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Thioredoxin-interacting Protein (TXNIP) Mediates Thioredoxin-dependent Antioxidant Mechanism in Endometrial Cancer Cells Treated With 1α,25-dihydroxyvitamin D_3

YESOL KIM[1], YEON-SUK KIM[1], MINJEONG KIM[2], JUN-MO KIM[3], HAE-HYEOG LEE[4] and TAE-HEE KIM[4]

[1]*Department of Interdisciplinary Program in Biomedical Science, Soonchunhyang University Graduate School, Asan, Republic of Korea;*
[2]*Department of Obstetrics and Gynecology, Bucheon St. Mary's Hospital, College of Medicine, The Catholic University of Korea, Bucheon, Republic of Korea;*
[3]*Department of Urology, Soonchunhyang University College of Medicine, Bucheon, Republic of Korea;*
[4]*Department of Obstetrics and Gynecology, Soonchunhyang University College of Medicine, Bucheon, Republic of Korea*

Abstract. *Background/Aim: To determine the mechanism of vitamin D_3-induced modulation of antioxidant-related factors in endometrial cancer, we investigated their role in apoptosis of human endometrial cancer cells exposed to vitamin D_3. Materials and Methods: The survival rate of human endometrial cancer cells was estimated after treatment with activated vitamin D_3. Reactive oxygen species (ROS) levels were measured using flow cytometry. The levels of VDR, Trx, TXNIP and apoptosis-related proteins were investigated using western blotting and immunocytochemistry in human tissues. Results: Treatment with D_3 induced apoptotic cell death and cell-cycle arrest by increasing ROS concentration. Vitamin D_3 inhibited proliferation of human endometrial cancer cells. It regulated intracellular ROS concentration in endometrial cancer cells via increased TXNIP expression. Conclusion: Antioxidant regulation via TXNIP is an important cell death mechanism in human endometrial cancer, and occurs via induction by vitamin D_3.*

Endometrial cancer accounts for most of the corpus carcinomas. Following cervical and ovarian cancer, endometrial cancer is ranked as the third most common gynecological cancer in South Korea. Age-standardized rates of endometrial cancer per 100,000 are 0.8% of the population with cancer, and 1.6% of those with gynecological cancer in 2013 (1). Age-standardized rate of endometrial cancer in Korean women has increased from 2.4 to 4.6 since 1990 (1, 2). However, endometrial cancer is the most common gynecological cancer among fertile women in the USA (3). The known major causes of endometrial cancer include endogenous hormones, excess body weight and lack of physical activity (4, 5). It is expected that the prevalence of endometrial cancer will be increased further due to westernized diet and lifestyle of Koreans (6).

Vitamin D_3, which acts as a hormone, can be obtained naturally *via* food intake or exposure to sunlight. Because vitamin D_3 has an important role in the prevention and treatment of various cancers such as breast cancer, multiple myeloma, ovarian cancer, vulvar cancer (7), blood vitamin D concentration is considered a potential prognostic marker (8-12). It is known that deficiency of vitamin D_3 is prevalent in stage III cancer according to the American Joint Committee on Cancer, and correlates with advanced stages of cancer (13). Vitamin D_3 plays a key role not only in proliferation, differentiation and immune responsiveness of endometrium (14), but also in the prevention and inhibition of endometrial cancer cells (15). The anti-cancer mechanisms mediated by vitamin D_3 include inhibition of gluconeogenesis, and induction of antioxidant activity (9, 13, 16-18). However, the response of endometrial cancer cells to antioxidant mechanisms induced by vitamin D_3 has yet to be elucidated.

The antioxidant activity used to maintain homeostasis of intracellular reactive oxygen species (ROS) is important for the survival of aerobic organisms. In the absence of efficient antioxidant activity, the concentration of intracellular ROS is elevated resulting in aging, mutations, or cell death.

Correspondence to: Tae-Hee Kim, MD, Ph.D., Department of Obstetrics and Gynecology, Soonchunhyang University Bucheon Hospital, 170 Jomaru-ro, Wonmi-gu, Bucheon 14584, Republic of Korea. Tel: +82 326215380, Fax: +82 260086874, e-mail: heeobgy@naver.com

Key Words: Endometrial cancer, vitamin D_3, thioredoxin, thioredoxin-interacting protein.

Thioredoxin (Trx) is a 12-kDa antioxidant protein with a catalytically active cystine S-S bridge. It is one of the major enzymatic antioxidants involved in defense mechanisms associated with thioredoxin reductase (TrxR). Because Trx binds and neutralizes ROS, it protects cells from oxidative stress (19-21). This antioxidant mechanism is linked to vitamin D_3 *via* thioredoxin-interacting protein (TXNIP/VDUP-1/TBP-2).

Vitamin D_3 metabolite, calcitriol, upregulates the transcription of TXNIP. Typically, TXNIP inhibits Trx *via* two mechanisms. It decreases the expression of Trx and inhibits the binding between Trx and ROS *via* competitive inhibition of the Trx active site (22). As a result, the increased levels of the TXNIP-Trx complex fail to regulate ROS generation effectively. In these conditions, cells become sensitive to oxidative stress, which triggers apoptosis (23). Inhibition of specific cancers by vitamin D_3 may be associated with the inhibition of antioxidant activity *via* TXNIP. However, its role in endometrial cancer is unknown.

This study investigated the antioxidant and apoptotic role of Trx and TXNIP in endometrial cancer upon treatment with vitamin D_3.

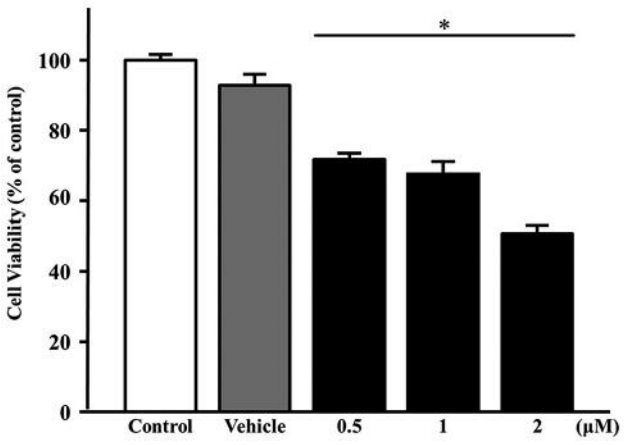

Figure 1. *Vitamin D_3 inhibited the proliferation of endometrial cancer cells. Ishikawa cells were treated with 0.5, 1, or 2 μM of vitamin D_3 for 24 h. Vitamin D_3 was dissolved in 0.2% (v/v) ethanol. Cell viability was evaluated by the MTT assay. When high dose of vitamin D_3 was used, cell viability was decreased. Only 50.8% of cells survived upon exposure to 2 μM of vitamin D_3 compared with the control group. In cell viability, three groups treated with vitamin D_3 showed statistical difference compared with the control group (*p<0.01).*

Materials and Methods

Reagents. The following reagents were utilized: Vitamin D_3 (1α, 25-dihydroxyvitamin D_3, D1530, Sigma-Aldrich Corp., St. Louis, MO, USA), Dulbecco's modified Eagle's medium-high glucose (DMEM, GenDEPOT Inc., Barker, TX, USA), fetal bovine serum (FBS, GenDEPOT Inc., Barker, TX, USA), Dulbecco's phosphate-buffered saline (DPBS, GenDEPOT Inc.), penicillin, ethanol, methyl thiazolyl tetrazolium (MTT, Sigma-Aldrich Corp.), DMSO, tris, sodium dodecyl sulfate (SDS, AMRESCO Inc., Solon, OH, USA), glycerol, phosphatase inhibitors (Sigma-Aldrich Corp.), protease inhibitors (Roche, Mannheim, Germany), methanol, Mayer's hematoxylin (ab128990, Abcam Inc., Cambridge, MA, USA), trypsin, H_2DCFDA (Thermo Fisher Scientific Inc., Waltham, MA, USA), skimmed milk, bovine serum albumin (BSA) solution, and Tween 20.

Ethical approval and sample collection. A total of ten endometrial tissues (five normal endometrium tissue and five endometrial cancer tissue) from endometrial cancer patients enrolled in a tertiary university hospital were analysed. This study was approved by local Institutional Review Boards (IRBs Number, SCHBC 2015-02-034) on March 2015.

Cell culture and treatment. The human endometrial cancer cell line Ishikawa purchased from ATCC was grown in DMEM, supplemented with 10% FBS, and 100 U/ml penicillin. Ishikawa cells were seeded in 100 mm culture plates, at a density of 5×10^5 cells/dish and incubated at 37˚C, in a humidified atmosphere with 5% CO_2. For further experiments, cells were cultured in 100 mm, 60 mm, 6-well, and 96-well plates. After incubation overnight with FBS-supplemented media, Ishikawa cells were starved *via* incubation with serum-free DMEM for 24 h. To estimate the effectiveness of activated vitamin D_3, 5 Ishikawa groups were exposed to 0, 0.5, 1, and 2 μM of Vitamin D_3 (1α, 25-dihydroxyvitamin D_3) dissolved in ethanol for 24 h. Control and vehicle groups were not treated with vitamin D_3. Ethanol, a solvent of vitamin D_3, was used at a concentration of 0.5% (v/v) in all groups. Unless otherwise stated, this treatment protocol was maintained. All the experiments were conducted at least three times.

Cell proliferation assay. Cell proliferation was determined *via* MTT using DMSO as a solvent. Ishikawa cells were seeded at 4,000 cells/well in 96-well plates. After starvation and vitamin D_3 treatment as mentioned above, 30 μl of MTT (5 mM/ml, w/v) solution were added to each well containing cells and incubated for 4 h at 37˚C. The media were carefully removed and 100 ml of DMSO were added into each well to dissolve the crystalized MTT. The absorbance was measured at 570 nm with a universal microplate Reader (Molecular devices, CA, USA).

ROS measurement. After vitamin D_3 treatment for 24 h, plates were washed with pre-warmed serum-free media and Dulbecco's Phosphate-Buffered Saline (DPBS). The positive control group was treated with 0.1mM H_2O_2 for 30 min before cell collection. Cells were harvested with trypsin and suspended in a medium containing 1 μM H2DCF-DA in DPBS, at 37˚C for 20 min. Following three washes with cold DPBS, the fluorescence intensity of the cells in each group was measured by flow cytometry (Coulter Corp., Miami, FL, USA).

Western blot. After vitamin D_3 treatment, the culture plates were washed twice with cold DPBS. Whole cells were lysed in RIPA buffer (50 mM Tris-HCl at pH 8.0, 150 mM sodium chloride, 1 mM EDTA, 1.0% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS) containing with phosphatase inhibitors and protease inhibitors on ice. The supernatants were collected after centrifugation at 12,000 rpm for 20 min at 4˚C. Detergent-compatible (DC) protein

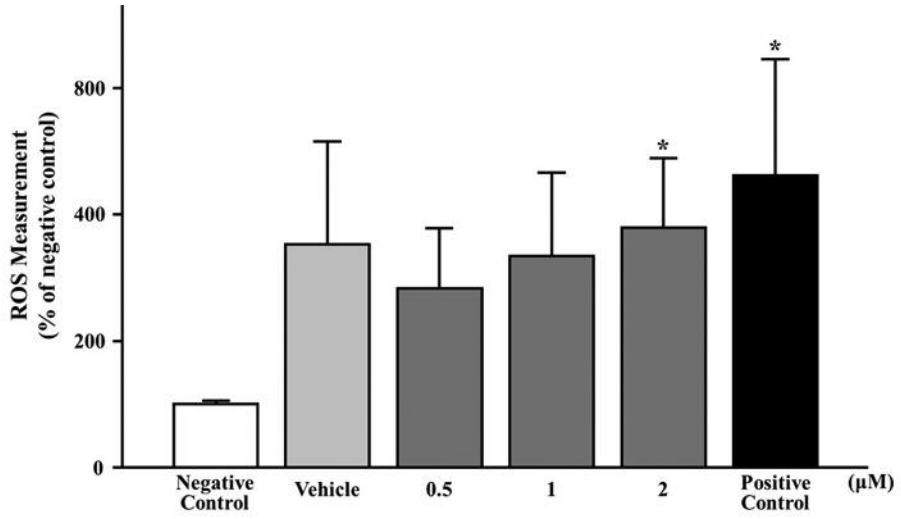

Figure 2. *Flow cytometry analysis of intracellular ROS using H2DCF-DA reagent. Cells were treated with different concentrations of vitamin D_3 for 24 h. Cells in the vehicle groups were exposed to the same amount of ethanol. After treatment, the medium was removed and replaced with H2DCF-DA reagent in all groups except the negative control, for flow cytometry analysis. The positive control group was exposed to H_2O_2 for 30 min before addition of H2DCF-DA. The levels of intracellular ROS were 4.61-fold higher in the positive control (H_2O_2) than in the negative control group (*p<0.05). Compared with the negative control group, ROS levels increased by 2.83-, 3.33-, and 3.78-fold (*p<0.05) at vitamin D_3 concentrations of 0.5, 1, and 2 μM, respectively.*

assay solution (Bio-Rad Laboratories, Berkeley, CA, USA) was used to measure protein concentration. The cell extracts were re-suspended using 5X sample buffer (0.6 mM Tris-HCl, 25% glycerol, 2% sodium dodecyl sulphate, 0.1% bromophenol blue, and 5% 2-mecaptopoethanol in distilled water) so that to obtain the same protein concentration in each group. The lysates were boiled at 100˚C, for 5 min and stored at –20˚C or –80˚C. The protein extracts of endometrial cancer cells were separated by SDS-polyacrylamide gel electrophoresis and transferred to PVDF membrane. Blots were blocked for 1 h in 5% skimmed milk or BSA solution in Tris-buffered saline (TBST, Tris-HCl of pH 7.4, glycerol, 1% Tween 20). Primary antibodies were diluted 1:1,000 to 2,000 in a blocking solution, and incubated overnight at 4˚C. After washing with TBST, the EXPOSE mouse- and rabbit-specific horseradish peroxidase (HRP)-conjugated secondary antibodies (sc-2004, sc-2005, Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted at a ratio of 1:5000 in blocking solution, were added and incubated at room temperature for 2 h. Enhanced chemiluminescence detection of western blot was performed using the western blotting Luminol Reagent (sc-2048, Santa Cruz Biotechnology) according to the manufacturer's protocol.

Primary antibodies used in this study were VDR (sc-13133, Santa Cruz Biotechnology, Inc.), TXNIP (sc-166234, Santa Cruz Biotechnology), Trx (ab86255, Abcam Inc., Cambridge, MA, USA), p27(anti-p27 KIP1, ab32034, Abcam Inc.), JAB1(sc-9074, Santa Cruz Biotechnology), and β-actin (ab133626, Abcam Inc.).

Immunocytochemistry. After vitamin D_3 treatment, plates were washed twice with cold DPBS and fixed for 10 min in cold 4% paraformaldehyde followed by permeabilization for 30 min with 0.3% H_2O_2 in methanol. Cells were blocked for 1 h in 5% normal goat serum-enriched TBS and incubated overnight at 4˚C with primary antibodies, diluted at a ratio of 1:200. After washing with TBS three times, the mouse- and rabbit-specific HRP and ABC complexes were incubated at RT for 30 min followed by evaluation of the immunocytochemical response using 3',3-diaminobenzidine (DAB) detection immunohistochemistry kit (ab80436, Abcam Inc.) at RT for 1 to 5 min. The specimens were counterstained with hematoxylin for 3 min and neutralized at 45˚C under tap water for 2 min.

Immunohistochemistry. Human tissues obtained during hysterectomies were separated from the organ (normal endometrium; specimen/endometrial cancer) and were fixed overnight in 4% cold PFA and embedded in paraffin. Four-micrometer serial sections were obtained and deparaffinized with xylene and alcohol as in routine processing experiments. Subsequently, the permeabilization procedure was carried out as described in immunocytochemistry above.

Statistical analysis. Student's T-test, Mann-Whitney *U*-test, and Kruskal Willis ANOVA were conducted for statistical analysis. Analyses were carried out using the SPSS Version 14 software (SPSS Inc., Chicago, IL, USA). All *p*-values were two-sided.

Results

Treatment of endometrial cancer cells with D_3 resulted in a dose-dependent inhibition of proliferation. To analyze the proliferation of Ishikawa cells, the MTT assay was used to measure mitochondria activity. Ishikawa cells were treated with 1.5 μM, 1 μM or 2 μM concentrations of 1α,25-dihydroxyvitamin D_3 and 0.2% (v/v) ethanol for 24 h. Compared with the control group, treatment with 0.5 μM, 1 μM, and 2 μM concentrations of activated vitamin D_3

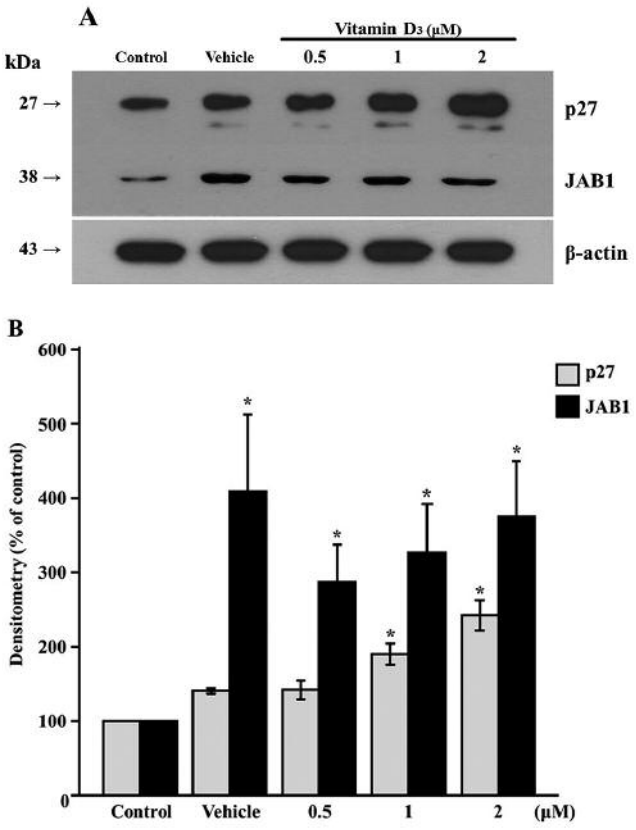

Figure 3. *Cell-cycle regulation proteins were increased by vitamin D_3 treatment. Each lane was loaded with 20 μg protein obtained from Ishikawa cell lysates. (A) Immunoblots were probed with anti-p27, anti-JAB1, and anti-β-actin antibodies. The p27 protein was increased following vitamin D_3 treatment (*$p<0.05$). The JAB1 protein expression was not affected by vitamin D_3. (B) The band densities are expressed as a percentage of the control group. The results of western blot analysis revealed that the levels of p27 proteins were increased in response to vitamin D_3 treatment (*$p<0.05$).*

reduced survival to 71.79%, 67.57%, and 50.79%, respectively (Figure 1). According to the results, vitamin D_3 induced death of endometrial cancer cells in a dose-dependent manner ($p<0.05$). Ethanol, which was used as the solvent for vitamin D_3, had no effect on proliferation ($p>0.05$).

Flow cytometry analysis of reactive oxygen species. Intracellular levels of ROS were increased in a dose-dependent manner following treatment of Ishikawa cells with vitamin D_3 (Figure 2). Vitamin D_3-treated groups showed increased fluorescence, close to the positive controls treated with H_2O_2. The positive control group showed 4.61-fold increase in ROS levels (*$p<0.05$) compared to the control group. The group treated with 2 μM vitamin D_3 showed a 3.78-fold increase compared to the non-treated negative group (*$p<0.05$).

Vitamin D_3 promoted apoptosis by regulating the expression and translocation of cell cycle regulation proteins. Western blot analysis indicated that treatment with vitamin D_3 resulted in increased levels of p27 but not of Jun activation domain-binding protein 1 (JAB1), both apoptosis-related proteins involved in cell cycle regulation (Figure 3A and B). The increase in p27 protein expression was not dependent on vitamin D_3 concentration.

JAB1 showed similar increase in the vehicle and control groups; however, a clear difference between the two groups was detected using immunocytochemistry (Figure 4). Each group showed different protein localization and morphology. Nuclear translocation of p27 and JAB1 was observed upon vitamin D_3 treatment. The morphology of Ishikawa cells was altered in each group. Cell nuclei and cytosol were shrunken following vitamin D_3 treatment.

Vitamin D_3 regulated the expression of apoptosis and redox-related proteins. Vitamin D_3 treatment resulted in enormous variation in the expression of VDR and TXNIP proteins between the control and vehicle groups (Figure 5A and B). Our western blotting results showed that VDR and TXNIP expression were greatly increased by vitamin D_3. However, the expression of thioredoxin protein was unaffected.

Immunocytochemical staining for each protein was carried out to assay for protein expression and localization (Figure 6). The control group showed high levels of thioredoxin but very low levels of VDR and TXNIP proteins. VDR and TXNIP protein expression was induced by vitamin D_3.

Differences in the expression of ROS-related proteins in normal endometrium and endometrial cancer tissues. Immunohistochemical staining for VDR, TXNIP and thioredoxin proteins was conducted in normal endometrium and endometrial cancer tissues. In these two groups, the expression of thioredoxin showed the biggest difference (Figure 7). In normal endometrium, the level of thioredoxin showed absence. But increased levels of thioredoxin are observed in endometrial cancer group. The two groups showed VDR expression.

Discussion

In this study, human endometrial cancer cells were treated with vitamin D_3 treatments and the expression of proteins associated with anti-oxidant mechanisms was examined. Further, we compared the expression of Trx and TXNIP proteins between human normal endometrial tissues and human endometrial cancer cells.

The levels of VDR, which is an indicator of vitamin D_3 effect on cells, and TXNIP were dramatically increased upon treatment with vitamin D_3 (Figures 5 and 6). However, the levels of Trx were not affected. The strong increase of VDR

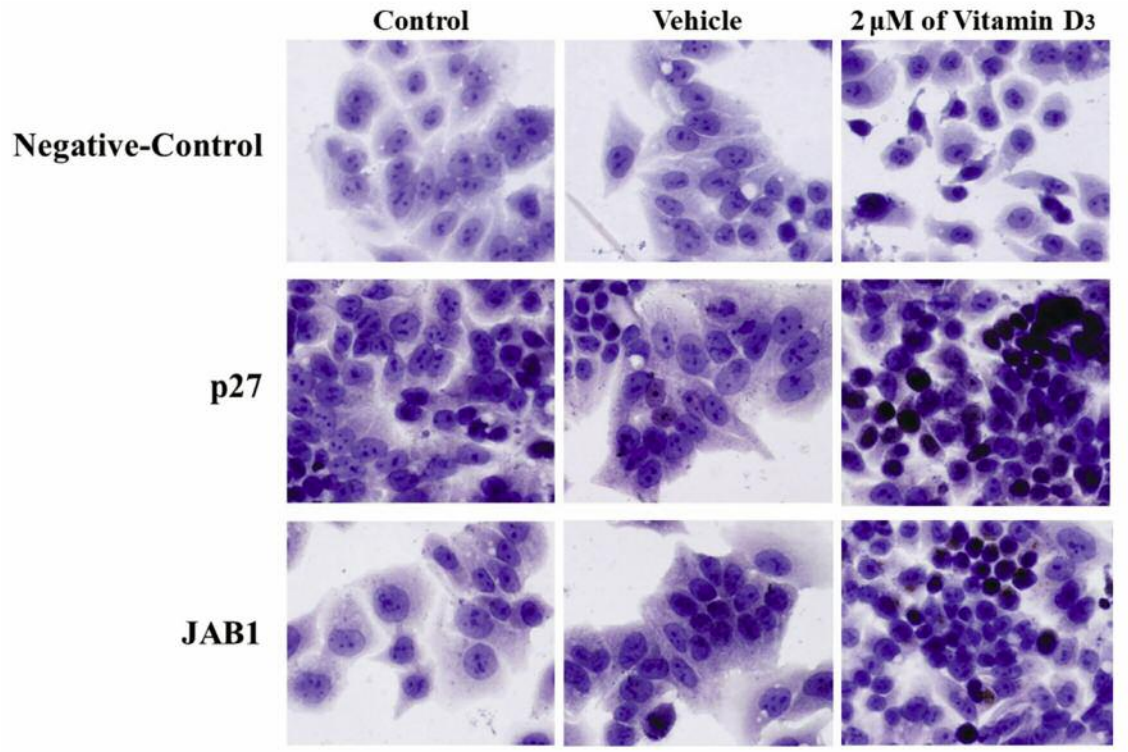

Figure 4. *Changes in the location of p27 and JAB1 protein by vitamin D_3 treatment. Immunocytochemistry (ICC) of protein and hematoxylin staining for nuclear detection. Ishikawa cells were exposed to ethanol in the control group, or vitamin D_3 in the experimental group. The control group was not exposed to vehicle or vitamin D_3. The antibodies to p27 and JAB1 were diluted 1:200. Only hematoxylin staining for nucleus was performed in the negative control. The two cell-cycle regulation proteins switched their location from cytosol to nuclei upon vitamin D_3 treatment.*

expression suggested that the administration of activated-vitamin D_3 was effective in endometrial cancer (7, 24). TXNIP interacts with Trx *via* binding with its active site. Compared with the dose-dependent increase in TXNIP, the Trx expression, however, remained unchanged after administration of vitamin D_3. These results indicate that vitamin D_3 does not directly regulate Trx, but indirectly *via* TXNIP.

We also demonstrated the differences in the expression of VDR, TXNIP and thioredoxin *via* immunohistochemistry in human tissue samples. Elevated expression of Trx and VDR was observed in human endometrial cancer tissues (Figure 7). In contrast, TXNIP expression was not detected in both normal endometrial and endometrial cancer tissues.

Our experimental data showed that vitamin D_3 caused cell-cycle arrest and inhibited the proliferation of endometrial cancer cells. Thus, ROS is strongly associated with the anti-cancer activity of vitamin D_3. Especially, TXNIP, which is induced by vitamin D_3 and inhibits the antioxidant function of Trx.

In human hepatocellular carcinoma (HCC), the expression levels of TXNIP were low and vitamin D_3 treatment stimulated the transcription of TXNIP (25). Administration of diethylnitrosamine to TXNIP-knockout mice increased hepatocarcinogenesis demonstrating that TXNIP suppressed TNF-α-induced NF-κB activation and transcription (26). This mechanism has also been confirmed in gastric carcinogenesis (27).

Our data combined with those of other studies, indicate that the incidence of endometrial cancer in humans may be related to ROS dysregulation, and vitamin D_3 sensitized endometrial cancer cells to TXNIP. Similar over-expression of Trx in human endometrial cancer tissues. The expression of Trx was significantly increased in cancer endometrium compared with normal endometrium regardless of VDR expression (Figure 7). However, TXNIP expression was low in both groups. The results of the in vitro experiments suggest that vitamin D_3 therapy inhibits the antioxidant effect of Trx in endometrial cancer, rendering the cells more vulnerable to intracellular ROS.

ROS are natural byproducts of aerobic respiration and energy production. The reactivity of ROS is attributed to the free radicals, which combine with fatty acids in the membrane and other biological molecules. Because of the reactivity, it is important to maintain the intracellular homeostasis of ROS.

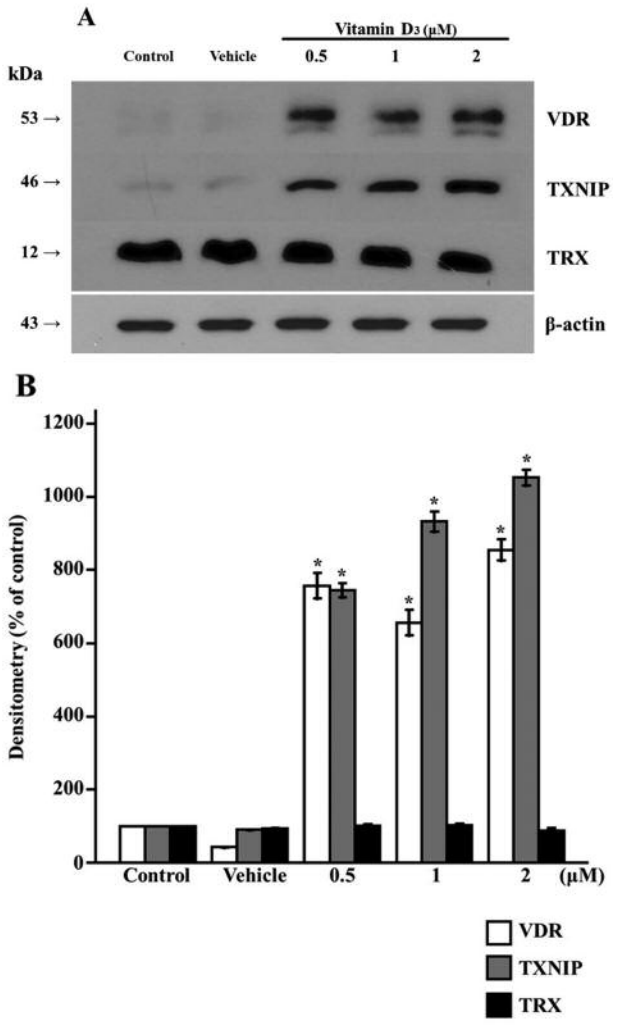

Figure 5. *Vitamin D_3 controlled the expression of VDR and TXNIP in endometrial cancer cells. Each lane was loaded with 10 μg protein derived from Ishikawa cell lysates. (A) Immunoblots were probed with anti-VDR, anti-TXNIP, anti-thioredoxin, and anti-β actin antibodies. The abundance of VDR and TXNIP was significantly increased by vitamin D_3 treatment, without differences in the expression of thioredoxin. (B) The band densities are expressed as a percentage of the control group. Densitometric analysis revealed that the expression of VDR and TXNIP proteins were increased by vitamin D_3 more than 6-fold but not by ethanol (*p<0.05).*

Cells utilize ROS as signaling molecules or eliminate them. Regulation of ROS is mediated *via* enzyme-dependent and enzyme-independent mechanisms. One of the antioxidant enzymes Trx is known to react with TXNIP.

Acute increase in the levels of ROS leads to DNA mutations, lesions, strand breaks, proteolytic cleavage, amino acid oxidation, and lipid peroxidation in cell and nuclear membranes, resulting in cell death. However, chronic low levels of ROS trigger cell signaling, increase cell proliferation and genetic mutations. Thus, increased cell proliferation and gene mutations are closely related to carcinogenesis.

Expression of p27 and JAB1 is associated with cell cycle regulation. The levels of these proteins are increased upon inhibition of cell proliferation. The expression of p27 and JAB1 was locally increased in endometrial cancer cells exposed to vitamin D_3.

P27 and JAB1 proteins are associated with cell cycle regulation. As endometrial cells die, the levels of p27 and JAB1 increase. p27 is known to act as a negative cell cycle regulator by interfering with the progression from G1 to S phase. It is considered as a tumor suppressor with anti-proliferative action, as it promotes apoptosis and cell cycle arrest (22, 23, 28). Especially, highly levels of p27 may be related to apoptotic signaling. JAB1 is widely known to regulate p27 stability and interact with p27 and TXNIP. Vitamin D_3 inhibits the translocation of p27 and JAB1 from nucleus to the cytosol, resulting in death *via* apoptosis. The effect of vitamin D_3 in endometrial cancer is mediated *via* control of apoptosis.

Vitamin D_3 has been associated with some cancers, such as breast, colorectal, and prostate cancers and correlated with the blood levels of 25(OH)vitamin D (9). The hormonal form of vitamin D_3 is calcitriol, which sensitizes breast cancer cells to ROS by destroying mitochondria, thereby inducing cell death (29), however, the related mechanisms are unknown.

Vitamin D deficiency is common in cancer patients. It is associated with the progression of cancers of the breast, vulva, prostate, pancreas, stomach, colon and rectum, and ovarian cancer (7). In breast cancer patients, elevated expression of VDR have been associated with increased progression-free survival and the overall survival rate compared with patients with moderate/negative VDR expression levels (7). In female cancers, however, prevalence of vitamin D deficiency has not been observed in ovarian cancer. The differences in mechanism between these cancers and endometrial cancer are unknown unlike breast cancer (10). Additional studies suggested that the incidence of endometrial cancer is not associated with circulating levels of vitamin D (30).

This study elucidated the relationship between vitamin D_3 and ROS in endometrial cancer. Vitamin D_3 inhibited the proliferation of human endometrial cancer cells. It also increased intracellular levels of ROS. The elevation of ROS maybe relevant to TXNIP expression regulation that the expression of cell death proteins is increased in endometrial cancer cells exposed to vitamin D_3.

Conflicts of Interest

The Authors declare that they have no conflicts of interest regarding this study.

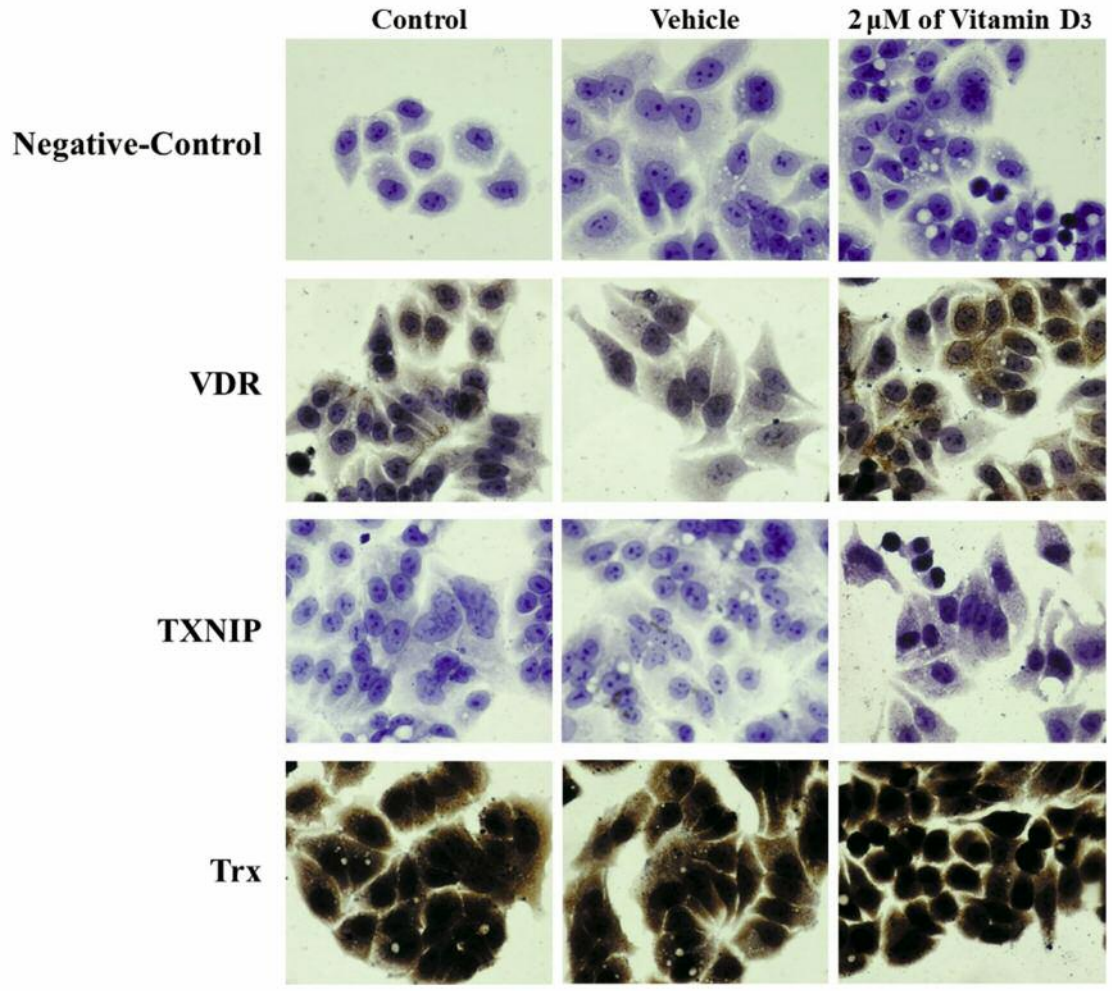

Figure 6. *Changes in the location of VDR, TXNIP and thioredoxin protein by vitamin D_3. Immunocytochemistry (ICC) of protein and hematoxylin staining for nuclear detection, were used. Ishikawa cells were exposed to ethanol in the control group, or vitamin D_3 in the experimental group. The control group was not exposed to vehicle or vitamin D_3. The antibodies to VDR, TXNIP, and thioredoxin were diluted 1:200, 1:100, and 400, respectively. Only hematoxylin staining was used for nucleus in the negative control. The expression of VDR and TXNIP was enhanced by vitamin D_3 when thioredoxin levels show no large variation in each group.*

Author's Contributions

Yesol Kim: Project development, data collection, manuscript writing; Yeon-Suk Kim: Data collection, data analysis, project development; MinJeong Kim: Data management, important intellectual input; Jun-Mo Kim: important intellectual input, project development; Hae-Hyeog Lee: Manuscript editing, conception and design; Tae-Hee Kim: Conception and design, manuscript editing, final approval of the submitted version.

Acknowledgements

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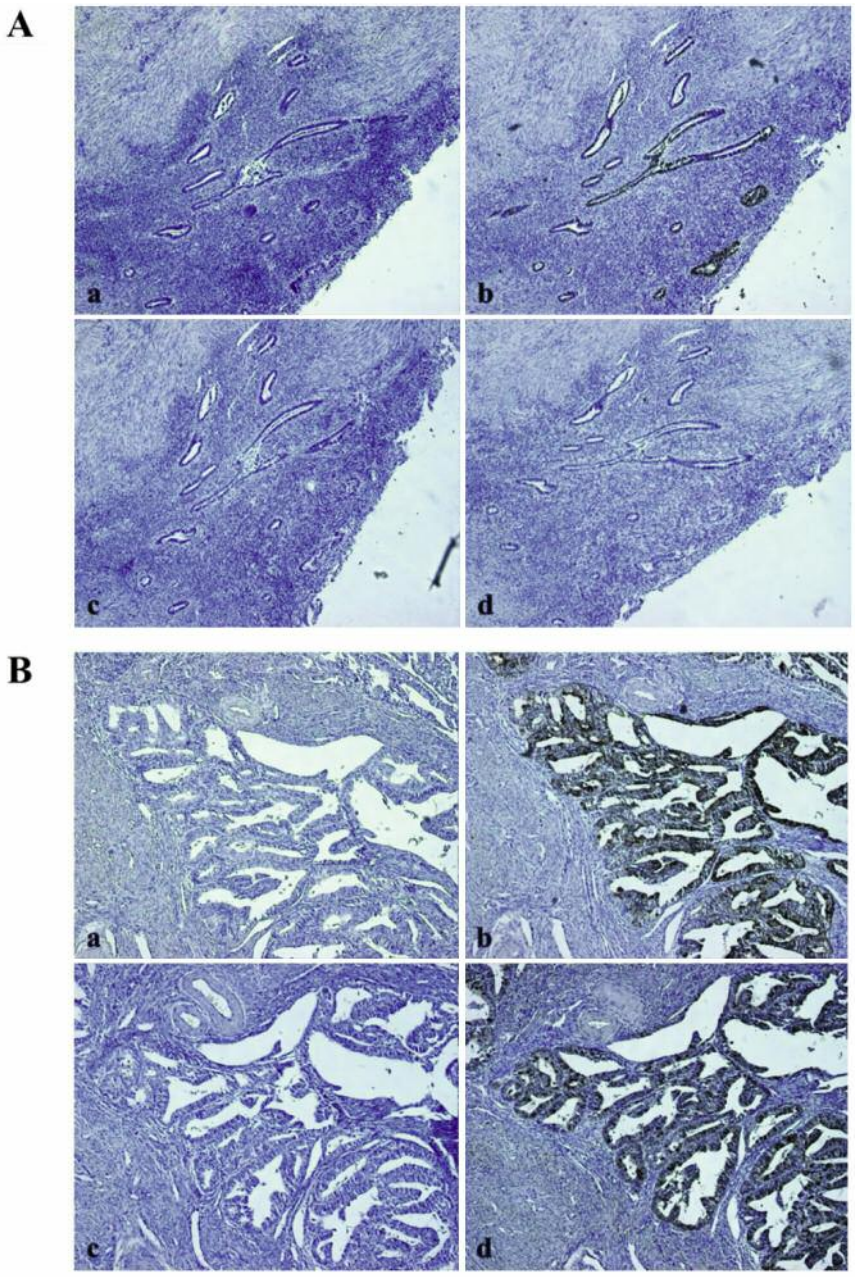

Figure 7. *Immunohistochemistry of normal endometrium and endometrial cancer tissues. Immunohistochemistry staining was conducted with human tissues: (A) normal endometrium group and (B) endometrial cancer group. Brown color was associated with each antibody-targeted protein, and blue-colored nuclei were stained with hematoxylin. (a) Antibody-negative control (only hematoxylin staining), (b) staining with anti-VDR antibodies, (c) staining with anti-TXNIP antibodies, and (d) staining with anti-thioredoxin antibodies.*

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