Benzo[a]pyrene promotes gastric cancer cell proliferation and metastasis likely through the Aryl hydrocarbon receptor and ERK-dependent induction of MMP9 and c-myc

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Abstract. Gastric cancer (GC) is the fifth most common cancer worldwide and the third leading cause of global cancer-related death. Benzo[a]pyrene (BaP), a Group I carcinogen categorized by the IARC, is a cumulative foodborne carcinogen and ubiquitous environmental pollutant with potent carcinogenic properties. However, the function and mechanism of BaP exposure on GC progression remains unclear. We investigated the role of BaP in human GC progression to identify potential mechanism underlining its carcinogenic activity. After exposure to various concentrations of BaP, human GC cells SGC-7901 and MNK-45 showed an increased capability of proliferation, migration and invasion. Further study indicated that BaP promotes the expression of matrix metalloproteinase-9 (MMP9) and c-myc at mRNA and protein level, and activates Aryl hydrocarbon receptor (AhR) and ERK pathway. Moreover, BaP-induced overexpression of MMP9 and c-myc were attenuated by the ERK inhibitor U0126 and AhR inhibitor resveratrol, respectively. These data suggest that BaP promotes proliferation and metastasis of GC cells through upregulation of MMP9 and c-myc expression, and this was likely mediated via the AhR and ERK signaling pathway.

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Introduction

Gastric cancer (GC) is the most common cancer worldwide and particularly prevalent in East Asia. Despite a steadily declining incidence, GC is still the third leading cause of global cancer-related death (1,2). Environmental agents, including a variety of known chemical carcinogens, play an important role in the initiation and progression of GC (3). As a representative member of the polycyclic aromatic hydrocarbon (PAH) family (4), BaP is generated as a result of the incomplete combustion of organic materials from various sources including charcoal-grilled and fried food, wood and tobacco smoke, residential heating, engine exhaust (5-7). Experimental evidence confirmed that BaP leads to tumors in multiple organs of experimental animals, and it has been listed as a human Group I carcinogen by the International Agency for Research on Cancer (IARC) (8,9). Human exposure to environmental BaP is almost unavoidable through ingestion and inhalation (10,11). It was reported that BaP absorbed into the body undergo metabolic activation by members of the cellular cytochrome P450 family. BaP metabolism generates several reactive, toxic metabolites that might covalently bind to DNA. The DNA adducts might eventually lead to gene mutation and carcinogenesis if left unrepaired (12).

Stomach, as a primary site of exposure to ingested BaP, is a common target for BaP induced carcinogenesis (13). Feeding laboratory animals with BaP can induce preneoplastic lesions (such as squamous cell papillomas) and cancer in the stomach (14-16). Early molecular events in the mouse stomach after exposure to BaP were explored by Labib *et al*, who documented altered expression of 414 genes, many of which are associated with tumorigenesis and tumor development (7).

BaP is a potent natural ligand of the AhR which is a ligand-activated transcription factor of the basic helix-loop-helix/Per-Arnt-Sim family (17). On binding to its ligands, AhR translocates into the nucleus and dimerizes with its partner molecule Aryl hydrocarbon receptor nuclear translocator (ARNT) (18,19). The AhR-ARNT heterodimer binds to a cognate xenobiotic response element (XRE) and induces a battery of gene expression (20,21). Early studies revealed both

pro-oncogenic and antitumorigenic role for AhR in multiple tumors (20,22). Some authors have shown that knockdown of the AhR resulted in decreased proliferation, invasion and migration of cancer cell lines (23,24); and transgenic mice that harbor a constitutively active AhR developed stomach tumors (25). On the contrary, it has been reported that mice losing AhR function spontaneously developed colonic tumors (26). The relationship between AhR pathway activation and GC progression is still unknown and need further elucidating.

The signaling kinase ERK, which is a key regulator in cancer progression, plays critical roles in the process of tumor proliferation and metastasis in a variety of cancer types (27). Recent studies identified a relationship between BaP exposure and ERK activation in breast and colon cancer (28,29). However, the relation between BaP activity and ERK pathway has yet to be established.

Epidemiologic study found that long-term exposure to BaP significantly increased the mortality in GC patients (30), suggesting that BaP plays a critical role in GC advancement. However, little is known about BaP in GC development and progression. In this study, we examined the impact of BaP exposure on human-derived GC cell lines of different differentiation properties, focusing on the biological effects of BaP on GC cell proliferation and metastasis, as well as the potential carcinogenic mechanism of BaP in GC.

Materials and methods

Cell culture and treatment. The human GC cell lines SGC-7901 and MNK-45 were obtained from the Cell Collection of the Chinese Academy of Sciences (Shanghai, China). These two cell lines were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS, Hyclone), 100 U/ml of penicillin and 100 μg/ml of streptomycin at 37°C in a 5% CO_2 incubator. BaP (Sigma-Aldrich, cat no. B1760), resveratrol (RSV; Sigma-Aldrich, cat no. V90038), ERK1/2 inhibitor U0126 (Cell Signaling, cat no. 9903S) were dissolved in dimethyl sulfoxide (DMSO) and diluted with RPMI-1640 to obtain the desired concentration. Final DMSO concentrations in cultures were ≤0.1%.

Cell proliferation assay. The cell proliferation was measured using Cell Counting Kit-8 (CCK-8) assay (Dojindo). Cells were exposed to various concentrations of BaP (i.e., 0.05, 0.1, 0.5, 1, 5 μM) and the vehicle (DMSO) for 48 h in 96-well plates. After treatment, 10 μl of CCK-8 was added to the medium and the cells were incubated for another 3 h. The optical density was measured at 450 nm using a microplate reader (Bio-Tek, USA).

Transwell migration assay and invasion assay. Migration assay, SGC-7901 and MNK-45 cells in serum-free medium were plated in the upper compartment of 24-well Transwell hanging cell culture insert with 8-μm micro-porous membranes (Millipore) in 24-well plates. The lower compartments of the plates were filled with RPMI-1640 containing 10% FBS. After incubated for 16 h with BaP, cells on the upper surface of the membrane were removed. Cells that migrated to the lower surface were fixed with 4% paraformaldehyde (PFA) and stained with 0.1% crystal violet. The stained cells were counted under a microscope.

The invasion assay was performed in a same way as the migration assays, except that the Transwell inserts were coated with Matrigel mixture (BD Biosciences) prior to plating of the cells and the cells were incubated for 48 h.

Real-time PCR. Total RNA was extracted from cells with TRIzol reagent (Takara, cat no. 9108). RNA concentration and purity were measured with microplate reader at A260 and A260/280, respectively. The Prime-Script™ RT-PCR kit (Takara, cat no. RR047A) was used to reverse-transcribe RNA into cDNA. qRT-PCR amplifications were performed with the Applied Biosystems 7500/7500 Fast Real-Time PCR Software (Applied Biosystems) using the SYBR® Premix Ex Taq™ II (cat no. RR820A). Reactions were carried out in a 20-μl reaction volume following the manufacturer's instructions. The thermal cycle conditions were as follows: 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 34 sec. GAPDH was used as an internal control. The primer sequences were: CYP1A1, forward, 5'-CCATGTCGGCCACGGAGTT-3' and reverse, 5'-ACAGTGCCAGGTGCGGGTT-3'; MMP9, forward, 5'-ACG CACGACGTCTTCCAGTA-3', and reverse, 5'-CCACCTGG TTCAACTCACTCC-3'; C-myc, forward, 5'-TACAACACCCG AGCAAGGAC-3' and reverse, 5'-AGCTAACGTTGAGGG GCATC-3'; GAPDH: forward, 5'-GCACCGTCAAGGCTGA GAAC-3', and reverse, 5'-TGGTGAAGACGCCAGTGGA-3'. The data were gathered on ABI 7500 One-Step Plus system.

Western blot analyses. Western blot analyses were conducted as previously described (31). After every specific treatment, cells were washed with precooled PBS and lysed on ice with 1 mM phenylmethanesulfonyl fluoride and RIPA buffer (Beyotime, cat no. P0033) containing protease inhibitors phosphatase inhibitors for the extraction of cellular protein. The supernatant was collected and stored in aliquots at -80°C until analysis by western blot analysis. The protein concentration was measured by BCA protein assay system, protein samples were loaded into each well and separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidenefluoride (PVDF) membranes. The blots were blocked with 5% skimmed milk in Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 h at room temperature and then incubated at 4°C overnight with primary antibodies against CYP1A1 (Abcam ab126828, 1:1,000), pERK (Abcam ab76165, 1:500), ERK (Abcam ab36991, 1:2,000), MMP9 (Abcam ab137867, 1:1,000) and c-myc (Abcam ab32072, 1:10,000). GAPDH (Bioworld Ap0063, 1:5,000) was used as a loading control. After being washed in TBST three times, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1:10,000) for 1 h at room temperature. The specific protein bands were visualized using SuperSignal West Pico Chemiluminescent Substrate and imaged using a VersaDoc imaging system (Bio-Rad).

Statistical analysis. The data are shown as the means ± standard deviation (SD). Statistical analysis was performed using the SPSS 19.0 (IBM, Armonk, NY, USA), and $P<0.05$ was considered to indicate statistically significant differences. The data were analyzed using ANOVA followed by Dunnett or Bonferroni t-test for multiple comparisons.
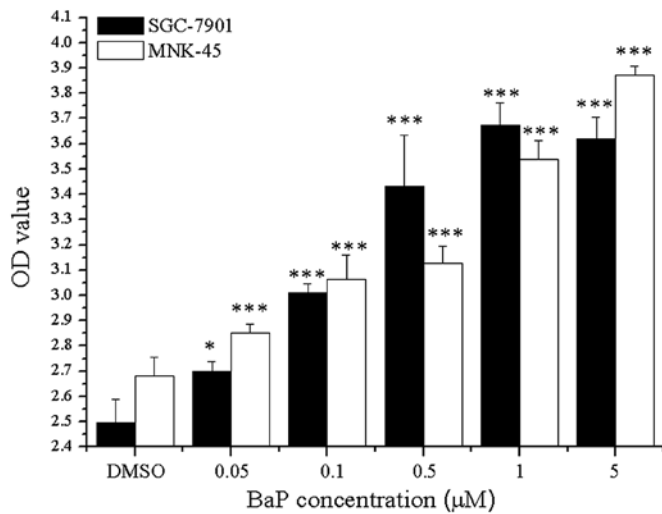
Figure 1. BaP treatment enhances proliferation of GC cells. SGC-7901 and MNK-45 cells exposed to various concentrations of BaP (as shown above) and vehicle (DMSO) for 48 h. Proliferation of BaP-exposed SGC-7901 and MNK-45 cells was increased in a dose-dependent manner compared with DMSO treated GC cells.

Results

BaP enhances proliferation and metastasis of GC cells. Cancer cell proliferation and metastasis are key indices of GC advancement and the major cause of death from the disease (32-34). Therefore, we explored whether BaP promotes the proliferation and metastasis of GC cells. After being treated with BaP, SGC-7901 and MNK-45 cells reached a faster growth rate as demonstrated by the CCK-8 cell proliferation assay (Fig. 1), The proliferation rate increased with higher dose of BaP, showing a dose-dependent effect of BaP on GC cell growth.

We also assessed the effects of BaP exposure on GC cell migration and invasion, the essential activities that allow tumor cells to spread to multiple organs through blood and lymph circulation. Transwell migration assay showed cell numbers were significantly increased on the lower surface in BaP-exposed SGC-7901 and MNK-45 cells, which indicated cell migration was enhanced by BaP treatment (Fig. 2). Similarly, BaP-treated SGC-7901 and MNK-45 cells also showed an increased ability of invasion (Fig. 3).
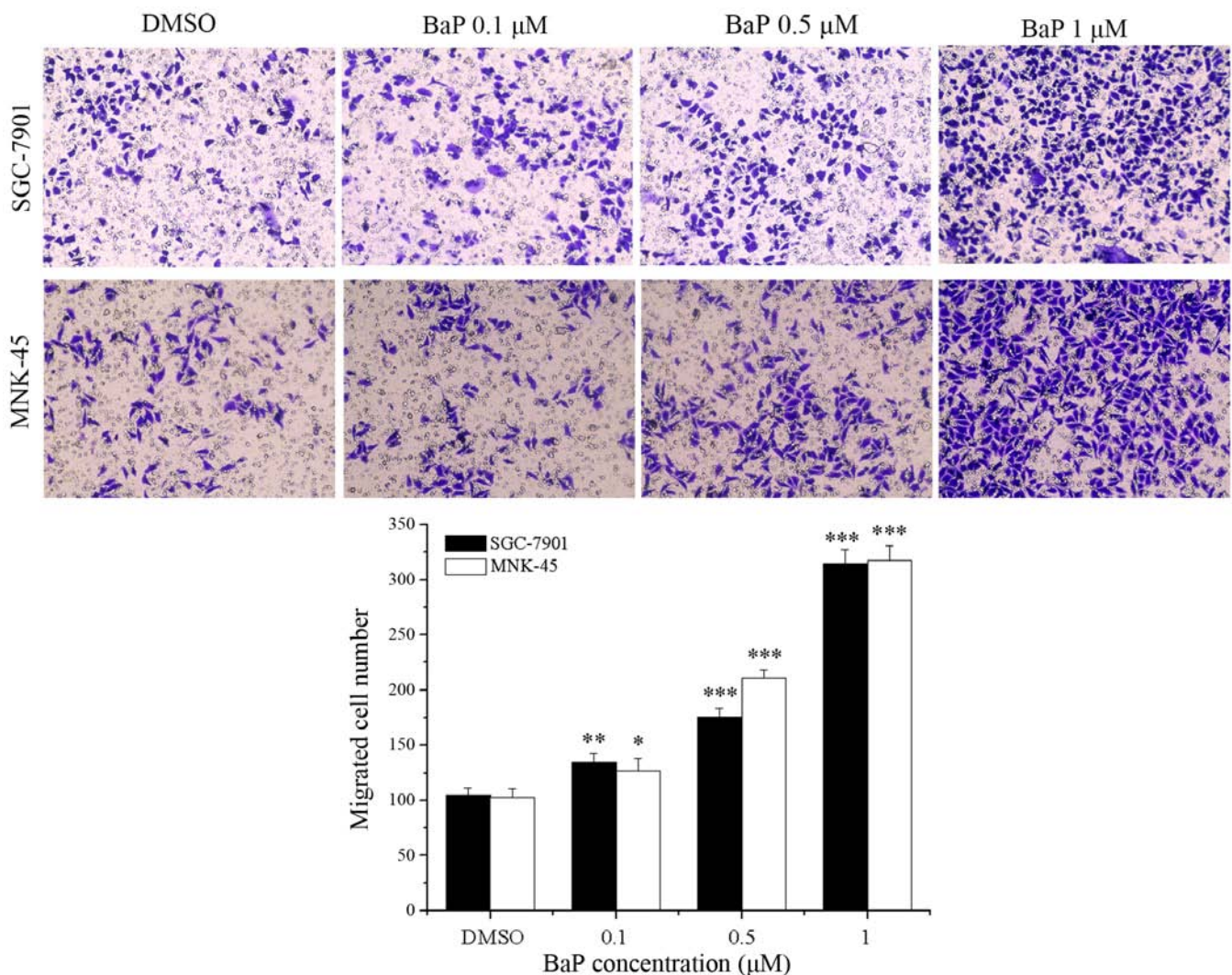

Figure 2. BaP treatment enhances migration of GC cells. SGC-7901 and MNK-45 cells exposed to various concentrations of BaP (as shown above) and vehicle (DMSO). After 16 h, Transwell migration assay showed increased number of GC cells migrated to lower chamber in responding to BaP. *P<0.05, **P<0.01, ***P<0.001, versus control group.
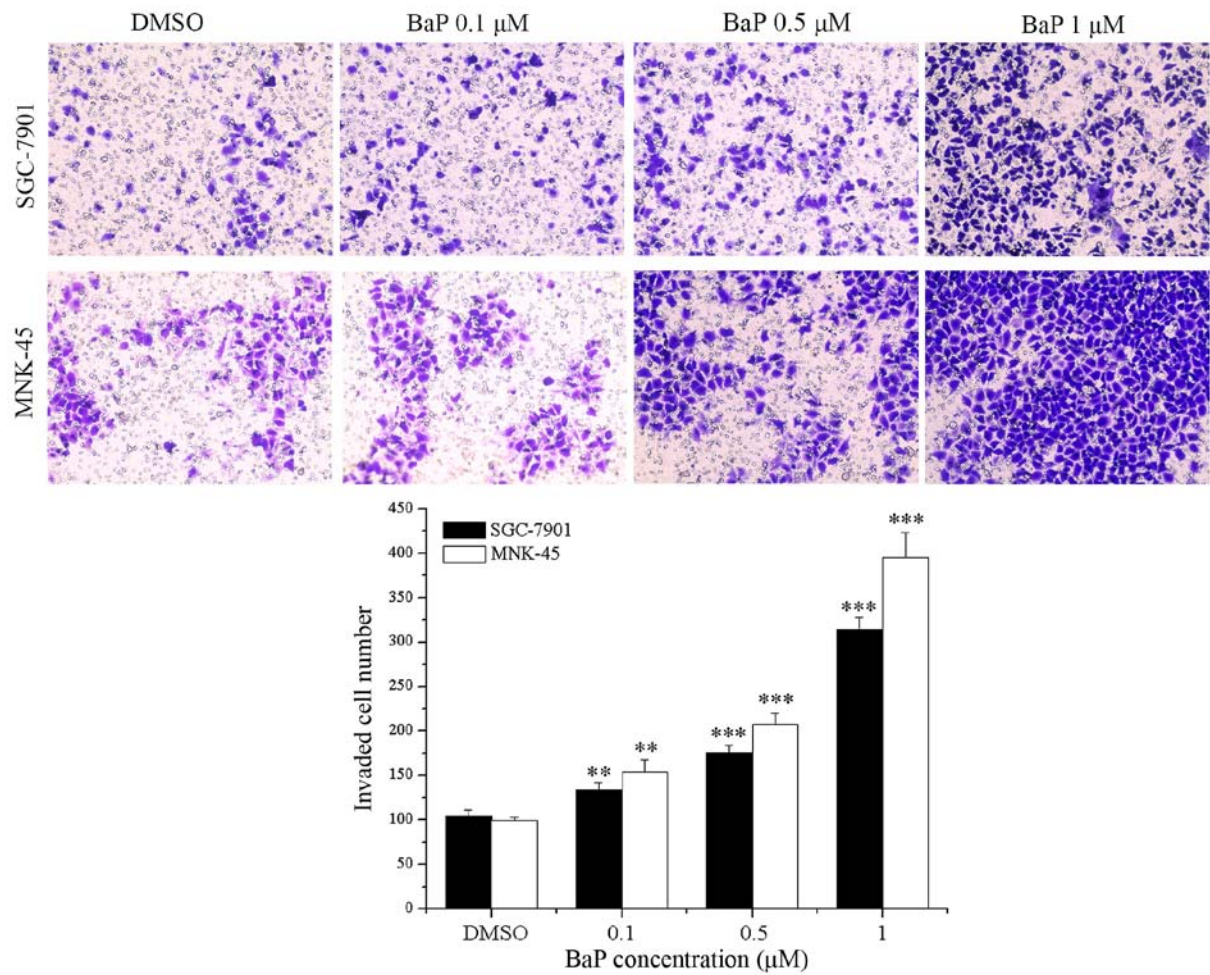
Figure 3. BaP treatment enhances invasion of GC cells. SGC-7901 and MNK-45 cells exposed to various concentrations of BaP (as shown above) and vehicle (DMSO). After 48 h, invasion assay showed increased invasive activity of GC cells. *P<0.05, **P<0.01, ***P<0.001, versus control group.
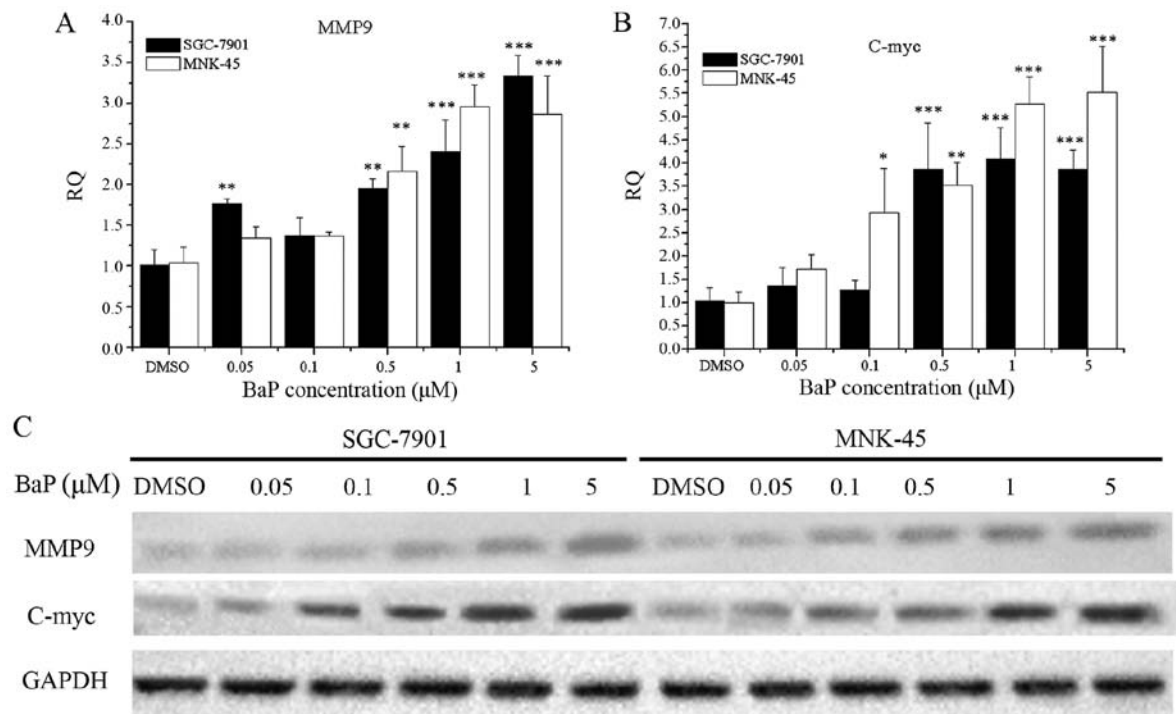

Figure 4. BaP promotes the expression of MMP9 and c-myc. SGC-7901 and MNK-45 cells were exposed to different concentrations (as shown above) of BaP for 48 h, (A and B) RT-PCR analysis of MMP9 and c-myc mRNA expression in a concentration-response. (C) Western blot analysis of MMP9 and c-myc protein expression in a concentration-response.
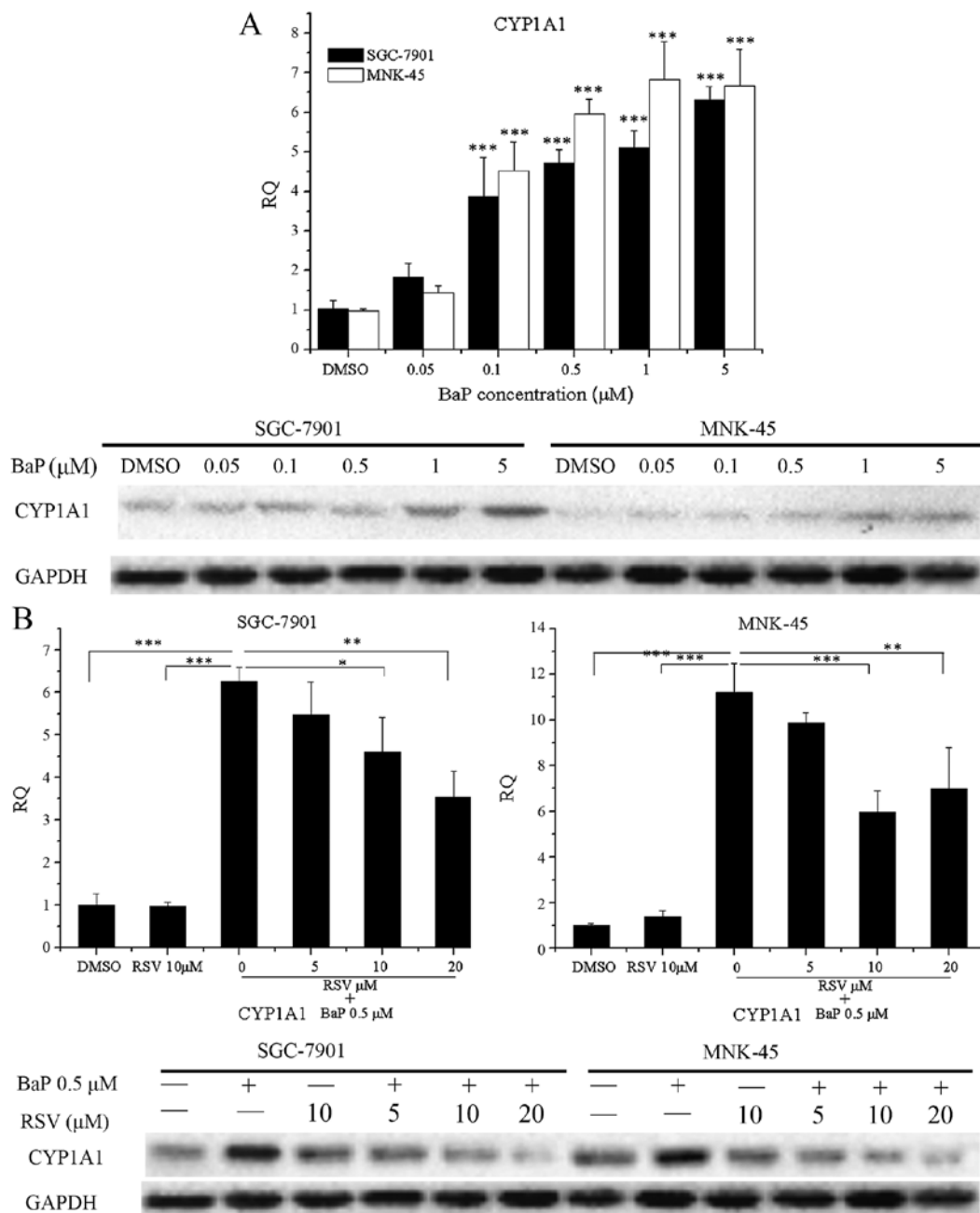
Figure 5. BaP activates the AhR pathway. SGC-7901 and MNK-45 cells were exposed to different concentrations (as shown above) of BaP for 48 h, (A) RT-PCR analysis of CYP1A1 mRNA expression in a concentration-response. Western blot analysis of CYP1A1 protein expression in a concentration-response. After co-treatment with BaP (0.5 μM) and AhR inhibitor RSV (at different concentrations as shown above) for 48 h, (B) mRNA expression of CYP1A1 was measured by RT-PCR. Protein expression of CYP1A1 was measured by western blot analysis. *P<0.05, **P<0.01, ***P<0.001.

BaP promotes the expression of MMP9 and c-myc. MMP9 is one of the type IV collagenase/gelatinases that can degrade extracellular matrix (ECM) components and is widely associated with tumor invasion and metastasis (35). A previous study suggested that BaP induced MMP9 expression in breast cancer cells (28). To determine if BaP has the same effect on MMP9 expression in GC cells, we examined the expression of MMP9 in BaP-treated SGC-7901 and MNK-45 cells using RT-PCR and western blotting. Similar to previous reports on breast cancer (28), our results clearly showed that BaP treatment enhanced MMP9 expression in a dose-dependent manner in GC cells (Fig. 4A and C).

C-myc, a classic oncogene, plays an important role in cancer initiation and maintenance (36). When activated in GC and other cancer cells, c-myc stimulates unlimited proliferation and growth of these cells (37-41). We therefore investigated whether c-myc is induced by BaP treatment in GC cells. As shown in Fig. 4B and 4C, the expression of c-myc was increased following BaP exposure at the mRNA and protein levels.

BaP activates AhR and ERK pathway. Previous studies have confirmed that BaP is an exogenous ligand of AhR (17), and in GC cells the AhR pathway can be activated by another AhR
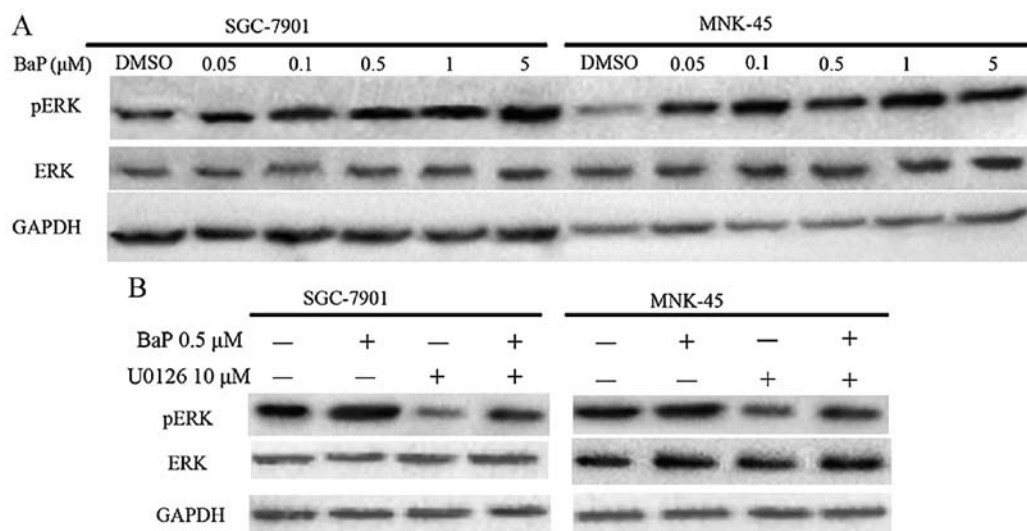
Figure 6. BaP activates the ERK pathway. SGC-7901 and MNK-45 cells were exposed to different concentrations (as shown above) of BaP for 48 h, (A) Western blot analysis of ERK protein expression and phosphorylation in a concentration-response. Cells pretreated with 10 μM ERK inhibitor U0126 for 2 h, then co-treated with BaP (0.5 μM) for 24 h, (B) ERK expression and phosphorylation were measured by western blot analysis.
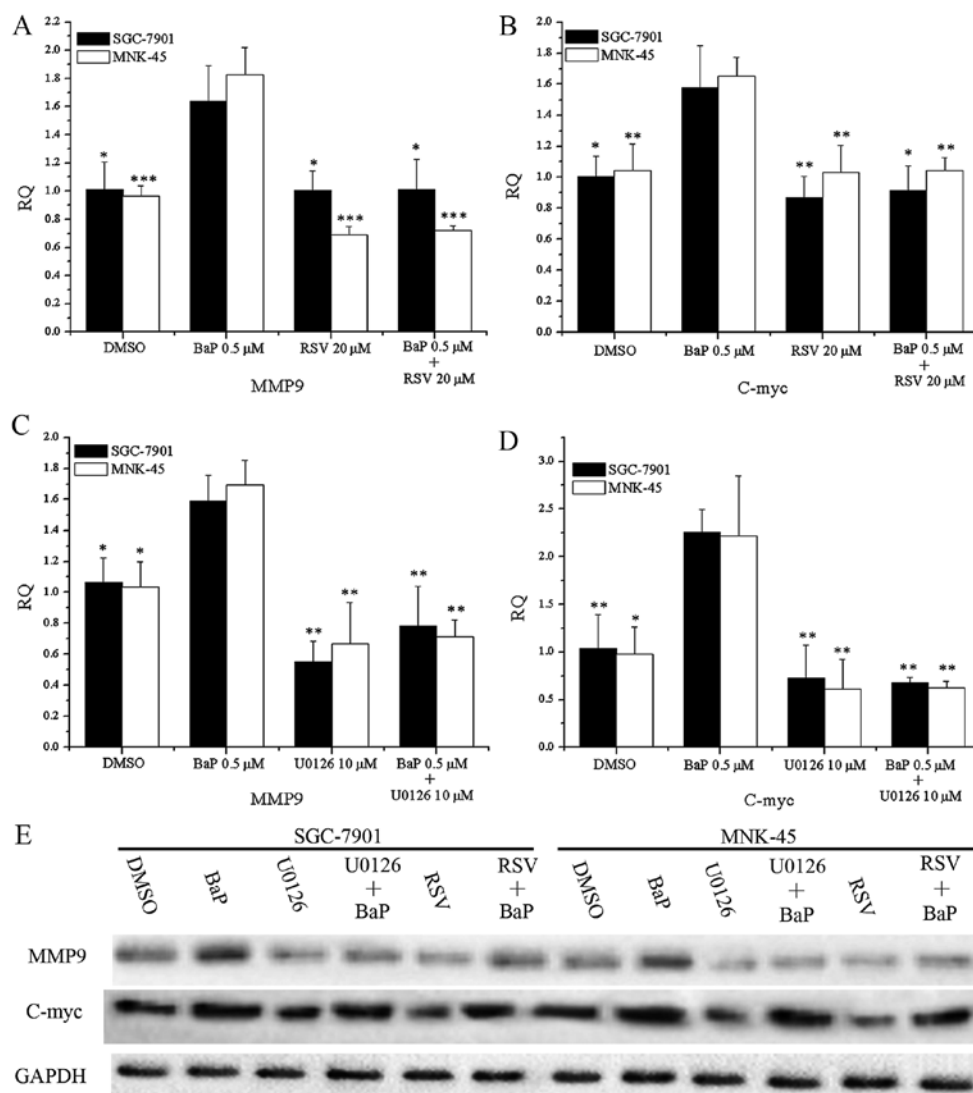

Figure 7. AhR and ERK mediate the expression of MMP9 and c-myc. SGC-7901 and MNK-45 cells were co-treated with BaP (0.5 μM) and RSV (20 μM) for 48 h, (A, B and E) the expression level of MMP9 and c-myc were detected by RT-PCR and western blot analysis. Cells pretreated with 10 μM U0126 for 2 h, then co-treated with BaP (0.5 μM) for 24 h, (C-E) the expression level of MMP9 and c-myc were also detected by RT-PCR and western blot analysis. *P<0.05, **P<0.01, ***P<0.001, versus the group of 0.5 μM BaP.
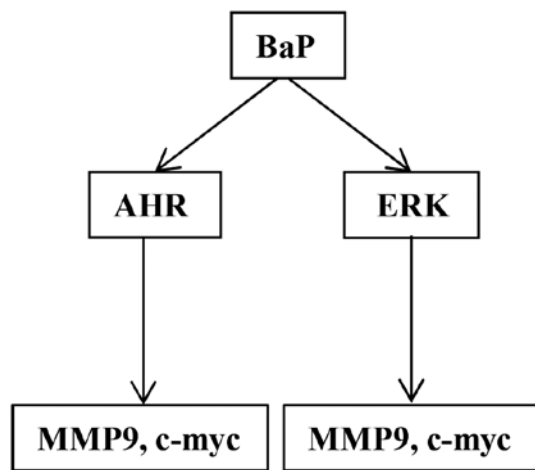
Figure 8. The molecular mechanism of BaP in GC cells. AhR and ERK pathway can be activated after BaP exposure, and activation of both pathways induces MMP9 and c-myc expression.

ligand 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (42). To verify whether AhR pathway can also be activated by BaP in GC cell lines, the expression of a classic target gene of AhR pathway, cytochrome P450 1A1 (CYP1A1), was detected by RT-PCR and western blotting (43). As shown in Fig. 5A, the expression of CYP1A1 mRNA and protein were elevated in both SGC-7901 and MNK-45 cell lines after BaP treatment. In addition, to clarify whether this BaP-induced CYP1A1 expression is AhR-dependent, AhR pathway was inhibited by an AhR antagonist RSV (42,44). As shown in Fig. 5B, RSV could reverse BaP-induced CYP1A1 expression. These data indicated that BaP can activate AhR pathway in GC cells.

The ERK pathway was also demonstrated to take part in BaP-induced cancer development (28). Western blot analysis showed that pERK protein expression in SGC-7901 and MNK-45 cells were induced by BaP treatment (Fig. 6A). Furthermore, U0126, a known ERK inhibitor, significantly blocked BaP-induced ERK activation (Fig. 6B).

Both AhR and ERK pathways mediate the expression of MMP9 and c-myc. To date, little is known about the impact of BaP on intracellular signal transduction in GC. As shown above, we demonstrated that BaP promoted the expression of MMP9 and c-myc and activated AhR and ERK pathways. Thus, we explored the potential relationship between the two oncogenes and these activated pathways. To elucidate whether the AhR activation really linked to MMP9 and c-myc overexpression, AhR antagonist RSV was used to block the AhR pathway. In the presence of RSV, the overexpression of MMP9 and c-myc induced by BaP were attenuated in SGC-7901 and MNK-45 cells (Fig. 7A, B, E), indicating that AhR pathway mediated the upregulation of MMP9 and c-myc expression by BaP.

In addition, we investigated the role of ERK pathway in the regulation of MMP9 and c-myc expression in BaP-treated GC cells. As shown in Fig. 7C, D and E, inhibition of ERK signaling by U0126 significantly attenuated BaP-induced MMP9 and c-myc expression at the mRNA and protein level. Therefore, the ERK pathway appears to participate in mediation of MMP9 and c-myc expression.

Discussion

GC is the most common cancer worldwide and the third leading cause of global cancer-related death (1, 2). As a Group I carcinogen categorized by the IARC, BaP has been confirmed to induce several types of cancers and play important roles in gastric carcinogenesis (8,15,16,30). In this study, we aimed to explore the effects of BaP on GC cell proliferation and metastasis and its regulatory mechanism.

C-myc is overexpressed in GC tissue and can promote GC proliferation and growth *in vivo* and *in vitro* (41,45). Our study found that BaP treatment increased proliferation of GC cell lines SGC-7901 and MNK-45. Additional evidence showed that exposure to BaP induced c-myc overexpression, which might promote GC cells proliferation. In regard to metastasis, ECM degradation by matrix metalloproteinases (MMPs) is known to facilitate tumor invasion and metastasis, and MMP9 is the most characterized member of MMPs with strong proteolytic activity in the ECM (35,46). In GC, MMP9 closely associates with the tumor grade and stage and plays important roles in tumor invasion and metastasis (47,48). We found that MMP9 was significantly induced after BaP exposure and BaP-treated SGC-7901 and MNK-45 cells showed stronger invasive and metastatic ability.

BaP induces gene mutation and causes deregulation of several proteins mainly through the AhR/CYP450 pathway, thus playing a carcinogenic role in multiple cancers (20). Previous studies have implied that AhR expression is significantly increased in GC tissues and GC cell lines, and its expression was associated with lymph node and distant metastasis in GC patients (49,50). *In vitro* study showed that AhR pathway activation promoted GC cell invasion (42), while blocking AhR pathway decreases GC cell growth and invasion (51). *In vivo* experiment also confirm that inhibition of AhR pathway suppresses GC growth and peritoneal dissemination (49). These results are consistent with our experiment. However, Yin *et al* reported that activation of AhR pathway by 3,3'-diindolylmethane inhibits GC SGC-7901 cell growth (44). This result is inconsistent with our experiment, the reason may be the difference of the AhR modulator. In addition, AhR can both inhibit and enhance tumor progression, whether AhR plays a pro-oncogenic or an antitumorigenic role likely depends on the cell type and the dominant pathway (20,22). Thus the underlying mechanism between these controversies remains to be further elucidated. Further molecular mechanism research showed AhR modulated c-Jun-dependent induction of MMP9 and Snail expression in GC (42,49). Our study showed that the AhR pathway was activated by BaP in GC cell lines and its activation might induce the expression of MMP9 and c-myc. The ERK pathway also has been reported to participate in BaP-induced tumor progression (28). ERK mediates multiple cellular signaling pathways and plays an important role in GC progression (52,53). However, no report on BaP and ERK in GC exists. This study suggests that BaP activates the ERK pathway and induces the expression of its downstream protein MMP9 and c-myc.

In conclusion, this study demonstrates that BaP promoted proliferation and metastasis of GC SGC-7901 and MNK-45 cells through upregulation of MMP9 and c-myc expression. Furthermore, AhR and ERK pathways can be activated after

BaP exposure, and activation of both pathways induces MMP9 and c-myc expression. However, as our study was performed only using GC cell line, further studies using normal gastric mucosa cells are needed, and *in vivo* studies must be conducted to further verify the findings reported in this study.

Acknowledgements

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