The role of vitamin D up-regulated protein 1 in epithelial ovarian cancer

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Summary

Purpose: The objective of this study was to verify the relationship between the expression of VDP1 and Jun activation domain-binding protein 1 (Jab1) and cyclin-dependent kinase inhibitor 1B (CDKN1B: p27kip1) in ovarian cancer cell strains. *Materials and Methods*: The ovarian cancer cell lines SK-OV-3, OVCAR-3 were plated and cultivated for 24 hours in a 37°C carbon dioxide incubator. Thereafter, vitamin D was added at various concentrations, and the cells were cultivated for an additional 24 hours. Using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, cell growth was checked, and changes in the protein level were observed using Western blotting, immunofluorescence and immunocytochemistry. *Results*: The authors found that the viability of the ovarian cancer cells was decreased by vitamin D, dose dependently. The results of Western blotting, immunofluorescence, and immunocytochemistry revealed that p27kip1 and VDUP1 increased, while Jab1 decreased, dose dependently. *Conclusion*: These findings show the relationship between p27kip1, Jab1 and VDUP1 following vitamin D treatment in ovarian cancer, indicating a correlation with cancer cell proliferation.

Key word: Epithelial ovarian neoplasms; Jab1; p27kip1; VDUP1 protein; Vitamin D.

Introduction

Epithelial ovarian cancer, which is almost incurable unless the disease is discovered early, shows the poorest prognosis among genital cancers [1, 2]. Despite decades of research, a clear mechanism and causative factor remain unknown. Hypotheses have been suggested, and continuous estrogen stimulation by ovulation has been considered a cause. Epidemiologically, it is known that the risk of ovarian cancer becomes critical by early menarche and delayed menopause [3-5]. Vitamin D binding to the vitamin D receptor allows calcium and phosphorus to be absorbed in the intestinal canal, playing a vital role in cell growth and function of bone [6, 7]. Regarding the inhibition of cell proliferation, the active form of vitamin D, vitamin 1, 25-dyhydroxyvitamin D3 or 1α, 25(OH)2D3 or calcitriol, is noted to be effective for the prevention and treatment of tumors [8].

Within cells, vitamin D up-regulated protein 1 (VDUP1) expression increases following exposure to hydrogen peroxide, UV rays or heat shock, lack of serum and transforming growth factor-β [9, 10]. VDUP1 is considered a spontaneous thioredoxin inhibitor, and studies are ongoing concerning the thioredoxin inhibitor as a new molecular targeted agent [11]. Until now, no study has focused on this topic in detail for ovarian cancer.

The cyclin-dependent kinase inhibitor 1B (CDKN1B: p27[kip1]) protein is encoded by a sthenic repressor gene of tumors, and its decrease indicates rapid progression and poor consequences in relation to anti-tumor agents. It is known that Jun activation domain-binding protein 1 (Jab1) aids p27[kip1] movement from the nucleus to the cytoplasm to destroy p27[kip1] [12]. Relationships between vitamin D and other cancers have been reported, with the exception of ovarian cancer. In this study, it is assumed that the expression levels of Jab1 and p27[kip1] in ovarian tumors have a negative correlation and are related to the progression and consequences of cancer. Meanwhile, VDUP1 interacts with Jab1 to inhibit it directly and interrupt p27[kip1] destruction and cell proliferation by Jab1 and p27[kip1] [13].

Thus, this study will focus on the relationship between the expression of VDUP1 and that of Jab1 and p27kip1 in ovarian cancer cells.

Materials and Methods

The human cancer cell lines OVCAR-3 and SK-OV-3 were used. OVCAR-3 cells were grown in RPMI-1640 supplemented with 20% fetal bovine serum (533-6955) and penicillin (100 mg/l) in a humidified atmosphere of 5% CO_2 at 37°C. SK-OV-3 cells were grown in RPMI-1640 supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% CO_2 at 37°C.

The effects of vitamin D treatment on cell proliferation were examined by the 3- (4,5-dimethylthiazol-2-yl) -2,5 -diphenyltetrazolium bromide (MTT) assay. OVCAR-3 cell and SK-OV-3 cells were plated and grown in 96-well plates at a concentration of 1.4×10^4 cells/well and 8×10^3 cells/well, respectively, for 24 hours. Cells were then treated with varying doses of $1,25(OH)_2$ vitamin D_3 for 24 hours. Viable cell densities were determined by metabolic conversion of the dye MTT. MTT (5 mg/ml) was added to the 96-well plates at a concentration of 40 μl/well, and the plates were incubated for an additional hour. The MTT reaction was terminated by the addition of 100 μl of dimethyl sulfoxide. Subsequently, the MTT assay results were read by measuring the absorption at 595 nm.

The lysate was prepared according to the manufacturer's instructions. All of the steps were carried out at 4°C. The protein concentrations were measured using a dye-binding protein assay based on the Lowry method. Cells were lysed in sodium dodecyl sulfate (SDS) sample buffer and run on 10% polyacrylamide gels, and then were transferred to nitrocellulose membranes using standard techniques with primary antibodies and horseradish peroxidase-conjugated secondary antibodies. The antibodies used were VDUP1, Jab1, and p27^{kip1}. Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system on X-ray film.

Cultured cells were rinsed briefly in phosphate-buffered saline (PBS; pH 7.4). The PBS was then aspirated, and the cells were covered to a depth of 2-3 mm with 2-4% formaldehyde for 15 minutes at room temperature. Cells were washed three times for five minutes in PBS and were covered with ice-cold 100% methanol (an adequate amount was used to cover the cells completely to a depth of 3-5 mm) and incubated in methanol for ten minutes at -20°C, followed by washing three times with PBS. The cells were then incubated in a humidified chamber with blocking buffer (5% normal goat serum + 0.3% Triton X-100 in PBS) for one hour at room temperature. The primary antibody was applied in a moist chamber overnight at 4°C. The primary antibodies used were mouse VDUP1 monoclonal antibody, mouse Jab1 monoclonal antibody, and rabbit anti-p27 KIP 1 monoclonal antibody. After three additional rinsing steps with PBS for five minutes, anti-rabbit IgG H&L antibody and anti-mouse IgG H&L antibody were added separately for one hour at room temperature in the dark. After being washed with PBS, coverslip slides were incubated with fluoroshield mounting medium with DAPI. All experiments were executed with control staining without the primary antibody to ensure that negative controls remained unstained.

Immunostaining was performed using the EXPOSE mouse and rabbit specific HRP/DAB detection IHC kit for Jab1, p27, and VDUP1. The primary antibodies used were mouse VDUP1 monoclonal antibody (1:250 dilution), mouse Jab1 monoclonal antibody (1:250 dilution), and rabbit anti-p27 KIP 1 monoclonal antibody (1:250 dilution). Negative controls were stained after omitting the primary antibody incubation step. For semiquantitative analysis of immunoreactivity, the H-score was used in the present study. The H-score was subsequently generated by adding the percentage of strongly stained nuclei (3×), percentage of moderately stained nuclei (2×), and percentage of weakly stained nuclei (1×), yielding a possible range of 0–300. The score was independently obtained by two of the observers. Three hundred cells were counted in randomly selected fields in each case.

For the assessment of apoptosis, cells were plated and treated with Vitamin D as indicated for 24 hours. Cells were washed twice in ice-cold Dulbecco's phosphate-buffered saline (DPBS) followed by centrifugation at 13,000 rpm for three minutes and

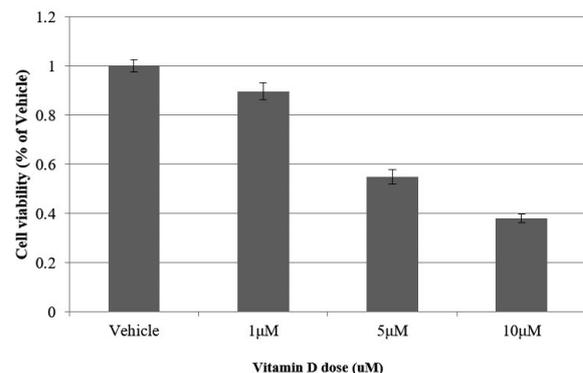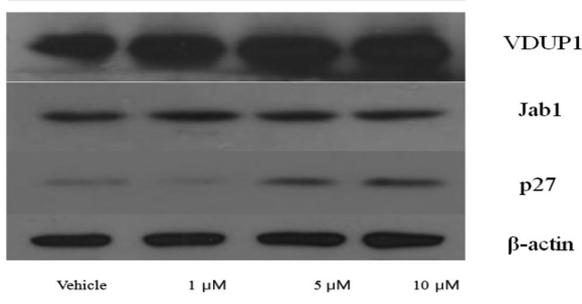## A. SK-OV-3 cell line
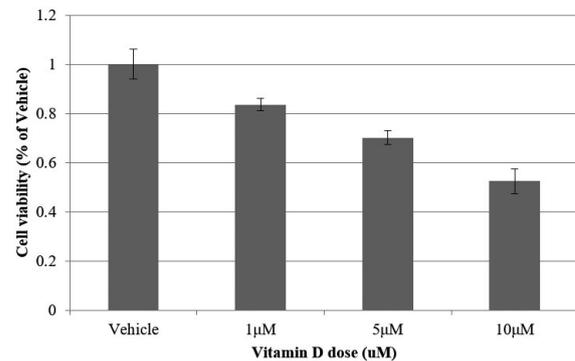
B. OVCAR-3 cell line

Figure 1. — Effect of vitamin D on cell viability in SK-OV-3 (A) and OVCAR-3 (B) ovarian cancer cells. Using the MTT assay, it was found that vitamin D induced SK-OV-3 and OVCAR-3 cell death in a dose-dependent manner. (A) After 24 hours, SK-OV-3 cell viability was reduced by vitamin D. Vitamin D was dissolved in ethanol to yield different concentrations: 1 μM, 5 μM, and 10 μM. The viability of SK-OV-3 cells treated with 1 μM vitamin D was about 83% compared with the survivability of vehicle dose-treated cells. (B) After 24 hours, the OVCAR-3 cell viability was reduced by vitamin D. Vitamin D was dissolved in ethanol to yield different concentrations: 1 μM, 5 μM, and 10 μM. The viability of OVCAR-3 cells treated with 1 μM vitamin D was about 89% compared with the survivability of vehicle dose-treated cells. Data were expressed as the percent of vehicle and mean ± standard deviation (SD).

resuspension in ice-cold DPBS containing 1× binding buffer. The cells were stained with Annexin V-FITC and propidium iodide according to the vendor's protocols and analyzed using a FACSAria flow cytometer.

Quantitation was performed using Image J. Statistical analysis was performed using SPSS Software (version 17.00). The present data were analyzed using Fisher's exact test and analysis of variance (ANOVA) followed by Duncan's post-hoc test. Differences were statistically significant at a p-value less than 0.05.
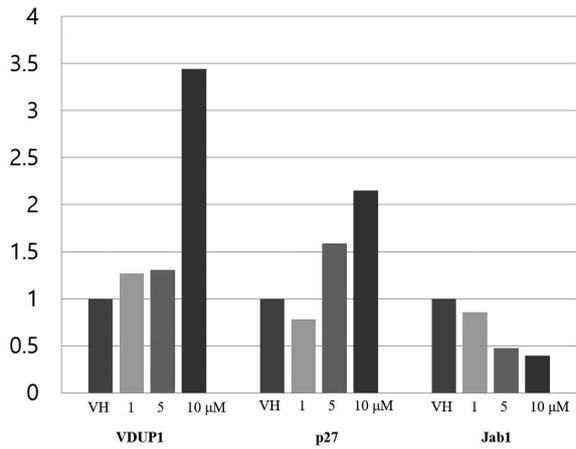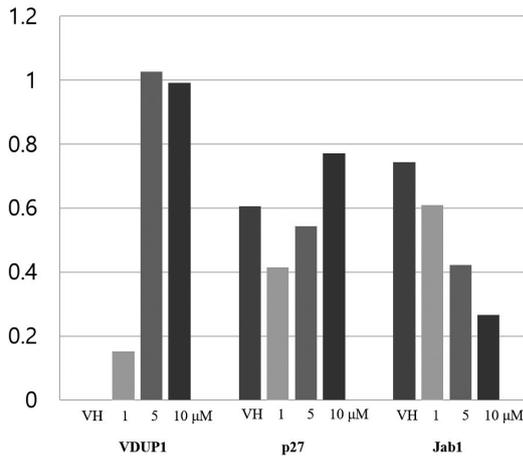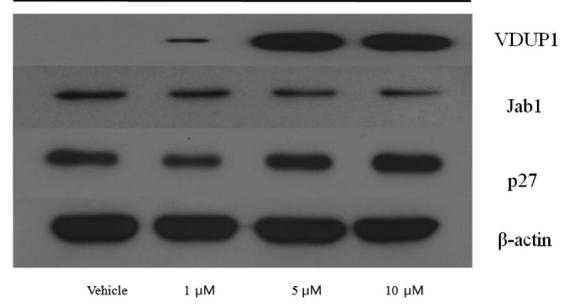
Figure 2. — Expression of VDUP1, p27kip1, and Jab1 in SK-OV-3 and OVCAR-3 cancer cells following treatment with vitamin D. Western blotting shows the effect of Vitamin D, which increased the expression of VDUP1, p27kip1, and decreased Jab1 in ovarian cancer cell lines. Cells were treated with 1 μM, 5 μM, and 10 μM vitamin D. β-Actin was used as an internal (loading) control. After carrying out Western blotting, quantification was performed for VDUP1, Jab1, and the p27kip1/ β-actin ratio.
(A) SK-OV-3 cell line. (B): OVCAR-3 cell line.

Results

In this study, the effect of VDUP1 expression was observed by injecting vitamin D into ovarian cancer cells. For the study, the cell lines SK-OV-3 and OVCAR-3 were used. Using the MTT assay, cell death in the cell lines was evaluated. Compared with the control group, Sk-OV-3 cells injected with 1 μm vitamin D showed 83% viability after 24 hours, and the viability decreased dose-dependently. Only 50% viability was found in cells treated with 10 μm vitamin D (Figure 1A).

OVCAR-3 treated with 1 μm vitamin D had 80% viability after 24 hours, and the viability decreased dose-dependently. Additionally, 40% viability was observed following treatment with 10 μm vitamin D (Figure 1B).

To find out whether vitamin D provokes apoptosis in SK-OV-3 and OVCAR-3 cells, Annexin V staining was checked (Figure 2).

In SK-OV-3 cells in which 10 μm vitamin D was in-

jected, the late apoptotic AY2 region had expanded after 24 hours, signifying the occurrence of apoptosis in the presence of 10 μm vitamin D compared with the vehicle. Similarly, in OVCAR-3 cells treated with 10 μm vitamin D, the late apoptotic AY2 region expanded after 24 hours. Thus, for treatment 10 μm vitamin D, apoptosis was observed.

After treatment of the ovarian cancer cell lines with vitamin D at different concentrations for 24 hours, the expression levels of VDUP1, Jab1 and p27[kip1] were observed by Western blot analysis (Figure 3). The expression levels of VDUP1 and p27[kip1] were increased, and that of Jab1 was decreased in vitamin D-treated SK-OV-3 cells (Figure 3A). The expression levels of VDUP1 and p27[kip1] increased while that of Jab1 decreased in vitamin D-treated OVCAR-3 cells (Figure 3B).

Using immunofluorescence, the expression levels of VDUP1, Jab1 and p27[kip1] in ovarian cancer cells were observed with different concentrations of vitamin D. In both

A. SK-OV-3 cell line
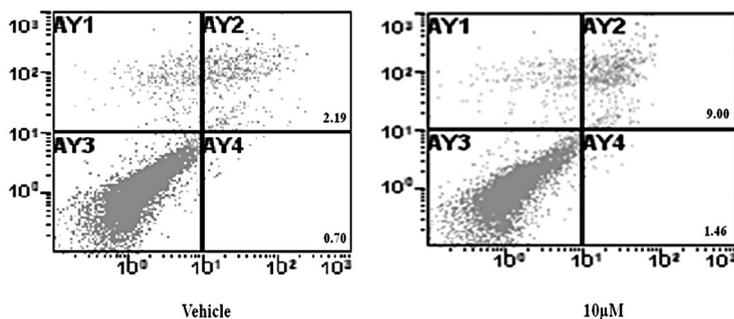
B. OVCAR-3 cell line
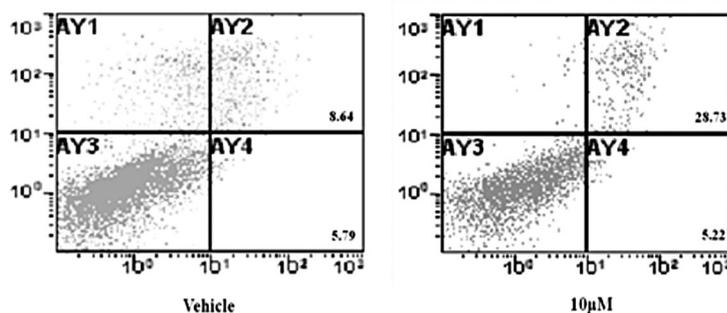

Figure 3. — Evaluation of the type of cell death after exposure to vitamin D. Cells were treated with vehicle and 10 μM vitamin D. The proportion or type of cell death was identified by the annexin V cell death assay. AY1, AY2, AY3, and AY4 denote necrotic, late apoptotic, viable (live) and early apoptotic regions, respectively. (A) SK-OV-3 cell line. (B) OVCAR-3 cell line.

cell lines, VDUP1 and p27^{kip1} showed clear expression following treatment with 10 μm vitamin D, while Jab1 only showed clear expression in the vehicle and was partially revealed in the cell lines following vitamin D treatment (Figure 4).

The H-score of VDUP1, Jab1, and p27^{kip1} expression was counted by immunocytochemistry in the vitamin D-treated ovarian cancer cell lines (Figure 5). Compared with the vehicle, p27^{kip1} showed an increasing tendency in vitamin D-treated SK-OV-3 cells (Figure 5A). Increased Jab1 was observed in OVCAR-3 cells treated with 10 μm vitamin D compared with the vehicle, and VDUP1 showed a significant increase at the 10-μm vitamin D concentration.

From the results of Western blotting, immunofluorescence, and immunocytochemistry, vitamin D showed a tendency to increase the expression of VDUP1 and p27^{kip1} but decrease the expression of Jab1 in ovarian cancer cells.

Discussion

Most cases of ovarian cancer are epithelial, showing Stage III or IV progressive lesions for 75% of patients at the time of diagnosis, and approximately 85% of patients die of the disease [14, 15]. Moreover, ovarian cancer is ranked as the sixth most common cancer and the most fatal one among female cancers [16, 17].

Regarding ovarian cancer, BRCA1 and BRCA2 gene mutations are considered to be typical causes, and patients with Lynch syndrome are reported to have a lower survival rate than patients without it [18, 19]. Additionally, only risk factors such as sterility, breast cancer, endometrial cancer, nulligravida, and obesity have been identified; in most cases, the causes are unknown. Intraperitoneal metastasis occurs most commonly, while vascular or lymphatic metastasis also exists [20].

Borderline ovarian tumors show no stromal invasion but a good prognosis regardless of a malignant cell's existence. Consequently, classification and treatment are not clearly established. As initial symptoms, most patients are symptom-free, and the remainder feel abdominal distension because of ascites. However, nearly all patients visit the hospital with cancer in progression. The blood CA-125 test or vaginal ultrasonography is used for early detection but neither is very useful. Only oral contraceptives are known as a prevention factor [21].

Classic therapy included platinum-taxane-based chemotherapy after performing staging laparotomy and tumor debulking to minimize retained tumor lesions. Despite these aggressive treatments, nearly 70% to 80% of patients show cancer recurrence after their first anti-cancer treatment. In summary, ovarian cancer is an intractable disease accompanied by severe side effects and a high relapse rate.

One of the reasons why ovarian cancer records a high
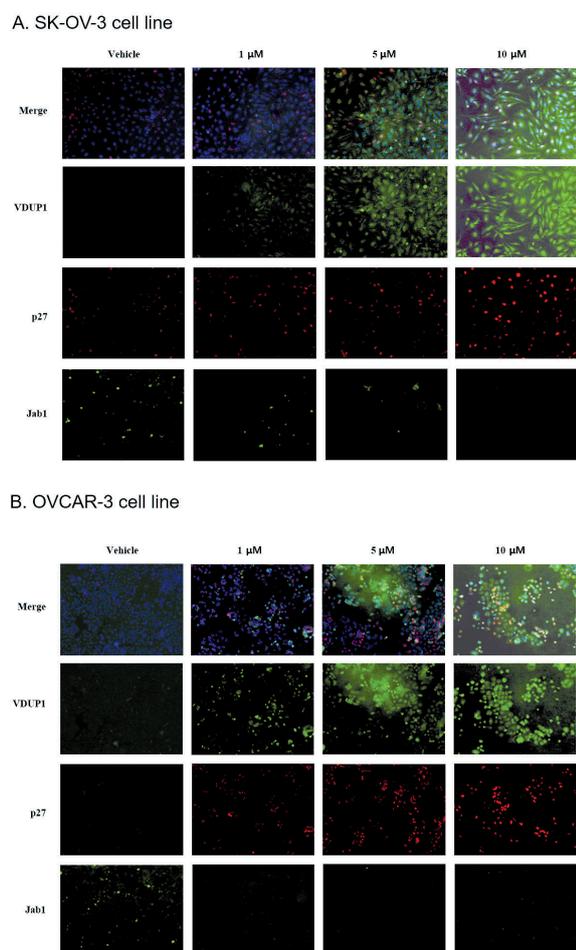
A. SK-OV-3 cell line

B. OVCAR-3 cell line

Figure 4. — Immunofluorescence staining of active VDUP1, p27kip1, and Jab1 in ovarian cancer cells after exposure to vitamin D. The data show the effect of Vitamin D, which increased the expression of VDUP1 and p27kip1, and decreased the expression of Jab1 in ovarian cancer cell lines. Cells were treated with 1 μM, 5 μM, and 10 μM vitamin D. For differential nuclear staining, VDUP1 (green) and p27kip1 (red) dye were employed.
(A) SK-OV-3 cell line. (B) OVCAR-3 cell line.

death rate is because existing anti-cancer treatment has a high risk of drug resistance leading to recurrence despite good susceptibility. Thanks to basic studies on ovarian cancer, the molecular mechanism of occurrence and growth of tumors are revealed, and genetic change induces tumor progression by affecting the signaling mechanism that controls cell growth and differentiation. Consequently, molecular targeted therapy suggests a potential approach for new treatment regarding the development of biological markers related to the prediction of consequences [22]. Another preventative treatment, low-dose aspirin, has been suggested [23].

Promising medications for epithelial ovarian cancer include temsirolimus, an anti-VEGF monoclonal antibody, bevacizumab, and olaparib, a Poly ADP Ribose Polymerase (PARP) inhibitor for patients with BRCA gene mutation [24, 25]. Thus, ovarian cancer is heterogeneous, and ideal treatment requires a delicate approach based on molecular studies.

Until now, according to the studies, vitamin D shows an inhibiting effect toward carcinoma in the breast, prostate, and colon [26]. From epidemiologic investigation, however, no correlation was found between ovarian cancer and the blood vitamin D level [27]. This study focuses on the carcinostatic effect of vitamin D in ovarian cancer.

Regarding the genomic mechanism, vitamin 1,25-dihydroxyvitamin D3 is synthesized by catalytic 1-α-hydroxylase. This enzyme exists in the kidney, lung, pancreas, and parathyroid affecting cell cycle arrest, induction of apoptosis, and cell differentiation, which are involved in different signaling processes [28].

Vitamin D not only reinforces the fertility of pregnancy by androgen management but also helps women undergoing in vitro fertilization procedures. Moreover, vitamin D is relevant to the management of fertility in men [29-31]. Additionally, sufficient synthesis of vitamin D has been reported to be beneficial for the mother's brighter skin during or after pregnancy [32]. A preventative effect on asthma has been confirmed as well [33].

According to the epidemiologic investigations and studies, the lack of vitamin D causes various cancers. In preclinical studies, vitamin D loss provoked a toxic effect, inhibition of cell proliferation and apoptosis [34].

The expression of VDUP1 has been reported to be regulated by various stress stimuli such as oxidant stress, ultraviolet radiation, and heart shock factor (HSF) [10, 35]. VDUP1 is involved in redox regulation and increases reactive oxygen species in fibroblasts by inhibiting the expression of thioredoxin [36]. The thioredoxin mechanism consists of thioredoxin (Trx), NADPH, and thioredoxin reductase (TrxR), which exist in almost all living cells. It plays an important role in the oxidation-reduction environment, cell growth, oxidant stress or defense toward apoptosis, and development of cancer.

Thioredoxin could be considered an antioxidant defense factor that protects the body from the attack of ROS and restores damaged protein. Meanwhile, it facilitates cancer's growth for cell growth, apoptosis, infinite proliferation, creation of blood vessels, and tissue infiltration. Regarding the facilitating mechanisms, there are DNA syntheses such as replication, repair, and DNA transcriptional factors activating DNA binding [37].

Only the reduced form of Trx by TrxR binds to ASK (apoptosis signal-regulation kinase) 1 and inhibits apoptosis function [38]. VDUP1 interacts with the catalytic active center of thioredoxin to render cells sensitive to apoptosis mediated by oxidant stress [16, 39]. In many malignant tumors, including ovarian tumors, it is observed that cyclin-dependent kinase inhibitors do not exist or are diminished,

A. SK-OV-3 cell line
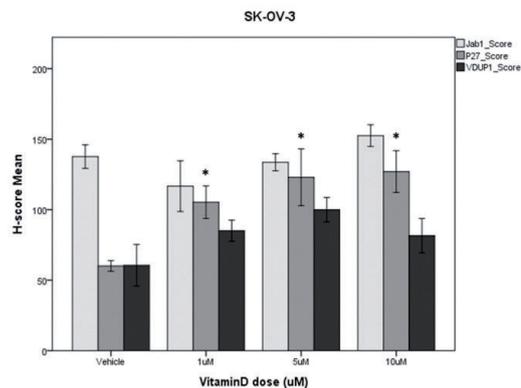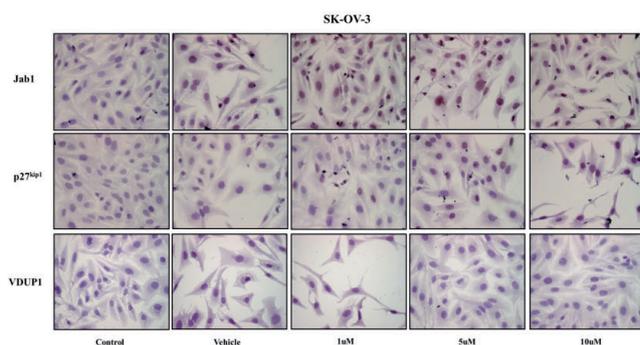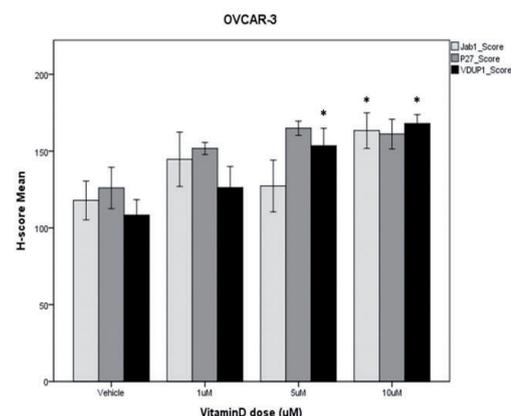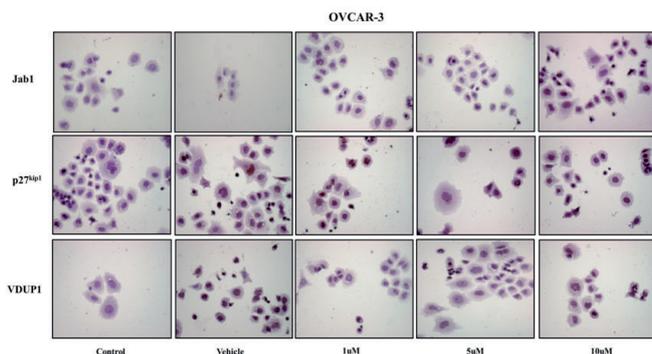

B. OVCAR-3 cell line

Figure 5. — Immunocytochemical localizations of active VDUP1, p27kip1, and Jab1 in ovarian cancer cells after exposure to vitamin D. Data are represented as the mean ± SD of the immunocytochemistry H-score calculated from five independent counts. *, **, and *** indicate significant differences at $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively, compared with the untreated vehicle group. The H-score value of the control group was zero. (A) SK-OV-3 cell line. (B) OVCAR-3 cell line.

including p27[kip1] [40-43].

p27[kip1] binds to and inhibits cyclin E/CDK2 to stop cell differentiation by halting the cell cycle at the G1 to S stage [44]. Therefore, loss of p27[kip1] indicates loss of the capacity to inhibit cell differentiation, which is relevant to the rapid growth of cancer and facilitation of cancer cells. Jab1 strengthens the expression of the c-Jun gene by inducing cell proliferation and destroys p27[kip1] by facilitating p27[kip1]'s movement from the nucleus to the cytoplasm by the ubiquitin/proteasome pathway [12].

VDUP1 is known to interact with Jab1 by directly inhibiting Jab1 and interrupting the destruction of p27[kip1] and cell proliferation [13]. It has also been noted that, in the process of vitamin D treatment increasing p27kip1, the Vitamin D receptor plays a role [45].

In the present study, vitamin D treatment seems to strengthen the stability and expression of p27kip1 in the ovarian cancer cell lines SK-OV-3 and OVCAR-3, similar to other cancer cell lines, like prostate cancer cells [46]. A mimetic diagram of the interrelationship between Vitamin

D, VDUP1, Jab1 and p27 is shown in Figure 6.

In conclusion, treatment of the ovarian cancer cell lines SK-OV-3 and OVCAR-3 with vitamin D (1,25 (OH)$_2$ vitamin D$_3$) leads to VDUP1 (vitamin D up-regulated protein 1) increase. In turn, VDUP1 functions as a negative regulator of Jab1 to interrupt p27[kip1]'s translocation and degradation in the nucleus. It has been suggested that vitamin D treatment in ovarian cancer has the potential to work as a preventative factor and an anti-cancer agent affecting the process of tumor development by the overexpression of VDUP1.
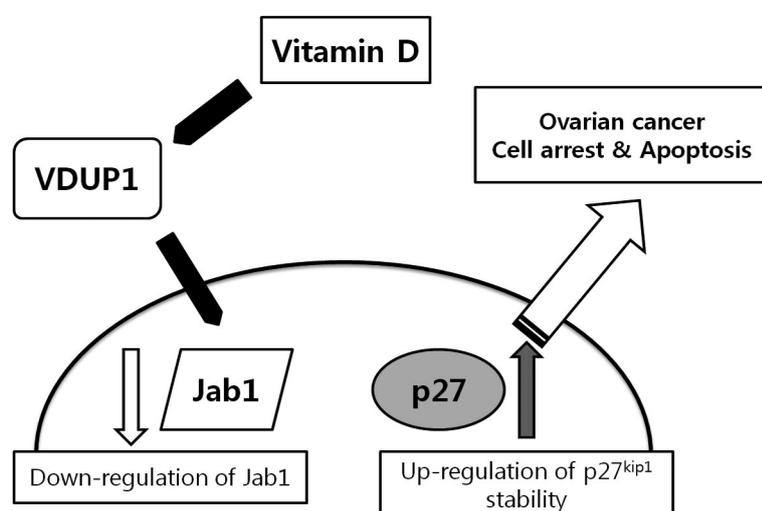
Figure 6. — The intracellular molecular mechanism of vitamin D is illustrated through the VDUP1 pathway. Through a series of mechanisms, VDUP1 induced by vitamin D blocks p27kip1 nuclear export mediated by Jab1.

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