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Original Article

Autophagy induced by vitamin D3 inhibits the proliferation of human bladder cancer cells

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Abstract: Understanding the pathogenic mechanisms of bladder cancer is important. Therefore in this study, to examine whether vitamin D3 could inhibit the proliferation of human bladder cancer cells by inducing autophagy, cell proliferation was examined using a CCK-8 assay and colony formation assays. Furthermore, autophagic activity was detected by western blotting and immunofluorescence assays. The effects of vitamin D3 on cell migration and cell cycle were also investigated. The results of the cell migration indicated that there was no significant difference between the groups treated with vitamin D3 compared with the control group. However, the cell cycle analysis showed that a greater number of bladder cancer cells were arrested in the S phase and G2/M phase following treatment with vitamin D3 compared with the control cells, indicating that the vitamin D3 inhibited the cell proliferation rate. Furthermore, the results of the CCK8 assay and colony formation assays also suggested that vitamin D3 inhibited cell proliferation. Western blot analysis showed that LC3-II protein expression was increased in cancer cells treated with vitamin D3, indicating that autophagy was activated by vitamin D3, stimulating the conversion of LC3-I to LC3-II. In addition, immunofluorescence analysis demonstrated that the number of autophagosomes was significantly increased in cancer cells treated with vitamin D3. In summary, the results indicate that vitamin D3 inhibited the proliferation of human bladder cancer and enhanced autophagic activity. Further studies are required to fully elucidate the pathogenic mechanisms involved in these effects.

Keywords: Bladder cancer, vitamin D3, autopagy, cell proliferation

Introduction

Bladder cancer is one of the most common cancers in Western countries, where it is the fourth and ninth most common cancer in men and women, respectively [1]. Approximately 75% of newly diagnosed patients have non-invasive bladder cancer, characterized by a high rate of recurrence and progression despite treatment with transurethral resection combined with intravesical chemotherapy. The remaining 25% of cases have muscle-invasive disease with poor outcomes despite systemic therapy [2]. Bladder cancer is caused by various genetic and epigenetic alterations, in addition to the direct and indirect effects of multiple risk factors, which have received greater research attention regarding the elucidation of the pathogenic mechanisms of bladder cancer [3].

Autophagy is an evolutionarily conserved biological process that degrades long-lived organelles and protein aggregates by fusion with lysosomes [4]. Autophagic activation is closely associated with various of human diseases, including cancer [5]. Previous studies have indicated that autophagy may be associated with cell survival mechanisms in bladder cancer [6, 7]. Furthermore, autophagy may be activated by anticancer agents, including tamoxifen, rapamycin, statins and vitamin D analogues, leading to the death of tumor cells [7, 8].

Vitamin D is a nutritional factor that may promote cell differentiation and decrease proliferation, invasion, angiogenesis and metastasis [9]. Furthermore, vitamin D3 is the main component of vitamin D in the human body, and is demonstrated to be the biologically active metabolite of vitamin D and best indicator of vitamin D levels [10]. To the best of our knowledge, there are no previous reports that have investigated the relationship between vitamin D3, autophagy and bladder cancer cell proliferation.

We hypothesize that vitamin D3 may inhibit the proliferation of human bladder cancer cells and induce autophagy. Therefore, the effect of vitamin D3 on the activation of autophagy was investigated in human bladder cancer cells, in addition to the effect on the proliferation of bladder cancer cells.

Materials and methods

Cell culture

The EJ and UMUC-3 human bladder carcinoma cell lines were used in our analysis, which were obtained from the Shanghai Institute for Biochemistry, Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI-1640 (Gibco Life Technologies, Carlsbad, CA, USA), supplemented with 10% fetal bovine serum, (BI, Israel) in humidified 5% CO_2 at 37°C. Trypsin solution (0.25%; BI, Israel) was used to detach cells from the culture flasks.

Half maximal inhibitory concentration (IC_{50}) of vitamin D3 in EJ cells

EJ cells were seeded in 96-well plate (4000/well) when in the logarithmic growth stage, then incubated with vitamin D3 (1, 1.5, 2, 2.5 and 3 μmol/ml) diluted in DMSO for 24 and 48 h. Then, each well was incubated with a mixture of CCK-8 solution and culture medium (10 μl CCK-8 solution and 90 μl culture medium) for 2 h. The absorbance at 450 nm was measured in each well. The inhibitory rate was calculated using the following formula: I (%) = (Ac-At)/Acx100. IC_{50} was obtained after calculation using SPSS 13.0 software (SPSS, Chicago, IL, USA). DMSO treatment was used as the control group.

Cell cycle analysis

Cells were stained with a Cell Cycle Detection kit (KeyGen, Nanjing, China) according to the manufacturer's protocols. Briefly, 70% ethanol was used to fix 1×10^6 cells, which were incubated with vitamin D3 for 24 h at 4°C. The cells were then washed twice with phosphate buffered saline (PBS) and incubated with 100 μg/ml RNase A for 30 min at 37°C. Then, the cells were stained with 50 μg/ml propidium iodide (PI) and protected from light. The results were analyzed using Modfit LT software (Verity Software House, Topsham, ME, USA).

Cell migration

Cell migration was measured using Transwell chamber assays (BD Biosciences, San Jose, CA, USA). In the Transwell assays, following treatment with vitamin D3 for 24 h, cells were trypsinized and seeded in the upper chambers at a density of 5×10^4 cells/well. A total of 500 μl RPMI-1640 medium with 10% FBS was added to the lower chamber. Migrated cells on the bottom surface were fixed with 4% formaldehyde methanol for 30 min, and non-migrated cells were removed by cotton swabs. The cells on the bottom surface of the membrane were stained with hematoxylin for 30 min. An optical microscope (200×, Olympus Corporation, Tokyo, Japan) was used to count the number of cells in five randomly selected fields.

Cells proliferation and morphology

EJ and UMUC-3 cells (800/well) were seeded in a 96-well plate with 100 μl medium. Cell proliferation was determined using a Cell Counting Kit-8 (CCK-8; Dojindo Molecular Technologies, Inc., Rockville, MD, USA) according to the manufacturer's protocols. Briefly, each well was incubated with a mixture of CCK-8 solution and culture medium (10 μl CCK-8 solution and 90 μl culture medium) for 2 h in 5% CO_2 and at 37°C. Subsequently, the absorbance was measured at 450 nm. The cell proliferation assay was performed after 24, 48, 72, 96 and 120 h of treatment with vitamin D3.

For the colony formation assay, cells were cultured for 12 days in a 6-well plate (500 cells/well). Cell colonies were washed with PBS twice, fixed with 4% formaldehyde and were stained with hematoxylin for 30 min. The number of colonies consisting of >50 cells were counted using a microscope after treatment with vitamin D3 for 24, 48, 72 and 96 h. Cell morphology was examined using an Optika inverted phase-contrast microscope (XDS-2; Optika SRL, Bergamo, Italy). Phase-contrast imaging was conducted on living cells without any fixation or treatment. The digital images of the living cells were recorded at each experimental time point and the most representative were selected. For both assessments, there were four replicates in each experiment and all experiments were repeated three times.

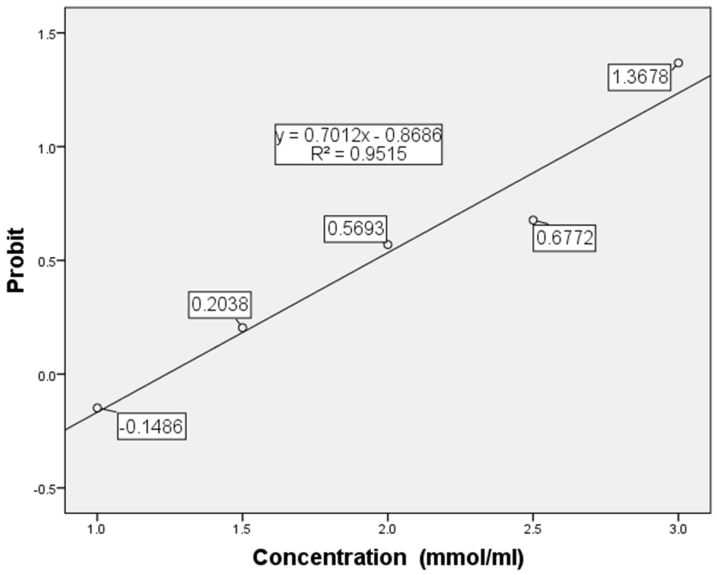

Figure 1. Half maximal inhibitory concentration (IC_{50}) of vitamin D3, calculated using the scatter diagram.

Western blot analysis

Cells were incubated with vitamin D3 at 37°C for 3, 6, 12 and 24 h and then incubated in ice-cold RIPA buffer [1 M Tris (pH 7.4), 5 M NaCl, 0.5 M EDTA (pH 8.0), 10% SDS, 10% DOS, and 10% NP40] with fresh protease inhibitor PMSF on ice for 30 min. The cells were scraped and the lysate was collected and centrifuged at 10,000 rpm at 4°C for 30 min. The liquid supernatants were collected, aliquoted and stored at -80°C for further use. Proteins (30 μg) were loaded in 12% SDS-PAGE gels and transferred onto a polyvinylidene difluoride (PVDF) membrane. The membranes were soaked in blocking buffer (5% skimmed milk) for 1 h at room temperature. The blots were washed with PBST. To probe for LC3 and GAPDH (all obtained from Proteintech, China), the membranes were incubated overnight at 4°C with the relevant antibodies, followed by appropriate HRP-conjugated secondary antibodies and enhanced chemiluminescence detection was performed (ECL, Pierce, Rockford, IL, USA). Triplicate gel images of identical samples were used for densitometry analysis.

Immunofluorescence

For immunofluorescence staining of talin and vinculin, EJ and UMUC-3 cells were treated with vitamin D3 for 6 and 12 h, and then fixed in 4% formaldehyde for 5 min (pH 7.4) on day 3 of the culture, and at day 7 of the culture for the other proteins that were stained for. Subsequently, the samples were washed three times in PBS for 5 min, pretreated with 1% bovine serum albumin in PBS containing 0.1% Triton X-100 for 1 h and then incubated in 1% Tween 20 for 20 min. After a 5-min wash in PBS, the primary antibody, mouse monoclonal anti-LC3 (dilution 1:200), was applied to the samples overnight at 4°C. Following this, the secondary antibody, Alexa Fluor 488-conjugated goat anti-mouse IgG, was incubated with the samples for 1 h at room temperature. A solution of 0.05% Tween-20 in PBS was used to wash the samples. The cell nuclei were counterstained with DAPI for 10 min. Following washing, an epifluorescence microscope (IX51, DP70 digital camera; Olympus Corporation) was used to capture images of the stained samples.

Statistical analysis

All data were analyzed with SPSS software 13.0 (SPSS, Chicago, IL, USA). The independent Student's t test and one-way ANOVA were used for comparisons between two and three groups, respectively. $P<0.05$ was considered to indicate a statistically significant difference.

Results

IC_{50} of vitamin D3 on bladder cancer cells

To evaluate the cytotoxicity of vitamin D3 to bladder cancer cells, EJ cells were treated with vitamin D3 at different concentrations, ranging from 1 to 3 μmol/ml, *in vitro* for 24 and 48 h, and the effect was subsequently evaluated using a CCK-8 assay. The results from the CCK-8 assay for cell viability indicated that vitamin D3 inhibited the growth and viability of EJ cells in a dose-dependent manner, and that the IC_{50} value of vitamin D3 was 1.226 μmol/ml (**Figure 1**).

Effect of vitamin D3 on the cell cycle in bladder cancer cells

To determine the effect of vitamin D3 on the cell cycle of bladder cancer cells, flow cytome-

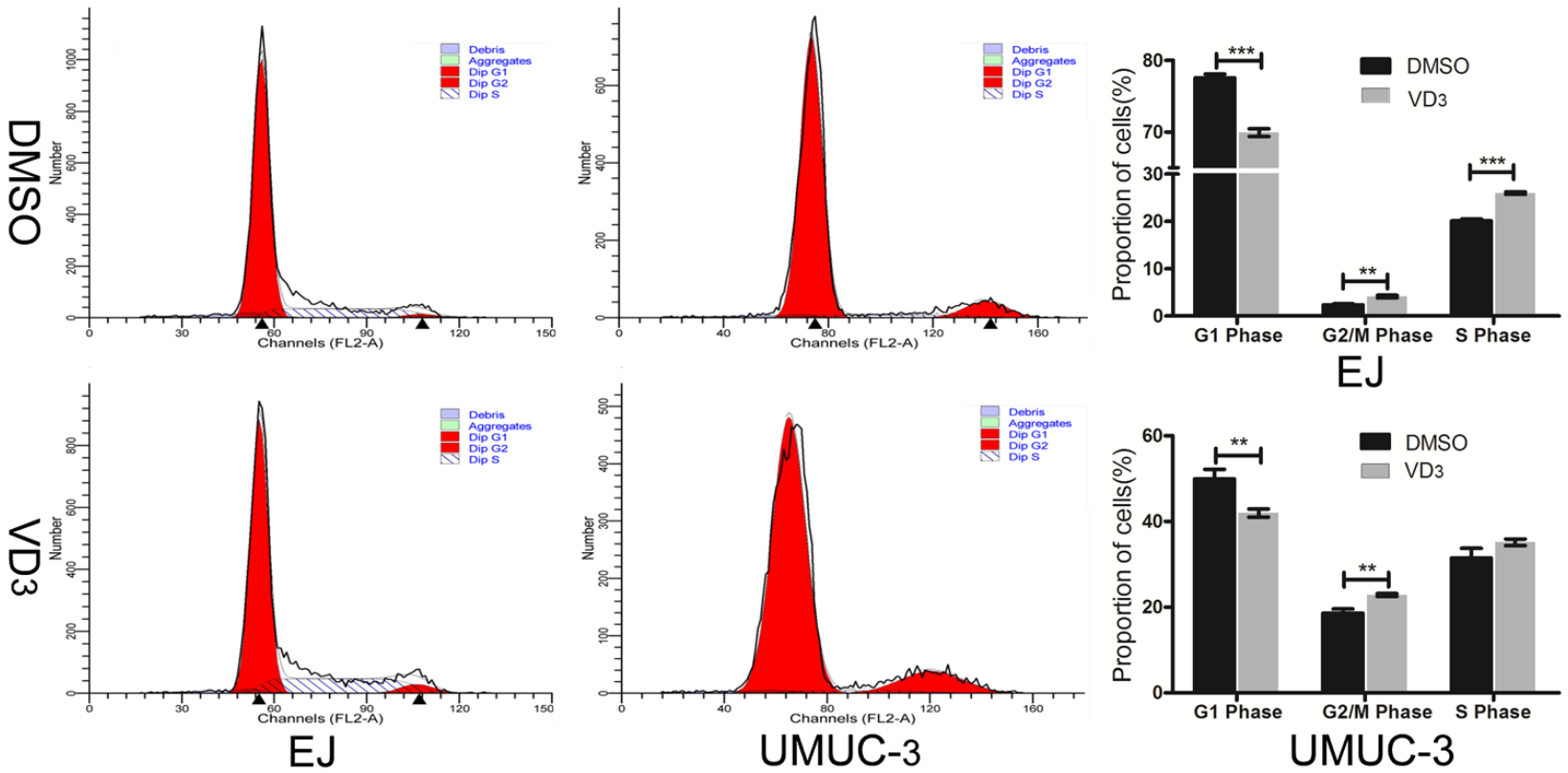

Figure 2. Flow cytometry analysis following vitamin D3 treatment. The analysis suggested that the proportion of S-phase cells (EJ, 25.99%; UMUC-3, 35.16%) and G2-M phase cells (EJ, 4.12%; UMUC-3, 22.86%) were markedly increased in the bladder cancer cell groups treated with vitamin D3 compared with the control groups (S-phase cells, EJ 20.11% and UMUC-3 31.45%; G2-M phase cells, EJ 2.35% and UMUC03 18.61%).

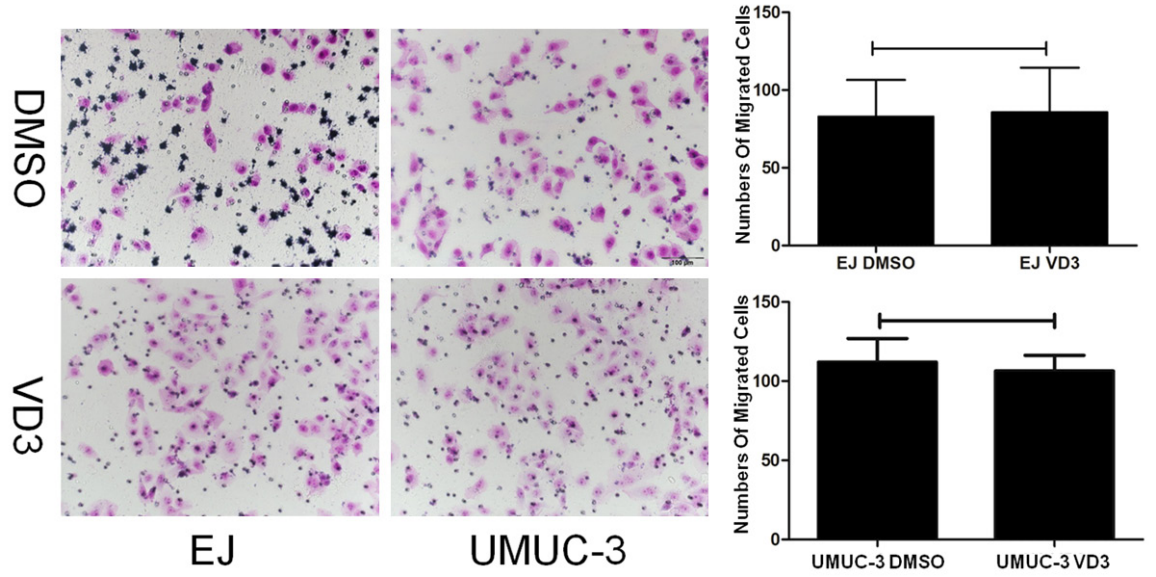

Figure 3. Analysis of cell migration following vitamin D3 treatment. The number of migrated cells was not significantly different between the vitamin D3-treated group and the control group, both in EJ and UMUC-3 cells (P>0.05).

try was conducted to examine the effects of vitamin D3 on EJ and UMUC-3 cell cycle distribution. The flow cytometry analysis suggested that the proportion of cells in S-phase (EJ, 25.99%; UMUC-3, 35.16%) and G2-M phase (EJ, 4.12%; UMUC-3, 22.86%) was markedly increased in the bladder cancer cells treated with vitamin D3 compared with the control groups (S-phase cells, 20.11% and 31.45% for EJ and UMUC-3 respectively; G2-M phase cells, 2.35% and 18.61% for EJ and UMUC-3 respectively; **Figure 2**), and the differences between the groups was statistically significant (P<0.05). This indicates that the EJ and UMUC-3 bladder cancer cells were arrested in the S phase and G2/M phase of the cell cycle following treatment with vitamin D3.

Effect of vitamin D3 on the migration of bladder cancer cells

To evaluate whether vitamin D3 influences the migration of human bladder cancer cells, the migration of bladder cancer cells was investigated using a Transwell assay. The results showed that there was no significant difference in the number of migrating cells between the vitamin D3-treated group and the control group in both EJ and UMUC-3 cells (P>0.05; **Figure 3**). This indicates that treatment with vitamin D3 had no significant effect on the migration of bladder cancer cells.

Effect of vitamin D3 on the proliferation of bladder cancer cells

The effect of vitamin D3 on the proliferation of bladder cancer cells was examined using the CCK-8 assay and a colony formation assay, coupled with analysis of cellular morphology. The proliferation of EJ and UMUC3 human bladder cancer cells was significantly inhibited by vitamin D3. **Figure 4A** indicates that vitamin D3 significantly inhibited bladder cancer cell proliferation compared with the control group, paticularly following treatment for 48 h. In the colony formation assay, the number vitamin D3-treated EJ and UMUC-3 cell colonies was significantly decreased compared with the negative controls (P<0.001; **Figure 4B** and **4C**). Furthermore, vitamin D3 induced an effect on the cellular morphology of EJ and UMUC-3 cells. As presented in **Figure 4D**, of the cancer cells treated with vitamin D3, no predominant clusters were observed and the culture was observed to be a monolayer of cells, regularly polygonal and uniform in morphology and size. No differences in the homogeneity, morphology and the size of the cells were observed. However, the number of cells was increased significantly faster in the DMSO group compared to the vitamin D3 group following 24 h of treatment, indicated by the absence of large empty spaces (**Figure 4D**). These results are consistent with the findings obtained by the CCK-8 and colony formation assays.

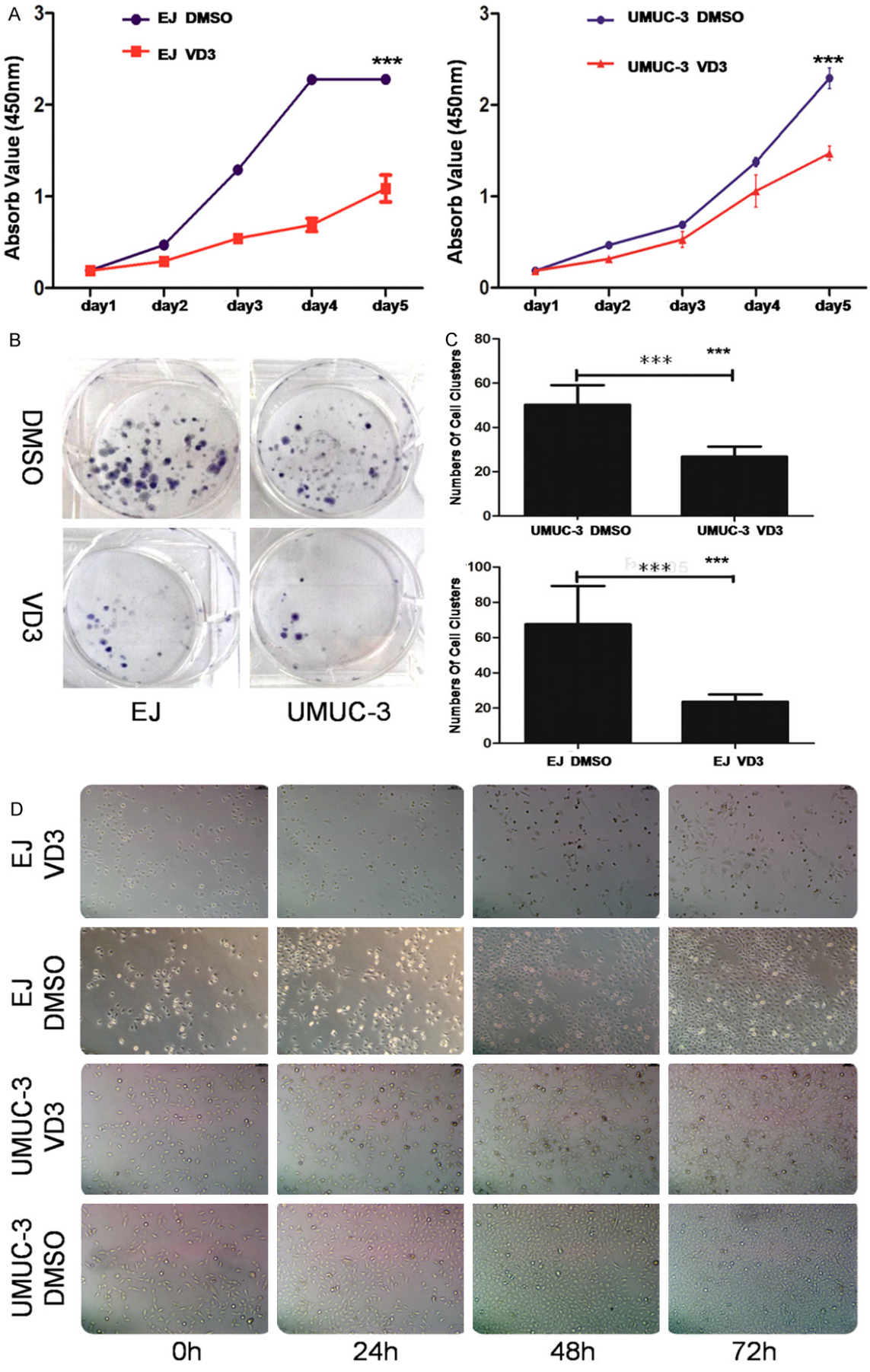
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Figure 4. Analysis of cell proliferation and colony formation following vitamin D3 treatment. (A) CCK8 assay showed that the cell proliferation of EJ and UMUC-3 cells was significantly inhibited following vitamin D3 stimulation. (B) The colony formation assay indicated that fewer colonies were stained in vitamin D3-treated cells, and (C) the differences were statistically significant (P<0.001). (D) Morphology of bladder cancer cells was taken by microscope at 0 h, 24 h, 48 h and 72 h, respectively.

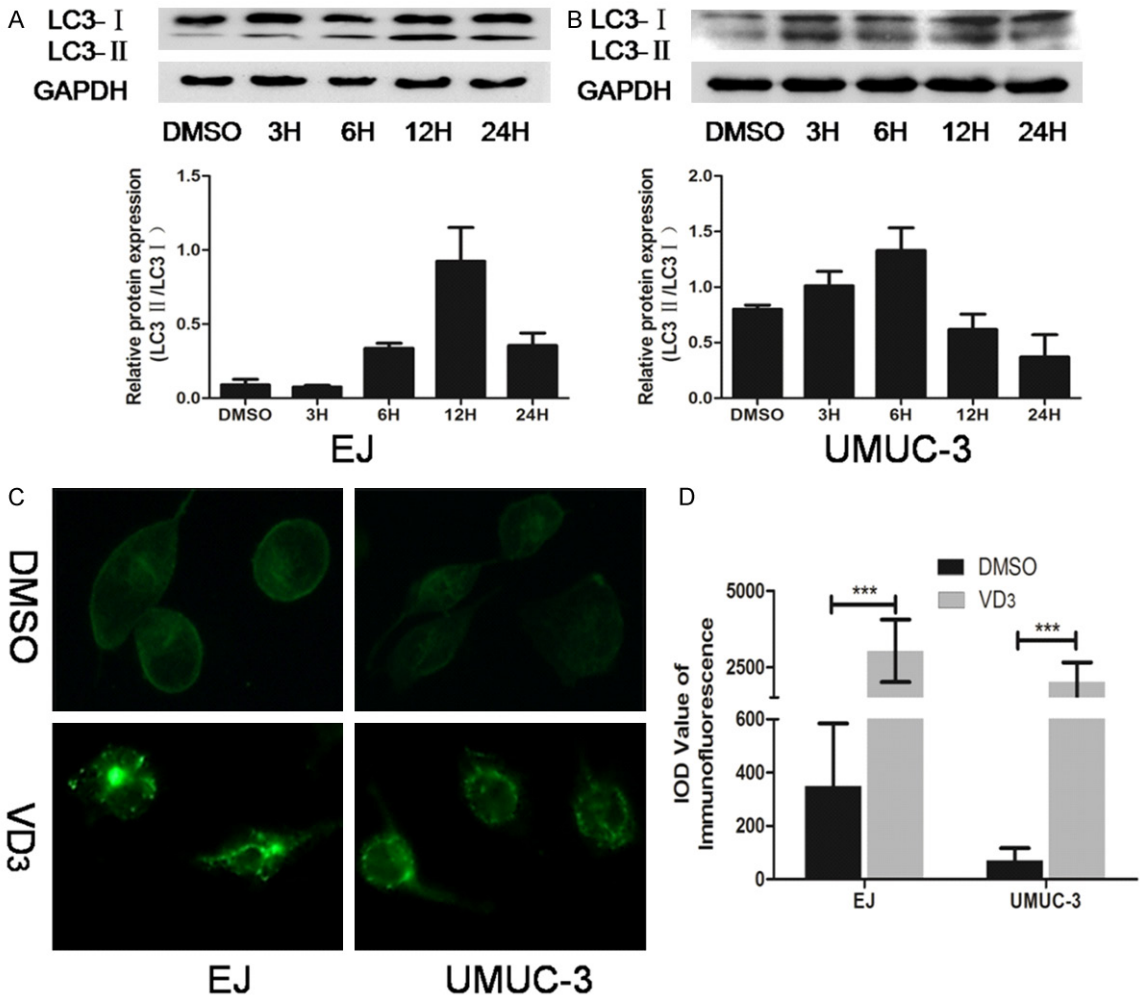

Figure 5. Western blotting and immunofluorescence analysis following vitamin D3 treatment. (A) The western blotting showed that there is a higher expression of LC3-II protein in EJ and UMUC-3 cells following vitamin D3 stimulation for 12 and 6 h respectively, (B) The differences were statistically significant (P<0.05). (C) Immunofluorescent staining indicated that autophagosomes were significantly increased in EJ and UMUC-3 cells treated with vitamin D3. The autophagosomes were indicated by the aggregation of LC protein (green) in vitamin D3-treated cells, and the differences were statistically significant (C) (P<0.005). (D) The differences of green fluorescence were statistically significant in EJ (P<0.001) and UMUC-3 cells (P<0.001).

Autophagy is induced by vitamin D3

It is well reported that autophagic activity is associated with the proliferation capability of bladder cancer cells, with this considered a key feature of its anticancer properties [6]. Therefore, we investigated whether vitamin D3 induced autophagy. The molecular basis of autophagy was analyzed by western blot analysis and immunofluorescence. Following treatment with vitamin D3, western blotting indicated that LC3-II protein expression was significantly increased in EJ and UMUC-3 cells, indicating that autophagy is activated by vitamin D3, stimulating the conversion of LC3-I to LC3-II (P<0.05; **Figure 5A** and **5B**). In the immuno-

fluorescence analysis, the autophagic response in bladder cancer cells to treatment with vitamin D3 was assessed by measuring the number of autophagosomes. The number of autophagosomes was significantly increased in EJ and UMUC-3 cells treated with vitamin D3 compared with the control group (**Figure 5C**).

Discussion

As advances in improving prognosis have been limited, there is a growing interest in exploring the mechanisms of tumorigenesis in bladder cancer [11, 12]. Autophagy is a biological process in which double-membrane vesicles that encapsulate cytoplasm and organelles fuse with lysosomes to degrade cellular components [13]. Autophagy is important for normal development and is involved numerous diseases, including infections, cancer and cardiovascular disease [5]. It has been previously reported that autophagy is an important survival mechanism for cancer cells, functioning to remove misfolded protein aggregates and abnormal organelles [14-16]. However, autophagy can also be activated by anticancer agents, such as vitamin D analogues (vitamin D3), resulting in autophagic cell death, which may be a mechanism of tumor cell death [17]. Therefore, it is unclear whether autophagy is a cell death mechanism in tumors, when it may also be a survival mechanism.

There is increasing interest in the use of vitamin D3 as a cheap and convenient supplement for disease prevention [18]. Lower serum vitamin D concentrations were previously reported to be associated with an increased risk of bladder cancer compared with higher serum levels [19]. Epidemiological evidence and preclinical studies in a number of cancer types, including bladder cancer, have indicated that vitamin D3 has antitumor activity and inhibitory effects on cancer cell proliferation, angiogenesis and tumor metastasis [20, 21]. Furthermore, vitamin D has been shown to decrease bladder cancer cell proliferation and bladder tumorigenesis in rats [6].

The association of vitamin D3 with autophagy has been previously demonstrated in various diseases. The inhibition of human myeloid leukemia cells by vitamin D3 was markedly improved by the upregulation of Beclin1 to trigger autophagy [22]. Sharma *et al* [23] identified a unique cytostatic function of autophagy that may be mediated by vitamin D3 to enhance the response to radiation in non-small cell lung cancer cells. In addition, autophagy has been shown to be triggered by a vitamin D3 analog, which induced apoptosis via a p53-independent mechanism involving p38 mitogen-activated protein kinase (AMPK) activation and AMPK kinase inactivation in B-cell chronic lymphocytic leukemia cells. Vitamin D3 also induced autophagy in breast cancer cells [22, 24]. However, the precise associations between vitamin D3, autophagy and bladder cancer cell proliferation remain unknown.

We hypothesize that vitamin D3 may inhibit the proliferation of human bladder cancer cells by the upregulation of autophagic activity. To investigate this, the IC_{50} value of vitamin D3 was determined for the subsequent analysis. The results of the cell migration assay indicated that there was no significant difference between the cell groups treated with vitamin D3 compared with the controls. However, the findings from the CCK-8 assay and colony formation assays suggested that vitamin D3 inhibited cell proliferation in the groups treated with vitamin D3 for 24 h. In addition, the cell cycle analysis indicated that an increased proportion of bladder cancer cells were arrested in the S phase and G2/M phase following treatment with vitamin D3 in comparison with the controls. Vitamin D3 may block the progression to S phase by acting on cyclin D, thereby inhibiting cell proliferation. It may also indirectly regulate the cell cycle by cross-talk with other signaling pathways mediated by growth factors, including insulin-like growth factor and epidermal growth factor [25, 26]. In addition, the western blot and immunofluorescence analyses indicated that the number of autophagosomes was increased in bladder cancer cells treated with vitamin D3. Together, the findings of the present study suggest that vitamin D3 inhibits bladder cancer cell proliferation and induces autophagy.

Currently, the pathophysiological mechanisms underlying vitamin D3, autophagy and bladder cancer cell proliferation are unclear. The vitamin D (1,25-dihydroxyvitamin D3) receptor (VDR) is highly expressed in metabolic tissues, including the bladder, kidneys and skin, and is moderately expressed in nearly all other tissues [9, 27]. It is a nuclear receptor that medi-

ates the majority of biological functions of vitamin D3. Activation of VDR signaling by vitamin D3 impacts a number of processes, including calcium metabolism, apoptosis, inflammation, infection and autophagy [28]. The role of autophagic activity on the survival of cancer cells is context-dependent, and can exhibit a tumor growth-suppressing or survival-promoting effect. The stimulation of autophagy in cancer cells has been observed in response to anticancer treatments, with the destruction of large proportions of the cytosol and organelles resulting in permanent cellular atrophy leading to the collapse of vital cellular functions [22, 29]. A potential explanation for our findings may be that autophagy is induced by vitamin D3 activation of VDR, p53, and possibly the AMPK-mTOR signaling pathway, to reduce cancer cell proliferation [9, 22, 30]. Furthermore, vitamin D3 may also induce a large increase in the number of autophagosomes, resulting in a reduction in cell proliferation and stimulating apoptosis, which is dependent on the activation of cytosolic Ca^{2+}/calmodulin-dependent protein kinase kinase β [25, 31]. In addition, vitamin D3 may inhibit cancer cell proliferation by inducing differentiation, which is associated with mechanisms involving the downregulation of protein kinase B and the subsequent activation of MAPK signaling, leading to CDKI upregulation [25].

There are a number of limitations of the present study. The vitamin D3 induction of autophagy was used to explore the role of autophagy in bladder cancer cell proliferation. Despite the widespread usage of vitamin D3, it may result in cytoplasmic accumulation of abnormal autophagosomes, which can be toxic to cells. More importantly, our experiments were conducted in bladder cancer cells. The efficacy of autophagy inhibition will be different *in vitro* and *in vivo*, due to unknown factors that affect the survival of cancer cells in their microenvironment [6]. Therefore, an *in vivo* efficacy study, such as a human bladder cancer xenograft model, is required to confirm our findings.

Conclusions

In summary, our results demonstrate that vitamin D3 arrested human bladder cancer cells in S phase and G2/M phase cell cycles, but did not affect cell migration. Furthermore, vitamin D3 was able to inhibit human bladder cancer cell proliferation and upregulate of autophagic activity. Further studies are required to fully elucidate the pathogenic mechanisms involved in these effects.

Acknowledgements

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Disclosure of conflict of interest

None.

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