JBACHS
Journal of Basic and Clinical Health Sciences

J Basic Clin Health Sci 2020; 4:128-132
https://doi.org/10.30621/jbachs.2020.1012

Original investigation

1,25-Dihydroxyvitamin D3 Induces N-Myc Downstream Regulated Gene-2 Expression In Papillary Thyroid Carcinoma Cells

Murat Sipahi[1], Didem Keleş Bartık[2], Mehmet Doruk[3], Fırat Bayraktar[4], Gülgün Oktay[5]

[1]Dokuz Eylül University, Institue of Health Sciences, Department of Biochemistry, İzmir, Turkey
[2]İzmir University of Economics, Vocational School of Health Services, Department of Medical Laboratory Techniques, İzmir, Turkey
[3]İzmir Bozyaka Training and Research Hospital, Division of Endocrinology and Metabolism, Department of Internal Medicine, İzmir, Turkey
[4]Dokuz Eylül University, Faculty of Medicine, Internal Medicine, Department of Internal Medicine Endocrinology And Metabolic Diseases, İzmir, Turkey
[5]Dokuz Eylül University, School of Medicine, Department of Medical Biochemistry, İzmir, Turkey

Address for Correspondence: Gülgün Oktay, **E-mail:** gulgun.oktay@deu.edu.tr
Received: 05.02.2020; **Accepted:** 10.03.2020; **Available Online Date:** 15.05.2020

Cite this article as: Sipahi M, Keleş Bartık D, Doruk M, Bayraktar F, Oktay G. 1,25-Dihydroxyvitamin D3 Induces N-Myc Downstream Regulated Gene-2 Expression In Papillary Thyroid Carcinoma Cells. J Basic Clin Health Sci 2020; 4:128-132.

ABSTRACT

Purpose: In addition to its role in serum calcium homeostasis, the anti-tumor function of 1,25-dihydroxyvitamin D_3 (calcitriol) in cancer development is well established. N-myc Downstream Regulated Gene 2 which functions as a tumor suppressor gene has recently been shown to be downregulated in various cancer leading to increased tumor incidence, progression and metastasis. The goal of this study was to investigate the possible effects of calcitriol treatment on NDRG2 expression in BCPAP papillary thyroid carcinoma cells.

Methods: The experiments were carried on human primary thyroid follicular epithelial cells (Nthy-ori-3-1), and human papillary thyroid carcinoma cells (BCPAP). The half maximal inhibitory concentration (IC_{50}) of calcitriol on BCPAP cells was determined by WST-1 assay. BCPAP cells were treated with 15 and 30μM calcitriol for 24, 48, and 72 hours, respectively. Basal NDGR2 expression in Nthy-ori-3–1 and BCPAP cells as well as the alterations on NDRG2 expression in calcitriol treated BCPAP cells were evaluated with western blot.

Results: A significant downregulation of NDRG2 was observed in BCPAP cells when compared to Nthy-ori-3–1 cells ($p<0.01$). IC_{50} dose of calcitriol was found to be 64, 54 and 43μM for 24, 48 and 72 hours, respectively. NDRG2 protein expression levels were significantly increased in 30μM calcitriol treated BCPAP cells after 48 hours ($p<0.05$).

Conclusions: Calcitriol induced NDRG2 protein expression in BCPAP cells. We predict that calcitriol increased NDRG2 protein levels in BCPAP cells via c-Myc repression, which is upregulated by aberrant Wnt/β-catenin signaling. Further investigation is required to enlighten the possible effect mechanisms of calcitriol in BCPAP cells.

Keywords: 1,25-Dihydroxyvitamin D_3, thyroid cancer, papillary thyroid cancer, N-myc downstream regulated gene 2

INTRODUCTION

Thyroid cancer is one of the most common endocrine neoplasia and the incidence is increasing worldwide. Most of the thyroid tumors arise from thyroid follicular epithelial cells which can be divided into four subclasses; 1) papillary thyroid cancer 2) follicular thyroid cancer, 3) Hurtle cell cancer and 4) anaplastic thyroid cancer. Medullary thyroid cancer, which is originated from non-follicular thyroid cells, accounts for 4% of thyroid cancer cases (1).

Papillary thyroid cancer (PTC), which accounts for 85–90% of all thyroid cancer cases, is the most common histological type of thyroid cancer. Due to its low progression rate and the positive role of radioactive iodine ablation, patients have a good prognosis with a high 10 year of survival rate (2).

Vitamin D is a fat-soluble vitamin and its major role is to increase the intestinal absorption of calcium and maintain proper serum calcium and phosphate concentrations which are essential for normal mineralization of bone (3). Calcitriol (1α, 25-Dihydroxyvitamin D_3) is the most active form of vitamin D and synthesis of endogenous calcitriol is a multistep process induced by ultraviolet rays from the sun in the skin. Molecular activity of calcitriol is dependent on binding to its receptor, Vitamin D Receptor (VDR), which is also a transcription factor present in both cytoplasm and nucleus (4). Binding of calcitriol to VDR induces the formation of calcitriol-VDR/Retinoid X Receptor (RXR) which is essential for translocation of calcitriol-RXR-VDR complex to nucleus. In nucleus, calcitriol-RXR-VDR complex interacts with vitamin D response elements (VDRE) and regulates several gene expression (5). The anti-cancer role of

calcitriol lies beneath the regulation of several genes which are involved in cell growth/proliferation (6), apoptosis (7), angiogenesis (8), inflammation (9) and differentiation (10).

N-myc Downstream Regulated Gene-2 (NDRG2), NDRG family (NDRG1–4) member (11), is located at chromosome 14q11.2 encoding a 41 kDa protein and its expression is down-regulated by c-Myc (12). *In vivo* and *in vitro* studies have indicated decreased expression of NDRG2 is in various cancers including colon (13), breast (14) and hepatocellular carcinoma (15). In gastric cancer, loss of NDRG2 expression is described as a hallmark of poor prognosis (16). These data support the notion that NDRG2 is a differentiation related gene downregulated in cancer. In this study, we investigated the effects of calcitriol on NDRG2 protein levels in BCPAP cells.

METHODS

Cell Culture

In this study, Nthy-ori-3-1, immortalised normal human primary thyroid follicular epithelial cells (EACC, 90011609) and BCPAP cells obtained from the tumor tissue of a 76-year-old woman with metastasizing papillary thyroid carcinoma in 1992 (ACC, 273) were used. Both cells were maintained in RPMI-1640 supplemented with 10% FBS (LONZA, Switzerland), 100 U/mL Penicillin/Streptomycin (LONZA, Switzerland) and 1% L-Glutamine (LONZA, Switzerland) in a 5% CO_2 environment at 37°C.

Cell Viability Assay

The half maximal inhibitory concentration (IC50) of calcitriol for BCPAP cells was evaluated with WST-1 test (Roche Applied Science, Germany). Calcitriol (Cayman Chemicals, Michigan) was prepared as stocks in ethanol (LONZA, Switzerland) and then diluted in culture medium to 1µM to 100µM.

BCPAP cells were seeded on 96 well plate at a density of 5.0 x 10^3 cells per well and incubated with 100 µl of medium with or without calcitriol (solvent control) in a 5% CO_2 environment at 37°C for 24, 48 and 72 hours. Due to low half-life of calcitriol (17), medium with calcitriol was renewed daily. WST-1 (10 µl) was added to each well and incubated at 37°C in a 5% CO_2 incubator for 2h. The absorbance was measured with microplate reader (BioTek, Vermont) at 450/655 nm. The data was expressed as a percentage of control.

Calcitriol Treatment

BCPAP cells were seeded on 25 cm^2 flasks at a density of 1.0 x 10^6. The cells were treated for 24, 48 and 72 hours with 2 ml of medium containing calcitriol (15 and 30µM) or ethanol (v/v) and the media were renewed daily.

Western blot analysis and antibodies

The cells were collected and lysed in RIPA lysis buffer (150 mM NaCl, 0.1% SDS, 1% NP-40, 20 mM Tris-HCl pH 7.5). The aliquots of the lysate were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) then transferred to PVDF membranes (Millipore, Billerica, MA). After blocking the PVDF membranes with 5% (w/v) nonfat dry milk in Tris-buffered saline Tween-20 (TBST), membranes were incubated with rabbit anti-human NDRG2 (1:1000, Cell Signaling) and rabbit anti-human α-actinin (1:3000, Cell Signaling) primary antibodies at 4°C overnight. The membranes were washed and incubated with anti-rabbit HRP-conjugated secondary antibodies for 1h at room temperature. Bands were detected by chemiluminescence assay visualized with Vilber Lourmat Chemi-Smart 5100 imaging system.

Statistical analysis

GraphPad Prism 8.2.1 (San Diego, CA, USA) software (LEAD Technologies Inc.) was used for statistical analysis and the paired t-test was applied. All experiments were performed in triplicate and all data were represented as mean ± standard error (SEM). Results were considered statistically significant when $p<0.05$.

RESULTS

Expression levels of NDRG2 in Nthy-ori-3–1 and BCPAP Cells

We compared NDRG2 protein expression by Western Blotting in Nthy-ori-3–1 and BCPAP cells. We observed significantly higher NDRG2 protein expression levels (3.3 fold) in Nthy-ori-3-1 cells compared to BCPAP cells ($p<0.01$) (Figure 1).

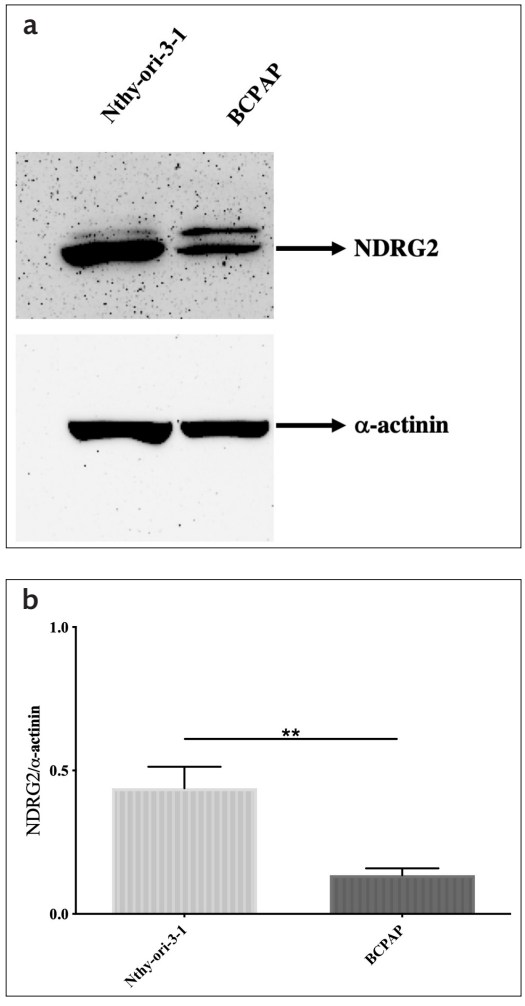

Figure 1. (a) Expression of basal NDRG2 expression in cell lines. **(b)** NDRG2 protein expression was significantly higher in Nthy-ori-3–1 cells compared to BCPAP cells (n=3). α-actinin served as a control to ensure equal loading. ** $p<0.01$.

Cell Viability

BCPAP cells were treated with calcitriol (1 μM to 100 μM) for 24, 48 and 72 hours. WST-1 test results showed that 64, 54 and 43μM of calcitriol treatment resulted in 50% reduction in cell viability at 24, 48 and 72 hours, respectively (Figure 2a). On the other hand, ethanol (solvent control) did not effect on cell viability (Figure 2b). 15 and 30μM of calcitriol were decided to be used for further experiments.

Alterations in NDRG2 Expression

BCPAP cells were treated with 15 and 30μM calcitriol for 24, 48 and 72 hours as described, previously. A significant increase in NDRG2 protein level was observed in BCPAP cells treated with 30μM calcitriol for 48 hours when compared to control and solvent control group (Figure 3). NDRG2 protein expression was also increased in BCPAP cells treated with 30μM calcitriol for 72 hours, however it was not significant.

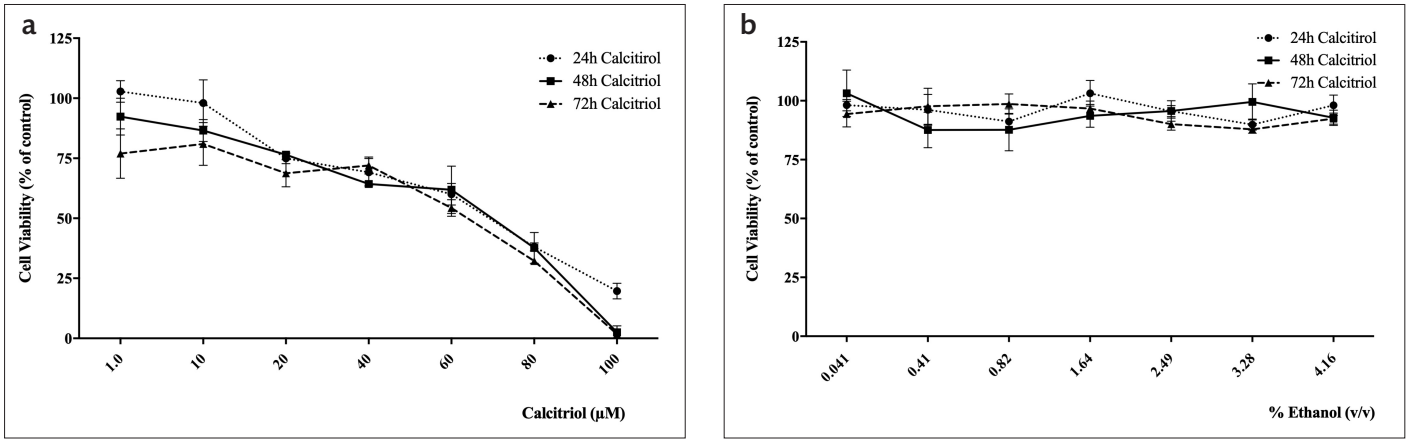

Figure 2. WST-1, cell viability assay was performed to detect IC_{50} values of calcitriol in BCPAP cells. (a) BCPAP cells were treated with 1μM to 100μM calcitriol for 24, 48 and 72 hours, respectively. IC_{50} dose of calcitriol was found to be 64, 54 and 43μM for 24, 48 and 72 hours, respectively. (b) BCPAP cells were treated ethanol (solvent control) for 24, 48 and 72 hours, respectively.

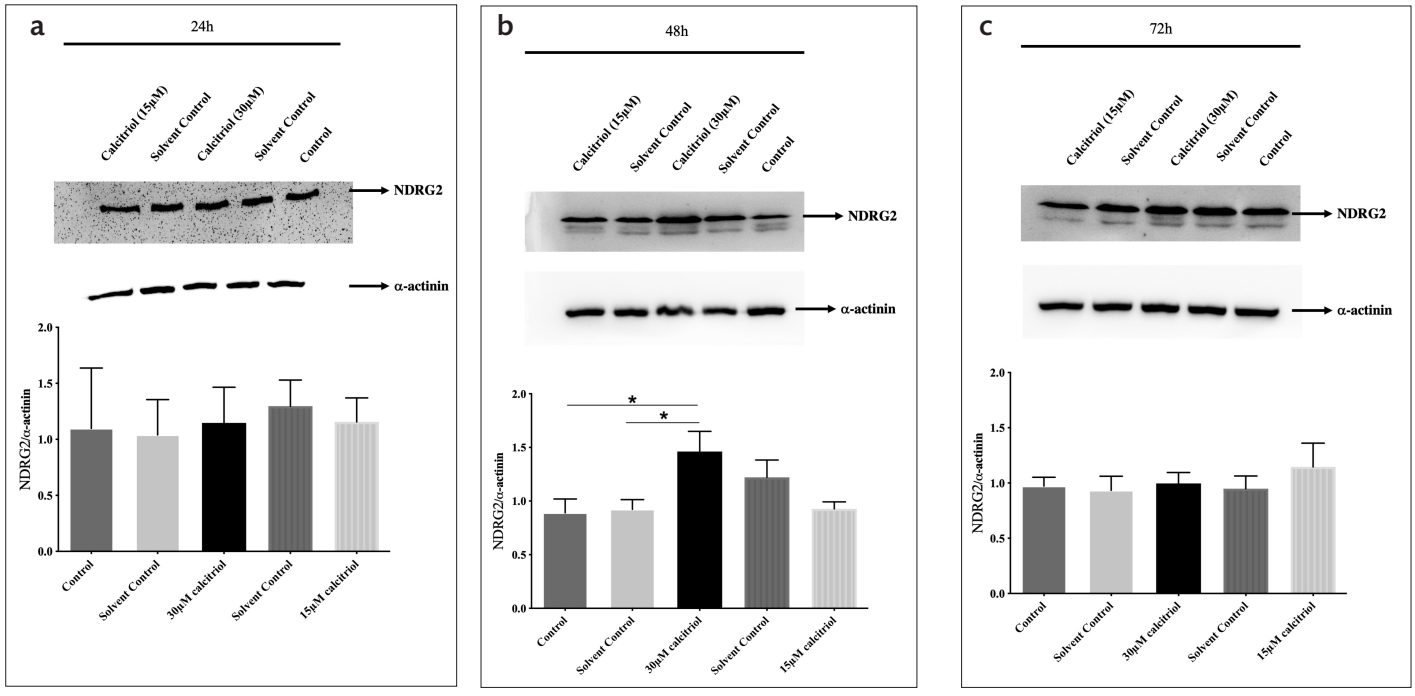

Figure 3. Alterations in NDRG2 protein levels in BCPAP cells after calcitriol treatment. Cell were treated with 15 or 30μM calcitriol for 24 **(a)**, 48 **(b)** and 72 **(c)** hours, respectively (n=3). α-actinin served as a control to ensure equal loading. * p<0.05.

DISCUSSION

Besides maintaining normal mineralization of bone, vitamin D is important for its tumor suppressing roles. There are numerous studies in which the importance of vitamin D metabolism, vitamin D receptor and calcitriol levels in thyroid cancer were investigated. Khadzkou et al. reported a significant increase in 1-alpha hydroxylase (CYP27B1) –vitamin D activating enzyme– in papillary thyroid carcinoma (PTC) tissues compared with normal tissues and especially high in areas of lymphocyte infiltration (18). A similar study was carried out by Clinckspoor et al. and

the decreased CYP24A1 (vitamin D catabolizing enzyme) and increased CYP27B1 protein expressions were observed in the primary PTC tissues with lymph node metastasis. In both studies, decrease in VDR protein levels was reported in tumor tissues obtained from lymph node metastasis compared to primary papillary thyroid tissues (19). These data suggest that a local vitamin D metabolism may compensate the decrease in calcitriol sensitivity due to VDR downregulation in primary PTC tumors prone to metastasis. Stepien et al. investigated a significant decrease in calcitriol concentration in peripheral blood serum of thyroid cancer patients. They also pointed out that the decrease in calcitriol concentration was lower in patients with earlier tumor stage which once again indicates the tumor suppressor roles of vitamin D metabolism and calcitriol in earlier stages of thyroid cancer (20). Okano et al. reported a significant decrease in DNA synthesis and c-Myc gene expression in papillary thyroid cancer cell line, NPA and Liu et al. observed a significant decrease in p27 degradation, which leads to G1-S arrest in numerous thyroid cancer cell lines after calcitriol treatment (21, 22). In another report, a 4-fold reduction in PTC tumor growth was observed in mice harboring a homozygous deletion of CYP24A1 when compared to normal counterparts (23).

In this study, first we compared the basal protein expression levels of NDRG2 to indicate that NDGR2 protein is lower in BCPAP cells when compared to Nthy-ori-3-1. We aimed to observe the effects of calcitriol on human papillary thyroid cancer cells rather than human primary thyroid follicular epithelial cells. Therefore, we addressed to question as to whether calcitriol treatment induced NDRG2 protein expression in BCPAP cells. Western blot results showed 30μM calcitriol treatment for 48 hours significantly upregulated NDRG2 protein expression in BCPAP cells. *In vivo* and *in vitro* studies investigating the effects of calcitriol on Wnt/β-catenin signaling pathway may shed the light on effects of calcitriol on NDRG2 expression in thyroid cancer. Uncontrolled activation of Wnt/β-catenin signaling pathway as well as defects in β-catenin degradation cause nuclear β-catenin accumulation, which leads to overexpression of invasion and proliferation related genes such as c-Myc (24). Larriba et al. reported that calcitriol treatment reduced c-Myc expression by promoting nuclear β-catenin export in colon cancer cells. However, alterations in NDRG2 expression after calcitriol treatment was not investigated in this study (25).

Moreover, Zhao et al. reported a significant increase in c-Myc expression and a significant decrease in NDRG2 expression in thyroid adenoma and carcinoma tissues compared to normal counterparts and suggested a negative correlation between two genes (26). Since numerous studies are showing that β-catenin dysregulation contributes to progression of thyroid tumors (27, 28), our hypothesis is that calcitriol treatment induced NDGR2 expression by suppressing c-Myc expression via inhibition of Wnt/β-catenin signaling pathway in BCPAP cells (Figure 4).

To our notion, this is the first *in vitro* study which investigates the effects of calcitriol on NDRG2 protein expression in papillary thyroid cancer. Further investigation is needed to determine whether induction of NDRG2 after calcitriol treatment is c-Myc and/or Wnt/β-catenin signaling pathway inhibition dependent.

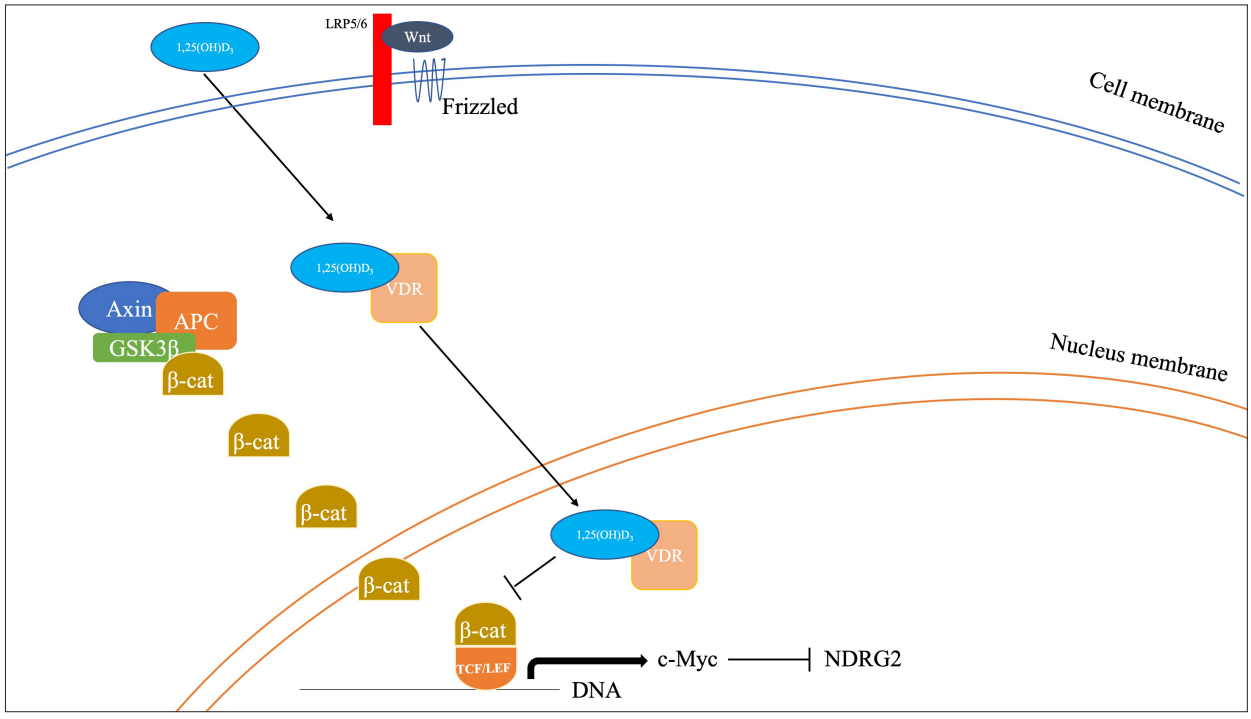

Figure 4. Schematic representation of repression of Wnt/β-catenin pathway by 1,25 (OH) D_3. Nuclear β-catenin accumulation is caused by mutation of APC, β-catenin, AXIN and GSK-3β genes or deregulated signaling from Wnt plasma membrane receptors which result in transcription of its target genes such as c-Myc, a known repressor of NDRG2.1,25 (OH) D_3 inhibits binding of β-catenin to TCF/LEF leading to decreased levels of c-Myc which can explain elevated levels of NDRG2 protein in calcitriol treated BCPAP cells.

Informed Consent: This is an in vitro study carried on cells which are grown under controlled conditions.

Peer-review: Externally peer-reviewed.

Author Contributions: Concept - GO, MS, DKB, MD, FB; Design - GO, MS, DKB; Supervision - GO, MS, DKB; Fundings - GO; Materials - GO, MS, DKB; Data Collection and/or Processing - GO, MS, DKB; Analysis and/or Interpretation - GO, MS, DKB, MD; Literature Search - GO, MS, DKB; Writing Manuscript - GO, MS, DKB; Critical Review - GO, MS, DKB, FB

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: This research was supported by a grant (no. 2018.KB.SAĞ.069) from Dokuz Eylül University Scientific Research Project Coordination Unit.

REFERENCES

1. Brown RL, de Souza JA, Cohen EE. Thyroid cancer: burden of illness and management of disease. J Cancer 2011;2:193–199. [CrossRef]
2. Hundahl SA, Fleming ID, Fremgen AM, Menck HR. A National Cancer Data Base report on 53, 856 cases of thyroid carcinoma treated in the U. S., 1985–1995. Cancer 1998;83:2638–2648. [CrossRef]
3. Ross AC, Manson JE, Abrams SA, et al. The 2011 report on dietary reference intakes for calcium and vitamin D from the Institute of Medicine: what clinicians need to know. J Clin Endocrinol Metab 2011;96:53–58. [CrossRef]
4. Moore DD, Kato S, Xie W, et al. International Union of Pharmacology. LXII. The NR1H and NR1I receptors: constitutive androstane receptor, pregnene X receptor, farnesoid X receptor alpha, farnesoid X receptor beta, liver X receptor alpha, liver X receptor beta, and vitamin D receptor. Pharmacol Rev 2006;58:742–759. [CrossRef]
5. Haussler MR, Whitfield GK, Haussler CA, et al. The nuclear vitamin D receptor: biological and molecular regulatory properties revealed. J Bone Miner Res 1998;13:325–349. [CrossRef]
6. Yang ES, Burnstein KL. Vitamin D inhibits G1 to S progression in LNCaP prostate cancer cells through p27Kip1 stabilization and Cdk2 mislocalization to the cytoplasm. J Biol Chem 2003;278:46862–46868. [CrossRef]
7. Jiang F, Bao J, Li P, Nicosia SV, Bai W. Induction of ovarian cancer cell apoptosis by 1, 25-dihydroxyvitamin D3 through the down-regulation of telomerase. J Biol Chem 2004;279:53213–53221. [CrossRef]
8. Chung I, Han G, Seshadri M, et al. Role of vitamin D receptor in the antiproliferative effects of calcitriol in tumor-derived endothelial cells and tumor angiogenesis in vivo. Cancer Res 2009;69:967–975. [CrossRef]
9. Van Waes C. Nuclear factor-kappaB in development, prevention, and therapy of cancer. Clin Cancer Res 2007;13:1076–1082. [CrossRef]
10. Palmer HG, Gonzalez-Sancho JM, Espada J, et al. Vitamin D(3) promotes the differentiation of colon carcinoma cells by the induction of E-cadherin and the inhibition of beta-catenin signaling. J Cell Biol 2001;154:369–387. [CrossRef]
11. Qu X, Zhai Y, Wei H, et al. Characterization and expression of three novel differentiation-related genes belong to the human NDRG gene family. Mol Cell Biochem 2002;229:35–44. [CrossRef]
12. Zhang J, Li F, Liu X, et al. The repression of human differentiation-related gene NDRG2 expression by Myc via Miz-1-dependent interaction with the NDRG2 core promoter. J Biol Chem 2006;281:39159–39168. [CrossRef]
13. Piepoli A, Cotugno R, Merla G, et al. Promoter methylation correlates with reduced NDRG2 expression in advanced colon tumour. BMC Med Genomics 2009;2:11. [CrossRef]
14. Liu N, Wang L, Liu X, et al. Promoter methylation, mutation, and genomic deletion are involved in the decreased NDRG2 expression levels in several cancer cell lines. Biochem Biophys Res Commun 2007;358:164–169. [CrossRef]
15. Lee DC, Kang YK, Kim WH, et al. Functional and clinical evidence for NDRG2 as a candidate suppressor of liver cancer metastasis. Cancer Res 2008;68:4210–4220. [CrossRef]
16. Choi SC, Yoon SR, Park YP, et al. Expression of NDRG2 is related to tumor progression and survival of gastric cancer patients through Fas-mediated cell death. Exp Mol Med 2007;39:705–714. [CrossRef]
17. Jones G. Pharmacokinetics of vitamin D toxicity. Am J Clin Nutr 2008;88:582S-586S. [CrossRef]
18. Khadzkou K, Buchwald P, Westin G, Dralle H, Akerstrom G, Hellman P. 25-hydroxyvitamin D3 1alpha-hydroxylase and vitamin D receptor expression in papillary thyroid carcinoma. J Histochem Cytochem 2006;54:355–361. [CrossRef]
19. Clinckspoor I, Hauben E, Verlinden L, et al. Altered expression of key players in vitamin D metabolism and signaling in malignant and benign thyroid tumors. J Histochem Cytochem 2012;60:502–511. [CrossRef]
20. Stepien T, Krupinski R, Sopinski J, et al. Decreased 1–25 dihydroxyvitamin D3 concentration in peripheral blood serum of patients with thyroid cancer. Arch Med Res 2010;41:190–194. [CrossRef]
21. Okano K, Usa T, Ohtsuru A, et al. Effect of 22-oxa-1, 25-dihydroxyvitamin D3 on human thyroid cancer cell growth. Endocr J 1999;46:243–252. [CrossRef]
22. Liu W, Asa SL, Fantus IG, Walfish PG, Ezzat S. Vitamin D arrests thyroid carcinoma cell growth and induces p27 dephosphorylation and accumulation through PTEN/akt-dependent and -independent pathways. Am J Pathol 2002;160:511–519. [CrossRef]
23. Zou M, Baitei EY, BinEssa HA, et al. Cyp24a1 Attenuation Limits Progression of Braf (V600E)-Induced Papillary Thyroid Cancer Cells and Sensitizes Them to BRAF (V600E) Inhibitor PLX4720. Cancer Res 2017;77:2161–2172. [CrossRef]
24. He TC, Sparks AB, Rago C, et al. Identification of c-MYC as a target of the APC pathway. Science 1998;281:1509–1512. [CrossRef]
25. Larriba MJ, Valle N, Palmer HG, et al. The inhibition of Wnt/beta-catenin signalling by 1alpha, 25-dihydroxyvitamin D3 is abrogated by Snail1 in human colon cancer cells. Endocr Relat Cancer 2007;14:141–151. [CrossRef]
26. Zhao H, Zhang J, Lu J, et al. Reduced expression of N-Myc downstream-regulated gene 2 in human thyroid cancer. BMC Cancer 2008;8:303. [CrossRef]
27. Cerrato A, Fulciniti F, Avallone A, Benincasa G, Palombini L, Grieco M. Beta- and gamma-catenin expression in thyroid carcinomas. J Pathol 1998;185:267–272. [CrossRef]
28. Garcia-Rostan G, Camp RL, Herrero A, Carcangiu ML, Rimm DL, Tallini G. Beta-catenin dysregulation in thyroid neoplasms: down-regulation, aberrant nuclear expression, and CTNNB1 exon 3 mutations are markers for aggressive tumor phenotypes and poor prognosis. Am J Pathol 2001;158:987–996. [CrossRef]