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Original Article

Downregulation of long non-coding RNA *UCA1* is associated with the anticancer effects of isorhapontigenin on human bladder cancer cells

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Abstract: Isorhapontigenin (ISO), the dominant active compound of the Chinese herb *Gnetum cleistostachyum*, has been shown for hundreds of years to exhibit several significant anti-cancer activities, and could possibly inhibit the proliferation of bladder cancer cells. However, details regarding the mechanism of its action are not well defined, especially regarding its regulation of the long non-coding RNA (lncRNA) of urothelial carcinoma antigen 1 (UCA1). To address this issue, human bladder cancer cell strain 5637 was chosen, and cell proliferation activity was assayed with a gradient of ISO for different treatment durations to screen for the optimal treatment concentration and duration. Subsequently, the mRNA expression level of UCA1 was assayed by real-time quantitative polymerase chain reaction (RT-qPCR), cell cycle was assayed by flow cytometry, cell migration activity was assayed by a cell wound healing test, and matrix metalloproteinase (MMP)-2 and MMP-9 expression level was determined by RT-qPCR and western blot analysis. As expected, with increasing concentrations of ISO, the survival rate of 5637 cells was significantly decreased, and treatment with 20 μM ISO for 72 h was validated as an optimal condition. After treatment with 20 μM ISO for 72 h, the mRNA expression level of UCA1 was significantly decreased, the cell cycle was significantly altered with a significant increase in the percentage of cells in G0/G1 phase, and a significant decrease in those in S and G2/M. Moreover, cell migration capacity was significantly decreased, and the mRNA and protein expression levels of MMP-2 and MMP-9 was significantly decreased. These results indicate that ISO could inhibit the proliferation of human bladder cells via downregulation of lncRNA of UCA1, and further downregulation of the cell cycle, cell migration, MMP-2, and MMP-9, not only providing a significant reference for studying the mechanism of human bladder cancer treatment, but also providing potential clinical value.

Keywords: Isorhapontigenin, bladder cancer cells, long non-coding RNA, urothelial carcinoma antigen 1, cell proliferation

Introduction

Bladder cancer is the ninth most common type of cancer in the world and the first most common urological cancer in China, with approximately 3.6×10^5 new cases every year [1-3]. Seventy to eighty-five percent of newly diagnosed cases are superficial tumors, which are mainly treated by transurethral resection of the bladder tumor (TURBT) [4-6]. However, the rate of tumor recurrence is high following the surgery. Several drugs are urgently needed to prevent tumor recurrence after TURBT which produces severe complications such as frequent micturition, urgent micturition, dysuria, hematuria, immune suppression, etc. [7-9]. Therefore, use of less complicated drugs that inhibit the proliferation and metastasis of bladder cancer cells is a novel method for the treatment of bladder cancer. Isorhapontigenin (ISO) is a new natural oligostilbene isolated from the Chinese herb *Gnetum cleistostachyum*, which has widespread pharmacological actions including anti-bladder cancer activity [10-12]. Previous research has clarified that active compound ISO could inhibit bladder carcinoma cell growth by downregulating cyclin D1 expression, however the detailed mechanism is still unknown [13-16].

Long non-coding RNAs (lncRNAs) are non-protein coding transcripts longer than 200 nucleo-

tides, and recent studies have demonstrated that lncRNAs are crucial for the regulation of epigenetics, cell cycle, and cell differentiation [17-21]. Furthermore, lncRNAs have been found to be involved in the pathogenesis of disease and development of cancer [22]. Urothelial carcinoma antigen 1 (UCA1) is an lncRNA which is commonly over-expressed in the human bladder transitional cell line BLZ-211 [20]. UCA1 is a potential biomarker in the urine for the diagnosis and prognosis of bladder carcinoma, and could promote the proliferation and tumorigenesis of bladder cancer [19, 20]. To further elucidate the mechanism of action of UCA1 in bladder cancer, human bladder cancer cell strain 5637 was treated at different time points with varying concentrations of ISO. The 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was used to determine optimal ISO concentrations and duration of treatment. Reverse transcription-polymerase chain reaction (RT-PCR) was used to detect UCA1 gene expression under optimal ISO treatment conditions, and flow cytometry (FCM) and wound healing assays were used to detect changes in cell cycle and migration capacity as a result of ISO treatment. Moreover, RT-qPCR and western blot analysis was used to determine the protein expression levels of matrix metalloproteinase (MMP)-2 and MMP-9 under different concentrations of ISO.

Materials and methods

Cell strains and culturing

The 5637 cell strain, which originated from human bladder cancer tissue, was prepared in our laboratory, and resurrected from liquid nitrogen using Roswell Park Memorial Institute-1640 medium (RPMI-1640, Hyclone, USA) supplemented with 10% (v/v) fetal bovine serum, and cultured to the logarithmic phase in a carbon dioxide (CO_2)-incubator with 5% CO_2 at 37°C for further usage.

Treatment of isorhapontigenin (ISO) on human bladder cancer cell strain 5637

The above-cultured 5637 cells were diluted to 5×10^3/ml, and inoculated on a 96 well-plate, and cultured in a CO_2-incubator with 5% CO_2 at 37°C until 60-70% confluence was reached. Subsequently, a total of 0.5 M ISO (Sigma, USA) was diluted, and incubated with 5637 cells at a final concentration of 0, 5, 10, 20, 40, or 60 μM. After incubation for 24, 48, or 72, cells were gathered for further assaying in triplicate.

Cell proliferation activity assay by 3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-2-H-tetrazolium bromide (MTT)

The above-gathered 5637 cells were transferred to a total of 80 μL no serum medium with 20 μL MTT (finally concentration: 5 mg/ml), and cultured in a CO_2-incubator with 5% CO_2 at 37°C for 4 h. Then, a total of 150 μL dimethyl sulfoxide (DMSO) was added to each well, and incubated for 10 min with shaking. Subsequently, the optical density (OD) at 490 nm was recorded with a microplate reader (Bio-Rad, USA), and the cell survival rate was calculated.

Real time quantitative polymerase chain reaction (RT-qPCR) assay

The optimal ISO concentration and culturing time was confirm based on the cell proliferation activity as above-mentioned, and cells were collected to extract the total RNA according to the manufacturer's instructions of a Total RNA Extraction Kit (Tiagen, Beijing, China); the purity and concentration of total RNA was confirmed. Then, the total RNA was reversely transcribed with a Reverse Transcription Kit (Toyobo, Japan) according to the manufacturer's instructions. The reaction mixture, including 10 μL 2× loading buffer, 1.2 μL oligo (dT), 2μL RNA, 0.2 μL MMLV, and 6.6 μL DEPC ddH_2O, was prepared and reacted at 65°C for 30 min, followed by 42°C for 30 min, and then by 85°C for 10 min. Subsequently, a total of 100 ng cDNA was used as a template in a RT-qPCR reaction using the SYBR® Premix Ex Taq™ kit (TAKARA, Japan), according to the manufacturers' instructions. The reaction mixture, including 12.5 μL 2× Primer Taq Mix, 1 μL forward primer, 1 μL reverse primer, 1 μL cDNA, and 9.5 μL ddH_2O, was prepared and the RT-qPCR was performed according to the following program: one cycle of 94°C for 5 min; 40 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 30 s, using the following primers: UCA1: pU: 5'-CTCTCCATTGGGTTCACCATTC-3' and pD: 5'-GCGGCAGGTCTTAAGAGATGAG-3'; 18S RNA: pU: 5'-CAGCCACCCGAGATTGAGCA-3' and pD: 5'-TAGTAGCGACGGGCGGTGTG-3'; The results were analyzed using the SDS 1.4 software (Applied Biosystems) based on $2^{-\Delta\Delta Ct}$, and histogram analysis was performed using the Origin 9.5 software (http://www.originlab.com/).

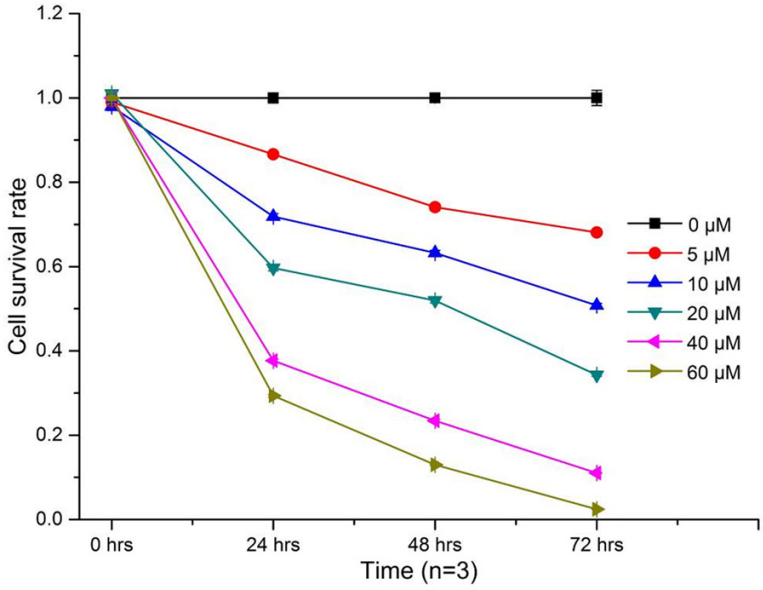

Figure 1. Cell proliferation assay of human bladder cancer cells by MTT. The image indicates that with increasing concentration of ISO, the cell survival rate was significantly decreased (*: $P < 0.05$; **: $P < 0.01$).

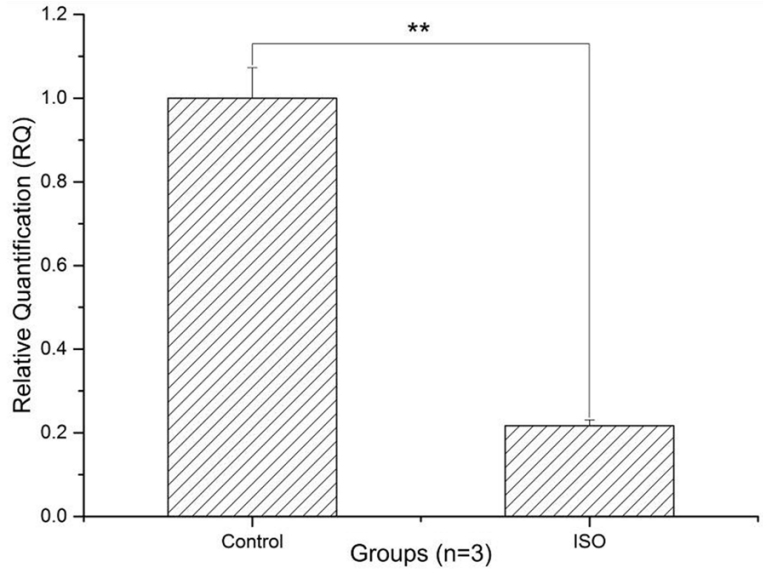

Figure 2. The mRNA expression level assay of UCA1 by RT-qPCR. The image indicates that after treatment with 20 μM ISO for 72 h, the mRNA expression level of UCA1 was significantly decreased (**: $P < 0.01$).

Additionally, for the RT-qPCR assay of MMP-2 and MMP-9, the following primers were used: MMP-2: pU: 5'-TCTGTGTTGTCCAGAGGCAA-3' and pD: 5'-TTGAAGCCAAGCGGTCTAAGT-3'; MMP-9: pU: 5'-CCTGGGCAGATTCCAAACCT-3' and pD: 5'-GTACACGCGAGTGAAGGTGA-3'; β-actin: pU: 5'-GTCATTCCAAATATGAGATGCGT-3' and pD: 5'-GCTATCACCTCCCCTGTGTG-3'. The above mentioned approach was followed.

Cell cycle assay by flow cytometry (FCM)

After treatment with ISO, 5637 cells were harvested and fixed with a total of 3 ml 75% ethanol overnight, and then centrifuged at 1500 rpm for 5 min before the supernatant was removed and washed twice with PBS (5 min/time). Subsequently, a total of 100 μL Rnase was added, the cells were incubated at 37°C for 30 min, and then a total of 400 μL propidium iodide (PI) was added and incubated at 4°C for 30 min without light. Subsequently, the cells were detected by FCM, and analyzed by Flow Jo 10.0 software (http://www.flowjo.com/).

Cell wound healing assay

For the cell wound healing assay, 5637 cells were cultured with normal culture medium until 70-80% confluence was reached. Subsequently, a wound (about 1 cm) was created by sterile tips, and carefully washed with serum-free medium to remove redundant cells. The above-selected optimal concentration of ISO was added on the wound to dry, and the cells were incubated in 5% CO_2 at 37°C for 0, 12, or 24 h. Images were collected to calculate the cell migration capacity.

Western blot assay

The total protein of the above-intervened 5637 cells was prepared, and measured by the BCA protein quantification kit (ZKP-C150045-1, Suzhou Zeke biotech Co., LTD, China). Roughly 35 mg total protein was fractionated by electrophoresis through 12.5% polyacrylamide gels, and transferred to polyvinylidenedifluoride (PVDF) membranes (GE Healthcare) following the manufacturers' instructions. The membrane was probed with the primary antibody, anti-MMP-2

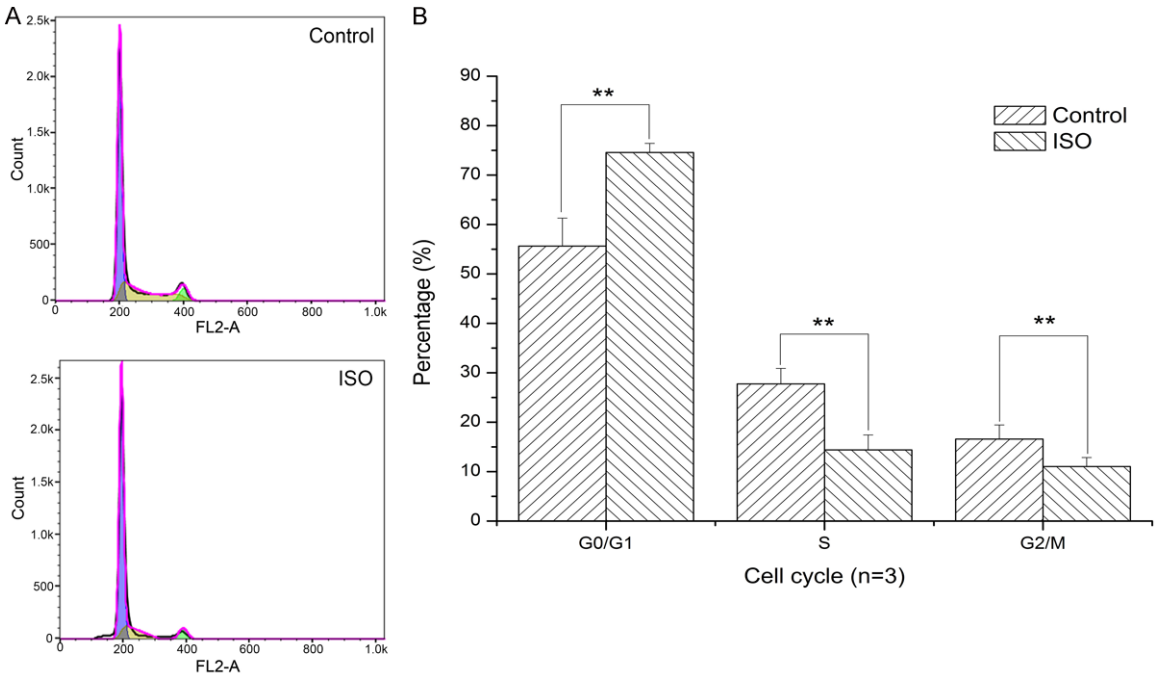

Figure 3. Cell cycle assay of human bladder cells by flow cytometry (FCM). The image indicates that after treatment with 20 μM ISO for 72 h, the cell cycle was altered with a significant increase in the number of cells in the G0/G1 phase, and a significant decrease in those in the S and G2/M phases (**: $P < 0.01$).

polyclonal antibody (ab37150, Abcam, USA), anti-MMP-9 polyclonal antibody (ab38898, Abcam, USA), or anti-β-actin monoclonal antibody (ab8226, Abcam, USA) for 1.5 h at room temperature. Subsequently, the membrane was incubated with horseradish peroxidase-conjugated goat anti-mouse secondary antibody (1:5,000 in TBST; Beijing Golden Bridge Biotechnology Company Ltd, China) at room temperature for 1 h. The chemiluminescence luminol reagent (ZKP-C150044-1, Suzhou Zeke biotech Co., LTD, China) was used to develop the immune-labeled bands on X-ray film, the optical density of the bands was quantified using the ImageJ 1.46 software (http://rsb.info.nih.gov/ij/download.html), and a histogram was generated using the Origin 9.5 software (http://www.originlab.com/).

Statistical analysis

All data are expressed as the mean ± standard deviation (SD). Statistical analysis was performed with a one-way ANOVA using SPSS software (version 21.0, http://spss.en.softonic.com/; Chicago, IL, USA), and Student's *t*-tests were performed in a group of two samples; $P < 0.05$ and $P < 0.01$ were considered to indicate significant differences and highly significant differences, respectively.

Results

Isorhapontigenin (ISO) could significantly inhibit the proliferation of human bladder carcinoma cells

To elucidate the inhibition of ISO on the proliferation of human bladder carcinoma cells, an ISO gradient panel, including 0, 5, 10, 20, 40, and 60 μM, was created, and the cell proliferation activity was assayed by MTT. As shown in **Figure 1**, when compared with the survival rate of human bladder carcinoma cells with 0 μM ISO treatment, the survival rate of human bladder carcinoma cells was significantly decreased with increasing concentrations of ISO, and after 24 h, the survival rates were 90, 75, 60, 40, and 30% for treatment with 5, 10, 20, 40, and 60 μM ISO, respectively. Similarly, after 48 h and 72 h of treatment, the survival rate of human bladder carcinoma cells was further decreased, and 20 μM ISO at 72 h treatment could inhibit roughly 60% of human bladder carcinoma cells, and therefore, this was chosen as the optimal treatment condition to be used in further evaluations.

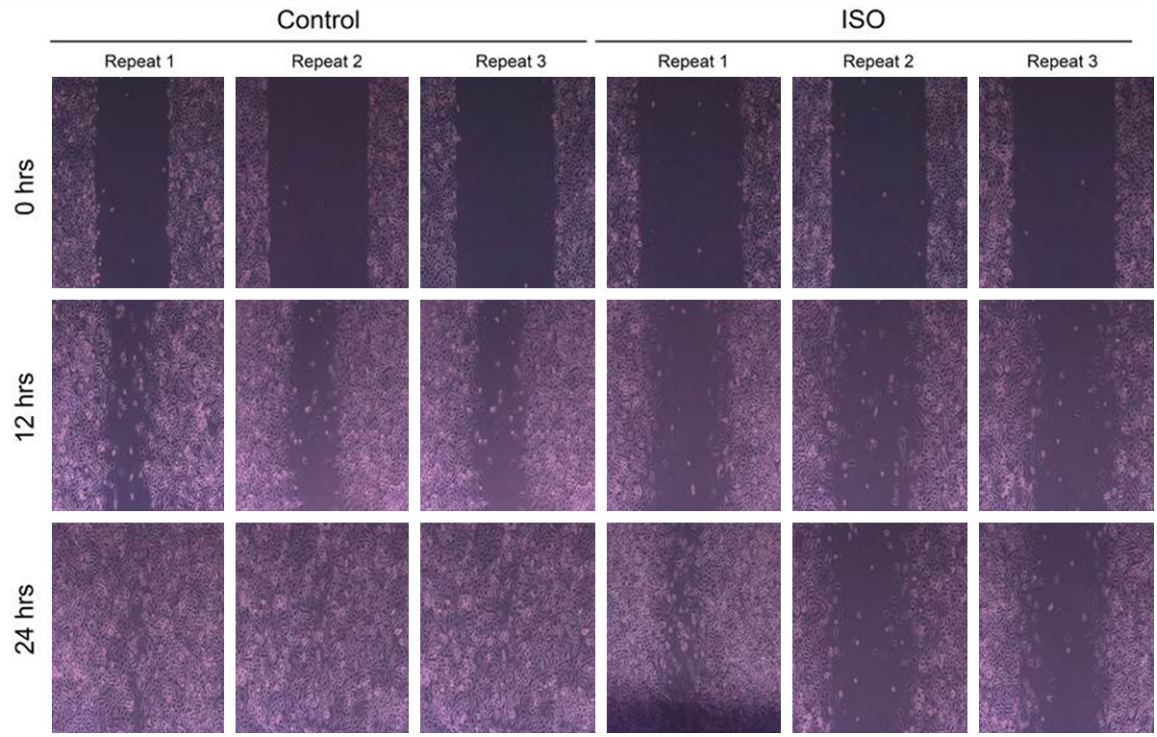

Figure 4. Cell migration capacity assay by cell wound healing. The images indicated that with prolonged culture time, the cell migration capacity was significantly decreased when compared with that of the control after treatment with 20 μM ISO (**: $P < 0.01$).

The mRNA expression level of UCA1 was significantly decreased following treatment with 20 μM ISO for 72 h

To elucidate the change in UCA1 expression after ISO treatment, RT-qPCR was chosen. After treatment with 20 μM ISO for 72 h, the UCA1 expression level was significantly decreased to the one fifth that of the control (**: $P < 0.01$, **Figure 2**).

The percentage of cells in the G0/G1 stage cells was significantly increased, and that of those in the S and G2/M staging cells was significantly decreased

To evaluate the alteration in cell cycle, human bladder carcinoma cells were collected to perform FCM, and the detailed information is shown of **Figure 3A** and **3B**. When compared with the control, the number of cells in the G0/G1 stage was significantly increased (**: $P < 0.01$), and the number of those in the S and G2/M stages was significantly decreased (**: $P < 0.01$).

Cell migration capacity was significantly decreased after treatment with 20 μM ISO for 72 h

In order to explore the change in cell migration capacity, the wound healing experiment was chosen. As the wound healing results in **Figure 4** show, as the incubating time increased, cells rapidly grew into the wound healing region, eventually completely covering the wound healing region after 24 h of culture in the control. However, cell migration capacity was significantly decreased with prolonged culturing time for cells treated with 20 μM ISO when compared with that of the control.

MMP-2 and MMP-9 expression levels were significantly decreased with the increasing concentrations of ISO

To elucidate the change in MMP-2 and MMP-9 expression after ISO treatment in human bladder carcinoma cells, the mRNA and protein expression levels were assayed by RT-qPCR and western blot, respectively. As shown in **Figure 5A** and **5B**, after treatment with 20 μM

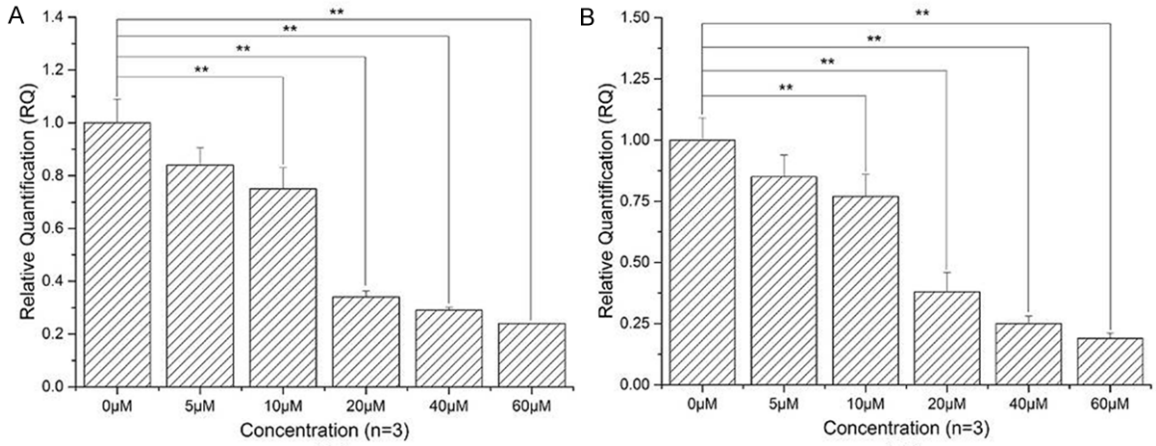

Figure 5. MMP-2 and MMP-9 mRNA expression level assay by RT-qPCR. A. RT-qPCR assay of the MMP-2 mRNA expression level. B. RT-qPCR assay of the MMP-9 mRNA expression level. The images indicate that with an increase in concentration of ISO, the MMP-2 and MMP-9 mRNA expression level was significantly decreased (*: $P < 0.05$; **: $P < 0.01$).

ISO for 72 h, the MMP-2 and MMP-9 mRNA expression level was significantly decreased (**: $P < 0.01$). Similarly, as shown in **Figure 6A-C**, the MMP-2 and expression level was significantly decreased after treatment with 20 μM ISO for 72 h, and continuously decreased with increasing ISO concentration; the histogram exhibited the same results (*: $P < 0.05$; **: $P < 0.01$).

Discussion

In the present study, the MTT assay demonstrated that ISO could inhibit the growth of human 5637 cells, and treatment with 20 μM ISO with 72 has an optimal inhibition condition. Subsequently, RT-PCR analysis showed that the mRNA expression level of UCA1 in the ISO treatment group was significantly decreased compared with that of the control. FCM analysis showed that the cell cycle in the ISO treatment group was significantly altered with a significant increase in the number of cells in the G0/G1 phase and a significant decrease of those in the S and G2/M phase. The cell wound healing assay results showed that the cell migration capacity in the ISO treatment group was significantly decreased compared with that of the control group. Furthermore, RT-qPCR and western blot showed that the expression of MMP-2 and MMP-9 was significantly decreased with an increase in ISO concentrations when compared with that of the control group.

Resveratrol is a natural compound extracted in Chinese herbal medicine that has diverse biological activities [23]. In recent years, more and more attention has been focused on this type of compound due to its novel anti-cancer activity [11, 24-26]. ISO is a new resveratrol analog that was isolated from the Chinese herb *Gnetum cleistostachyum*. The latest research has revealed that ISO could inhibit bladder cancer UMUC3 cell growth via downregulation of Cyclin D1 [14]. However, the mechanism of action of ISO treatment was not fully understood. To evaluate the optimal inhibition concentration of ISO in human bladder cancer, we first examined the effects of ISO on bladder cancer 5637 cells, and found that the survival rate of 5637 cells was significantly decreased with prolonged treatment time. Similarly, the survival rate of 5637 cells was significantly decreased with increasing ISO treatment concentrations. In the follow-up study, we chose the optimal treatment condition as 20 μM ISO for 72 h.

LncRNAs are a large and diverse kind of transcript RNA molecule with a length of more than 200 nucleotides that do not encode proteins [27]. However, more and more evidence has shown that several lncRNAs are involved in the pathogenesis of diseases including in cancer development, due to their function in the regulation of chromatin, gene expression, and translation control [28]. UCA1 is a kind of lncRNA which regulates key cancer pathways at transcriptional, post-transcriptional, and epigenetic levels [29]. As our previous study demonstrated, over-expression of UCA1a could upregulate FZD5, WNT5A, ARNT2, PDGFB, and

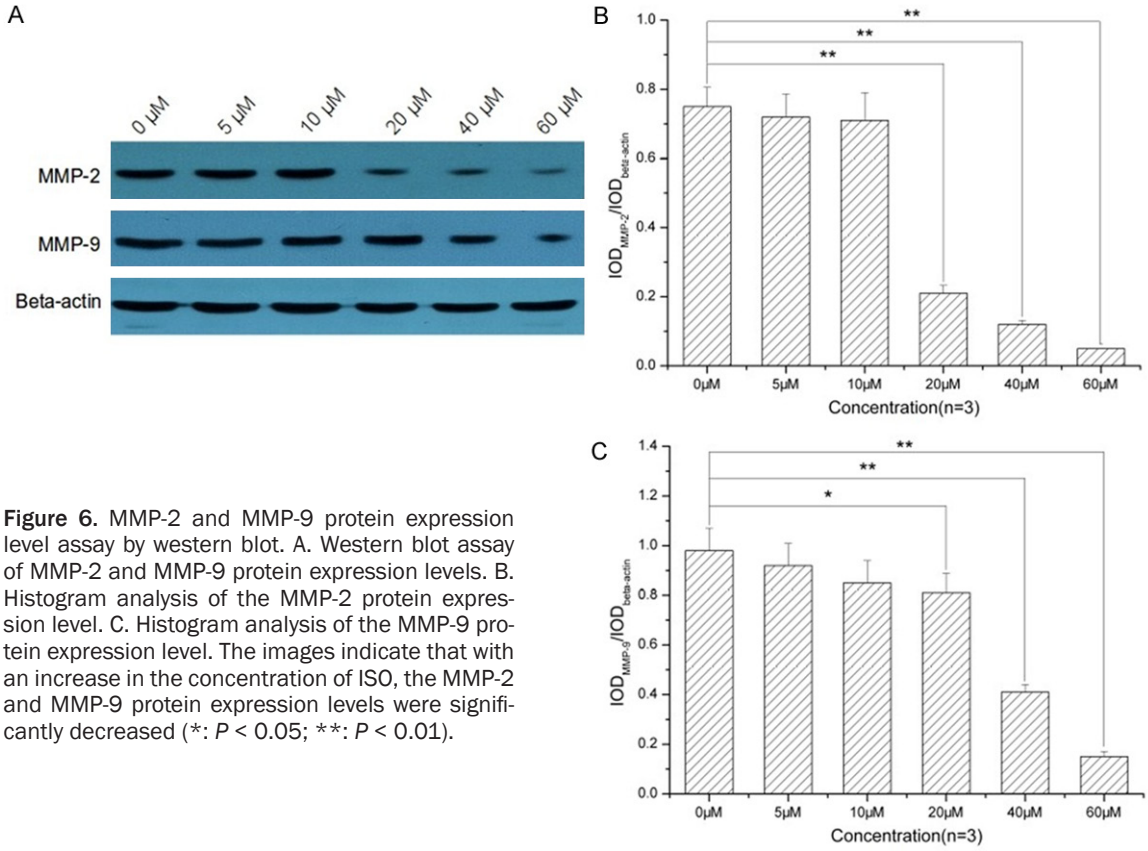

Figure 6. MMP-2 and MMP-9 protein expression level assay by western blot. A. Western blot assay of MMP-2 and MMP-9 protein expression levels. B. Histogram analysis of the MMP-2 protein expression level. C. Histogram analysis of the MMP-9 protein expression level. The images indicate that with an increase in the concentration of ISO, the MMP-2 and MMP-9 protein expression levels were significantly decreased (*: $P < 0.05$; **: $P < 0.01$).

LAMA3 by at least 2-fold [30]. Nevertheless, a few studies have indicated a role of UCA1 in bladder cancer progression. In our study, RT-PCR results showed that the mRNA expression level of UCA1 was significantly decreased after treatment with 20 μM ISO for 72 h, which indicated that ISO could inhibit the proliferation of human bladder cells via downregulation of UCA1 lncRNA.

The cell cycle and cell migration are a vital biological process which is influenced by the occurrence and metastasis of tumors [31]. Exploration of the changes in cell cycle phase and cell migration capacity is a fundamental method to evaluate the effect of drugs. In our study, the FCM cell cycle assay showed that the cell cycle in the ISO treatment group was altered with a significant increase in the number of cells in the G0/G1 phase and a significant decrease in those in S and G2/M phases compared with that of the control. The cell wound healing assay showed that cells gradually grew into the wound healing region with prolonged incubation time and the cell migration capacity in the ISO treatment group was significantly decreased compared with that of the control group.

Matrix metalloproteinases (MMPs) are a family of calcium-dependent zinc-containing endopeptidases that are involved in various physiological and pathological processes [32-34]. MMP-2 and MMP-9 are MMP family members which play major roles in cell behaviors such as cell proliferation, migration, differentiation, angiogenesis, apoptosis, and host defense [35, 36]. Several studies have shown that MMP-2 and MMP-9 are overexpressed in varied tumors and are associated with their biological behavior [37, 38]. In our study, western blot analysis showed that protein expression of MMP-2 and MMP-9 was significantly decreased with increasing ISO concentrations. Therefore, our study indicated that ISO could inhibit the proliferation of human bladder cells by decreasing the expression of MMP-2 and MMP-9.

In summary, the present study demonstrated that ISO inhibits bladder carcinoma cell growth via downregulation of UCA1 lncRNA, which is subsequently followed by further downregula-

tion of the cell cycle phases, cell migration capacity, and MMP-2 and MMP-9 expression. Obviously, there are some limitations to this study, such as the limited sample size, and the analysis was only at the cellular level. Future studies will further explore the target proteins of UCA1 using the RNA pull down method, and evaluate the mechanisms of action of ISO in animal experiments.

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Disclosure of conflict of interest

None.

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