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The Effects of Orientin on Proliferation and Apoptosis of T24 Human Bladder Carcinoma Cells Occurs Through the Inhibition of Nuclear Factor-kappaB and the Hedgehog Signaling Pathway

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Data Interpretation D
Manuscript Preparation E
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Background: Orientin is a flavone isolated from medicinal plants used in traditional Chinese medicine (TCM), which suppresses the growth of cancer cells *in vitro*. The effects of orientin in bladder cancer cells remains unknown. This study aimed to investigate the effect of orientin on proliferation and apoptosis of T24 human transitional cell bladder carcinoma cells *in vitro* in the presence of an agonist and an inhibitor of nuclear factor-kappaB (NF-κB).

Material/Methods: T24 cells were cultured and divided into four study groups: an untreated control group; a group treated with 100 μM orientin; a group treated with 100 μM orientin with NF-κB agonist, phorbol 12-myristate 13-acetate (PMA); and a group treated with 100 μM orientin and the NF-κB inhibitor, IκBα. The MTT assay was performed to assess cell viability, and flow cytometry evaluated the cell cycle. The expression of proteins in the Hedgehog signaling pathway and inflammatory cytokines were determined by Western blot and enzyme-linked immunosorbent assay (ELISA).

Results: Orientin inhibited the proliferation of T24 cells, caused cell cycle arrest, reduced cell viability, and inhibited the expression of inflammatory mediators. Treatment of T24 cells with orientin inhibited the expression of NF-κB and components of the Hedgehog signaling pathway, and the NF-κB agonist, PMA, reversed these effects.

Conclusions: Treatment of T24 human bladder carcinoma cells *in vitro* with orientin inhibited cell proliferation and promoted cell apoptosis by suppressing the Hedgehog signaling pathway and NF-κB.

MeSH Keywords: Apoptosis • Cell Proliferation • Urinary Bladder Neoplasms

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Background

Worldwide, transitional cell carcinoma of the bladder, or bladder cancer, is a common malignancy with high morbidity, particularly in male patients. The tumor is classified histologically and superficial, without muscle invasion, and muscle-invasive [1–3]. Invasive bladder cancer has a high recurrence rate. It is important for patients who have been treated surgically with transurethral resection to attend regular follow-up examinations in the months following surgery.

The predisposing factors for bladder cancer include exposure to industrial chemicals, smoking, and increasing age [4,5]. Treatment for bladder cancer includes radiation, chemotherapy, immunotherapy, localized surgery, and radical cystectomy with urinary diversion [5–8]. Currently, research on bladder cancer has resulted in some progress in improvements in treatment [9]. However, the survival rates for patients with bladder cancer have remained unchanged for the past three decades [10]. There is a need for improvements in the prevention, diagnosis, and treatment of bladder cancer as patient survival rates need to improve. Radiation therapy and chemotherapy are associated with side effects, and the discovery for new drugs with high efficacy and low toxicity are still required.

Drug discovery requires an understanding of the underlying mechanism in cancer. Signaling pathways, including nuclear factor-kappaB (NF-κB) signaling, play an important role in bladder cancer, as tumor cell proliferation is suppressed by NF-κB *in vitro* and *in vivo* [11]. Some factors that inhibit NF-κB signaling pathways may affect tumor cell proliferation and migration, including epigallocatechin-3-gallate (EGCG), which downregulates NF-κB, and nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα), which is an inhibitor of NF-κB. The topical effect of Bacillus Calmette-Guérin (BCG) in bladder cancer is enhanced by curcumin via downregulation of NF-κB [12]. Also, Hedgehog signaling is associated with the progression of bladder cancer [13]. Therefore, the NF-κB signaling pathway and the Hedgehog signaling pathway in bladder cancer were chosen for further investigation in this study.

Traditional Chinese medicine (TCM) has been used for centuries to treat human disease. Compounds extracted these medicines and herbs have been developed successfully in modern clinical practice, including paclitaxel, vinblastine, camptothecin, and artemisinin. Orientin is a flavonoid C-glycoside extracted from herbs and plants, including *Passiflora edulis*, millet, and bamboo leaves [14,15]. Orientin possesses a variety of biological activities in cancer, inflammation, and as an antioxidant [16–18]. Anticancer effects have been shown by flavonoid C-glycosides [19], and cell migration in human breast cancer cells was inhibited *in vitro* by orientin [20]. However, the effects of orientin on bladder cancer remain unknown. Therefore, the aim of this study was to investigate the effect of orientin on proliferation and apoptosis of T24 human transitional cell bladder carcinoma cells *in vitro* in the presence of an agonist, phorbol 12-myristate 13-acetate (PMA), and an inhibitor, IκBα, of NF-κB.

Material and Methods

Cell culture

T24 human transitional cell bladder carcinoma cells were purchased from Cobioer (Nanjing, China). Cells were cultured in a 96-well culture plate at 1×10^5 cells/ml in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin, at 37°C in a humidified incubator with 5% CO_2.

T24 cells were cultured and divided into four study groups: an untreated control group; a group treated with 100 μM orientin; a group treated with 100 μM orientin with NF-κB agonist, phorbol 12-myristate 13-acetate (PMA) (10 ng/ml) (Sigma-Aldrich, St. Louis MO, USA); and a group treated with 100 μM orientin and the NF-κB inhibitor, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (IκBα).

MTT assay

T24 cells in the logarithmic growth phase were digested using trypsin. Then, 1 mL of complete medium was added to terminate digestion. The cell suspension was centrifuged, and the cells were collected. After cell counting, the cell density was adjusted to 3.5×10^4 cells/ml. Then, 100 μl cells were seeded into 96-well culture plate. After the cells became adherent, orientin at concentrations of 10, 20, 50, 100, 500, and 1000 μM were added to the culture medium, and 10 μL of MTT was added to each well of the cells at 24 h, 48 h, and 72 h. After treated with MTT, cells were cultured for another 4 h. A RNE90002 microplate reader (REAGEN LLC., Moorestown, NJ, USA) was used to measure the absorbance.

Cell cycle detection

The cells at the logarithmic growth phase were digested by 0.5 ml of 0.25% pancreatic enzymes. After centrifuging, the cells were collected and adjusted to 1×10^5 cells/ml, and 100 μl of cells were added to six-well plates. Orientin, at increasing doses of 10, 20, 50, 100, 500, and 1000 μM, were added to the culture medium. After 72 h, the cells were trypsinized and washed three times in PBS. The cells were suspended in PBS with 50 μg/ml of propidium iodide (PI) (Sigma-Aldrich, St. Louis MO, USA) and 100 μg/ml of RNase A. The cells were

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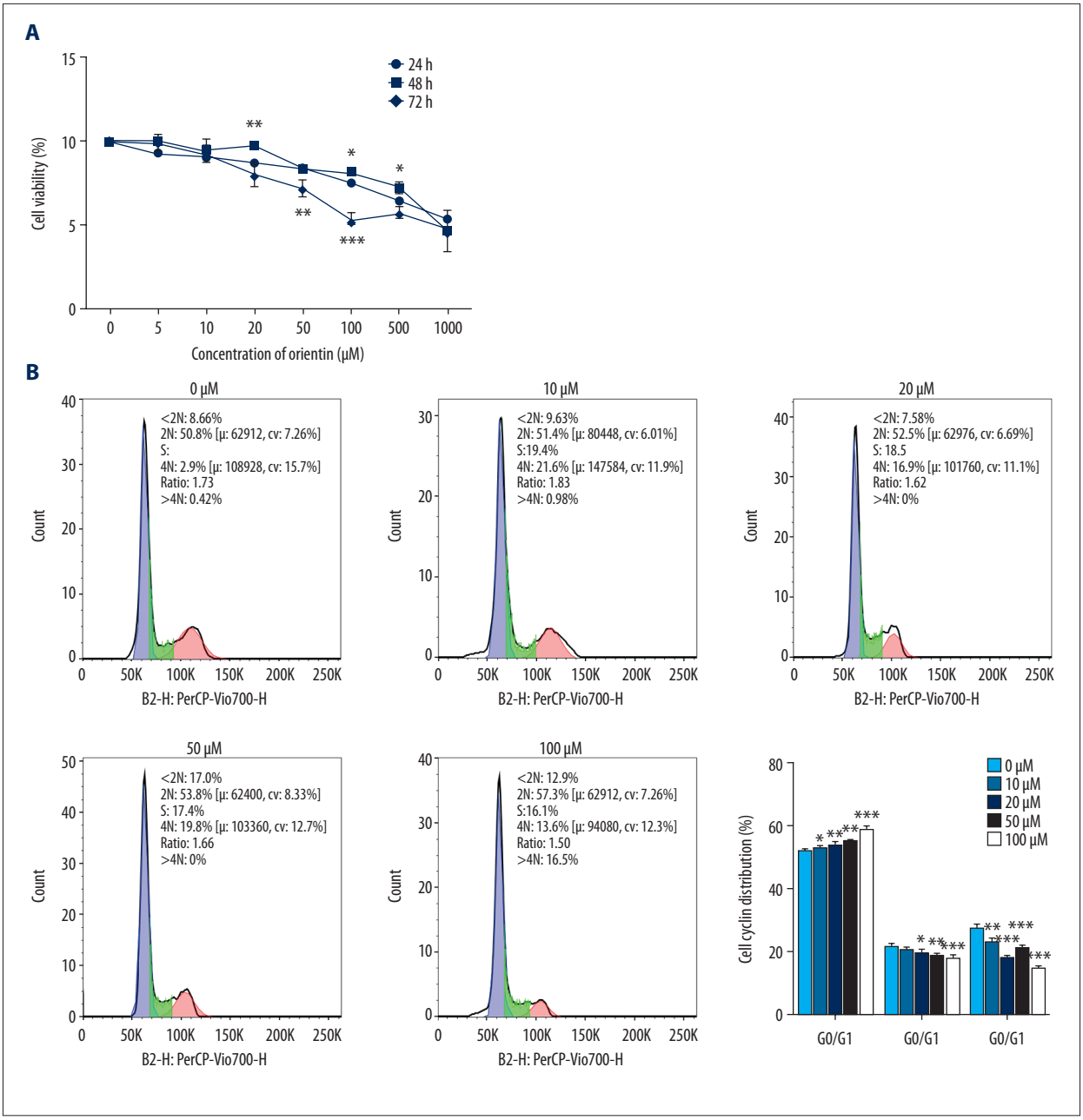

Figure 1. The effects of orientin on cell viability and the cell cycle in T24 human bladder carcinoma cells *in vitro*. The effect of orientin on cell viability at 24 h, 48 h, and 72 h (**A**) and on the cell cycle (**B**). * P<0.05, ** P<0.01 and *** P<0.001 *vs.* the control group (0 μM).

incubated at 4°C in the dark for 30 minutes. Flow cytometry was performed using the BD FACSCalibur flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The results were analyzed by FlowJo version 10 software.

The expression of tumor necrosis factor α (TNFα), interleukin-1 (IL-1), and IL-6

After the cells were treated with increasing concentrations of orientin, the level of TNFα, IL-1, and IL-6 in the supernatants of the T24 cells were quantified using enzyme-linked immunosorbent assay (ELISA) kits, according to the manufacturer's instructions. The cells in the different groups were lysed and then centrifuged to obtain the supernatants. The levels of

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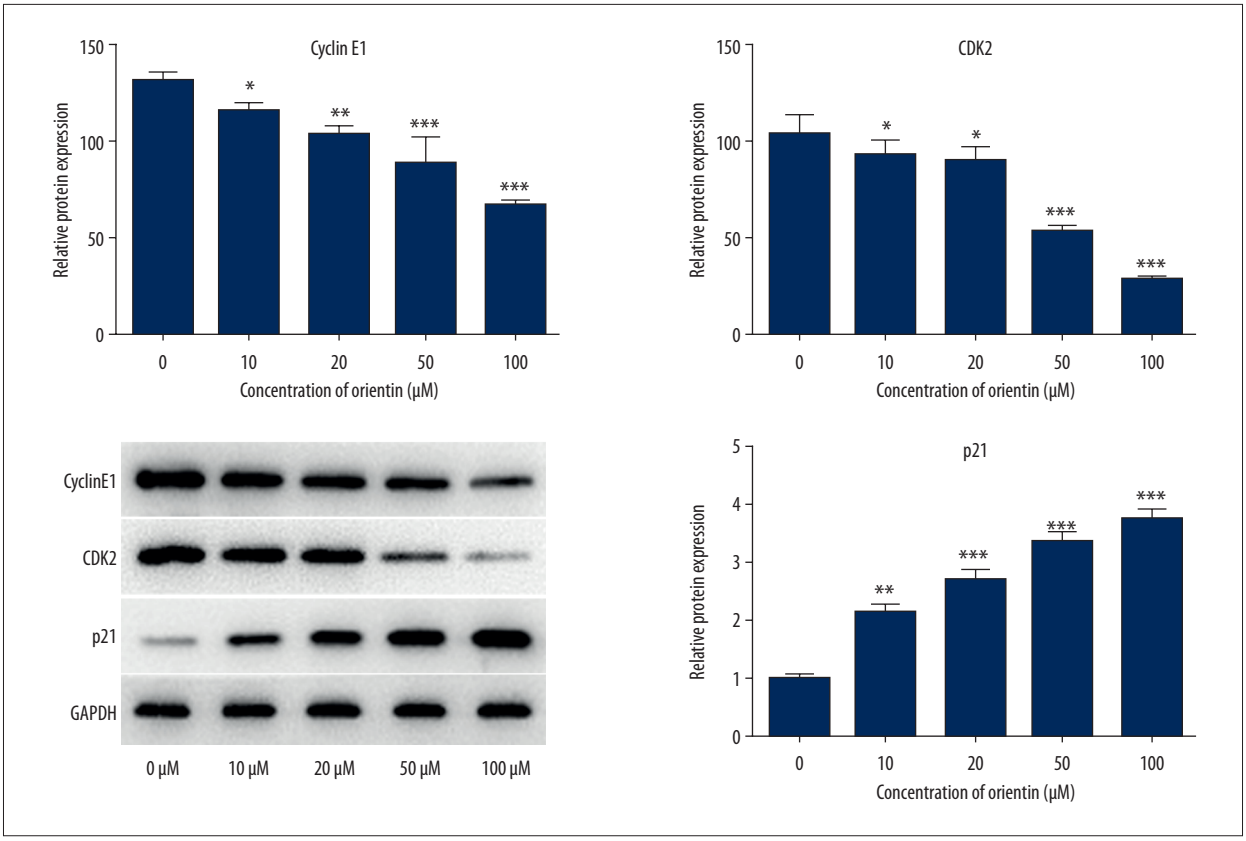

Figure 2. The effects of orientin on the expression of CDK2, cyclin E1, and P21 in T24 human bladder carcinoma cells *in vitro*. The effect of orientin on the expression of CDK2, cyclin E1, and P21; * P<0.05, ** P<0.01 and *** P<0.001 *vs.* the control group (0 μM).

TNFα, IL-1, and IL-6 in the supernatants of each group were determined by ELISA.

Western blot

After treatment with increasing doses of orientin, the T24 cells were lysed to obtain the total protein. The samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene fluoride (PVDF) membranes and blocked with 5% dried skimmed milk powder. The membranes were incubated with the primary antibody overnight. After washing for three times, the membranes were then incubated with the secondary antibody. The Odyssey CLx Imaging System (Li-Cor Biosciences, Lincoln, NE, USA) was used to analyze the protein expression.

Statistical analysis

Data were analyzed using SPSS software version 10.0 (IBM, Chicago, IL, USA) and GraphPad Prism version 8.0 (GraphPad Software, La Jolla, CA, USA). Data were presented as the mean±standard deviation (SD). Student's t-test determined differences between groups. P-values <0.05 represented a significant difference between groups.

Results

Orientin inhibited T24 cell proliferation and arrested the cell cycle

T24 cell viability was evaluated by the MTT assay. As shown in Figure 1A, the cells treated with orientin showed reduced viability in a dose-dependent manner. When the cells were treated with 100 μM orientin, the cell viability at 72 h was significantly lower than that at 48 h, and at 24 h. There was no significant difference in cell viability between 100 μM orientin and 1000 μM orientin. As shown in Figure 1B, as the concentration of orientin increased, there was a reduction of cells in the S phase and an accumulation of cells in G0-G1 phase. Also, the expression of the cell cycle proteins, CDK2 and cyclin E1, which have a crucial role in cell transition from the G1 phase to the S phase, were significantly reduced compared with the control group (0 μM) (Figure 2). P21 expression, which mediated cell-cycle arrest at the G1 or G2 phase, increased with

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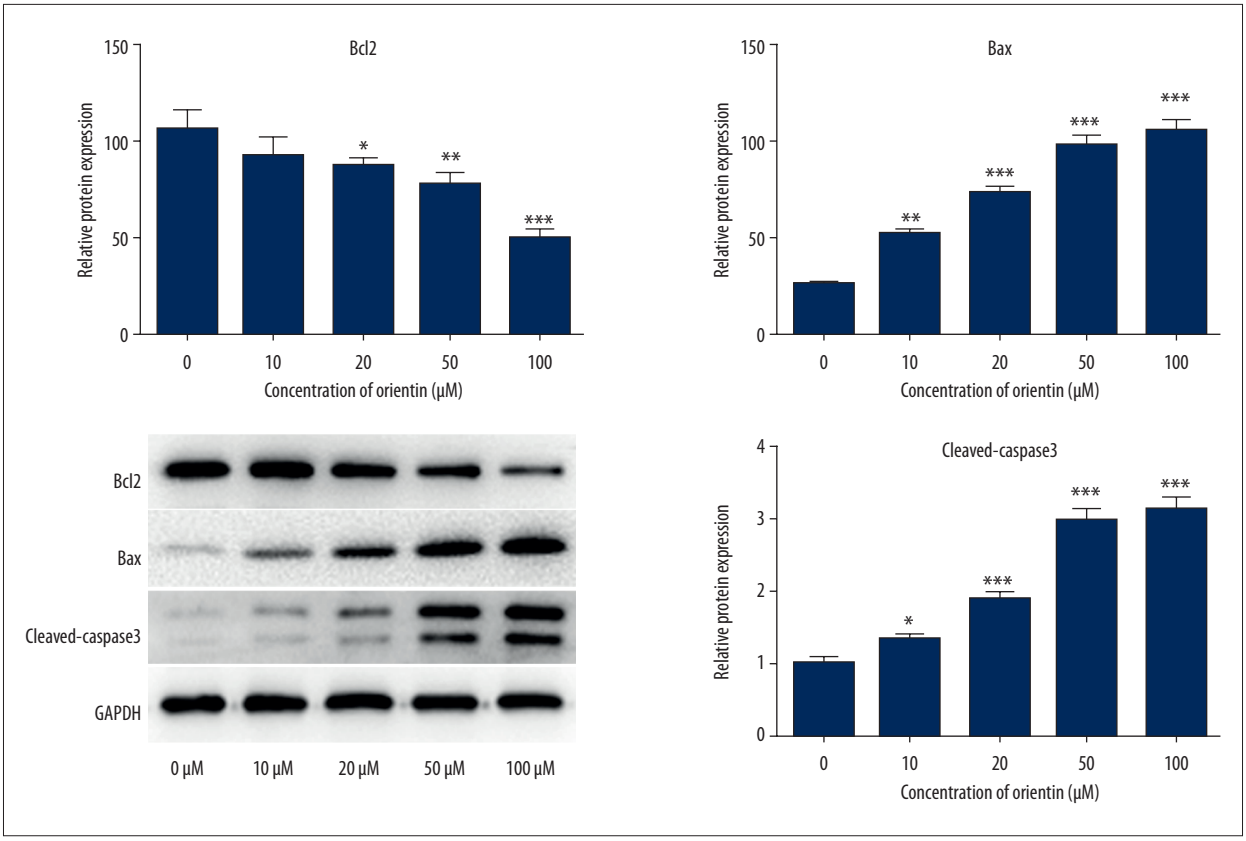

Figure 3. The effects of orientin on the expression of apoptosis-associated proteins, Bcl-2, BAX, and cleaved caspase-3 in T24 human bladder carcinoma cells *in vitro*. The effect of orientin on the expression of apoptosis-associated proteins, Bcl-2, BAX, and cleaved caspase-3. * P<0.05, ** P<0.01 and *** P<0.001 *vs.* the control group (0 μM).

increasing concentrations of orientin. These results supported that orientin had an inhibitory effect on cell proliferation by arresting the cell cycle in T24 cells *in vitro*.

Orientin promoted apoptosis of T24 cells *in vitro*

As shown in Figure 3, apoptosis-associated proteins investigated. The expression of Bcl-2, an anti-apoptosis protein, decreased with increasing concentrations of orientin. However, the pro-apoptotic proteins, including BAX and cleaved-caspase 3, were increased with increasing concentrations of orientin. These results indicated that orientin promoted apoptosis of T24 cells *in vitro* in a dose-dependent manner.

Orientin inhibited the expression of inflammatory cytokines in T24 cells *in vitro*

As shown in Figure 4, the levels of TNFα, IL-1, and IL-6 increased with increasing concentrations of orientin. The proteins in the nuclear factor-kappaB (NF-κB) signaling pathway were also evaluated (Figure 5). P-P65, an activation factor of the NF-κB signaling pathway, was reduced. Following treatment with the NF-κB inhibitor, IκBα, the expression of inflammatory cytokines increased. These findings indicated the expression of inflammatory mediators in T24 cells *in vitro* were inhibited by orientin in a dose-dependent manner.

Orientin inhibited the expression of Hedgehog signaling proteins way by the inhibition of NF-κB in T24 cells *in vitro*

As shown in Figure 6, the protein expression of shh, p-smo, smo, and Gli2 in the Hedgehog signaling pathway in the group of T24 cells treated with 100 μM of orientin were significantly downregulated compared with the control group. After treatment with 100 μM of orientin in the presence of the NF-κB agonist, phorbol 12-myristate 13-acetate (PMA), the relevant proteins in the Hedgehog signaling pathway were upregulated, compared with the group treated with orientin alone. These findings showed that the inhibitory effect of orientin on protein expression in the Hedgehog signaling pathway in T24 cells *in vitro* was through the inhibition of NF-κB.

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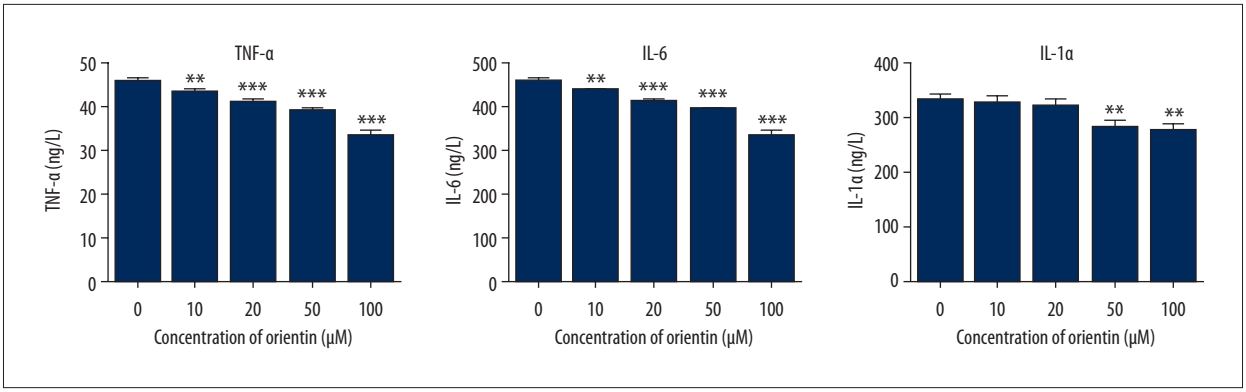

Figure 4. The effects of orientin on the expression of TNFα, IL-1, and IL-6 in T24 human bladder carcinoma cells *in vitro*. The effect of orientin on level of TNFα, IL-1, and IL-6; ** P<0.01 and *** P<0.001 *vs.* the control group (0 μM).

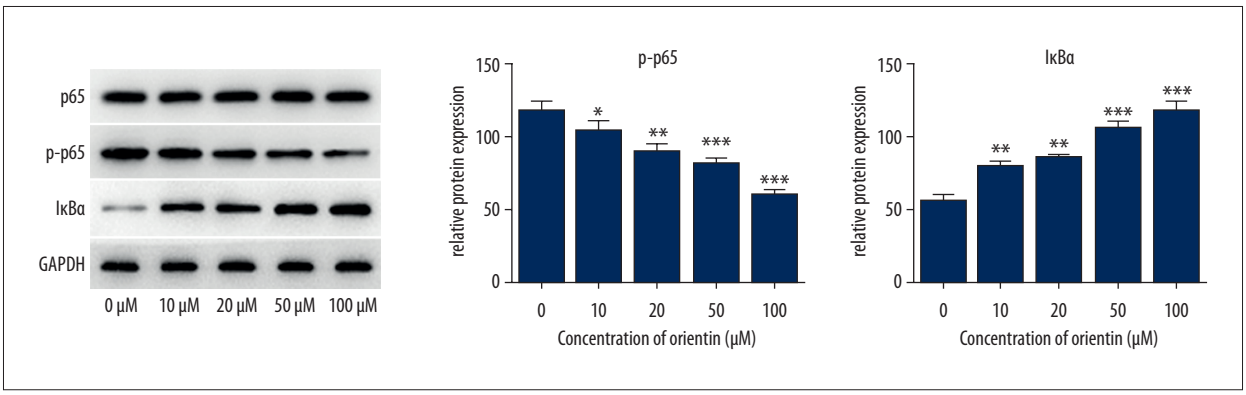

Figure 5. The effects of orientin on the expression of the NF-κB inhibitor, IκBα, and p-P65 in T24 human bladder carcinoma cells *in vitro*. The effect of orientin on the expression of the NF-κB inhibitor, IκBα, and p-P65; * P<0.05, ** P<0.01 and *** P<0.001 *vs.* the control group (0 μM).

Discussion

Worldwide, bladder cancer is the ninth most common cancer, but in China, it ranks sixth [21,22]. Bladder cancer is characterized by a high rate of recurrence and metastasis. Therefore, there is an urgent need to develop highly effective drugs to treat bladder cancer. Orientin is a flavone isolated from medicinal plants, and is used in traditional Chinese medicine (TCM). Orientin suppresses the proliferation of cancer cells and increases apoptosis *in vitro*, including in hepatoma cell lines [23]. A previously published study showed that orientin significantly inhibited proliferation and promoted apoptosis in esophageal carcinoma cells [24]. Orientin was reported to inhibit the proliferation and promote apoptosis of breast cancer cells [25]. The findings from the present study, of T24 human bladder carcinoma cells *in vitro*, showed that orientin had anti-proliferation and pro-apoptotic effects on bladder cancer cells. These effects were mediated through the inhibition of protein expression of the Hedgehog signaling pathway by suppressing nuclear factor-kappaB (NF-κB).

In this study, T24 cell proliferation was inhibited by orientin in a dose-dependent manner. Cells remained in the G0-G1 phase of the cell cycle, and the S phase was reduced, indicating the cell-cycle arrest was caused by orientin. Also, CDK2 and cyclin E1, which were transitional markers of cells from the G1 phase to the S phase, was significantly reduced by orientin. Also, P21, a mediator of cell-cycle arrest at the G1 or G2 phase, were increased by orientin. These findings supported the anti-proliferation effect of orientin in T24 cells *in vitro*. Apoptosis-associated proteins were increased, and the anti-apoptosis protein, Bcl-2, was reduced, which supported the effect of orientin in promoting apoptosis in T24 cells *in vitro* (Figure 3).

NF-κB is a transcription factor that is capable of regulating biological processes involved in malignancy, including cell proliferation, migration, and angiogenesis by inducing transcription of target genes [26–29]. Orientin has previously been shown to have an anti-proliferative effect in colorectal cancer and to inhibit NF-κB signaling [30]. In the present study, compared with the control group (0 μM), P-P65 was reduced. The use of the NF-κB inhibitor, IκBα, reversed these effects, confirming

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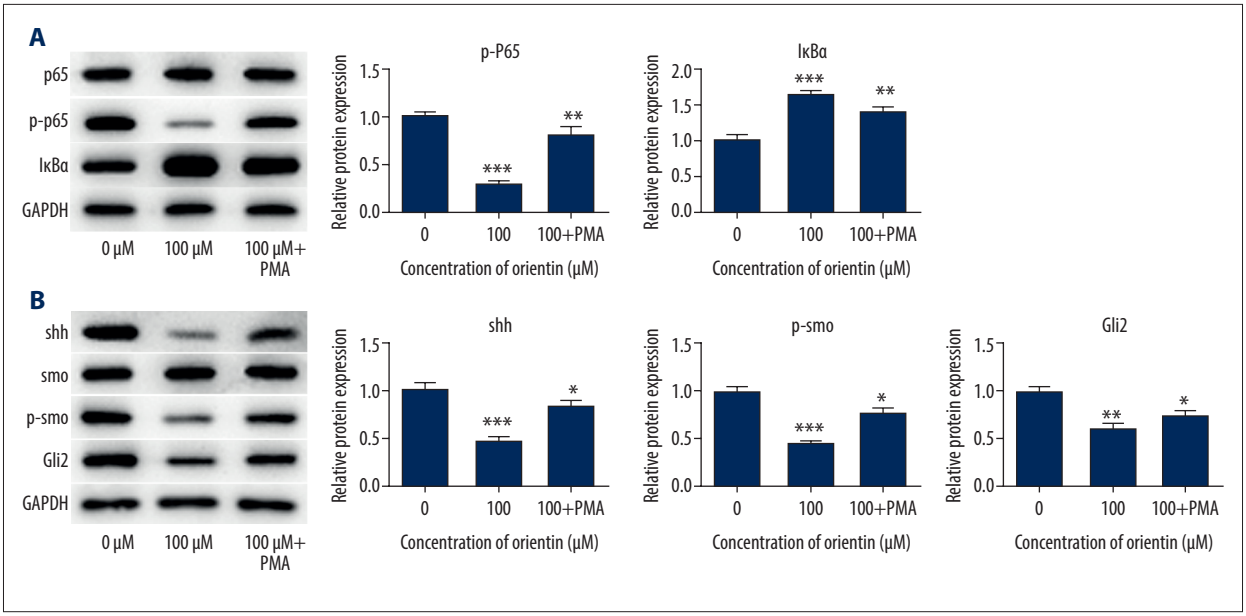

Figure 6. The effect of orientin on the expression of proteins in the Hedgehog signaling pathway following inhibition of nuclear NF-κB in T24 human bladder carcinoma cells *in vitro*. The expression of the NF-κB inhibitor, IκBα, and p-P65 in the different groups (**A**). The expression of proteins in the Hedgehog signaling pathway in the different groups (**B**); * P<0.05, ** P<0.01 and *** P<0.001 *vs.* the control group (0 μM).

that the NF-κB signaling pathway was inhibited by orientin in T24 cells *in vitro*.

NF-κB is reported to promote the expression of the Shh signaling protein to activate the Hedgehog signaling pathway in pancreatic carcinoma cells [31]. NF-κB/Shh was previously shown to have a pro-proliferation effect, and a positive association between the expression of NF-κB p65 and Shh was shown in surgical resection specimens of pancreatic cancer [32]. The relationship between the NF-κB signaling pathway and the Hedgehog signaling pathway in T24 bladder cancer cells treated with orientin requires further study. However, in the present study, using a single human bladder cancer cell line, T24, orientin inhibited the expression of proteins in the Hedgehog signaling pathway by inhibiting NF-κB. This result was reversed following treatment with the NF-κB inhibitor, IκBα, (Figure 6). These preliminary *in vitro* findings, using a single human bladder cancer cell line, showed that orientin had an anti-proliferation effect by inhibiting the expression of Hedgehog signaling pathway proteins by suppressing the NF-κB pathway. These preliminary findings require further study using more human bladder cancer cell lines and should be supported with future *in vivo* studies with animal xenograft models.

Conclusions

This study aimed to investigate the effects of orientin on proliferation and apoptosis of T24 human transitional cell bladder carcinoma cells *in vitro* in the presence of an agonist and an inhibitor of nuclear factor-kappaB (NF-κB). Treatment of T24 human bladder carcinoma cells with orientin inhibited cell proliferation and promoted cell apoptosis by suppressing the Hedgehog signaling pathway and NF-κB. Future *in vivo* pharmacodynamic studies are needed to investigate the role of orientin in bladder cancer.

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