

## RESEARCH ARTICLE

# OK-432 Suppresses Proliferation and Metastasis by Tumor Associated Macrophages in Bladder Cancer

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### Abstract

OK-432, a Streptococcus-derived anticancer immunotherapeutic agent, has been applied in clinic for many years and achieved great progress in various cancers. In the present study, we investigated its anticancer effect on bladder cancer through tumor associated macrophages (TAMs). MTS assay validated OK-432 could inhibit proliferation in both T24 and EJ bladder cell lines. OK-432 also induced apoptosis of bladder cancer cells *in vitro*. Consequently, we demonstrated that OK-432 could suppress the bladder cancer cells migration and invasion by altering the EMT-related factors. Furthermore, using SD rat model, we revealed that OK-432 inhibited tumor growth, suppressed PCNA expression and inhibited metastasis *in vivo*. Taken together, these findings strongly suggest that OK-432 inhibits cell proliferation and metastasis through inducing macrophages to secrete cytokines in bladder cancer.

**Keywords:** OK-432 - bladder cancer - proliferation - apoptosis - TAMs

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### Introduction

Bladder cancer is the ninth most common cancer diagnosis worldwide, with more than 330,000 new cases each year and more than 130,000 deaths per year, with an estimated male-female ratio of 3.8:1.0. At any point in time, 2.7 million people have a history of urinary bladder cancer (Ploeg et al., 2009). Superficial bladder cancer accounts for approximately 70% of all bladder cancer cases. The standard treatment for patients with superficial bladder cancer is transurethral resection (TUR) of tumors. However, approximately 60% to 70% of these tumors will recur, with 25% showing progression to a higher stage or grade (Schenk-Braat and Bangma, 2005). Intravesical chemotherapy is widely used as an adjuvant therapy to prevent recurrence and progression of superficial disease after TUR (Wu et al., 2009). Bacille Calmette-Guerin (BCG) is the first choice to intravesical instillation for non-muscle-invasion bladder cancer (NMIBC) (Newton et al., 2014), but its side effects must be considered. Data from Ayari et al suggested that patients with high level of infiltration by tumor associated macrophages (TAMs) in the cancer area don't respond as well to BCG immunotherapy (Ayari et al., 2009).

Tumor associated macrophages (TAMs) are key players in the tumor microenvironment (Mantovani et al., 2011). Thus, a role of these cells in promoting proliferation and survival of tumor cells, angiogenesis, matrix remodeling and metastasis has been described (Mantovani et al., 2008;

Qian and Pollard, 2010), and a high number of TAMs was associated with short survival and poor prognosis in many tumors (Zhang et al., 2012). Few reports already point to a role of macrophages in epithelial-mesenchymal-transition (EMT) which is a central process in early tumorigenesis (Lin et al., 2006; Bonde et al., 2012). These alterations are regarded as prerequisite for cell dissociation from the epithelial context, cell dissemination and finally formation of metastasis.

OK-432, which is a penicillin-killed and lyophilized preparation of a low-virulence strain of Streptococcus pyogenes (group A), has been successfully used as an immunotherapeutic agent against many types of malignancies, including head and neck cancer, stomach cancer, lung cancer, pancreatic cancer and colorectal cancer (Yamaguchi et al., 2003; Yoo et al., 2010; Nakamoto et al., 2011; Tano et al., 2012; Tano et al., 2013; Wada et al., 2014). It has been reported that OK-432 elicits anti-tumor effects by stimulating immunocompetent cells such as macrophages, T cells, and natural killer (NK) cells, and by inducing helper T-cell 1 (Th1) type cytokines, such as IFN- $\gamma$ , TNF- $\alpha$ , IL-6, IL-8, IL-10, IL-12, and IL-18 (Oshimi et al., 1980; Saito et al., 1982; Fujimoto et al., 1997), which can augment cytotoxic T lymphocytes to antitumor.

In this study, we investigated whether OK-432 exerts its anti-tumor activities through stimulated TAMs to produce cytokines to prompt the cancer cells apoptosis and inhibit the cancer cells metastatic. Our results

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demonstrated for the first time that OK-432 could inhibit proliferation and suppress metastasis *in vitro* and *vivo*.

## Materials and Methods

### Reagents

OK-432 (1KE equals 100  $\mu$ g of dried *streptococci*) was donated by Shandong Luya pharmaceutical company (Shandong, China). Antibodies against E-cadherin, Vimentin, snail and GAPDH were purchased from Sigma-Aldrich Technology, Inc. (St. Louis, Missouri). Annexin V-FITC Apoptosis Detection kits were from keygen Biotech, Inc. (Nanjing, China). Phorbolmyristate acetate (PMA) was purchased from Sigma (St. Louis, MO, USA). Human TNF- $\alpha$  and IL-10 ELISA kits were obtained from Affymetrix ebioscience (Santa Clara, CA, USA). CD68 antibody was purchased from Abcam (Abcam, MA, USA).

### Cell culture and animals

Human bladder cancer cell lines (EJ, T24) and Human monocyte cell line THP-1 were purchased from ATCC. ALL of T24, EJ and THP-1 cell lines were maintained in RPMI1640, supplied with 10% fetal bovine serum. THP-1 Cells were cultured in six-well plates for 48 h in the presence of 100 nM PMA, which allowed them to differentiate into adherent macrophages. The MNU and control groups received 0.20 ml 0.9% physiological saline. The OK-432 group received 0.5 ke/kg (1ke equals 100  $\mu$ g of dried *streptococci*) OK-432.

### Conditional medium

THP-1 cells were cultured in RPIM-1640 with 10% FBS for 24 h, then treated with 100 nM PMA for another 48h, discharged the supernatant, changed to ok-432 (0.1, 0.5, 1 ke/ml) medium for 24 h and collected supernatant. The supernatant was collected by centrifugation, and then filtered with 0.22 $\mu$ m pore size filter. TAM-CM was consisted with medium supplied with 10% fetal bovine serum and the supernatant at a ratio of 4:6.

### Immunofluorescence analysis

THP-1 cells were treated with 100 nM PMA for 48h, changed to ok-432 (0.1, 0.5, 1 ke/ml) medium for 24h. The cells were fixed in 4% formaldehyde in phosphate-buffered saline (PBS), permeabilized with ice cold acetone for 3 min, and then blocked in 5% milk-10% FCS-0.3% bovine serum albumin-0.3% Triton X-100 in PBS for 1h at room temperature. CD68 was visualized by using a 1:200 dilution of the anti-CD68 antibody overnight (Abcam, MA, USA), followed by probing with a 1:800 dilution of Alexa Fluor 488 donkey anti rabbit IgG (H+L) 2 mg/mL (Invitrogen, Carlsbad, CA, USA). Cell nuclei were visualized by DAPI staining. Samples were analyzed by Zeiss axiovert 200 using AxioVision Rel.4.5 software (Carl Zeiss, Jena, Germany).

### Cell viability test

T24 and EJ cells were seeded in 96-well culture plates. Cells were treated with diluted conditioned medium derived from supernatant of TAM-CM. Cell growth rate was determined by MTS assay. Briefly, culture media

were replaced with fresh DMEM medium containing MTS stock solution and incubated at 37°C for two hours. At the end of incubation, Colorimetric analysis using a 96-well microplate reader was performed at wavelength 490 nm. The experiments were performed in triplicate.

### Detection of apoptosis by flow cytometric analysis

Cells (EJ and T24) were exposed to different doses of TAM-CM for 24 h. Cells were then collected and subjected to Annexin V and propidium iodide (PI) staining using an Annexin V-FITC Apoptosis Detection Kit, following the protocols provided by the manufacturer. Apoptotic cells were then analyzed by flow cytometry (BD FACSCalibur, San Jose, CA).

### Migration and invasion assay

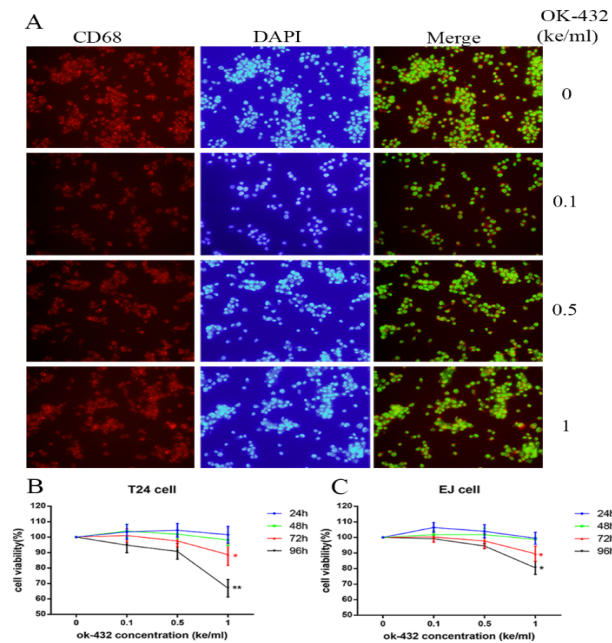
A transwell cell culture chamber (Corning, Bedford, MA, USA) with PET membranes (24-well insert, 8- $\mu$ m pore size, Millipore) supplemented with 10% fetal bovine serum was added to each well of the plate to act as a chemoattractant in the lower chamber. Then the cells were suspended at a free medium of  $4 \times 10^5$  cells/mL in medium with different concentrations of TAM-CM treated with for 4hs, and 0.2 mL of each was added to the top chamber. Cells were incubated for 24h, and those that did not migrate through the pores were removed by scraping the upper surface of the membrane with a cotton swab. Cells that had migrated to the lower surface of the membrane were fixed for 30 min in 100% methanol and stained with 0.5% crystal violet for 15 min. The cells that invaded through the insert were counted in five random fields and expressed as the average number of cells per field. These experiments were done in triplicate and performed a minimum of three times. Differently, the culture chamber was coated with Matrigel in invasion assay, dried and reconstituted at 37°C with free fetal bovine culture medium.

### Western blot analysis

Cells were harvested at 24h following different concentration TAM-CM treatment. After blocking the membranes with 5% BSA in PBS for 2h at room temperature, the membranes were incubated with appropriate dilutions of specific primary antibodies overnight at 4°C. After washing, the blots were incubated with anti-rabbit, anti-mouse, anti-goat IgG HRP for 1 h. The blots were developed in ECL mixture (Beyotime, Nantong, China).

### Animal studies

N-methyl-N-nitrosourea (MNU) induced rat bladder tumor model utilized has been previously described with some modifications. We used 30 female SD rats at age 8 weeks obtained from the Experimental Animal Center of Huazhong University of Science and Technology. They remained sedated for approximately 45 minutes after bladder catheterization via a 20-gauge angiocatheter to prevent spontaneous micturition. Cancer induction and control, including 4 doses from weeks 0 to 8. A total of 20 rats received 2 mg MNU dissolved in 0.2 ml 1 M sodium citrate (pH 6.0). Another 10 rats received 0.20 ml 0.9%



**Figure 1. (A) Protein Expression of CD68 after Macrophages Treated with Various Concentration OK-432(0, 0.1, 0.5, 1 ke/ml).** OK-432 inhibited bladder cancer cells proliferation through inducing macrophages. **(B)** T24 cells treated with various dose TAM-CM for different time (24h, 48h, 72h, 96h), the cell viability of T24 was analyzed by MTS assay. **(C)** The same procedure treated with EJ cells, MTS examined the cell viability of EJ cells. \* $p < 0.05$ , \*\* $p < 0.01$

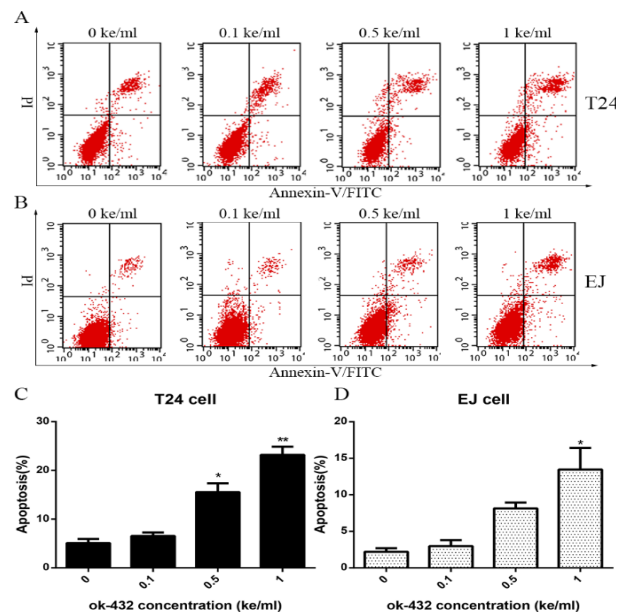
physiological saline (controls), each every other week on weeks 0, 2, 4 and 6. Treatment, including 6 doses from weeks 8 to 16 rats were divided into 2 groups with 10 each group. The MNU and control groups received 0.20 ml 0.9% physiological saline. The OK-432 group received 0.5 ke/kg OK-432. Doses were dissolved in 0.20 ml 0.9% physiological saline and administered weekly for 8 weeks. After 16 weeks of the protocol (cancer induction, control and treatment) all rats were sacrificed. The bladder was collected and processed for histopathological and immunological evaluation. Animal care and protocols were approved by the Institutional Animal Care and Use Committee of Huazhong University of Science and Technology, and all the animal experiments were performed in accordance with the NIH Guidelines on the Use of Laboratory Animals.

#### Immunohistochemical (IHC) staining

Tumor tissues obtained from euthanized SD rats were fixed in 10% (v/v) formaldehyde in PBS, embedded in paraffin, and cut into 5-mm sections. When stained, tumor tissue sections were deparaffinized in xylene solution and rehydrated using gradient ethanol concentrations. The tissue sections were stained with specific antibodies against E-ca (1:400) and vimentin (1:200). The sections incubated with secondary antibodies in the absence of primary antibodies were used as negative control. Hematoxylin was used for counterstaining. Slides were viewed and photographed under a light microscope.

#### Statistical analysis

All statistical analyses were performed using SPSS 19.0 software. Quantitative data are presented as



**Figure 2. OK-432 Induces T24 and EJ Cells Apoptosis through TAMs.** (A) T24 cells were pretreated with 0, 1, 2, 4 ke/ml OK-432 for 24h, then T24 cells were harvested. Different concentration group cells were stained with Annexin-V and PI, and analyzed by Flow Cytometre. **(B)** EJ cells were treated with previous procedure and analyzed by Flow Cytometre. **(C)** and **(D)** Quantification of the panel A and B apoptosis rate. \* $p < 0.05$ , \*\* $p < 0.01$

Mean $\pm$ SE and the differences among the control and test groups were compared by one-way ANOVA, followed by Dunnett's test for separate comparisons. In the comparison involving only two groups, Student's t test was used.  $P < 0.05$  was considered statistically significant.

## Results

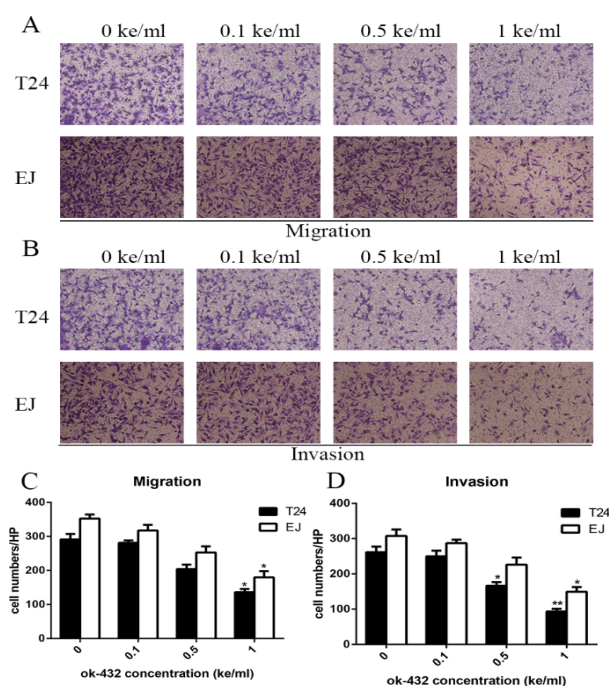
### Successful induction of TAMs

Human cell line THP-1, which upon PMA-stimulation shows a macrophage-like phenotype (Daigneault et al., 2010). In our study, we treated with 100 nM PMA for 48h, changed to ok-432 (0.1, 0.5, 1 ke/ml) medium for 24 h, and then analysed the expression of CD68 as a macrophages surface marker on the macrophages (Figure 1A). Surprisingly, CD68 was highly expressed both in control and ok-432 group, which indicated that the phenotype of macrophages did not change whether the cells were treated with OK-432 or not.

### OK-432 Inhibited the bladder cancer cells proliferation through TAMs

To elucidate OK-432 Inhibited the Bladder Cancer Cells Proliferation through TAMs, we collected the supernatant of the macrophages treated with OK-432 of different concentration, and then prepared the conditional medium as above methods. T24 and EJ cell lines were future incubated with conditional medium in the presence with various concentrations. The cell viabilities analyzed by MTS at different time points (24h, 48h, 72h, 96h). As shown in Figure 1B and 1C, low dose OK-432 (ranged 0.1 ke/ml to 0.5 ke/ml) inhibited the bladder cancer cells proliferation inconspicuously, while high dose OK-





**Figure 3. OK-432 Effect on Bladder Cancer Migration and Invasion through TAMs.** (A) T24 cells treated with were different concentrations of TAM-CM for 4hs, then harvested, after which  $5 \times 10^4$  cells were seeded into the upper compartments of chambers. Migration was analyzed using chamber assays with Falcon cell culture inserts. Invasive properties were analyzed using Falcon cell culture inserts covered with 50 $\mu$ l of Matrigel per filter. For both assays, the lower chambers contained conditioned media (serum-free medium L), which was used as a chemoattractant. After incubation for 24 h, the cells invading the lower surface were counted microscopically. The results are representative of 5 independent experiments. Panel (A) and (C) were shown the number of migration cells. Panel (B) and (D) were shown the number of invasion cells. \* $p < 0.05$ , \*\* $p < 0.01$  vs controls

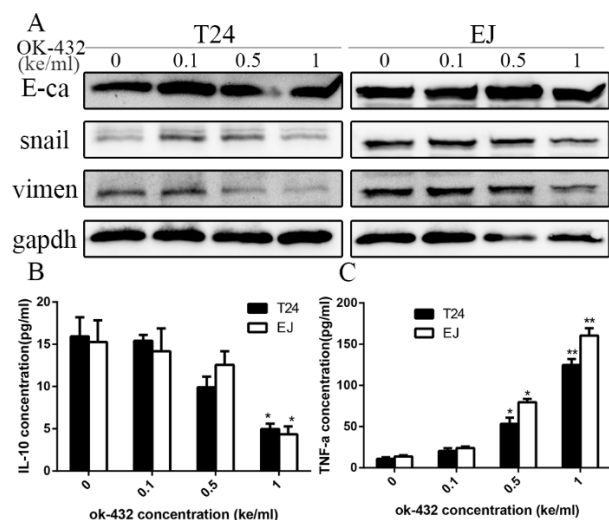
432significantly decreased the T24 and EJ cells viability, which showed a time- and dose-dependent manner.

#### OK-432 Potentiates Apoptosis of Bladder Cancer Cells through TAMs

To investigate the effect of OK-432 on bladder cancer cells through TAMs, we examined the apoptosis after T24 and EJ cells treated with various concentrations conditional medium for 24 hours, and then stained with Annexin-V and Propidium iodide, and analyzed using flow cytometer. In Figure 2A, the apoptosis rate of T24 cells which treated with 0, 0.1, 0.5, and 1 ke/ml OK-432 was 5.08%, 6.55%, 15.53%, and 23.2% respectively(Figure 2A), and the apoptosis rate of EJ cells was observed to increase shown dose-dependent (Figure 2B). 1 ke/ml OK-432 increased the apoptosis rate more significantly than control group (Figure 2C-D). Hence, we validated that OK-432 prompted the bladder cancer cell apoptosis through TAMs.

#### OK-432 Inhibited the Bladder Cancer Cells Migration and Invasion through TAMs

In my future study, we investigated the correlation OK-432 on bladder cancer cells migration and invasion



**Figure 4. (A) After Treated with Various TAM-CM for 24h, T24 and EJ Cells were Harvest, Total Protein were Analyzed by Western-Blotting.** EMT related proteins were analyzed. The expression of E-Cadherin, Vimentin and snail were examined by western-blot. (B) and (C) IL-10 and TNF- $\alpha$  in the macrophages supernatant treated by various OK-432(0, 0.1, 0.5, 1 ke/ml) were analysed by enzyme linked immunosorbent assay(ELISA). \* $p < 0.05$ , \*\* $p < 0.01$  vs controls

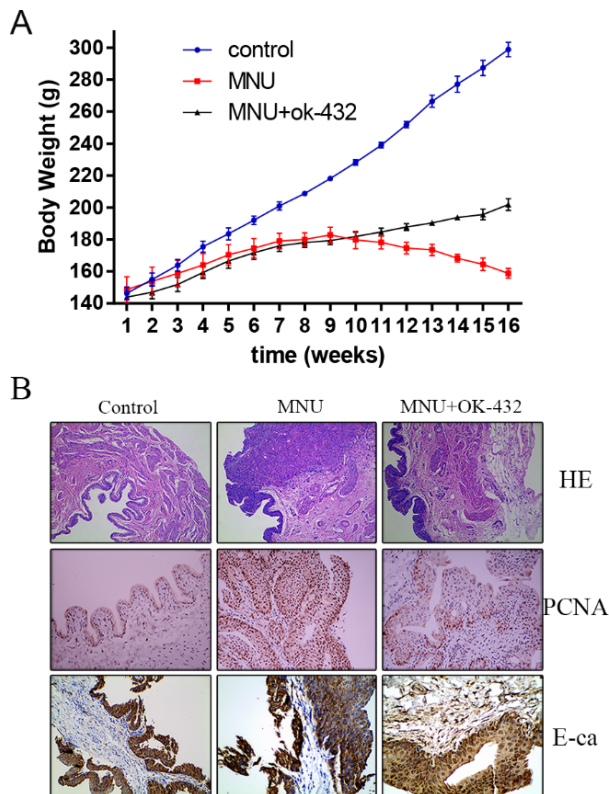
through TAMs. T24 and EJ cells were treated with various concentrations TAMs for 6 hours were applied to transwell assay. 24 hours later, during the migration test, we observed that TAMs from high dose OK-432 (1 ke/ml) inhibited T24 and EJ cells significantly (Figure 3A and 3C), and less effect on low dose group (0, 0.1ke/ml) comparing with control group. In addition, both results showed dose-dependent with OK-432. Invasion assay was conducted with the same results (Figure 3B and 3D).

#### Expression of EMT proteins treated by TAMs

We next examined whether co-culture with TAMs induced alterations of EMT markers for bladder cancer cells. Western-blot showed that protein expressions of mesenchymal markers vimentin and snail were higher in T24 cells co-cultured with TAMs than those in monoculture (Figures 4A). Conversely, the protein expressions of epithelial marker E-cadherin was decreased in co-cultured compared with monoculture T24 cells (Figures 4A). Similarly, altered expression of EMT markers was also observed in EJ cells. Thus, co-culture with TAMs increased the expression of mesenchymal markers but diminished those of epithelial markers in bladder cancer cells.

#### Elisa kits assayed the secreted cytokines treated TAMs

To investigate the mechanism of OK-432 inhibit the bladder cancer cells proliferation and metastasis, we assayed the secreted cytokines in the supernate derived from TAMs treated with various concentration OK-432. Interestingly, we detected that the distribution of IL-10 decreased with the concentration of OK-432 up-regulated in T24 and EJ cells, especially in down-regulated significantly in 1 ke/ml group (Figure 4B). Conversely, the concentration of TNF- $\alpha$  show a dose-dependent up-regulate both in the T24 and EJ Cells (Figure 4C).



**Figure 5. OK-432 Inhibited the Bladder Cancer Growth and Metastatic *in vivo*.** (A) Body weight of rats were measured during the course of experiments. (B) HE stained in bladder tissues. IHC staining for examining levels PCNA and E-Ca proteins expressions. Data are shown as mean of 10 rats results in each group. \* $p < 0.05$ , \*\* $p < 0.01$  vs controls

#### OK-432 Suppressed the Bladder Cancer Growth and metastatic *in vivo*

In our previous study, we validated OK-432 inhibited bladder cancer cell proliferation and prompted cell apoptosis, metastatic *in vitro*. To investigate the effect of OK-432 on bladder cancer *in vivo*, we conducted our research on SD rats. Firstly, we established bladder cancer rats model according to MNU methods. Next, MNU group rats received OK-432 treatment for 8 weeks, while other groups received saline. Together 16 weeks later, all rats sacrificed, removed the bladder tissues and analyzed by IHC. Interesting, our IHC results coincided with our previous study. E-ca and PCNA protein were examined in IHC. As shown in figure, body weight of all rats were calculated during the experiments (Figure 5A), and the expression of E-ca down-regulated while PCNA up-regulated treated with OK-432 (Figure 5B). Taken together, the SD ratmodel results confirmed OK-432 suppressed tumor growth *in vivo*.

## Discussion

OK-432 as an anti-tumor agent has been applied in clinic for many years, including ovarian cancer, hepatocellular carcinoma, lung cancer, gastric cancer, head and neck cancer (Tano et al., 2012). However, the mechanism has not been clearly clarified. Macrophages in the tumor microenvironment play an important role

in tumor cell survival. They influence the tumor cell to proliferate, invade into surrounding normal tissues and metastasize to local and distant sites. And tumor associated macrophages not only facilitate tumorigenesis transformation of CTPC-induced BEAS-2B cells, but also promote tumor growth, angiogenesis and metastasis in a nude mice model (Zhang et al., 2014).

Hence, we hypothesis OK-432 may induce the TAMs secreted cytokines, which against the bladder cancer proliferation and metastatic, and then attempt to elucidate the mechanism of ok-432. EMT is an important biological process of tumor progression. The transition of epithelial-to-mesenchymal cells (EMT) is highly important for morphological changes to occur during embryonic development, and accumulating evidence suggests it also contributes to the progression of primary tumors toward a metastatic state (Biddle and Mackenzie, 2012). Epithelial cell markers, such as E-cadherin, are diminished during EMT while mesenchymal cell markers are up-regulated. Transcription factors that repress E-cadherin expression, including Snail and Slug, are involved in this transition (Liu et al., 2012), and EMT markers have been identified in tumor tissue following pancreatic resection (Javle et al., 2007). Macrophages within tumor tissue greatly contribute to the aggressive progression of cancer by releasing cell-stimulating growth factors and cytokines (Allavena and Mantovani, 2012).

In the present study, pro-inflammatory cytokine TNF- $\alpha$  was detected in the macrophages supernatant treated with various concentrations OK-432, and which showed a dose-dependent up-regulate. It was indicated that OK-432 can stimulus the macrophages to secrete the cytokines TNF- $\alpha$ . Subsequently we assayed the bladder cancer cells proliferation under TAM-CM treated *in vitro*. Interestingly, TAM-CM could suppress the T24 and EJ cells proliferation, especially in 1ke/ml OK-432 treated macrophages for 72h and 96h group ( $p < 0.05$ ), which showed that OK-432 could suppress the bladder cancer cells proliferation through inducing macrophages to secrete TNF- $\alpha$ .

*In vitro*, we assessed the apoptosis of T24 and EJ cells under treat with TAM-CM. The apoptosis rate of T24 and EJ cells was 23.2% and 13.46% treated with 1ke/ml OK-432, respectively, which is higher than control group significantly ( $p < 0.01$ ,  $p < 0.05$ ). TNF- $\alpha$  is a pro-inflammatory cytokine produced by multiple immune and non-immune cells, including lymphocytes, mast cells, endothelial cells, fibroblasts and adipocytes. It functions in the regulation of diverse physiological cellular events, including cell proliferation, differentiation and apoptosis as well as various inflammatory processes (Bazzoni and Beutler, 1996; Liu, 2005).

In the future study, we assayed the T24 and EJ cells migration and invasion applying transwell kits. It was showed that TAM-CM can inhibit the bladder cancer migration and invasion. Western-blotting assayed that OK-432 can up-regulate E-Ca protein level, and down-regulate snail and vimentin expression through TAMs. To confirm TAM-CM during EMT of bladder cancer cells, the role of IL-10 was investigated. The positive correlation between serum IL-10 levels and cancer progression and

the localization of IL-10 in advanced metastases proposes IL-10 has a crucial role within the tumor (Sato et al., 2011). Hence, we believed that OK-432 can inhibited bladder cancer cells migration and invasion through inducing macrophages to secret IL-10.

*In vivo*, bladder cancer was modeled by MNU intravesical. Then, Treatment group SD rats received OK-432 intravesical, while other groups received saline for 8 weeks. Interestingly, in the study, IHC confirmed that OK-432 can inhibit PCNA protein expression, and prompted bladder cancer cells E-Ca expression. We believed that OK-432 can suppress bladder cells proliferation and inhibit bladder cells metastatic *in vivo*.

Taken together, we have shown that OK-432 exhibits the suppression of IL-10, and promoting the expression of TNF- $\alpha$  through TAMs, which are associated with anti-proliferative, pro-apoptotic and anti-invasive effects. Furthermore, OK-432 could be an interesting lead compound for the modulation of inflammatory diseases as well as certain cancers in which inhibition of anti-cancer activity may be desirable.

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