Vitamin D receptor knockdown attenuates the antiproliferative, pro-apoptotic and anti-invasive effect of vitamin D by activating the Wnt/β-catenin signaling pathway in papillary thyroid cancer

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Received October 16, 2019; Accepted April 23, 2020

DOI: 10.3892/mmr.2020.11522

Abstract. Vitamin D and the vitamin D receptor (VDR) complex have been reported to inhibit the growth of several types of tumor; however, their function in papillary thyroid cancer (PCT) remains unknown. In addition, the Wnt/β-catenin signaling pathway was discovered to serve a critical role in the pathology of PCT. Therefore, the present study aimed to determine the role of the VDR and its association with Wnt/β-catenin signaling in vitamin D-treated PTC cells. VDR expression was detected in human PTC cells (including MDA-T120, MDA-T85, SNU-790 and IHH4 cells) and thyroid follicular cells (Nthy-ori 3-1 cells). SNU-790 and IHH4 cells were infected with KD-VDR or negative control (KD-NC) lentiviruses, treated with $1,25(OH)_2D3$ (the active form of vitamin D), and subsequently referred to as the KD-VDR&vitD and KD-NC&vitD groups, respectively. Additionally, PTC cells infected with KD-NC and not treated with $1,25(OH)_2D3$ were used as the normal control and referred to as the KD-NC group. VDR mRNA and protein expression levels were increased in MDA-T120, SNU-790 and MDA-T85 cells compared to Nthy-ori 3-1 cells, whereas in IHH4 cells, VDR mRNA and protein expression levels were similar to Nthy-ori 3-1 cells. In SNU-790 and IHH4 cells, cell proliferation and invasion were decreased in the KD-NC&vitD group compared with the KD-NC group, but increased in the KD-VDR&vitD group compared with the KD-NC&vitD group. Cell apoptosis was increased in the KD-NC&vitD group compared with the KD-NC group, and decreased in the KD-VDR&vitD group compared with the KD-NC&vitD group. Furthermore, the expression levels of Wnt family member 3 and catenin β1 were decreased in the KD-NC&vitD group compared with the KD-NC group, but increased in the KD-VDR&vitD group compared with the KD-NC&vitD group. In conclusion, the present study revealed that VDR-KD attenuated the antiproliferative, pro-apoptotic and anti-invasive effects of vitamin D in PTC by activating the Wnt/β-catenin signaling pathway.

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Key words: vitamin D receptor, vitamin D, cellular functions, Wnt/β-catenin, papillary thyroid cancer

Introduction

Thyroid cancer is one of the most commonly diagnosed endocrine carcinomas and has an increasing incidence; the incidence and mortality rates are ~9.61/100,000 people and 0.35/100,000 people, respectively, in China annually (1,2). Papillary thyroid cancer (PTC) is the most common type of thyroid cancer and accounts for ~90% of all thyroid cancer cases (3). Although the majority of patients with PTC benefit from surgery, levothyroxine and radioactive iodine therapy, certain patients still experience a high rate of metastasis and recurrence (4-7). Therefore, understanding the mechanisms underlying the development and progression of PTC may aid the development of novel therapies in the future.

The gene encoding the vitamin D receptor (VDR), which is expressed by epithelial cells in both normal and malignant thyroid glands, is located on chromosome 12q13.1 (8). Previous studies have revealed that vitamin D, which can be hydroxylated to the active form $1,25(OH)_2D3$ (referred to as vitamin D or D3 in the present study), activates VDR (9,10). Furthermore, VDR expression is associated with cancer risk and mortality in various types of cancer, including breast, lung and colorectal cancer (11-14). Other studies suggested that the vitamin D and VDR complex binds to p21 and p27 proteins, which suppress DNA synthesis, inactivate mitogenic signals and inhibit tumor progression (15-18). The vitamin D level is decreased in PTC tissues compared with paired non-cancerous tissues, and is found to inhibit the proliferation of PTC cells (19). Furthermore, a previous study has revealed that VDR is upregulated in human PTC tissues compared with normal thyroid tissues (20). In addition, in our preliminary experiments, VDR was upregulated in human PTC tissues compared with human thyroid tissues and was also upregulated in PTC cell lines compared with thyroid follicular cells (Pang *et al* unpublished data). Furthermore, the Wnt/β-catenin signaling pathway was reported to serve

a critical role in mediating the development and progression of PTC (21,22). However, whether VDR influences tumor progression and regulates the Wnt/β-catenin signaling pathway through its interaction with vitamin D in PTC cells remains unknown. Therefore, the present study explored the role of vitamin D and the influence of VDR knockdown (KD) on cell proliferation, apoptosis, invasion and Wnt/β-catenin signaling in vitamin D-treated PTC cells.

Materials and methods

Cell culture. The human PTC cell lines MDA-T120 and MDA-T85 were purchased from the American Type Culture Collection (ATCC), SNU-790 was purchased from the Korean Cell Line Bank (Korean Cell Line Research Foundation) and IHH4 was purchased from the Japanese Collection of Research Bioresources Cell Bank. The human thyroid follicular cell line Nthy-ori 3-1 was purchased from Sigma-Aldrich (Merck KGaA). 293T cells were purchased from the ATCC. Nthy-ori 3-1, MDA-T120, SNU-790 and MDA-T85 cells were cultured in RPMI-1640 medium (Sigma-Aldrich; Merck KGaA) supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich; Merck KGaA). IHH4 and 293T cells were cultured in DMEM (Sigma-Aldrich; Merck KGaA) supplemented with 10% FBS. All cells were maintained in a humidified incubator at 37˚C and 5% CO_2.

Gene expression omnibus (GEO) datasets. The GEO datasets GSE33630 (23), GSE27155 (24), GSE3467 (25) and GSE3678 (26) were downloaded from the GEO database (http://www.ncbi.nlm.nih.gov/geo) (18) to assess VDR expression levels in PTC and healthy tissues. The datasets comprised 49 PTC tumor samples and 45 normal thyroids samples from GSE33630, 51 PTC tumor samples and 4 normal samples from GSE27155, 9 PTC tumor samples and 9 paired normal thyroid tissues from GSE3467, and 7 PTC samples and 7 paired normal thyroid tissue samples from GSE3678.

Lentivirus construction and infection. KD-VDR and non-targeting negative control (KD-NC) plasmids were cloned into the LV-H1/GFP&Puro plasmid and constructed by Shanghai GenePharma Co., Ltd. The sequence for VDR-KD was 5'-CGG CCTGAGATCAATCACATTTAACTCGAGTTAAATGTG ATTGATCTCAGGTTTTT-3' and for KD-NC was 5'-CAC CGTTCTCCGAACGTGTCACGTCGAAACGTGACACGT TCGGAGAA-3'. Then, 0.8 μg KD-VDR and KD-NC plasmids, as well as the packaging plasmids pRSV/pREV (Addgene, Inc.), pCMV-VSV-G (Addgene, Inc.) and pMDLG/pRRE (Addgene, Inc.), were co-transfected into $2x10^5$ 293T cells using Lipofectamine® 2000 reagent (Thermo Fisher Scientific, Inc.) and the lentiviral particles were harvested after 48 h by centrifugation (30,000 x g; 4˚C; 4 h). A total of $1x10^5$ SNU-790 and $1x10^5$ IHH4 cells were infected with the lentiviral particles (MOI=20 and 40, respectively) in the presence of 8 μg/ml polybrene (Sigma-Aldrich; Merck KGaA) at 37˚C for 24 h, and then stably infected cells were selected using puromycin (Thermo Fisher Scientific, Inc.) for 7 days.

1,25(OH)$_2$D3 treatment. KD-VDR and KD-NC cells that were maintained in RPMI-1640 medium or DMEM (depending on cell type), supplemented with 10% FBS containing 10 nM D3 (Sigma-Aldrich; Merck KGaA) were referred to as the KD-VDR&vitD and KD-NC&vitD groups, respectively. Whereas, KD-NC cells cultured without D3 were referred to as the KD-NC group.

Cell Counting Kit-8 (CCK-8) assay. At 0, 24, 48 and 72 h post-treatment, cell proliferation was determined using the CCK-8 assay (Dojindo Molecular Technologies, Inc.), according to the manufacturer's instructions. Briefly, $5x10^3$ cells/well were seeded into 96-well plates and incubated at 37˚C. Subsequently, 10 μl CCK-8 and 90 μl serum-free medium (RPMI-1640 medium or DMEM depending on the cell type) were added to each well and incubated at 37˚C for 2 h. The optical density (OD) value was measured using a microplate reader at a wavelength of 450 nm (BioTek Instruments, Inc.).

Flow cytometric analysis of apoptosis. At 48 h post-treatment, cells were stained using an Annexin V-FITC Apoptosis Detection kit (BD Biosciences) according to the manufacturer's instructions. Briefly, cells in each group were collected by centrifugation (850 x g; 5 min; room temperature), and then washed with PBS. Subsequently, the cells were suspended in 100 μl binding buffer, and incubated at 4˚C with 5 μl Annexin V and 5 μl propidium iodide in the dark for 15 min. Following the incubation, 400 μl binding buffer was added and the apoptotic cells (early + late apoptotic cells were analyzed using a CytoFLEX™ flow cytometer (Beckman Coulter, Inc.) and FlowJo 7.0 software (FlowJo LLC). PI^-/Annexin V^+ cells represented early apoptotic cells and PI^+/ Annexin V^+ cells represented late apoptotic cells.

Transwell Matrigel assay. The invasive ability of SNU-790 and IHH4 cells was detected at 24 h post-transfection. Briefly, $4x10^4$ transfected cells in serum-free media (RPMI-1640 medium or DMEM depending on the cell type) were seeded into the upper chambers of Transwell plates (pore size, 8-μm), precoated at 37˚C for 1 h with Matrigel (BD Biosciences). RPMI-1640 medium or DMEM (depending on the cell type) supplemented with 10% FBS was plated into the lower chamber. Following incubation for 24 h at 37˚C, the non-invasive cells remaining on the upper membrane were removed with cotton wool and the invasive cells were fixed with 4% formaldehyde for 15 min at room temperature and stained with 0.1% crystal violet for 15 min at room temperature. Stained cells were visualized using an IX73 inverted microscope (magnification, x200; Olympus Corporation) and analyzed using Image-Pro Plus 6.0 software (Media Cybernetics, Inc.).

Reverse transcription-quantitative (RT-q)PCR. Total RNA was extracted from the cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and was reverse transcribed into cDNA using the PrimeScript™ RT Master mix (Takara Biotechnology Co., Ltd.) at 37˚C for 20 min, and then 85˚C for 5 sec. qPCR was subsequently performed using the TB Green Fast qPCR mix (Takara Biotechnology Co., Ltd.) and the following thermocycling parameters: Initial denaturation at 95˚C for 30 sec; followed by 40 cycles of denaturation at 95˚C for 5 sec, annellation and extension at 61˚C for 15 sec. VDR, Wnt3 and CTNNB1 mRNA expression levels were

Table I. Primers used for reverse transcription-quantitative PCR.

Gene	Primer sequence (5'→3')
Vitamin D receptor	F: GCCGCATCACCAAGGACAA
	R: TCTGAGCAGGAGGAGGAGGA
Wnt family member 3	F: AGCCTGACTTCCGTGCCATC
	R: CTTCTCCGTCCTCGTGTTGTG
Catenin β1	F: GCCATTACAACTCTCCACAACCT
	R: GACAGATAGCACCTTCAGCACTC
GAPDH	F: GACCACAGTCCATGCCATCAC
	R: ACGCCTGCTTCACCACCTT

F, forward; R, reverse.

Table II. Antibodies used for western blotting analysis.

A, Primary antibody[a]	Cat. no.	Dilution
Anti-VDR; mouse polyclonal	ab89626	1:1,000
Anti-Wnt3; mouse polyclonal	ab169175	1:1,000
Anti-CTNNB1; mouse monoclonal	ab22656	1:1,500
Anti-GAPDH; mouse monoclonal	ab8245	1:5,000
B, Secondary antibody[a]		
Goat anti-mouse IgG-HRP	ab205719	1:10,000

[a]All antibodies were purchased from Abcam. CTNNB1, catenin β1; HRP, horseradish peroxidase; Wnt3, Wnt family member 3; VDR, vitamin D receptor.

quantified using the $2^{-\Delta\Delta Cq}$ method (27) and normalized to the internal reference gene GAPDH. The primers used for the qPCR are presented in Table I.

Western blotting. Total protein was extracted using RIPA buffer (Sigma-Aldrich; Merck KGaA). The protein concentration in each sample was measured using the Bicinchoninic Acid Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc.) and 20 μg protein/lane was separated on 4-12% NuPAGE Bis-Tris Gels (Thermo Fisher Scientific, Inc.). The separated proteins were transferred to polyvinylidene fluoride membranes (EMD Millipore) and blocked with 5% BSA (Thermo Fisher Scientific, Inc.) at 37˚C for 2 h. The membranes were subsequently incubated with the primary antibodies overnight at 4˚C. Subsequently, the membranes were incubated with the secondary antibody for 90 min at 37˚C. Protein bands were visualized using the Novex ECL Chemiluminescent Substrate Reagent kit (Thermo Fisher Scientific, Inc.) and X-ray film (Kodak). The antibodies and respective dilutions used in the present study are presented in Table II.

Statistical analysis. Data are presented as the mean ± standard deviation. Statistical analyses were performed using GraphPad Prism software (version 7.02; GraphPad Software, Inc.). Comparisons between a control group and other experimental groups were determined by one-way ANOVA followed by Dunnett's test. Comparison between two groups was assessed using the unpaired Student's t-test or paired t-test if appropriate. Multiple comparisons among groups were assessed by one-way ANOVA followed by Tukey's test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Expression of VDR in human PTC and thyroid follicular cells. Comparisons between the control thyroid follicular cell line Nthy-ori 3-1 and four PTC cell lines, revealed that VDR mRNA expression levels were increased in MDA-T120, SNU-790 and MDA-T85 cells compared with Nthy-ori 3-1 cells, but similar between IHH4 and Nthy-ori 3-1 cells (Fig. 1A). Similarly, western blotting revealed that VDR protein levels were increased in MDA-T120, SNU-790 and MDA-T85 cells comparing with Nthy-ori 3-1 cells, but similar IHH4 and Nthy-ori 3-1 cells (Fig. 1B and C). These data indicated that VDR was upregulated in human PTC cell lines MDA-T120, SNU-790 and MDA-T85 compared with the control thyroid follicular cell line Nthy-ori 3-1. Considering the observation that VDR expression was upregulated in SNU-790 cells, and the finding that VDR expression was similar between IHH4 cell line and thyroid follicular cells, SNU-790 and IHH4 cells were chosen in the following functional experiments to determine the influence of KD-VDR in vitamin D-treated PTC cells.

Expression of VDR in PTC and healthy tissues. GEO datasets were used to compare VDR expression in PTC and healthy tissues (Fig. S1). VDR expression was increased in PTC tissue compared with healthy tissue in the GSE33630, GSE27155, GSE3467 and GSE3678 datasets. These data demonstrated that VDR was upregulated in PTC tissues compared with healthy tissues.

KD-VDR in PTC cells. VDR expression was assessed in SNU-790 and IHH4 cells stably infected with KD-VDR and KD-NC plasmids. Comparison between the two groups showed that VDR mRNA and protein expression levels were decreased in KD-VDR SNU-790 cells compared with KD-NC SNU-790 cells (Fig. 2A and B, respectively). Similarly, VDR mRNA and protein expression levels were decreased in KD-VDR IHH4 cells compared with KD-NC IHH4 cells (Fig. 2C and D, respectively). These results suggested that PTC cells stably infected with KD-VDR or KD-NC lentiviruses were successfully established.

Effect of vitamin D and KD-VDR on cell proliferation. To investigate the effect of KD-VDR on cell proliferation in vitamin D-treated PTC cell lines, CCK-8 assays were conducted; it was revealed that in SNU-790 (Fig. 3A) and IHH4 cells (Fig. 3B), cell proliferation was decreased in the KD-NC&vitD group at 48 and 72 h compared with the KD-NC group, and increased in the KD-VDR&vitD group at 48 and 72 h compared with the KD-NC&vitD group. These data suggested that KD-VDR infection attenuated the antiproliferative effect of vitamin D treatment in PTC cells.

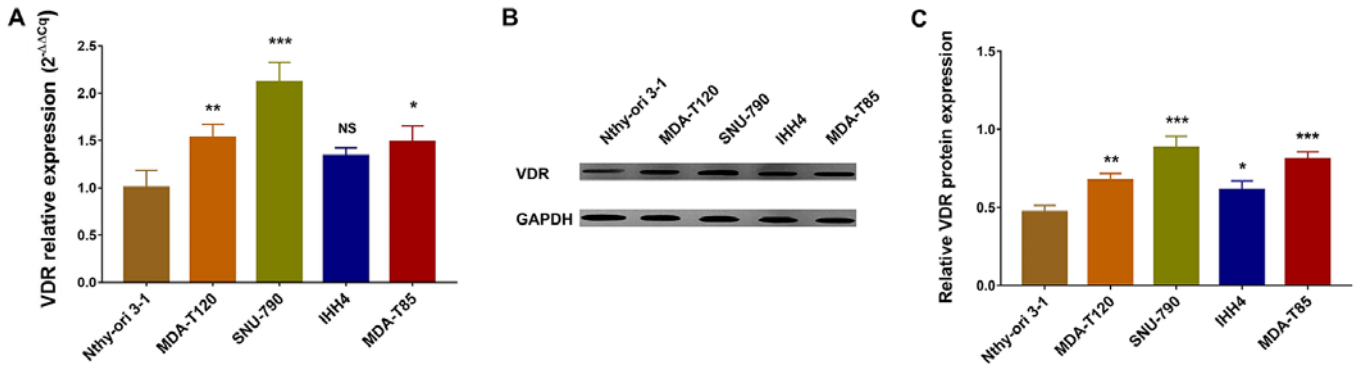

Figure 1. Detection of VDR expression in PTC and thyroid follicular cells. Comparison of VDR (A) mRNA and (B) protein expression levels between PTC cell lines (MDA-T120, SNU-790, IHH4 and MDA-T85) and the control human thyroid follicular cell line Nthy-ori 3-1 was determined by one-way ANOVA followed by Dunnett's test. (C) Semi-quantification of the expression levels presented in part. (B) *P<0.05; **P<0.01; ***P<0.001 vs. Nthy-ori 3-1 cells. NS, not significant; PTC, papillary thyroid cancer; VDR, vitamin D receptor.

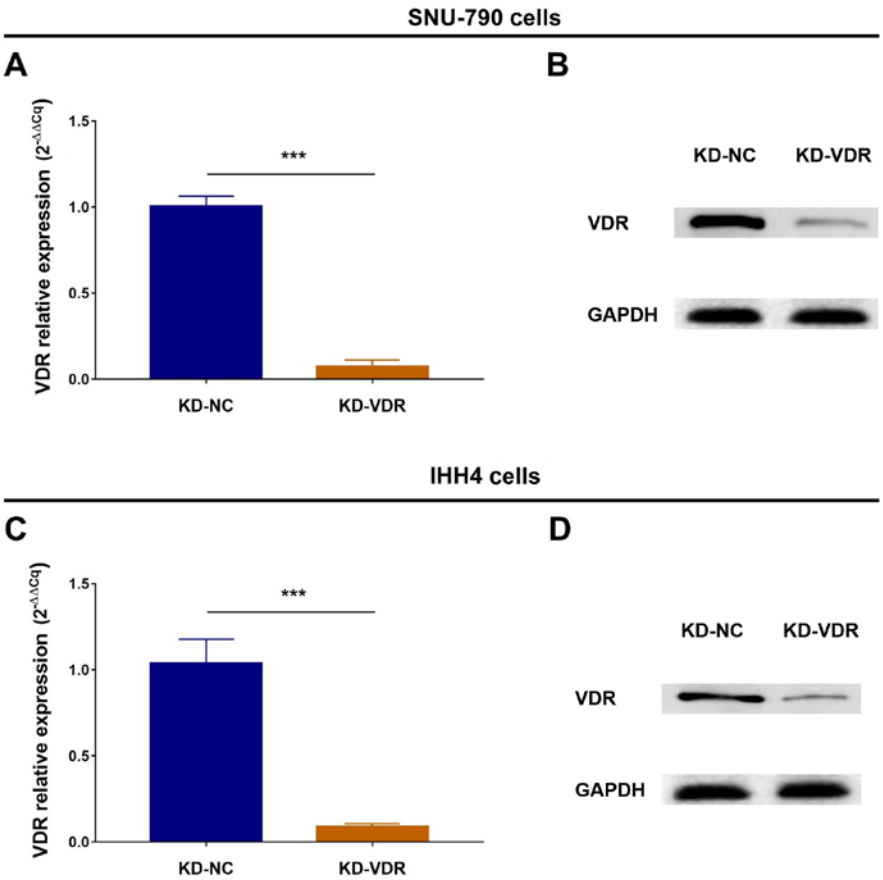

Figure 2. VDR expression in papillary thyroid cancer cells after knockdown. Comparison of VDR (A) mRNA and (B) protein expression levels between KD-NC and KD-VDR infection in SNU-790 cells. Comparison of VDR (C) mRNA and (D) protein expression in KD-NC and KD-VDR infection in IHH4 cells. Comparison between the two groups was assessed using the unpaired Student's t-test. ***P<0.001. KD, knockdown; NC, negative control; VDR, vitamin D receptor.

Effect of vitamin D and KD-VDR on apoptosis. To determine the effect of KD-VDR on the rate of cell apoptosis in vitamin D-treated PTC cell lines, flow cytometric analysis of apoptosis was performed; in SNU-790 (Fig. 4A) and IHH4 cells (Fig. 4B), cell apoptosis was increased in the KD-NC&vitD group compared with the KD-NC group, whereas cell apoptosis was decreased in the KD-VDR&vitD group compared with the KD-NC&vitD group. These data indicated that KD-VDR attenuated the pro-apoptotic effect of vitamin D treatment in PTC cells.

Effect of vitamin D and KD-VDR on cell invasion. To elucidate the effect of KD-VDR on cell invasion in vitamin D-treated PTC cell lines, Transwell Matrigel assays were performed, which revealed that in SNU-790 (Fig. 5A) and IHH4 (Fig. 5B) cells, invasion was decreased in the KD-NC&vitD group compared with the KD-NC group, but increased in the KD-VDR&vitD group compared with the KD-NC&vitD group. These data suggested that KD-VDR attenuated the anti-invasive effect of vitamin D treatment in PTC cells.

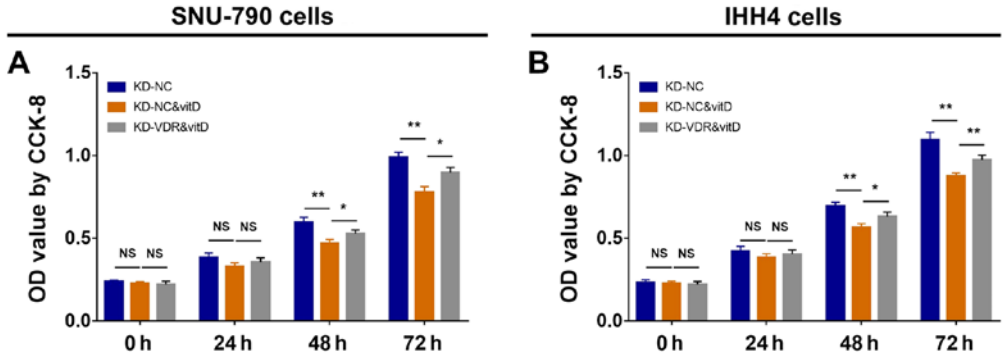

Figure 3. Cell proliferation in SNU-790 and IHH4 cells after KD-VDR and vitD treatment. CCK-8 assays for proliferation were performed, and OD values were compared between stably infected (A) SNU-790 and (B) IHH4 cells treated with or without vitD at 0, 24, 48 and 72 h. Multiple comparisons among groups were assessed by one-way ANOVA followed by Tukey's test. *P<0.05; **P<0.01. CCK-8, Cell Counting Kit-8; KD, knockdown; NC, negative control; NS, not significant; OD, optical density; VDR, vitamin D receptor; vitD, vitamin D.

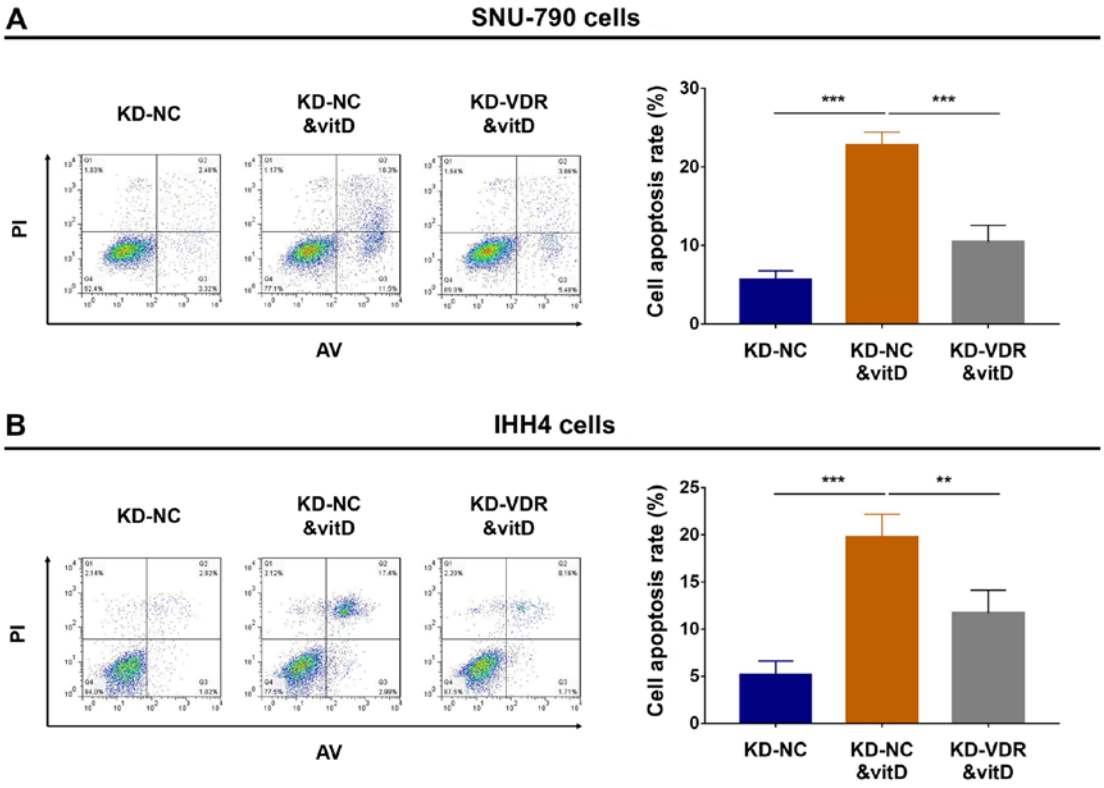

Figure 4. Apoptosis in SNU-790 and IHH4 cells after KD-VDR and vitD treatment. Comparison of apoptotic rates between the stably infected (A) SNU-790 and (B) IHH4 cells treated with or without vitD. Multiple comparisons among groups were assessed by one-way ANOVA followed by Tukey's test. **P<0.01; ***P<0.001. NC, negative control; KD, knockdown; VDR, vitamin D receptor; vitD, vitamin D.

Effect of vitamin D and KD-VDR on the Wnt/β-catenin signaling pathway. To determine the effect of KD-VDR on the Wnt/β-catenin signaling pathway in vitamin D-treated PTC cell lines, RT-qPCR and western blotting assays were performed. The results revealed that in SNU-790 cells, Wnt3 and CTNNB1 mRNA expression levels were decreased in the KD-NC&vitD group compared with the KD-NC group, but increased in the KD-VDR&vitD group compared with the KD-NC&vitD group (Fig. 6A and B). Wnt3 and CTNNB1 protein expression levels exhibited a similar trend (Fig. 6C and D). Similarly, in IHH4 cells, Wnt3 and CTNNB1 mRNA expression levels were decreased in the KD-NC&vitD group compared with the KD-NC group, but increased in the KD-VDR&vitD group compared with the KD-NC&vitD group (Fig. 6E and F), and Wnt3 and CTNNB1 protein expression levels exhibited a similar trend (Fig. 6G and H). These data indicated that KD-VDR attenuated the inhibitory effect of vitamin D on the Wnt/β-catenin signaling pathway in PTC cells.

Discussion

Vitamin D is synthesized through a series of steps, starting from a cholesterol precursor molecule that is transformed into the vitamin D hormone precursor (vitamin D_3) and subsequently hydroxylated to its most active hormone form, $1,25(OH)_2D3$, in the liver and kidney (14). Vitamin D binds to VDR when it enters

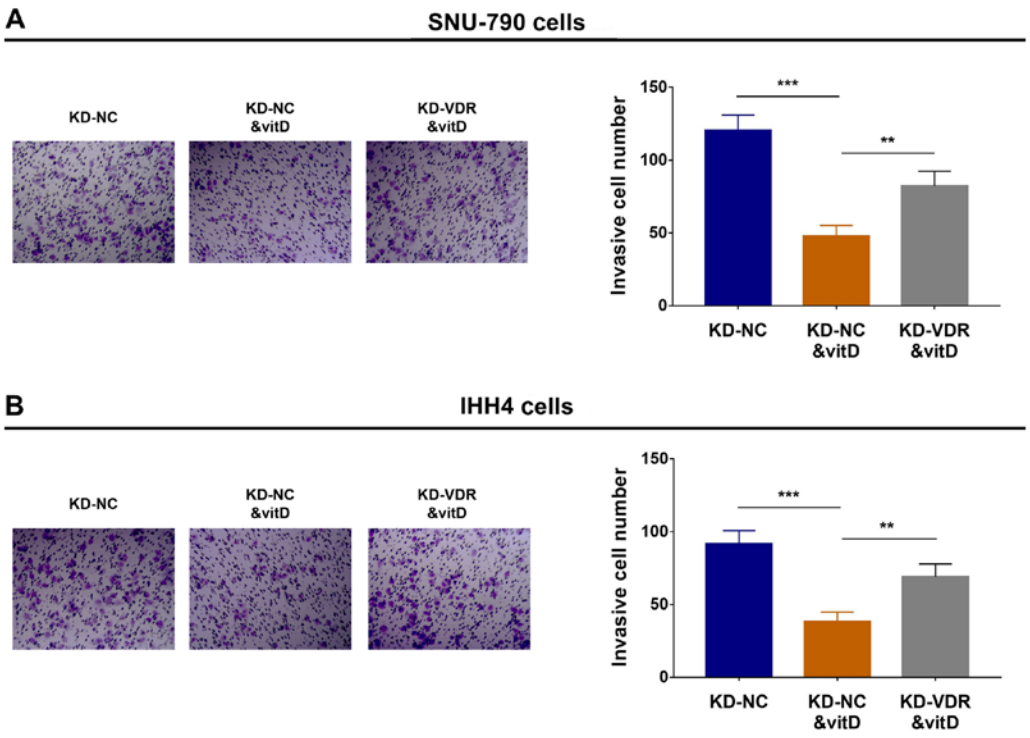

Figure 5. Cell invasion in SNU-790 and IHH4 cells after KD-VDR and vitD treatment. Comparison of the number of invading cells between the stably infected (A) SNU-790 and (B) IHH4 cells treated with or without vitD. Multiple comparisons among groups were assessed by one-way ANOVA followed by Tukey's test. **P<0.01; ***P<0.001. NC, negative control; KD, knockdown; VDR, vitamin D receptor; vitD, vitamin D.

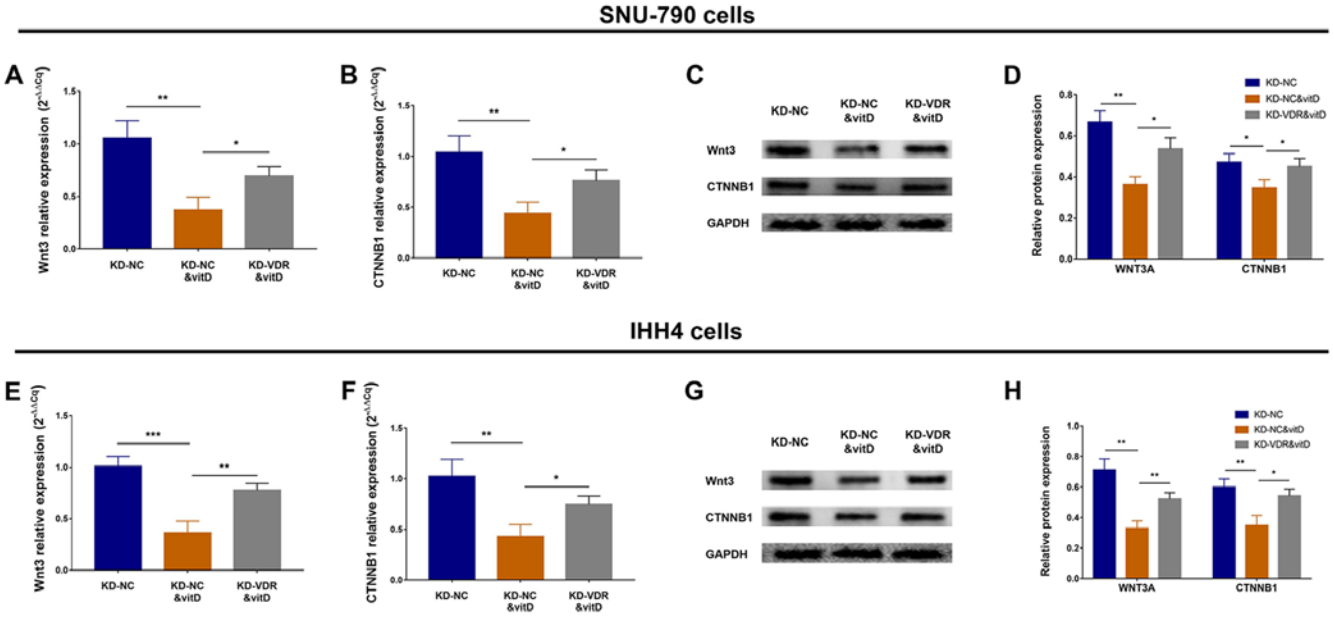

Figure 6. Wnt3 and CTNNB1 expression levels in SNU-790 and IHH4 cells after VDR KD and vitD treatment. Stably transfected SNU-790 cells were treated with or without vitD and the expression levels of (A) Wnt3 mRNA, (B) CTNNB1 mRNA and (C) Wnt3 and CTNNB1 proteins were detected. (D) Semi-quantification of the expression levels from part. (C) Stably transfected IHH4 cells were treated with or without vitD and the expression levels of (E) Wnt3 mRNA, (F) CTNNB1 mRNA, (G) Wnt3 and CTNNB1 proteins were detected. (H) Semi-quantification of the expression levels presented in part. (G) Multiple comparisons among groups were assessed by one-way ANOVA followed by Tukey's test. *P<0.05; **P<0.01; ***P<0.001. CTNNB1, catenin β1; KD, knockdown; NC, negative control; VDR, vitamin D receptor; vitD, vitamin D; Wnt3, Wnt family member 3.

the cells, forming a complex that is reported to influence cell cycle regulation by decreasing DNA synthesis, inactivating mitogenic signals, reducing expression of c-Myc and inhibiting cell differentiation, which inhibit tumor formation in several types of cancer, including breast, colorectal and lung cancer (13,28,29). For example, in breast cancer, vitamin D modulates the intracellular kinase pathways (including p38 mitogen activated protein kinase and ERK), represses the expression of proto-oncogenes (such as c-Myc) and decreases the proliferation of breast cancer cells (15,30). In PTC, vitamin D is associated with decreased

PTC risk, and vitamin D inhibits PTC development and progression (19). Furthermore, VDR is reported to be upregulated in PTC tissues compared with normal thyroid tissue (20,31). However, the role of VDR in the development and progression of PTC has not been fully elucidated. The present study analyzed VDR expression in human PTC and normal thyroid follicular cells, and revealed that VDR mRNA and protein expression levels were increased in PTC cells compared with thyroid follicular cells, which was consistent with previous studies (20,31).

Previous studies had revealed that VDR has the potential to regulate cancer cellular activities and influence tumor progression and metabolism by binding to vitamin D (15,20,32,33). For example, in vitamin D-treated breast cancer cells, silencing of VDR promotes cancer cell motility and invasiveness by increasing the expression of proteins involved in cell adhesion, proliferation, cytoskeletal organization (33). The present study explored the modulatory effects of vitamin D on cellular activities in PTC cells, and further investigated whether KD-VDR affected the actions of vitamin D in PTC cells. In the current study, SNU-790 and IHH4 cells were selected for the functional experiments, based on the rational that among the MDA-T120, MDA-T85, SNU-790 and IHH4 cell lines, VDR was more highly expressed in SNU-790 cells compared with the human thyroid follicular cells Nthy-ori 3-1. Therefore, this cell line was chosen as it could show an increased phenotype after VDR knockdown over the remaining cell lines. By contrast, VDR expression was similar between IHH4 cell line and the human thyroid follicular cells. Therefore, IHH4 cell line was selected to explore the regulatory role of VDR knockdown on this cell line expressing lower VDR levels compared to the remaining PTC cell lines. In the present study, the two PTC cell lines were selected to exclude the cell line-dependent phenomenon for the investigation of the effects of VDR knockdown on cell proliferation, apoptosis, invasion and Wnt/β-catenin signaling in vitamin D-treated PTC cells. In the current study, KD-VDR attenuated the antiproliferative, pro-apoptotic and anti-invasive effects of vitamin D in PTC cells. In a previous study, vitamin D was shown to reduce cyclin-dependent kinase activity, regulate the expression of the plasminogen activator system and increase E-cadherin expression in prostate cancer (14). Vitamin D may therefore inhibit PTC cell proliferation and invasion in a similar manner. A different previous study revealed that VDR was upregulated in PTC cells compared with thyroid follicular cells and that the vitamin D and VDR complex exhibited anti-tumor properties (34). Therefore, KD-VDR might decrease the anti-tumor effect by reducing the number of vitamin D/VDR complexes in PTC cells. The present study revealed that KD-VDR may attenuate the effect of vitamin D on the oncogenic Wnt/β-catenin signaling pathway in PTC and inhibit cell proliferation and invasion but promote cell proliferation.

A previous study revealed that cell proliferation, apoptosis and epithelial-mesenchymal transition are mediated by VDR-activated vitamin D and the Wnt/β-catenin signaling pathway in renal cancer (32). Furthermore, in uterine fibroid cells, vitamin D reduced the expression levels of Wnt4 and β-catenin, whereas VDR silencing promoted the expression levels of Wnt4 and β-catenin, further promoting cell proliferation and extracellular matrix production (17). Furthermore, in breast and prostate cancer, VDR silencing was associated with the attenuation of the Wnt/β-catenin signaling pathway, along with the downregulation of downstream genes such as cyclin D1 and interleukin-6 (35). In addition, studies have demonstrated the involvement of the Wnt/β-catenin signaling pathway in cell growth, invasion and metastasis in several types of cancer, including PTC (36). A previous study revealed that activation of the Wnt/β-catenin signaling pathway contributes to the progression of PTC by promoting cell proliferation and invasion (22). Therefore, the present study explored the regulatory effect of vitamin D on Wnt/β-catenin signaling, and to further investigate the regulatory effect of KD-VDR on the Wnt/β-catenin signaling pathway in vitamin D-treated PTC cells. The results revealed that vitamin D suppressed the expression of Wnt3 and CTNBB1 in PTC cells, and that KD-VDR attenuated the inhibitory effect of vitamin D on the expression of Wnt3 and CTNBB1 in PTC cells. This suggested that KD-VDR might attenuate the antiproliferative, pro-apoptotic and anti-invasive effects of vitamin D by activating the Wnt/β-catenin signaling pathway in PTC cells. However, the mechanism by which VDR alone regulates PTC cells was not investigated in the present study. However, similar to mechanisms reported in breast and prostate cancer, inhibiting the VDR may activate specific downstream promotors of Wnt/β-catenin signaling pathway in PTC cells, which further stimulates Wnt/β-catenin signaling (34,37).

Nonetheless, there are several limitations to the present study; i) The effect of KD-VDR was only investigated in vitamin D-treated PTC cells, therefore further studies are required to determine the effect of KD-VDR in vitamin D-treated normal thyroid cell lines; and ii) the current study did not investigate the effect of VDR alone in PTC cells, hence further cellular experiments are required.

In conclusion, KD-VDR attenuated the antiproliferative, pro-apoptotic and anti-invasive effect of vitamin D in PTC cells possibly by activating the Wnt/β-catenin signaling pathway. The results obtained in the present study suggested that VDR may be a novel and promising therapeutic target in the treatment of PTC, which might be applied in future clinical practice and improve the clinical outcomes of patients with PTC.

Acknowledgements

Not applicable.

Funding

This study was supported by the Subject of Heilongjiang Provincal Health and Family Planning Commission (grant no. 2018043) and the Youth Project of Haiyan Fund (grant no. JJQN2017-11).

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

RP made substantial contributions to the design of the present study. Data acquisition and interpretation was conducted by RP, YX, XH, BL and JY. PR and JY critically revised the manuscript for intellectual content. All authors agree to be accountable for all aspects of the work in ensuring that questions related to the

accuracy or integrity of the work are appropriately investigated and resolved. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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