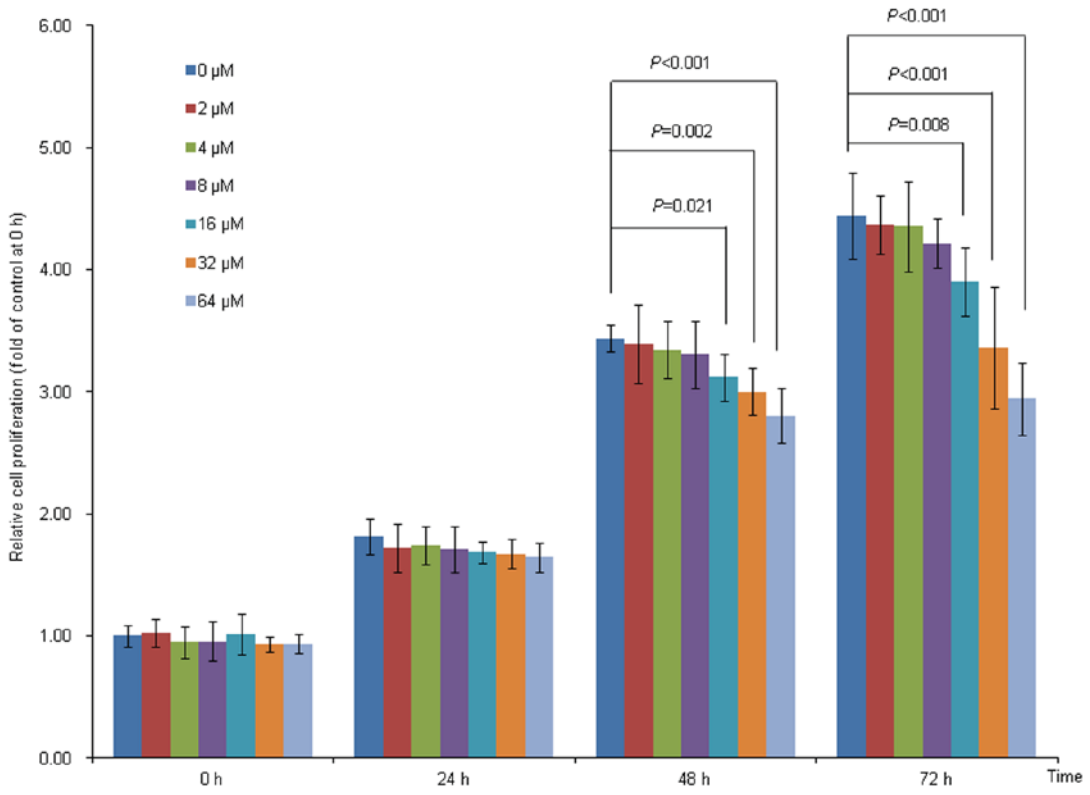

Figure 1. Effects of BaP treatment on Hep-G2 cell proliferation. Hep-G2 cells were treated with different concentrations of BaP (0, 2, 4, 8, 16, 32 or 64 μM) for 0, 24, 48 or 72 h, and an MTT assay was performed. Relative cell proliferation rates were determined by normalizing with that of the solvent control at 0 h. Data are expressed as the mean ± standard deviation. BaP, benzo(a)pyrene.

groups (2, 4 and 8 μM), compared with the solvent control group (Fig. 2). Furthermore, BaP exposure (8 μM) markedly increased the expression of p-ERK protein at 24, 48 and 72 h, compared with the solvent control (Fig. 2). BaP treatment had no obvious effects of total ERK expression, and tubulin was used as loading control.

The effects of BaP treatment on Hep-G2 cell migration and invasion. Fig. 3 demonstrated the effects of BaP treatment on Hep-G2 cell migration and invasion *in vitro*. The results demonstrated that Hep-G2 cells treated with BaP (2 and 4 μM) migrated more quickly to heal the scratched wounds than the cells treated with DMSO (Fig. 3A and B). Furthermore, the results of the present study indicated that Hep-G2 cells treated with BaP (2 and 4 μM) had an enhanced ability to invade through the Matrigel matrix, compared with cells treated with DMSO (Fig. 3C and D).

Increased ERK activity is required for BaP-induced Hep-G2 cell migration and invasion. As described earlier, BaP treatment induced the expression of p-ERK protein and enhanced the migration and invasion abilities of Hep-G2 cells. To further investigate whether or not ERK signaling serves an important role in the BaP-induced migration and invasion of Hep-G2 cells, wound-healing and Transwell invasion assays were performed in the presence of BaP and a known ERK inhibitor, U0126. The results data demonstrated that the ERK inhibitor, U0126, significantly inhibited the migration and invasion of Hep-G2 cells. As demonstrated in Fig. 4A, the scratched wound width was markedly wider in the U0126-treated group (+) than that in the U0126-untreated group (-) at 48 h (Fig. 4A). When the migration ability was represented as the healing width, it was observed that the healing width was markedly decreased in U0126-treated group (+) as compared with U0126-untreated group (-) at 48 h (Fig. 4B). The number of invaded cells was markedly decreased in U0126-treated group (+), compared with the U0126-untreated group (-) (Fig. 4C and D). Furthermore, the results of the present study demonstrated that blocking of ERK activation abolished the abilities of the migration (Fig. 4A and B) and invasion (Fig. 4C and D) of Hep-G2 cells induced by treatment with BaP, which suggested that Hep-G2 cell migration and invasion induced by BaP treatment may be mediated by ERK activation.

BaP-induced vimentin protein expression is modulated by ERK activation. Several proteins, including matrix metalloproteinase 2 (MMP2), p-Akt, E-cadherin, N-cadherin and vimentin serve important roles in the invasion and metastasis of liver cancer (31-34). Whether or not these proteins were involved in the downstream targets of ERK signaling in Hep-G2 cells treated with BaP was subsequently investigated. The expression of these proteins in the presence of BaP and the ERK inhibitor, U0126, was detected. The results demonstrated that the increased expression of vimentin protein was observed in the BaP treatment group, compared with the solvent control, which was inhibited by treatment with U0126 (Fig. 5). No effect on the expression

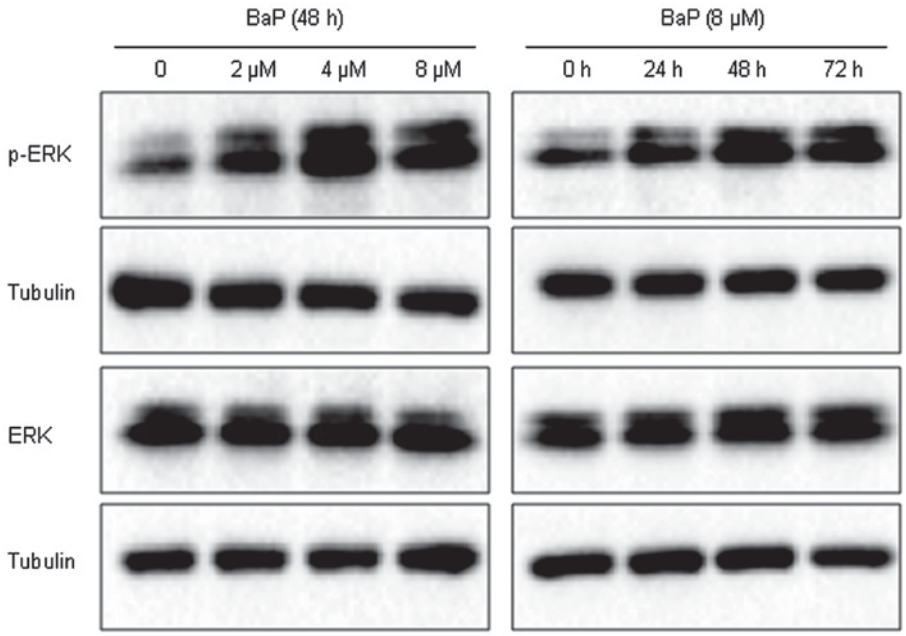

Figure 2. Effects of BaP treatment on the expression of p-ERK protein. Hep-G2 cells were treated with various concentrations of BaP (0, 2, 4 or 8 μM) for 48 h or 8 μM of BaP for 24, 48 or 72 h. Western blot analysis was performed to detect the expression of p-ERK protein. BaP, benzo(a)pyrene; p-ERK, phosphorylated extracellular signal-regulated kinase.

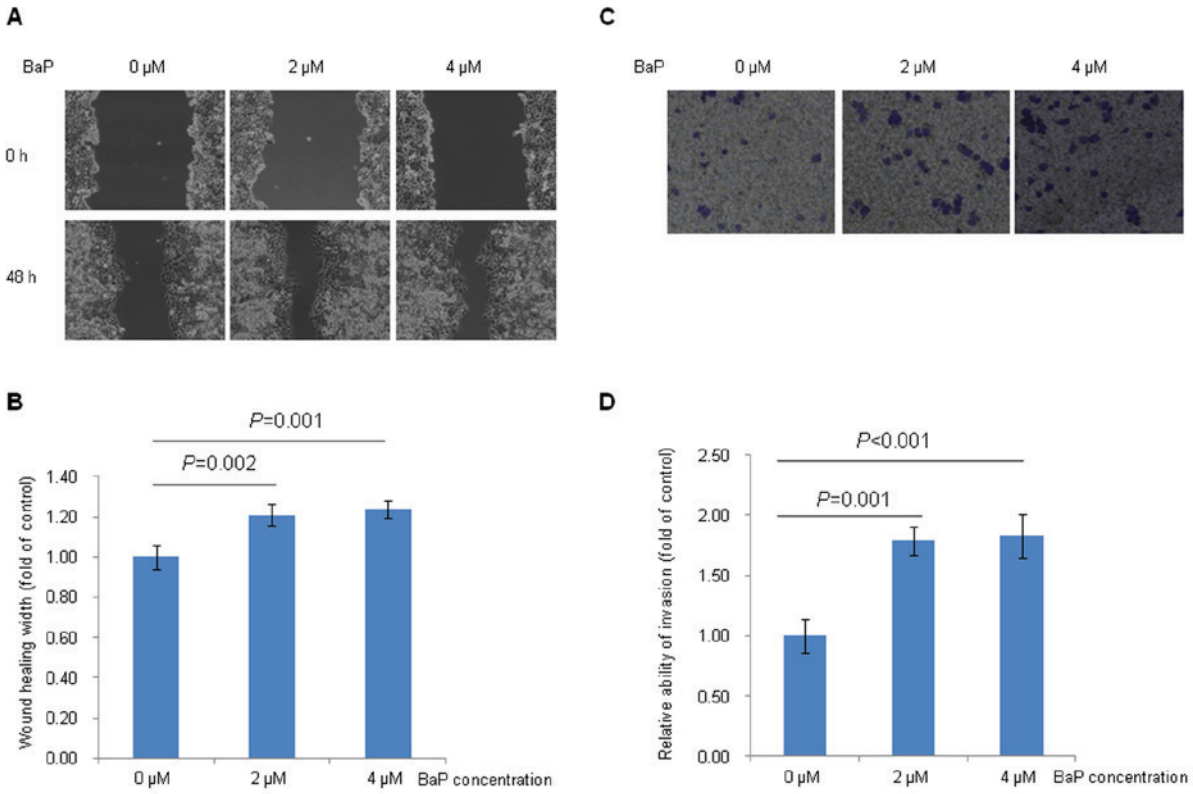

Figure 3. Effects of BaP treatment on Hep-G2 cell migration and invasion. Wound-healing assay was (A) performed and (B) quantified to evaluate the effects of BaP treatment on Hep-G2 cell migration. Cells were wounded by scratching and treated with BaP (0, 2, or 4 μM) for 48 h. Images were captured at 0 and 48 h. The healing width was calculated as the wound width at 0 h minus the wound width at 48 h and was normalized to the control. Values are expressed as the mean ± standard deviation. Transwell invasion assay was (C) performed and (D) quantified to assess the effects of BaP treatment on Hep-G2 cell invasion. Cells were harvested and seeded into the upper chamber with Matrigel and treated with BaP (0, 2 or 4 μM) for 24 h. The number of migrated cells was counted and normalized to the control. Values are expressed as the mean ± standard deviation. BaP, benzo(a)pyrene.

of proteins, including β-catenin, p-Akt, MMP2, E-cadherin and N-cadherin in Hep-G2 cells was observed followed treatment with BaP (Fig. 5). Additionally, the expression of additional proteins, including TGFB-induced factor (TGIF),

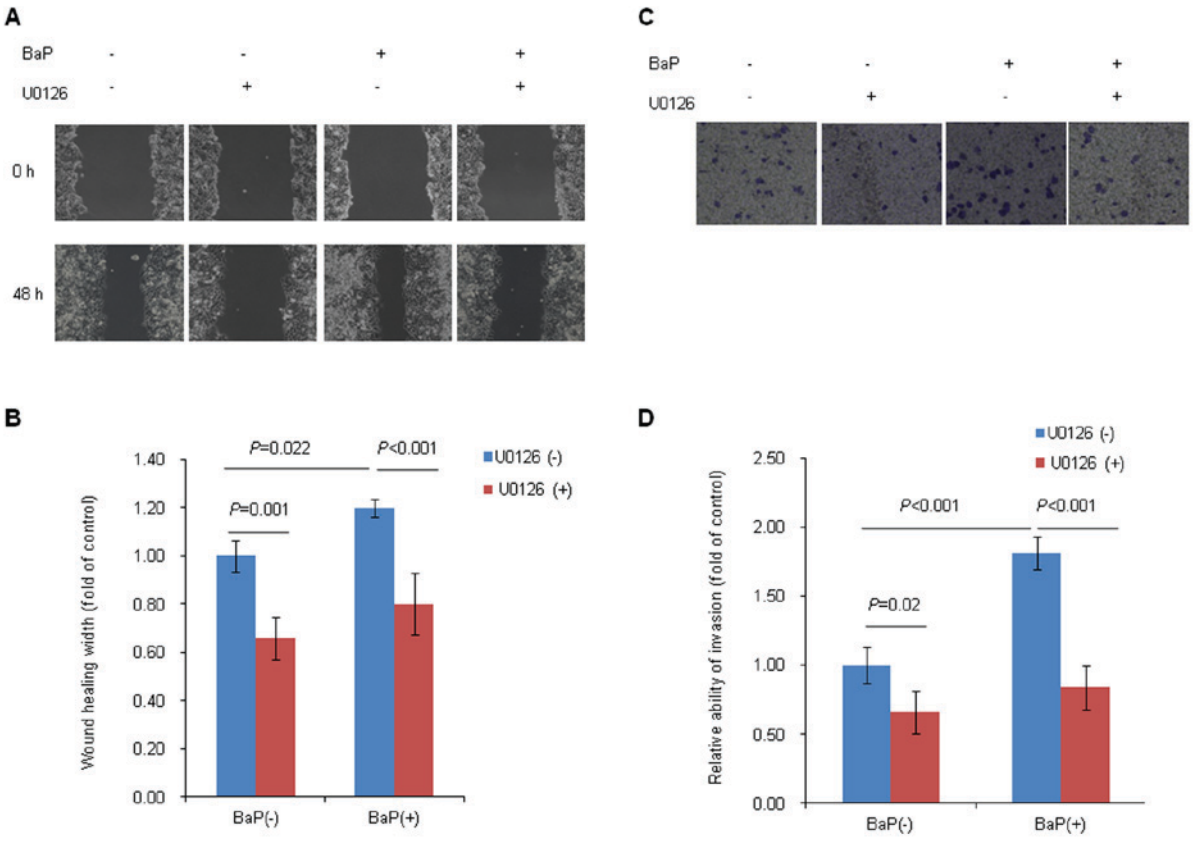

Figure 4. ERK activation is required for BaP-induced Hep-G2 cell migration and invasion. Wound-healing assay was (A) performed and (B) quantified to evaluate the potential role of p-ERK in BaP-induced Hep-G2 cell migration. Cells were wounded by scratching and cultured in the presence of BaP (4 μM) or U0126 (20 μM) for 48 h. Images were captured at 0 and 48 h. The healing width was calculated as the wound width at 0 h minus the wound width at 48 h and was normalized to the control. Values are expressed as the mean ± standard deviation. Transwell invasion assay was (C) performed and (D) quantified to assess the potential role of p-ERK in BaP-induced Hep-G2 cell invasion. Cells were harvested and seeded into the upper chamber with Matrigel and were cultured in the presence of BaP (4 μM) or U0126 (20 μM) for 24 h. The number of migrated cells was counted and normalized to the control. Values are expressed as mean ± standard deviation. ERK, extracellular signal-regulated kinase; BaP, benzo(a)pyrene; p-ERK, phosphorylated ERK.

p-STAT3, Twist1 and STIM1, was also detected in Hep-G2 cells induced by BaP, but BaP treatment did not have any notable effects on the expression of these proteins (data not shown).

Discussion

It has been reported that several key molecular events are implicated in BaP-induced hepato-carcinogenesis. Bolotina *et al* (35) demonstrated that BaP-dependent activation of transcript factors nuclear factor (NF)-κB and activator protein 1 (AP-1) was associated with tumor promotion in hepatoma cell cultures. Cui *et al* (36) demonstrated that pregnane X receptor regulated the AhR/CYP1A1 pathway and protected liver cells from BaP-induced DNA damage. Marrone *et al* (37) reported that treatment of HepaRG cells with BaP resulted in specific changes in the expression of microRNAs compared with their non-carcinogenic analogues. Souza *et al* (38) demonstrated that BaP and its major metabolites deregulated metastatic markers via non-genotoxic and genotoxic mechanisms and activated the inflammatory pathway (NF-κB signaling and cytokine-cytokine receptor interaction) in Hep-G2 cells. BaP also induced strong repression of genes involved in cholesterol and fatty acid biosynthesis in Hep-G2 cells (38).

Previous studies have documented the association between BaP exposure and cancer cell invasion and metastasis. Ueng *et al* (39) reported that BaP treatment enhanced the invasive ability of lung cancer CL5 cells *in vitro*. Wang *et al* (30) indicated that BaP treatment may promote A549 lung cancer cell migration and invasion. Miller *et al* (40) reported that BaP treatment increased the invasion abilities of human breast cancer MDA-MB-231 cells. The present study observed that BaP treatment enhanced the abilities of migration and invasion in liver cancer Hep-G2 cells *in vitro*. The results of the present study are consistent with previous observations (16). Ba *et al* (16) observed that chronic BaP exposure was able to promote liver cancer cell migration and invasion *in vivo* and *in vitro*. Taken together, the results of the present study indicated that exposure to BaP had effects on liver cancer metastasis and progression.

Numerous studies have demonstrated that ERK signaling is involved in BaP-induced carcinogenesis. For example, Patten Hitt *et al* (41) demonstrated that BaP activated ERK, which was involved in cell proliferation, in colon adenocarcinoma HT29 cells (41). Wang *et al* (42) reported that BaP-induced cell cycle progression occurred via the ERK-induced checkpoint kinase 1 pathway activation in human lung cancer cells. Kometani *et al* (43) reported that the EGFR tyrosine kinase inhibitor reduced the cellular proliferation and the level of

phosphorylation of ERK1/2, which is a downstream signal of the EGFR in BaP-treated A549 cells. The present study observed that BaP treatment increased the level of p-ERK protein expression in Hep-G2 cells, and the ERK inhibitor, U0126, blocked BaP-induced Hep-G2 cell migration and invasion, which suggested that ERK activation may mediate BaP-induced migration and invasion in Hep-G2 cells. Two previous studies have demonstrated that elevated ERK activity is required for BaP-induced migration and invasion in human breast cancer MDA-MB-231 and MCF-7 cells (29,44). Taken together, the results of the present study and previous studies suggested that ERK signaling served an important role in the migration and invasion of different types of cancer cells induced by BaP.

Vimentin, a type III intermediate filament protein, is expressed in mesenchymal cells. Vimentin is often used as a marker of liver cancer metastasis. Hu *et al* (45) reported that overexpression of vimentin was significantly associated with liver cancer metastasis. Wei *et al* (46) reported that silencing of glucose-regulated protein 78 enhanced liver cancer cell migration through regulation of vimentin. Wang *et al* (47) demonstrated that long non-coding RNA AOC4P suppressed liver cancer metastasis by enhancing vimentin degradation and inhibiting epithelial-mesenchymal transition. The results of a study undertaken by Dong *et al* (34) indicated that osteopontin promoted epithelial-mesenchymal transition of liver cancer cells through regulating vimentin. The present study observed that BaP treatment increased the expression of vimentin protein, which was attenuated by inhibition of ERK activity.

There are several limitations to the present study. To begin with, Hep-G2 cells were treated with BaP (2, 4 and 8 μM) for 48 h, which may be different from long-term exposure to low concentrations of BaP for humans under normal living conditions. Furthermore, although the results of the present study demonstrated that BaP exposure increased the expression of p-ERK at times between 24 and 72 h, while the effects at times between 0 and 24 h were not investigated and require investigation in future studies. Additionally, the ERK inhibitor was revealed to attenuate BaP-induced vimentin protein expression. However, the underlying mechanisms regarding how p-ERK regulates vimentin protein expression were not fully addressed in the present study and should be a focus of future studies. Furthermore, the potential role of ERK in BaP-induced migration and invasion was only investigated at the cellular level. Further studies on this topic based on animal experiments and population should be performed to verify the results of the present study.

The Hep-G2 cell line was originally thought to be a hepatocellular carcinoma cell line, but was later revealed to derive from a hepatoblastoma (28), and is most frequently used to study the invasion and metastasis of liver cancer (48-52). In the present study, the Hep-G2 cell line was used to investigate the potential role of p-ERK in BaP-induced invasion and migration of liver cancer cells. Although this cell line was reported to be misidentified as hepatocellular carcinoma cell, this issue is unlikely to affect the outcomes of the present study.

In summary, to the best of our knowledge, the present study was the first to demonstrate that BaP exposure promoted Hep-G2 cell migration and invasion by upregulating p-ERK protein expression. The present study, in part, enriched understanding of the mechanisms on the increased risk of liver cancer metastasis among people who are exposed to BaP.

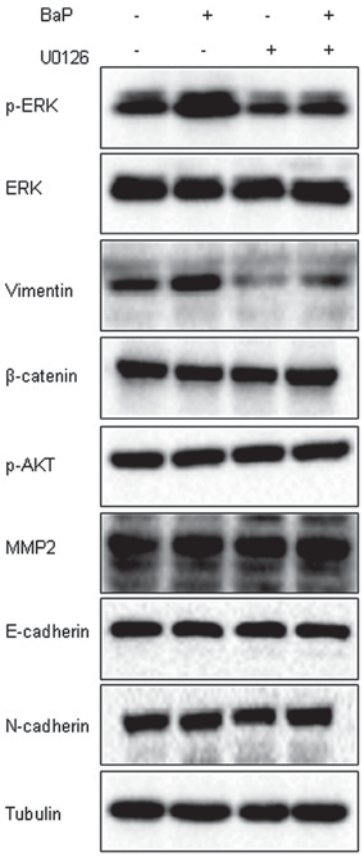

Figure 5. BaP-induced vimentin protein expression is modulated by ERK activation. Cells were seeded in 60-mm plates and treated with 4 μM BaP or 20 μM U0126 for 48 h. Western blot analysis was performed to analyze the level of protein expression. BaP, benzo(a)pyrene; ERK, extracellular signal-regulated kinase; p, phosphorylated; Akt, protein kinase B.

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Availability of data and materials

The datasets generated/ analyzed during the present study are included in the published article.

Authors' contributions

YW contributed to study design, implementation, experiments, data analysis, and manuscript writing. YW, TP and LL contributed to western blot analysis. YW, LL, and HW contributed to the cell proliferation assay, wound healing assay and invasion assay. HY and DZ contributed to data analysis.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

All authors declare that they have no competing interests.

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