Vitamin D receptor expression and its clinical significance in papillary thyroid cancer

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Directed by Professor Kee-Hyun Nam

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ABSTRACT

Vitamin D receptor expression and its clinical significance in papillary thyroid cancer

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Background: The vitamin D receptor (VDR) is one of the essential components in the vitamin D signaling pathway and has been known to play a role in anticancer activity. This study aimed to evaluate the association between VDR and serum vitamin D, and its clinical significance in papillary thyroid cancer (PTC).

Methods: VDR protein and mRNA expression in 73 PTC tissues were compared with that in normal and benign tissues. Further, the association of VDR with vitamin D enzymes, and markers of cell proliferation and metastasis was investigated. The level of VDR expression was evaluated for the correlation with serum vitamin D levels and clinicopathologic characteristics of patients with PTC. Data from 501 patients with PTC from The Cancer Genome Atlas (TCGA) database were analyzed to confirm and support our findings.

Results: Increased VDR protein and mRNA expression were observed in PTC compared with that in normal and benign tissues. However, lower VDR protein expression was identified in high TNM stage PTC, which was associated with low p21 protein expression. Lower relative VDR mRNA

expression in PTC was associated with low serum 25-hydroxyvitamin D level. The TCGA database showed a positive correlation among mRNA expression of VDR, CYP24A1, and p21.

Conclusions: We demonstrated an association between decreased VDR protein expression and advanced stage PTC, and a correlation between low VDR mRNA expression with low serum 25(OH)D level. Overall, this study demonstrates that low VDR expression in PTC may be positively correlated with low serum vitamin D level and disease aggressiveness. Further large, prospective studies are needed to validate the potential anticancer effect of VDR in thyroid cancer.

Key words: vitamin D, vitamin D receptor, thyroid cancer, thyroid surgery

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I. INTRODUCTION

The understanding of vitamin D has been evolving since its discovery in the early 20th century, from being a simple vitamin to a steroid pro-hormone[1]. It has been known to play an important role in bone mineralization by regulating calcium and phosphate metabolism. Previous studies have demonstrated that vitamin D deficiency, which is common worldwide, could be associated with non-skeletal conditions, including muscle weakness, metabolic diseases, cardiovascular disorders, autoimmune diseases, and infectious diseases[2-5]. The associations between low serum 25-hydroxyvitamin D (25(OH)D), a biomarker of vitamin D status, and several malignancies including colorectal cancer, prostate cancer, breast cancer, and head and neck squamous cell carcinoma have also been reported, focusing on the potential anticancer effects of vitamin D[6-18]. In thyroid cancer, there has been controversy over the correlation between serum 25(OH)D level and clinicopathologic characteristics of thyroid cancer[19-23].

Calcitriol (1,25-dihydroxyvitamin D_3, $1,25(OH)_2D_3$) is a potent activated form of vitamin D and is tightly regulated through a complex process involving the vitamin D-activating enzyme 1-α-hydroxylase (also named

CYP27B1) and 24-hydroxylase (also named CYP24A1). The enzyme 1-α-hydroxylase is responsible for the final hydroxylation step from 25(OH)D toward $1,25(OH)_2D_3$ while 24-hydroxylase is the key enzyme in the inactivation of $1,25(OH)_2D_3$. The action of calcitriol occurs mainly through its binding to the nuclear vitamin D receptor (VDR), which acts as a hormone-regulated transcription factor[24,25].

Several studies have focused on the dynamics of VDR and calcitriol-related enzymes in cancer tissues[26,27], suggesting that VDR plays a critical role in the anticancer mechanism of calcitriol. VDR expression in cancer tissue has been shown to have an anticancer effect, and its clinical significance and prognostic value in several cancers has been consistently reported[14,28-33]. Meanwhile, there have not been many reports on the relationship among serum vitamin D, VDR, and thyroid cancer. A few studies have discussed the overall vitamin D metabolism in normal, benign, and malignant thyroid tissues[34-36].

Based on the studies mentioned above, we aimed to investigate VDR expression in papillary thyroid cancer (PTC) and evaluate its clinical significance. We hypothesized that low VDR expression in PTC may be associated with low serum vitamin D levels and aggressive clinicopathologic features. In this study, we first investigated the expression of VDR, CYP27B1, CYP24A1, and markers of cell proliferation (i.e., p21, a cell cycle regulator) and metastasis (i.e., E-cadherin, a cell-to-cell adhesion marker) in human thyroid tissues, from normal, benign, to PTC. Second, we correlated the expression profiles of VDR in PTC with serum vitamin D levels and other clinicopathologic characteristics.

II. MATERIALS AND METHODS

1. Study participants and sample collection

A prospective study was conducted on patients with thyroid tumors who had visited the Department of Surgery in the Yonsei University College of Medicine for surgery from April 2017 to July 2018. Patients who were diagnosed with PTC or benign thyroid tumor by fine-needle aspiration biopsy were included in this study. Patients were excluded if they had 1) any medications that might alter vitamin D metabolism (including calcium and vitamin D supplementation); 2) a disease that could affect serum vitamin D levels (including renal disorders, liver disease); 3) abnormal thyroid function; 4) a history of previous neck surgery or irradiation; 5) any prior cancer history; or 6) declined to participate in the study.

The indication for surgery was based on clinical findings, and thyroid surgery was performed by a single surgeon. Blood samples were obtained within one month prior to surgery. Serum levels of 25(OH)D, thyroid-stimulating hormone (TSH), free thyroxine (fT4), and triiodothyronine (T3) were measured simultaneously. We also obtained serum $1,25(OH)_2D_3$ levels in patients with cancer to study its relationship with serum 25(OH)D levels and other clinicopathologic features in PTC. According to a recent criteria, serum 25(OH)D levels <20 ng/mL are defined as deficient, levels from 20 to 29.9 ng/mL are insufficient, and levels ≥30 ng/mL are sufficient[37]. We categorized the patients into two groups by their serum vitamin D levels. A 20 ng/mL cut-off was used for serum 25(OH)D (deficient or not deficient) and a 40 ng/mL (a median value) cut-off was utilized for serum $1,25(OH)_2D_3$.

Clinical characteristics and demographic data were collected by retrospective chart review, extracting the following patient data: age at the time of surgery, sex, histologic type of primary tumor, tumor size, tumor multiplicity, tumor bilaterality, extrathyroidal extension, BRAF (serine/threonine-protein

kinase B-Raf) V600E mutations, and the Tumor, Node, Metastasis (TNM) classification. Tumor size was defined as the greatest tumor diameter based on histopathology results. A cut-off value of ≥1 cm was used for tumor size since a tumor size of <1 cm was used in the definition of papillary thyroid microcarcinoma. The TNM classification system of the American Joint Committee on Cancer (AJCC) eighth edition was used for the staging system. The T stage was classified into 1/2 or 3/4, the N stage into N0 vs. N1, and the tumor stage into I/II or III/IV.

Thyroid tissues were obtained from patients for immunohistochemistry (IHC) analysis, and real-time quantitative polymerase chain reaction (qPCR). Biopsy samples were collected from a central location in malignant and benign tumors to obtain a pure tumor sample. Malignant and benign tumors were present simultaneously in four patients, and both tissues samples were taken. Normal thyroid tissues exhibiting normal morphology were taken from the contralateral lobe of thyroid tumor specimens for use as a control. Paraffin-embedded tissues (for IHC analysis) from 92 patients and snap-frozen thyroid tissues (for qPCR analysis) from 68 patients were collected. All procedures performed in this study were in accordance with the ethical standards of the Institutional Review Board of Yonsei University College of Medicine (4–2016–0657) and the 1964 Declaration of Helsinki or comparable ethical standards. All patients provided written informed consent.

2. Immunohistochemistry

Immunohistochemistry staining for VDR, CYP27B1, CYP24A1, p21, and E-cadherin was performed on paraffin-embedded sections from 73 PTC, 23 benign, and 25 normal thyroid tissues. Thyroid tissues were isolated and fixed with 10% formalin. Tissues were cut into 4-μm thick sections and were assessed for expression of the target proteins through IHC. Deparaffinization, rehydration, and epitope retrieval were performed using citrate buffer, pH 6.0

(Sigma-Aldrich, Saint Louis, MO, USA). After blocking of endogenous peroxidase activity using 3% hydrogen peroxidase (Dako, Glostrup, Denmark) and an incubation period with 10% donkey serum, the sections were incubated with the primary antibodies: rabbit anti-VDR antibodies (ab134826; Abcam, Cambridge, UK; diluted at 1:200), mouse anti-CYP27B1 antibodies (sc515903; Santa Cruz Biotechnology, TX, USA; diluted at 1:200), mouse anti-CYP24 antibodies (sc365700; Santa Cruz Biotechnology; diluted at 1:200), rabbit anti-p21 antibodies (ab109520; Abcam; diluted at 1:200), and rabbit anti-E-cadherin antibodies (ab15148; Abcam; diluted at 1:50). After rinsing with phosphate-buffered saline (PBS), incubation with secondary antibodies was performed for 60 min. Subsequently, detection using the Dako REAL Envision Detection system was performed and the peroxidase activity was revealed using the DAB substrate (Dako). Finally, sections were counterstained with hematoxylin, rinsed, and viewed with mounting medium (Dako).

Immunohistochemical findings were interpreted by a single independent investigator (JSKoo). Protein expression of nuclear VDR, cytoplasmic VDR, and nuclear p21 were quantified as the percent of stained nuclei per 100 cells. For cytoplasmic p21, CYP24A1, CYP27B1, and E-cadherin, staining intensity were scored as follows: 0 (no staining), 1 (weak), 2 (modest), or 3 (strong) (Figure 1). For the two-group comparisons, we used the cut-off levels of 50% for nuclear and cytoplasmic VDR expression[38], and 10% for nuclear p21 expression[39] to classify into high or low expression. For cytoplasmic p21, CYP27B1, CYP24A1, and E-cadherin, a score of 0-1 was categorized as negative, and a score of 2-3 was considered as positive (Figure 2).

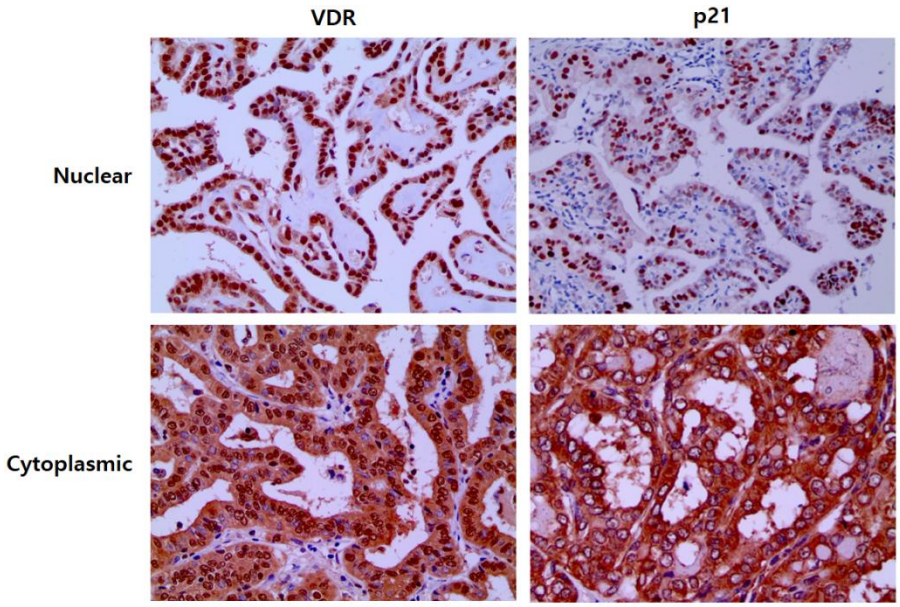

Figure 1. Protein expression of nuclear VDR, cytoplasmic VDR, nuclear p21, and cytoplasmic p21 in papillary thyroid cancer. (400×)

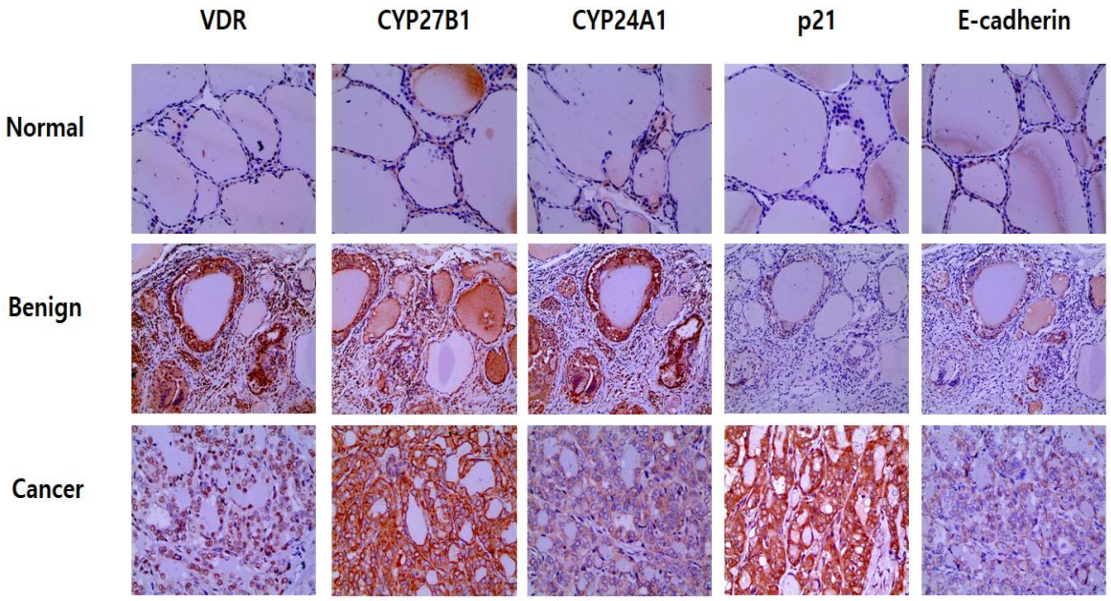

Figure 2. Protein expression profiles of VDR, CYP27B1, CYP24A1, p21, and E-cadherin in normal, benign, and papillary thyroid cancer. (400×)

3. Real-time quantitative PCR (qPCR)

Tissue samples obtained during thyroidectomy in 68 patients were immediately snap-frozen in liquid nitrogen and stored at –80°C. Total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. RNA was quantified using a Spectrophotometer NanoDrop 2000 (Nanodrop Technologies, Wilmington, DE, USA). Sample purity was confirmed by measuring A260/280 ratios while sample quality was assessed using 1% agarose gel electrophoresis. The acceptable purity as indicated by A260/280 is greater than 1.8, and RNA samples that showed smear or degradation were excluded. The fresh 38 PTC-normal pairs and 10 benign-normal pairs were subjected to qPCR analyses[40].

RNA was reverse transcribed to cDNA using an AccuPower RT Premix Kit (Bioneer Inc., Daejeon, South Korea) for experiments. qPCR was performed with an EvaGreen Q Master Mix (LaboPass, Seoul, Korea). Primer sequences for the target genes VDR, CYP27B1, CYP24A1, p21, and E-cadherin are listed in Table 1. Gene expression was measured by qRT-PCR using a StepOnePlus™ real-time PCR machine (Applied Biosystems, CA, USA). All reactions were performed in duplicate. The relative expression was analyzed by normalizing the data to β-Actin mRNA levels.

TABLE 1. Real-Time Quantitative PCR primer sequences for the target genes in the experiment.

Target Gene	Primer Sequences (5'-3')
β-Actin	Forward: CTACCTCATGAAGATCCTCACCGA
	Reverse: TTCTCCTTAATGTCACGCACGATT
VDR	Forward: TGGAGACTTTGACCGGAACG
	Reverse: GGGCAGGTGAATAGTGCCTT
CYP24A1	Forward: TGGGTTCCTTTGAGTCGGTG
	Reverse: TCCACGGTTTGATCTCCAGC
CYP27B1	Forward: TCTTCCCTTTGGCTTTGGCA

	Reverse: GTCTTGGGTCTAACTGGGGC
p21	Forward: GCGACTGTGATGCGCTAATG
	Reverse: GAAGGTAGAGCTTGGGCAGG
E-cadherin	Forward: CACCACGGGCTTGGATTTTG
	Reverse: TGGGGGCTTCATTCACATCC

All sequences are listed in the 5'-3' direction.

In the present study, data are presented as the fold change in target gene expression of the tumors relative to its expression in the counterpart normal tissue. Results of real-time PCR data were represented as Ct values, where Ct was defined as the threshold cycle number of PCRs at which the amplified product was first detected. The average Ct was calculated for both the target genes and β-Actin, and the ΔCt was determined as the mean of the duplicate Ct values for the target gene minus the mean of the duplicate Ct values for β-Actin. The ΔΔCt represented the difference between the paired tissue samples, as calculated by the formula ΔΔCt = ΔCt of tumor - ΔCt of normal. The N-fold differential expression in the target gene of a tumor sample compared to that in the normal sample counterpart was expressed as $2^{-\Delta\Delta CT}$ [41,42]. The cut-off level of two (a median value) for the N-fold differential expression of VDR was used to categorize the PTC patients into two groups for further comparative analyses to determine the relationship with clinicopathologic features.

3. Data from The Cancer Genome Atlas thyroid cancer database

Publicly available mRNA sequencing (RNA-Seq) data of 501 patients with thyroid cancer from The Cancer Genome Atlas (TCGA) database (version 2016_01_28; https://gdac.broadinstitute.org) were analyzed[12,43]. RNA-Seq data of VDR, CYP27B1, CYP24A1, p21, and E-cadherin expressions of PTC were retrieved for further evaluation.

4. Statistical analyses

The baseline data is presented as the number and percentage for categorical variables and as the mean ± standard deviation for continuous variables, unless otherwise specified. Continuous variables were compared using Student's t test for two group comparisons. Categorical variables were compared using the chi-square ($\chi 2$) test or Fisher's exact test. The Pearson correlation coefficient was determined in RNA-Seq data of 501 patients with thyroid cancer from the TCGA database using simple linear regression analysis. P-values less than 0.05 were considered statistically significant. All data were processed and statistically analyzed using IBM SPSS Statistics for Windows, version 25.0 (IBM Corp., Armonk, NY, USA).

III. RESULTS

1. Immunohistochemistry

Vitamin D metabolism, cell proliferation, and metastasis were evaluated in 73 PTC, 23 benign, and 25 normal samples by comparing protein expressions of VDR, CYP24A1, CYP27B1, p21, and E-cadherin. A total of 25 pairs of cancer-normal tissues from the same patient were first compared (Table 2). Higher nuclear VDR expression was found in 68.0% of PTC, compared to 20.0% of normal thyroid tissues (P = 0.001). Positive CYP27B1 expression was significantly higher in PTC compared with that in normal thyroid tissues (92.0% vs. 52.0%, P = 0.004). Additionally, nuclear p21 expression was also found to be significantly increased in the PTC group compared with that in the normal thyroid group (28.0% vs. 4.0%, P = 0.049). There was no significant difference in the protein expressions of cytoplasmic p21, cytoplasmic VDR, CYP24A1, and E-cadherin.

We expanded our investigation to include all tissue samples (Table 3). We conducted the intergroup comparison for further analysis, and a significant difference was noted in the expression of several target proteins. Analyses between 73 PTC and 25 normal tissues revealed higher protein expression in nuclear VDR (57.5% vs. 20.0%, P = 0.001) and cytoplasmic VDR (23.3% vs. 0%, P = 0.005) in the PTC group. Moreover, there was also a significant increase in nuclear p21 (41.0% vs. 4.0%, P <0.001) and E-cadherin expression (52.1% vs. 16.0%, P = 0.002) in the PTC group compared with those in the normal tissue group.

We then compared 73 PTC and 23 benign thyroid tissue groups, wherein PTC demonstrated higher protein expression in nuclear VDR (57.5% vs. 33.3%, P = 0.009), cytoplasmic VDR (23.3% vs. 0%, P = 0.010), nuclear p21 (41.0% vs. 0%, P = 0.001), and E-cadherin (52.1% vs. 16.7%, P = 0.011), similar to the aforementioned comparison. Comparative analyses between 23 benign and 25 normal samples yielded no significant difference in the

expression of target proteins (Table 3).

TABLE 2. Protein expression profiles of 25 paired papillary thyroid cancer and normal tissues.

Expression	Normal (*n*=25)	Cancer (*n*=25)	*P* value
VDR - Nuclear			
0-50	20 (80.0)	8 (32.0)	0.001
51-100	5 (20.0)	20 (68.0)	
VDR - Cytoplasmic			
0-50	25 (100.0)	25 (100.0)	NA
51-100	0 (0.0)	0 (0.0)	
CYP27B1			
Negative	12 (48.0)	2 (8.0)	0.004
Positive	13 (52.0)	23 (92.0)	
CYP24A1			
Negative	22 (88.0)	18 (72.0)	0.289
Positive	3 (12.0)	7 (28.0)	
P21 - nuclear			
0-10	24 (96.0)	18 (72.0)	0.049
11-100	1 (4.0)	7 (28.0)	
P21 - cytoplasmic			
Negative	22 (88.0)	18 (72.0)	0.289
Positive	3 (12.0)	7 (28.0)	
E-cadherin			
Negative	21 (84.0)	16 (64.0)	0.196
Positive	4 (16.0)	9 (36.0)	

Values are expressed as number (%).

VDR, vitamin D receptor.

TABLE 3. Protein expression profiles of the thyroid tissue samples, stratified by histology.

Expression	Normal (*n*=25)	Benign (n=23)	Cancer (n=73)
VDR - Nuclear			
0-50	20 (80.0)	17 (73.9)	31 (42.5) **/#
51-100	5 (20.0)	6 (26.1)	42 (57.5)
VDR - Cytoplasmic			
0-50	25 (100.0)	23 (100.0)	56 (76.7) **/#
51-100	0 (0.0)	0 (0.0)	17 (23.3)
CYP27B1			
Negative	12 (48.0)	11 (47.8)	24 (32.9)
Positive	13 (52.0)	12 (52.2)	49 (67.1)
CYP24A1			
Negative	22 (88.0)	21 (91.3)	62 (84.9)
Positive	3 (12.0)	2 (8.7)	11 (15.1)
P21 - nuclear			
0-10	24 (96.0)	22 (95.7)	43 (58.9) **/##
11-100	1 (4.0)	1 (4.3)	30 (41.1)
P21 - cytoplasmic			
Negative	22 (88.0)	22 (95.7)	64 (87.7)
Positive	3 (12.0)	1 (4.3)	9 (12.3)
E-cadherin			
Negative	21 (84.0)	18 (78.3)	35 (47.9) **/#
Positive	4 (16.0)	5 (21.7)	38 (52.1)

Values are expressed as number (%).

$^{**}p < 0.01$ vs. normal, $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ vs. benign.
VDR, vitamin D receptor.

The association of VDR protein expression with the clinicopathologic characteristics of 73 PTC patients was investigated (Table 4). High nuclear VDR expression was significantly associated with high nuclear p21 expression (P = 0.023). The TNM stage was found to be significantly associated with nuclear VDR expression, wherein a significant decrease in nuclear VDR expression was observed in PTC TNM stages 3 and 4 (P = 0.017). Further analyses revealed that the expression of all target proteins in PTC TNM stage 3 and 4 samples were similar to those seen in normal thyroid tissues (Data not shown). There was no significant association between nuclear VDR and other features, such as serum 25(OH)D, serum $1,25(OH)_2D_3$, age, sex, tumor size, tumor multifocality, tumor bilaterality, extrathyroidal extension, TNM stages, and BRAF mutation. There was also no significant relationship observed among cytoplasmic p21, cytoplasmic VDR, E-cadherin, CYP27B1, and CYP24A1. Serum vitamin D levels, both 25(OH)D and $1,25(OH)_2D_3$, showed no significant correlation with other clinicopathologic features (Table 5 and 6).

TABLE 4. Clinicopathological characteristics of patients with papillary thyroid cancer according to vitamin D receptor expression level.

	Nuclear VDR Expression		
	0-50% (N = 31), n (%)	51-100% (N = 42), n (%)	P-value
Age (yr), mean ± SD	46.74 ± 14.21	43.98 ± 13.22	0.395*
Age (yr)			
<55	20 (64.5)	34 (81.0)	0.114†
≥55	11 (35.5)	8 (19.0)	

Sex			
Male	9 (29.0)	13 (31.0)	0.860†
Female	22 (71.0)	29 (69.0)	
25(OH)D (ng/mL)	19.41 ± 8.40	18.00 ± 7.30	0.446*
25(OH)D (ng/mL)			
<20	22 (71.0)	30 (71.4)	0.966†
≥20	9 (39.0)	12 (28.6)	
$1,25(OH)_2D_3$ (ng/mL)	40.39 ± 18.66	48.56 ± 22.05	0.100*
$1,25(OH)_2D_3$ (ng/mL)			
<40	16 (51.6)	15 (35.7)	0.174†
≥40	15 (48.4)	27 (64.3)	
Tumor size (cm)	1.29 ± 0.98	1.25 ± 0.82	0.866*
Tumor size			
≤1cm	16 (51.6)	22 (52.4)	0.948†
>1cm	15 (48.4)	20 (47.6)	
Multifocality			
Negative	23 (74.2)	30 (71.4)	0.793†
Positive	8 (25.8)	12 (28.6)	
Bilaterality			
Negative	27 (87.1)	36 (85.7)	1.000†
Positive	4 (12.9)	6 (14.3)	
Extrathyroidal extension			
Negative	10 (32.3)	16 (38.1)	0.607†
Positive	21 (67.7)	26 (61.9)	
T-stage			
T1-T2	11 (35.5)	18 (42.9)	0.525†

T3-T4	20 (64.5)	24 (57.1)	
Regional lymph node			
N0	17 (54.8)	25 (59.5)	0.689†
N1	14 (45.2)	17 (40.5)	
Distant metastasis			
M0	30 (96.8)	39 (92.9)	0.632†
M1	1 (3.2)	3 (7.1)	
TNM stage group			
I-II	18 (58.1)	35 (83.3)	0.017†
III-IV	13 (41.9)	7 (16.7)	
BRAF mutation			
Absent	6 (19.4)	7 (16.7)	0.767†
Present	25 (80.6)	35 (83.3)	
VDR - Cytoplasmic			
Negative to positive 0-50%	21 (67.7)	35 (83.3)	0.119†
Strong Positive 51-100%	10 (32.3)	7 (16.7)	
CYP27B1			
Negative 0-1	10 (32.3)	14 (33.3)	0.923†
Positive 2-3	21 (67.7)	28 (66.7)	
CYP24A1			
Negative 0-1	27 (87.1)	35 (83.3)	0.657†
Positive 2-3	4 (12.9)	7 (16.7)	
P21 - Nuclear			
Negative 0-10%	23 (74.2)	20 (47.6)	0.023†
Positive 11-100%	8 (25.8)	22 (52.4)	
P21 - Cytoplasmic			
Negative 0-1	30 (96.8)	34 (81.0)	0.069†

Positive 2-3	1 (3.2)	8 (19.0)	
E-cadherin			
Negative 0-1	17 (54.8)	18 (42.9)	0.311†
Positive 2-3	14 (45.2)	24 (57.1)	

*P-values were calculated using Student's t-test. Data are expressed as mean ± SD.

†P-values were calculated using $\chi 2$ test. Data are expressed as number (%).

25(OH)D, 25-hydroxyvitamin D; $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3 (calcitriol).

TABLE 5. Clinicopathological characteristics of patients with papillary thyroid cancer according to serum 25(OH)D level.

	Serum vitamin D, 25(OH)D (ng/mL)		P-value
	0-20 (N = 52), n (%)	20- (N = 21), n (%)	
Age (yr), mean ± SD	44.42 ± 13.53	46.95 ± 14.00	0.476*
Age			
<55	39 (75.0)	15 (71.4)	0.753†
≥55	13 (25.0)	6 (28.6)	
Sex			
Male	16 (30.8)	6 (28.6)	0.853†
Female	36 (69.2)	15 (71.4)	
$1,25(OH)_2D_3$ (ng/mL)	40.19 ± 18.89	57.23 ± 21.23	0.001*
$1,25(OH)_2D_3$ (ng/mL)			
<40	29 (55.8)	2 (9.5)	<0.001†
≥40	23 (44.2)	19 (90.5)	
Tumor size (cm)	1.27 ± 0.93	1.26 ± 0.79	0.961*
Tumor size			

≤1cm	26 (50.0)	12 (57.1)	0.580†
>1cm	26 (50.0)	9 (42.9)	
Multifocality			
Negative	36 (69.2)	17 (81.0)	0. 309†
Positive	16 (30.8)	4 (19.2)	
Bilaterality			
Negative	43 (82.7)	20 (95.2)	0.153†
Positive	9 (17.3)	1 (4.8)	
Extrathyroidal extension			
Negative	17 (32.7)	9 (17.3)	0.412†
Positive	35 (67.3)	12 (57.1)	
T-stage			
T1-T2	18 (34.6)	11 (52.4)	0.160†
T3-T4	34 (65.4)	10 (17.6)	
Regional lymph node			
N0	27 (51.9)	15 (71.4)	0.689†
N1	25 (48.1)	6 (28.6)	
Distant metastasis			
M0	48 (2.3)	21 (100.0)	0.191†
M1	4 (7.7)	0 (0)	
TNM stage group			
I-II	36 (69.2)	17 (81.0)	0.309†
III-IV	16 (30.8)	4 (19.0)	
BRAF mutation			
Absent	8 (15.4)	5 (23.8)	0.394†
Present	44 (84.6)	16 (76.2)	
VDR - Nuclear			

Negative to positive 0-50%	22 (42.3)	9 (42.9)	0.966†
Strong Positive 51-100%	30 (57.7)	12 (57.1)	
VDR - Cytoplasmic			
Negative to positive 0-50%	41 (78.8)	15 (71.4)	0.497†
Strong Positive 51-100%	11 (21.2)	6 (28.6)	
CYP27B1			
Negative 0-1	18 (34.6)	6 (28.6)	0.619†
Positive 2-3	34 (65.4)	15 (71.4)	
CYP24A1			
Negative 0-1	43 (82.7)	19 (90.5)	0.400†
Positive 2-3	9 (17.3)	2 (9.5)	
P21 - Nuclear			
Negative 0-10%	32 (61.5)	11 (52.4)	0.472†
Positive 11-100%	20 (38.5)	10 (47.6)	
P21 - Cytoplasmic			
Negative 0-1	45 (86.5)	19 (90.5)	0.643†
Positive 2-3	7 (13.5)	2 (9.5)	
E-cadherin			
Negative 0-1	24 (46.2)	11 (52.4)	0.630†
Positive 2-3	28 (53.8)	10 (47.6)	

*P-values were calculated using Student's t-test. Data are expressed as mean ± SD.

†P-values were calculated using $\chi 2$ test. Data are expressed as number (%).

25(OH)D, 25-hydroxyvitamin D; $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3 (calcitriol).

TABLE 6. Clinicopathological characteristics of patients with papillary thyroid cancer according to serum 1,25(OH)D_3 level.

	Serum vitamin D3, 1,25(OH)D_3 (ng/mL)		P-value
	0-40 (N = 52), n (%)	40- (N = 21), n (%)	
Age (yr), mean ± SD	44.42 ± 13.53	46.95 ± 14.00	0.476*
Age			
<55	22 (71.0)	32 (76.2)	0.615†
≥55	9 (29.0)	10 (23.8)	
Sex			
Male	8 (25.8)	14 (33.3)	0.488†
Female	23 (74.2)	28 (66.7)	
25(OH)D (ng/mL)	40.19 ± 18.89	57.23 ± 21.23	0.001*
25(OH)D (ng/mL)			
<20	29 (55.8)	2 (9.5)	<0.001†
≥20	23 (44.2)	19 (90.5)	
Tumor size (cm)	1.27 ± 0.93	1.26 ± 0.79	0.961*
Tumor size			
≤1cm	14 (45.2)	24 (57.1)	0.311†
>1cm	17 (54.8)	18 (42.9)	
Multifocality			
Negative	21 (67.7)	32 (76.2)	0. 424†
Positive	10 (32.3	10 (23.8)	
Bilaterality			
Negative	25 (80.6)	38 (90.5)	0.227†
Positive	6 (19.4)	4 (9.5)	
Extrathyroidal			

extension			
Negative	11 (35.5)	15 (35.7)	0.984†
Positive	20 (64.5)	27 (64.3)	
T-stage			
T1-T2	11 (35.5)	18 (42.9)	0.525†
T3-T4	20 (64.5)	24 (57.1)	
Regional lymph node			
N0	14 (45.2)	28 (66.7)	0.066†
N1	17 (54.8)	14 (33.3)	
Distant metastasis			
M0	28 (90.3)	41 (97.6)	0.176†
M1	3 (9.7)	1 (2.4)	
TNM stage group			
I-II	23 (74.2)	30 (71.4)	0.793†
III-IV	8 (25.8)	12 (28.6)	
BRAF mutation			
Absent	5 (16.1)	8 (19.0)	0.747†
Present	26 (83.9)	34 (81.0)	
VDR - Nuclear			
Negative to positive 0-50%	16 (51.6)	15 (31.7)	0.174†
Strong Positive 51-100%	15 (48.4)	27 (64.3)	
VDR - Cytoplasmic			
Negative to positive 0-50%	25 (80.6)	31 (73.8)	0.495†
Strong Positive 51-100%	6 (19.4)	11 (26.2)	
CYP27B1			

Negative 0-1	12 (38.7)	12 (28.6)	0.362†
Positive 2-3	19 (61.3)	30 (71.4)	
CYP24A1			
Negative 0-1	25 (80.6)	37 (88.1)	0.379†
Positive 2-3	6 (19.4)	5 (11.9)	
P21 - Nuclear			
Negative 0-10%	20 (64.5)	23 (54.8)	0.402†
Positive 11-100%	11 (35.5)	19 (45.2)	
P21 - Cytoplasmic			
Negative 0-1	25 (80.6)	39 (92.9)	0.117†
Positive 2-3	6 (19.4)	3 (7.1)	
E-cadherin			
Negative 0-1	16 (51.6)	19 (45.2)	0.590†
Positive 2-3	15 (48.4)	23 (54.8)	

*P-values were calculated using Student's t-test. Data are expressed as mean ± SD.

†P-values were calculated using $\chi 2$ test. Data are expressed as number (%).

25(OH)D, 25-hydroxyvitamin D; $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3 (calcitriol).

2. Real-time quantitative PCR (qPCR)

Comparative analysis was conducted for the relative tumor-normal mRNA expression values in 38 PTC and 10 benign tissues, along with their normal counterpart. The VDR gene showed a markedly higher relative expression in PTC, compared with that in benign tumors (20.24 ± 7.63 vs. 3.41 ±1.58, P = 0.037). The other genes, CYP27B1, CYP24A1, p21, and E-cadherin, yielded no evident difference in relative mRNA expression between PTC and benign tumors (Figure 3).

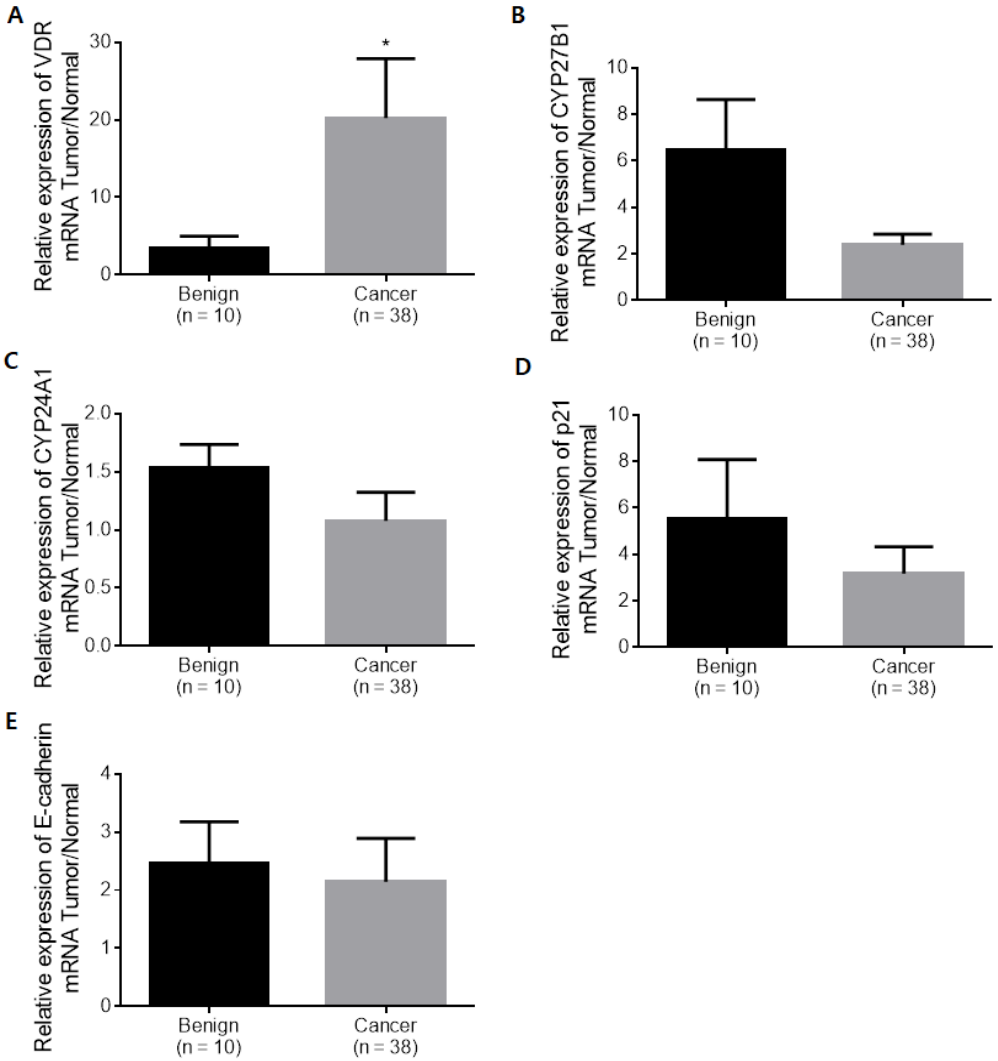

Figure 3. Relative tumor-normal mRNA expression levels of the target genes (A) VDR, (B) CYP27B1, (C) CYP24A1, (D) p21, and (E) E-cadherin in papillary thyroid cancer and benign tumor.

Data are expressed as the mean ± standard error of the mean; $^{*}p < 0.05$; VDR, vitamin D receptor.

To corroborate the consistency of VDR expression patterns, we

examined the correlation of relative cancer-normal mRNA expression of VDR, CYP27B1, CYP24A1, p21, and E-cadherin with VDR protein expression in PTC. Increased mRNA expression of the VDR in cancer was observed in patients with higher (>50) nuclear VDR protein expression (31.21 ± 12.16 vs. 3.43 ± 1.04, P = 0.033). The other genes showed no association with nuclear VDR protein expression (Figure 4).

We proceeded with further investigation to determine the relationship between the relative cancer-normal mRNA expression level of VDR genes and the clinicopathologic characteristics of 38 PTC patients (Table 7). The group with ≤2-fold cancer-normal VDR mRNA expression ratio had a lower mean serum 25(OH)D level (22.42 ± 9.56 vs. 16.01 ± 5.56, P = 0.017) and had more patients with serum vitamin 25(OH)D deficiency (47.4% vs. 84.2%, P = 0.038). However, serum $1,25(OH)_2D_3$ level was similar regardless of the relative VDR mRNA expression levels (48.81 ± 25.67 vs. 47.50 ± 19.43, P = 0.860) (Figure 5).

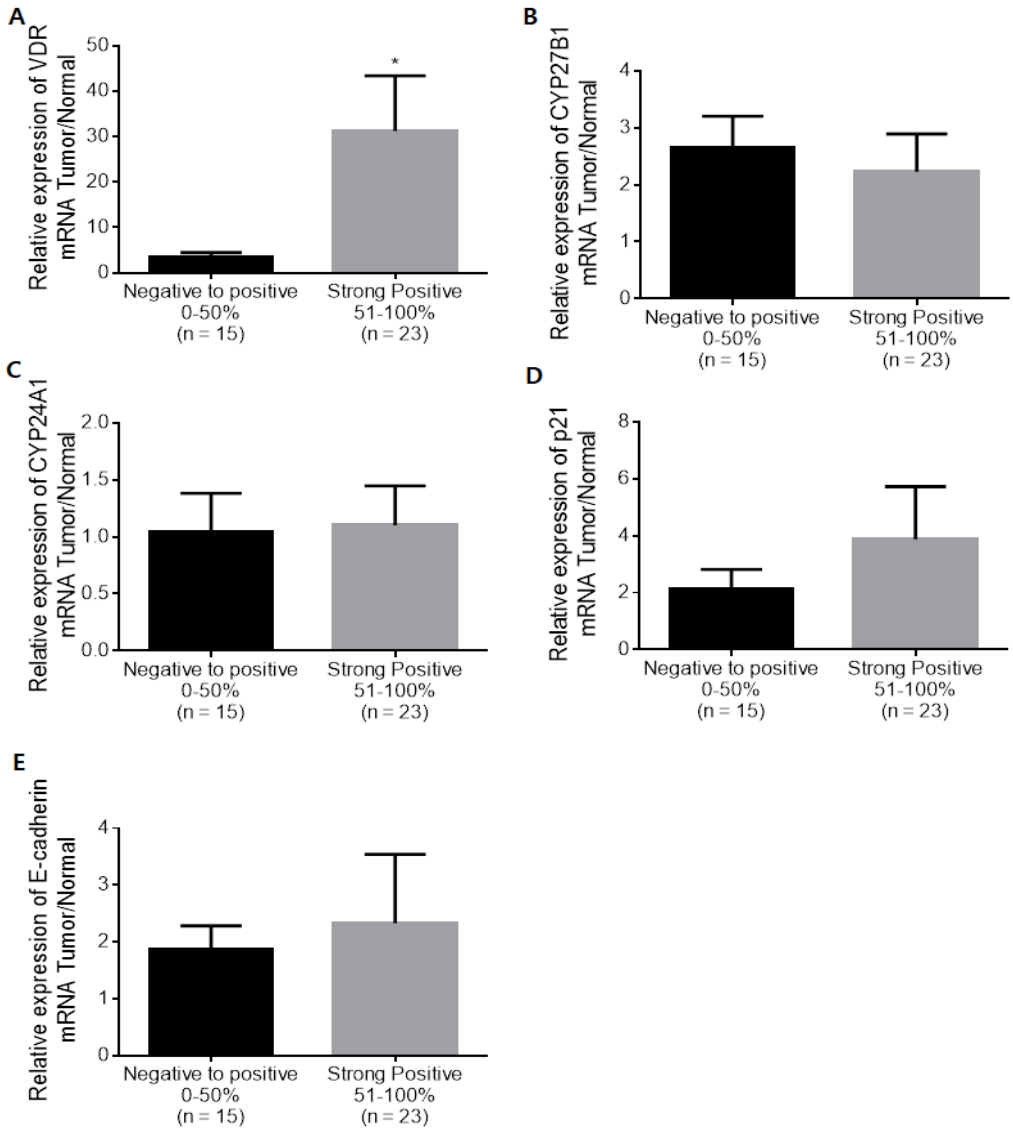

Figure 4. Correlation between mRNA expression profile of the target genes (A) VDR, (B) CYP27B1, (C) CYP24A1, (D) p21, and (E) E-cadherin with VDR protein expression.

Data are expressed as the mean ± standard error of the mean; $^{*}p < 0.05$; VDR, vitamin D receptor.

TABLE 7. Clinicopathological characteristics of patients with papillary thyroid cancer according to vitamin D receptor mRNA relative expression level.

	VDR mRNA relative expression level		
	T/N < 2 (N = 19), n (%)	T/N ≥ 2 (N = 19), n (%)	P-value
Age (yr), mean ± SD	48.81 ± 12.66	42.53 ± 12.61	0.731*
Age			
<55	16 (84.2)	15 (78.9)	1.000†
≥55	3 (15.8)	4 (21.1)	
Sex			
Male	4 (21.1)	6 (31.6)	0.714†
Female	15 (78.9)	13 (68.4)	
25(OH)D (ng/mL)	16.01 ± 5.56	22.42 ± 9.56	0.017*
25(OH)D (ng/mL)			
<20	16 (84.2)	9 (47.4)	0.038†
≥20	3 (15.8)	10 (52.6)	
$1,25(OH)_2D_3$ (ng/mL)	40.39 ± 18.66	48.56 ± 22.05	0.100*
$1,25(OH)_2D_3$ (ng/mL)			
<40	8 (42.1)	7 (36.8)	0.740†
≥40	11 (57.9)	12 (63.2)	
Tumor size (cm)	1.06 ± 0.54	1.57 ± 1.28	0.117*
Tumor size			
≤1cm	10 (52.6)	9 (47.4)	0.746†
>1cm	9 (47.4)	10 (52.6)	
Multifocality			
Negative	12 (63.2)	13 (68.4)	0.732†

Positive	7 (36.8)	3 (31.6)	
Bilaterality			
Negative	13 (68.4)	17 (89.5)	0.232†
Positive	6 (31.6)	2 (10.5)	
Extrathyroidal extension			
Negative	7 (36.8)	7 (36.8)	1.000†
Positive	12 (63.2)	12 (63.2)	
T-stage			
T1-T2	7 (36.8)	8 (42.1)	0.740†
T3-T4	12 (63.2)	11 (57.9)	
Regional lymph node			
N0	13 (68.4)	12 (63.2)	0.732†
N1	6 (31.6)	7 (36.8)	
Distant metastasis			
M0	18 (94.7)	18 (94.7)	1.000†
M1	1 (5.3)	1 (5.3)	
TNM stage group			
I-II	13 (68.4)	13 (68.4)	1.000†
III-IV	6 (31.6)	6 (31.6)	
BRAF mutation			
Absent	5 (26.3)	3 (15.8)	0.693†
Present	14 (73.7)	16 (84.2)	
VDR - Nuclear			
Negative to positive 0-50%	7 (36.8)	8 (42.1)	0.740†
Strong Positive 51-100%	12 (63.2)	11 (57.9)	
VDR - Cytoplasmic			

Negative to positive 0-50%	15 (78.9)	14 (73.7)	1.000†
Strong Positive 51-100%	4 (21.1)	5 (26.3)	
CYP27B1			
Negative 0-1	5 (26.3)	5 (26.3)	1.000†
Positive 2-3	14 (73.7)	14 (73.7)	
CYP24A1			
Negative 0-1	18 (94.7)	15 (78.9)	0.340†
Positive 2-3	1 (5.3)	4 (21.1)	
P21 - Nuclear			
Negative 0-10%	9 (47.4)	10 (52.6)	0.746†
Positive 11-100%	10 (52.6)	9 (47.4)	
P21 - Cytoplasmic			
Negative 0-1	17 (89.5)	16 (84.2)	1.000†
Positive 2-3	2 (10.5)	3 (15.8)	
E-cadherin			
Negative 0-1	12 (63.2)	7 (36.8)	0.105†
Positive 2-3	7 (36.8)	12 (63.2)	

* P-values were calculated using Student's t-test. Data are expressed as mean ± SD.

†P-values were calculated using $\chi 2$ test. Data are expressed as number (%).

25(OH)D, 25-hydroxyvitamin D; $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3 (calcitriol).

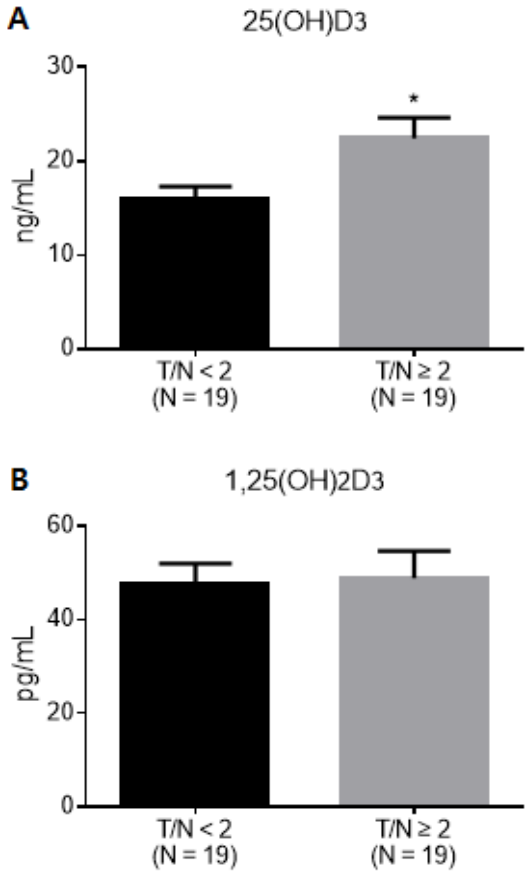

Figure 5. Correlation between relative cancer-normal mRNA expression of VDR genes and (A) serum 25(OH)D and (B) serum $1,25(OH)_2D_3$ levels.
Data are expressed as the mean ± standard error of the mean; $^{*}p < 0.05$; VDR, vitamin D receptor.

3. TCGA thyroid cancer data according to the VDR mRNA expression status

Due to restricted mRNA expression profiles in our analyses, we obtained RNA-Seq data of PTC patients from the TCGA thyroid cancer database. We further investigated vitamin D metabolism, cell proliferation, and metastasis in 501 PTC patients, evaluating the relationship of VDR mRNA expression profiles with CYP27B1, CYP24A1, p21, and E-cadherin. VDR

mRNA expression demonstrated a positive association with p21 (r = 0.109, P = 0.015) and CYP24A1 (r = 0.215, P < 0.001), and a negative association with E-cadherin (r = -0.129, P = 0.004). However, VDR mRNA expression showed no significant relationship with CYP27B1 (r = -0.05, P = 0.269) (Figure 6).

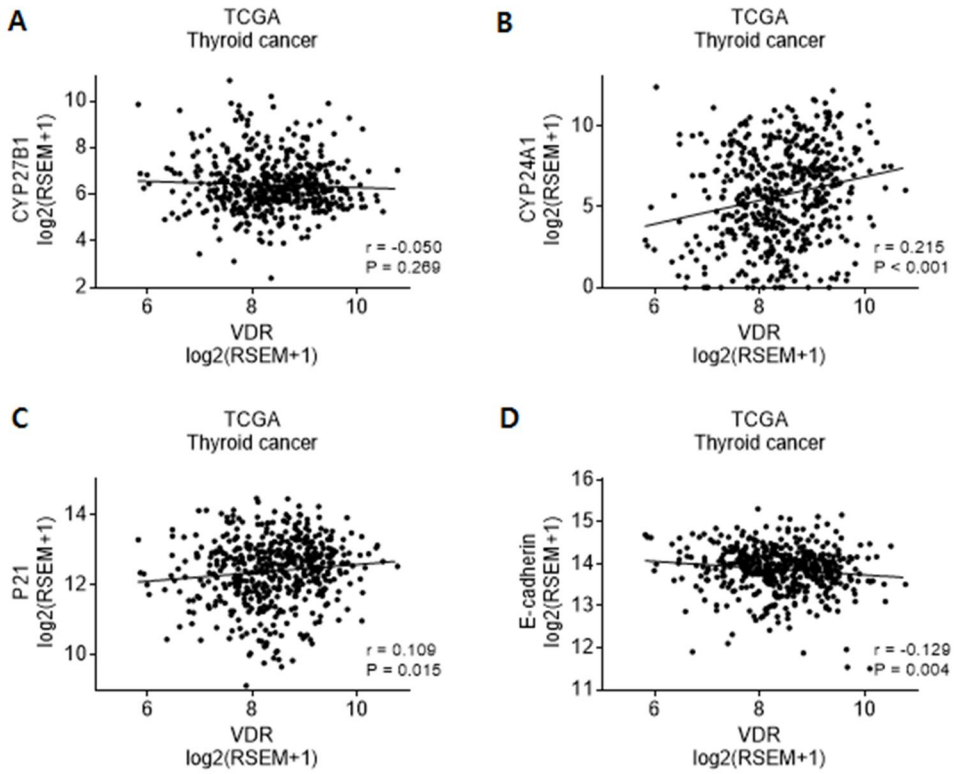

Figure 6. mRNA expression of (A) CYP27B1, (B) CYP24A1, (C) p21, and (D) E-cadherin in the RNA-Seq data of 501 patients with thyroid cancer from The Cancer Genome Atlas database.

*p < 0.05; VDR, vitamin D receptor.

IV. DISCUSSION

In this study, we have identified relevant components in vitamin D metabolism for their effect in thyroid cancer. The presence of VDR in normal thyroid has been previously described[44]. PTC samples showed enhanced protein and mRNA expressions of VDR and vitamin D related enzymes when compared to normal and benign human thyroid tissue, suggesting a potential antitumor response[34,35]. In our study, we evaluated the protein and mRNA expression profiles of VDR, CYP24A1, and CYP27B1 in normal, benign, and PTC tissues and assessed their anti-proliferation, anti-adhesion, and anti-invasion characteristics in cancer cells.

In IHC analysis, we evaluated VDR protein expression in different cellular compartments (i.e., nucleus and the cytoplasm). Several studies have reported the expression pattern of VDR in various cancers, and a few have evaluated the clinical significance of either nuclear or cytoplasmic VDR expression in cancer[45-48]. Particularly, one study reported that IHC staining was generally cytoplasmic in thyroid cancer and was more intense near the tumor capsule[34]. Another study revealed that nuclear VDR expression in PTC samples was negatively correlated with STAT3 hyperphosphorylation, an indicator of worse clinicopathologic characteristics[48].

In our study, nuclear VDR expression was higher than cytoplasmic VDR expression in most PTCs, compared with that in normal and benign samples. Cytoplasmic VDR expression was found only in few patients with PTC, but it was significantly enhanced in PTC compared with that in normal and benign tissues. VDR action seems consistent throughout the vitamin D pathway and may contribute to the anticancer activity of vitamin D[11,25]. Furthermore, nuclear VDR expression was higher than cytoplasmic VDR expression, implying that VDR activity occurred mainly in the nucleus. Nuclear VDR expression was significantly decreased in TNM stage 3 and 4 PTC, indicating that the calcitriol-VDR complex had a reduced anticancer

effect.

We analyzed the protein expression of vitamin D-related enzymes CYP27B1 and CYP24A1 in relation to VDR. Comparison of 73 PTC with 25 normal samples revealed elevated CYP27B1 and CYP24A1 expression in PTC compared to that in normal tissue, although it was not statistically significant. Previous studies have demonstrated elevated CYP27B1 and VDR expression in thyroid cancer, and enhanced CYP24A1 expression in benign thyroid tissue than in normal or in PTC[34,35]. Since CYP27B1 plays a role in calcitriol synthesis, enhanced VDR and CYP27B1 expression can lead to a magnified vitamin D action in thyroid cancer. In our study, CYP27B1 elevation in PTC compared with paired normal tissue is a finding comparable with that from previous studies.

Evaluating mRNA expression level was more challenging compared to protein level analysis because a substantial number of thyroid tissues were excluded during the quality control process. Nevertheless, VDR expression was typically increased in PTC than in normal and benign tissues, both in protein and mRNA levels. In detail, the relative mRNA expression level of VDR in PTC was higher than the protein expression level, which might result from the compartmentalization of VDR protein expression into the nucleus and cytoplasm. This could lead to a higher increase in overall VDR mRNA expression. Moreover, higher VDR protein expression in PTC was evident in patients with high cancer-normal mRNA expression ratio, which supports the augmented expression of VDR in PTC throughout the experiments, in both protein and mRNA levels. These findings are consistent with previous studies that described the potential anticancer effect of VDR in thyroid cancer[34,35].

There has been substantial research on increased vitamin D mechanisms in other benign and malignant tumors elucidating the anti-proliferative, pro-apoptotic, and dedifferentiating effects of VDR, but there are variations in study findings[11,14,16,27,36,49-53]. In our study, the VDR expression

was mostly consistent with that from previous studies, but disparate results were observed for the relationship between VDR protein and mRNA levels. Some results for enzymes were also inconsistent[34,35]. These discrepancies may be attributed to methodological differences, variation in the number of subjects, protein and mRNA instability, and tissue-specific results[27]. Further experimental research is needed to confirm these results.

The genomic mechanism of calcitriol action through VDR, as well as its antitumor action, has been widely studied. Calcitriol is also identified to regulate specific signaling pathways, such as those in colon, breast, and prostate cancer. Through these pathways, calcitriol plays a role in the anti-proliferation, pro-apoptosis, dedifferentiation, anti-inflammation, anti-angiogenesis, anti-invasion, and anti-metastasis of cancer[11,25,54,55]. Recently, Pang et al. suggested that VDR knockdown attenuates the anti-proliferative, pro-apoptotic and anti-invasive effect of vitamin D in PTC by activating the Wnt/β-catenin signaling pathway[56]. Zhang et al. also showed that calcitriol enhances Doxorubicin-induced apoptosis in PTC cells by regulating the VDR/PTPN2/p-STAT3 pathway[48]. We hypothesize that increased VDR expression in PTC tissues in our study is caused by similar mechanisms, which may also be impaired in advanced stage PTC.

Thyroid tumorigenesis and progression are also known to be affected by defects in cell cycle regulators (e.g., cyclin D1, cyclin-dependent kinase inhibitor, p21[57]) and cell-to-cell adhesion molecules (e.g., E-cadherin, transmembrane glycoprotein, epithelial cadherin[58]). Previous literature has demonstrated that calcitriol inhibits cell proliferation through cell cycle arrest in by activating p21 and p27, particularly in the G0/G1 phase[54,59]. Several studies have reported that E-cadherin is involved in the invasion and metastasis of thyroid cancer, but the results were inconsistent[60,61].

The ambivalent function of p21 has been recognized in prior experimental studies owing to its cytoplasmic localization, demonstrating pro-

apoptotic activity in the nucleus and anti-apoptotic activity in the cytoplasm[57,62]. Thus, we evaluated p21 protein expression separately according to nuclear and cytoplasmic expression. Our study showed a positive correlation between nuclear p21 and nuclear VDR protein expression, which were increased in PTC compared to that in both normal and benign samples. The human p21 gene contains VDR binding promoter regions, and is a transcriptional target for the calcitriol-VDR complex[11,63]. Liu et al. demonstrated that calcitriol-induced p27 activation in thyroid cancer cells was accomplished by VDR-mediated regulation of p27 phosphorylation and degradation through the action of the phosphatase and tensin homologue deleted (PTEN) gene[59]. Although p21 mRNA expression did not demonstrate notable findings, the positive correlation between nuclear p21 and nuclear VDR protein expression may develop into potential anti-proliferative effect in thyroid cancer.

E-cadherin showed enhanced protein expression in PTC compared with that in both normal and benign tissues, but our mRNA study revealed a similar increase in mRNA expression level in both PTC and benign tumors compared with that in normal tissue. Even though the role of E-cadherin in thyroid cancer remains unclear, its elevated protein expression in PTC compared with that in benign tumor may suggest its anticancer potential in our study. Previous studies have shown that VDR activation by calcitriol induces E-cadherin expression. This promotes the translocation of β-catenin from the nucleus to the plasma membrane, and competes with T-cell transcription factor 4 (TCF4) for β-catenin binding, which inhibits the Wnt/β-catenin/TCF4 signaling pathway. This also supports its possible anti-invasion and anti-metastasis roles in thyroid cancer.

Further analyses of 501 PTC samples from the TCGA database was performed, and it revealed diverse results. VDR mRNA expression was positively correlated with p21 and CYP24A1. This agrees with a previous

study that reported a decreased VDR and CYP24A1 mRNA expression in the PTC N1 stage, accompanied by a decreased p21 expression[35]. The authors observed a positive correlation in VDR and CYP24A1 with p21, which suggests the reduced anti-proliferative effect of VDR and p21 in advanced thyroid cancer. In this study, we observed a similar correlation and clinical significance, wherein decreased nuclear VDR was associated with decreased nuclear p21 protein expression and advanced PTC TNM stage. The N stage was not significantly correlated, unlike previous studies, but lower VDR expression in an advanced stage may explain the loss of anti-proliferative, dedifferentiating functions in aggressive thyroid cancer.

Moreover, Clinkspoor et al. showed that VDR protein expression was primarily lost in anaplastic thyroid cancer (ATC) tissues, which was more evident in those with high Ki67 expression (>30%) or with distant metastasis[35]. In human thyroid cancer cell lines, a similar finding was observed by Somjen et al. The study discovered that VDR expression was down-regulated in ATC (ARO), up-regulated in PTC (NPA), and not affected in follicular thyroid cancer cell cine (MRO) after pre-treatment with vitamin D less-calcemic analog[64]. The anti-proliferative function of VDR appears to decline not only in the advanced stages of thyroid cancer, but also in aggressive cell-types.

Repressed VDR action may be explained by the impaired vitamin D and VDR signaling pathways. Moreover, some studies have investigated the counteraction of cancer cells via multiple mechanisms to restrain VDR expression in several cancers, including colon and breast cancer. First, the snail family transcriptional repressor (SNAIL), which is involved in tumor invasion and metastasis, was shown to suppress VDR expression by binding with E-boxes in the proximal promoter region of the VDR gene to inhibit its transcription[65]. Second, the tumor-suppressor gene, p53, is known to promote VDR transcription. However, p53 mutant cells can also regulate VDR responses by directly binding to VDR and redirecting its transcriptional

program to apoptosis[66,67]. Third, a RAS oncogene mutation was found to inhibit VDR expression by suppressing VDR transcription[68]. Fourth, epigenetic silencing of VDR has also been reported in cancer. CpG island methylation in the VDR promoter region was associated with reduced VDR expression[69]. Lastly, the involvement of microRNA (miRNA) was reported to control VDR expression in cancer[70]. In thyroid cancer, the mechanism for decreased VDR expression in advanced stages has not been clearly defined. Further investigation is needed to identify the specific mechanism of VDR action present in advanced thyroid cancer.

Meanwhile, another study that analyzed the TCGA data reported contradictory results on the clinical significance of VDR[71]. Although we similarly found that VDR mRNA expression was elevated in PTC than that in normal thyroid tissue, the previous study reported that overexpression of VDR mRNA was associated with the classic and tall cell subtype, AJCC stage IV, and lower recurrence-free survival. They explained their findings in the context of impaired VDR expression on the cell membrane, wherein the VDR mRNA may be overexpressed in response to decreased VDR protein expression in damaged cellular membranes. To resolve the conflicting results, further experimental validation is essential to prove the effects of VDR on thyroid cancer.

Regarding the vitamin D pathway, combined vitamin D and VDR can postulate similar roles in anticancer action and can raise reasonable speculation of the positive correlation between them. We hypothesized a positive correlation of serum vitamin D level and VDR expression in PTC, and a low level of these two factors could be associated with the aggressiveness of PTC. Our investigation yielded varying results. The lower mRNA expression level of VDR in PTC compared with that in normal tissues was associated with a low serum 25(OH)D level. On the other hand, VDR protein expression was not associated with serum vitamin D levels, and no clinicopathologic significance

in PTC was found with serum vitamin D levels. We assume that this disparity primarily comes from the compartmentalized expression of VDR protein and relative mRNA expression level. The different nature between the mRNA and its protein, including different half-lives and the posttranscriptional modification process, can also lead to unequal results. The several factors can also affect both protein and gene expressions, such as unstable environmental factors that can influence the expression level (chemicals, temperature, light, and etc.), unstable co-binding proteins (promotor, ribosome, and etc.), and patient factors (age, gender, hormones, and etc.)[27,72,73]. As for the quantification of the protein expression, another experimental technique for protein detection, such as western blot, can be considered.

Nevertheless, the positive correlation between relative VDR mRNA expression and serum 25(OH)D level in PTC indicates similar antitumor actions on thyroid cancer. There have been varying results in the correlation between serum vitamin D level and aggressiveness and prognosis of thyroid cancer[19-23], and there have only been a few reports on the relationship of serum vitamin D levels and VDR in several cancers[74-76]. Since serum 25(OH)D is the best biomarker for vitamin D status[2], our findings are clinically meaningful. The positive correlation between VDR mRNA expression and serum 25(OH)D level may be a clue for the positive correlation between serum vitamin D deficiency and lower VDR expression in PTC, which may be linked to tumor aggressiveness. Further evaluation with larger cohorts is required to validate these results.

This study has several limitations. First, a majority of the study subjects (71.2%, 51/72) were deficient in serum 25(OH)D. Vitamin D deficiency has become common worldwide and in South Korea[77,78], making it difficult to recruit patients with sufficient serum vitamin D levels. Second, serum vitamin D levels were not adjusted to BMI and seasonal variation for the analysis. However, we excluded patients who had abnormal thyroid function, liver

disease, and kidney abnormalities. Third, in mRNA expression analyses, we excluded a substantial number of tissues due to the qualification control procedure for accurate results, resulting in a smaller number of specimens. Fourth, the assessment of the association between serum vitamin D, VDR expression and patient prognosis (i.e., overall survival and recurrence) was not feasible in this study. The use of VDR expression levels in thyroid tissues and serum vitamin D levels as a prognostic indicator in thyroid cancer has not been established, and requires further evaluation.

Despite these limitations, this study is meaningful because, firstly, a detailed evaluation of VDR and p21 protein expression was performed since both nuclear and cytoplasmic expression was analyzed. Second, although with some restrictions, a considerable number of thyroid tissues was examined to study the overall vitamin D metabolism in PTC, in terms of both protein and mRNA levels. Third, we obtained data from the TCGA database to support and validate our results. Fourth, this is the first report demonstrating the positive correlation among low VDR expression, low serum 25(OH)D level, and aggressiveness of thyroid cancer.

V. CONCLUSION

In this study, we demonstrated the elevated protein and mRNA expression of VDR in PTC compared with that in normal and benign tissues. However, lower protein expression of nuclear VDR was identified in high TNM stage PTC, which was associated with low nuclear p21 protein expression. This study provides further evidence for the potential anti-proliferative effects of VDR in PTC, which is diminished in aggressive thyroid cancer. Moreover, lower VDR mRNA expression in PTC was associated with low serum 25(OH)D levels. Overall, this study suggests a positive correlation among low VDR expression, serum vitamin D deficiency, and disease aggressiveness in PTC.

We believe that this study will contribute to a better understanding of vitamin D metabolism and the clinical significance of VDR expression in PTC. Large, prospective studies are needed to validate the potential anticancer effect of VDR in thyroid cancer. We recommend that the usefulness of vitamin D and VDR as prognostic markers as well as the therapeutic potential of vitamin D in thyroid cancer should be further investigated in future research.

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ABSTRACT (IN KOREAN)

유두갑상선암에서 비타민 D 수용체 발현과 그의 임상적 의의

<지도교수 남 기 현>

연세대학교 대학원 의학과

김 민 지

목적: 유두상 갑상선암에서 비타민 D 수용체와 혈중 비타민 D, 그리고 그 임상 양상과의 관련성을 평가하고, 비타민 D 수용체의 암 억제효과 여부를 평가하고자 하였다.

대상과 방법: 총 73명의 유두상 갑상선암 조직에서 비타민 D 수용체의 단백질 및 유전자 발현 정도를 반대편 정상 갑상선 조직 및 양성 갑상선 종양 조직과 비교해 보았다. 또한 이들의 비타민 D 관련 효소들 (CYP27B1, CYP24A1)과 세포 주기 억제 인자 (p21) 및 세포 부착 인자 (E-cadherin)와의 관련성에 대해 규명해보았다. 혈중 비타민 D 및 임상 양상과의 관련성도 평가하였다. 그리고, 이들의 특성을 암유전체 지도 (The Cancer Genome Atlas, TCGA) 데이터베이스의 501명 유두상 갑상선암 환자 자료에서도 확인해 보았다.

결과: 유두상 갑상선암에서 비타민 D 수용체의 단백질 및 유전자 발현 정도가 정상 및 양성 갑상선 조직에 비해 증가되어 있었다. 비타민 D 수용체 단백질의 낮은 발현은 진행된 병기에서 낮은 p21 인자의 발현과 관련이 있었다. 정상 조직 대비 유두상 갑상선암 비타민 D 수용체 유전자의 상대 정량이 높은 군에서 혈중 비타민D 도 높은 특성을 보였다. 암유전체 지도 데이터베이스에서는 비타민 D 수용체와 CYP24A1, p21이 양의 상관관계를 보였다.

결론: 유두상 갑상선암에서 비타민 D 수용체의 낮은 발현은 낮은 혈중 비타민 D 및 진행된 병기와 관련성이 있을 가능성을 확인하였다. 비타민 D 수용체의 암 억제 효능을 입증하기 위해서는 대규모 전향 연구가 더 필요하겠다.

핵심되는 말: 비타민 D 수용체, 유두상 갑상선암, 혈중 비타민 D, 갑상선 수술