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Cigarette smoke extract induces epithelial–mesenchymal transition of human bladder cancer T24 cells through activation of ERK1/2 pathway

Xin Sun[a,1], Qifei Deng[b,1], Zhaofeng Liang[c], Zhiqi Liu[a], Hao Geng[a], Li Zhao[a], Qirui Zhou[d], Jie Liu[a], Jiaxing Ma[a], Daming Wang[a], Dexin Yu[a,*], Caiyun Zhong[e,*]

[a] Department of Urology, The Second Affiliated Hospital of Anhui Medical University, 80 Feicui Rd, Hefei, Anhui 230032, PR China
[b] Department of Urology, Anhui Provincial Children's Hospital, 39 East Wangjiang Road, Hefei, Anhui 230032, PR China
[c] Jiangsu Key Laboratory of Medical Science and Laboratory Medicine, School of Medicine, Jiangsu University, Zhenjiang, Jiangsu 212013, PR China
[d] Anhui International Travel Health Care Center, Anhui Entry-Exit Inspection and Quarantine Bureau, 329 Tunxi Road, Hefei, Anhui 230022, PR China
[e] Department of Nutrition and Food Safety, School of Public Health, Nanjing Medical University, 818 East Tianyuan Road, Nanjing, Jiangsu 211166, PR China

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ABSTRACT

Bladder cancer is a common genitourinary malignant disease worldwide. Abundant evidence has shown that cigarette smoke (CS) is a crucial risk factor for bladder cancer. Nevertheless, the mechanism underlying the relationship between cigarette smoking and bladder cancer remains unclear. In the present study, we investigated the effects of cigarette smoke extract (CSE) on mitogen-activated protein kinase (MAPK) pathway activation and EMT alterations in human bladder cancer T24 cells, and the preventive effect of extracellular regulated protein kinases 1 and 2 (ERK1/2) inhibitor U0126 was further examined. Our results illustrated that CSE exposure induced morphological change of human bladder cancer T24 cells, enhanced migratory and invasive capacities, reduced epithelial marker expression and elevated mesenchymal marker expression. Meanwhile, exposure of T24 cells to CSE resulted in activation of ERK1/2 pathway as well as activator protein 1 (AP-1) proteins. Interestingly, treatment with ERK1/2 inhibitor U0126 effectively abrogated CSE-triggered EMT and ERK1/2/AP-1 activation. These findings provide novel insight into the molecular mechanisms of CS-associated bladder cancer and may open up new avenues in the search for potential target of bladder cancer intervention.

1. Introduction

Bladder cancer is the sixth most common malignancies worldwide [1], and accounting for an estimated 261 000 new cases diagnosed and 115 000 deaths each year [2]. In China, bladder cancer is the first leading cause of death among urinary malignant tumors [3]. Environmental and/or occupational factors have been proposed to be involved in different cancer types including bladder cancer [4]. Specifically, cigarette smoking has long been known as a major risk factor for bladder cancer [5]. Although many previous epidemiological and experimental studies have revealed the positive link between CS and bladder cancer as well as other cancers [2,6–8], the molecular pathogenesis remains largely unknown.

Epithelial–mesenchymal transition (EMT) is a crucial pathophysiological process in embryonic development as well as cancer development [9–11]. During the EMT process, cells lose their epithelial traits and acquire mesenchymal features. Evidences have suggested that EMT is critically involved in the tumor invasion and metastasis in addition to facilitating tumorigenic progress. Abundant researches showed that exposure of cells to carcinogens, such as arsenite, benzo(a)pyrene-diolepoxide and methylnitrosourea, induces EMT during malignant transformation [12–14]. TS has been documented to promote EMT [15,16]. Nonetheless, the underlying mechanisms by which CS induces EMT are poorly understood.

The mitogen-activated protein kinases (MAPKs) belong to a family of serine/threonine kinases that contain three major subfamilies: extracellular regulated protein kinases 1 and 2 (ERK1/2), the Jun N-terminal kinases (JNKs), and p38 [15,16].

Abbreviations: CSE, Cigarette smoke extract; CS, Cigarette smoke; EMT, Epithelial–mesenchymal transition; MAPK, Mitogen-activated protein kinase; ERK1/2, extracellular regulated protein kinases 1 and 2; AP-1, activator protein 1; DMSO, dimethyl sulfoxide; MTT, thiazolyl blue tetrazolium bromide; qRT-PCR, quantitative reverse transcription-polymerase chain reaction.

* Corresponding authors.
E-mail addresses: yudx_urology@126.com (D. Yu), cyzhong@njmu.edu.cn (C. Zhong).

[1] These authors contributed equally to this work.

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The ERK1/2 pathway has been confirmed to play a pivotal role in tumorigenic process [18]. The essential function of ERK1/2 in regulating EMT has been well established. Some reports have revealed that ERK1/2 promote EMT in various epithelial cells [19–21]. Recently, an in vivo study also indicated that CS enhanced ERK1/2 activation in bladder tissue of mice [22].

Herein, the aim of this study was to investigate the function and the molecular mechanism of MAPK pathways involved in the CSE-induced EMT alterations of human bladder cancer T24 cells. We found that CSE induced EMT alterations and stimulated the activity of ERK1/2 pathway in T24 cells. Meanwhile, decreased ERK1/2 pathway by U0126 reversed CSE-triggered EMT alterations of T24 cells. These novel findings indicate the important role of ERK1/2 activity in CS-associated cancer progression and may open new avenues in search for potential interventional target of CS-associated bladder cancer.

2. Materials and methods

2.1. Chemicals and reagents

Dimethyl sulfoxide (DMSO) and methanol were obtained from Merck (Reading Township, NJ, USA). RPMI 1640 medium, fetal bovine serum (FBS), phosphate-buffered saline (PBS), antibiotics, and trypsin were obtained from HyClone (Logan, UT, USA). The primary antibodies for phosphorylated ERK1/2, phosphorylated c-Jun, and phosphorylated c-Fos were obtained from Cell Signaling Technology (Beverly, MA). Antibodies for E-cadherin, ZO-1, Vimentin, N-cadherin, Snail, ERK1/2 inhibitor (U0126), p38 inhibitor (SB203580), and JNK inhibitor (SP600125) were also purchased from Cell Signaling Technology (Beverly, MA, USA). GAPDH antibody was from Biogot Technology (Nanjing, China). Primers for E-cadherin, ZO-1, Vimentin, N-cadherin, and GAPDH were synthesized by Invitrogen (Carlsbad, CA).

2.2. Cell culture and treatments

Human bladder cancer T24 cells were obtained from American Type Culture Collection (ATCC, Wiltshire, USA) and maintained in RPMI-1640 medium supplemented with 10% FBS, penicillin, and streptomycin. Cells were seeded in 25-cm^2 flasks and incubated in a CO_2 incubator at 37 °C, with 5% CO_2 and 95% filtered air. The medium was changed every other day. When the cells reached 80–90% confluency, they were treated with various concentrations of CSE, or with U0126 (5 μM), SB203580 (10 μM), or SP600125 (5 μM) for 6 days. All experiments were carried out three times.

2.3. Preparation of CSE

CSE was freshly prepared for each experiment by combusting one commercial cigarette according to the reported method [23,24]. Commercial cigarettes (Hongtashan filter-tipped cigarettes made in Yunnan, China; each contain 12 mg tar and 1.1 mg nicotine) were smoked. By application of a vacuum, mainstream smoke was drawn through 10 ml of prewarmed (37° C) FBS-free RPMI-1640 medium supplemented with penicillin and streptomycin at a rate of 5 min/cigarette. The obtained solution was referred to as having 100% strength. Then the CSE stock solution was filtered through a 0.22-μm pore size filter and diluted to the desired concentration with treatment medium. The resulting CSE was applied to T24 cell cultures within 30 min of preparation. The control solution was prepared using the same protocol, except that the cigarettes were unlit.

2.4. Cell toxicity assay

T24 cells were seeded in 96-well plates at a plating density of 2×10^3 cells per well in 200 μl of medium and incubated overnight with RPMI 1640 containing 10% FBS. Then the cells were exposed to various concentrations of CSE prepared as previously outlined for 6 days, and the cell viability was determined by MTT assay. For the present study, media containing various concentrations of CSE were changed every day. Six days later, MTT stock solution was added to each well to solubilize the formazan crystals, and plates were incubated for an additional 4 h at 37° C. Afterwards, MTT solution in the medium was removed and the crystals were solubilized in dimethylsulfoxide (DMSO). Absorbance was measured at 490 nm using a microplate reader. All measurements were performed in triplicate.

2.5. Transwell invasion and migration assay

T24 cells were pretreated for 6 days, and then the cells were transferred to Transwell chambers. Briefly, a total of 100 μl of serum-free medium (containing 1×10^4 cells) were added to the upper chambers of the Transwell system (24 wells, 8-mm pore size; Millipore, Billerica, MA, USA) coated with 2 mg/ml Matrigel to form a matrix barrier, whereas the lower compartment was filled with 800 μl of RPMI-1640 supplemented with 10% FBS. After incubation at 37° C for 48 h, the T24 cells remaining inside the upper chamber were removed with cotton swabs. The cells on the lower surface of the membrane were stained with 0.1% crystal violet after fixation with methanol and then imaged under a light microscope. Five fields of each chamber were randomly selected, and the cell numbers were counted under a microscope. For the migration assay, the cells were seeded into upper chambers that were not coated with Matrigel. The following steps in the assay were the same in the invasion assay. After 48 h, the cells on five randomly selected fields in the lower surface were counted, and the cell numbers were then subjected to statistical analysis.

2.6. Western blots

T24 cells were harvested, washed with ice-cold PBS and lysed in RIPA buffer (Thermo Scientific, USA). Concentrations of the precipitated proteins in cell lysates were measured with BCA Protein Assay (Pierce, Rockford, IL, USA). Proteins were fractionated by electrophoresis through 10% SDS-polyacrylamide gels and were transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). After blocking with 5% milk, the membranes were blocked and incubated with indicated primary antibodies and secondary antibodies. The blots were subsequently developed using an enhanced chemiluminescence detection kit (Amersham Biosciences, USA) and exposed to film. The levels of target protein bands were densitometrically determined using Image Lab Software 3.0. The variation in the density of bands was expressed as fold changes compared with the control in the blot after normalization to GAPDH. Presented blots are representative of three independent experiments.

2.7. RNA extraction and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was isolated by RNAiso Plus according to the manufacturer's instructions (TaKaRa, Japan). 2 μg of total RNA was transcribed into cDNA using AMV reverse transcriptase (Takara) following the manufacturer's protocol. qRT-PCR was performed using the Power SYBR Green Master Mix (TaKaRa, Japan) and an ABI 7300 real-time PCR detection system (Applied Biosystems, CA). The primers used were as follows: E-cadherin, forward 5′-