

Original Article

Trichostatin A promotes bladder cancer cell apoptosis and inhibits cell autophagy through MEK/ERK signaling pathway

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Abstract: Histone acetylation enzyme inhibitor is a kind of high-efficiency and low toxicity drug for cancer. Therefore, as histone acetylation enzyme inhibitor, it is very important to explore the mechanism of trichostatin A (TSA) for bladder cancer. In this study, cell vitality was detected by MTT assay; Cell ultrastructure was observed by optical microscopy and electron microscopy; Real-time quantitative PCR was used to detect expression of Caspase 3, Bcl-2, ATG5 and Beclin1 genes; Flow cytometry detected cell apoptosis; Western blotting test expression of HDAC4, P62, LC3B, p-ERK, ERK, P53, Caspase-3, Bax and Bcl-2, respectively. Transmission electron microscopy showed that T24 cells and EJ cells appeared to be massive autophagosomes after 24 h TSA treatment. Compared with control group, there was no change in Caspase-3, and Bcl-2 was obviously decreased and ATG5 was significantly increased after 24h TSA treatment both T24 cells and EJ cells. However, Caspase-3, BCL-2 and ATG5 and Beclin-1 were significantly decreased compared with control group after 48 h. Western blotting indicated that compared with the control group, P53, Bax, and Caspase-3 were dramatically increased, and Bcl-2 was significantly reduced; moreover, P62 was gradually increased with the increase of doses, and LC3-I/LC3-II ratio gradually increased; p-ERK and ERK expression were significantly reduced. Likely, the role of TSA is consistent with specific inhibitor PD98059 on ERK1/2 pathway. Our results indicated that TSA can promote bladder cancer cell apoptosis and inhibit cell autophagy by blocking MEK/ERK signaling pathway.

Keywords: Trichostatin A, TSA, bladder cancer, autophagy, apoptosis

Introduction

Bladder cancers are the second most common urologic tumor, its incidence ranked the 11th in the malignant tumor all over the world and it is increasing at the rate of 300,000 per year [1, 2]. According to the 2002 TNM classification, the patients of non-muscle-invasive bladder cancer, confined to the mucosa or submucosa approximately accounted for 75-85% [3]; the remaining 25% of new bladder cancer patients were muscle-invasive.

The treatments of bladder cancer mainly are traditional surgery and chemotherapy. After receiving the first transurethral resection of bladder cancer for patients with non-muscle invasive bladder cancer, recurrence rate is as high as 50% to 80% within five years, and 14%

of patients often appear tumor progression or metastasis. Moreover, the survival rate is reduced for bladder cancer patients with progression or metastasis: the survival rate of 5-year is 52-77% for T2 stage patients, patients with T3 stage is 40-64%, and T4 stage patients or patients with positive lymph node is 26-44% [4].

Therefore, it is necessary to receive perfusion chemotherapy or immune treatment for bladder cancer patients. For high-risk T1G3 bladder cancer or cancer in situ, patients are mainly used BCG vaccine to prevent tumor recurrence and progression; while bladder cancer patients with Ta, T1G1 or T1G2, mainly adopt a few adverse effects chemotherapy drugs. However, patients who received postoperative chemotherapy still have high recurrence rates: thio-

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pa with 44%, adriamycin 39%, mitomycin C with 36%, epirubicin with 39%, respectively [5]. For muscle invasive bladder cancer patients, the 5-year survival rate is 10%-15%, cisplatin-based chemotherapy is the standard treatment, which mainly contain the MVAC (methotrexate, vinblastine, adriamycin and cisplatin) and GC (gemcitabine and cisplatin) schemes, yet the adjuvant chemotherapy can only increase the survival rates of 5% [6, 7]. Therefore, it needs to explore a new treatment strategies to improve the survival of patients with bladder cancer.

Autophagy is a highly conserved self-digestive process in eukaryotic cells. To maintain cellular homeostasis, cytoplasmic proteins and damaged organelles are delivered to lysosomes for degradation in this process. Emerging evidence demonstrated that autophagy could play an important role in cancer therapy, but it is still debatable whether autophagy causes survival or death in different cancer cells [8-10]. Although several anticancer drugs have been proved to regulate crosstalk of autophagy and apoptosis in bladder cancer cells, the role of histone deacetylase inhibitors has been little studied.

In this study, we explored the effect of histone deacetylase inhibitors trichostatin A (TSA) in mediating autophagy and apoptosis in bladder cancer cells. We found that TSA promoted bladder cancer cells apoptosis by inhibiting cell autophagy. Furthermore, we found that the MEK/ERK signaling pathway played a major role in the regulation of autophagy in bladder cancer cells.

Materials and methods

Cell culture

Human bladder transitional cell cancer cell lines (T24 and EJ) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). T24 and EJ cells were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 units/ml streptomycin at 37°C in 5% CO₂.

Cell viability assay

Cells were plated in 96-well plates at 5×10³ cells per well at 24 h before treatment. Cells were treated with TSA at 25 nmol/l, 50 nmol/l,

100 nmol/l, 200 nmol/l and 400 nmol/l for 72 h, respectively; dimethylsulfoxide (DMSO) as control. After 72 h, 20 μl 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, 5 mg/ml) was added to each well and the plate was incubated at 37°C for 4 h. Finally, the medium of each well was completely removed. 150 μl of the dimethylsulfoxide solution was added into each well; and then the above solution was shaken up at room temperature. Cell viability was analyzed by using MTT assay. The absorbance of the samples was measured using a ELISA reader at 490 nm wavelength. The relative cell viability was calculated according to the following formula: [(mean value A₄₉₀ of experimental group-mean value A₄₉₀ of blank control group)/(mean value A₄₉₀ of control group-mean value A₄₉₀ of blank control group)] ×100%.

Cell ultrastructure

Cells were seeded in 6-well plates with RPMI 1640 supplemented with 10% fetal bovine serum at a density of 2×10⁶ cells/well and were treated with 200 nmol/l TSA. Then, the cells were fixed in 2.5% glutaraldehyde, fixed in 1% osmium tetroxide, dehydrated using gradient ethanol and embedded in epoxyresin. After that, 50-nm-thick ultrathin sections were cut, followed by staining with lead citrate and uranyl acetate. The sections were observed under JEM-1230 TEM (JOEL Ltd, Tokyo, Japan).

Flow cytometry analysis

To detect apoptosis, the cells were seeded in 6-well plates at 2×10⁶ cells/well and cells were received 100 nmol/l, 200 nmol/l and 400 nmol/l TSA treatment for 48 h, respectively. After that, cell apoptosis was examined by using flow cytometry.

RNA isolation and quantitative real-time PCR

Total RNAs were isolated using Trizol reagent (Invitrogen, Grand Island, NY). After quantified by a Nano Drop ND-1000 spectrophotometer, 500 ng RNA was reversely transcribed into cDNA according to the instructions provided by Takara reverse transcription kit (Takara, China). Quantitative real-time PCR (Q-RT-PCR) assay was conducted using a Bio-Rad CFX96 system with SYBR green to determine the

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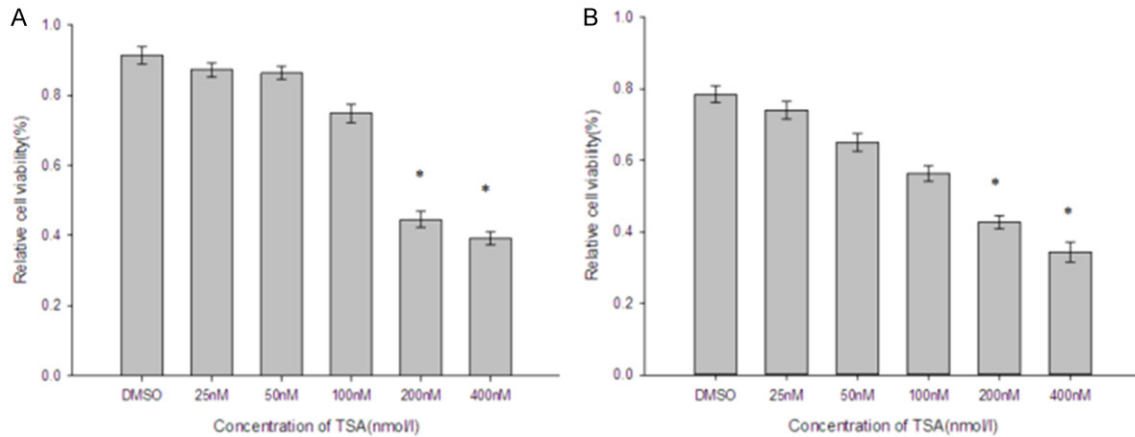


Figure 1. The antiproliferative effects of trichostatin A on different bladder cancer cell lines. Graph showing antiproliferative effects in T24 and EJ cell lines after 72 h trichostatin A treatment (A, T24 cells, *vs control, $P < 0.05$; B, EJ cells, *vs control, $P < 0.05$).

mRNA expression level of a gene of interest. Expression level were normalized to the expression of GAPDH RNA. (All primers used are listed in [Table S1](#)).

Western blot analysis

The human bladder cancer cell lines (T24 and EJ) were seeded in a 25 cm² cell culture flask at a density of 1×10^5 cells/cm². T24 and EJ cells were treated with 100 nmol/l TSA, 200 nmol/l TSA, PD98059 and U0126 (Sigma-Aldrich Inc, St. Louis, USA) after 24 h of incubation, respectively. After 48 h treatment, cells were harvested and lysed in a RIPA lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China). Proteins were electrophoresed on 10-12% SDS polyacrylamide gels and were transferred to polyvinylidene difluoride membranes (Hybond-P; GE Health Care, Buckinghamshire, UK). Blots were blocked with 5% nonfat dry milk in TBS-T (Tris-buffered saline and 0.1% Tween-20, pH 7.4) at room temperature for 30 min. The primary antibodies used were: mouse anti-p62 monoclonal antibody (mAb) (Cell Signaling Technology, Massachusetts, USA), rabbit antimicrotubule-associated protein 1 light chain 3 (LC3) polyclonal antibody (pAb) and anti-P53 (Abcam plc, Cambridge, UK), and mouse anti- β -actin mAb (Bioworld Technology, St. Louis Park, USA), rabbit anti-HDAC4, anti-Caspase 3, anti-Bax, anti-Bcl-2, anti-p-ERK, anti-ERK and anti- β -actin (Beyotime Institute of Biotechnology, Shanghai, China). The secondary antibodies used were: horseradish peroxidase-conju-

gated antibodies against rabbit IgG (ZSGB-BIO, Beijing, China), and mouse IgG (ZSGB-BIO, Beijing, China). Immunoreactive bands on the blots were visualized using enhanced chemiluminescence substrates (ECL Plus; GE Healthcare).

Statistics analysis

All data are presented as mean \pm SD (standard derivation). Statistical analysis was carried out using SPSS 13.0 with the level of significance set at $P < 0.05$. Difference between two groups was performed using Student's t-test. One-way ANOVA was used to compare among groups when the means follows the normal distribution.

Results

TSA inhibits proliferation of bladder cancer cells

MTT test was used to detect the effects of different dose TSA on T24 and EJ cells. Culture medium with DMSO was considered as control. As is shown from **Figure 1**, there was no obvious effect on cells with 0.04% DMSO; And TSA produced obvious inhibitory effect on T24 and EJ cells when its concentrations were more than 100 nmol/l ($P < 0.05$).

TSA induces apoptosis in vitro

To explore the mechanism of the TSA on bladder tumor cells damage, we used flow cytometry to detect cells apoptosis before and after

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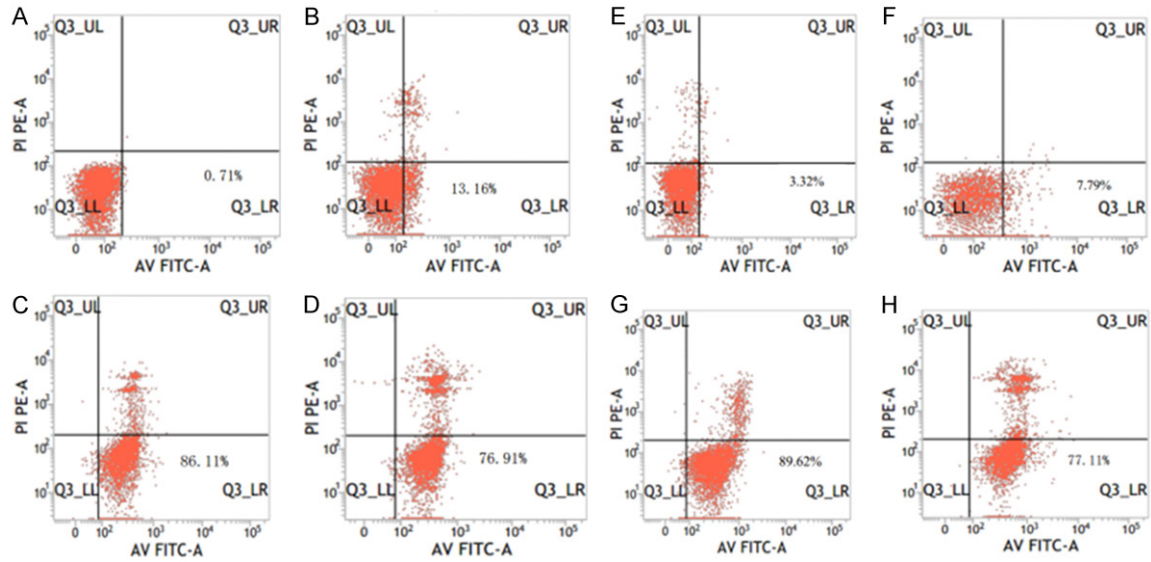


Figure 2. Effect of trichostatin A on bladder cancer cells apoptosis. After trichostatin A treatment 48 h, flow cytometry detected cell apoptosis in EJ cells and T24 cells (A = Control group; B = 100 nmol/L trichostatin A; C = 200 nmol/L trichostatin A; D = 400 nmol/L trichostatin A; E = Control group; F = 100 nmol/L trichostatin A; G = 200 nmol/L TSA; H = 400 nmol/L trichostatin A).

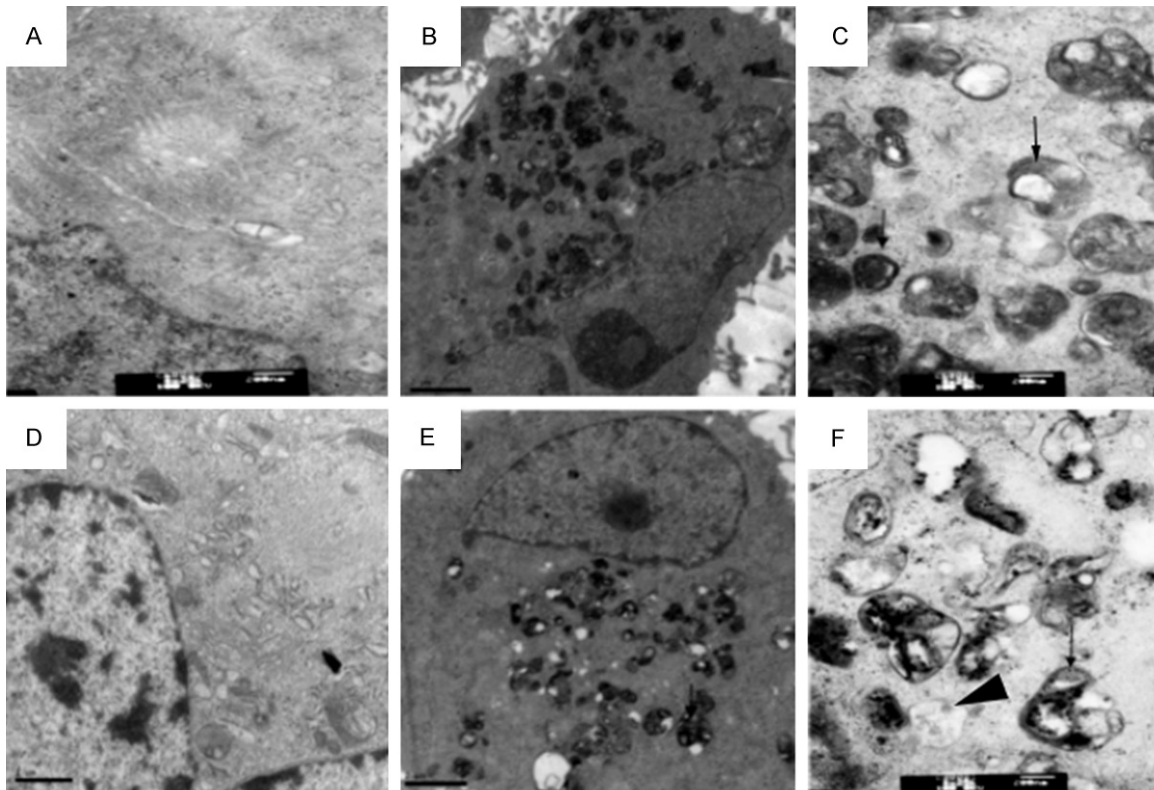


Figure 3. The cell ultrastructural features. The cell ultrastructural features of 200 nmol/L trichostatin A on different bladder cancer cell lines after 24 h by transmission electron microscopy (Upper, EJ cells: A = Control group, 25000 \times ; B = 200 nmol/L trichostatin A group, 10000 \times ; C = EJ cells group, 25000 \times . Down, T24 cells: D = Control group, 20000 \times ; E = 200 nmol/L trichostatin A group, 10000 \times ; F = EJ cells group, 25000 \times . The arrows indicated autophagy body or autophagy-lysosome).

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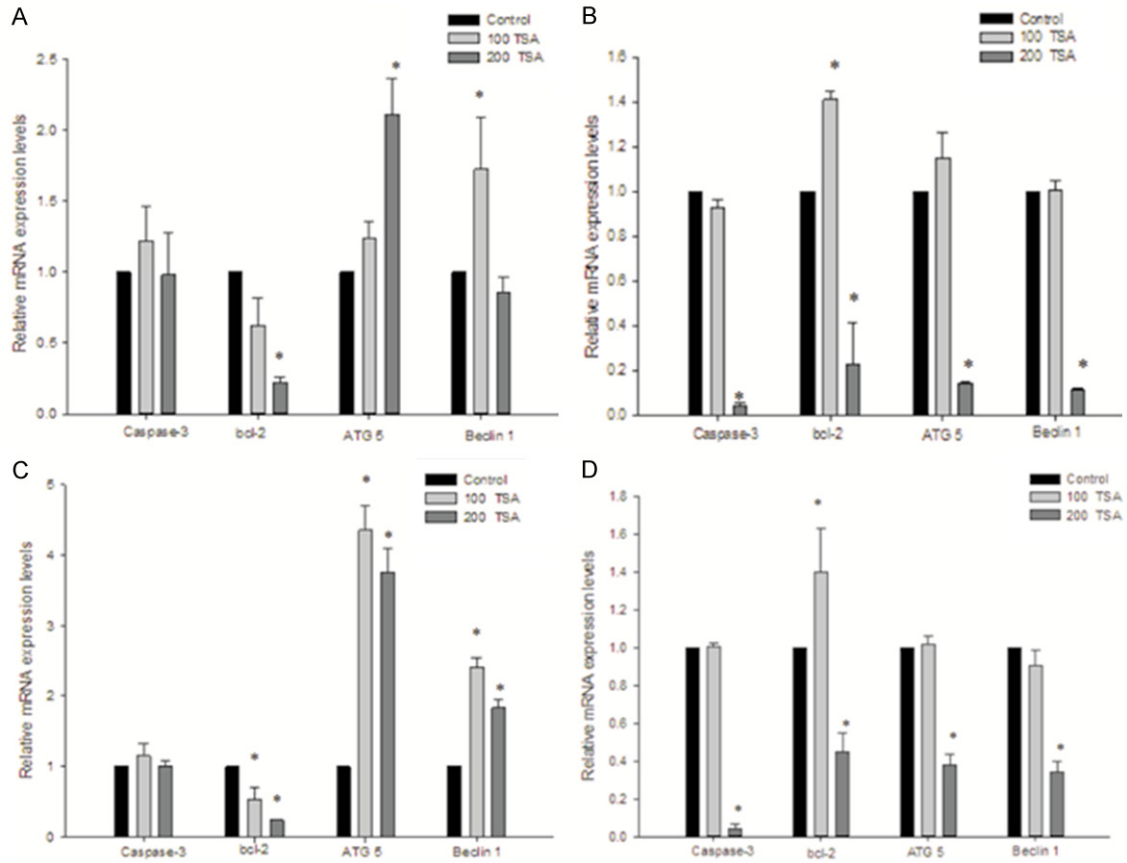


Figure 4. Changes in autophagy and apoptosis-related genes. After trichostatin A treatment, RT-PCR detected the expression of Caspase-3, bcl-2, ATG5 and Beclin 1 in different bladder cancer cells (Upper, EJ cells: A = trichostatin A treatment 24 h; B = trichostatin A treatment 48 h. Down, T24 cells: C = trichostatin A treatment 24 h; D = trichostatin A treatment 48 h).

TSA treatment. The results were shown in the **Figure 2**: After 48 h treatment with TSA, EJ and T24 cells appeared significant early apoptosis and late apoptosis cells in a dose dependent way (**Figure 2**). Early apoptosis rate were 0.71%, 13.16%, 86.11% and 76.91% in EJ cells respectively; While early apoptosis rate were 3.32%, 7.79%, 89.62% and 77.11% in T24 cells respectively.

Early cell morphology characteristics of autophagy

Since transmission electron microscope is the gold standard of observing cell autophagy, we used it to observe bladder cancer cells autophagy before and after TSA treatment. The figures showed that after 24 h, cells appeared autophagosome in EJ and T24 bladder cancer cells receiving 200 nmol/l TSA (**Figure 3**).

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In order to further investigate the relationships between autophagy and apoptosis in the process of TSA treatment, we used RT-PCR to measure the expression of autophagy related genes ATG5, Beclin1 and apoptosis related gene Caspase 3, the Bcl-2 in bladder cancer cells (**Figure 4**).

The data illustrated that after 200 nmol/l TSA treatment on EJ cells, there were no remarkable changes in the expression of Caspase-3 and Beclin-1 at 24 h ($P > 0.05$); while BCL-2 expression was decreased, ATG5 was significantly increased ($P < 0.05$); After 48 h, Caspase 3, BCL-2, ATG5 and Beclin-1 were significantly decreased ($P < 0.05$). Similarly, for T24 cells, there also were no obvious changes in the expression of Caspase-3 at 24 h ($P > 0.05$);

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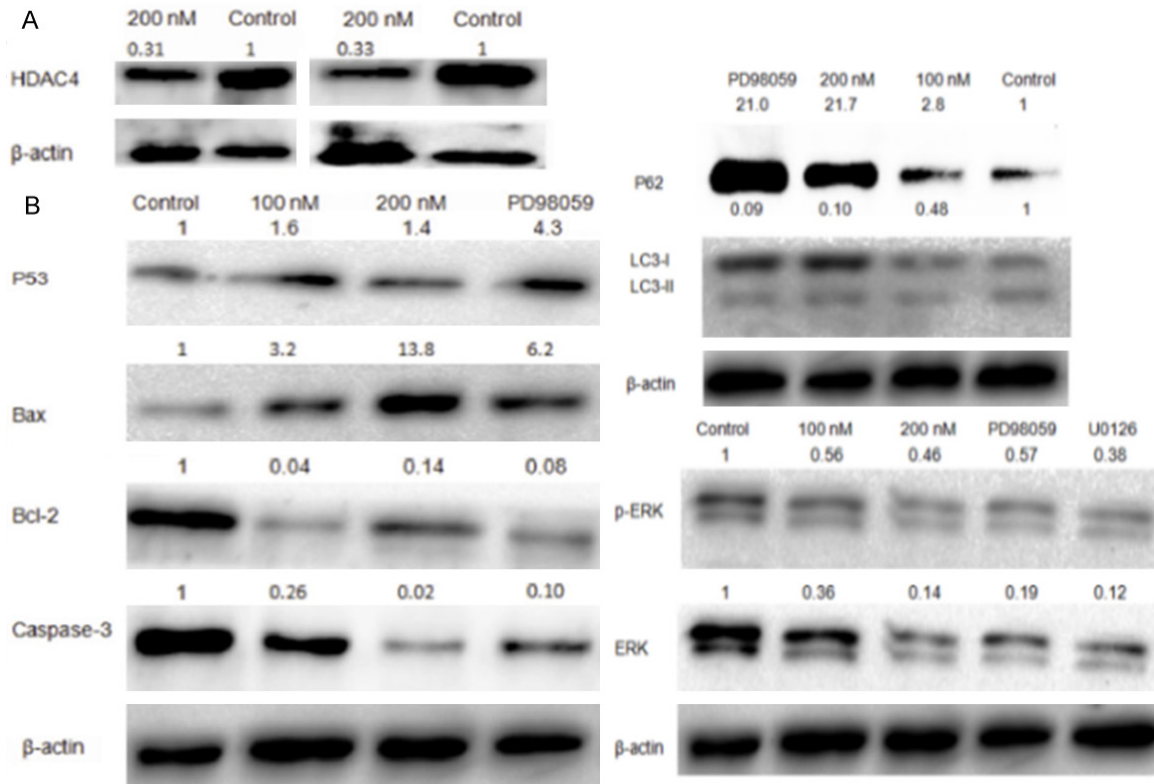


Figure 5. Relationship of cell acetylation, apoptosis, autophagy and MEK/ERK signaling pathway, PD98059 and U0126 as MEK/ERK signaling pathway special inhibitor (A) After 200 nmol/L TSA treatment, Western blotting detected the expression of HDAC4 in different bladder cancer cells in 48 h (Left, T24 cells; Right, EJ cells). (B) EJ cells were treated by 100 nmol/l, 200 nmol/l TSA and PD98059, respectively, western blotting detected P53, Bax, Bcl-2 and Caspase-3 expression after 48 h; β -actin as internal contrast, * $P < 0.05$; and western blotting detected P62 and LC3 expression after 48 h; β -actin as internal contrast, *versus 100 TSA, $P < 0.05$). EJ cells were treated by 100 nmol/l, 200 nmol/l TSA, U0126 and PD98059, respectively, western blotting detected p-ERK and ERK expression after 48 h; β -actin as internal contrast, *versus control, $P < 0.05$).

while BCL-2 expression was decreased, ATG5 and Beclin-1 were significantly increased ($P < 0.05$); After 48 h, Caspase 3, BCL-2, ATG5 and Beclin-1 were significantly decreased ($P < 0.05$).

Relationship of cell apoptosis, autophagy and MEK/ERK signaling pathway

Compared with the control group, HDAC4 acetylation expression notably dropped in T24 and EJ cells with 200 nmol/l TSA after 48 h (**Figure 5**); Meanwhile, anti-apoptosis protein Bcl-2 expression and total Caspase-3 protein was significantly decreased; pro-apoptosis protein P53 and Bax expression were obvious increased; and we further found that autophagy related protein P62 expression increased and LC3-II/LC3-I ratio decreased in EJ cells; This suggests that the cell autophagy was gradually suppressed. MEK1/2 specific inhibitor PD98059 also produced similar effects (**Figure 5**).

In order to explore the possible molecular mechanism of TSA in preventing cell autophagy, we tested the autophagy related pathways MEK-ERK expression (**Figure 5**). The figure showed that compared with the control group, p-ERK and ERK expression were significantly decreased by 100 nmol/l or 200 nmol/l TSA in EJ cells; it was in accordance with the role of specific ERK1/2 inhibitor PD98059 and U0126.

Discussion

Under normal circumstances, autophagy remains at a low level in order to maintain cellular homeostasis, including cell metabolism and energy requirement [10, 11]. However, under pathological conditions, many diseases can occur aberrant autophagy, such as metabolic diseases, neurodegenerative diseases, infectious diseases, inflammation and tumors [12-16]. Especially in cancer, because of the par-

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ticular tumor microenvironment, the autophagy performed a complex dual roles during the anti-cancer treatment [17, 18]. On the one hand, autophagy as a tumor suppressor, it can be a negative regulator of tumor formation and progression, thus inducing autophagy can inhibit tumor cell survival; on the other hand, autophagy can promote tumor cell survival, inhibiting protective autophagy displays a good anti-tumor effects. In addition, the crosstalks between autophagy and apoptosis play an important role in the process of anti-tumor therapy [19]. Autophagy gene Beclin1, also known as Atg6, can boost the formation of cancer; and autophagy-related protein Bcl-2 can be bound to the Beclin1 location forming Bcl-2/Beclin1 complex, and further promote autophagy [20, 21]. In most cases, autophagy is closely related to mammalian target of rapamycin complex 1 (mTORC1), PI3K-Akt protein can inhibit mTORC1 expression by Rheb protein inactivity, and ultimately promote the survival of cancer cells [22, 23]. Moreover, activation of Ras can indirectly regulate PI3K-Akt-mTORC1 axis, it is achieved through interaction with the catalytic subunit of PI3K p110 α and strengthening the role of Ras-Raf-MAPK signaling pathway. Ras activation can be bind to the Raf protein and subsequently makes MEK1/2 and ERK1/2 phosphorylation [24].

In this study, our group discussed the role of autophagy in TSA killing bladder cancer cells. The data illustrated that TSA inhibited bladder cancer cells growth by apoptosis and autophagy: at the beginning, bladder cancer cells adapted the changes in surrounding by autophagy; with the extension of time, cell autophagy was gradually suppressed by the use of high concentrations TSA. Expression of pro-apoptotic protein Bax and Caspase-3 increased gradually, anti-apoptotic protein Bcl-2 expression was significantly decreased, tumor suppressor protein P53 expression was obviously upregulated. The mechanisms of TSA in inhibiting autophagy were also initially examined and found that TSA could inhibit autophagy through MEK/ERK signaling pathway. ERK is extracellular regulated protein kinases of the RAS/RAF/MEK/ERK signaling pathway, including ERK1 and ERK2, ERKs regulate cell proliferation, differentiation and survival. TSA via MEK/ERK signaling pathway to inhibit autophagy was confirmed by the signaling pathway special inhibitor PD98059 and U0126.

The present studies showed that the mechanisms of autophagy-related tumor protective effect may be attributed to the following factors. Autophagy can make tumor cells adapt to the hypoxia microenvironment: Degenhardt et al demonstrated that hypoxia can specifically induce autophagy in cancer cells [25]. Besides, further research revealed the molecular mechanisms of hypoxia and autophagy. Hypoxia inducible factor -1 α is a key regulatory factor of cell hypoxia stress response; it is highly expressed in the hypoxic tumor cells so as to induce autophagy [26]. Bcl-2/BNIP3, HIF-1 α downstream molecules, can induce mitochondrial autophagy, and also it can prevent damaged mitochondrial release pro-apoptotic proteins, which promotes cell survival and inhibition of apoptosis [27]. Zhang et al. reported that specifically inhibiting the expression of P53 protein dramatically reduced the expression of LC3II and DRAM [28]. In Bax/Bak double knockout embryonic fibroblasts, apoptosis-inducing agents etoposide treatment caused cell death; however, along with the emergence of a large sum of autophagosomes, cell death was significantly weakened after knockdown autophagy gene atg5 and beclin1. Autophagy also are induced in overexpression Bcl-2/Bcl-xl Hela cells [25]. These suggest that autophagy can be activated by Bcl-2 overexpression [29].

In this study, with the inhibition of autophagy, P53 protein is upregulated, while Bcl-2 protein is reduced. These data suggest that TSA can inhibit autophagy, which may be related to P53 up-regulation and Bcl-2 down-regulation, and it causes apoptosis by promoting the release of apoptotic proteins. Moreover, TSA inhibits bladder cancer cells autophagy by MEK/ERK signaling pathway. These findings not only provide a new theoretical basis of histone deacetylase inhibitors in treating bladder cancer, but also provide experimental data for the TSA as an adjunct therapy. In addition, we have witnessed the tumor suppressor protein P53 and Bcl-2 involved in the regulation of autophagy process, but the specific mechanism is still unclear and requires further explored.

To sum up, our results indicated that the TSA can promote bladder cancer cell apoptosis by inhibiting cell autophagy, and it inhibits cell autophagy by blocking MEK/ERK signaling pathway.

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Disclosure of conflict of interest

None.

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Table S1. All used primers data

Gene	Genebank ID	Forward (5'-3')	Reverse (5'-3')	Product Size (bp)
Caspase-3	NM_004346.3	GACTCTGGAATATCCCTGGACAACA	AGGTTTGCTGCATCGACATCTG	140
Bcl-2	NM_000633.2	AACATCGCCCTGTGGATGAC	AGAGTCTTCAGAGACAGCCAGGAG	146
Beclin 1	NM_003766.3	AACCAACGTCTTTAATGCAACCTTC	AGCAGCATTAAATCTCATTCCATTCC	121
ATG 5	NM_004849.2	TTGAATATGAAGGCACACCACTGAA	GCATCCTTAGATGGACAGTGCAGA	151
GAPDH	NM_002046.3	GCACCGTCAAGGCTGAGAAC	TGGTGAAGACGCCAGTGA	138