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Original Article

The mechanism by which cisplatin, mitomycin, and doxorubicin inhibit cell growth in bladder cancer

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Abstract: Bladder cancer indicates an advanced and metastatic disease with high disease specificity and a low overall survival rate. Post-surgical treatment using chemotherapeutic agents shows a high prognostic recurrence rate, while combinational drug treatments demonstrate promising effects in reducing the recurrence rate in post-operative bladder cancer. In this study, we evaluated the inhibitory effects of single and combinational treatments of three drugs (cisplatin, mitomycin, and doxorubicin) on the growth of T24 cells in bladder cancer *in vitro*. The inhibitory effects on the proliferation of T24 cells were measured by conducting the MTT assay, and the apoptosis of T24 cells was evaluated by flow cytometry and Hoechst 33258 staining. The expression of the protein was examined by performing the Western blotting assay. The MTT assay showed a considerably lower growth of cancer cells in the combinational treatment groups than in cells in the single-drug treatment groups, which was further confirmed by the results of the Western blotting and flow cytometry assays. We also found that the drug-induced apoptosis of T24 cells was regulated by the Caspase protein family. Therefore, the combined treatment of cisplatin, mitomycin, and doxorubicin might reduce the risk of the recurrence of bladder cancer. This study evaluated the efficacy of the combination of three drugs in the treatment of bladder cancer.

Keywords: Bladder cancer, cisplatin, mitomycin, doxorubicin

Introduction

Bladder cancer endangers human life and health. In the past 10 years, epidemiological studies have shown that, along with the continuous improvement in diagnosis and treatment strategies, more than 300,000 new cases of bladder cancer arise annually, with more men affected than women [1]. In European countries, the mortality rate of bladder cancer is higher in men than in women, while the incidence in women is also rising. In the United States, the incidence rate in white people is considerably higher than that in black people, but the mortality rate is similar. In Canada, the incidence of bladder cancer is half of that in the United States. The mortality and incidence rates of bladder cancer in Asian countries have remained stable or decreased in the past few years [2]. However, in African countries, due to poor medical facilities and the environment, have incidence and mortality rates of bladder cancer that are increasing among many young individuals, which might be related to unhealthy eating habits and poor public health conditions [3].

In China, 80,500 new cases of bladder urothelial carcinoma (which is the sixth most prevalent male malignant tumor in China), from 62,100 men and 18,400 women were reported [4]. Around 32,900 deaths (25,100 men and 7,800 women) occurred due to bladder urothelial carcinoma [2]. With economic development in China, the improvement of human health and living habits has been gaining attention, especially the prevention and treatment of diseases in public health institutions and among individuals [5]. In this study, we evaluated the inhibitory effects of single and combinational treatments of three drugs (cisplatin, mitomycin, and doxorubicin) on the growth of bladder cancer

Table 1. The concentration and drug combination used in the MTT assay

Drug		C0	C1	C2	C3	C4	C5	C6	C7
ADM	ng/mL	0	100	150	200	250	300	350	400
DDP	μg/mL	0	1.25	2.5	5	10	20	40	80

T24 cells *in vitro* to provide recommendations for reducing the recurrence and mortality rates of bladder cancer in clinical practice, and thus, enhancing the quality of life of the patients.

Materials and methods

Materials

Dimethyl sulfoxide (DMSO) was purchased from Sigma (USA), sterile PBS and agarose were purchased from Solarbio Biotechnology Co., Ltd. (Beijing), cyanine/streptomycin double-antibody were obtained from Gansu Second People's Hospital, and 10% fetal bovine serum (FBS) was purchased from Zhejiang Tianhang Biotechnology Co., Ltd. Doxorubicin (H14023879) was purchased from Shanxi Pude Pharmaceutical Co., Ltd. Cisplatin (H37021358) was purchased from Qilu Pharmaceutical Co., Ltd. Mitomycin (H33020786) was purchased from Zhejiang Haizheng Pharmaceutical Co., Ltd.

Cell culture and drug preparation

The human bladder transitional carcinoma cell line T24 was purchased from the National Collection of Authenticated Cell Cultures (Shanghai). The T24 cells were cultured in DMEM/high glucose medium (Hyclone) containing 10% fetal bovine serum (FBS) and 1% double-antibody. The cells were passaged when 95% confluence was reached.

Doxorubicin (ADM), Cisplatin (DDP), and Mitomycin (MMC) were dissolved in ddH_2O to obtain a stock solution of 1 mg/mL and stored at 4°C.

MTT assay

The T24 cells were cultured in a CO_2 incubator until the confluence reached 85%. The adherent cells were then collected using trypsin. A fresh culture medium was added to resuspend the cells to obtain a final cell density of 1×10^5 cells/mL, and 100 μL of the cell suspension was added to all the wells of a 96-well plate and cultured overnight. After the cells adhered to the bottom of the wells, they were treated for 24 h using drugs or drug combinations as shown in **Table 1**. To measure cell viability, 10 μL of MTT was added to each well and cultured for 4 h. The culture medium was then discarded and 100 μL of DMSO was added to dissolve the die at 37°C for 15 min. The absorbance was measured at 490 nm with a microplate reader.

Hoechst 33258 staining

The T24 cells in the logarithmic growth stage were cultured with DMEM/high sugar medium containing penicillin, streptomycin, and 10% fetal bovine serum until 85% confluence. The cells were digested and collected using trypsin after centrifugation with 2 mL of fresh medium. Additional fresh culture medium was added to make a cell density of 1×10^5/mL, and then, 100 μL of cell suspension was added to each well of a 96-well plate. After the cells were completely attached to the bottom overnight, they were treated with different drugs as mentioned above. The cells were collected, fixed, and stained using Hoechst 33258. Briefly, 20 μL of Hoechst 33258 was evenly coated on the cells, which were stained at room temperature for 5 min. The cells were washed three times with PBS for 1 min, and then, a fluorescent microscope was used to record the images.

Flow cytometry assay

After trypsin digestion, the cells were collected and centrifuged at 800 rpm for 5 min. Fresh medium (2 mL) was added to resuspend the cells. The cell density was counted and adjusted to 1×10^5/mL by adding fresh culture medium. Next, 100 μL cell suspension was added to each well of the 96-well plate. After the cells were completely attached to the bottom overnight, the drugs were added. The volume of the added drugs was 10% of the volume of the cell culture medium. After 24 h of culture, the cells were washed twice with PBS, centrifuged at 1,000 rpm for 5 min, and the supernatant was discarded. Av/PI staining was performed for cell apoptosis analysis following the instructions provided with the flow cytometry kit.

Western blotting assay

Western blotting was performed following the method used in our previous study, with some

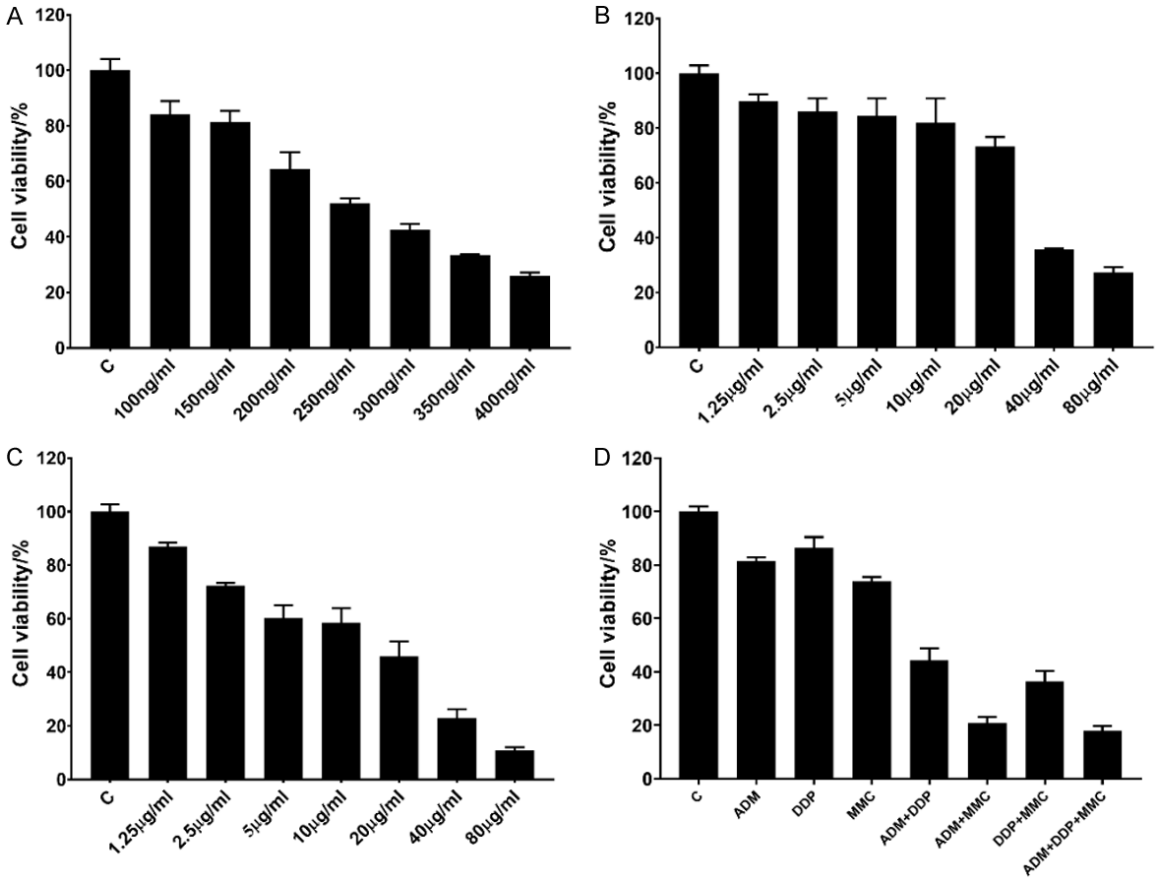

Figure 1. The inhibitory effects of ADM, DDP, MMC, and their combinations on the growth of T24 cells. A: ADM; B: DDP; C: MMC; D: Drug combination.

modifications [6]. Briefly, T24 cells in the logarithmic growth stage were cultured and treated with drugs as described above. Next, the cells were harvested with trypsin, lysed in RIPA supplemented with 0.1% PMSF on ice, and centrifuged at 12,000 *g* for 10 min at 4°C. The supernatants were collected and total protein levels were quantified using the BCA protein assay kit. Then, the cell lysates were mixed with loading buffer and boiled, vortexed, centrifuged, and subjected to SDS-PAGE electrophoresis. Finally, they were transferred onto a PVDF membrane following a standard protocol. After blocking with 5% non-fat milk in TBST (50 mM Tris-HCl at pH 8.0, 150 mM NaCl, 0.1% Tween 20), the membrane was incubated with primary and secondary antibodies, respectively. The signals were detected using an ECL kit and analyzed using the Image J software.

Statistical analysis

Statistical analyses were conducted using SPSS 19.0. The differences between groups were determined by performing a one-way ANOVA. Tukey's multiple comparison test was conducted to determine significant differences among multiple comparisons. All differences among and between groups were statistically significant at $P < 0.05$.

Results and discussion

Inhibitory effects of the three drugs on T24 cells

Bladder cancer is a malignant tumor that affects more than 400,000 patients annually worldwide. The incidence is higher in men than in women [7, 8]. The results of the MTT assay showed that the inhibitory effect of cisplatin combined with doxorubicin was higher than that of cisplatin alone. The half-maximal inhibitory concentration (IC50) values of the ADM, DDP, and MMC alone were 257.7 ng/mL, 32.7 μg/mL, and 10.8 μg/mL, respectively (**Figure 1A-C**). The inhibition results of all the tested

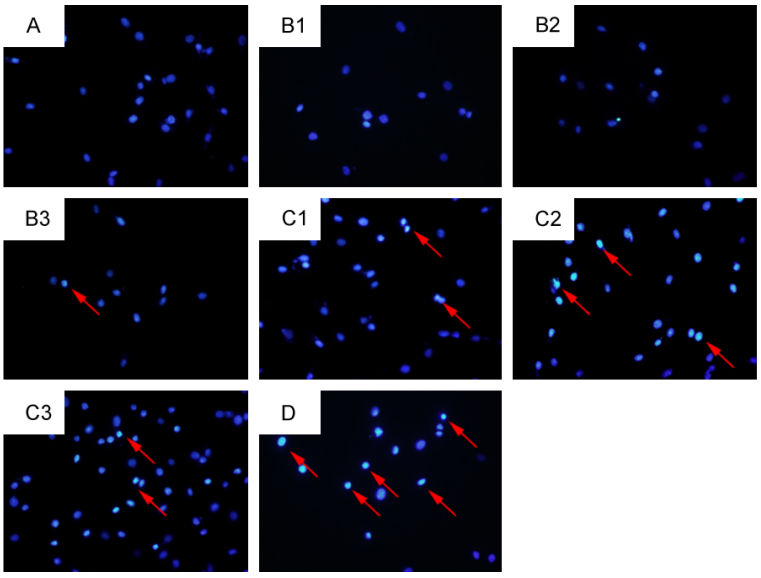

Figure 2. Hoechst 33258 staining of T24 cells treated with ADM, DDP, MMC, and their combinations. A: Control group; B1: ADM; B2: DDP; B3: MMC; C1: ADM + DDP; C2: ADM + MMC; C3: DDP + 2.5 MMC; D: ADM + DDP + MMC. The red arrows show the location of apoptotic bodies.

groups, including ADM (150 ng/mL), DDP (2.5 μg/mL), and MMC (2.5 μg/mL), are shown in **Figure 1D**. The above concentration of each drug was used to form combinational treatment groups, including the ADM + DDP, ADM + MMC, DDP + MMC, and ADM + DDP + MMC groups (**Figure 1D**). The results showed that the inhibitory effect of the combination of ADM + MMC was similar to that of the three drugs, which indicated that these two combinations had the strongest inhibitory effect on T24 cells (**Figure 1D**). This preliminary study also showed a positive correlation between the inhibitory effects of the drugs on bladder cancer cell growth and the doses of the drugs.

Effects of the three drugs and their combinations on cell apoptosis

The recurrence rate of bladder cancer is very high. Although there are several causes of bladder cancer, smoking is one of the main causes [9, 10]. To explain the recurrence, Hoechst 33258 staining followed by fluorescence microscope detection was conducted to evaluate programmed cell apoptosis. The results showed that the inhibitory effects of drug combinations were significantly stronger than that of any single drug in inducing cell apoptosis (**Figure 2**). Moreover, the apoptotic phenotypes of the three-drug combination group were different from those of the two-drug combination groups, which further showed that the inhibitory effect of the drug combination on the growth of T24 cells was better than individual drug treatments.

The flow cytometry assay was conducted to further examine cell apoptosis in T24 cells treated with ADM, DDP, MMC, and their combinations. About 77% of the cells were apoptotic after treatment with the doxorubicin and cisplatin combination, while 70.3% and 1.03% of the cells were apoptotic after treatment with doxorubicin only and cisplatin only, respectively (**Figure 3**). These findings indicated that doxorubicin plays a more important role in inducing the apoptosis of T24 cells. Additionally, the drug combinations induced apoptosis mostly during the late stage of apoptosis (**Figure 3**). We found similar results after administering a combination of drugs to T24 cells, which further confirmed that the drug combinations had strong inhibitory effects on the growth of bladder cancer cells.

Effects of the three drugs and their combinations on Caspase-3 expression in T24 cells

Mitomycin, doxorubicin, and cisplatin inhibit T24 cells mainly by regulating the AQP1, Channel, and Caspase family proteins [11]. Caspase-3 is involved in the inhibition of bladder cancer [12]. The results of the Western blot analysis of t-Caspase-3 and c-Caspase-3 in T24 cells treated with different drug combinations showed that the combination of doxorubicin and mitomycin was significantly more effective than the other combinations (**Figure 4**). The inhibitory effect of cisplatin on T24 Caspase-3 protein expression was significantly lower than the inhibitory effect of the other drugs, and the inhibitory effects of drug combinations were significantly higher than those of any single-drug treatment. Some studies also found that the abnormal expression of some growth-essential genes were associated with a poor prognosis of bladder cancer, and these genes might be potential targets for the precise treatment of bladder cancer [13]. This preliminary

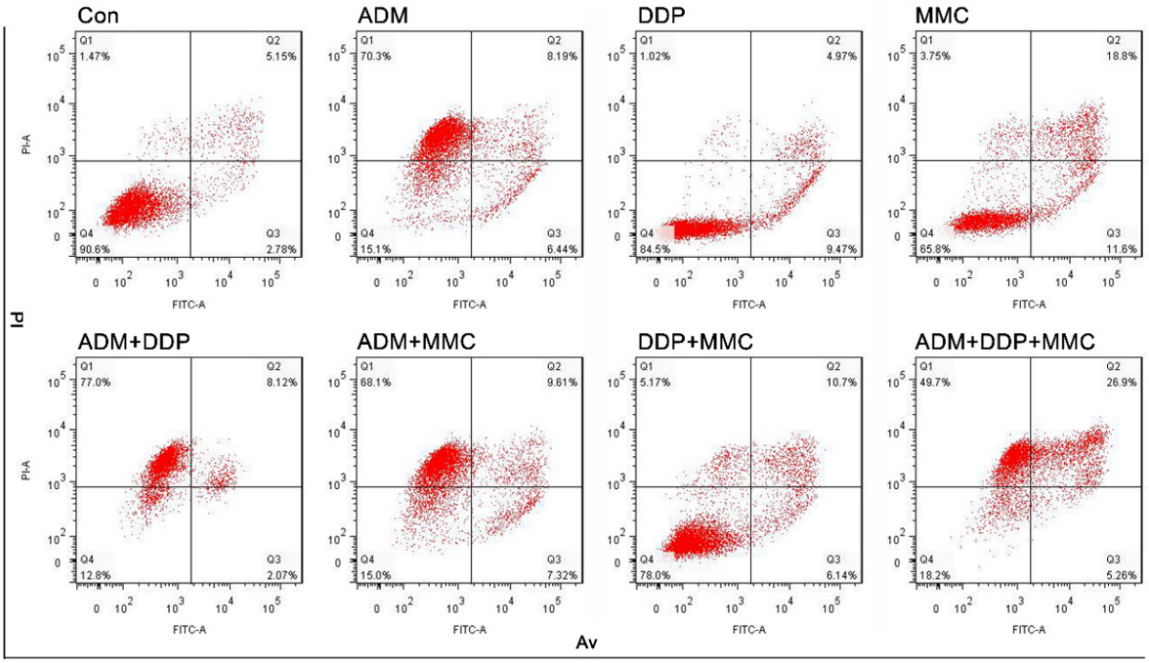

Figure 3. A flow cytometry analysis was performed to evaluate the apoptosis of T24 cells treated with ADM, DDP, MMC, and their combinations.

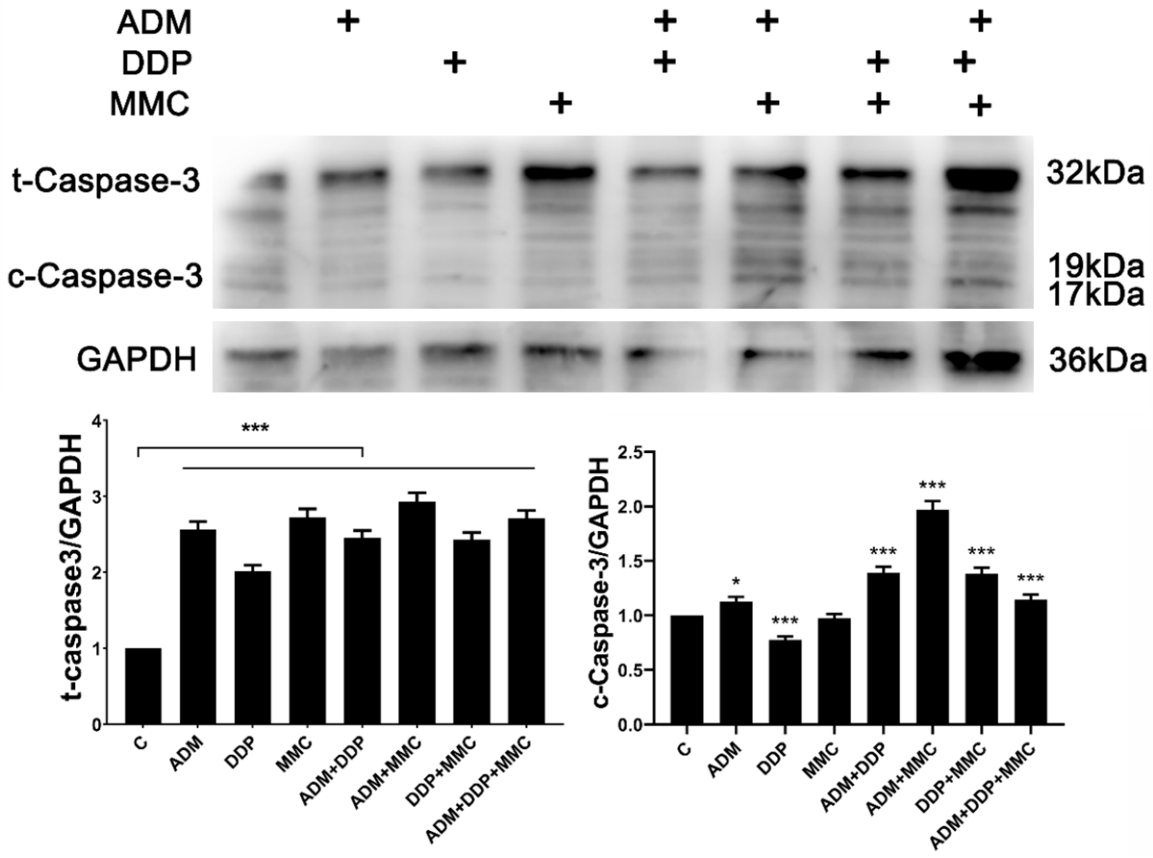

Figure 4. Caspase protein expression in T24 cells treated with ADM, DDP, MMC, and their combinations.

study provided a scientific basis for further research on the mechanism and targeted therapy of bladder cancer.

Conclusions

Bladder cancer indicates an advanced and metastatic disease with high disease specificity and a low overall survival rate. Post-surgical treatment shows a high prognostic recurrence rate of bladder cancer, while chemotherapy with combined drug treatment shows a considerably lower recurrence rate than single-drug treatment [14]. In the therapeutic regimen for most solid tumors, chemotherapy is the best option for treating metastatic diseases. However, bladder cancer is chemo-resistant, and the success of treatment after using multiagent routines, including cisplatin, is modest [8]. Our study revealed that the combined treatment with cisplatin and doxorubicin showed higher inhibitory effects than cisplatin alone. We also showed that the inhibitory effects of the drugs on the growth of the bladder cancer cell lines were in a concentration-dependent and time-dependent manner and might depend on the induction of cell apoptosis. The findings in this study further indicated that stronger tumor inhibition is possible when cisplatin is combined with other chemotherapeutic agents.

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Disclosure of conflict of interest

None.

Abbreviations

ADM, Doxorubicin; DDP, Cisplatin; MMC, Mitomycin; GPED, Gansu Province Education Department; MTT, 3-(4,5)-dimethylthiahiazo(-z-y1)-3,5-diphenytetrazo-Liumromide; CAPF, Chinese Armed Police Force.

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References

[1] Steinmaus CM, Nuñez S and Smith AH. Diet and bladder cancer: a meta-analysis of six dietary variables. Am J Epidemiol 2000; 151: 693-702.

[2] Saginala K, Barsouk A, Aluru JS, Rawla P, Padala SA and Barsouk A. Epidemiology of bladder cancer. Med Sci (Basel) 2020; 8: 15.

[3] Hamdi Y, Abdeljaoued-Tej I, Zatchi AA, Abdelhak S, Boubaker S, Brown JS and Benkahla A. Cancer in Africa: the untold story. Front Oncol 2021; 11: 650117.

[4] Chinese guidelines for diagnosis and treatment of urothelial carcinoma of bladder 2018 (English version). Chin J Cancer Res 2019; 31: 49-66.

[5] Li X, Lu JP, Hu S, Cheng KK, De Maeseneer J, Meng QY, Mossialos E, Xu DR, Yip WN, Zhang HZ, Krumholz HM, Jiang LX and Hu SS. The primary health-care system in China. Lancet 2017; 390: 2584-2594.

[6] Zhao LH, Duan ZY, Wang Y, Wang MZ, Liu Y, Wang X and Li HY. Protective effect of Terminalia chebula Retz. extract against Aβ aggregation and Aβ-induced toxicity in Caenorhabditis elegans. J Ethnopharmacol 2021; 268: 113640.

[7] Alradhi M, Safi M, Tao S, Al-Danakh A, Almoiliqy M, Baldi S and Li X. Surgery improves survival in bladder signet-ring cell carcinoma-a population-based study. Ther Adv Urol 2022; 14: 17562872221079473.

[8] Antoni S, Ferlay J, Soerjomataram I, Znaor A, Jemal A and Bray F. Bladder cancer incidence and mortality: a global overview and recent trends. Eur Urol 2017; 71: 96-108.

[9] Park JY. Chapter 13 - Risk factors for recurrence and progression of nonmuscle invasive bladder cancer. In bladder can*cer*. In: Ku JH, editor. Academic Press: 2018. pp. 187-199.

[10] Mari A, Campi R, Tellini R, Gandaglia G, Albisinni S, Abufaraj M, Hatzichristodoulou G, Montorsi F, van Velthoven R, Carini M, Minervini A and Shariat SF. Patterns and predictors of recurrence after open radical cystectomy for bladder cancer: a comprehensive review of the literature. World J Urol 2018; 36: 157-170.

[11] Guo H, Li FP, Xu WG, Chen JJ, Hou YC, Wang CX, Ding JX and Chen XS. Mucoadhesive cationic polypeptide nanogel with enhanced penetra-

tion for efficient intravesical chemotherapy of bladder cancer. Adv Sci (Weinh) 2018; 5: 1800004.
[12] Adamo A, Dal Collo G, Bazzoni R and Krampera M. Role of mesenchymal stromal cell-derived extracellular vesicles in tumour microenvironment. Biochim Biophys Acta Rev Cancer 2019; 1871: 192-198.
[13] Amala A and Emerson IA. Identification of target genes in cancer diseases using protein-protein interaction networks. Netw Model Anal Health Inform Bioinform 2019; 8: 2.
[14] Yang LS, Shan BL, Shan LL, Chin P, Murray S, Ahmadi N and Saxena A. A systematic review and meta-analysis of quality of life outcomes after radical cystectomy for bladder cancer. Surg Oncol 2016; 25: 281-97.