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Original Research Article

Viscum album extract suppresses cell proliferation and induces apoptosis in bladder cancer cells

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Abstract

Purpose: *To evaluate the effect of Viscum album (VA) extract on the progression of bladder cancer (BC) and its effect on the proliferation and apoptosis of T24 and J82 bladder cancer cells.*
Methods: *3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT assay) was conducted to examine the proliferation of bladder cancer cells. Flow cytometry (FCM) was employed to assess changes in the cell cycle of bladder cancer cells. The expression levels of proliferating cell nuclear antigen (PCNA), CLND1 (cyclin D1), p21, and p27 in control and VA extract-treated (100, 200, or 300 μg/mL) T24 and J82 cells were measured by immunoblot assay. The effects of VA extract on T24 or J82 cell apoptosis were evaluated using FCM. Immunoblot assay was performed to evaluate Bcl-2, Bax, and cleaved caspase 3 expression in control or VA extract-treated bladder cancer cells. In addition, the effect of VA extract on Axl-AKT pathways was also evaluated by immunoblot assay.*
Results: *Viscum album extract treatment significantly blocked bladder cancer cell proliferation and induced cell cycle arrest. In addition, VA extract stimulated bladder cancer cell apoptosis. Moreover, this study found that VA extract suppressed Axl-AKT pathways in bladder cancer.*
Conclusion: *Viscum album extract exerts anti-proliferation and pro-apoptosis effects on bladder cancer cells. These abilities render Viscum album extract as promising agent in bladder cancer treatment.*

Keywords: *Viscum album, Bladder cancer, Proliferation, Apoptosis, Cell cycle arrest*

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INTRODUCTION

Bladder cancer is one of the most high metastastic malignancies worldwide and exhibits high mortality. Approximately 4,000,000 new cases are diagnosed each year, making it second only to prostate cancer [1,2]. The most common type is bladder urothelial carcinoma, which accounts for approximately 90 % of bladder cancers [3,4]. Common treatment strategies for bladder cancer are surgical resection, radiotherapy, chemotherapy, and combinatorial therapy [5]. However, because of the poor early diagnosis capacity and the high metastatic potential of advanced bladder cancer, the development of new treatments remains

imperative [6,7]. In recent years, targeted therapy for bladder cancer has shown great potential to improve patient's survival. However, additional therapeutic agents need to be developed [8].

Traditional Chinese medicine is a precious resource of China [9]. The efficacy of multiple Chinese herbal medicines has been confirmed by modern scientific research [10]. *Viscum album* (VA) is a traditional Chinese semi-parasitic evergreen shrub belonging to the mistletoe family. Its main active components include glycoproteins, mistletoe toxins, flavonoids, alkaloids, polysaccharides, and lipids [11,12]. *Viscum album* extract has several published benefits, such as promoting microcirculation and anti-tumour effects [13,14].

In recent years, the anti-tumour effect of VA has been widely confirmed in multiple studies [11,15]. Previous studies indicated that VA inhibits the development of several cancers, such as liver cancer, lung cancer, brain glioma, leukaemia, and melanoma [14,16-18]. In leukaemia, VA inhibits cell proliferation as well as induces apoptosis [19]. Additionally, in SK-hep1 cells, VA promotes cell death by inducing G1 phase arrest [20]. Recently, VA has gained increasingly more attention in the quest for effective anti-tumour components derived from plants.

However, the possible roles of VA in the development and metastasis of BC remains unclear.

EXPERIMENTAL

Antibodies

The following antibodies were purchased from Abcam (Cambridge, UK) unless otherwise stated. Rabbit anti-PCNA antibody (1:1000 dilution, ab92552); rabbit anti-Cyclin D1 antibody (1:2000 dilution, ab16663); rabbit anti-p21 antibody (1:2000 dilution, ab109520); rabbit anti-p27 antibody (1:2000 dilution, ab92741); rabbit anti-Bcl-2 antibody (1:1000 dilution, ab32124); rabbit anti-Bax antibody (1:1000 dilution, ab32503); rabbit anti-cleaved caspase 3 antibody (1:2000 dilution, ab13585); rabbit anti-Axl antibody (1:2000 dilution, ab227871); rabbit anti-AKT antibody (1:1000 dilution, ab179463); rabbit anti-AKT(phospho S473) antibody (1:2000 dilution, ab8123); rabbit anti-Axl (phospho S473) antibody (1:2000 dilution, ab8123); rabbit phospho-Axl (p-Axl, Tyr702, #5724, Cell Signaling, Beverly, MA, USA); rabbit pAKT (p-AKT, Ser473, #4060, Cell Signaling); mouse anti-β-actin antibody (1:1000 dilution, ab8226); goat Anti-Rabbit IgG (1:5000 dilution, ab97051; and goat Anti-mouse IgG (1:5000 dilution, ab6789).

Cell culture

The two human bladder cell lines T24 and J82 were bought from the American Type Culture Collection (ATCC). T24 and J82 cells were cultured in RPMI1640 and MEM culture medium (Gibco, CA, USA), respectively, added with 10 % FBS (Gibco) in a 5 % CO_2 incubator.

MTT assay

Both T24 and J82 bladder cancer cells were added into several 96-well plates at a density of 1000. After 24 h, BC cells were treated with VA extract at doses of 100, 200, or 300 μg/mL. Subsequently, the cells were cultured for 12, 24, or 48 h, and 400 μL MTT was added into the culture medium. After washing with PBS twice, the cells were extracted with DMSO (100 μL) and the absorbance value at 570 nm wavelength was measured.

Cell cycle assay

Ethanol-fixed T24 and J82 cells were treated with propidium iodide (PI) and RNase A at room temperature for 30 min. The stages were analysed using a FACS Calibur flow cytometer. The percentages of cells in each phase, including G0/G1, S, and G2/M phases were calculated.

Western blot assay

Total proteins in cells were extracted from T24 or J82 cells by NP-40 reagent, and were detected through SDS-PAGE and transferred onto PVDF membranes, and subsequently blocked with 5 % fat-free milk in TBST buffer. The membranes were incubated with appropriate primary antibodies for 2 h. After four washes with TBST, the membranes were incubated in TBST buffer with secondary antibodies for 1 h. The blots were then visualized.

Determination of cell apoptosis

T24 or J82 cells were treated with VA extract and then resuspended and washed twice with PBS. Subsequently, the cells were treated with annexin V-FITC agent for 10 min at room temperature and with propidium iodide (PI) agent for 20 min in the dark. After filtering with a strainer, the cells were manually analyzed using a FACS Calibur flow cytometer (BD Biosciences).

Statistical analysis

GraphPad 6.0 software was used for statistical analysis in this study. All results are presented as mean ± SD (n = 3). Student's t-test was performed for statistical comparison, with $p < 0.05$ considered significant.

RESULTS

VA extract treatment significantly suppressed proliferation of bladder cancer cells *in vitro*

The results showed that VA extract significantly reduced the proliferation and growth of T24 and J82 cells in a time- and dose-dependent manner (Figure 1 A, B). Therefore, VA extract may serve as potential anti-proliferative agent for bladder cancer cells.

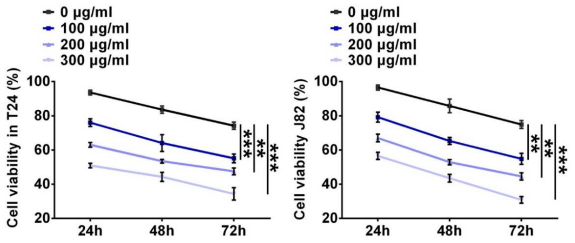

Figure 1: The effect of *Viscum album* on bladder cancer cell proliferation *in vitro*. MTT assays revealed differences in cell viability between controls and VA extract-treated (100, 200, and 300 μg/mL) T24 and J82 cells at 24, 48, and 72 h. $P < 0.05$ was considered statistically significant; $^{**}p < 0.01$, $^{***}\ p < 0.001$, compared to 0 μg/mL group

VA extract stimulates the arrest of cell cycle in bladder cancer cells

Due to the significant effects of VA on cell proliferation, this study further investigated whether VA affected the cell cycle of bladder cancer cells. Both J82 and T24 cells were incubated with control or VA extract in different concentrations (100, 200, and 300 μg/mL) for 48 h. The effects of VA extract on the cell cycle were evaluated. Interestingly, it was noticed that the percentage of VA extract-treated cells in G0/G1 phase was markedly increased when compared to control cells, whereas S phase cells in the VA treatment group were significantly decreased (Figure 2 A). In addition, modest changes were found in the percentage of G2/M phase cells between control and VA extract groups (Figure 2 A). Therefore, the cell cycle was arrested in VA-treated T24 or J82 cells compared to control cells. Treatment with an obvious high concentration of VA extract (300 μM) led to more significant cell cycle arrest than did low concentrations of VA extract, suggesting a concentration-dependent effect of VA extract on the cell cycle in bladder cancer.

This study further explored the effect of VA on the cell cycle of BC cells. Immunoblot assays were performed to evaluate the expression levels of Proliferating Cell Nuclear Antigen (PCNA), CLND1, p21, and p27 in control or VA extract-treated T24 and J82 cells. PCNA and CLND1 are markers of proliferating cells, whereas p21 and p27 are indicative of cell cycle arrest. Consistent with the flow cytometry results, a marked decrease expression of PCNA and cyclin D1 was observed in VA-treated cells (Figure 2B). On the other hand, the expression of p21 and p27 in VA-treated T24 and J82 cells were significantly increased (Fig. 2B), suggesting that treatment with VA extract stimulated cell cycle arrest in bladder cancer cells.

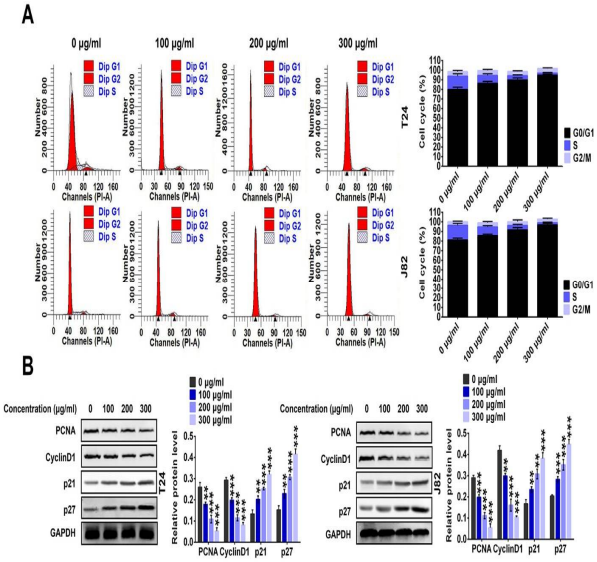

Figure 2: Effect of *Viscum album* extract on bladder cancer cell cycle. (A). J82 and T24 cells were treated with VA extract at concentrations of 100, 200 or 300 μg/mL for 48 h. Cell cycle phases were subsequently evaluated. Percent cells in different phases in the control and VA treatment groups are shown. (B). Immunoblot assays revealed the expression levels of PCNA, CLND1, p21, and p27 in control or VA extract-treated T24 and J82 cells. $P < 0.05$ was considered significant; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}P < 0.001$, compared to 0 μg/mL group

VA extract treatment contributes to apoptosis of bladder cancer cells *in vitro*

This study next assessed the potential effects of VA extract on T24 and J82 cell apoptosis *in vitro*. The results from cell apoptosis assays revealed the induction of apoptosis in VA extract-treated T24 and J82 cells in a concentration-dependent manner (Figure 3 A). To further confirm these

previous findings, we detected the expression levels of apoptosis marker Bcl-2, Bax, and cleaved caspase 3. Bcl-2 expression decreased slowly with increase in VA extract concentrations, while Bax was gradually increased (Figure 3 B). It was also showed that cleaved caspase 3 expression was increased upon VA extract treatment (Figure 3 B). Taken together, these results demonstrate the role of VA extract in apoptosis stimulation in bladder cancer.

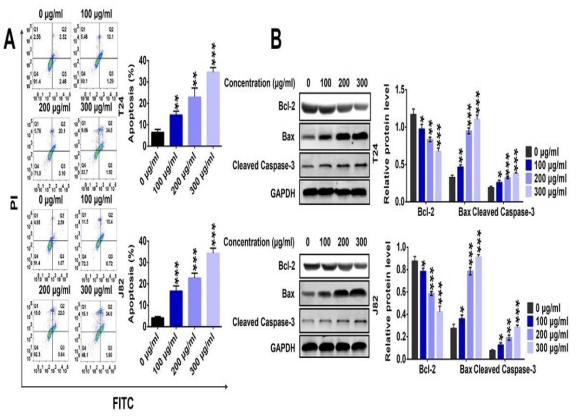

Figure 3: VA promotes cell apoptosis of bladder cancer cells *in vitro*. (A). Flow cytometric (FCM) analysis of apoptosis cells in VA extract-treated T24 and J82 cells. The proportion of apoptotic cells was quantified. (B). Immunoblot assays showing the expression of apoptosis markers including Bcl-2, Bax, and cleaved caspase 3 in control and VA extract-treated T24 and J82 cells; *$p < 0.05$, **$p < 0.01$, and *** $p < 0.001$ were considered significant, respectively

VA extract treatment suppressed Axl-AKT pathways in bladder cancer

The above results confirm the tumour inhibition activity of VA extract in bladder cancer, mainly through its effects on cell cycle progression and apoptosis. This study subsequently explored the regulatory mechanism underlying the suppression of bladder cancer by VA extract. Previous studies have indicated that VA inhibits cancer development through the Axl-AKT signaling pathway. Therefore, immunoblot assays were conducted to detect the levels of Axl, p-Axl, AKT, and pAKT in two types of bladder cancer cells (T24 and J82 cells) treated with control or VA extract (100, 200, and 300 μg/mL) for 48 h.

Immunoblot data showed a decrease in Axl expression and modest changes in AKT expression in these bladder cancer cells between the control and VA extract groups (Figure 4). However, a significant drop in the expression levels of p-Axl and pAKT were evaluated in T24 and J82 cells treated with VA extract (Figure 4). This study further observed decreases in the levels of p-Axl/Axl and p-AKT/AKT following VA extract treatment (Figure 4). Thus, VA extract treatment led to a decrease in Axl phosphorylation, which in turn led to a decrease in AKT phosphorylation, thereby inhibiting cell proliferation and inducing apoptosis through the AKT signalling pathway.

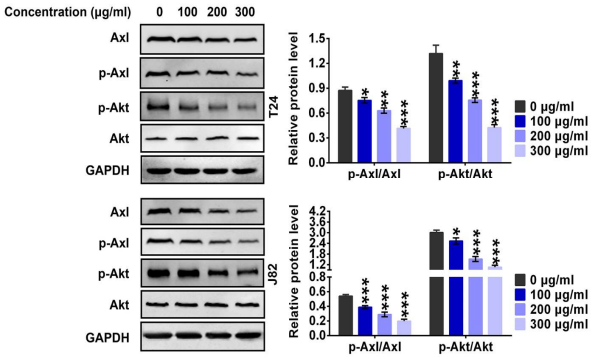

Figure 4: Effect of *Viscum album* extract on the activation of Axl-AKT pathways in BC cells. Immunoblot analysis results of the expression and phosphorylation of various proteins in T24 and J82 cells (left), and the quantitative results (right). $P < 0.05$, **P < 0.01, and *** $p < 0.001$

DISCUSSION

Bladder cancer is a high invasiveness malignancy in the urinary system, with high mortality and morbidity [3]. Unfortunately, the early symptoms of bladder cancer are not obvious, and at the time of diagnosis, patients are often at an advanced stage when bladder cancer is highly metastatic [21]. Surgical removal of bladder cancer often results in recurrence and chemotherapy has significant side effects [22]. Therefore, it is important to develop new therapeutic drugs and methods.

Chinese herbal medicine is rich in content and variety and many drugs have potential anti-tumour effects that are worthy of further study [23]. The present work found that VA, a traditional Chinese herbal medicine, has potent anti-proliferation and pro-apoptosis effects on bladder cancer cells and could be thought as a potential therapeutic methods for bladder cancer. However, the target of VA and the molecular mechanisms by which it inhibits bladder cancer require further investigation.

In this study, results from MTT assay and FCM assay revealed that VA extract treatment blocked cell survival and induced cell cycle arrest. Immunoblot assays also confirmed these results. Proliferating Cell Nuclear Antigen (PCNA)and CLND1 are cell cycle-related proteins that are often expressed at high levels in tumour tissues

and suggest a poor prognosis [24]. However, p21 and p27 are generally believed to promote cell cycle arrest, which is consistent with the results of this study [25]. Similarly, the anti-proliferation effects of VA and induction of cell cycle arrest have been observed in other tumours. In lung cancer, VA has an anti-proliferative effect and overcomes the resistance of cancer cells to cisplatin and erlotinib [26]. In leukaemia cells, VA exerts an anti-proliferative effect and promotes cell cycle arrest and apoptosis [17].

In addition, VA induced cell cycle arrest and further inhibit cell proliferation and the development of hepatocellular carcinoma [27]. This study also observed a pro-apoptosis effect of VA in bladder cancer; including various changes in the levels of Bcl-2, Bax, and cleaved caspase 3 expression, further confirming the FCM results. Similar to the above observations, another study indicated that VA induces apoptosis of rat glioma cells, mainly by regulating the expression of Hsp27 [16]. However, the detailed molecular mechanism underlying the synergistic regulation by VA of cell growth and apoptosis remains to be further studied.

On the other hand, this study also observed significant effects of VA extract on the Axl-AKT pathways in this study. The AKT signalling pathway is abnormal in a variety of tumours and results in dysregulation of cell proliferation, invasion, and apoptosis, further affecting the occurrence and development of tumours [28]. Anexelekto receptor tyrosine kinase regulates tumour development by affecting the AKT signalling pathway [29]. Several published studies confirmed the role of Axl in tumour progression, suggesting that Axl is a potential therapeutic target [30]. This study found that Axl expression and Axl and AKT phosphorylation levels were significantly reduced by VA treatment, demonstrating that the Axl-AKT signalling pathways were significantly affected, thereby affecting the growth, proliferation, and apoptosis of bladder cancer cells. Meanwhile, VA also affects lung cancer cell proliferation through the regulation of Axl expression [26]. Taken together, the findings of this work confirm that Axl could be a potential target of VA.

This present work observed an anti-proliferation effect of VA extract in bladder cancer cells *in vitro*. In addition, VA extract was further found to induce cell cycle arrest in bladder cancer, with decreased expression of PCNA and cyclin D1 and upregulation of p21 and p27. Similar to previous studies, this study found that in bladder cancer cells, VA extract promoted cell apoptosis *in vitro*; this was confirmed by the downregulation of Bcl-2 and cleaved caspase 3 and the upregulation of Bax.

CONCLUSION

This work revealed the anti-survival and pro-apoptosis roles of *Viscum album* extract on bladder cancer cells. Therefore, as a traditional Chinese herbal medicine, VA extract can serve as a novel and promising therapeutic agent for the treatment of bladder cancer.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was performed by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Shengyang Zhao, Mang Ke, and Ting Huang designed all the experiments and revised the paper. Tao Hong and Hongyuan Yu performed the experiments, Xianjun Zhang wrote the paper, and Mang Ke approved the final draft.

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