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Original Article

Inhibition of tobacco smoke-induced bladder MAPK activation and epithelial-mesenchymal transition in mice by curcumin

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Abstract: Tobacco smoke (TS) has been shown to cause bladder cancer. Epithelial-mesenchymal transition (EMT) is a crucial pathophysiological process in cancer development. MAPK pathways play central roles in tumorigenesis including EMT process. Curcumin is a promising chemopreventive agent for several types of cancers. In the present study we investigated the effects of TS on MAPK pathway activation and EMT alterations in the bladder of mice, and the preventive effect of curcumin was further examined. Results showed that exposure of mice to TS for 12 weeks resulted in activation of ERK1/2, JNK, p38 and ERK5 MAPK pathways as well as AP-1 proteins in bladder. TS reduced mRNA and protein expression levels of epithelial markers E-cadherin and ZO-1, while mRNA and protein expression levels of the mesenchymal markers vimentin and N-cadherin were increased. Curcumin treatment effectively attenuated TS-triggered activation of ERK1/2, JNK and p38 MAPK pathways, AP-1 proteins and EMT alterations in bladder tissue. These results suggest the protective effects of curcumin in TS-induced MAPK activation and EMT, thus providing new insights into the chemoprevention of TS-associated bladder cancer.

Keywords: Tobacco smoke, curcumin, bladder cancer, epithelial-mesenchymal transition, MAPK pathways

Introduction

Bladder cancer (BC) is the sixth most common malignancies worldwide [1], which is predominantly a disease of developed countries such as Europe, North America and Australia, 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 malignancies [3, 4].

Many factors are strongly associated with increased risk of bladder cancer, including tobacco smoke (TS), dietary factors, environmental factors, drinking water contaminants such as chlorinated byproducts and arsenic, and use of pioglitazone [5, 6]. TS is one of the leading causes of bladder cancer. Current tobacco smokers have an approximately four-fold higher risk of bladder cancer than non-smokers [7]. It is estimated that TS accounts for 23% of all female bladder cancer, whereas in men 50% of the cancer is attributed to TS [2]. TS contains more than 5000 constituents [8, 9]. Among them, many compounds found in TS are known to induce free radicals, possess toxic and carcinogenic activities [10]. These ingredients of TS contribute to its carcinogenic potential, including transformation and progression of cancer. Although enormous progress in understanding its molecular mechanisms leading to bladder cancer development has been made, the molecular pathogenesis remains largely unknown.

Epithelial-mesenchymal transition (EMT) is a crucial pathophysiological process in embryon-

ic development as well as cancer development [11, 12]. During EMT process, cells lose their epithelial traits and acquire mesenchymal features. Evidences have suggested that, in addition to facilitating tumor invasion and metastasis, EMT is also critically involved in the initiation of tumorigenesis by promoting cell malignant transformation. Exposure of cells to carcinogens, such as arsenite, benzo (a) pyrene-diolepoxide, methylnitrosourea and tobacco smoke, induces EMT during transformation and tumor formation [13-16]. TS has been documented to promote EMT [17-19]. TS-induced EMT has been found to regulate early events in carcinogenesis: down-regulation of epithelial cadherin, loss of cell-cell adhesion and apical-basal polarity, as well as increased mobility of cells. Nonetheless, the underlying mechanisms by which TS induces EMT are poorly understood.

The mitogen-activated protein kinases (MAPKs) belong to a family of serine/threonine kinases that play central roles in tumorigenic process [20, 21]. There are four major subfamilies have been identified: extracellular regulated protein kinases 1 and 2 (ERK1/2), the Jun N-terminal kinases (JNKs), p38, and ERK5. Recently, some groups reported that JNKs, p38, ERK1/2 regulate EMT [22-24]. ERK5 is the lesser studied MAPK pathway. Its functions in cancer oncogenesis and EMT regulation have also been explored [25, 26].

Curcumin [7-bis (4-hydroxy-3-methoxypentyl)-1, 6-hepadiene-3, 5-dione, 1], a yellow coloring agent, is the principal active component of the plant Curcuma longa. It has long been used throughout Asia as a food additive and a traditional herbal medicine. Curcumin has garnered attention in Western medicine due to its anti-inflammatory, antioxidant, anticancer and chemopreventive properties [27-29]. Evidence obtained from in vitro and in vivo studies indicates that curcumin has a therapeutic potential in preventing and treating several chronic diseases including various types of cancer [30-32]. However, its effect on TS-induced bladder EMT has not been defined.

The aim of the present study was to investigate TS modulation of MAPK activation and EMT in the bladder tissue of mice, and the preventive effects of curcumin against TS-induced alterations was further examined. Findings from this study indicate the chemopreventive effect of curcumin in TS-associated bladder pathological alterations.

Materials and methods

Chemicals and reagents

Curcumin was purchased from Sigma (St. Louis, MO, purity: 99.0%). The primary antibodies for phosphorylated JNK, phosphorylated p38, phosphorylated ERK1/2, phosphorylated ERK5, phosphorylated c-Jun, phosphorylated c-Fos, E-cadherin, N-cadherin and Vimentin were obtained from Cell Signaling Technology (Beverly, MA). Antibody for ZO-1 was from Santa Cruz Biotechnology (Santa Cruz, CA). GAPDH antibody was from Biogot Technology (Nanjing, China) Primers for E-cadherin, ZO-1, N-cadherin, Vimentin and GAPDH were synthesized by Invitrogen (Carlsbad, CA). Sources of other materials are noted accordingly in the text.

Mice and TS exposure

Eight-week-old male BALB/c mice weighing 18-22 g were purchased from the Animal Research Center of Nanjing Medical University. All mice were allowed to acclimate for 1 week prior to the onset of experimental exposure. The mice were housed in polypropylene cages, maintained on a 12-hour light/dark cycle, 22±0.5°C room temperature, 40-60% relative humidity, and free access to water and AIN-76A diet. Animals were handled in accordance with the recommendations in the guidelines of the Animal Care and Welfare Committee of Nanjing Medical University. The study protocol was approved by the Committee on the Ethics of Animal Experiments of Nanjing Medical University.

Mice were exposed to tobacco smoke in a smoking apparatus. One filterless commercial cigarette (Hongtashan, one of the most consumed cigarette in China, contains 12 mg tar and 1.1 mg nicotine per cigarette) was combusted to generate TS by a smoke machine, which smoked the cigarettes and pumped the mainstream cigarette smoke from burning cigarettes at a constant rate (each cigarette took 5 min to burn out). The smoke was delivered to whole-body exposure chambers with target concentration of total particulate matter (TPM) of 85 mg/m^3. Animals were exposed for 6 hours daily for 12 consecutive weeks. Animals in the control group were exposed to filtered, condi-

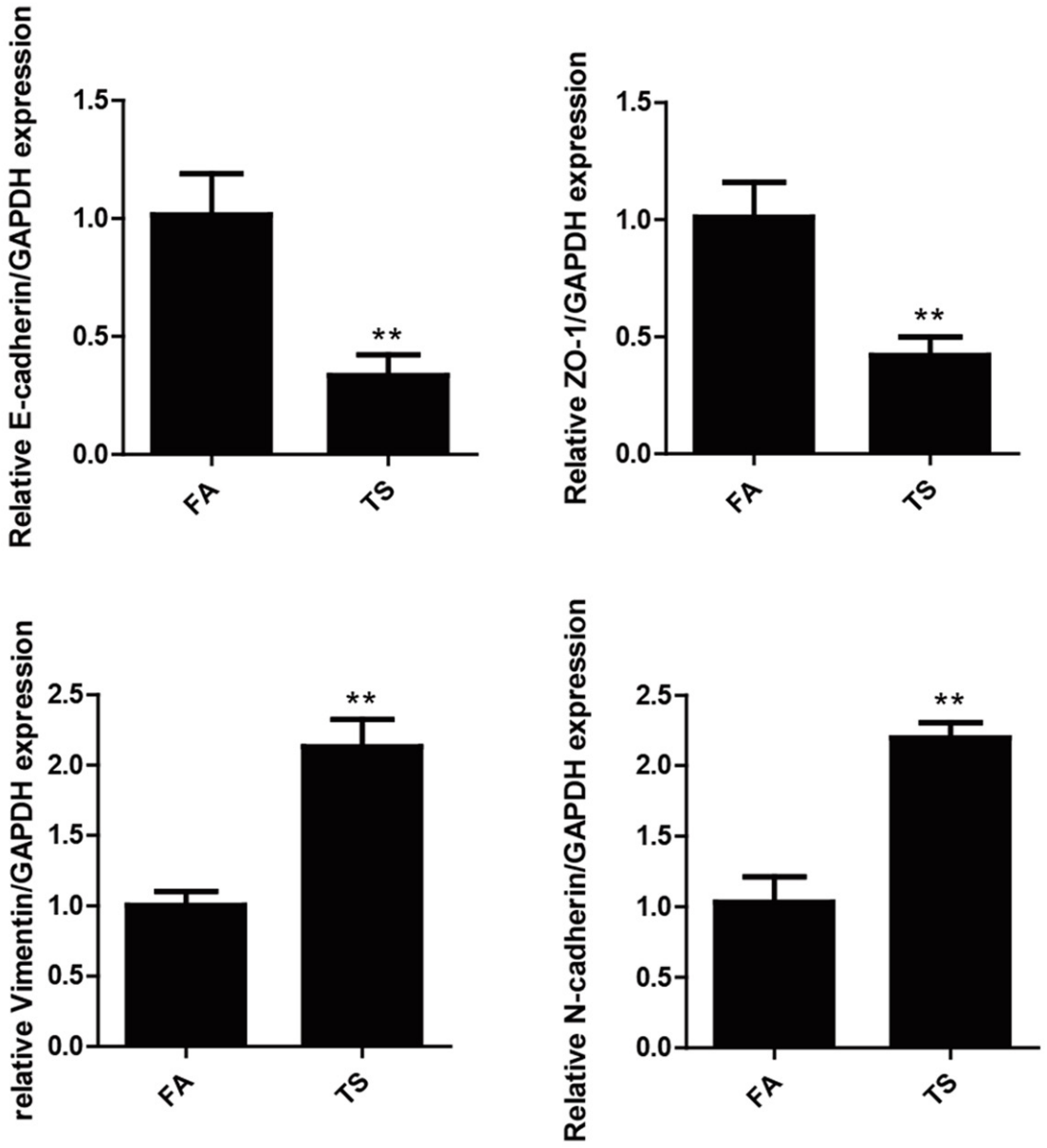

Figure 1. TS altered the mRNA expression levels of EMT markers in the bladder of mice exposed to TS for 12 weeks. TS reduced mRNA levels of E-cadherin and ZO-1, and induced mRNA levels of Vimentin and N-cadherin in the bladder, detected by quantitative reverse transcriptase-polymerase chain reaction after normalization to glyceraldehyde 3-phosphate dehydrogenase. Data are expressed as mean ± SD. **P < 0.01, compared with FA control.

tioned air. Ten mice were randomly assigned into each group. The exposures were monitored and characterized as the followings: carbon monoxide (14.72±2.89 mg/m^3), TSP (0 mg/m^3) for the control group; carbon monoxide (184.07±23.51 mg/m^3), TSP (83.53±5.63 mg/m^3) for TS exposure group. After the last TS exposure, mice were sacrificed and the bladder tissues were isolated, frozen and stored at -80°C until analysis.

Curcumin treatment of mice

In a separate set of animal study, mice were treated daily with curcumin [50 or 100 mg/kg body weight (BW), p.o.] as previously reported [32-34]. Mice were randomly divided into four groups (n = 8 per group): filtered air group; TS-exposed group, mice were exposed to TS; TS + Cur 50 mg/kg BW, mice were treated with 50 mg/kg BW curcumin and exposed to TS; TS

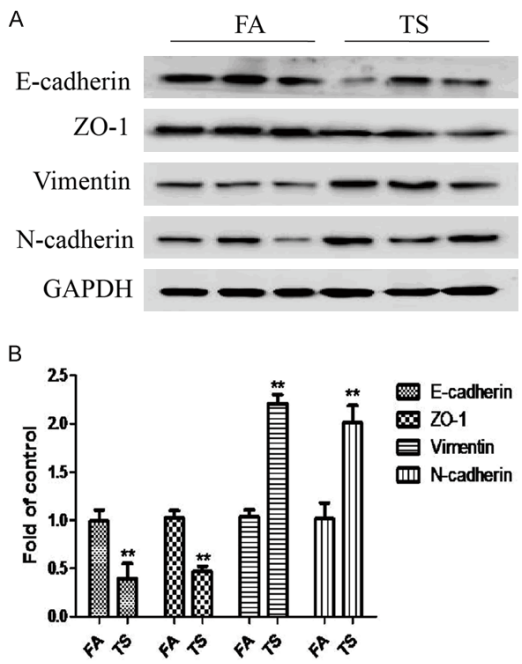

Figure 2. TS altered the protein expression levels of EMT markers in the bladder of mice exposed to TS for 12 weeks. A. TS decreased the protein levels of E-cadherin and ZO-1, and increased the protein levels of Vimentin and N-cadherin in the bladders. Glyceraldehyde 3-phosphate dehydrogenase was used as loading control for Western blotting. B. Densitometric analyses of Western blotting. Data are expressed as mean ± SD. **P < 0.01, compared with FA control.

+ Cur 100 mg/kg BW, mice were treated with 100 mg/kg BW curcumin and exposed to TS. The administration dosages of curcumin were based on the measurements of mouse body weight and the amount of diet consumption. Animals were weighed every three days. Mice were exposed to filtered air or TS with a target concentration of 85 mg/m^3 TPM for 12 weeks. Following the completion of exposure, mice were sacrificed and bladder tissues were collected for analysis.

Western blot analysis

Bladder tissues were homogenized in a lysate buffer (5 mmol/L EDTA, 50 mmol/L Tris, 1% SDS, pH 7.5, 10 μg/mL aprotinin, 1% sodium deoxycholate, 1% NP-40, 1 mM PMSF, 1% Triton-X 100, and 10 μg/mL leupeptin) and then centrifuged at 4°C for 20 min. Protein concentrations were measured with the BCA Protein Assay (Pierce, Rockford, IL). Fifty micrograms of proteins were fractionated by electrophoresis through 7.5-10% SDS-PAGE and were transferred to PVDF membrane (Millipore, Billerica, MA). The membranes were blocked with 5% defatted milk and subsequently probed with primary antibody overnight at 4°C, and then incubated with horseradish peroxidase-conjugated secondary antibody. GAPDH served as the loading control. For densitometric analyses, protein bands on the blots were measured by the use of Eagle Eye II software.

Quantitative real-time PCR

Total RNA was isolated by RNAiso Plus according to the manufacturer the manufacturer's instructions (TaKaRa, Japan). Two micrograms of total RNA was reverse transcribed into cDNA using AMV Reverse Transcriptase (Promega, Madison, WI). 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'-TCGACACCCGATTCAAAGTGG-3' and reverse 5'-TTCCAGAAACGGAGGCCTGAT-3'; ZO-1, forward 5'-GCAGCCACAACCAATTCATAG-3' and reverse 5'-GCAGACGATGTTCATAGTTTC-3'; Vimentin, forward 5'-CCTTGACATTGAGATTGCCA-3' and reverse 5'-GTATCAACCAGAGGGAGTGA-3'; N-cadherin forward 5'-ATCAAGTGCCATTAGCCAAG-3' and reverse 5'-CTGAGCAGTGAATGTTGTCA-3'; GAPDH, forward 5'-GCTGCCCAACGCACCGAATA-3' and reverse 5'-GAGTCAACGGATTTGGTCGT-3'. All of the primers were synthesized by Invitrogen (Carlsbad, CA). The levels of mRNA expression for each gene were normalized by its respective GAPDH. Fold changes in gene expression were calculated by a comparative threshold cycle (Ct) method using the formula $2^{-(\Delta\Delta Ct)}$.

Statistical analysis

Statistical analyses were performed with SPSS 16.0. All data were expressed as mean ± standard deviation. One-way ANOVA was used for comparison of statistical differences among multiple groups, followed by the LSD significant difference test. Unpaired Student t test was also used for the comparison between two groups. A value of $P < 0.05$ was considered significantly different.

Results

TS altered the expression of EMT markers in bladder tissues of mice

TS is one of the key risk factors for bladder cancer, and TS-induced EMT is critically involved in TS-associated malignant transfor-

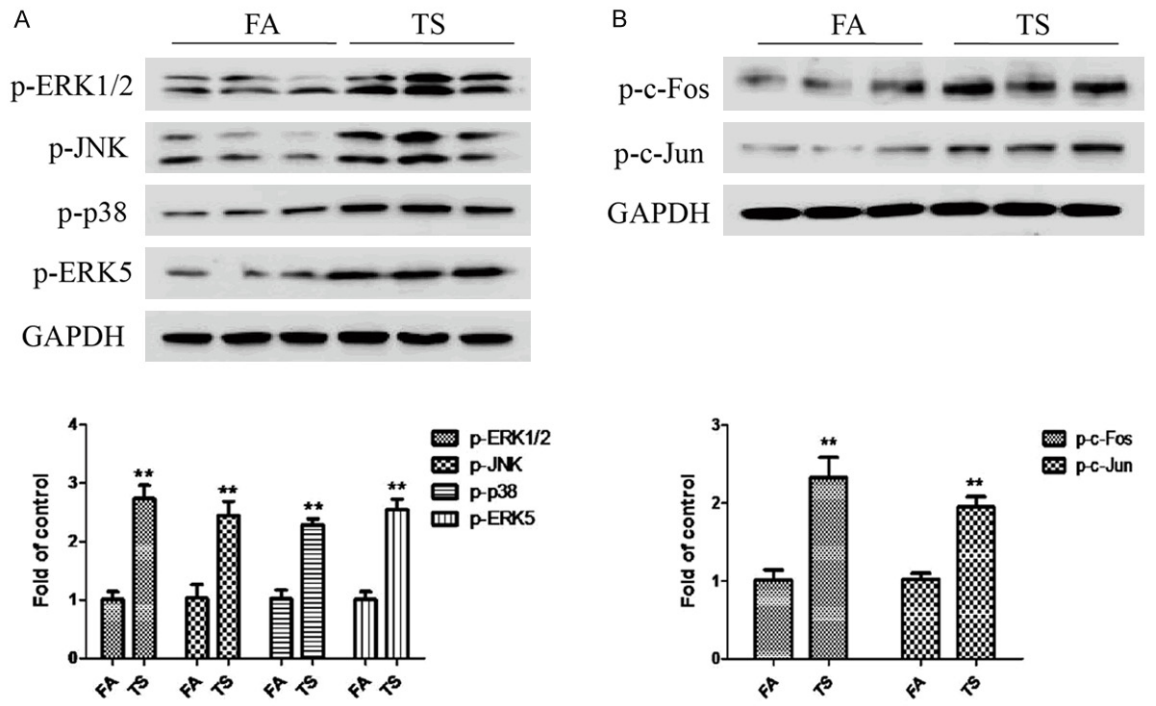

Figure 3. TS increased MAPKs activation in the bladder of mice. A. Western blotting analyses of phosphorylated ERK1/2, phosphorylated JNK, phosphorylated p38 and phosphorylated ERK5. B. Western blotting analyses of phosphorylated c-Fos and phosphorylated c-Jun. Data are expressed as mean ± SD. **P < 0.01, compared with FA control.

mation. We investigated whether TS induces EMT-like changes in an animal model. Mice were exposed to TS for 12 weeks, and the expression of the epithelial markers E-cadherin and ZO-1, and the mesenchymal markers Vimentin and N-cadherin in bladder tissues were examined. qRT-PCR results revealed that TS exposure decreased the bladder mRNA expression levels of E-cadherin and ZO-1, and elevated mRNA expression levels of Vimentin and N-cadherin (**Figure 1**). Moreover, Western blot analyses further showed that TS exposure reduced E-cadherin and ZO-1 protein expression levels, and increased Vimentin and N-cadherin protein levels (**Figure 2**).

TS increased MAPK/AP-1 activation in bladder tissue of mice

To determine whether TS-elicited bladder EMT alterations is associated with change in MAPK activation, the expression levels of phosphorylated ERK1/2, phosphorylated JNK, phosphorylated p38 and phosphorylated ERK5, the indicators of MAPK activation status, were measured. It was found that TS activated ERK1/2, JNK, p38 and ERK5 MAPK pathways (**Figure 3A**). Meanwhile, TS exposure increased the activation of AP-1 protein in the bladder of mice, as indicated by elevated levels of phosphorylated-c-Jun, phosphorylated c-Fos and Fos B (**Figure 3B**).

Curcumin attenuated TS-triggered bladder EMT change in mice

In order to determine the effects of curcumin on TS-mediated EMT in the bladder, mice were received curcumin (50 or 100 mg/kg BW, p.o.) and exposed to TS for 12 weeks. **Figures 4** and **5** show that TS-induced alterations in mRNA and protein expressions of the EMT markers, including decreases of the epithelial markers E-cadherin and ZO-1, and increases of the mesenchymal markers Vimentin and N-cadherin, were effectively attenuated with 100 mg/kg BW curcumin treatment. These data suggested that curcumin prevented TS-induced bladder EMT changes in vivo.

Curcumin suppressed TS-induced MAPK/AP-1 activation in mouse bladder

To explore the influence of curcumin on TS-mediated activation of MAPK/AP-1 pathway in

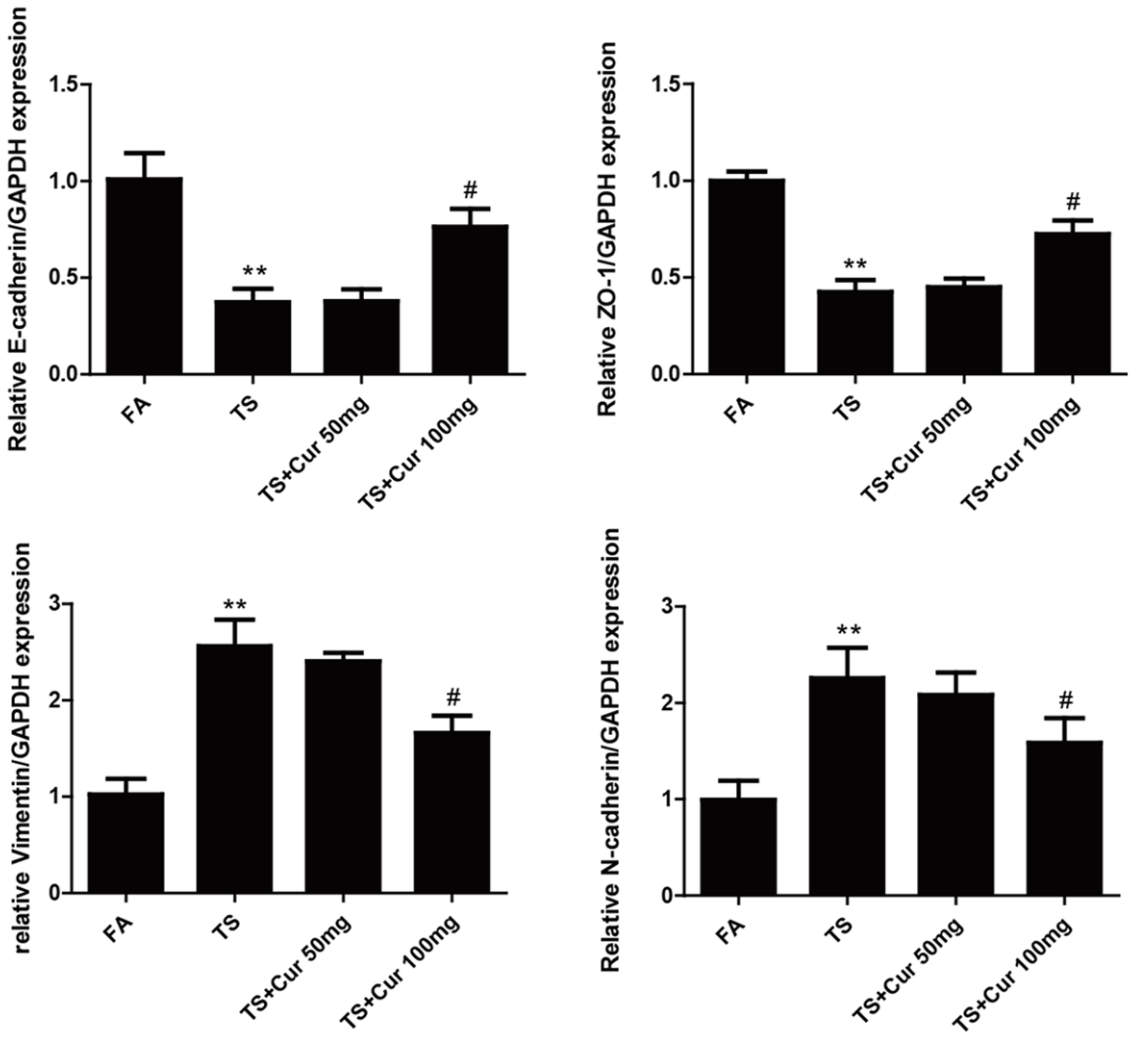

Figure 4. Curcumin attenuated TS-induced alterations in the mRNA expression of EMT markers in the bladder of mice. qRT-PCR analyses of E-cadherin, ZO-1, Vimentin and N-cadherin mRNAs. Data are expressed as mean ± SD. **P < 0.01, compared with FA control; #P < 0.05, compared with TS. FA = filtered air; TS = tobacco smoke; Cur = curcumin.

bladder tissue, we further examined the changes in MAPK/AP-1 activation following curcumin treatment. Western blot analyses showed that 100 mg/kg BW curcumin inhibited TS-induced ERK1/2, JNK and p38 activation in a dose-dependently manner, although ERK5 activity was not significantly suppressed by curcumin treatment (**Figure 6A**). Treatment of curcumin also significantly decreased TS-induced AP-1 activation (**Figure 6B**).

Discussion

Bladder cancer is one of the leading causes of cancer-related death in the world. The relationship between the occurrence of bladder cancer and TS has been established [35, 36]. TS is a major cause of bladder cancer, which promotes the initiation and progression of bladder cancer. Nevertheless, the mechanisms by which TS causes the development of bladder cancer remain to be elucidated. In the present study we revealed that TS induced EMT alteration in the bladder of mice. We further showed that TS-induced bladder EMT was associated with activation of MAPK pathways and AP-1 proteins. Meanwhile, our data indicated that curcumin prevented TS-triggered MAPK/AP-1 activation and effectively attenuated TS-induced bladder EMT changes in vivo.

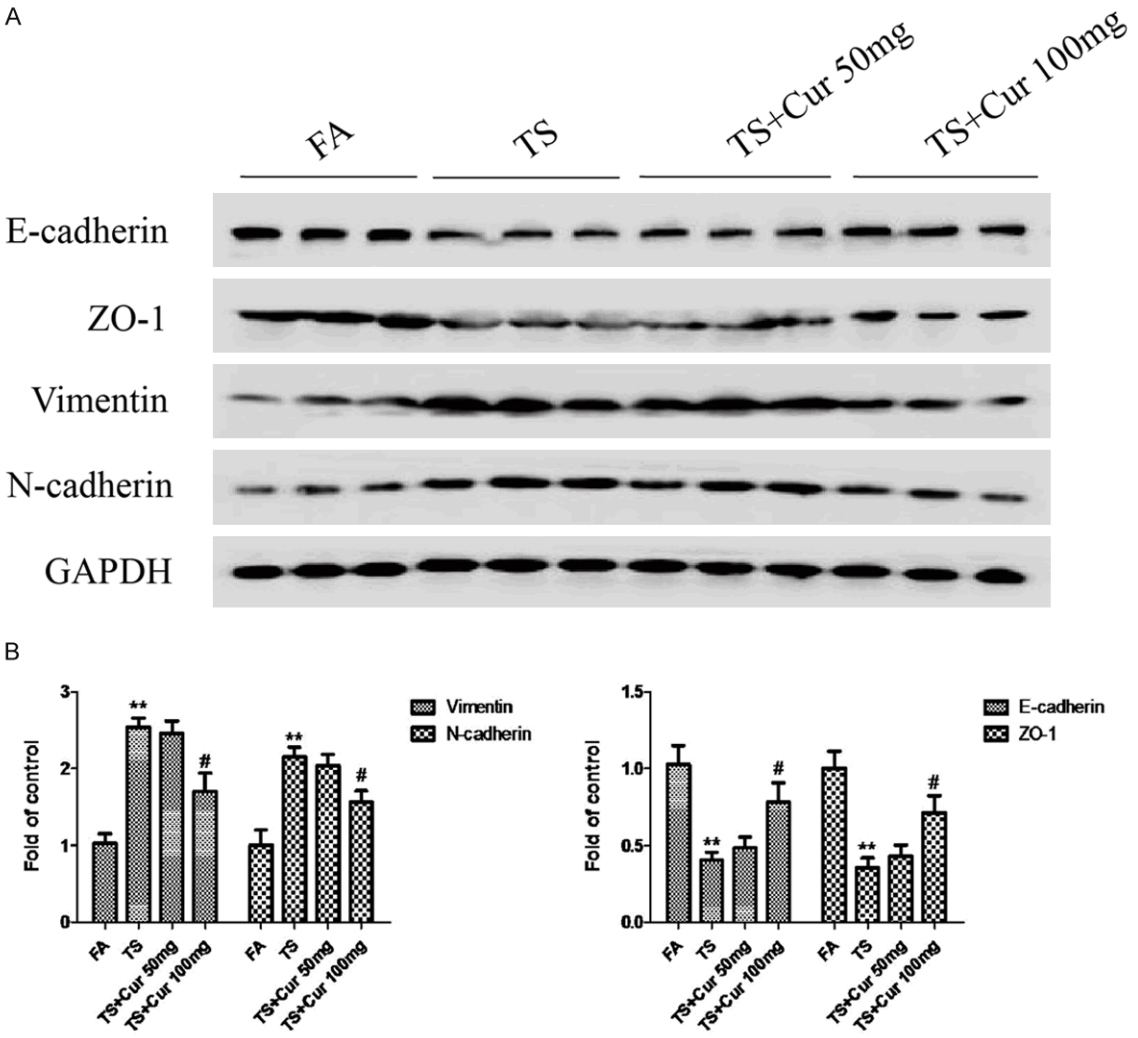

Figure 5. Curcumin attenuated TS-induced alterations in the protein expression of EMT markers in mice. A. Western blotting analyses of E-cadherin, ZO-1, Vimentin and N-cadherin proteins. B. Densitometric analyses of Western blotting. Data are expressed as mean ± SD. **P < 0.01, compared with FA control; #P < 0.05, compared with TS. FA = filtered air; TS = tobacco smoke; Cur = curcumin.

EMT is a crucial process in cancer development. Evidences have revealed that exposure of cells to carcinogens induces EMT during transformation and tumor formation [13-16], suggesting the critical involvement of EMT in the initiation of tumorigenesis by promoting cell malignant transformation. In agreement with previous reports, we showed in the present study that exposure to TS induced EMT in the bladder of mice. TS altered the expression of EMT markers, including decreased epithelial markers E-cadherin and ZO-1, and increased mesenchymal markers Vimentin and N-cadherin. Our data revealed that TS triggered bladder EMT *in vivo*.

Several cell signaling factors have been implicated in EMT. As the proto-oncogenic signaling, MAPK pathways are implicated in important cellular processes including gene expression, proliferation, apoptosis, angiogenesis, cell motility and differentiation. It has been established that MAPK pathways play central roles in tumorigenic process by promoting the development and progression of cancer. Evidences show that ERK1/2, JNK, p38 and ERK5 promote EMT [22-26]. In the present study we showed that TS-induced bladder EMT was associated with upregulation of ERK1/2, p38, JNK and ERK5 activation in vivo. Meanwhile, we also found that TS increased the acti-

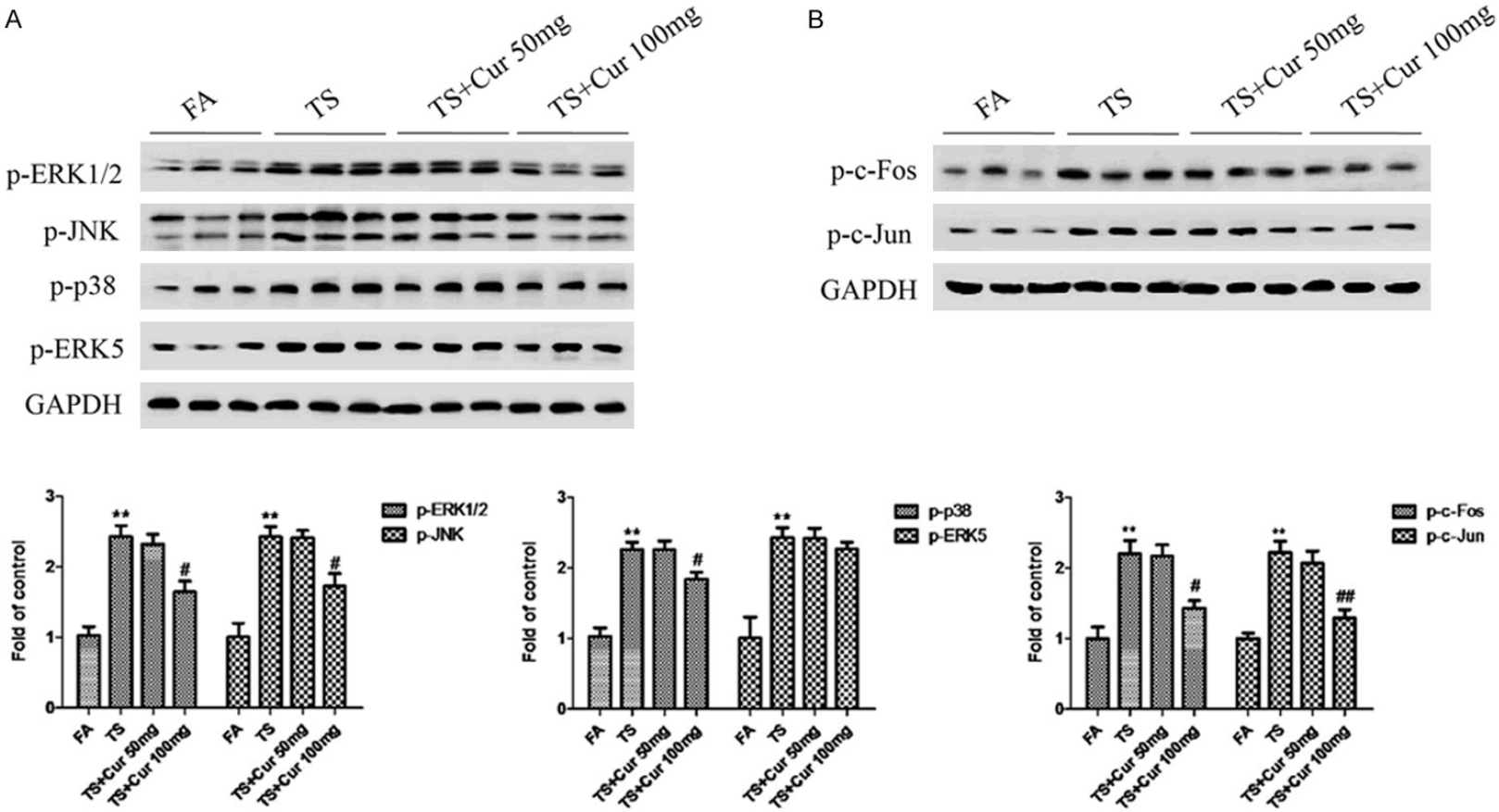

Figure 6. Curcumin suppressed TS-induced MAPK/AP-1 activation in the bladder of mice. A. Western blotting analyses of phosphorylated ERK1/2, phosphorylated JNK, phosphorylated p38 and phosphorylated ERK5. B. Western blotting analyses of phosphorylated c-Fos, phosphorylated c-Jun and FosB. Data are expressed as mean ± SD. $^{**}P < 0.01$, compared with FA control; $^{\#}P < 0.05$, compared with TS; $^{\#\#}P < 0.01$, compared with TS. FA = filtered air; TS = tobacco smoke; Cur = curcumin.

vation of AP-1 proteins. These data suggest that MAPK/AP-1 activation is involved in TS-induced bladder EMT.

Curcumin is a dietary polyphenol that has various biological activities and excellent tolerance when administered systemically [37, 38]. Some studies have reported the safety of curcumin as well as its anticancer activities in relation to many cancers [39-41]. The concentrations of curcumin used in our study were 50 and 100 mg/kg BW/day. A similar dose of curcumin was shown to be effective in animal studies [34, 42]. This is equivalent to 3-6 g per adult, a dose similar to that used in humans [43]. After treatment with 50 or 100 mg/kg BW/day dose of curcumin for 12 weeks, the effect of curcumin on TS-induced changes in the mRNA and protein expression of the EMT markers, including decreases of the epithelial markers and increases of the mesenchymal markers, was examined. Our results illustrated that TS-triggered EMT alteration in the bladder of mice was effectively attenuated by 100 mg/kg BW/day dose of curcumin. Moreover, our results further revealed that administration of curcumin at 100 mg/kg BW dose in the 12-week period prevented TS-mediated activation of ERK1/2, JNK and p38 MAPK pathways. Meanwhile, TS-induced AP-1 activation was suppressed following curcumin treatment. Thus, these data suggested the protective effect of curcumin against TS-induced MAPK activation and EMT changes in the bladder tissue.

In summary, the present study suggests that TS-induced MAPK activation and EMT changes in bladder tissue is effectively attenuated by curcumin treatment. Findings from this study could provide new insights into the molecular pathogenesis and chemoprevention of TS-associated bladder pathological alterations.

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Disclosure of conflict of interest

None.

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