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Benzidine induces epithelial–mesenchymal transition in human uroepithelial cells through ERK1/2 pathway

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ABSTRACT

Prolonged benzidine exposure is a known cause of urothelial carcinoma (UC). Benzidine-induced epithelial-to-mesenchymal transition (EMT) is critically involved in cell malignant transformation. The role of ERK1/2 in regulating benzidine-triggered EMT has not been investigated. This study was to investigate the regulatory role of ERK1/2 in benzidine-induced EMT. By using wound healing and transwell chamber migration assays, we found that benzidine could increase SV-HUC-1 cells invasion activity, western blotting and Immunofluorescence showed that the expression levels of Snail, β-catenin, Vimentin, and MMP-2 were significantly increased, while, the expression levels of E-cadherin, ZO-1 were decreased. To further demonstrate the mechanism in this process, we found that the phosphorylation of ERK1/2, p38, JNK and AP-1 proteins were significantly enhanced compared to the control group (*$P < 0.05$). Afterward, treated with MAPK pathways inhibitors, only ERK inhibitor (U0126) could reduce the expression of EMT markers in SV-HUC-1 cells, but not p38 and JNK inhibitor (SB203580, SP600125) , which indicated that benzidine induces the epithelial–mesenchymal transition in human uroepithelial cells through ERK1/2 pathway. Taken together, findings from this study could provide into the molecular mechanisms by which benzidine exerts its bladder-cancer-promoting effect as well as its target intervention.

1. Introduction

Bladder cancer is the most common malignancy of the urinary tract, with 73,000 new cases and 15,210 deaths in 2013 in the US alone [1]. Benzidine, which belongs to aromatic amines, is classified by the International Agency for Research on Cancer (IARC) as definite human carcinogens on the basis of sufficient evidence of bladder cancer in animals and human beings [2,3]. Benzidine can be found in products made by the chemical, dye, and rubber industries, as well as in hair dyes, paints, fungicides, plastics, metals, motor vehicle exhaust, and industrial pollutant emissions [4–6]. Previous studies showed that food dyes (including Red 40, Yellow 5, and Yellow 6) have been contaminated with benzidine and that they could cause hypersensitivity reactions [7]. Thus, benzidine still constitutes a great hazard for human health. Previously, many epidemiological investigations about benzidine were researched, but little was used to investigate how benzidine leads to bladder cancer. Therefore, we try to characterize benzidine-induced carcinogenesis.

The epithelial–mesenchymal transition (EMT) is a multistep process in which epithelial cells lose their epithelial characteristics and gain mesenchymal characteristics, including loss of cell–cell adhesion, increased motility and invasiveness, resistance to apoptosis and changes in cellular morphology, it was only a simple morphological change, but also associates with the transcriptional or expressional alteration of some epithelial and mesenchymal genes [8,9]. Evidences have suggested that, in addition to facilitating tumor invasion and metastasis, EMT is also critically involved

Abbreviations: SV-HUC-1, SV-40 immortalized human uroepithelial cells; EMT, epithelial–mesenchymal transition; MTT, thiazolyl blue tetrazolium bromide; RT-PCR, reverse transcription-polymerase chain reaction.

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in the initiation of tumorigenesis. Exposure of cells to carcinogens induces EMT during malignant transformation [10–14]. TS has been documented to promote EMT which regulates early events in lung carcinogenesis [13,15–21]. Nonetheless, the underlying mechanisms by which benzidine induces EMT are poorly understood.

The EMT includes a series of complex changes, including many signaling pathways involved in it, such as the Wnt, Notch, and MAPK signaling pathways [22]. The MAPKs are include extracellular signal-regulated kinases ERK1/2, JNKs and the p38 family and are activated through three-tier kinase signaling cascades [23]. The activation of the MAPK pathways have been associated with the activation of many transcription factors, resulting in the increased expression of numerous genes involved in tumor cell proliferation, apoptosis, angiogenesis, metastasis, and the tumor progression [24–27].

Here, we aimed to investigate the role of MAPK signaling in low benzidine concentration-induced EMT. In previous studies, most of them were used to research the EMT in cancer cell lines. Now, we through treating normal bladder epithelial cells (SV-HUC-1) with a low concentration of benzidine, we try to demonstrate how benzidine induce carcinogenesis in bladder.

2. Materials and methods

2.1. Chemicals and reagents

Benzidine (4, 4′-diaminobiphenyl; ≥98.0%, RT), dimethyl sulfoxide (DMSO), methanol were obtained from Merck (Reading Township, NJ, USA). Other chemicals were of the highest purity available from Sigma–Aldrich (St. Louis, MO, USA). The kinase inhibitors U0126, SB203580, and SP600125 were obtained from Cell Signaling Technology (Beverly, MA, USA). Growth media (Ham's F12 medium), fetal bovine serum (FBS), phosphate-buffered saline (PBS), antibiotics, and trypsin were obtained from HyClone (Logan, UT, USA). Antibodies to phospho-ERK1/2, phospho-JNK, and phospho-p38 were from Cell Signaling Technology. Antibodies to p-c-Jun, p-c-Fos, Snail, E-cadherin, and β-catenin were also from Cell Signaling Technology. Antibodies to ZO-1, Vimentin, and MMP-2 were from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2. Cell culture and treatments

SV-40 immortalized human uroepithelial cell line (SV-HUC-1) obtained from the American Type Culture Collection (ATCC, Wiltshire, USA) grown in 25-cm^2 flasks (initial density, 1×10^5 cells/ml). Cells were maintained in Ham's F-12 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 units/ml streptomycin at 37 °C and 5% CO_2 humidified incubator. After culture for 12 h, cells were either exposed to different concentrations of benzidine, or were treated with U0126 (10 μM), SB203580 (10 μM), or SP600125 (5 μM). SV-HUC-1 cells were stimulated with benzidine or inhibitors for 4 days. The final concentrations of benzidine were 0, 0.001, 0.005, 0.01, 0.05, and 0.1 μM. All experiments were carried out three times.

2.3. MTT assay

Cell viability (as determined by mitochondrial enzyme functions) was assayed by MTT conversion to Formosan. In brief, 5×10^4 cells per well were seeded in 96-well plates. After culture for 12 h, cells were grown to around 80% confluence, treated with 100 μl growth medium in the presence of 0.1% DMSO, benzidine (10^{-6}–200 μM) for 4 days. Cell viability was then assessed using MTT assay. Ten microliter of MTT solution (5 mg/mL) was added to each well, and the plates were further incubated for 4 h at 37 °C. Medium containing MTT was removed, and precipitants were solubilized in DMSO. Absorbance was measured at 490 nm using a microplate reader. All measurements were performed in triplicate.

2.4. Wound healing assay

SV-HUC-1 cells were pretreated with control medium or medium containing benzidine (0.005 or 0.01 μM) for 4 days, and then cells were seeded in 6-well plates (2×10^5 cells/well) and incubated for 24 h in serum-free medium before wounding. The scratch wound was generated on the surface of the plates using a pipette tip. Photographic images were taken from SV-HUC-1 cells at 0, 12, and 24 h.

2.5. Transwell chamber migration assay

SV-HUC-1 cells were pretreated for 4 days, and then the cells were transferred to transwell chambers. Serum was added to the bottom wells of the chambers to induce cell migration. After 24 h, cells that had migrated through the membrane were stained with 0.5% methylrosaniline chloride solution and counted. Cells were counted in five random fields and counts were expressed as 1% of the average number of cells/field under a light microscope.

2.6. Western blots

For western blot analysis, 6×10^6 cells per dish were seeded in 100 mm plastic tissue culture dishes. After culture for 12 h, cells were either exposed to different concentrations of benzidine for 4 days, or were treated with either the U0126 (10 μM), SB203580 (10 μM), or SP600125 (5 μM) inhibitors. Proteins (50 μg per lane) were separated by 10% SDS–polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Bedford, MA, USA). After blocking in 5% fat-free dry milk in Tween20 Tris-buffered saline (TBST), membranes were incubated with primary antibodies overnight at 4 °C, washed in TBST, and then incubated with goat-rabbit peroxidase-conjugated secondary antibodies. Protein bands were detected using the Immobilon western chemiluminescent HRP substrate kit.

2.7. Reverse transcription-polymerase chain reaction (RT-PCR)

For RNA analysis, 6×10^6 cells per dish were seeded in 100-mm plastic tissue culture dishes. After culture for 12 h, cells were exposed to benzidine. After another 4 days of culture, cells were harvested and total RNA was extracted with TRIzol reagent according to the manufacturer's instructions. Total RNA was reverse-transcribed using the Easy RT-PCR kit according to the manufacturer's protocol. The relative expression levels of E-cadherin, ZO-1, and Vimentin were analyzed by RT-PCR with GAPDH as a loading control. A 20 μl total reaction mixture included 10 μl SYBR premix, 0.4 μl Rox, 7.8 μl dH_2O, 0.4 μl forward and 0.4 μl reverse primers

Table 1
Primer sequences of target genes.

Gene name	Primer sequence
Vimentin	Forward 5′-GAGAACTTTGCCGTTGAAGC-3′
	Reverse 5′-GCTTCCTGTAGGTGGCAATC-3′
E-cadherin	Forward 5′-TCGACACCCGATTCAAAGTGG-3′
	Reverse 5′-TTCCAGAAACGGAGGCCTGAT-3′
ZO-1	Forward 5′-GCAGCCACAACCAATTCATAG-3′
	Reverse 5′-GCAGACGATGTTCATAGTTTC-3′
GAPDH	Forward 5′-GCTGCCCAACGCACCGAATA-3′
	Reverse 5′-GAGTCAACGGATTTGGTCGT-3′

(primer sequences for each target gene are shown in Table 1), and 1 μl cDNA sample. Each sample was run in triplicate. The RT-PCR program included an initial denaturation step at 95 °C for 15 s, followed by 40 cycles of amplification and quantification at 95 °C for 10 s, 60 °C for 30 s, and72°Cfor 30 s. At the end of the program, a melting curve analysis was performed.

2.8. Immunofluorescence

After benzidine treatment for 4 days, SV-HUC-1 cells were fixed in 4% paraformaldehyde for 15 min at room temperature, and were washed three times with TBSTx. Then, membranes were ruptured with 0.1% TritonX-100 for 30 min. Slides were blocked in 5% BSA containing TBSTx for 1 h at room temperature, and then were incubated with a monoclonal E-cadherin (1:150 dilution) and Vimentin (1:100 dilution) antibodies in 5% BSA overnight at 4 °C. After washing with TBSTx, cells were incubated with secondary antibodies for 1 h at room temperature. Finally, cells were counterstained with 4,6′-diamidino-2-phenylindole (DAPI) before mounting, then cells were washed three times with TBSTx. Stained cells were mounted with fluorescent mounting medium (Dako, Carpinteria, CA, USA). The fluorescent images were obtained using a confocal laser scanning microscope (LSM700; Carl Zeiss Meditec, Göttingen, Germany).

2.9. Statistical analysis

Results were expressed as mean ± S.D. from at least three independent experiments. Statistical analysis was performed according to Student's *t*-test by one-way analysis of variance. The Student's *t*-test was used to compare the labeling index and relative density for the different treatments. Significant difference was taken as $^{*}P < 0.05$ or $^{**}P < 0.01$.

3. Results

3.1. Screening of nontoxic dose of benzidine with SV-HUC-1 cells

To test the effects of different concentrations of benzidine treatment on SV-HUC-1 cells, the cells were treated with a range of concentrations (0–50 μM) of benzidine for 4 days. Based on the MTT results, the concentrations of benzidine (>1 μM) appeared to have a cell toxicity on SV-HUC-1 cells (Fig. 1A). Therefore, 0.001–0.1 μM benzidine were selected as the concentration for the following experiments.

3.2. Benzidine induces EMT in SV-HUC-1 cells

EMT process is manifested by alterations in cell morphology, migration and invasion capacity. Treatment of SV-HUC-1 cells with benzidine for 4 days resulted in significant morphological change from epithelial round-shaped to spindle-like mesenchymal form (Fig 1B). To further examine the effect of benzidine on EMT, wound healing assay and transwell assays were carried out to analyze SV-HUC-1 cells migratory capacities in response to benzidine. Benzidine treatment significantly increased SV-HUC-1 cell migration (Fig. 1C and D). The transwell chamber migration assay was performed to detect on the number of migrating cells (Fig. 1E). To determine whether molecular alterations of EMT occurred in benzidine treated cells, the expression levels of EMT markers were determined. Exposure of SV-HUC-1 cells to benzidine resulted in increased protein expression levels of Snail, β-catenin, Vimentin, and MMP-2, while the protein expression levels of E-cadherin and ZO-1 were reduced by western blots analyses (Fig. 2A). Additionally, levels of E-cadherin, ZO-1, and Vimentin mRNA were analyzed by RT-PCR (Fig. 2B–D), and the results showed that the expression of E-cadherin and ZO-1 were reduced while Vimentin were enhanced. To further confirm the benzidine induced EMT in SV-HUC-1 cells, we also examined cell surface markers of E-cadherin, an epithelial marker, and Vimentin, a mesenchymal marker. We chose 0.005uM and 0.01 μM benzidine to treat SV-HUC-1 cells for 4 days, the expression of E-cadherin markedly decreased while Vimentin increased (Fig. 2E), which was consisted with the cell surface marker change in EMT and was consistent with those of the Western blots assay.

3.3. Benzidine activates MAPK/AP-1 pathways in SV-HUC-1 cells

To test whether benzidine could activate the MAPK/AP-1 pathways, total and phospho-ERK, phospho-p38, and phospho-JNK protein levels were measured after benzidine treatment in SV-HUC-1 cells. The cells were treated with benzidine concentrations ranging from 0 to 0.1 μM for 4 days. Notably, increased levels of phosphorylated ERK1/2, p38, and JNK occurred in the absence of any changes in total ERK1/2, p38, or JNK levels (Fig. 3A). These data indicated that benzidine could induce the phosphorylation of ERK1/2, p38, and JNK in SV-HUC-1. AP-1 is composed of either homo- or hetero-dimers between members of the Jun and Fos families. We detected significant increases in p-c-Jun and JunB levels, while JunD levels were not significantly affected. The Fos family, including p-c-Fos, FosB, and Fra-1, were up regulated (Fig. 3B).

3.4. ERK1/2/AP-1 pathway was involved in the induction of EMT by benzidine

To interrogate which intracellular signaling cascade the Benzidine-mediated EMT might be coupled to, the involvement of the MAPK pathways were studied. SV-HUC-1 cells were treated with MAPK pathways inhibitors such as ERK1/2 inhibitor (U0126), p38 inhibitor (SB203580), and JNK inhibitor (SP600125). Results showed that ERK1/2 inhibitor (U0126) significantly attenuated the process of epithelial mesenchymal transformation (EMT) induced by benzidine (Fig. 4A), but not p38 and JNK inhibitors (Fig. 4B and C). Collectively, these results suggested that the induction of EMT by benzidine in SV-HUC-1 cells required MAPK pathways participation.

4. Discussion

We first found the EMT through treating normal bladder epithelial cell (SV-HUC-1) with a low concentration of benzidine. No previous studies had investigated the effect of benzidine on AP-1-regualted EMT markers. Therefore, this study provides the first report to explore the role of MAPK/AP-1 pathways in benzidine-induced EMT.

Firstly, we have demonstrated that benzidine can induce the EMT. EMT is a crucial process in cancer development. Some studies revealed that exposure of cells to carcinogens induces EMT during transformation and tumor formation [10–14], suggesting the important role of EMT in the initiation of tumorigenesis by promoting cell malignant transformation. Xu et al. revealed that EMT is involved in arsenite-induced transformation of human bronchial epithelial cells [11,12]. Tellez et al. demonstrated that exposure of human bronchial epithelial cells to tobacco carcinogens benzo(a) pyrene-diolepoxide and methylnitrosourea induces EMT, which is an early manifestation during transformation process, participating in cancer initiation and promoting the clonal expansion of pre-malignant cells [10]. In agreement with previous reports, we showed in the present study that exposure to benzidine induced

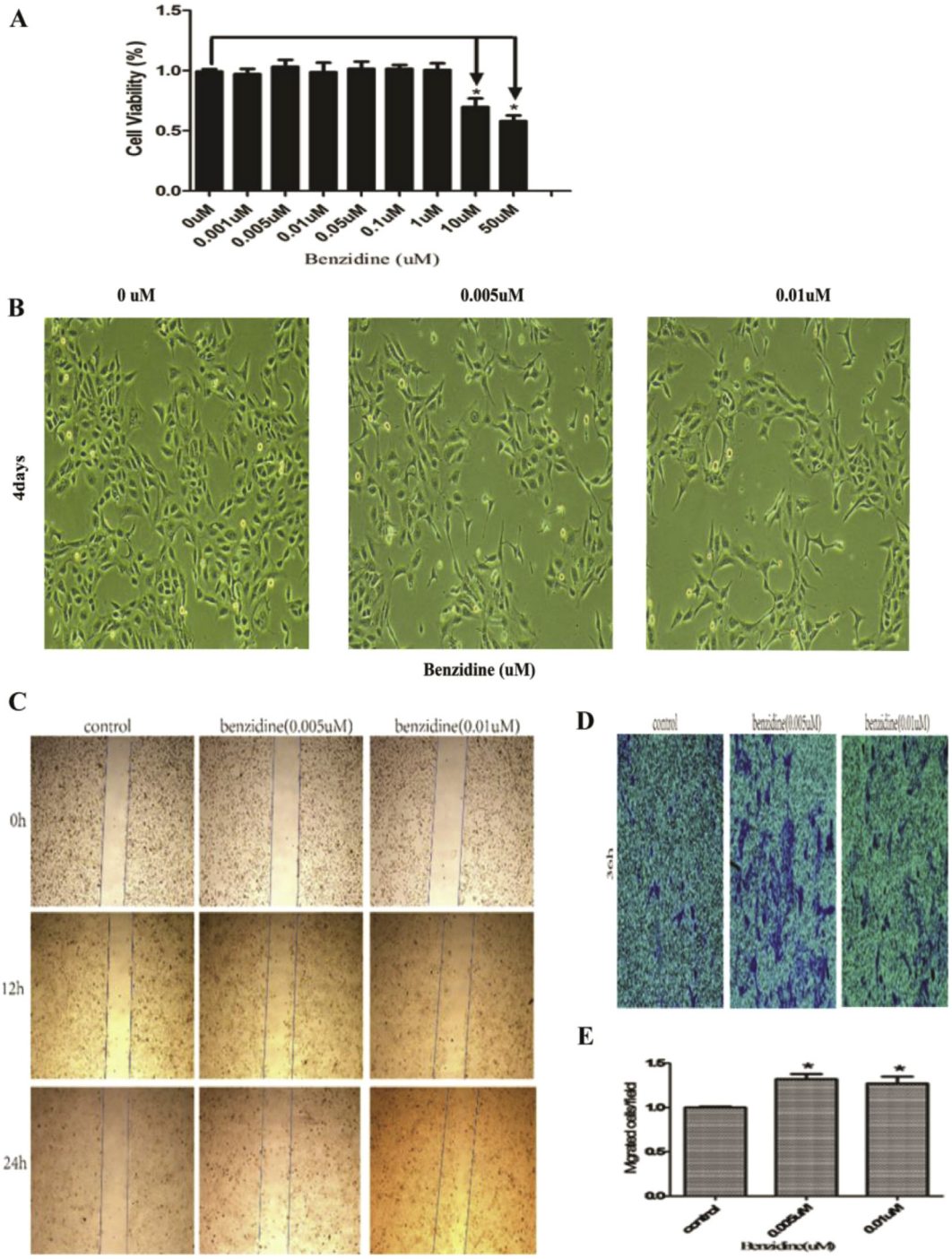

Fig. 1. Benzidine exposure induces cell migration of normal bladder epithelial cells (SV-HUC-1). (A) Effect of benzidine on cell viability of SV-HUC-1 cells. SV-HUC-1 cells were treated with various concentrations of benzidine for 4 days and cell viability was measured by MTT assay. (B) Benzidine induced morphological change from epithelial to spindle-like mesenchymal shape, as shown by morphological examination of SV-HUC-1 cells following benzidine treatment for 4 days. (C) Benzidine enhanced migratory capacity of SV-HUC-1 cells, as determined by wound healing assay. (D) Benzidine enhanced invasive capacity of SV-HUC-1 cells, determined by transwell invasion assay. (E) Quantification of migrating cells. *P < 0.05 vs controls.

EMT in SV-HUC-1 cells, as manifested by morphological change from epithelial to mesenchymal form, increased migratory capacities, as well as alterations in the expression of EMT markers, including decreased epithelial markers E-cadherin and ZO-1, and increased mesenchymal markers Snail, Vimentin, bete-catenin, and MMP-2. Taken together, our data revealed that benzidine triggered EMT.

Previous studies have shown that the initiation of the EMT is dependent on the concomitant activity of a variety of signal-transduction pathways, including MAPK pathways, for the morphogenic process of the EMT [22,28]. Some studies find that MAPK activation is a key modulator in the progression of renal diseases and is thought to occur in various kinds of cells, including mesangial cells [29] and vascular endothelial cells [30]. However, little is known about the activation MAPK in normal bladder

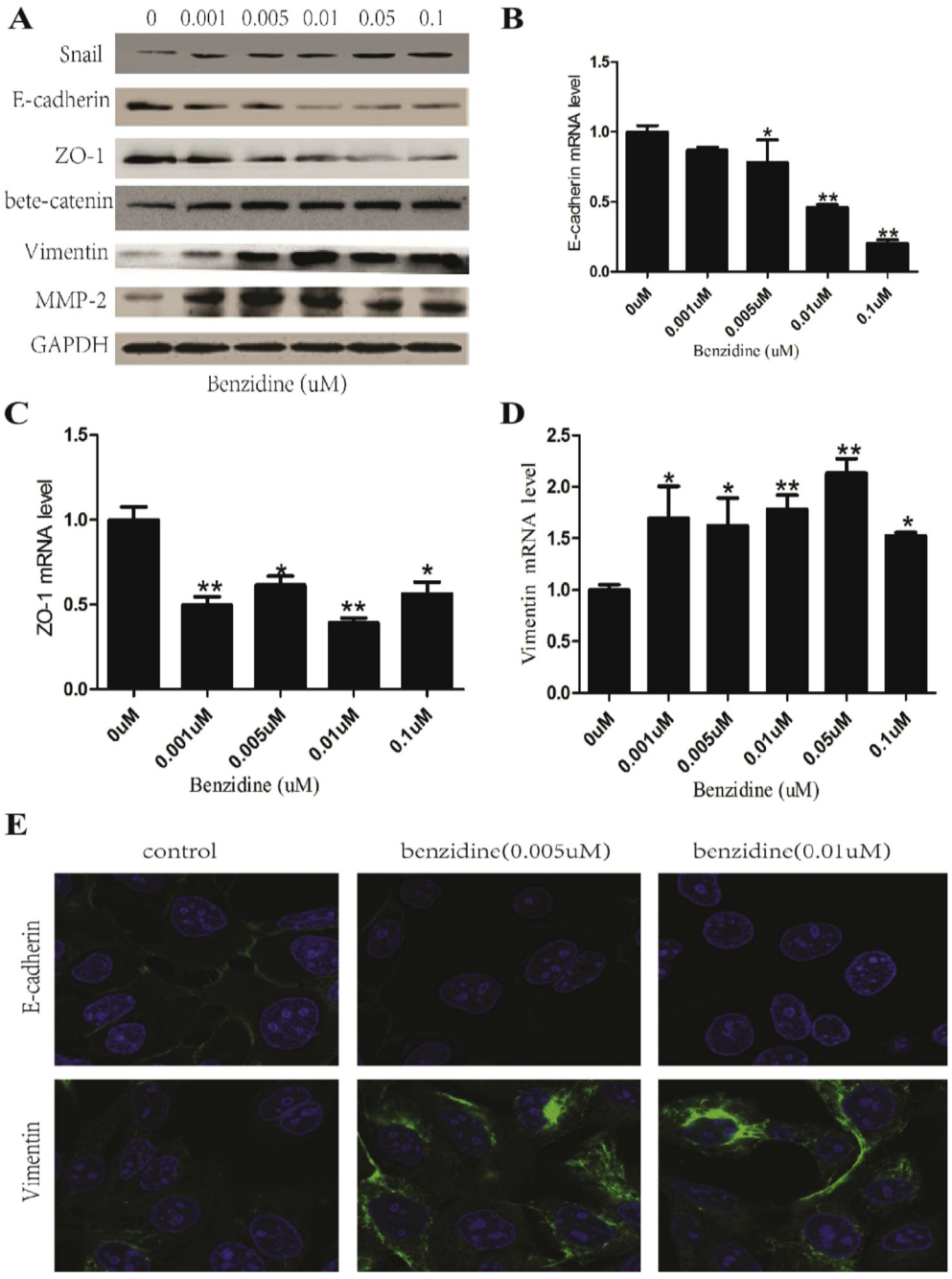

Fig. 2. Benzidine alters the expression of EMT markers in normal bladder epithelial cells (SV-HUC-1). (A) Benzidine exposure decreased the protein expression of epithelial markers E-cadherin and ZO-1, and increased protein expression of Snail, bete-catenin, Vimentin, and MMP-2 by Western blotting. (B, C) E-cadherin, ZO-1mRNA levels are reduced in response to benzidine by quantitative real-time PCR. (D) Benzidine exposure induced Vimentin mRNA upregulation by quantitative real-time PCR. (E) SV-HUC-1 cells after 0.005, 0.01 μM benzidine treatment were subjected to fluorescence staining, results showed that the expression of E-cadherin markedly decreased and Vimentin increased. *$P < 0.05$; **$P < 0.01$, vs. control. One representative image of three independent experiments is shown.

epithelial cell. But a wealth of data that has supported the central role of the p38 MAPK pathway in high glucose-induced cell damage [31–34]. Therefore, we focused on the MAPK pathways to determine whether it mediates benzidine-induced EMT. Our results had showed that benzidine could activate the three MAPK pathways, further supported that the role of MAPK in EMT by three MAPK pathways inhibitors findings. Results showed that the EMT of SV-HUC-1 cells mainly through activating the ERK1/2 pathway. However, the P38 and JNK/MAPK pathways have not obviously involved in the induction of EMT in our current study. So we guess that P38 and JNK/MAPK pathways may just effect the cell proliferation and it will be further to confirm in our future study.

Furthermore, our data also show that benzidine activates AP-1 through three distinct MAPK signaling pathways, including ERK1/2, p38, and JNK. Recent investigations have demonstrated that JNK plays an important role in AP-1 activation. Once activated, JNK phosphorylates Jun and increases AP-1 transcriptional activity to promote the invasive growth and metastasis of various tumors, such as breast cancer, squamous cell carcinoma (SCC), and melanoma [35,36]. It has been established that AP-1 activity plays a

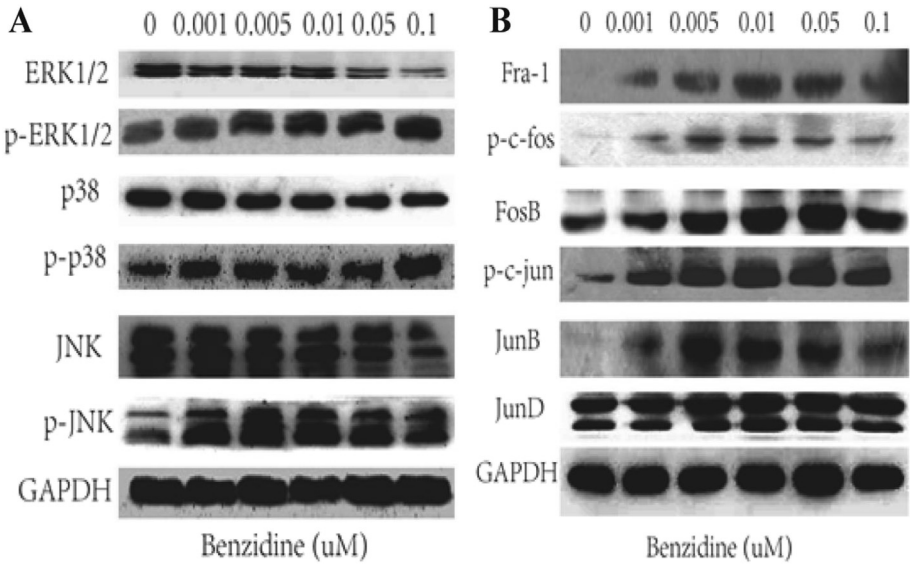

Fig. 3. Benzidine activates MAPK/AP-1 pathways in SV-HUC-1 cells. (A) Total and phospho-ERK1/2, phospho-p38, and phospho-JNK protein levels were determined by Western blotting using specific antibodies. We detected changes in the levels of p-ERK1/2, p-p38 and p-JNK protein, especially at concentrations of 0.005 and 0.01 μM. (B) Western blotting analysis of p-c-Jun, Jun B, Jun D, Fra-1, p-c-Fos, and FosB. Fra-2 was not detectable. Data is expressed as mean ± SD of three independent experiments for each treatment.

central role in the process of tumorigenesis [37]. AP-1 mediates many physiological processes, such as proliferation, survival, differentiation, apoptosis, and transformation [38]. To our knowledge, data on the function of AP-1 in benzidine-induced EMT is lacking. In this study we showed that benzidine-mediated ERK1/2 increased the activation of AP-1 proteins p-c-Fos and p-c-Jun; on the contrary, ERK1/2 inhibition decreased p-c-Fos and p-c-Jun activation, suggesting the modulation of ERK1/2 on AP-1. It is noteworthy that the precise mechanism by which ERK1/2 regulates AP-1 activation is unknown at present time, and future studies are warranted to

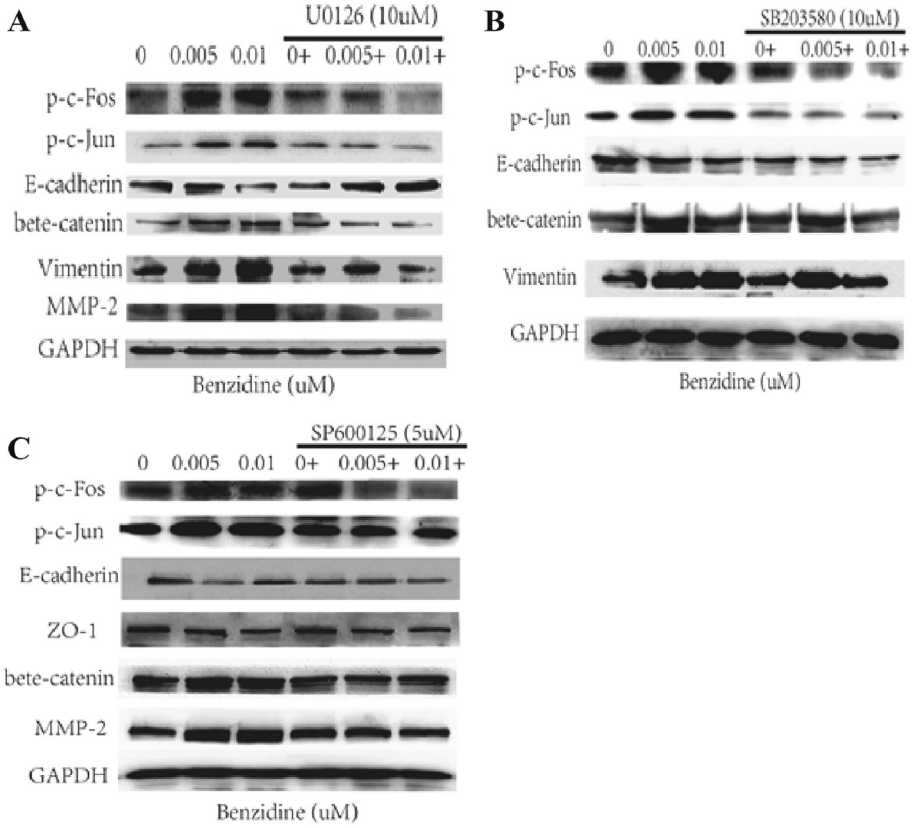

Fig. 4. ERK1/2 pathway was involved in the induction of epithelial mesenchymal transformation by benzidine. (A) E-cadherin protein expression was down regulated when cells were used ERK1/2 inhibitor (U0126). However, the bete-catenin, Vimentin, and MMP-2 proteins expression were increased by using the ERK1/2 inhibitor. (B, C) p38 and JNK inhibitors were not obviously. One representative image of three independent experiments is shown.

define the underlying mechanism of ERK1/2 regulation on AP-1 protein.

In conclusion, we demonstrated significant effects of benzidine that resulted in the initiation of EMT, which was mainly through the activation of ERK1/2 pathway. These findings indicated the important role of ERK1/2 pathway in benzidine-induced pathologies and provide new insights into the molecular mechanisms that underlie pathologies induced by benzidine including bladder diseases and cancer.

Conflict of interest

The authors declare no conflict of interest.

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