Int J Clin Exp Med 2017;10(12):16771-16779
www.ijcem.com /ISSN:1940-5901/IJCEM0056633

Original Article

Paris saponin VII inhibits epithelial-to-mesenchymal transition in ovarian cancer cell migration and invasion via the GSK-3β/β-catenin signaling pathway

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Received May 2, 2017; Accepted October 26, 2017; Epub December 15, 2017; Published December 30, 2017

Abstract: Metastasis is the main cause of death in ovarian cancer. Targeting the process of metastasis is a strategy of ovarian cancer therapy. As a traditional Chinese medicine, *Trillium tschonoskii* Maxim has been used for the treatment of many diseases, including cancer. However, no evidence has been shown for the anti-metastatic effect of Paris saponin VII (PS VII) extracted from *T. tschonoskii* on ovarian cancer cells, as well as its underling mechanisms. This study aimed to demonstrate the role of PS VII on the migration and invasion of ovarian cancer cells. Viability and proliferation of ovarian cancer cells were determined by the MTT assay. By using the scratch wound healing and Transwell assays, we detected migration and invasion of the cells, respectively. Furthermore, expression of epithelial-to-mesenchymal transition (EMT) markers was detected after stimulation with PS VII. Also, expressions of GSK-3β and β-catenin were detected by Western blot analysis. Finally, an inhibitor of GSK-3β was used to verify the effect of PS VII on expressions of EMT markers and β-catenin. We found that the proliferation, migration, and invasion of A2780 ovarian cancer cells were inhibited by using PS VII. PS VII up-regulated E-cadherin and down-regulated N-cadherin and vimentin in a dose-dependent manner. PS VII also exerted its ability to activate GSK-3β as well as inhibiting expression of β-catenin and its nuclear translocation. Finally, by applying a GSK-3β inhibitor, lithium chloride (LiCl), we determined the roles of GSK-3β in expression of EMT markers and its transcription factor. Taken together, these results demonstrated that PS VII inhibited EMT and reduced the invasion of ovarian cancer cells via the GSK-3β/β-catenin signaling pathway.

Keywords: Paris saponin VII, migration, invasion, ovarian cancer cells, GSK-3β

Introduction

Ovarian cancer is one of the most common cancers in women worldwide with the highest mortality rate of gynecological malignancies. In 2013, there were more than two million new cases and one million mortalities worldwide [1, 2]. Despite some advances in surgery followed by neoadjuvant chemotherapy on localized ovarian cancer, the mortality of ovarian cancer patients, due to the highly invasive nature, of the cancer cells, still remains high [3]. As a result, about 70%-80% of patients who have distant metastasis die within 5 years of diagnosis [4, 5]. Therefore, there is an urgent need to determine the molecular mechanisms of ovarian cancer metastasis and to identify novel drugs for a better treatment outcome.

Ovarian cancer cells are highly invasive, and the inhibition of their invasion may be effective in the treatment of ovarian cancer. To acquire invasive abilities, cancer cells undergo epithelial-mesenchymal transition (EMT), a unique phenotypic change which is a morphological conversion process [6]. EMT is a highly conserved cellular process which is characterized by loss of cell adhesion, inhibition of the epithelial molecule E-cadherin, and acquisition of mesenchymal markers N-cadherin and vimentin [7]. EMT was first described as a feature of embryogenesis. Recently, it was reported in mechanisms of carcinoma invasion and metastasis [7, 8]. Among the mediators that regulate EMT, glycogen synthase kinase (GSK-3β), was shown to play an important role [9, 10]. Previous studies reported the crucial role of GSK-3β in modulat-

ing cell functions, such as cytoskeleton maintenance, gene transcription, migration, and invasion [11-13]. Among them, a study demonstrated that GSK-3β plays an important role in β-catenin degradation and nuclear translocation [12]. Previous studies demonstrated the role of GSK-3β/β-catenin in regulating the metastasis of various type of tumors [9, 14, 15].

Recent studies showed that natural products were reliable sources in anti-tumor therapeutic pharmaceutical drug development [16]. Among of them, *Trillium tschonoskii* Maxim has long been used in ameliorating pain and treating hypertension, and neurasthenia, as well as cancer [17]. Paris saponin VII (PS VII) (**Figure 1A**) is a type of steroidal saponin which is extracted from *T. tschonoskii*. Previous studies revealed that PS VII possesses an anti-tumor property in various types of cancer. PS VII can inhibit the growth of colorectal cancer cells through the Ras pathway and suppress their metastasis by modulating MMP-2 and MMP-9 production [18-20]. PS VII was also shown to inhibit the viability and induce apoptosis of breast cancer cells [21]. Another study found that PS VII can suppress the growth of cervical cancer Hela cells [22]. Moreover, PS VII was shown to have the capacity of suppressing the migration and invasion of lung cancer cells [23]. However, no direct evidence has been shown as to whether PS VII has any effect on ovarian cancer migration, invasion and EMT, as well as its mechanisms.

The present study was designed to evaluate the role of PS VII in ovarian cancer cell migration and invasion, as well as EMT and its underlying molecular mechanisms.

Materials and methods

Cell lines and reagents

Human A2780 ovarian cancer cell lines were purchased from the American Type Culture Collection (ATCC) and were routinely cultured in RPMI-1640 containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in 5% CO_2 at 37°C. Rabbit anti-Akt, phosphor-Akt, Rabbit anti-GSK-3β, and phosphor-GSK-3β antibodies were purchased from Cell Signaling Technology (USA). Rabbit anti-E-cadherin, N-cadherin, vimentin, and β-catenin antibodies were purchased from Abcam (Cambridge, UK). PS VII with a purity of >98% was purchased from PureOne Biotechnology (Shanghai, China). Lithium chloride (LiCl), a selective inhibitor of GSK-3β, was purchased from Sigma-Aldrich (USA).

MTT assay of cell viability and proliferation

A2780 ovarian cancer cell viability and proliferation were examined with the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. A2780 cells (5000 cells/well) in 100 μL medium were seeded into 96-well plates. After stimulation with PS VII of various concentrations (0, 0.5, 1, 2.5, 5, and 10 μM), for various times (0, 3, 6, 12, 24, and 48 h), and 20 μl MTT (5 mg/ml) was added into each well. After incubation for 4 h, 100 μl of dimethyl sulfoxide (DMSO) was added to each well for another 15 min. Finally, the absorbance values were determined by a microplate luminometer (Bio-Rad, CA, USA) at 490 nm.

Western blot

After stimulation with PS VII, A2780 cells were collected and lysed. Total cell protein concentrations were examined by using the BCA protein assay kit (Beyotime Biotechnology, China). Proteins from cell lysates were loaded in 12% SDS-PAGE gels. After electrophoresis proteins were transferred to PVDF membranes (Millipore, USA) and blocked with 5% fat-free milk at room temperature for 1 h. After incubation with the indicated primary antibodies overnight at 4°C, the membranes were washed with TBST and then incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Finally, immune complexes were detected with ECL reagents, and the blots were quantified by densitometric analysis using the Alpha Imager 2200.

GSK-3β kinase assay

A fluorescence peptide substrate-based assay was used to evaluate GSK-3β kinase activity (Omnia Ser/Thr Recombinant kit, Invitrogen). The GSK-3β complex was prepared from equal amounts of cell lysates by immunoprecipitation. Then it was incubated with 10 μM of Ser/Thr peptide substrate in the kinase reaction buffer (containing 1 mM ATP and 1 mM DTT) for 20 min at 30°C. The fluorescent intensity was

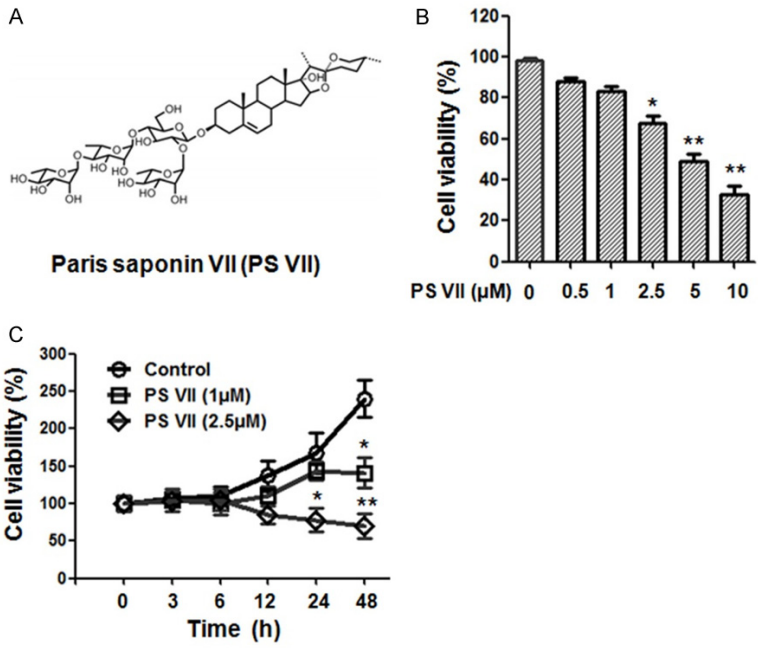

Figure 1. The chemical structure of Paris saponin VII (PS VII) and the effect of PS VII on A2780 ovarian cancer cell proliferation. A. Chemical structure of PS VII. B. After treatment with PS VII for 24 h at various concentrations (0, 0.5, 1, 2.5, 5, and 10 μM), the viability of the A2780 cells was detected by MTT assay. C. After treatment with 0, 1 and 2.5 μM PS VII for different times (0, 3, 6, 12, 24, and 48 h), MTT assay was used to examine the viability of A2780 cells. The results represent the mean ± SD from three independent experiments. *P<0.05, **P<0.01 vs. the control group.

recorded by measuring the *A*485 in a 96-well plate. The relative GSK-3β activity was calculated by using untreated cells (equal to 1).

Scratch wound healing assay

A scratch wound healing assay was used to evaluate the migration ability of the A2780 cells. Briefly, A2780 cells (1×10^6/well) were seeded in 6-well plates cultured with RPMI-1640 supplemented with 10% FBS. When confluency was reached, each well was straight scratched with a 200 μl pipette tip. To evaluate the effects of PS VII on the migration of A2780 cells, 1 μM PS VII was added to the plates. After 24 h of incubation, the wound healing areas were photographed and then the distance between the two cell edges was analyzed by ImageJ software.

In vitro invasion assay

To access the effect of PS VII on the invasive ability of A2780 cells, the Transwell system was used. A2780 cells were cultured in Boyden chambers, with 8-μm pore filter inserts, in 24-well plates (Corning Costar). The pore inserts were pre-coated overnight with Matrigel (BD Biosciences, USA). A2780 cells (1×10^5 cells/well) were suspended in 100 μl RPMI-1640 supplemented with 1% FBS and were added to the upper chamber. 200 μl RPMI-1640 with 10% FBS and 1 μM PS VII was added to the lower chamber. After 24 h of incubation, the cells that had attached to the lower surface were fixed with methanol and then stained with 0.1% crystal violet. Finally, 5 random high-power fields (magnification, ×200) of each sample were selected and counted to determine the average number of invasive cells.

Statistics

The data are shown as the mean ± standard deviation (SD). All of the experiments were repeated at least three times. Comparisons among values for all groups were performed by one-way analysis of variance (ANOVA). The Holm's t-test was used for analysis of differences between different groups. Differences were considered to be significant at P<0.05.

Results

Inhibitory effect of PS VII on the cell viability of ovarian cancer cells

The structure of PS VII is shown in **Figure 1A**. To verify the role of PS VII on the cell viability of A2780 ovarian cancer cells, the MTT assay was used as described in the materials and methods. As shown in **Figure 1B**, different doses (0, 0.5, 1, 2.5, 5, and 10 μM) of PS VII were added to the cultured A2780 cells. At a dose of 2.5, 5, and 10 μM, PS VII significantly suppressed the viability of A2780 cells after 24 h treatment. However, at doses below 2.5 μM (0.5 and 1 μM), the inhibitory effect was not significant. We selected 1 and 2.5 μM of PS VII to treat the cells at different times (0, 3, 6, 12, 24, and 48 h). As shown in **Figure 1C**, after 24 h treatment, PS VII significantly suppressed the cell

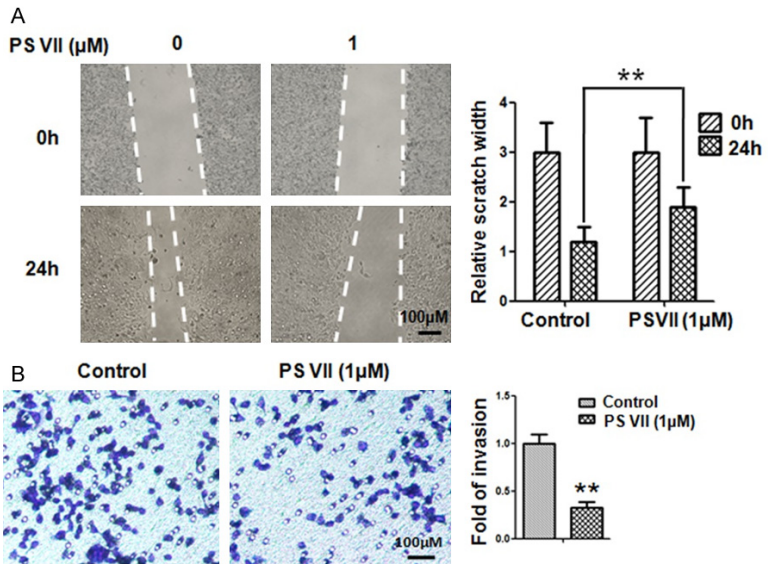

Figure 2. PS VII inhibits the migration and invasion of A2780 ovarian cancer cells. A. A2780 cells were treated with PS VII at a concentration of 1 μM for 24 h and then assessed by a wound healing assay. B. A2780 cells were treated with PS VII at a concentration of 1 μM for 24 h. The effect of PS VII on invasion of A2780 ovarian cancer cells was assessed by Transwell assay. The bar graph represents the results of three independent experiments, **p<0.01 vs. the control group.

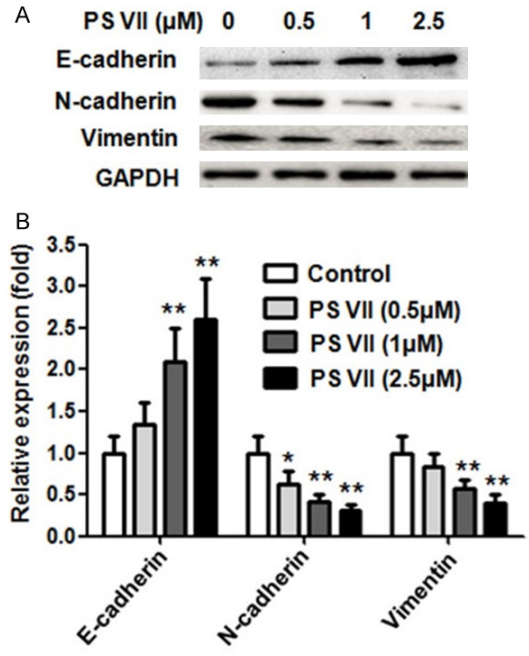

Figure 3. PS VII increases expression of E-cadherin and reduces expressions of N-cadherin and vimentin of A2780 ovarian cancer cells. A. A2780 cells were treated with PS VII at various concentrations (0, 0.5, 1, and 2.5 μM) for 24 h and the levels of E-cadherin, N-cadherin, and vimentin protein expressions were determined by Western blotting analysis. B. Statistical analysis of the Western blotting results. *P<0.05, **P<0.01 vs. the control group. Data shown are means ± SD from three independent experiments.

viability at a dose of 2.5 μM, while 1 μM PS VII did not inhibited cell viability. However, after 48 h treatment, 1 and 2.5 μM of PS VII significantly suppressed cell viability. Based on these results, we chose PS VII at a dose of 1 μM for 24 h in the following migration and invasion experiments so that the effect of cell viability was excluded.

Migration and invasion are inhibited by PS VII in ovarian cancer cells

A wound healing assay was used to evaluate the role of PS VII on the migration of A2780 ovarian cancer cells. A2780 cells were treated with 1 μM PS VII for 24 h. As shown in **Figure 2A**, migration of A2780 cells was inhibited by PS VII (1 μM). The results of the wound healing assay revealed that healing over the scratch was significantly deceased with treatment of PS VII. To further explore the role of PS VII on A2780 cell invasion, a Transwell assay was used. A2780 cells were treated with 1 μM PS VII for 24 h. As shown in **Figure 2B**, the invasive ability of A2780 cells were suppressed by PS VII (1 μM). The results of the Transwell assay suggested that PS VII can inhibit the invasion of ovarian cancer cells.

PS VII altered expressions of EMT markers in ovarian cancer cells

Much evidence showed that EMT plays an important role in the process of cancer cell invasion and metastasis [24, 25]. We explored the effect of PS VII on expressions of EMT markers (E-cadherin, N-cadherin, and vimentin) in A2780 cells. Various doses of PS VII (0, 0.5, 1, and 2.5 μM) were added to A2780 cells for 24 h. As shown in **Figure 3A** and **3B**, PS VII treatment significantly increased expression of the epithelial marker E-cadherin and decreased expressions of the mesenchymal markers N-cadherin and vimentin in A2780 cells. These results indicated that PS VII may alter expressions of EMT markers in a dose-dependent manner. The above results also suggested that the inhibitory effect of PS VII on A2780 cell

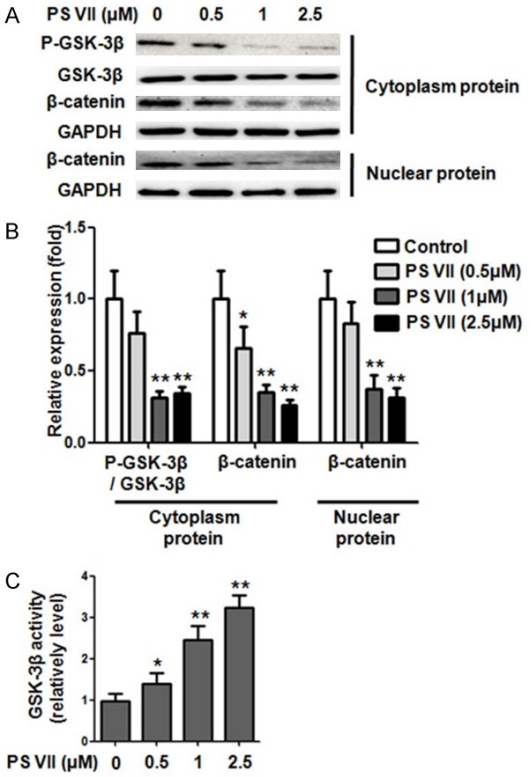

Figure 4. PS VII modulates the GSK-3β/β-catenin signaling pathway in A2780 ovarian cancer cells. A. A2780 cells were stimulated with PS VII at various concentrations (0, 0.5, 1, and 2.5 μM) for 24 h, and expressions of p-GSK-3 and β-catenin in the cytosol and β-catenin in the nucleus were analyzed by Western blotting. B. Statistical analysis of the Western blotting results. C. A2780 cells were stimulated with PS VII at various concentrations (0, 0.5, 1, and 2.5 μM) for 24 h, and GSK-3 activity was determined by GSK-3β Kinase Assay. *P<0.05, **P<0.01 vs. the control group. Data shown are means ± SD from three independent experiments.

invasion and migration may be associated with EMT.

PS VII activates GSK-3β and inhibits β-catenin expression and nuclear translocation

GSK-3β, known to be a survival factor for cancer, plays an important role in various cancer developments. Modulation of EMT through the GSK-3β/β-catenin signaling pathway was demonstrated in tumorigenesis and cancer progression [14, 26, 27]. To verify the effect of PS VII on the GSK-3β/β-catenin signaling pathway, we stimulated A2780 cells with various doses of PS VII (0, 0.5, 1, and 2.5 μM) for 24 h. Then the cytosol and nuclear proteins were extracted, followed by detection of expressions of phosphor-GSK-3β and β-catenin. As shown in **Figure 4A** and **4B**, the relative expression of phospho-GSK-3β in the cytosol was significantly down-regulated in a dose-dependent manner. Expressions of cytosol and nuclear β-catenin proteins were also significantly down-regulated in a dose-dependent manner. To further assess the result, the effect of PS VII on GSK-3β activity was detected by GSK-3β kinase assay. The result demonstrated that PS VII over a range of concentrations (0, 0.5, 1, and 2.5 μM) can significantly promote GSK-3β activity in A2780 cells. These results suggested that PS VII can activate GSK-3β and inhibit β-catenin expression and nuclear translocation. These results implied that the GSK-3β/β-catenin pathway may be involved in PS VII-induced inhibition of EMT in ovarian cancer cells.

The GSK-3β/β-catenin pathway is involved in the anti-metastatic mechanism of PS VII in ovarian cancer cells

To further investigate whether the effect of PS VII on EMT was attributed to GSK-3β/β-catenin signaling, LiCl (a GSK-3β inhibitor) was chosen to pre-treat the cells for 1 h before PS VII application in order to block GSK-3β activation. As shown in **Figure 5A** and **5B**, inhibition of GSK-3β activity by LiCl significantly reversed PS VII-induced E-cadherin, N-cadherin, and vimentin expressions. These results suggested that GSK-3β activation is critical for EMT. The results further indicated that PS VII exerted its anti-metastatic effect and that mediation of EMT might be GSK-3β -dependent.

To further investigate the anti-metastatic mechanism of PS VII, we detected the effect of LiCl on expression of β-catenin in the presence and absence of PS VII. As shown in **Figure 5A** and **5B**, expressions of β-catenin in the cytosol and nucleus were significantly reversed by using the GSK-3β inhibitor. The results indicated that PS VII suppressed expression of β-catenin and its nuclear translocation at least in part through the GSK-3β pathway. Taken together, these results strengthen the idea that inhibition of metastasis by PS VII is modulated by inhibition of EMT through the GSK-3β/β-catenin signaling pathway in ovarian cancer cells.

Discussion

Metastasis is regarded as a leading cause of mortality in most cancer patients. Thus, revealing the mechanisms of metastasis and seeking effective agents to inhibit metastasis are the

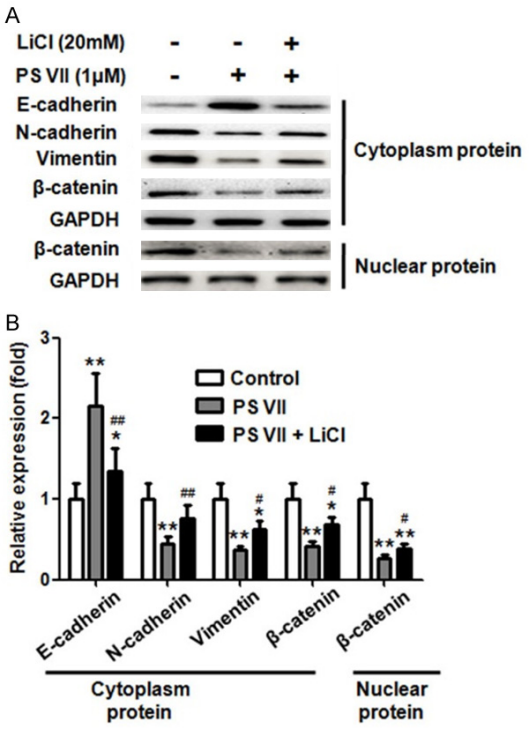

Figure 5. Inhibition of GSK-3β on PS VII-mediated epithelial-to-mesenchymal transition (EMT) markers and expression of β-catenin in A2780 ovarian cancer cells. A. A2780 cells were treated with PS VII (1 μM) and/or LiCl (20 mM) for 24 h, then expressions of E-cadherin, N-cadherin, vimentin and β-catenin in the cytosol and β-catenin in the nucleus were assessed by Western blotting assay. B. Statistical analysis of the Western blotting results. The results represent the mean ± SD from three independent experiments. *P<0.05, **P<0.01 vs. the control group; #P<0.05, ##P<0.01, compared with the cells treated with PS VII.

most crucial issues in cancer research. As a type of steroidal saponin extracted from *T. tschonoskii*, PS VII has been recently reported to have the ability to suppress cell proliferation, migration, and invasion in colorectal, breast, lung, and cervical cancer cells [18-23]. Although previous studies demonstrated the anti-cancer effect of PS VII, the role of PS VII on ovarian cancer cell migration and invasion needs to be clearly elucidated, as well as EMT and its detailed molecular mechanisms. In our study, we first examined the effect of PS VII on the viability of ovarian cancer cells. Our results found that 1 μM PS VII significantly suppressed cell proliferation after 48 h of treatment, while there was no significant effect before 24 h. Therefore, we selected 24 h as the stimulation time-point and 1 μM as the stimulation concentration in the following experiments involving cell migration and invasion, so that the influence of proliferation was excluded. For the first time, we revealed that PS VII could effectively suppress ovarian cancer cell migration and invasion by using wound healing and Transwell assays. These results demonstrated the anti-metastatic activity of PS VII.

The metastasis of ovarian cancer is a complex and multi-step process which includes migration, invasion, and adhesion [28]. EMT is a unique phenotypic change through which epithelial cells lose their polarity and cell-cell adhesion and acquire the migratory and invasive properties of mesenchymal cells. EMT is characterized by inhibition of the epithelial marker E-cadherin and acquisition of the mesenchymal markers N-cadherin and vimentin. After gaining a mesenchymal phenotype through EMT, cancer cells can invade adjacent tissues, break through the basement membrane, and then enter the bloodstream [29]. Previous studies revealed the role of EMT in ovarian cancer progression by increasing matrix metalloproteinase production and promoting dissemination, thus leading to poor prognosis in ovarian cancer patients [30-32]. In the present study, we explored the specific role of PS VII and the underlying relationship between PS VII and EMT in ovarian cancer cells. It was found that PS VII up-regulated expression of the epithelial marker E-cadherin and down-regulated expressions of the mesenchymal markers N-cadherin and vimentin in a dose-dependent manner after incubation with PS VII for 24 h. Our results indicated that PS VII inhibited EMT in ovarian cancer cells.

GSK-3β, a crucial signal transduction pathway, was reported to regulate a diverse range of cell functions, such as cytoskeleton maintenance and gene transcription [11, 12]. Expression of GSK-3β is associated with cell migration and invasion [13]. The activity of GSK-3β is modulated by a site-specific phosphorylation of Tyr216/Ser9 residues, and the phosphorylation of GSK-3β leading to its inactivation [33]. Furthermore, GSK-3β activity is crucial for β-catenin degradation and nuclear translocation which is essential for carcinogenesis [12]. Previous studies showed the role of GSK-3β/β-catenin in regulating the metastasis of various types of tumors, such as renal cancer, gastric cancer, breast cancer, lung cancer, and hepatocellular carcinoma, by modulating EMT [9, 14,

15, 27, 34]. In this study, the GSK-3β activity in ovarian cancer cells was determined by Western blot and GSK-3β kinase assay. Our results found that expression of phosphor-GSK-3β was decreased while GSK-3β activity was increased when treated with PS VII. Furthermore, expression of β-catenin in the cytosol and nucleus was detected by Western blot. We found that expressions of cytosol and nuclear β-catenin proteins were significantly down-regulated in a dose-dependent manner. These results suggested that PS VII can activate GSK-3β and inhibit β-catenin expression and nuclear translocation. Our results also implied that the GSK-3β/β-catenin pathway may be involved in PS VII-induced suppression of EMT in ovarian cancer cells.

To verify that the GSK-3β/β-catenin signaling pathway was involved in PS VII-induced suppression of EMT in ovarian cancer cells, we blocked GSK-3β activity by using the GSK-3β inhibitor LiCl. It was found that inhibition of GSK-3β activity by LiCl significantly reversed PS VII-induced E-cadherin, N-cadherin, and vimentin expressions. These results indicated that GSK-3β activation is critical for EMT and the role of PS VII might be GSK-3β-dependent. Also, blockade of GSK-3β activity further reversed expression of β-catenin in the cytosol and nucleus. These results demonstrated that PS VII suppressed expression of β-catenin and its nuclear translocation via the GSK-3β pathway.

In summary, the present study for the first time suggested that PS VII suppressed the migration and invasion of ovarian cancer cells by inhibiting EMT. Also, the inhibitory effect of PS VII in EMT was modulated through the GSK-3β/β-catenin signaling pathway. Taken together, PS VII inhibits the invasion of ovarian cancer cells by inhibiting EMT via the GSK-3β/β-catenin signaling pathway. Therefore, our findings demonstrated that PS VII is a promising agent in ovarian cancer therapy. Further *in vivo* research needs to be done.

Acknowledgements

This study was supported by the Medical and Health Development of Science and Technology Plan Project of Shandong Province (no. 2016WS0740) and Medical and Health Development of Science and Technology Plan Project of Zibo City (no. 2016kj010059). Thanks to Dr. Edward C. Mignot, Shandong University, for linguistic advice.

Disclosure of conflict of interest

None.

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